Light Microscopy

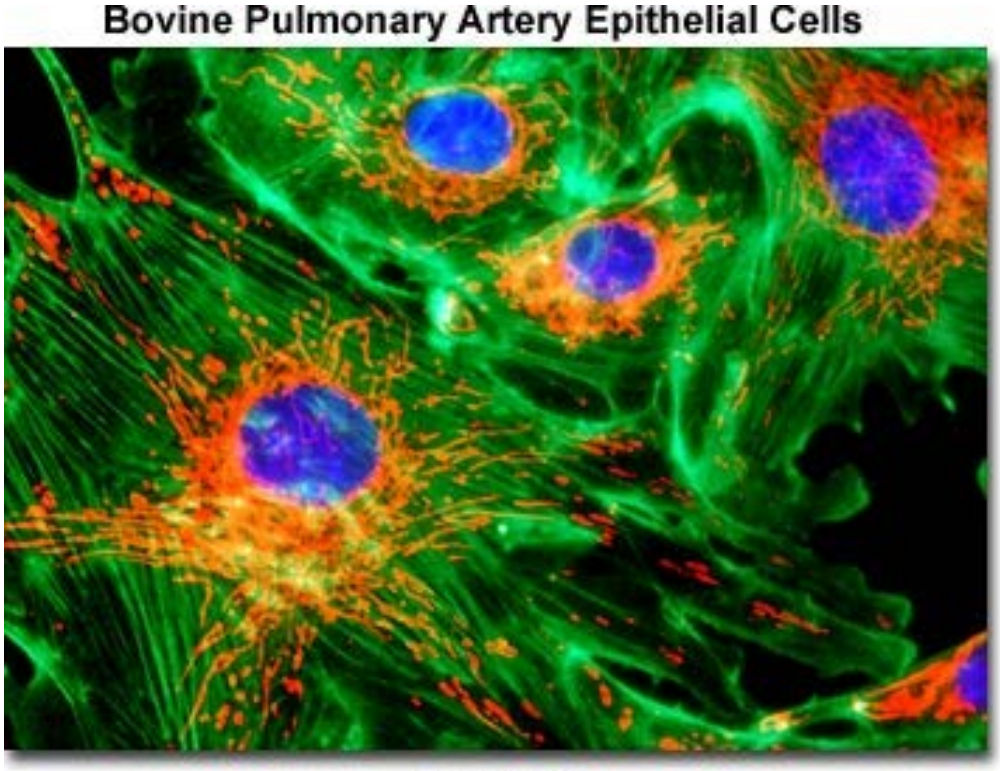


Figure 1

Need for microscopy to measure smaller and fainter objects in cells

Play cell size tutorial!

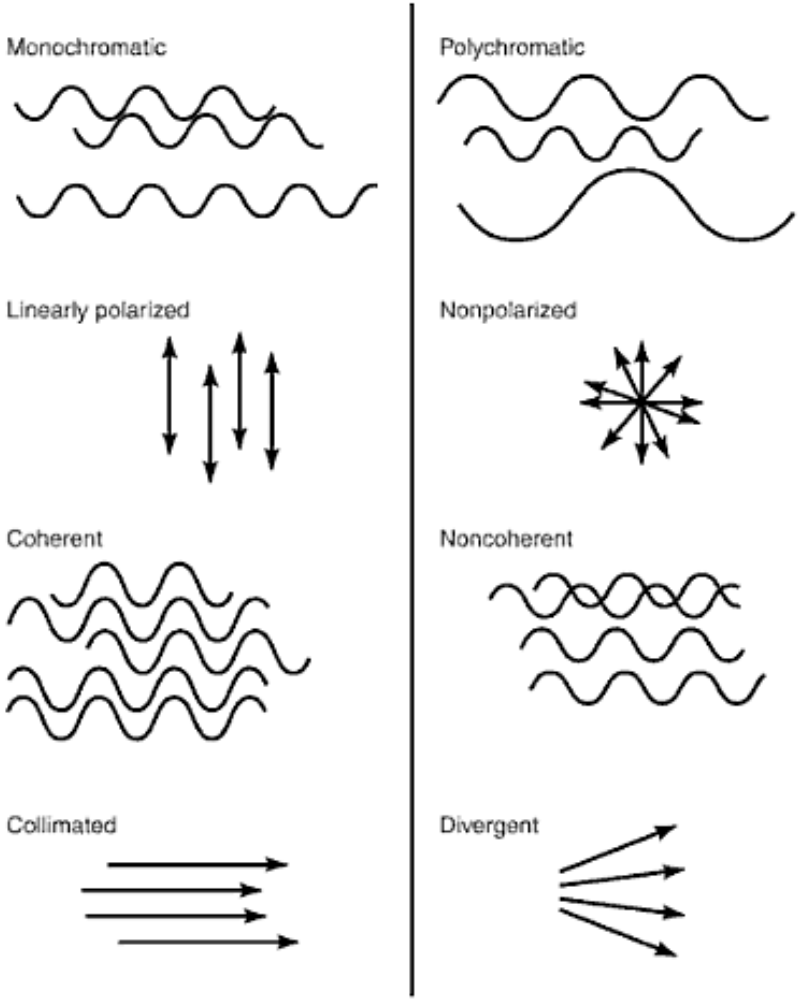
Quality of Light

wave

vector

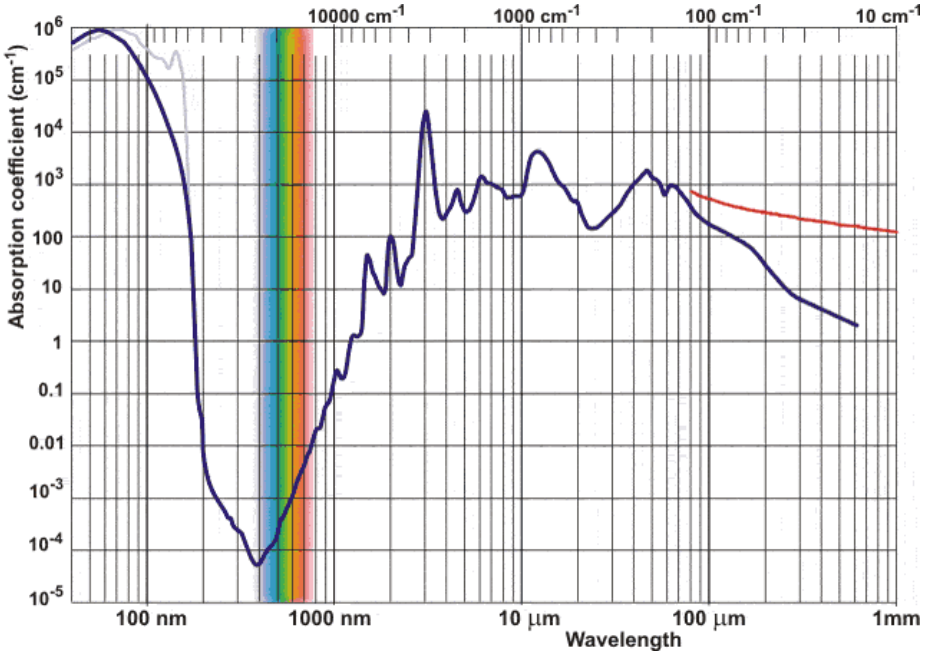
phase

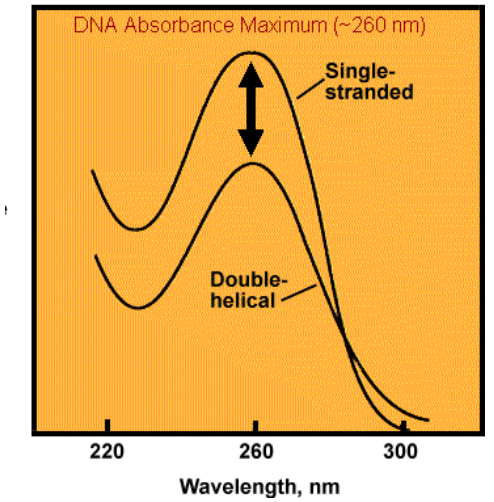
beam



Effects of Light on Cells

Absorption of Water



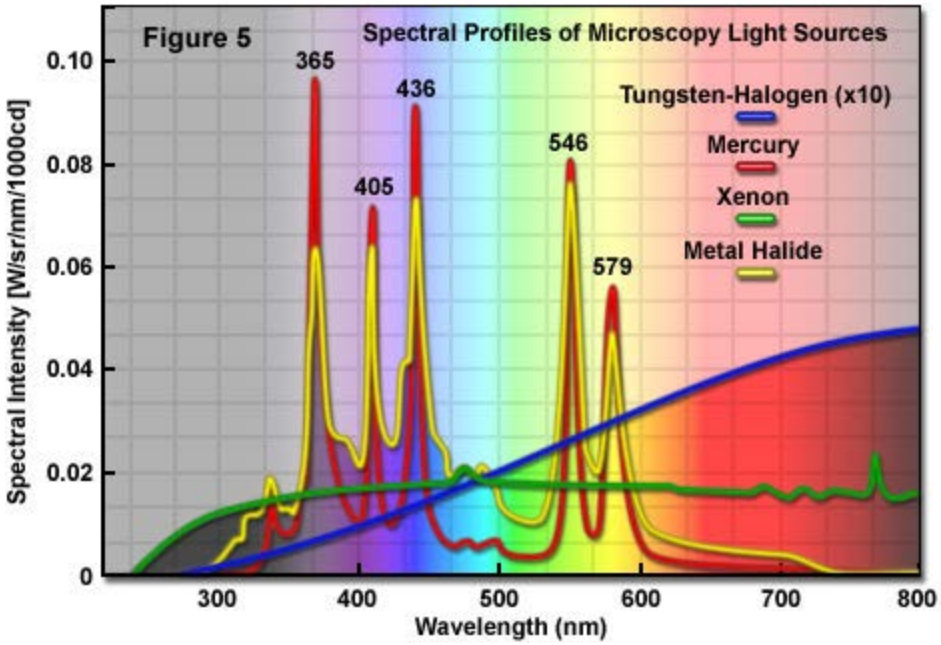


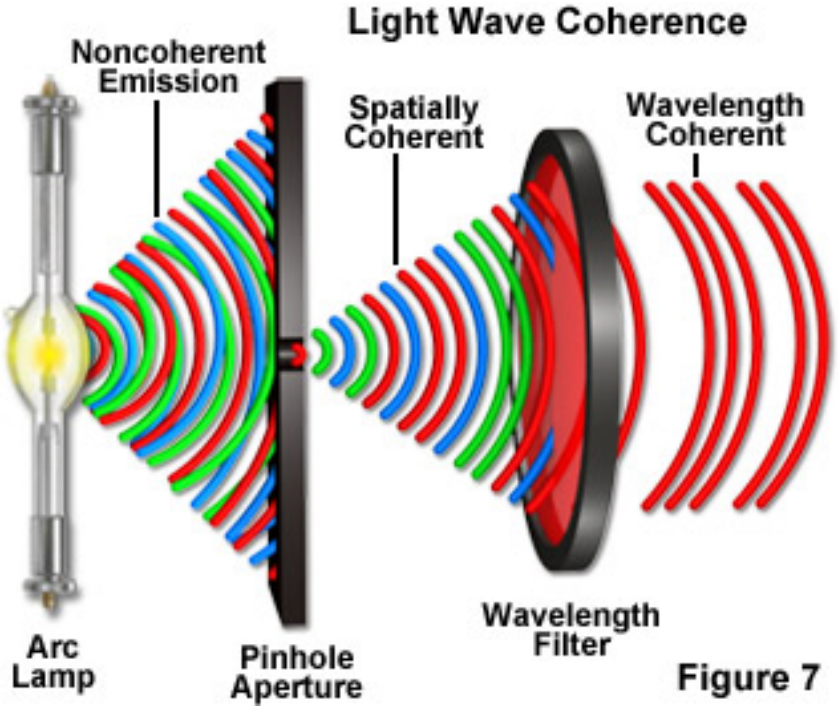
VIS light is ideal for live cell imaging

Light Sources

LAMPS

Point source
Slightly coherent
Polychromatic
Unpolarized





LASERS

Collimated
Highly coherent
Monochromatic
Linearly polarized

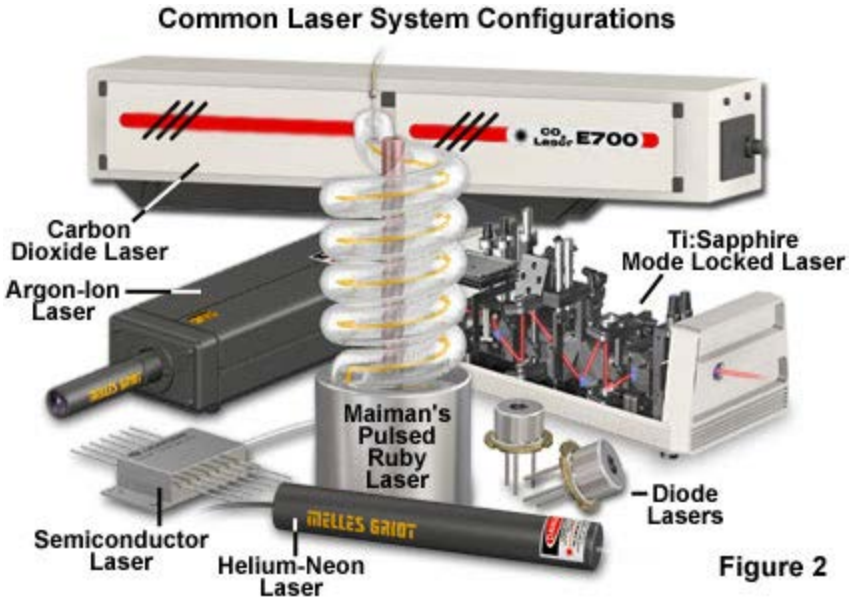
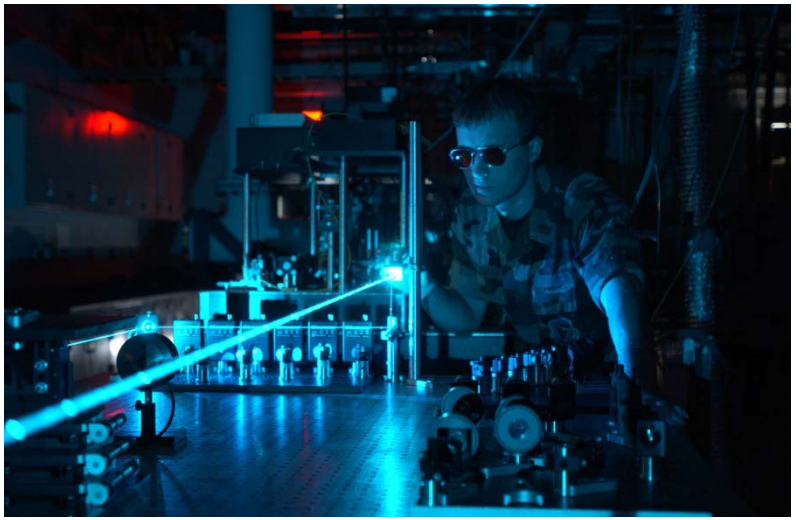


Figure 2



How Lasers Generate Coherent Light

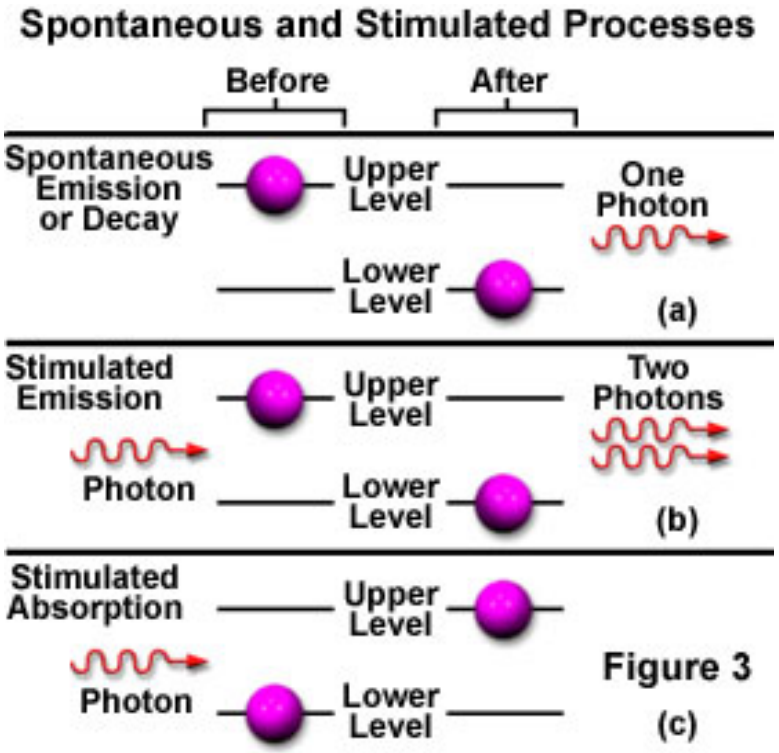


Figure 3

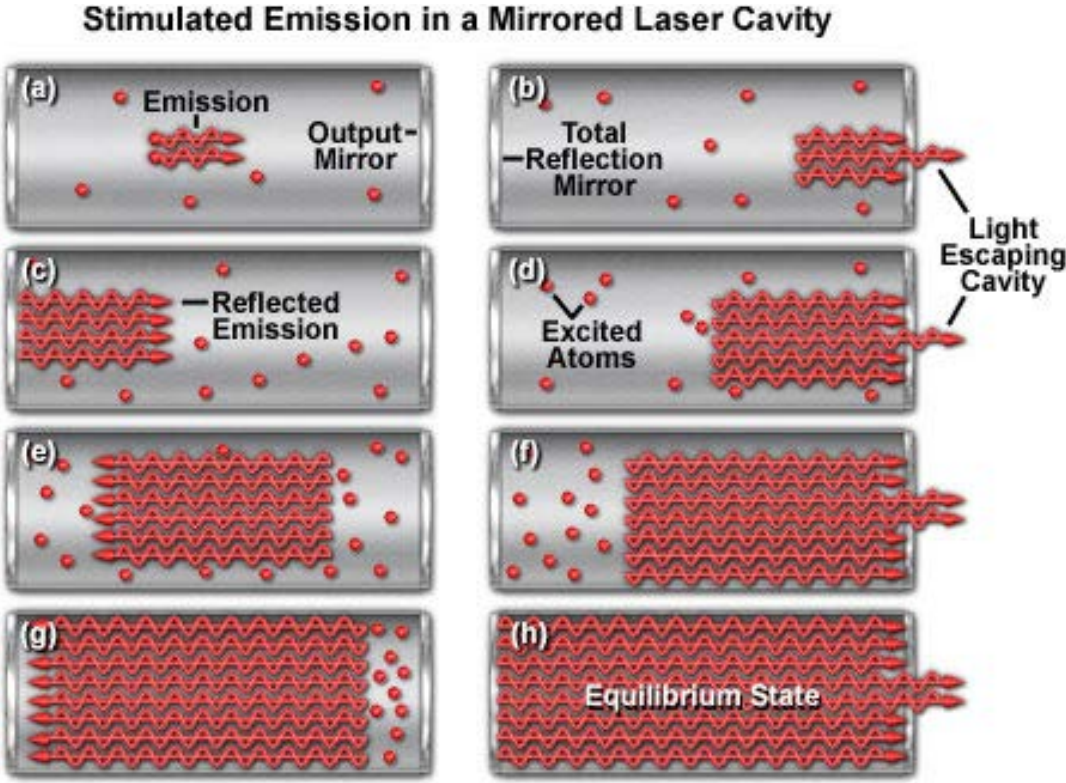
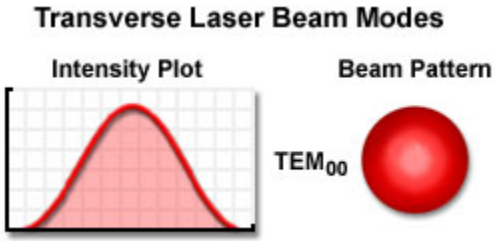
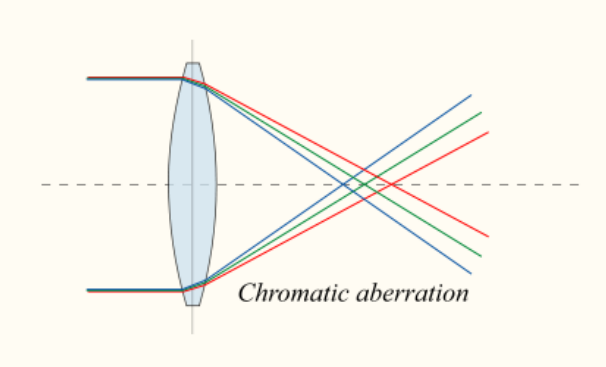


Figure 6

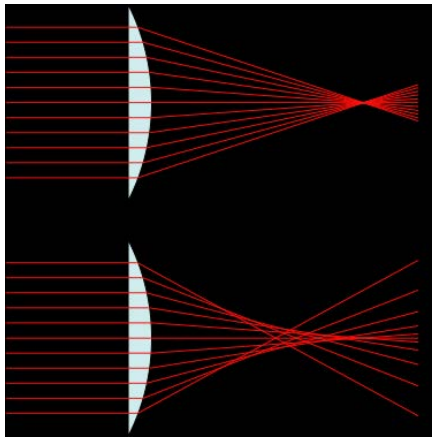
RESONANCE **N • λ = 2 • (Cavity Length)** where **N** is an integer, and λ is the wavelength.



Lens Aberrations



refraction of light



Spherical aberration

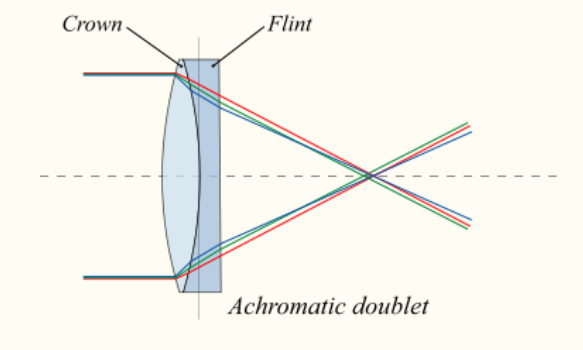
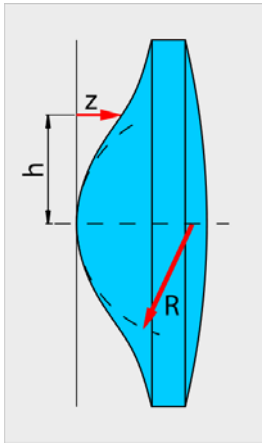
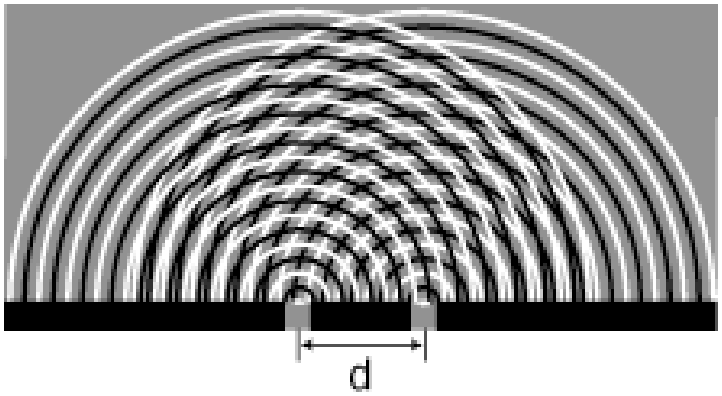


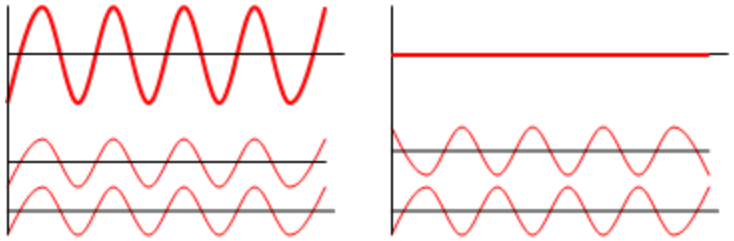
Figure 1



Aspheric biconvex

Light as a Wave: Interference



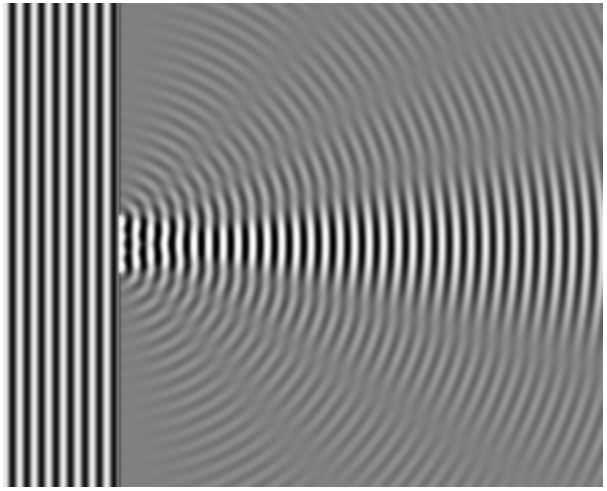


Destructive interference

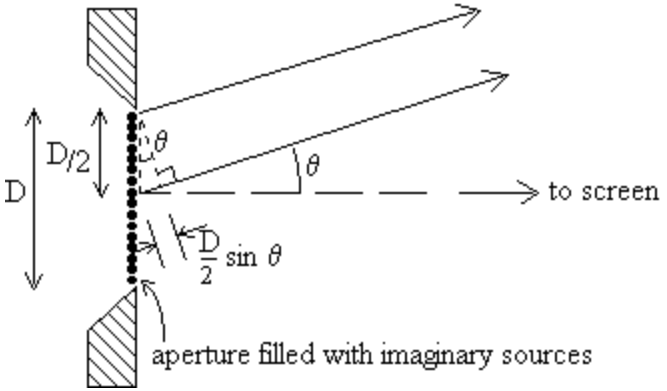
Constructive interference

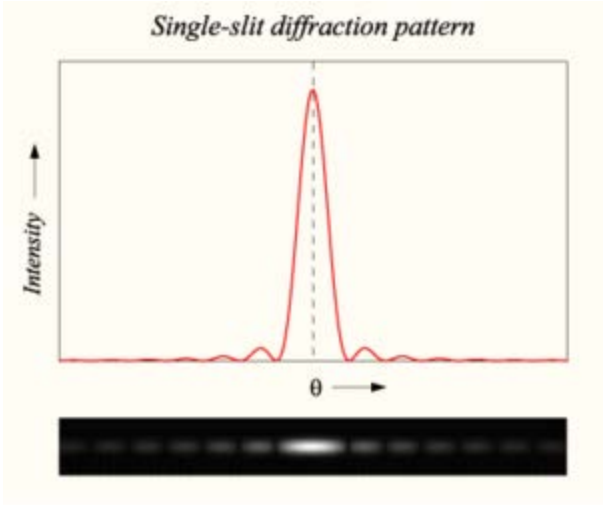
Interference Pattern is observed when the two beams are out of phase!

Diffraction



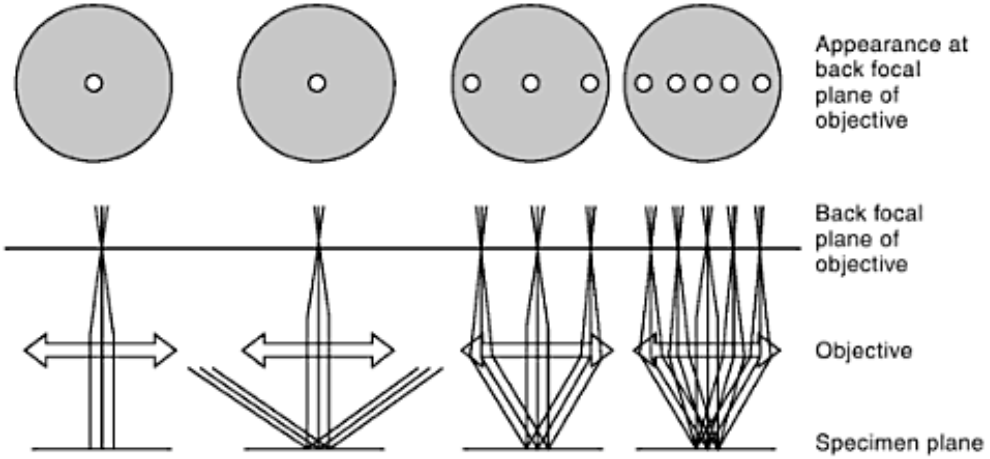
diffraction

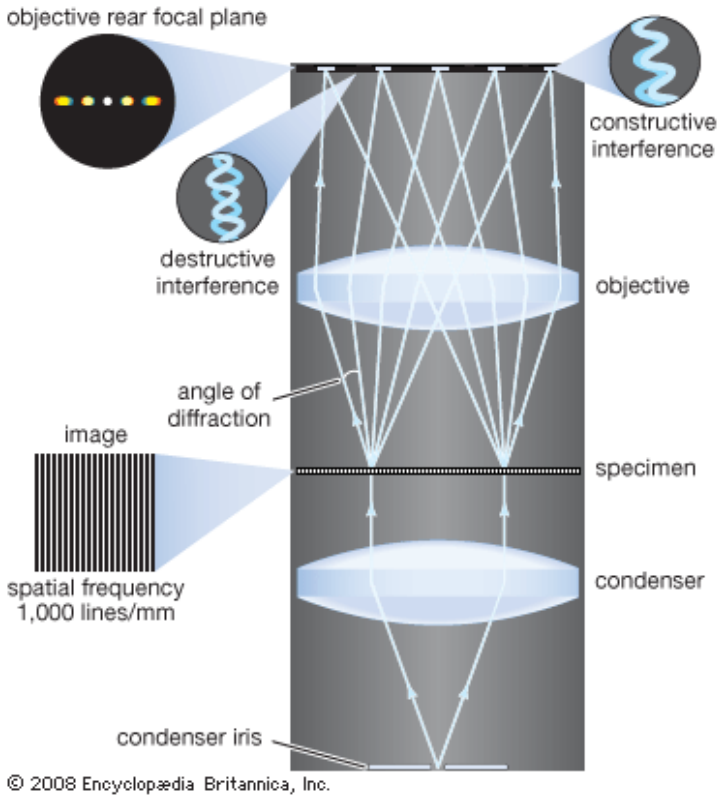




$$\frac{D}{2}\sin\theta = \frac{\lambda}{2}$$ First minimum

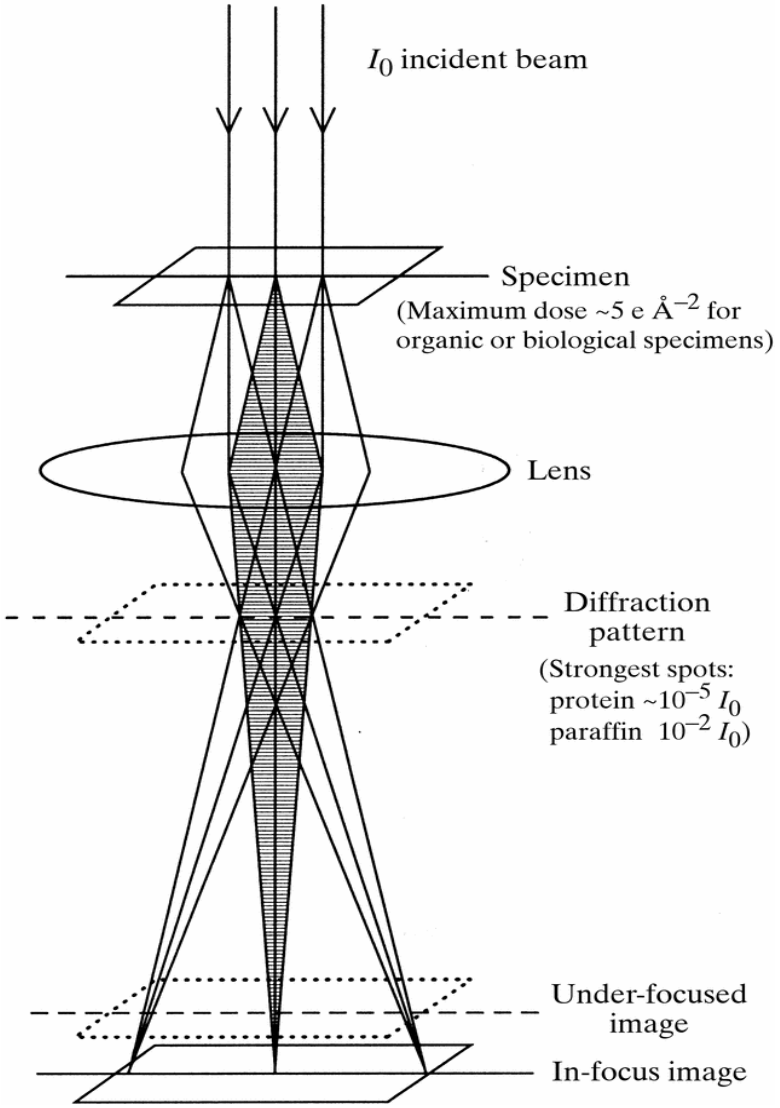
Objective Back Focal Plane





1. Light is diffracted by the specimen
2. Collection of diffracted light by the objective
3. Interference of diffracted and nondiffracted rays

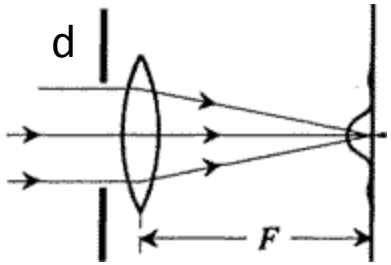
Image

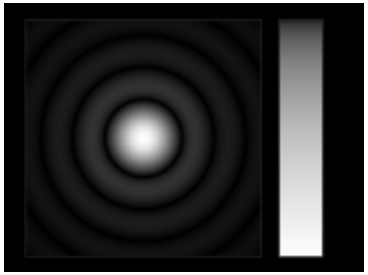


Interference between 0th order and higher order diffracted rays in the image plane generates image contrast

Interference of diffracted rays is the inverse transform of diffraction

Circular Aperture Diffraction

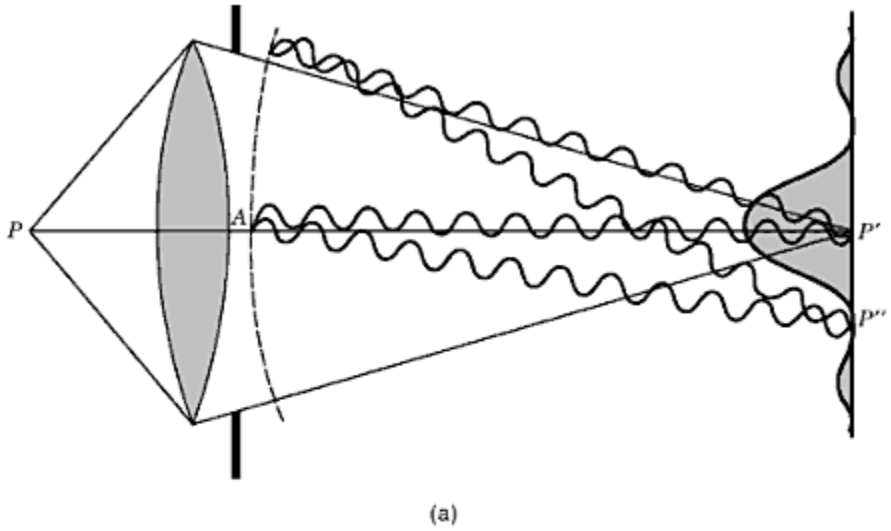




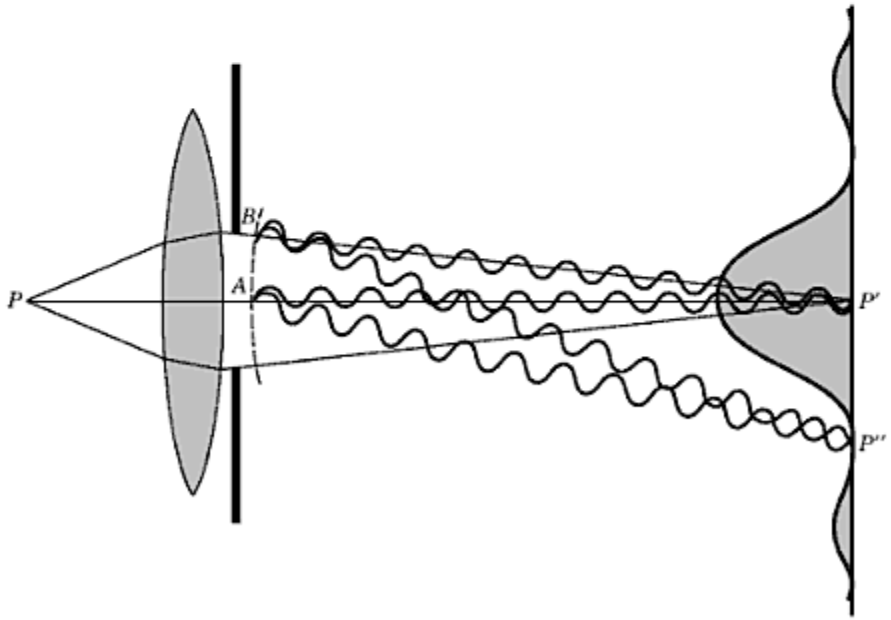
$$I(\theta) = I_0 \left(\frac{2J_1(ka \sin\theta)}{ka \sin\theta} \right)^2 = I_0 \left(\frac{2J_1(x)}{x} \right)^2$$ $k = 2\pi / \lambda$, d=2a

First minima $$\sin\theta = 1.22 \frac{\lambda}{d}$$

Aperture Angle



(a)



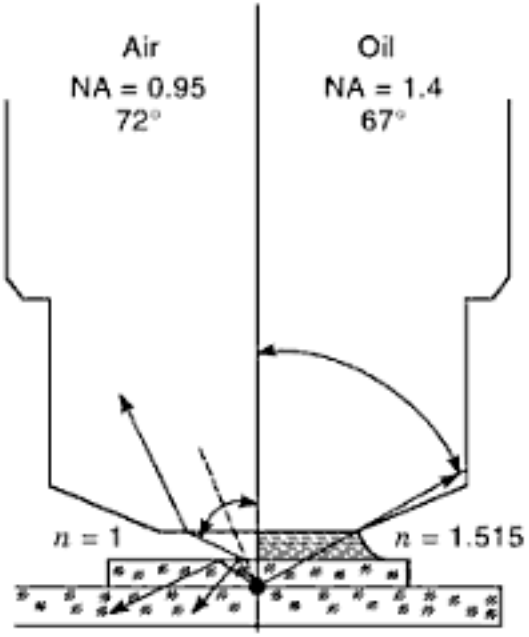
(b)

The width of the spot depends on the aperture size, or aperture angle determines the spot size.

Numerical Aperture

$NA = n.\sin\theta$

$n_{air} * \sin\theta_c = n_{glass} * \sin\theta_i$
$1 = 1.515 * \sin\theta_i \quad \theta_i = 40°$

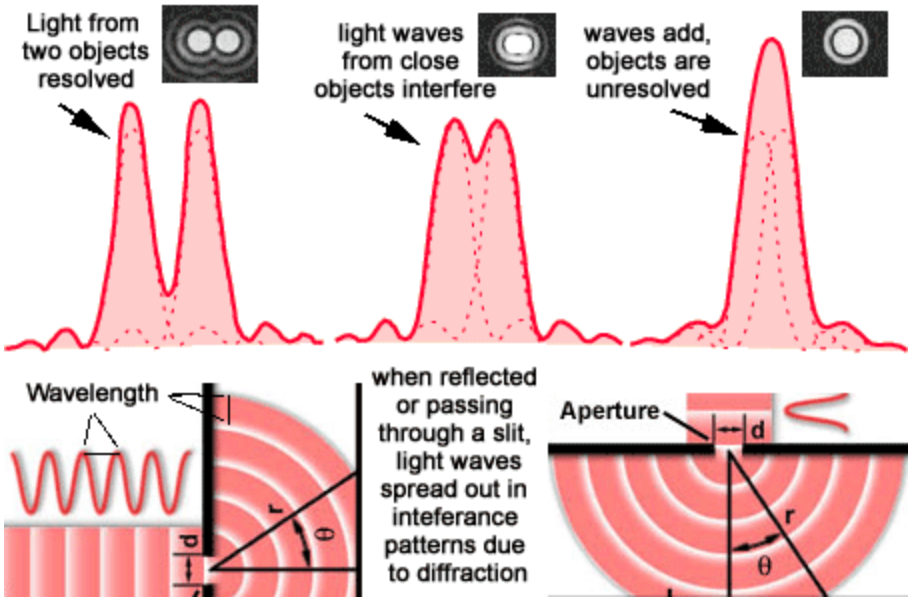


$\theta_i = 72°$
NA limit is 1.49

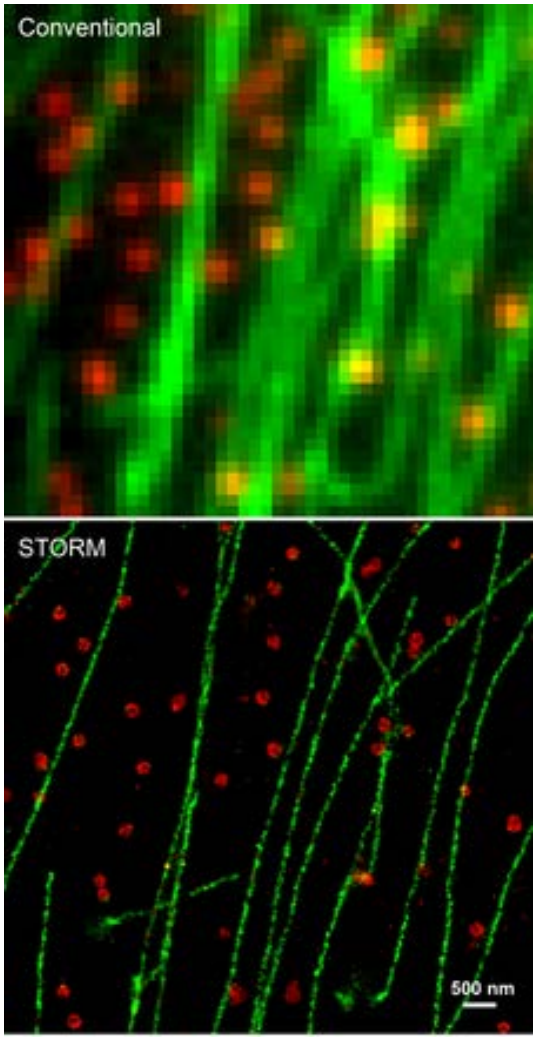
$d = 1.22 * \lambda / (2 * NA)$, where d is minimum resolved distance

Resolution Limit of the Light Microscope

Abbe's Limit



d = 1.22*λ/(2*NA) ~250 nm in VIS region



zhuang.harvard.edu

Infinity Optics

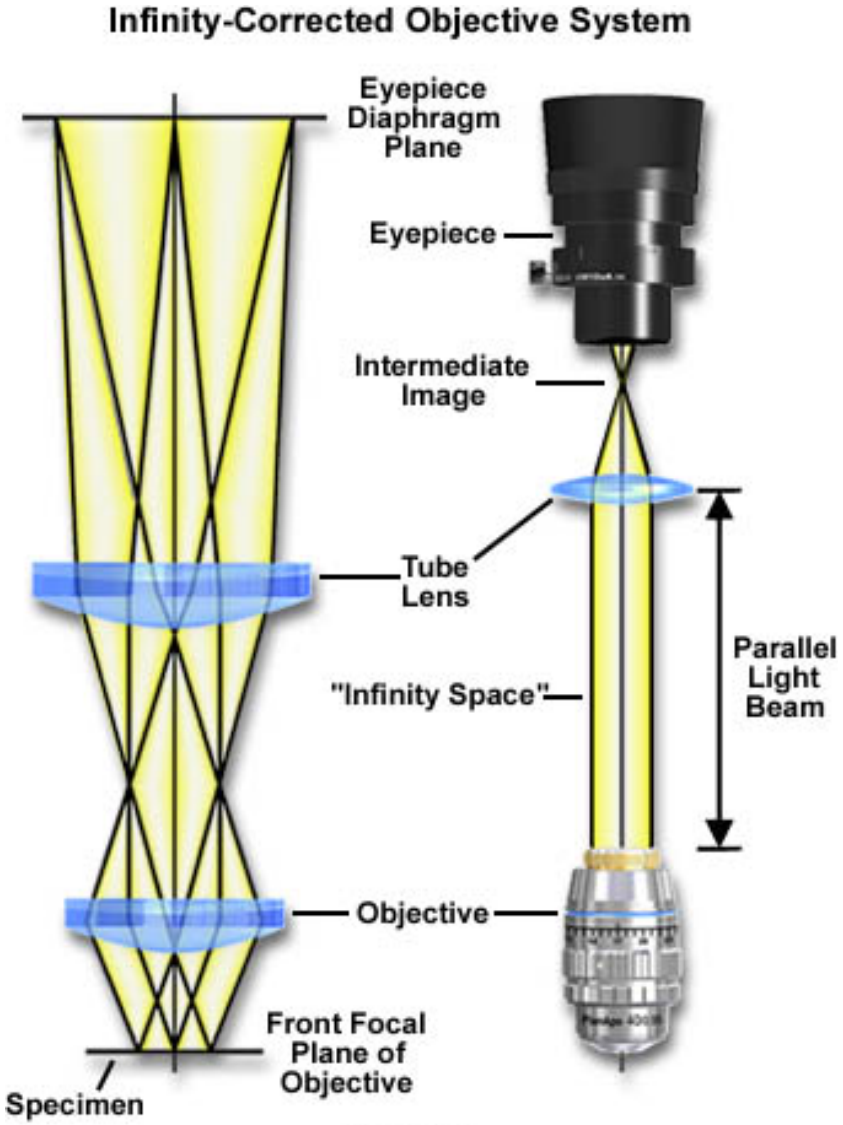


Figure 3

Microscope systems with **finite tube length** were produced in the past.

When objectives and tube lengths are mismatched, image quality often suffers due to the introduction of **spherical aberrations** because the ***optical tube length*** is changed

When optical element is inserted is placed in the light path between the back of the objective and the eyepiece, the mechanical tube length changes

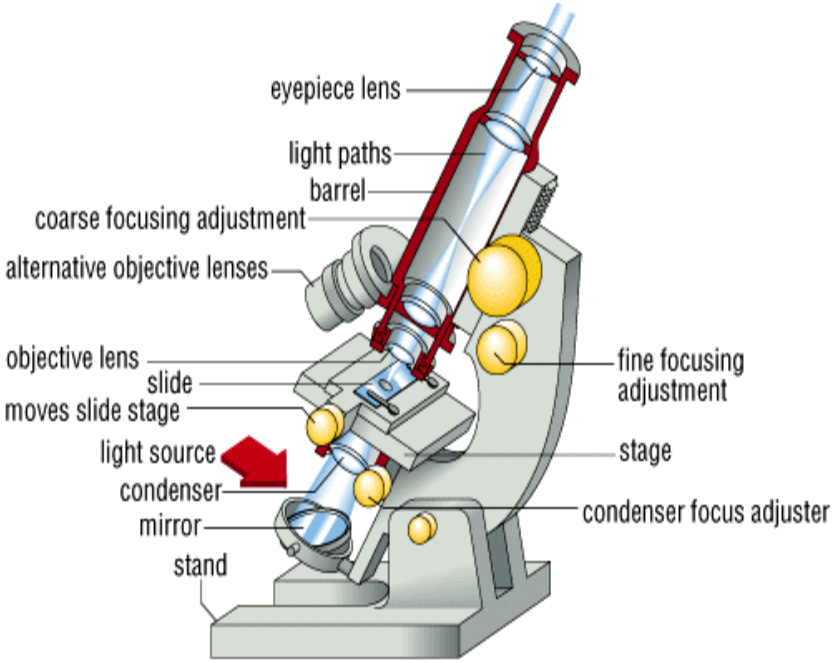
infinity-corrected objectives generate emerging parallel rays and provide flexibility to the user to modify the path.

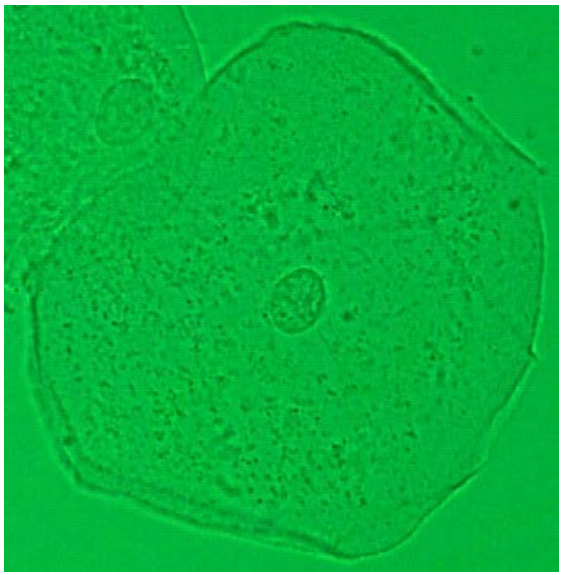
Bright Field Microscopy

Simplest of all microscopy techniques

Limitations of standard optical microscopy: (bright field microscopy) lie in three areas;

- The technique can only image dark or strongly refracting objects effectively.
- Diffraction limits resolution to approximately 0.2 micron.
- Out of focus light from points outside the focal plane reduces image clarity.





Most biological specimens do not absorb much light to produce contrast, but they diffract the light and cause a phase shift of the transmitted wave.

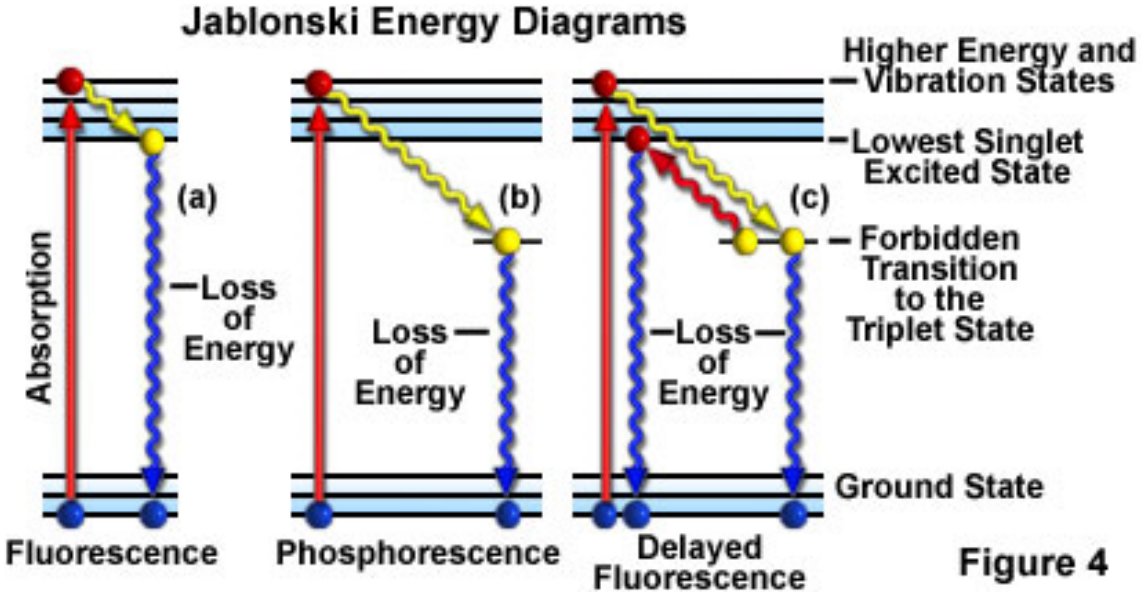
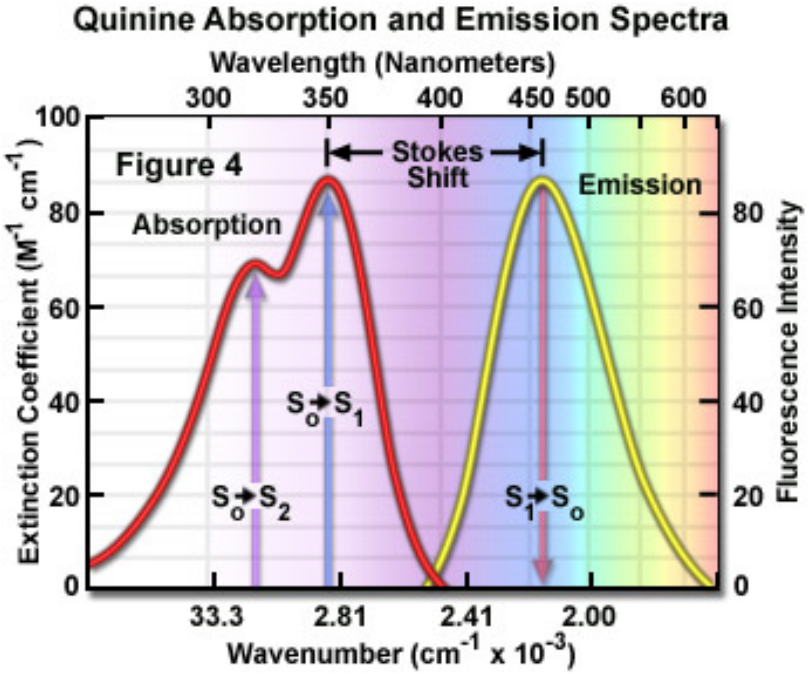

Jablonski Energy Diagrams
Higher Energy and Vibration States
Lowest Singlet Excited State
Forbidden Transition to the Triplet State
Ground State
(a)
(b)
(c)
Absorption
Loss of Energy
Loss of Energy
Loss of Energy
Fluorescence
Phosphorescence
Delayed Fluorescence

Figure 4


Quinine Absorption and Emission Spectra
Wavelength (Nanometers)
300
350
400
450
500
600
Figure 4
Stokes Shift
Absorption
Emission
$S_o \rightarrow S_1$
$S_o \rightarrow S_2$
$S_1 \rightarrow S_o$
Extinction Coefficient (M^{-1} cm^{-1})
Fluorescence Intensity
0
20
40
60
80
100
33.3
2.81
2.41
2.00
Wavenumber (cm^{-1} x 10^{-3})

Photobleaching and Blinking of Fluorophores

•Molecules with electrons in the triplet state are chemically reactive
•Since the rate of conversion is slow, molecules stay in the triplet state a long time (**spin conversion)**
•**Dissolved oxygen** (ground state is triplet) is highly reactive with fluorophores in the triplet state, leading to free radicals (singlet oxygen) that are toxic to cells.
•In photobleaching, fluorophore permanently loses the ability to fluoresce due to photon-induced chemical damage and covalent modification

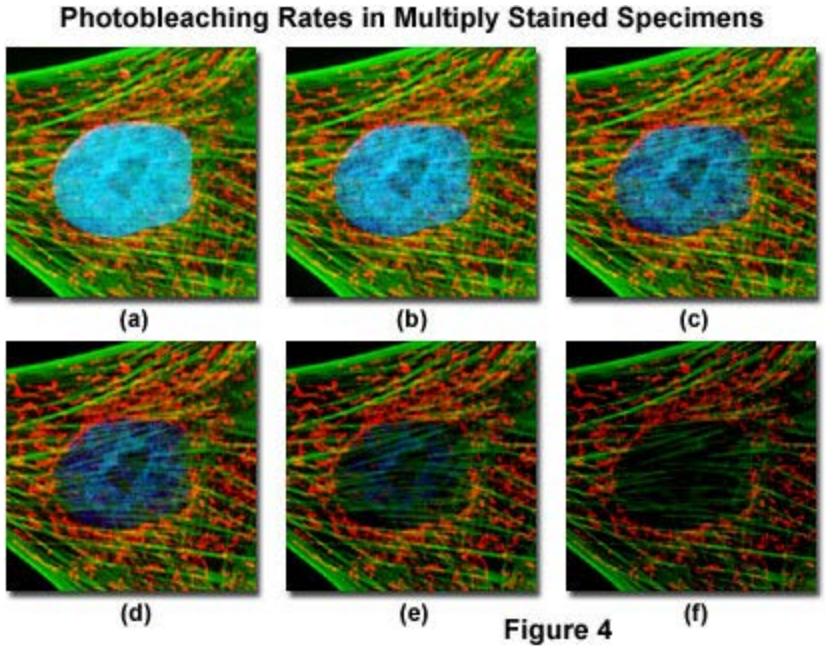


Figure 4

Photobleaching can be reduced by limiting the exposure time of fluorophores to illumination or by lowering the excitation energy (***low signal***)

Solution can be deoxygenated by antioxidative reagents (gluocose oxidase, ascorbic acid)

Modern Fluorophore Technology

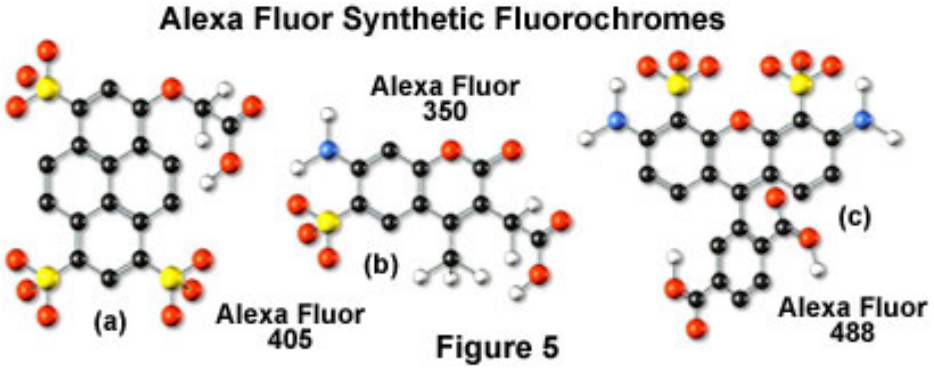


Figure 5

fluorescence intensity remains stable for long periods of time

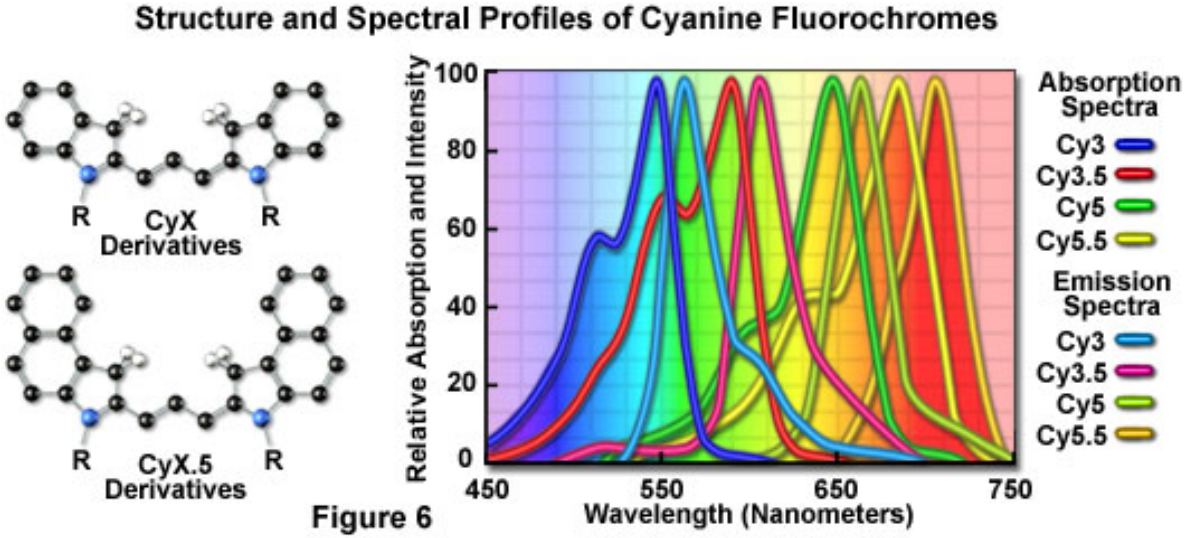


Figure 6

- High quantum yield
- High extinction coefficient
- Less pH sensitivity
- Enhanced photostability
- High duty ratio
- Less blinking
- Water solubility
- Matching absorption maxima
- Membrane permeable
- maleimides, succinimidyl esters, and hydrazides
- conjugated to phalloidin, G-actin, and rabbit skeletal muscle actin
- conjugates to lectin, dextrin, streptavidin, avidin, biocytin, and a wide variety of secondary antibodies

Quantum Dots

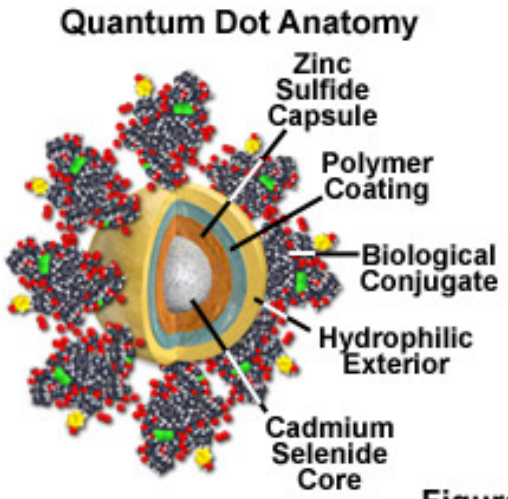
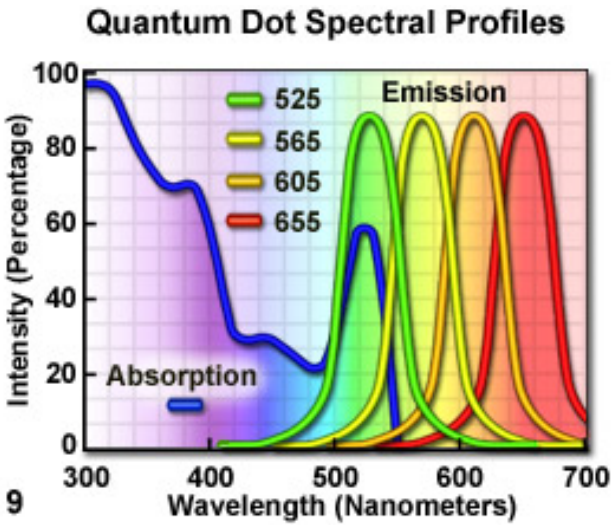
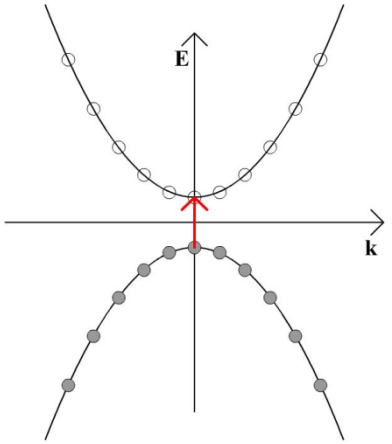


Figure 9





The absorbed photon creates an electron-hole pair that quickly recombines with the concurrent emission of a photon having lower energy.

Nanometer-sized crystals of purified semiconductors (CdSe)
ZnS surface coating improves brightness
Hydrophilic polymer shell coating (water solubility)
Biological conjugation (antibody, 6His, Streptavidin, wheat germ agglutinin

Long-term photostability (20 min)
High fluorescence intensity levels (20-50 fold)
Multiple colors with single-wavelength excitation
Narrow emission spectra (30nm FWHM)

Big Size (20-40 nm)
Multivalency (20-50 sites)
Toxicity to cells

The Fluorescent Protein Color Palette

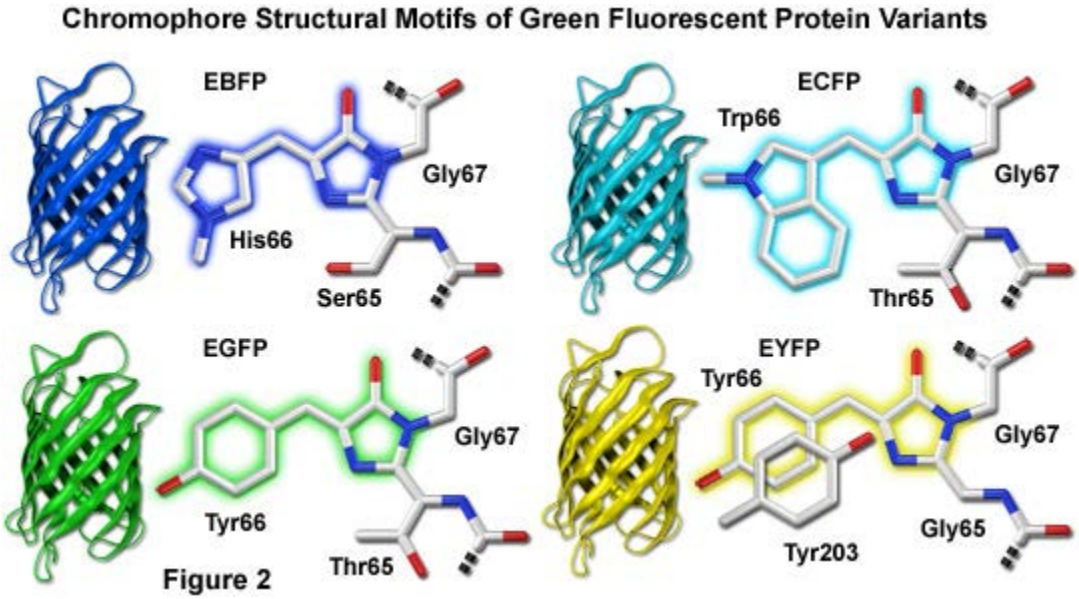


Figure 2

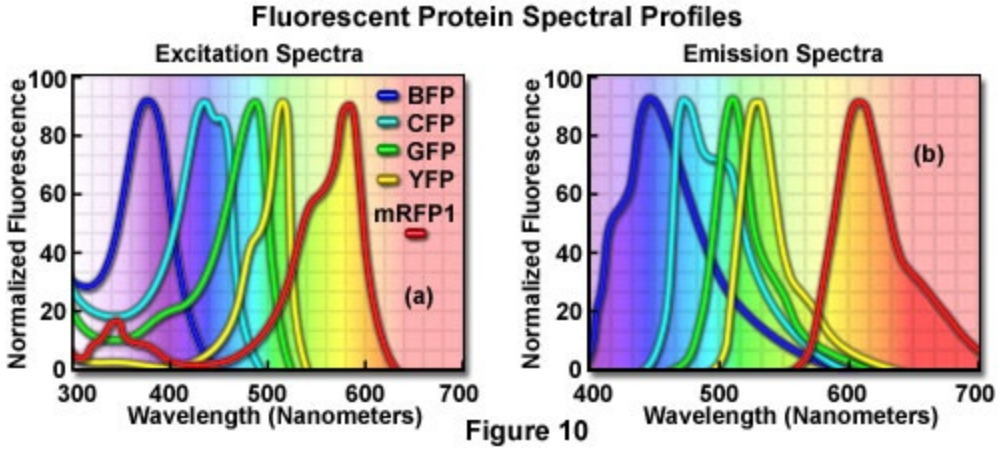
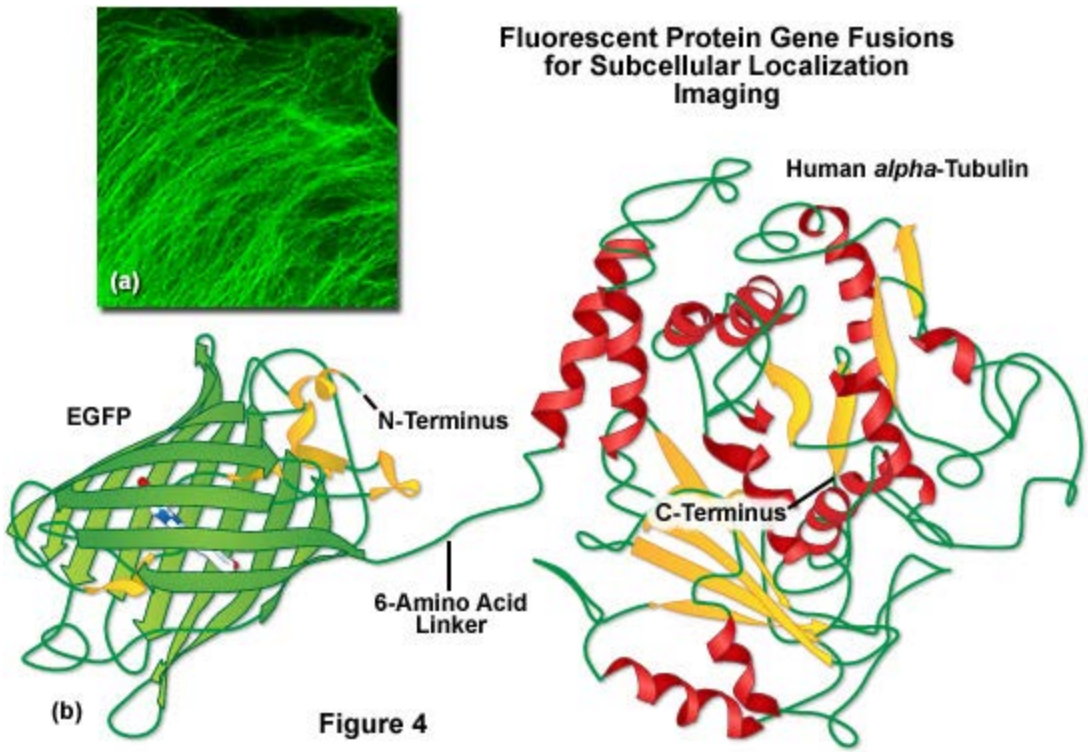


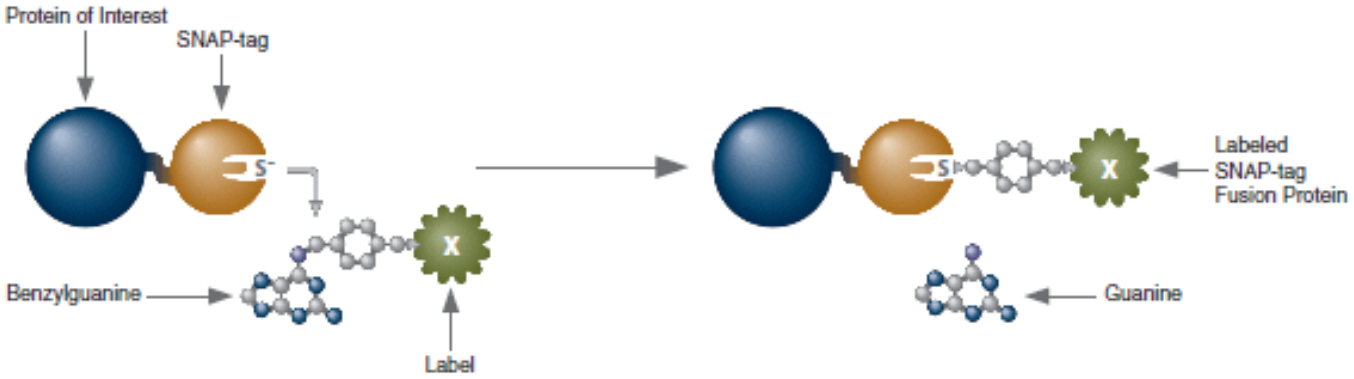
Figure 10



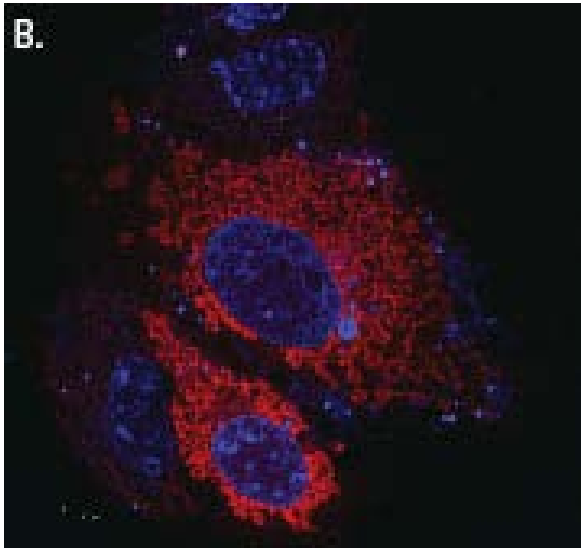
Remaining Issues:

- Fluorescent proteins are dim and susceptible to photobleaching
- Low expression is not observable because of cell autofluorescence
- GFP cannot be fused into every gene/genetic complications
- GFP size can be an issue for certain applications
- GFP dimerization

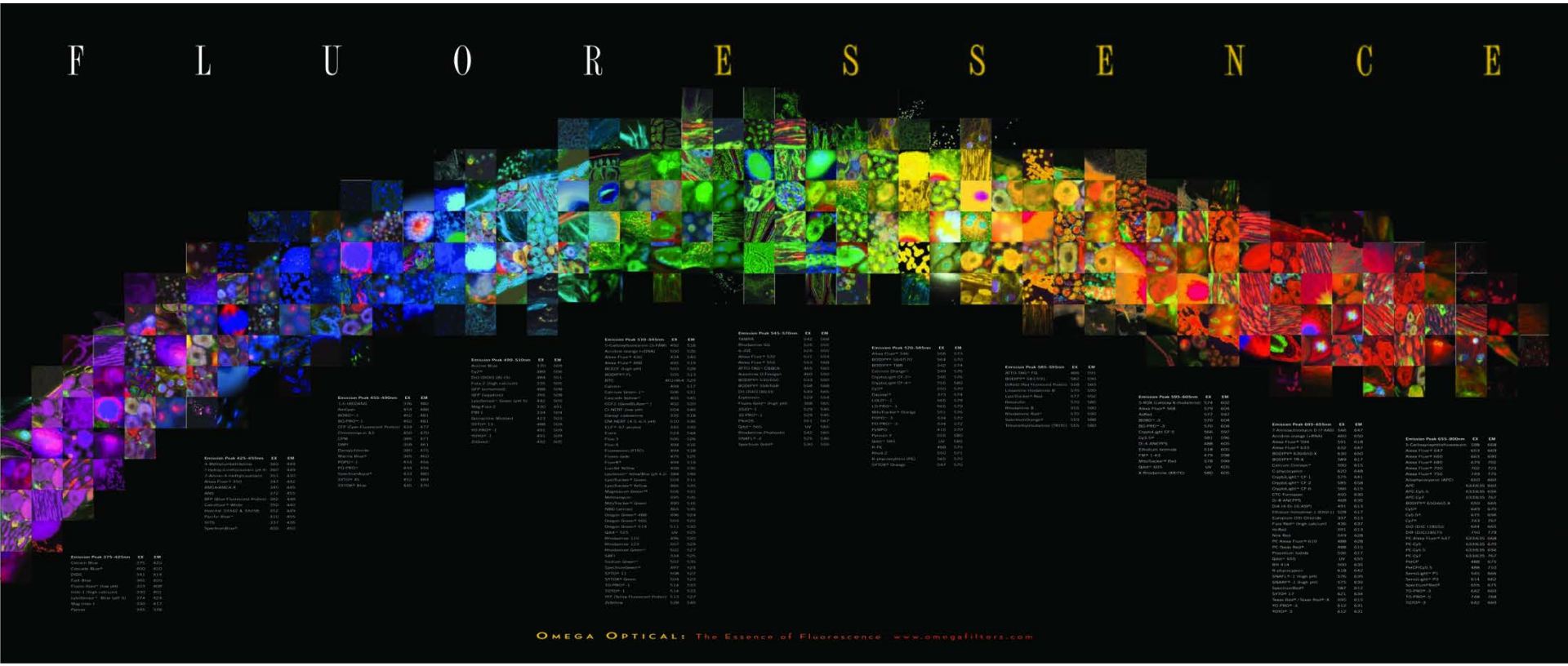
Genetic Tags



SNAP Tag is ~20 kD mutant of a DNA repair protein



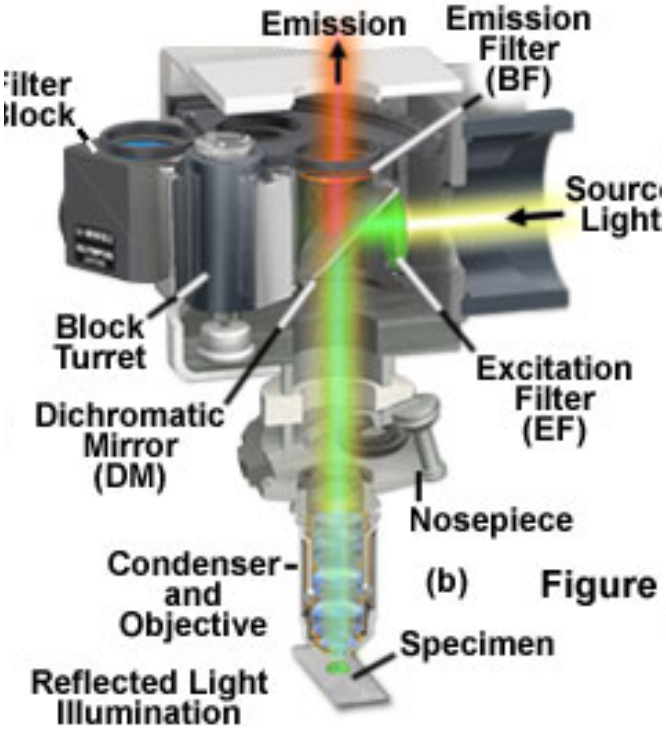
Mitochondrial cytochrome oxidase (red, SNAP)
Nuclei (blue, Hoechst)



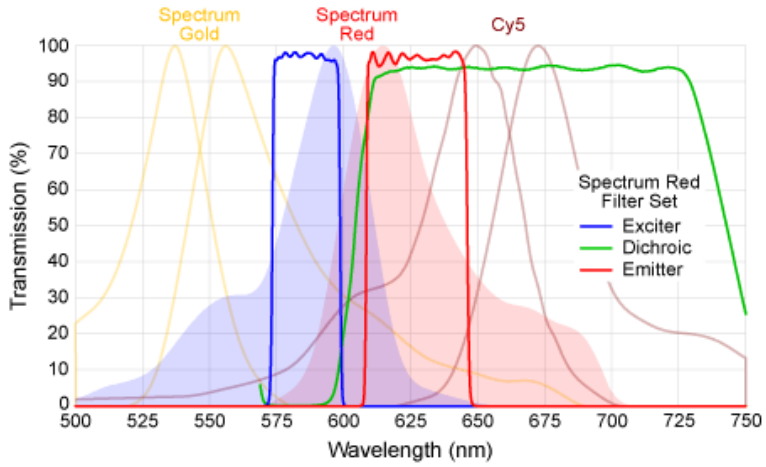
Fluorophores are catalogued and described according to their absorption and fluorescence properties, including the spectral profiles, wavelengths of maximum absorbance and emission, and the fluorescence intensity of the emitted light.

Chemically reactive forms of many dyes are commercially available

Standard Filter Sets



(b) Figure



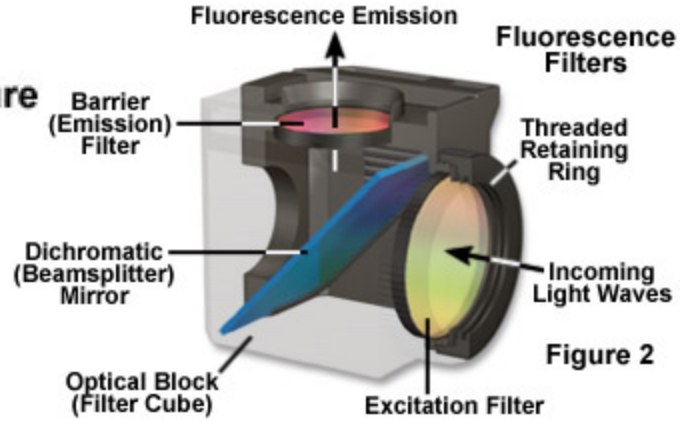
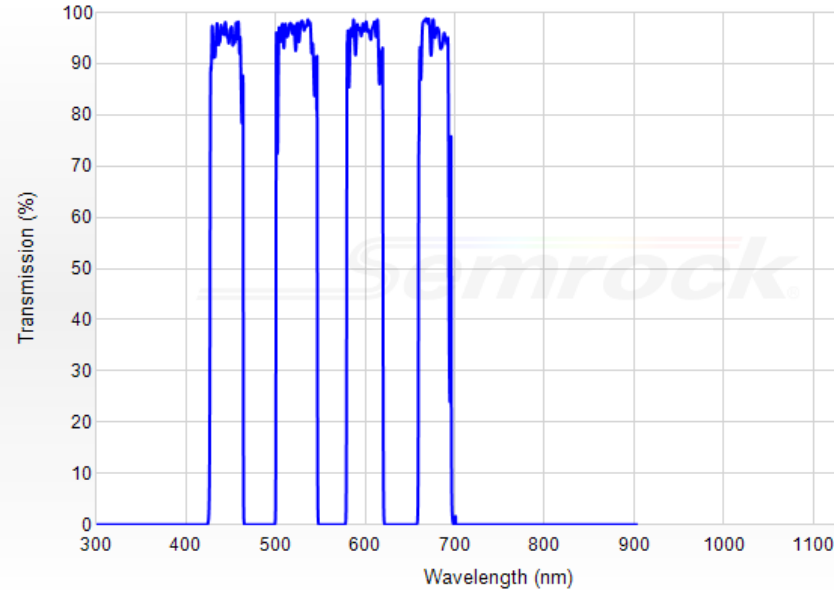


Figure 2

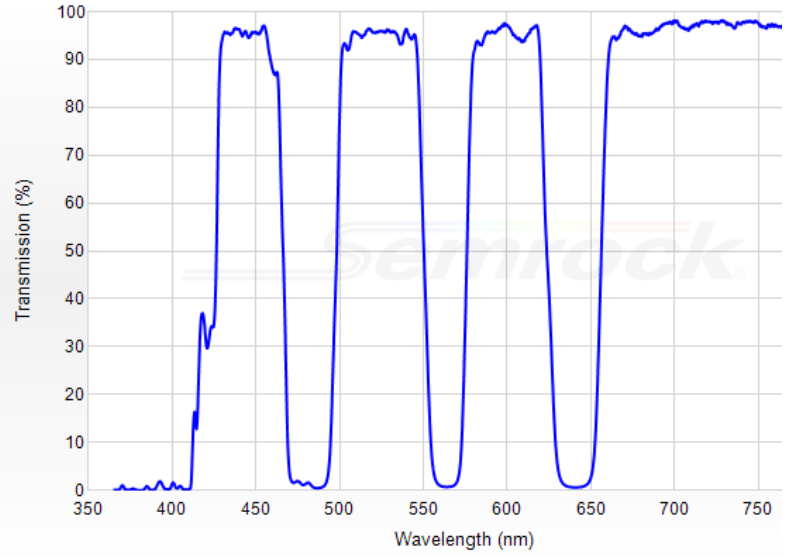
- If a lamp is used for excitation, narrow-band (10-20 nm) excitation filter is used to generate monochromatic light.
- Laser emission is reflected from dichroic mirror and sent to the sample.
- Fluorescence signal from the specimen is transmitted by the dichroic and filtered by emission filter.

Multi-Color Imaging

EMITTER

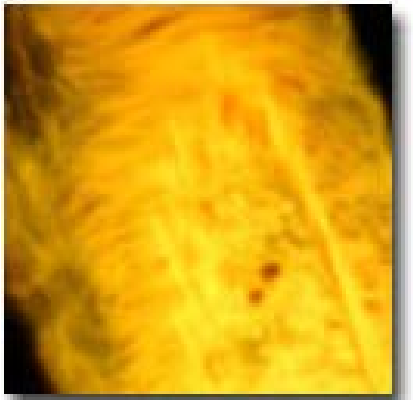


MIRROR

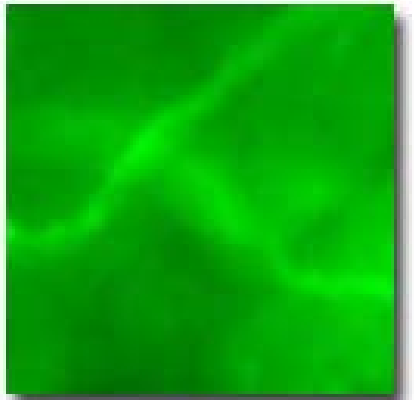


Sequential excitation with laser lines with multi-band emission and dichroic filter set.

Epi Fluorescence Images



human medulla

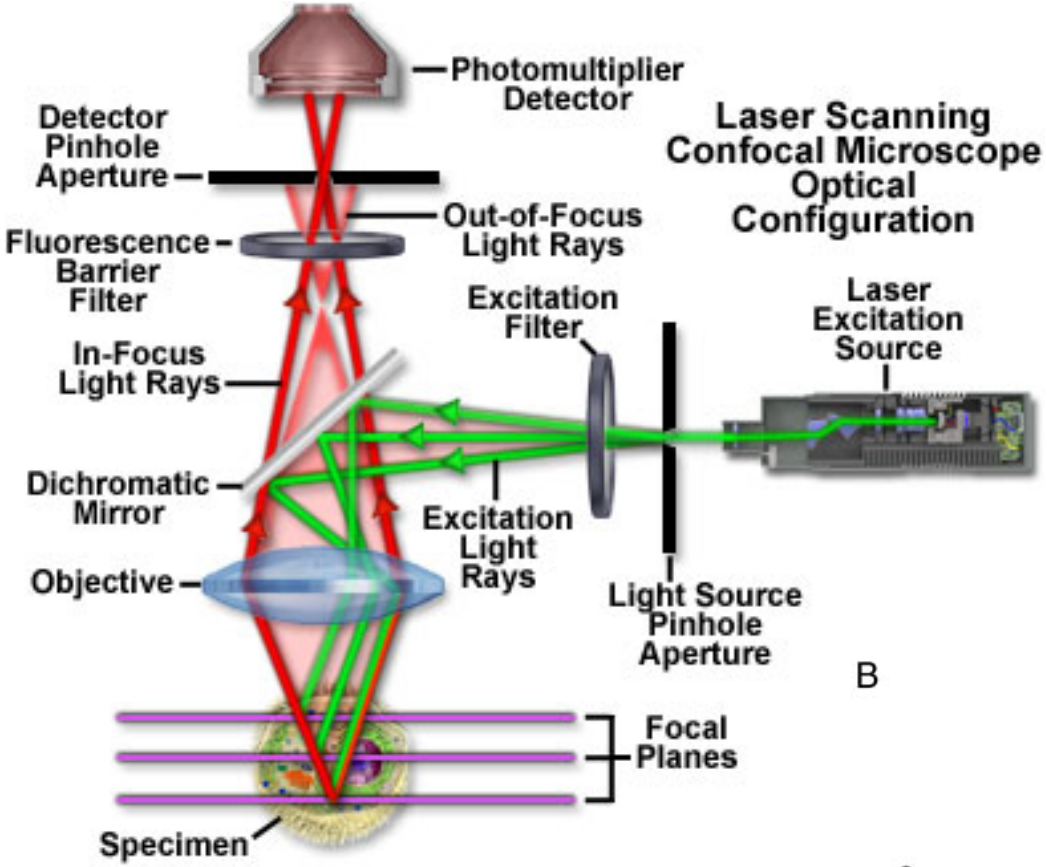


rabbit muscle fibers

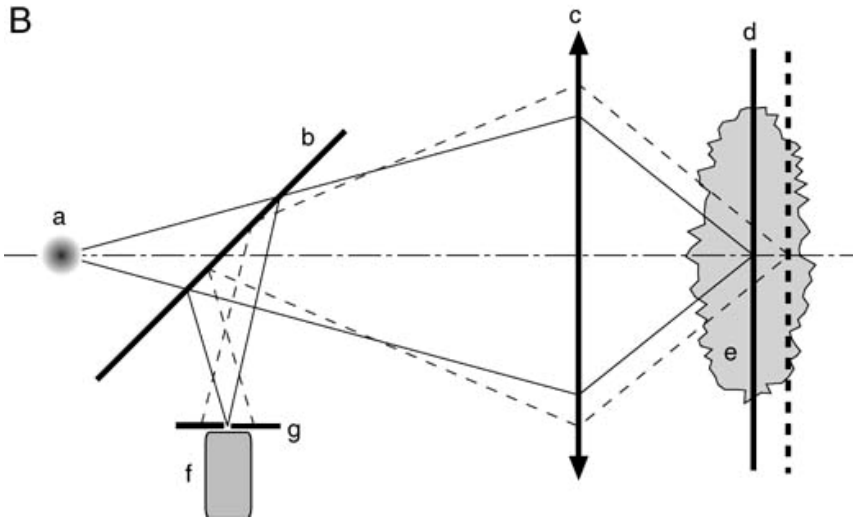
sunflower pollen grain

- Images are bright, but blurry and low contrast
- Epi-illuminator excites the whole thickness of the specimen, unable to control the depth of the field
- Bright fluorescent signal from out-of-focus objects give low-contrast
- Autofluorescence (fluorescence signal from unlabeled objects) of the cell increases the background

Components of a Confocal Microscope



- Image is formed by raster scanning of the focused laser beam.
- Magnification is generated by scanning step size.
- Fluorescence signal is detected by single channel detector PMT.
- Digital pixels are generated by analog to digital converter.



Confocal and Widefield Fluorescence Microscopy

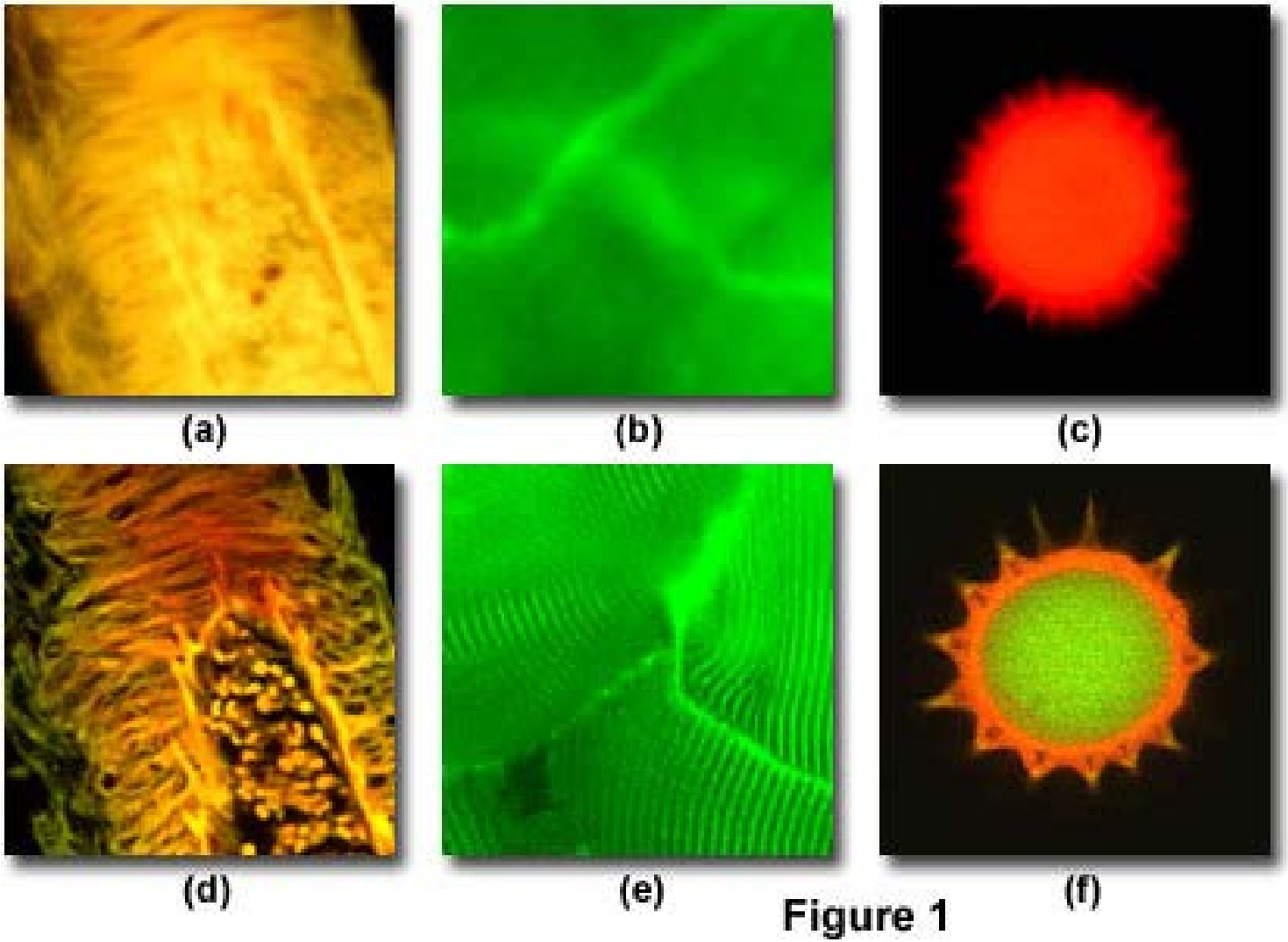


Figure 1

Superior image contrast and clarity

3D Scanning Confocal Microscopy

•The primary advantage of laser scanning confocal microscopy is the ability to serially produce thin (0.5 to 1.5 micrometer) optical sections through fluorescent specimens
•Objective can be moved in z direction with a piezoelectric motor to take z stack images.

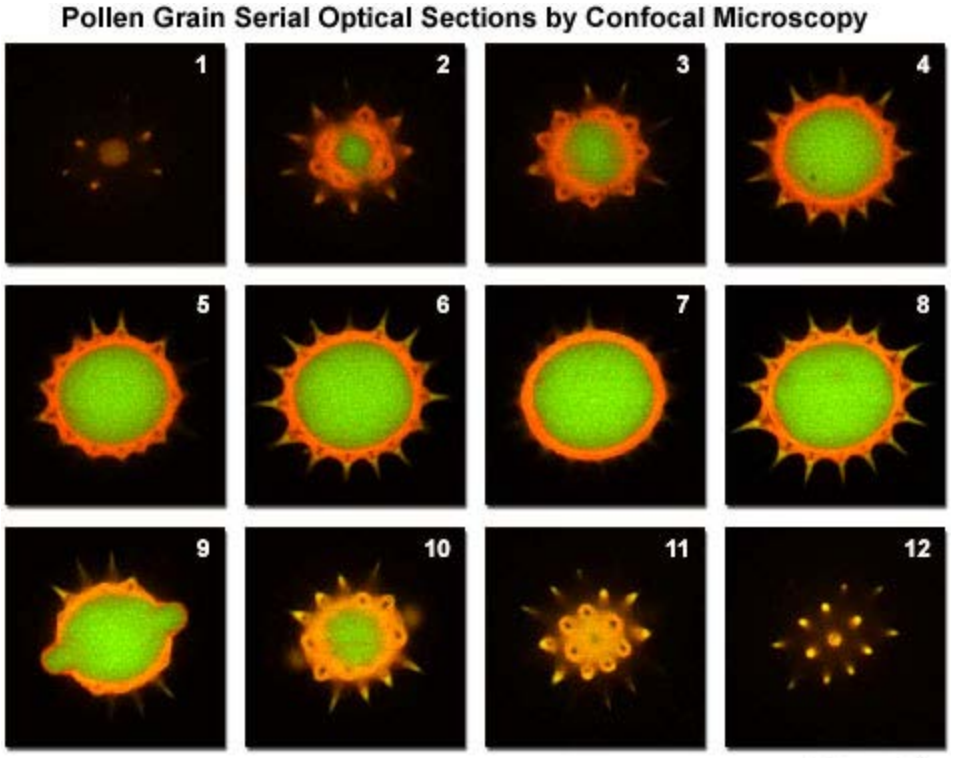


Figure 6

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

Multi Photon Confocal Microscopy

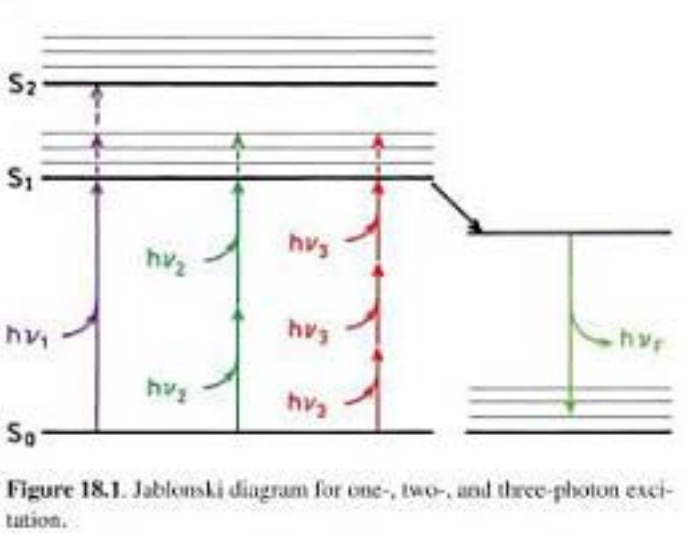
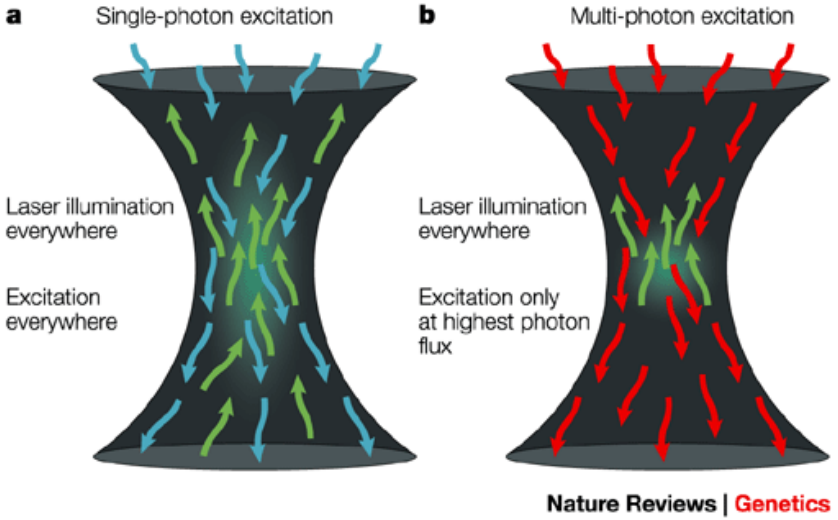
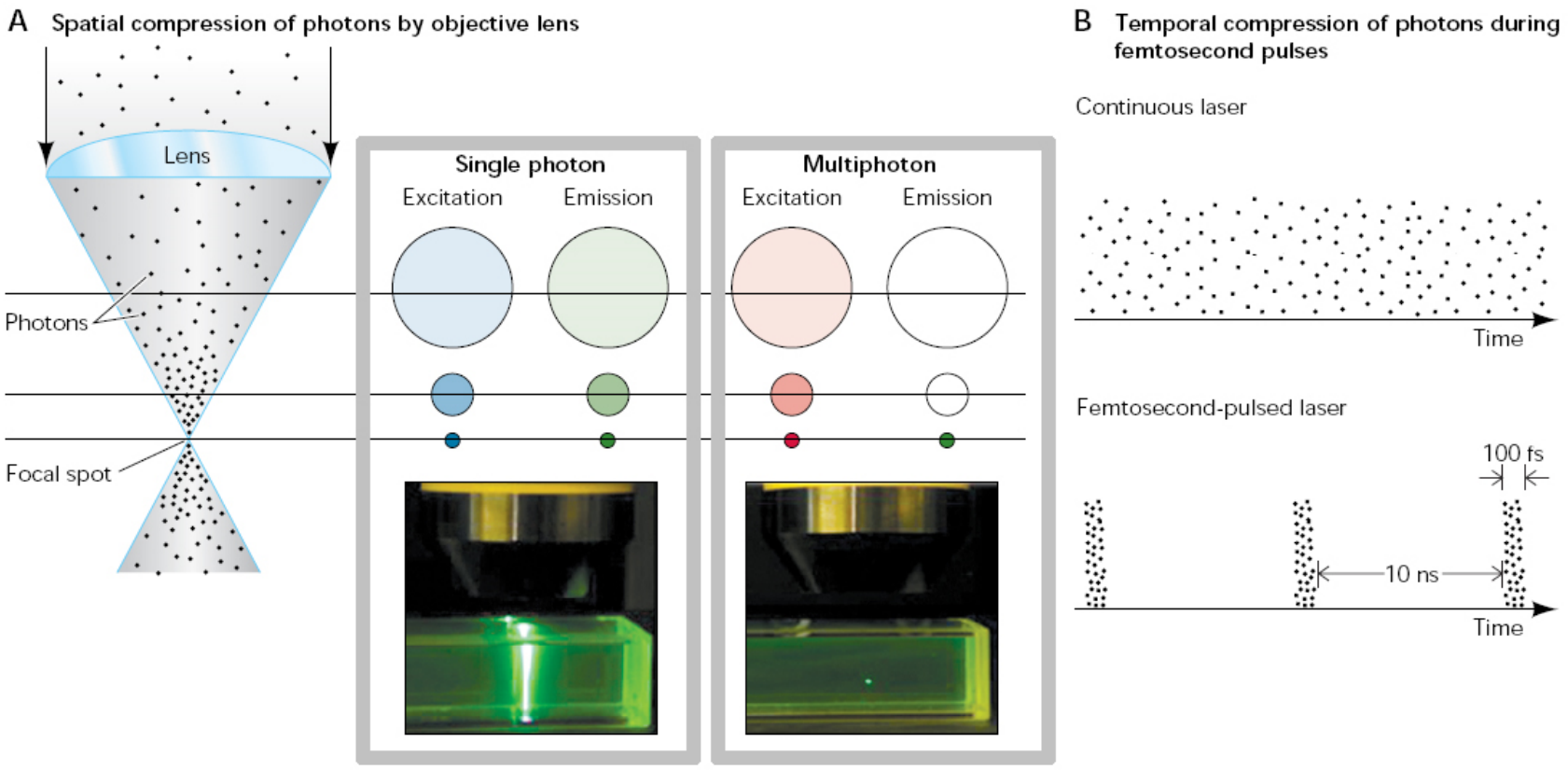


Figure 18.1. Jablonski diagram for one-, two-, and three-photon excitation.



- Illumination with intense 800 nm light can excite a fluorophore that is normally excited at 400 nm
- Two photons must be absorbed simultaneously
- Absorption efficiency is proportional to I^2 of the laser.



Pulsed infrared lasers (Ti-Sapphire) compress photons in time domain and effectively increase the intensity for 2-photon absorption.
Excitation occurs only in focus, no pinhole is required to exclude out of focus fluorescence.
The use of near IR permits examination of thick specimens, up to 0.5 mm.

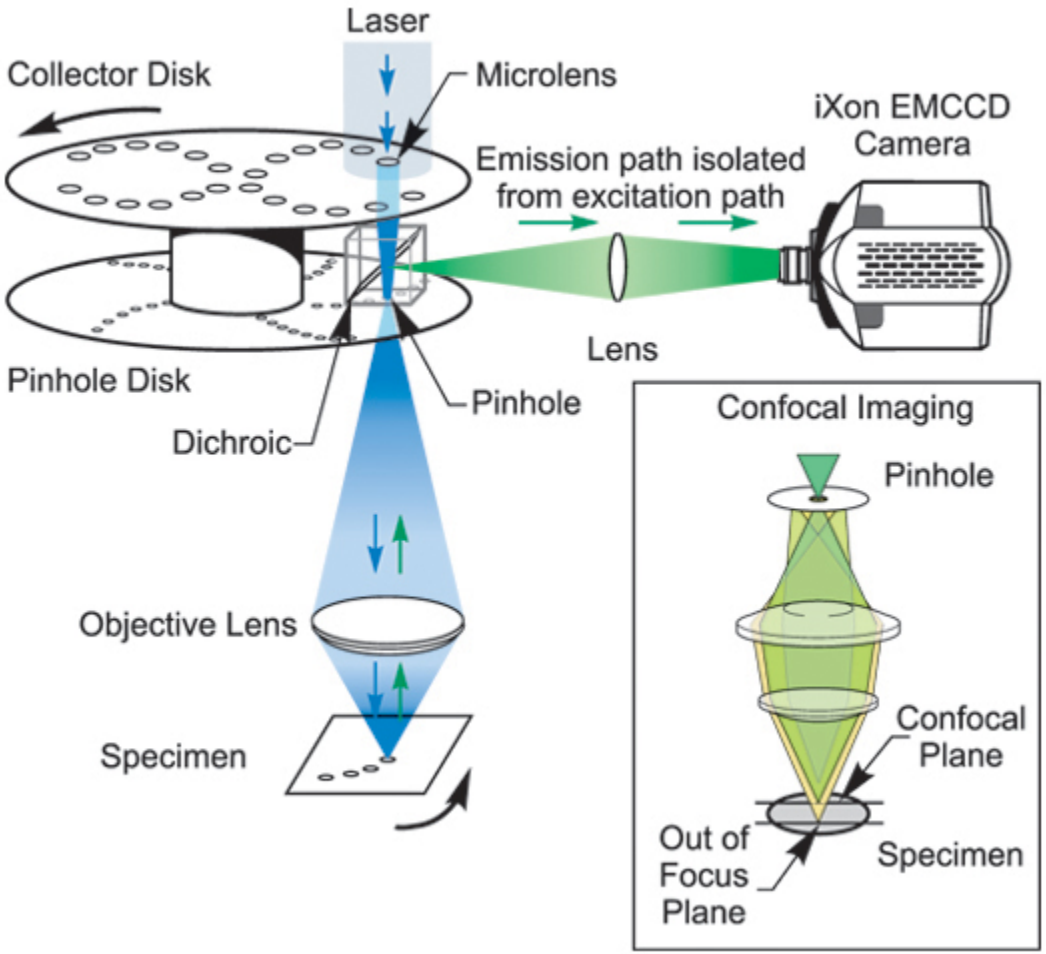
One Photon vs. Two Photon Confocal Microscopy

- **Image Resolution:** No difference
- **Thick Specimens:** Two Photon can study 10 times thicker specimens than one-photon.
- **Thin Specimens:** Two Photon slightly increases the photobleaching of dyes.
- **Absorption Spectrum:** Two-photon absorption spectrum can be different from single-photon.
- **Focal Spot Size:** Two-photon can be used effectively for localized photochemistry applications. UV-switchable probes (caged-GFP or caged-ATP).
- **Lasers:** Two photon requires mode-locked ultrafast lasers. Ti-Sapphire covers 720-900 nm. NdYLF is at 1047 nm.

Limitations of Two Photon Microscopy:

- Cost, alignment
- Local heating of water.
- Phototoxicity of IR excitation and near-UV emission.
- Development of new ultrafast lasers that cover all of the wavelengths for common probes.

Spinning Disk Confocal Microscope



- Raster scanning method slows down image acquisition
- A Nipkow disk contains thousands of small pinholes arranged in rows of outwardly spiraling tracks.
- Pinholes of the Nipkow disk have very low light transmission.
- Yokogawa introduced microlenses in pinholes to boost light gathering efficiency.
- Every point in the specimen receives same amount of illumination.
- Live cell imaging with confocal microscopy!

Resonance Energy Transfer

a fluorophore in the excited state (termed the **donor**) may transfer its excitation energy to a neighboring chromophore (the **acceptor**) non-radiatively through long range dipole-dipole interactions over distances measured in nanometers.

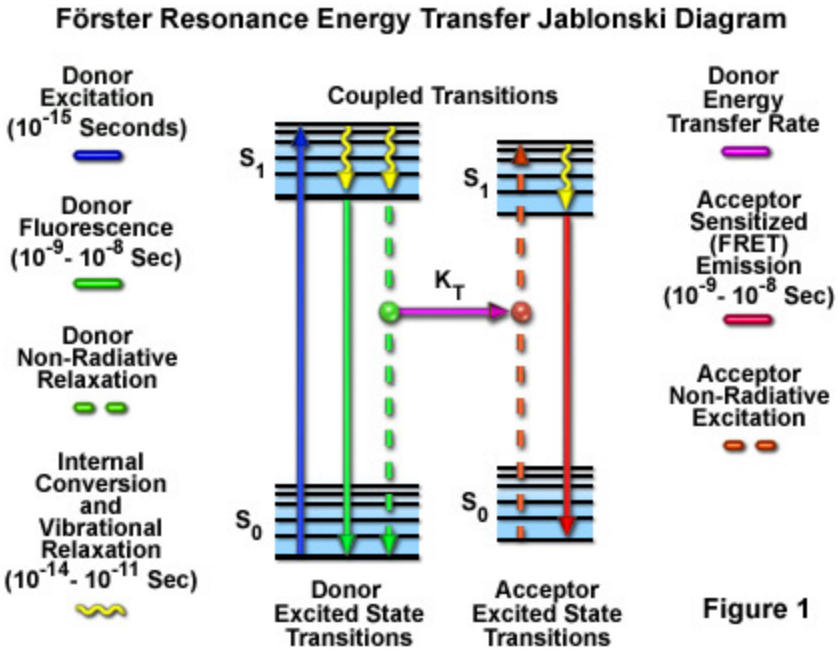
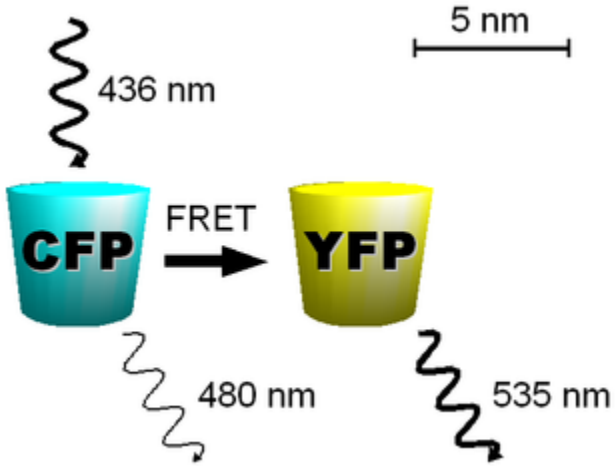


Figure 1



Spectral Overlap of FRET Pairs

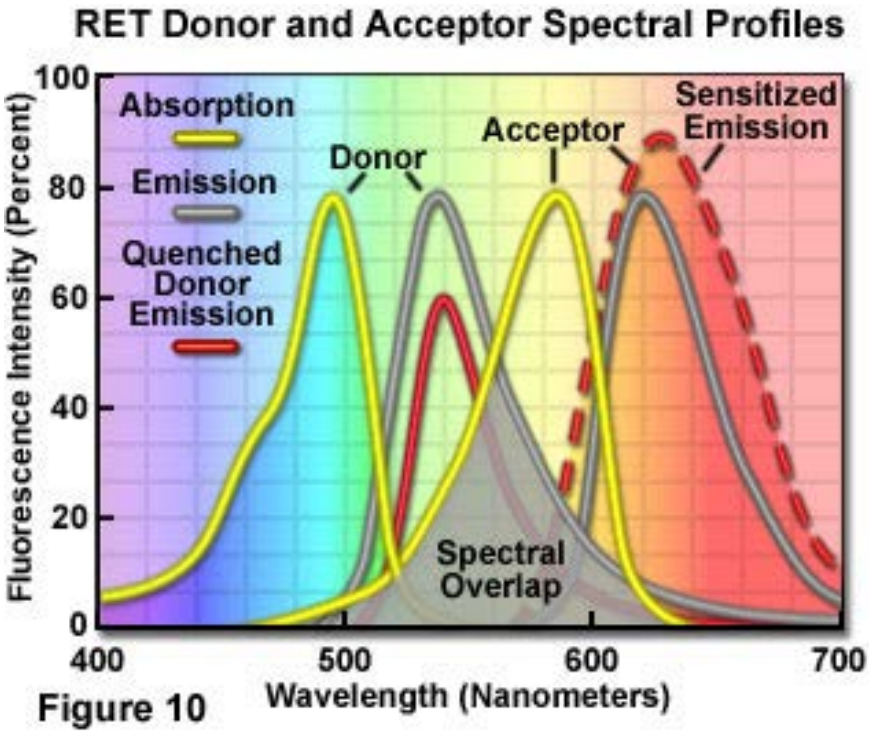
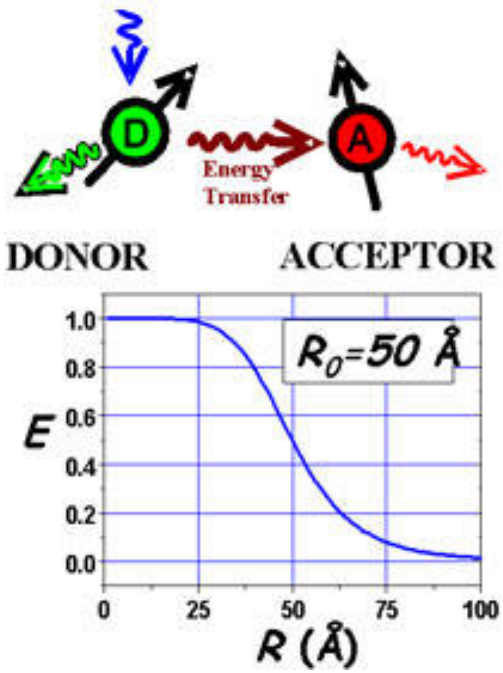


Figure 10

the emission spectrum of the donor fluorophore overlaps the absorption spectrum of the acceptor (there is no intermediate photon involved)

$$J = \int f_{\mathrm{D}}(\lambda)\, \epsilon_{\mathrm{A}}(\lambda)\, \lambda^4\, d\lambda$$ the spectral overlap integral

FRET Efficiency

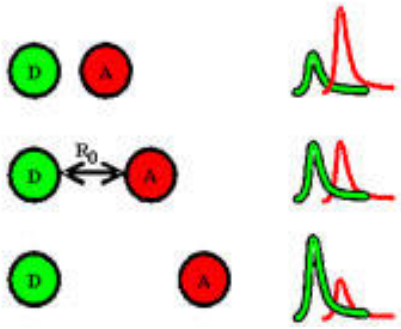


Energy Transfer Efficiency

$$E = \frac{1}{1+(R/R_0)^6}$$

R_0=50% transfer efficiency distance
3nm~7nm

"Spectroscopic Ruler"



$$R_0^{\,6} = \frac{9\,Q_0\,(\ln 10)\kappa^2\,J}{128\,\pi^5\,n^4\,N_A}$$

Q_0 is the fluorescence quantum yield of the donor in the absence of the acceptor, κ^2 is the dipole orientation factor, n is the refractive index, N_A is Avogadro's number,

If the FRET pairs have larger overlap, energy transfer occurs at longer distances (10 nm max).

Factors Affecting FRET Measurements

- FRET measurements of protein-protein interactions in which one partner might be in excess concentration.
- the measurement of a small level of FRET against a background of fluorescent labels that are not undergoing FRET
- In some cases, the acceptor can be directly excited with light in the wavelength region chosen to excite the donor
- fluorescence from the donor can leak into the detection channel for the acceptor fluorescence, especially when the emission spectral profiles of the donor and acceptor exhibit significant overlap.
- the level of a FRET signal can be reduced if the two fluorophores are not properly aligned (for instance, having a K^2 value of approximately zero) or if they are simply not positioned within the Förster radius (greater than 6 nanometers).

FRET Techniques in Cell Biology Applications

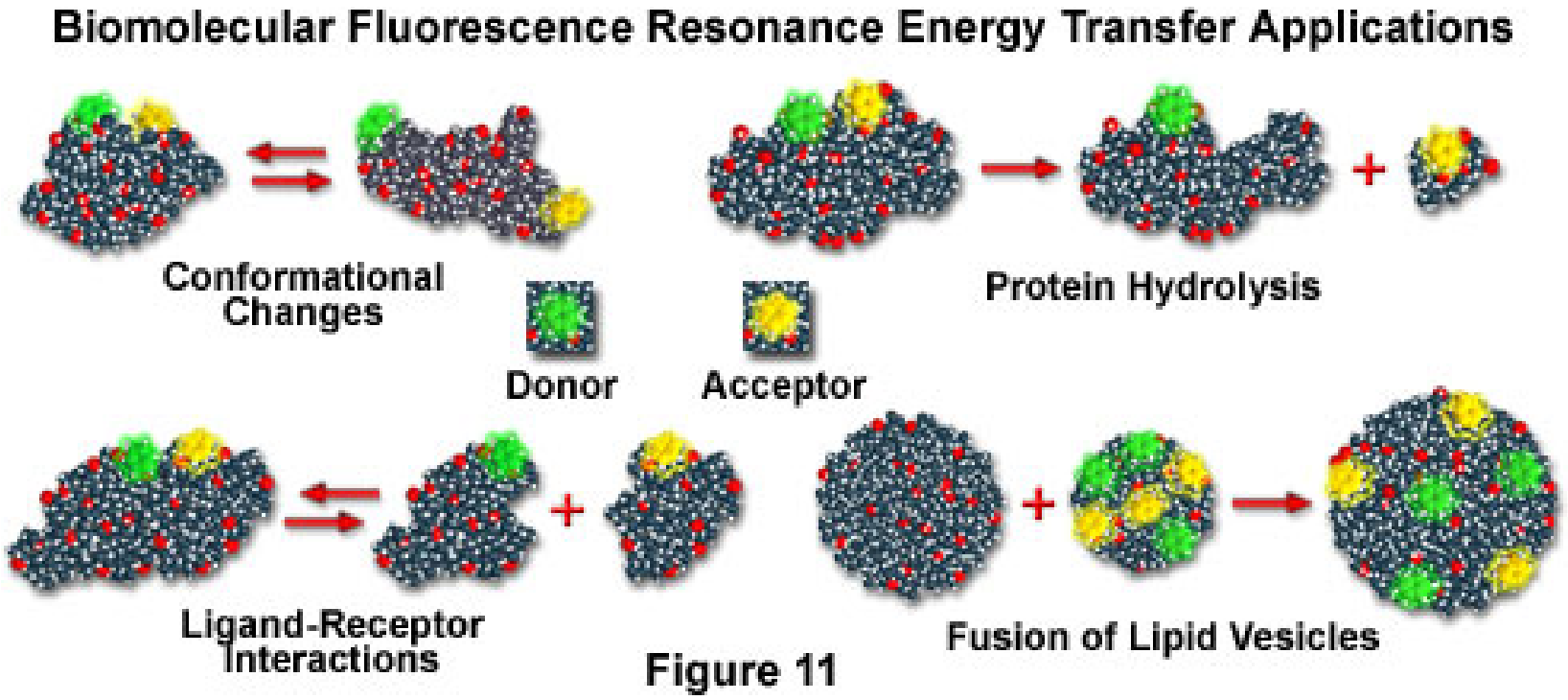
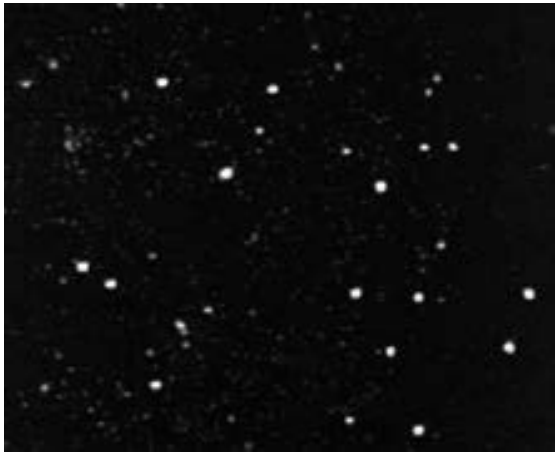


Figure 11

Single Molecule Fluorescence



Single tetramethyl rhodamine molecules observed under TIRF using the Apo100x (N.A. 1.65)

What can be done?

1. Avoid ensemble averaging!

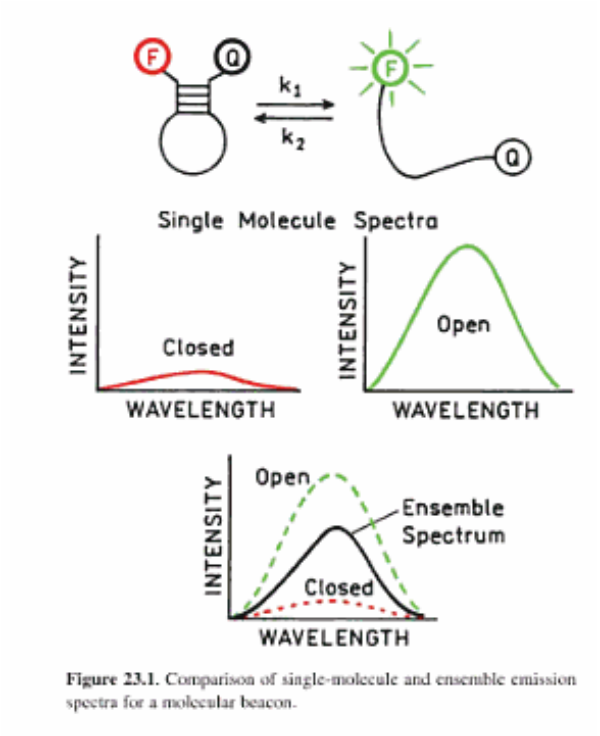
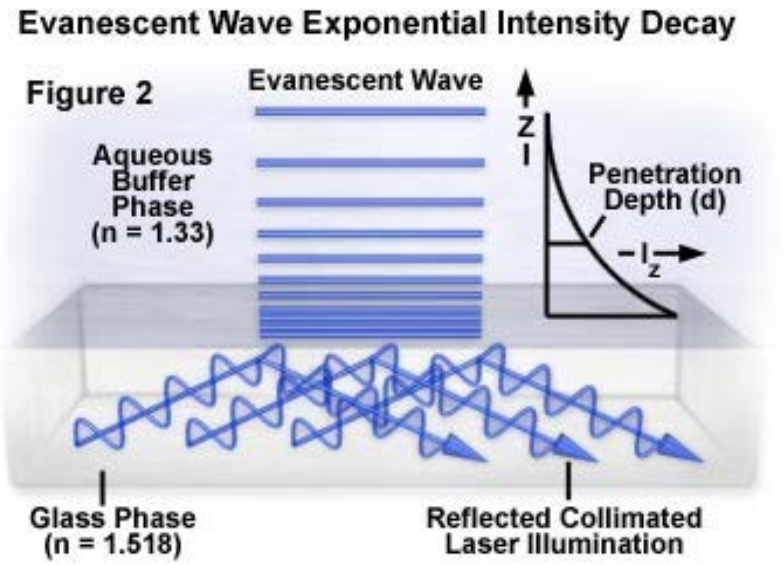
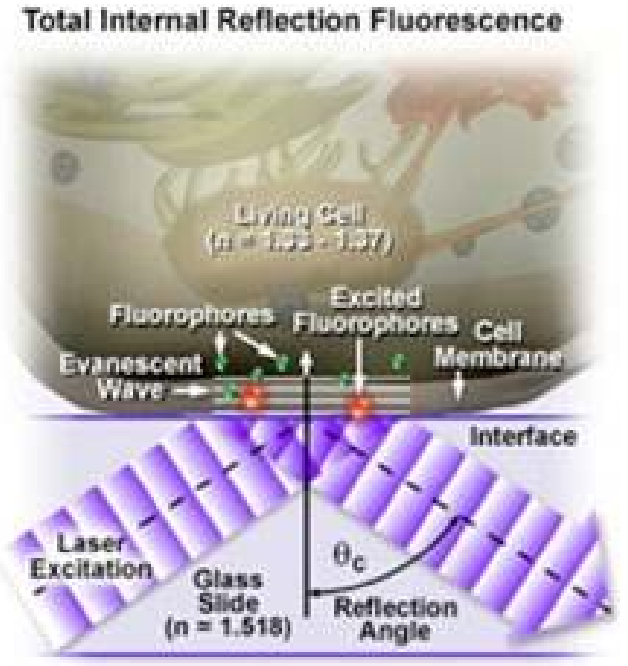


Figure 23.1. Comparison of single-molecule and ensemble emission spectra for a molecular beacon.

2. probing the dynamic interactions of nucleic acids and proteins,
3. tracking single particles over microns of distances,
4. deciphering the rotational motion of multisubunit systems.

Total Internal Reflection Fluorescence Microscopy



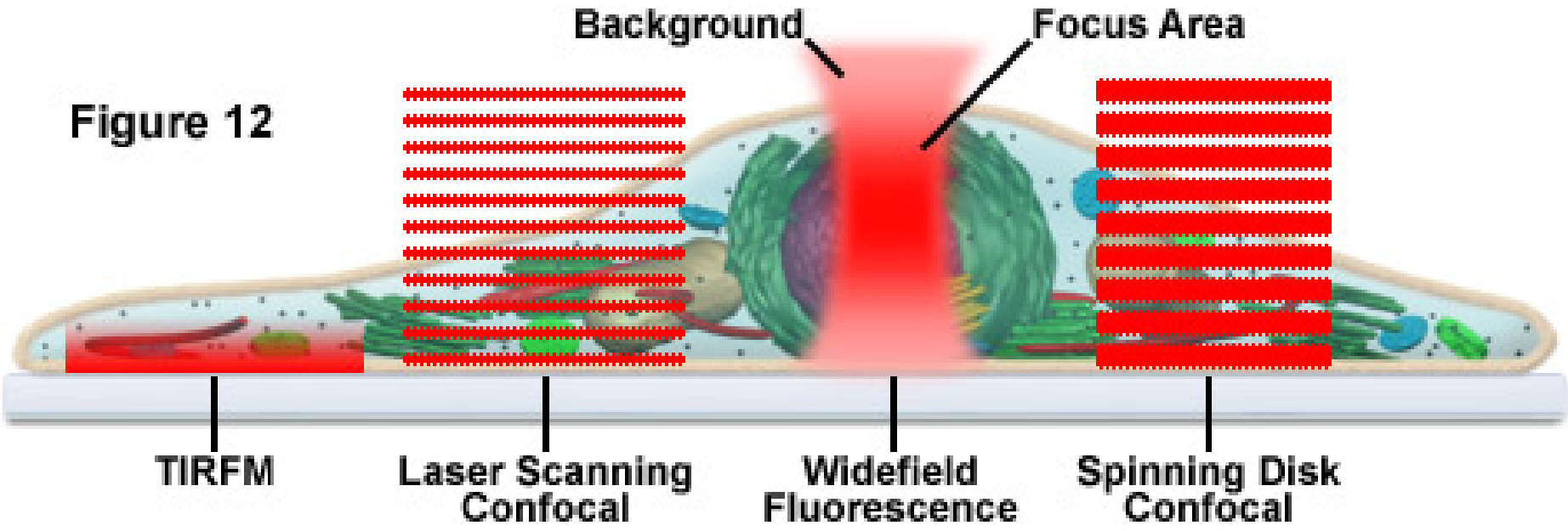


$d = I_0/4\pi(n_1^2\sin^2 - n_2^2)^{-1/2}$ ~80 nm

Critical angle is 65.7°

Instead of sending the light directly to the sample, it is total internally reflected from the glass water interface.
No energy is transmitted to water.
However, incoming waves generate an evanescent filed at the surface.

Figure 12


Fluorescence Imaging Modes in Live-Cell Microscopy
Background
Focus Area
TIRFM
Laser Scanning Confocal
Widefield Fluorescence
Spinning Disk Confocal

TIRF vs Wide Field

Fluorescent Beads and Cheek Cell

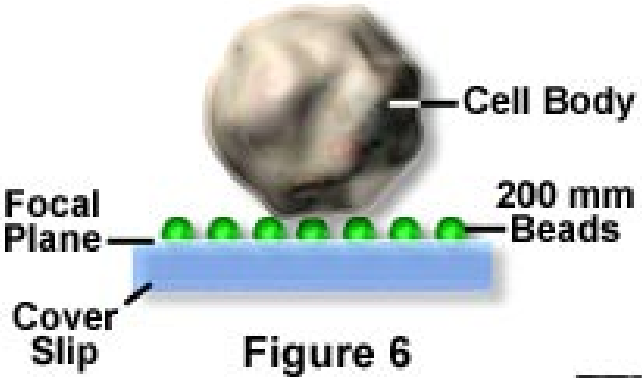
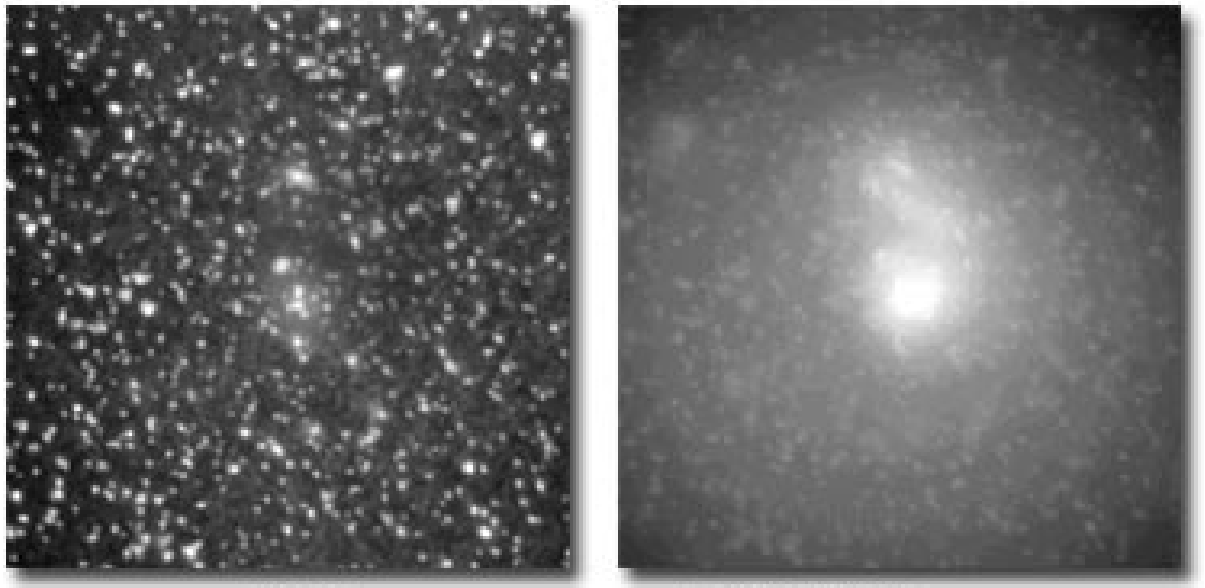


Figure 6

Fluorescent Beads and Cheek Cell



TIRFM Widefield Fluorescence

Figure 7

High Signal to Noise Ratio Imaging

- The background signal has effectively been reduced by constraining the illumination to a shallow depth (TIRF or confocal)
- Fluorophores have become brighter and more photostable by carefully tuning the solution conditions.
- Innovations in CCDs have achieved high quantum yield and zero-dead time together with a sub-millisecond time resolution.
- Advances in objective lenses [high numerical aperture (NA), high magnification, and low aberrations]
- Laser technologies (high-stability, single-mode lasers with various wavelengths)

An ideal Single Molecule Fluorophore

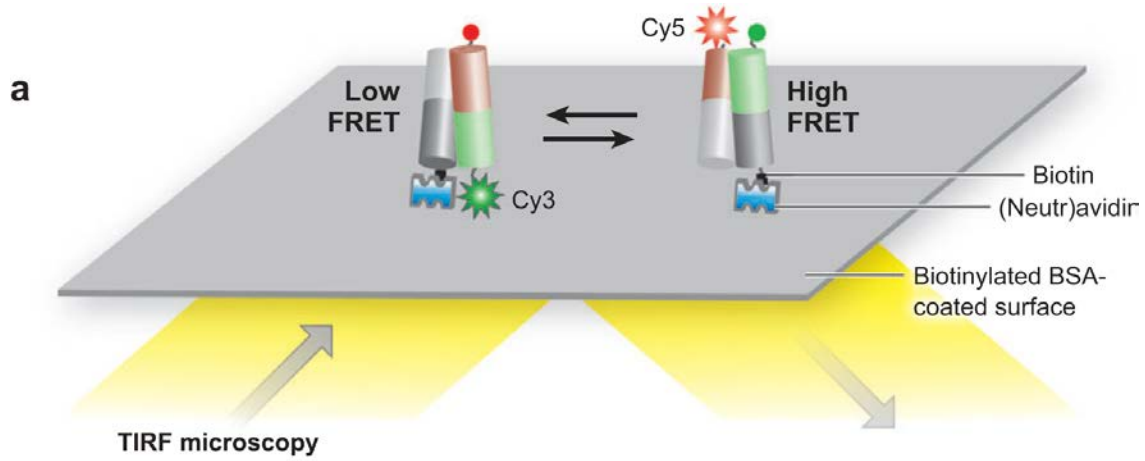
(*a) has* high absorption and fluorescence quantum yield

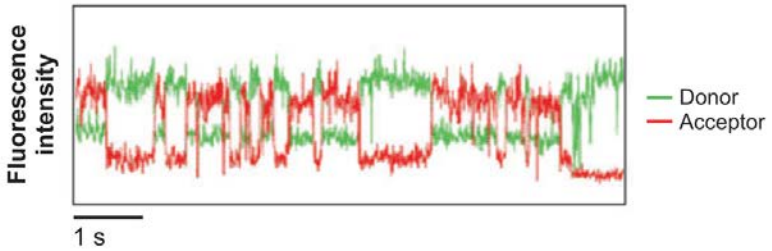
(*b) shows steady emission intensity,*

(*c) does not perturb the host molecule*

(*d) stays photoactive over a long time under* intense illumination.

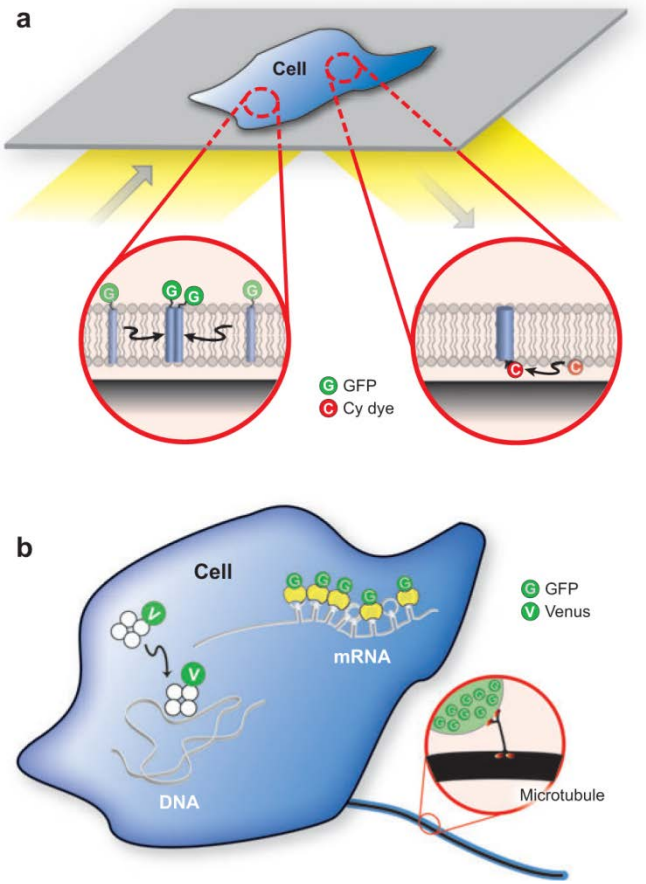
Applications: Single Molecule FRET on Surface Tethered Molecules



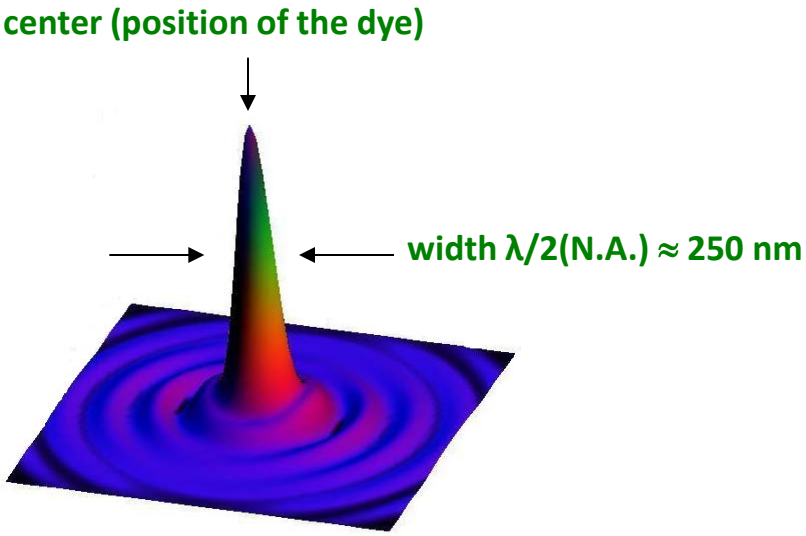


Holliday junctions) are tethered via biotin-neutravidin conjugation on the bovine serum albumin (BSA)-coated surface. The conformational dynamics of Holliday junction is shown in a fluorescence time trace

Single Molecule Imaging *in vivo*



Single Molecule Fluorescent Particle Tracking

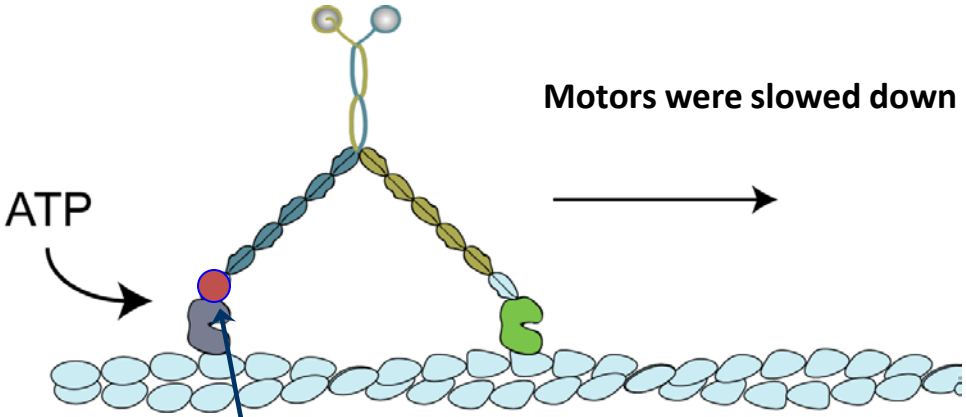


Dye molecules can be localized with **1 nm precision** by collecting sufficient number of photons

$$\sigma_{\mu_i} = \sqrt{\left(\frac{s_i^2}{N} + \frac{a^2/1}{N} + \frac{28\pi s_i^4 b^2}{a^2 N^2}\right)}$$

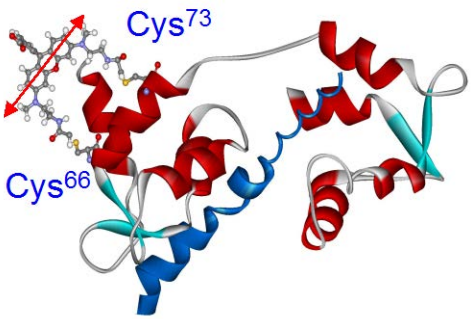
Thompson et. al. Biophys. J. (2000)

Myosin V FIONA assay *in vitro*

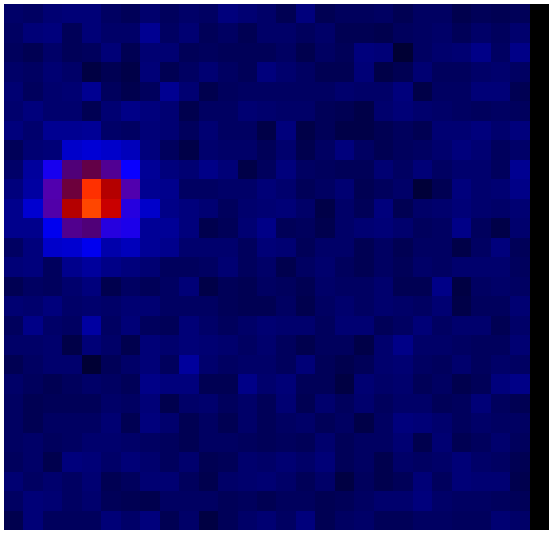


Motors were slowed down by limited [ATP]

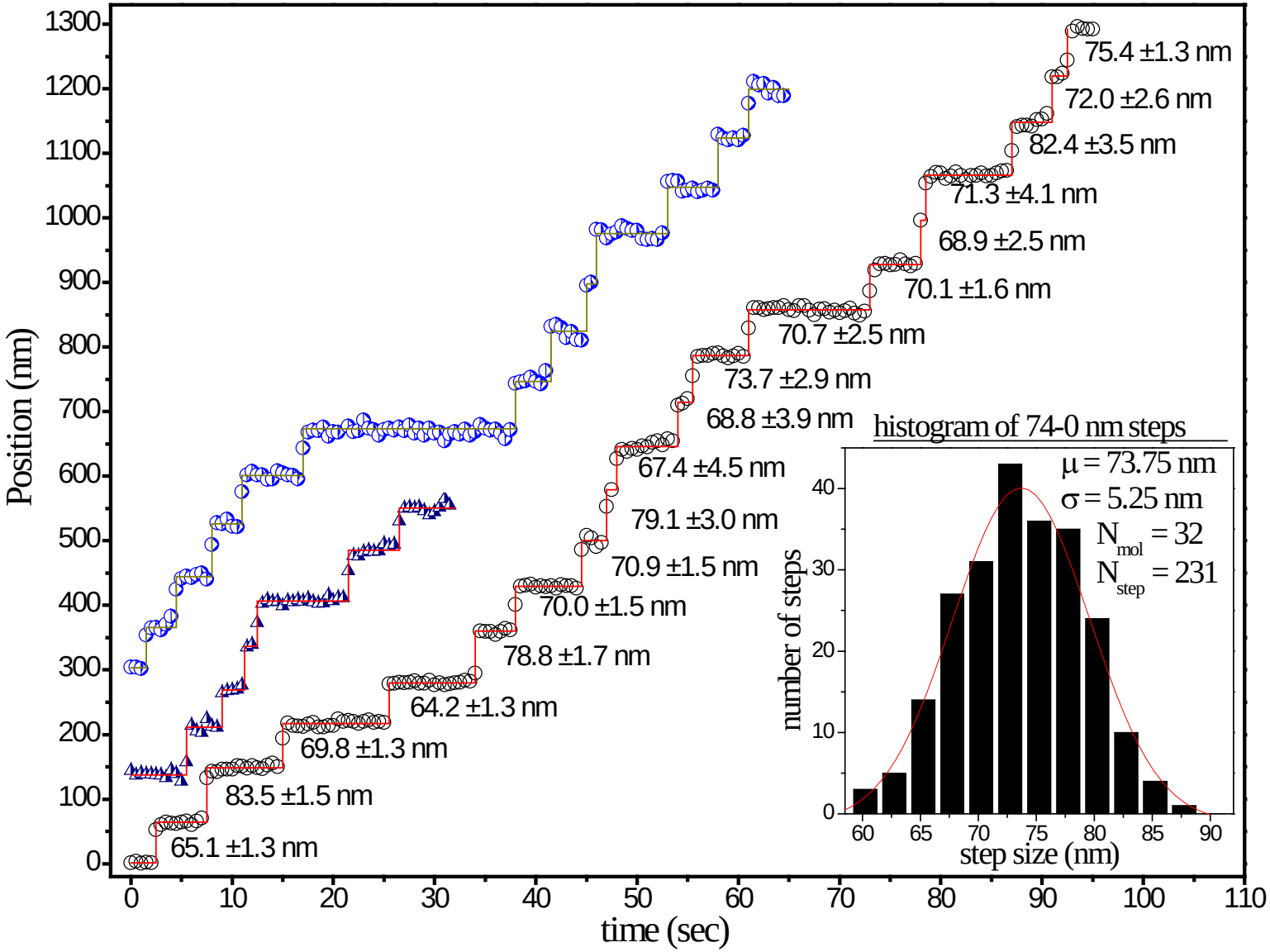
Br-labeled calmodulins were exchanged into the myosin V lever arm



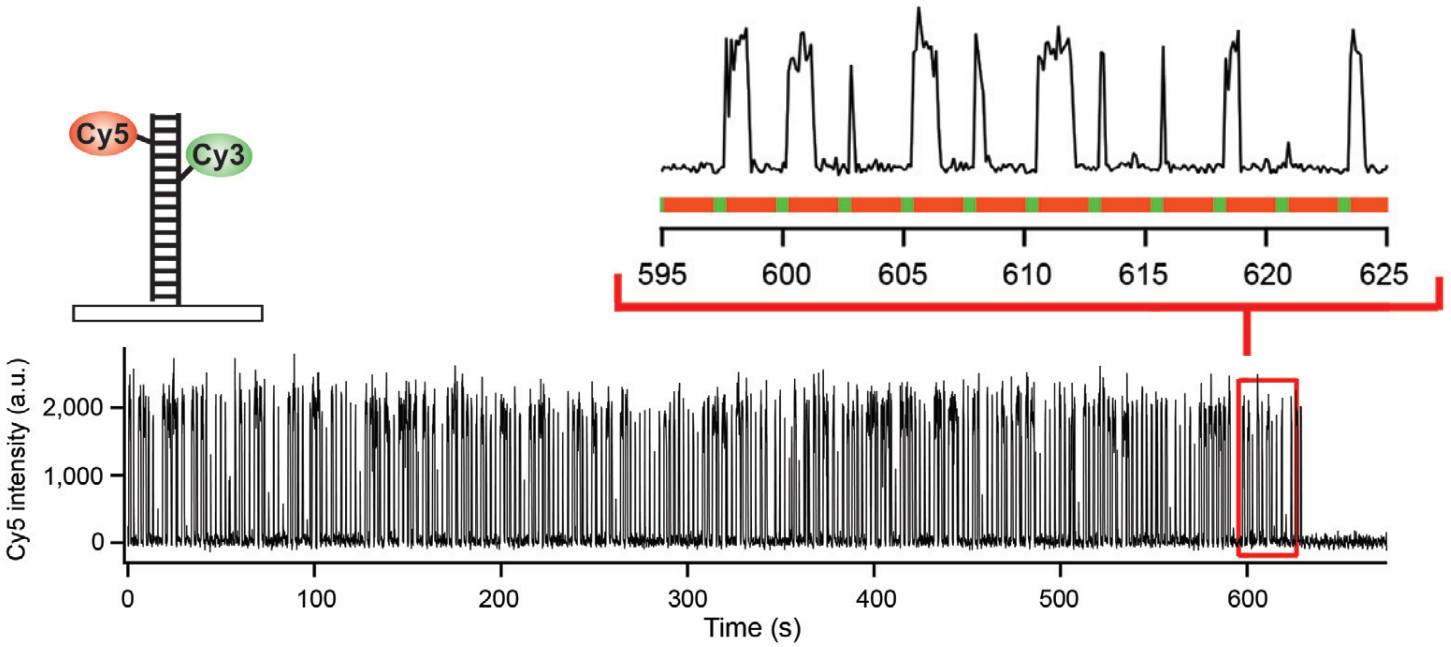
Bi-functional Rhodamine (Br)



Myosin V Walks Hand-over-Hand

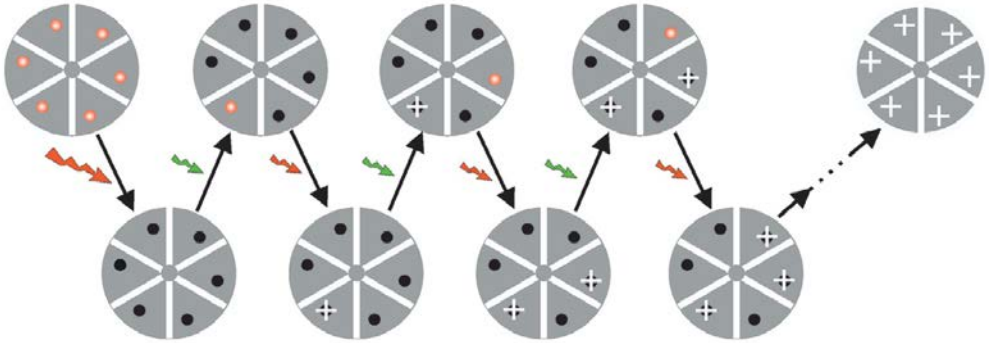


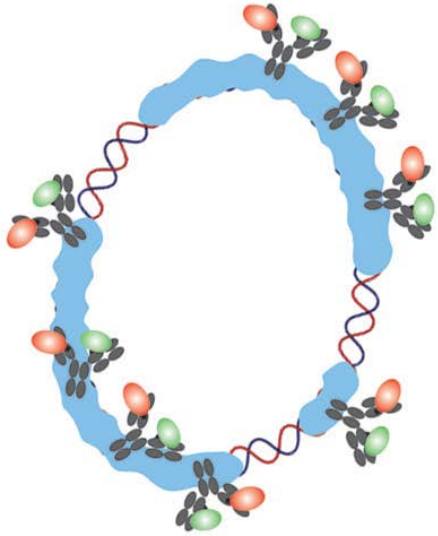
Stochastic Optical Reconstruction Microscopy (STORM)

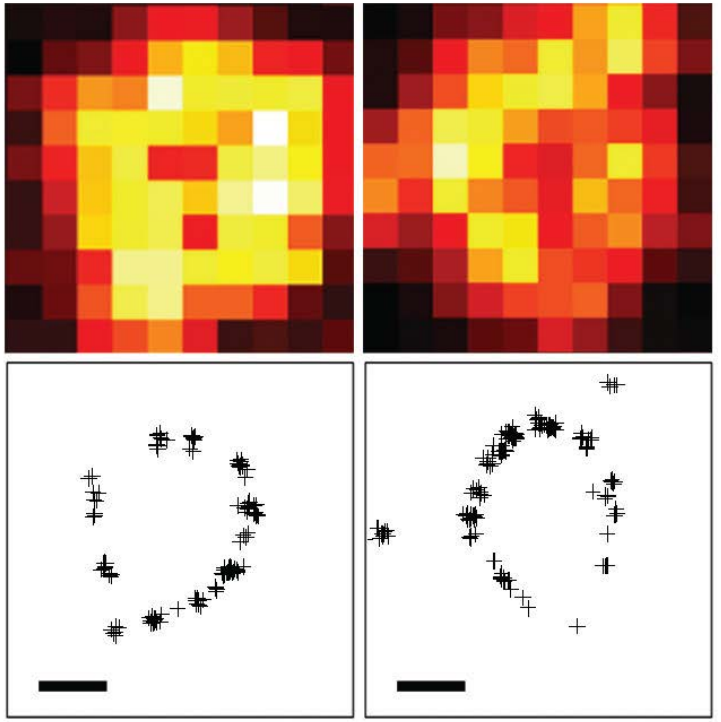


- Red light excites Cy5, which fluoresces and then photobleaches (dark state)
- Green light reactivates Cy5 if it is in close proximity to Cy3.
- This cycle can be repeated hundreds of times.

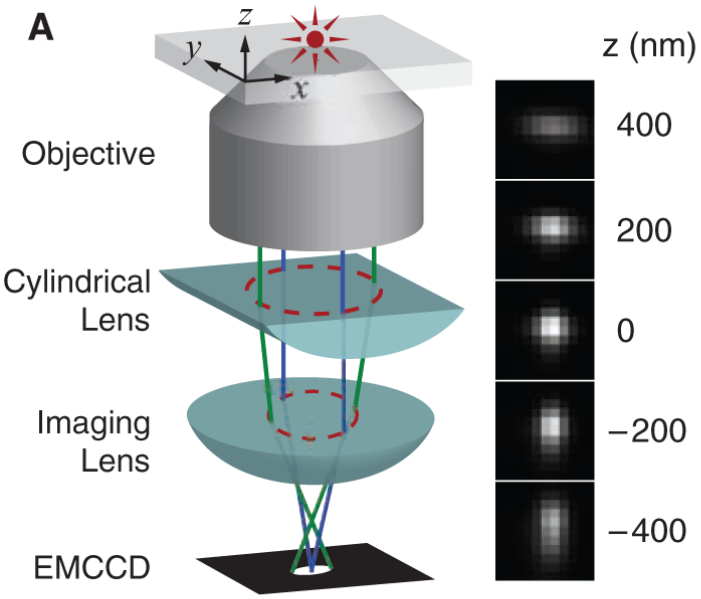
Stochastic Photoactivation

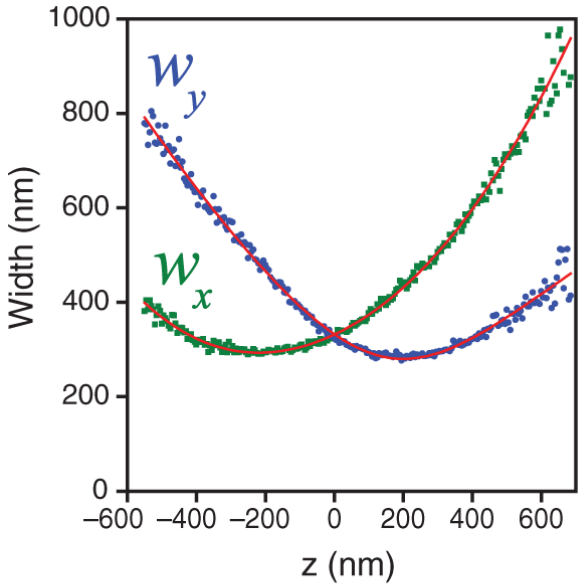


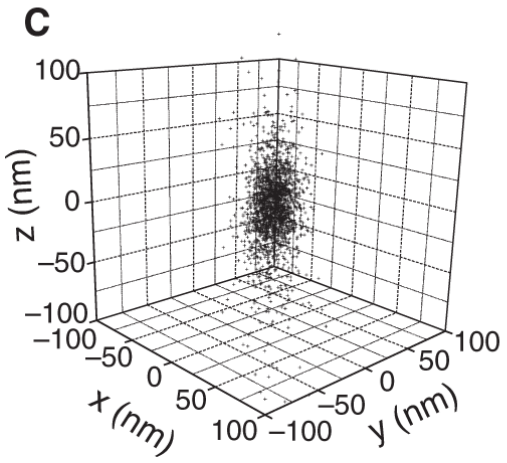


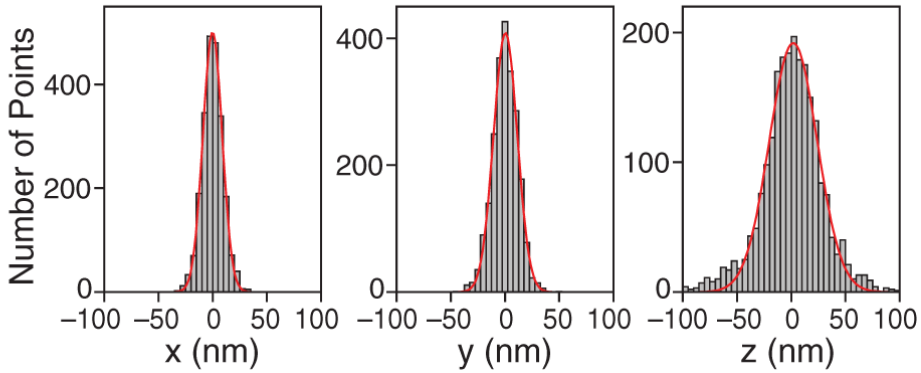


3D STORM

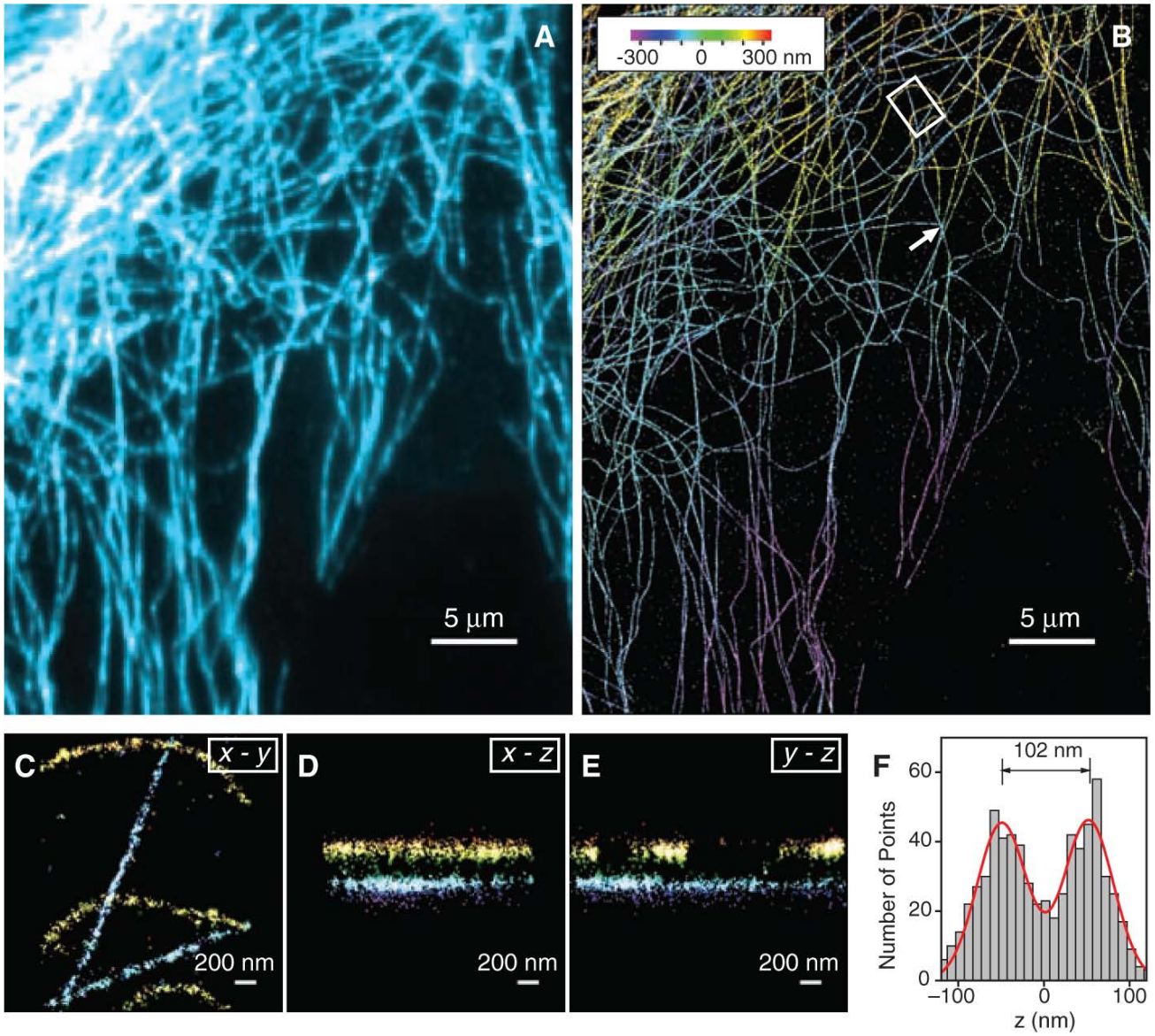

A
z
y
x
Objective
Cylindrical Lens
Imaging Lens
EMCCD
z (nm)
400
200
0
−200
−400

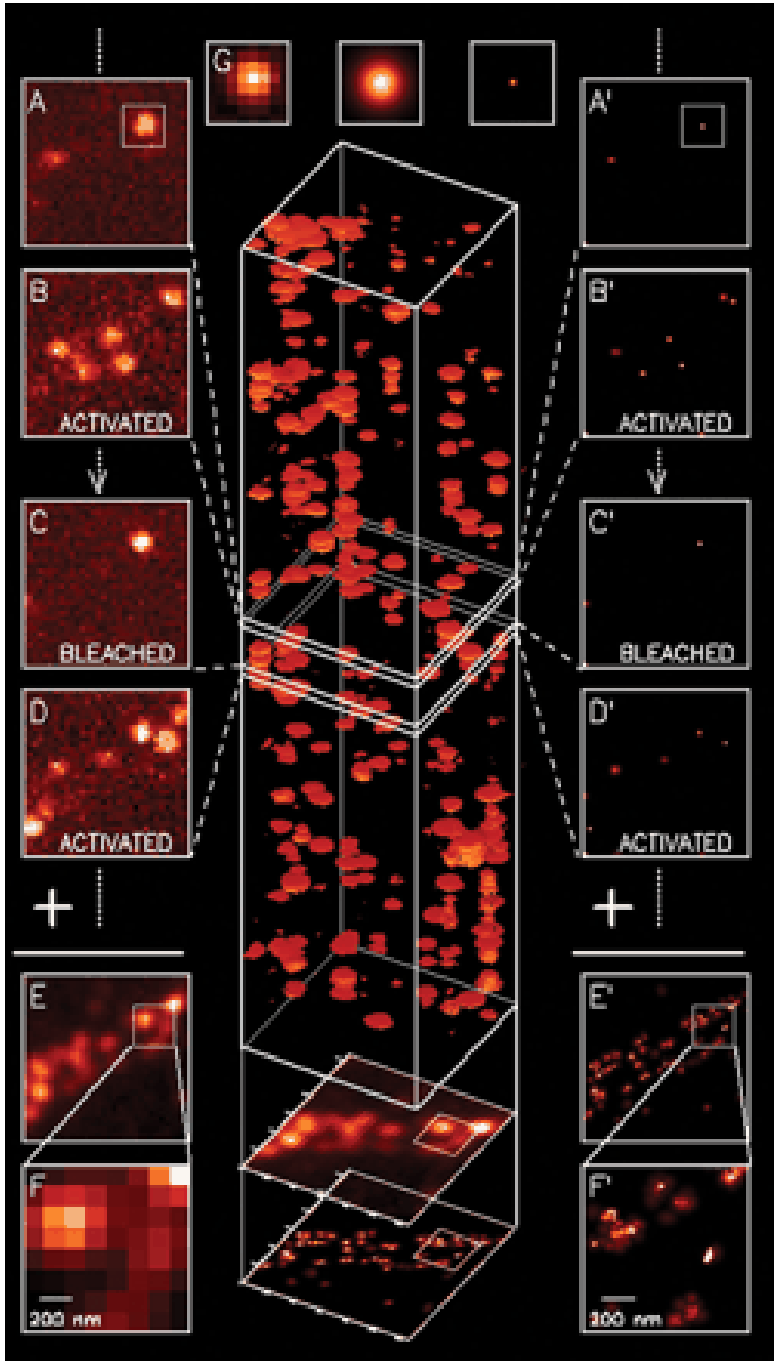

B
1000
800
600
400
200
0
Width (nm)
w_y
w_x
−600 −400 −200 0 200 400 600
z (nm)


C
100
50
0
−50
−100
z (nm)
−100 −50 0 50 100
x (nm)
−100 −50 0 50 100
y (nm)


Number of Points
400
200
0
−100 −50 0 50 100
x (nm)
400
200
0
−100 −50 0 50 100
y (nm)
200
100
0
−100 −50 0 50 100
z (nm)

Microtubule Imaging in 3D





Photoactivated Light Microscopy (PALM)

A sparse subset of PA-FP molecules that are attached to proteins of interest and then fixed within a cell are activated (A and B) with a brief laser pulse at λ_{act} = 405 mm and then imaged at λ_{exc} = 561 mm until most are bleached (C).

This process is repeated many times (C and D).

Summing the molecular images across all frames (E and F).

If the location of each molecule is first determined, the molecule can be plotted as a Gaussian that has a standard deviation equal to the uncertainty $\sigma_{x,y}$.

Repeating with all molecules across all frames (A' through D') and summing the results yields a superresolution image (E' and F').