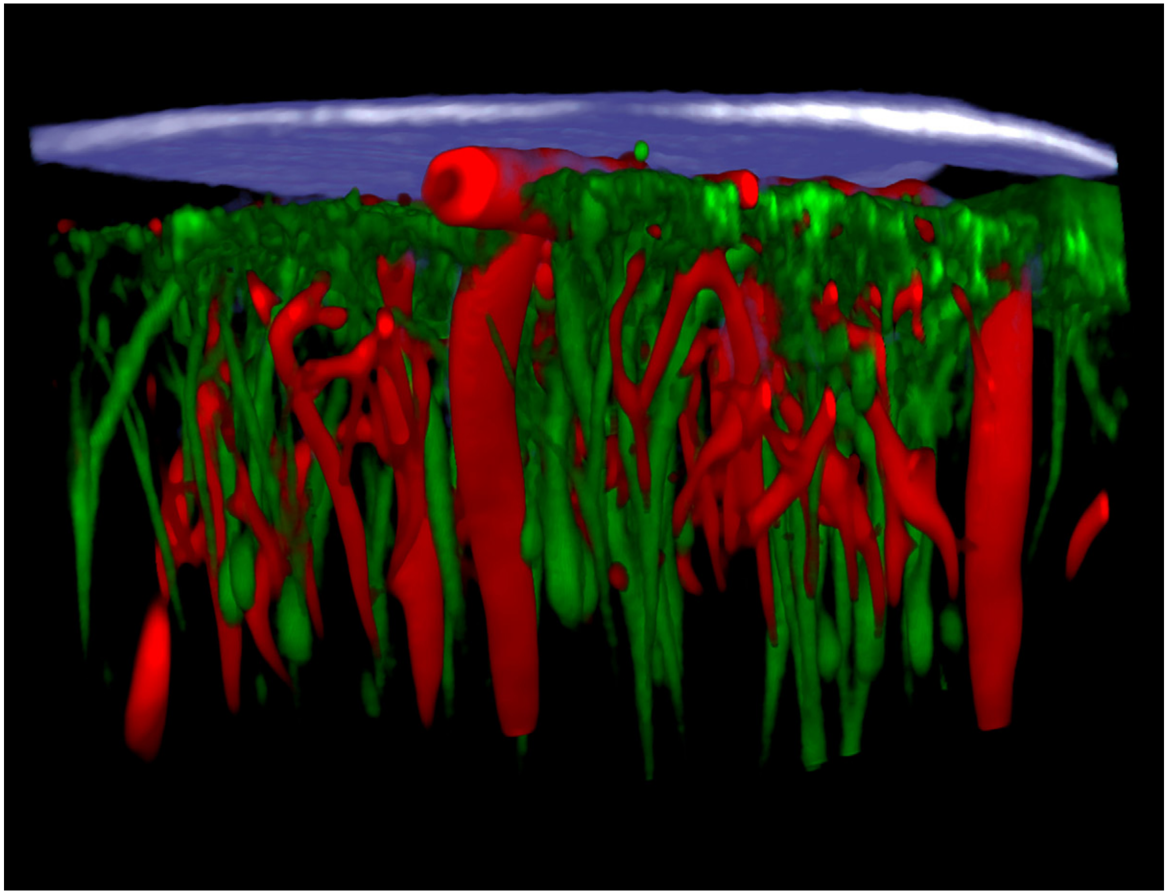
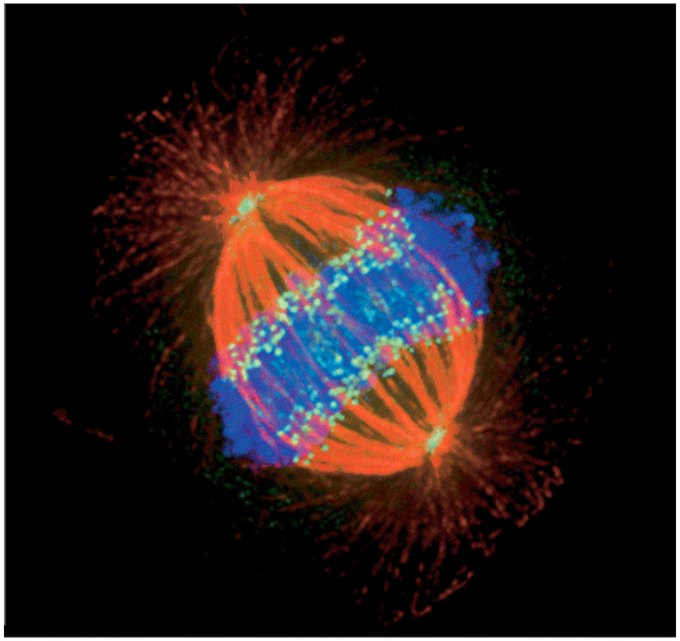
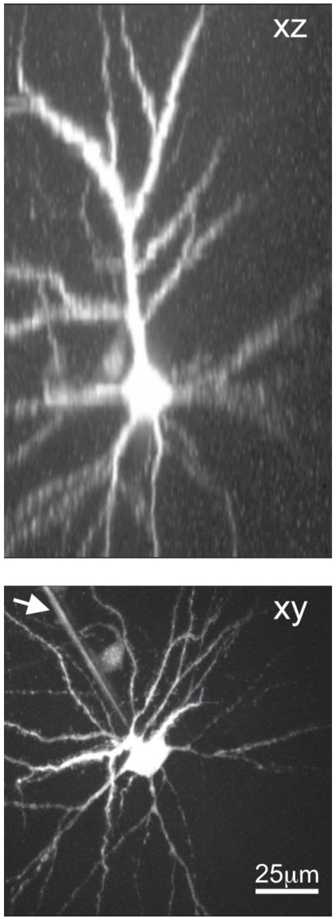
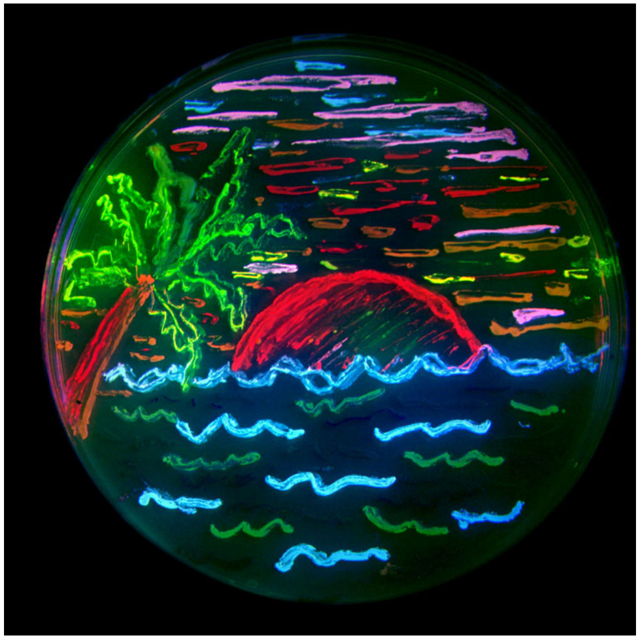
Idiosynchratic primer on principles of light microscopy for neuroscience

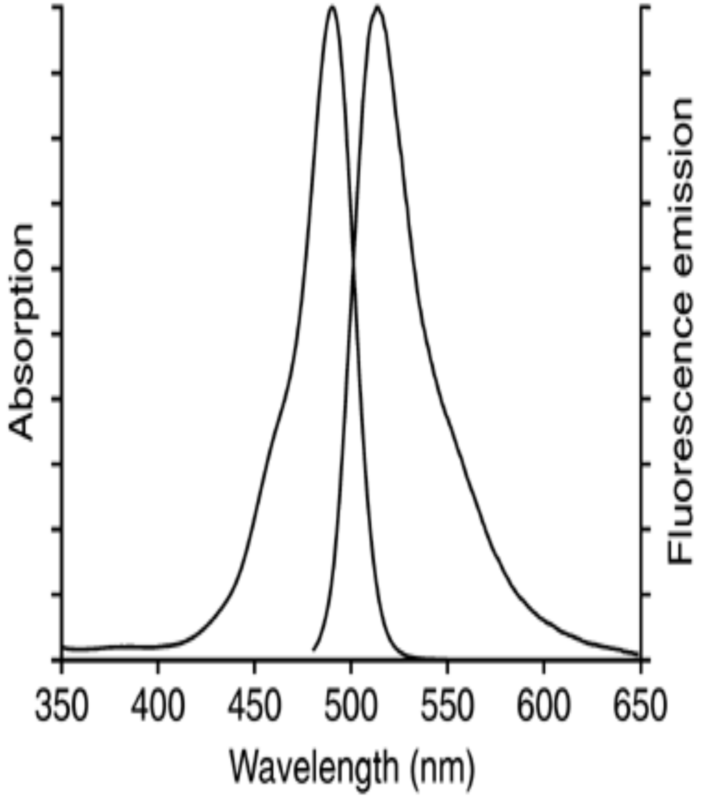


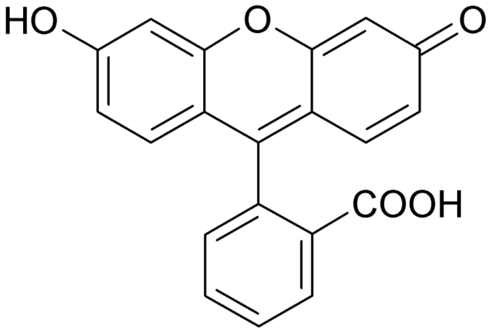
Fluorescence: Contrast based on absorption and incoherent emission







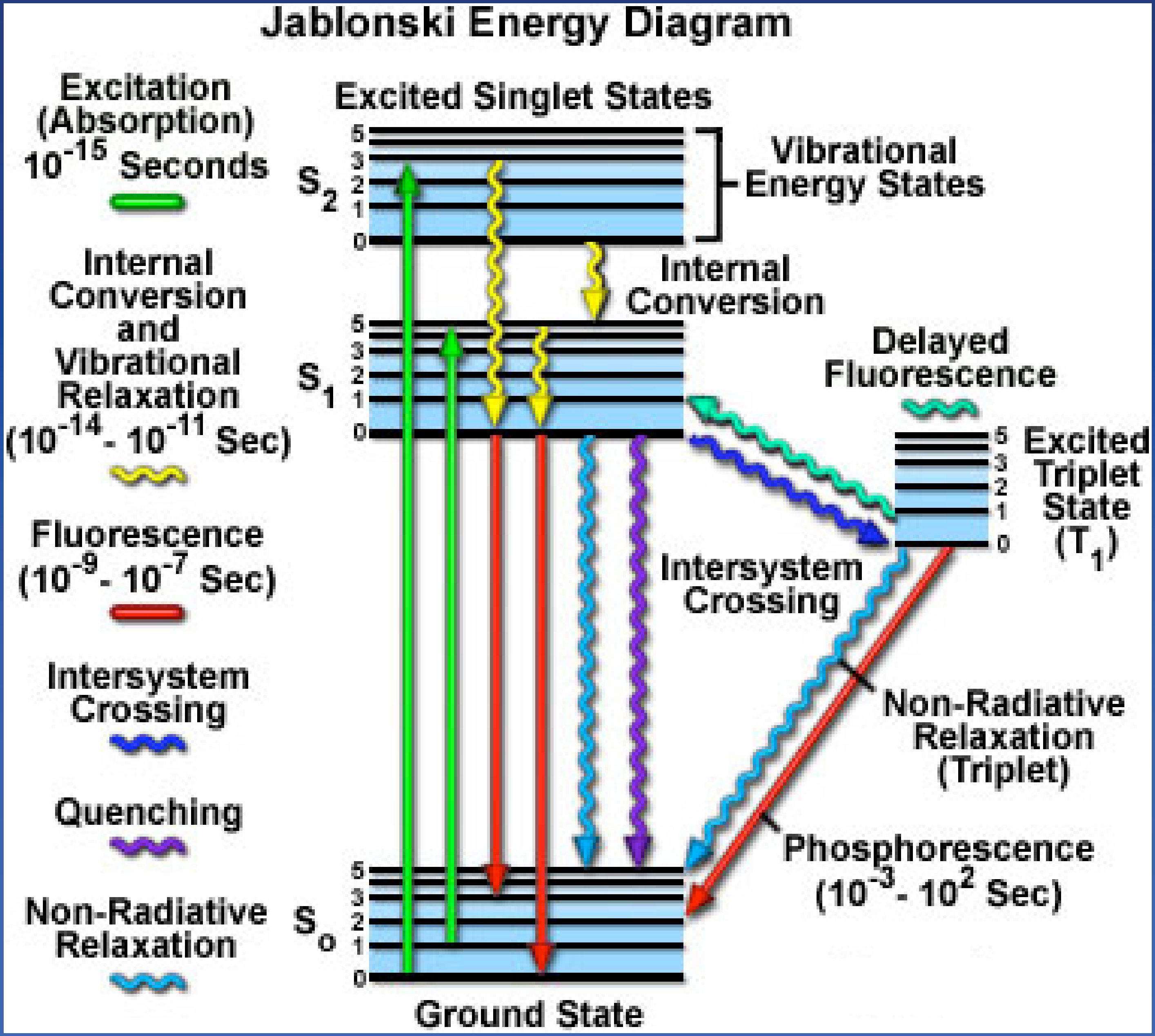




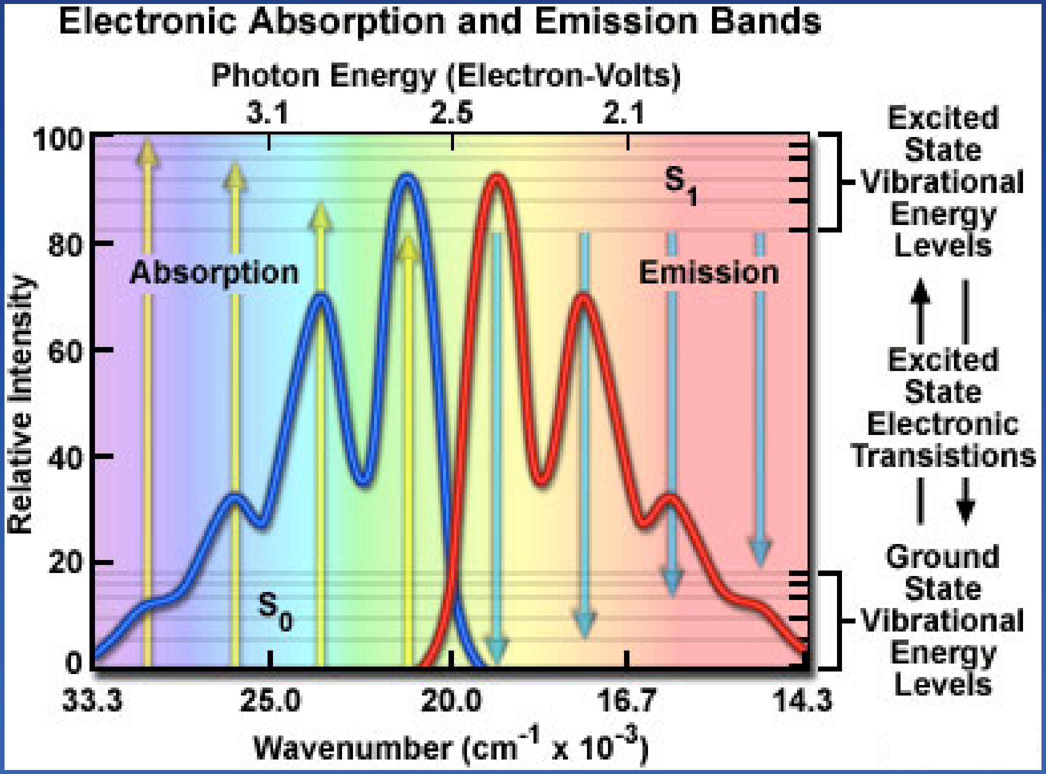
$\varepsilon = 9 \times 10^4\ M^{-1}cm^{-1}$ at $\lambda = 500$ nm

Rule of thumb: $\varepsilon_{maximum} \sim$ 20,000 x (number of rings)

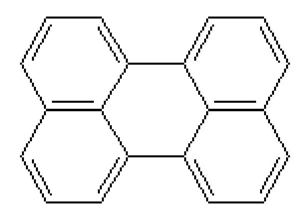
Example: $\varepsilon = 90{,}000\ M^{-1}cm^{-1}$ at $\lambda = 500$ nm for fluorescein


Jablonski Energy Diagram
Excitation (Absorption) 10^{-15} Seconds
Internal Conversion and Vibrational Relaxation (10^{-14} - 10^{-11} Sec)
Fluorescence (10^{-9} - 10^{-7} Sec)
Intersystem Crossing
Quenching
Non-Radiative Relaxation
Excited Singlet States
S_2
S_1
S_o
5
3
2
1
0
Vibrational Energy States
Internal Conversion
Delayed Fluorescence
Excited Triplet State (T_1)
Intersystem Crossing
Non-Radiative Relaxation (Triplet)
Phosphorescence (10^{-3} - 10^{2} Sec)
Ground State

Fluorescence in a "stiff" molecule



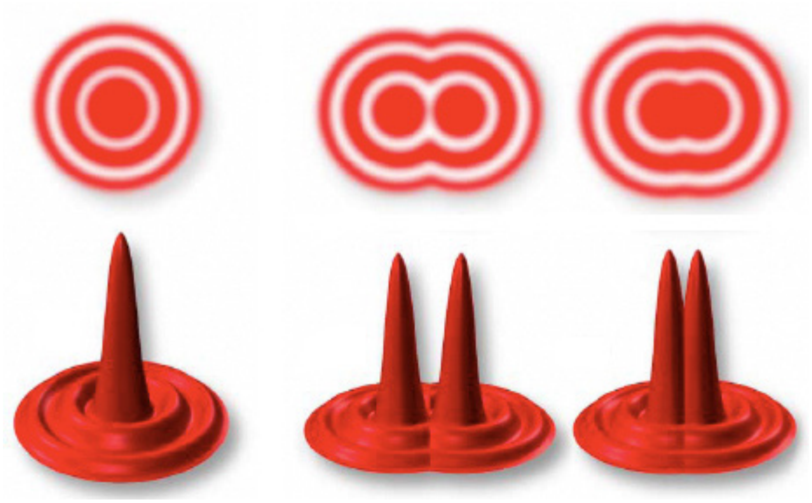
- Band structure due to |0⟩ and |1⟩ vibrational levels
- Ground state vibrational levels not populated at kT
- Mirror symmetry due to similarity of |0⟩ and |1⟩ vibrational modes



perylene

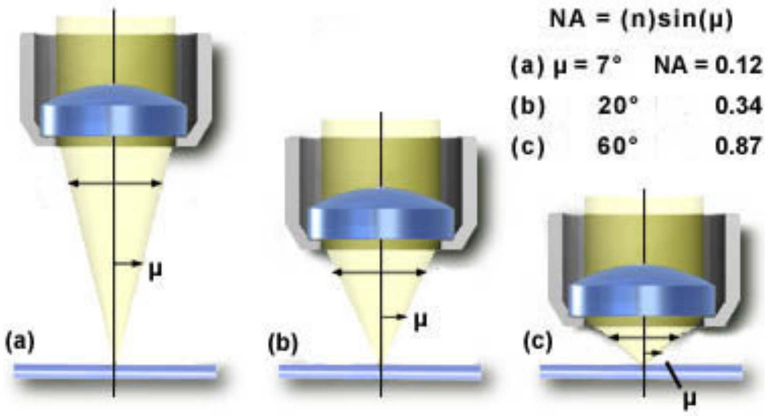
Spatial resolution: Set by the wavelength and geometry

What does a point object look like? Expect to visualize it as a dot of order one wavelength



First zero of pattern, J(r)/r, at r = λ x (0.6 / NA).

What is numerical aperture (NA)?



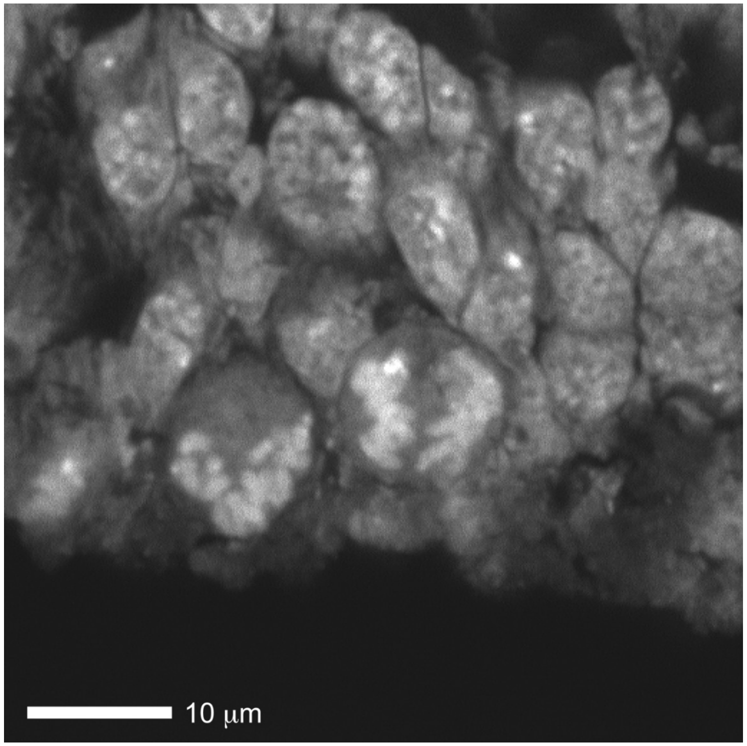
NA = (n)sin(μ)

	μ	NA
(a)	μ = 7°	NA = 0.12
(b)	20°	0.34
(c)	60°	0.87

At the very best, NA = 1 and r = 0.3 µm for green light.

Better resolution for samples embedded in high index media.

Sampling theorem requires ≥ 2 samples per resolution unit, so we need to sample at 0.15 µm.



For the example CCD with 7200 pixels on edge, the maximum field is 7200 x 0.15 µm = 1.1 mm.

In practice, most lenses do not have such a large fields

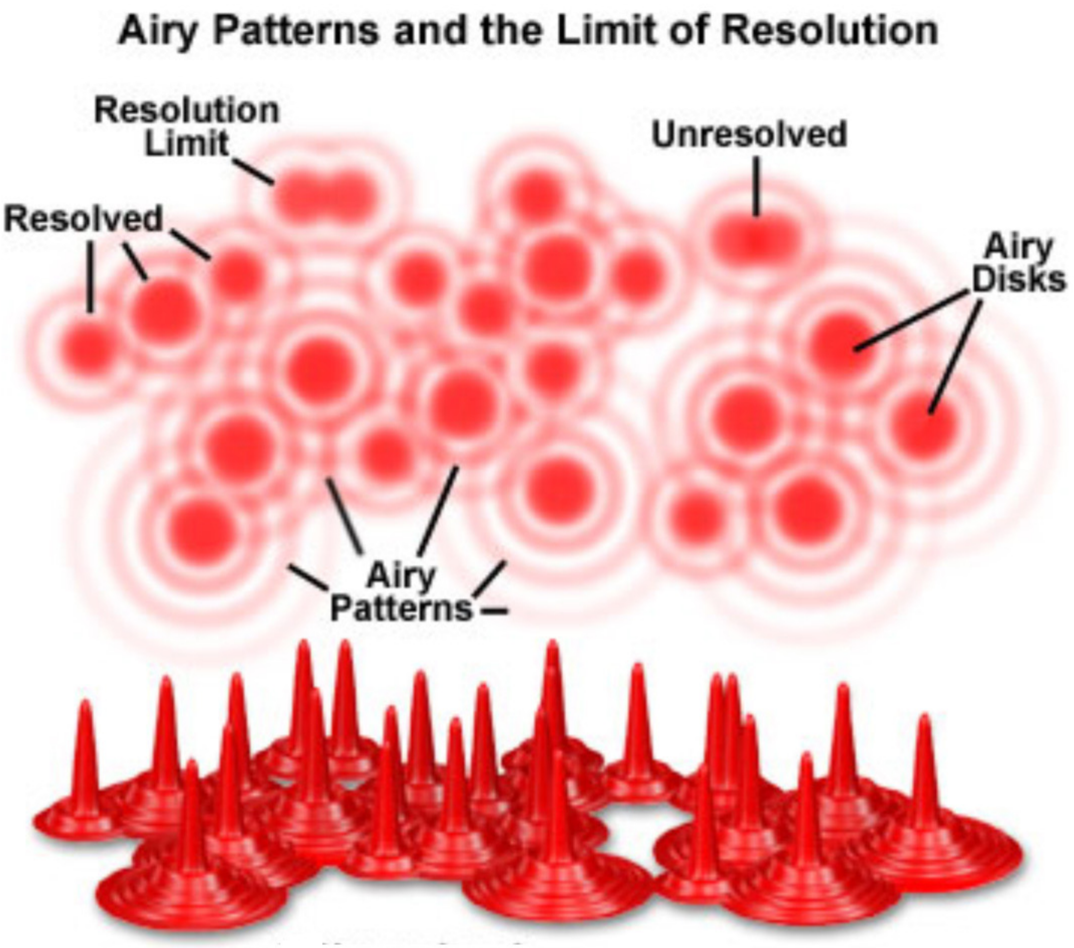
***e.g.*, Zeiss 40X 1.2 NA water objective has 500 µm field and r = 0.25 µm.**

This implies a CCD resolution of $(500/0.25/2)^2 = (4000)^2$ ~ 16 Mpixels; quite reasonable.

Detection versus discrimination

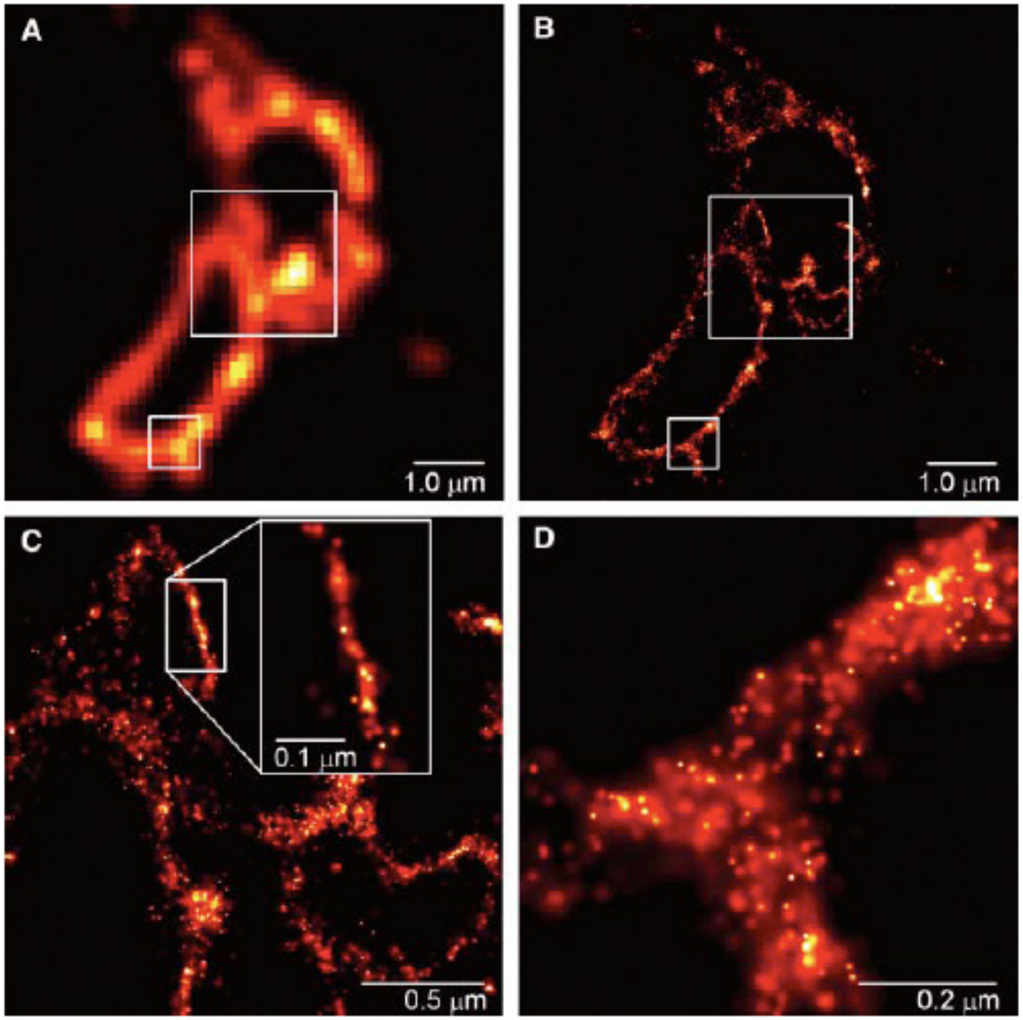
Detection has arbitrary accuracy.

Resolution implies a minimum distance between identical objects



PALM

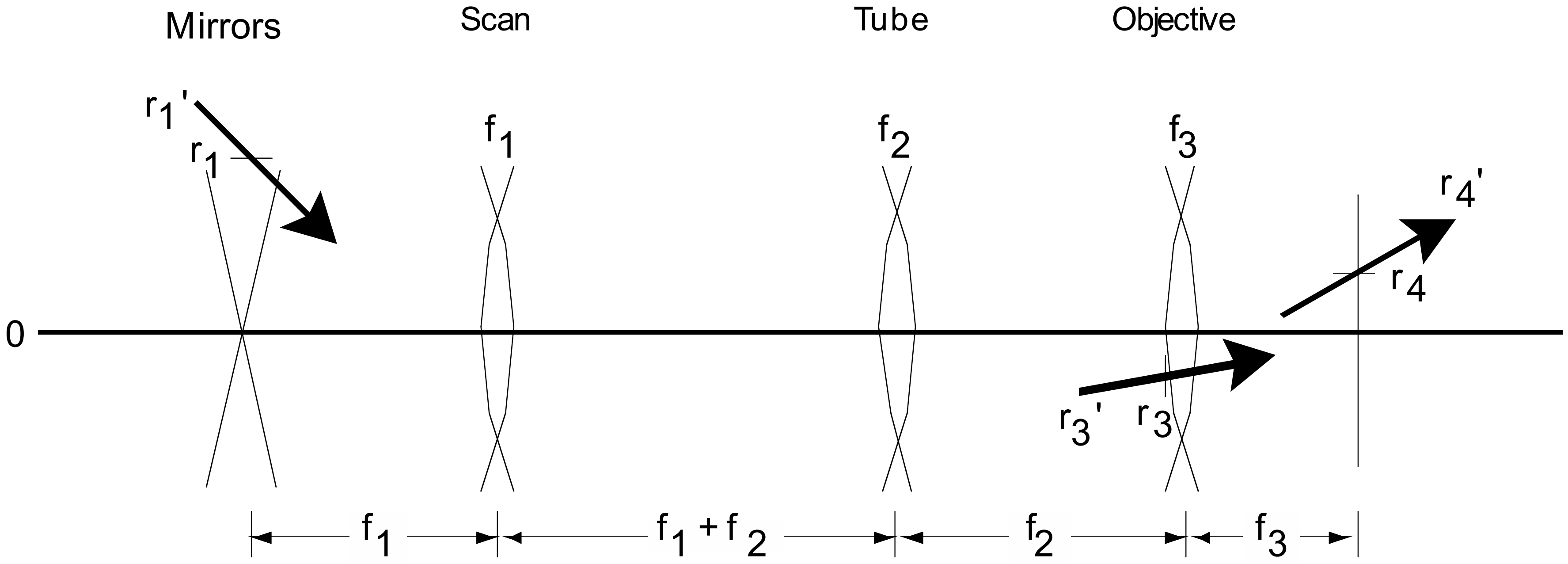
Imaging of overlapping, identical fluorophores that switched on at low density



Betzig, Patterson, Sougrat, Lindwasser, Olenych, Bonifacino, Davidson, Lippincott-Schwartz & Hess (Science 2006)
Rust, Bates & Zhuang (Nature Methods 2006)

Scanning microscopy for optical sectioning

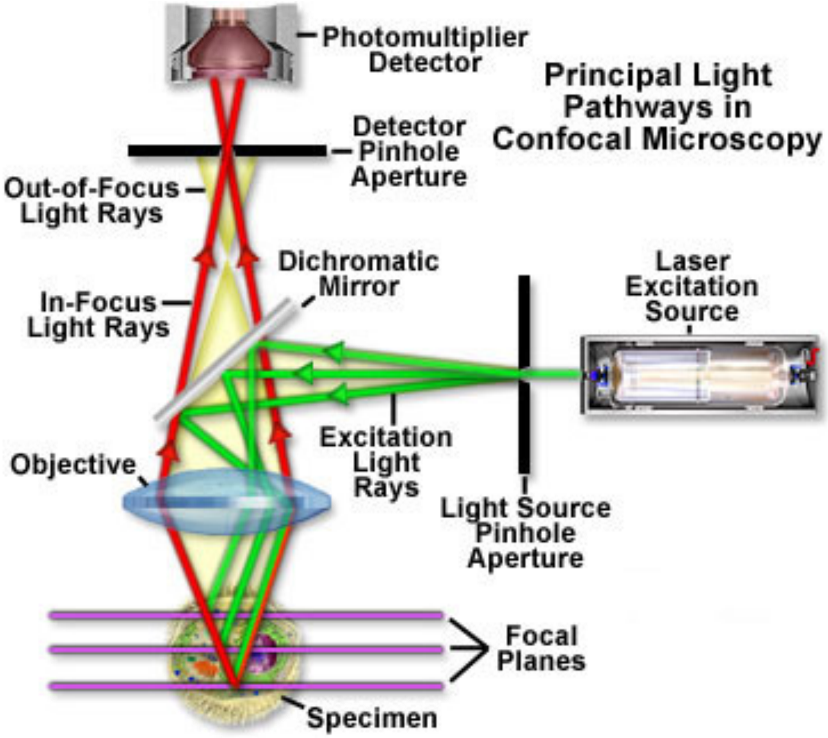
Pixel-by-pixel excitation for confocal, multiphoton, harmonic, STED, … , microscopy

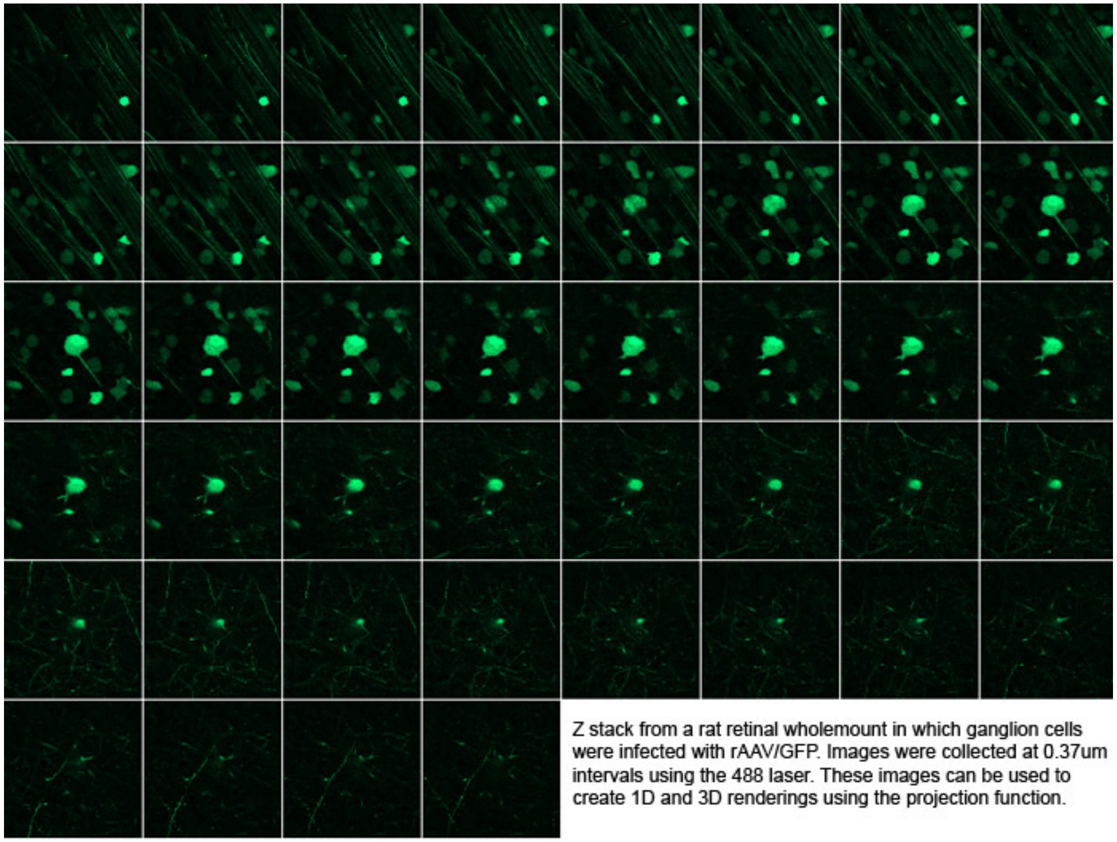


Change in slope of the mirror, r_1', is turned into a change in position in the focal plane, r_4,

with $r_4 = -f_3 \frac{f_1}{f_2} r_1'$

Confocal scanning microscopy (Minsky, Amos, …., *ça* 1960)

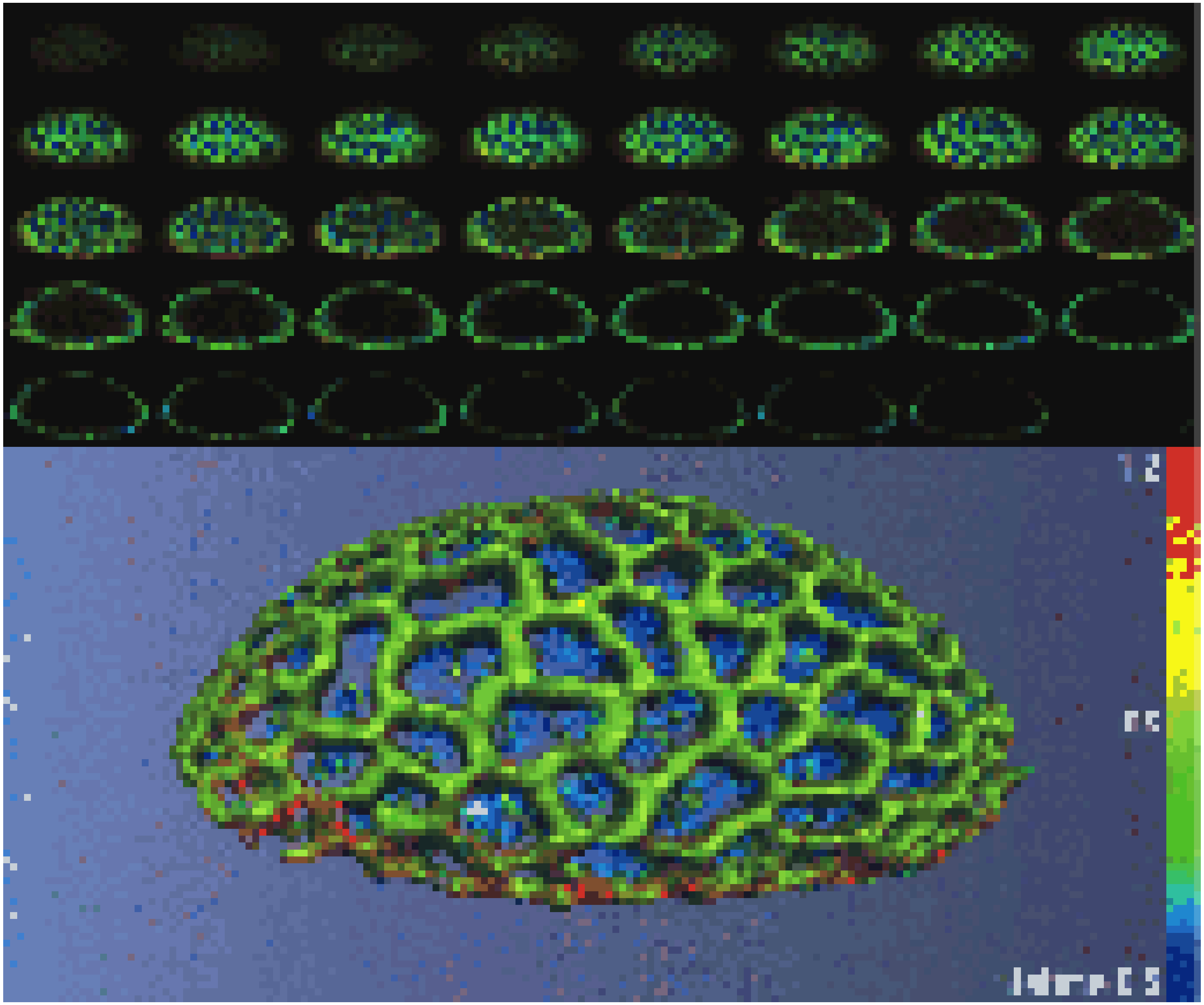




Z stack from a rat retinal wholemount in which ganglion cells were infected with rAAV/GFP. Images were collected at 0.37um intervals using the 488 laser. These images can be used to create 1D and 3D renderings using the projection function.

Each image has a depth of field that is given by $\Delta z \sim 2\lambda / (NA)^2$

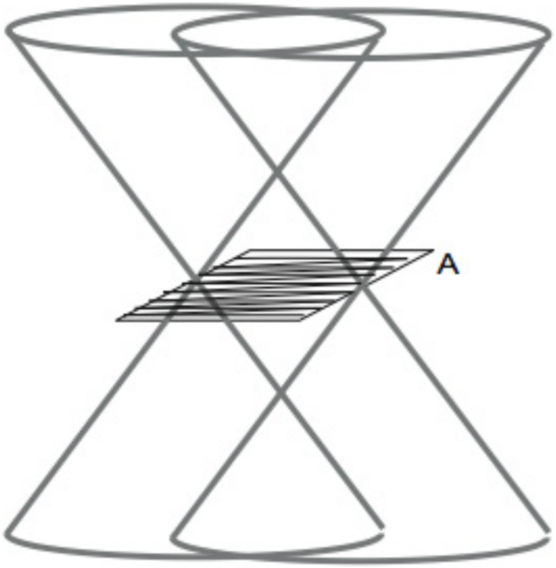
Images must be reassembled to form a 3-D view

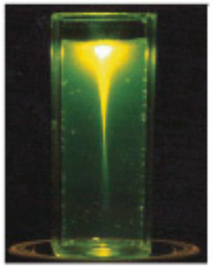
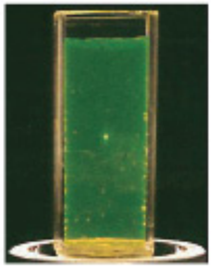

1.2
0.5

Two-photon laser scanning microscopy (Denk *et al*. Science 1990)

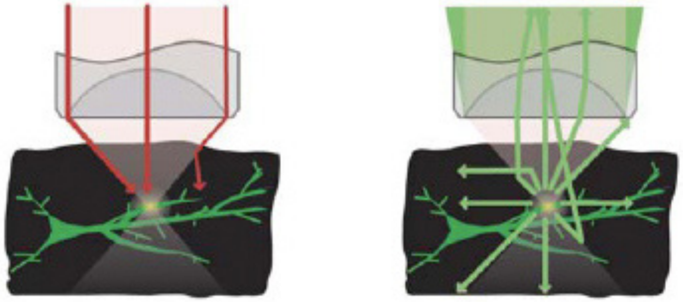
Integrated Absorption Probability

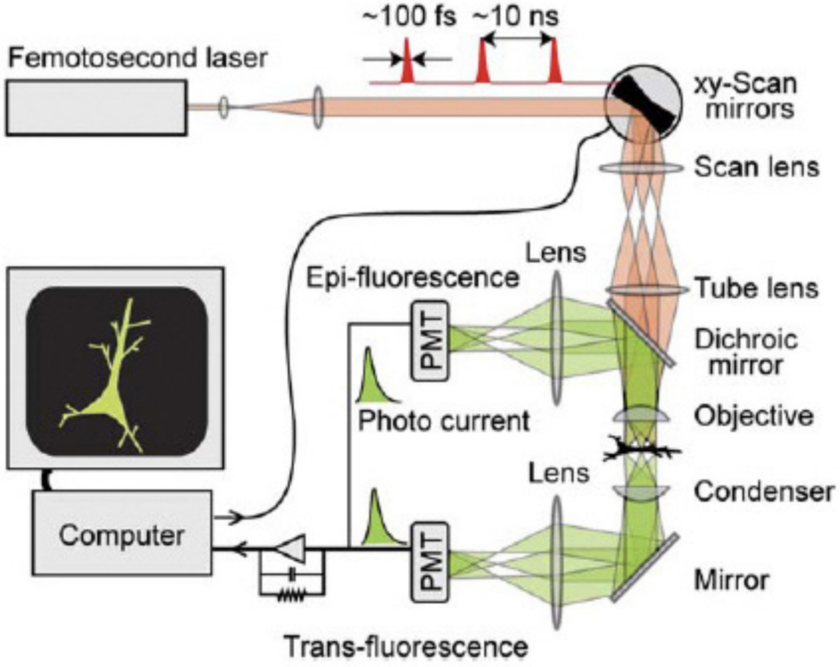
Area A is scanned in a time t with power P

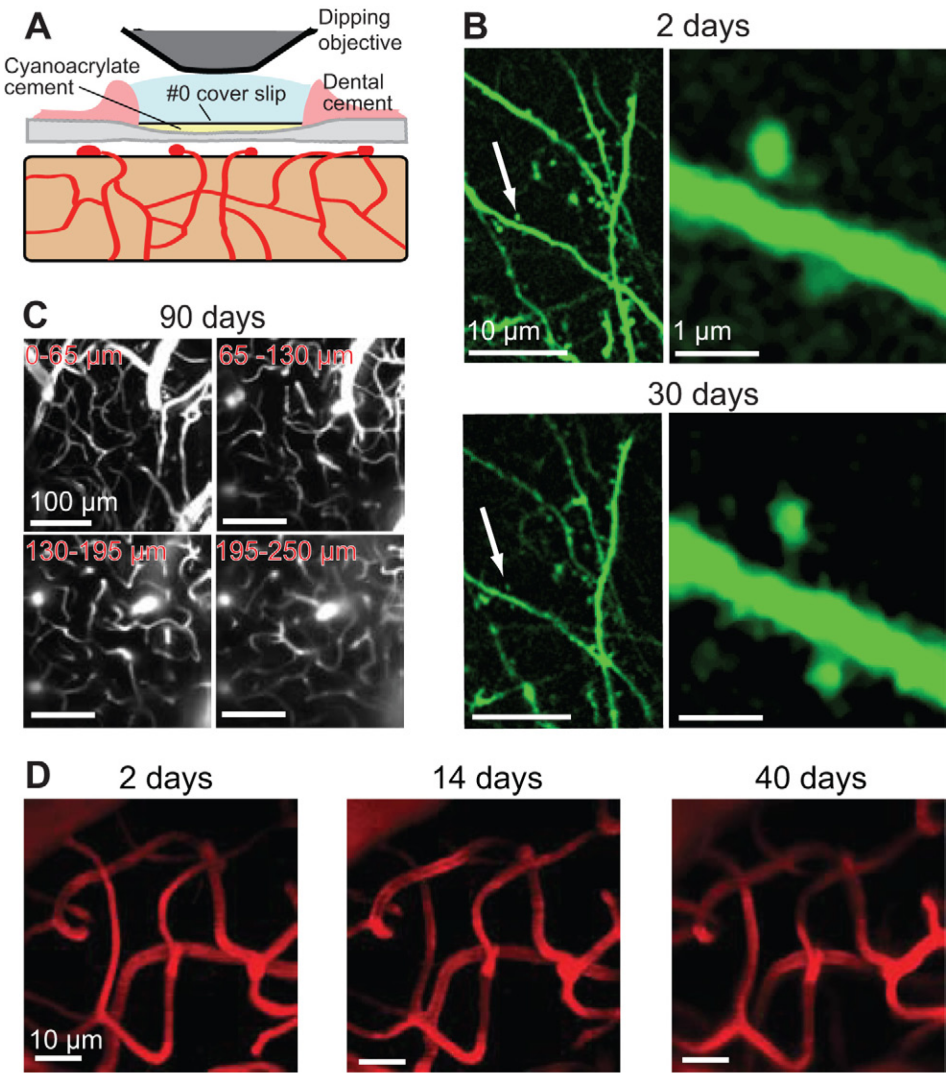


	One photon ($N_{absorption} \sim P\ t$)	Two photon ($N_{absorption} \sim P^2\ t$)
Out of focus	$\frac{P_o}{A} A = P_o$	$\left(\frac{P_o}{A}\right)^2 A = \frac{P_o^2}{A}$
In focus	P_o	P_o^2
In focus Out of focus	~ Constant 	$\frac{1}{A}$ 

In vitro and *in vivo* two-photon microscopy for structural studies

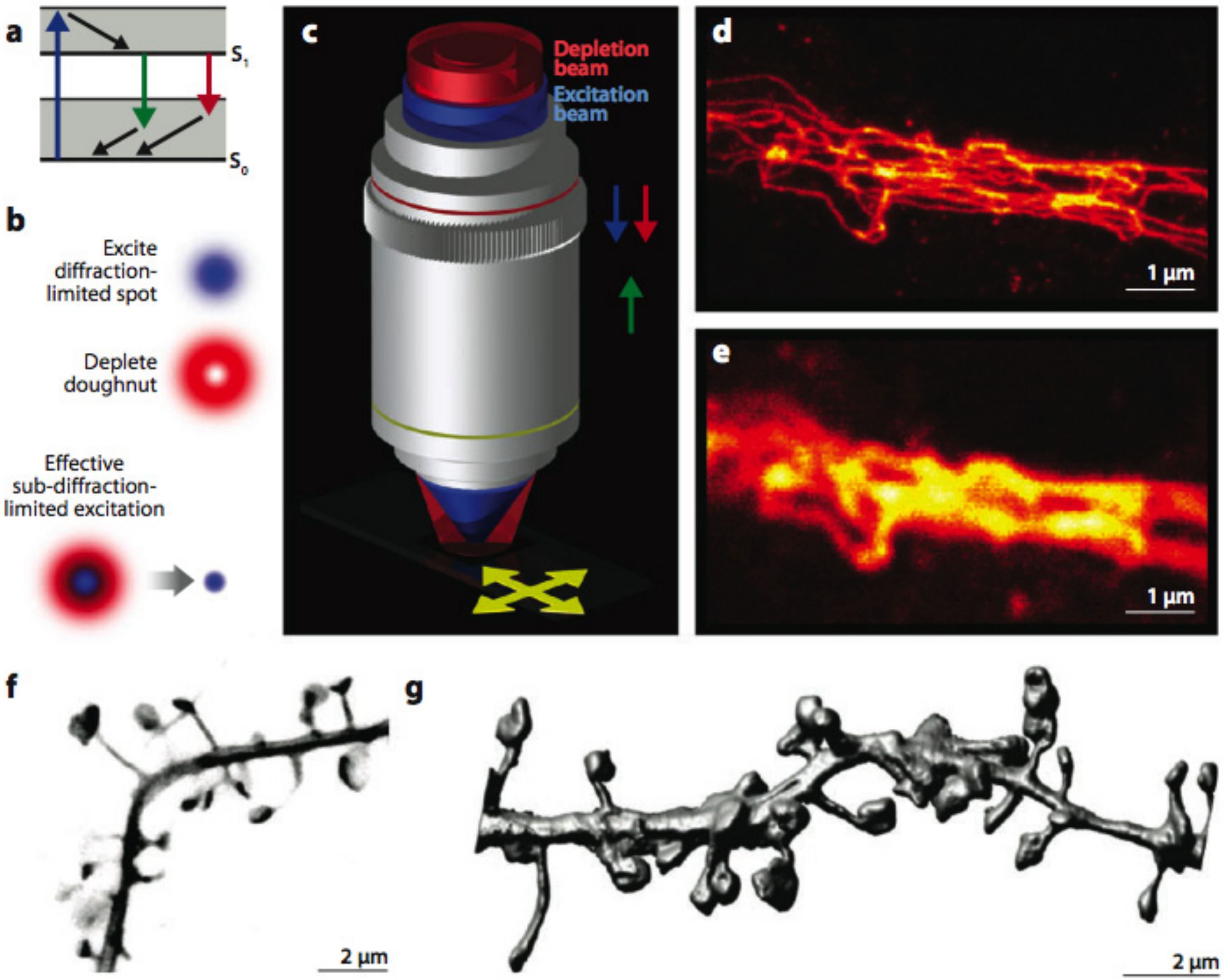




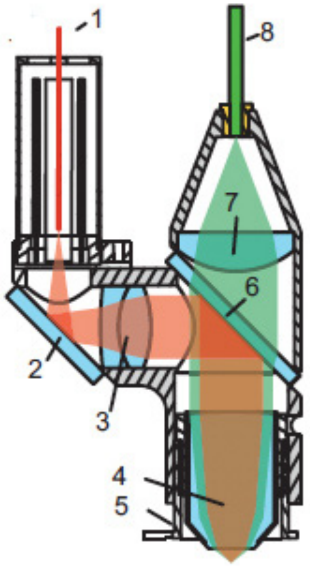


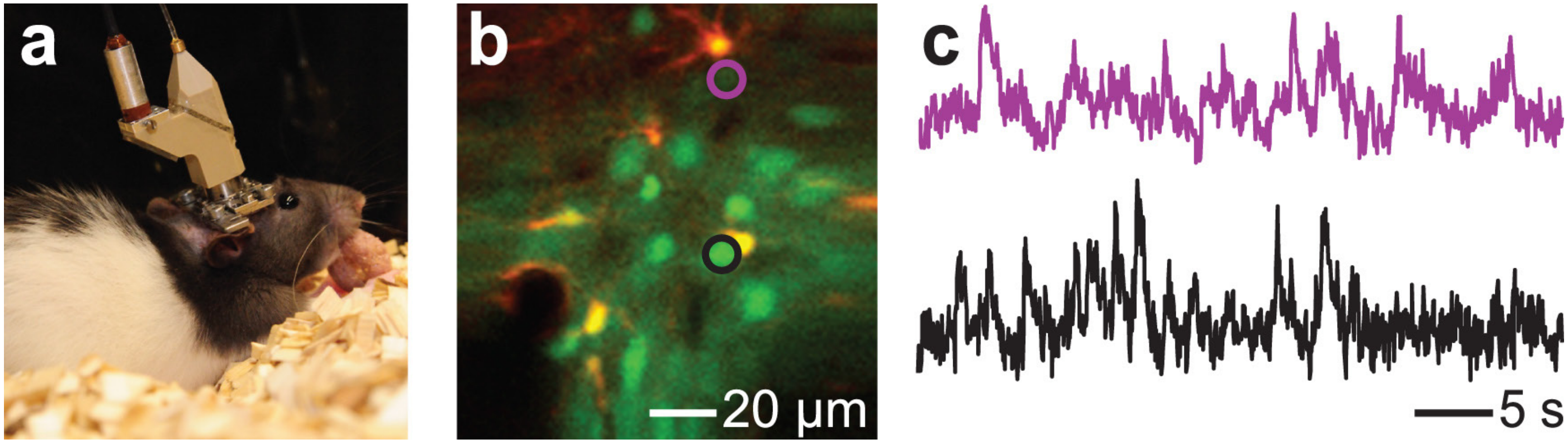
Currently, one typically images 500 µm deep and can image to 1000 µm with special equipment.

Super-resolution scanning microscopy through stimulated emission: STED (Hell & coworkers 1994)



*T*wo-photon microscopy with free-ranging animals for cell function



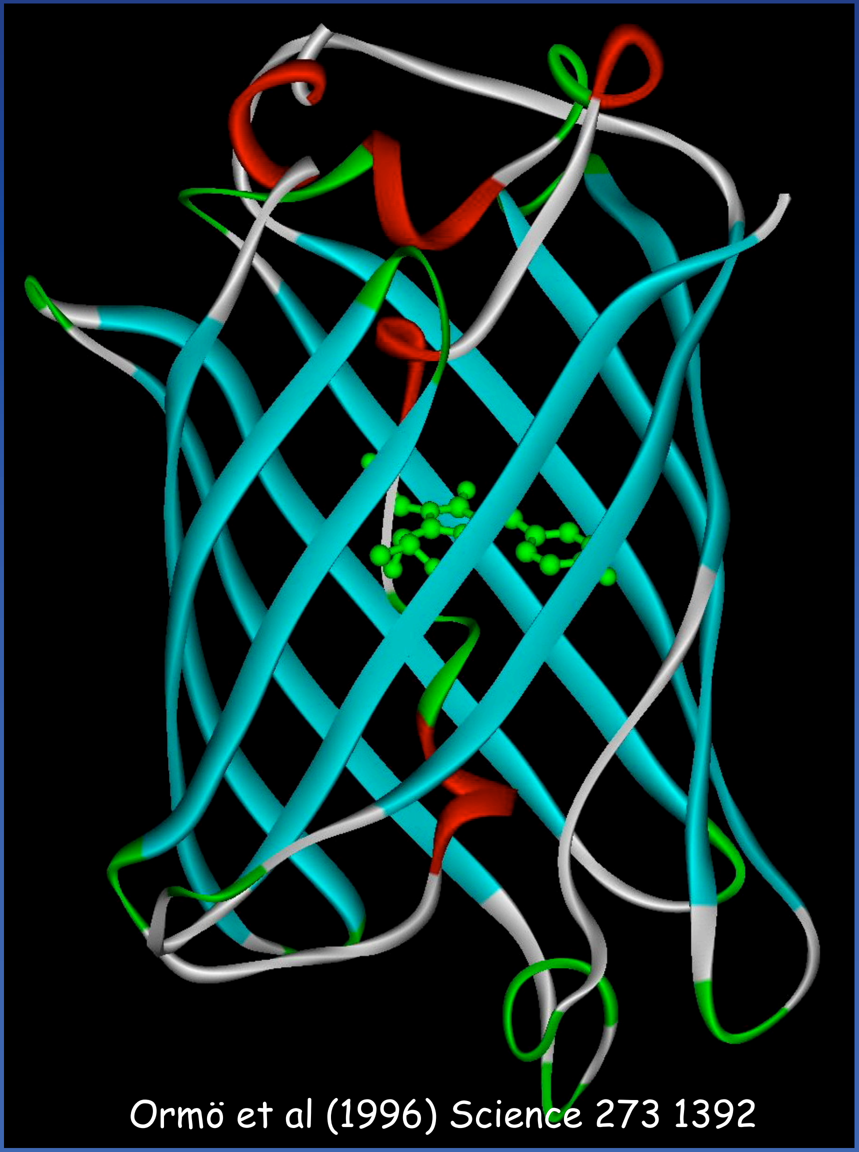


Sawinski, Wallace, Greenberg, Grossmann, Denk & Kerr (2009 PNAS)

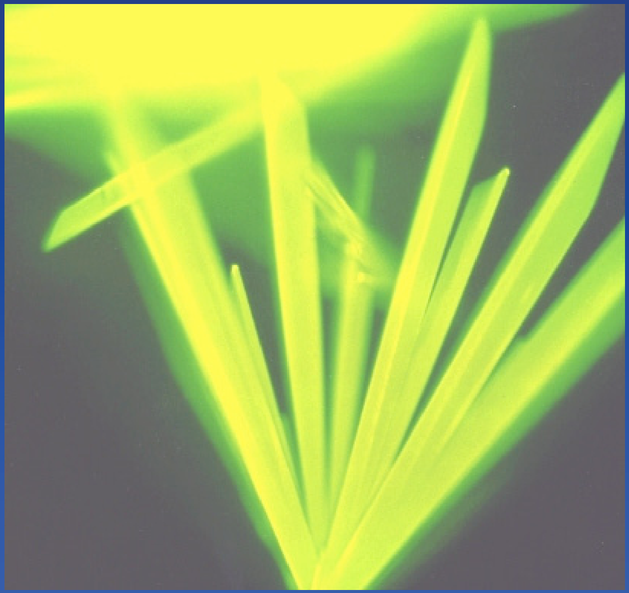
The World of XFPs

www.uwphoto.no © Erling Svensen

Structure of S65T GFP (EGFP)

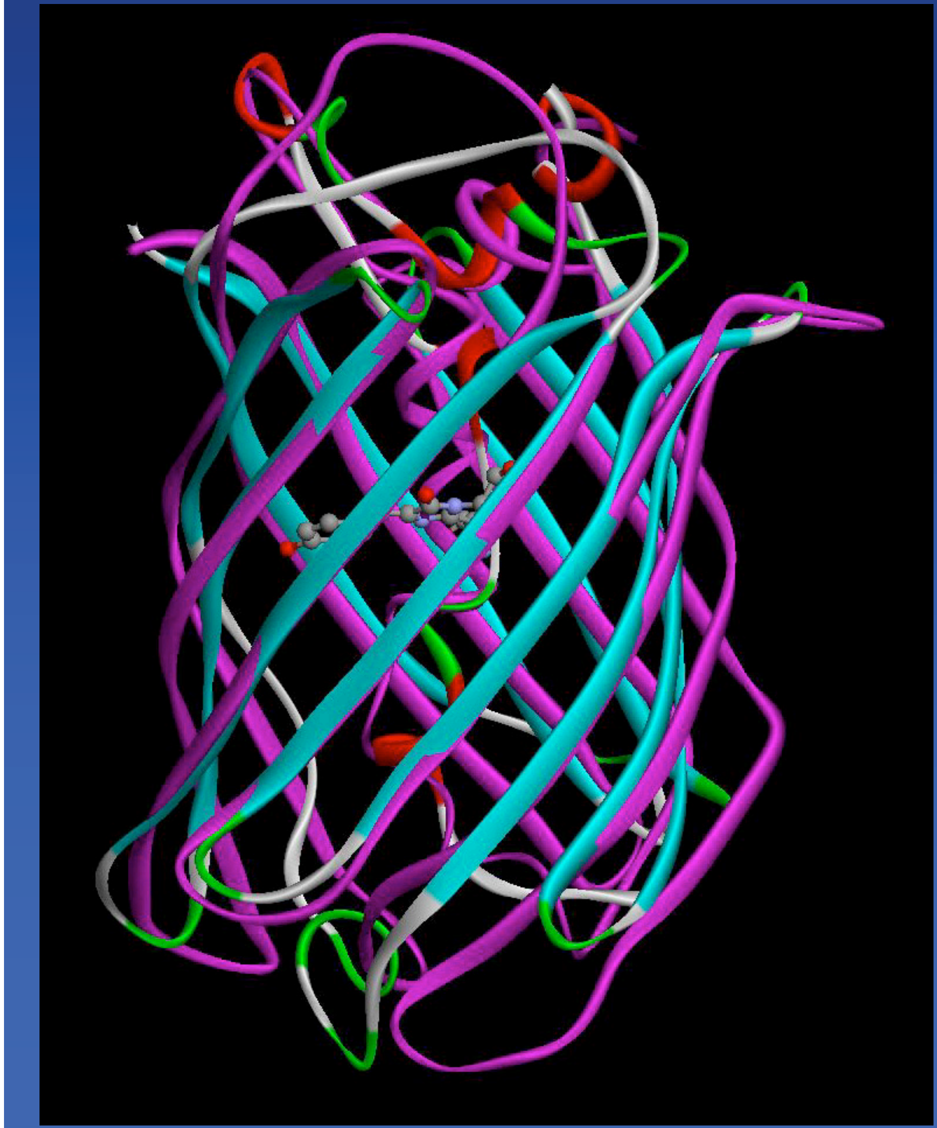


Ormö et al (1996) Science 273 1392



- 11 stranded ß-barrel surrounds chromophore on central helix
- Chromophore rigidly held and protected from environment
- Protein fold required for fluorescence

THE GFP fold is not unique



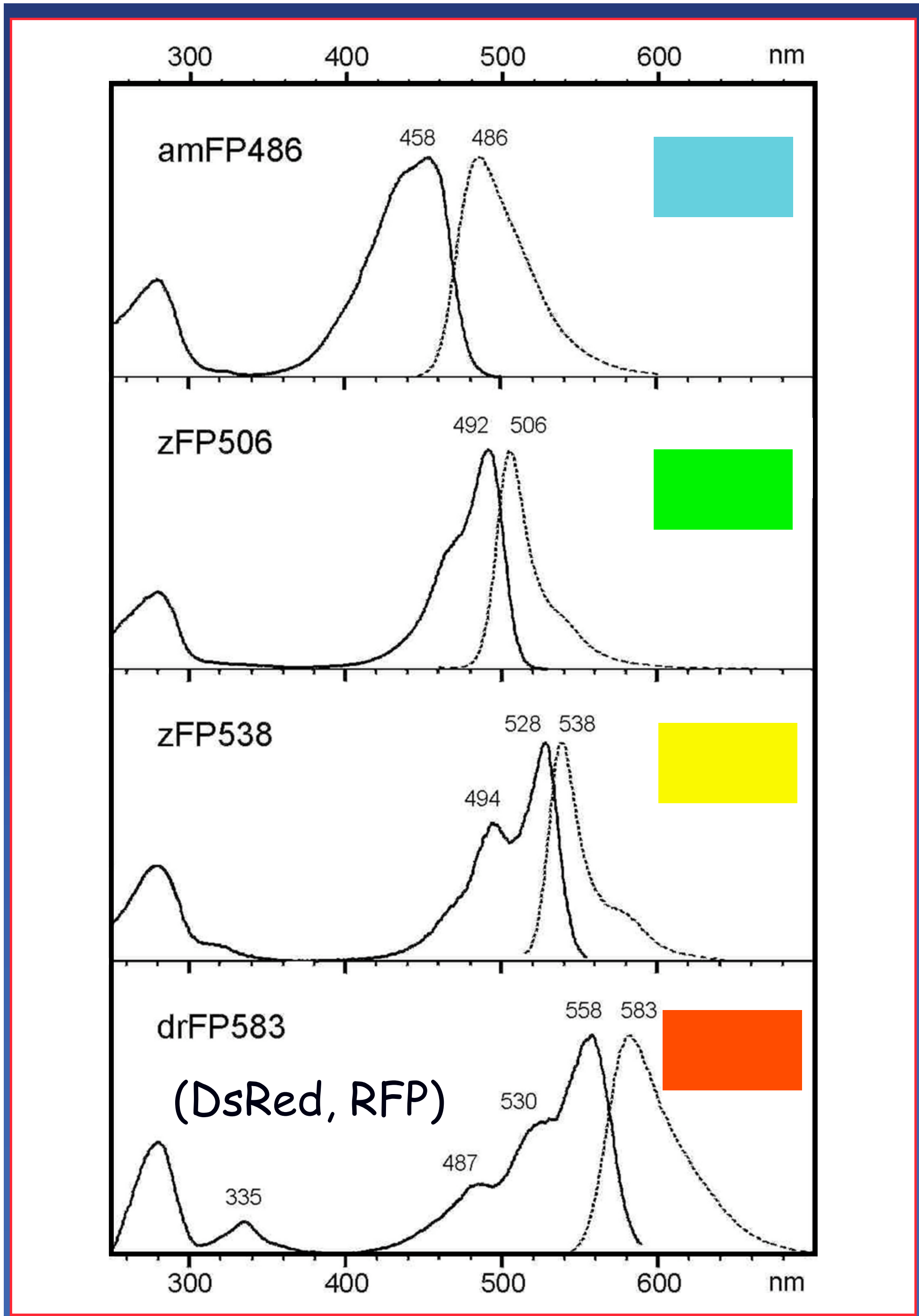
purple:

Nidogen-1 G2 domain of mouse basement membrane complex

cyan: GFP

Hopf, et al. Nat Struct Biol. 2001 8: 634.

1999: Reef GFPs

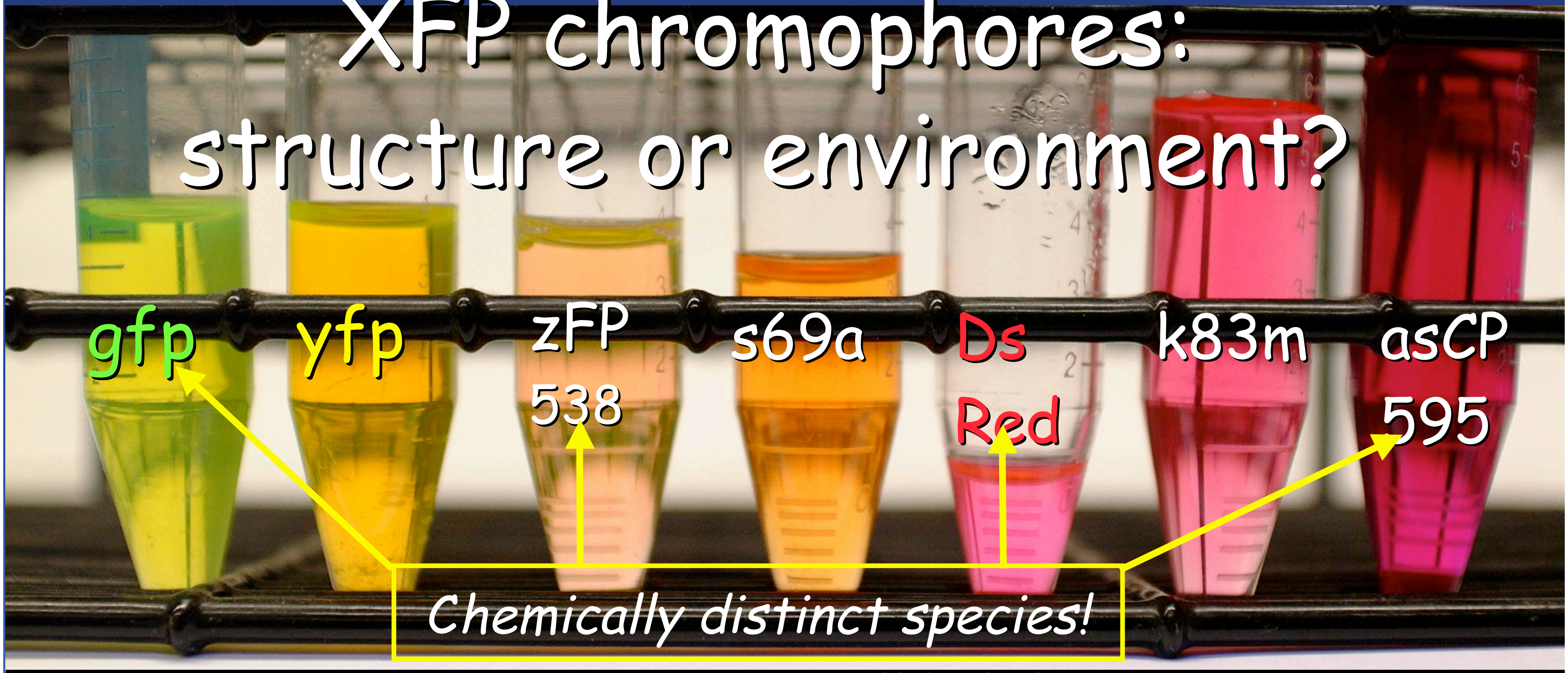


Red mushroom coral

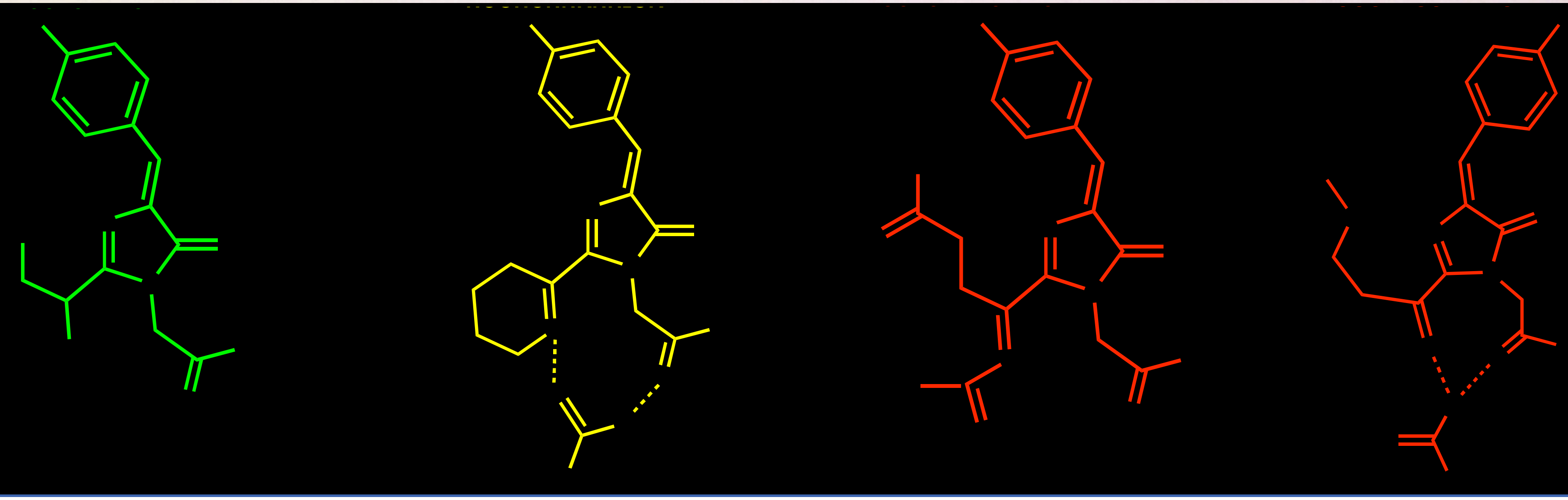
Four color classes,
same chromophore!
(Xaa-Tyr-Gly)

Matz et al. (1999) Nature Biotech. 17, 969-973

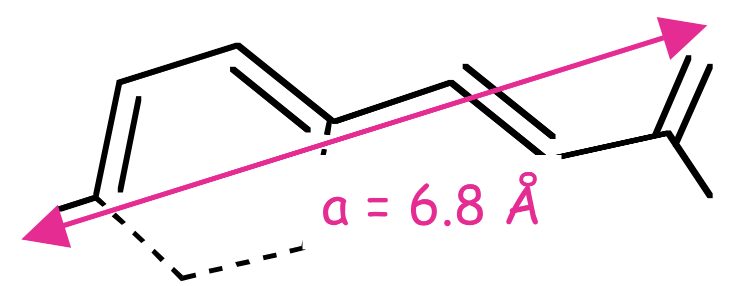
XFP chromophores: structure or environment?

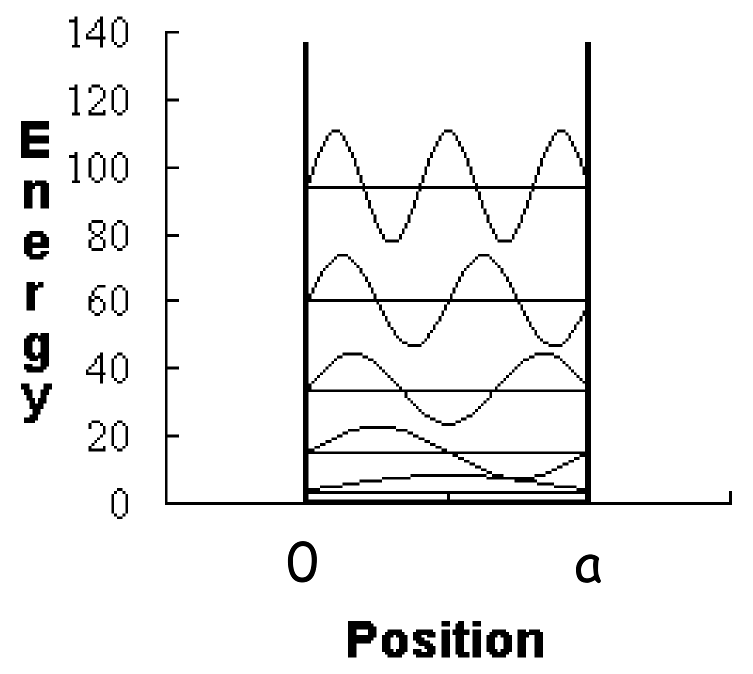


Chemically distinct species!



"Infinite potential well" approximation: electron in a box





$E_n = n^2h^2/8m_ea^2$

Light absorption:

$E_{1-2} = 3h^2/8m_ea^2$

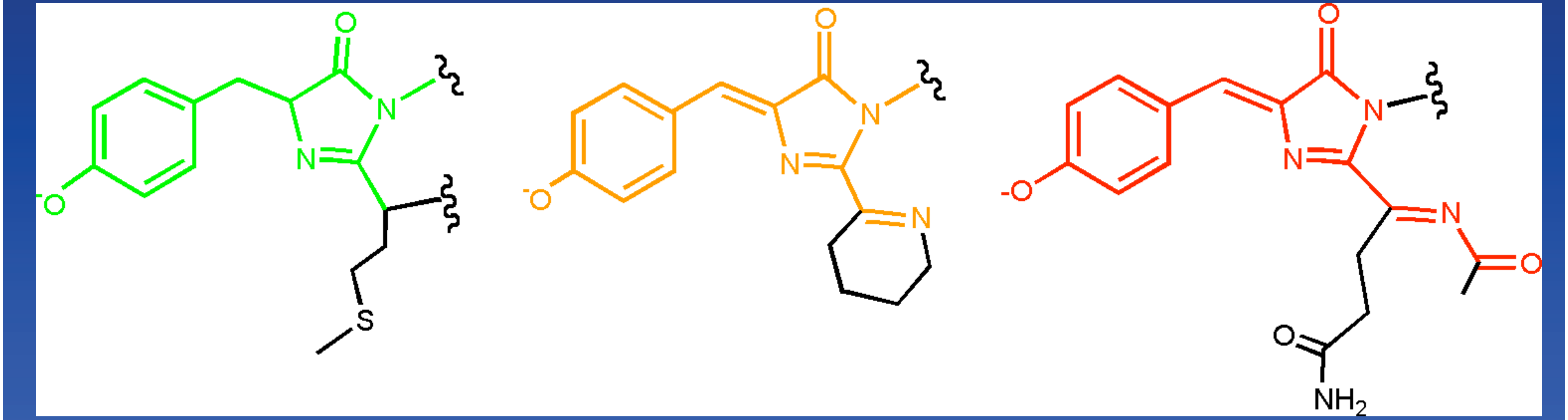
$E = h\nu$ *(Einstein-Planck)*

λ_{obs} = 475 nm (anion)

λ_{calc} = $\underline{505\ \text{nm}}$

longer molecule ->
longer wavelengths

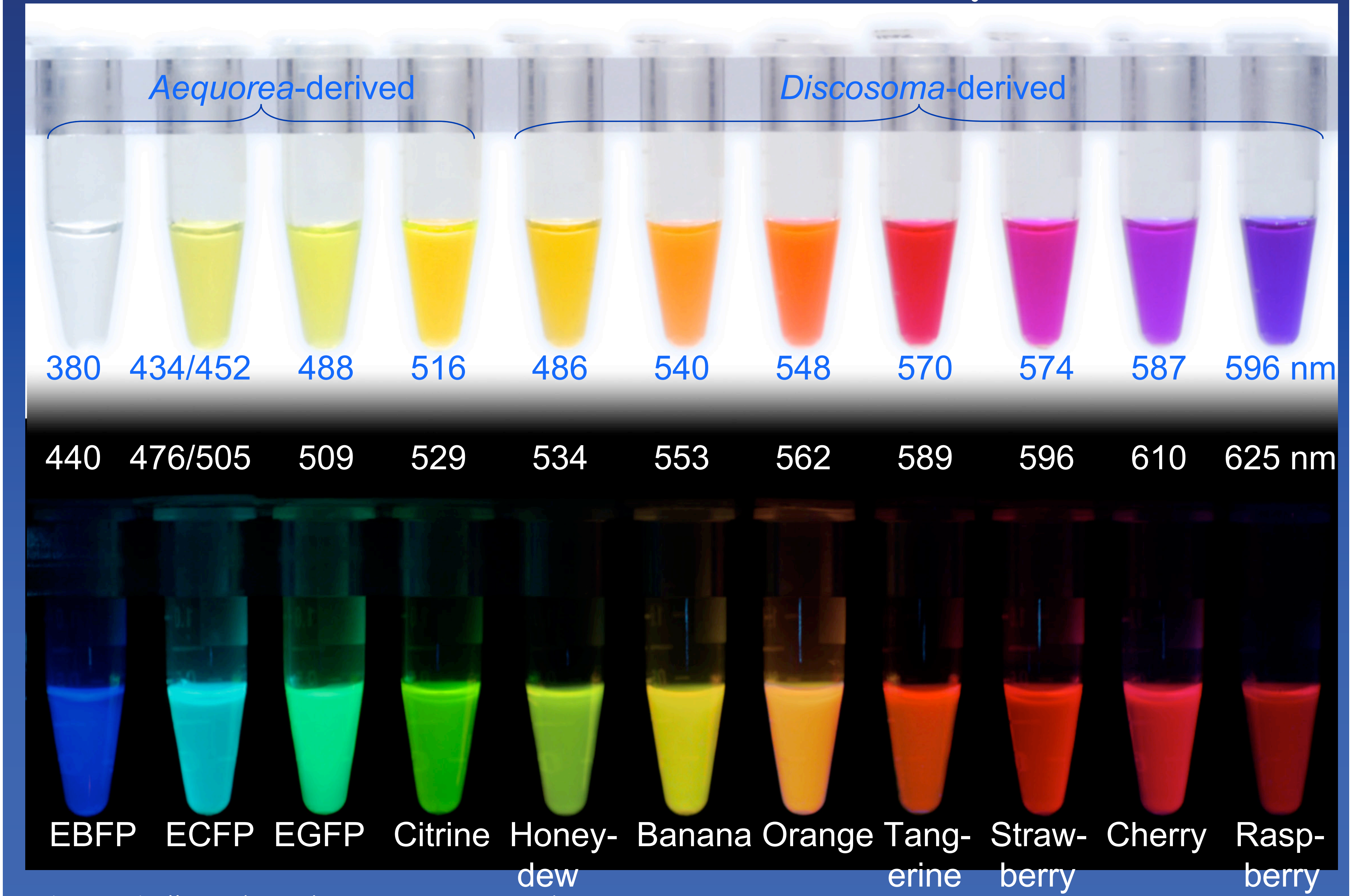
Structural basis for color class



510 nm	538 nm	585 nm
GFP	zFP538	DsRed

Model: extent of chromophore conjugation
is responsible for the emission class

Intoduction to fluorescence: Example of XFPs



Rob Campbell, Nathan Shaner, Lei Wang and Roger Y. Tsien

Biosensor design with GFP

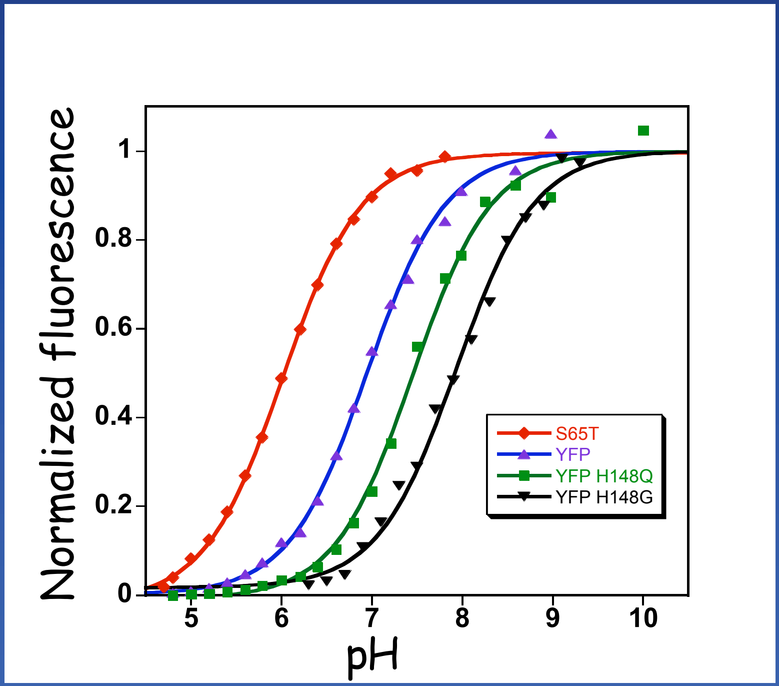

Ormö et al (1996) Science 273 1392

"The bulge", tolerates insertion of ENTIRE PROTEINS!

His148 interacts with chromophore

"The bulge" can control fluorescence

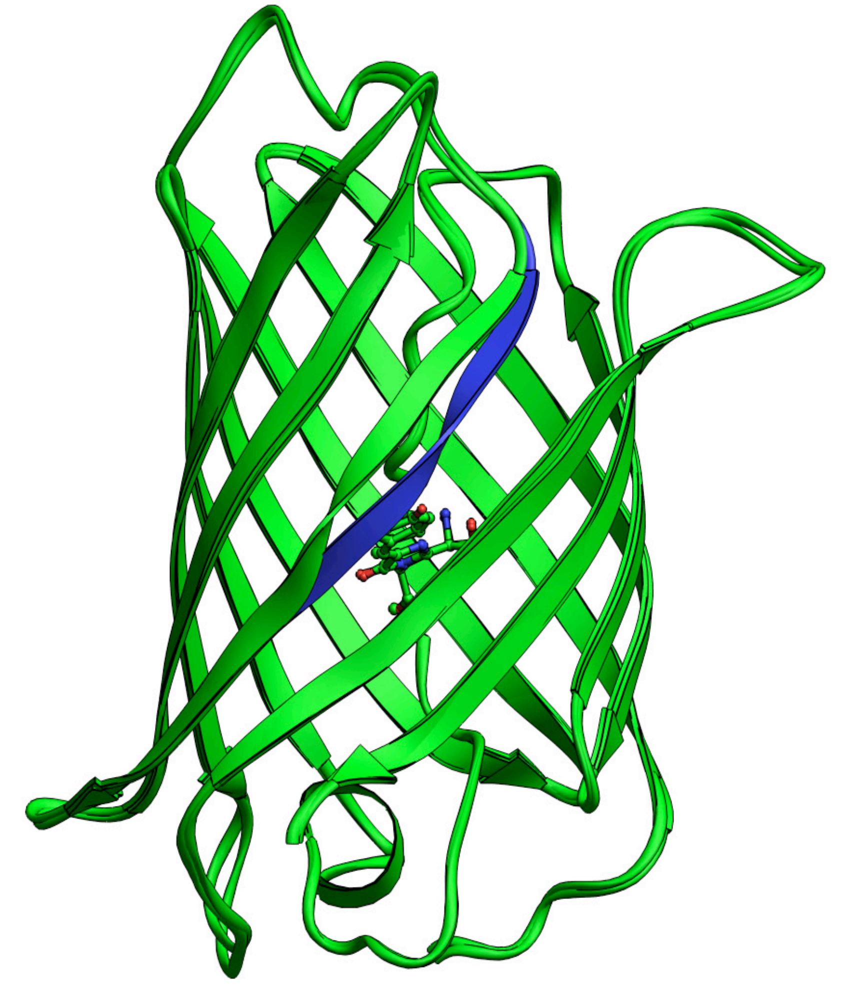
Mutants are pH sensitive

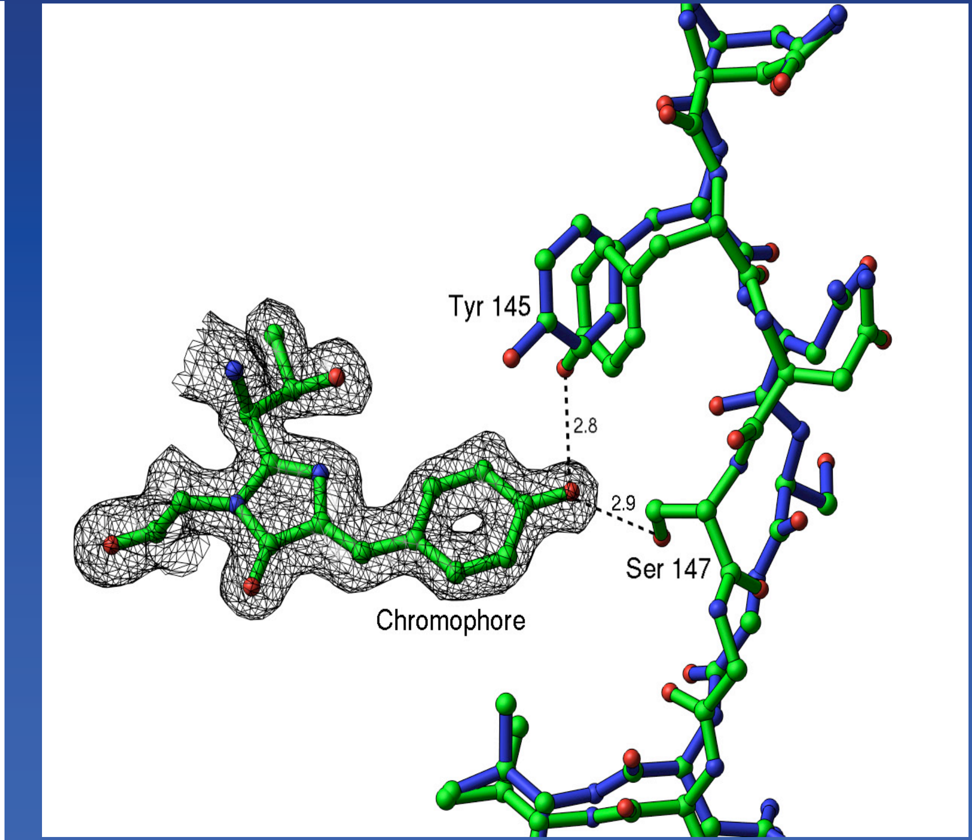


Mutant	pKa
S65T	6.0
YFP	6.9
YFP/H148Q	7.4
YFP/H148G	7.9
S65T/H148D	7.9

- Crystal structures show that chromophore titrates directly.
- Span the physiological range of 5 - 9. *But Dark at low pH*

Crystal structures of deGFP1 at high pH (1.8 Å) and low pH (1.5 Å) pH

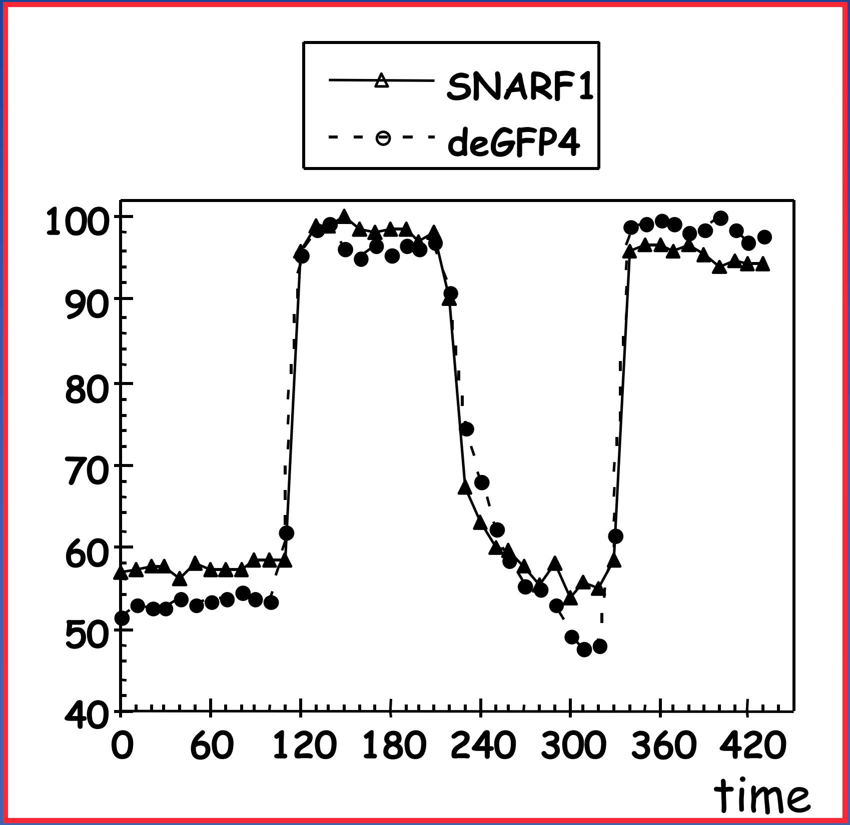




Hanson et al. Biochemistry 41, 15477 (2002)

strand rearrangement at "the bulge"

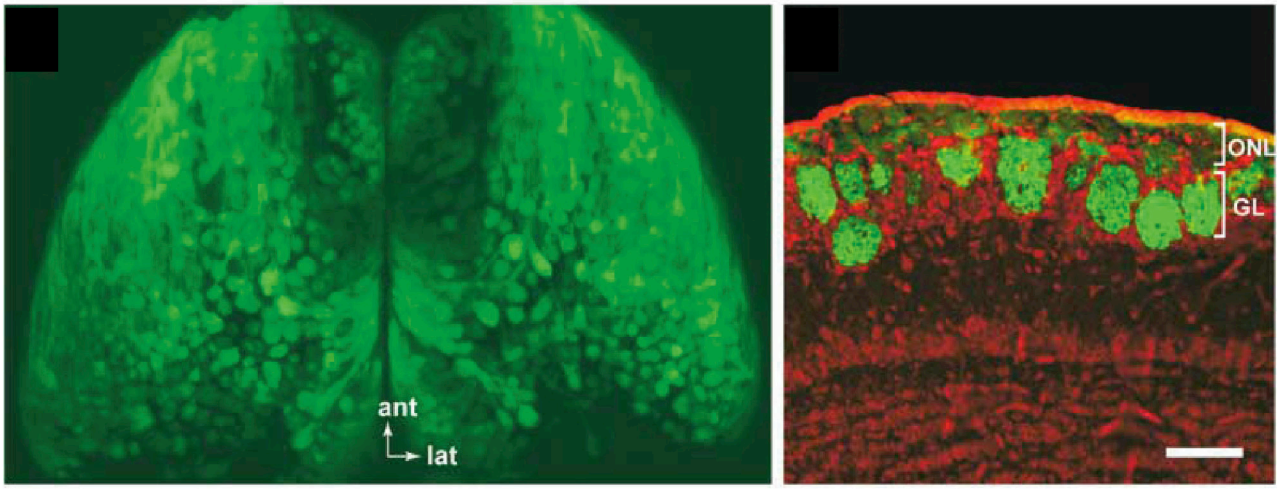
Δ pH Single-Cell Imaging
deGFP4 vs SNARF-1

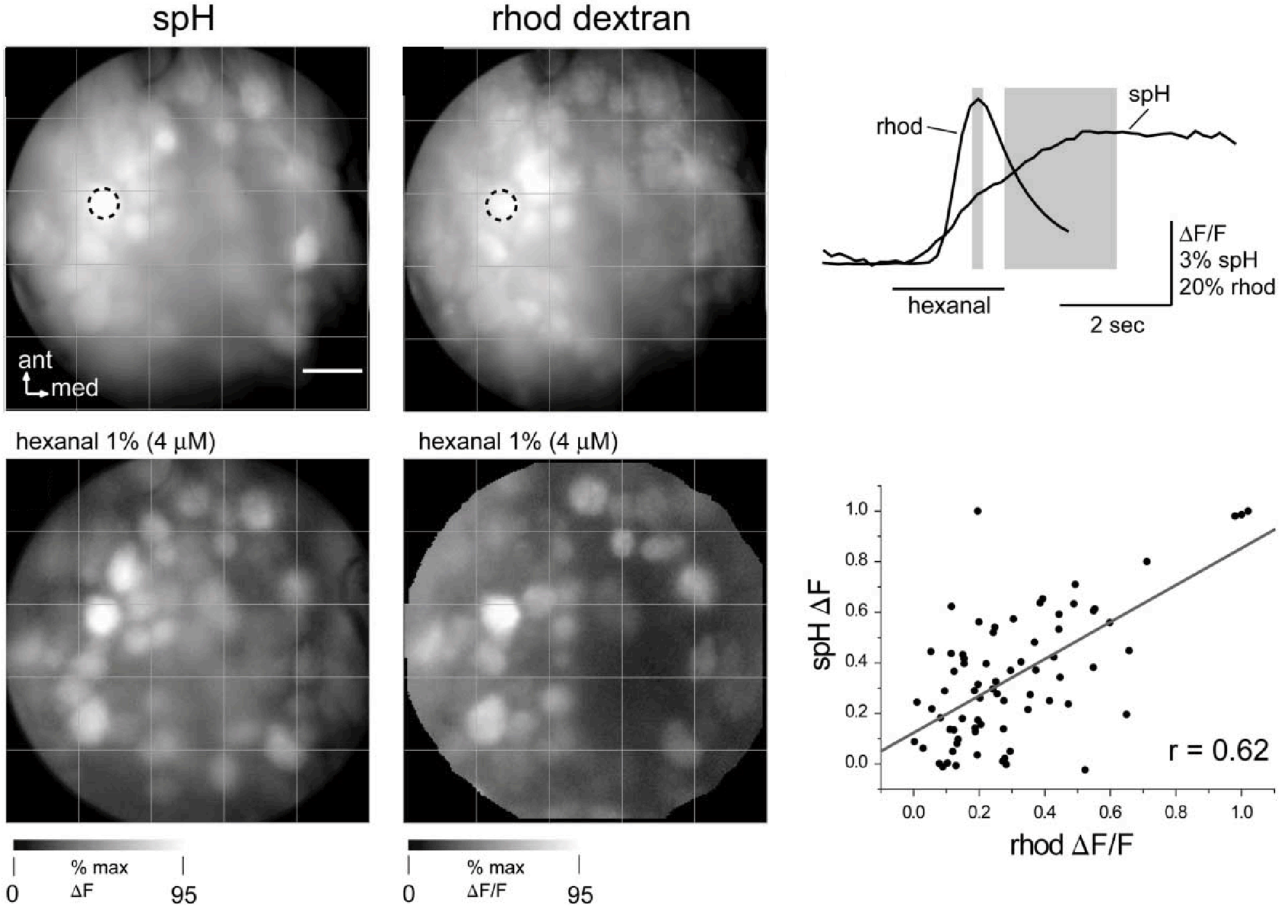


deGFP4 has comparable response to commercial dye. BUT, it can be targeted to organelle!

Hanson et al.
(Biochemistry)

SynaptoPhluorin as a Tool to Map Odor Responses in Olfactory Receptor Neurons *In Vivo*





T. Bozza, J. P. McGann, P. Mombaerts and M. Wachowiak, Neuron 2004