http://spectrum.mit.edu/wp-content/images/2011-fall/smarter-quantum-dots.jpg

Module 2 overview

lecture	*lab*
1. Introduction to the module	1. Start-up protein eng.
2. Rational protein design	2. Site-directed mutagenesis
3. Fluorescence and sensors	3. DNA amplification
4. Protein expression	4. Prepare expression system
SPRING BREAK	
5. Review & gene analysis	5. Induce protein
6. Purification and protein analysis	6. Purify protein
7. Binding & affinity measurements	7. Characterize expression
8. High throughput engineering	8. Assess protein function

Lecture 3: Fluorescence and sensors

I. Basics of fluorescence
   A. Important applications
   B. Energy levels and spectra
   C. Emission, quenching, and energy transfer

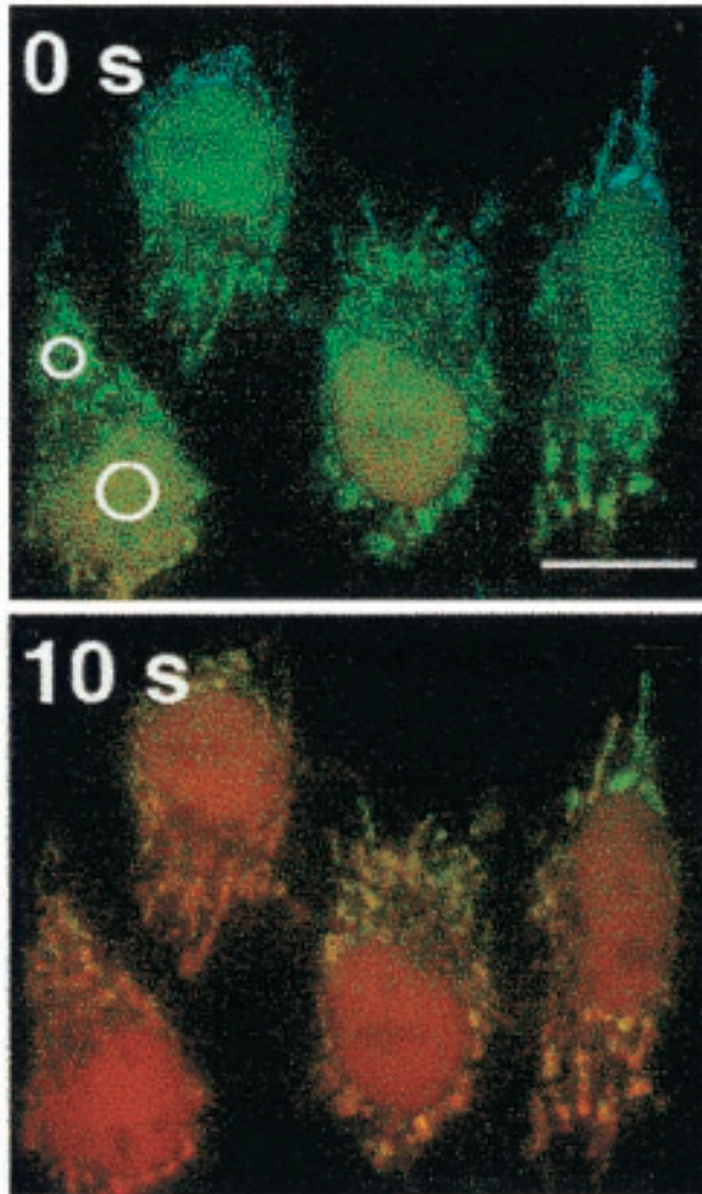
II. Fluorescent calcium sensors
   A. Properties of calcium sensors
   B. Applying Ca^{2+} sensors for imaging
   C. Genetically-encoded sensors

http://www.caliperls.com

www.fireflysci.com

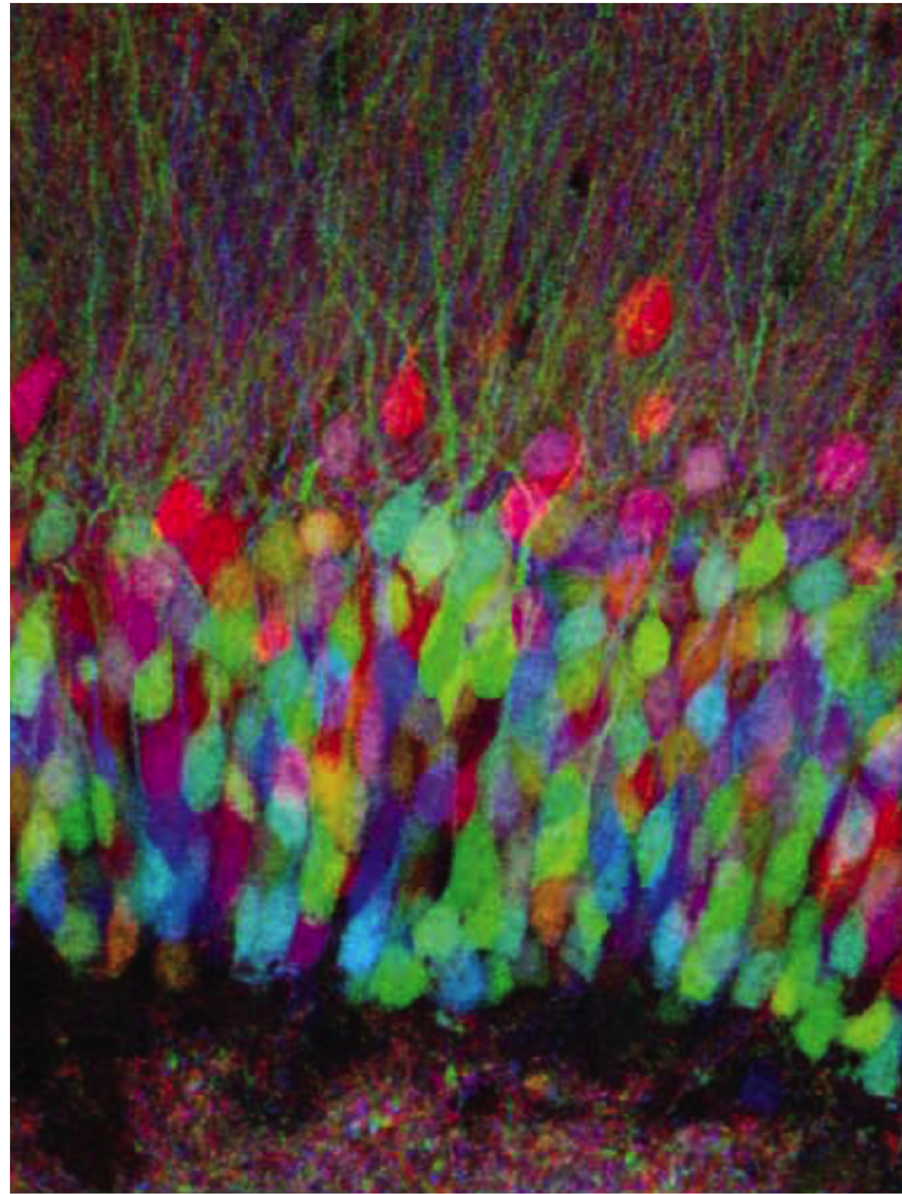
in vitro assays

functional imaging



Nagai *et al.* (2001) *PNAS*

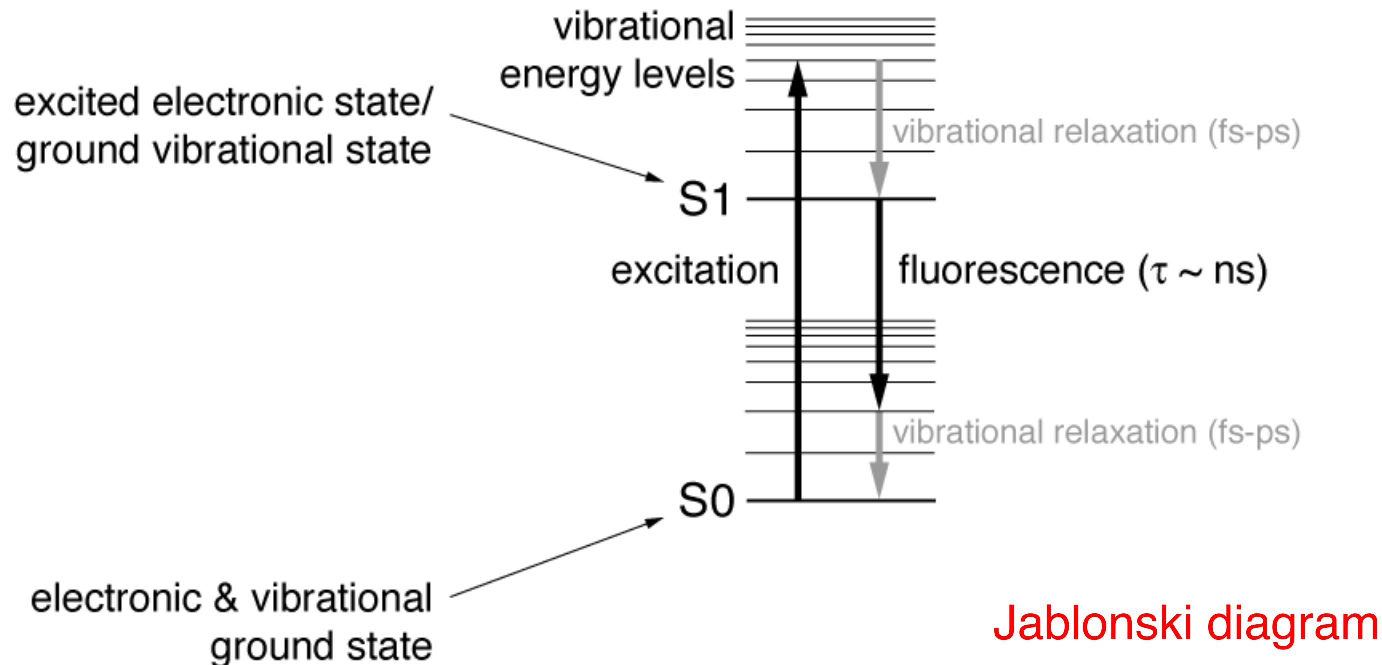
anatomical imaging & histology



Livet *et al.* (2007) *Nature*

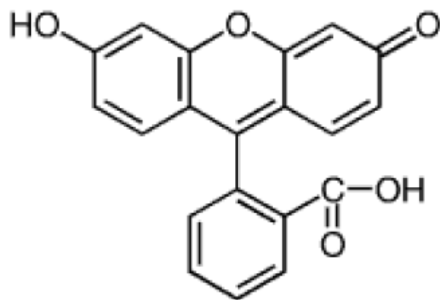
Introduction to fluorescence

Fluorescence arises from transitions among molecular energy levels:

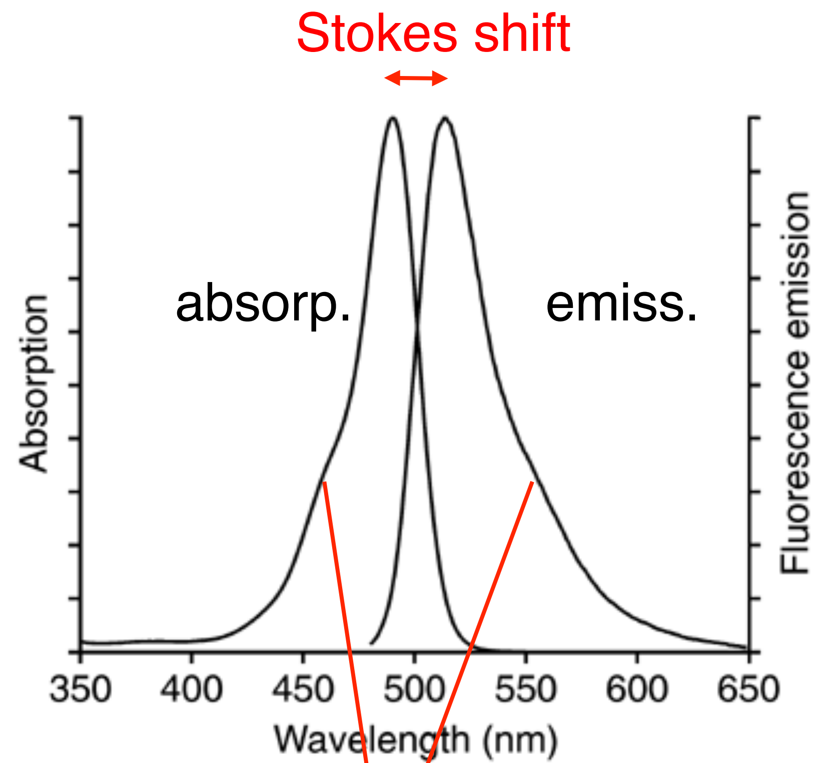


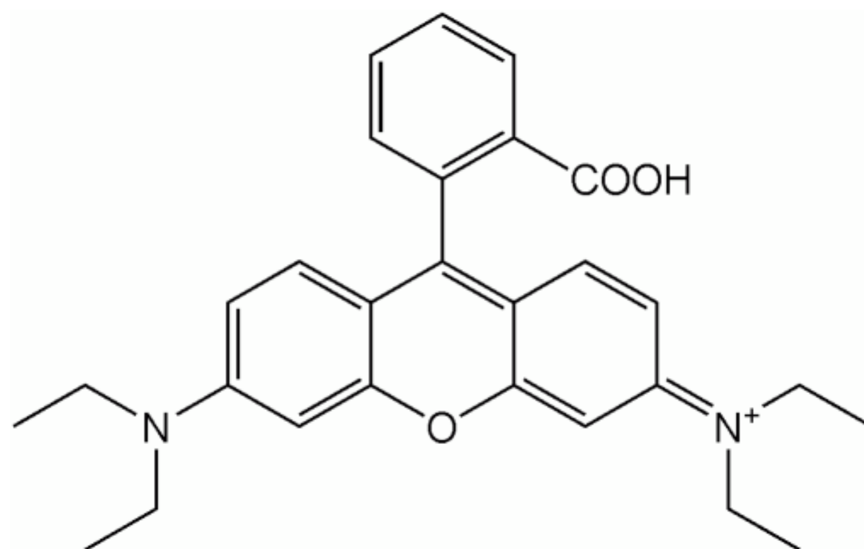
- electronic energy level transitions correspond to visible wavelengths
- vibrational energy level transitions correspond to infrared wavelengths
- rotational energy levels are coupled to vibrations and account for the smooth appearance of absorption/emission spectra

Fluorescence spectra for a typical fluorophore



fluorescein





rhodamine B
λ_{em} = 565 nm

Decay of excited electrons can occur by radiative and nonradiative processes. If N is the fraction of fluorophore in the excited state, and Γ and k are radiative and nonradiative decay rates, respectively:

$$\frac{dN}{dt} = -\left(\Gamma + k\right)N$$

such that

$$N = N_0 e^{-\left(\Gamma + k\right)t} = N_0 e^{-t/\tau}$$

where τ is the fluorescence lifetime, incorporating both Γ and k:

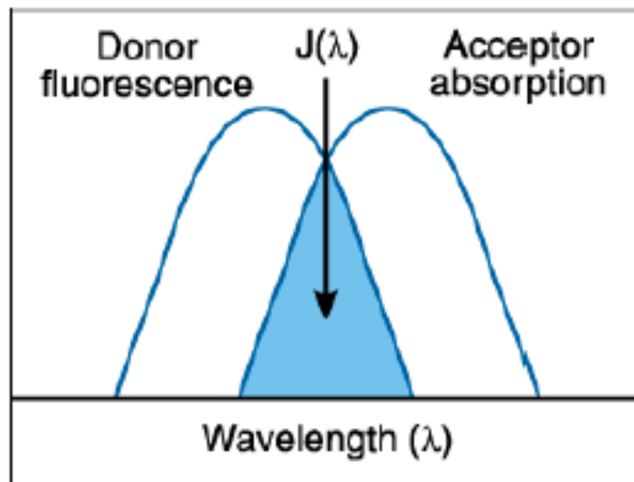
$$\tau = \frac{1}{\Gamma + k}$$

τ_0 describes the fluorescence lifetime in the absence of nonradiative decay. The efficiency of a fluorophore is quantified by its quantum yield Q:

$$Q = \frac{\Gamma}{\Gamma + k} = \frac{\tau}{\tau_0}$$

One of the main routes of nonradiative decay is a process called quenching, which results in environmental sensitivity for many fluorescent molecules, and underlies the mechanism of several sensors

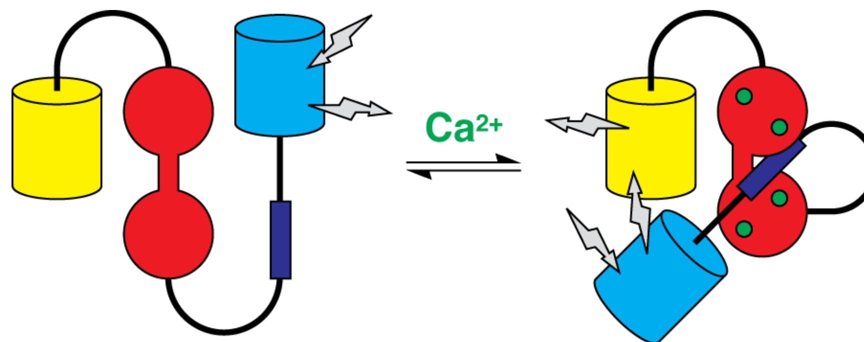
Fluorescence resonance energy transfer (FRET) can take place when the absorption spectrum of an "acceptor" overlaps with the emission spectrum of a "donor," and *geometry favors dipolar coupling between the fluorophores.*



The distance at which 50% of excited donors are deexcited by the FRET mechanism is defined as the Förster radius (usu. 10-100 Å):

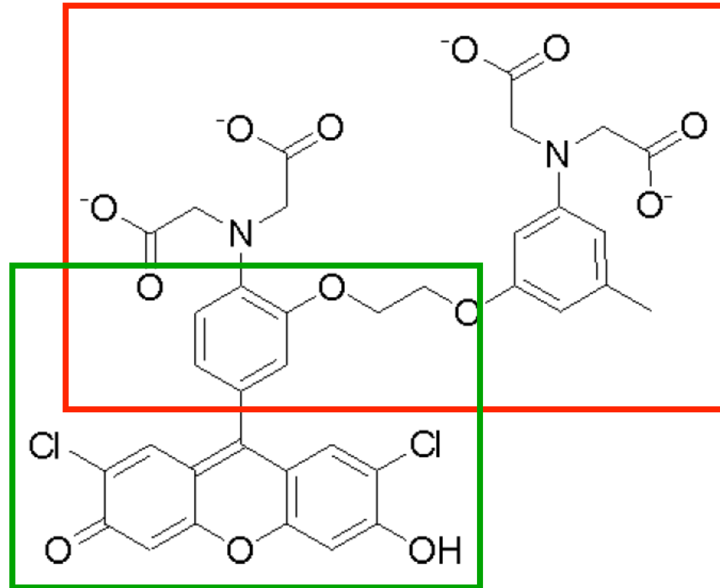
$$R_0 = \left[8.8 \times 10^{12} \cdot \kappa^2 \cdot n^{-4} \cdot QY_D \cdot J(\lambda)\right]^{1/6}$$

FRET efficiency is defined as: $$E = \left[1 + \left(r/R_0\right)^6\right]^{-1}$$

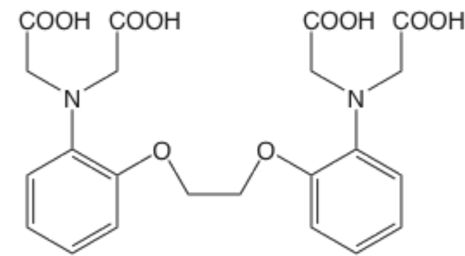


Fluorescent calcium sensors

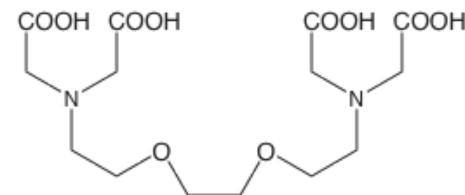
A typical calcium sensor consists of a calcium sensitive component attached to one or more fluorescent moieties:



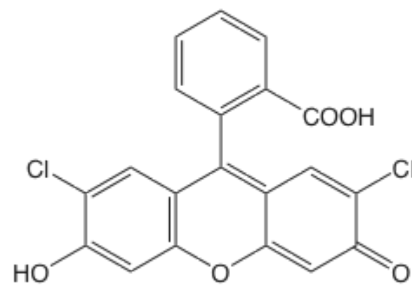
Fluo-3



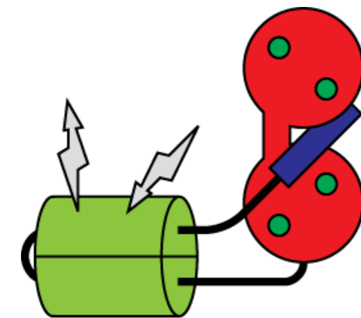
BAPTA



EGTA

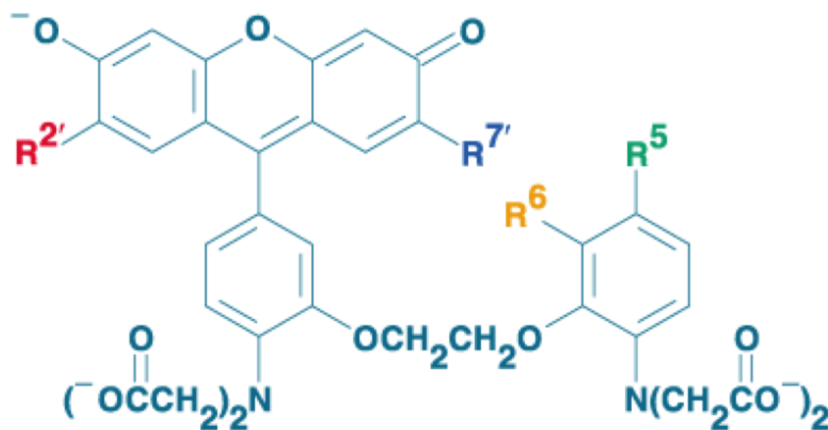


dichlorofluorescein

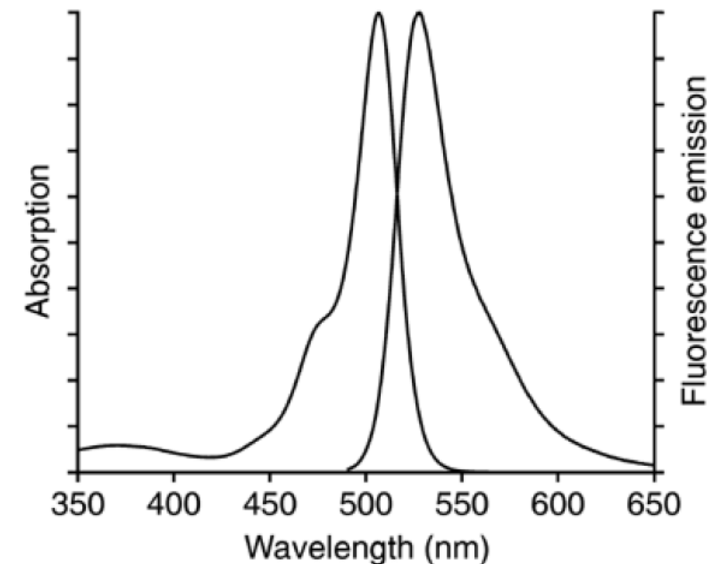


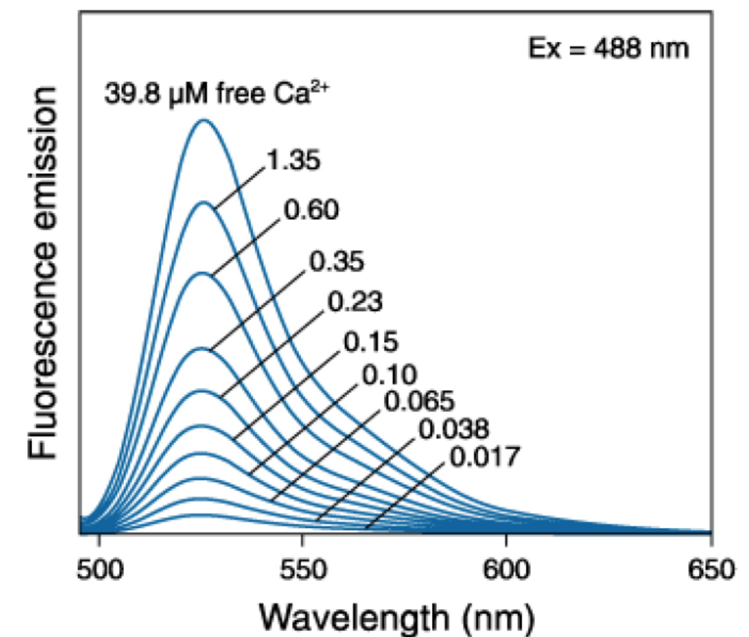
Fluo dyes:

- visible absorption/emission wavelengths
- virtually no emission in absence of Ca^{2+}
- range of calcium affinities



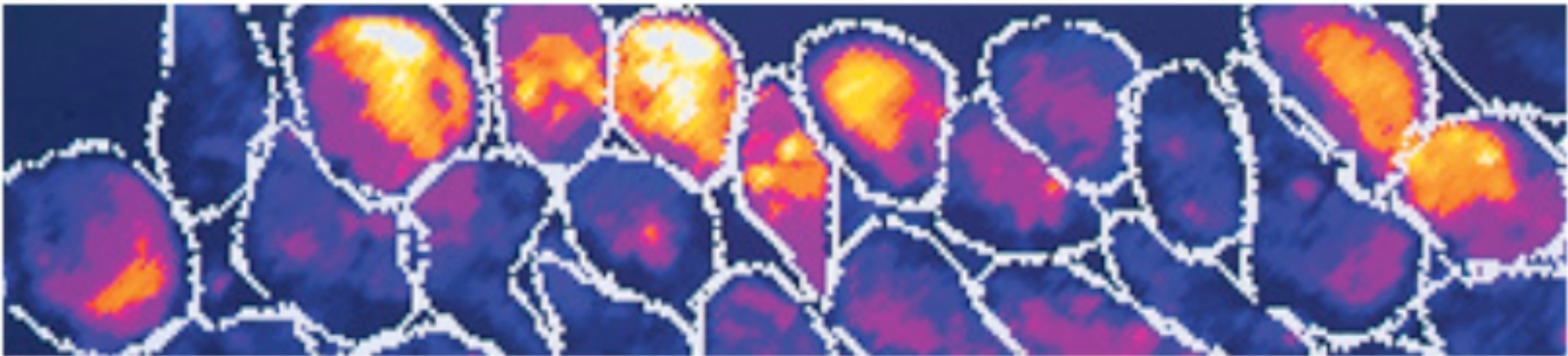
Indicator	$K_d(Ca^{2+})$	$R^{2'}$	$R^{7'}$	R^5	R^6
Fluo-3	0.39 µM	Cl	Cl	CH_3	H
Fluo-4	0.35 µM	F	F	CH_3	H
Fluo-5F	2.3 µM	F	F	F	H
Fluo-5N	90 µM	F	F	NO_2	H
Fluo-4FF	9.7 µM	F	F	F	F





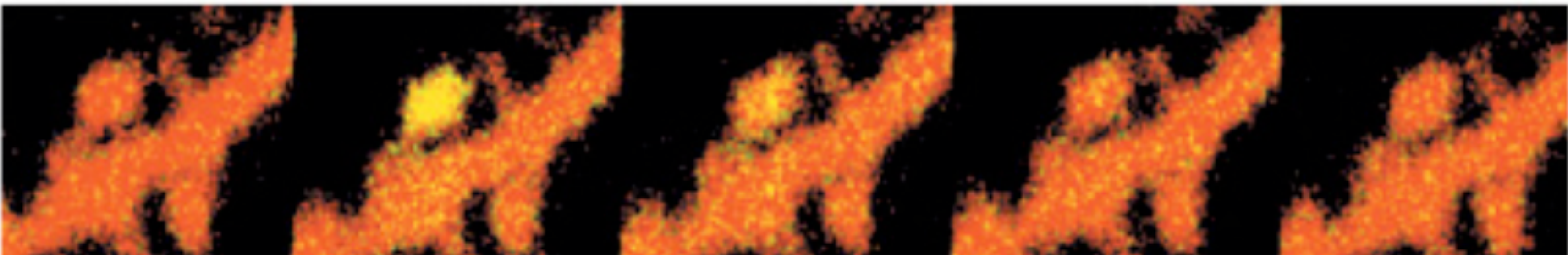
Sensors with different calcium affinities (K_d values) may be appropriate for different applications:

spontaneous Ca^{2+} fluctuations in *Xenopus* embryo



fluo-3 (0.39 μM)

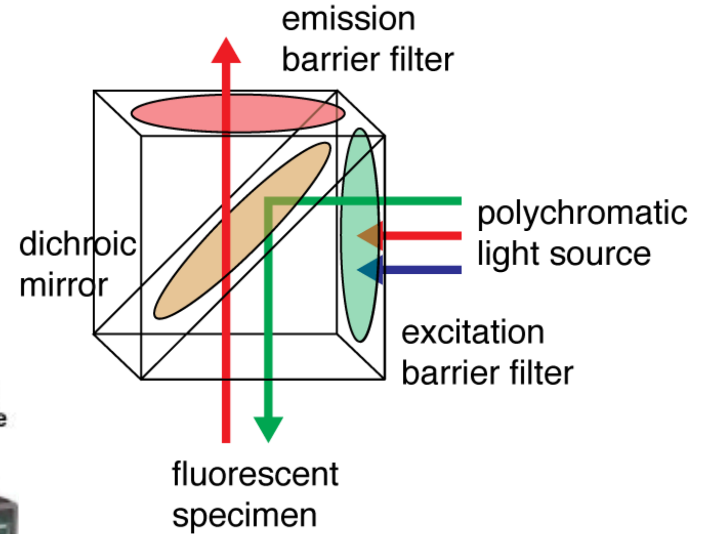
calcium transients in dendritic spines



fluo-5F (2.3 μM)

www.invitrogen.com

imaging with fluorescence

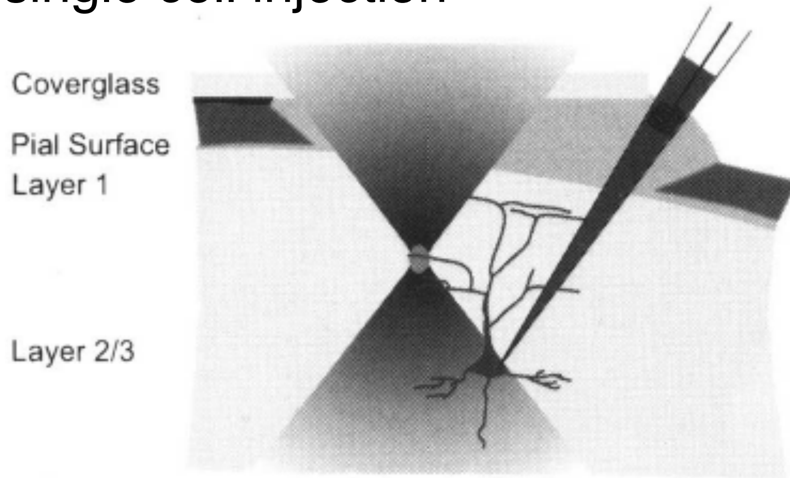


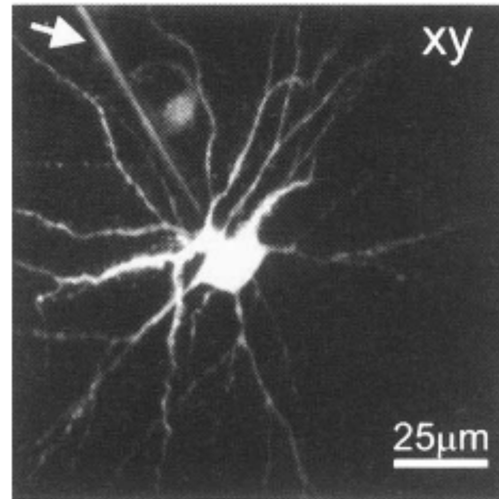
epifluorescence microscope geometry

confocal and 2 photon microscopy use tricks to improve resolution and tissue penetration

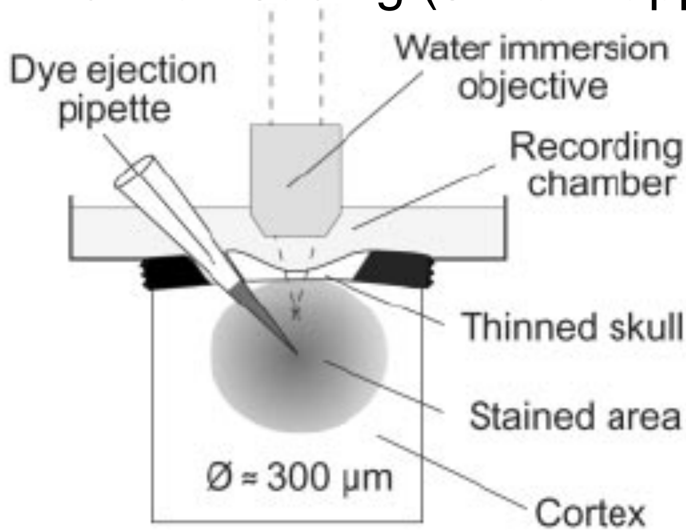
How are calcium dyes applied to cells?

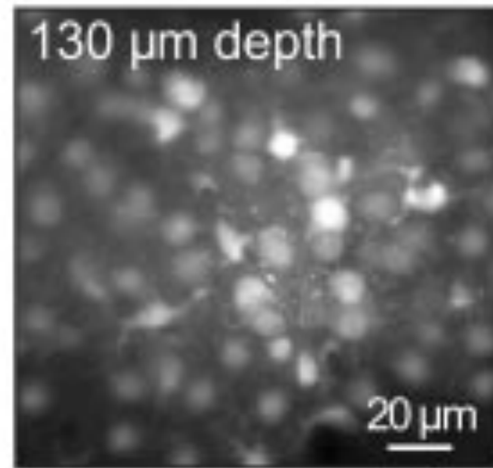
single cell injection



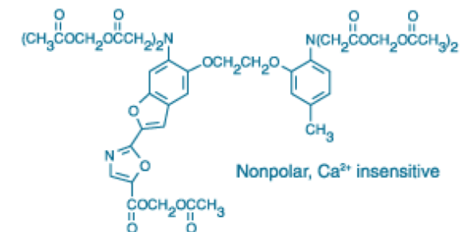


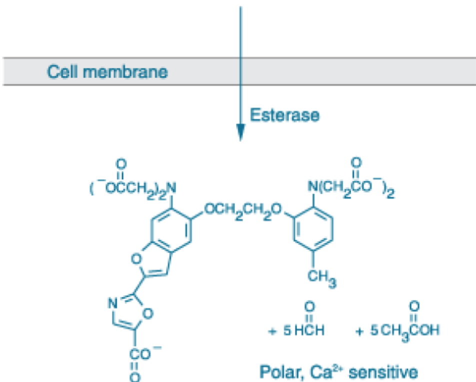
multicellular loading (or bath application)





AM-esters





limitation	effect on experiments	solution
dye binds to intracellular proteins and does not function	loss of fluorescence responses, alteration of calcium sensitivity	alter localization/ solubility, *e.g.* using dextran conjugate
dye leaks from cell or is sequestered into inappropriate cellular compartments	loss of fluorescence responses, higher background fluorescence	use dextran conjugates or targeted indicators, ratiometric imaging
dye bleaches over the course of experiments	loss of fluorescence responses	lower imaging duty cycle, select dyes with low bleaching, ratiometric imaging

Ratiometric imaging

Suppose you measure fluorescence intensity from a cell, but you don't know either how much dye is present or what the calcium concentration is; you have one equation in two unknowns ($[L]_{tot}$ and $[Ca^{2+}]$):

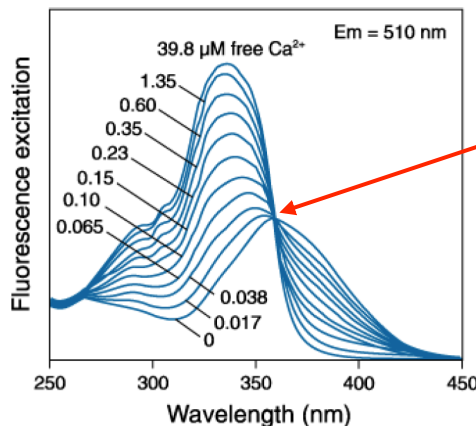
$$F_{tot} = F_{Ca^{2+}}[L \cdot Ca^{2+}] + F_{free}\left([L]_{tot} - [L \cdot Ca^{2+}]\right)$$

$$\text{where} \quad [L \cdot Ca^{2+}] = \frac{[L]_{tot}}{\left(1 + K_d / [Ca^{2+}]\right)}$$

The trick is to combine measurements at the first wavelength with measurements at another wavelength, to get a second equation:

$$F^*_{tot} = F^*_{Ca^{2+}}[L \cdot Ca^{2+}] + F^*_{free}\left([L]_{tot} - [L \cdot Ca^{2+}]\right)$$

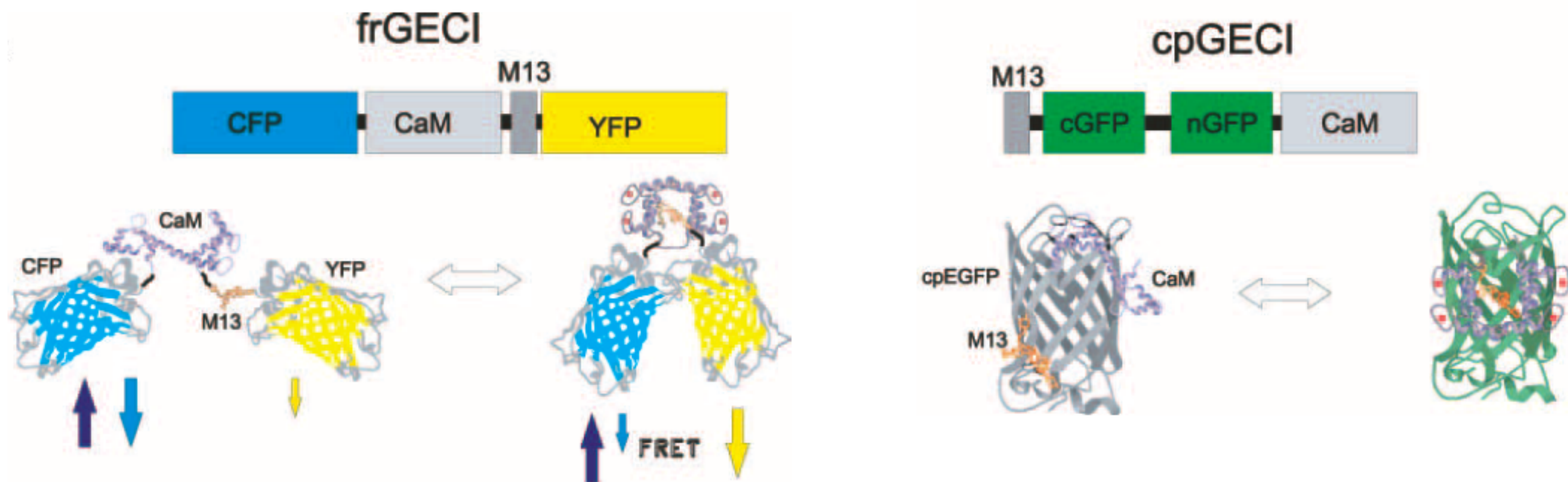
$$= F^*[L]_{tot} \qquad (\text{if } F^* \text{ is independent of } [Ca^{2+}])$$



"isosbestic point"

The ratio F/F^* is independent of $[L]_{tot}$ and depends only on the calcium concentration.

limitation	effect on experiments	solution	
dye binds to intracellular proteins and does not function	loss of fluorescence responses, alteration of calcium sensitivity	alter localization/ solubility, *e.g.* using dextran conjugate	
dye leaks from cell or is sequestered into inappropriate cellular compartments	loss of fluorescence responses, higher background fluorescence	~~use dextran conjugates or targeted indicators, ratiometric imaging~~	**use proteins**
dye bleaches over the course of experiments	loss of fluorescence responses	~~lower imaging duty cycle, select dyes with low bleaching, ratiometric imaging~~	**use proteins**



Genetically-encoded calcium sensors:

- CaM + single XFPs (pericams, camgaroos, GCaMPs)
- FRET-based CaM-XFP fusions (CaMeleons)
- troponin C based

Advantages of genetically-encoded calcium indicators:

- noninvasive delivery (expression within cells)
- constant resynthesis (limited effect of bleaching)
- targeted expression

Protein sensors genes can be introduced by making transgenics, or by *in vivo* transfection (viral, electroporation, “biolistics,” *etc.*).

Dombeck *et al.* (2007) *Neuron 56:* 43-57