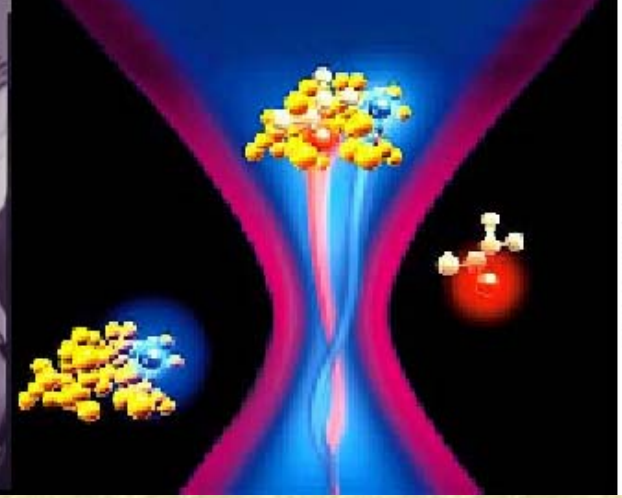
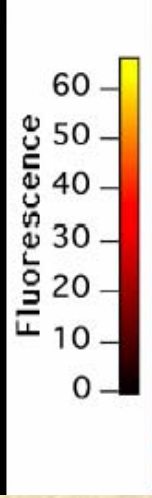
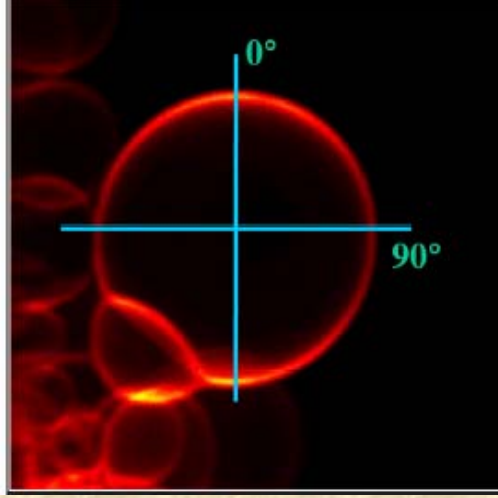






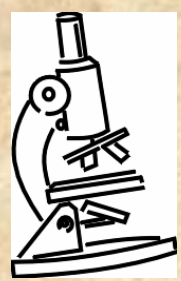
Fluorescence Workshop
UMN Physics
June 8-10, 2006

Fluorescence Microscopy and Fluorescence Correlation Spectroscopy

Joachim Mueller

Fluorescence Microscopy

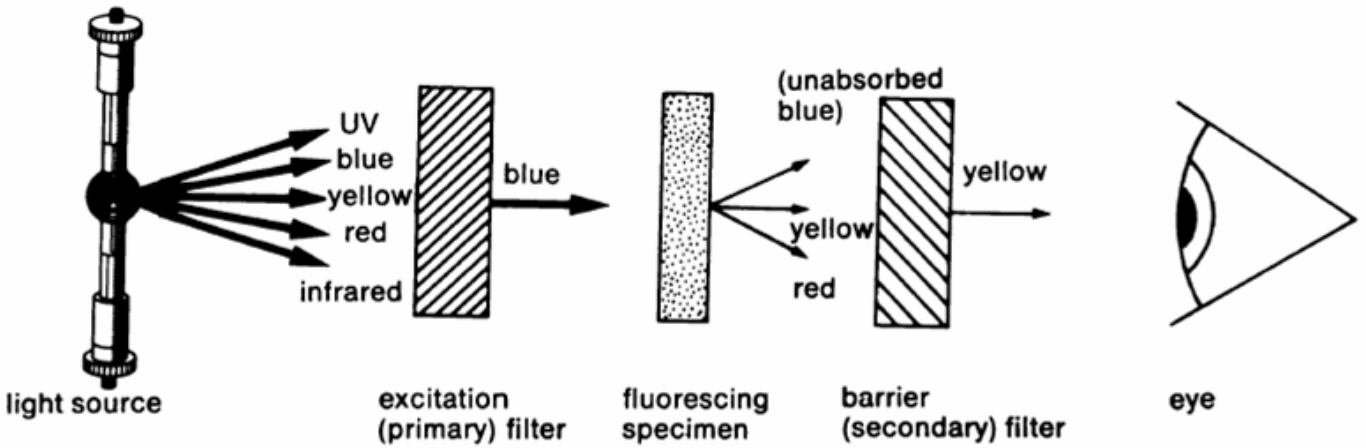
Use a microscope as a fluorometer



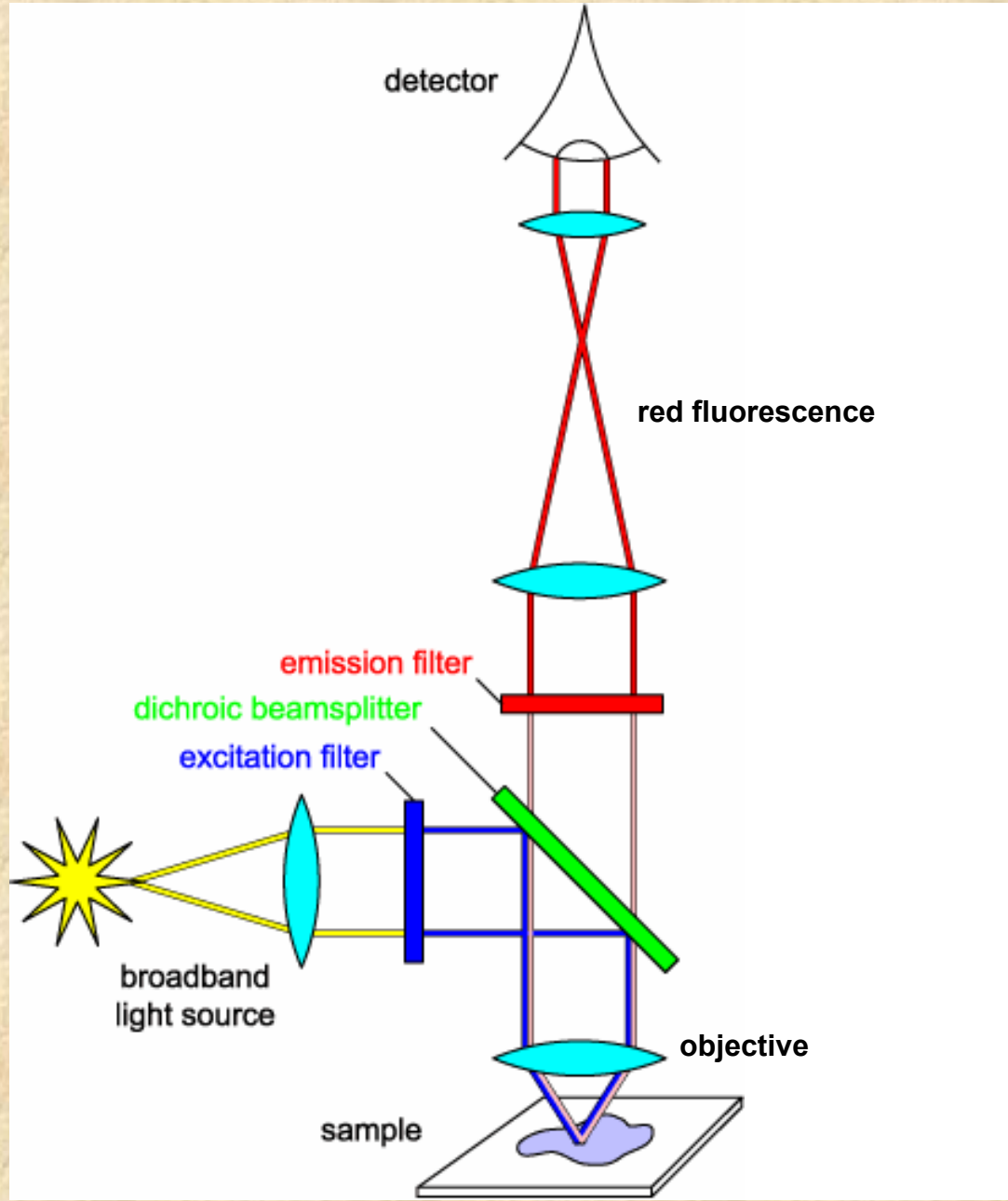
Advantages:

- superb optics
- very high collection efficiency
- imaging
- allows single cell measurements
- single molecule experiments

The microscope as a filter fluorometer with focusing optics



Basic design of a fluorescent microscope



Filters

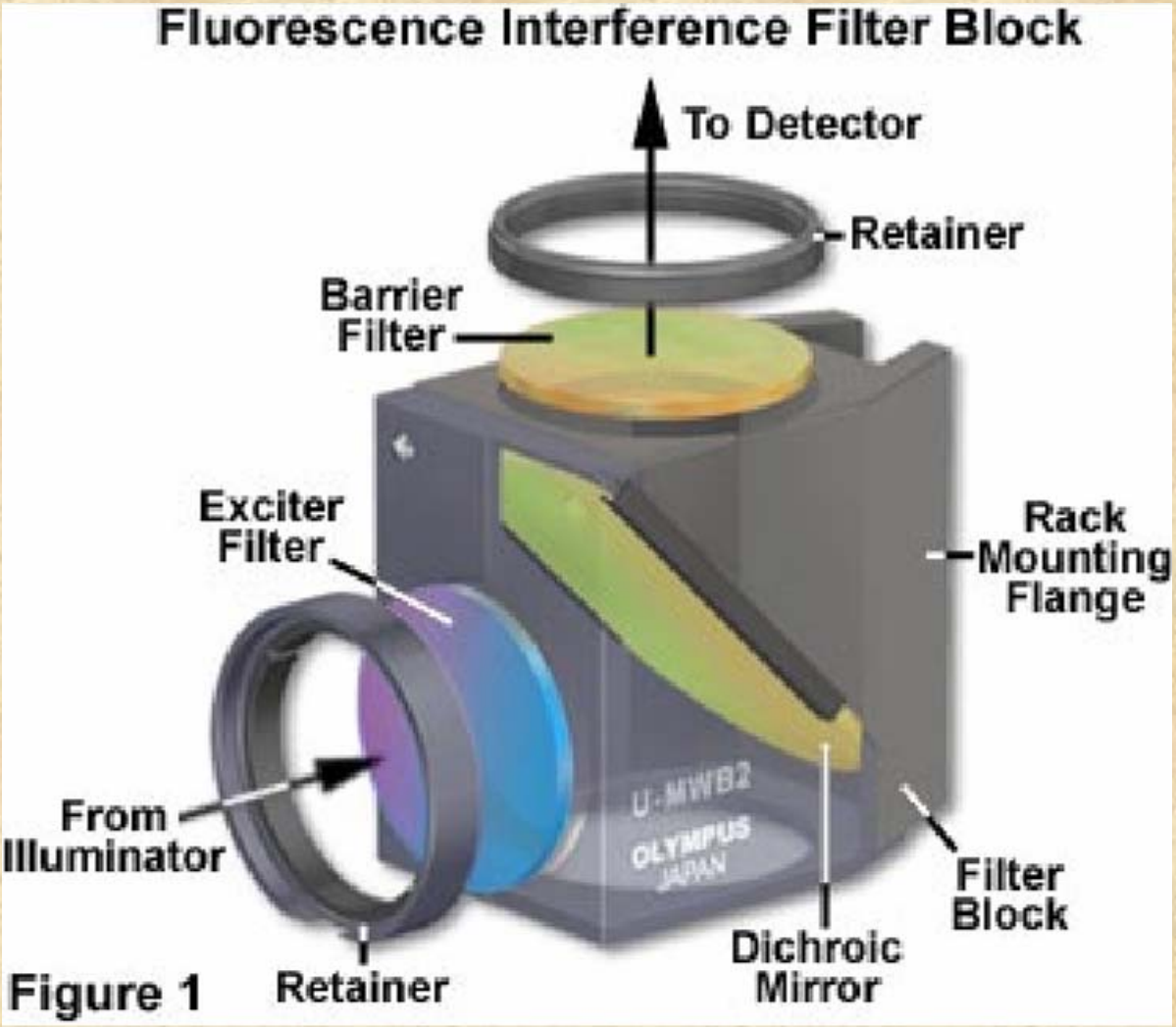
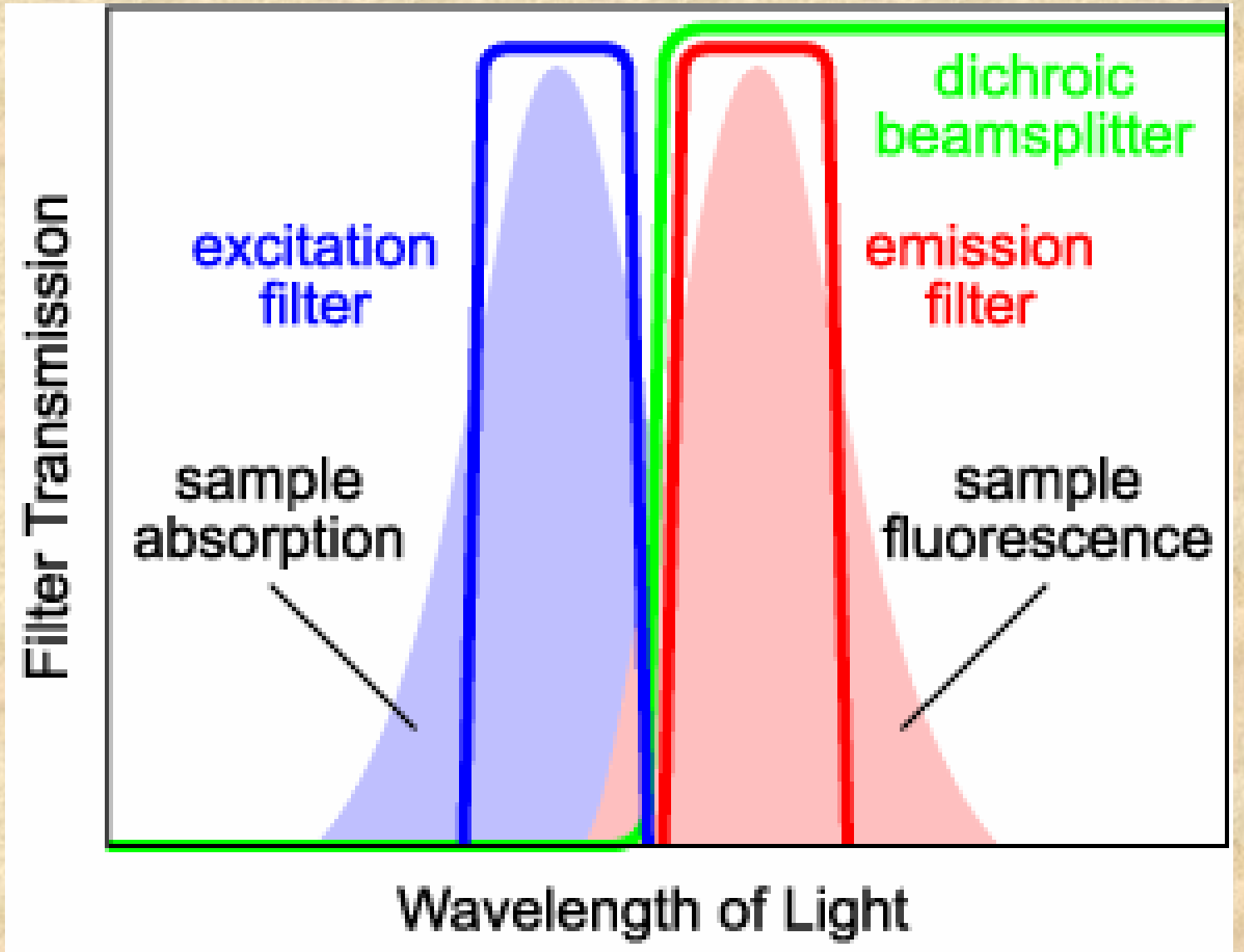
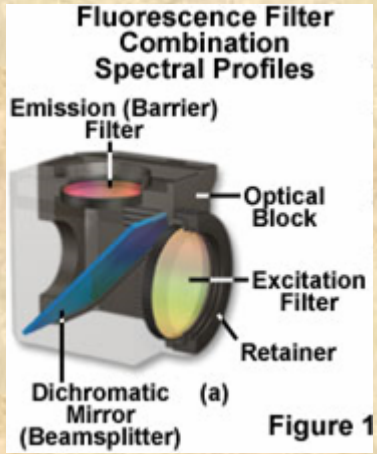
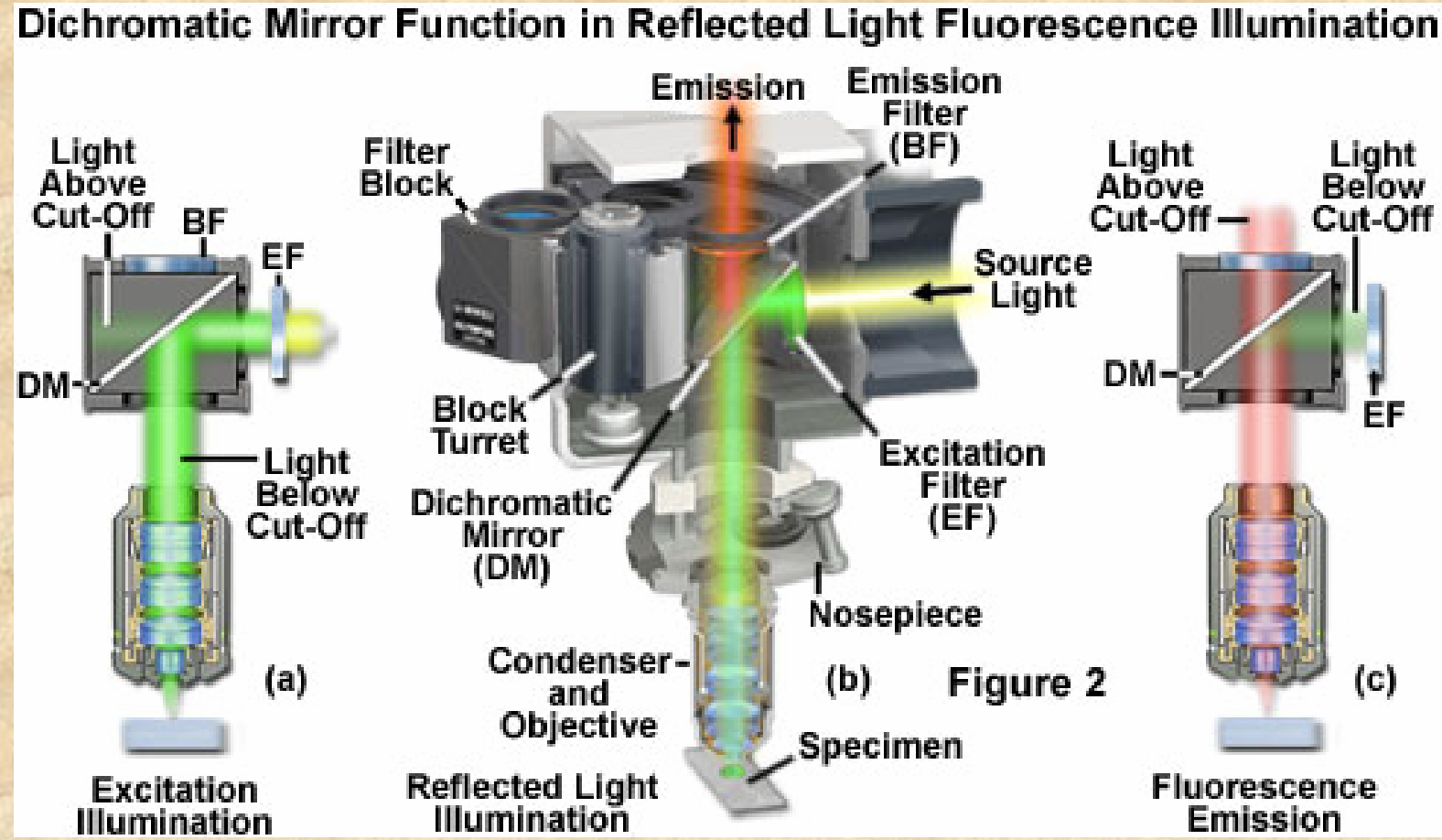


Figure 1

Selecting Filters






Dichromatic Mirror Function in Reflected Light Fluorescence Illumination
Light Above Cut-Off
BF
EF
DM
Light Below Cut-Off
(a)
Excitation Illumination
Emission
Emission Filter (BF)
Filter Block
Source Light
Block Turret
Dichromatic Mirror (DM)
Excitation Filter (EF)
Nosepiece
Condenser and Objective
Specimen
(b)
Reflected Light Illumination
Figure 2
Light Above Cut-Off
Light Below Cut-Off
DM
EF
(c)
Fluorescence Emission

Anatomy of a Fluorescence Microscope

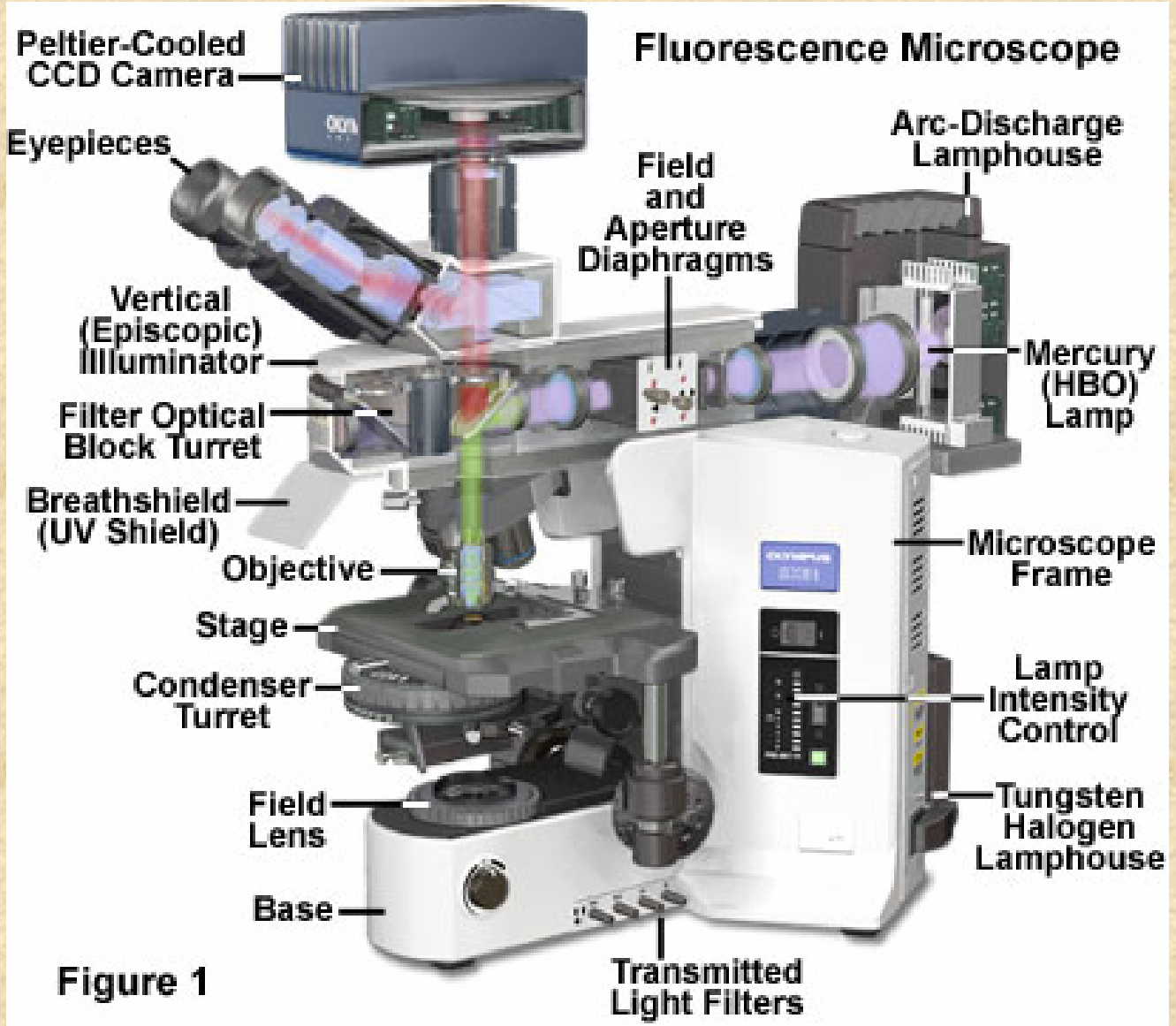
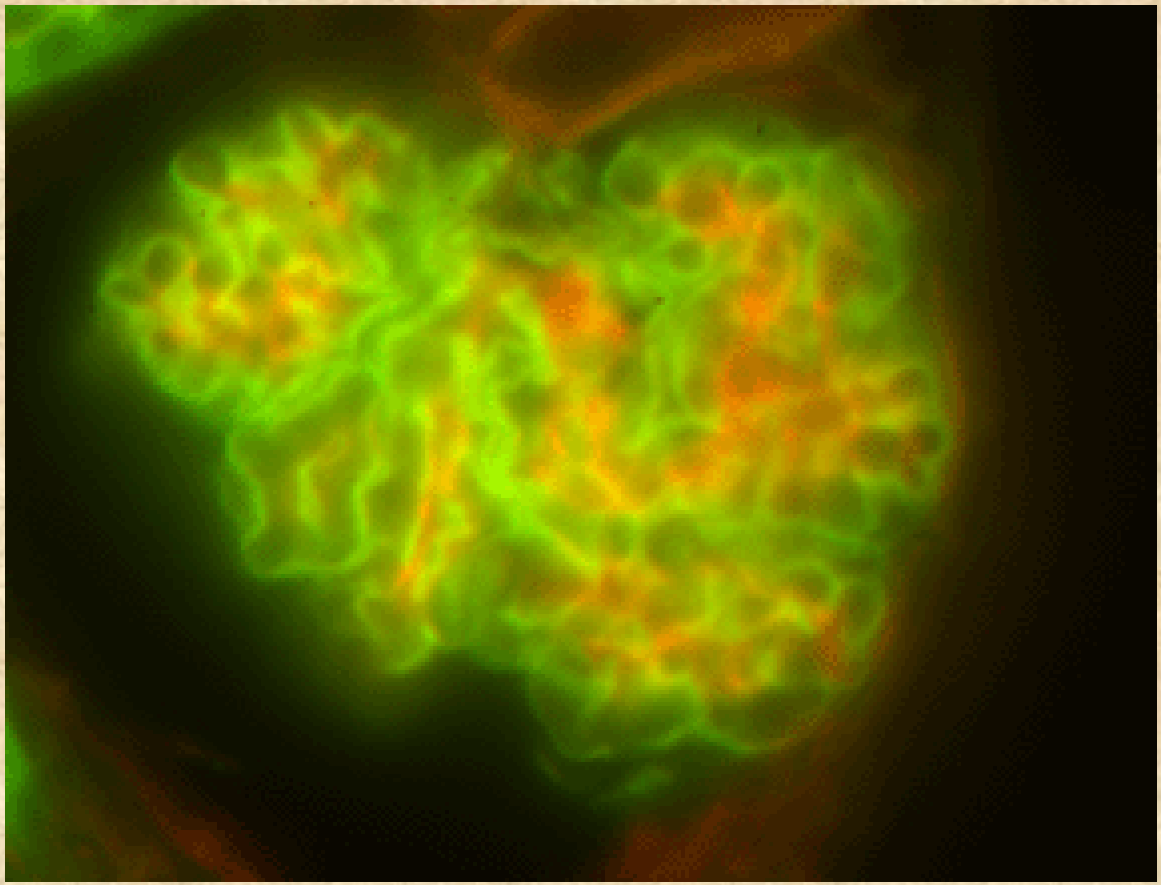


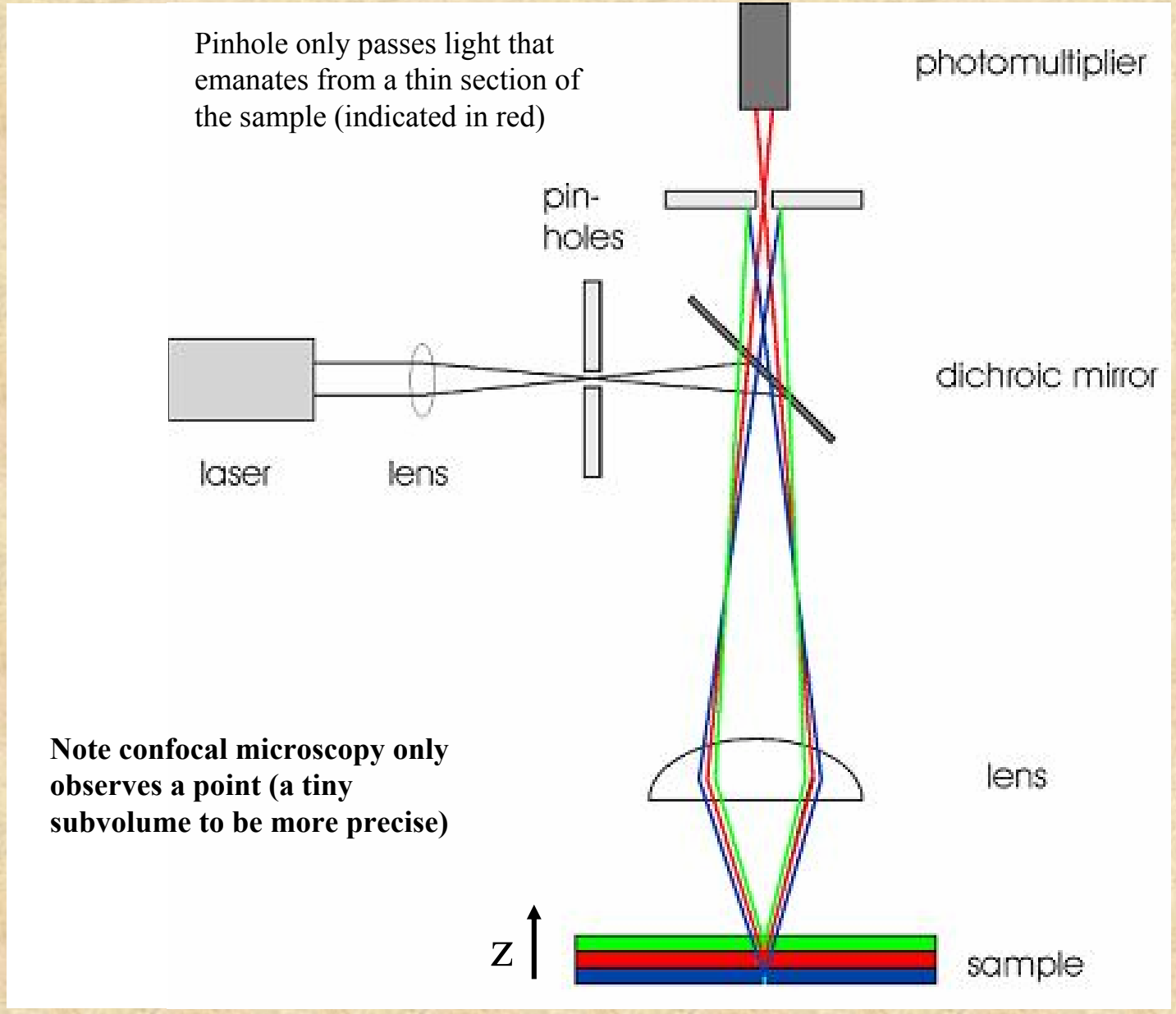
Figure 1

Widefield-fluorescence microscope image



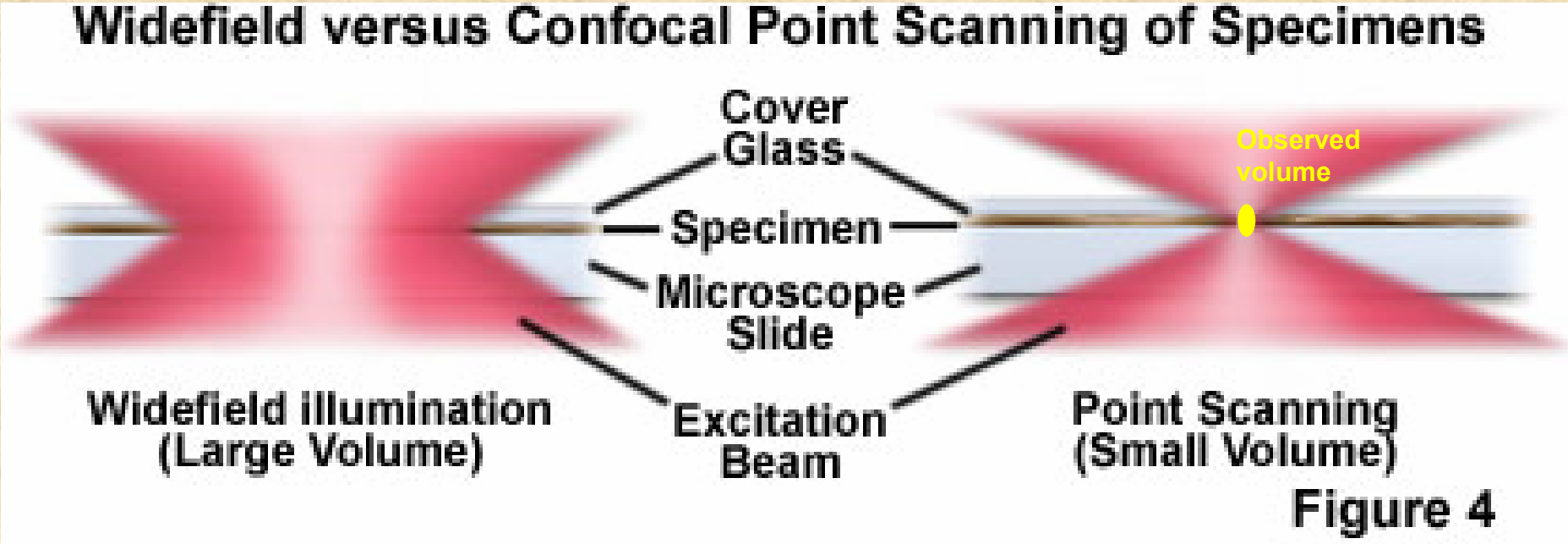
Widefield fluorescence image of a 16 micron thick section of fluorescently-labeled mouse kidney.

Principle of Confocal Microscopy

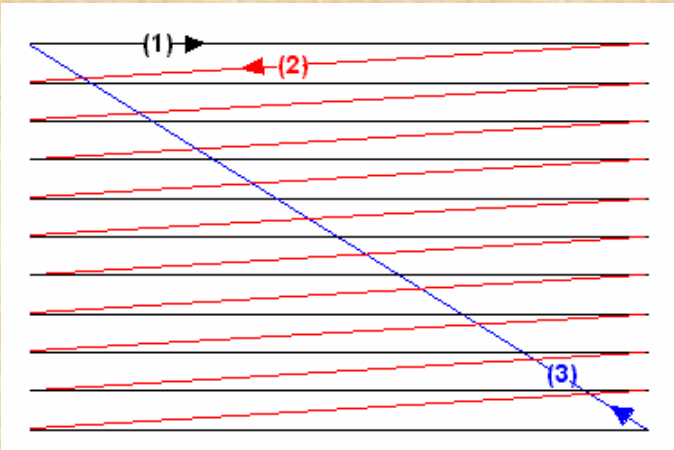


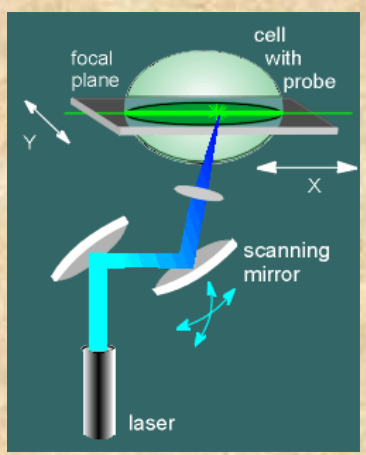
Note confocal microscopy only observes a point (a tiny subvolume to be more precise)

Laser Scanning Confocal Microscopy (LSCM):



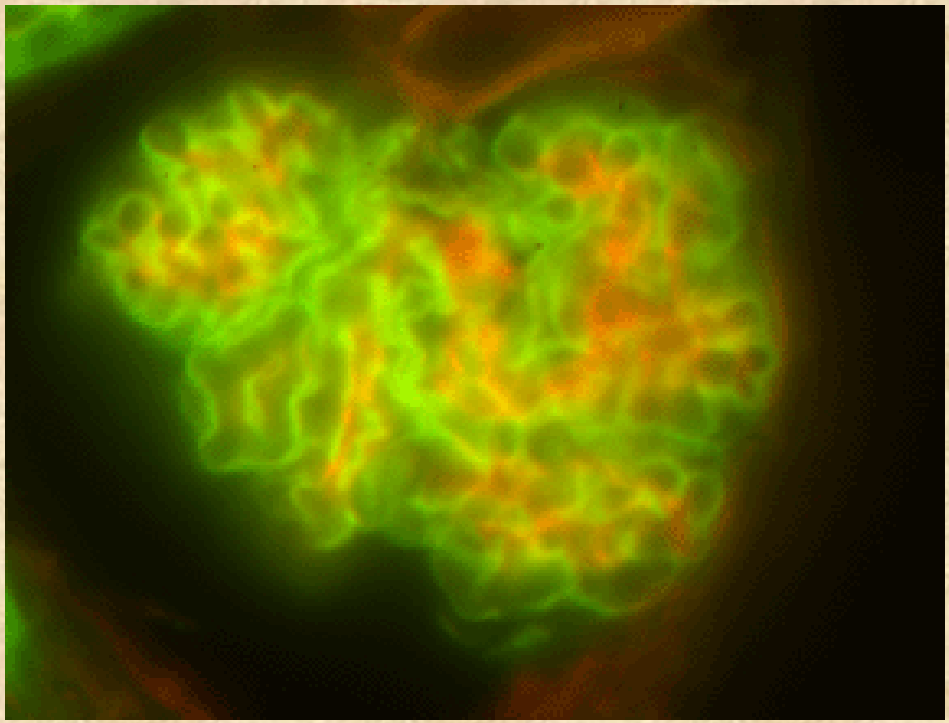
Need to perform a raster scan to build up an image point by point





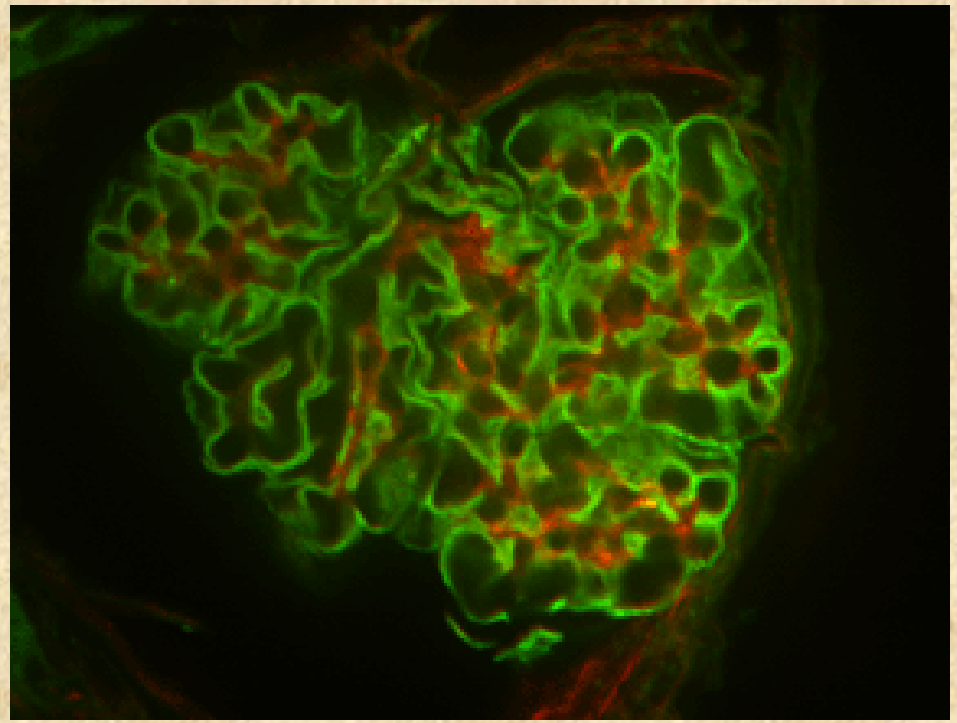
Two electronically driven scan mirrors move the laser spot on the sample in a raster-like fashion.

Widefield Image



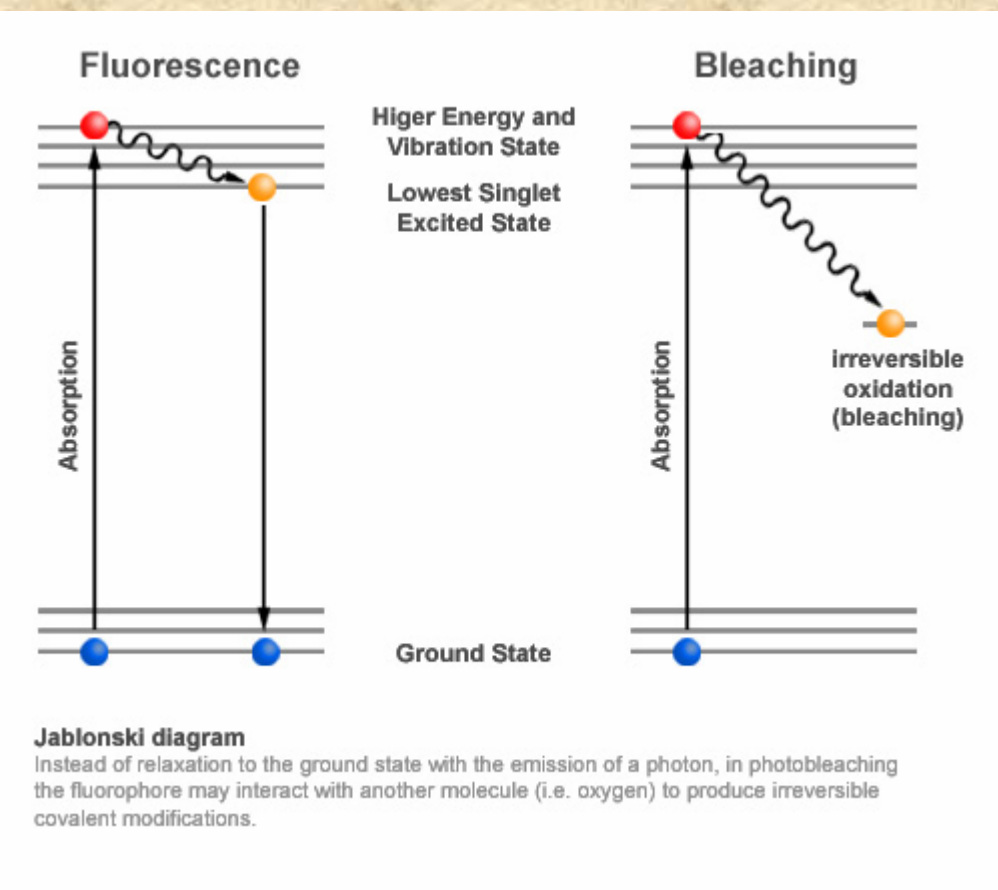
Widefield fluorescence image of a 16 micron thick section of fluorescently-labeled mouse kidney.

Confocal Image



The same specimen show on the left, taken with a confocal microscope.

Photobleaching

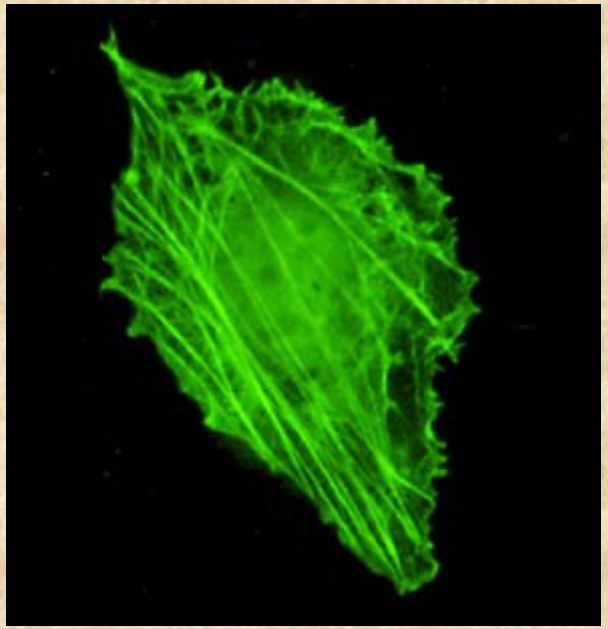


Jablonski diagram
Instead of relaxation to the ground state with the emission of a photon, in photobleaching the fluorophore may interact with another molecule (i.e. oxygen) to produce irreversible covalent modifications.

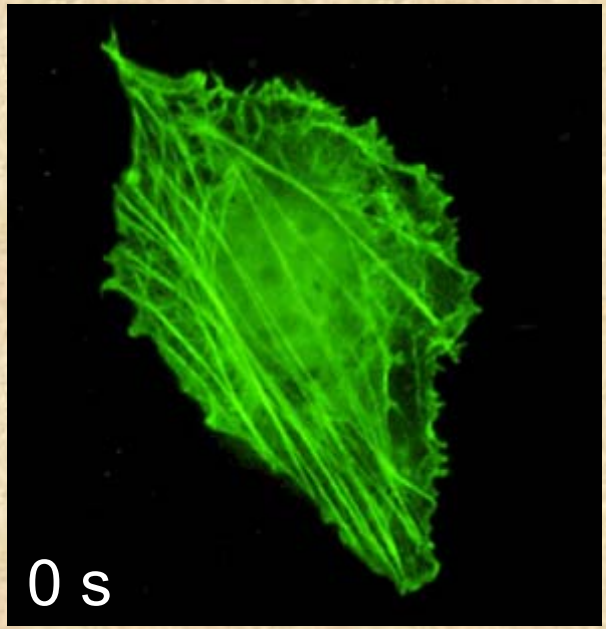
The average number of excitation and emission cycles that occur for a particular fluorophore before photobleaching is dependent upon the molecular structure and the local environment. Some fluorophores bleach quickly after emitting only a few photons, while others that are more robust can undergo thousands or millions of cycles before bleaching.

Photobleaching

original image

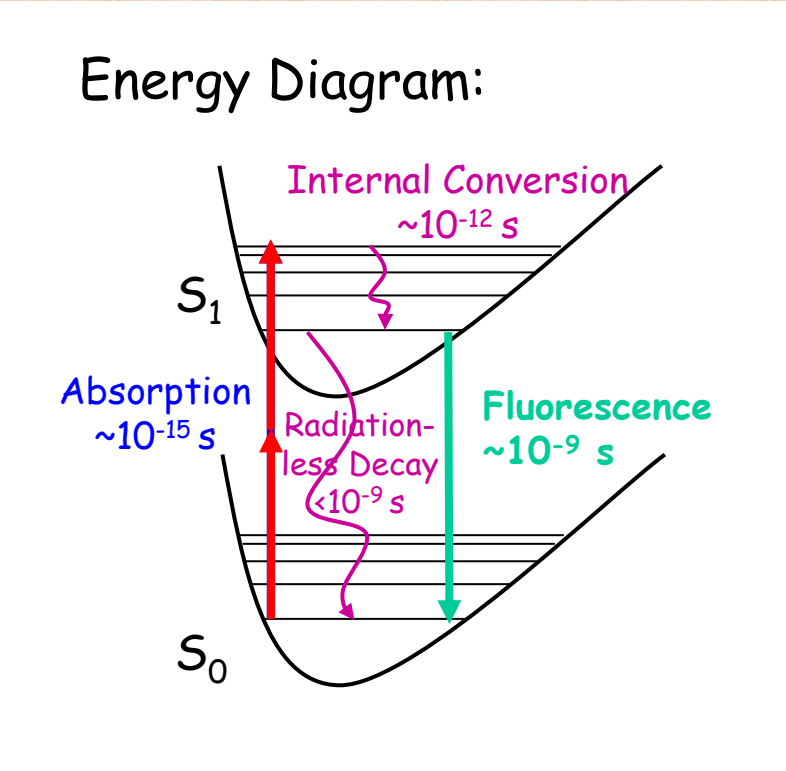


Photobleached image



Two-Photon Microscopy: Principle

Now consider two-photon absorption

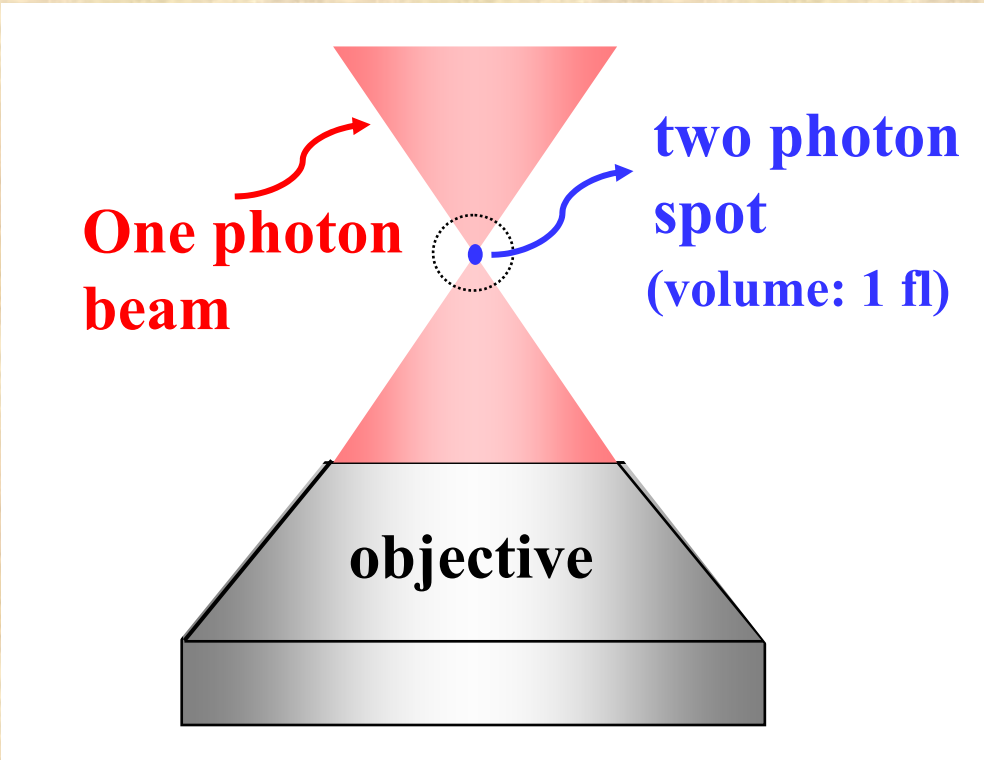


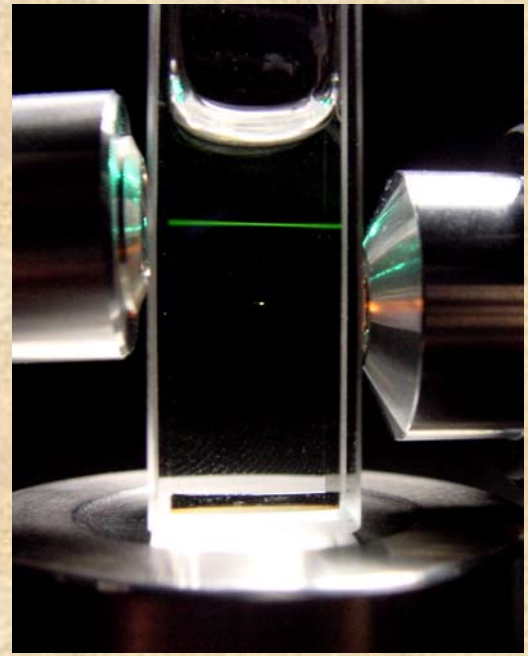
Two-photon absorption is an optical nonlinear process

Two-Photon Fluorescence

Simultaneous absorption of two-photons is a rare process:

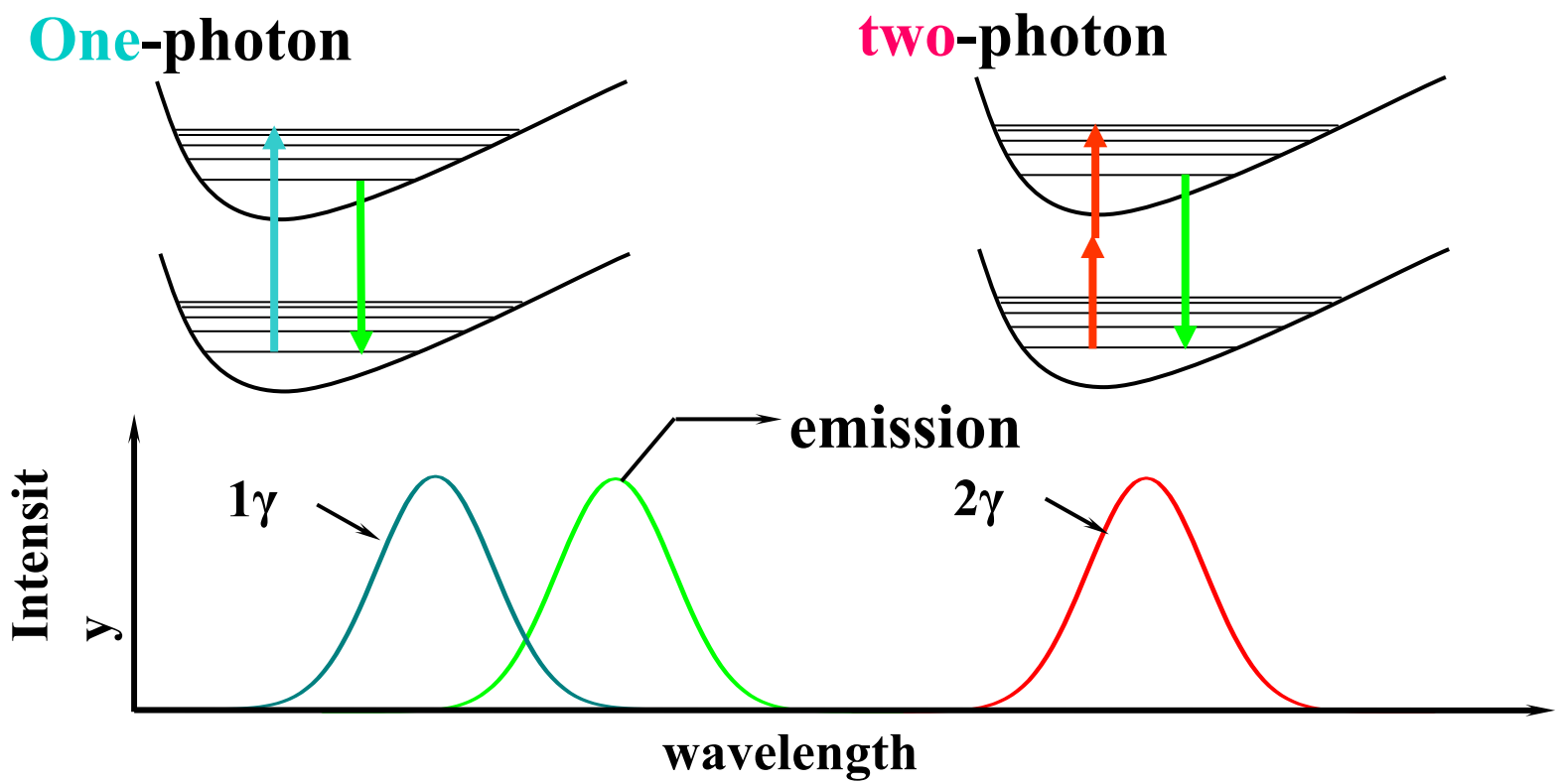
Maximize two-photon effect by increasing the photon flux
- spatially by focusing the light
- temporally (ultrafast laser pulses)





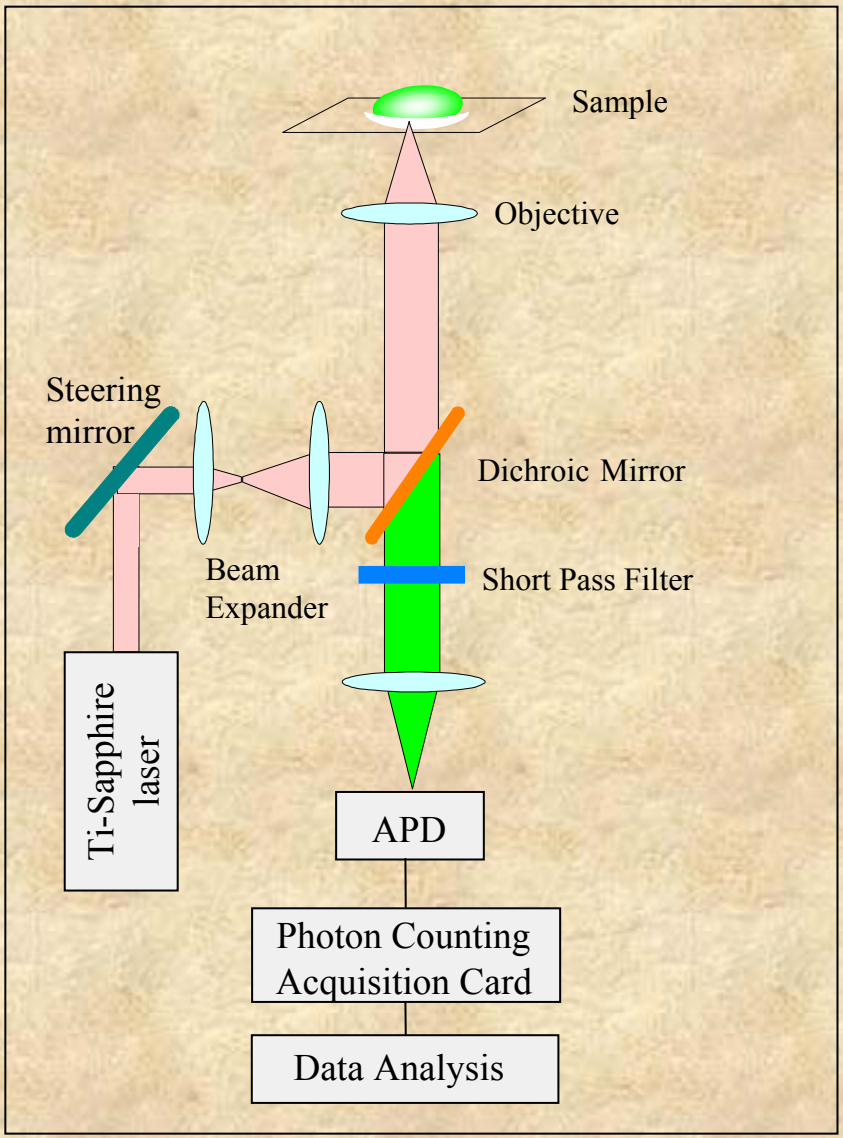
Inherent 3 - dimensional optical sectioning effect!

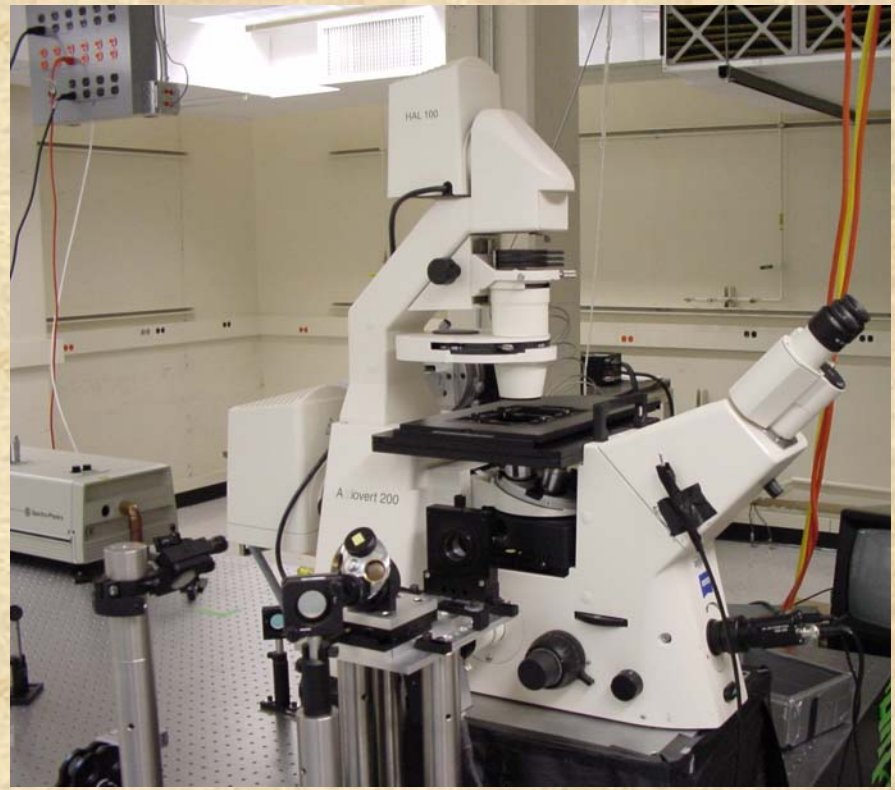
Two-photon spectroscopy



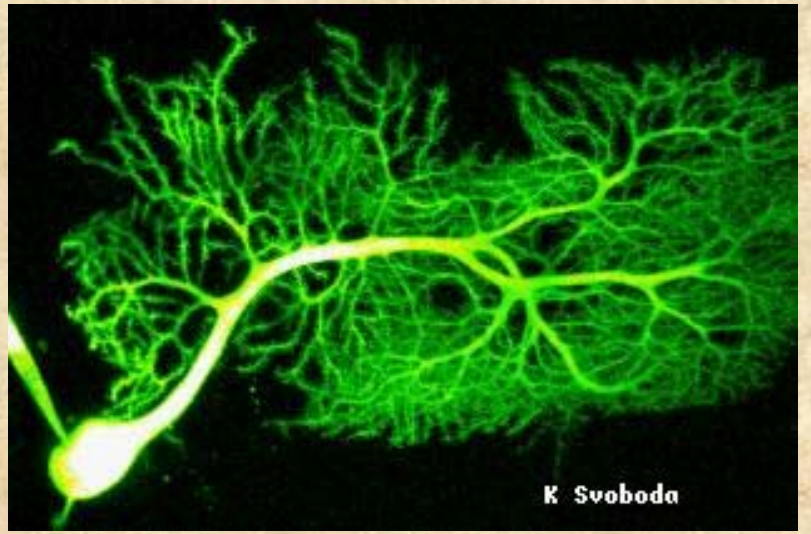
Two-photon absorption is spectrally well separated from the fluorescence! Note that Raman of the solvent will not occur within the fluorescence emission spectrum.

Two-photon Instrumentation





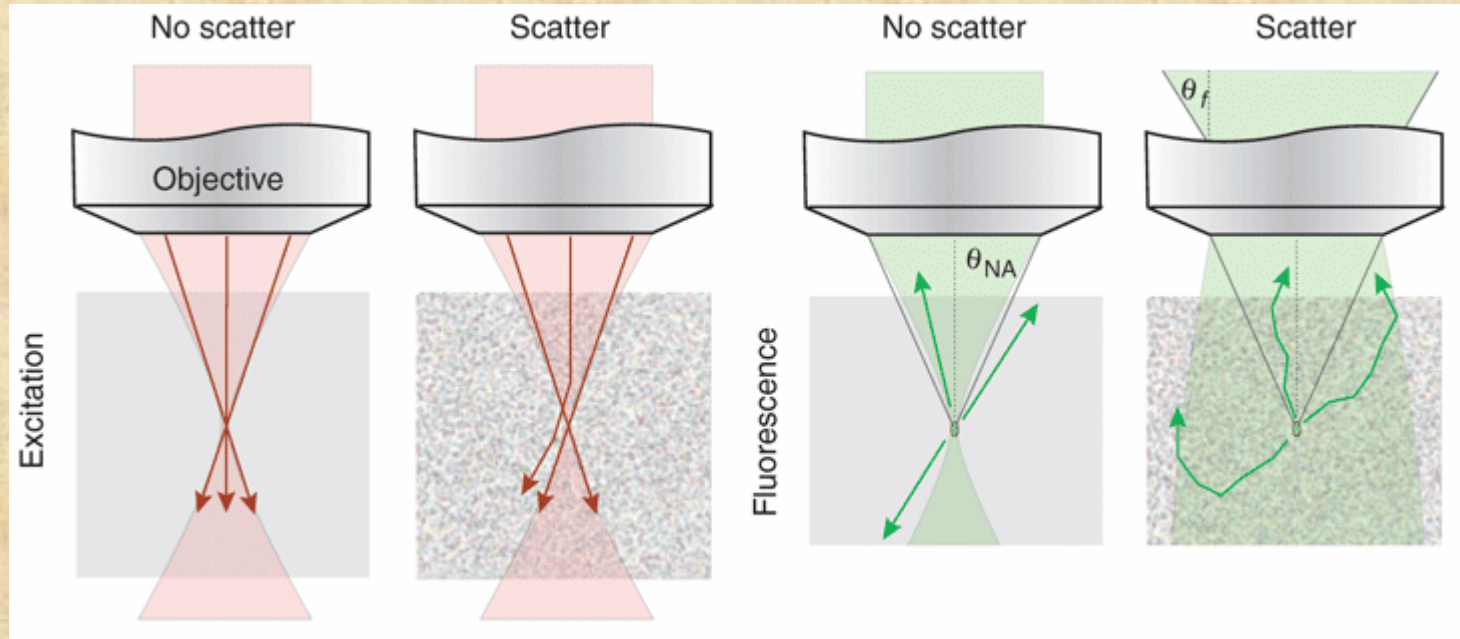
Two-photon Imaging



Purkinje neurone in a living brain slice filled with fluorescein dextran imaged with two-photon excitation laser scanning microscopy (Svoboda, Cold spring Harbor Laboratories)

Two-photon image resolution is essentially the same as that of confocal microscopy. However, imaging in the presence of significant scatter (such as in thick tissue) requires two-photon excitation.

Also note that photobleaching in two-photon microscopy is strictly restricted to the excitation volume!



Denk, *Nature Methods* **2**, 932 - 940 (2005)

Fluorescence Fluctuation Spectroscopy

Q: How many fluorescent molecules are (on average) in the two-photon (or confocal) volume of the microscope?

A: That depends on the concentration. At a high concentration there are more molecules in the volume than at low concentrations.

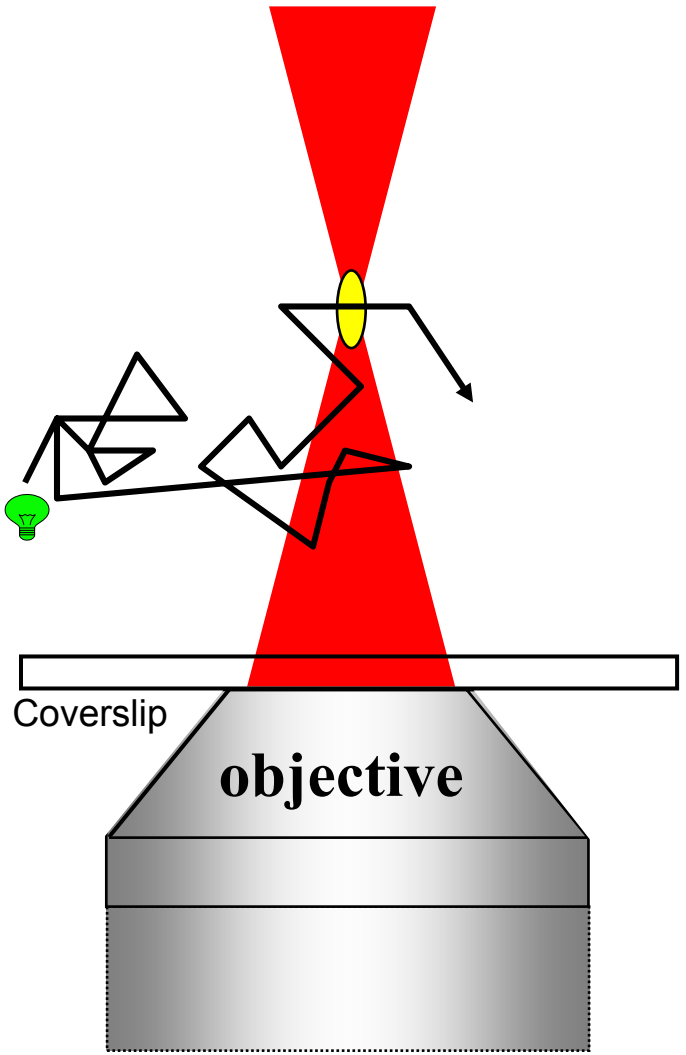
Q: Ok, many proteins in cells have nanomolar concentrations. How many proteins (assuming c = 1 nM) are now in the volume?

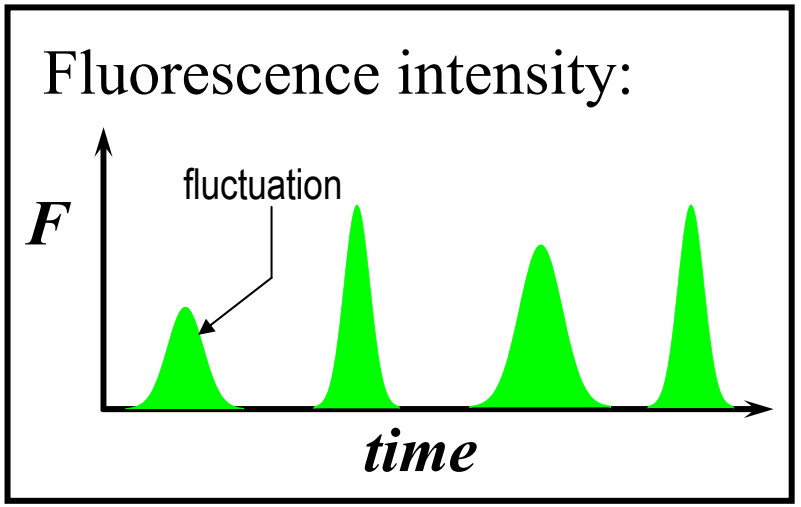
A: Let me calculate … (Volume is 1 femtoliter, Avogadro's number is 6×10^{23}, c = 1 nM). The number I get is a **single molecule** per observation volume. Well that's ok, fluorescence is very sensitive and can detect single molecules.

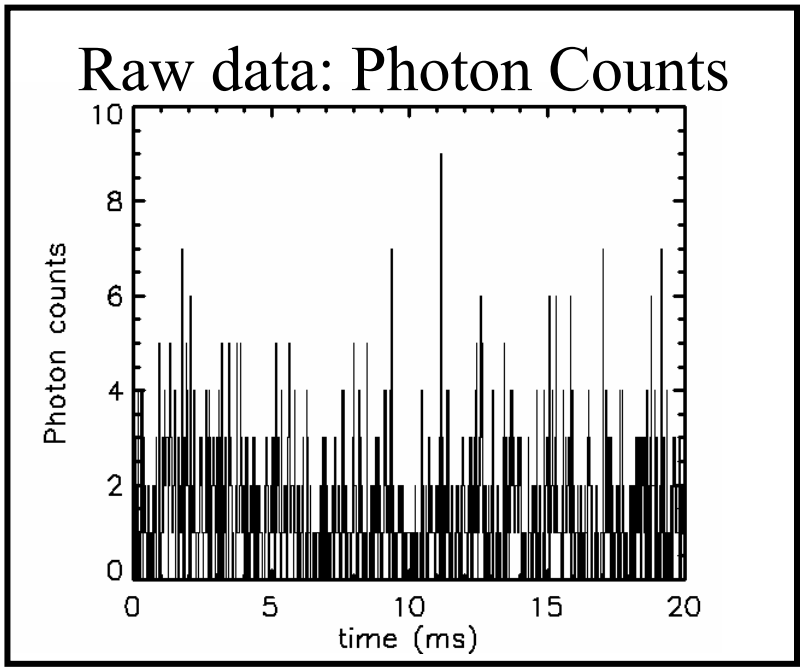
Q: Proteins in a solution (and in a cell) are typically mobile. They diffuse around. What will happen if the single molecule moves around? Also a single molecule is in the volume on average. Is there a chance that sometimes there will be two or no molecules in the volume?

A: Yes, the number of molecules will fluctuate as they diffuse in and out of the observation volume. Because two molecules produce more fluorescence than a single molecule there will be fluctuations in the fluorescence intensity.

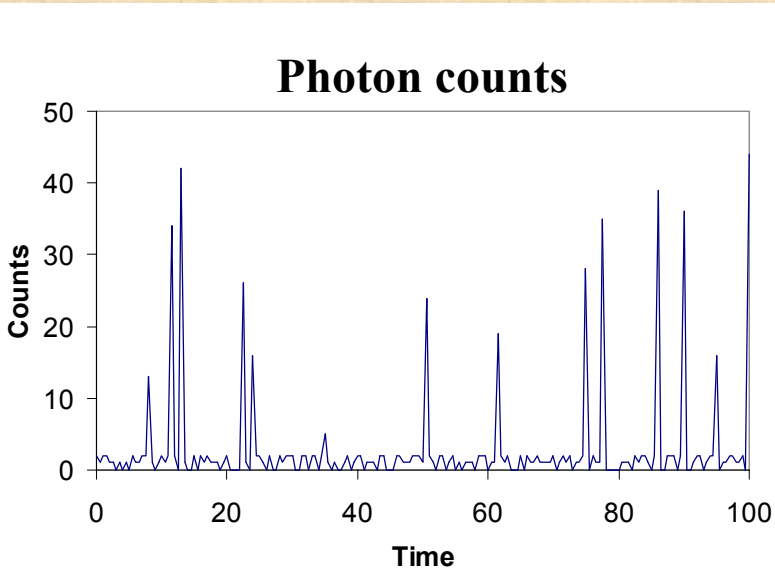
Fluorescence Fluctuation Spectroscopy

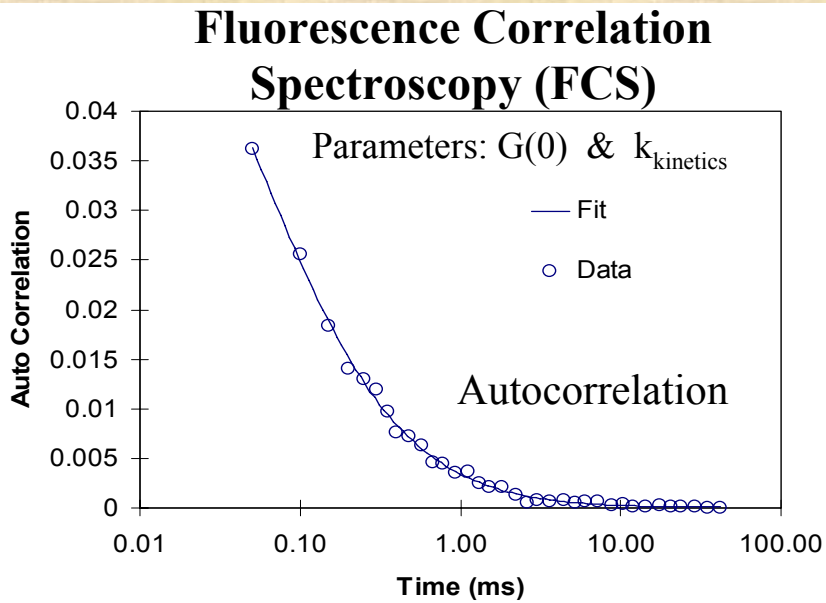


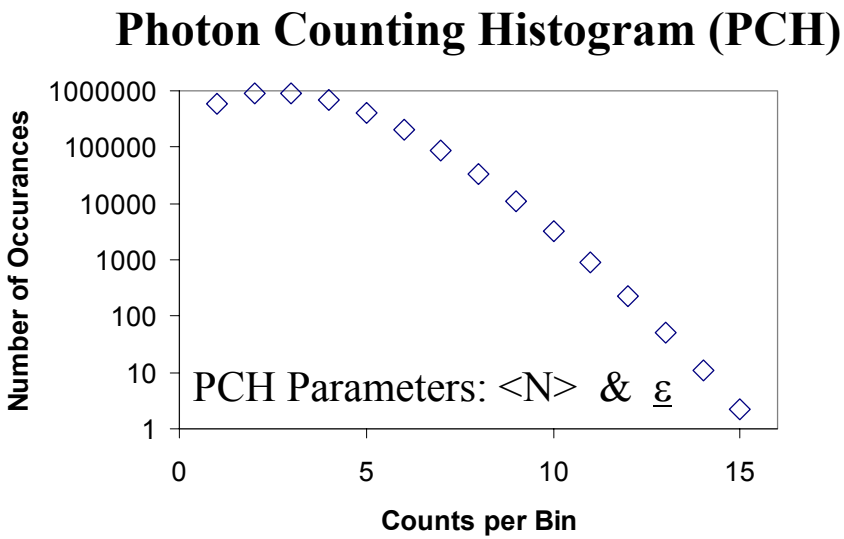




Statistical Analysis of the Fluctuations required



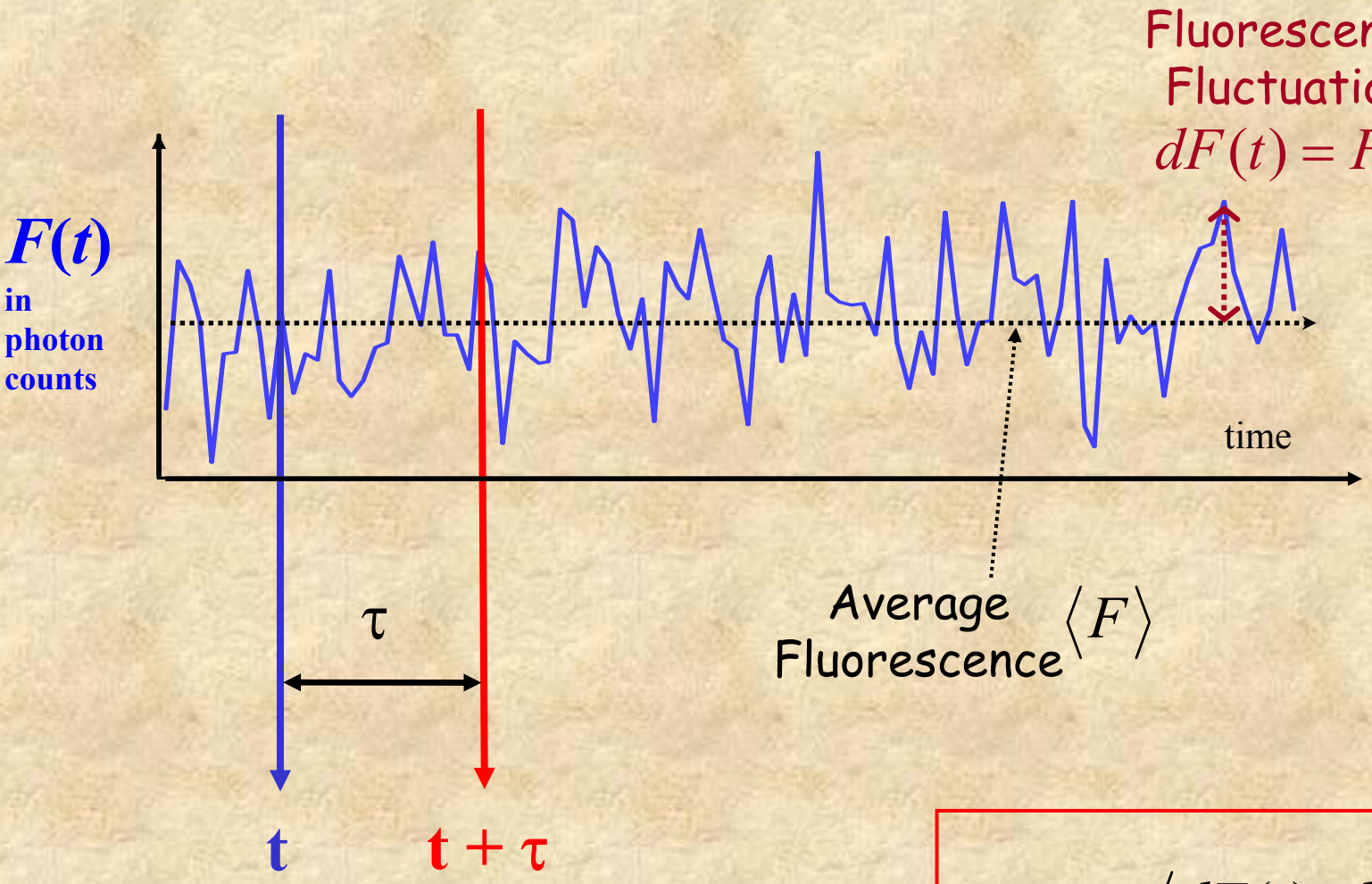




Autocorrelation Function:

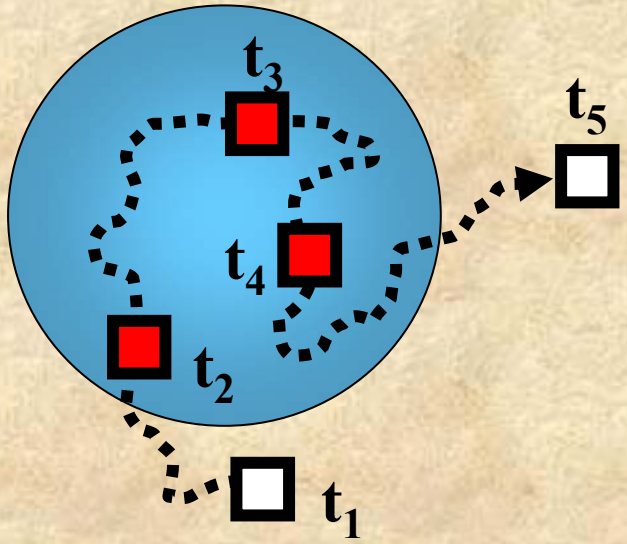
$$G(\tau) = \frac{\langle dF(t) \cdot dF(t+\tau) \rangle}{\langle F \rangle^2}$$

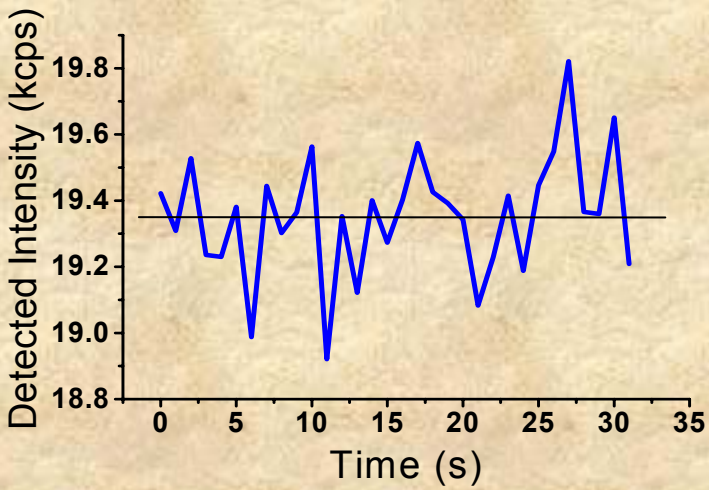
Calculating the Autocorrelation Function



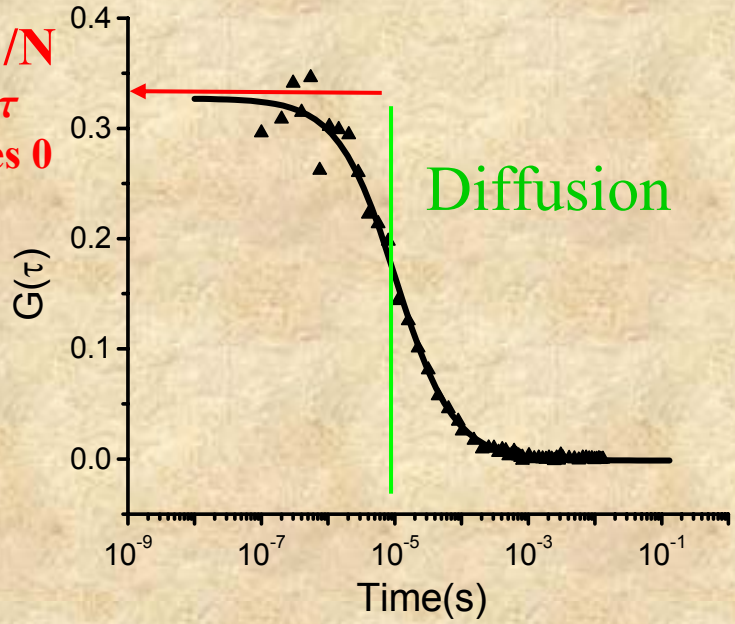
$$G(\tau) = \frac{\langle dF(t) \cdot dF(t+\tau) \rangle}{\langle F \rangle^2}$$

The Autocorrelation Function



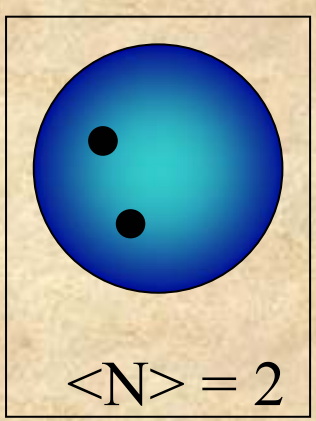


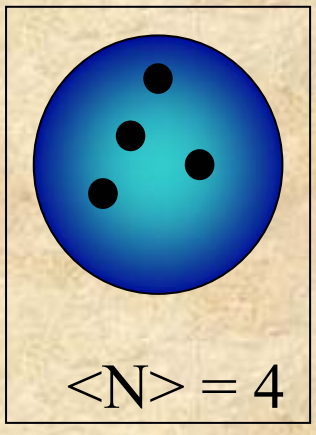
G(0) ∝ 1/N as time τ approaches 0

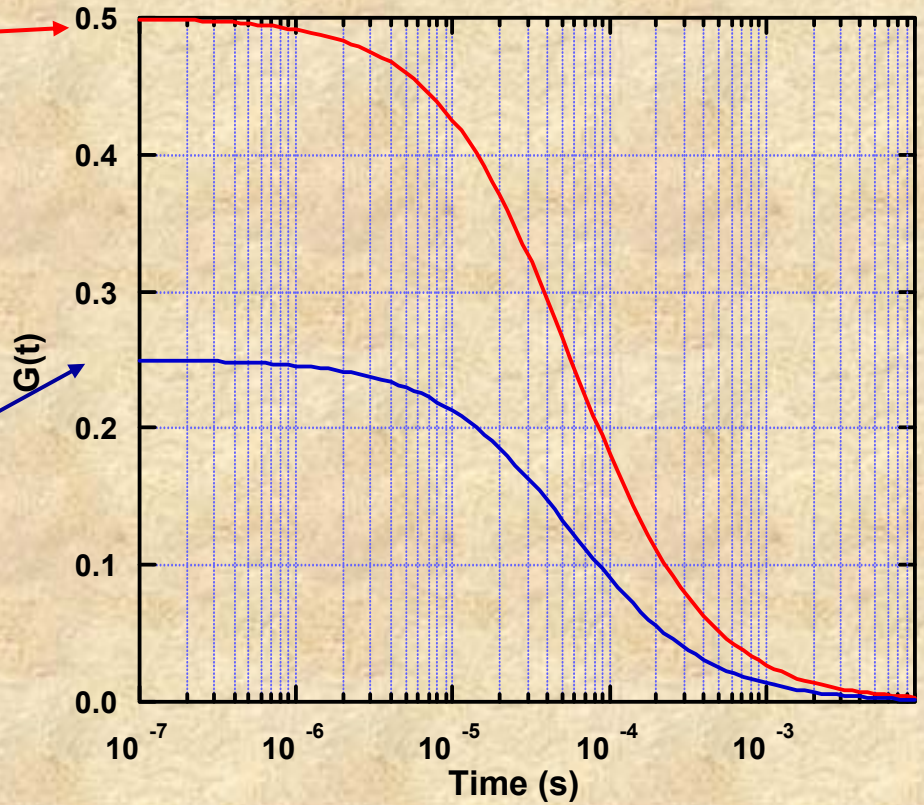


$$G(\tau) = \frac{\langle dF(t)\, dF(t+\tau) \rangle}{\langle F \rangle^2}$$

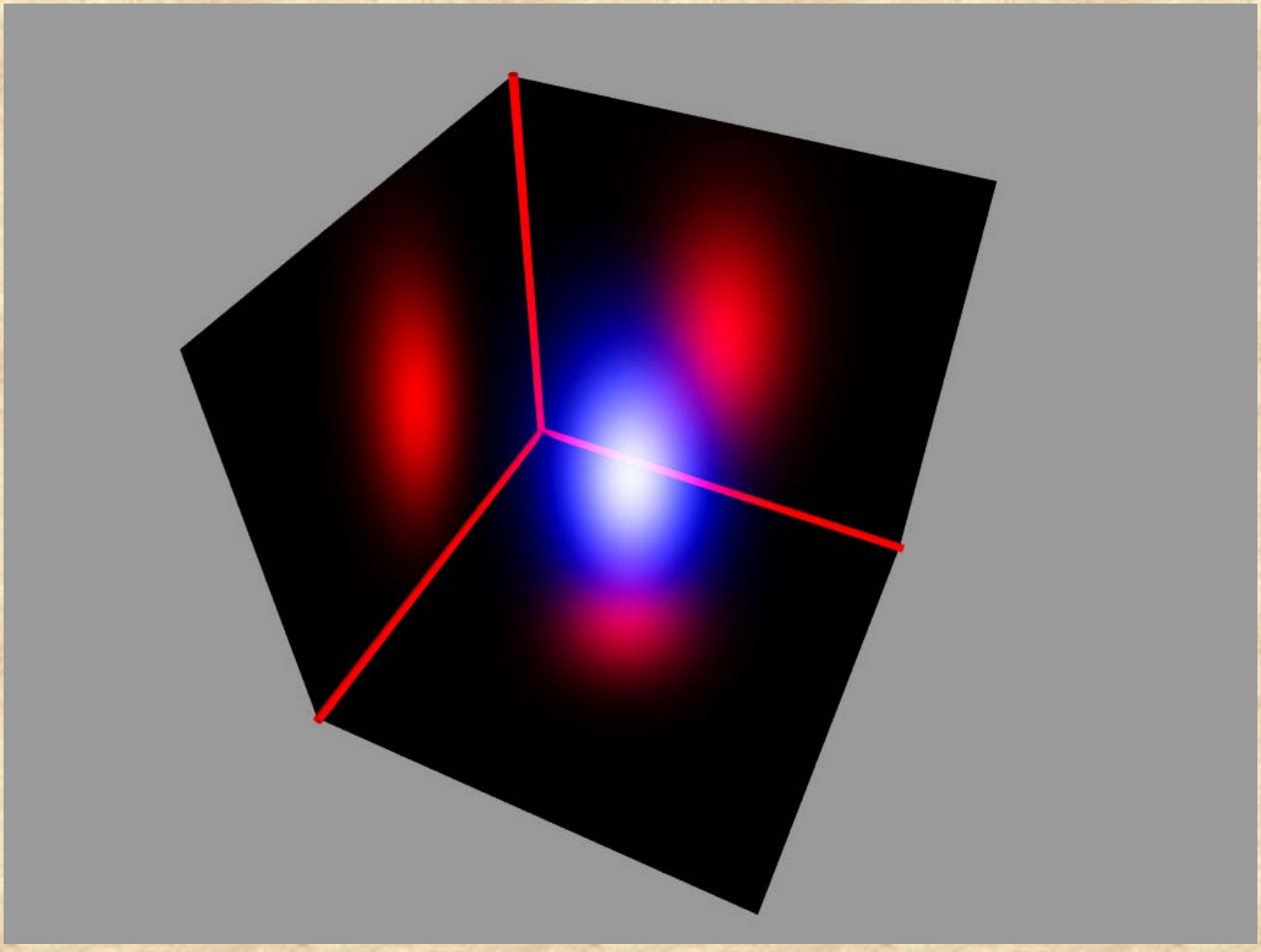
The Effects of Particle Concentration on the Autocorrelation Curve







What about the excitation (or observation) volume shape?



Correlation function of diffusing molecules

For a 3-dimensional Gaussian excitation volume:

$$G(\tau) = \frac{1}{\sqrt{8}} \frac{1}{N} \left(1 + \frac{8D\tau}{w_0^2}\right)^{-1} \left(1 + \frac{8D\tau}{z_0^2}\right)^{-1/2}$$

1-photon equation contains a 4, instead of 8

N: average number of particles inside volume
D: Diffusion coefficient
w_o: radial beam waist of two-photon laser spot
z_o: axial beam waist of two-photon laser spot

The Effects of Particle Size on the Autocorrelation Curve

Diffusion Constants

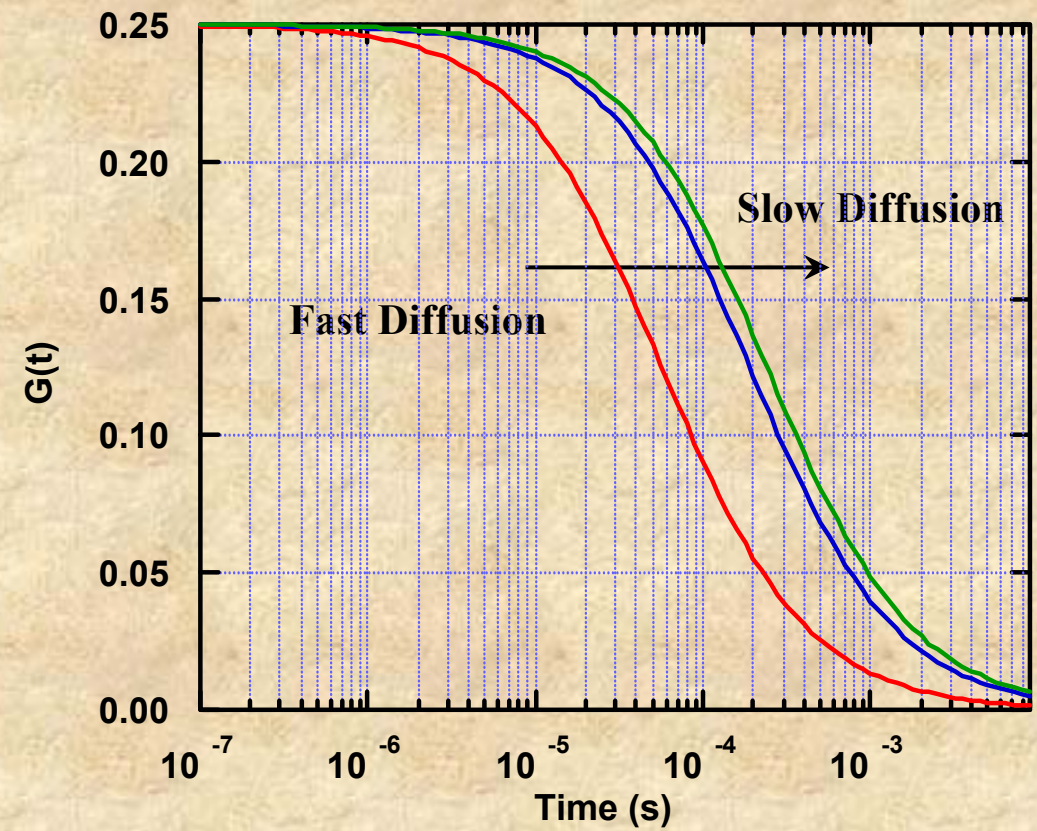
300 um^2/s
90 um^2/s
71 um^2/s

Stokes-Einstein Equation:

$$D = \frac{k \cdot T}{6 \cdot \pi \cdot \eta \cdot r}$$

and

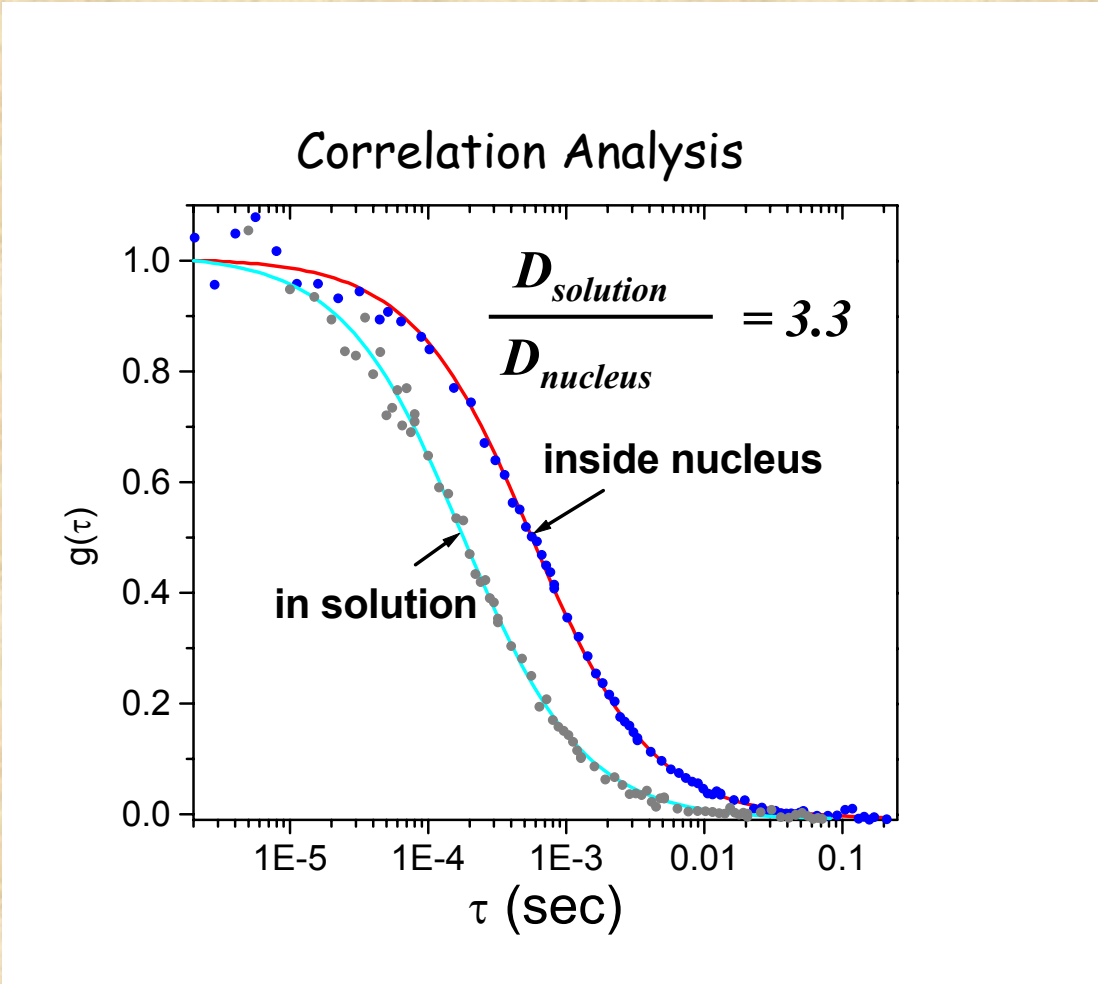
$$MW \propto Volume \propto r^3$$



Monomer --> Dimer

Only a change in D by a factor of $2^{1/3}$, or 1.26

FCS inside living cells

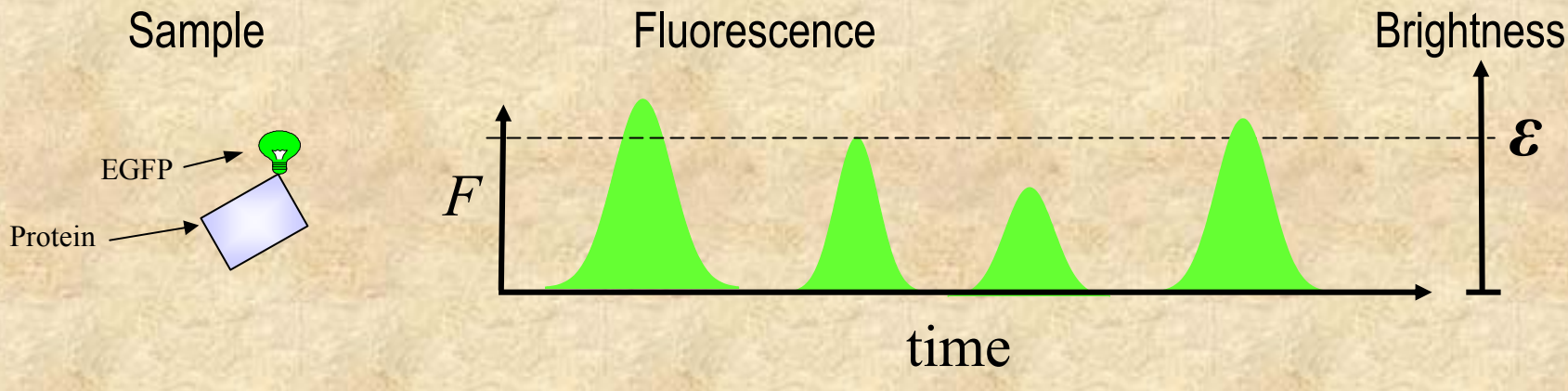


Measure the diffusion coefficient of Green Fluorescent Protein (GFP) in aqueous solution in inside the nucleus of a cell.

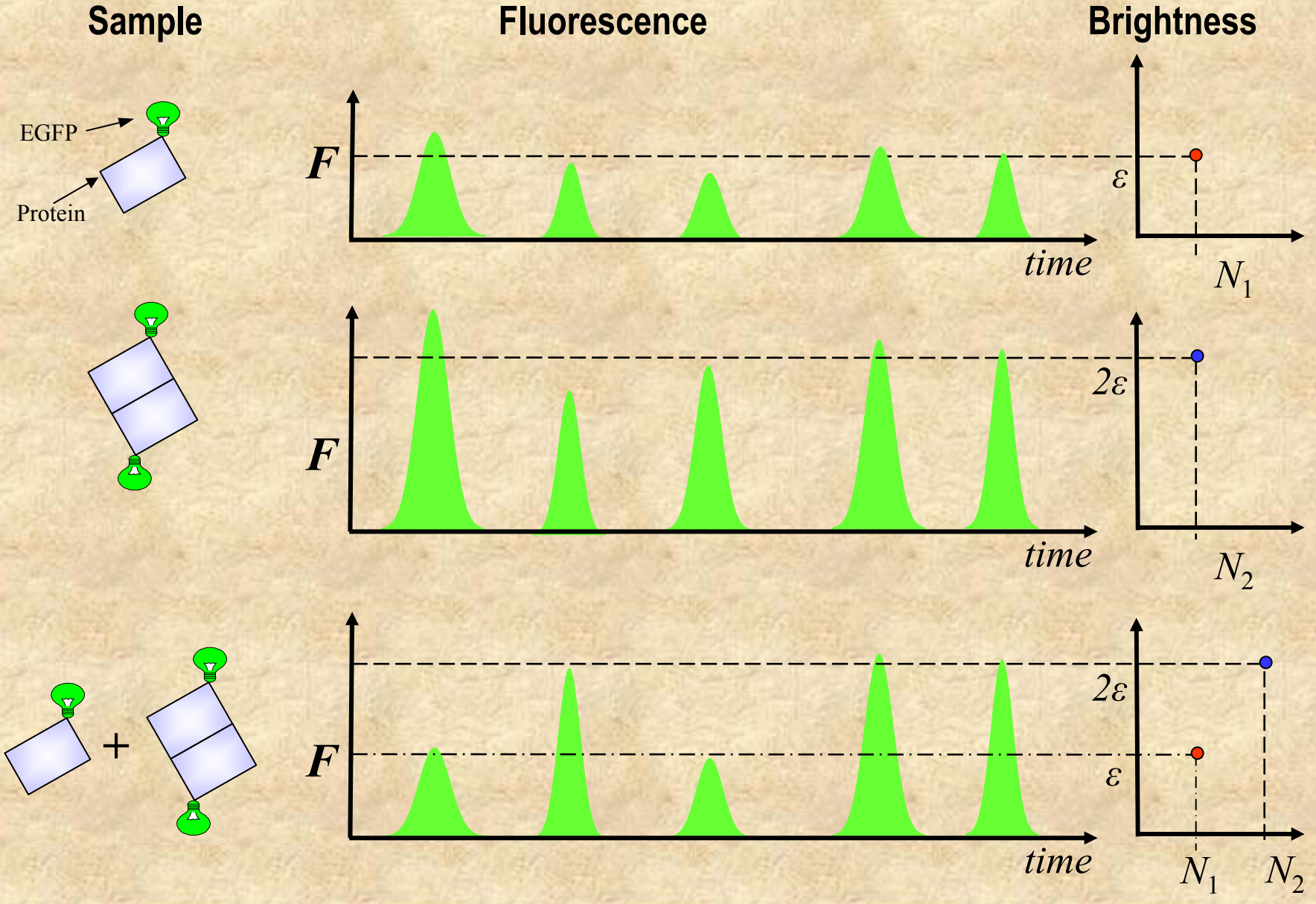
Statistical Analysis: Brightness

Brightness ε is the **average fluorescence intensity** of a single particle

Illustration:



Brightness Encodes Stoichiometry



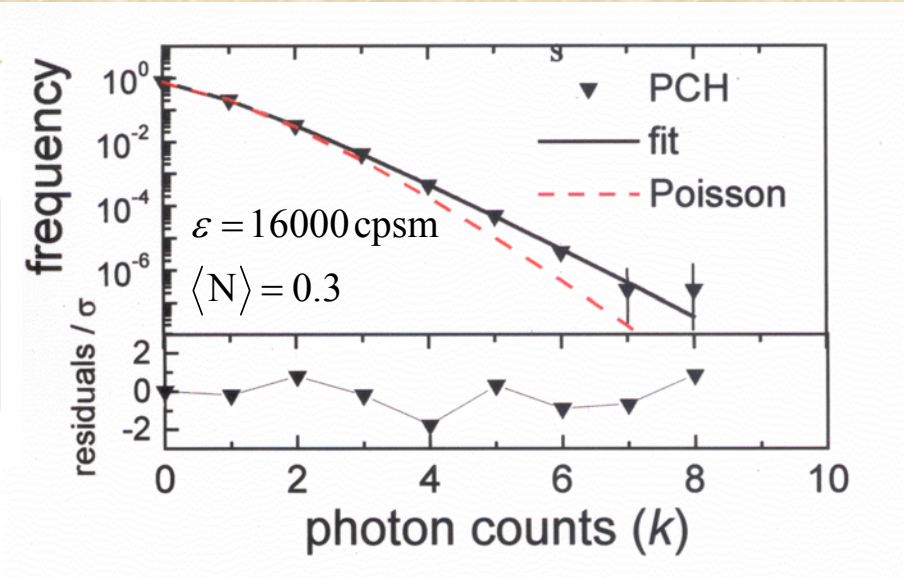
Photon Counting Histogram (PCH)

Aim: To resolve species from differences in their molecular brightness

Molecular brightness ε : The average photon count rate of a single fluorophore

PCH: probability distribution function $p(k)$

where $p(k)$ is the probability of observing k photon counts



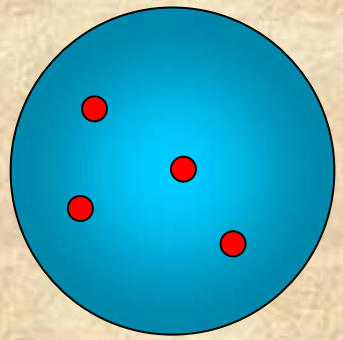
Single Species:

$$p(k) = PCH(\varepsilon, \langle N \rangle)$$

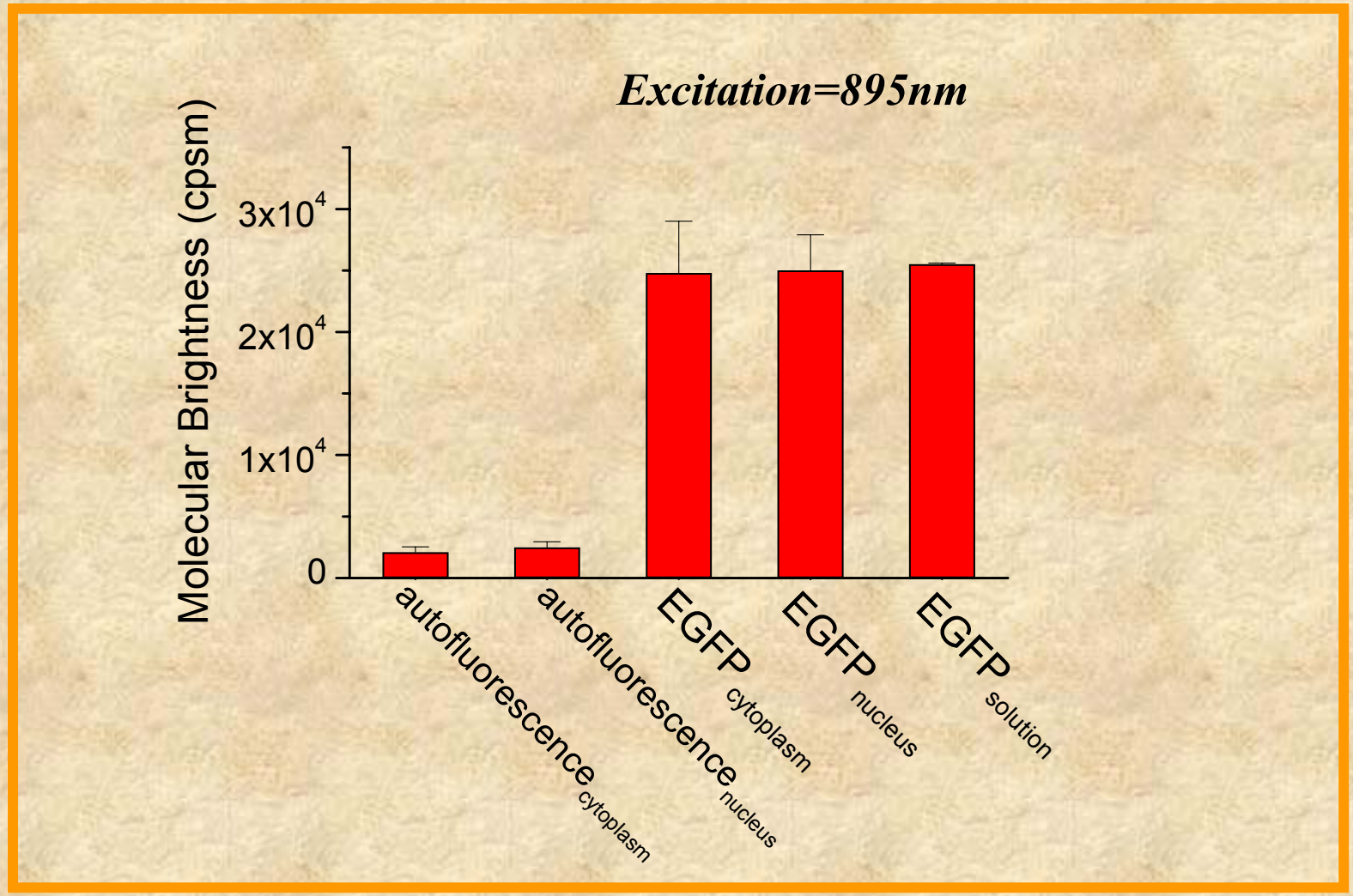
Note: PCH is Non-Poissonian!

Sources of Non-Poissonian Noise

- Detector Noise
- Diffusing Particles in an Inhomogeneous Excitation Beam*
- Particle Number Fluctuations*
- Multiple Species*



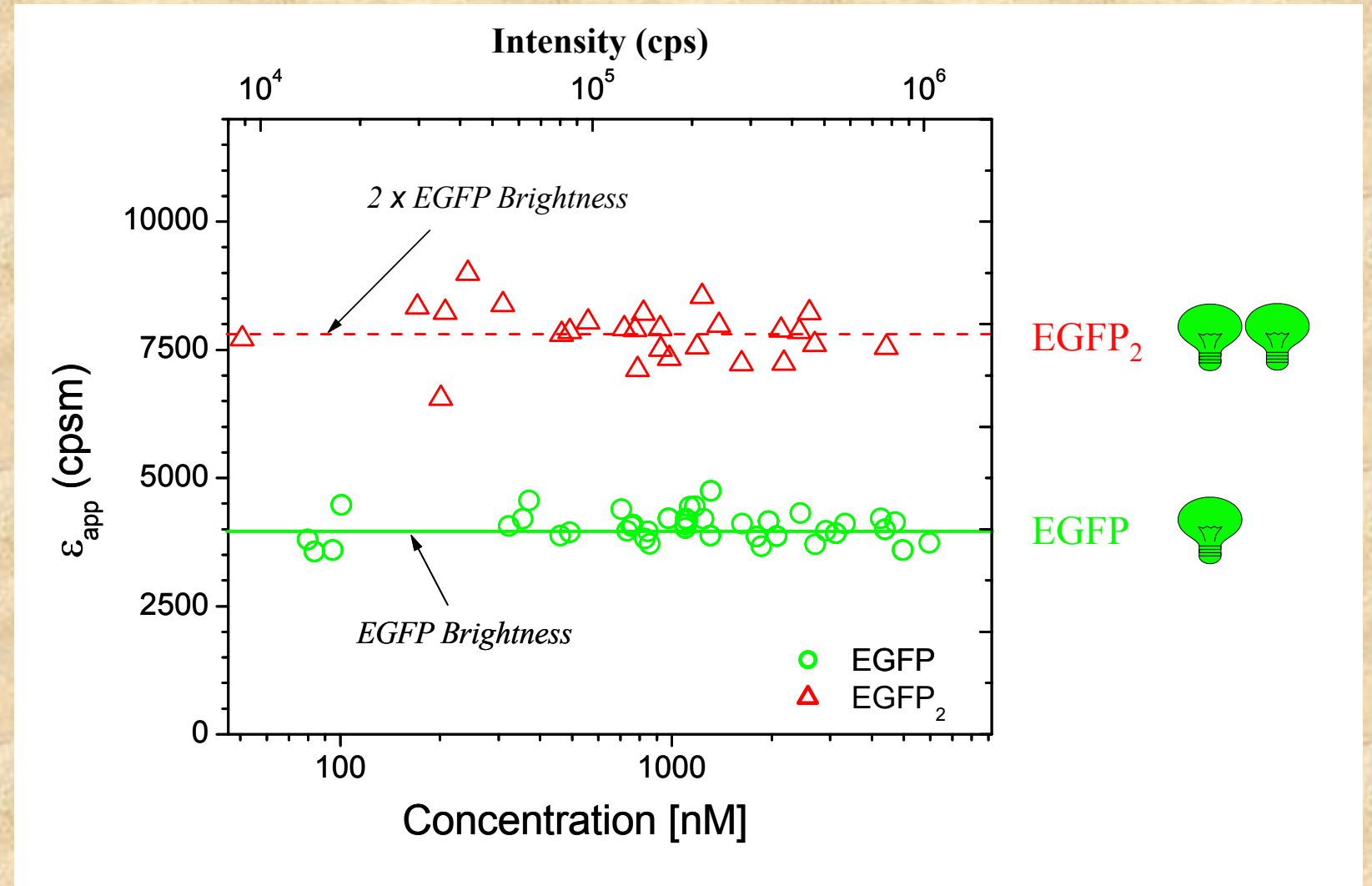
PCH in cells: Brightness of EGFP



The molecular brightness of EGFP is a factor ten higher than that of the autofluorescence in HeLa cells

Chen Y, Mueller JD, Ruan Q, Gratton E (2002) Biophysical Journal, 82, 133 .

Brightness and Stoichiometry



Brightness of $EGFP_2$ is twice the brightness of EGFP

Chen Y, Wei LN, Mueller JD, PNAS (2003) 100, 15492-15497

The End