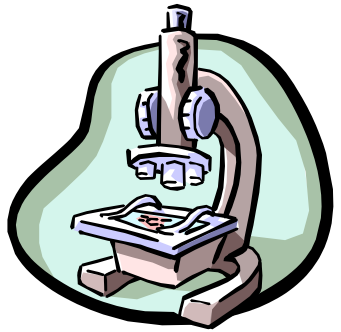
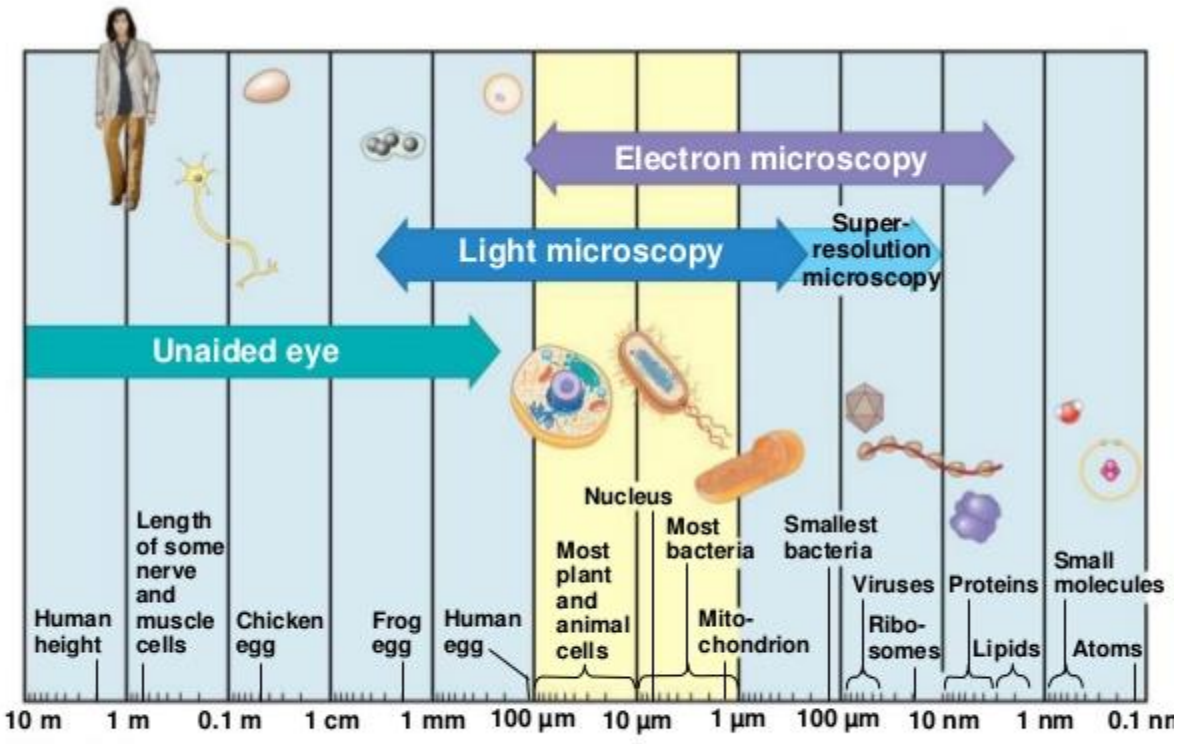
# COMPARATIVE BRAIN EVOLUTION

Optical Microscopy

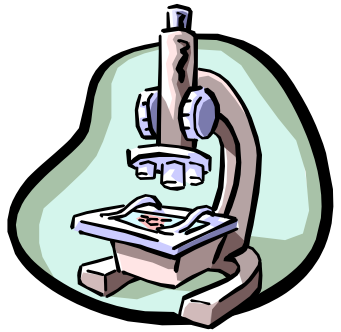
# WHY MICROSCOPY ?






- Microscopes are essential for biological studies
- Light microscopes: cellular resolution
  - bright-field (stains)
  - dark-field
  - phase contrast
  - fluorescence (stains)
- Super resolution microscopy: subcellular resolution

# Timeline of the Microscope

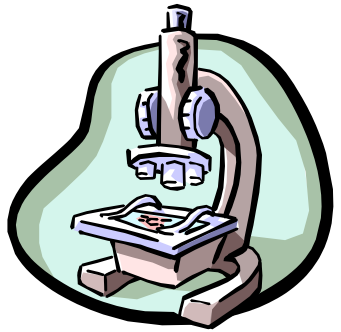


## Abbe's Law

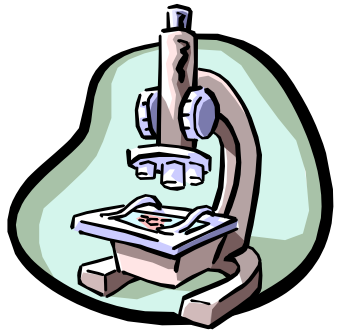
$$d = \frac{\lambda}{2\, n \sin\theta}$$

"minimum resolving distance (d) is related to the wavelength of light (lambda) divided by the Numeric Aperture, which is proportional to the angle of the light cone (theta) formed by a point on the object, to the objective".

# Some Definitions



- Magnification: increase of an object's apparent size
- Resolution: power to show details clearly
- Both are needed to see a clear image



## Magnification

enlargement of an object

compare size of image to actual size of object

## total magnification

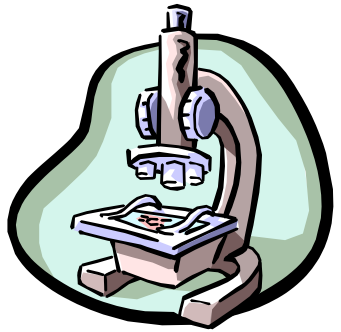
ocular power x objective power = total magnification

naked eye

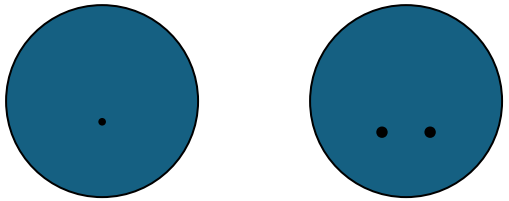
20x magnification

Magnification is NOT ALWAYS related with resolution

# Resolution power to show details clearly



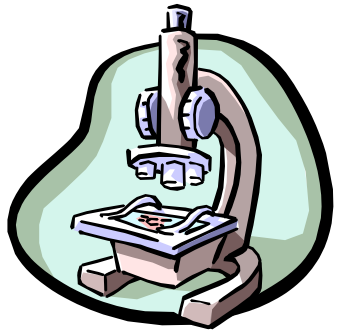
- **Resolution** – capacity to show 2 points that are close together as separate



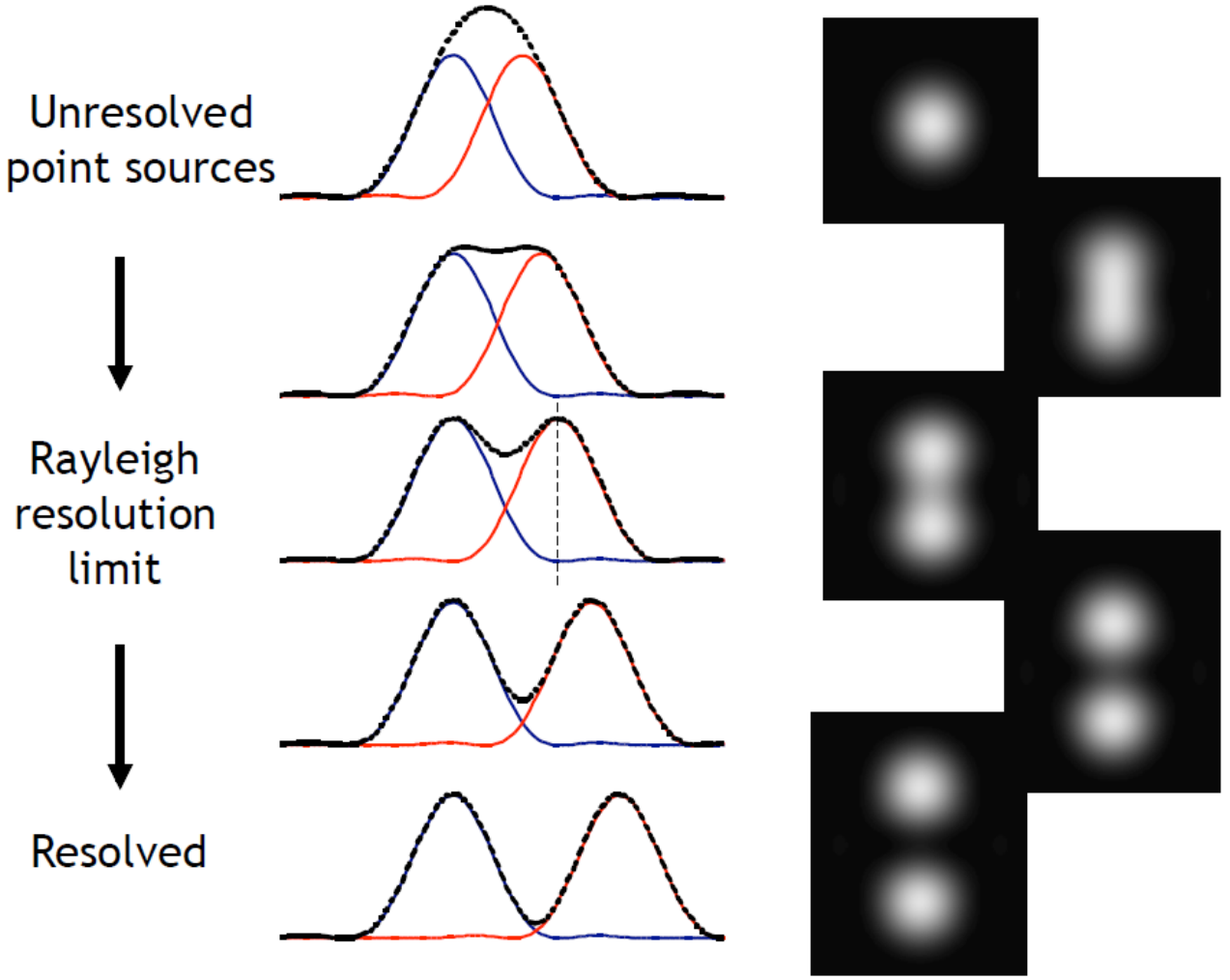
10x 1000x

**Poor Resolution** = Blurry Image

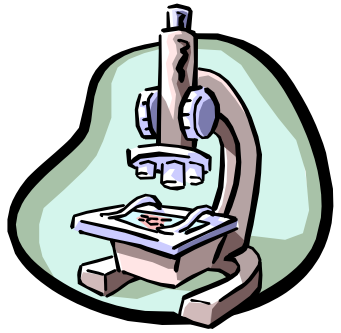
**Good Resolution** = Clear Image



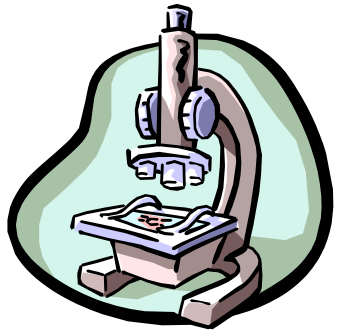
# Resolution


Unresolved
point sources
Rayleigh
resolution
limit
Resolved

# Some Definitions

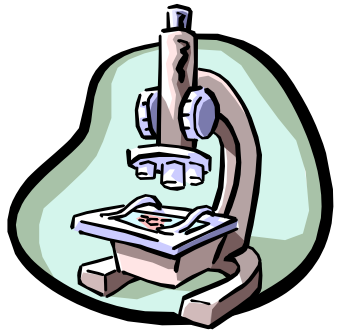


- **Absorption**
  - When light passes through an object the intensity is reduced depending upon the color absorbed. Thus the selective absorption of white light produces colored light.
- **Refraction**
  - Direction change of a ray of light passing from one transparent medium to another with different optical density. A ray from less to more dense medium is bent perpendicular to the surface, with greater deviation for shorter wavelengths
- **Diffraction**
  - Light rays bend around edges - new wavefronts are generated at sharp edges - the smaller the aperture the lower the definition
- **Dispersion**
  - Separation of light into its constituent wavelengths when entering a transparent medium - the change of refractive index with wavelength, such as the spectrum produced by a prism or a rainbow



# The characteristics of objectives


Figure 1



# Your friend - the objective

Objectives can be classified into transmitted light and reflected-light (Epi) versions.

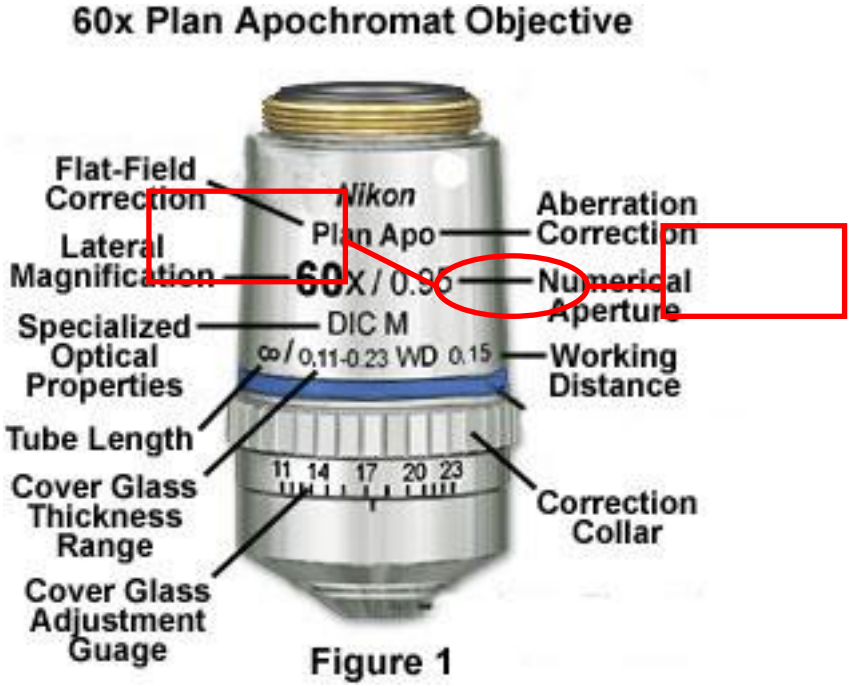
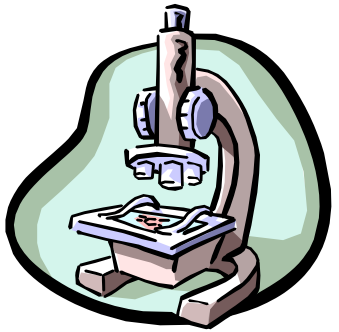



Figure 1


Figure 1

# Numerical Aperture (N.A.)



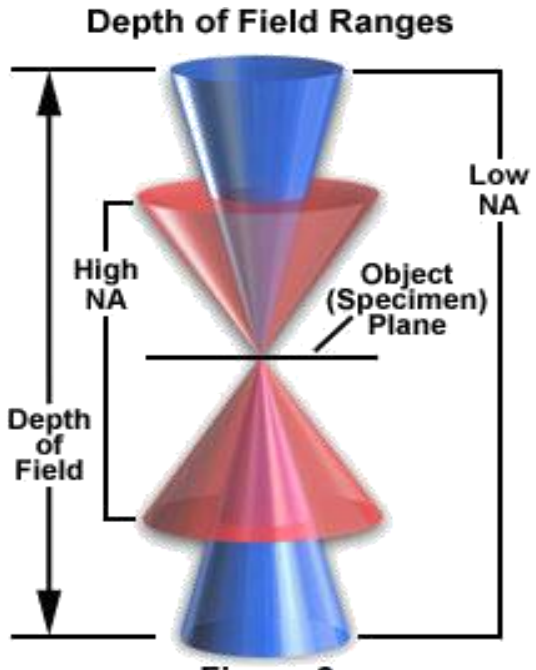


Figure 2

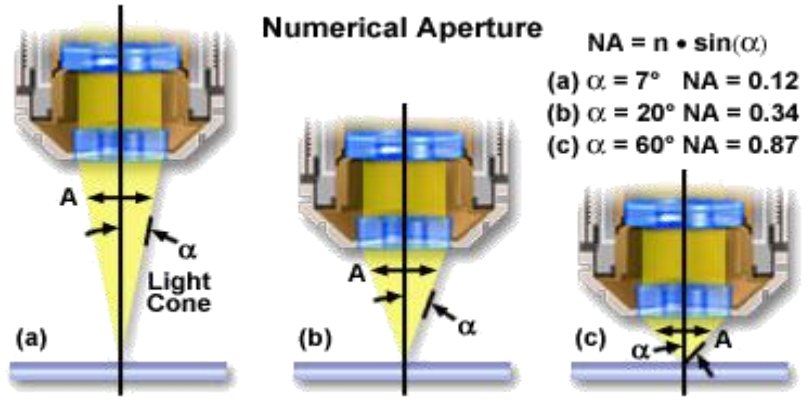


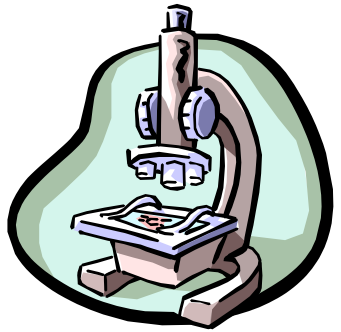
Figure 1

**Numerical Aperture = N.A. = n · sin α**

α is half the opening angle of the objective.

n is the refractive index of the immersion medium used between the objective and the object.

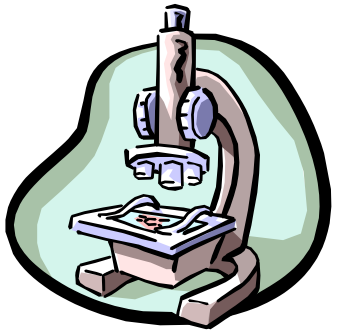
(n = 1 for air; n = 1.51 for oil or glass)

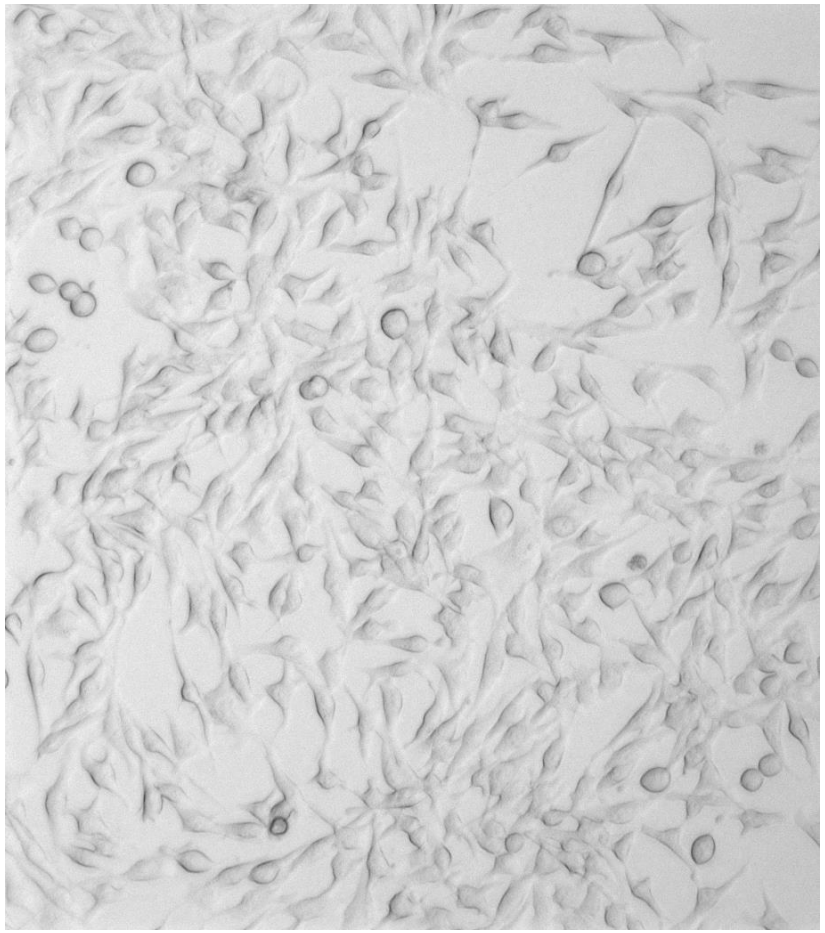


# Light microscopy

Magnifying is not seeing

# How can we use the properties of light to create contrast?





Which properties can be used?

Absorption

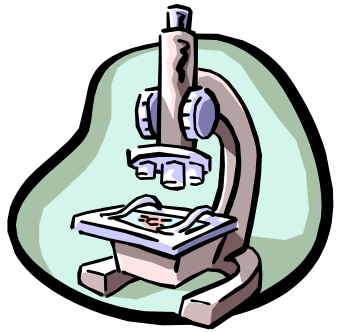
Scattering

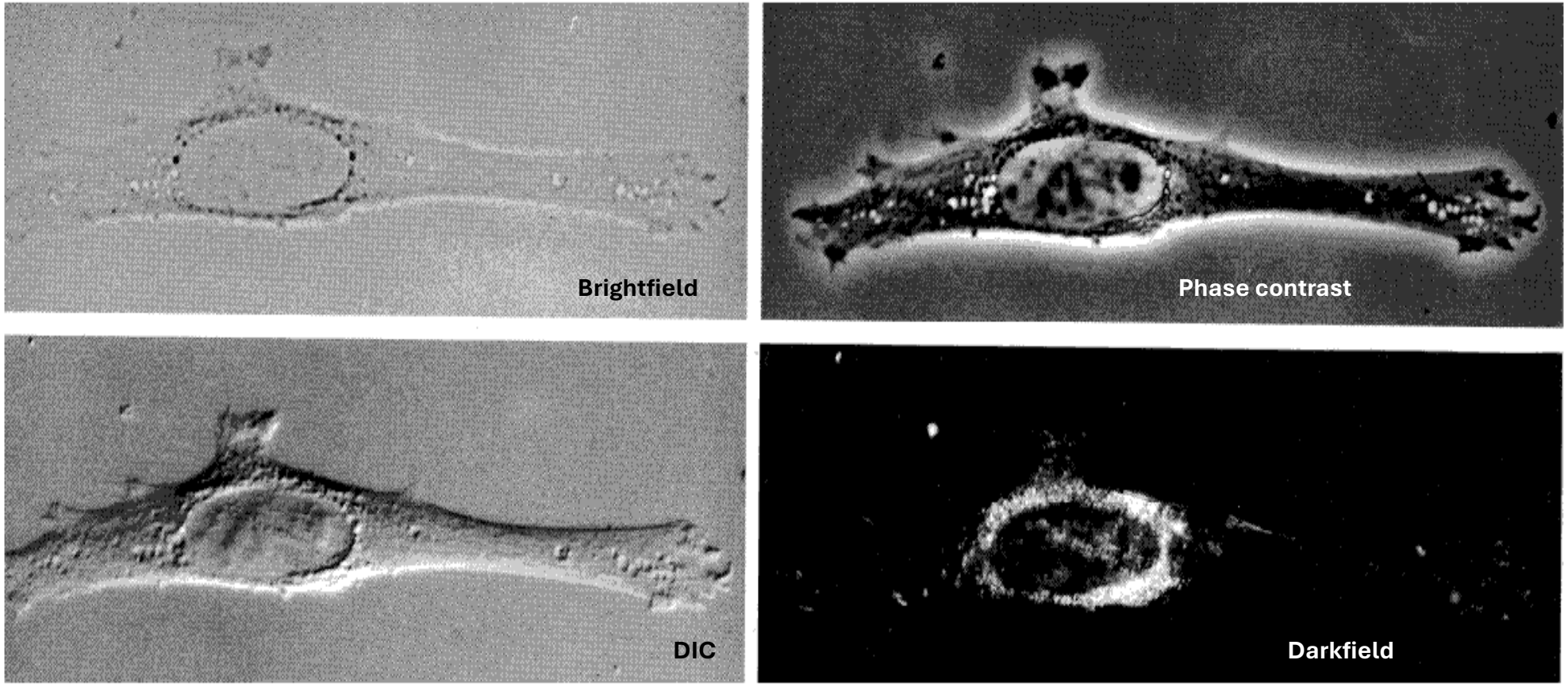
Refraction

Phase

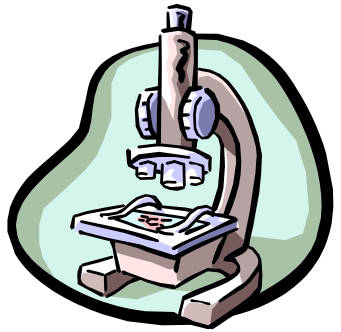
Polarization

# Contrasting techniques





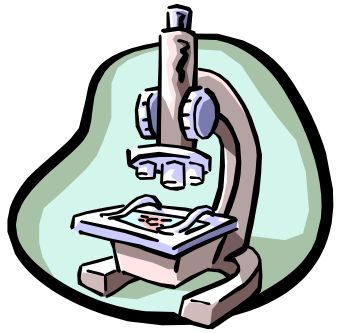

Taken from: http://fig.cox.miami.edu/~cmallery/150/Fallsyll.htm



# Contrasting techniques

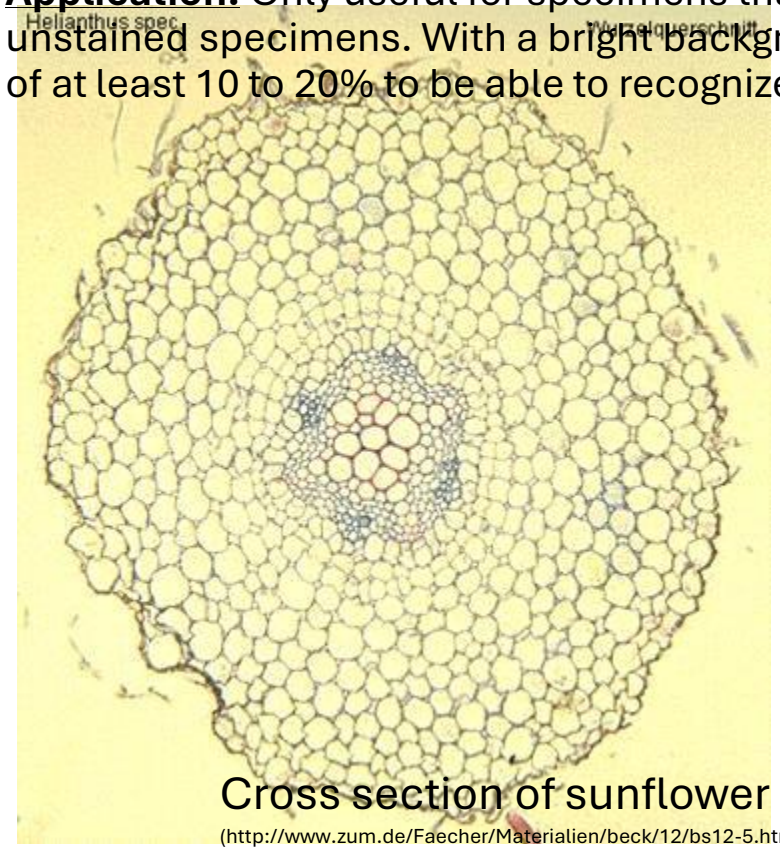
- Brightfield
- Darkfield
- Phase Contrast
- Polarization Contrast
- Differential Interference Contrast (DIC)

# Brightfield



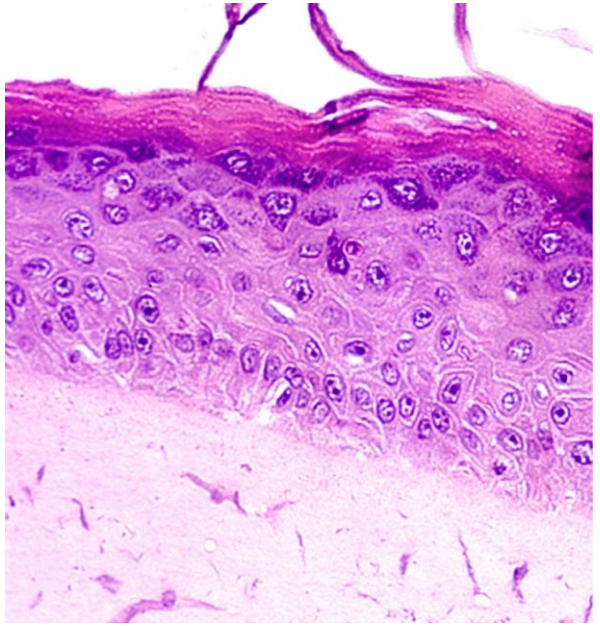
**Principle**: Light is transmitted through the sample and absorbed by it.

**Application:** Only useful for specimens that can be contrasted via dyes. Very little contrast in unstained specimens. With a bright background, the human eye requires local intensity fluctuations of at least 10 to 20% to be able to recognize objects.



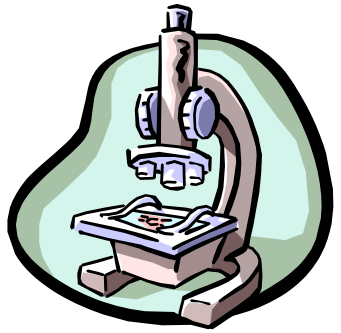

Cross section of sunflower root

(http://www.zum.de/Faecher/Materialien/beck/12/bs12-5.htm)

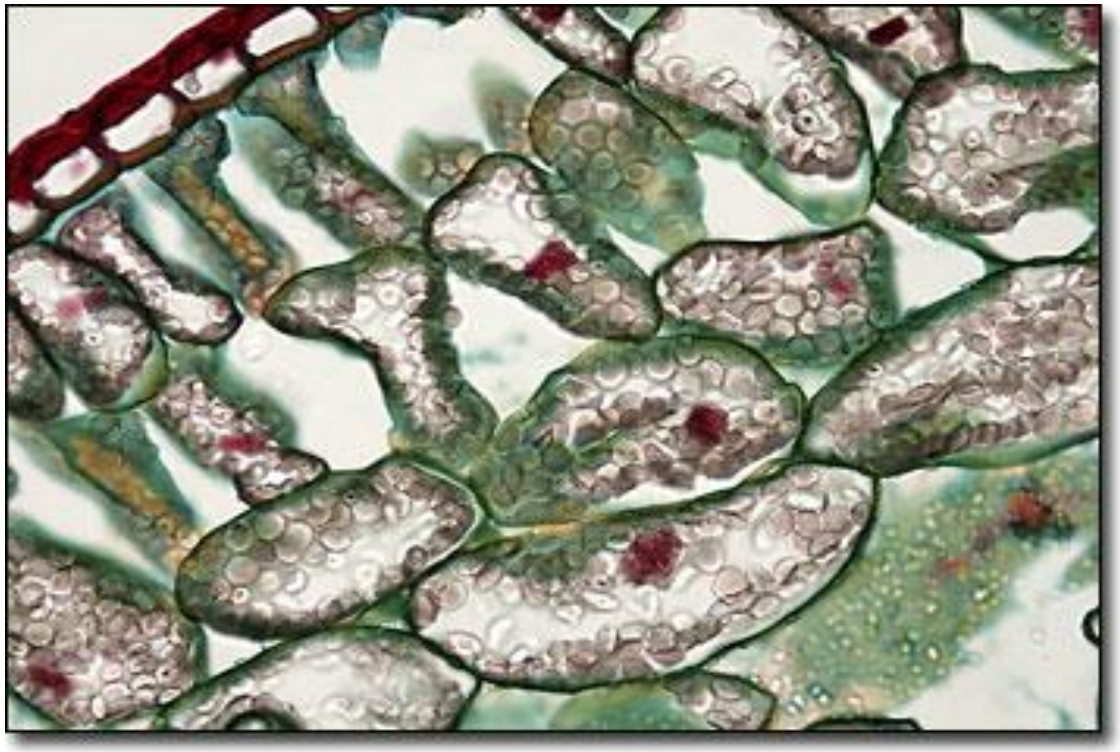


Piece of artificially grown skin

(www.igb.fhg.de/.../dt/PI_BioTechnica2001.dt.html)

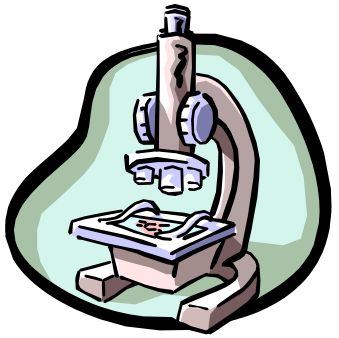


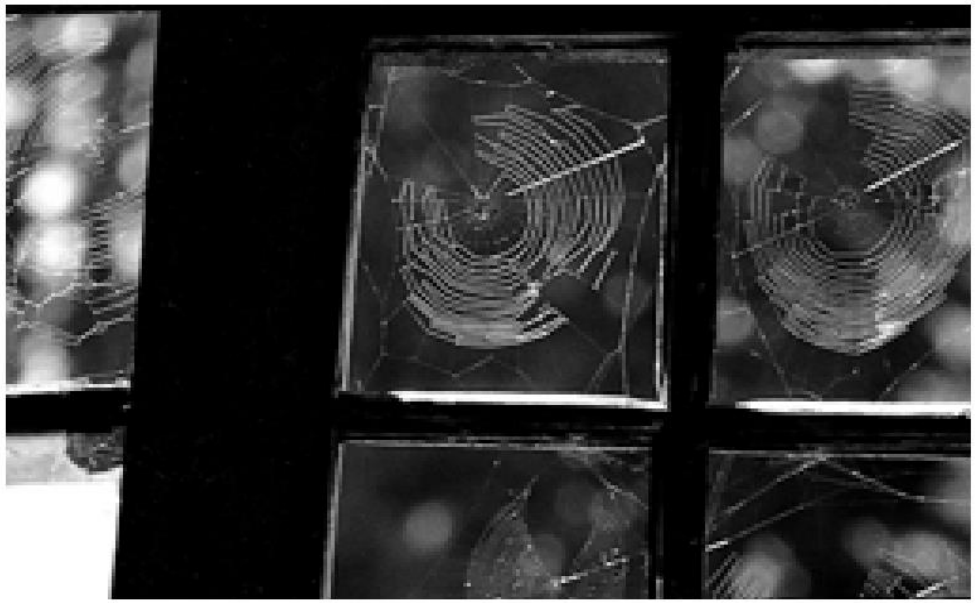
# Bright Field



- Bright Field is the most universal technique used in light microscope.
- Usually used in samples with colorimetric staining or good contrast.

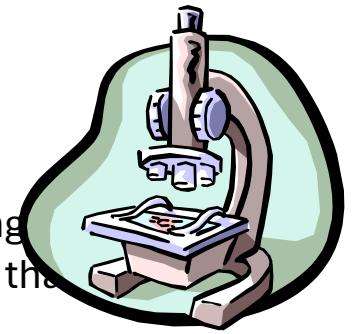
# Dark Field





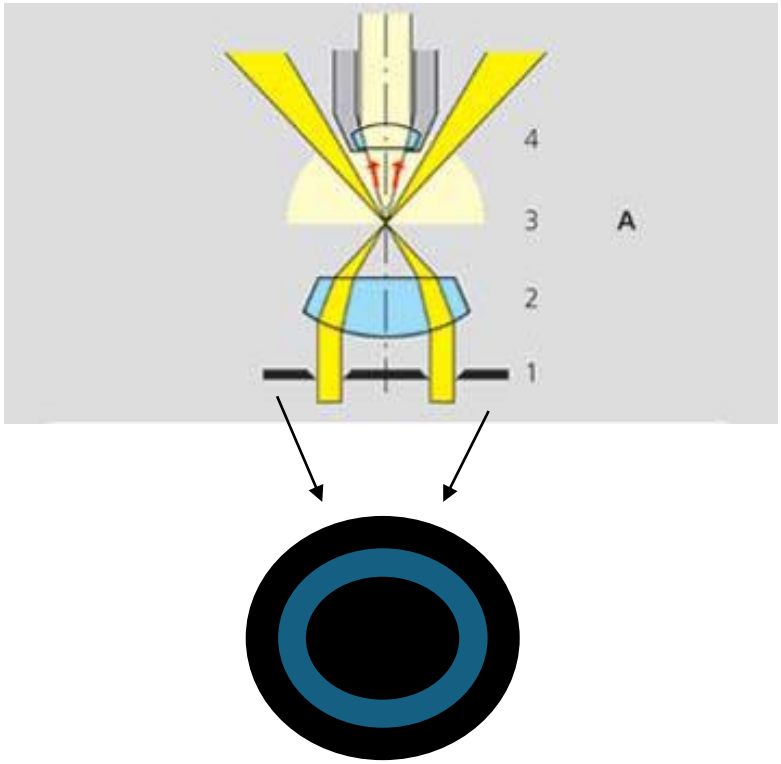
- Fine structures can often not be seen in front of a bright background.

# Darkfield

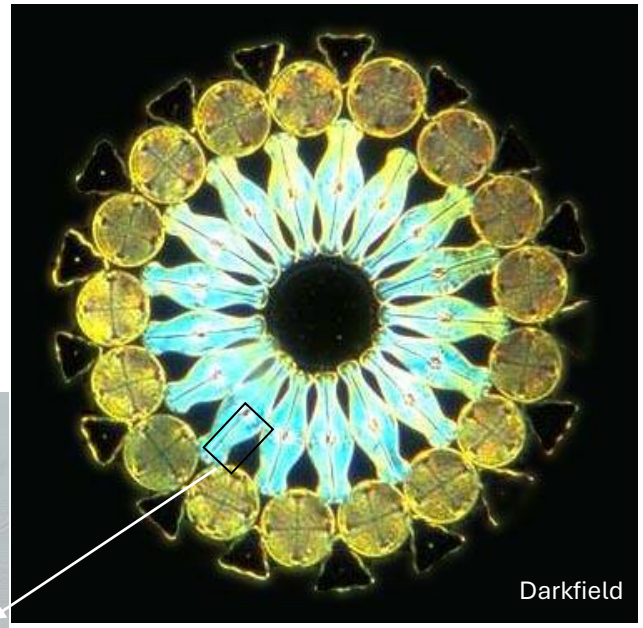


**Principle:** The illuminating rays of light are directed through the sample from the side by putting a dark disk into the condenser that hinders the main light beam to enter the objective. Only light that is scattered by structures in the sample enters the objective.

**Application**: People use it a lot to look at Diatoms and other unstained/colourless specimens







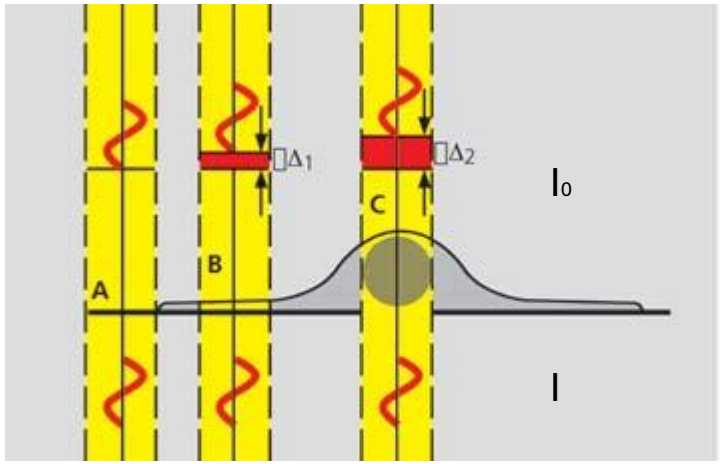

Symbiotic Diatom colony

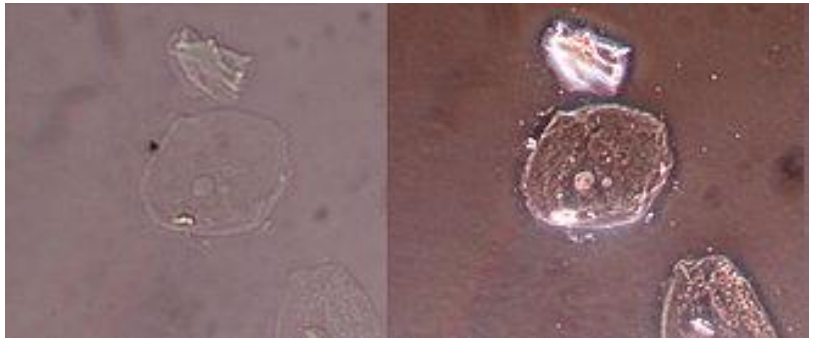
(www1.tip.nl/~t936927/making.html)

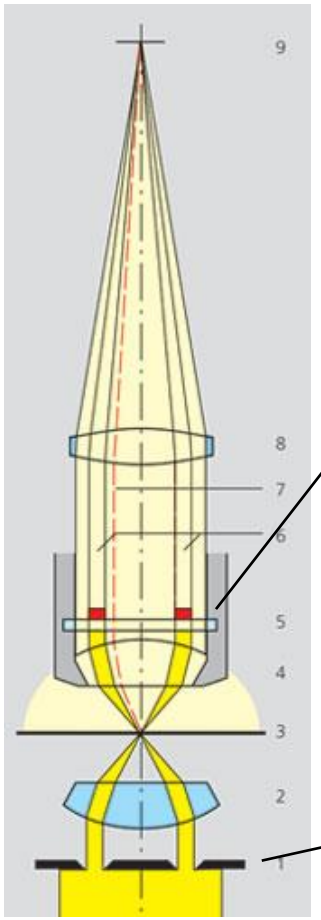
→ *we do not have microscopes set up for darkfield*

# Phase contrast in theory

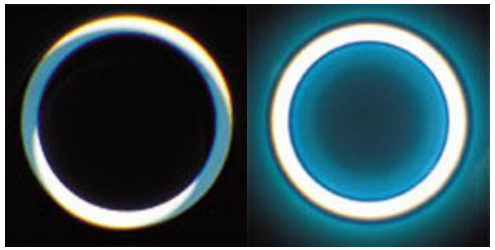
**Principle**: Incident light [Io] is out of phase with transmitted light [I] as it was slowed down while passing through different parts of the sample and when the phases of the light are synchronized by an interference lens, a new image with greater contrast is seen.





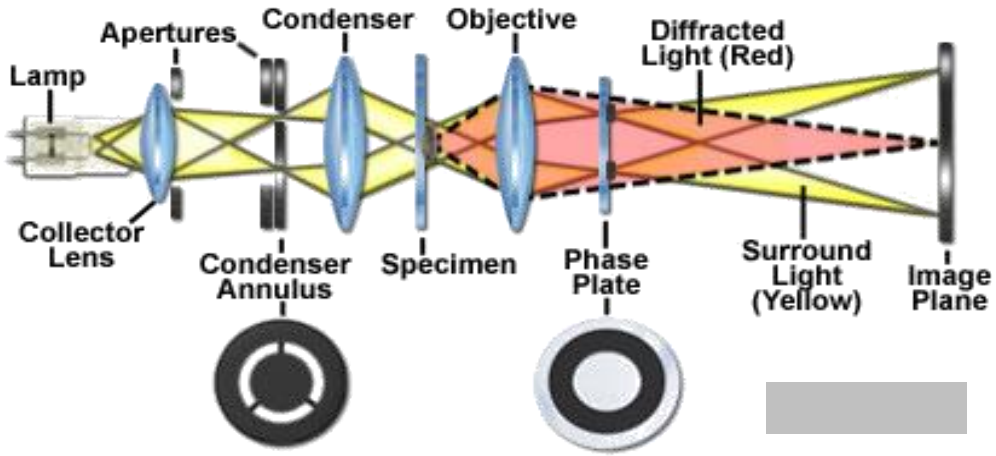

Phase ring

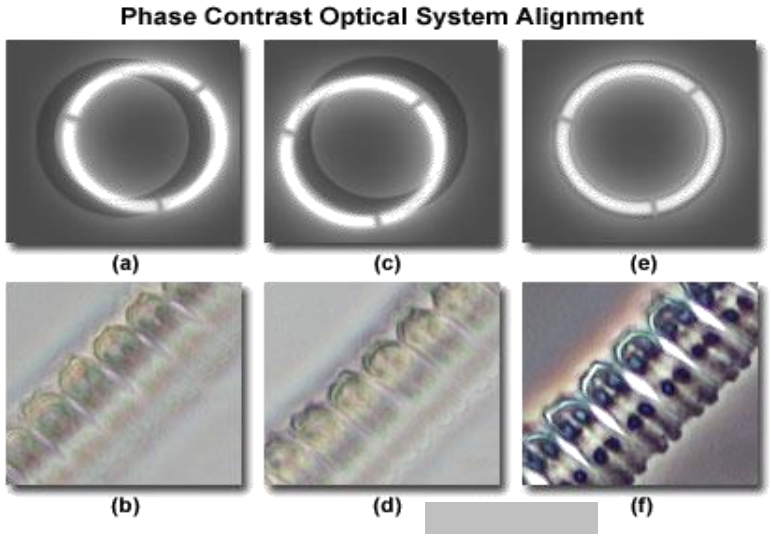


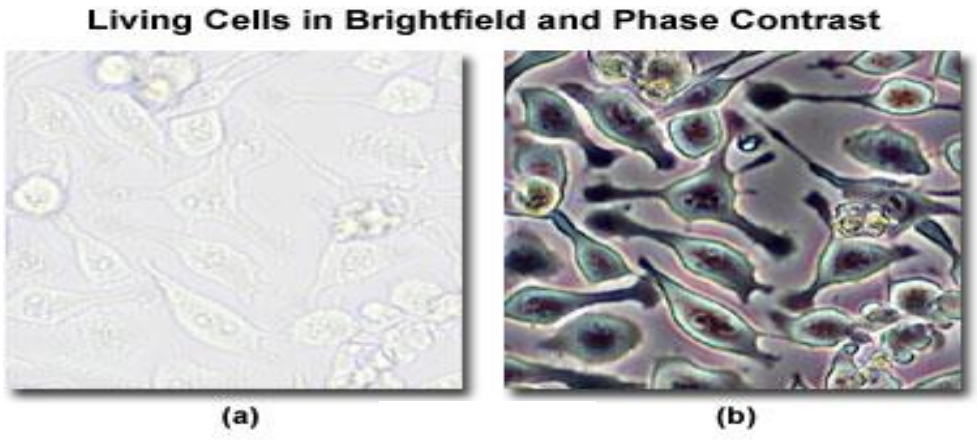
not aligned aligned

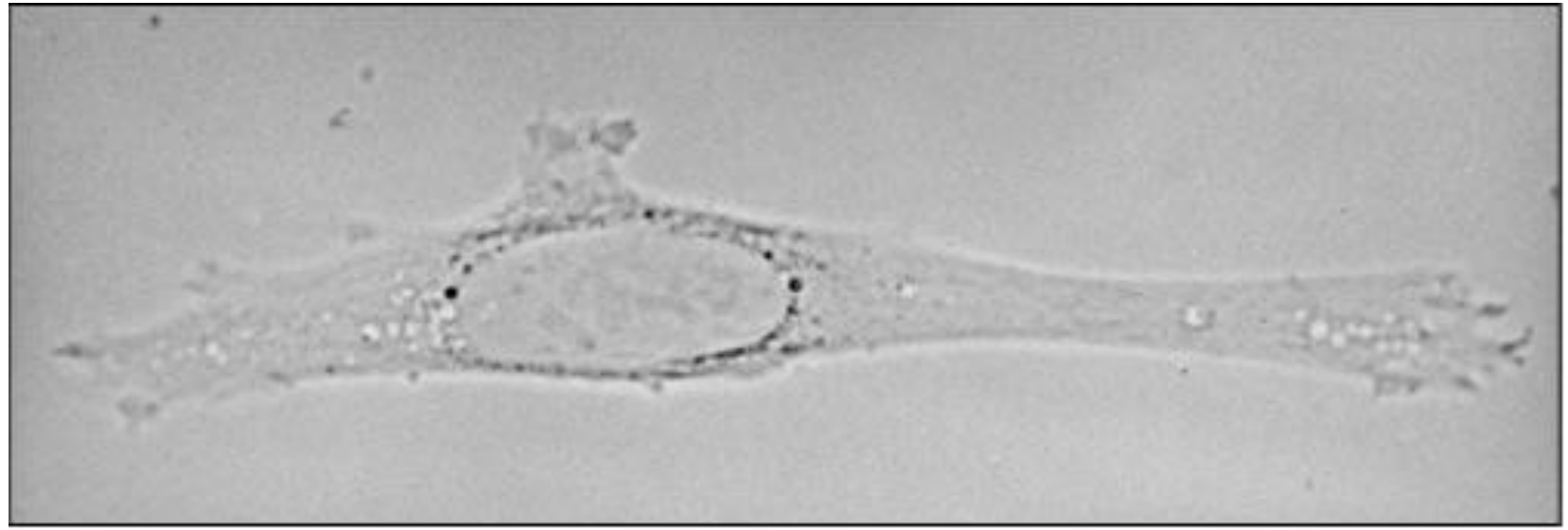
Phase stops

# Phase Contrast

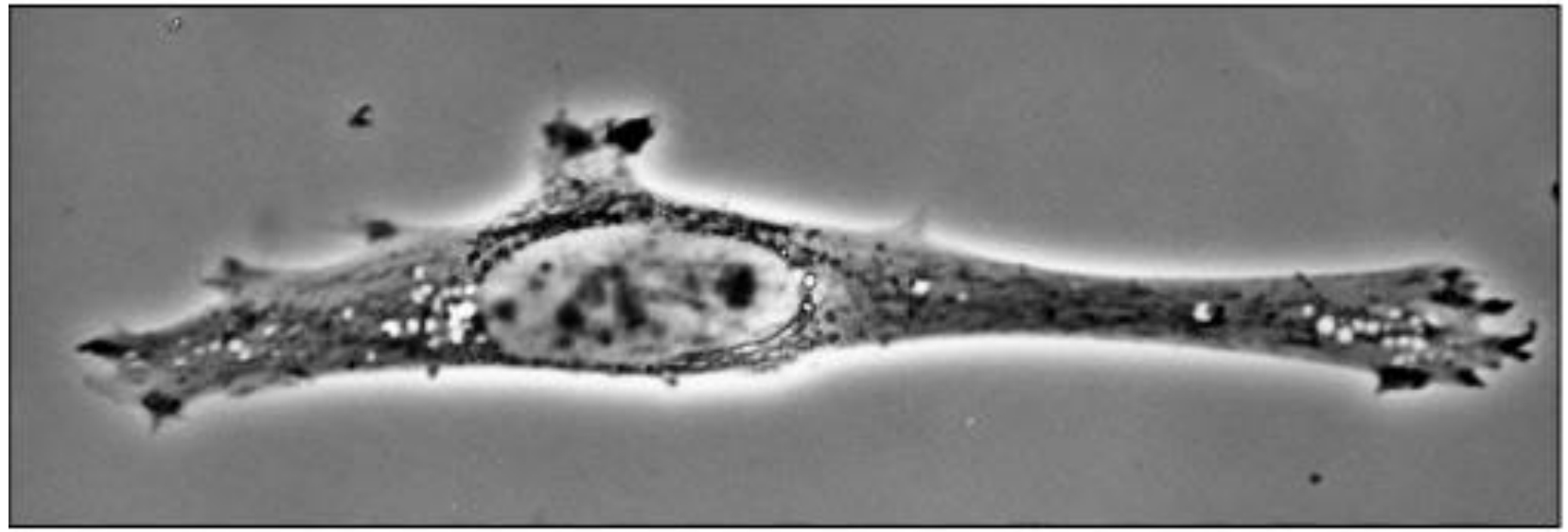




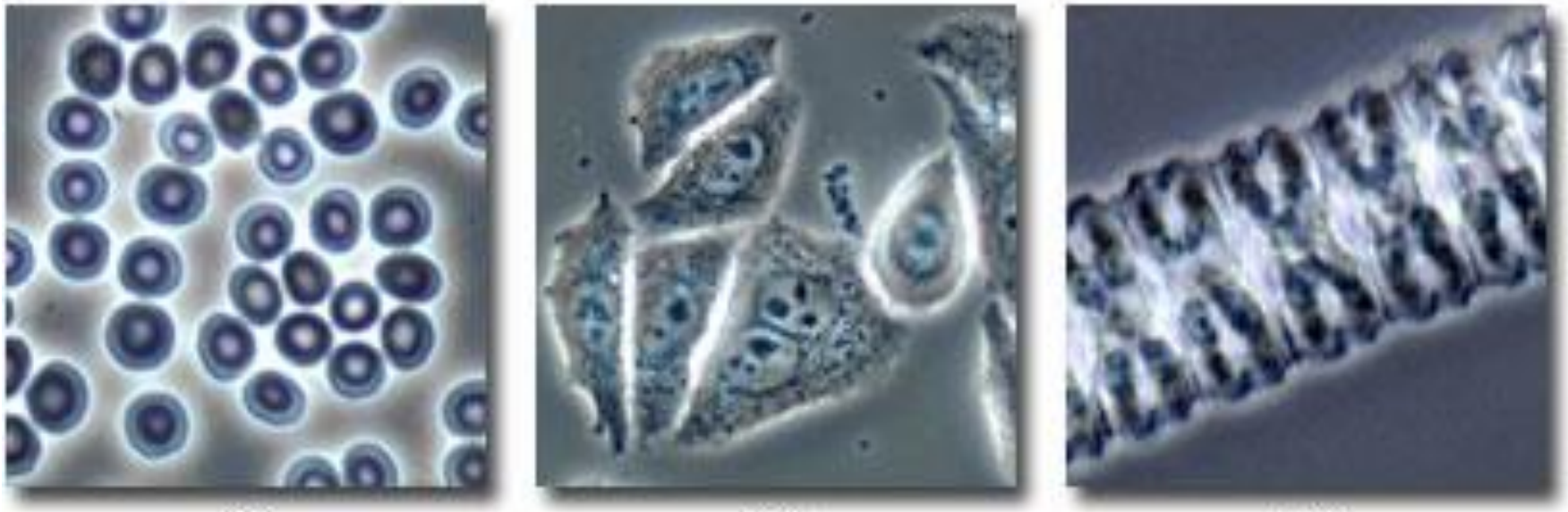
(A)



(B)

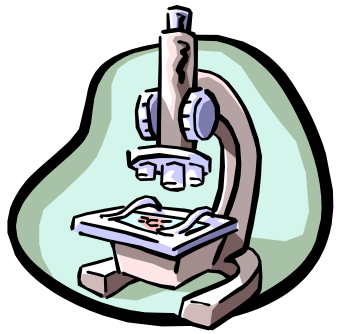
50 µm

## Halos in Phase Contrast Microscopy

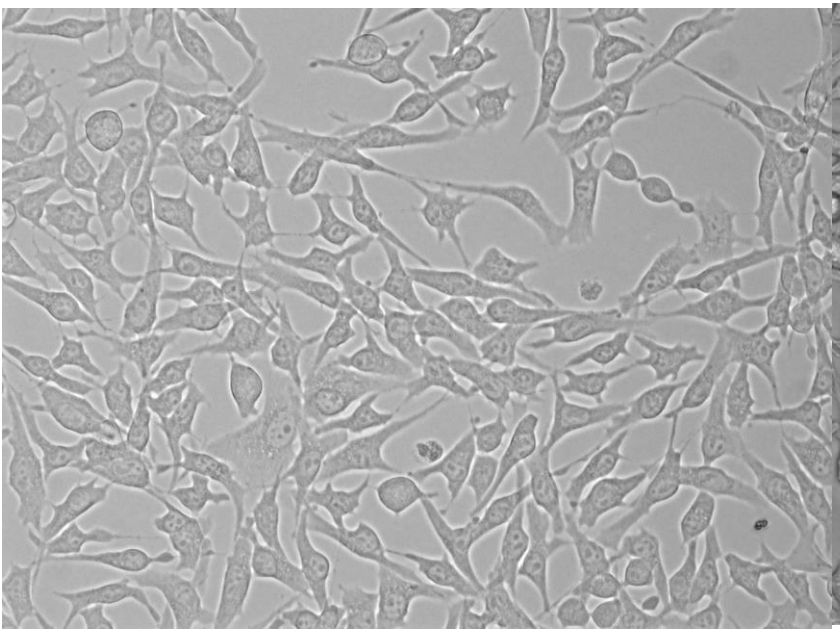


(a) (c) (e)

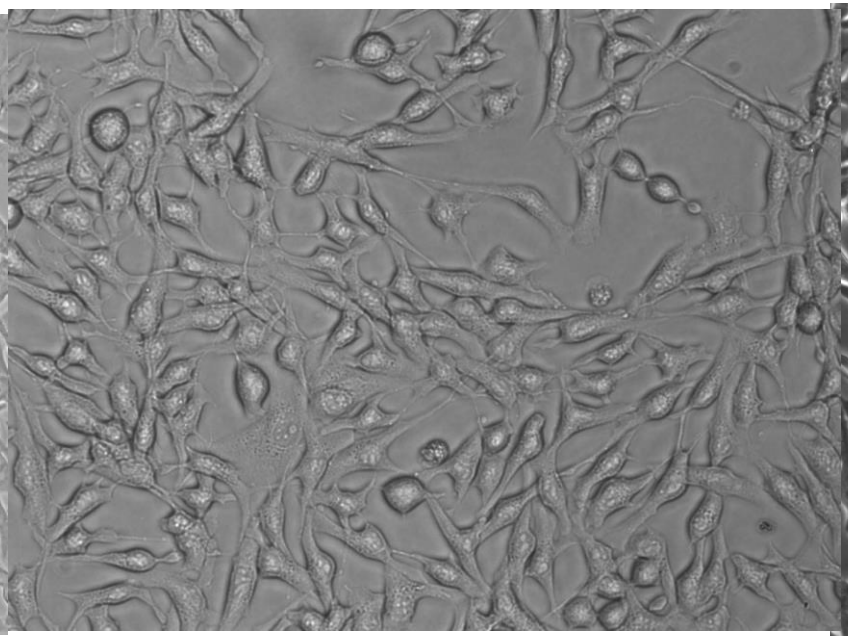
# Phase contrast in practice



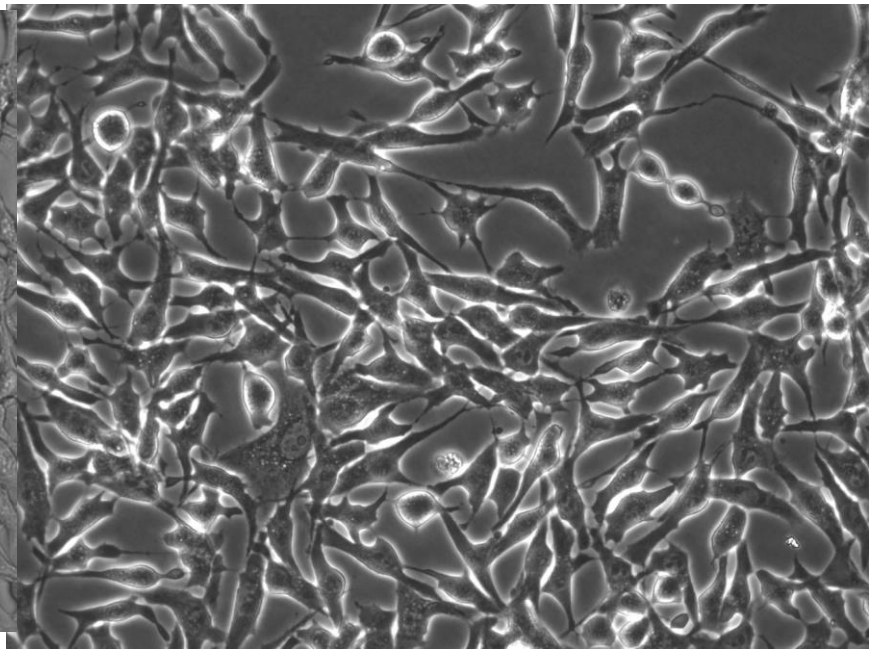
**Application**: Phase contrast is the most commonly used contrasting technique All tissue culture microscopes and the time-lapse microscopes are set up for phase.



**brightfield**



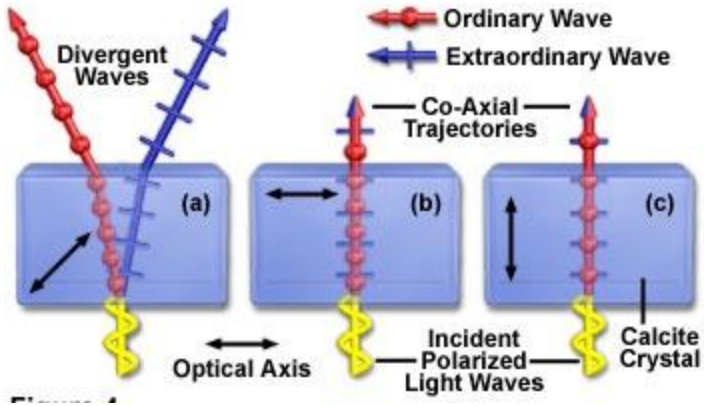
**wrong phase stop**



**right phase stop**

# DIC or Nomarsky

## Birefringence




Bi-Refraction in Calcite Crystals

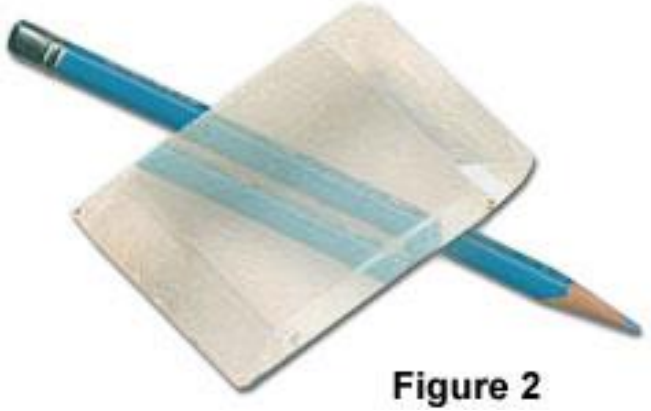
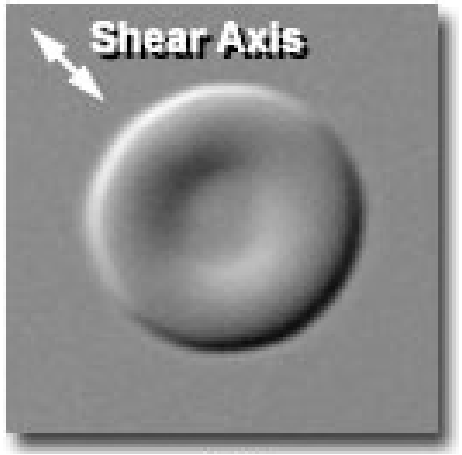


Figure 2

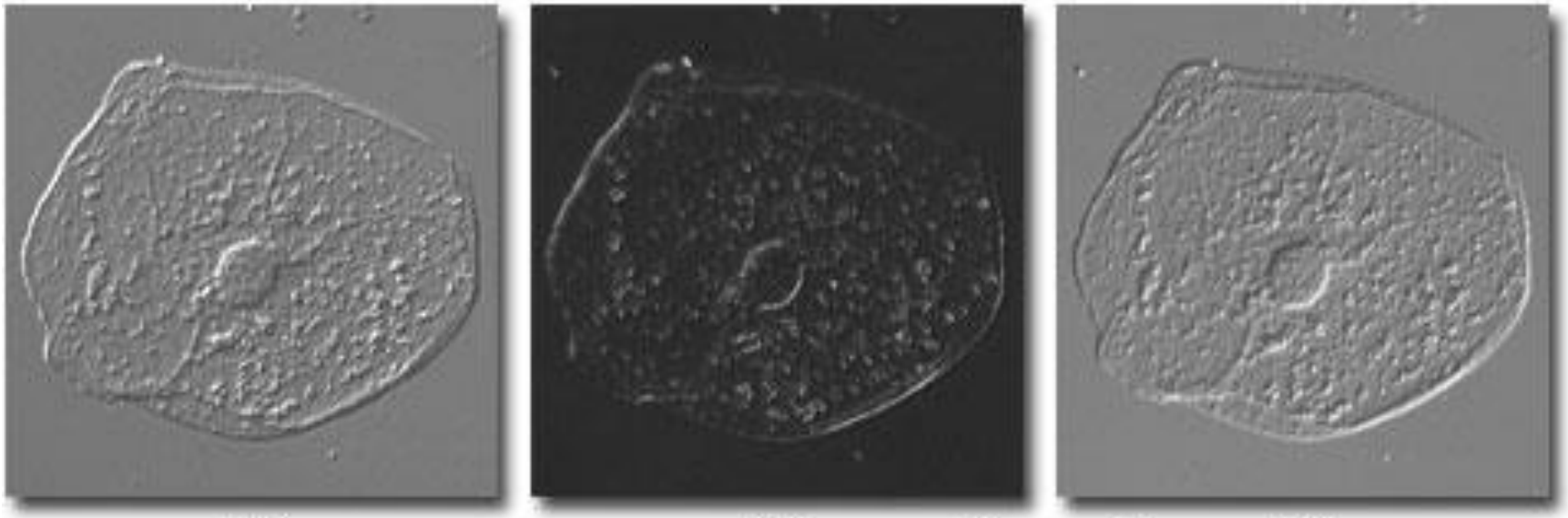
- Birefringent materials have different indices of refraction for light polarized parallel or perpendicular to the optical axis.
- Two beams with orthogonal polarization are produced if illumination is at an angle to optical axis

# Features of a DIC image




1. Contrast is directional
2. Contrast highlights edges
3. One end brighter, other is dimmer giving a pseudo – 3D image

## Positive and Negative Bias in Differential Interference Contrast



(a) (b) (c)

Figure 8

## Effect of Specimen Orientation on DIC Images

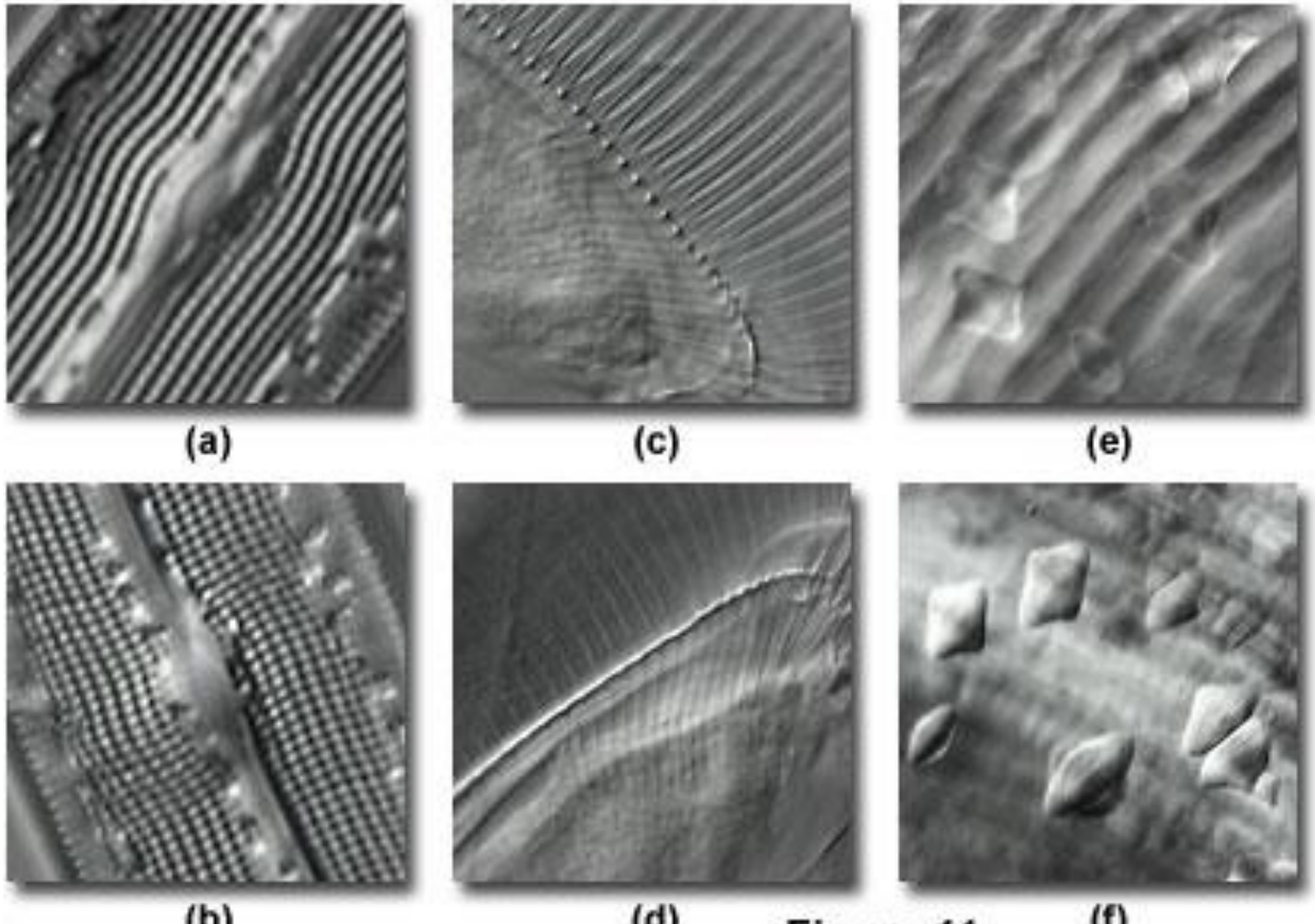
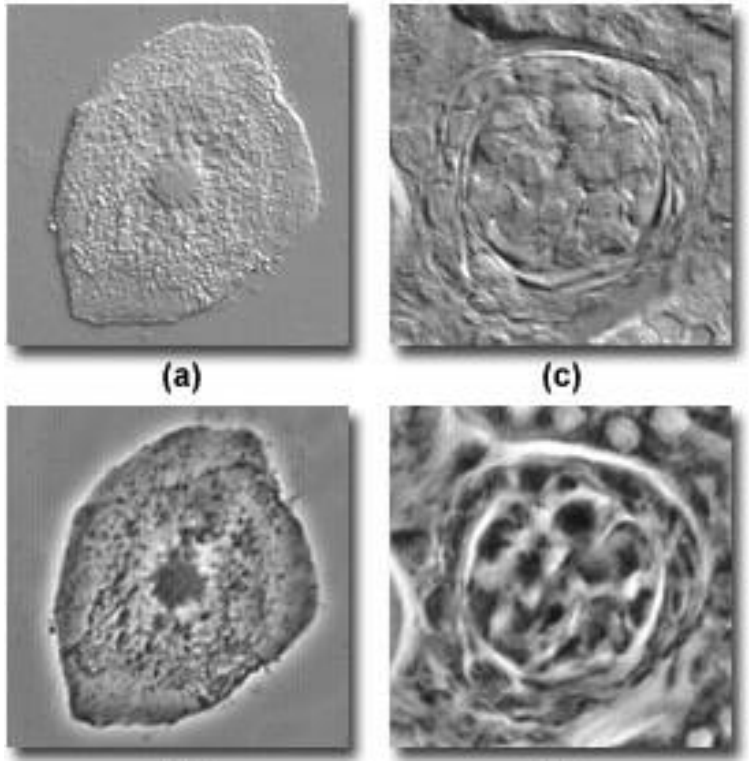


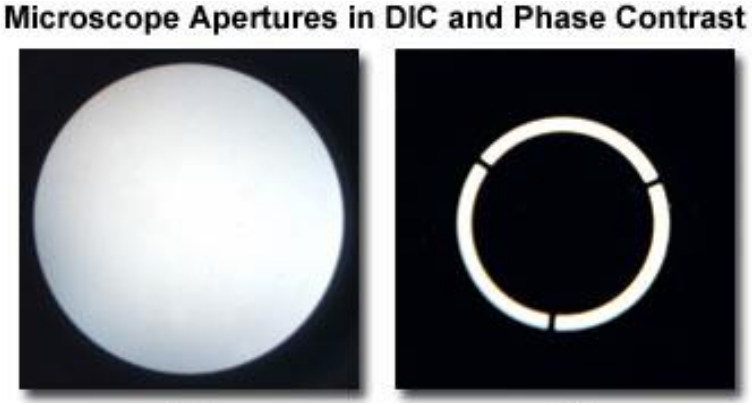

Figure 11

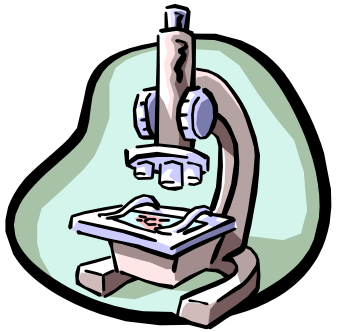
# DIC is higher resolution than phase contrast

DIC

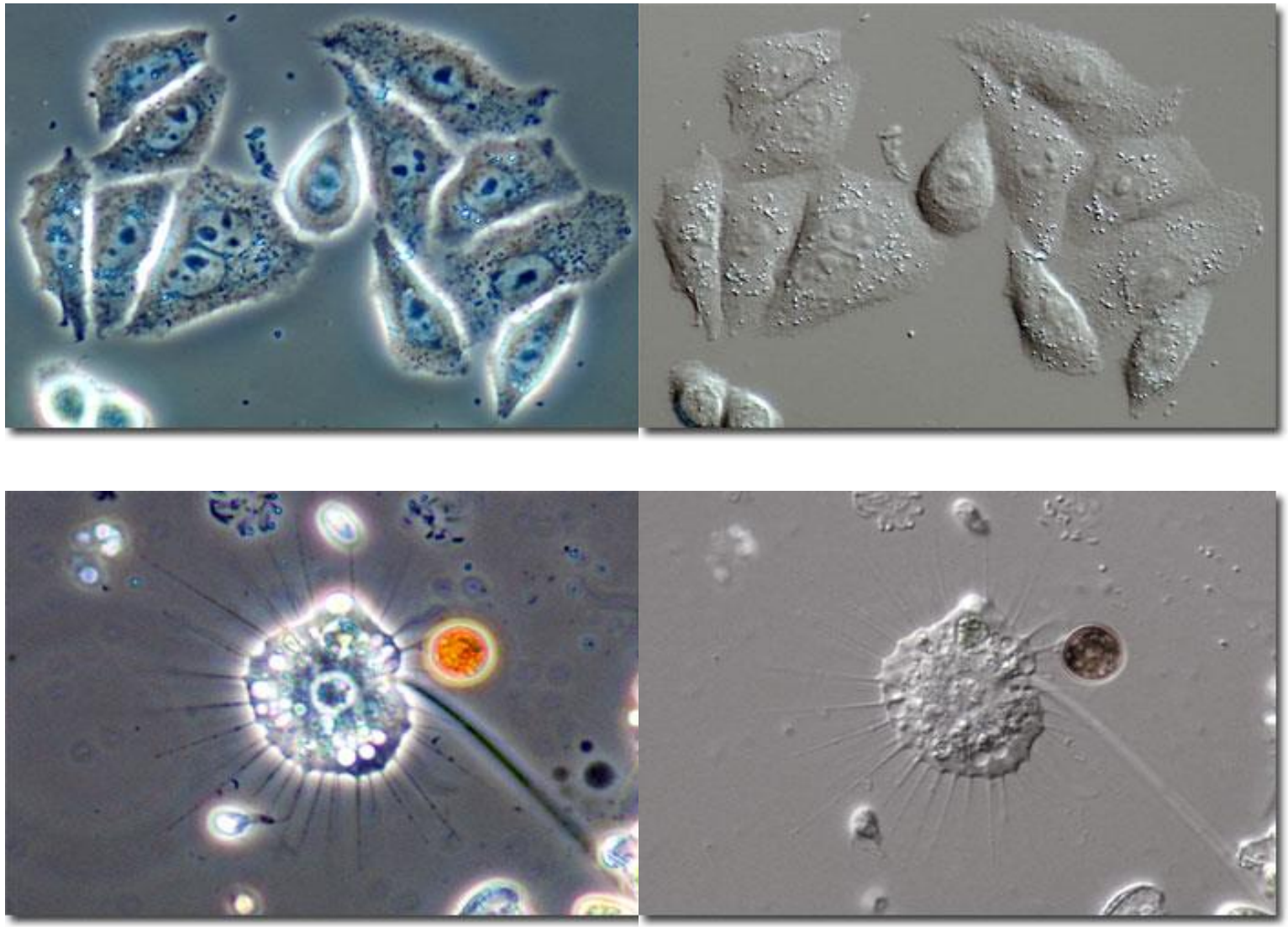
Phase





HeLa Cell Culture



Heliozoans (*Actinophrys sol*)

## Halos in Phase Contrast and DIC Microscopy

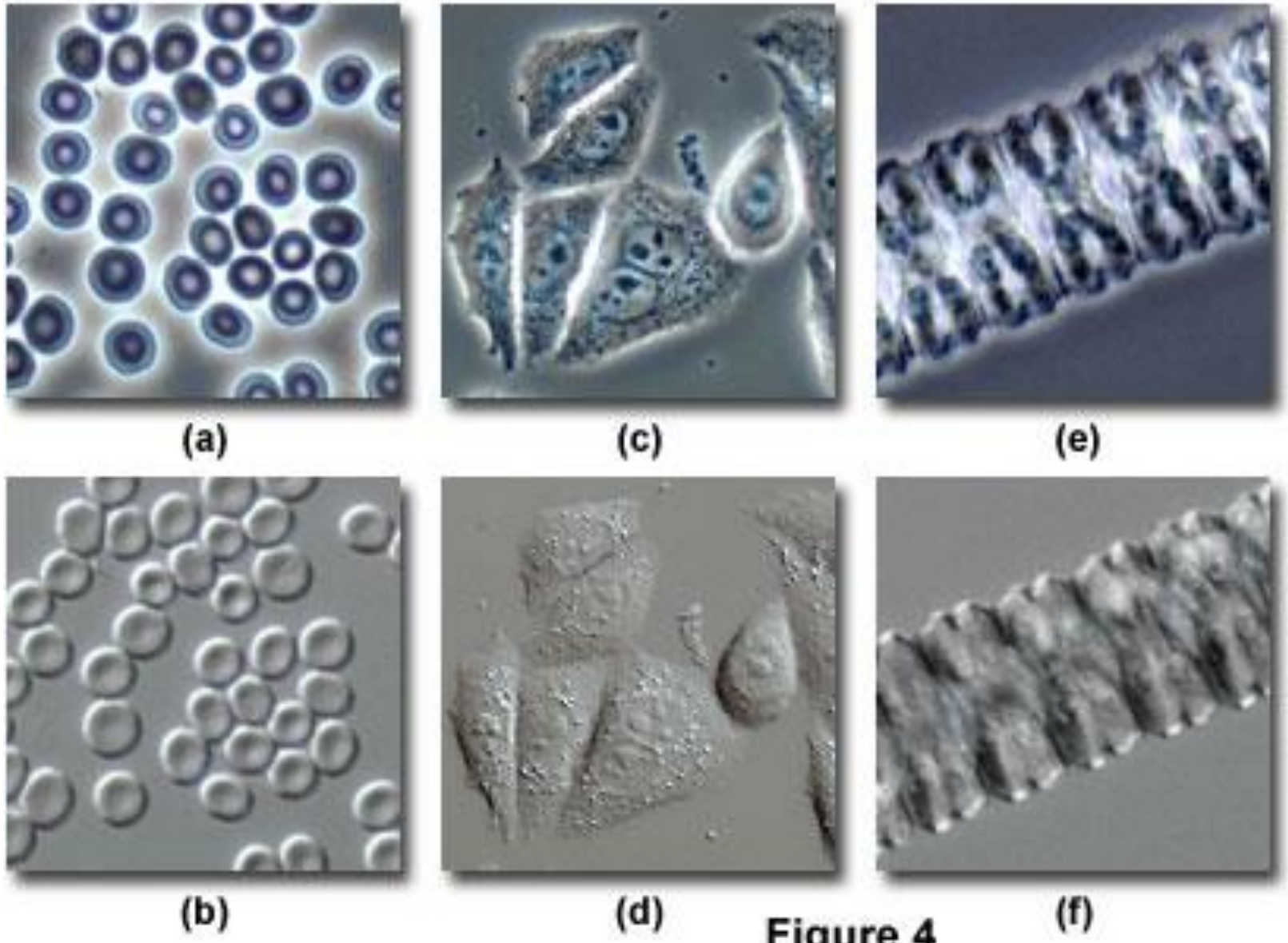



**Figure 4**

## Transparent Specimens in Phase Contrast and DIC

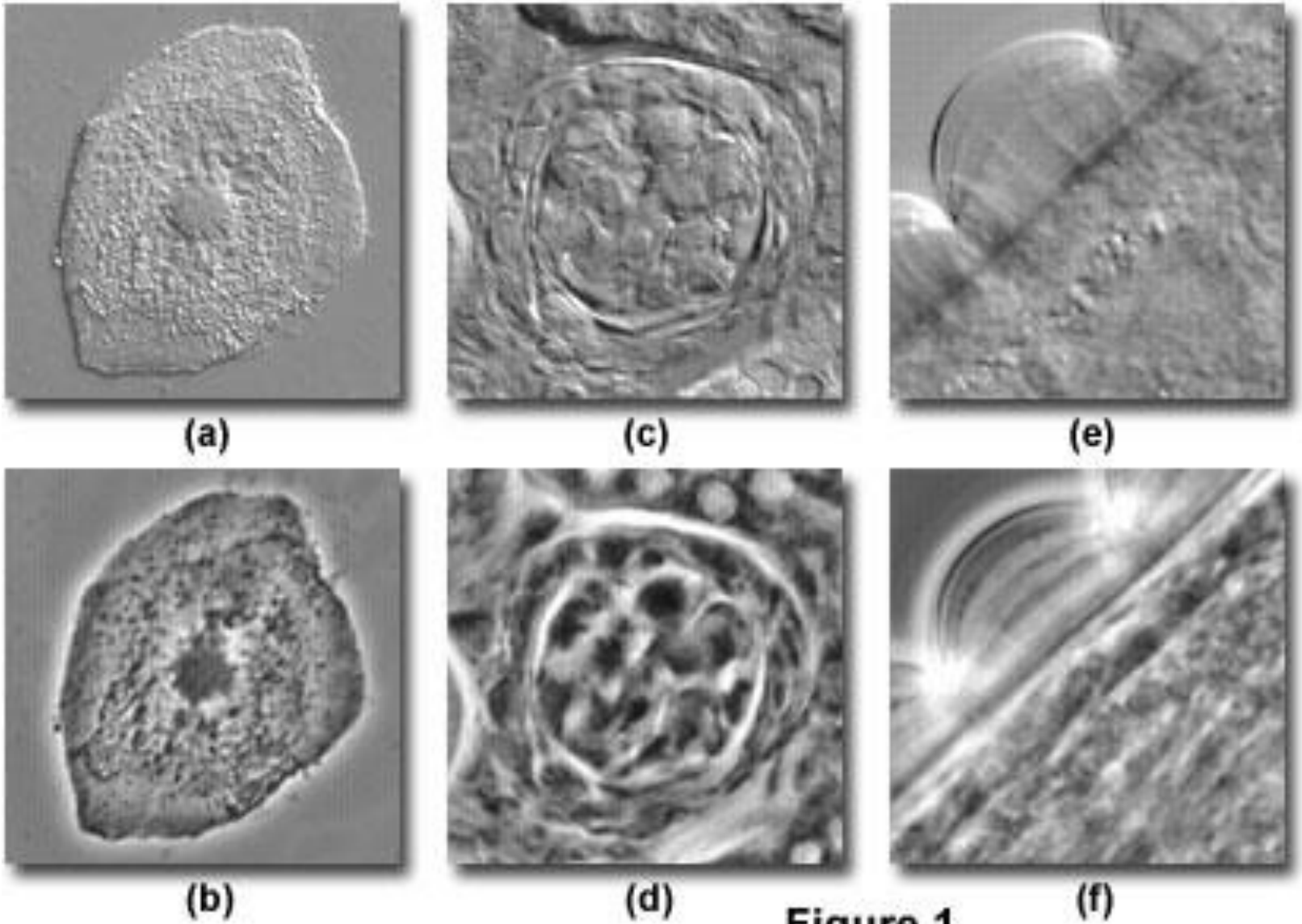



Figure 1

## Birefringent Specimens in Phase Contrast and DIC

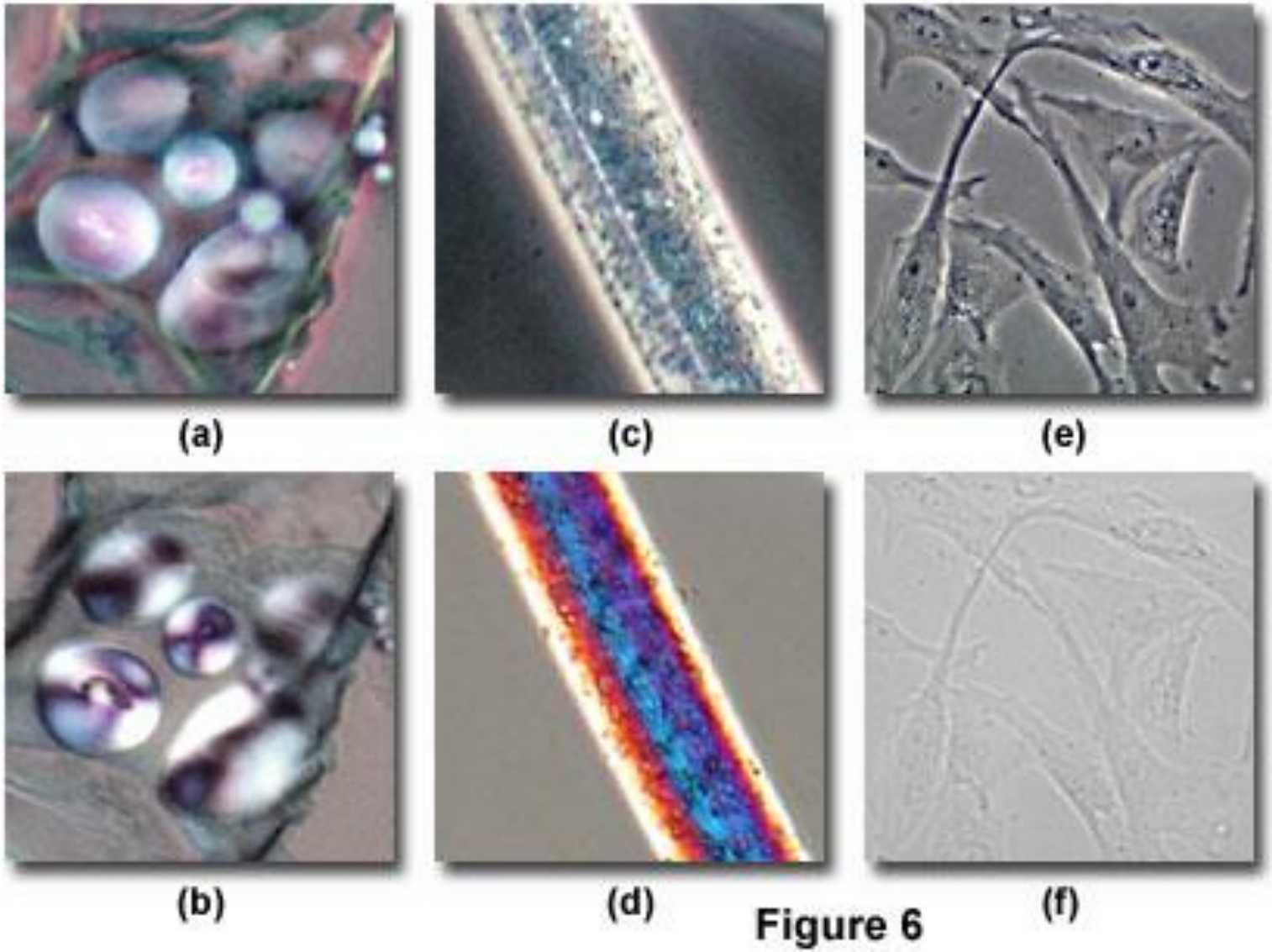
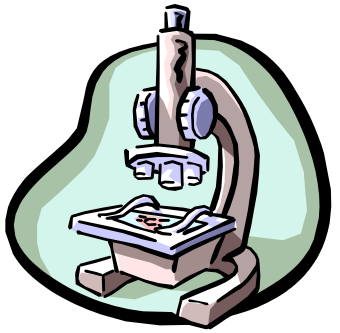



Figure 6

# Contrasting techniques - a summary



- Brightfield -absorption

  Light is transmitted through the sample. Only useful for specimens that can be contrasted via dyes. Very little contrast in unstained specimens.

- Darkfield -scattering

  The illuminating rays of light are directed from the side so that only scattered light enters the microscope lenses, consequently the cell appears as an illuminated object against the view.

- Phase Contrast- phase interference

  Incident light [Io] is out of phase with transmitted light [I] and when the phases of the light are synchronized by an interference lens, a new image with greater contrast is seen

- Polarization Contrast -polarization

  Uses polarized light for illumination. Only when the vibration direction of the polarized light is altered by a sample placed into the light path, light can pass through the analyzer. The sample appears light against a black background.

- Differential Interference Contrast (DIC) – polarization + phase interference

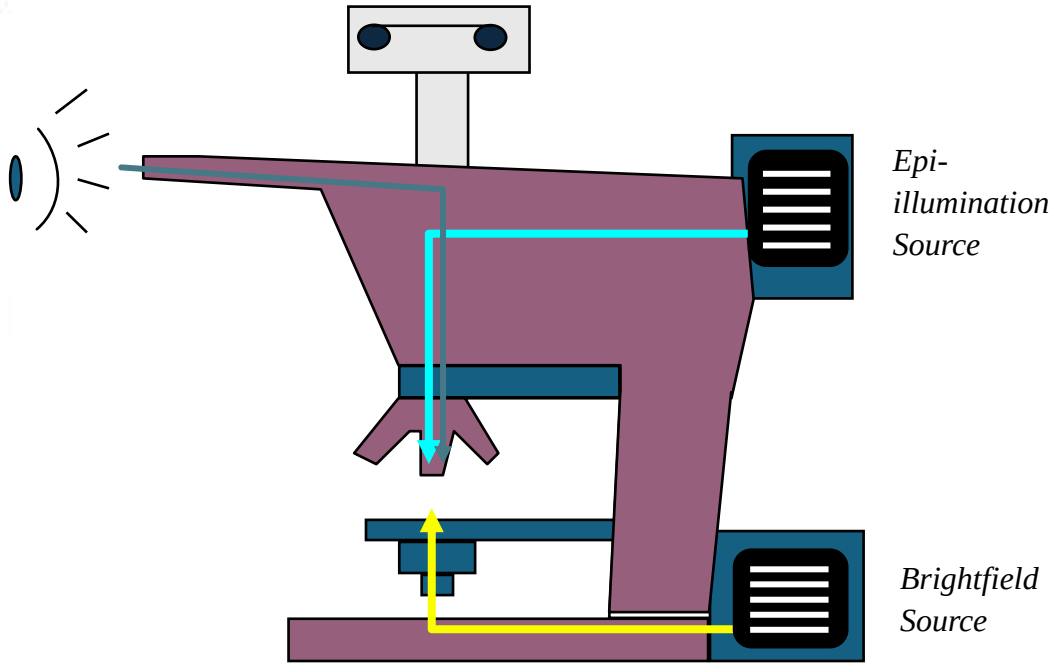
  Also known as Nomarski microscopy. Synchronizing of the different phases of incident and transmitted light is done by a set of special condenser lens mounted below the stage of a microscope

- Fluorescence Contrast (->Ireen)

# Upright Scope

Nikon Eclipse E600
with U-III Film
Camera System
(circa early 1990s)

*Image from Nikon promotional materials*



# Inverted Microscope

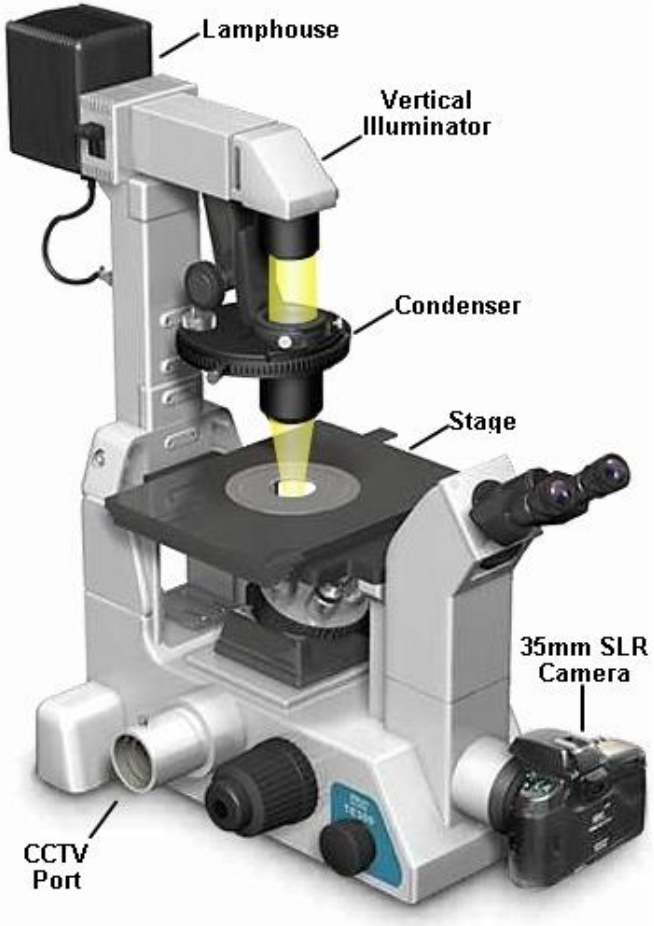
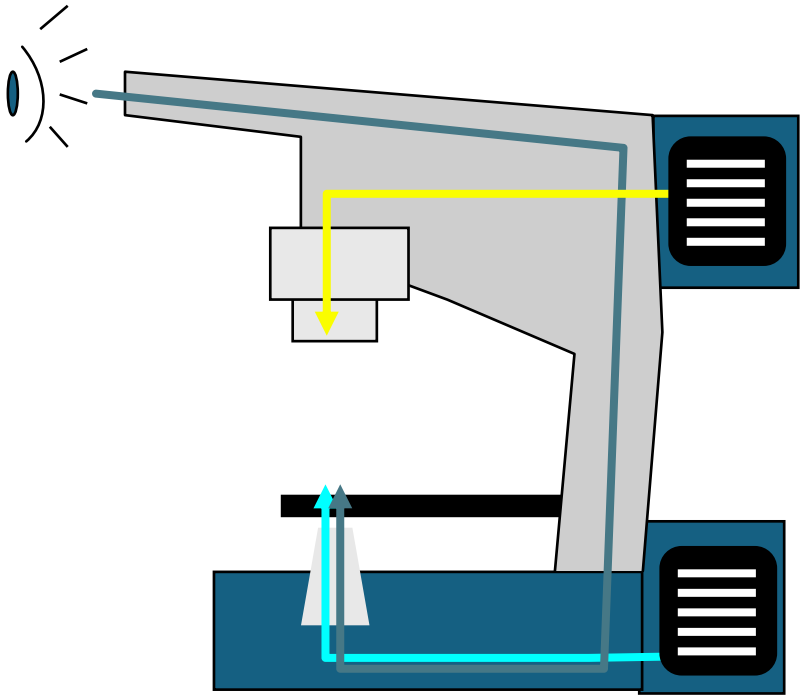
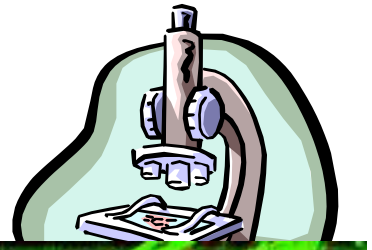



*Image from Nikon promotional materials*

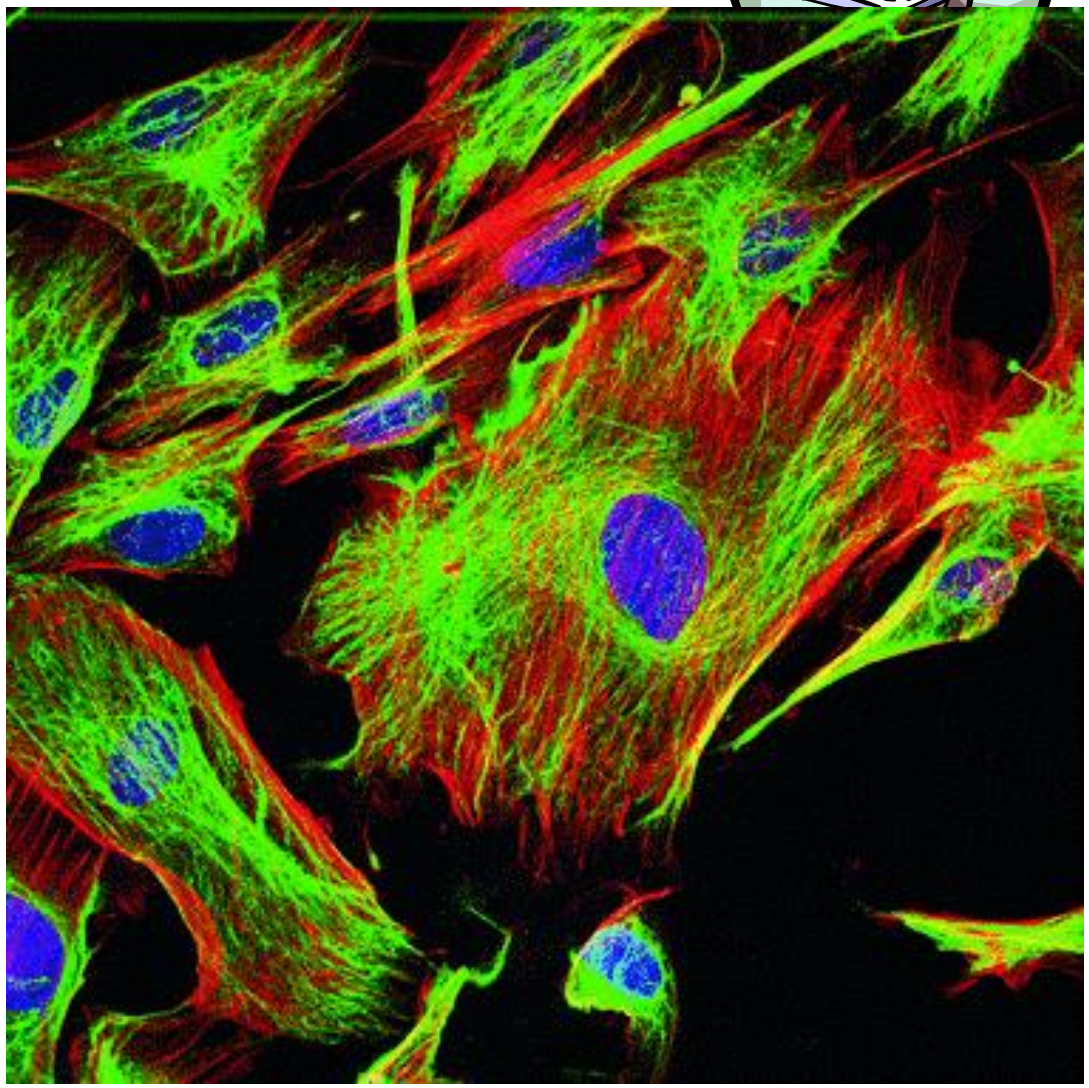


# Why fluorescence?

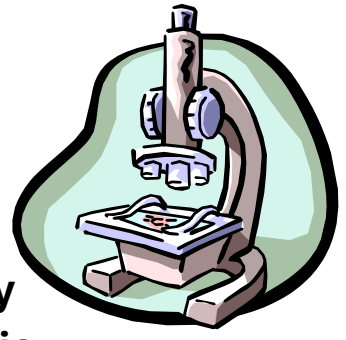


High resolution

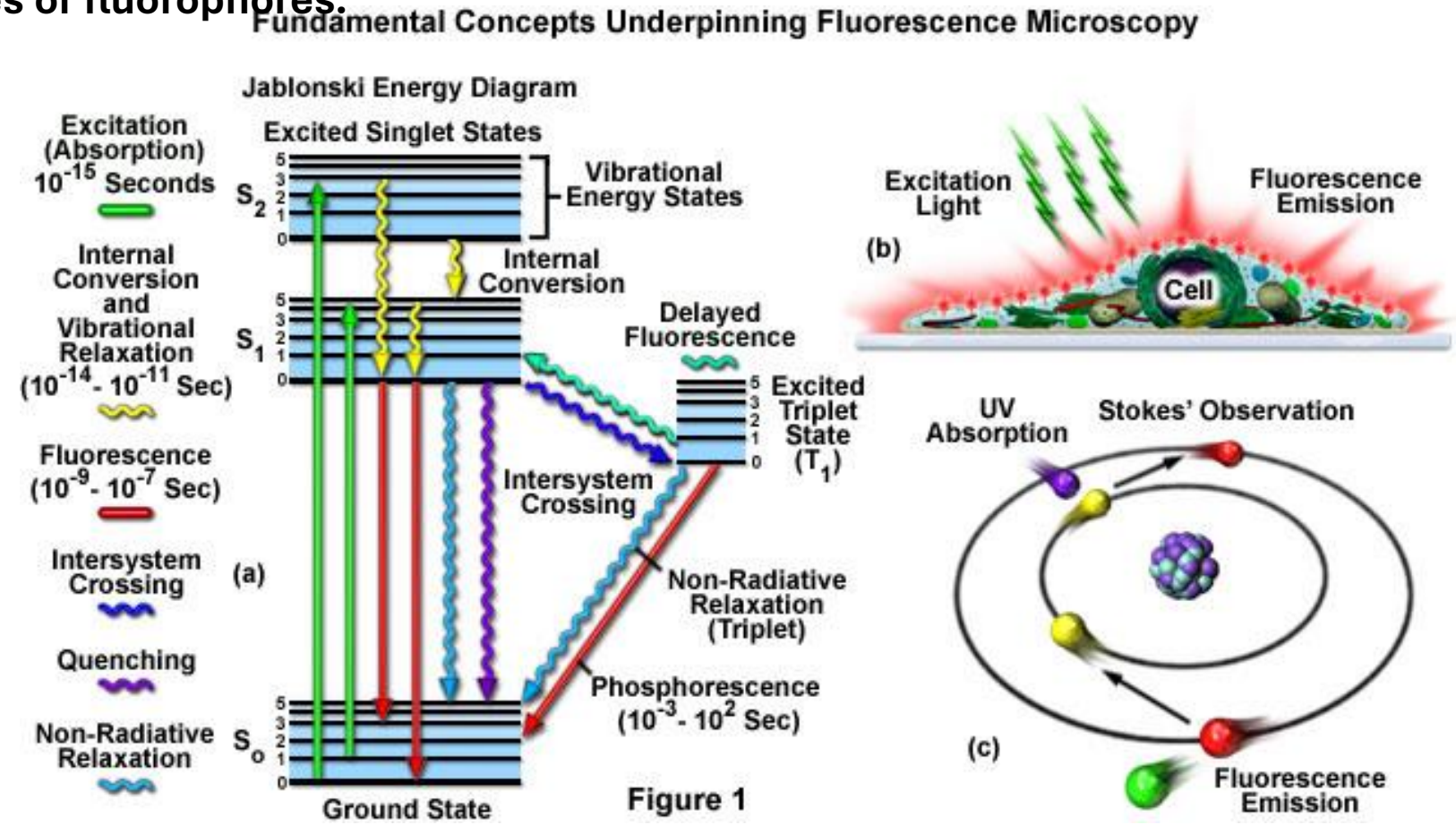
**High contrast**
**High specificity**
**Quantitative**
**Live Cell Imaging**



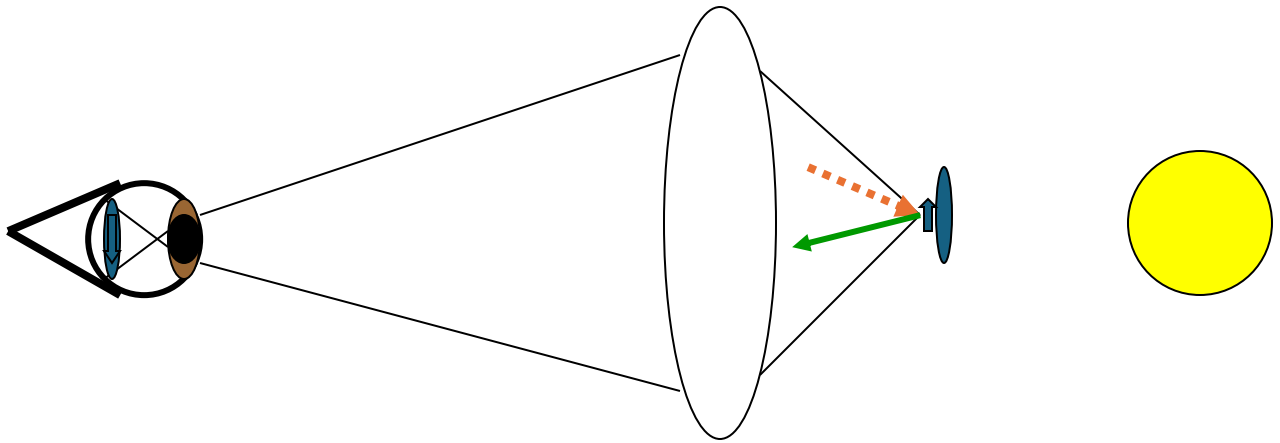
# What is Fluorescence ?



**Molecules absorbing the energy of electromagnetic radiation will jump to a higher energy level. When certain excited molecules return to the ground state they emit radiation. This phenomenon is known as fluorescence. Fluorescent molecules are known as fluorochromes or fluorophores.**



# Transmittance is subtractive while fluorescence is additive



# Fluorescence Principle

- When certain compounds are illuminated with high energy light, they then emit light of a different, lower frequency. This effect is known as **fluorescence**.
- Often specimens show their own characteristic autofluorescence image, based on their chemical makeup.
- The key feature of fluorescence microscopy is that it employs reflected rather than transmitted light, which means transmitted light techniques such as **phase contrast** and **DIC** can be combined with fluorescence microscopy.

# Fluorescence

The **longer** the wavelength the **lower** the energy

The **shorter** the wavelength the **higher** the energy
e.g. UV light from sun causes the sunburn
not the red visible light

# Working in greater detail

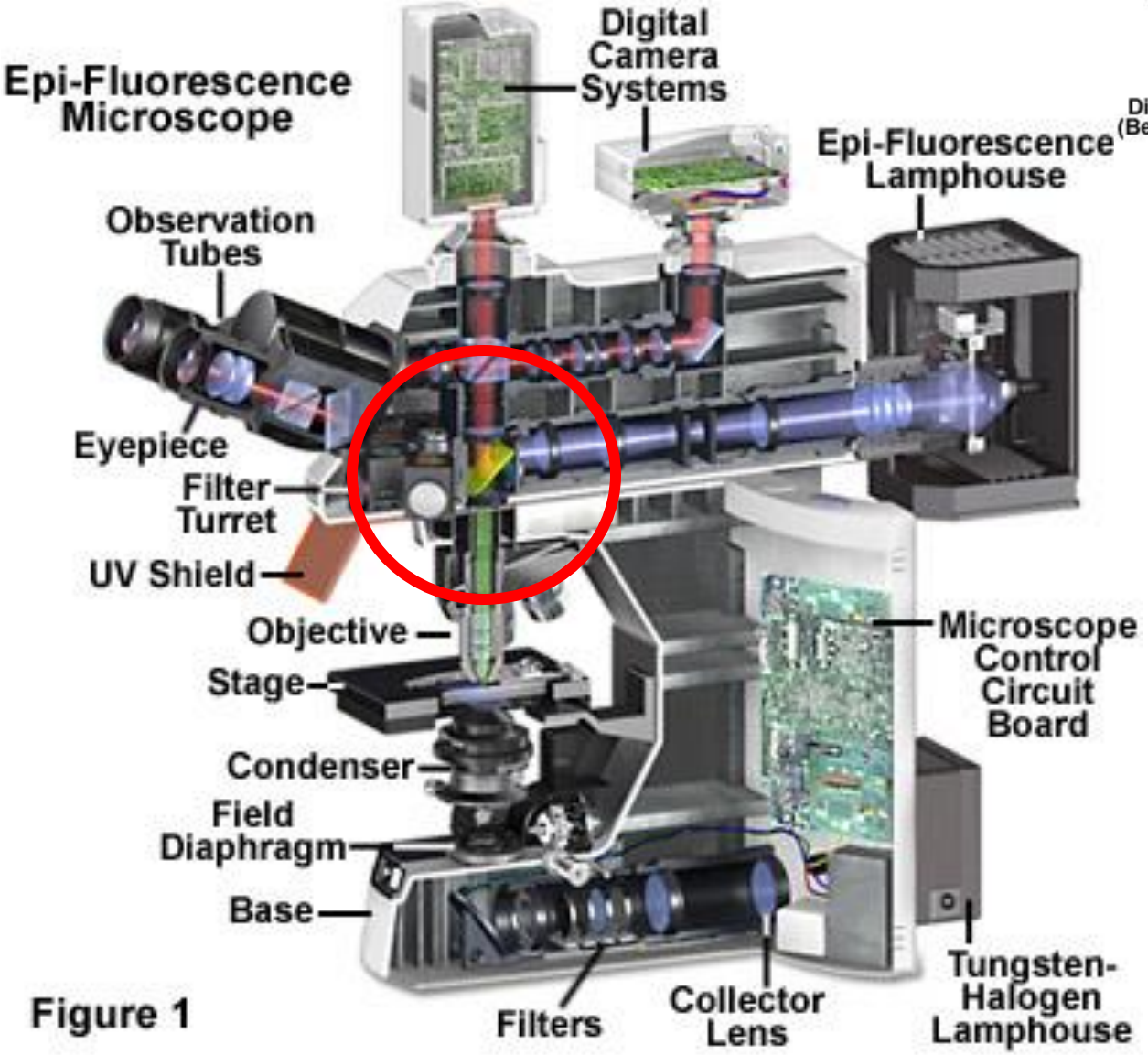



Figure 1

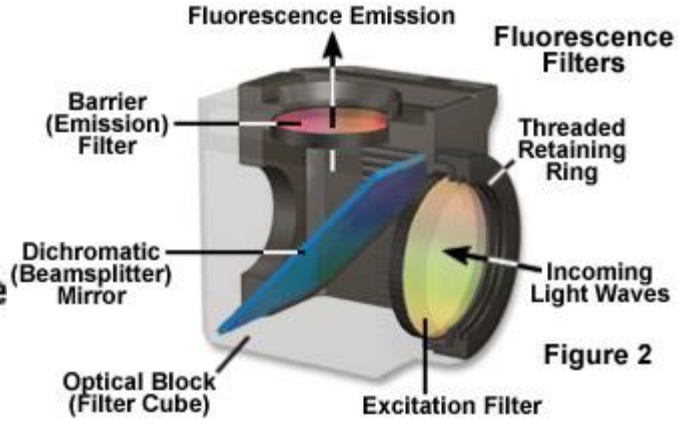
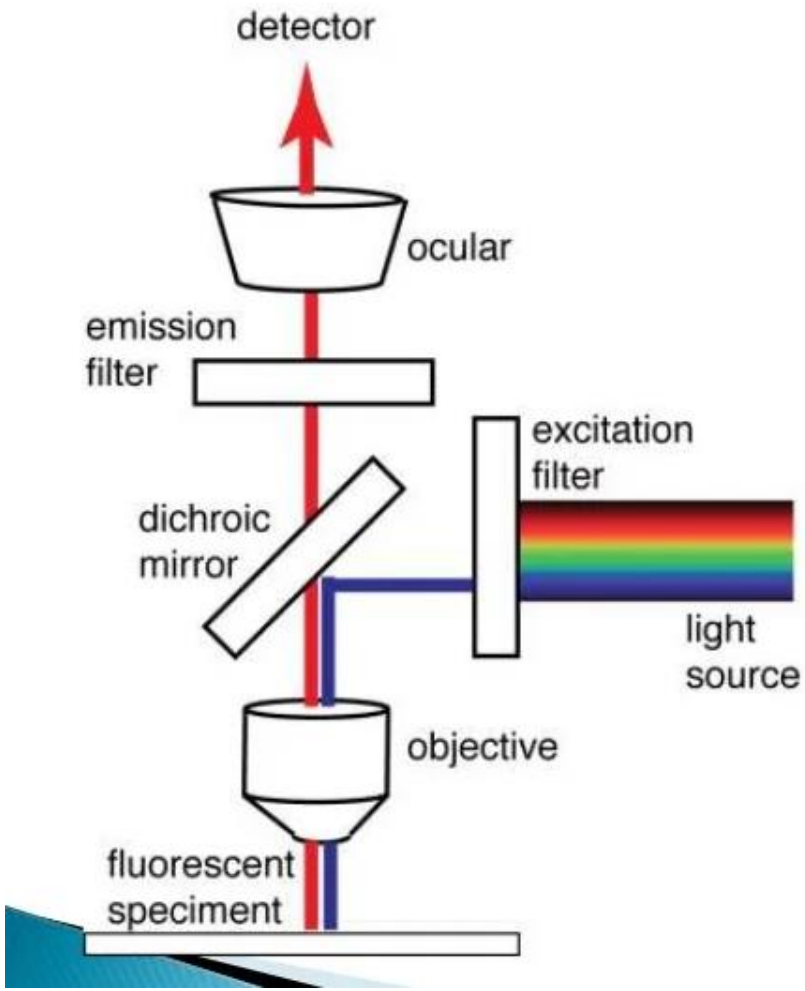


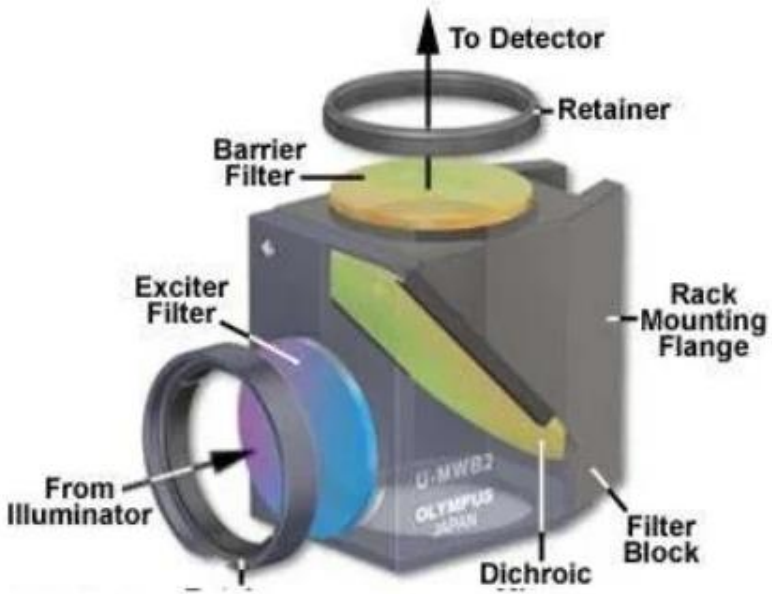

Figure 2

1. Excitation light travels along the illuminator perpendicular to the optical axis of the microscope

2. The light then impinges upon the excitation filter where selection of the desired band and blockage of unwanted wavelength occurs.

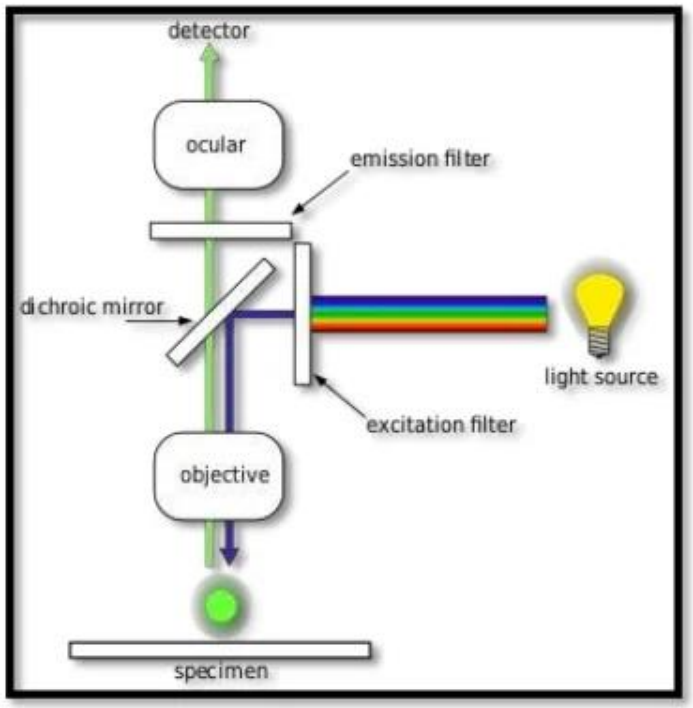
# FLUORESCENCE MICROSCOPE:



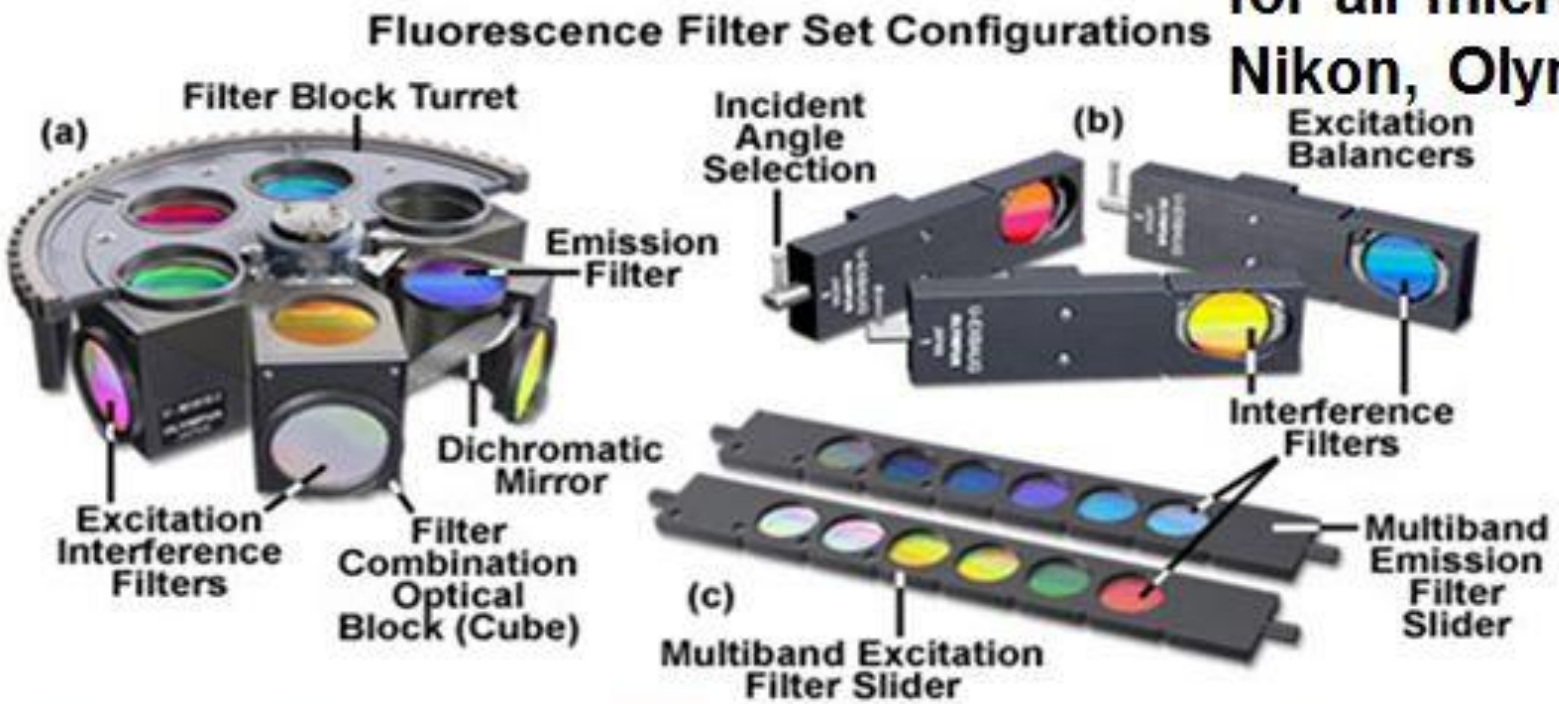



# EPIFLUORESCENCE MICROSCOPY

- Epifluorescence microscopy is a method of fluorescence microscopy that is widely used in life sciences.
- The excitory light is passed from above(or,for inverted microscope,from below),through the objective lens and then onto the specimen instead of passing it first throgh the specimen.
- The fluorescence in the specimen then gives rise to the emitted light which is focused to the detector by the same objective lens that is used for excitation.



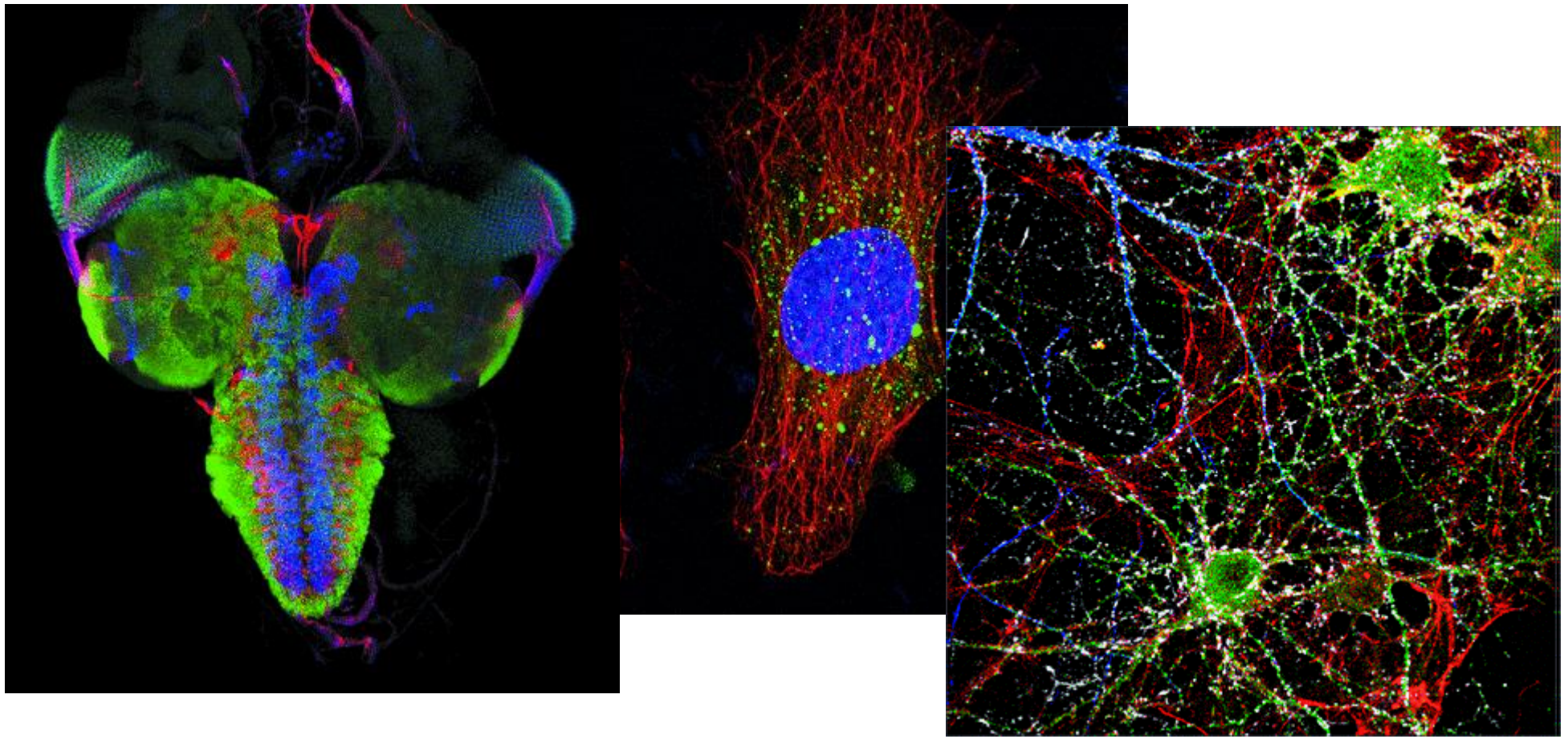
# for all microscope brands (Leica, Nikon, Olympus & Zeiss)

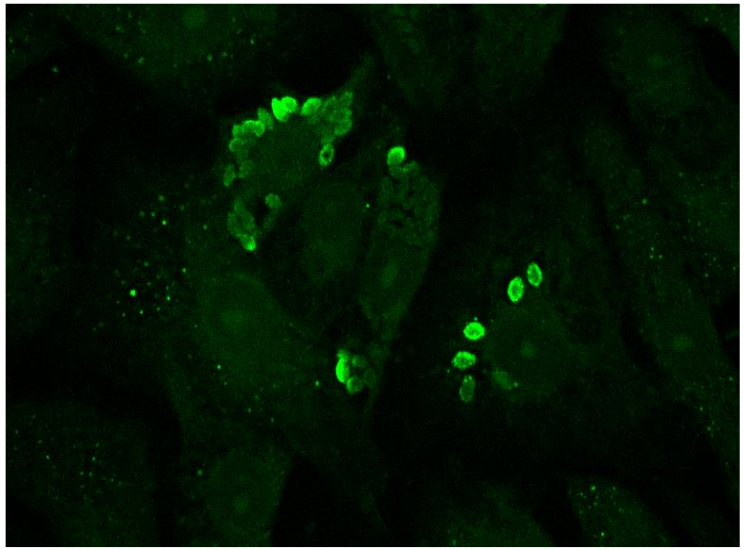


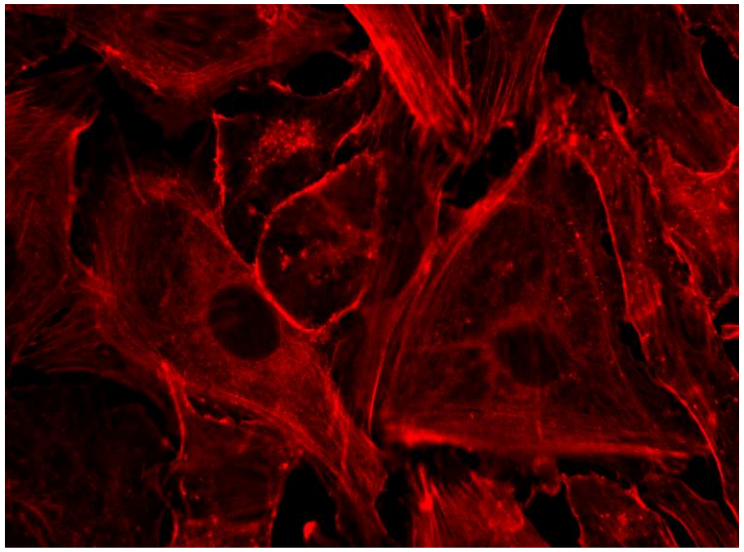


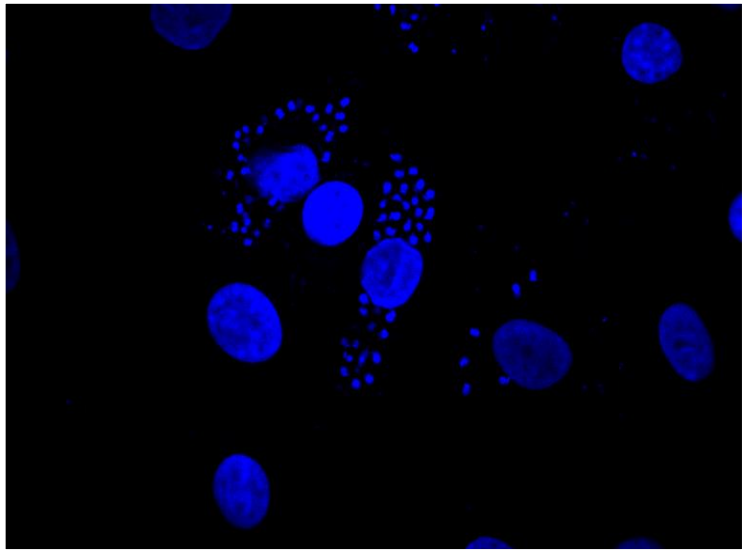


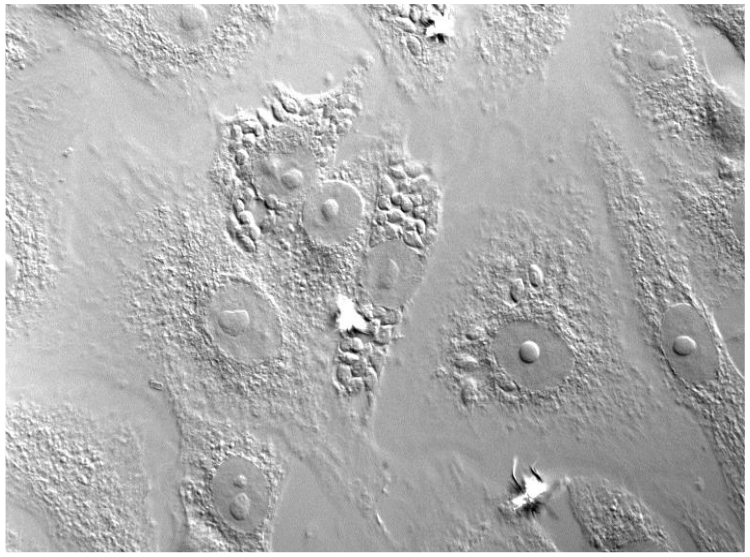


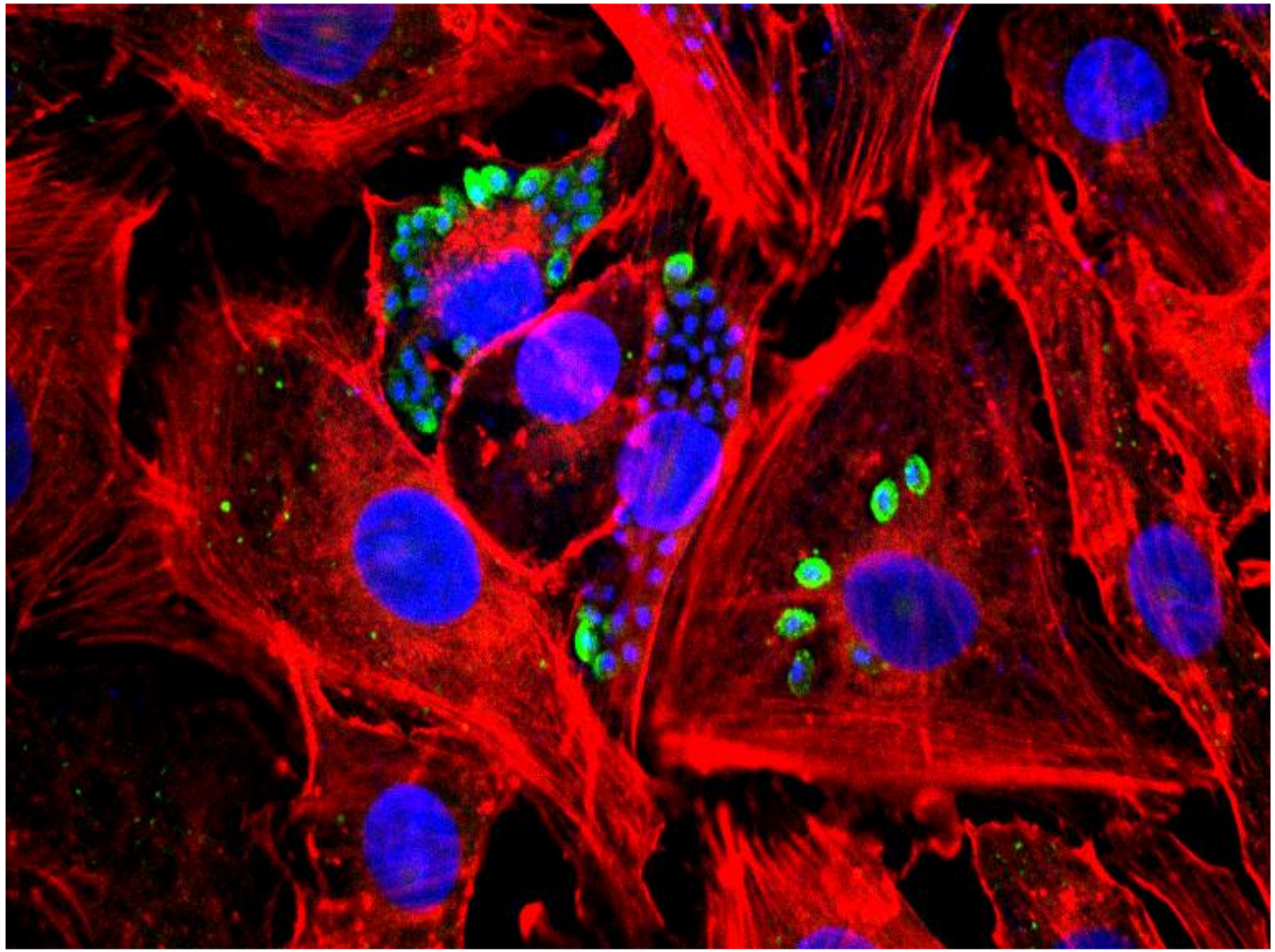


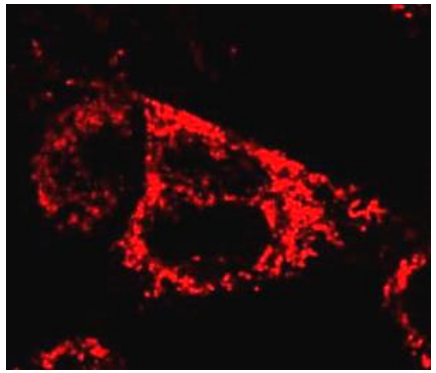


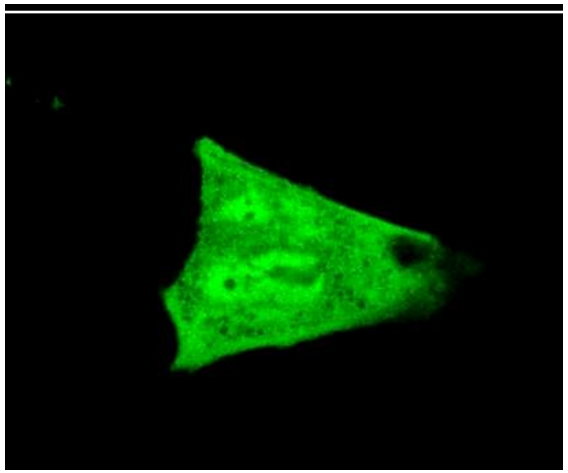


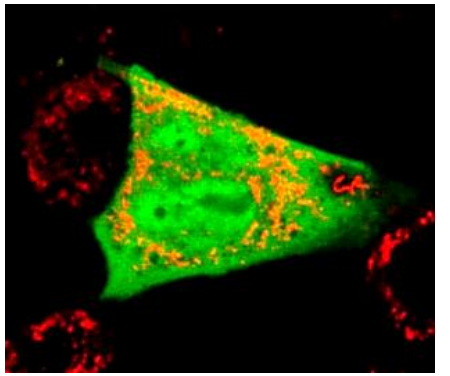


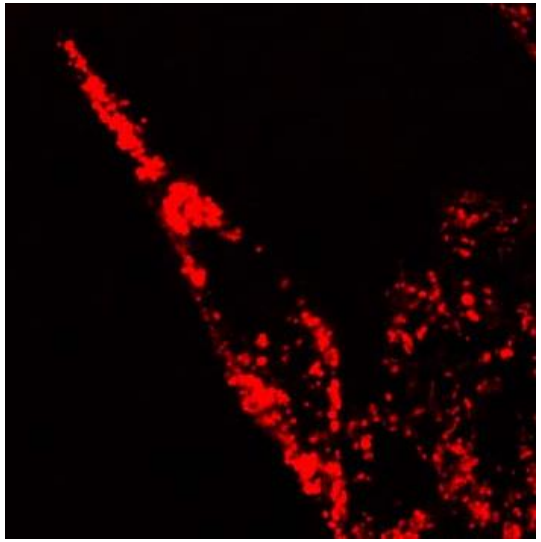
Actin - Rhodamine-phalloidin
Antibody to *T.cruzi* - FITC
DNA - Dapi

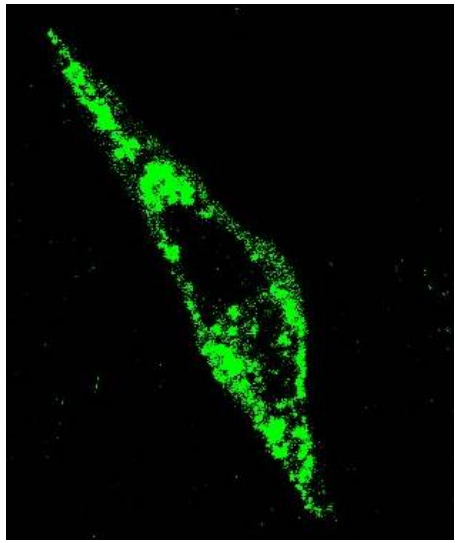












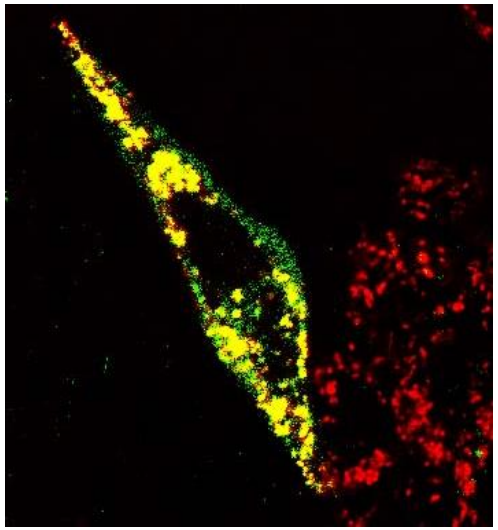
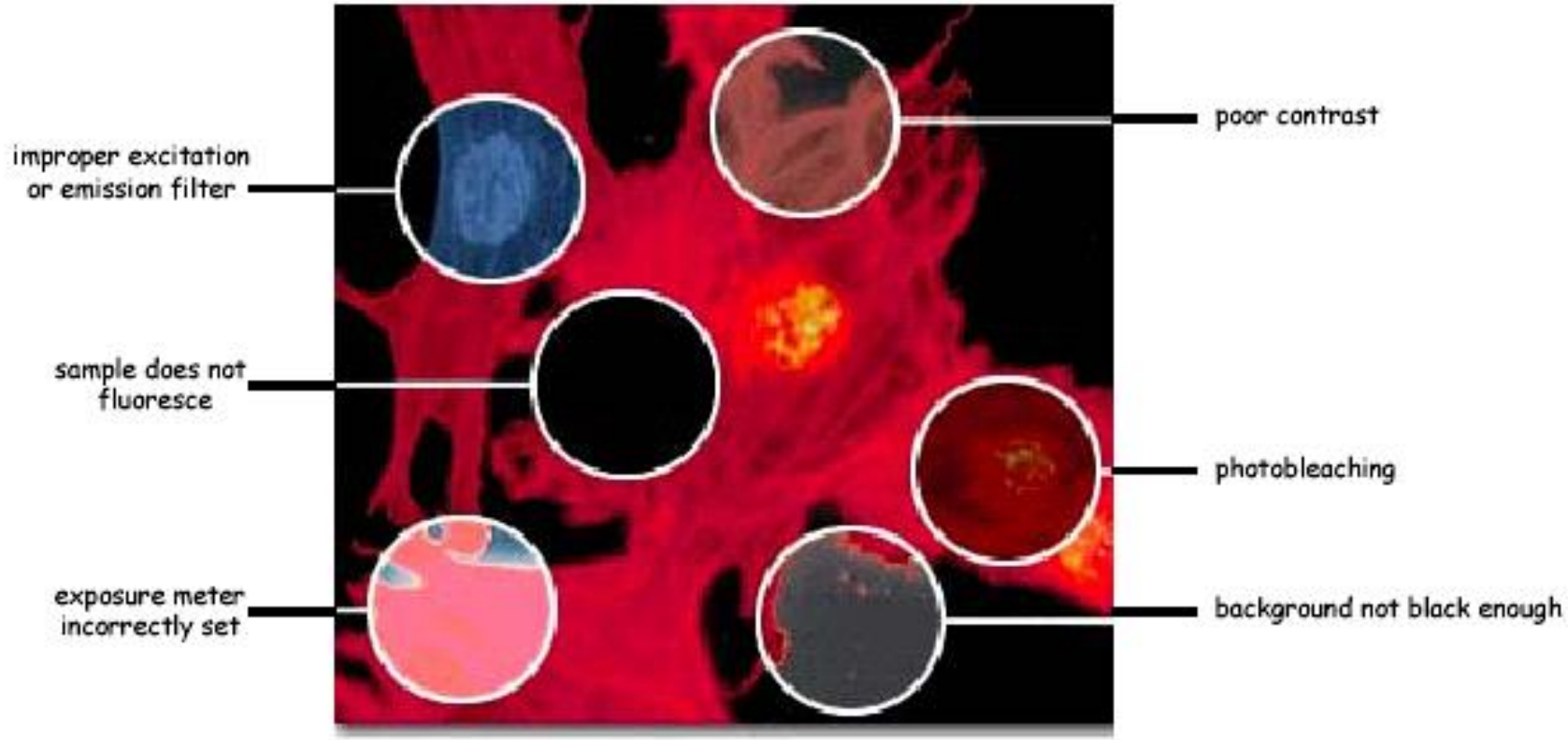


Figure 3: Problems with Fluorescence microscopy



# COMPONENTS

- Typical components of a fluorescence microscope are:
- the **light source** (xenon arc lamp or mercury-vapor lamp),
- the **excitation filter**,
- the **dichroic mirror** and
- the **emission filter**.

# Fluorescence Excitation Spectra

**Intensity**

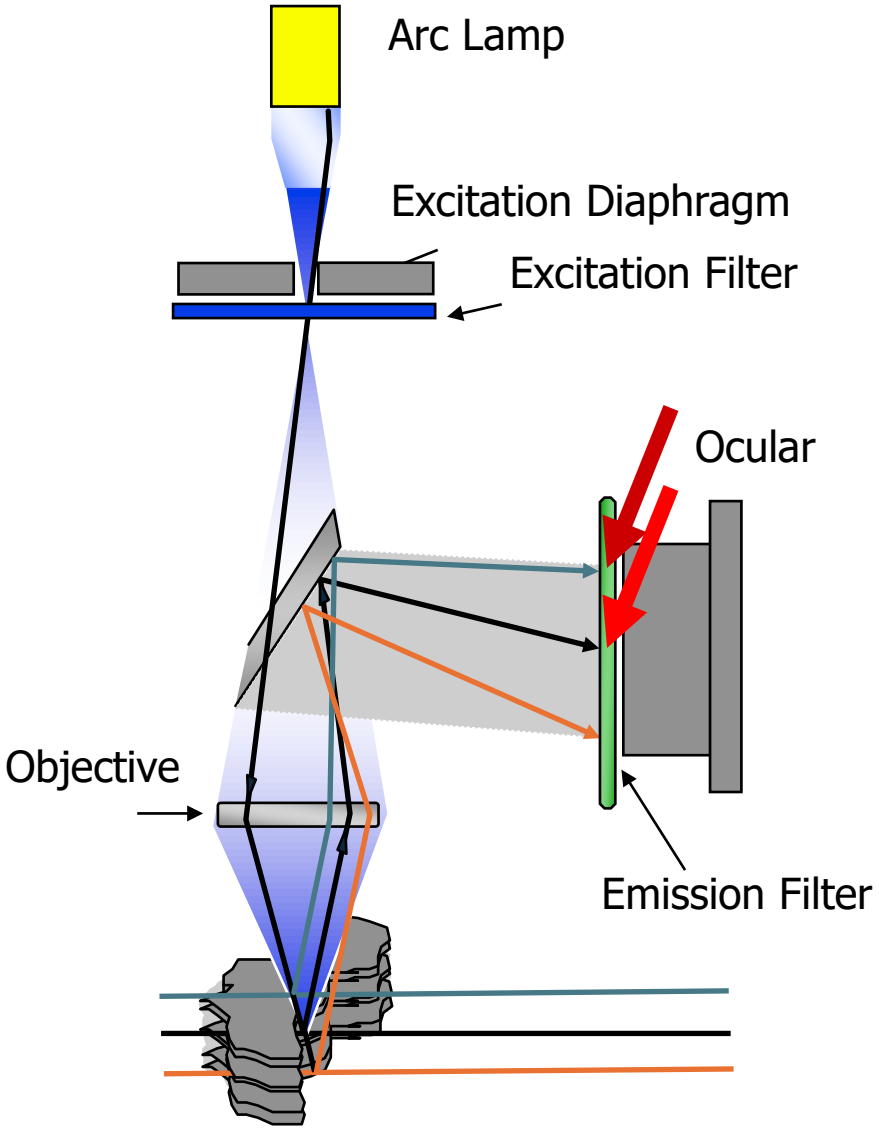
related to the **probability** of the event

**Wavelength**

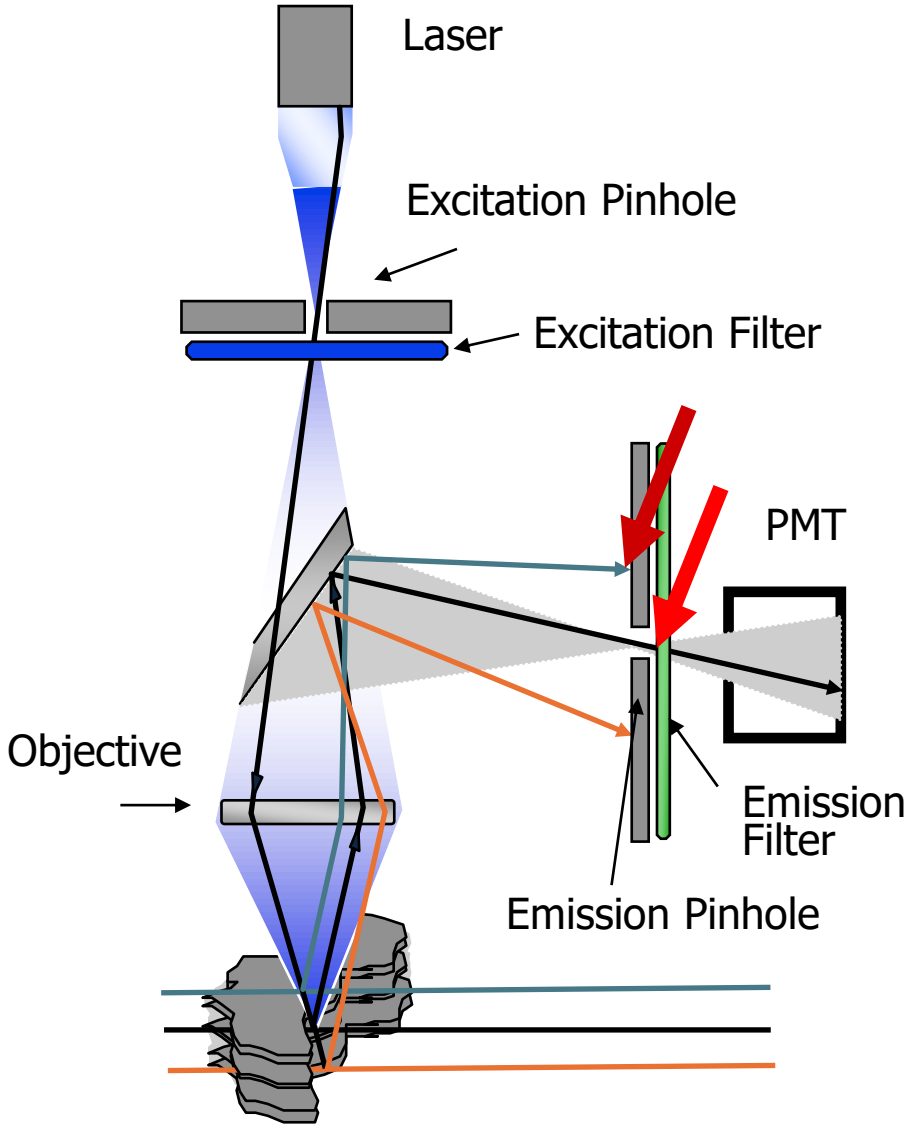
the **energy** of the light absorbed or emitted

# *Confocal Laser Scanning Microscopy (CLSM)*

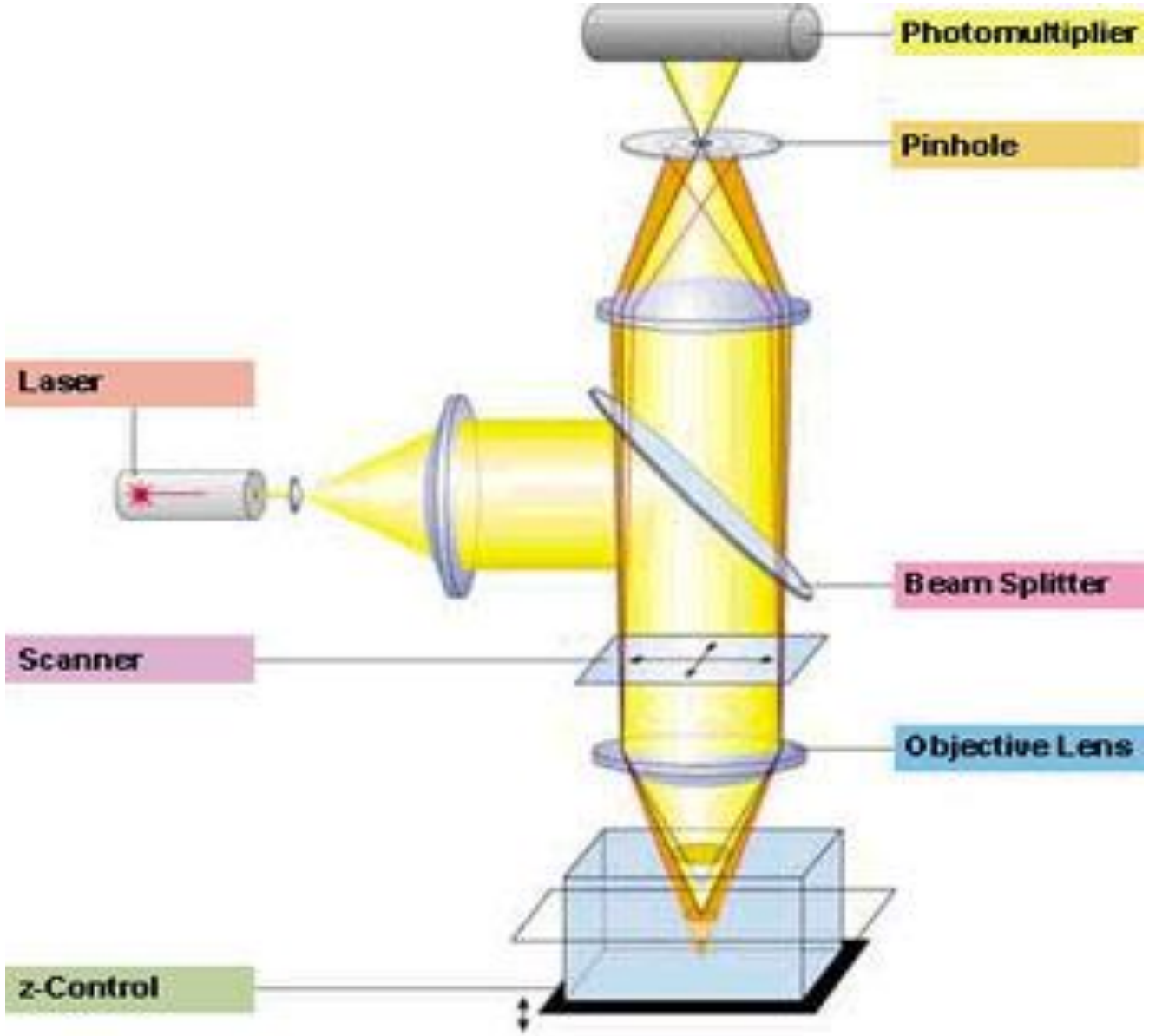
## Fluorescence Microscope



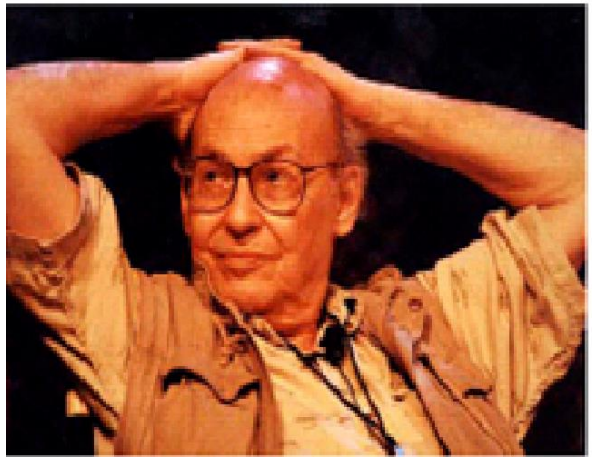

## Confocal Fluorescence Microscope

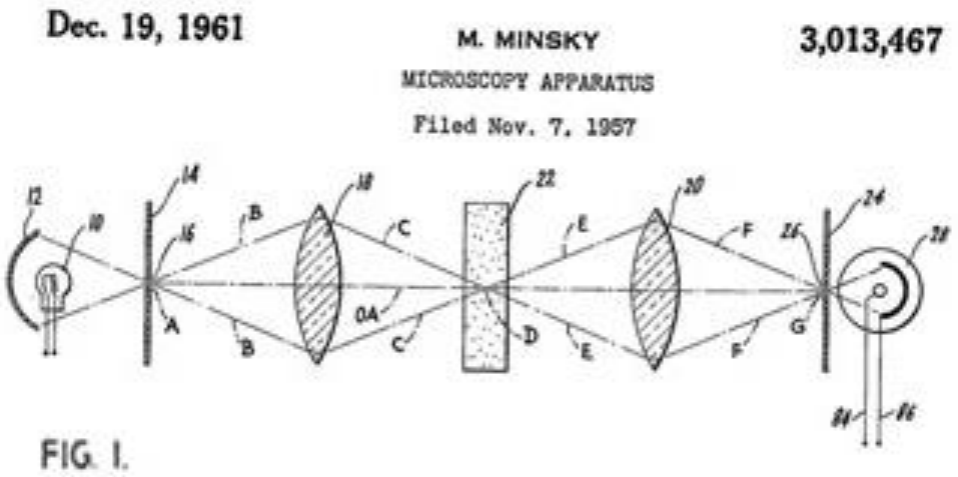


# Simplified Optics of a Confocal Microscope

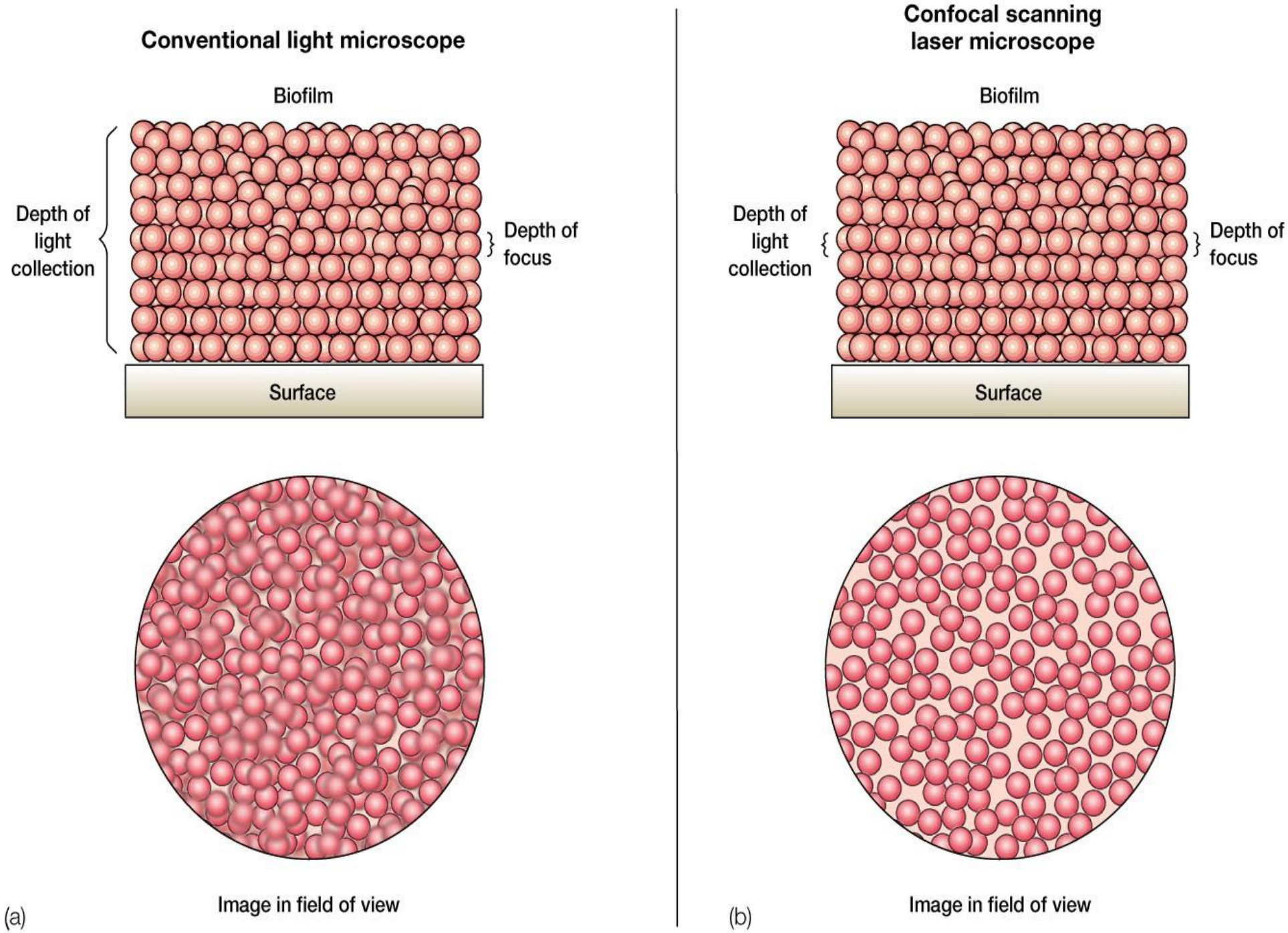


- *The optical details of the confocal microscope are complex, but the basic idea is simple.*
- *CLSM is classified under Single beam scanning microscopy, as the specimen s illuminated and scanned using only 1 light source*
- *MARVIN MINSKY awarded confocal patent focal scanning microscope in 1957, US PATENT 301467*

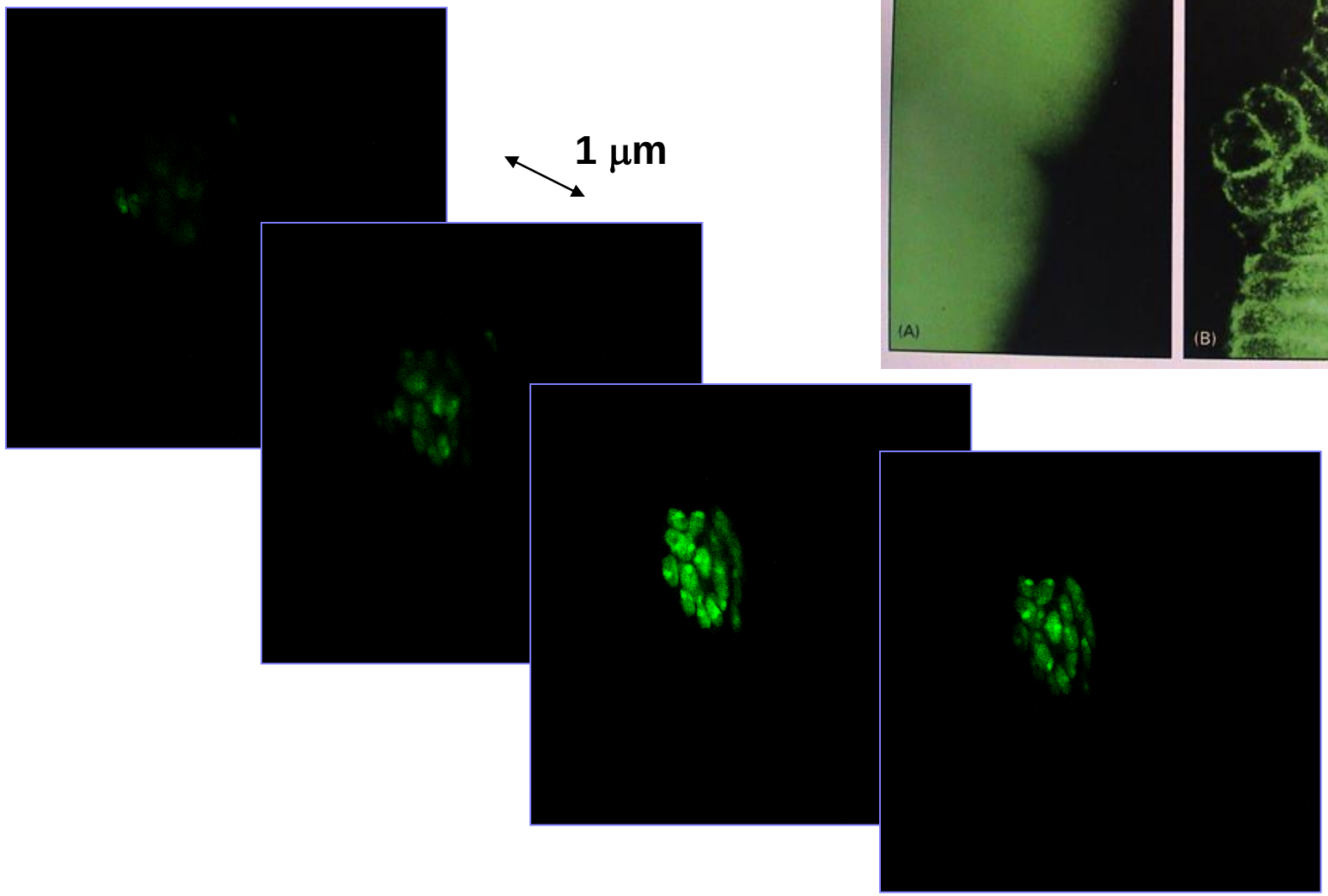




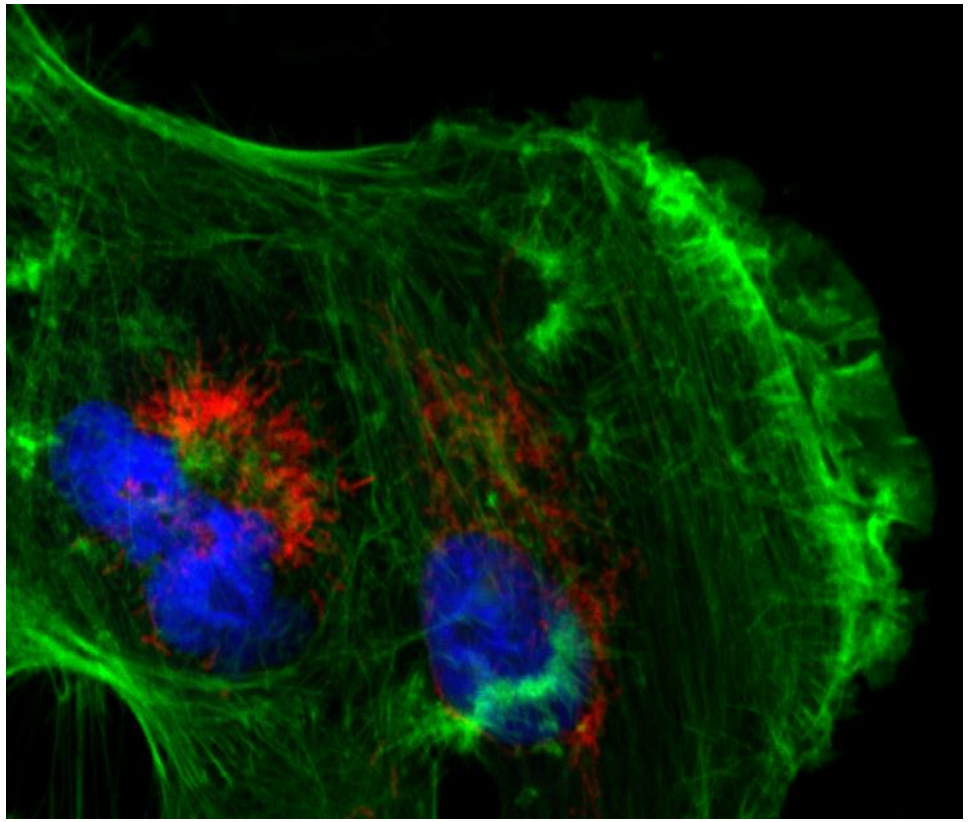
# WIDEFIELD FLUORESCENCE IMAGING


Conventional light microscope
Biofilm
Depth of light collection
Depth of focus
Surface
Image in field of view
(a)
Confocal scanning laser microscope
Biofilm
Depth of light collection
Depth of focus
Surface
Image in field of view
(b)

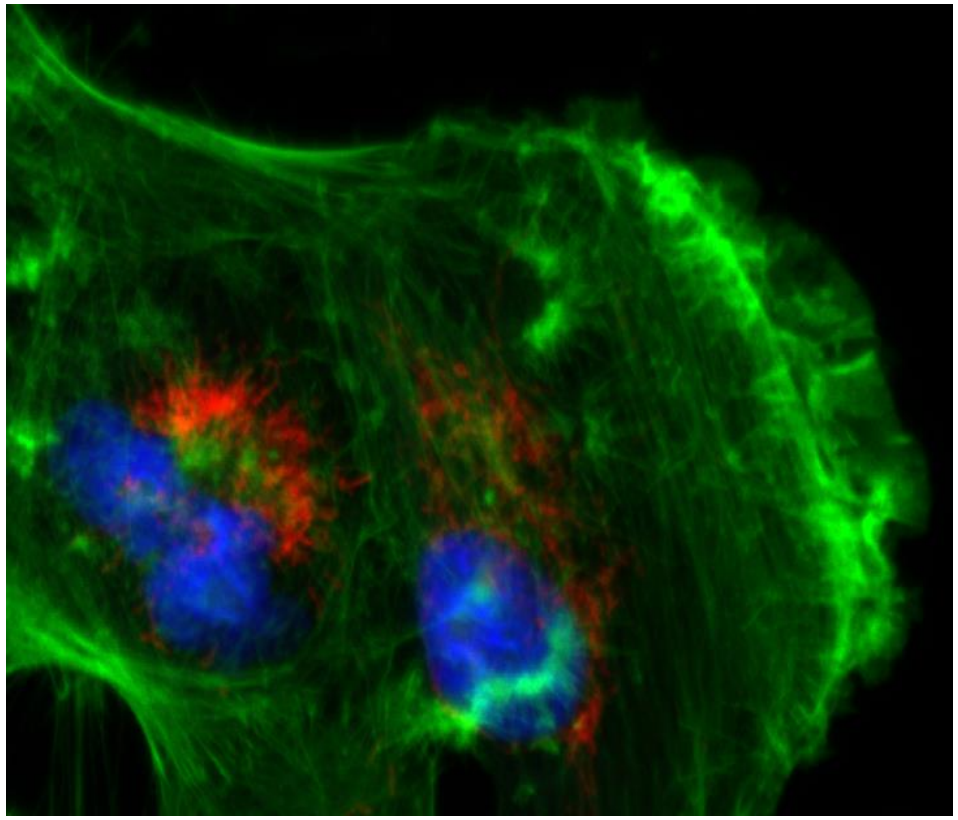
# Optical sectioning



# Confocal vs. Widefield



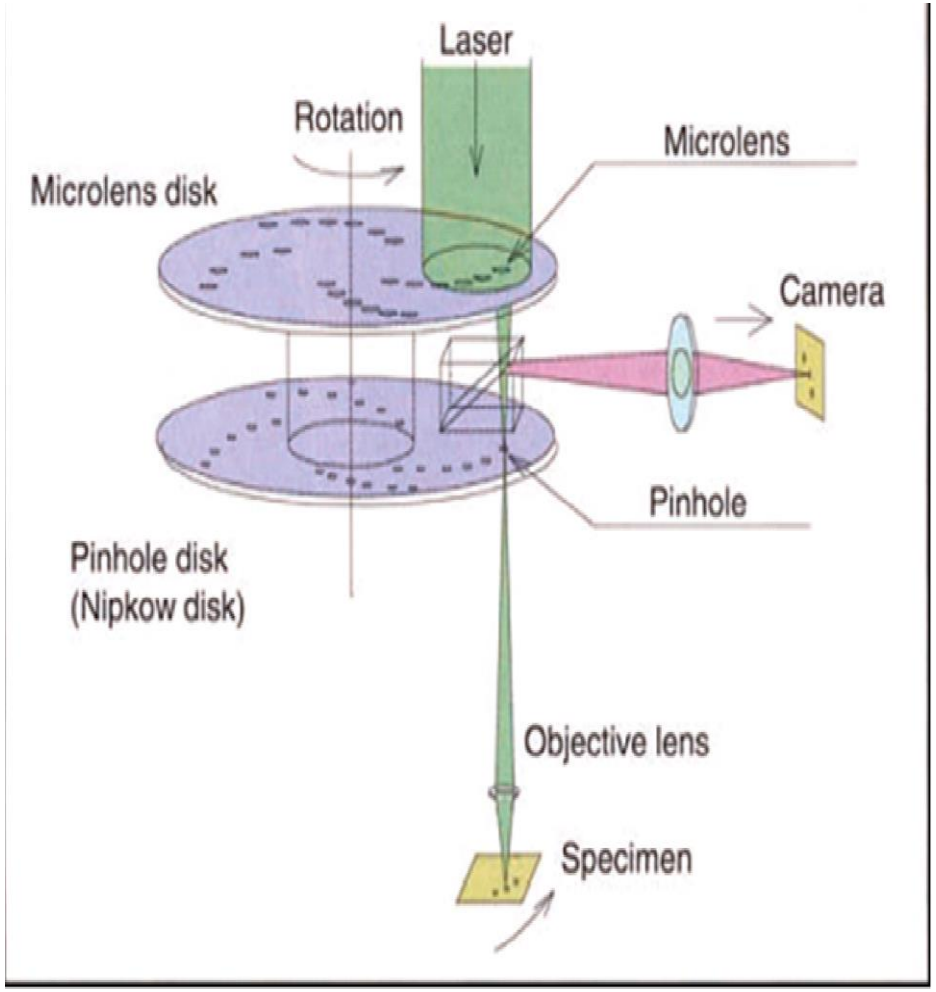
Confocal



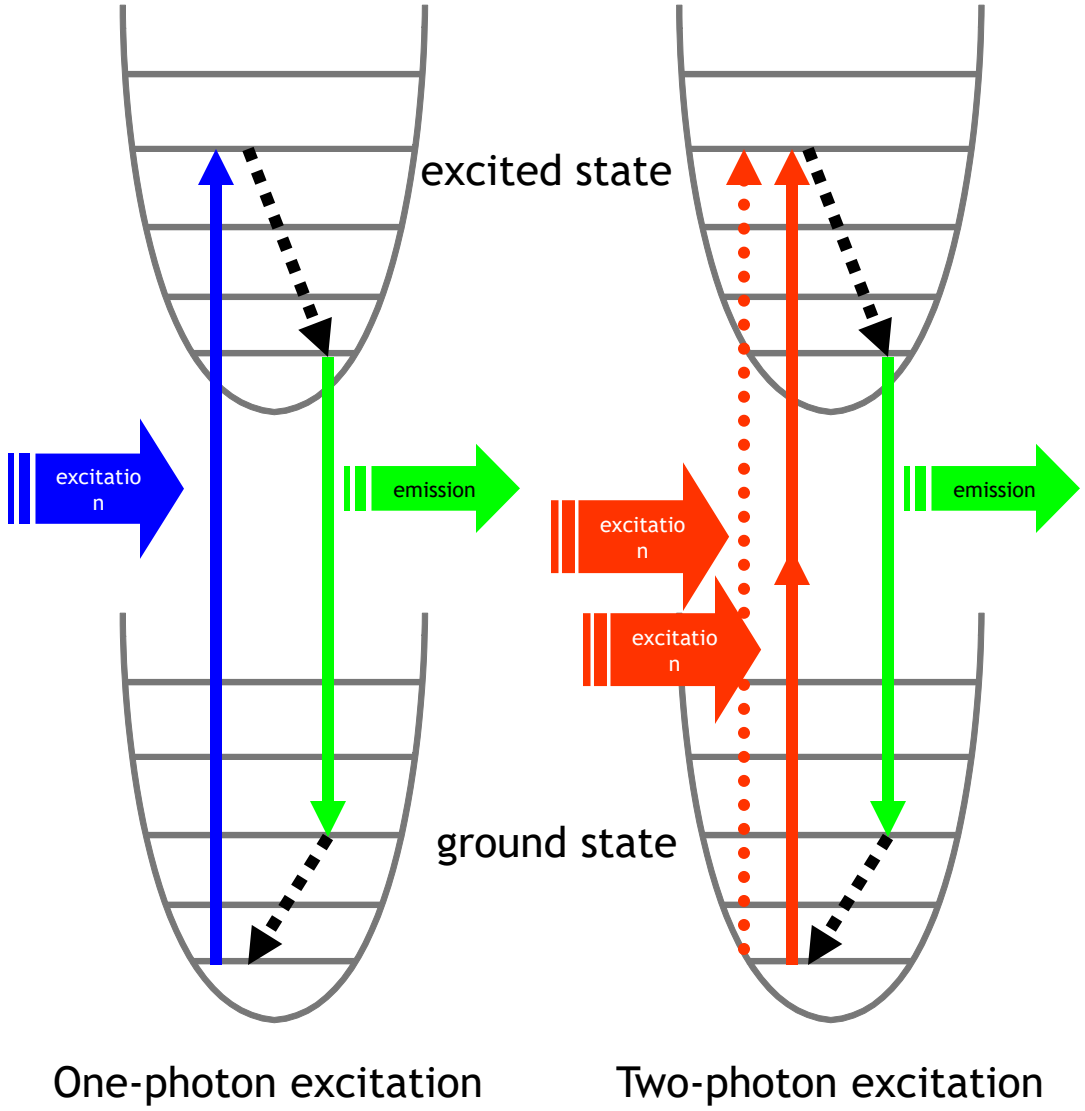
Widefield

Tissue culture cell with 60x / 1.4NA objective

# Spinning Disk Confocal

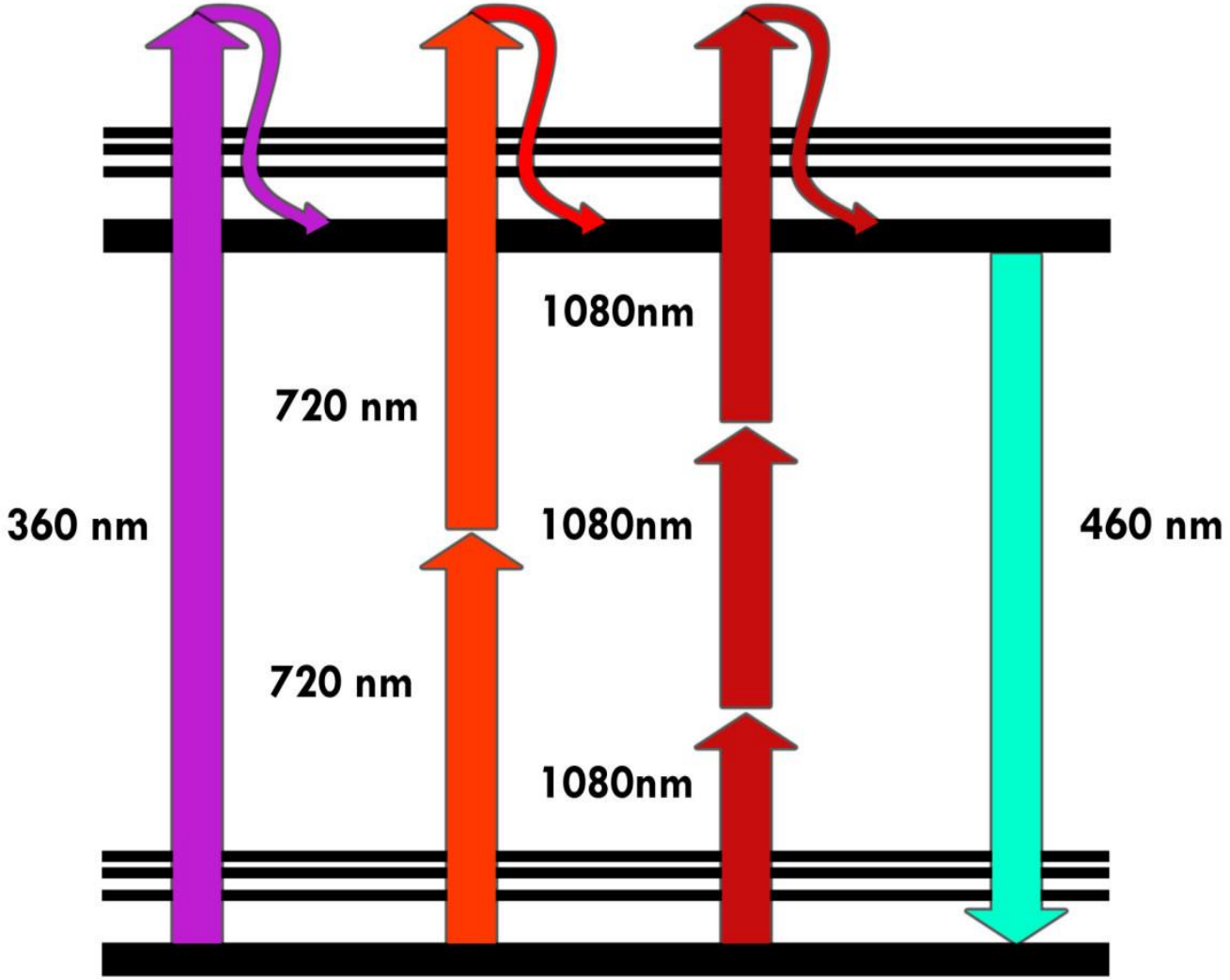


# 2-photon excitation

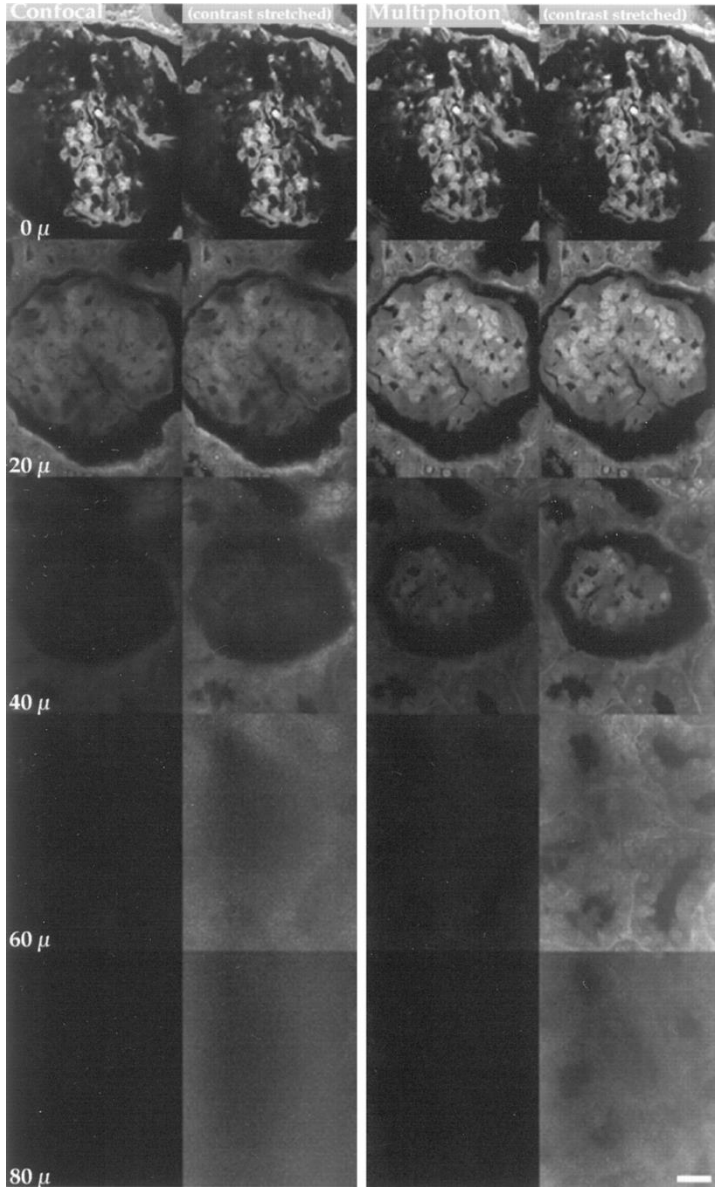



- Two-photon excitation occurs through the absorption of two lower energy photons via short-lived intermediate states.
- After either excitation process, the fluorophore relaxes to the lowest energy level of the first excited electronic states via vibrational processes.
- The subsequent fluorescence emission processes for both relaxation modes are the same.

# From 2-photon to multiphoton...



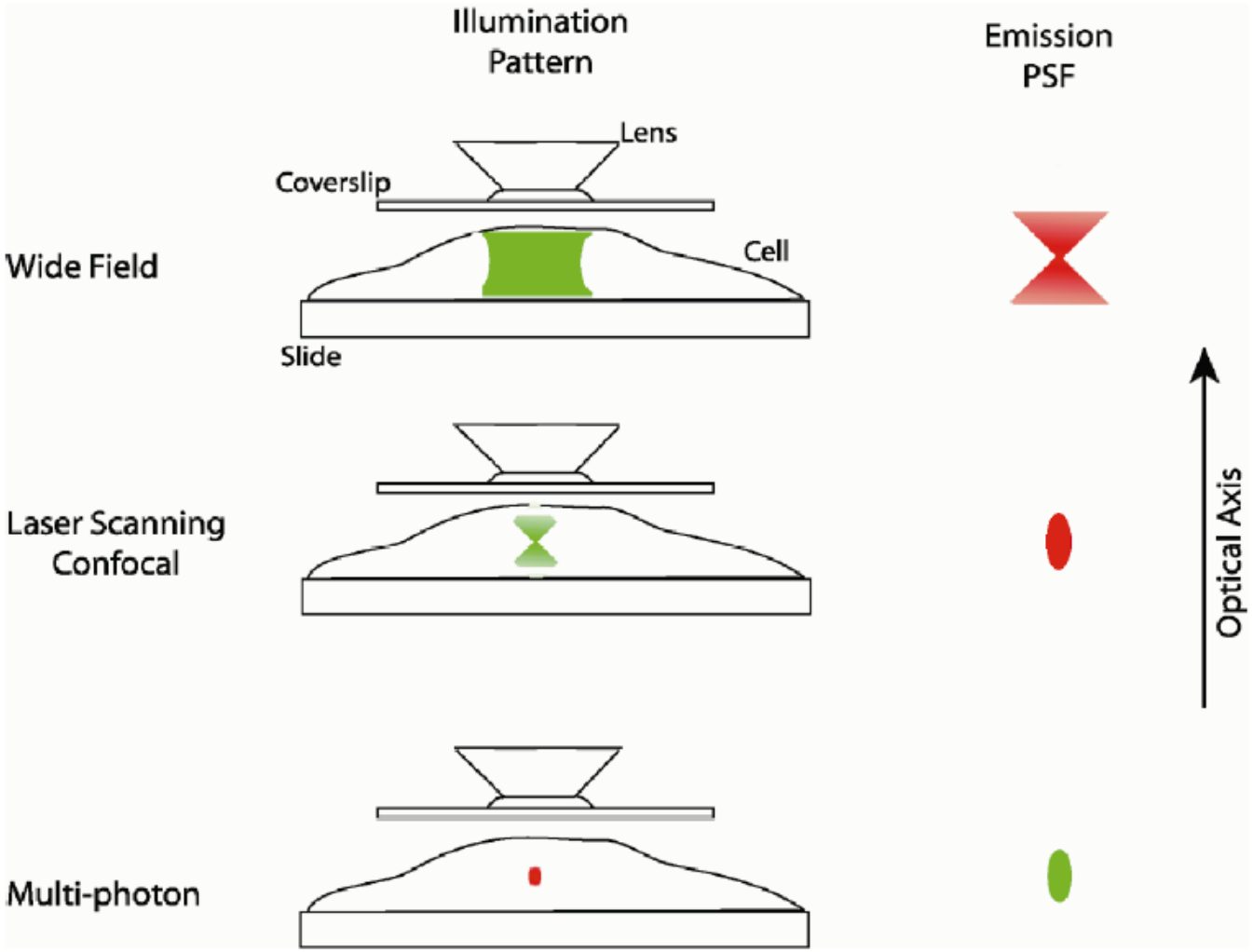
# Penetration depth




Comparison of imaging penetration depth between confocal and multiphoton microscopy. Optical sections through a glomerulus from an acid-fucsin-stained monkey kidney pathology sample imaged by confocal microscopy with 2 μW of 532-nm light (*left, columns 1 and 2*) and multiphoton microscopy with 4.3 mW of 1047-nm light (descanned; *right, columns 3 and 4*) were compared. At the surface, the image quality and signal intensity are similar; however, at increasing depth into the sample, signal intensity and quality of the confocal image falls off more rapidly than the multiphoton image. Images were collected at a pixel resolution of 0.27 μm with a Kalman 3 collection filter. Scale bar, 20 μm.

Centonze VE, White JG. Multiphoton excitation provides optical sections from deeper within scattering specimens than confocal imaging. Biophys J. 1998 Oct;75(4):2021-24.

# Wide-field vs. confocal vs. 2-photon

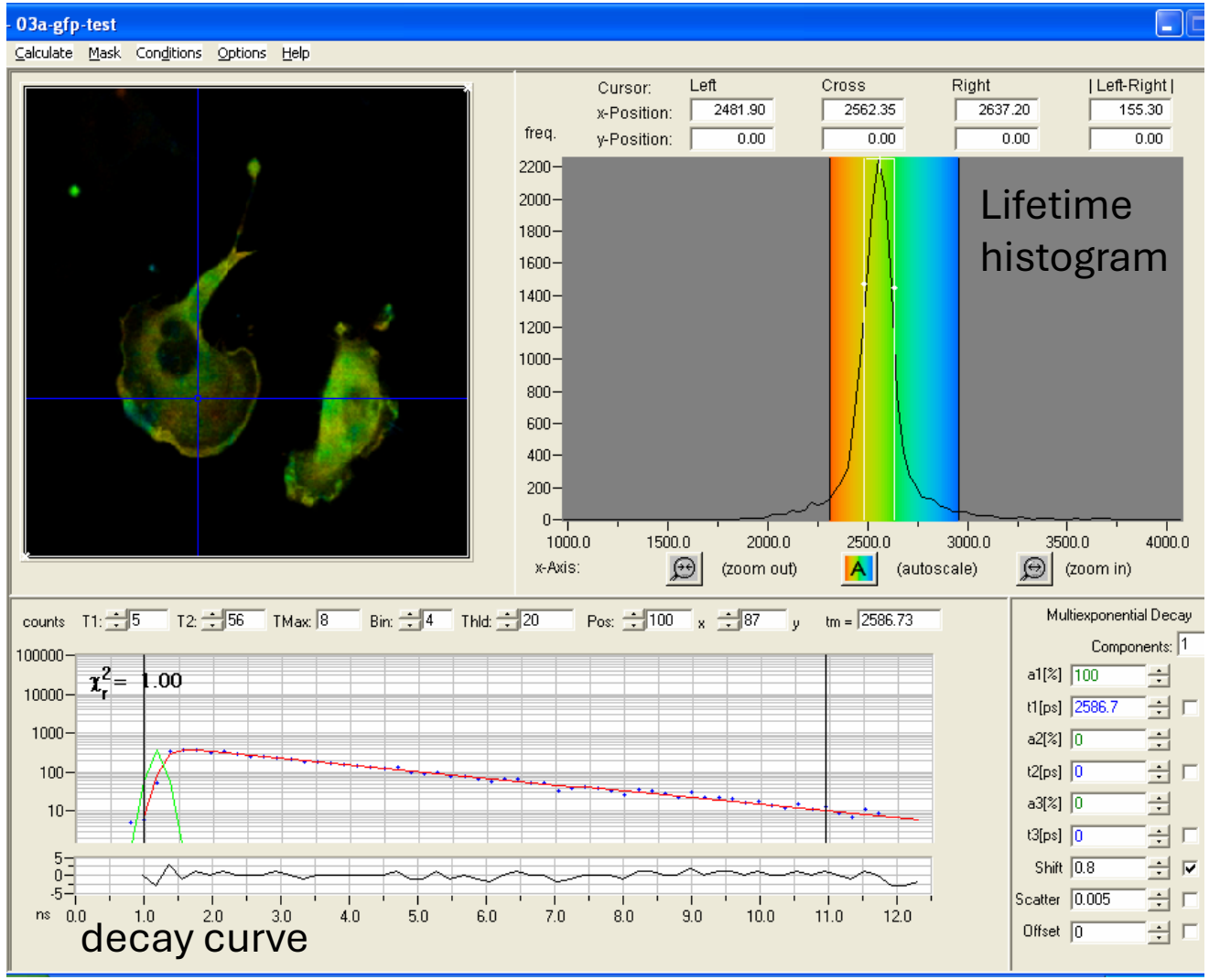



Drawing by P. D. Andrews, I. S. Harper and J. R. Swedlow

# Special applications:

- FRET and FLIM
- FRAP and photoactivation
- **TIRF**

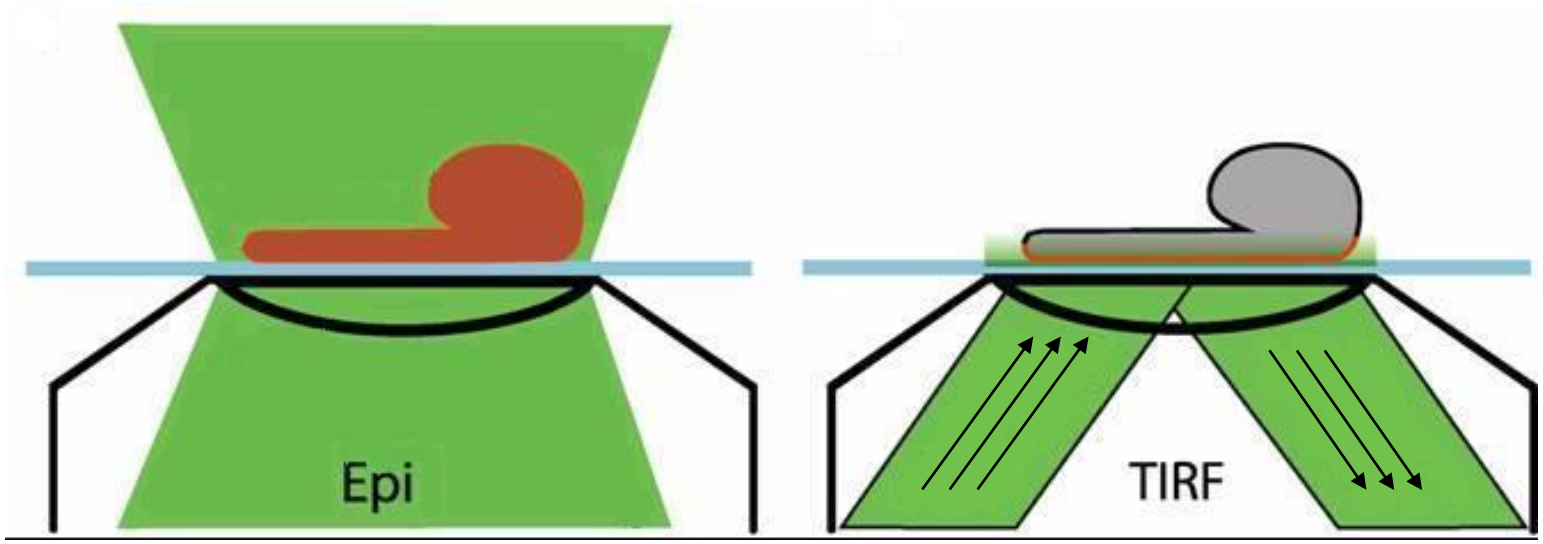
# FLIM




Excitation of many electrons at the same time → count the different times when they are falling back down (i.e. photons are emitted)

lifetime = ½ of all electrons are fallen back

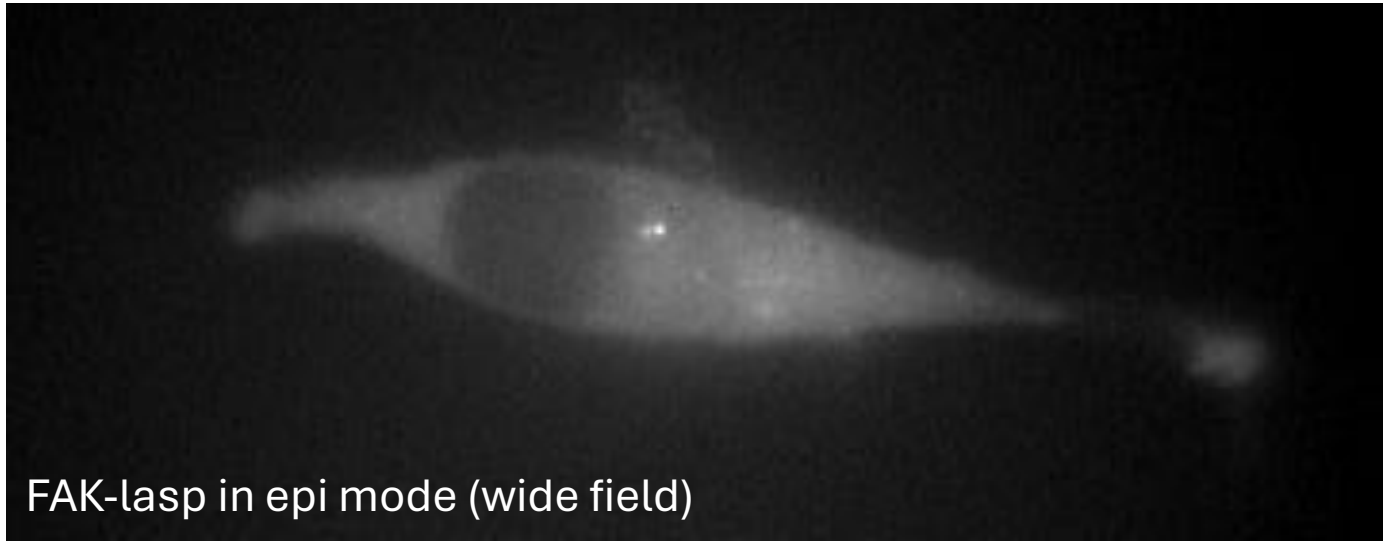
# TIRF (Total Internal Reflection Fluorescence)



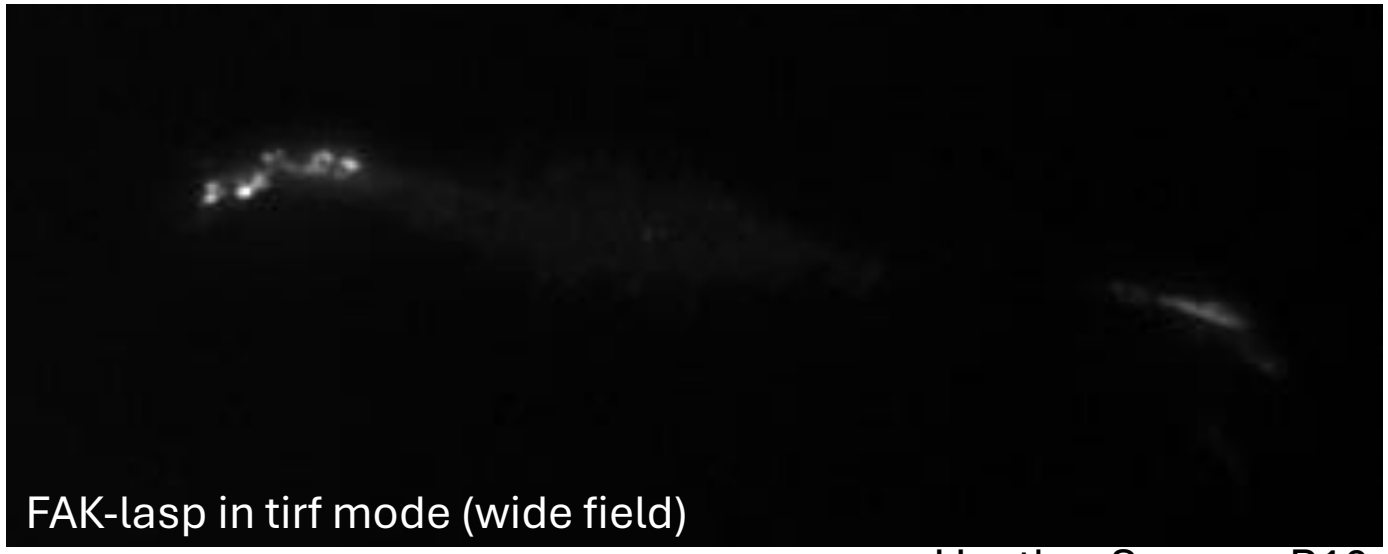

You need:

- TIRF objectives with high NA
- TIRF condensor, where you are able to change the angle of illumination
- Glass coverslips

# TIRF vs epi



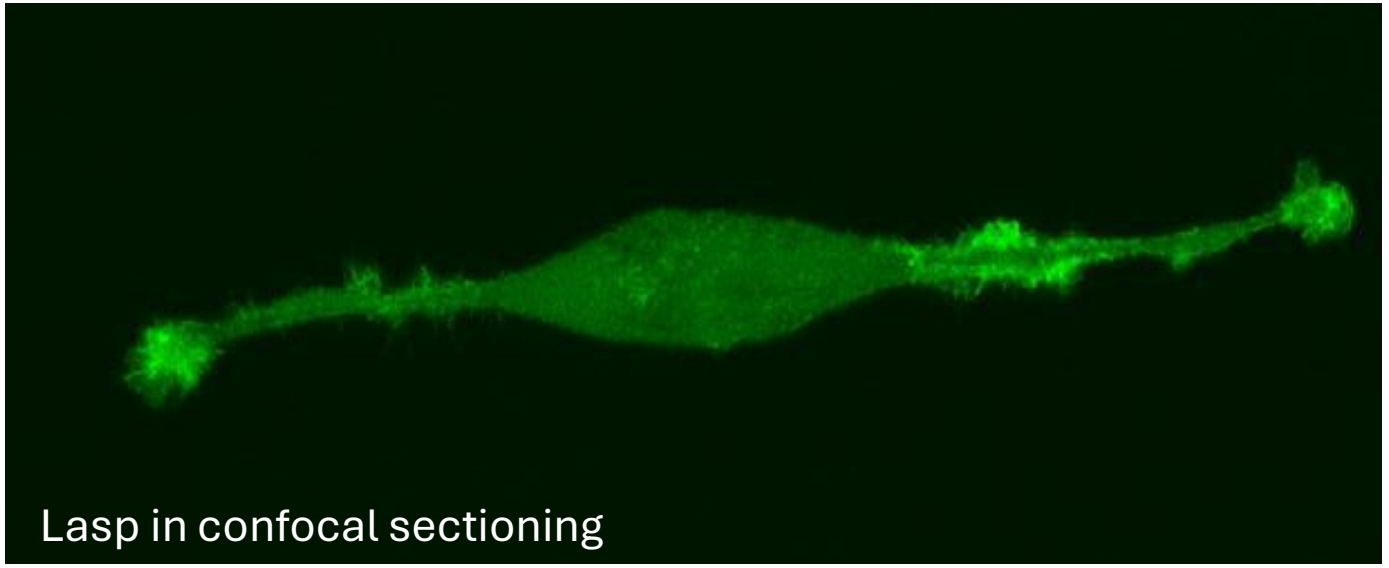
FAK-lasp in epi mode (wide field)

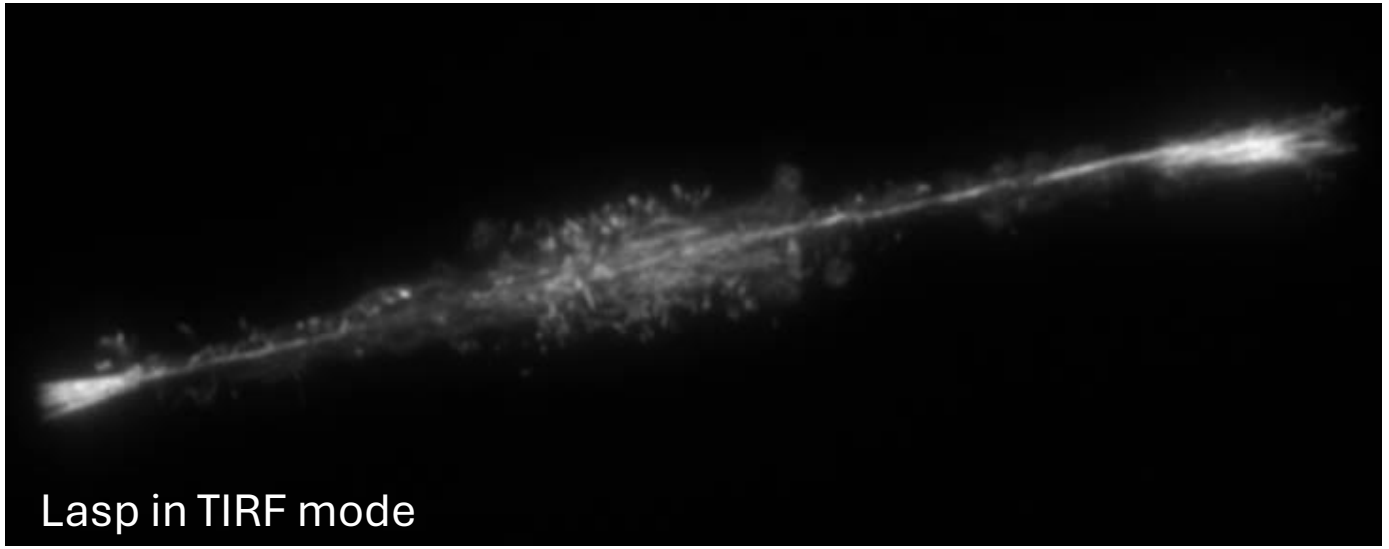


FAK-lasp in tirf mode (wide field)

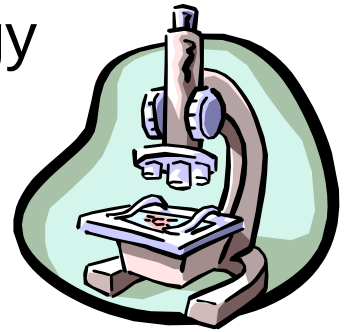
Heather Spence, R10

# TIRF vs epi




Heather Spence, R10



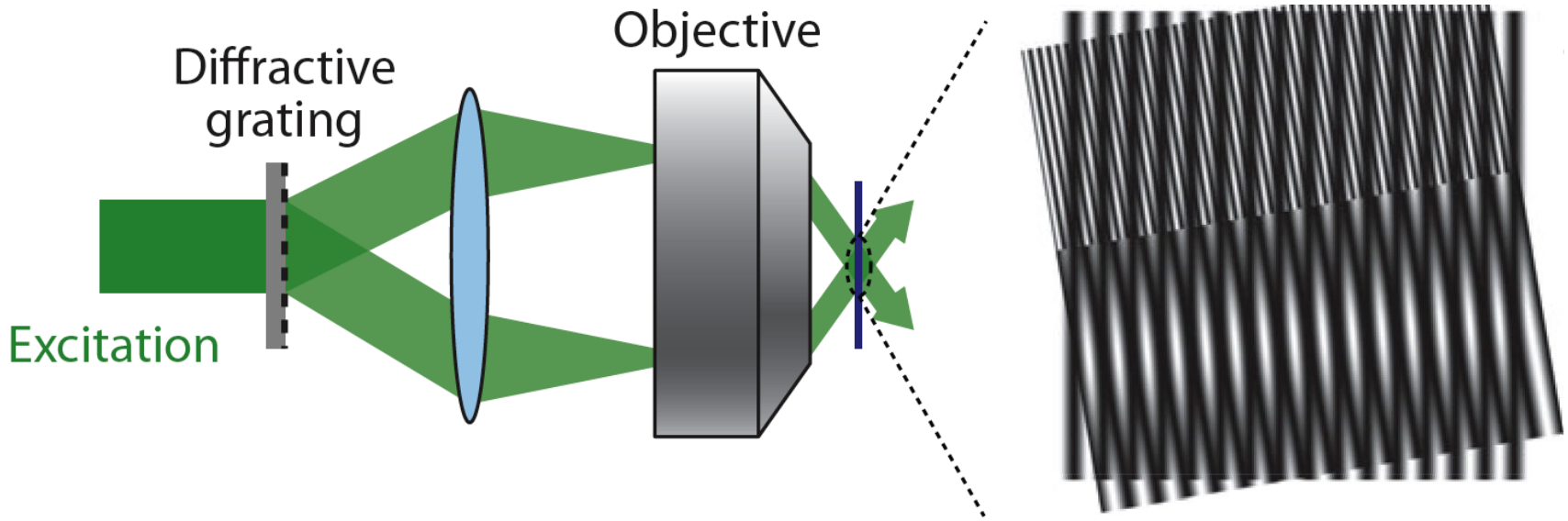
# Timeline of the Microscope

## Abbe's Law

$$d = \frac{\lambda}{2\, n \sin\theta}$$

"minimum resolving distance (d) is related to the wavelength of light (lambda) divided by the Numeric Aperture, which is proportional to the angle of the light cone (theta) formed by a point on the object, to the objective".

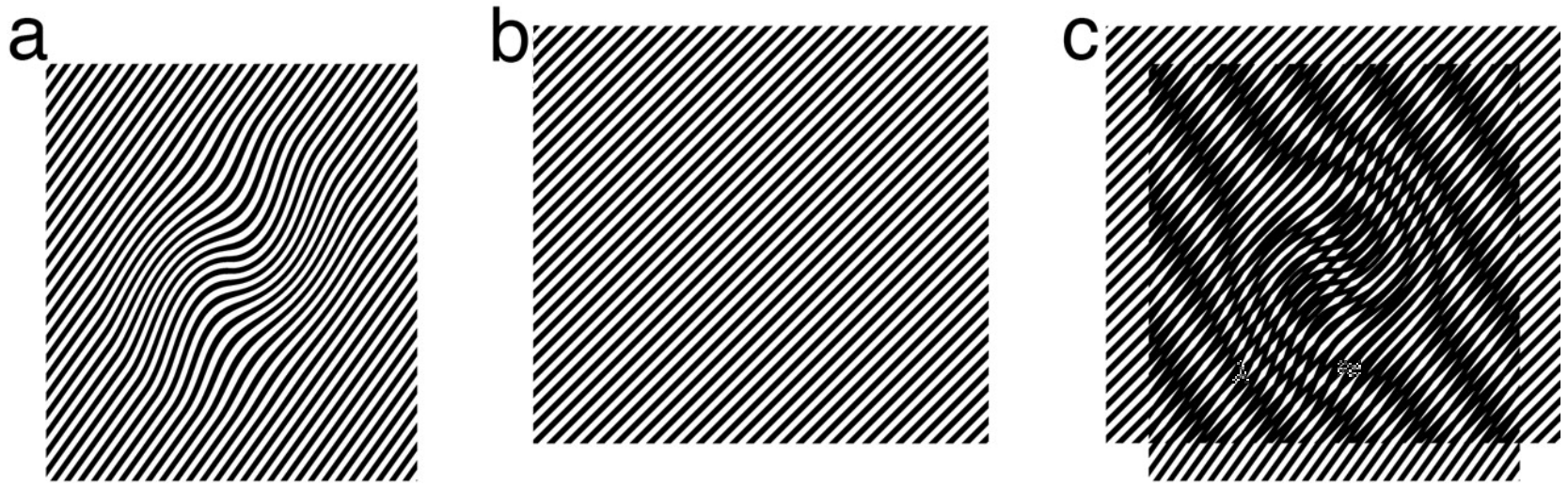
# Structured-Illumination Micropscopy (SIM)




100 nm resolution
possible

# Resolution extension through the moiré effect

If the illumination contains a spatial frequency $\mathbf{k}_1$, then each sample frequency $\mathbf{k}$ gives rise to moiré fringes at the difference frequency $\mathbf{k} - \mathbf{k}_1$. Those fringes will be observable in the microscope if $|\mathbf{k} - \mathbf{k}_1| < k_0$



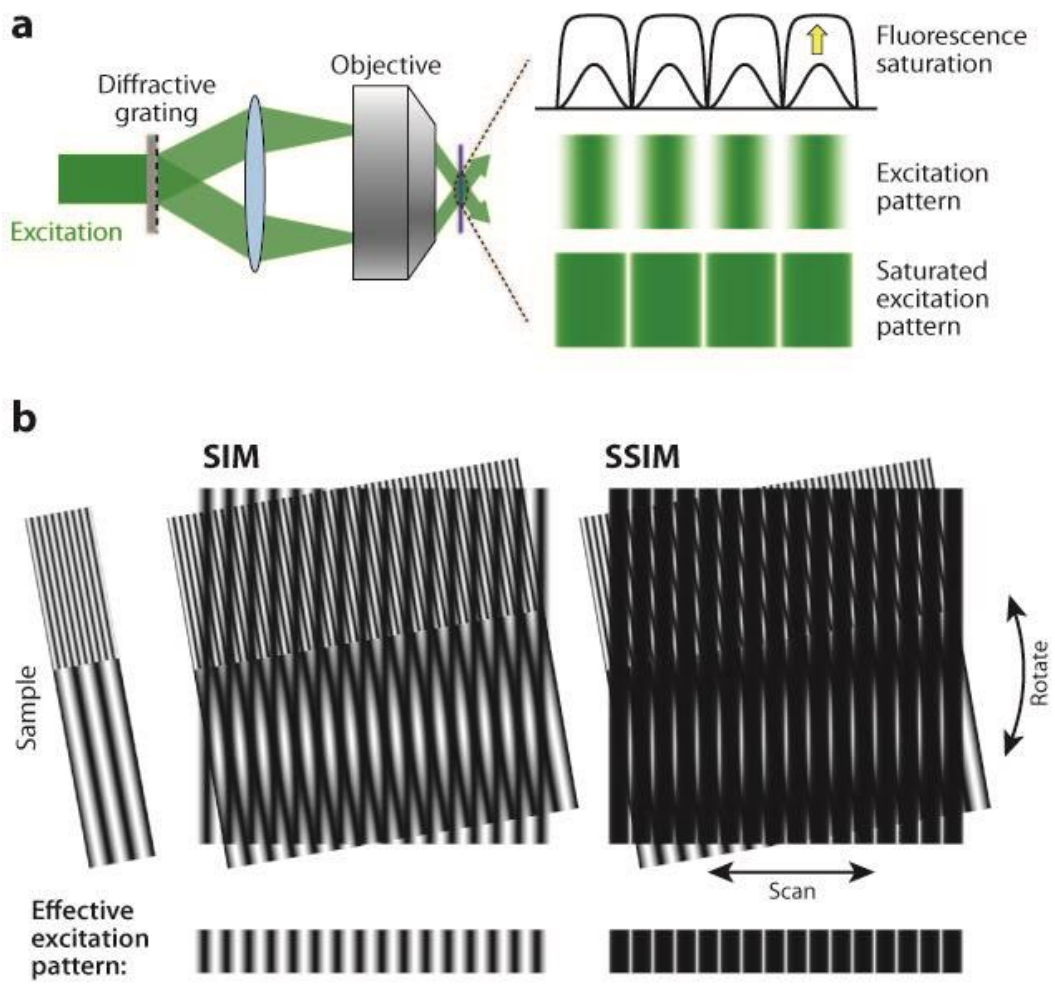

If an unknown sample structure (*a*) is multiplied by a known regular illumination pattern (*b*), moiré fringes will appear (*c*). The Moiré fringes occur at the spatial difference frequencies between the pattern frequency and each spatial frequency component of the sample structure and can be coarse enough to observe through the microscope even if the original unknown pattern is unresolvable. Otherwise-unobservable sample information can be deduced from the fringes and computationally restored.

Gustafsson, M.G.L. (2005) Proc. Natl. Acad. Sci. USA 102, 13081-13086



The word moiré is French (from the past participle of the verb moirer, meaning to water).

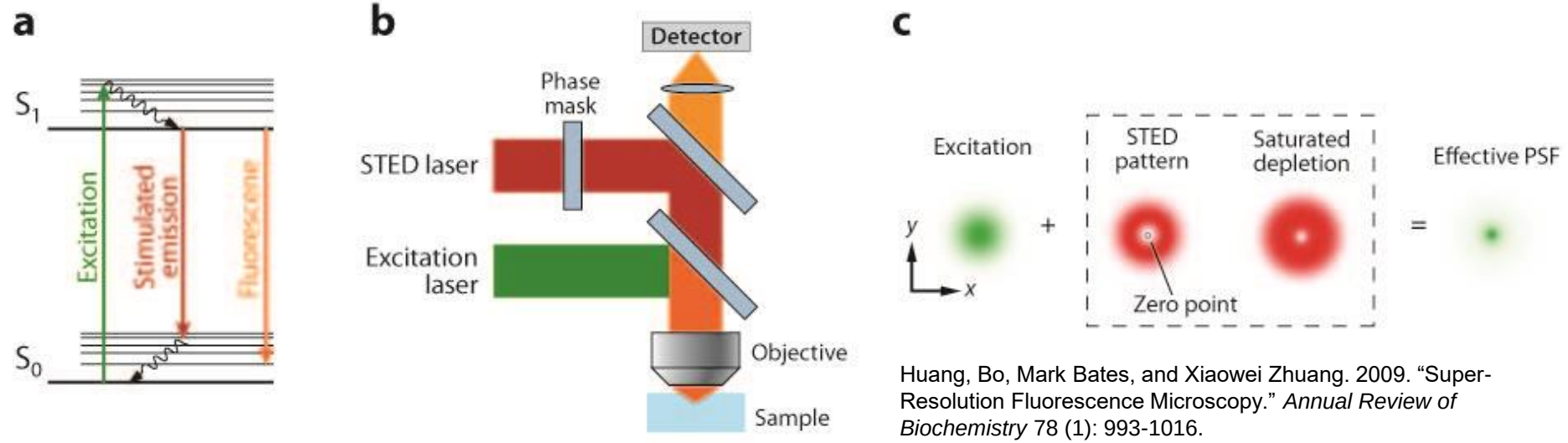
# Saturated structured illumination




Huang, Bo, Mark Bates, and Xiaowei Zhuang. 2009. “Super-Resolution Fluorescence Microscopy.” *Annual Review of Biochemistry* 78 (1): 993-1016. doi:10.1146/annurev.biochem.77.061906.092021.

(*a)* A diffractive grating in the excitation path splits the light into two beams. Their interference after emerging from the objective and reaching the sample creates a sinusoidal illumination pattern with alternating peaks and zero points. Strong excitation light saturates the fluorescence emission at the peaks without exciting fluorophores at the zero points, leading to sharp dark regions in the excitation pattern. (*b)* When a sinusoidal illumination pattern is applied to a sample, a moiré pattern at a significantly lower spatial frequency than that of the sample can be generated and imaged by the microscope (SIM panel, *lower part*). Multiple images that resulted from scanning and rotating the excitation pattern are then used to reconstruct the sample structure. SSIM introduces a high-frequency component into the excitation pattern, allowing features far below the diffraction limit to be resolved

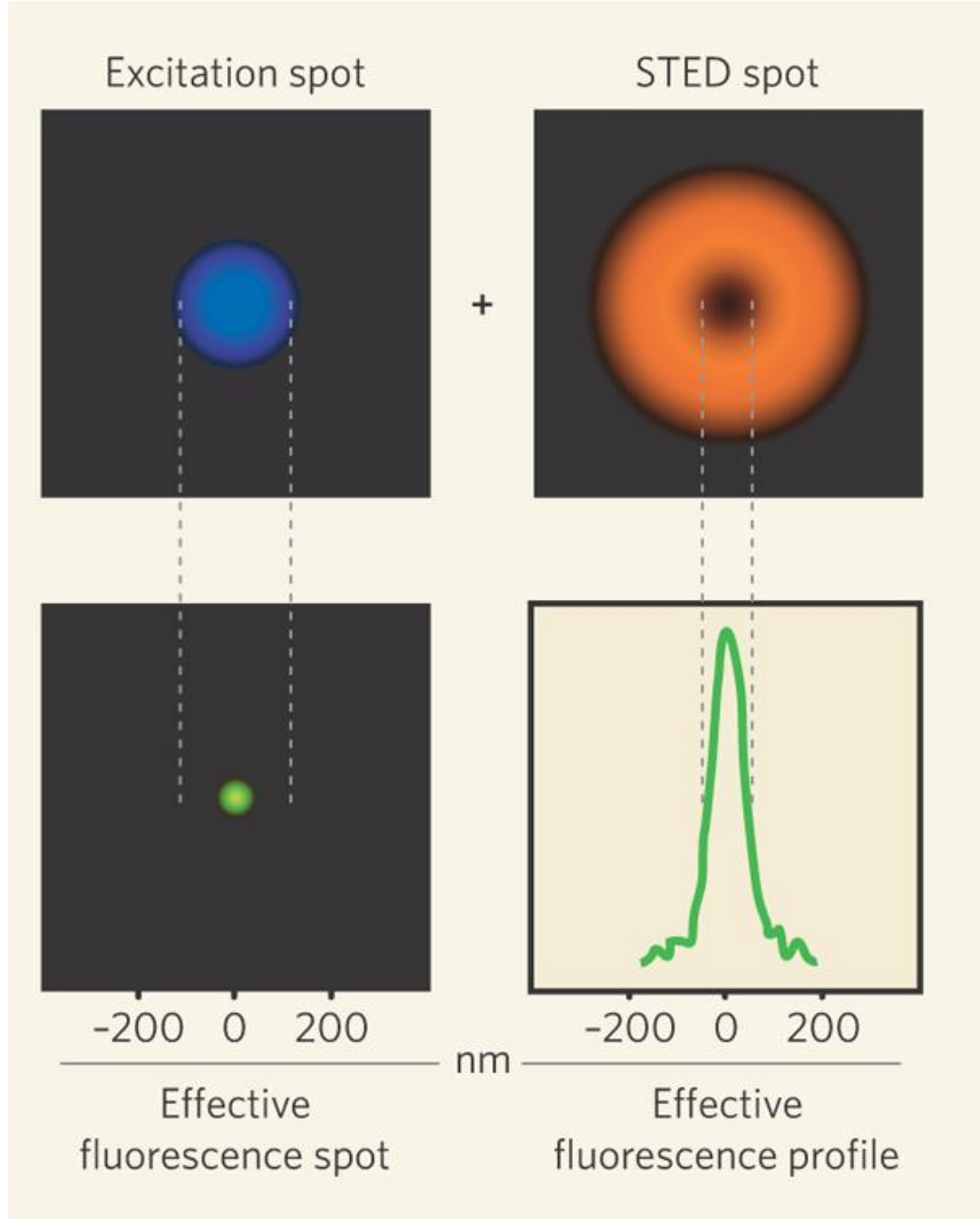
# The principle of stimulated emission depletion (STED) microscopy



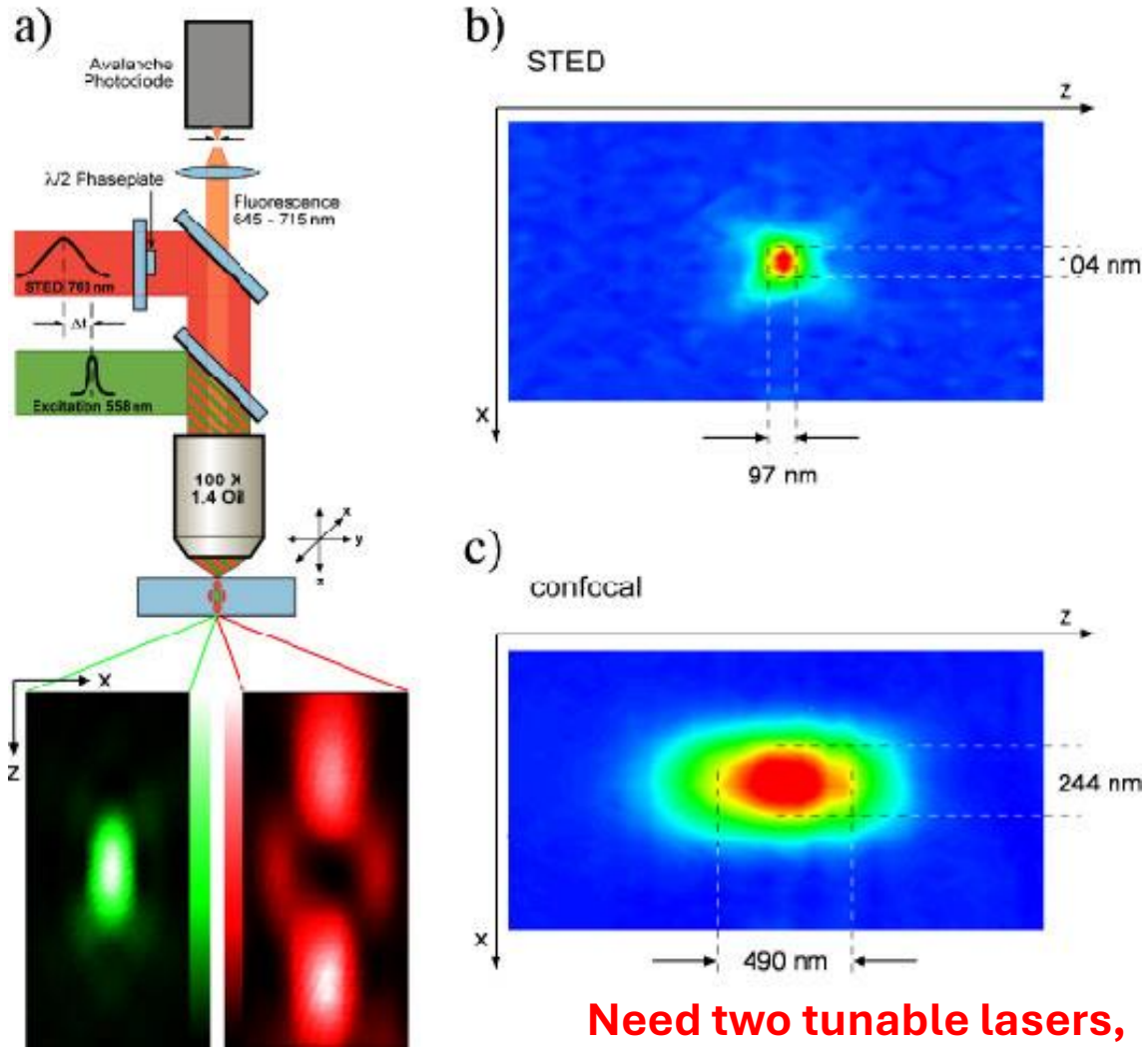

Huang, Bo, Mark Bates, and Xiaowei Zhuang. 2009. “Super-Resolution Fluorescence Microscopy.” *Annual Review of Biochemistry* 78 (1): 993-1016.

[redacted]

to a decreased size of the effective point spread function (PSF).


Excitation spot
STED spot
+
-200
0
200
nm
-200
0
200
Effective
fluorescence spot
Effective
fluorescence profile

# STED Experimental Setup and PSF's

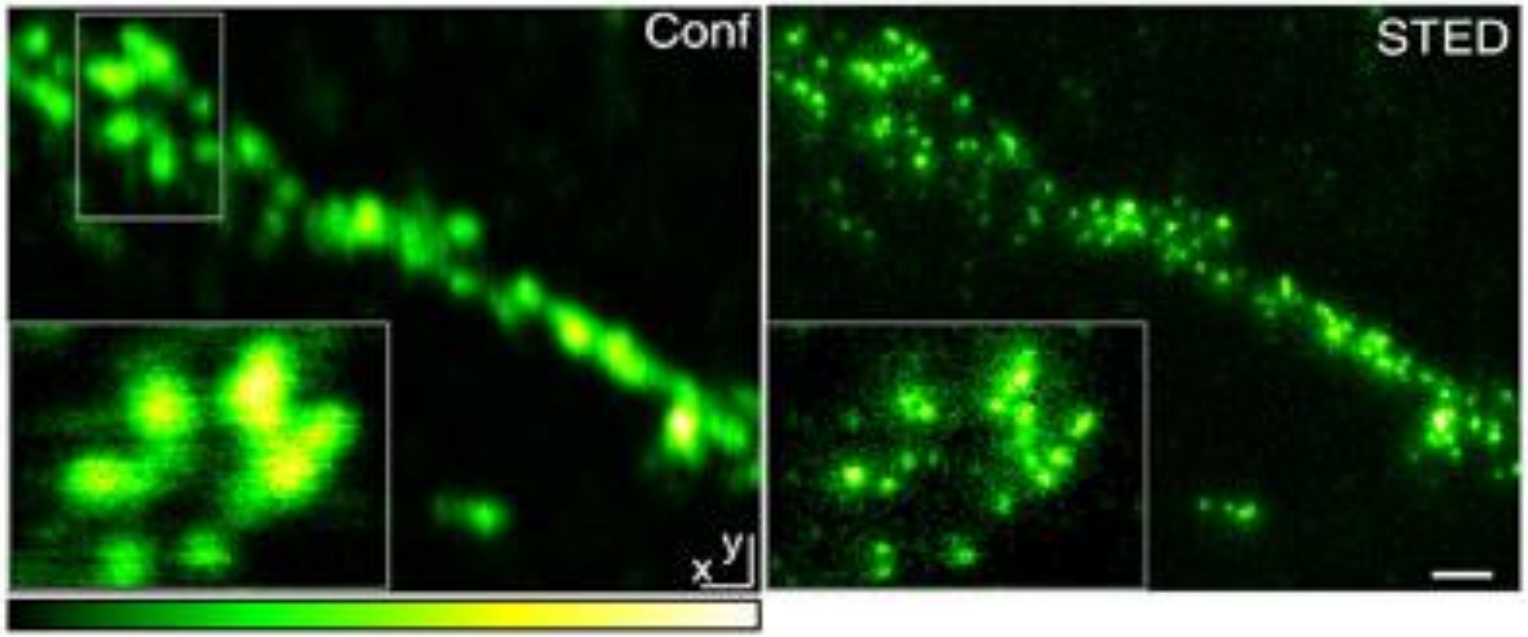



**100 nm**
**Axial and lateral**
**PSFs**

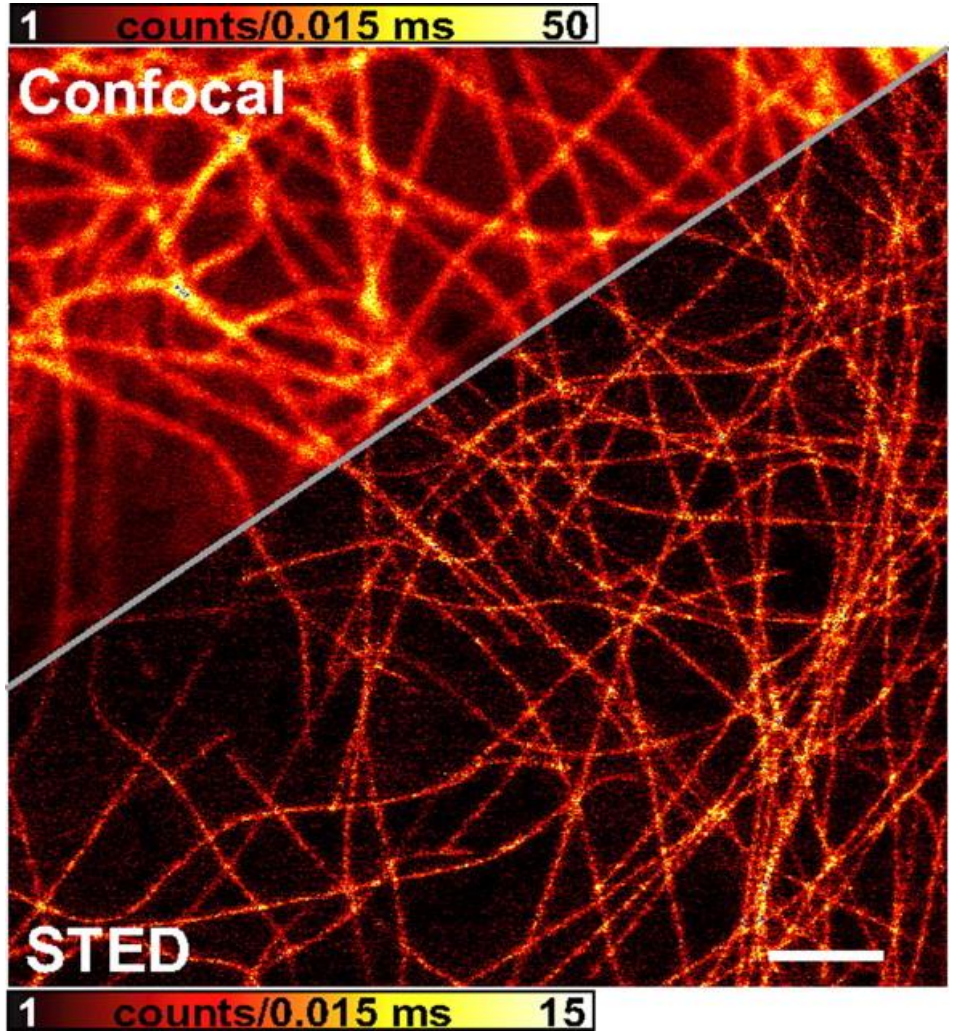
**Need two tunable lasers,**
**Overlapped spatially, temporally**
**And synchronized**

Hell et al

# RESOLUTION IMPROVEMENT IN STED

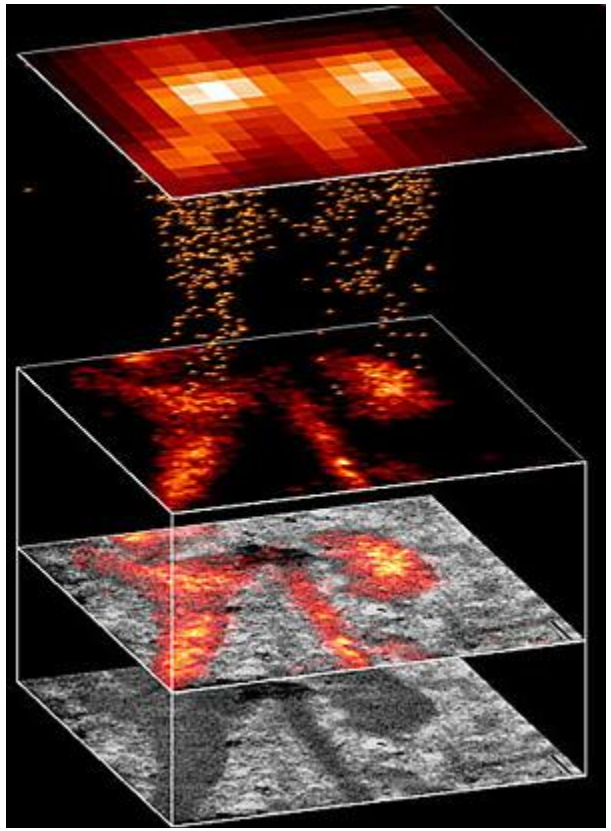


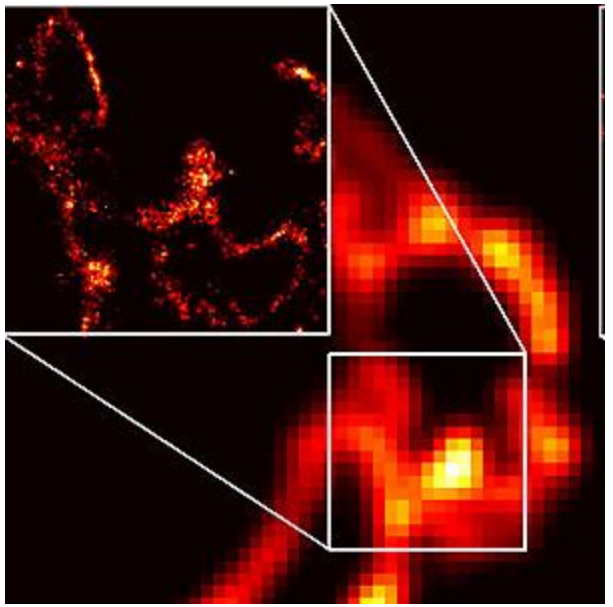
# Example: Subdiffraction resolution fluorescence imaging of microtubules

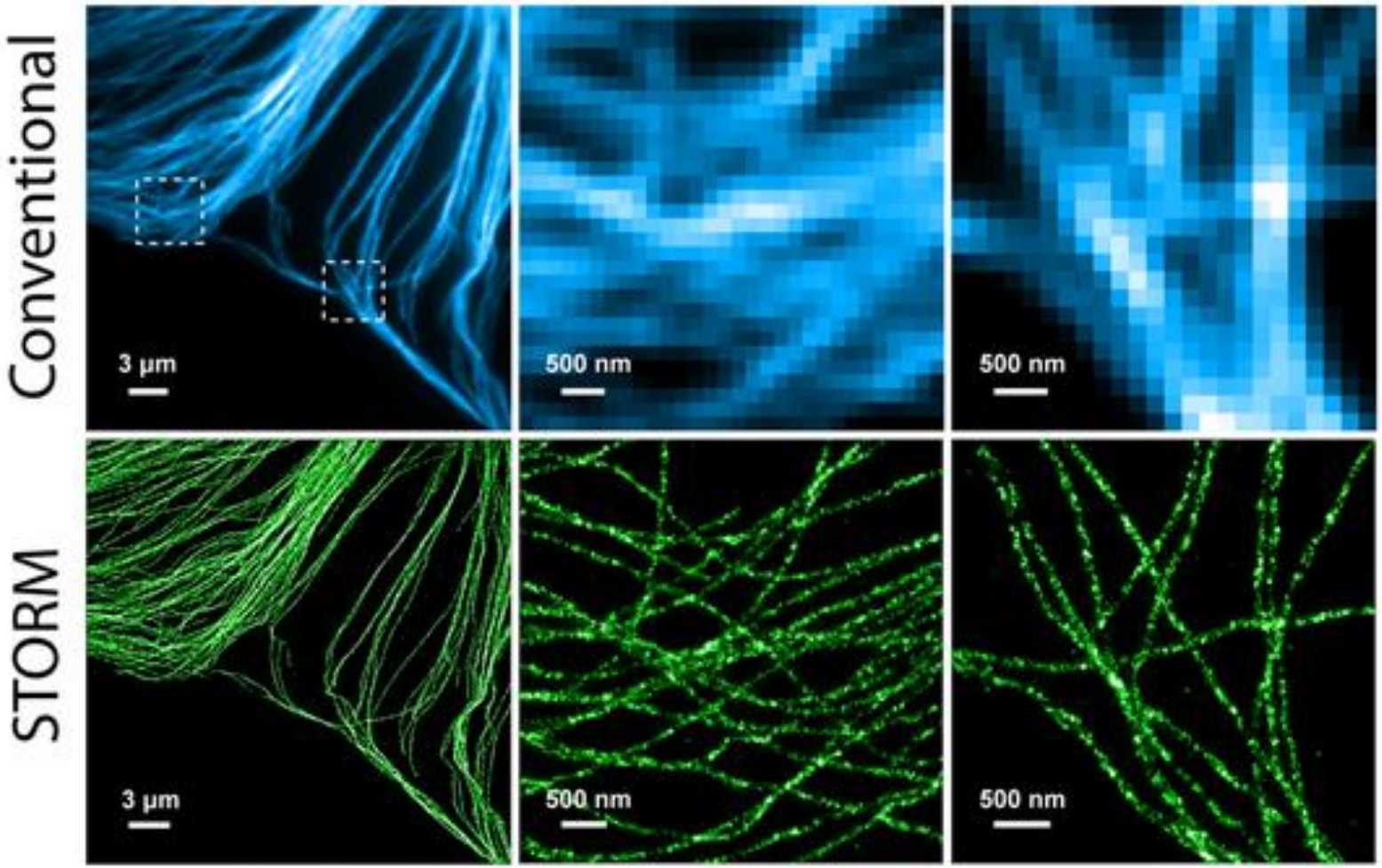



Hein B et al. PNAS 2008;105:14271-14276

# Photo-activation Localization Microscopy (PALM)

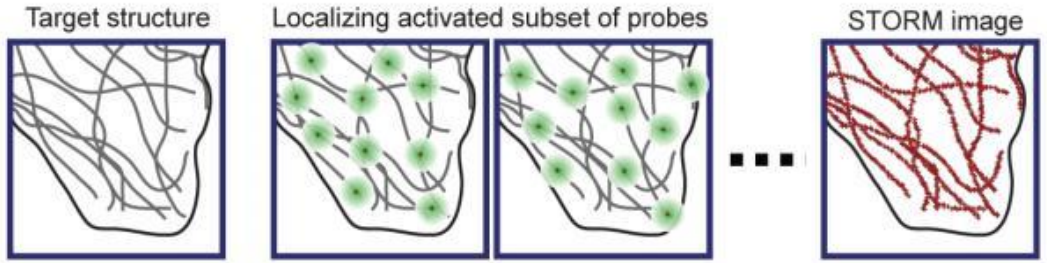




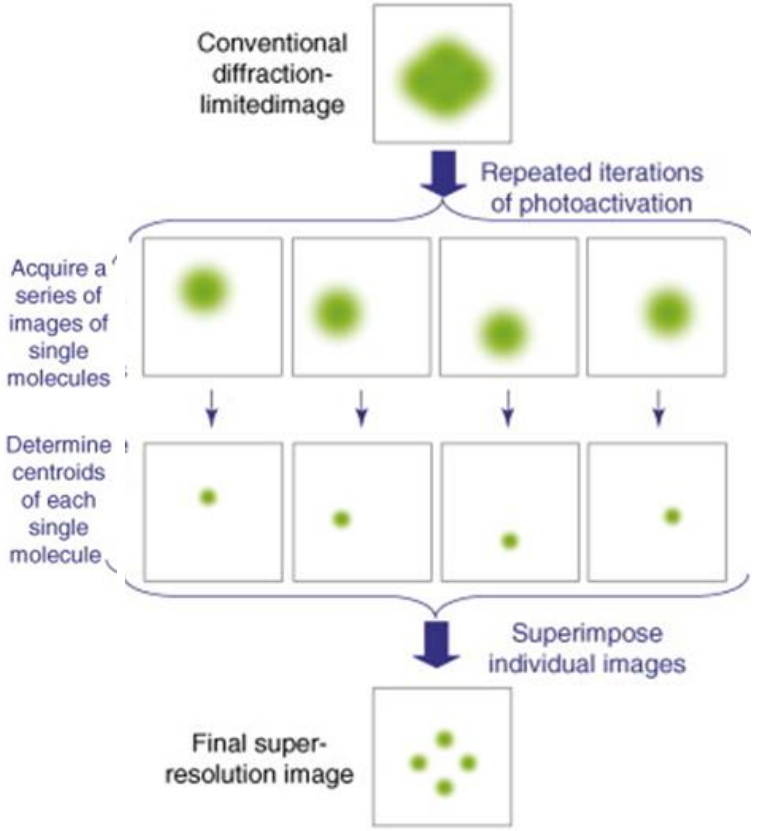

Conventional
STORM
3 µm
500 nm
500 nm
3 µm
500 nm
500 nm

# Stochastic optical reconstruction microscopy (STORM) or (fluorescence) photoactivation localization microscopy ((F)PALM)

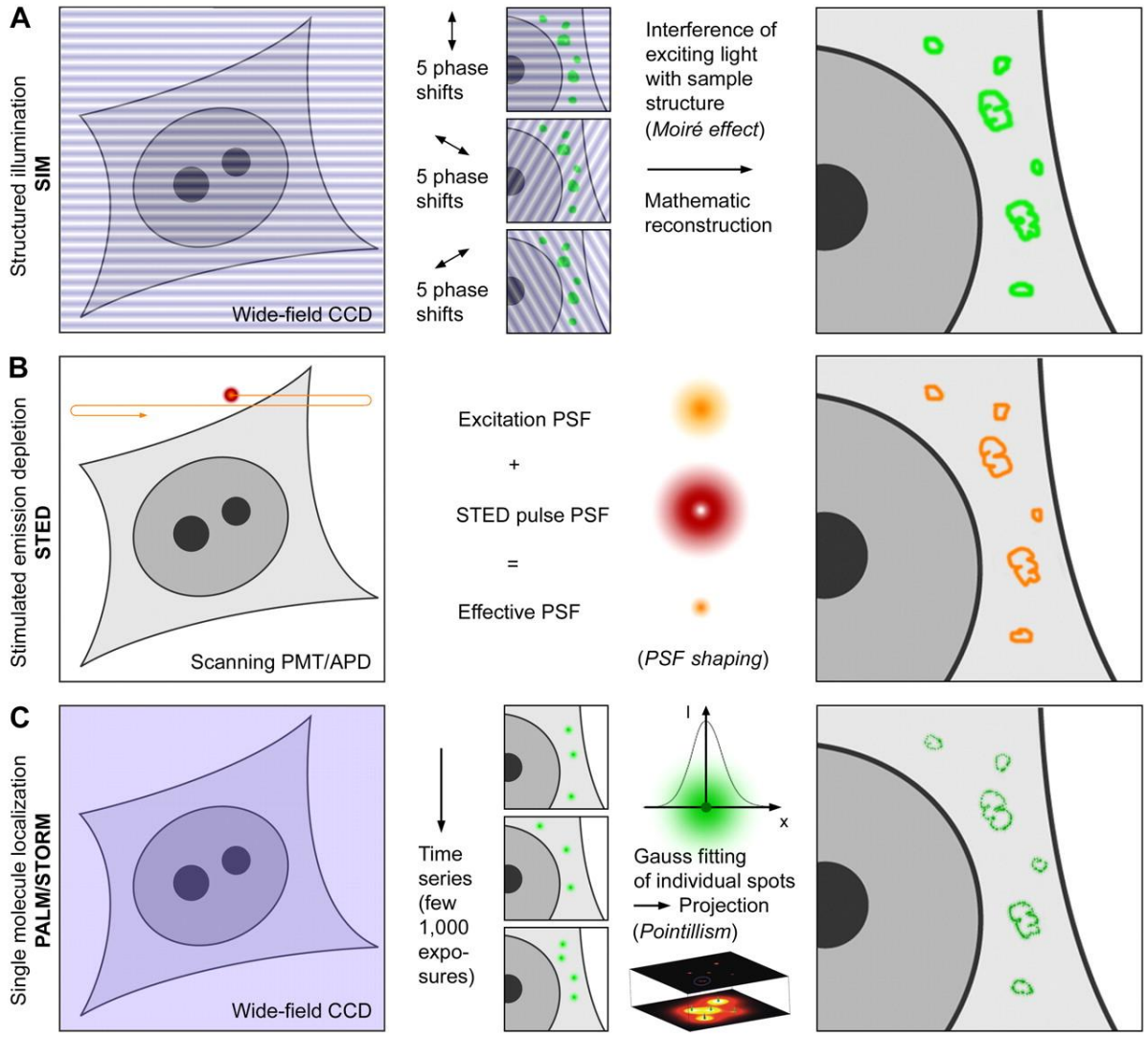
Zhuang, Xiaowei. 2009. “Nano-imaging with Storm.” *Nature photonics* 3 (7): 365-367. doi:10.1038/nphoton.2009.101.




Different fluorescent probes marking the sample structure are activated at different time points, allowing subsets of fluorophores to be imaged without spatial overlap and to be localized to high precision. Iterating the activation and imaging process allows the position of many fluorescent probes to be determined and a super-resolution image is then reconstructed from the positions of a large number of localized probe molecules.



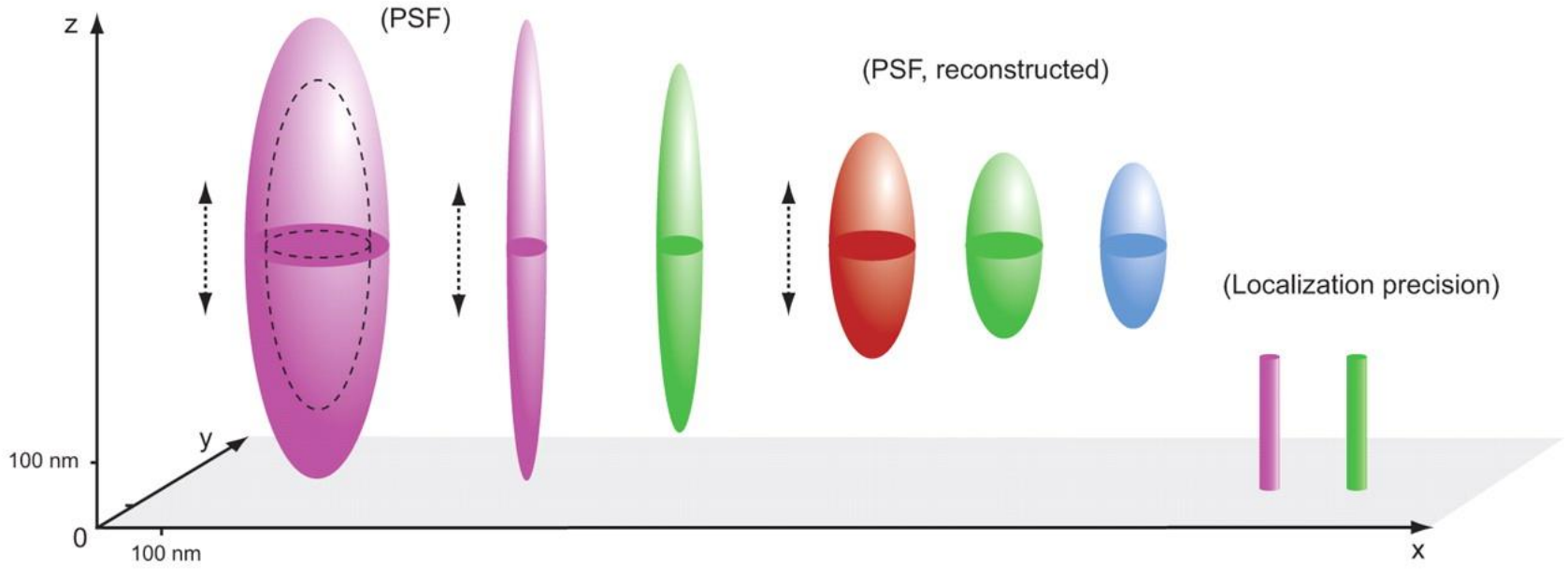
# Super-resolution imaging principles.




**Schermelleh L et al. J Cell Biol 2010;190:165-175**

# Resolvable volumes obtained with current commercial super-resolution microscopes.

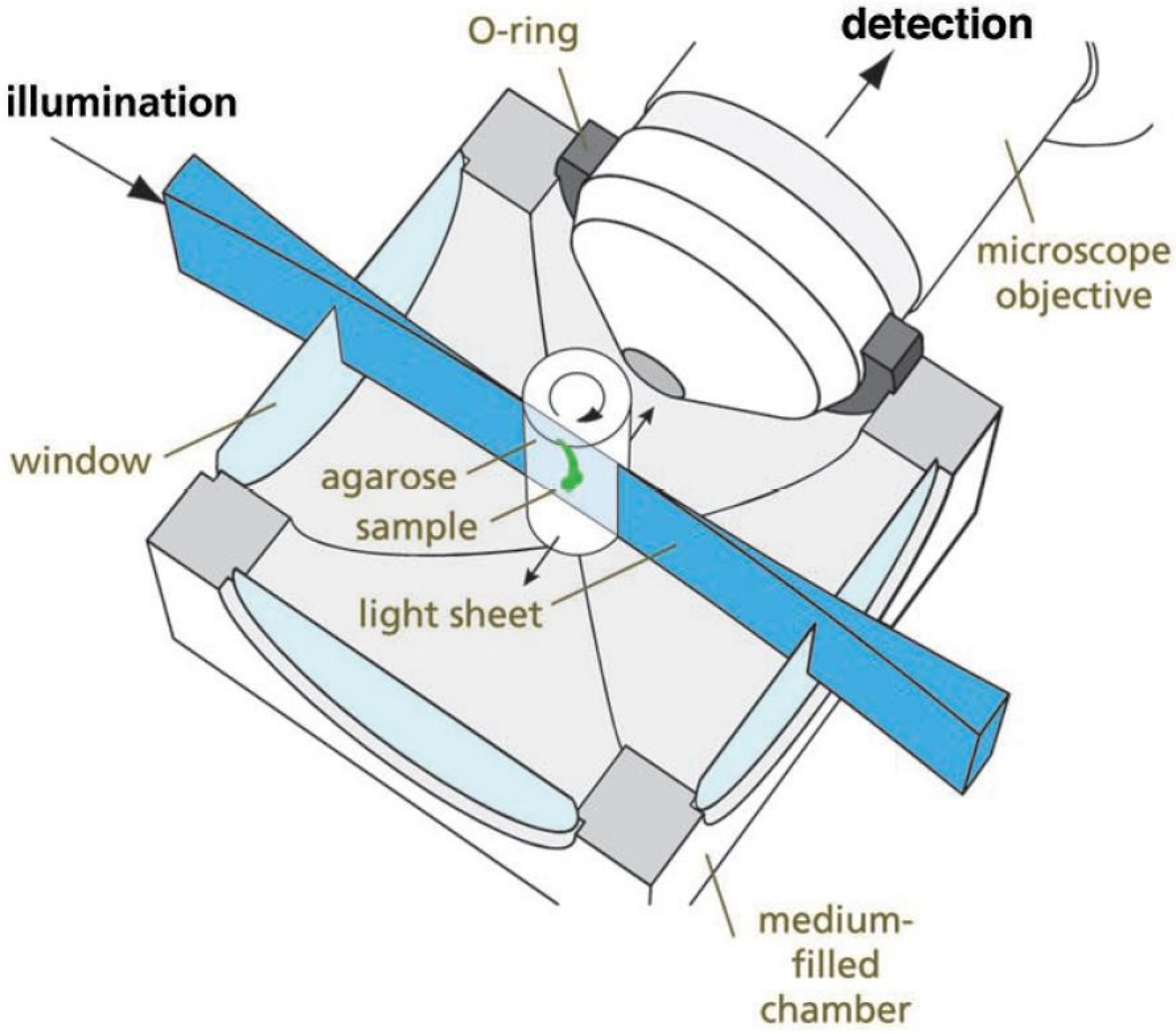
| | CLSM | STED | CW-STED | 3D-SIM | | | PALM/STORM | |
|---|---|---|---|---|---|---|---|---|
| ~$\lambda_{em}$ [nm] | 460-670 | 670 | 520 | 620 | 520 | 460 | 670 | 520 |




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ~$D_{x,y}$ [nm] | 180-250 | 60 | 70 | 130 | 110 | 100 | 30 | |
| ~$D_z$ [nm] | 500-700 | 700 | 560 | 340 | 280 | 250 | 140 | (TIRF-range) |
| ~$V_{x,y,z}$ [*$10^{-3}$ µm$^3$] | 10-23 | 1.3 | 1.5 | 3.0 | 1.8 | 1.3 | 0.1 | |

Schermelleh, Lothar, Rainer Heintzmann, and Heinrich Leonhardt. 2010. “A guide to super-resolution fluorescence microscopy.” *The Journal of Cell Biology* 190 (2) (July 26): 165 -175. doi:10.1083/jcb.201002021.

# Selective Plane Illumination Microscopy (SPIM)

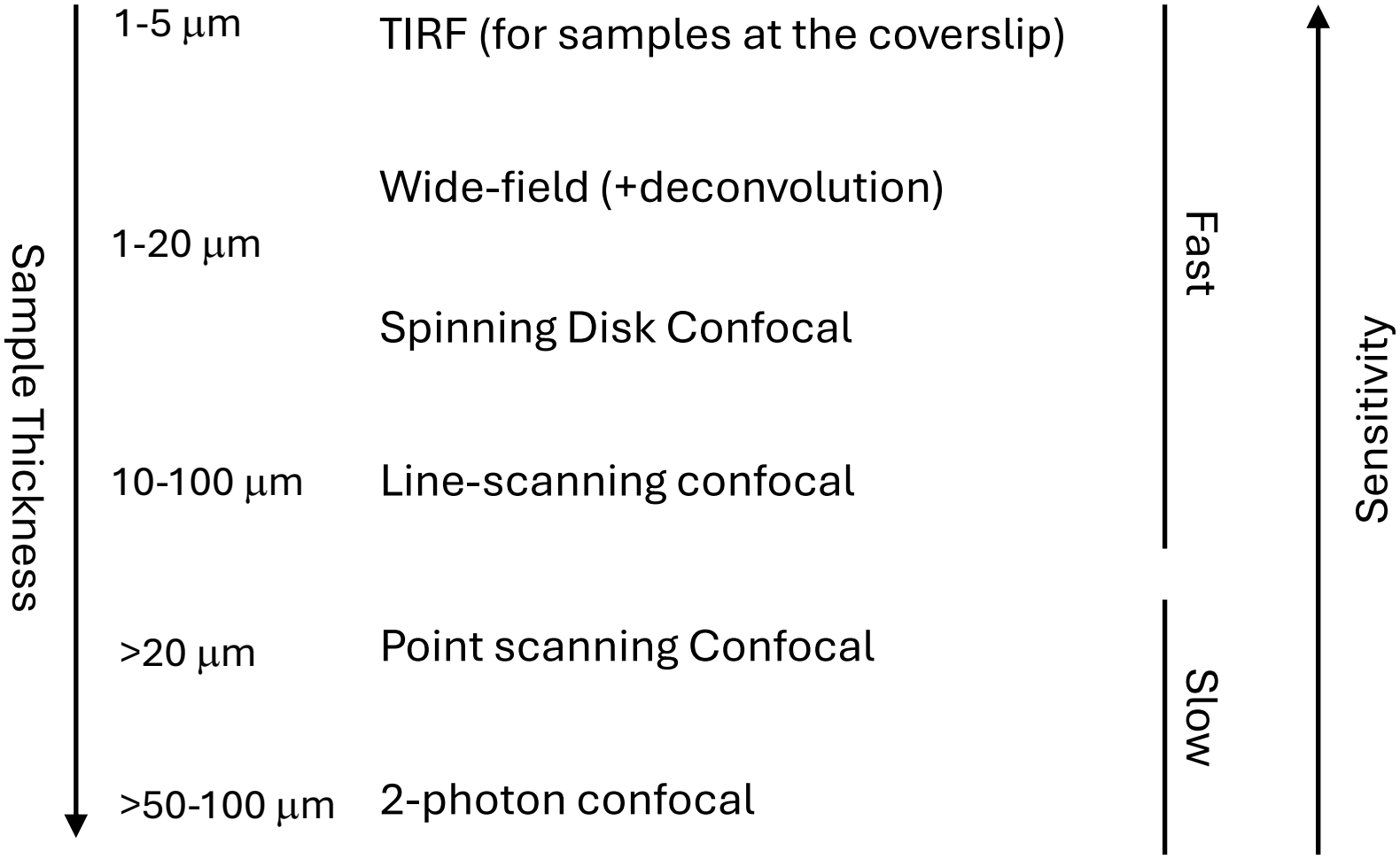


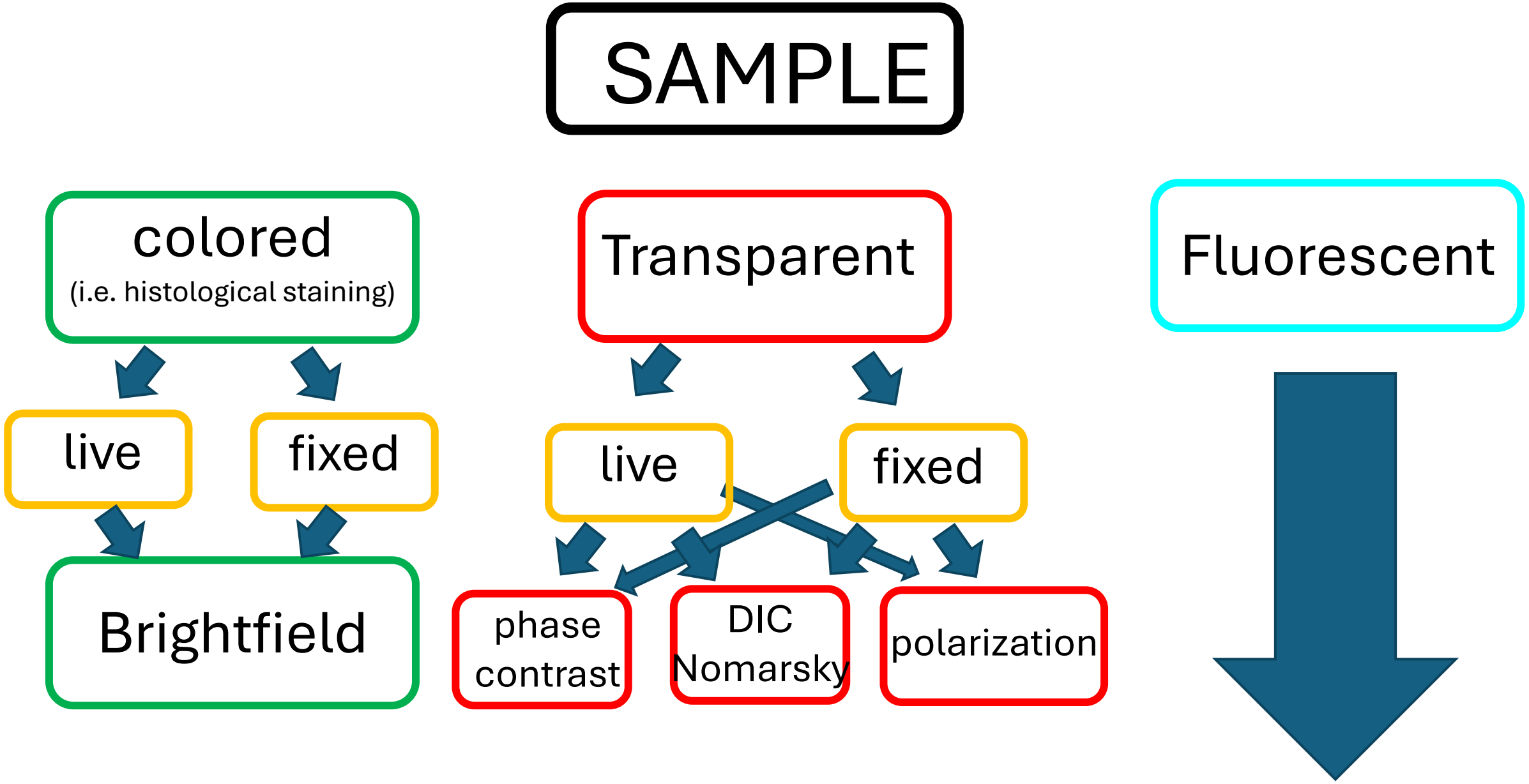

Jan Huisken,* Jim Swoger, Filippo Del Bene, Joachim Wittbrodt, Ernst H. K. Stelzer*, **Optical Sectioning Deep Inside Live Embryos by Selective Plane Illumination Microscopy,** Science, Vol. 103, p. 1007-1009, 2004

# Summary/comparison

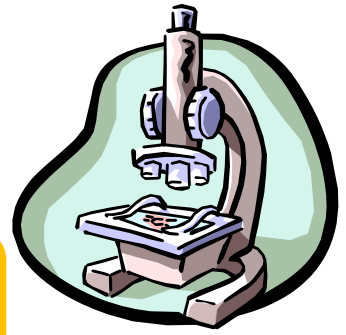
| method | excitation | detection | sectioning | use |
|---|---|---|---|---|
| Wide field | Whole sample | Whole sample | No sectioning | Simple fluorescence samples |
| confocal | Whole sample | One z-plane | 350-500nm | High contrast images, optical sectioning |
| 2-Photon | One z-plane | One z-plane | 500-700nm | Deep tissue imaging, optical sectioning |
| | | | | |
| FLIM/FRET | | | | Protein interactions |
| FRAP + photoactivation | 405 laser (UV) | | | dynamics/mobility |
| TIRF | Only bottom plane | Only bottom plane | 150-200nm | Membrane dynamics |

# WHICH IMAGING TECHNIQUE SHOULD I USE?




SAMPLE
colored
(i.e. histological staining)
live
fixed
Brightfield
Transparent
live
fixed
phase contrast
DIC Nomarsky
polarization
Fluorescent

# SAMPLE fluorescent



## live

### “Slim”

- epifluo
- TIRF

### “Thick”

- Confocal Spinning disk/LSM
- Structured illumination /Light sheet
- STED

## fixed

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- STORM / PALM
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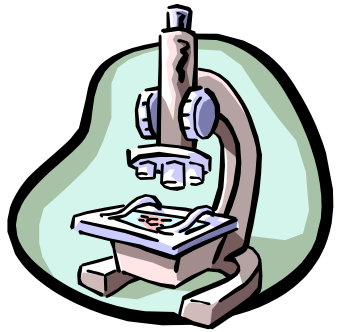
Please consider the single techniques limitations!

# Timeline of the Microscope



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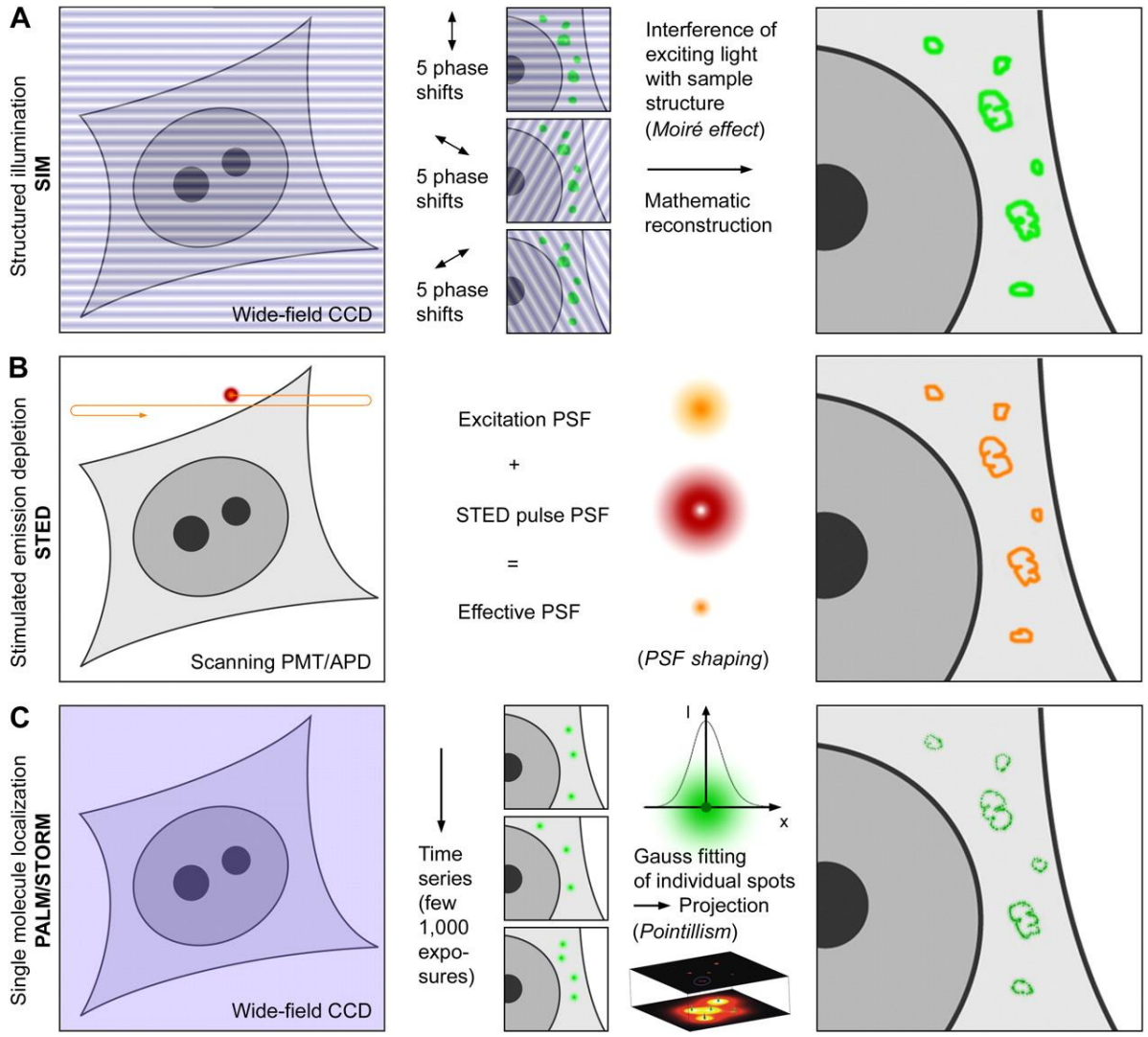


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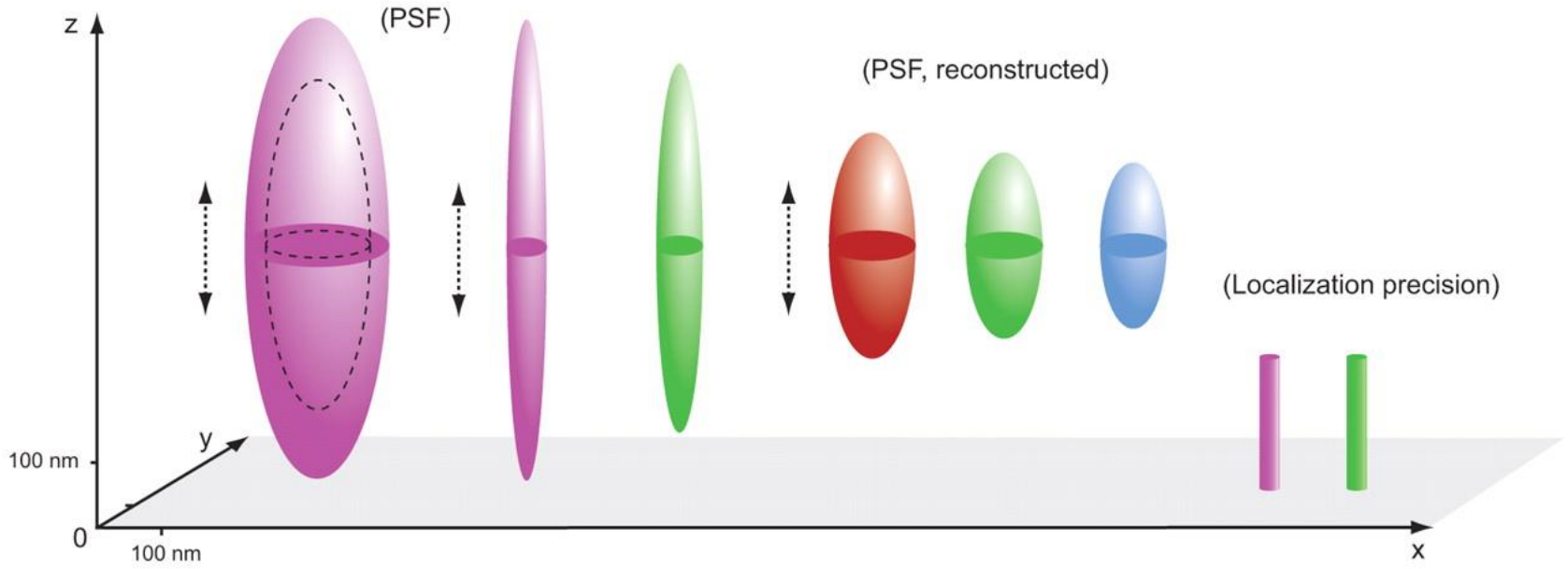


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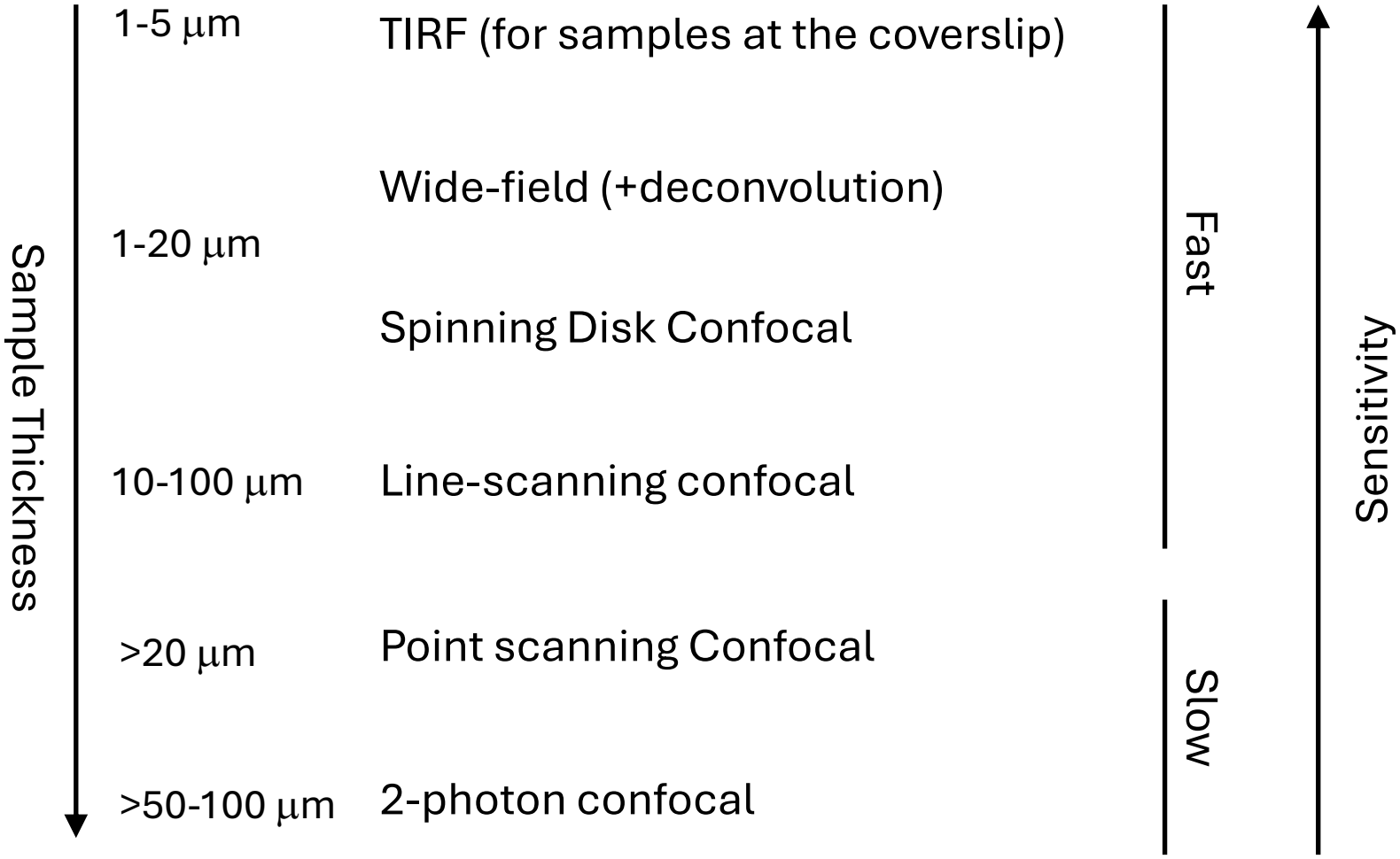

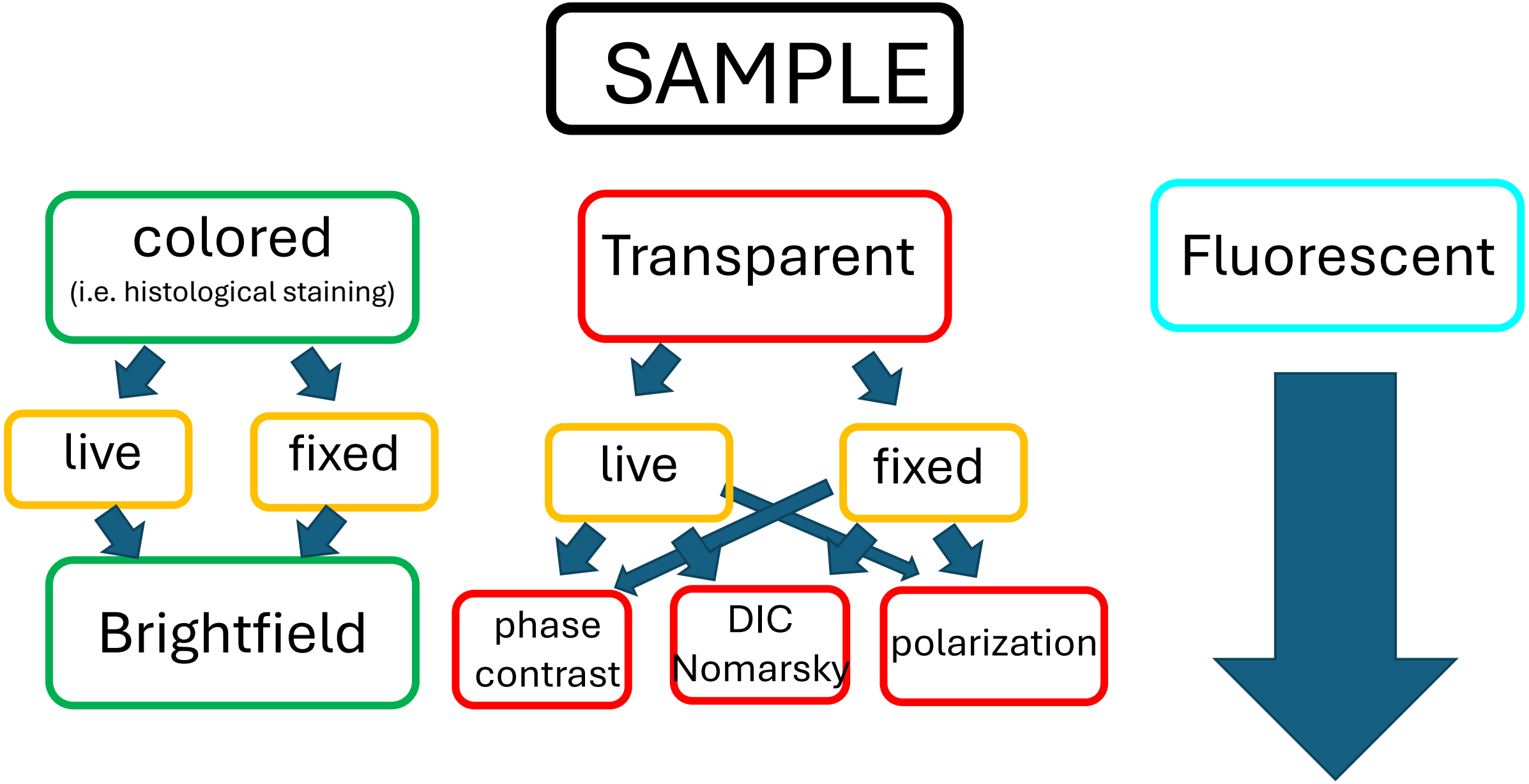
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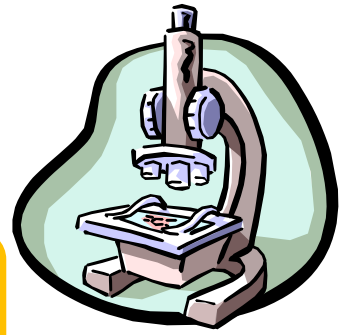
(i.e. histological staining)
live
fixed
Brightfield
Transparent
live
fixed
phase contrast
DIC Nomarsky
polarization
Fluorescent

# SAMPLE fluorescent



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