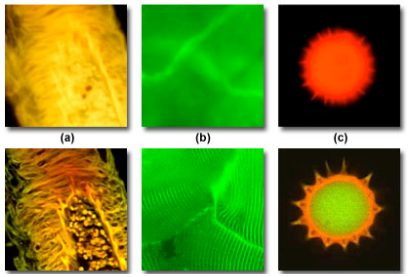
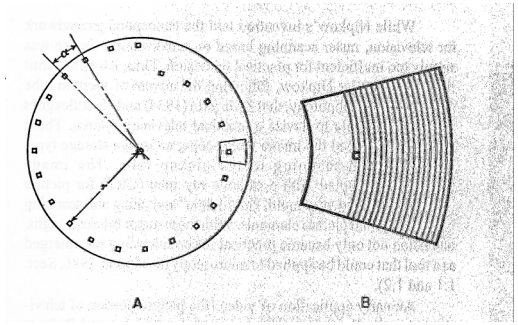
Widefield Fluorescence Microscopy



Confocal Fluorescence Microscopy

Nipkow Disk



Invented in 1884 by Paul Nipkow,a contemporary of Abbe.

Central element of an early incarnation of the television.

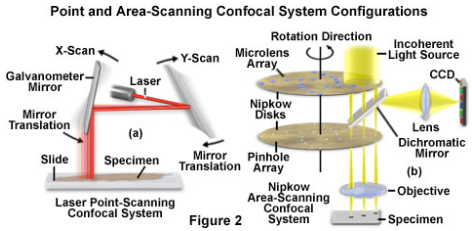
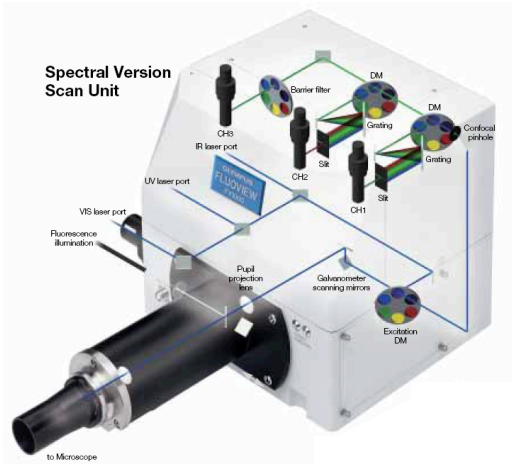
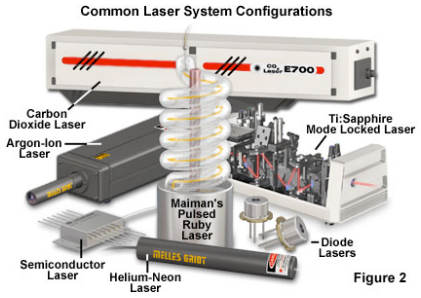


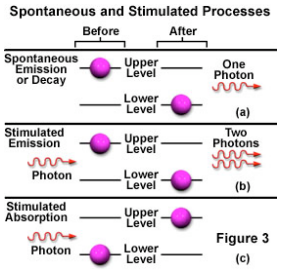
Figure 2

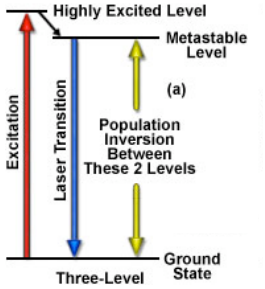




Light Amplification by Stimulated Emission of Radiation

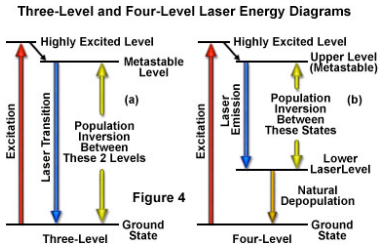
1917	Albert Einstein	stimulated emission possible
1950s	Charles Townes	built first "MASER"
	Gordon Gould	coined term LASER
1960	Theodore Maiman	built first LASER (ruby)





Stable LASER output requires establishment and maintenance of a "population inversion" of excitable electrons.

Must "pump" the medium with either electrical or photonic energy



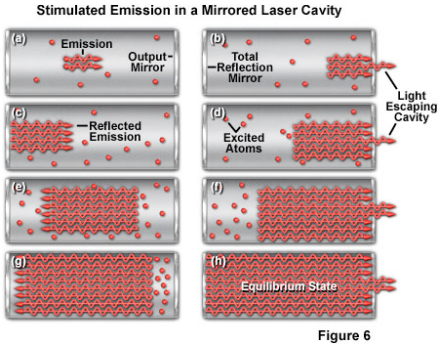
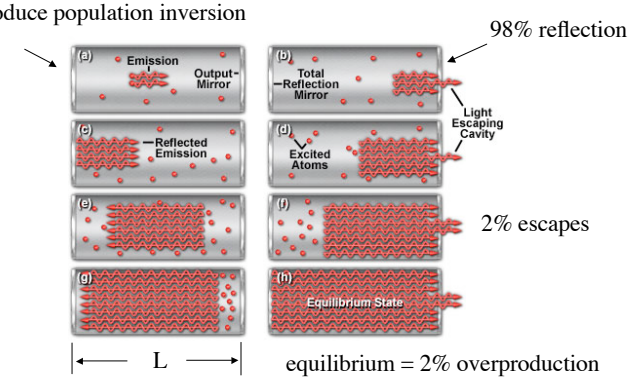


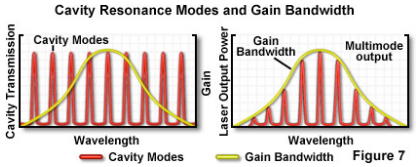
Figure 6

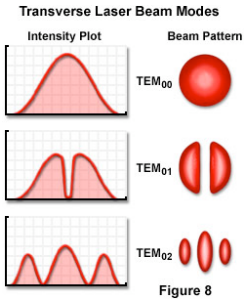
pumping = supply of energy to produce population inversion

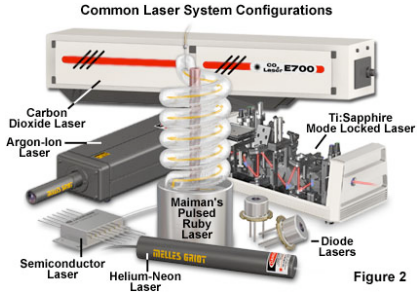


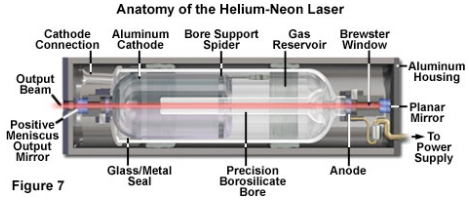
equilibrium = 2% overproduction

L / λ = integer









~ 10,000 - 40,000 hours

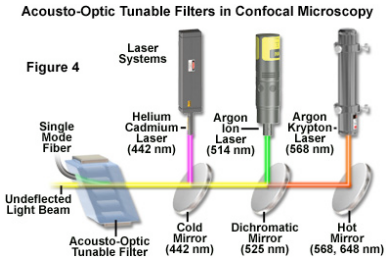
Laser Lines

Table 1.1. Principle emission lines of gas lasers useful for confocal laser scanning microscopy

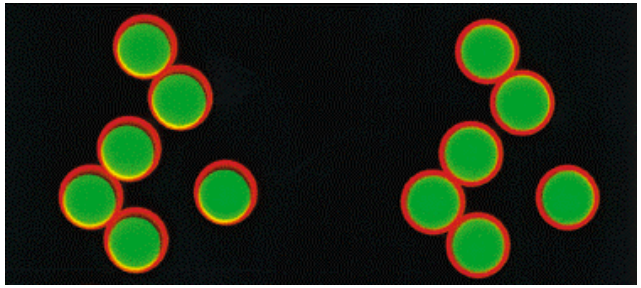
Laser	Wavelength (nm)			
	UV	Blue	Green	Red
Helium–cadmium	325	442		
Helium–cadmium (RGB)		442	534, 538	636
Low power argon ion		488	514	
Water-cooled argon ion	351, 364	457, 488	514, 528	
Argon–krypton mixed gas		488	568	647
Helium–neon (green)			543	
Helium–neon (red)				633

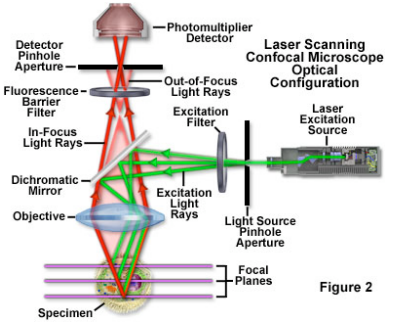
RGB, red, green and blue; UV, ultraviolet.

1152



Alignment





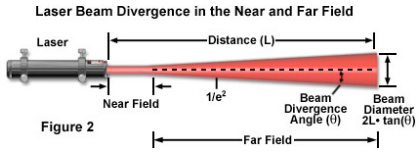


Figure 2

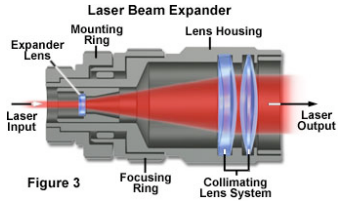


Figure 3

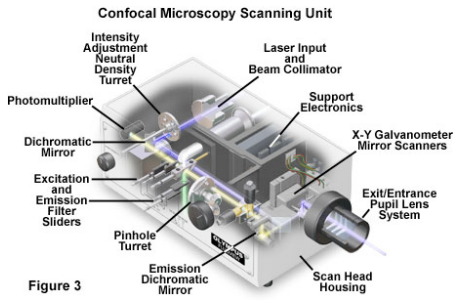


Figure 3

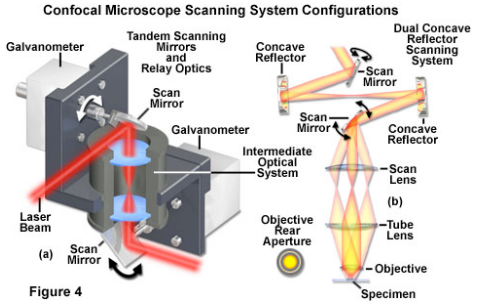
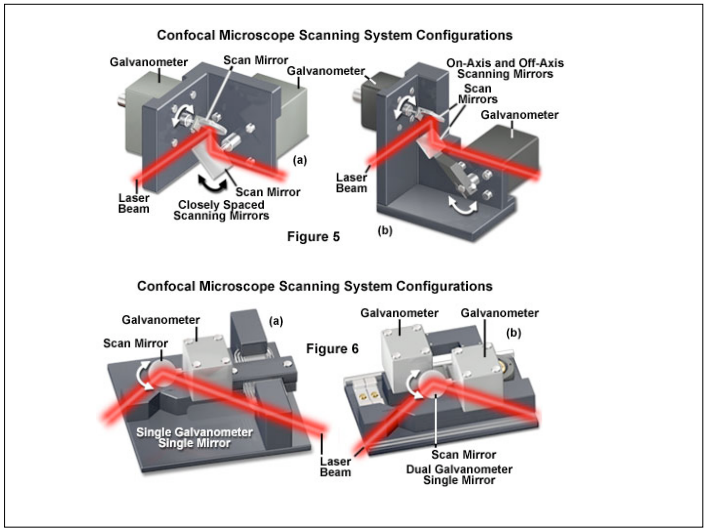
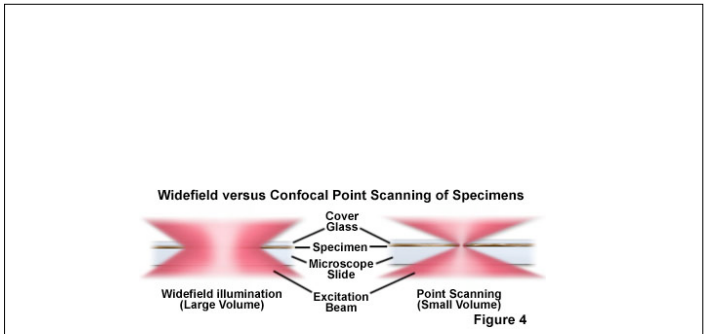
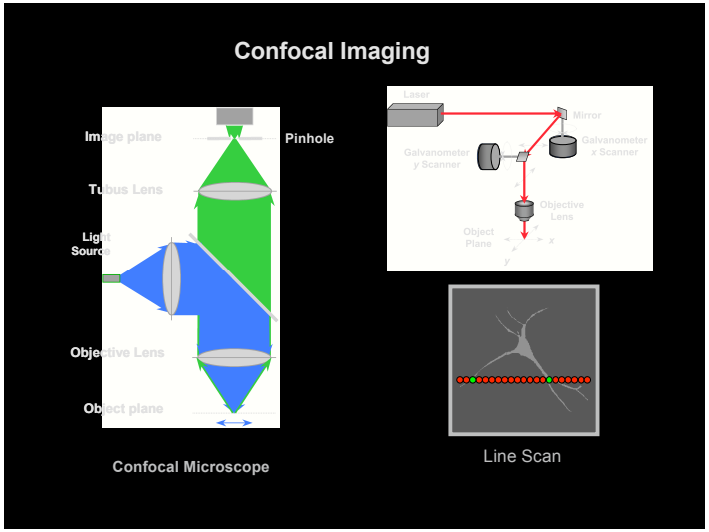
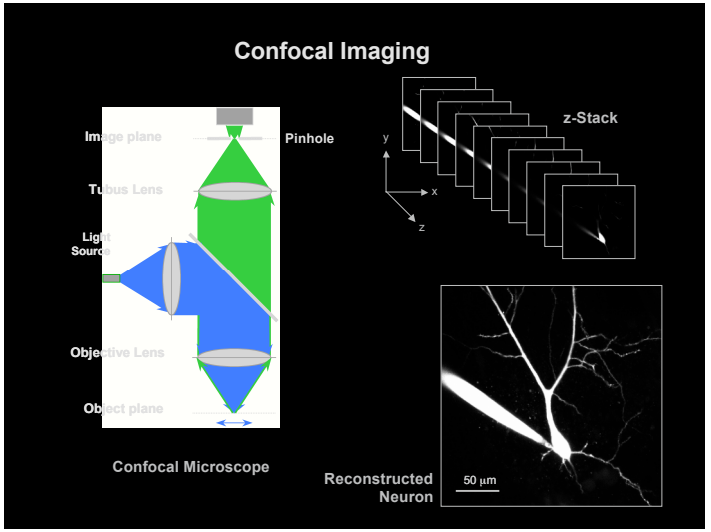


Figure 4


Confocal Microscope Scanning System Configurations
Galvanometer
Scan Mirror
Galvanometer
(a)
Laser Beam
Scan Mirror
Closely Spaced Scanning Mirrors
On-Axis and Off-Axis Scanning Mirrors
Scan Mirrors
Galvanometer
Laser Beam
(b)
Figure 5
Confocal Microscope Scanning System Configurations
Galvanometer
(a)
Scan Mirror
Single Galvanometer Single Mirror
Figure 6
Galvanometer
Galvanometer
(b)
Laser Beam
Scan Mirror
Dual Galvanometer Single Mirror


Widefield versus Confocal Point Scanning of Specimens
Cover Glass
Specimen
Microscope Slide
Widefield illumination (Large Volume)
Excitation Beam
Point Scanning (Small Volume)
Figure 4


Confocal Imaging
Pinhole
Tubus Lens
Objective Lens
Object plane
Laser
Mirror
Galvanometer y Scanner
Galvanometer x Scanner
Objective Lens
Object Plane
Confocal Microscope
Line Scan


Confocal Imaging
Pinhole
Tubus Lens
Objective Lens
Object plane
z-Stack
Confocal Microscope
Reconstructed Neuron
50 µm

Pollen Grain Serial Optical Sections by Confocal Microscopy

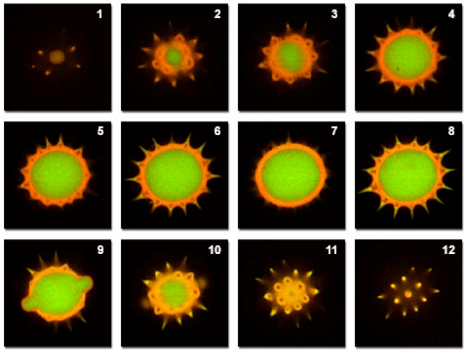
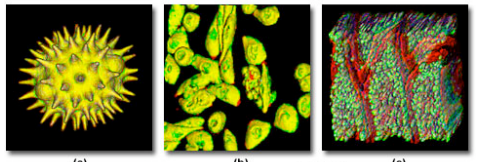


Figure 6

Three-Dimensional Volume Renders from Confocal Optical Sections



(a) (b) (c)

Figure 7

Side-On Photomultiplier Anatomy

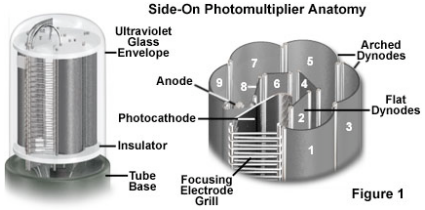


Figure 1

Common Photomultiplier Dynode Chain Configurations

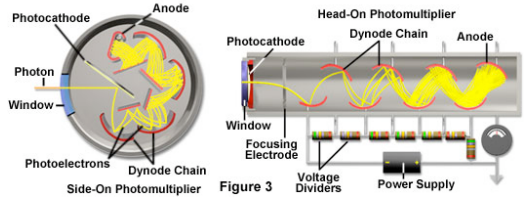
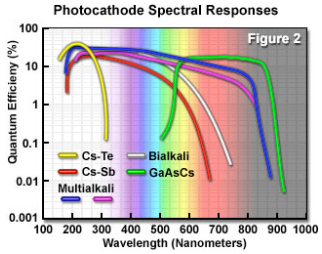
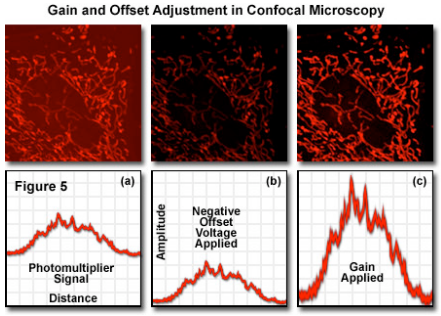


Figure 3





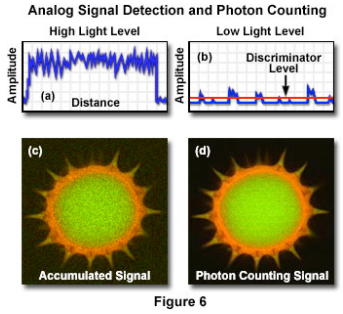
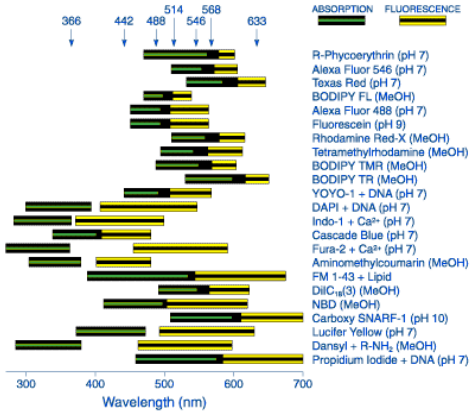


Figure 6

What to look for in a fluorophore

- Fluorescence Spectrum
- Quantum Yield
- Extinction Coefficient
- Stability (Photobleaching)
- Sensitivity to Environment
- Toxicity
- Reactivity
- Solubility

Fluorophores



Common Fluorophores in Widefield and Confocal Microscopy

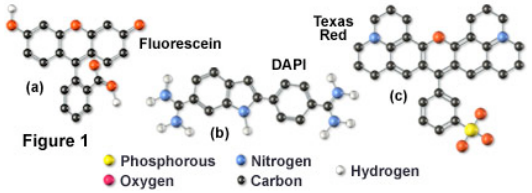


Figure 1

Traditional Nuclear and General Purpose Fluorophores

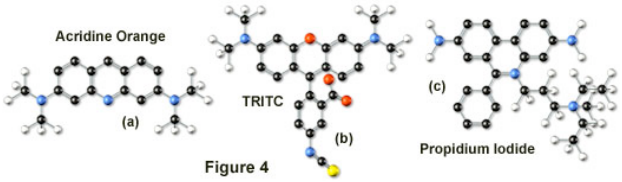


Figure 4

Alexa Fluor Synthetic Fluorochromes

Figure 5

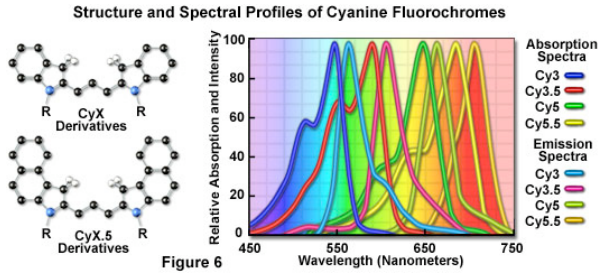


Figure 6

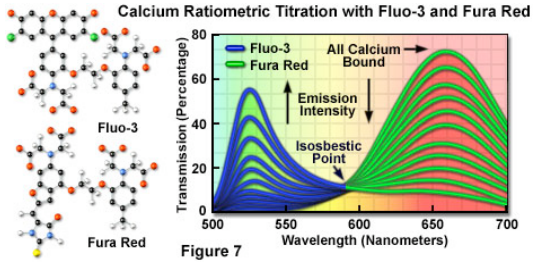


Figure 7

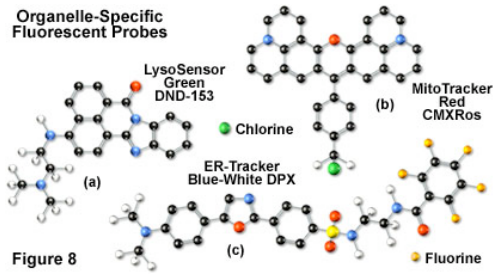


Figure 8

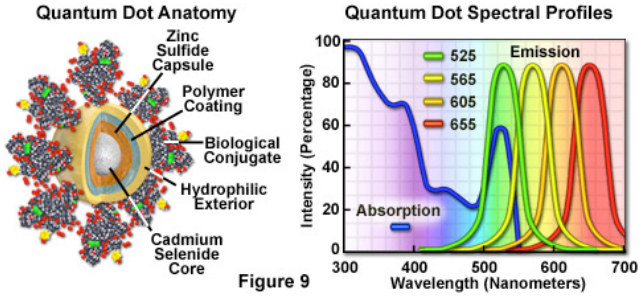
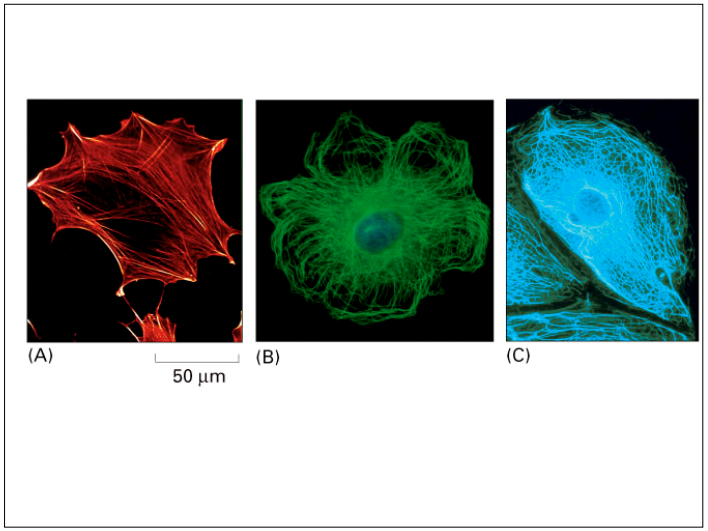
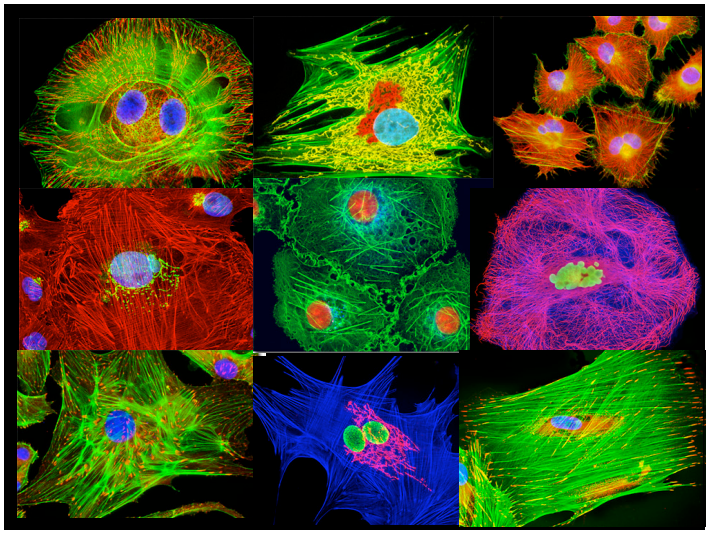
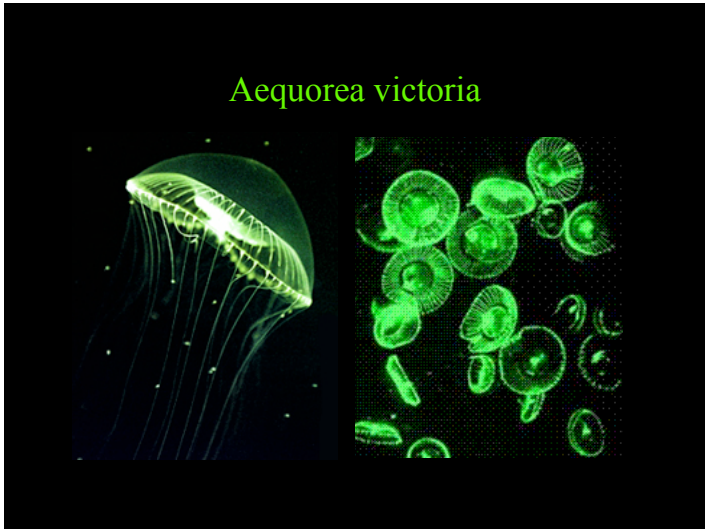


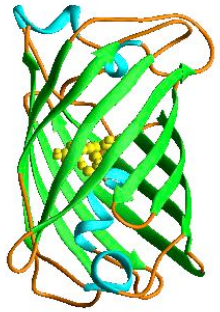
Figure 9

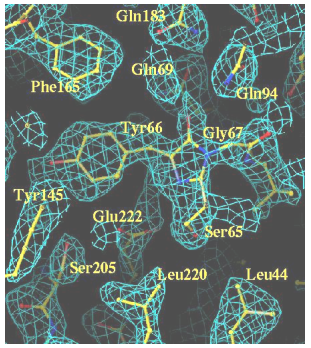




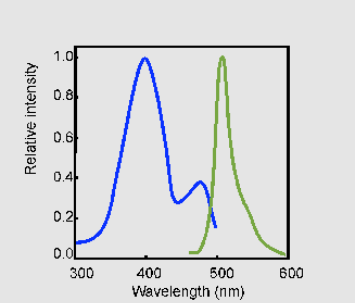


Green Fluorescent Protein
Aequorea Victoria

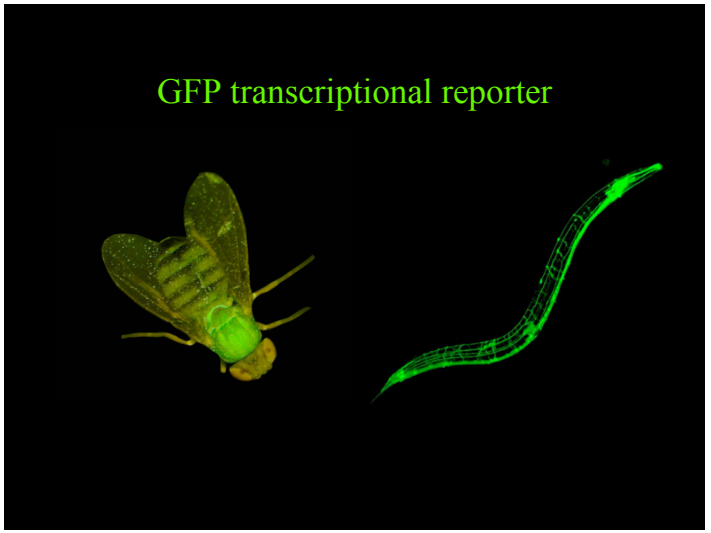


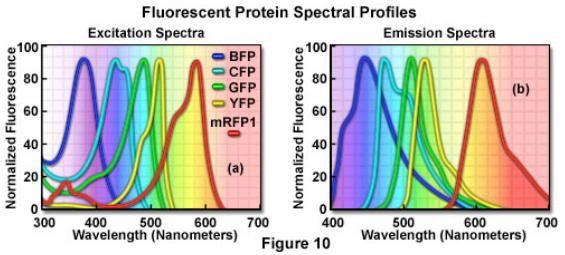


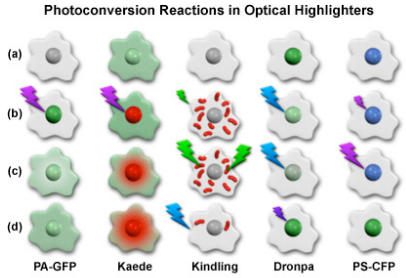
Green Fluorescent Protein

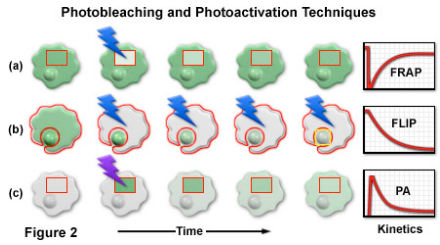


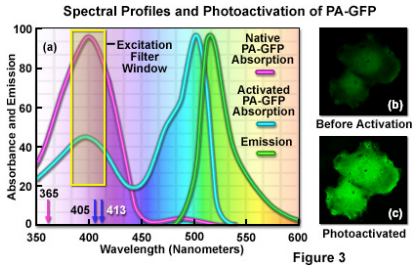
The excitation spectrum of native GFP from *A. victoria* (blue) has two excitation maxima at 395 nm and at 470 nm. The fluorescence emission spectrum (green) has a peak at 509 nm and a shoulder at 540 nm.

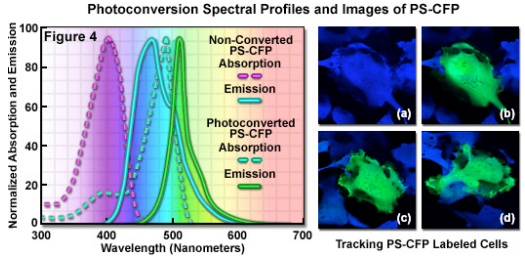




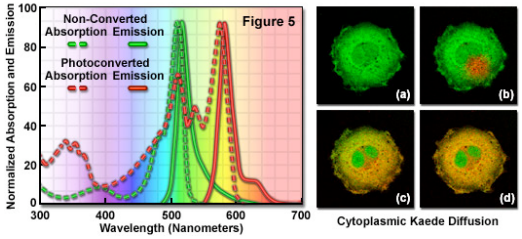

Photoconversion Reactions in Optical Highlighters
(a)
(b)
(c)
(d)
PA-GFP
Kaede
Kindling
Dronpa
PS-CFP
Figure 1


Photobleaching and Photoactivation Techniques
(a)
(b)
(c)
FRAP
FLIP
PA
Figure 2
Time
Kinetics


Spectral Profiles and Photoactivation of PA-GFP
(a)
Excitation Filter Window
Native PA-GFP Absorption
Activated PA-GFP Absorption
Emission
365
405
413
Absorbance and Emission
Wavelength (Nanometers)
(b)
Before Activation
(c)
Photoactivated
Figure 3


Photoconversion Spectral Profiles and Images of PS-CFP
Figure 4
Non-Converted PS-CFP Absorption
Emission
Photoconverted PS-CFP Absorption
Emission
Normalized Absorption and Emission
Wavelength (Nanometers)
(a)
(b)
(c)
(d)
Tracking PS-CFP Labeled Cells

Photoconversion Spectral Profiles and Images of Kaede Fluorescent Protein



Kaede and Eos Chromophore Photoconversion Mechanism

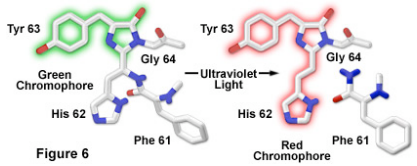


Figure 6

Tracking Organelle Movements with Kindling Fluorescent Protein

Figure 7

DsRed and Kindling Fluorescent Protein Chromophores

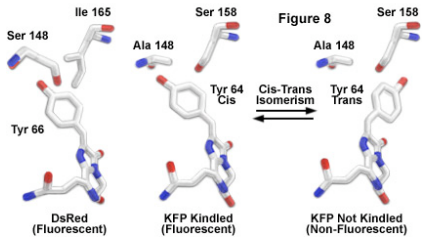


Figure 8

Reversible Photoswitching of Dronpa Fluorescent Protein

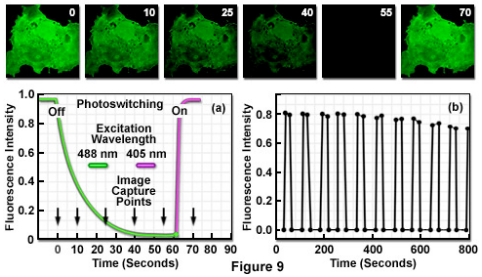


Figure 9

Brightness Levels and Images of Optical Highlighter Proteins

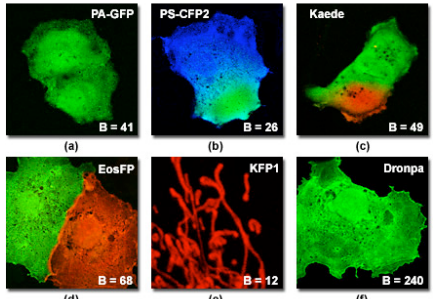


Figure 10

Combined Use of Phase Contrast and Fluorescence Illumination

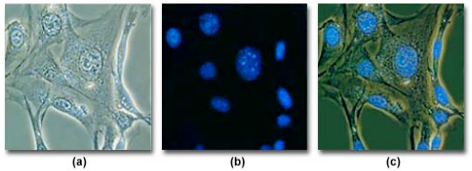


Figure 3

Combined Use of DIC and Fluorescence Illumination

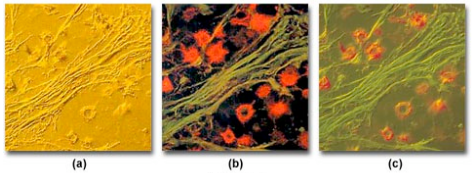


Figure 4

Inhibition of Photobleaching and Specimen Fading

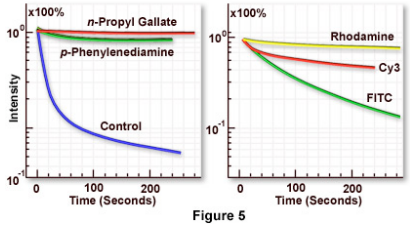
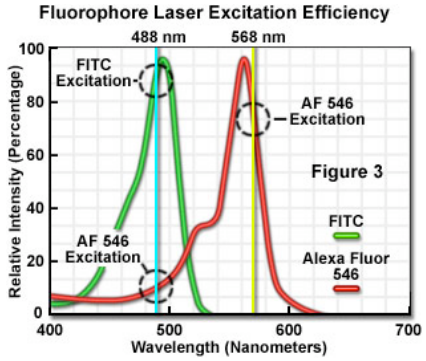
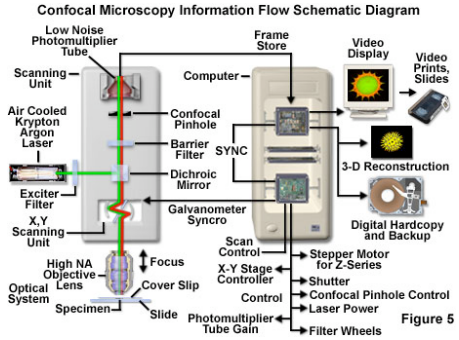
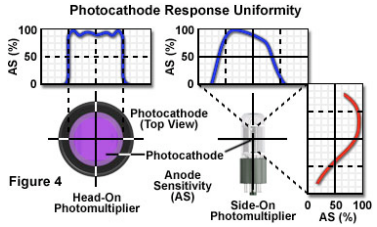


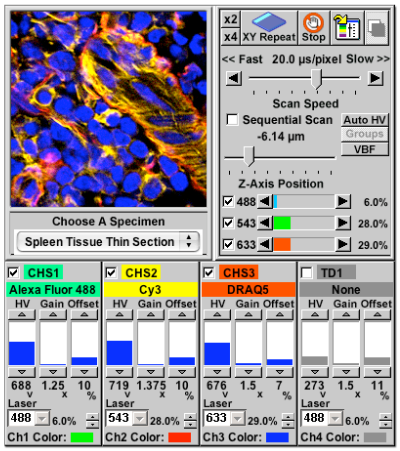
Figure 5

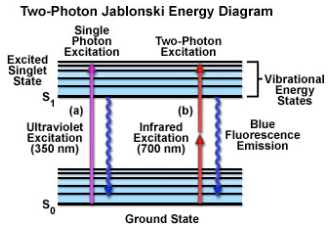






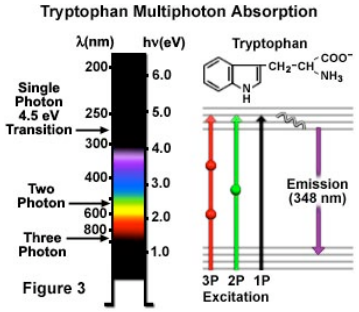
http://www.olympusconfocal.com/java/confocalsimulator/index.html

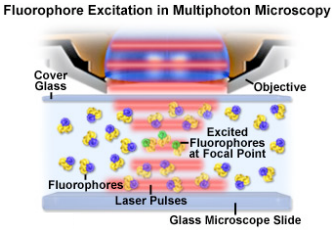


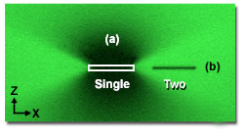


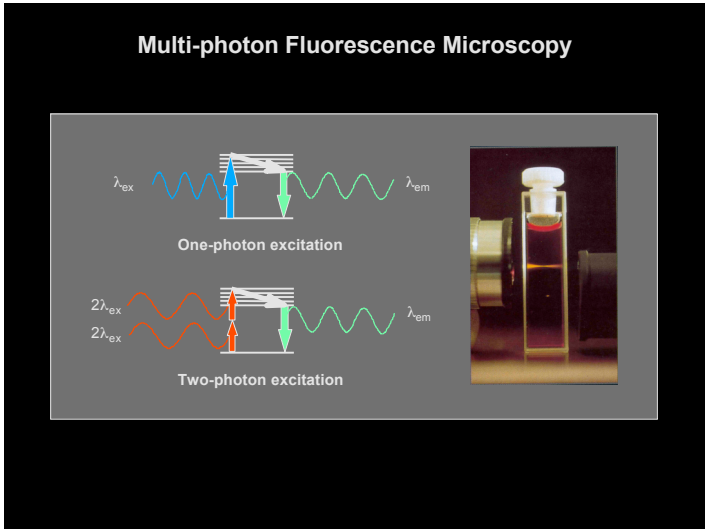
2-photon excitation (2PE)

- Both photons must arrive within < 10^{-18} sec
- Fluorescence emission varies with the square of excitation intensity
- Requires photon density ~ 10^6 x greater than for single photon exc
- 3 photon excitation also possible (~ 10 x greater than for 2PE)





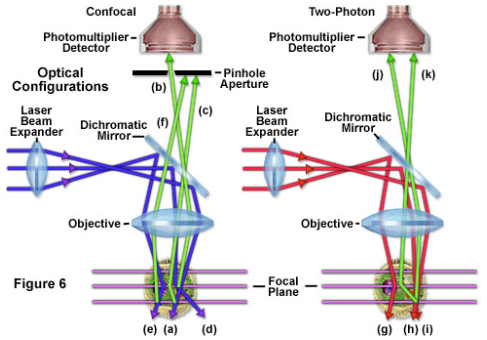




•Absence of out-of-focus absorption allows more of the excitation light photons to reach the desired specimen level.

•The red and infrared light employed in two-photon excitation undergoes less scattering than light that is bluer in color (shorter wavelengths).

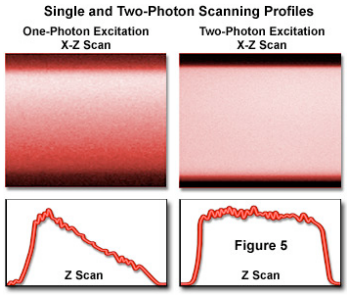
•The effects of light scattering are less detrimental to two-photon microscopy than to confocal microscopy



•Absence of out-of-focus absorption allows more of the excitation light photons to reach the desired specimen level.

•The red and infrared light employed in two-photon excitation undergoes less scattering than light that is bluer in color (shorter wavelengths).

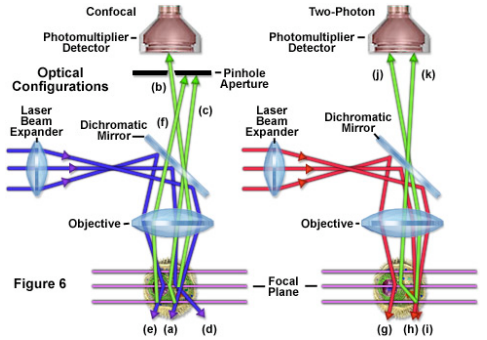
•The effects of light scattering are less detrimental to two-photon microscopy than to confocal microscopy

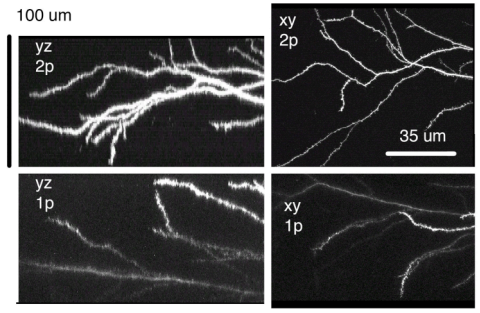


•Absence of out-of-focus absorption allows more of the excitation light photons to reach the desired specimen level.

•The red and infrared light employed in two-photon excitation undergoes less scattering than light that is bluer in color (shorter wavelengths).

•The effects of light scattering are less detrimental to two-photon microscopy than to confocal microscopy





Titanium-Sapphire Laser

Mode-Locked, Pulsed Laser

Tunable between 700 – 1200 nm

100 fsec pulses @ 100 MHz

Support frequency doubling to 350 – 600 nm

Costs ~ $160,000