BIOLOGICAL PHYSICS

CHAPTER 5 –OPTICAL MICROSCOPES

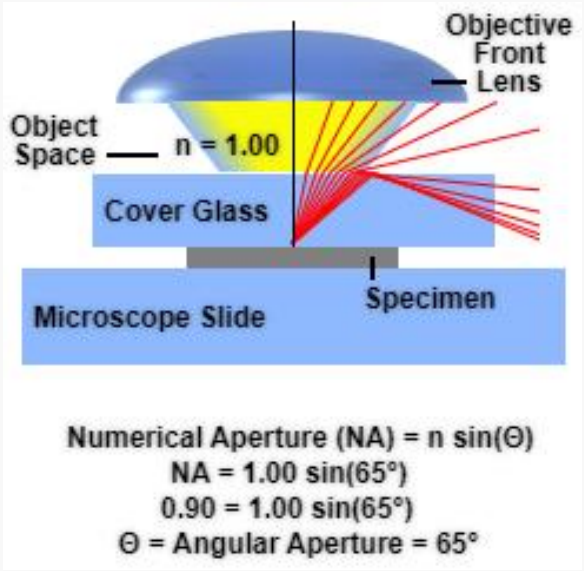
Poopalasingam Sivakumar
Department of Physics, SIUC

Recapitulate (f-number and Numerical Aperture

$$f_{number} = \frac{f}{D}$$

The NA of a lens is exhibited the difference in solid angles (cone) of light accepted.

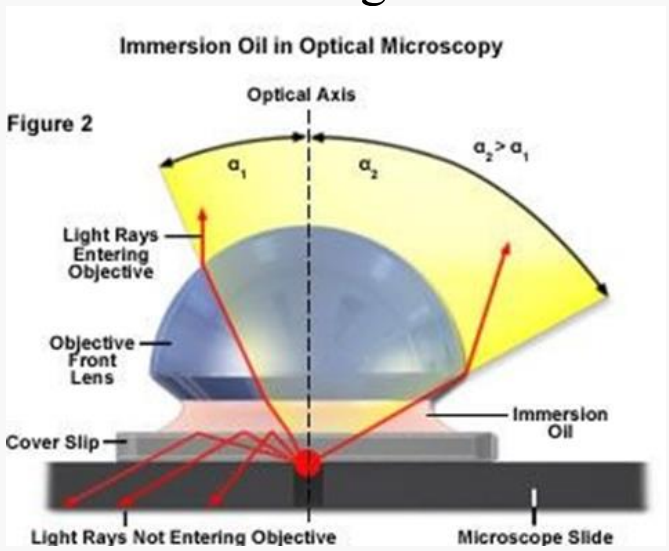
$$image\ brigtness \propto \frac{1}{(f_{number})^2} = (NA)^2$$

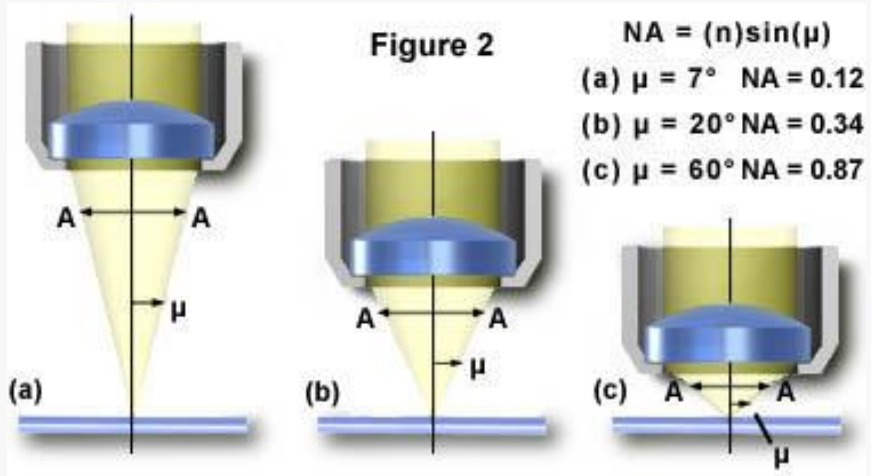


Lens in a medium

$$NA = n \sin \alpha$$

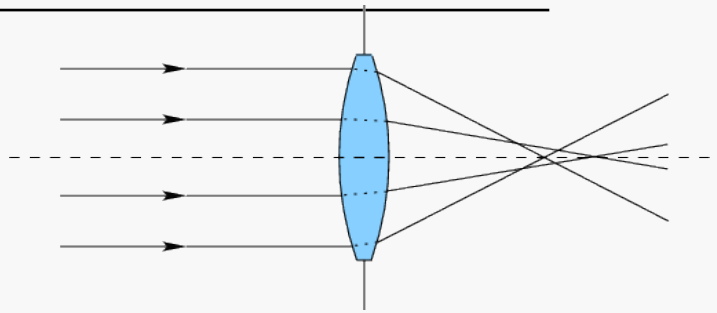
Here, n is the index of refraction of the intervening medium b-t object and lens and α is half-angle.

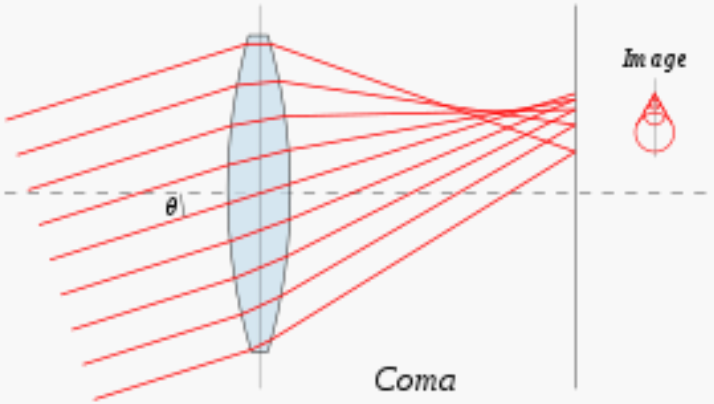


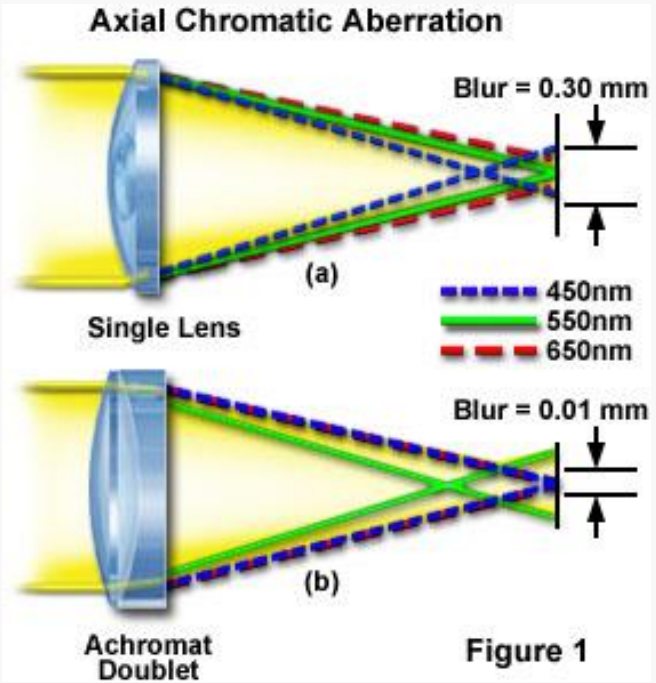


Recapitulate of Last Class (Aberration)

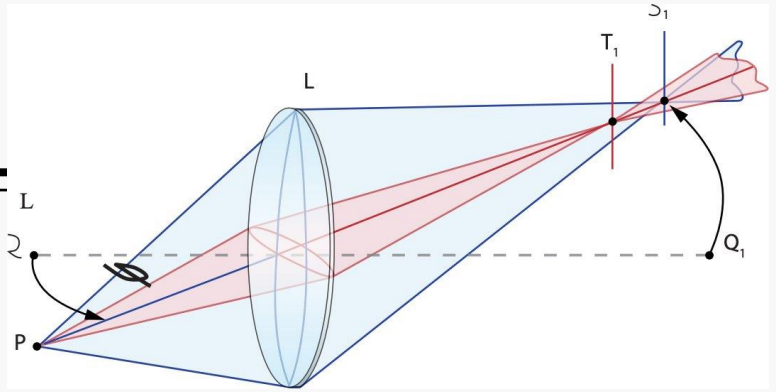
- **Spherical aberration** due to higher order terms of sine function in Snell's law.
- **Chromatic Aberration**: failure of lens to focus all the colors to the same point.
- **Comatic (Coma) Aberration** from rays from an off-axis point of light where appearing to have a tail (coma) like a comet. It is due to certain optical design or imperfection in the lens or other components that results in off-axis point source.



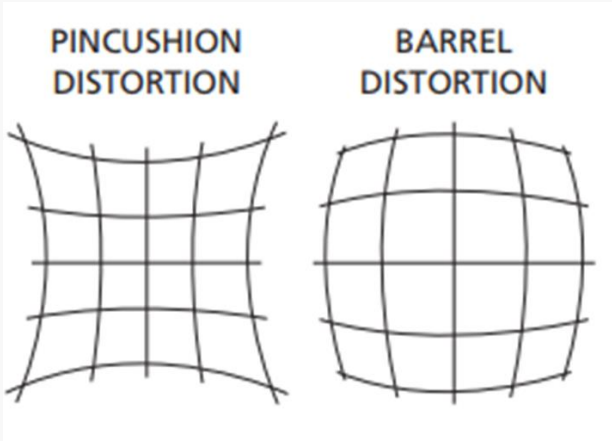


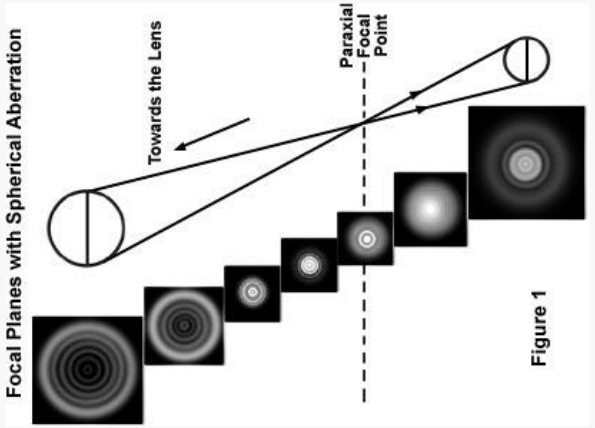


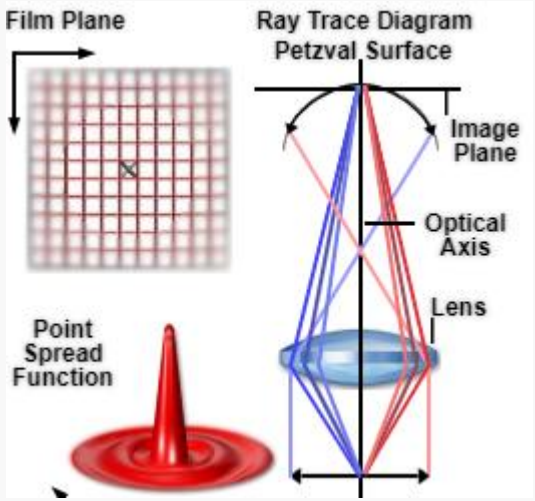
Recapitulate of Last Class (Aberration)



- **Astigmatism Aberration**: similar to comatic aberration, however not sensitive to aperture size and depend more strongly on the oblique angle of the light beam. (vision defect is a result of different lens curvature in different plane)
- **Geometric Distortion Aberrations** is typically observed stereoscopic microscopy. It arises due to differences in focal lengths and magnification via different part of the lens. It doesn't present in thin lens but can occur in thick simple lenses.
- **Field Curvature Aberration**: image is perfect only on curved image plane.
- **Focus Depth and Spherical Aberration**: formed by the constructive and destructive interference of light wave







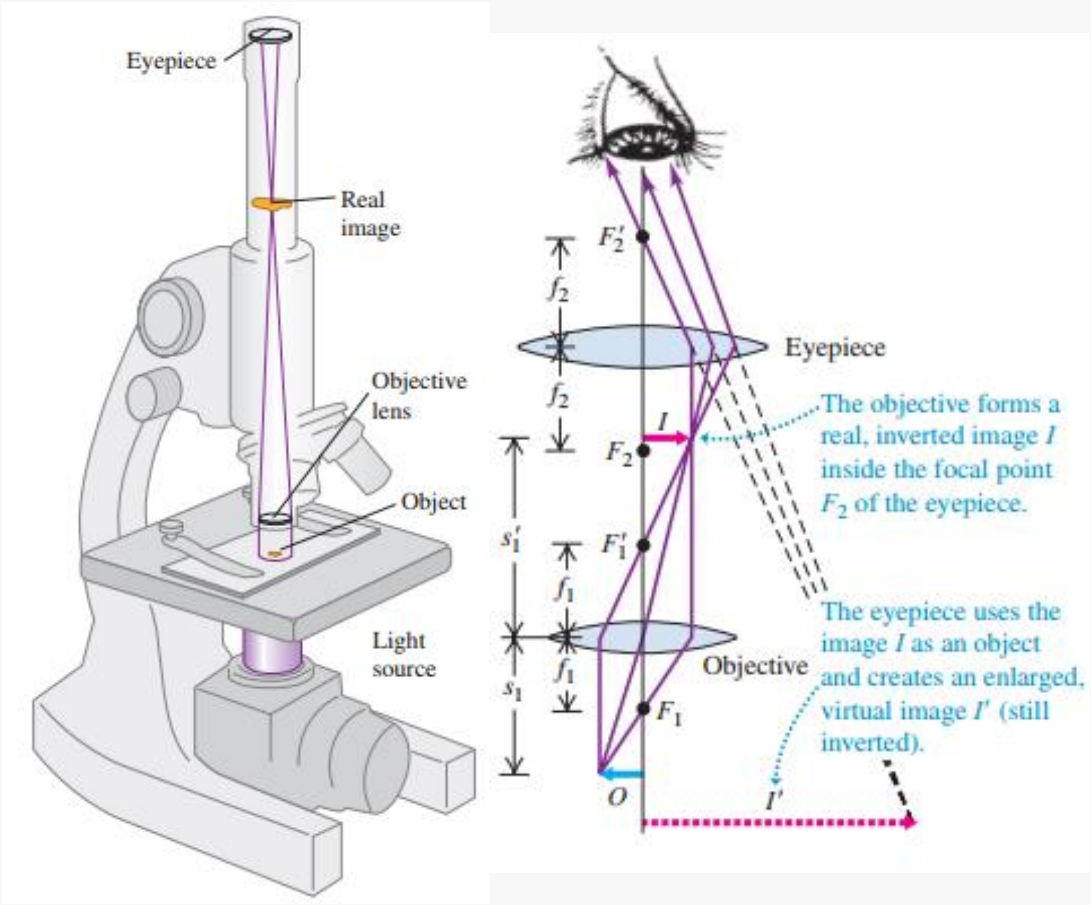
Recapitulate of Last Class (Optical Microscopy)

Magnification of microscopes is from product of two factors:

- Lateral magnification M_1 of the object.
- 2nd is angular magnification M_2 of the eyepiece, which relates the angular size of the virtual image.

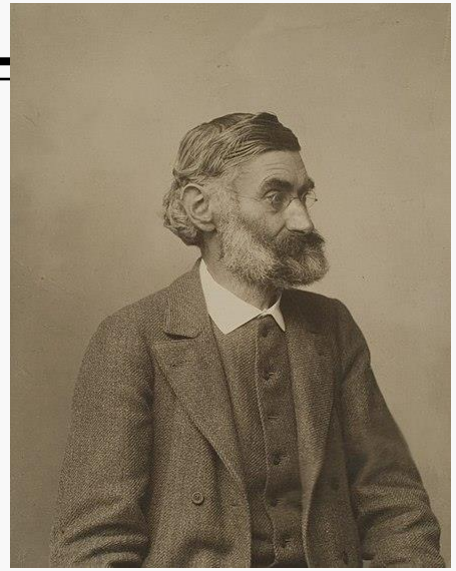
$$M = M_1 M_2$$

$$M = \frac{(25\ cm)\ s_i}{f_1 f_2}$$



Theory of Image Formation

- Although compound microscope theories were invented in 1800, however, the experimental limitation had been prevented the development of compound microscope due to higher aberration effect.
- Improvement in optical apparatus in 19th century allowed to minimize the spherical aberration. Microscopes that can perform in the match of theoretical limit were possible.
- Abbe was hired by Carl Zeiss to improve the manufacturing process of optical instrument in 1866. Until then, image of microscope produced based on trial and error; some having exceptional optical performance but others having undesirable features.



Ernst Karl Abbe (1840-1905), German Physicist

Resolution limit formula engraved in Ernst Abbe Memorial in Jena

Theory of Image Formation

- Abbe invented the apochromatic lens which eliminates both the primary and secondary color distortion.
- Abbe condense used for microscope illumination and designed the first refractometer.
- The modern theory of image formation of microscope was founded in 1873 by the German Physics Ernst-Abbe.

Abbe: The microscope image is the interference effect of a diffraction phenomenon.

Microscope by Carl Zeiss (1879) with optics by Abbe.

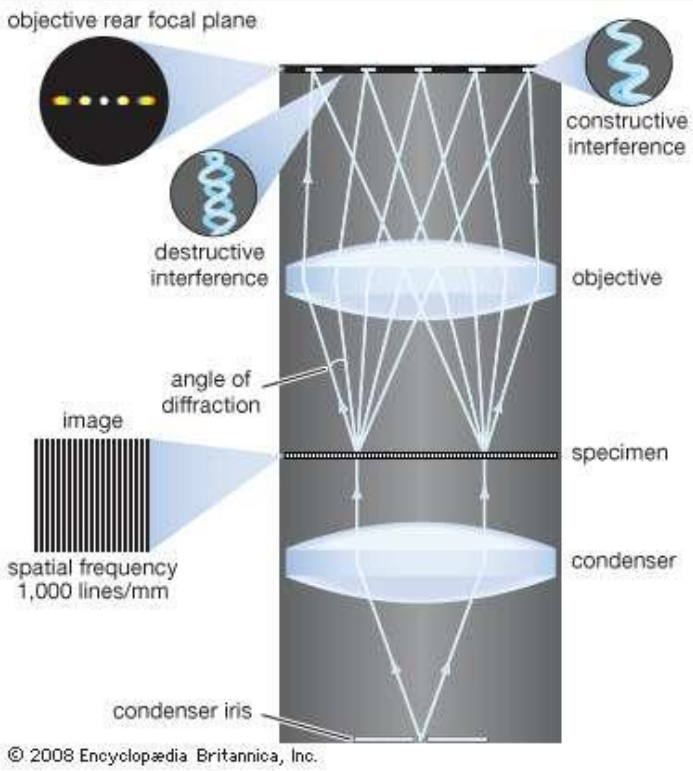
Theory of Image Formation (Abbe Theory)

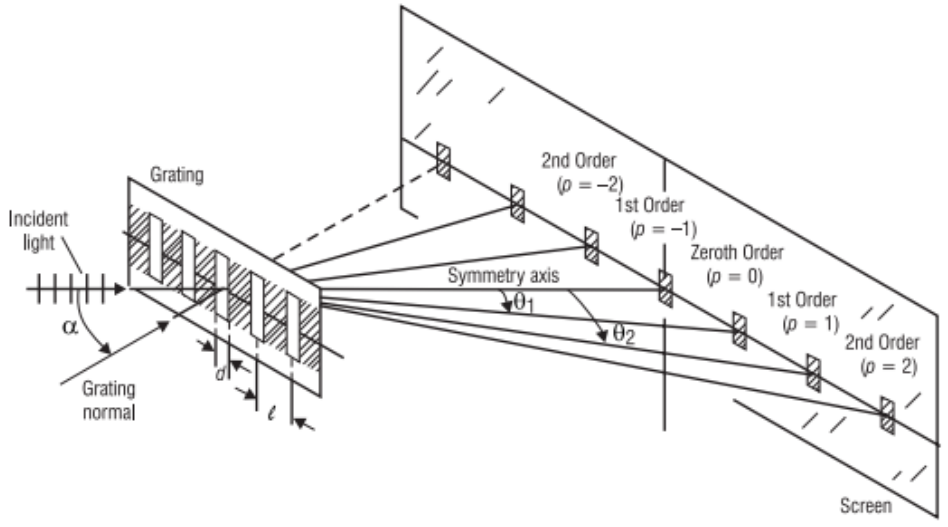
- Image of a specimen is formed due to diffraction. The specimen is seen by the light as a complex superposition of grating with varying grating constants.
- The intensity pattern on the screen is a superposition of the diffraction effect from each slit as we as interference effect of the light from all the adjacent slits.
- If the distance between the grids decreases (i.e. the grating constant decreases) the diffraction angles will increase.

$$\ell(\sin\alpha + \sin\theta_p) = p\,\lambda$$

Where α is angle of incidence of light and θ_p is angle of locating the Pth -order fringe. For incident on the grating along the grating normal $\alpha = 0$

$$\ell \sin\theta_p = p\,\lambda$$





Theory of Image Formation

$$\sin\alpha = m\frac{\lambda}{d}$$

When objective just accept the zero and the two first order maxima, m =1

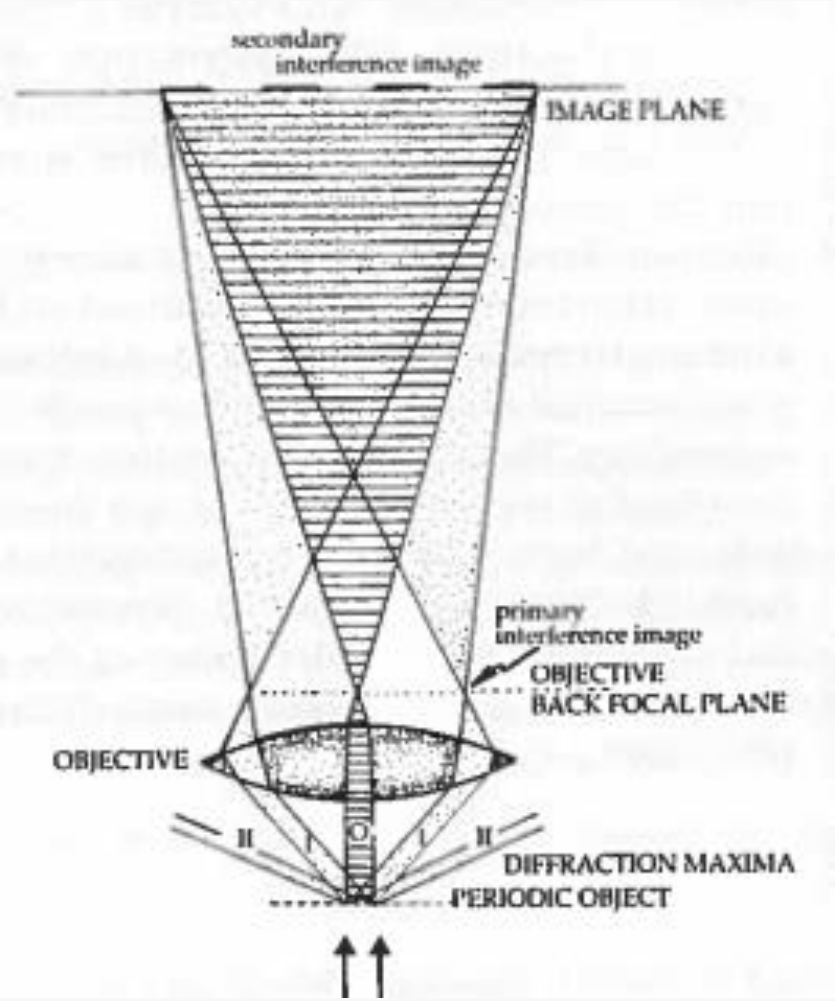
$$d = \frac{\lambda}{\sin\alpha} \rightarrow d = \frac{\lambda}{n\sin\alpha} = \frac{\lambda}{NA}$$

Where n is the refractive index of the medium between grating and lens and NA numerical aperture of the lens.

Abbe Resolution for microscopy

$$d = \frac{\lambda}{2NA}$$

Later refined by Lord Raleigh in 1896 as separation necessary between two Air patterns.



Resolution and Airy Pattern

- Resolution of optical microscope is the smallest distance between two point on a specimen can be distinguished as two separate entities. We can relate it to magnification of microscope; however, it is associated with the diffraction phenomena due to nature of light.

- When light from various point of a specimen passes through the object and reconstituted as an image. These various points of specimen appear in the image as a pattern (not points) know as **Airy pattern** (named after Sir George Biddell Airy, mathematician and Astronomer, British). This phenomenon due to diffraction or scattering of light as it passes through minute parts and spaces in the specimen and circular back aperture of objective.

- The limit at which **Airy disks** can be resolved into separate entities is often called the **Rayleigh criterion** (named after John William Strutt, 3rd Baron Raleigh, theoretical and experimental physics).

- One way to increase the resolving power of the microscope is to use immersion liquid.

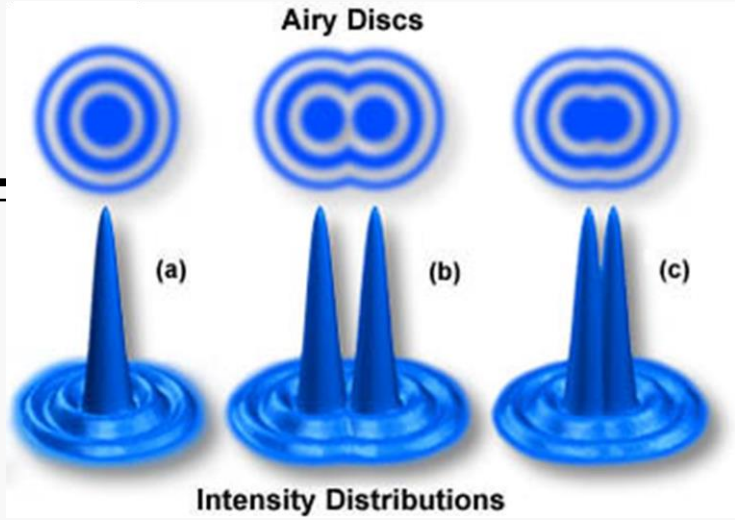


Illustration of hypothetical **Airy disk.** Central maximum (0^{th} order) surrounded by 1^{st} , 2^{nd} ,3^{rd} , etc. decreasing brightness

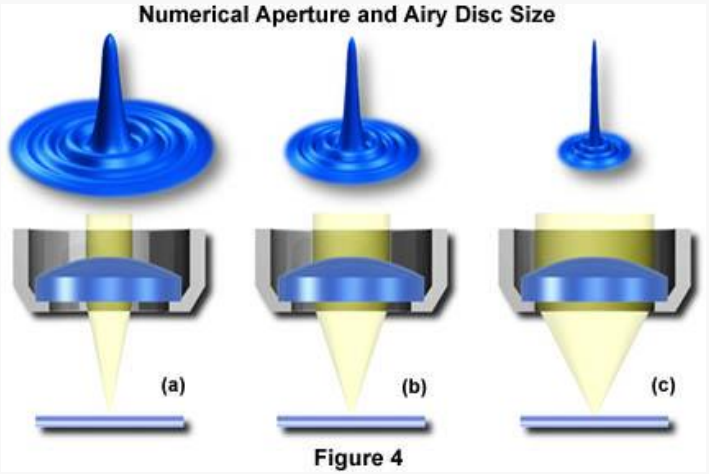
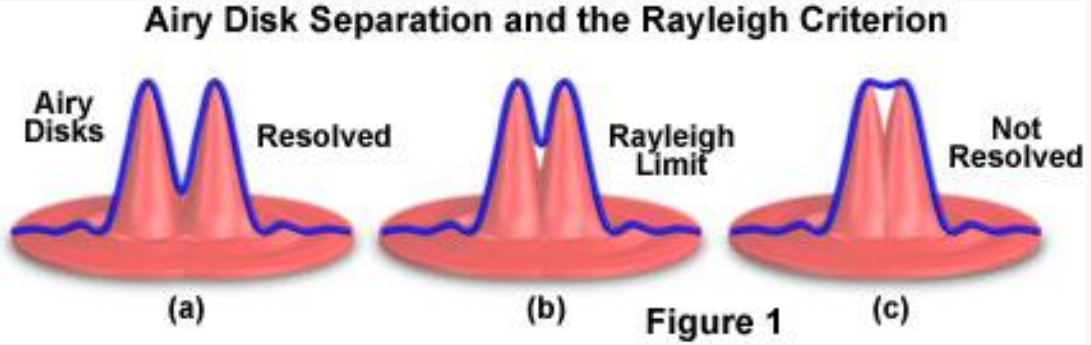


Illustration of effect of NA on size of **Airy disk** using same focal length but differing NA.

Resolution and Airy Pattern



Generally, Raleigh criterion is considered as accepted criterion for minimum resolvable detail.

Single slit $\sin\theta = \frac{\lambda}{d} \rightarrow$ Resolution $R = \frac{\lambda}{2\,NA}$

Circular Aperture $\sin\theta = 1.22\frac{\lambda}{d} \rightarrow$ Resolution $R = 0.61\frac{\lambda}{NA}$

Microscope Resolution $R = 1.22\frac{\lambda}{NA_{obj}+NA_{cond}}$

Where NA_{obj} is the NA of the objective and NA_{cond} is the NA of the condenser. One can notice that the magnification doesn't appear as a factor in any of those equations, because NA and wavelength of light determine the resolution.

Optical Microscopy

- highly sensitive light detector such as a high-efficiency charge-coupled device (CCD), or sometimes a photomultiplier tube (PMT) system (a total amplification of $\sim 10^8$ is possible) in which used in confocal microscopy.
- Another technique used as a single detector is avalanche photodiode (APD) which is an alternative to a PMT. The total multiplication of signal is $> 10^3$ which is less than a PMT, however capable of single photon detection with an advantage of a much smaller footprint, permitting in some cases a 2D array .
- Most of the sensitive light microscopes using electron multiplying CCD(EMCCD) detection or complementary MOS (CMOS) technology.

Photomultiplier Tube (PMT)

- A photomultiplier tube, useful for light detection of very weak signals. The detector works by amplifying the electrons generated by a photocathode exposed to a photon flux.

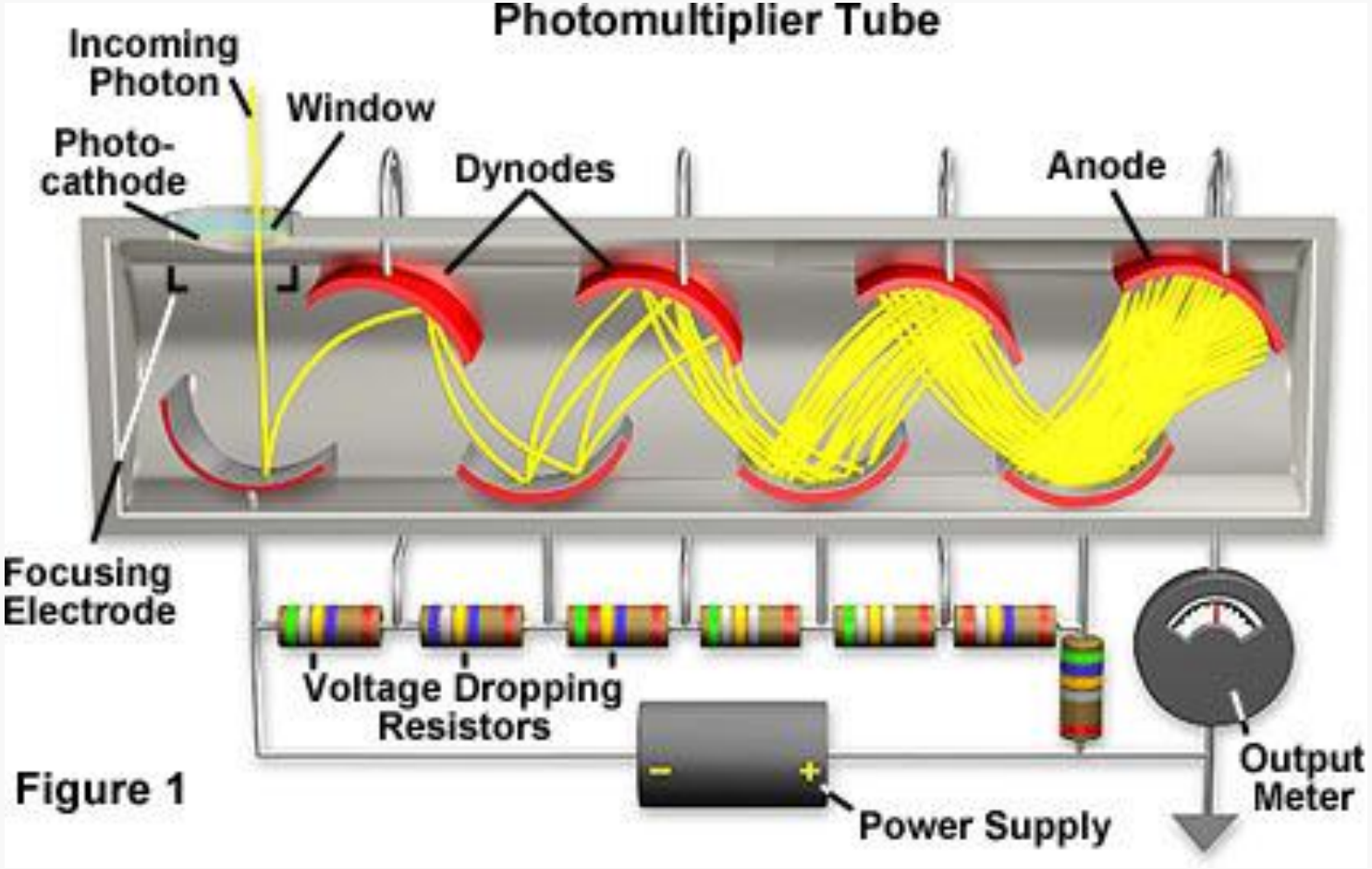


Figure 1

Optical Microscopy

- Non-fluorescence Microscopy: essential part of instrument for life science however, the major drawback is poor contrast since most of the material (living organism) is water (~60%). Several adaptations to the basic to improve the image contrast.

- Fluorescence Microscopy: although fluorescence has several advantages over other techniques this has its disadvantages too. The most prominent being photobleaching, quenching and photodamage. This leads to low SNR and degrade image contrast. Till date, there has been not a single technique that can completely eliminate these effects

Bright-Field microscopy

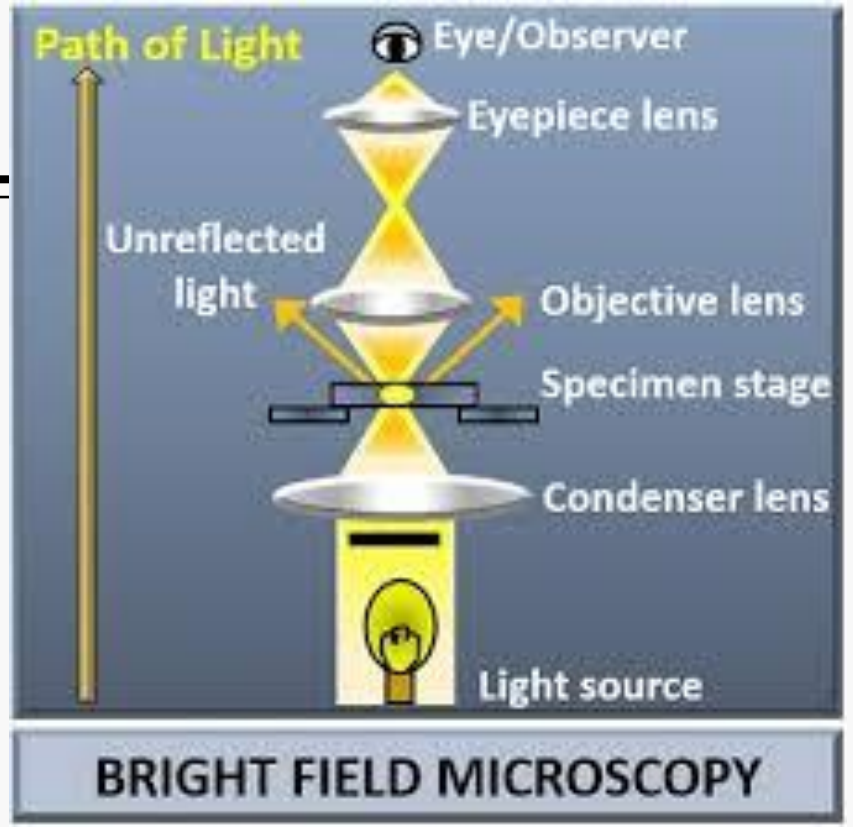
Simplest of all the optical microscopy where it used an illumination techniques.

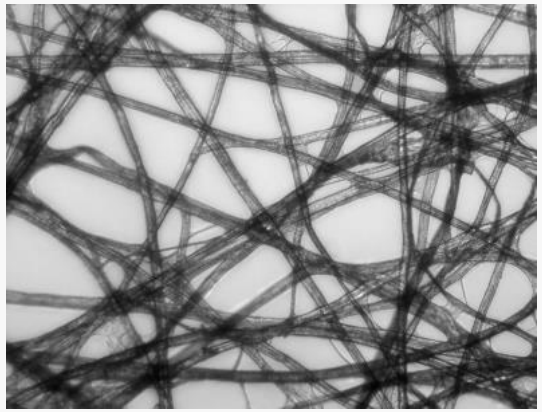
Sample illumination is transmitted (illuminated from below and observed from above) white light, and contrast in the sample is caused by attenuation of the transmitted light in dense areas of the sample.

Background is bright, but specimen is dark

Disadvantages:

- Very low contrast. And limited magnification (around 1300 ×)
- Cannot be used to observe living specimens of bacteria.
- Requires a strong light source for high magnification application. Intensity can produce and damage the specimens or kill living microorganism





Bright-field illumination image of Tissue Paper

Dark-Field microscopy

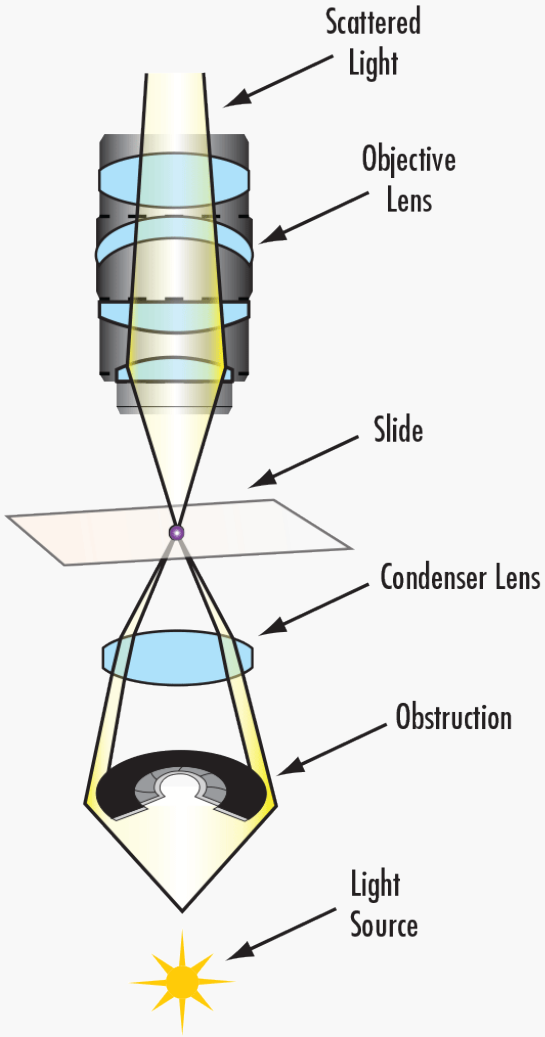
Produces an image that is the opposite of bright-field:

- Bright specimen on a dark background

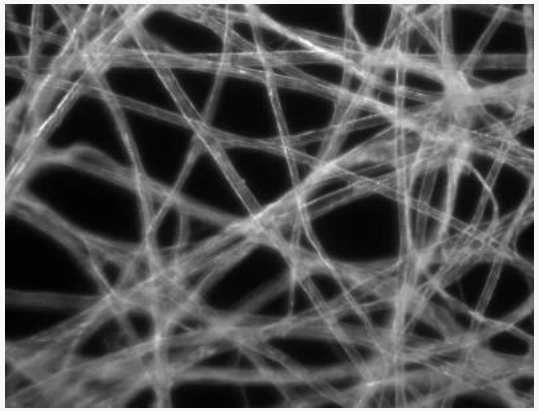
How it works:

- Direct (unscattered) light is blocked
- Only light scattered by the specimen reaches the objective

The primary goal of this technique is to enhance the contrast of an unstained sample. It is a incredibly powerful, yet simple, for live cellular analysis.

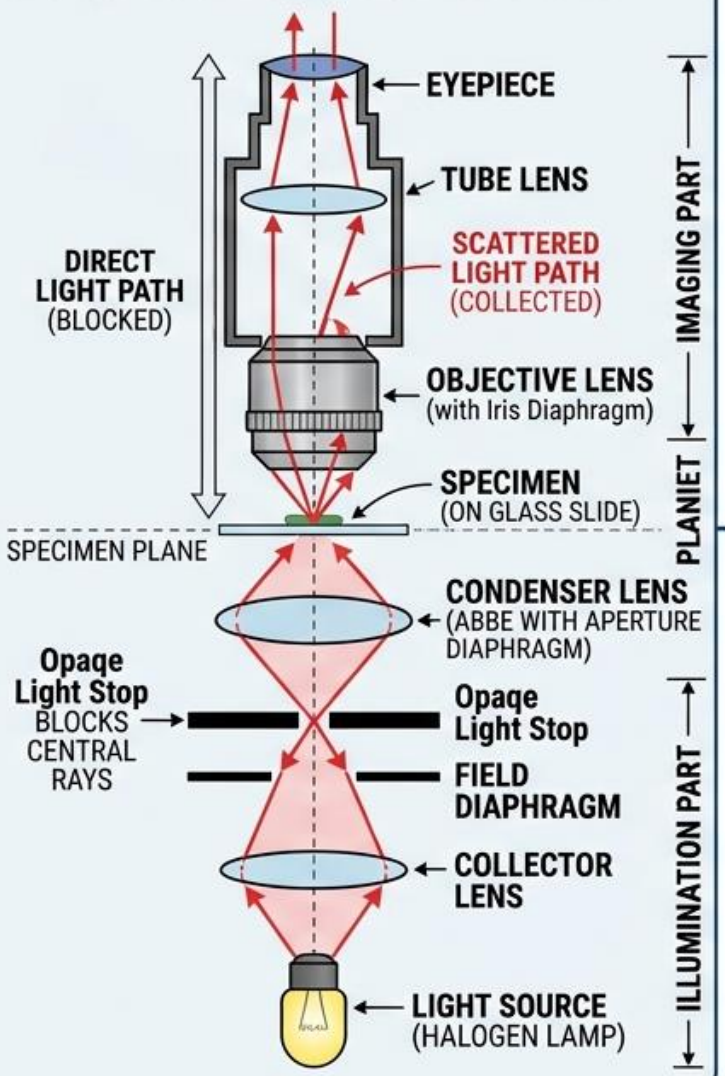


Dark-field illumination image of Tissue Paper



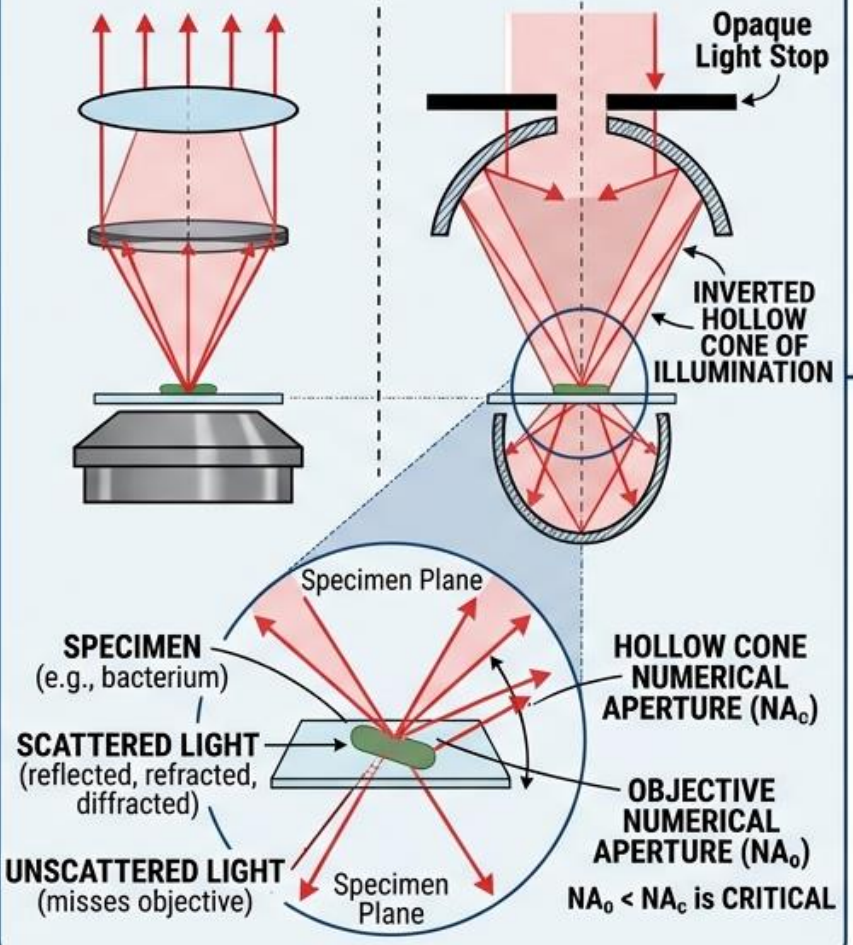
SCHEMATIC DIAGRAM: DARK-FIELD MICROSCOPY SYSTEM

1. SYSTEM OVERVIEW & LIGHT PATH

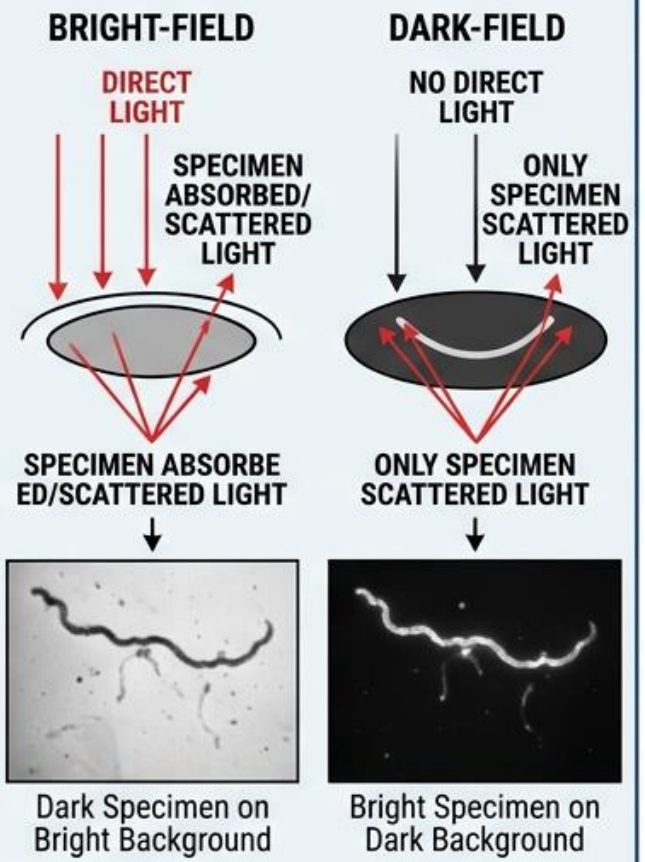


2. KEY COMPONENT: DARK-FIELD CONDENSER

(Comparison)



3. FORMATION OF IMAGE



BASIC PRINCIPLE:
By blocking direct light, only scattered light from optical discontinuities in the specimen forms the image, significantly enhancing contrast for unstained, transparent samples.

Phase Contrast microscopy

- It was developed by Frits Zernike in 1934. It is most frequently used method in biology which used for cell culture and live cell imaging. It utilizes differences in refractive index inside a biological sample, which varies as a function of spatial location (1.35- 1.45)
- The phase of the light propagating through a region of sample, length of sample (vertical) Δz is few microns, will be retarded. Retardation of phase when passing through the cells roughly around a **quarter of a wavelength** $\left(\frac{\lambda}{4}\right)$ to that light passing through the largely aqueous environment in between cells. So, the optical length become

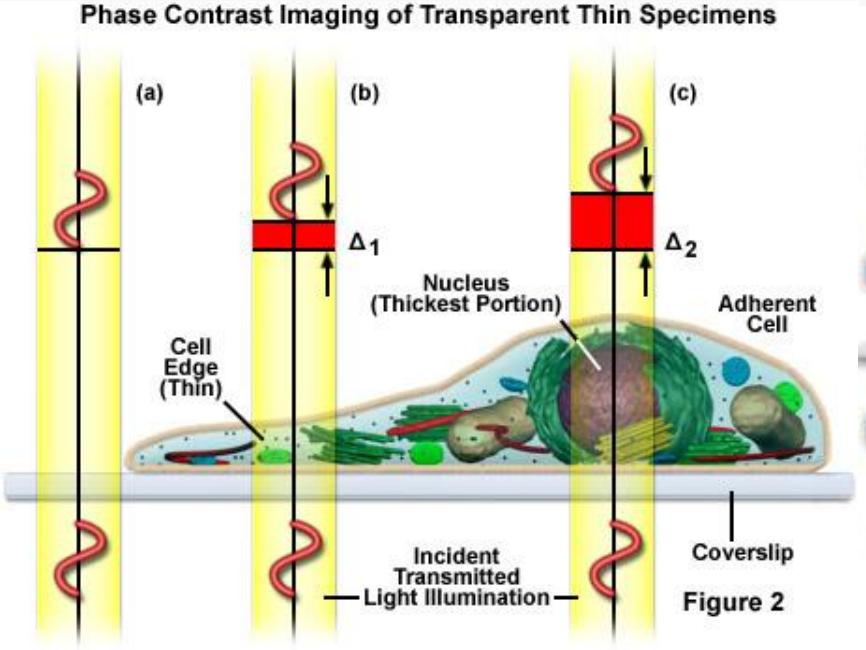
$$\Delta z = \frac{\lambda n_w}{4(n_t - n_w)}$$

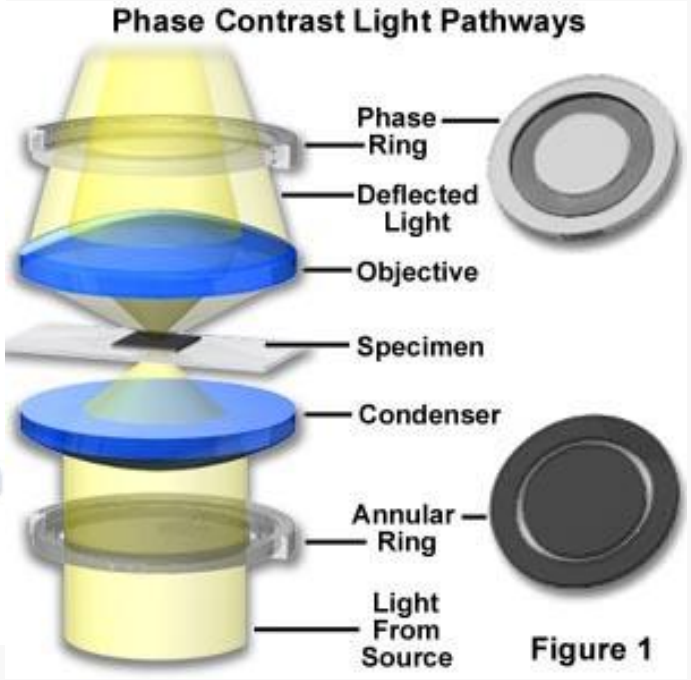
- Unfortunately, our eye as well as camera film cannot detect the phase differences ($\lambda/4$). We are sensitive to colors or intensity.

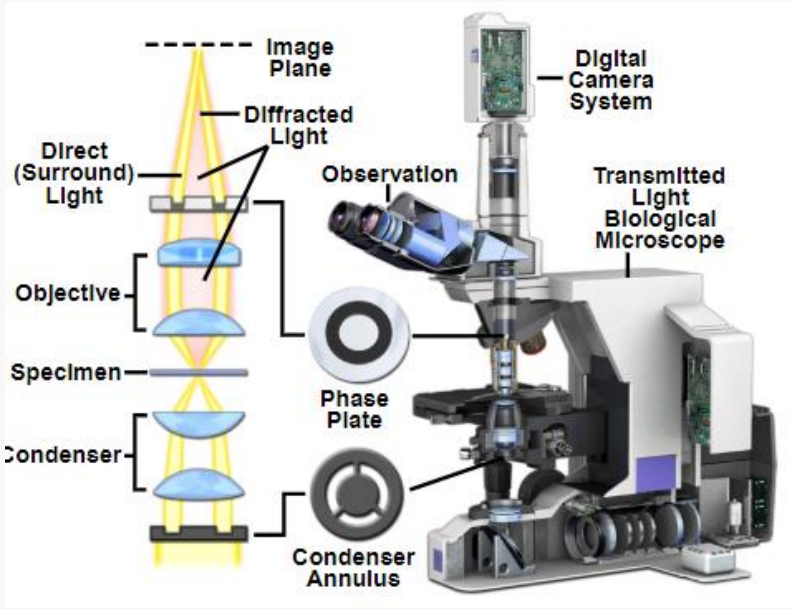
- The change of phase is converted into changes in amplitude

Phase Contrast microscopy

- A phase ring converts this retardation into a half wavelength phase shift (a condition of destructive interference).
- This achieved by introducing a half wavelength phase increase in the ring ($+ve$ phase contrast) by having an extra thickness of glass in which background appears darker relative to the foreground of sample or more commonly by introducing further half wavelength phaser retardation in the ring in which sample appear brighter relative to the background





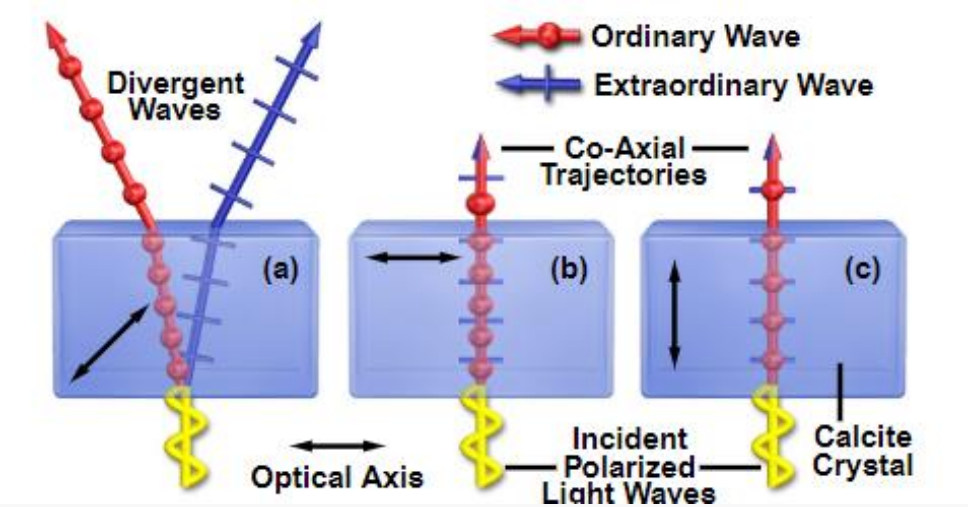


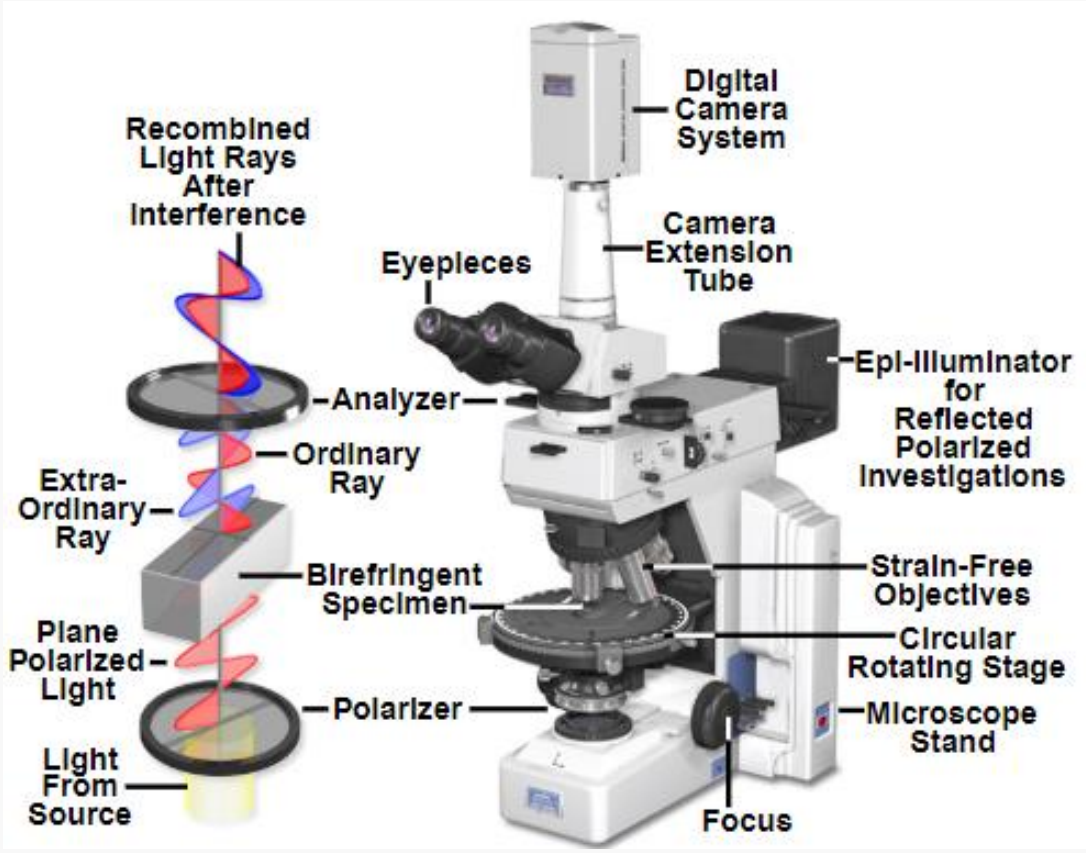
Polarized Light Microscopy

- Polarized light is a contrast-enhancing technique that improves the quality of the image obtained with birefringent materials.
- Refractive index of sample influence of the orientation of polarization E-field vector of the incident light. It is often due to repeating structural features in a sample.
- This microscope is excellent tool for generating images of these biological liquid crystal features. It is primarily employed in crystallography that why typically restricted to geologists, mineralogists, and chemists.
- This microscope is excellent tool for generating images of these biological liquid crystal features

Polarized Light Microscopy

- Figure below exhibits the different optical path lengths subsequently shifted in phase relative to one another. (a) for oblique case; and (b) for the situation where incident light is perpendicular to the optical axis of a birefringent crystal, and (c) parallel to the optical axis





Differential Interference Contrast (DIC) Microscopy

- In 1952, Georges Nomarski (French Optics) developed the techniques as improvement over phase contrast microscopy. Here, Georges modified the Wollaston (split unpolarized incident light into two orthogonally polarized outputs) for detecting optical gradients in specimens and converting them into intensity differences.

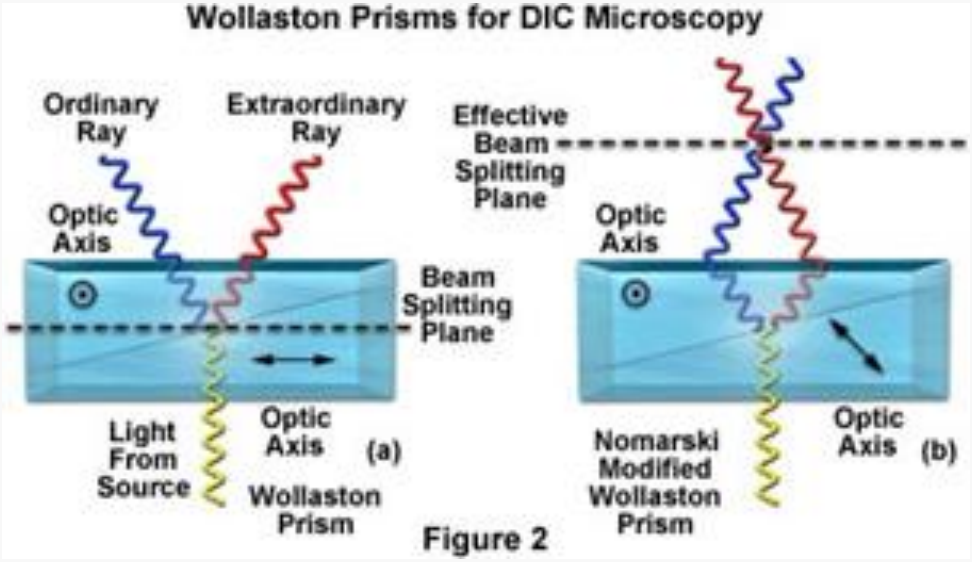


Figure 2

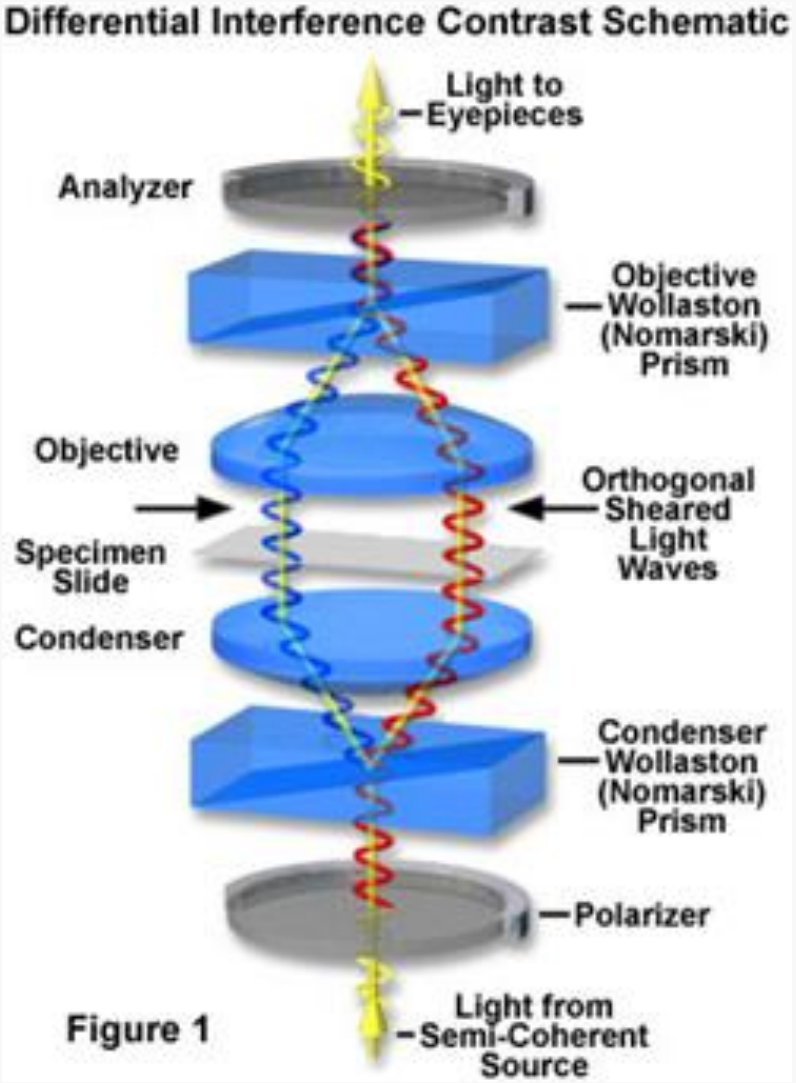
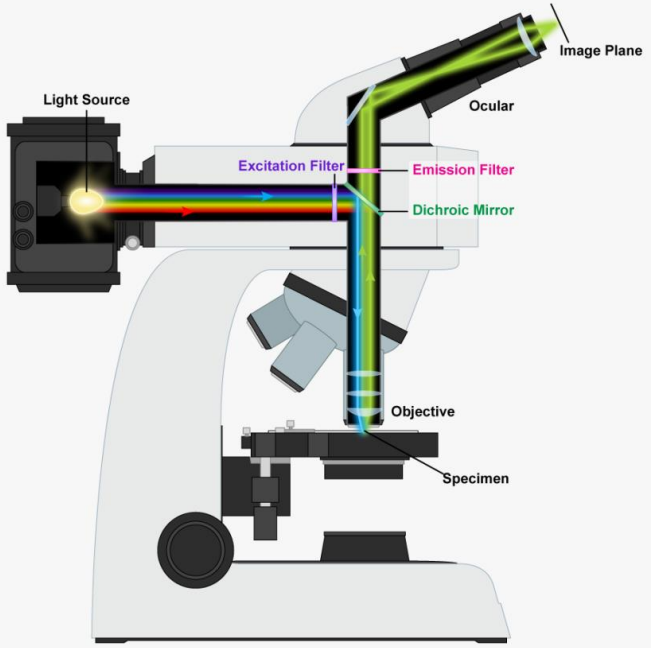


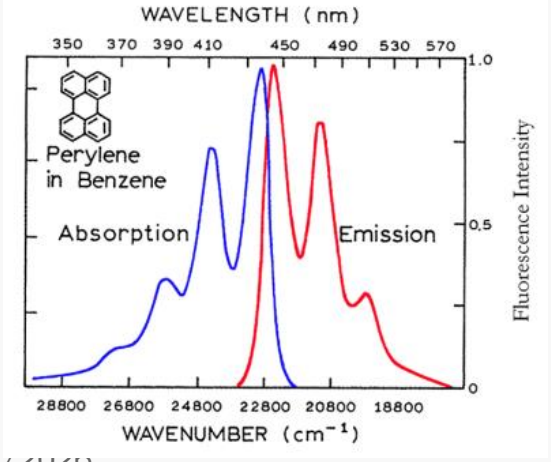
Figure 1

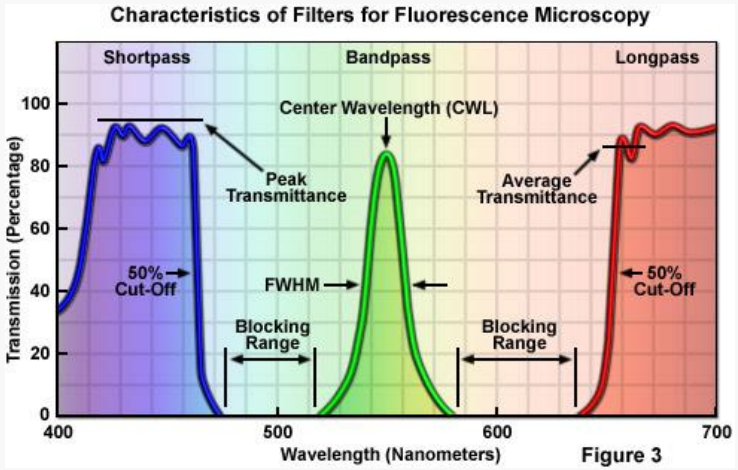
Fluorescence Microscope

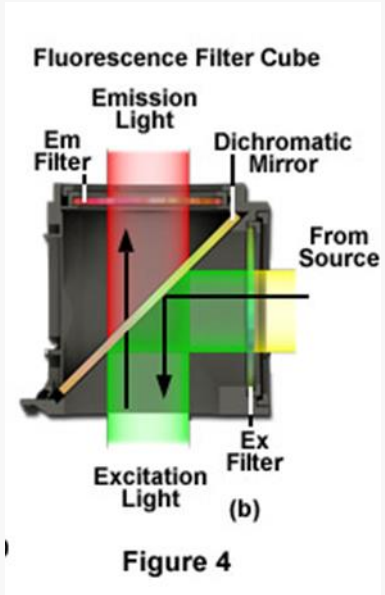


- The basic function of a fluorescence microscope is to irradiate the specimen with a desired and specific band of wavelengths and then to separate the much weaker emitted fluorescence from the excitation light.

- Vibrational energy is lost when electrons relax from excited state bac to ground state. Because of this energy lost, the emission of spectrum of excited fluorophore is usually shifter to longer wavelength which is called **Stokes' Shift (law)**. As **Stokes's shift** value increase which allows to separate excitation from emission light via fluorescence filter.



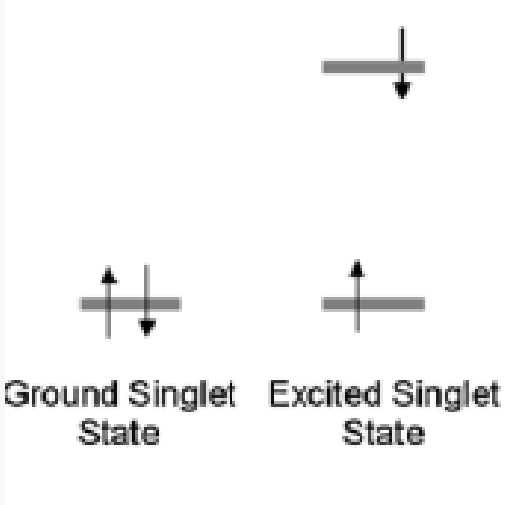


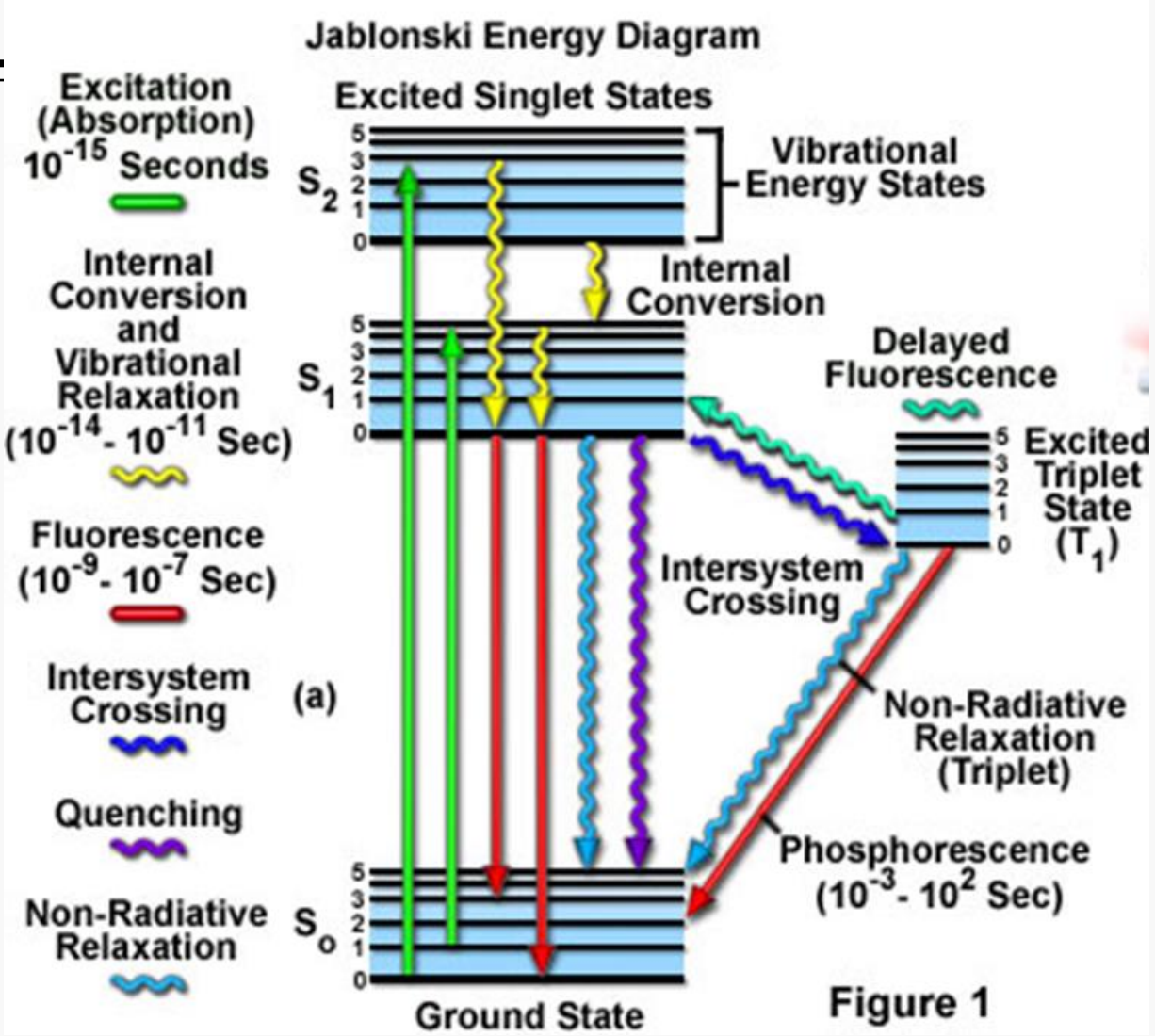


Fluorescence Microscope

Energy level diagram for a molecule showing pathways for the deactivation of an excited state.

Fluorescence activity can be schematically illustrated with the classical Jablonski diagram, first proposed by Professor Alexander Jablonski (polish physicist (1898-1980)) in 1935 to describe absorption and emission of light





Fluorescence Microscope

- Phosphorescence is emission of light from triplet excited states, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transition from triplet state to the singlet ground state are forbidden and the emission rates are slow (10^3 to 10^0 s^{-1}), so the phosphorescence lifetimes are typically milliseconds to seconds.

- Fluorescence is the result of a three-stage process that occurs in certain molecules (generally polyaromatic hydrocarbons or heterocycles) called fluorophores or fluorescent dyes

Fluorescence Microscope

Electrons pair up and net spin (opposite spin) is zero which is called **singlet state**. When electron pair has same spin (spin in same direction) is called **triplet state**. The state is determine using the spin angular momentum total electrons spin ($L_s = 2S + 1$):

i. Singlet State: if the total spin S is zero (e.g. S= 1/2 + (-1/2) = 0), the spin angular momentum is one ($L_s = 2(0) + 1 = 1$), only one state exists, then it is called **singlet state.**
ii. Doublet State: if the total spin S is one half (e.g. S= 1/2), the spin angular momentum is one ($L_s = 2\left(\frac{1}{2}\right) + 1 = 2$), two states exist then it is called **doublet state.**
iii. Triplet State: if the total spin S is one (e.g. S= 1/2 + 1/2 = 1), the spin angular momentum is one ($L_s = 2(1) + 1 = 3$), three states exist then it is called **singlet state.**

Fluorescence

- Fluorescence typically occurs from aromatic molecules. E.g. **Quinine**, present in tonic water, can be observed a faint blue glow at the surface when the tonic water is exposed to sun light. Here, quinine in tonic water is excited by the UV light from the sun. Upon return to the ground state the quinine emits blue light with a wavelength near 450 nm.
- One can notice that the first known fluorophore, quinine, was responsible for stimulating the development of the first spectrofluorometers that appeared in the 1950s.

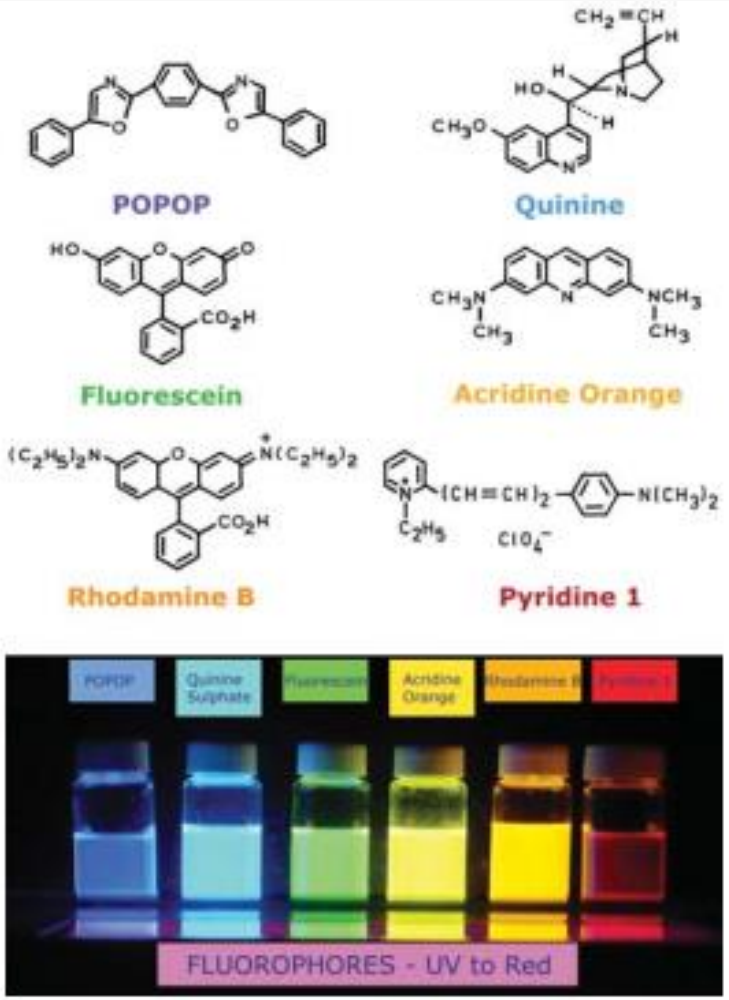


Figure 1.1. Structures of typical fluorescent substances.

Excitation Source

Energy level diagram for a molecule showing pathways for the deactivation of an excited state

Table 10.3 Common Sources of Electromagnetic Radiation		
Source	Wavelength Region	Useful for...
H_2 and D_2 lamp	continuum source from 160–380 nm	molecular absorption
tungsten lamp	continuum source from 320–2400 nm	molecular absorption
Xe arc lamp	continuum source from 200–1000 nm	molecular fluorescence
nernst glower	continuum source from 0.4–20 μm	molecular absorption
globar	continuum source from 1–40 μm	molecular absorption
nichrome wire	continuum source from 0.75–20 μm	molecular absorption
hollow cathode lamp	line source in UV/Visible	atomic absorption
Hg vapor lamp	line source in UV/Visible	molecular fluorescence
laser	line source in UV/Visible/IR	atomic and molecular absorption, fluorescence, and scattering

Excitation Source

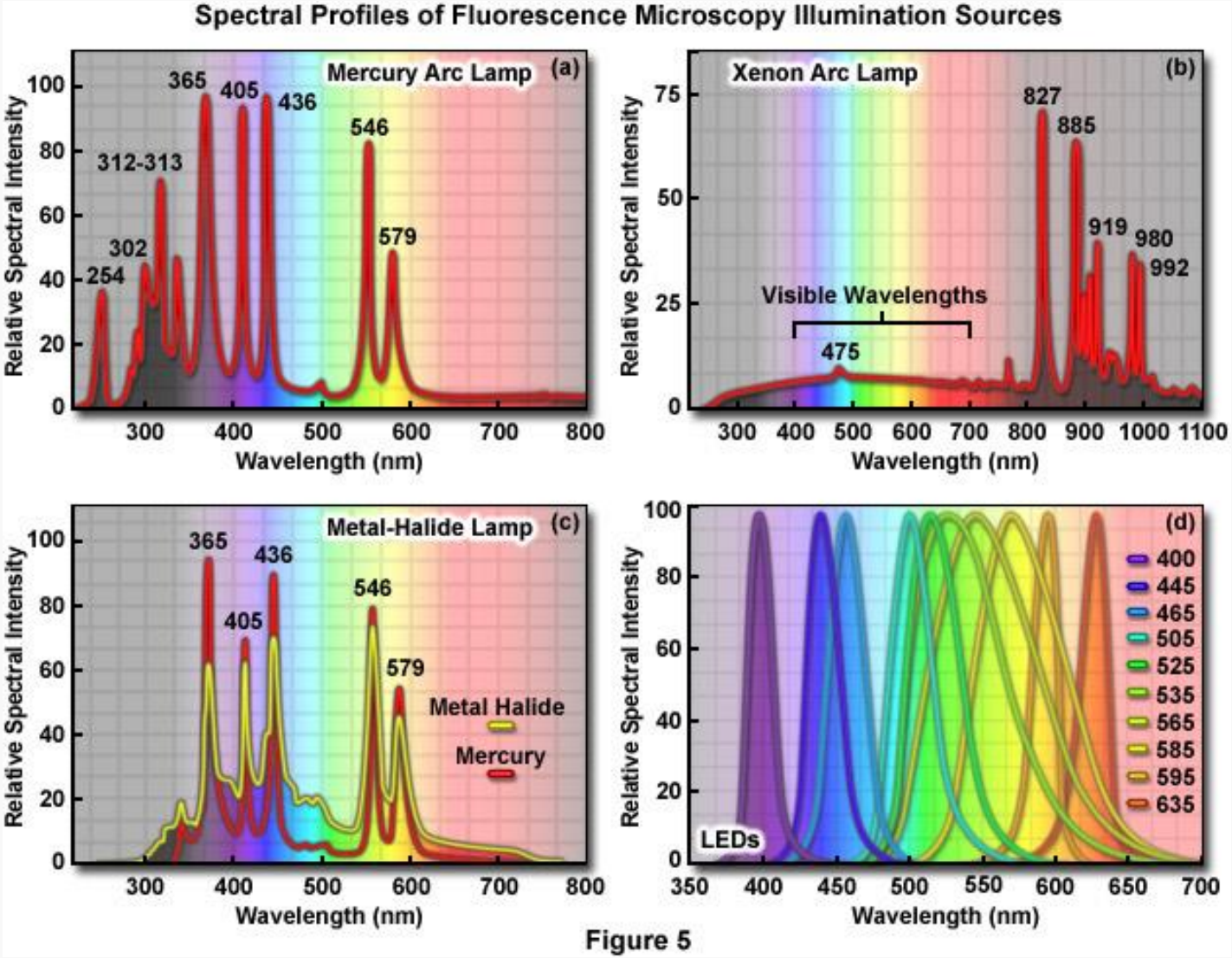


Figure 5

Widefield Fluorescence Microscopy

- Most widely used fluorescence microscopy techniques and the easiest fluorescence imaging mode.
- In contrast to confocal microscopy, whole specimen is exposed to light in widefield fluorescence microscopy.
- Fluorescence signals from all focal planes are detected, which leads to lower contrast in thick samples like spheroids and tissue. Therefore, widefield microscopy is best applied with thin specimens with low background autofluorescence, like adherent cells

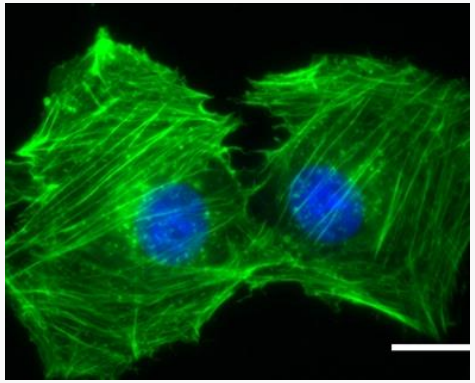
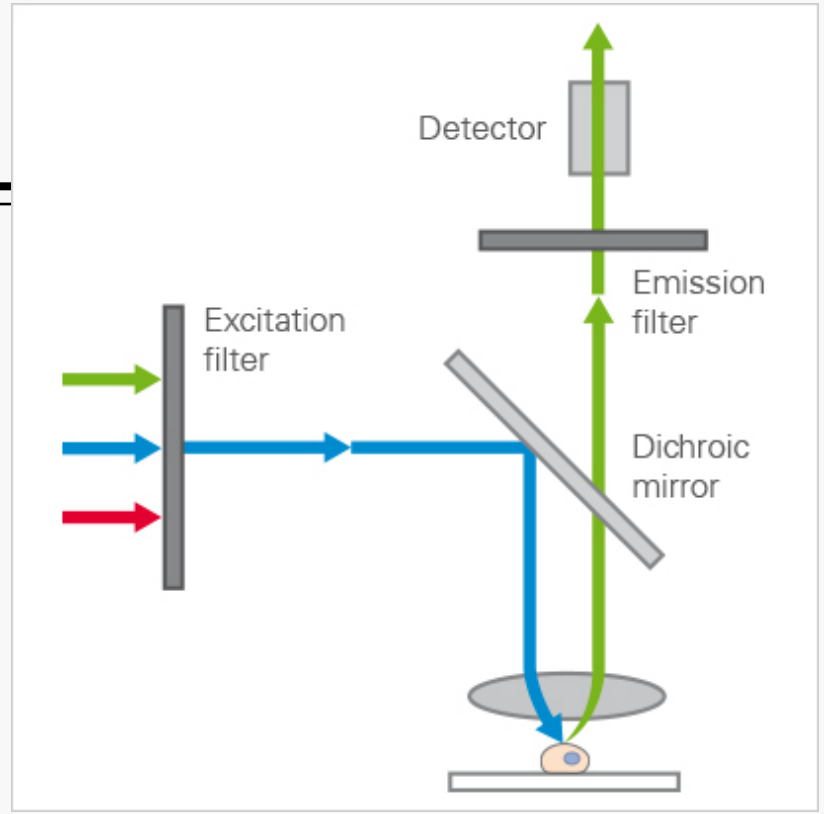


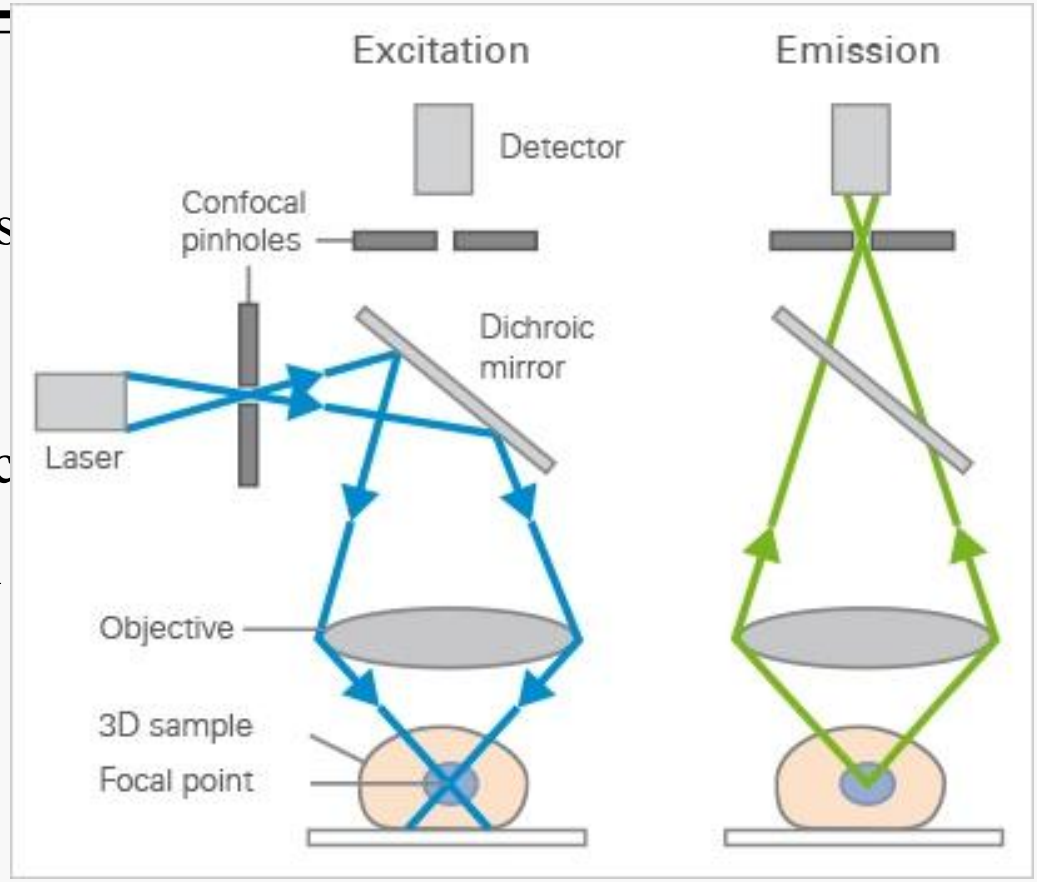
Image from Fluorescence Microscopy: proteins inside cell



Light pathways in a widefield microscope

Confocal Microscopy

- Similar to the widefield microscope, the confocal microscope uses fluorescence optics. Instead of illuminating the whole sample at once, laser light is focused onto a defined spot at a specific depth within the sample.
- This leads to the fluorescent emission from specific location. A pinhole inside the optical pathway cuts off signals that are out of focus, thus allowing only the fluorescence signals from the illuminated
- By scanning the specimen in a raster pattern, images of one single optical plane are created. 3D objects can be visualized by scanning several optical planes



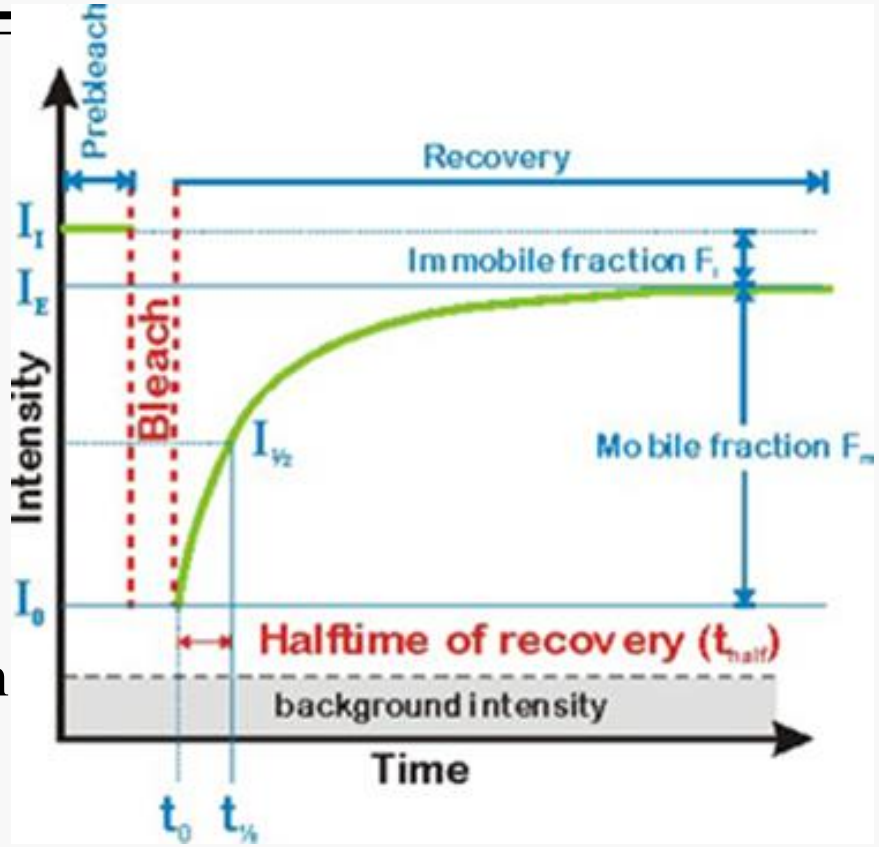
Excitation and emission light pathways in a basic confocal microscope configuration.

Two-Photon and Multiphoton Microscopy

- Two-photon (also called multiphoton) microscopy can be used for live cell imaging of thick biological specimens.
- It can used for live cell imaging of thick bio samples (molecule can be visualized as deep as 1 mm). It has several advantages over confocal microscopy where higher wavelength leading to less photobleaching or photodamage (especially on living samples).
- Fluorophore is excited with two or three photons of a higher wavelength when they hit the fluorophore at the very same time. (typically, within several femtoseconds); allowing low energy IR photons to excite standard fluorophores,

Fluorescence Recovery After Photobleaching (FRAP) Microscopy

- FRAP was developed by Axelrod and coworkers in 1970 to study protein mobility in living cells (2D or 3D mobility).
- The fluorescently labeled molecules in a small region of the cell are irreversibly photobleached using high laser power, followed by monitoring the subsequent movement of the surrounding non-bleached fluorescent molecules into the photobleached region using low laser power.
- FRAP recovery curve (exponential equation) gives information about the mobility of a molecule and the fraction of immobile.

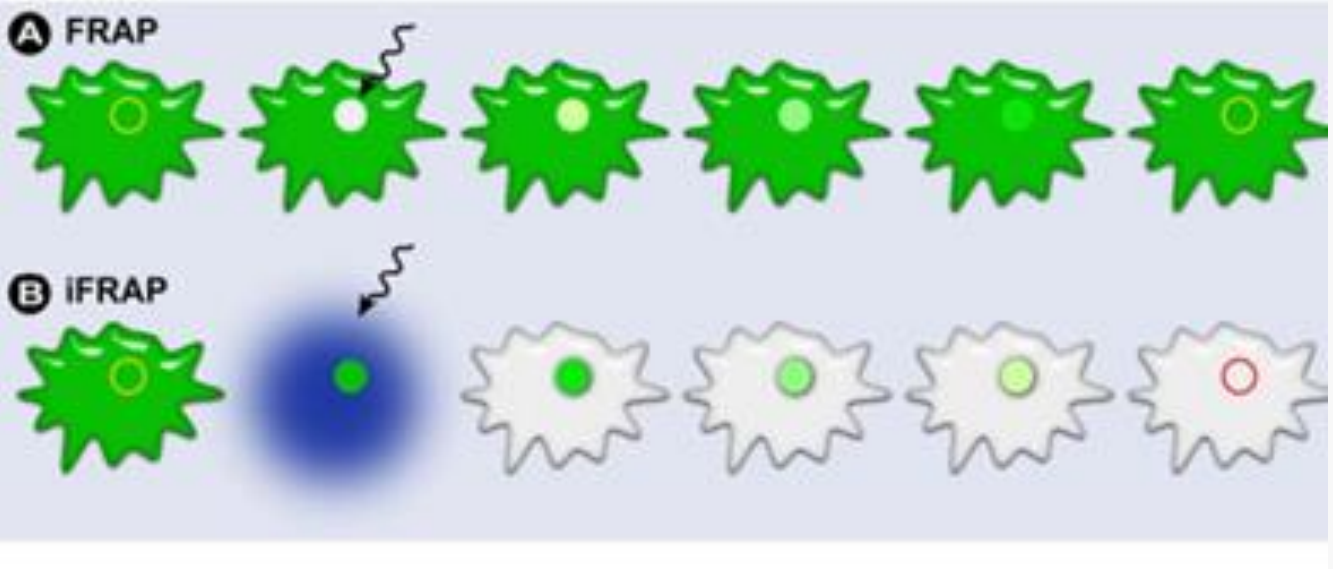


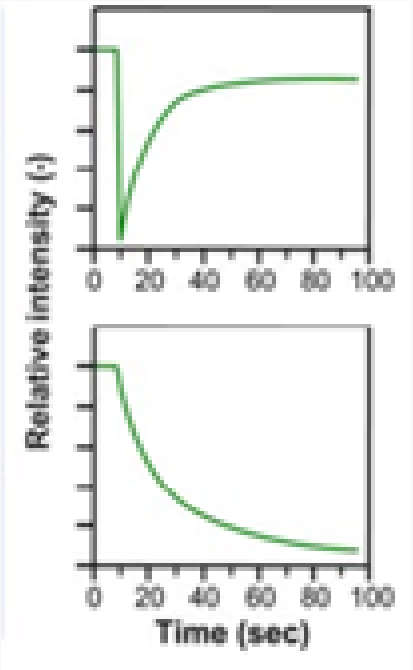
$$I_t = M_f(1 - e^{-\tau t})$$

Where M_f is mobile fraction and $(1 - M_f)$ is immobile fraction.

Substitution of $I_t = \frac{1}{2}$ M_f gives: $\tau_{1/2} = \frac{\ln(0.5)}{-\tau}$

Fluorescence Recovery After Photobleaching (FRAP) Microscopy





(A) (FRAP) A region of interest (ROI) is selected, bleached with an intense laser beam, and the fluorescence recovery in the ROI is measured over time. (**B**) In iFRAP, the reverse is done, and a ROI is selected to remain intact, while the rest of the cell is bleached. This is particularly useful when studying dynamic movement in organelles such as the nucleus

Fluorescence Loss in Photobleaching (FLIP) Microscopy

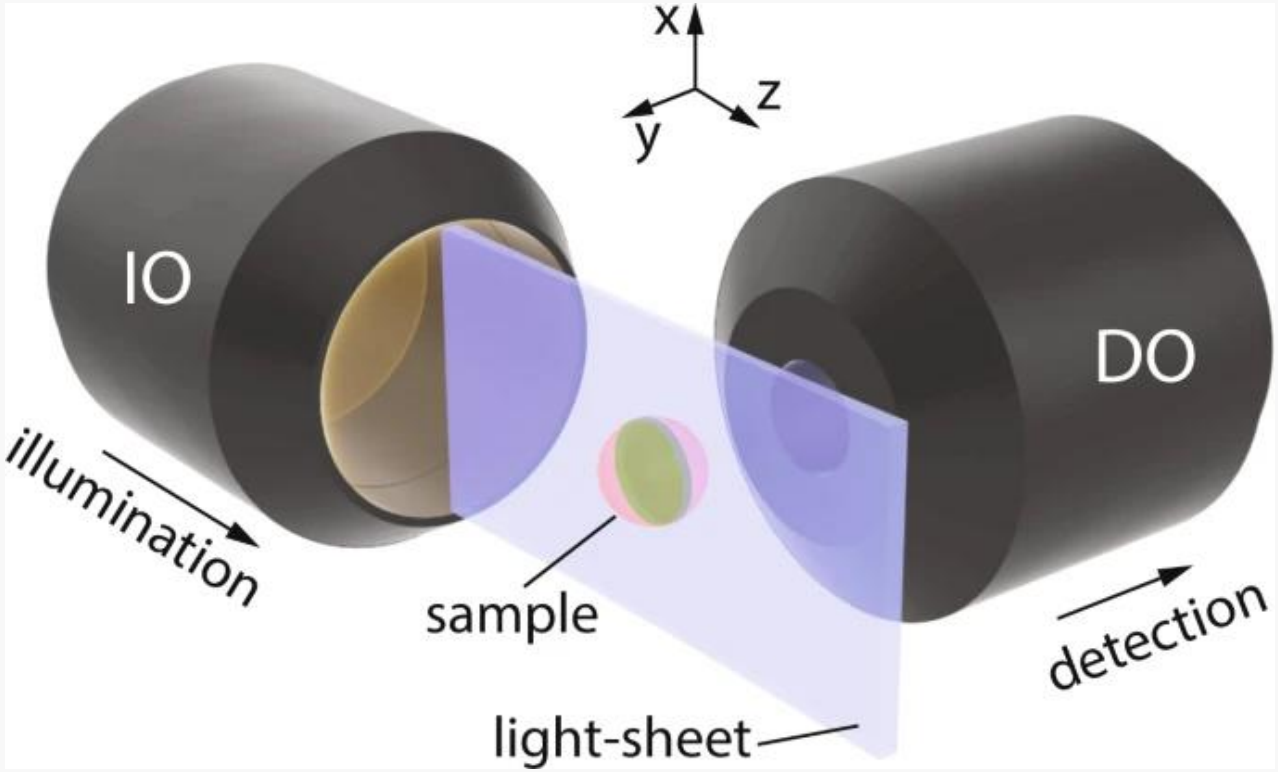
- Repetitive bleaching of a selected region of interest (ROI) during the entire monitoring period and the fluorescence intensity in regions outside the selected bleached area is measured.
- The decline in fluorescence intensity in the surrounding regions is due to bleaching of fluorochromes that move through the ROI during the repetitive bleaching process.
- The drop in fluorescence intensity outside the bleached region is caused by a steadily increasing population of bleached, non-fluorescent molecules within the cell and thus provides quantitative data on their molecular mobility

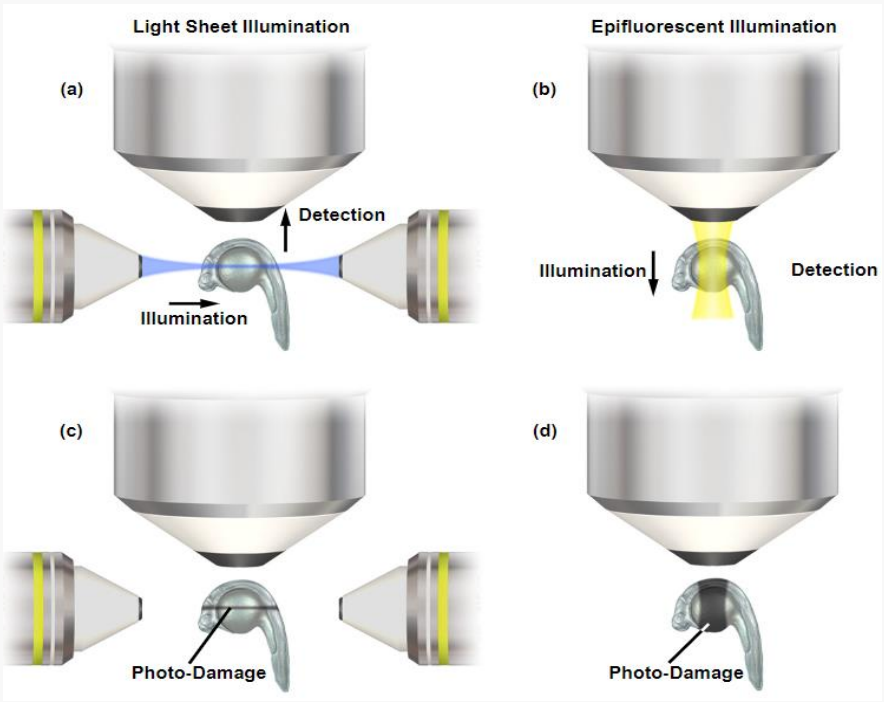
Light Sheet Fluorescence Microscopy (LSFM)

LSFM is rooted in a technique termed ‘ultramicroscopy’ pioneered in 1902 by Richard Adolf Zsigmondy, an organic chemist and experimental physicist, and Henry Siedentopf, an optical physicist.

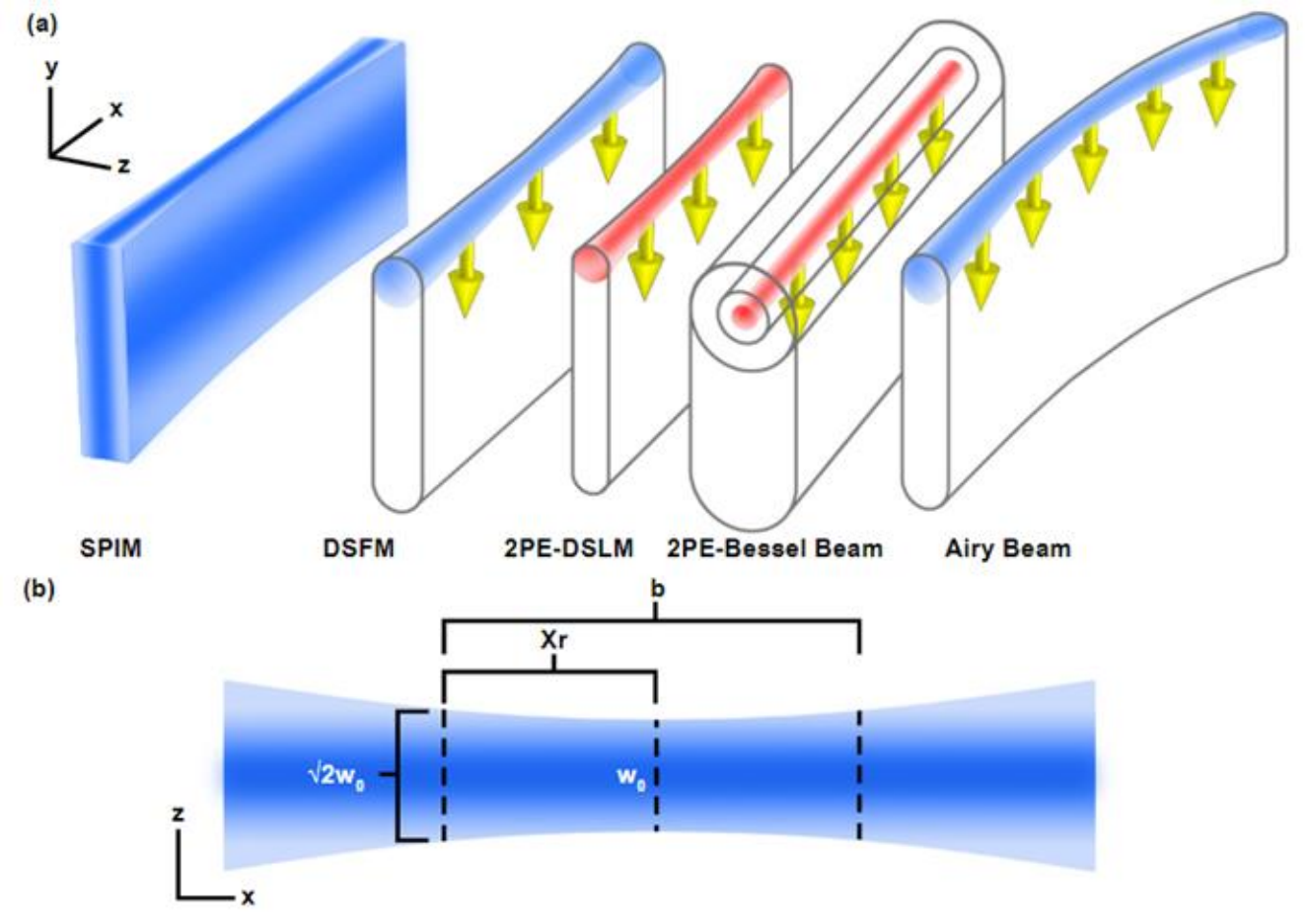
Light Sheet Fluorescence Microscopy (LSFM)

Excellent method for 3D and live imaging of large specimens over extended periods of time, utilizing a unique planar illumination approach that minimizes photobleaching and phototoxicity. In life science,





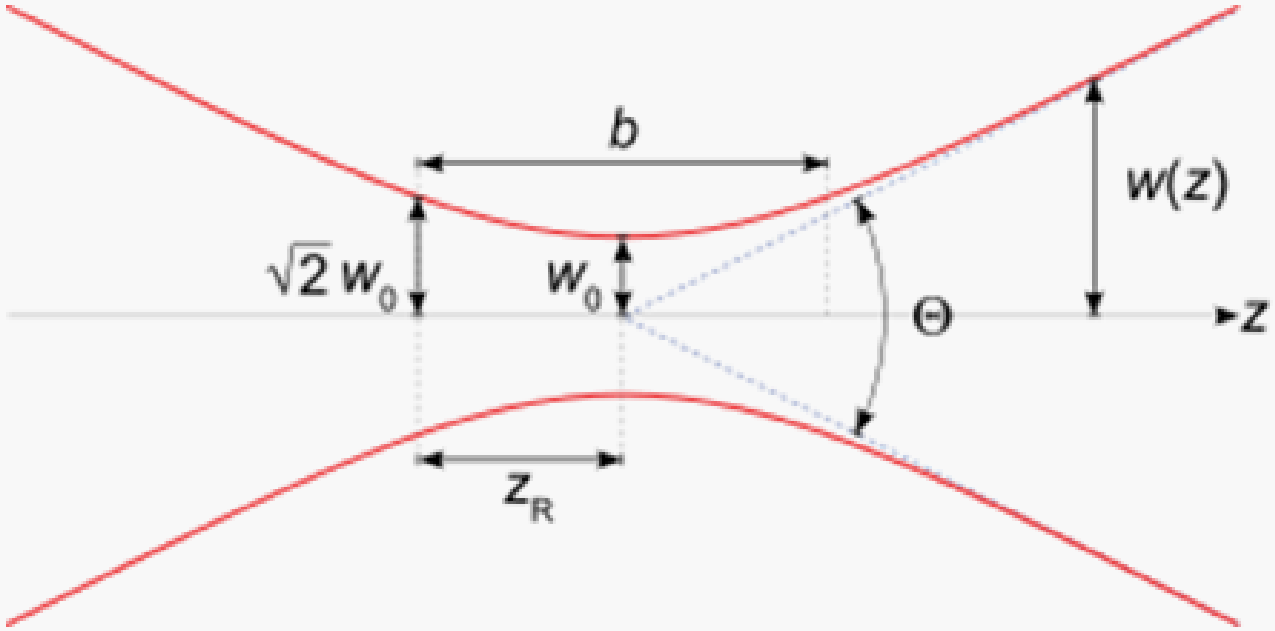
Light Sheet Fluorescence Microscopy (LSFM)



Illustrations of different types of light sheets:

A planar light sheet (SPIM), a digitally scanned light sheet with arrows denoting the direction of scanning (DSLM), a two-photon excitation DSLM (2PE-DSLM) with slightly reduced range, and a 2PE-Bessel Beam light sheet with extended range and reduced contribution from side lobes (larger gray outline represents extent of Bessel Beam side lobes).

Light Sheet Fluorescence Microscopy (LSFM)



Gaussian beam width $w(z)$ as a function of the distance z along the beam. Where w_0 is beam waist, b is depth of focus, z_R is Rayleigh Range, and Θ is total angular spread..

Light Sheet Fluorescence Microscopy (LSFM)

Beam waist (w_0) specifying the thickness of the most tightly focused mid-point of the illumination profile.

$$w_0 = \frac{1.4 f \lambda}{2\, D_{lens}}$$

The spot size (w) evolve with a position z along the beam which is given by:

$$w(z) = w_0 \sqrt{1 + \left(\frac{z}{z_R}\right)^2}$$

where $\boldsymbol{z_R}$ is **Rayleigh range**, which is:

$$\boldsymbol{z_R = \frac{\pi w_0^2 n}{\lambda}}$$

Light Sheet Fluorescence Microscopy (LSFM)

For $\boldsymbol{z \gg z_R}$, the parameter $\boldsymbol{w(z)}$ increase linearly with z, beam is cone-shape. The angle between lines along that cone (see Fig 4-13) is:

$$\boldsymbol{\theta = \lim_{z\to\infty} \arctan\left(\frac{w(z)}{z}\right) \approx \frac{\lambda}{\pi n w_0}}$$

The total angular spread of the beam is given by

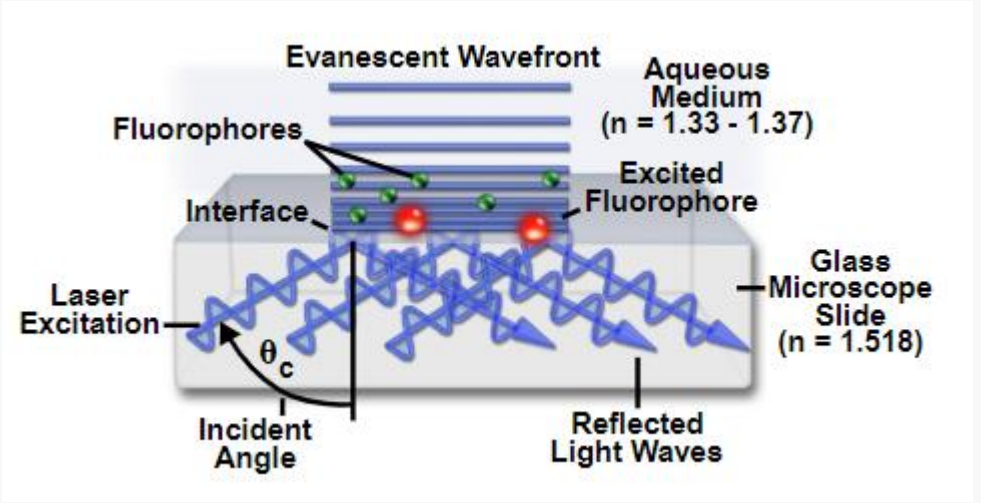
$$\boldsymbol{\Theta = 2\theta}$$

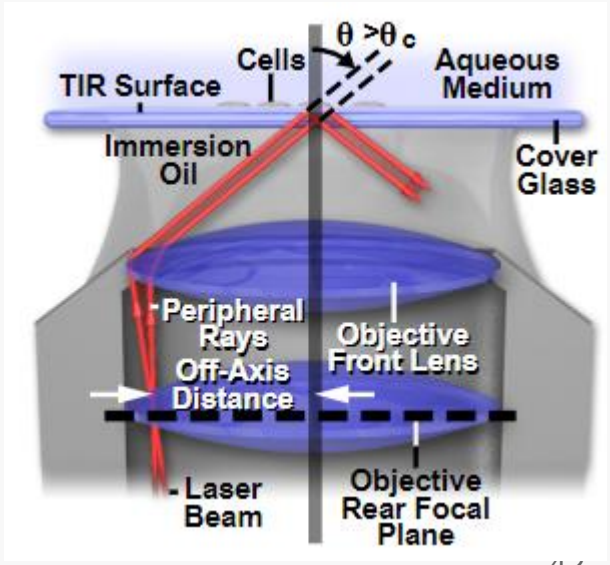
Confocal parameter or depth of focal (b) of a light sheet, which defines the distance through which the sheet can be said to provide near-homogenous planar illumination:

$$b = 2\, z_R = \frac{2\pi w_0^2}{\lambda}$$

Total Internal Reflection Fluorescence (TIRF)

- A useful technique for visualizing membrane (not for structure located deep).
- It exploits the unique properties of an induced **evanescent wave** or **field** (oscillating electric and/or magnetic field doesn't propagate as an EM wave but as oscillating charge, decay exponentially) in a limited specimen region immediately adjacent to the interface between two media having different refractive indices.
- Area of Evanescent field is able to excite fluorophores which is close to the glass/sample interface. It can be about 100-200 nm deep into the specimen.





Total Internal Reflection Fluorescence (TIRF)

At critical angle:

$$n_1 \sin\theta_c = n_2 \sin 90^o \rightarrow \theta_c = \arcsin\left(\frac{n_2}{n_1}\right)$$

The intensity as a function of distant from the interface is:

$$I(z) = I_0 \exp(-\beta z)$$

Where I_0 is the energy at the interface and

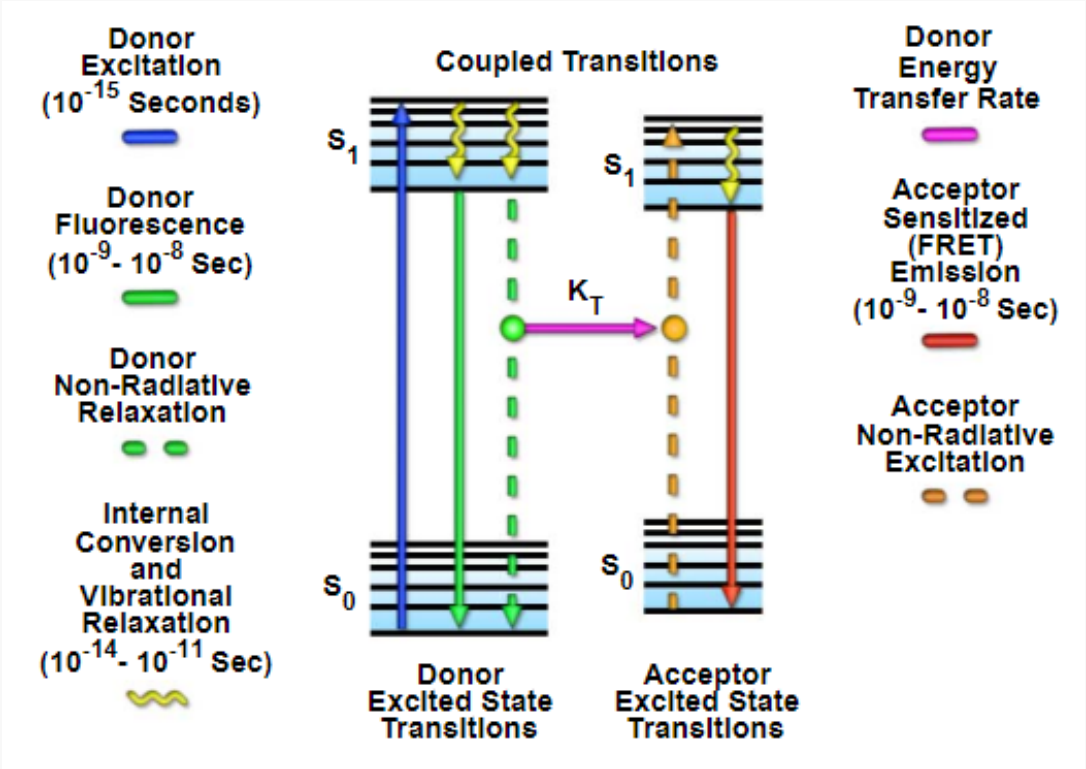
$$\beta = \frac{4\pi}{\lambda} \sqrt{(n_1 \sin\theta)^2 - n_2^2}$$

Two-Photon and Multiphoton Microscopy

- Two-photon (also called multiphoton) microscopy can be used for live cell imaging of thick biological specimens.
- It can used for live cell imaging of thick bio samples (molecule can be visualized as deep as 1 mm). It has several advantages over confocal microscopy where higher wavelength leading to less photobleaching or photodamage (especially on living samples).
- Fluorophore is excited with two or three photons of a higher wavelength when they hit the fluorophore at the very same time. (typically, within several femtoseconds); allowing low energy IR photons to excite standard fluorophores,

Förster Resonance Energy Transfer (FRET) Microscopy

- FRET is a process where energy is transferred non-radiatively (via long-range dipole-dipole coupling) from an excited donor fluorochrome to another molecule or acceptor.
- It depends on the distance between donor and acceptor (physical interaction of the two molecules): Only occur if the distance between donor and acceptor is less than approximately 10 nm.
- Popular method for examining interaction in situ.
- Because resolution of a fluorescence microscope is several hundred times less than the size of proteins, co-localization often leads to questionable results.

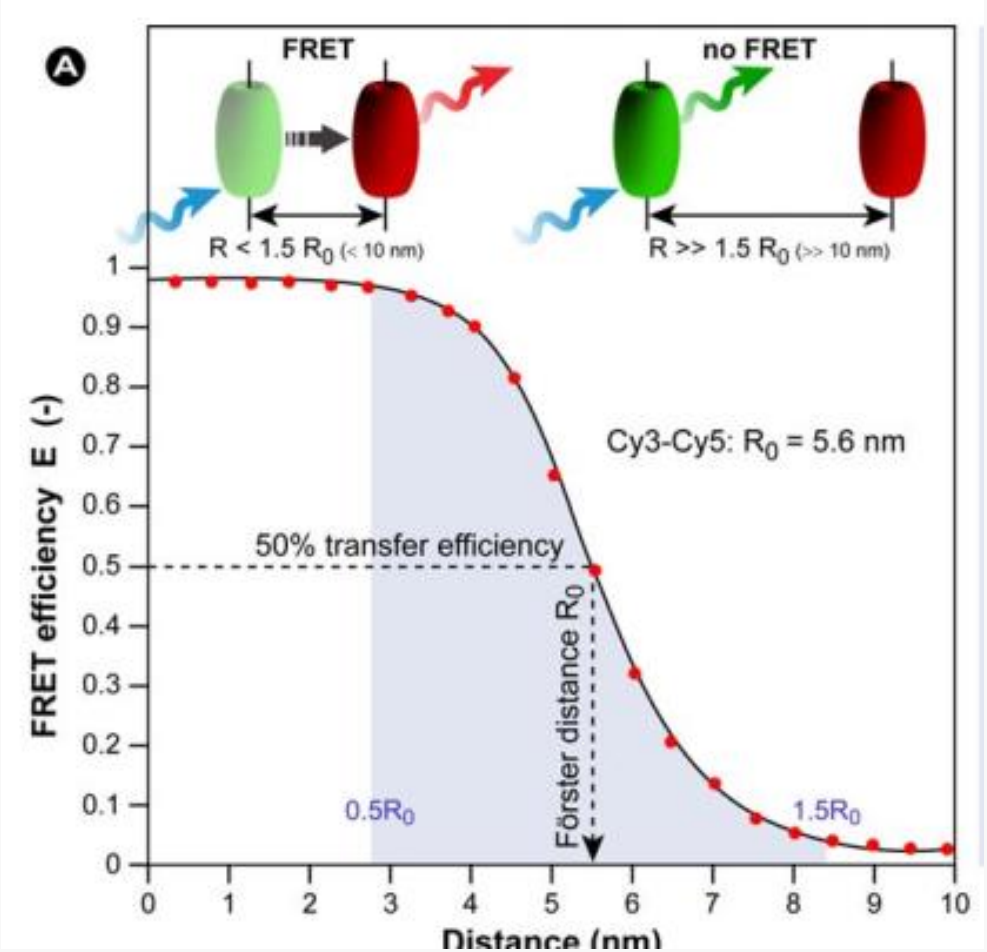


FRET energy transfer using Jablonski diagram.

Förster Resonance Energy Transfer (FRET) Microscopy

- In FRET, donor and acceptor pair must be in proximity of less than 10 nm for the detection of donor fluorescence.
- The figure show the intensity of donor emission decreases as distance.
- Forster (resonance energy transfer) Theory states the efficiency of energy transfer. An expressed for the rate constant of transfer k_T

$$k_T(R) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6$$

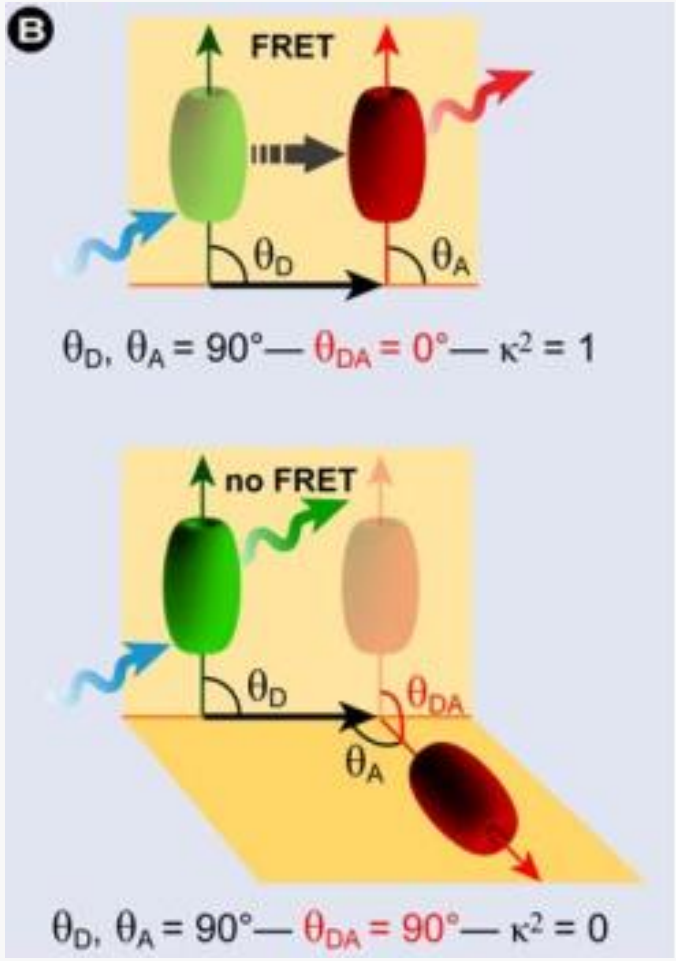


Förster Resonance Energy Transfer (FRET) Microscopy

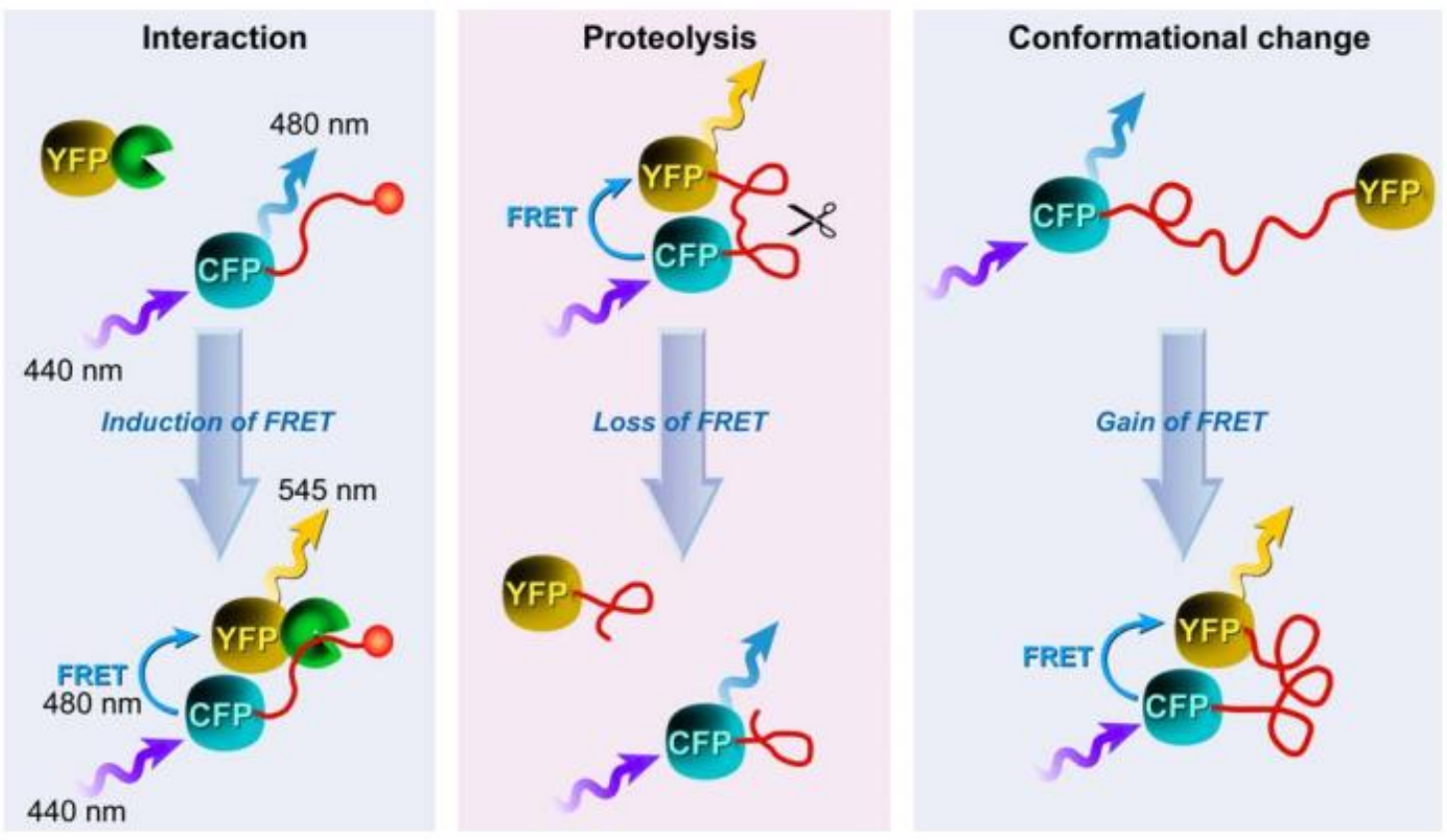
- FRET efficiency (*EFRET*), depends on the physical distance between donor and acceptor, the spectral overlap of the donor emission spectrum and the acceptor absorption spectrum, and the relative orientation of the donor emission dipole moment and the acceptor absorption dipole moment. The efficiency *EFRET* :

$$E_{FRET} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \rightarrow r = R_0 \left(\frac{1}{E_{FRET}} - 1\right)^{1/6}$$

Where Förster radius (*R*0) is the characteristic distance where 50% FRET efficiency occurs, which can be calculated from the spectroscopic and mutual dipole orientational parameters of the donor and acceptor.



Possible Approaches for Developing FRET Biosensor



(a) Interaction of the labeled proteins results in a FRET signal, which was previously not present because the separation between donor and acceptor was too large;

(b) proteolysis of an intramolecularly labeled biomolecule leads to separation of donor and acceptor beyond 10 nm and a concomitant loss in FRET signal,

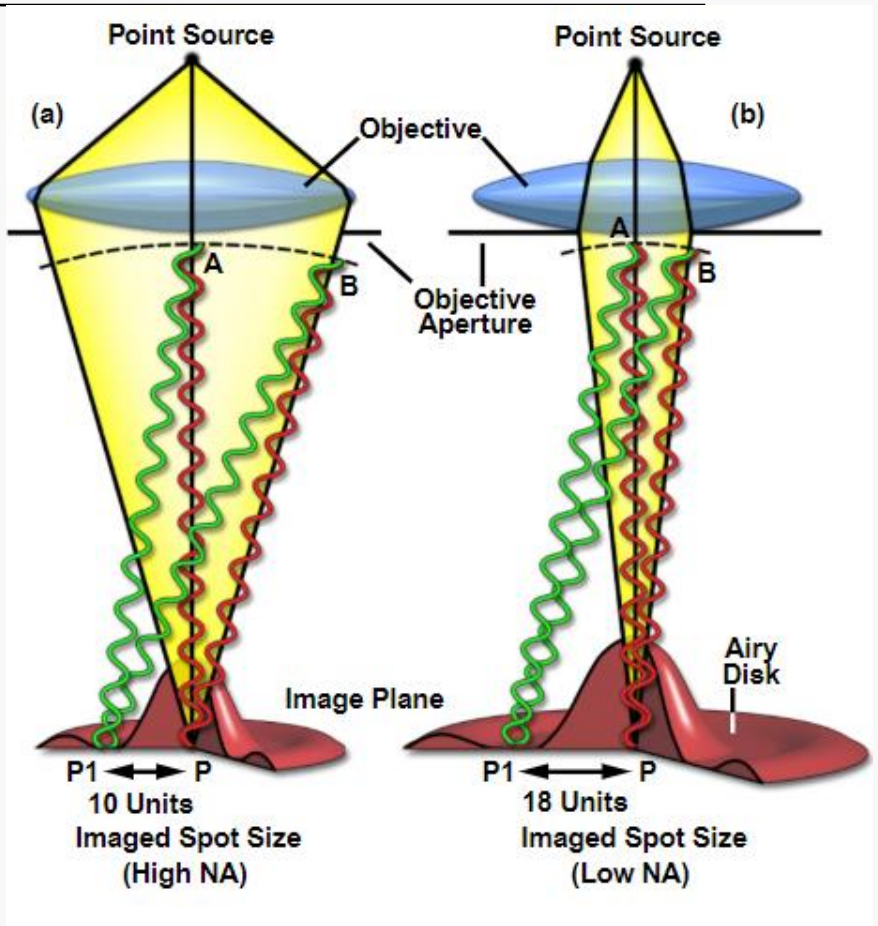
(c) an intramolecularly labeled biomolecule undergoes a conformational change upon stimulation with a ligand or binding of a substrate, resulting in an increase in FRET

Super-Resolution Microscopy

- Diffraction Limit and Resolution of Power of a Microscopy
- Introduction to Super-Resolution Microscopy
- Super-resolution Microscopy Techniques:
  - Stimulated emission depletion (STED)
  - Saturated structured illumination microscopy (SSIM)
  - Reversible Saturable Optical Linear Fluorescence Transitions (RESOLFT)
  - Photoactivated localization microscopy (PALM)
  - Fluorescence photoactivation localization microscopy (FPALM)
  - Stochastic optical reconstruction microscopy STORM

Resolution Microscopy

Resolution depends on the wavelength and NA. The resolution limitations are often referred as the **diffraction** barrier which restrict the ability of optical instruments to distinguish between two points. It is typically about half the wavelength of light used to image the specimen. The maximal resolution is about 200 nm for VIS source.



Resolution limit imposed by wave nature of light: diffraction limit

Diffraction Limit and Resolving Power

In terms of resolution, the radius of the diffraction Airy disk in the lateral

$$Abbe\ Resolution_{x,y} = \frac{\lambda}{2NA}$$

Rayleigh criterion are directly related to the properties and geometry of the point-spread function:

$$Rayleigh\ Resolution_{x,y} = \frac{1.22\lambda}{2NA} = 0.61\frac{\lambda}{NA}$$

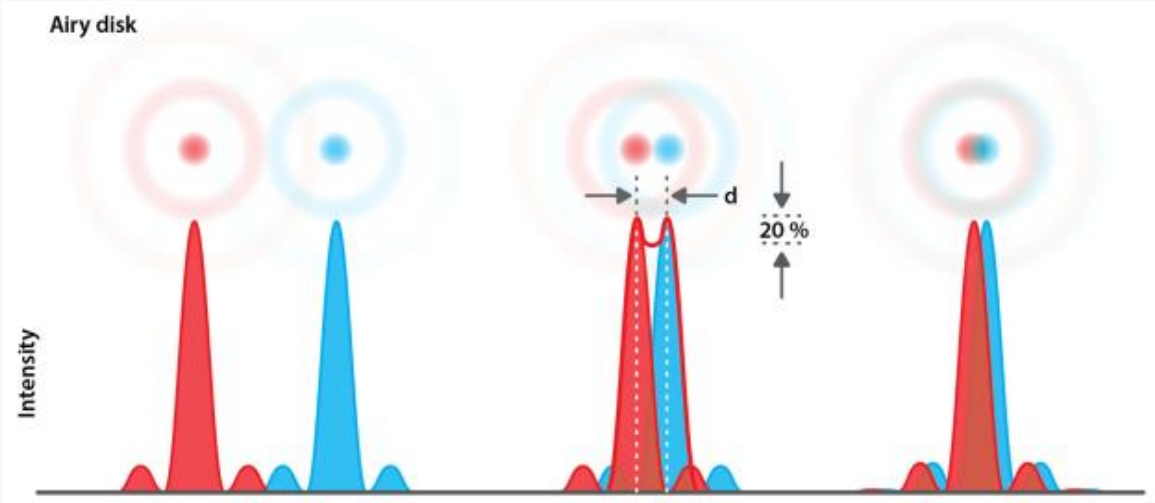


Fig-2 Rayleigh criterion for lateral axial resolution.

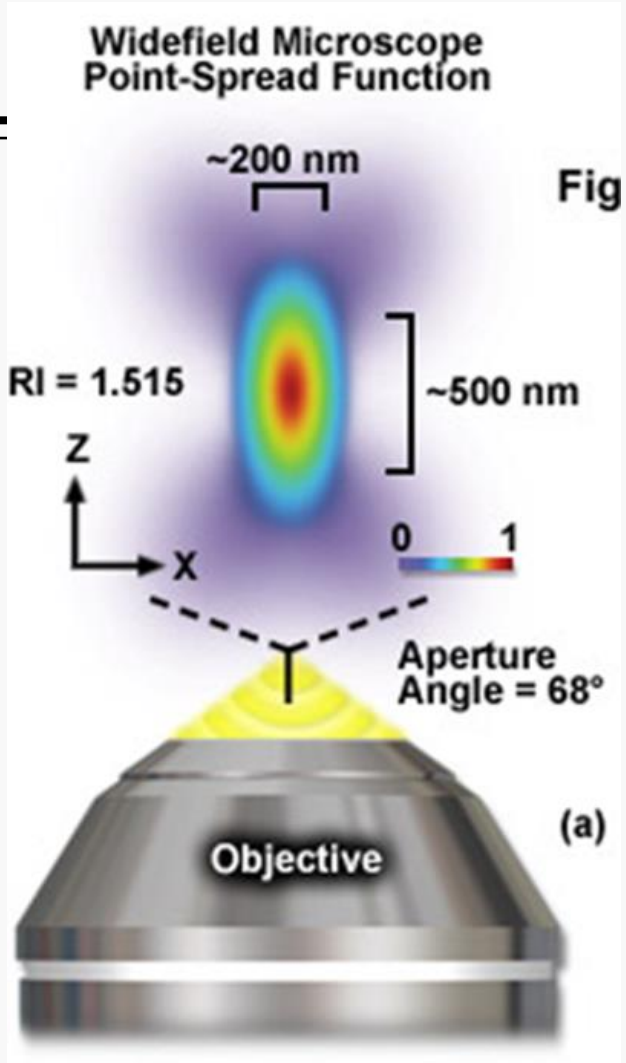
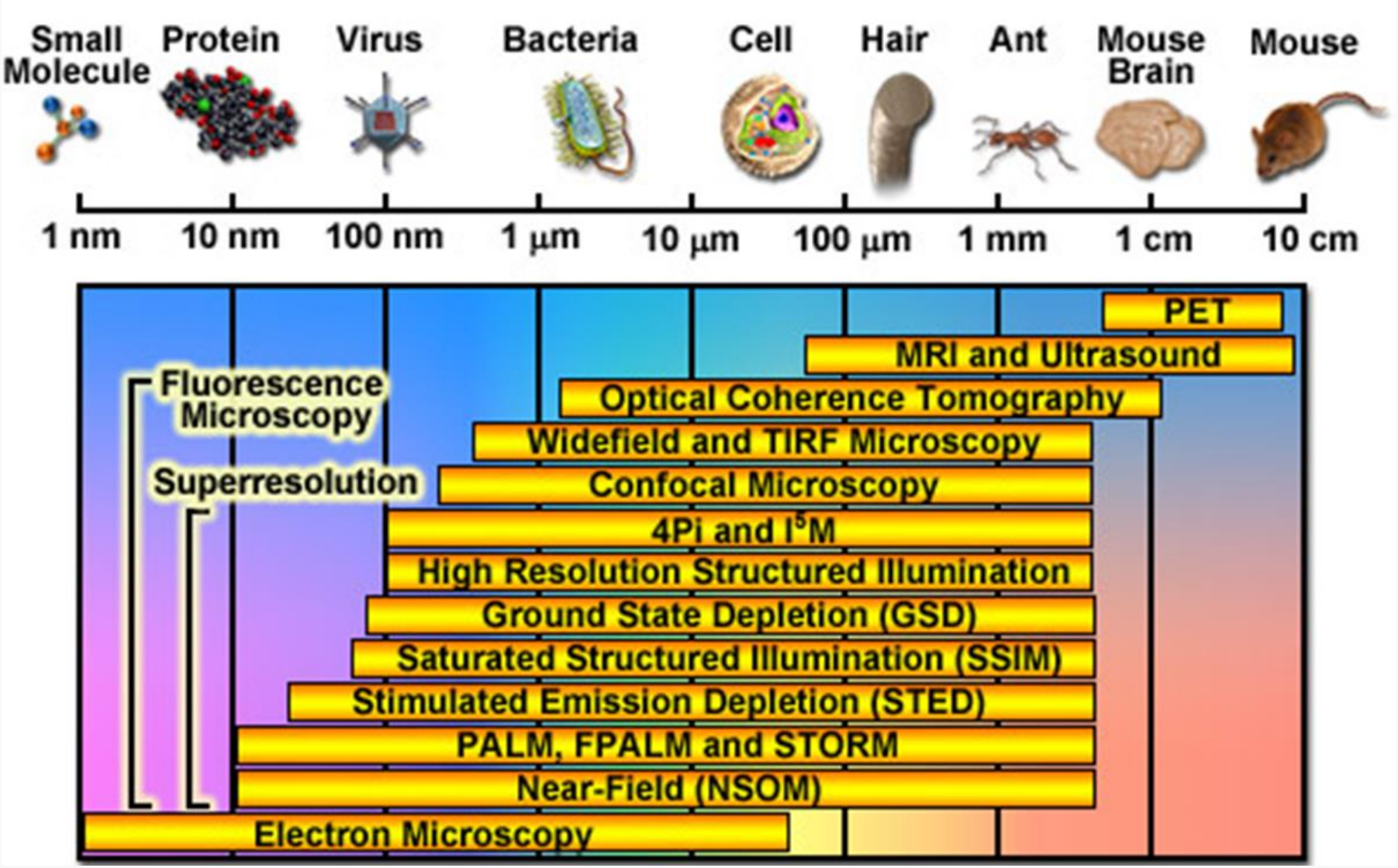


Fig-1: Illustration of lateral and axial resolution by objective lens.

Spatial Resolution of Biological Imaging Methods

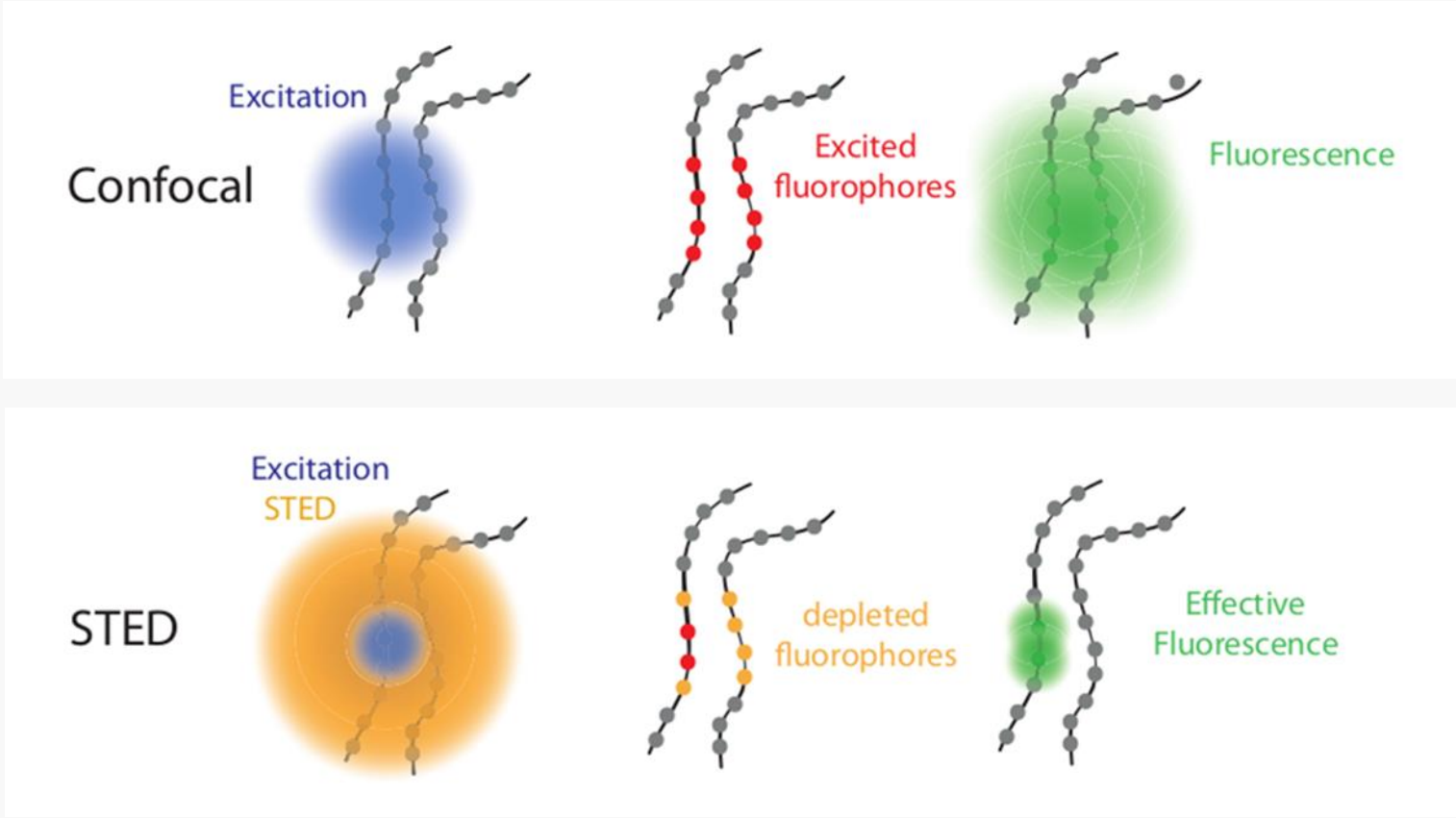


Super-Resolution Microscopy

- Super-resolution microscopy breaks the diffraction barrier, enabling “nanoscopy” with substantially improved optical resolution of down to 5–20 nm.
- Enables the visualization of structures (below diffraction limit) in living cells that cannot be resolved using standard widefield or confocal fluorescence microscopy.
- There are several super-resolution microscopies, and each of them has its advantage and disadvantage. Next, we will briefly discuss the various super-resolution microscopy techniques:
  - Stimulated emission depletion (STED)
  - Saturated structured illumination microscopy (SSIM)
  - Reversible Saturable Optical Linear Fluorescence Transitions (RESOLFT)
  - Photoactivated localization microscopy (PALM)
  - Fluorescence photoactivation localization microscopy (FPALM)
  - Stochastic optical reconstruction microscopy STORM

Stimulate Emission Depletion (STED) Microscopy

Stimulate Emission Depletion (STED) microscopy, first described by Stefan Hall, is a powerful imaging method which uses localized excitation method to be breaking the diffraction barrier to improve the lateral spatial resolution.



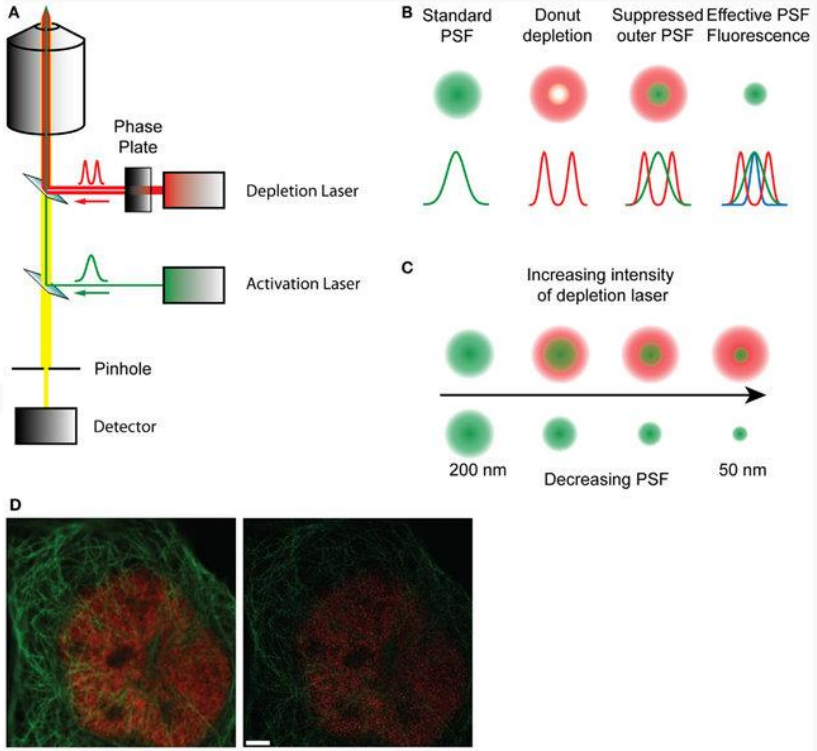
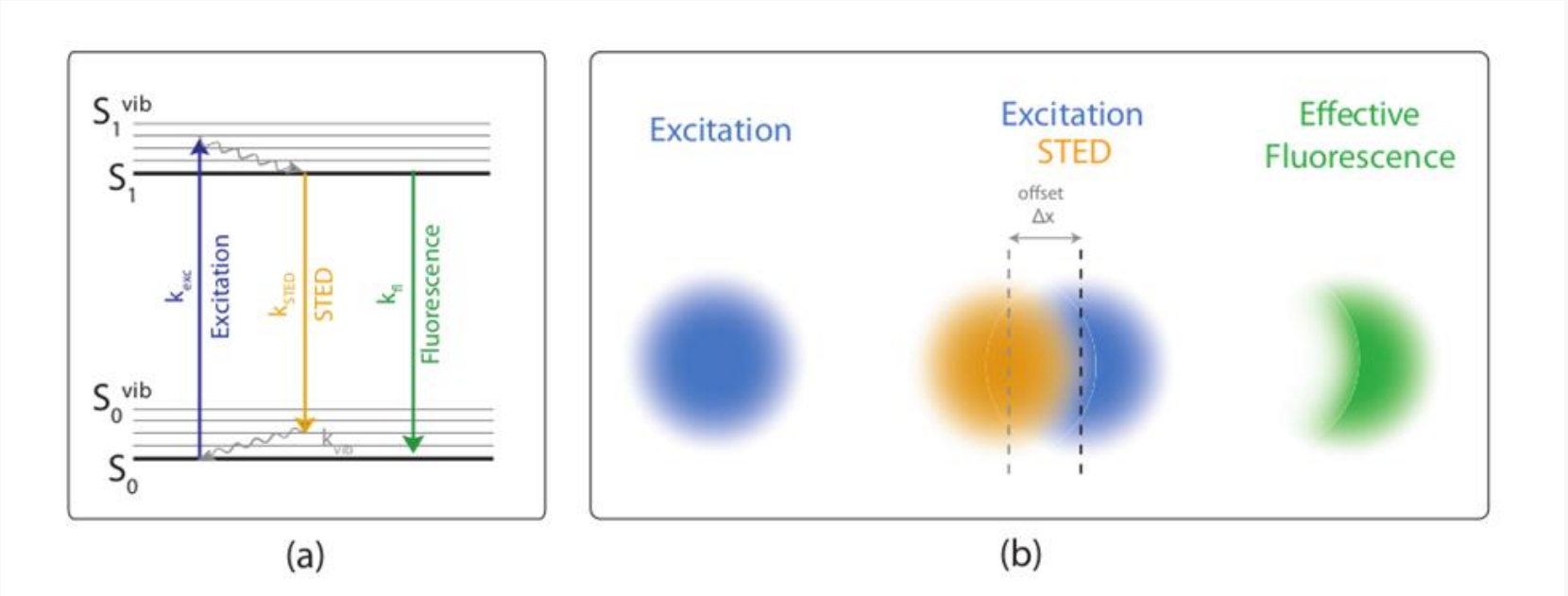


Illustration of spatial resolution of confocal microscope and STEM microscope

Stimulate Emission Depletion (STED) Microscopy

Stimulate Emission Depletion (STED) microscopy is a powerful imaging method which uses localized excitation method to be breaking the diffraction barrier to improve the lateral spatial resolution.



5 (a) Jablonski diagram illustrating the process of excitation, stimulated emission and fluorescence. (b) concept of STED

Stimulate Emission Depletion (STED) Microscopy: Beam Shaping

Beam shaping required to create a STEM beam in such a way the beam has a symmetrically high intensity profile around a zero-intensity center in the focus. A doughnut-like shape allows efficient and symmetrical confinement of the depleted region. Here, the beam must shape such that focused light interferes destructively in the center of focus.

.

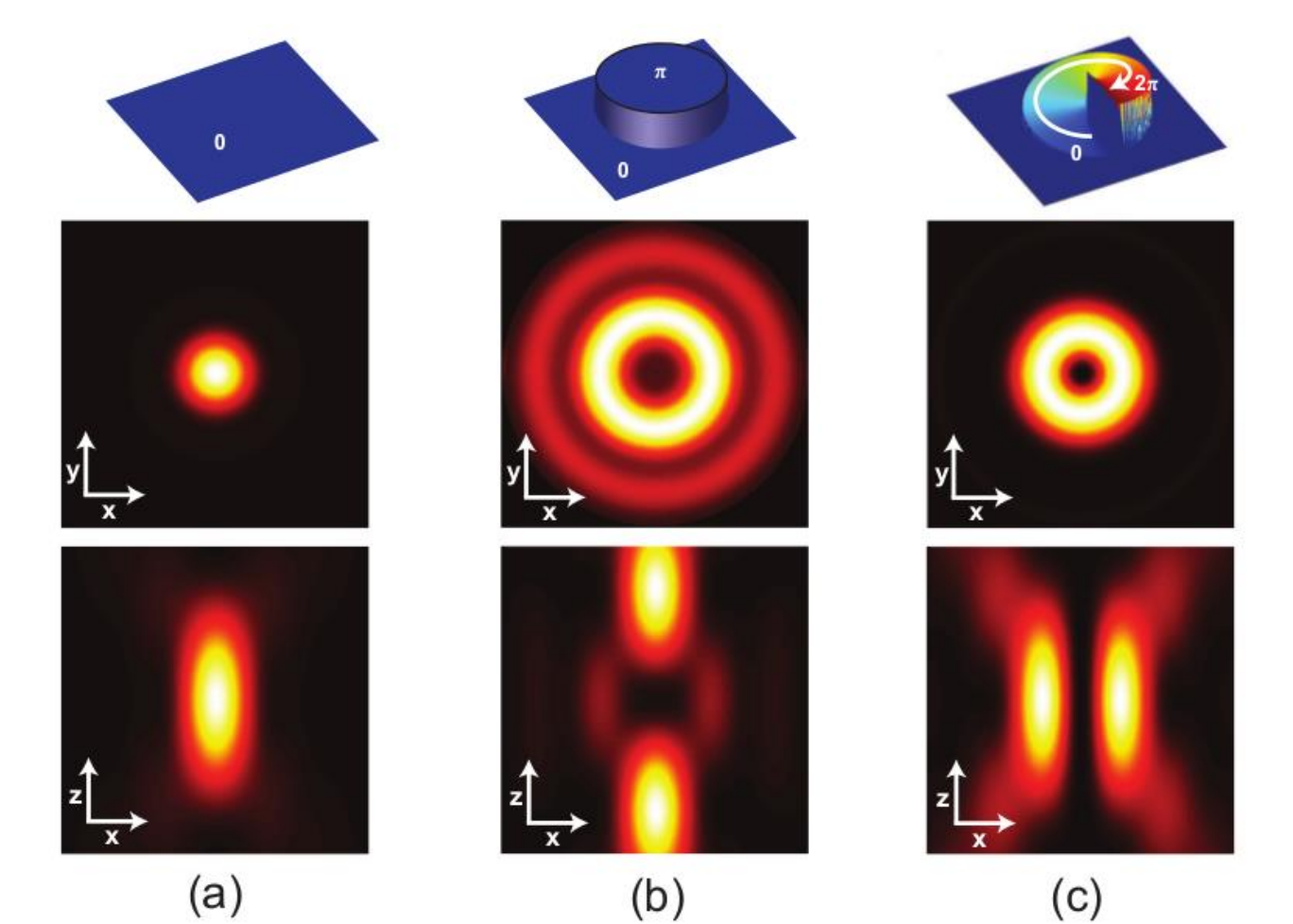


Illustration of beam shaping for STED microscopy.

Stimulate Emission Depletion (STED) Microscopy: Resolution

We have learned in the previous slide that overlap of a doughnut shaped STED beam leads to an increase in resolution. The spatial resolution is dependent on the STED excitation intensity. The effective fluorescent spot can be described mathematically as

$$\text{FWHM} \approx \frac{\lambda}{2\, n \sin\theta \sqrt{1 + \frac{I_{STED}^{max}}{I_{sat}}}} = \frac{\lambda}{2\, NA \sqrt{1 + \frac{I_{STED}^{max}}{I_{sat}}}}$$

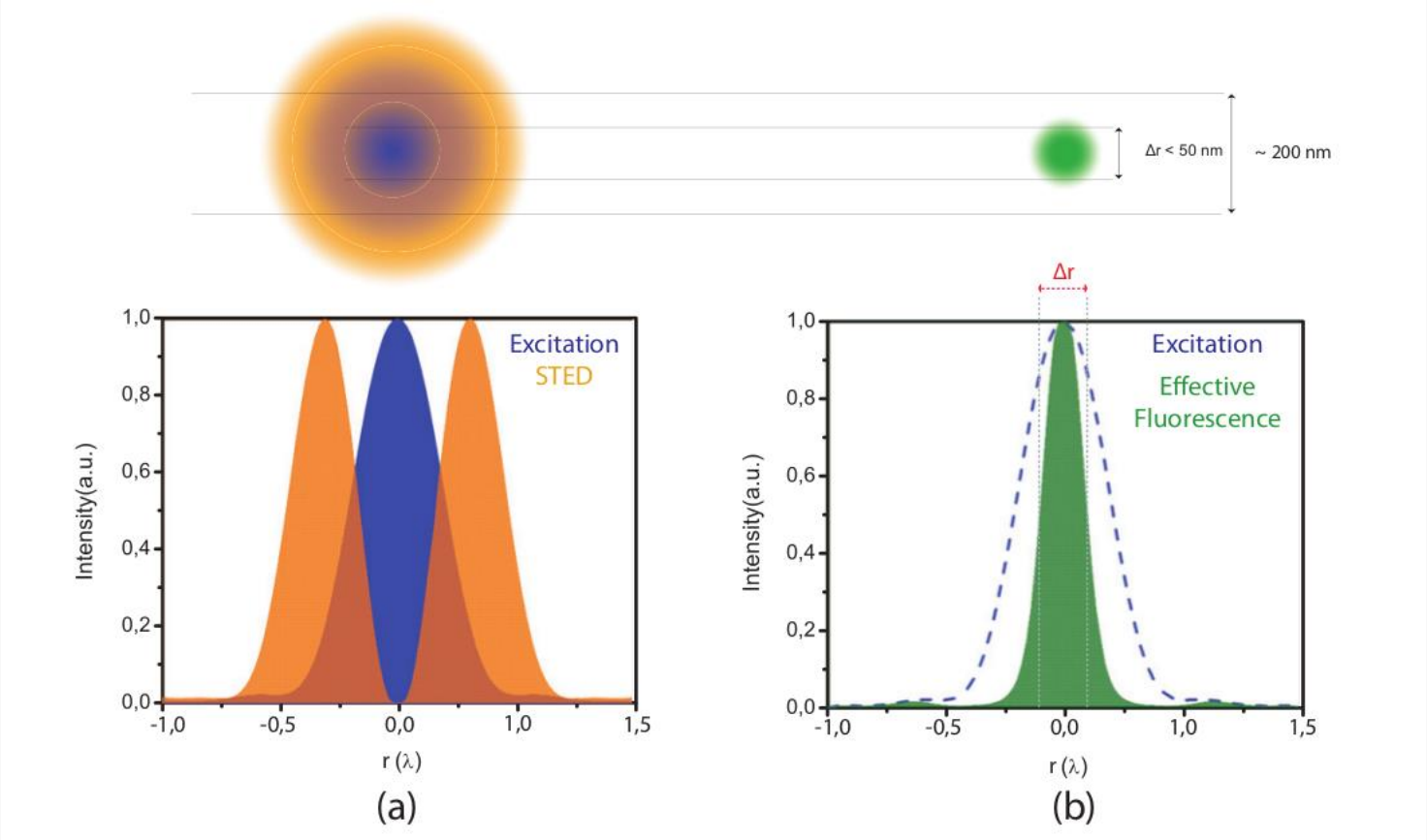
Where I_{sat} is the saturation intensity of the fluorophore and I_{STED}^{max} is the intensity of STED laser. Also note that when the STED intensity $I_{STED}^{max} = 0$ the above equation reduces to Abbe's diffraction limit.

.

Stimulate Emission Depletion (STED) Microscopy

In practice, however, the extreme intensities required to reach extreme high resolutions would lead to unwanted effects such as photo-bleaching, optical trapping, multi-photon absorption, sample heating or even sample destruction.

.



Stimulate Emission Depletion (STED) Microscopy

STED microscopy allows super-resolution imaging in the 50nm range. Unfortunately, this increased optical resolution also leads to a drawback: because many fluorophores are depleted by the depletion laser, this also results in a lower signal (fewer photons) being captured by the detector. Because of the Poisson nature of photon statistics, the signal-to-noise ratio of the resulting image will decrease compared to normal confocal imaging. Therefore, the SNR value in STED images will be much lower compared to their confocal counterpart.

.

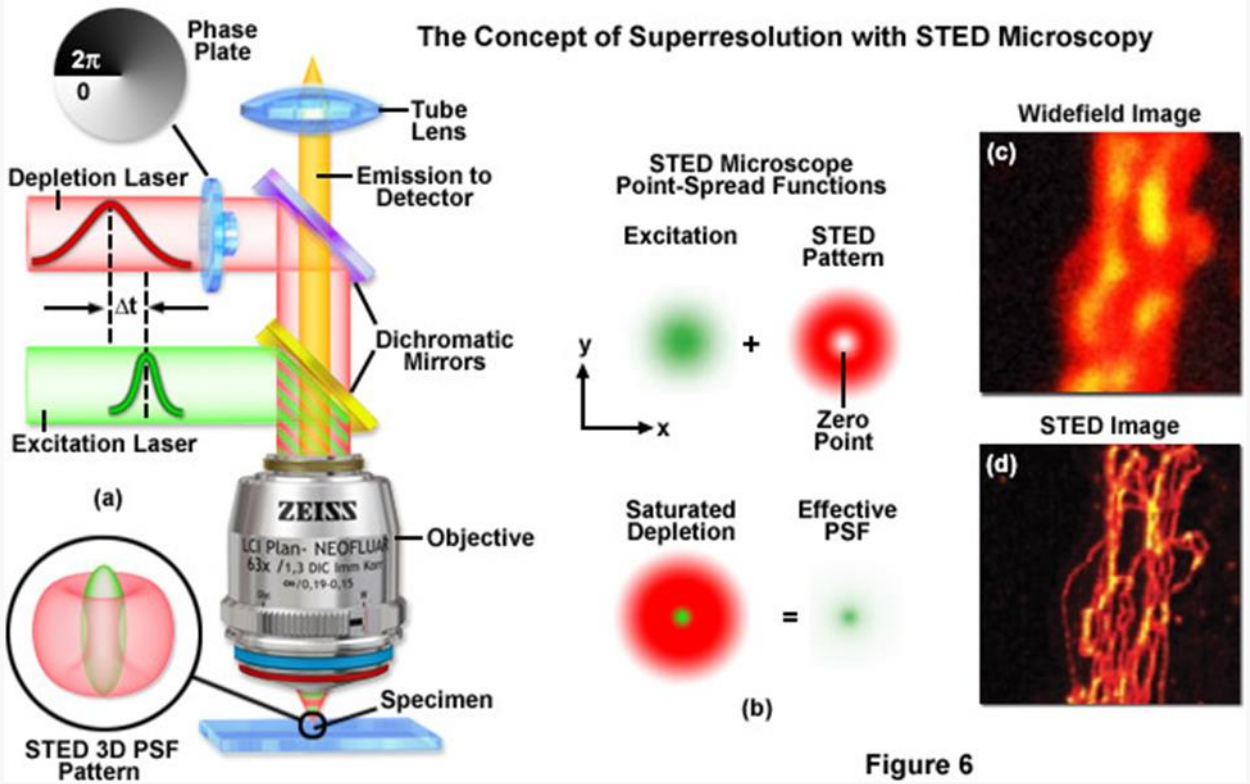


Figure 6

Saturated Structured Illumination Microscopy (SSIM)

Saturated Pattern Excitation Microscopy (SPEM) and Saturated structured illumination microscopy (SSIM) is a non-linear method deplete the fluorophore ground state by saturated excitation to generate a sinusoidal emission pattern that is recorded on an area-array CCD detector. Experimental verification of the concept of SSIM was demonstrated by Mats Gustaffon in 2005 with resolution better than 50 nm.

.

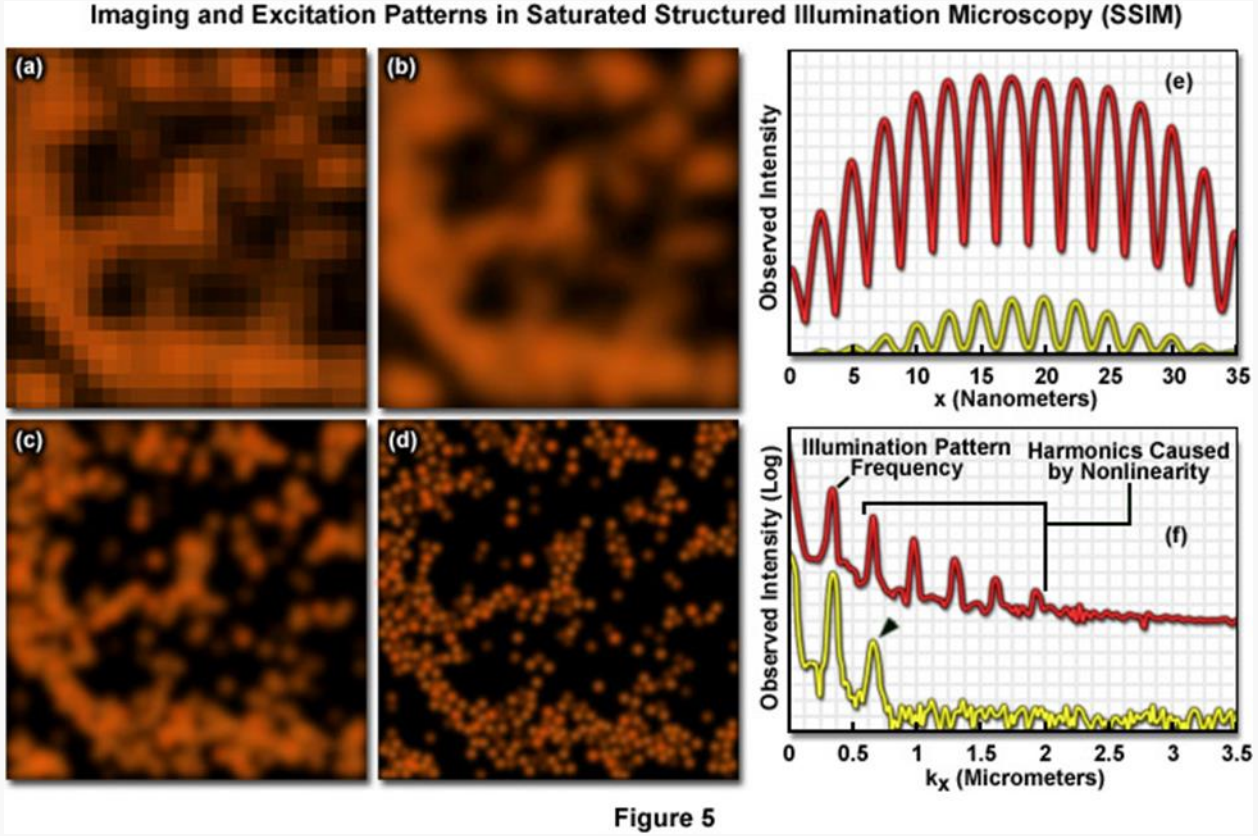


Figure 5

Reversible Saturable Optical Linear Fluorescence Transitions (RESOLET)

Reversible (or switchable) Saturable Optical Linear Fluorescence Transitions (RESOLFT) uses the mechanism of switching principle. No like STED, the switching mechanism does no longer need to be purely electronically, but through conformational changes of the molecules.

This scheme focuses on fluorescent probes that can be reversibly photo-switched between a fluorescent "on" state and a dark "off" state (or between any two states **A** and **B**). The exact nature of these states is variable and can be the ground and excited singlet states.

The RESOLFT concept also includes switching isomerization states (such as *cis-trans*) and other optically bi-stable transitions in fluorophores.

.

Reversible Saturable Optical Linear Fluorescence Transitions (RESOLET)

Extremely high intensities are necessary for switching off the excited singlet state with STED (using what is termed a **depletion laser**), but switching to a metastable triplet or similar dark state (GSD) requires a light intensity between a thousand and a million times lower.

.

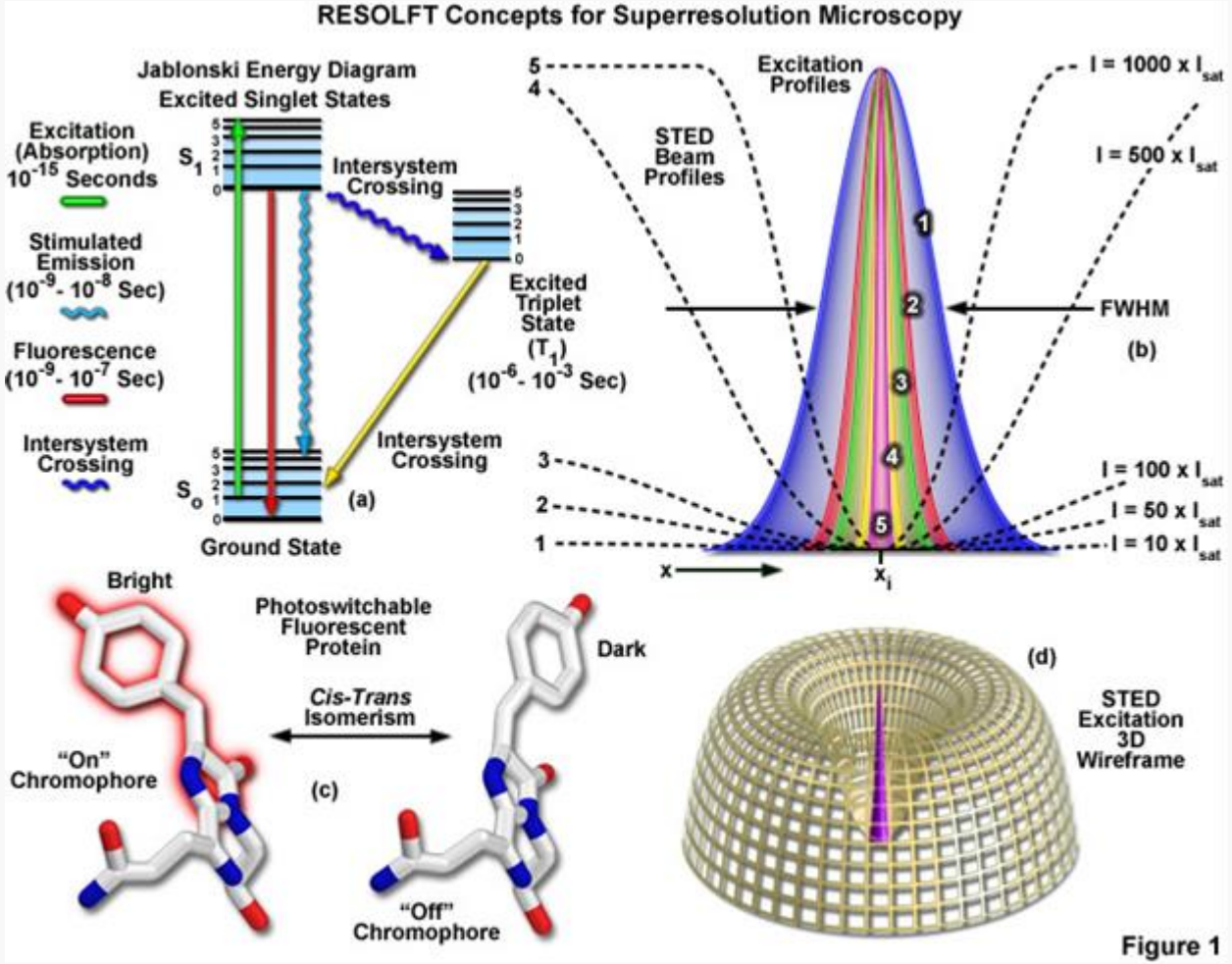


Figure **Error! No text of specified style in document.**-1 RESOLFT concepts for super-resolution microscopy.

Near-Field Optical Microscopy

- Concepts of **near-field** and **far-field**, which have been "borrowed" from electromagnetic radiation theory developed for antenna technology and applied to microscopy.

- In near-field microscopy, the specimen is imaged within a region having a radius much shorter than the illumination wavelength.
- In contrast, far-field microscopy positions the specimen many thousands of wavelengths away from the objective (often a millimeter or more) and is limited in resolution by diffraction of the optical wavefronts as they pass through the objective rear aperture. Most of the conventional microscopes are far-field.
- Near-field microscopes circumvent the diffraction barrier by exploiting the unique properties of evanescent waves.
- Resolution is limited only by the physical size of the aperture rather than the wavelength of illuminating light, such that lateral and axial resolutions of 20 nm and 2 to 5 nm, respectively, can be achieved.

Thank you very much for your attention