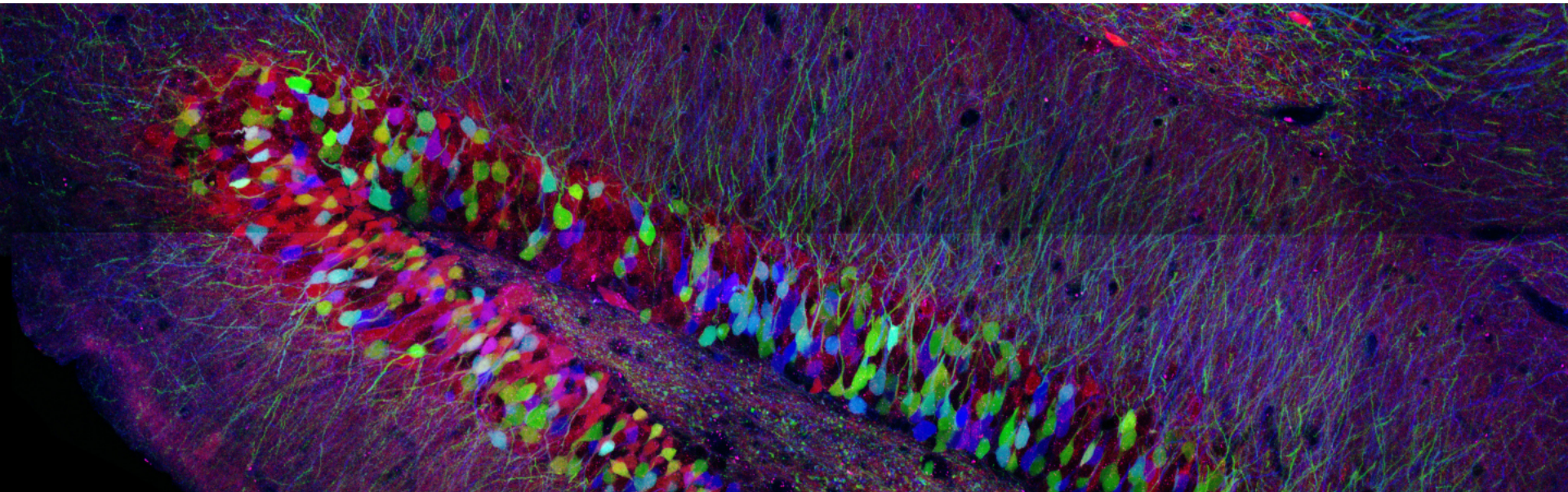
Applied Neuroscience

Columbia Science Honors Program

Fall 2016

Imaging Techniques in Neuroscience



Imaging Techniques in Neuroscience

Objective: Review of Microscopy Methods in Neuroscience

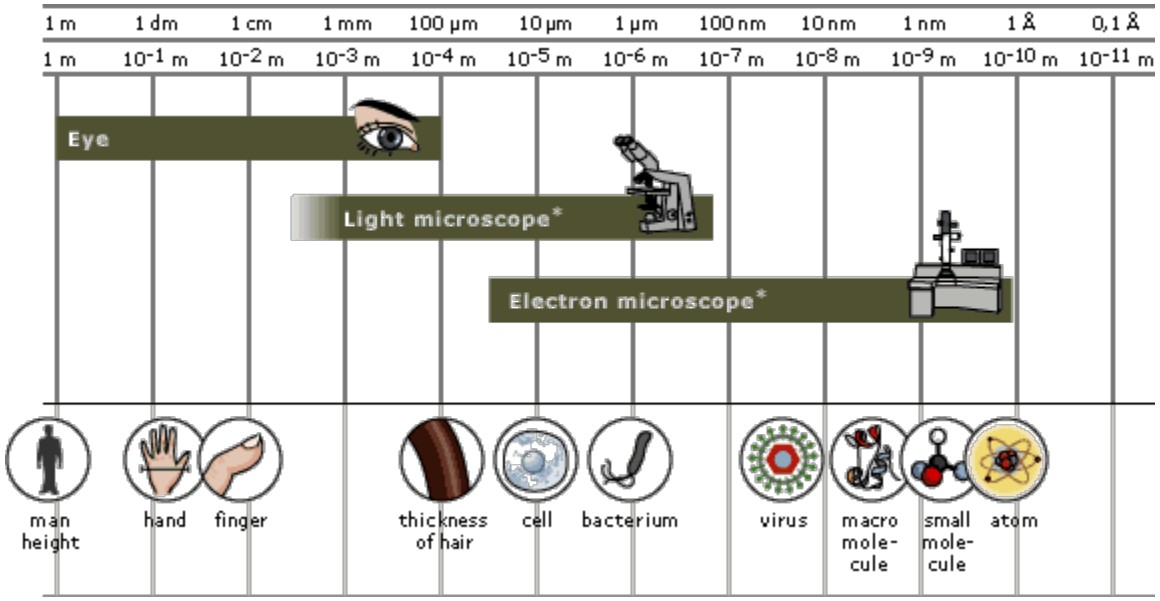
Agenda:

1. Fundamentals of Imaging
   - *Basics of Optics*
   - *Principles of Fluorescence*
2. Chemistry of Fluorophores
3. Ca^{++} Imaging
4. Imaging Techniques
   - *Phase Contrast*
   - *DIC*
   - *Polarized Light*
   - *Confocal*
   - *Multi-Photon*
   - *Super-Resolution*

Optics for Microscopy

What are microscopes?

They are specialized optical instruments used to look at objects that are too small to seen with the naked eye.

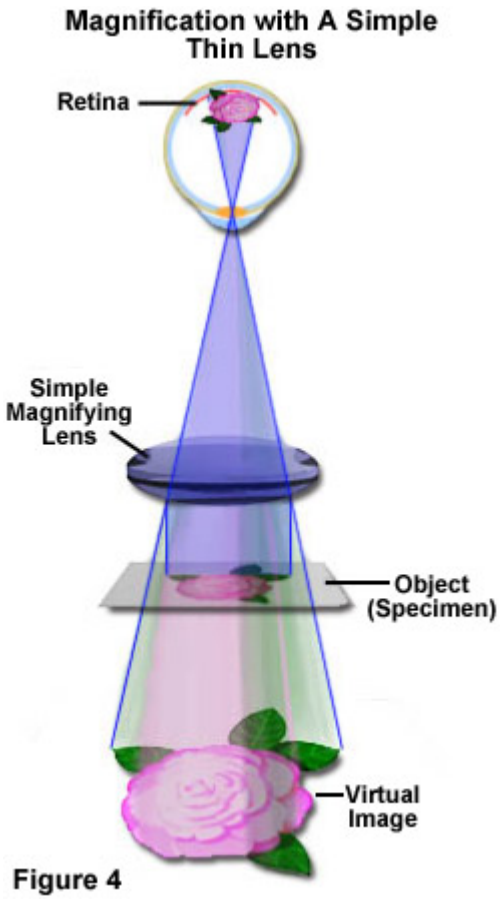


* Light microscope includes phase contrast and fluorescence microscopes. Electron microscope includes transmission electron microscope.

Magnification

Simple microscope

Contains ≥ 1 convex lenses that allowed a specimen to be focused by the magnifier, positioned between the object and the eye.



Developed over 500 years ago.

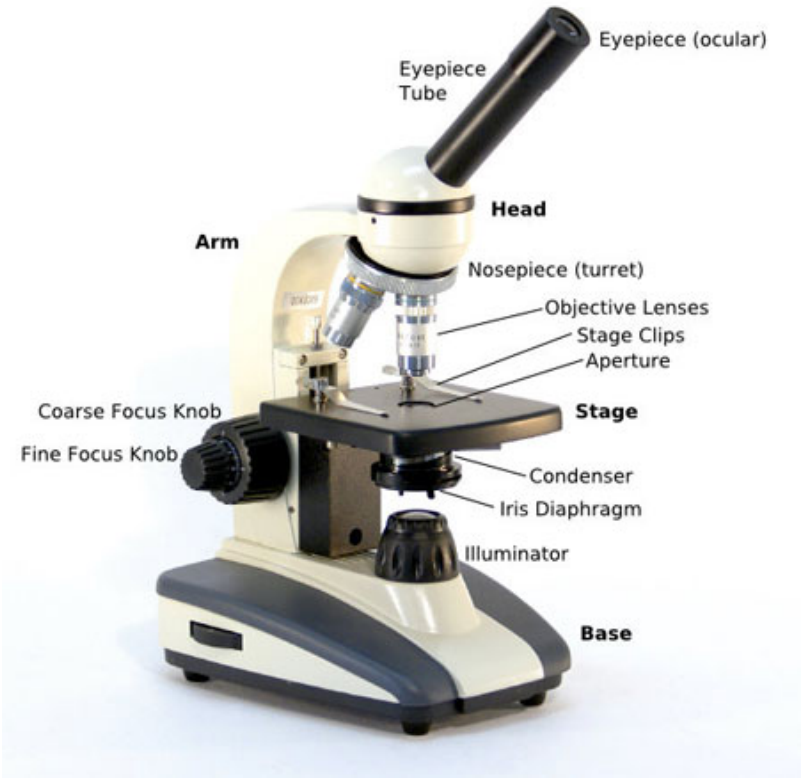
More than a 8-fold or 10-fold magnification with a simple microscope is not useful because:

1. **Small field of view**
2. **Lens needs to be brought very close to the eye**

Magnification

Compound microscope

Composed of 2 convex lenses aligned in series (an objective, which is closer to the sample and an eyepiece, which is closer to the observer's eye)



Developed in the 1600s.

Two-stage magnification

Total *magnification = Objective Magnification x Eyepiece Magnification*

Used to view smaller specimens like cell structures.

How are images formed with a microscope?

Optical components of a microscope

Microscope Optical Train Components

MICROSCOPE COMPONENT	ATTRIBUTES
Illuminator	Light Source, Collector Lens, Field Diaphragm, Heat Filters, Light Balancing Filters, Diffuser, Neutral Density Filters
Light Conditioner	Condenser Iris, Darkfield Stop, Aperture Mask, Phase Annulus, Polarizer, Off-Center Slit Aperture, Nomarski Prism, Fluorescence Excitation Filter
Condenser	Numerical Aperture, Focal Length, Aberrations, Light Transmission, Immersion Media, Working Distance
Specimen	Slide Thickness, Cover Glass Thickness, Immersion Media, Absorption, Transmission, Diffraction, Fluorescence, Retardation, Birefringence
Objective	Magnification, Numerical Aperture, Focal Length, Immersion Media, Aberrations, Light Transmission, Optical Transfer Function, Working Distance
Image Filter	Compensator, Analyzer, Nomarski Prism, Objective Iris, Phase Plate, SSEE Filter, Modulator Plate, Light Transmission, Wavelength Selection, Fluorescence Barrier Filter
Eyepiece	Magnification, Aberrations, Field Size, Eye Point
Detector	Human Eye, Photographic Emulsion, Photomultiplier, Photodiode Array, Video Camera

Some components are image-forming elements, others modify the illumination of the specimen and have filtering or transforming functions.

How are images formed with a microscope?

Perfect lens is an ideally corrected glass element that is free of aberration and focuses light onto a single point.

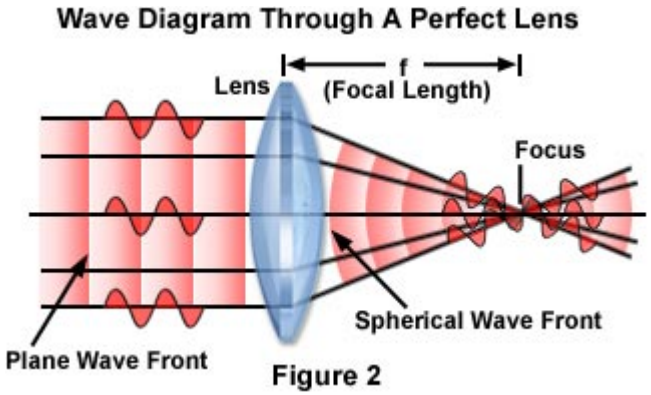


Figure 2

When studying lenses, wave-like property of light is often ignored and light travels in straight lines called rays.

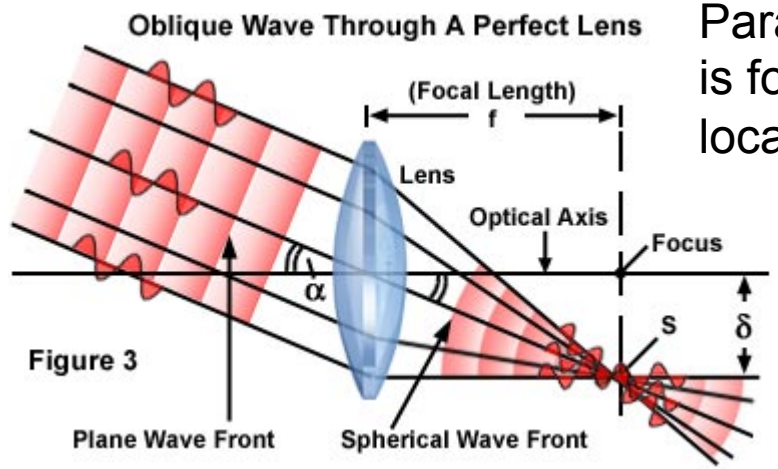


Figure 3

Parallel beam of light passes through the lens and is focused (by refraction) onto a point source located at the focal point (focus) of lens.

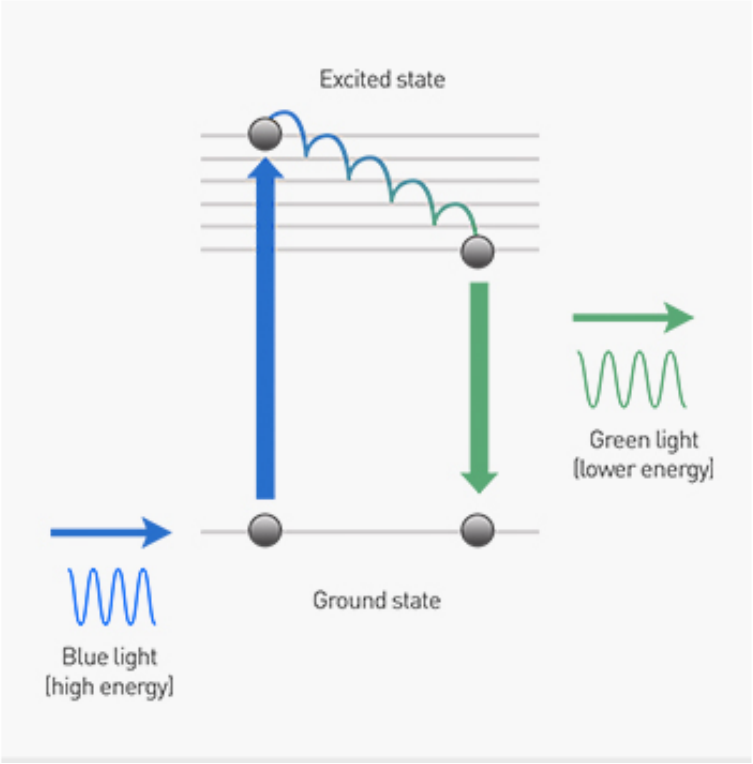
Types of Emission

1. **Fluorescence:**
   Return from excited singlet state to ground state and does not require change in spin orientation

2. **Phosphorescence:**
   Return from a triplet excited state to a ground state and requires a change in spin orientation

Emissive rates of fluorescence are many orders of magnitude faster than that of phosphorescence

Jablonkski Diagram



Simplified Jablonski Diagram
Showing the energy state change of a fluorophore's electron as it undergoes fluorescence, with the corresponding change in the color of light

Photon: packet of energy that makes up light

Emission: electrons are excited, which results in a rapid loss of energy

longer wavelength and different color

Emitted photons are used to create images in data collection.

Absorption: energy of photons are taken up electrons, which results in an increase in internal energy

Fluorophores

Fluorophore: a fluorescent chemical compound that can re-emit light upon light excitation

- *contain many combined aromatic groups*

Chromophore: the portion of a compound responsible for its color

(chemistry) group of atoms in a molecule in which the electronic transition responsible for a given spectral band is located

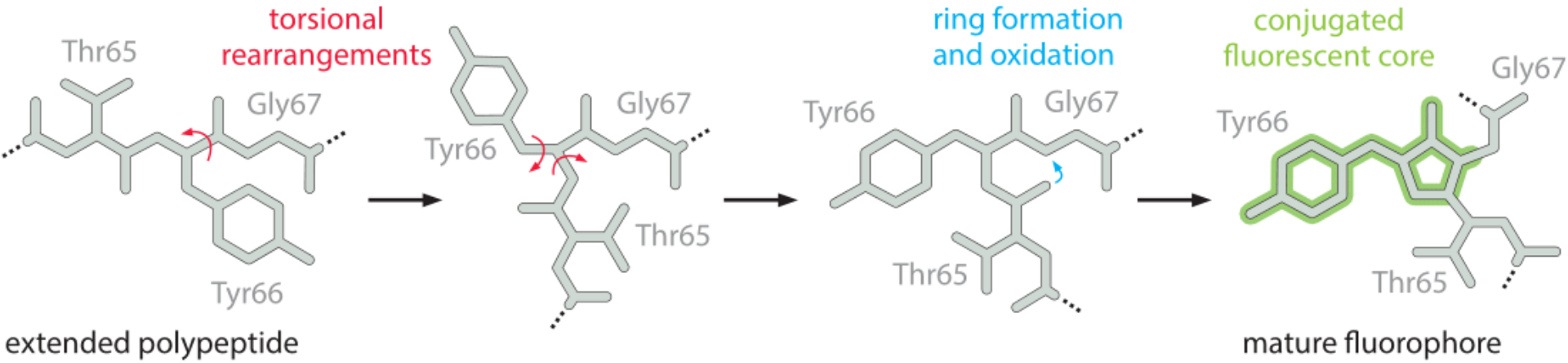
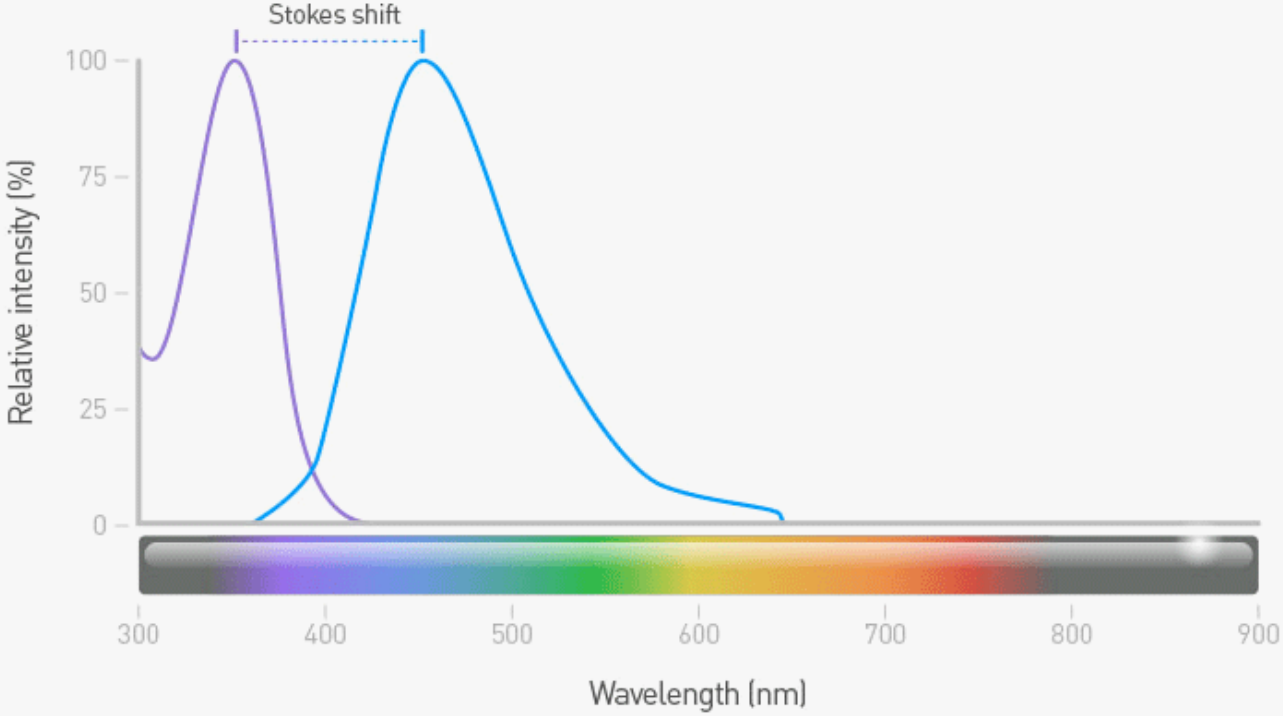


Diagram of Chromophore Formation in Maturing GFP

Stokes Shift



1. Fluorophore is a dipole, surrounded by water.
2. Dipole moment changes when fluorophore is excited but water molecules do not adapt to this quickly.
3. With vibrational relaxation, water re-aligns dipole moment.

Stokes Shift: difference (in wavelength or frequency units) between positions of the band maxima of the absorption and emission spectra of the same electronic transition

1. Vibrational relaxation or dissipation
2. Solvent Re-organization

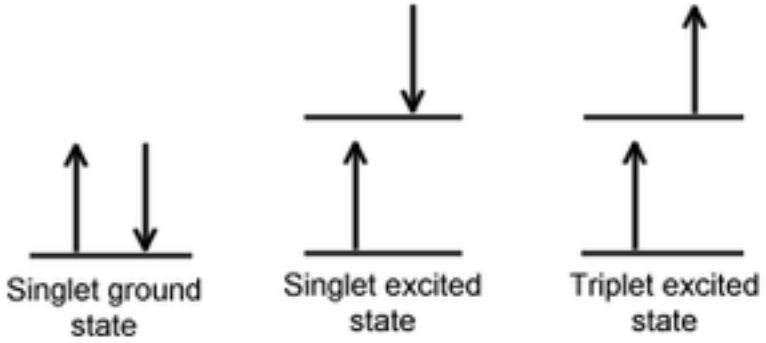
Principles of Fluorescence

Luminescence: Emission of photons from electronically excited states

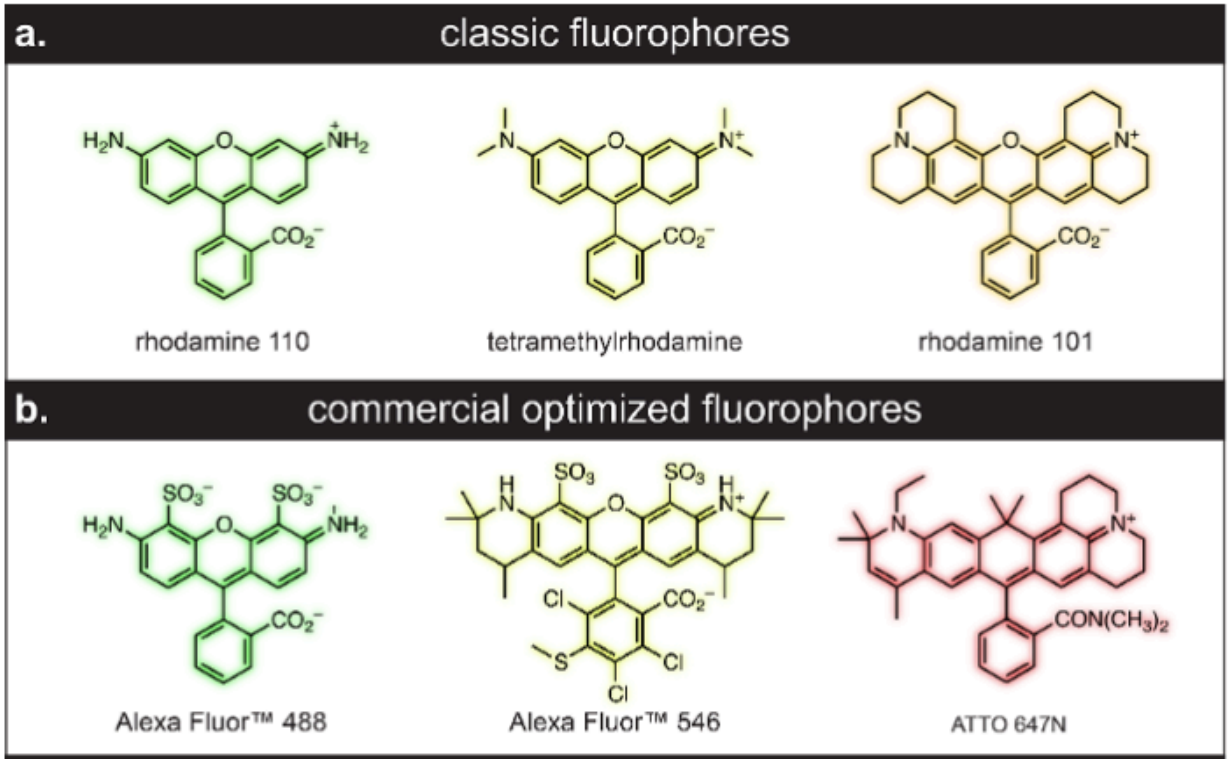
1. Relaxation from singlet excited state
2. Relaxation from triplet excited state

Singlet excited state: Electrons in higher energy orbital has the opposite spin orientation relative to electron in the lower orbital

Triplet excited state: Excited valence electron may spontaneously reverse its spin (slip flip). This is known as *intersystem crossing*. Electrons in both orbitals now have the same spin orientation.



Fluorophores



DAPI, Fluorescein, Rhodamine

Cy2, Cy3, Cy5, Alexa Fluor Dyes

A. Classic Fluorophores

B. Commercial Optimized Fluorophores

Chemical Stability, Photo-stability, Bright, Narrow Excitation and Emission Peaks, No Unspecific Binding, Measurable Lifetime (~1-10 nanoseconds)

Fluorophores: Physical Fluorophores

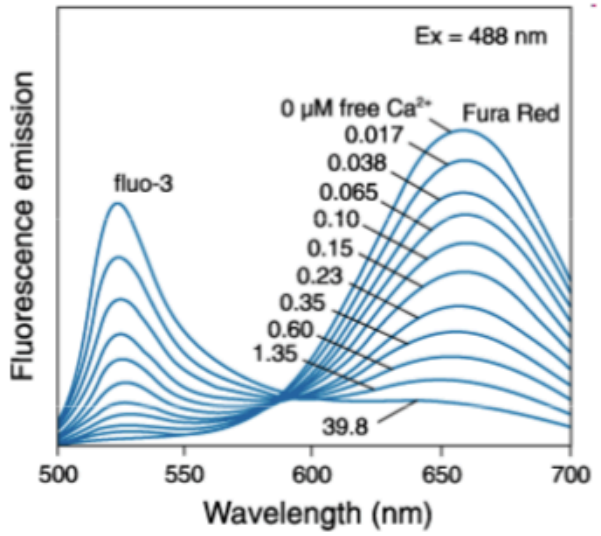
Advantages:

1. Tunable to any wavelength
2. Large Stokes shift
3. Extreme photo-stability

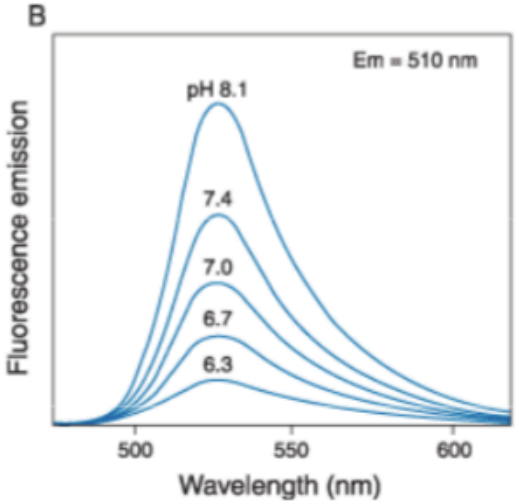
Disadvantages:

1. Quenching
2. Photo-bleaching
3. Large Size

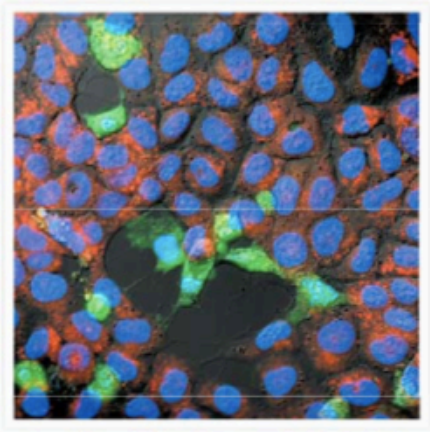
Fluorophores: Environmental Sensors



Ca²⁺ indicator Fura-Red

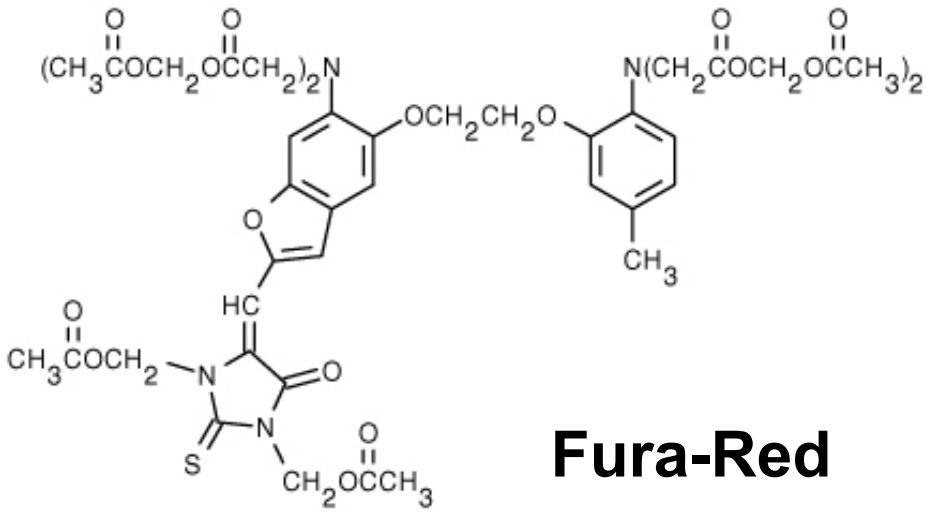


pH indicator BCECF



[image: Molecular Probes]

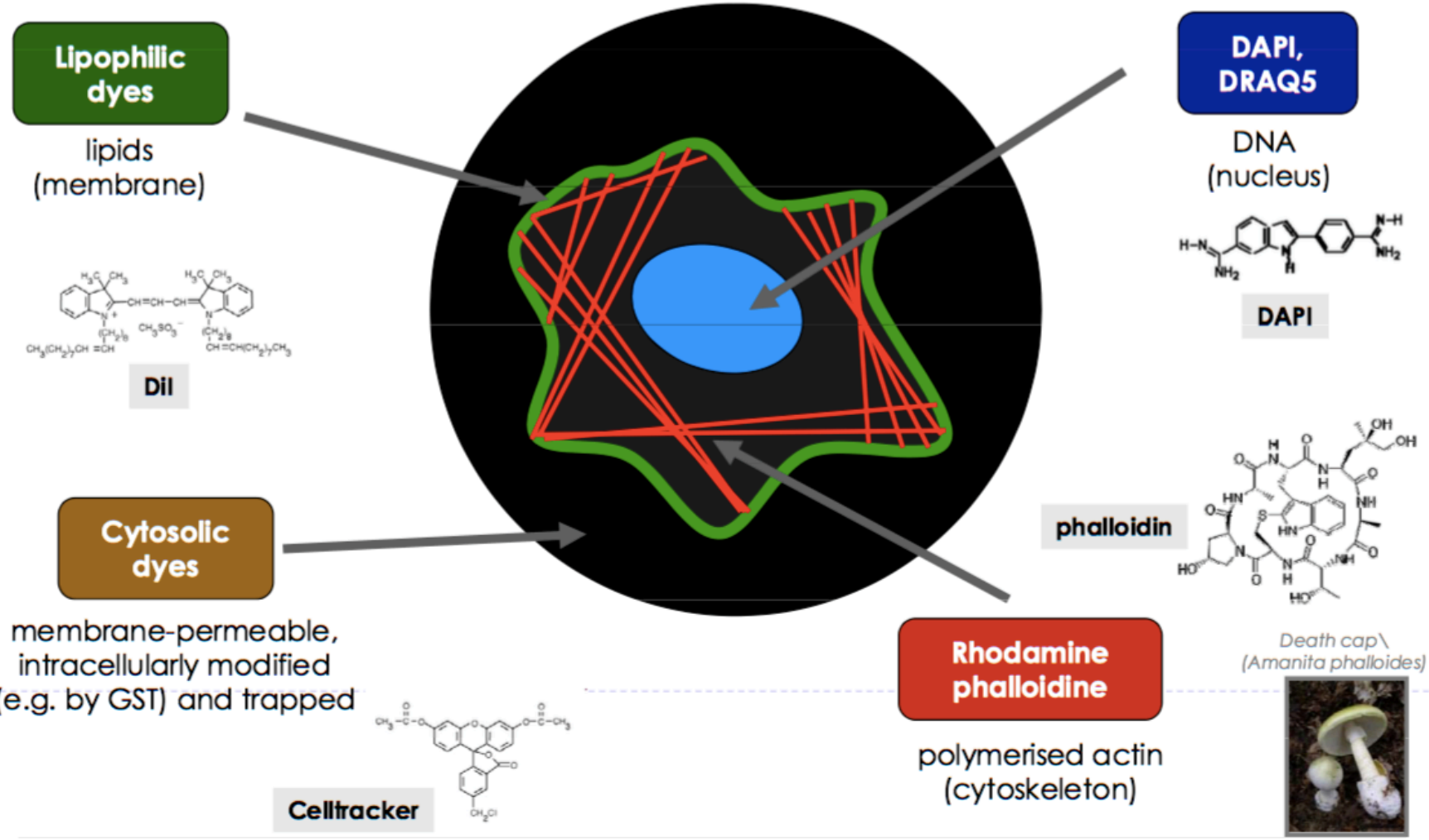
apoptosis indicator
rhodamine 110, bis
-L-aspartic acid amide



Fura-Red

- Ion Concentrations
- pH
- Reactive Oxygen, NO species
- Membrane Potential
- Cell Viability
- Uptake
  - Phagocytosis
  - Endocytosis
  - Receptor Internalization

Fluorophores with Binding Specificity



Fluorescent Staining: Antibodies

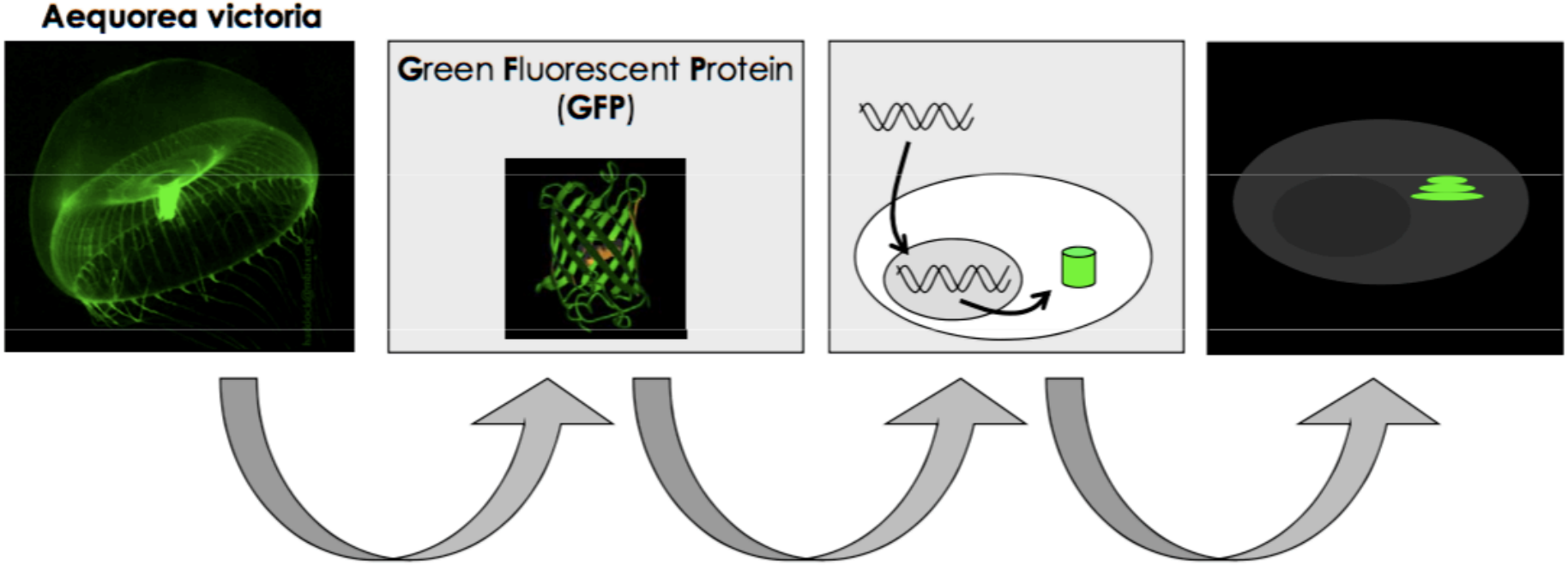
Antibody Labeling – To Consider:

- Antibodies are large proteins
- Must detect **native** protein (not **denatured**, as for Western Blots)
- Must only detect **one** single protein
- Labeling Density
  - Too dense: **auto-quenching**
  - Too sparse: ideal for single-molecule analysis in **STORM**

Controls:

- No Primary Antibody (Unspecific background from secondary)
- **Positive Control:** High expression of protein in question
- **Negative Control:** No expression of protein in question
- **GFP fusion** of protein in question
  - GFP and antibody must give some staining
  - (Ideal: Induced Translocation)

Green Fluorescent Protein (GFP)



Green Fluorescent Protein (GFP)

Osama Shimomura first isolated GFP from the jellyfish *Aequorea victoria* and discovered that it glowed bright under ultraviolet light.

Martin Chalfie demonstrated the value of GFP genetic tag for biological phenomena.

Roger Tsien contributed to our general understanding for how GFP fluoresces and extended the color palette.

Structure of GFP

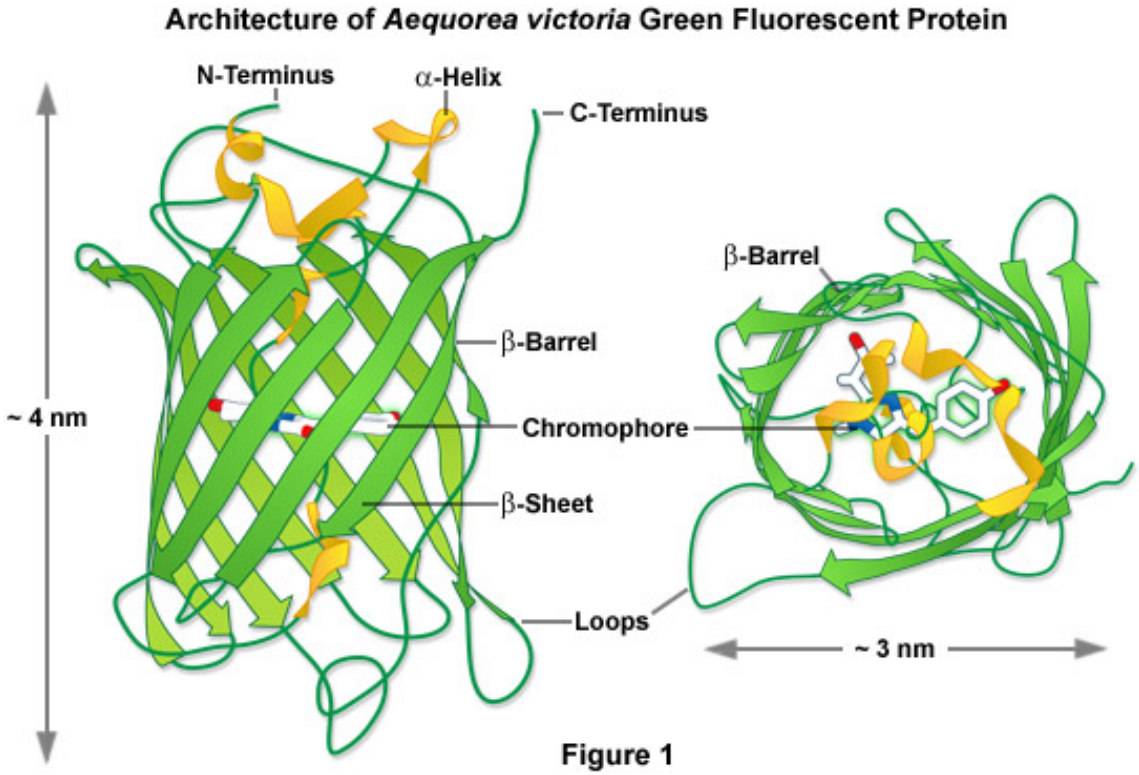
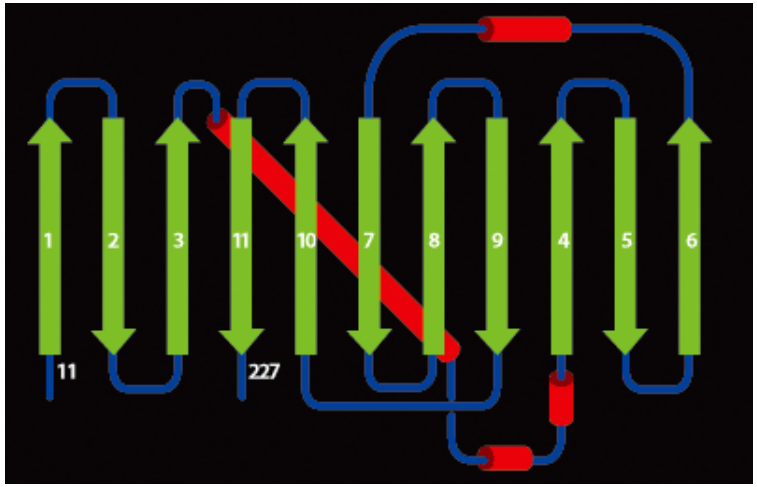


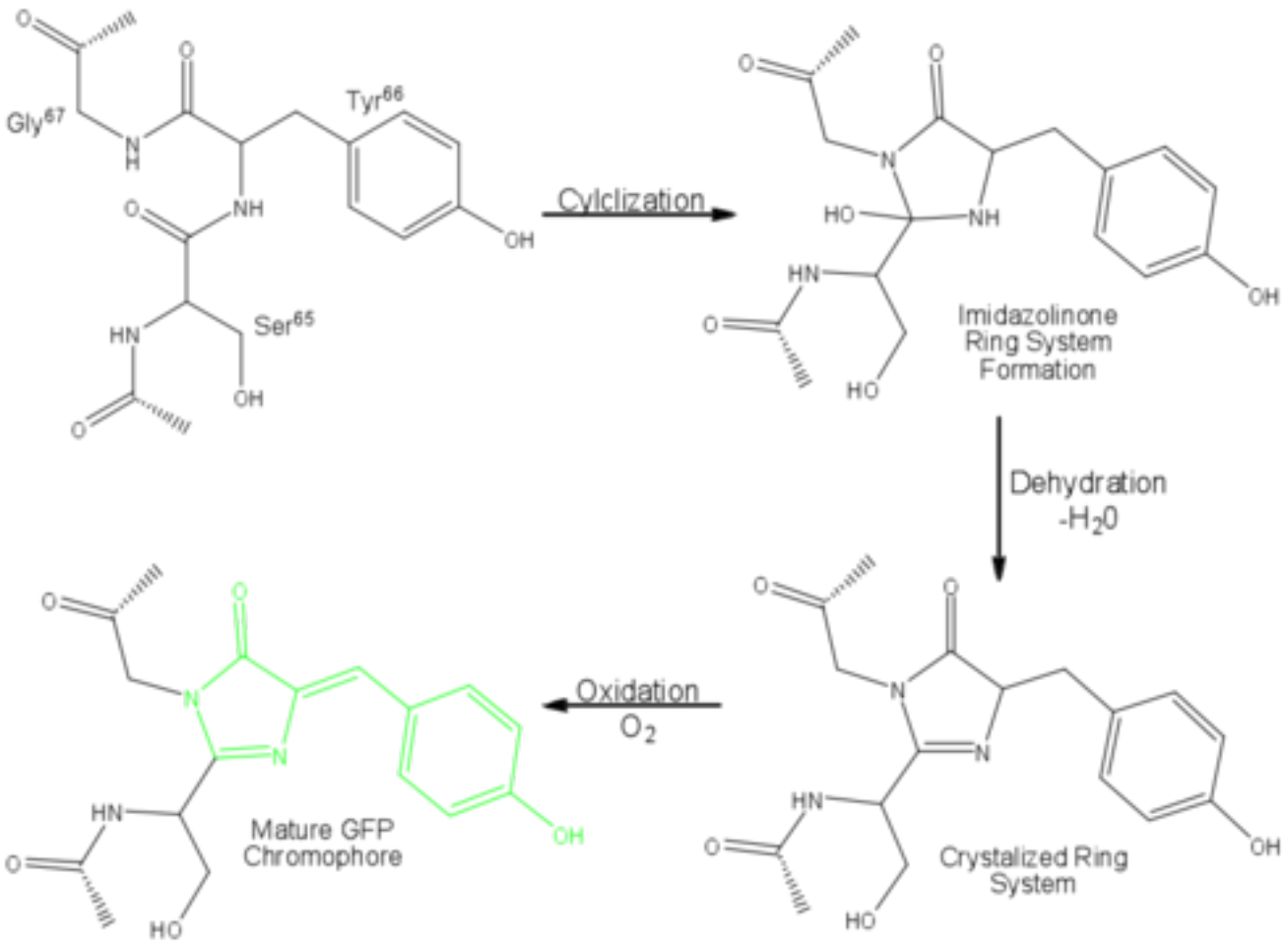
Figure 1



Main Motif: β-barrel

- 11 anti-parallel β-strands form a compact cylinder
- Inside the β-structure, there is the chromophore
- Rigid barrel protects the chromophore against photo-chemical damage and the passage of unwanted, diffusible ligands

GFP Chromophore Maturation



Three amino acid residues in the central α-helix constitute the fluorophore of GFP: $Ser^{65}Tyr^{66}Gly^{67}$

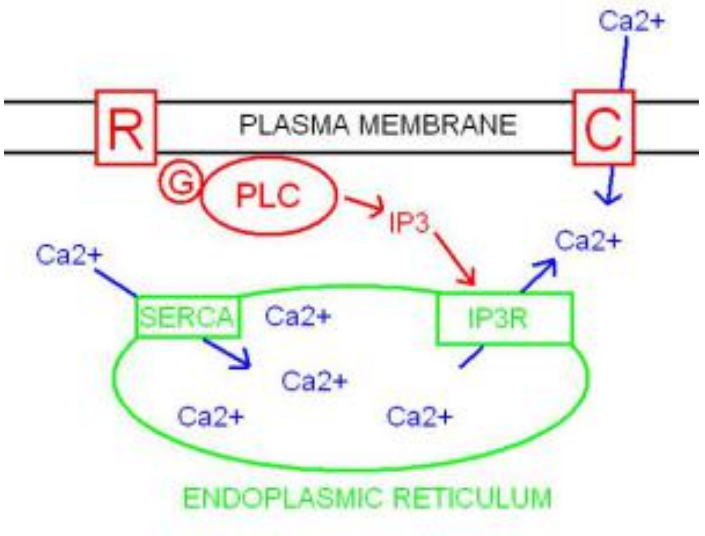
Roger Tsien discovered that this tri-peptide sequence is post-translationally modified by internal cyclization and oxidation.

Calcium Imaging

Ca^{2+} are important ions in neurons – they can regulate gene expression, cause neurotransmitter release and facilitate synaptic plasticity

They can enter the cytoplasm of a neuron from:

1) **Extracellular environment**
2) **Intracellular stores**



Voltage gated calcium channels (C): when a neuron becomes active, its membrane is depolarized and Ca^{2+} can enter the cytoplasm.

When Ca^{2+} enter the cytoplasm, SERCA proteins pump them into the ER. Intracellular store of Ca^{2+} can be released when IP3 is created, which in turn stimulates IP3 receptors (a calcium channel).

Increase in free Ca^{2+} = increased neural activity

Calcium Imaging

Visualizing the flux of Ca2+ within a neuron is useful

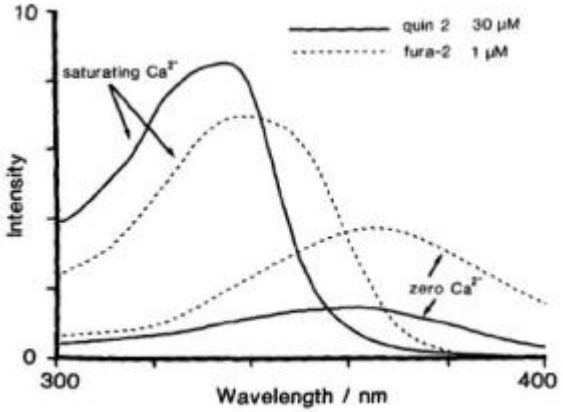
Altered Ca2+ signaling has been implicated in many diseases like schizophrenia, Alzheimer's and Huntington's

Calcium imaging allows Ca2+ concentration to be detected as changes in fluorescence

Roger Tsien

1985: Roger Tsien's group chemically linked a molecule that could bind Ca2+ to a molecule with fluorescent properties

Resulting molecule would have different fluorescent properties when bound to Ca2+ than unbound → created fura-2



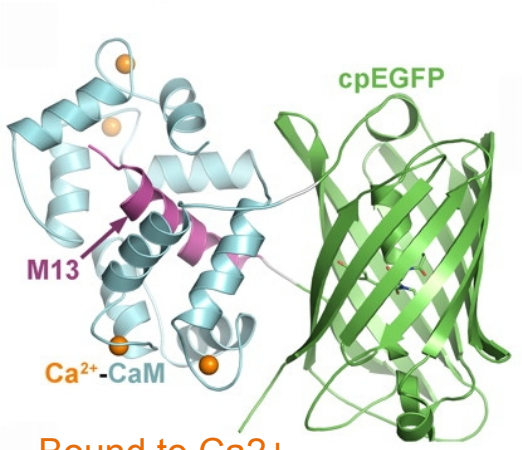
Calcium Imaging

Name	Kd (nM)	Examples of In Vivo Applications	Representative References
Chemical Calcium Indicators			
Oregon Green BAPTA-1	170	Mouse neocortex, mouse hippocampus, mouse olfactory bulb, rat neocortex, rat cerebellum, ferret neocortex, cat neocortex, zebrafish	Dombeck et al., 2010, Sullivan et al., 2005, Ohki et al., 2005, Li et al., 2008, Greenberg et al., 2008,Rochefort et al., 2011, Sumbre et al., 2008, Wachowiak et al., 2004
Calcium Green-1	190	Mouse neocortex, mouse olfactory bulb, honeybee, turtle, zebrafish, rat neocortex	Dombeck et al., 2009, Oka et al., 2006, Galizia et al., 1999,Wachowiak et al., 2002, Brustein et al., 2003, Svoboda et al., 1997
Fura-2	140	Mouse neocortex	Sohya et al., 2007
Indo-1	230	Mouse neocortex	Stosiek et al., 2003
Fluo-4	345	Mouse neocortex, Xenopus larvae	Sato et al., 2007, Demarque and Spitzer, 2010
Rhod-2	570	Mouse neocortex, Zebrafish	Takano et al., 2006, Yaksi et al., 2009
X-rhod-1	700	Mouse neocortex	Nagayama et al., 2007
Genetically Encoded Calcium Indicators			
Camgaroo 1		Drosophila	Yu et al., 2003
Camgaroo 2		Drosophila, mouse olfactory bulb	Yu et al., 2003, Hasan et al., 2004
Inverse pericam	200	Zebrafish, mouse olfactory bulb	Hasan et al., 2004, Li et al., 2005
GCaMP 2	840	Mouse olfactory bulb, mouse cerebellum	Fletcher et al., 2009, Díez-García et al., 2005
GCaMP 3	660	Mouse neocortex, mouse hippocampus, Drosophila, C. elegans	Tian et al., 2009, Dombeck et al., 2010, Seelig et al., 2010, Tian et al., 2009
Yellow Cameleon 3.6	250	Mouse neocortex	Lütcke et al., 2010
Yellow Cameleon Nano	15–50	Zebrafish	Horikawa et al., 2010
D3cpV	600	Mouse neocortex	Wallace et al., 2008
TN-XL	2200	Drosophila, macaque	Mank et al., 2006, Heider et al., 2010
TN-L15	710	Mouse neocortex	Heim et al., 2007
TN-XXL	800	Drosophila, mouse neocortex	Mank et al., 2008, Mank et al., 2008

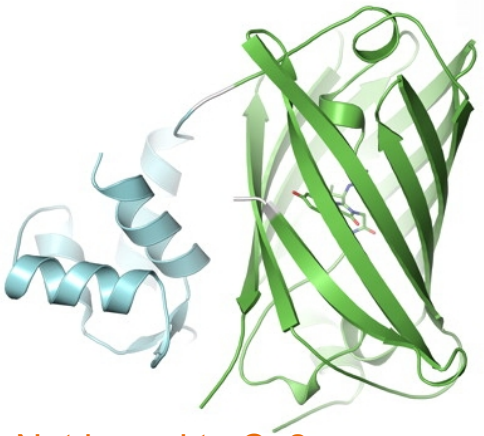
Calcium Imaging

GCaMP: genetically encoded calcium indicator (GECI)

It's a fusion of green fluorescent protein (GFP), calmodulin and M13 (peptide sequence from myosin light chain kinase)



Bound to Ca2+

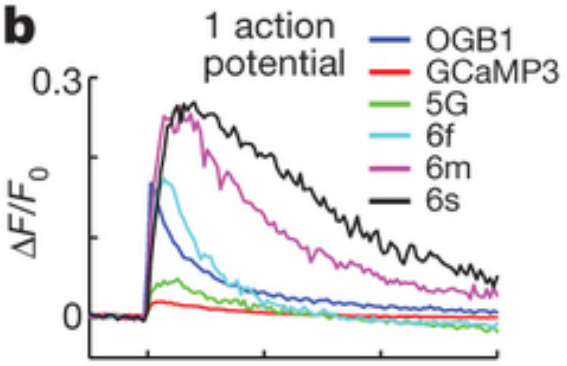


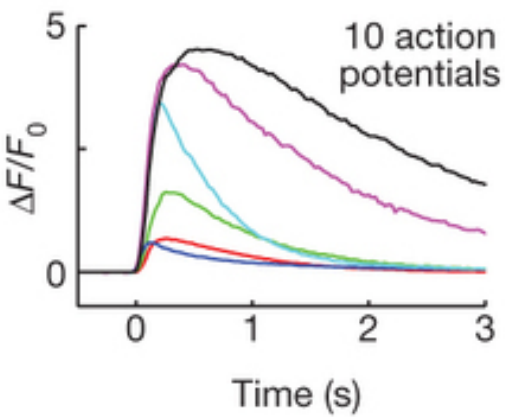
Not bound to Ca2+

There are now many GCaMP variants:

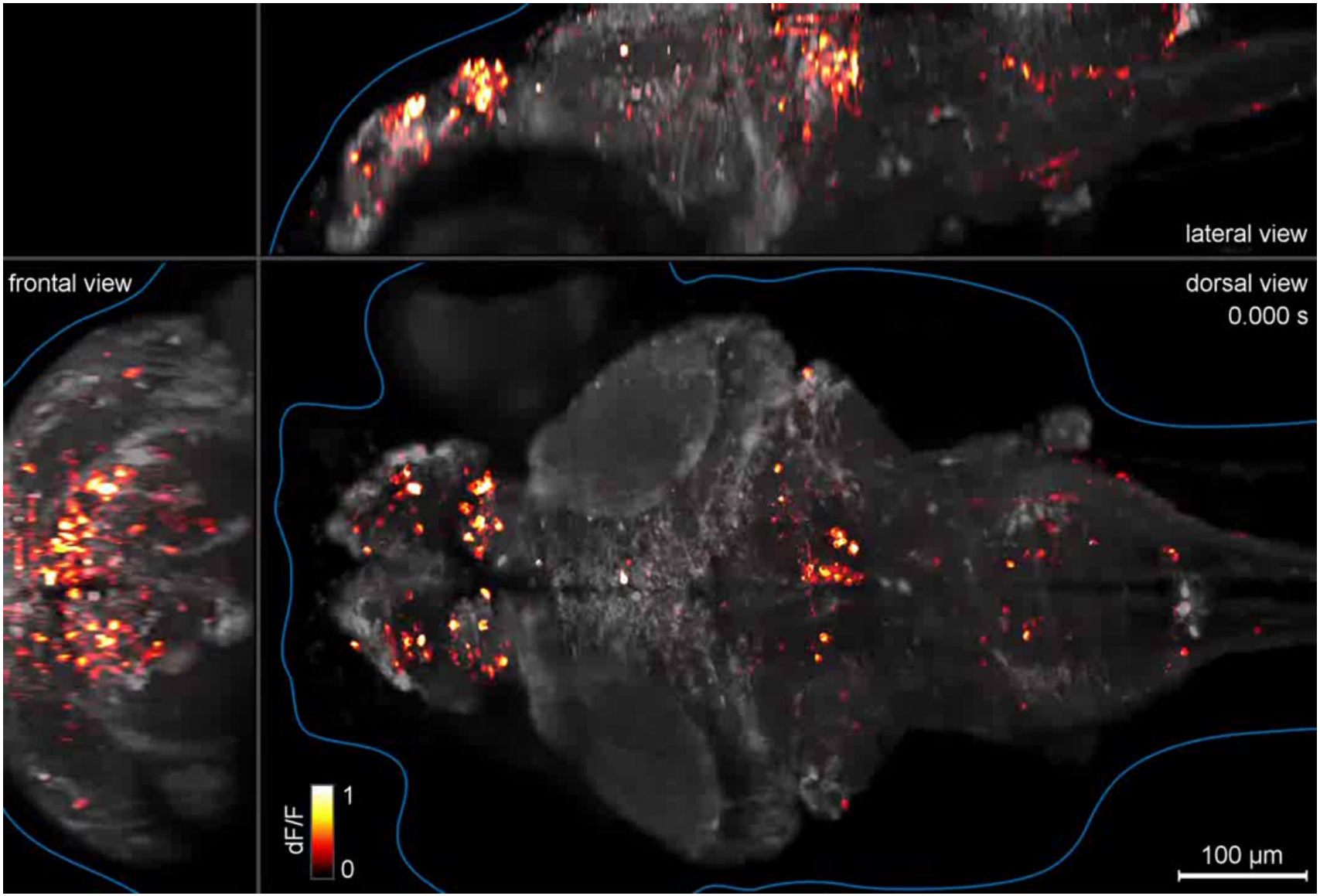
Three ultrasensitive ones are GCaMP6s, 6m and 6f (slow, medium and fast kinetics, respectively)

More sensitive sensors have slower kinetics.



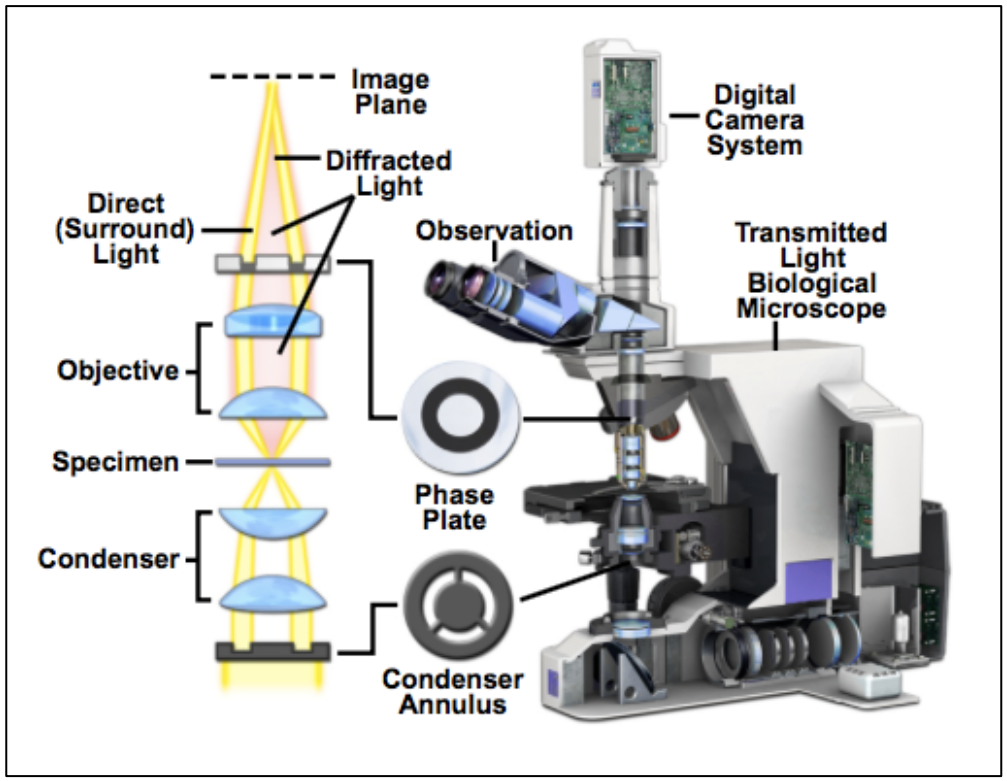


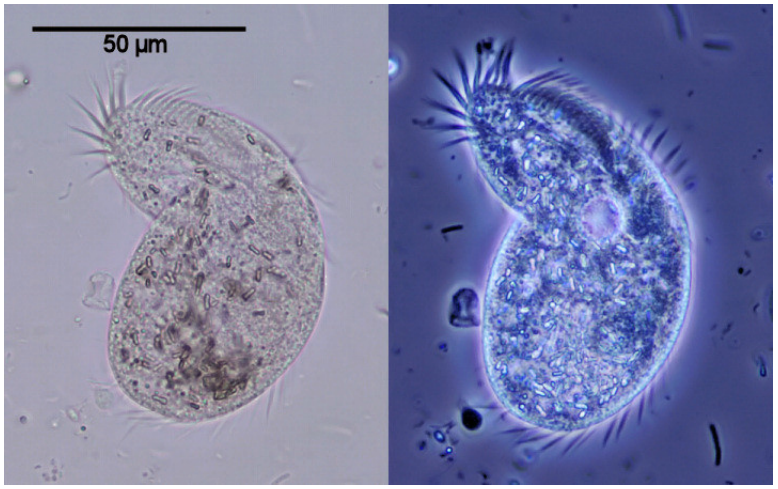
Calcium Imaging



Misha Ahrens' lab

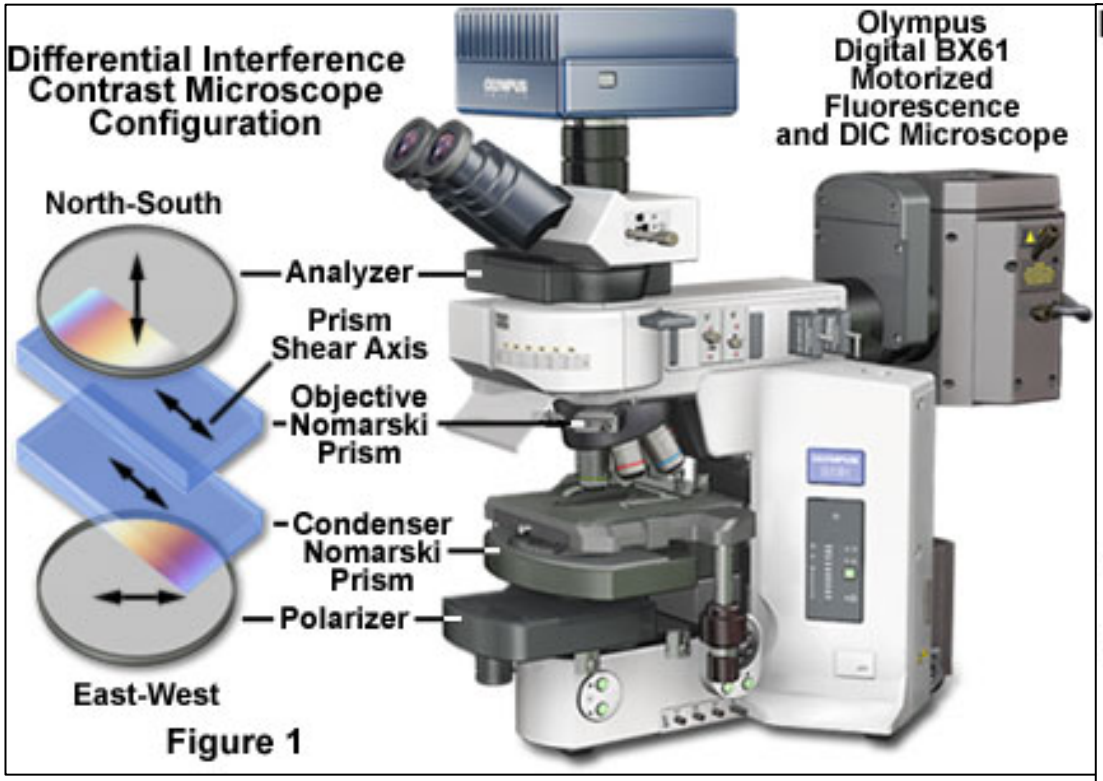
Phase-Contrast Microscopy

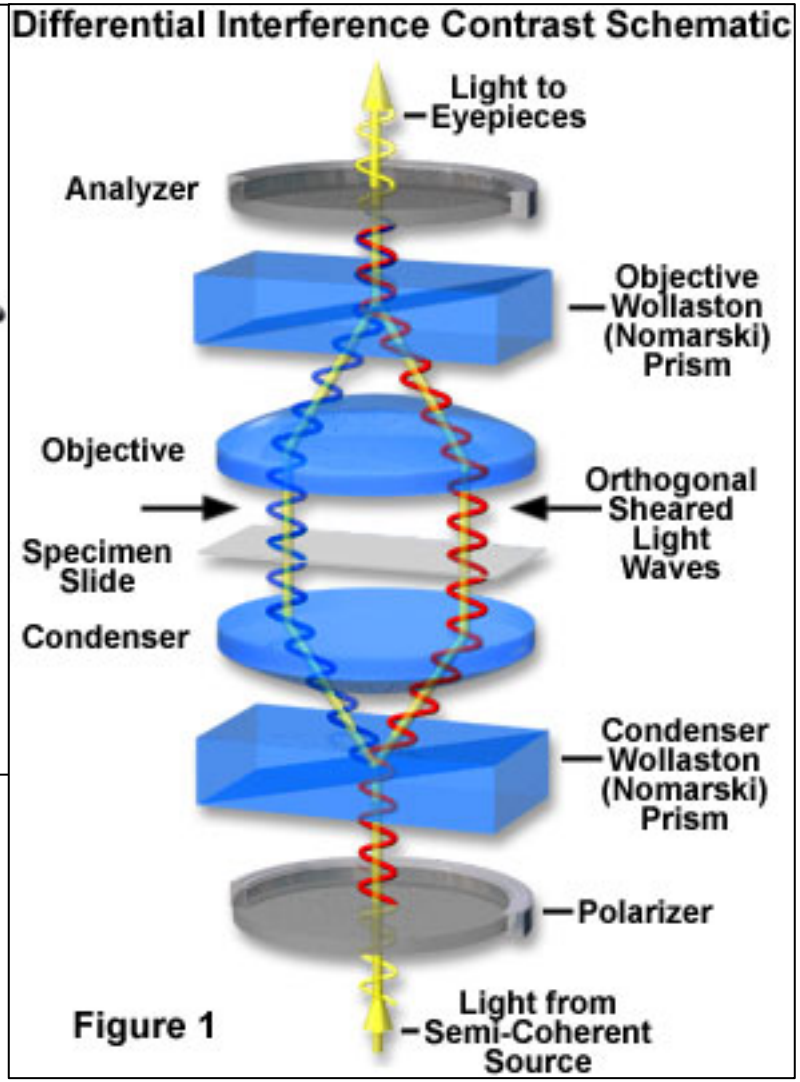




Phase-Contrast Microscopy: technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. Phase shifts alone are invisible but are visible when brightness varies.

Differential Interference Contrast Microscopy





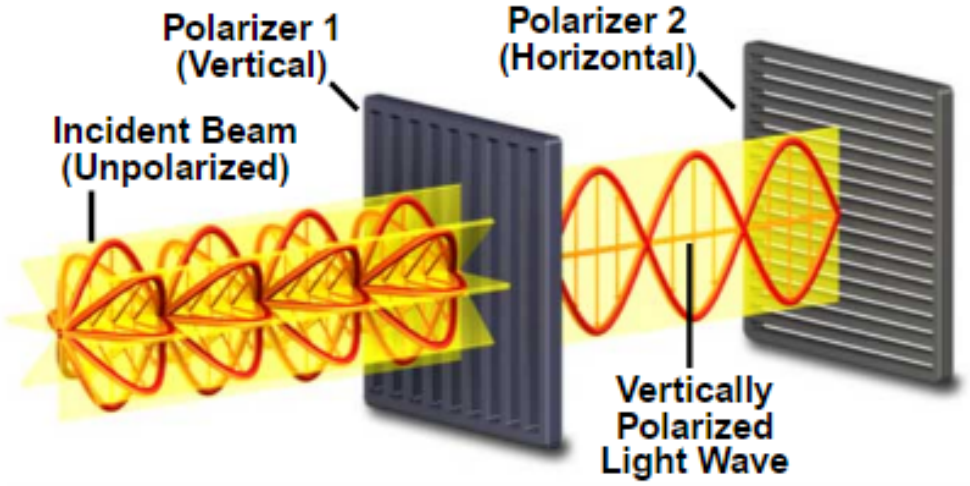
Differential Interference Contrast Microscopy:

technique used to enhance the contrast in unstained, transparent samples

Polarized Light Microscopy

Polarized Light Microscopy: contrast-enhancing technique that improves the quality of the image obtained with birefringent materials. Often used to look at minerals and crystals.

Birefringence: optical property of a material having a refractive index that is dependent upon the polarization and direction of light



Light waves have electric field vectors that vibrate in all planes that are perpendicular to the direction of propagation. By polarizing light, the vectors are restricted to a single plane.

Polarized Light Microscopy

Anisotropic materials: has properties that are directionally dependent (i.e. refractive index)

Polarized light microscope is designed to observe and photograph specimens that are visible due to their optically anisotropic character.

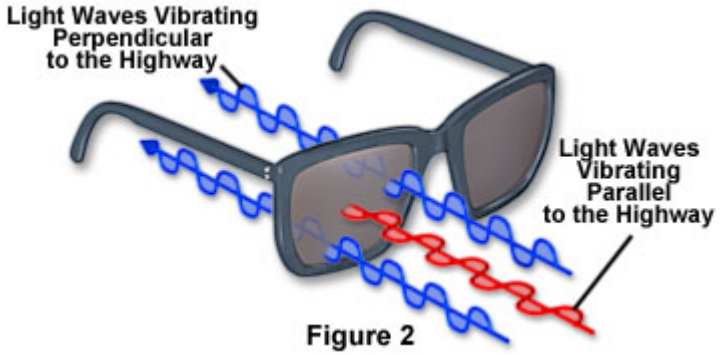
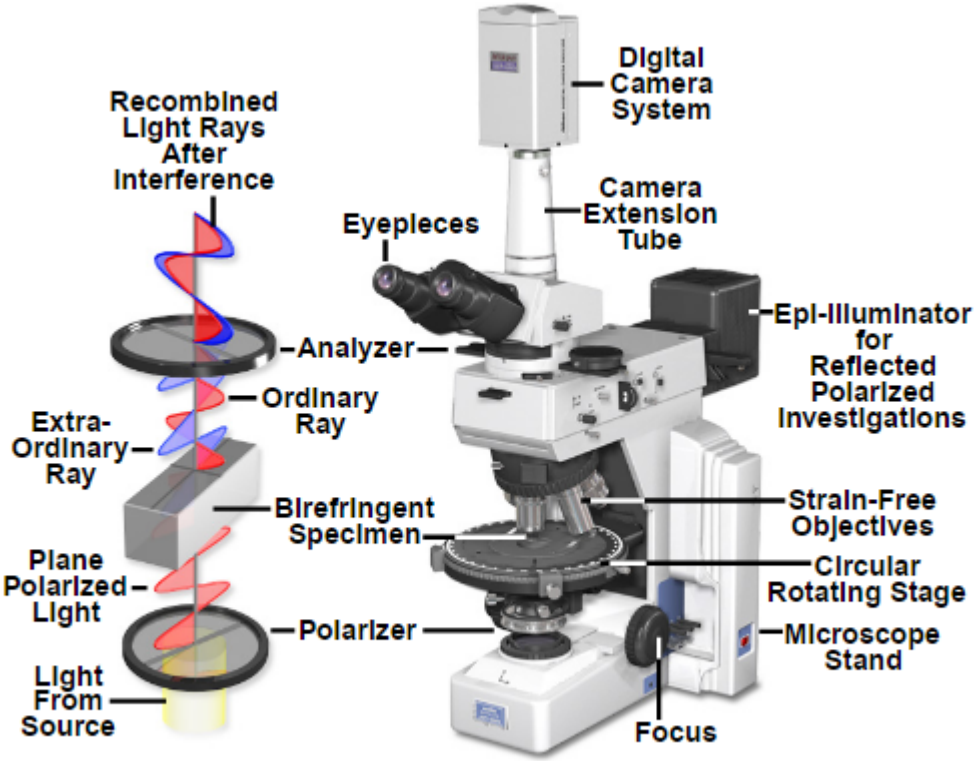
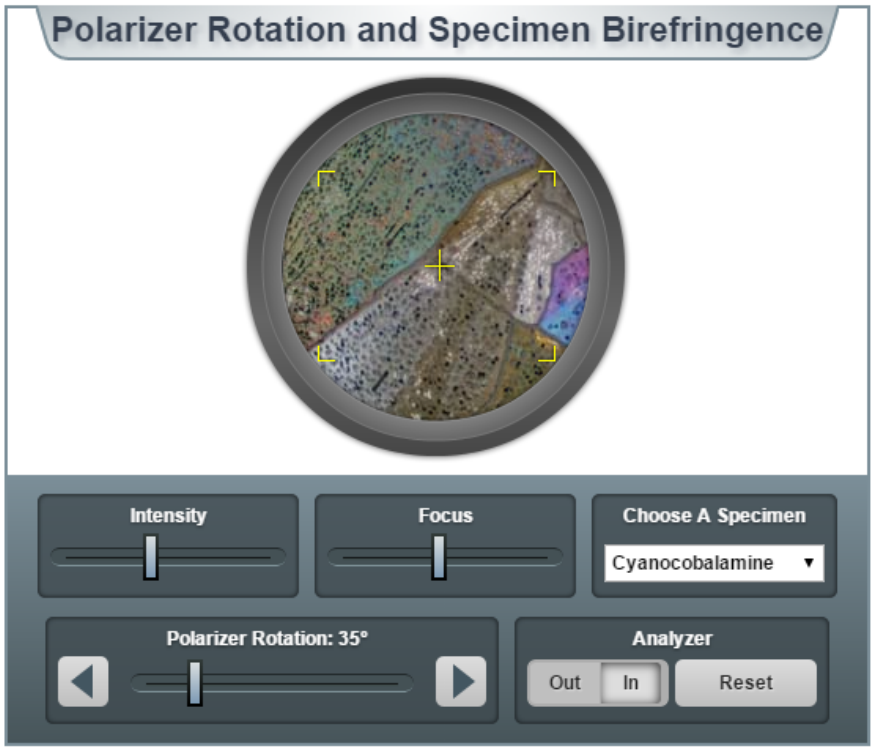
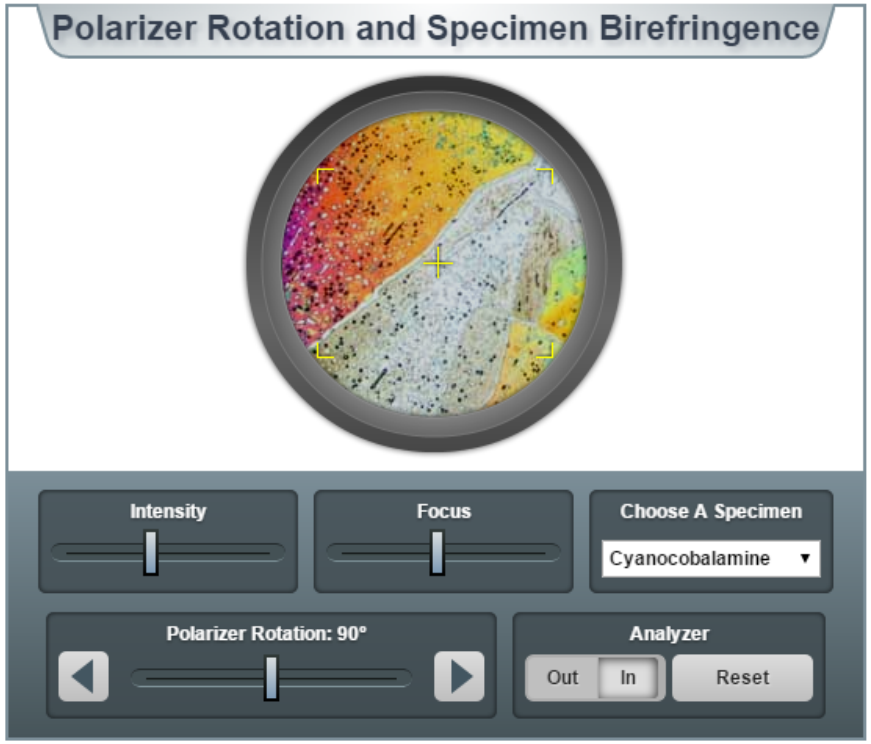


Figure 2



Polarized Light Microscopy





Confocal Microscopy

Confocal Microscopy: Technique that allows the ability to control depth of field, elimination of background information away from focal plane (reduces image degradation) and the ability to collect optical sections from thick specimens

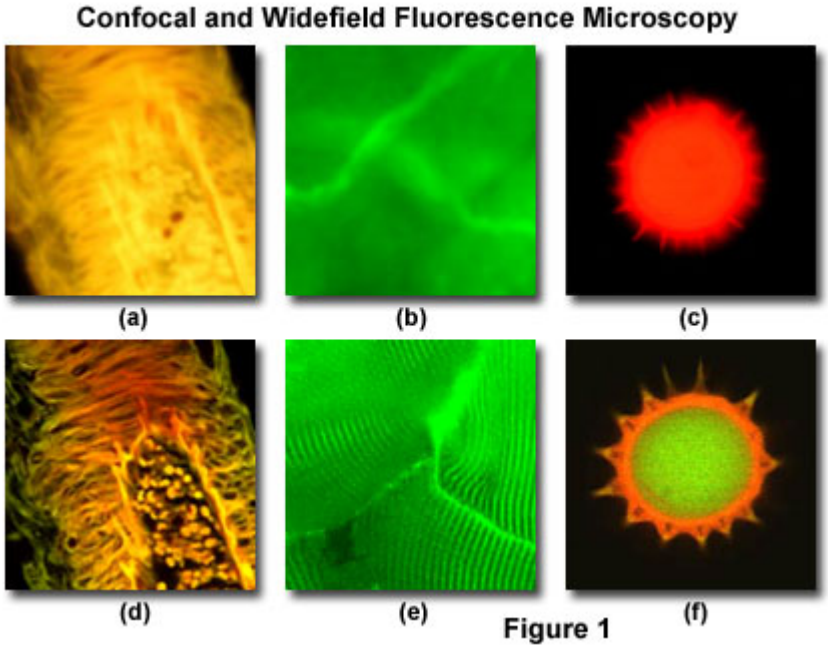


Figure 1

Confocal uses spatial filtering techniques to eliminate out-of-focus light or glare in specimens whose thickness >> plane of focus

Confocal Microscopy

How does it work? Light emitted by laser excitation source passes through pinhole aperture that is situated in a conjugate plane with a scanning point on the specimen and a second pinhole aperture positioned in front of the detector.

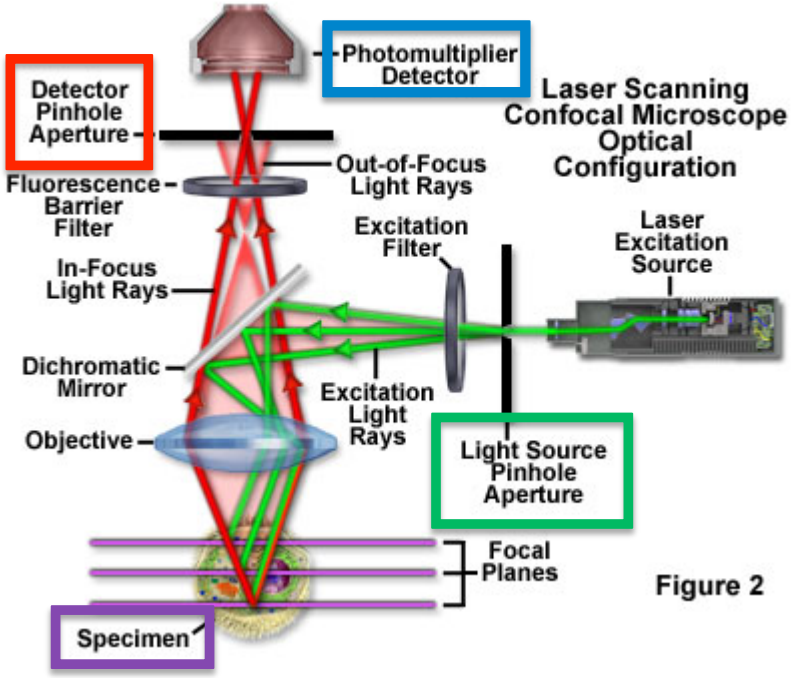


Figure 2

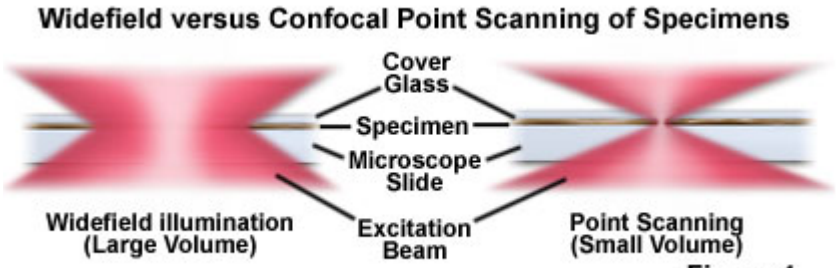


Figure 4

Confocal Microscopy

Advantages of confocal microscopy:

1. Able to serially produce thin optical sections (0.5-1.5 um) for specimens (50 um or more)
2. Contrast and definition are improved due to reduction in background fluorescence and increased signal to noise
3. Sections can be collected laterally and vertically (optically and with software tricks, respectively)
4. Once a z-stack is collected, 3D volume renderings can be created

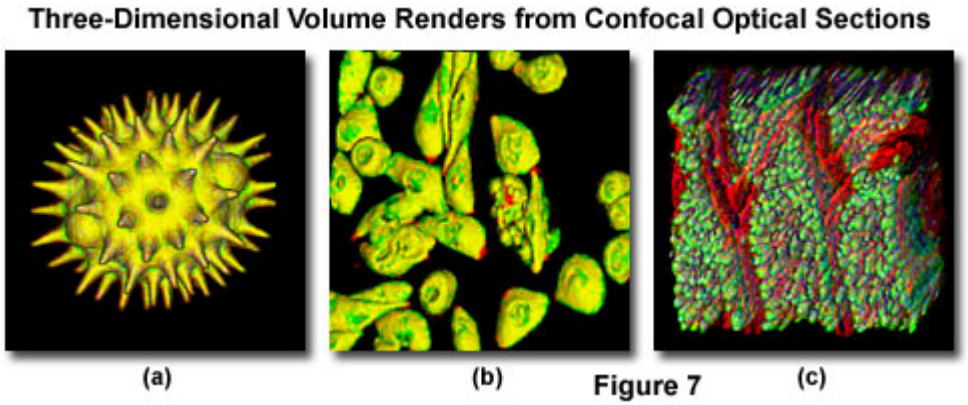


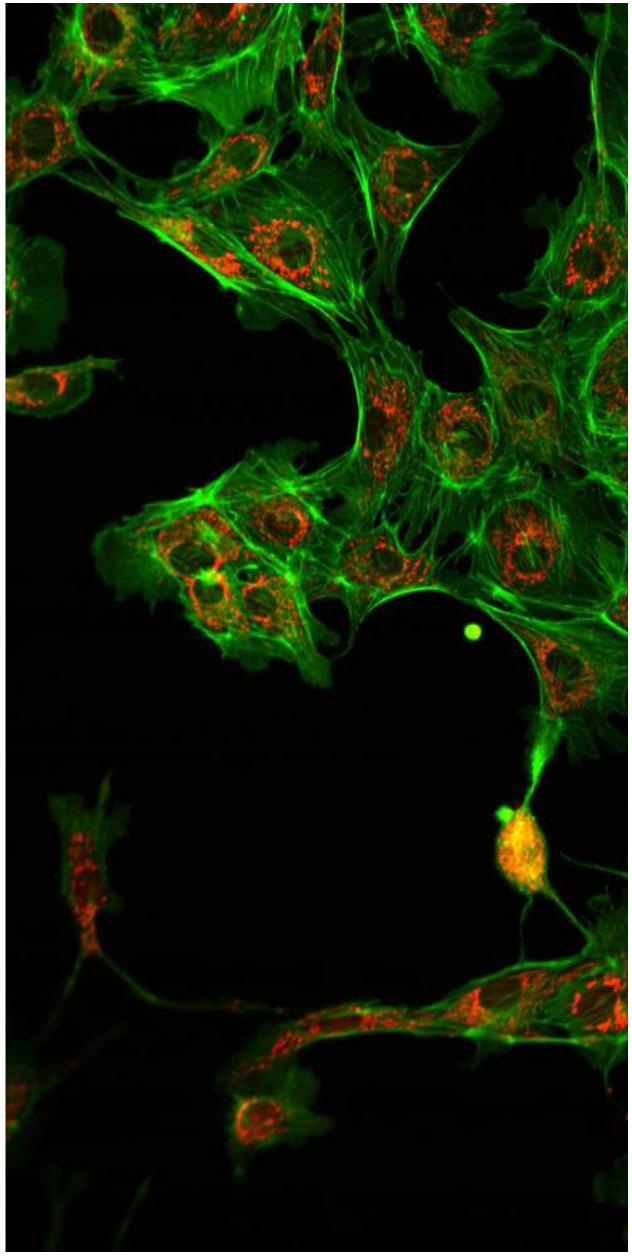
Figure 7

5. Magnification can be altered electronically

Confocal Microscopy

Disadvantages of confocal microscopy:

1. Limited number of excitation wavelengths (making lasers is difficult and expensive in UV region)
2. High intensity laser irradiation to living cells/tissues is harmful
3. They are expensive (order of magnitude higher than widefield microscopes)
4. Speed of imaging large samples
5. Learning curve

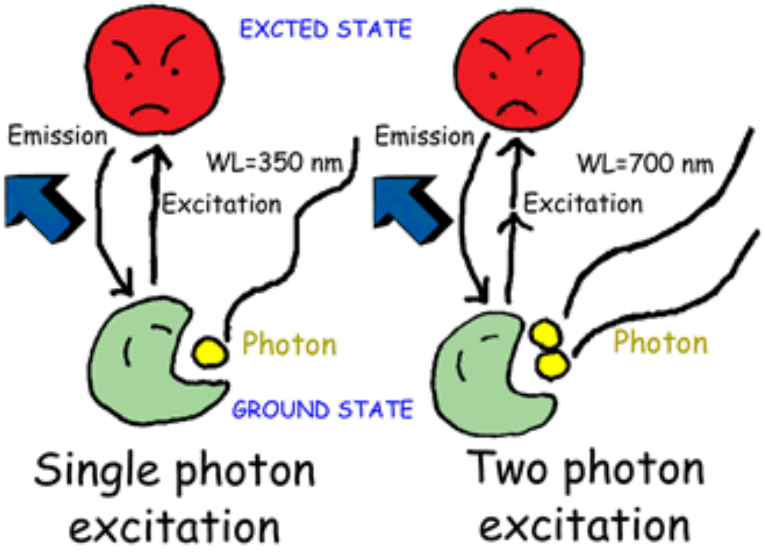


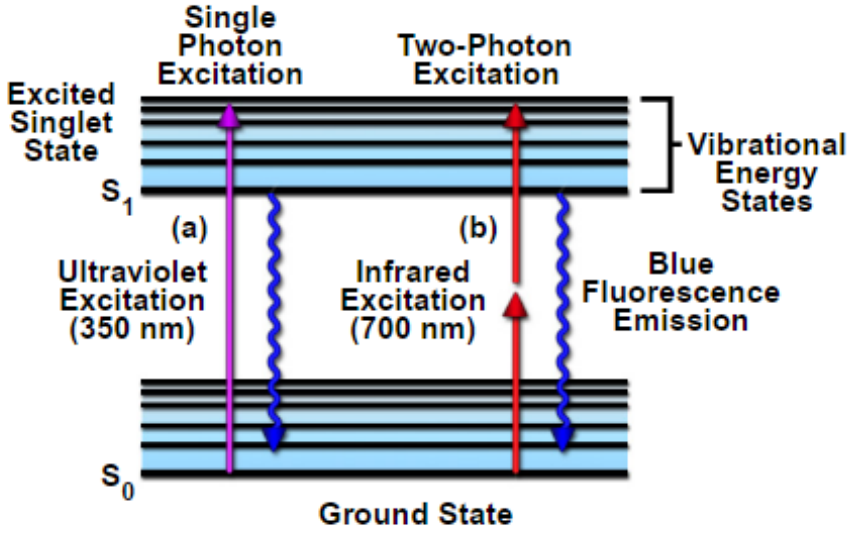
Multiphoton Microscopy

Multiphoton Microscopy: Also called two-photon excitation microscopy or non-linear microscopy. It is an alternative to confocal microscopy that has many advantages for 3D imaging.

Depends on the probability of two photons being absorbed at the same time.

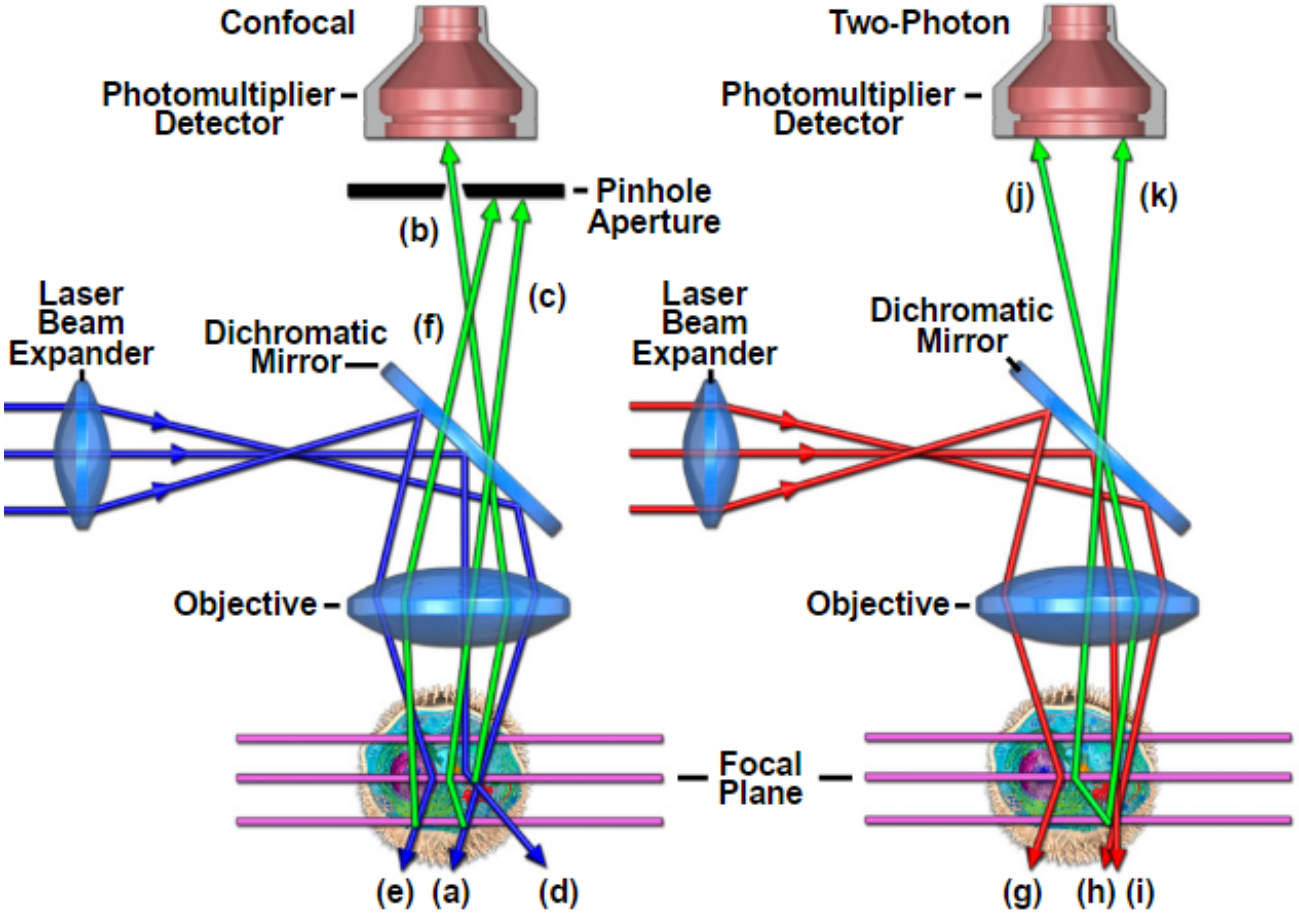
What is the difference?





Multiphoton Microscopy

Optical arrangement:



Multiphoton Microscopy

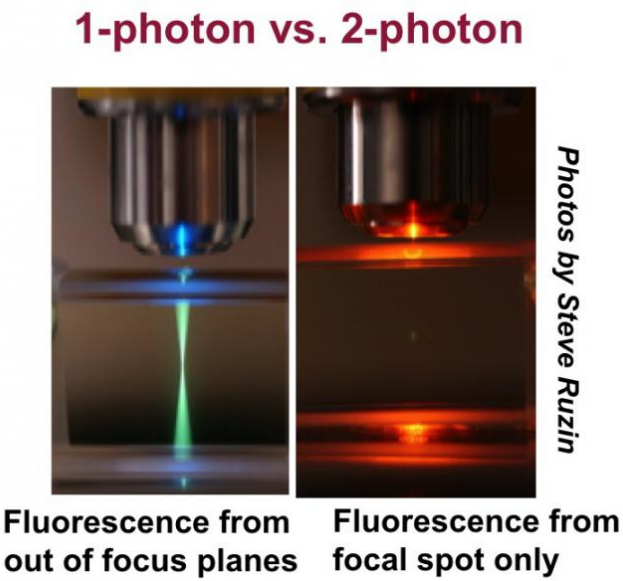
Advantages:

1. Significantly less absorption in biological specimens, making technique better for thick specimens
2. Confocal microscopy uses emission pinhole aperture to reject out of focus light. In thick specimens, scattering will still happen. Two-photon limits excitation volume, requiring no pinhole aperture. Signal loss is thus minimized.
3. Two photon induces less photo bleaching and photodamage.
4. Wider gap between excitation and emission wavelengths makes it easier to reject excitation light.
5. Light-mediated chemical reactions, like photoactivation, can be done in very small areas (since photodamage only occurs in a small volume).

Multiphoton Microscopy

Disadvantages:

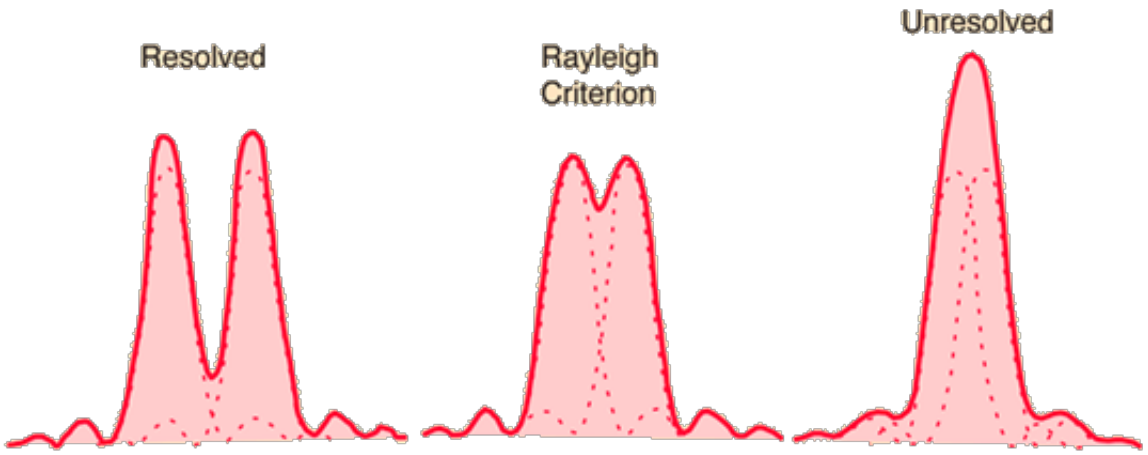
1. Resolution of two-photon is lower than that of confocal
   Abbe's law = resolution of microscopy system is inversely proportional to the wavelength of light used
2. Potential for thermal damage to specimen if it contains chromophores that absorb the excitation wavelengths

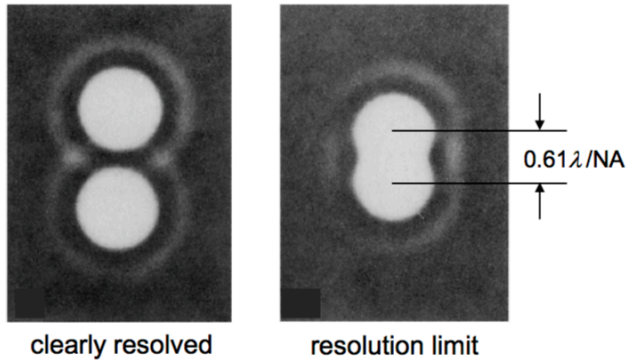


3. Two-photon excitation spectra is not fully characterized for all fluorophores

Numerical Aperture and Resolution

The resolution of an optical microscope is determined by the **wavelength (λ)** and the **numerical aperture (NA)** of the objective.





Rayleigh Criterion: criterion for the minimally resolvable detail

An imaging process is said to be diffraction-limited when the first diffraction minimum of the image of one source point coincides with the maximum of another

Resolution: 0.61λ/ NA

For dry objectives:

NA < 1.0

For immersion objectives:

NA < 1.5

Why does optical resolution have a limit?

Higher spatial frequency components lead to larger diffraction angles. The diffraction caused by *very high spatial frequencies turns the light out of the collection angle of the objective.*

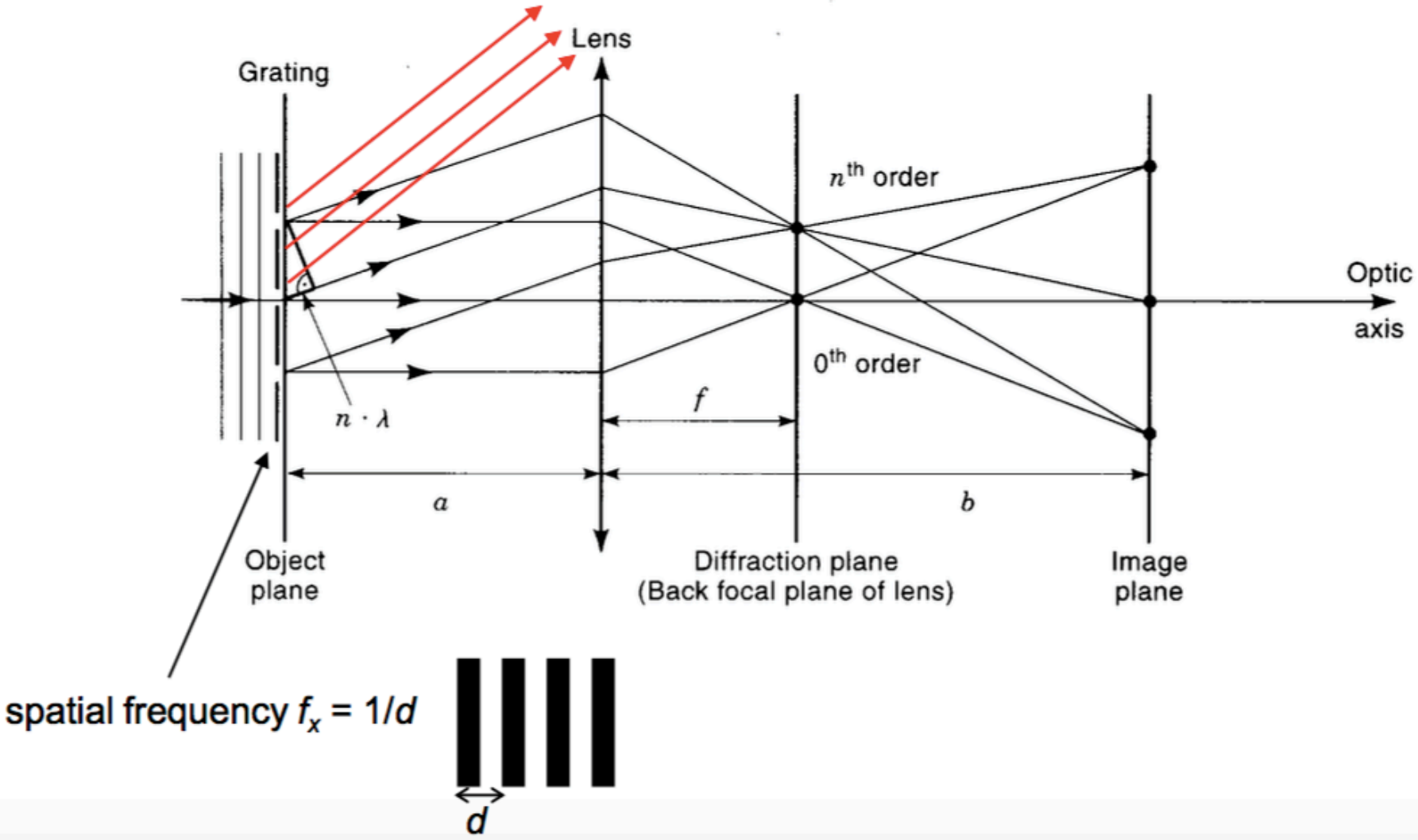
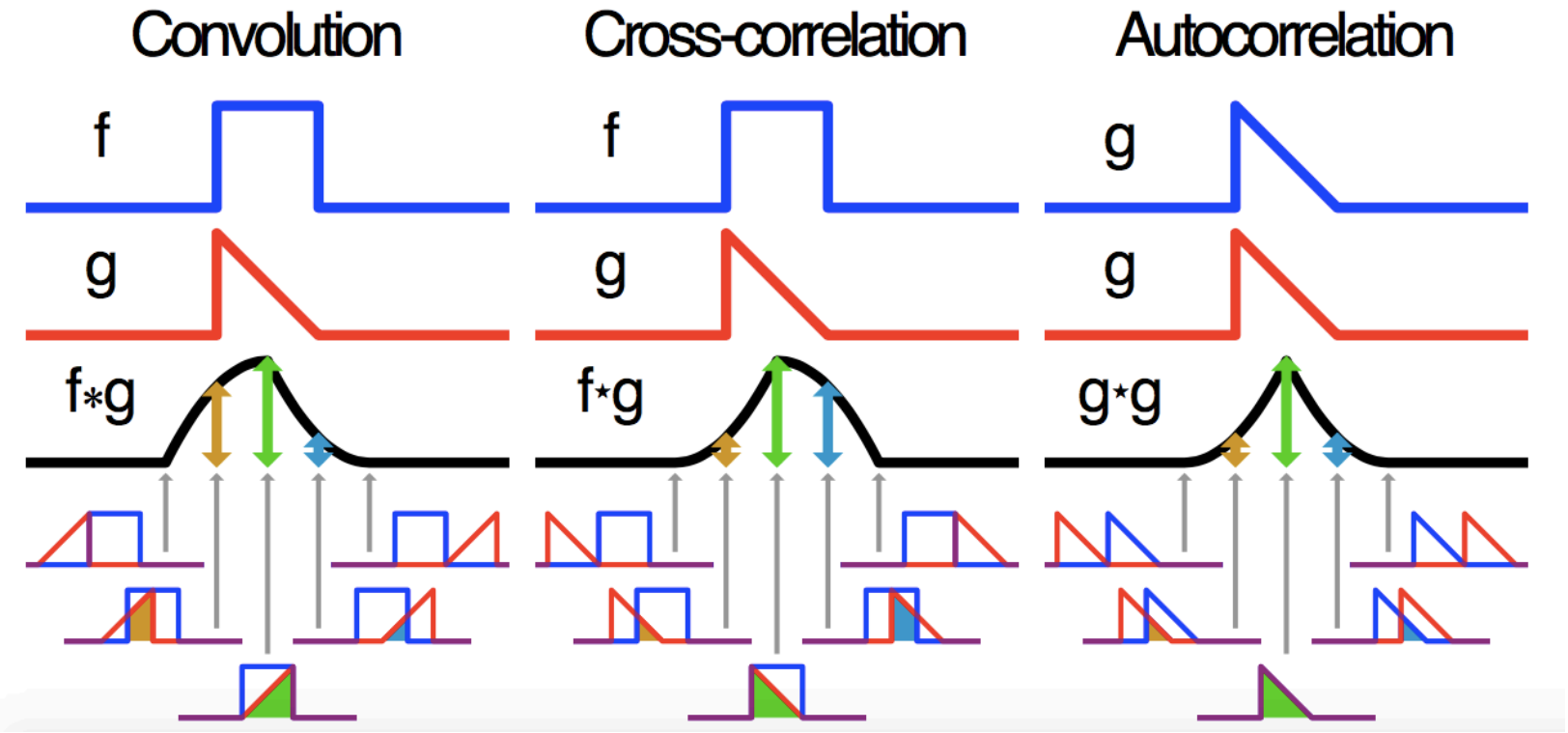


Image Convolution



Convolution: a mathematical operation on two functions (*f and g*) that produces a third function

Cross-Correlation: measure of similarity of two series as a function of the lag of one relative to the other

Auto-Correlation: correlation of a signal with itself at different points in time

Image Deconvolution

*Computing the inverse of the convolution operation is known as **deconvolution.***

Deconvolution: computationally intensive image processing technique that is used for improving the contrast and resolution of digital microscopy images

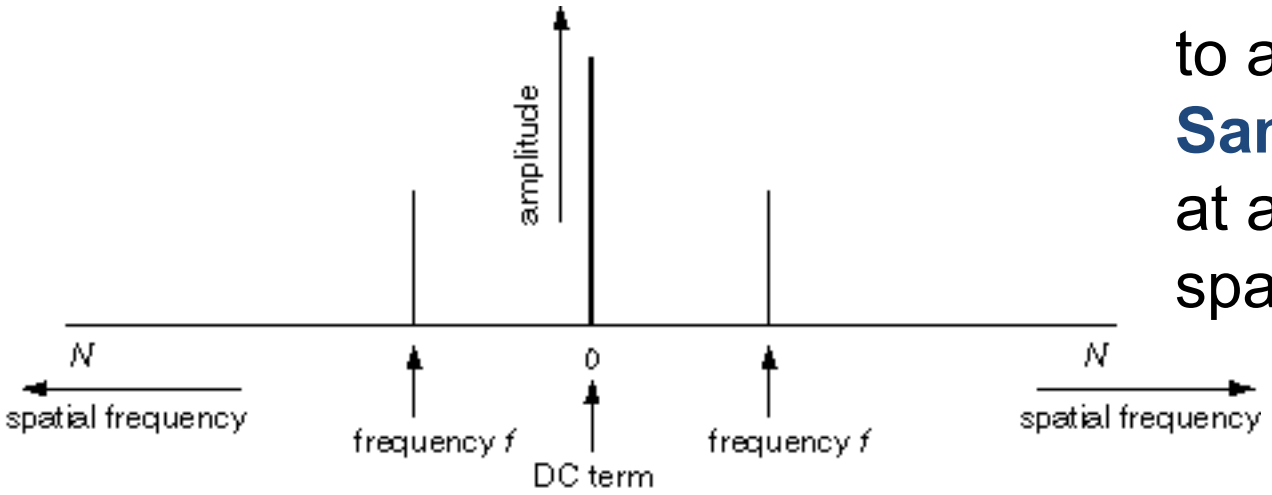
Object of deconvolution is to find the solution of a convolution equation of the form:

$$f \times g = h$$

h is a recorded signal
f is a signal we wish to recover, but has been convolved with some other signal ***g*** before we recorded it.

Deconvolution is usually performed by computing the Fourier Transform of the recorded signal ***h*** and transfer function ***g.***

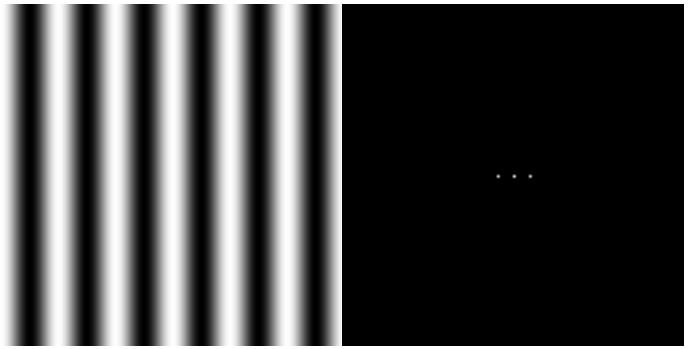
Fourier Transform



Sampling: reduction of a continuous signal to a discrete signal
Sample: set of values at a point in time or space

Fourier Theory: states any signal, in our case visual images, can be expressed as a sum of a series of sinusoids

Nyquist Frequency: highest spatial frequency that can be encoded in a digital image

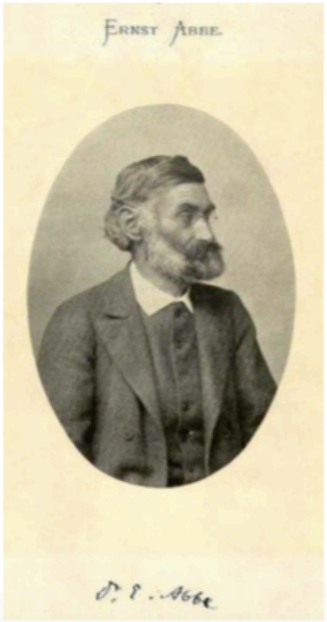


Left:
Brightness Image
Right:
Fourier Transform

Abbe's Image Formation Theory

Gratings represent the Fourier components (spatial frequency) of an object.

The diffraction patterns of all the Fourier components interfere at the image plane to form the image.



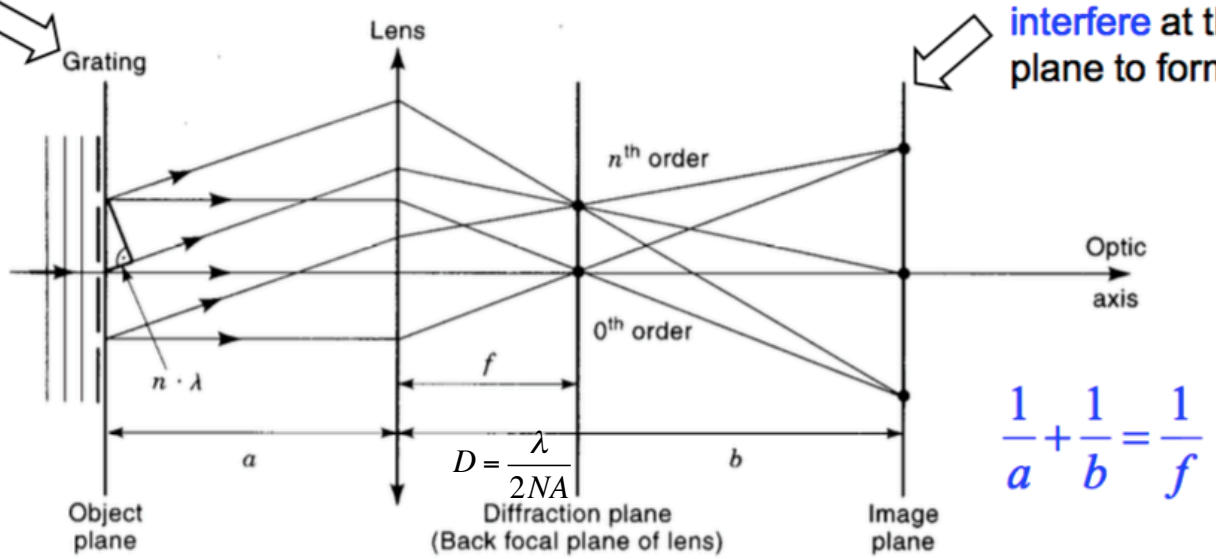


Figure 5-14

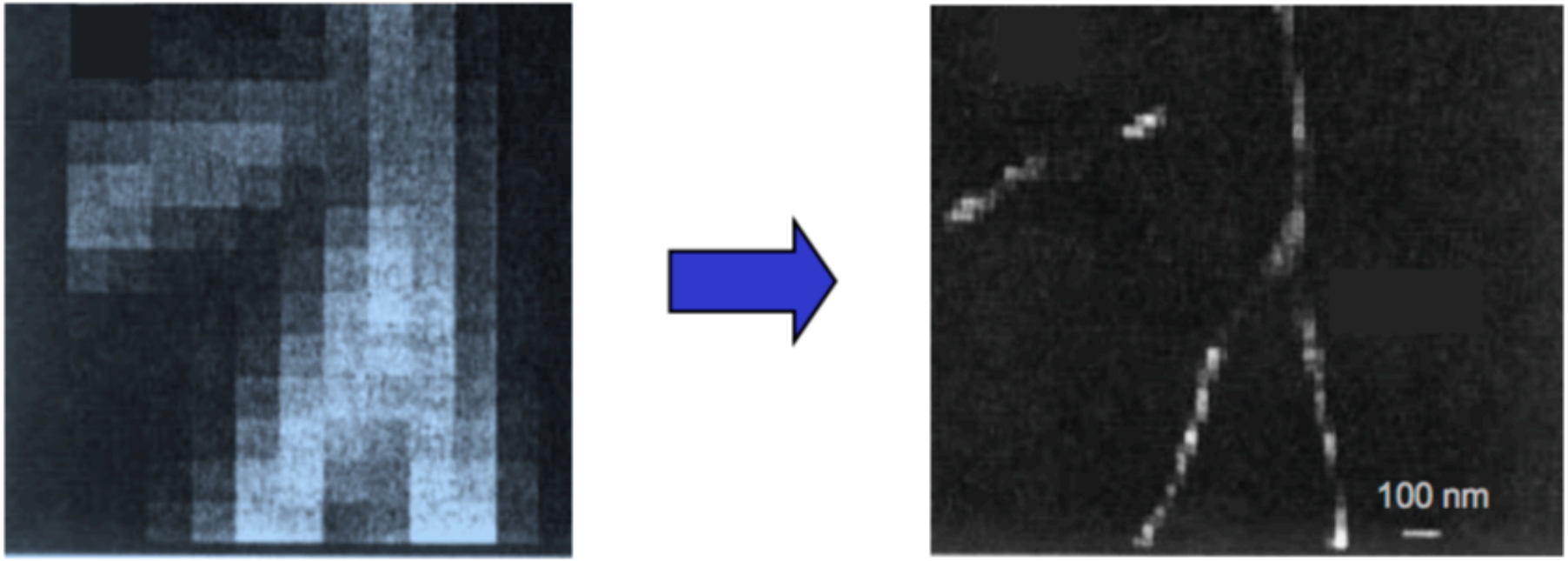
Abbe's theory for image formation in a light microscope. An objective lens focused on a grating ($2f > a > f$) in the object plane produces a magnified real image of the grating in the image plane. The diffraction plane is located at $1f$ in the back aperture of the lens. An incident planar wavefront is shown. Diffracted nth-order and nondiffracted 0th-order rays are separated in the diffraction plane, but are combined in the image plane.

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

D is diffraction limit
λ is wavelength of light
NA is numerical aperture

Super-Resolution On Fluorescent Images

To approach super resolution, we need **high contrast** (provided by fluorescent labeling).



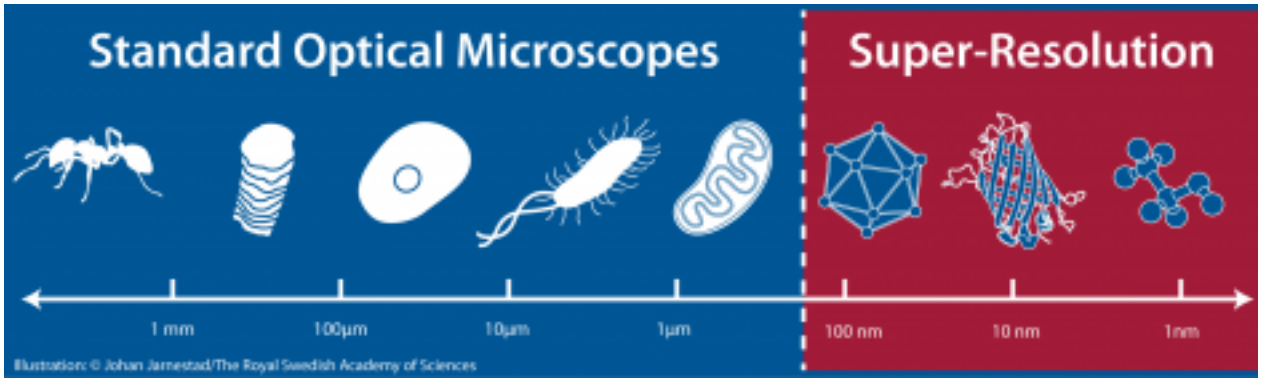
Sample: microtubules in a rat kidney cell

Deconvolution is used to achieve a superior resolution. In above image, 2000 iterations were used to achieve ~50 nm lateral resolution.

Super-Resolution Microscopy

Super-Resolution Microscopy: Super-resolution techniques allow images to be taken with a higher resolution than the diffraction limit.

1. **Deterministic Super-Resolution:**
   Fluorophores show a non-linear response to excitation, which can be exploited to enhance resolution (i.e. STED)
2. **Stochastic Super-Resolution:**
   Chemical complexity of many molecular light sources gives them a complex temporal behavior, which can be used to make many close-by fluorophores emit light at separate times and thereby become resolvable in time. (i.e. STORM)



The Nobel Prize in Chemistry 2014

The Royal Swedish Academy of Sciences has decided to award the Nobel Prize in Chemistry for 2014 to

Eric Betzig

Janelia Farm Research Campus,
Howard Hughes Medical Institute,
Ashburn, VA, USA.

Stefan W. Hell

Max Planck Institute for Biophysical Chemistry, Göttingen, and German Cancer Research Center, Heidelberg, Germany

William E. Moerner

Stanford University,
Stanford, CA, USA

"for the development of super-resolved fluorescence microscopy"

Stimulated Emission Depletion (STED) Microscopy

STED Microscopy: a technique that creates super-resolution images by the selective deactivation of flurophores, minimizing the area of illumination of the focal point, and thus enhancing the achievable resolution for a given system

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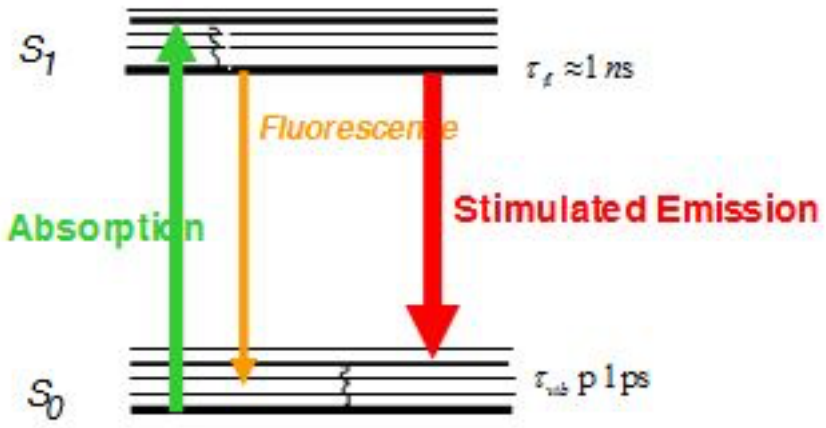
   Fluorophores show a non-linear response to excitation, which can be exploited to enhance resolution (i.e. STED)
2. **Stochastic Super-Resolution:**
   Chemical complexity of many molecular light sources gives them a complex temporal behavior, which can be used to make many close-by fluorophores emit light at separate times and thereby become resolvable in time. (i.e. STORM)

Stimulated Emission Depletion (STED) Microscopy



Jablonski Diagram showing redshift of stimulated photon. This redshift allows the stimulated photon to be ignored.

$$D = \frac{\lambda}{2n\sin\alpha\sqrt{1+\frac{I}{I_{sat}}}}$$

Fluorescence: excitation of an electron results in a loss of a energy

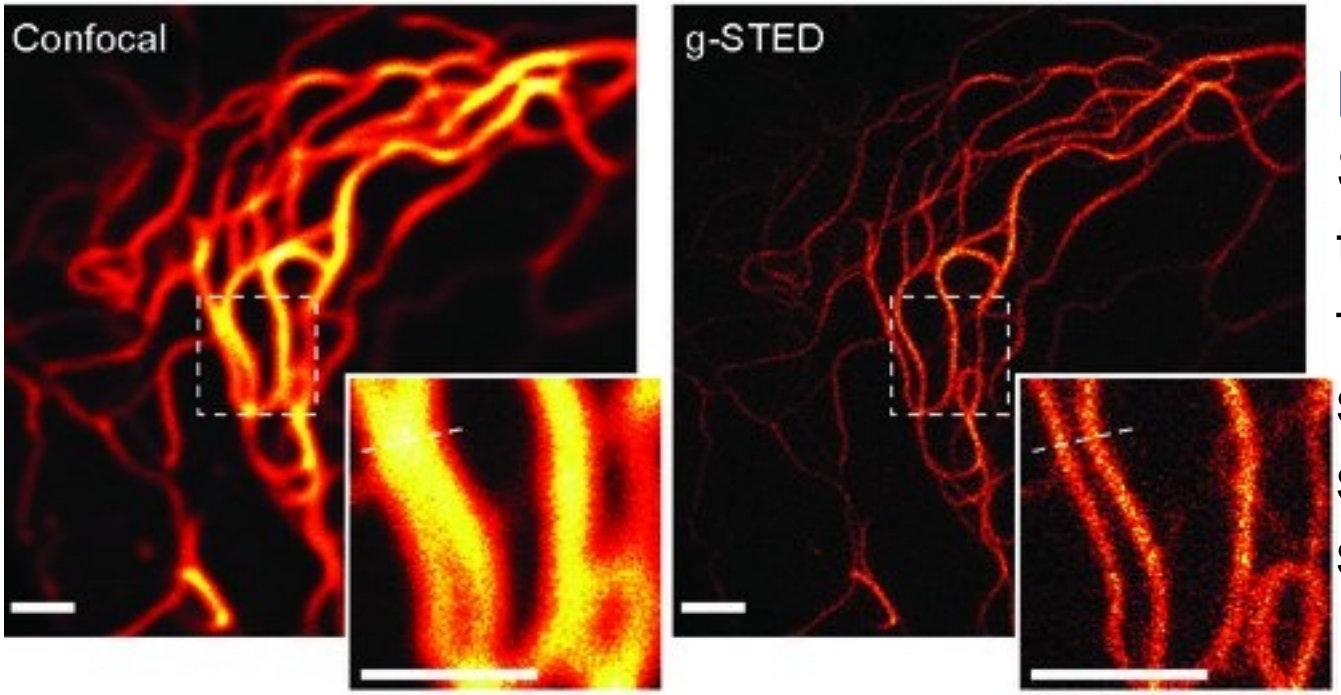
Stimulated Emission: STED interrupts this process before the photon is released. Excited electron is forced to relax in a higher vibration state than the fluorescence transition would enter, which lowers energy and causes photon to be red-shifted.

n is refractive index

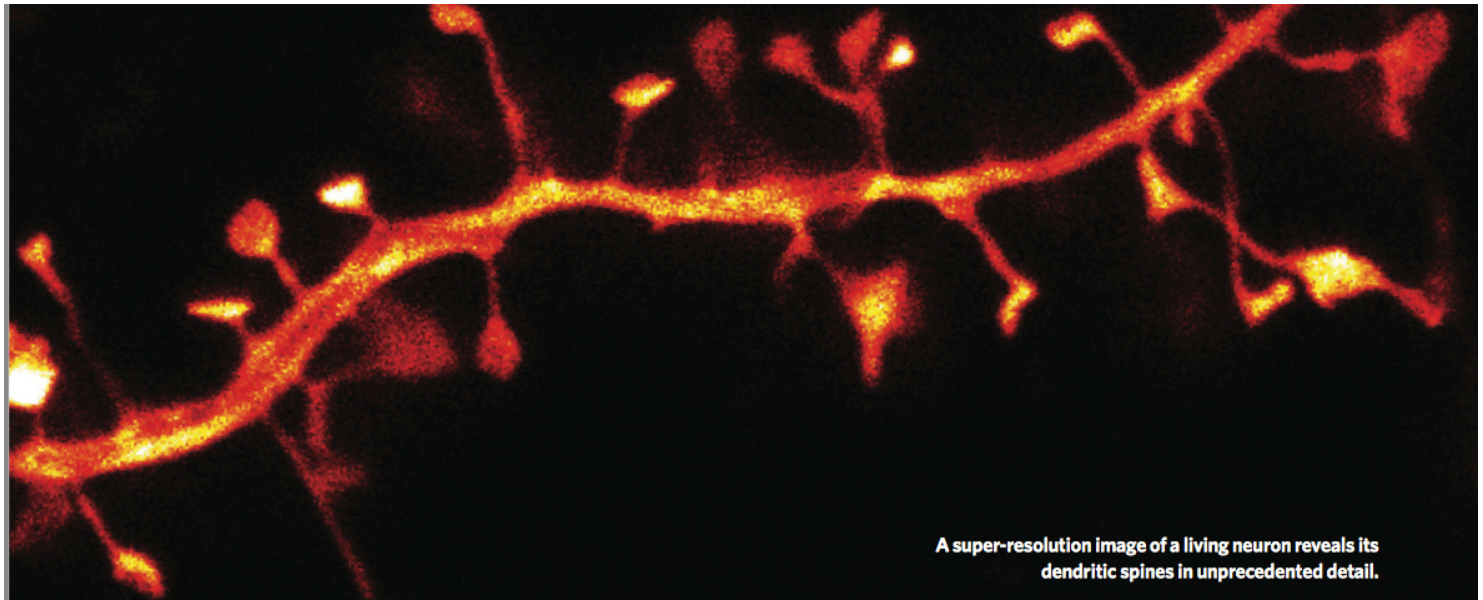
I is intracavity intensity (arrangement of mirrors for light waves)

$\mathbf{I_{sat}}$ is saturation intensity

Stimulated Emission Depletion (STED) Microscopy

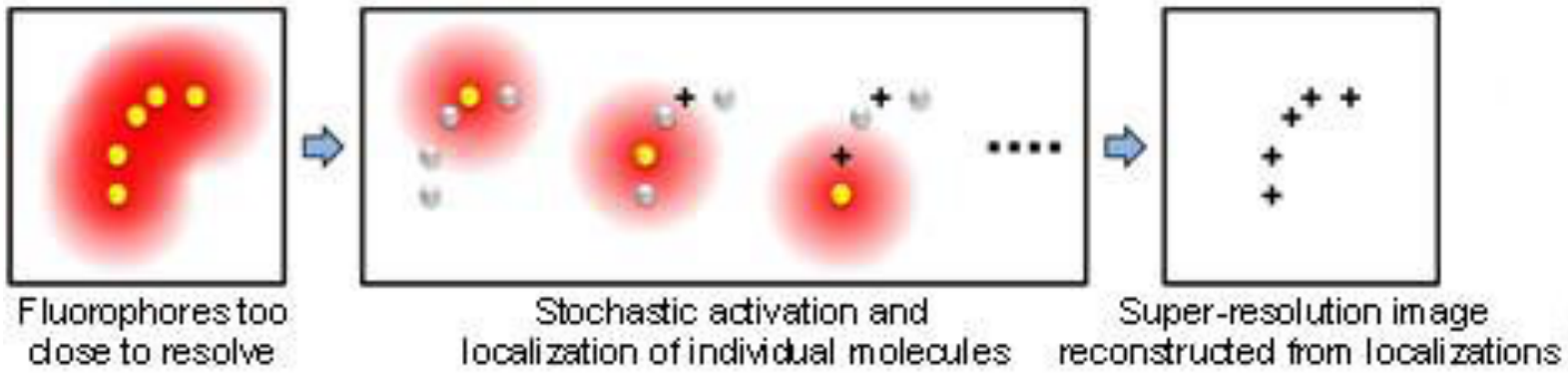


Dendritic Spine: Small protrusion that receives input from axon. It serves as a storage site for synaptic strength.

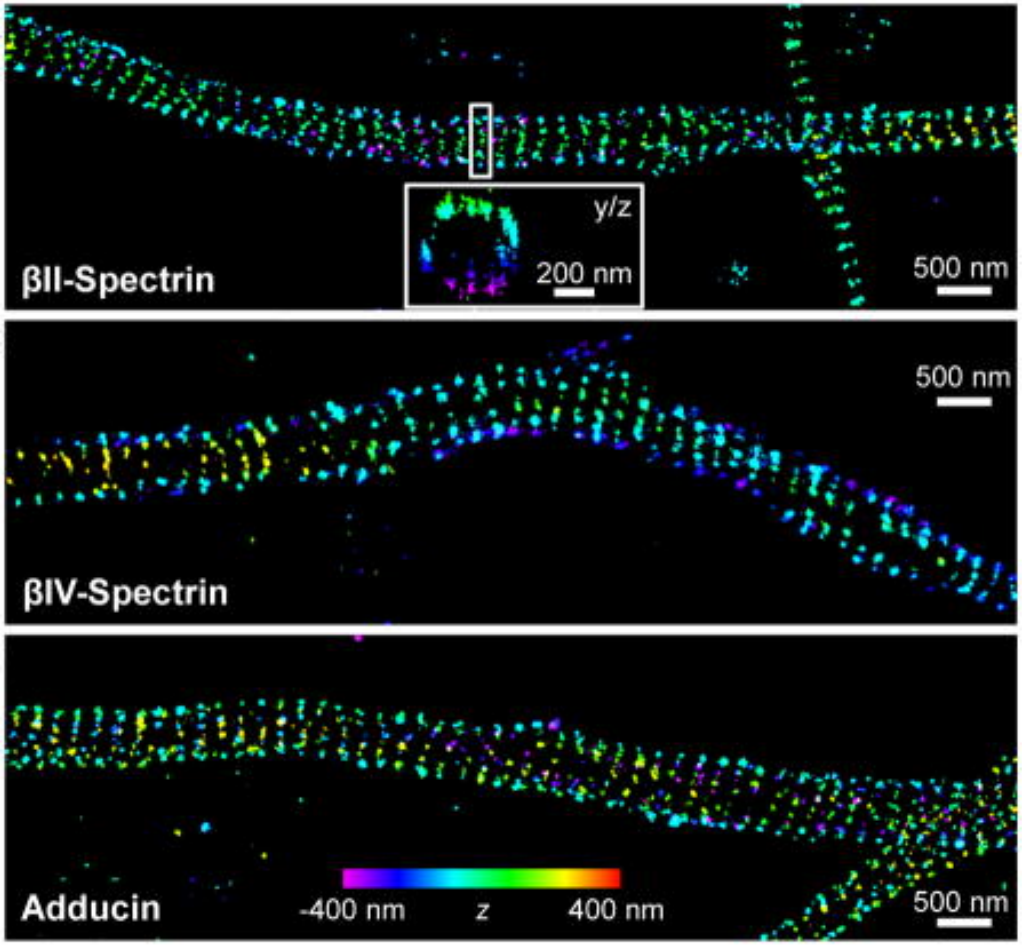


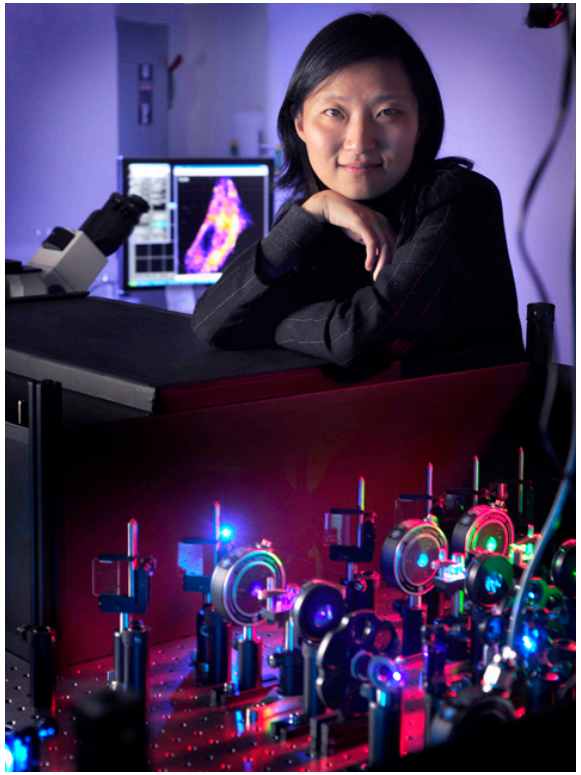
Stochastic Optical Resolution Microscopy (STORM)

STORM: a technique that uses a photo-switchable molecule to generate emission of photons in a precise location before it is deactivated by photo-bleaching. The sparsely activated fluorescent molecules must be separated by a distance that exceeds the Abbe diffraction limit (**~250 nm**).



Stochastic Optical Resolution Microscopy (STORM)





Xiaowei Zhuang

Periodicity in Cytoskeletal Structures of Axons

Spectrin: maintains the stability and structure of membrane

Adducin: located at spectrin-actin junction and binds calmodulin