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. Gene analysis & induction
6. Purification and protein analysis	6. Characterize expression
7. Binding & affinity measurements	7. Assay protein behavior
8. High throughput engineering	8. Data analysis

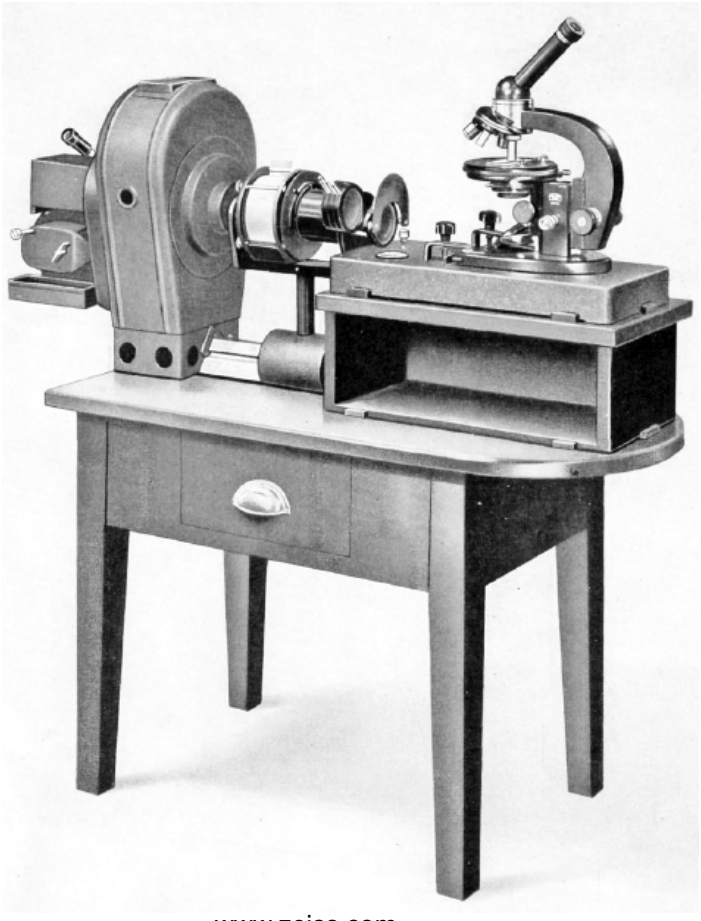
Lecture 3: Fluorescence and sensors

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 in cells
   C. *In vivo* limitations and remedies
   D. Advantages of genetically-encoded sensors

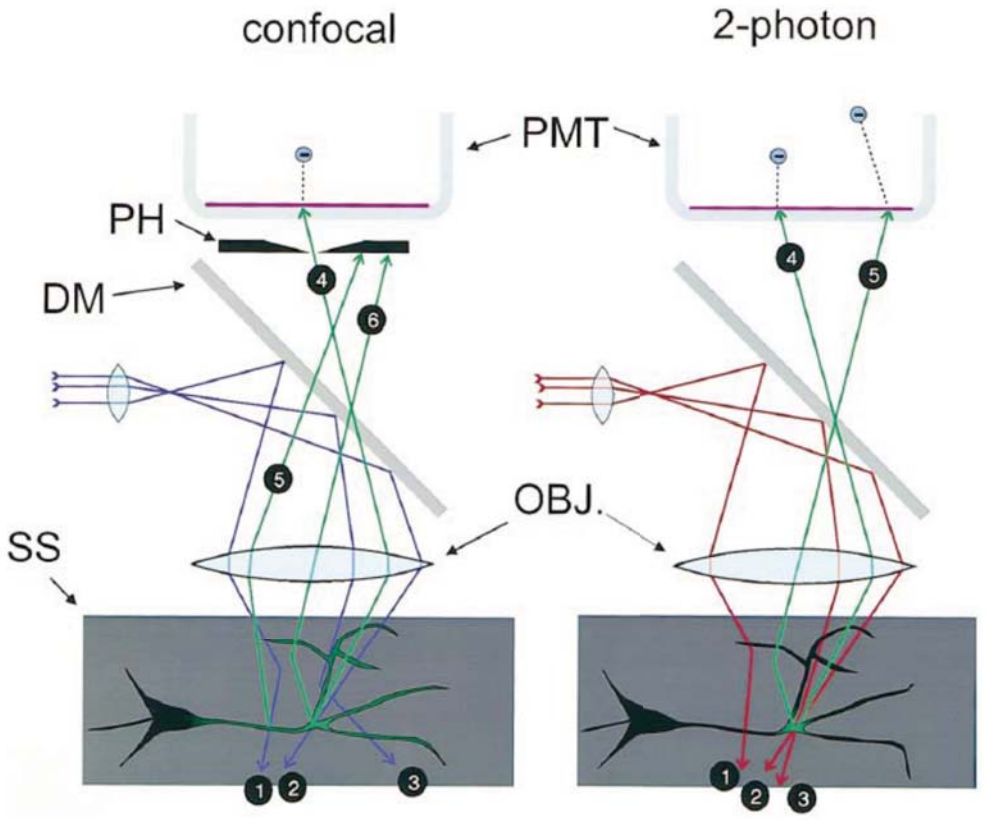
fluorescence/luminescence microscopy

H. Lehmann & S. von Prowazek (1913)



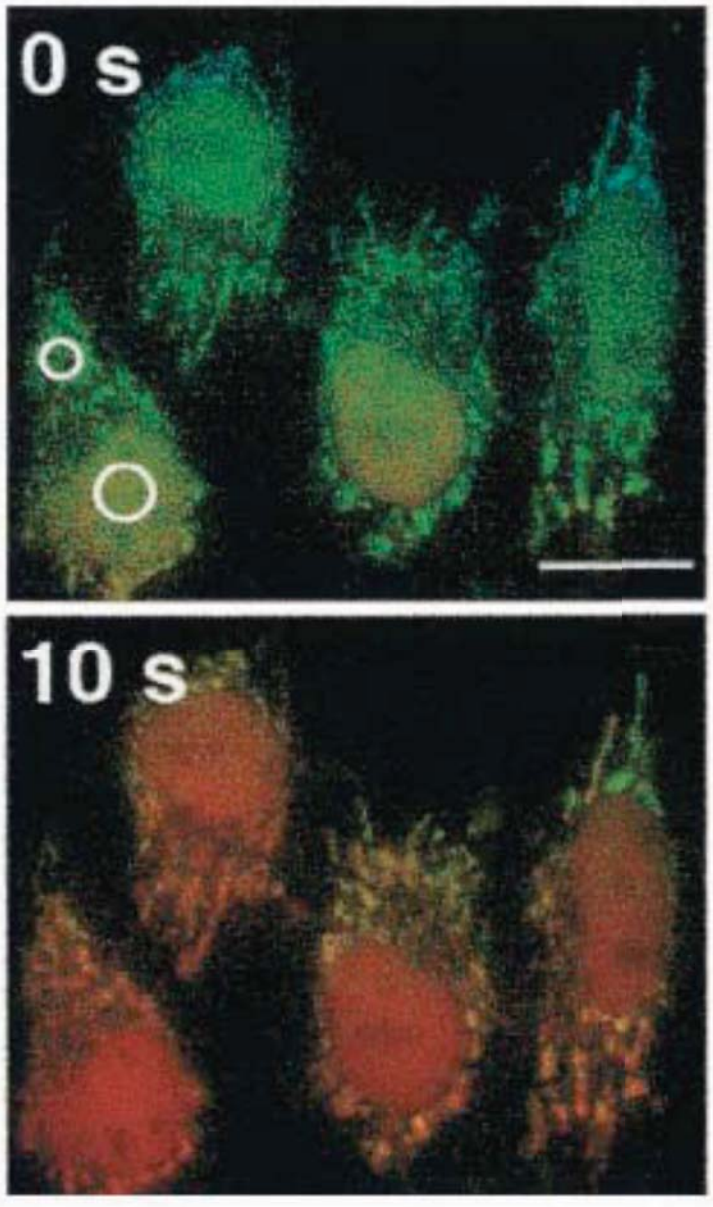
www.zeiss.com

laser scanning microscopy



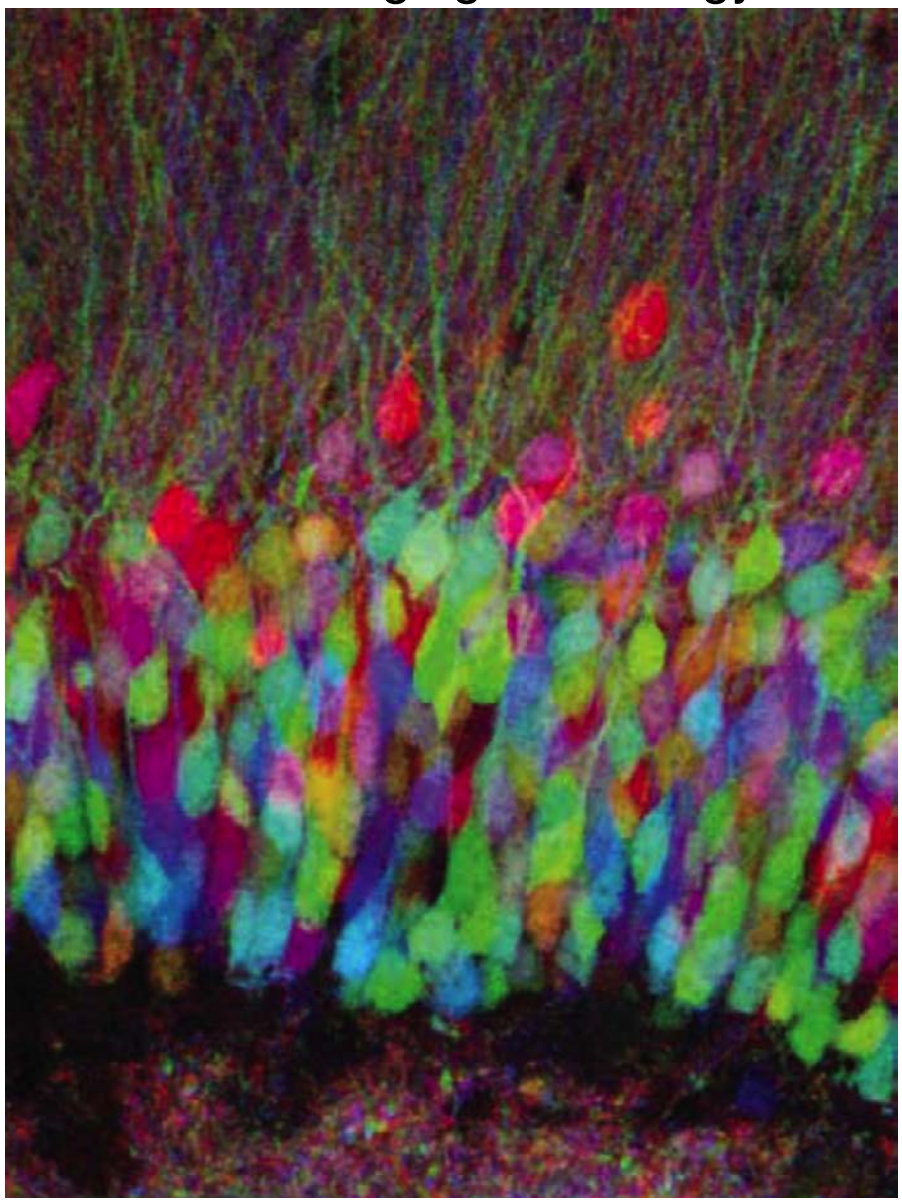
Denk & Svoboda (1997) *Neuron*

functional imaging



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

anatomical imaging & histology



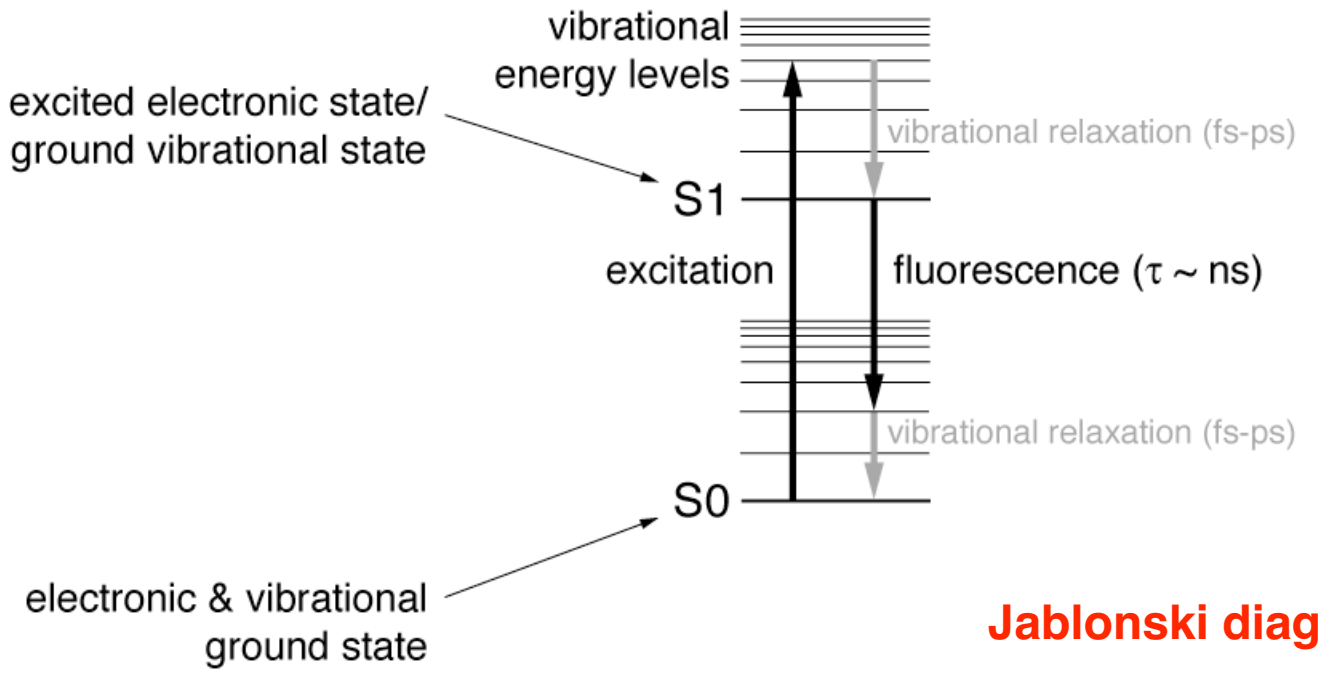
Livet *et al.* (2007) *Nature*

www.caliperls.com

in vitro assays

Introduction to fluorescence

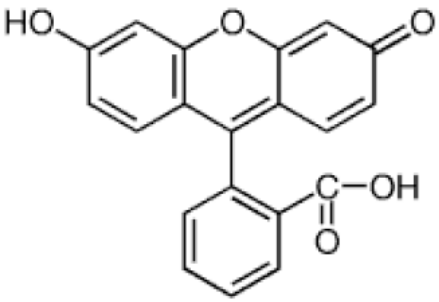
Fluorescence arises from transitions among molecular energy levels:



Jablonski diagram

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

Fluorescence spectra for a typical fluorophore



fluorescein

Public domain image.

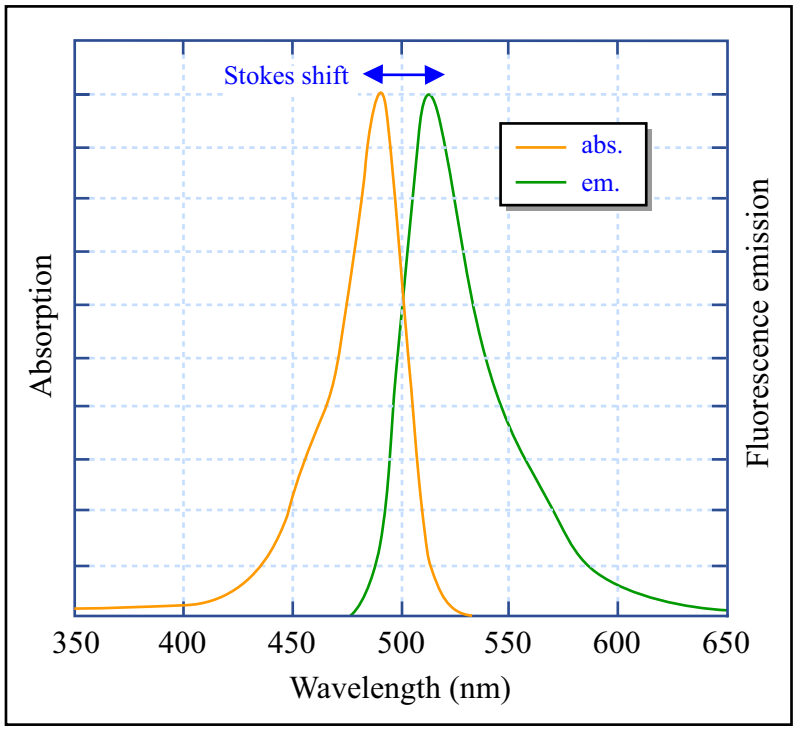
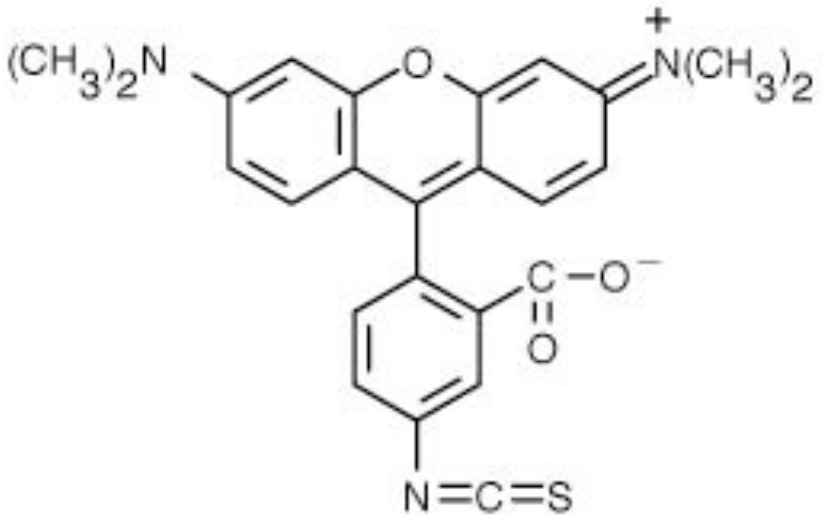


Image by MIT OpenCourseWare.

- small organics like fluorescein are the most common fluorophores
- in general, the larger the aromatic ring system, the longer the wavelength for excitation and emission
- quantum dots are ~10 nm particles that exhibit narrower emission bands and less “bleaching” than organic dyes
- some atoms (lanthanides) exhibit fluorescence as well



tetramethylrhodamine isothiocyanate

λ_{em} = 580 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:

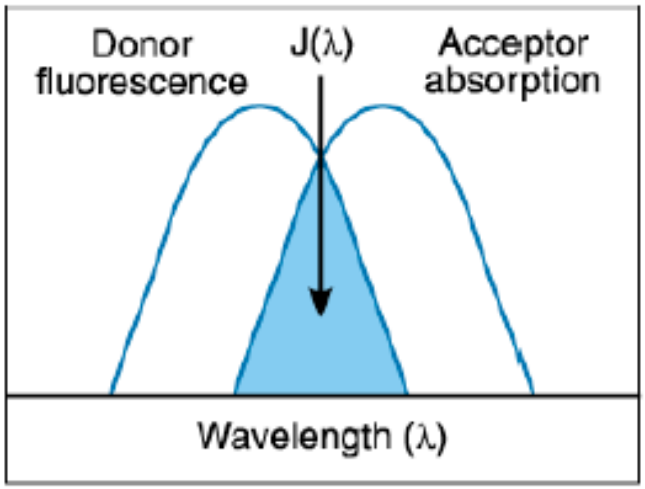
$$\tau = \frac{1}{\Gamma + k}$$

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.*



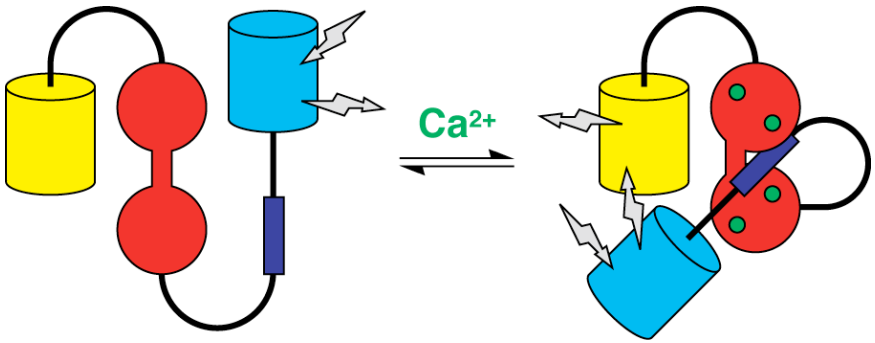
Courtesy of Life Technologies, Carlsbad, CA. Used with permission.

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:

$$E = \left[1 + \left(r / R_0 \right)^6 \right]^{-1}$$



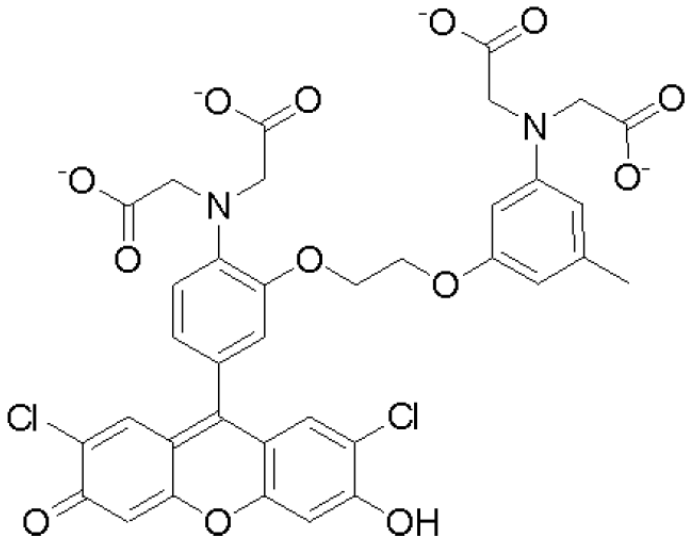
Fluorescent calcium sensors

A wide variety of fluorescent calcium dyes are available. They differ along several axes:

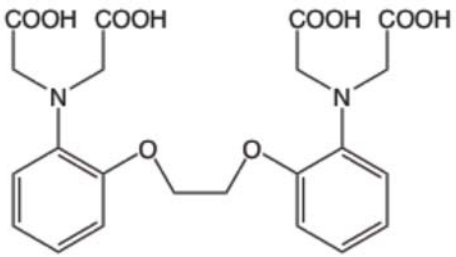
- calcium affinity
- absorbance and emission properties
- structural properties (*e.g.* protein *vs.* small molecule, membrane permeability, binding and localization)

Indicators with each set of properties may be suitable for specific experiments.

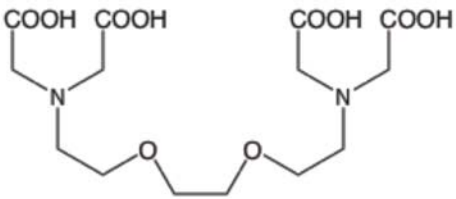
A typical calcium sensor consists of a calcium sensitive component attached to one or more fluorescent moieties:



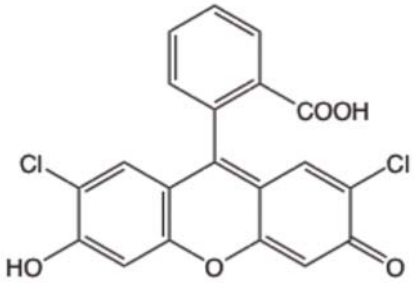
Fluo-3



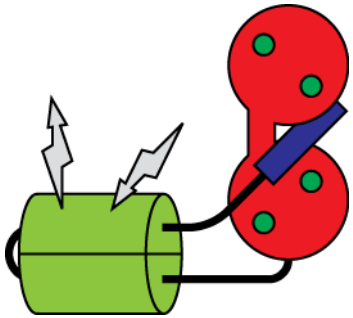
BAPTA



EGTA

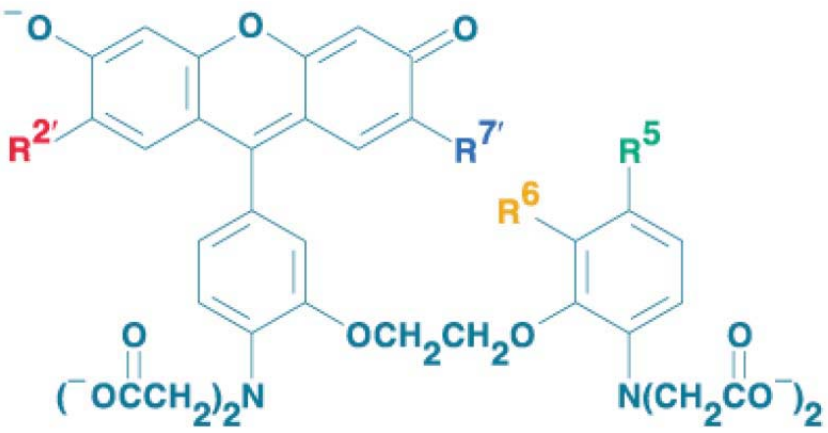


dichlorofluorescein



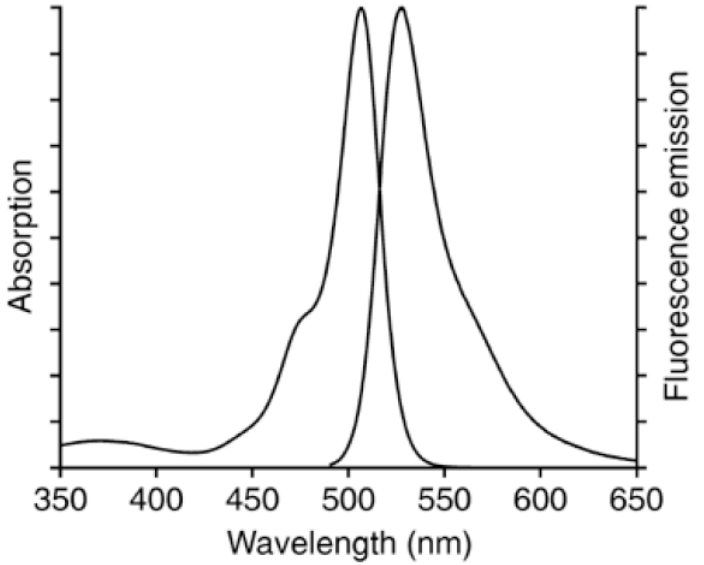
Fluo dyes:

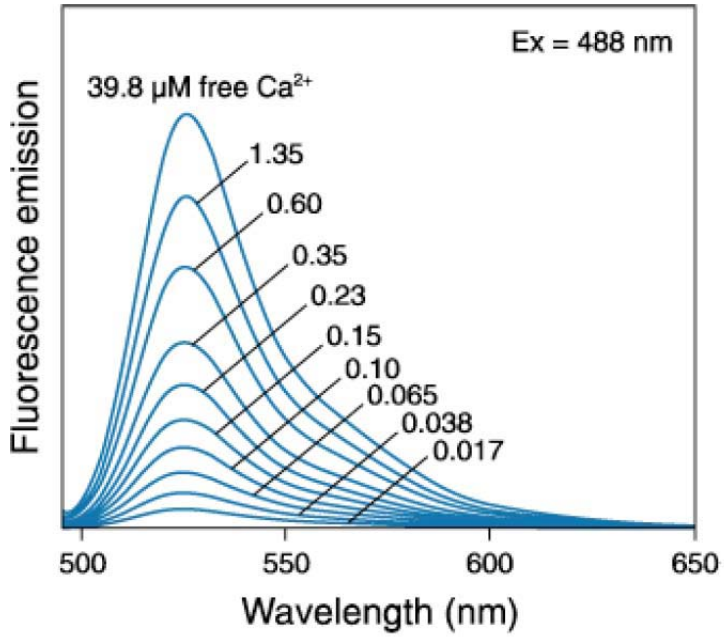
- visible absorption/emission wavelengths
- virtually no emission in absence of Ca^{2+}
- range of calcium affinities



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

Fluo-3 Spectra





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

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

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See http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Image-Gallery/Image-Detail.2404.html.

calcium transients in dendritic spines

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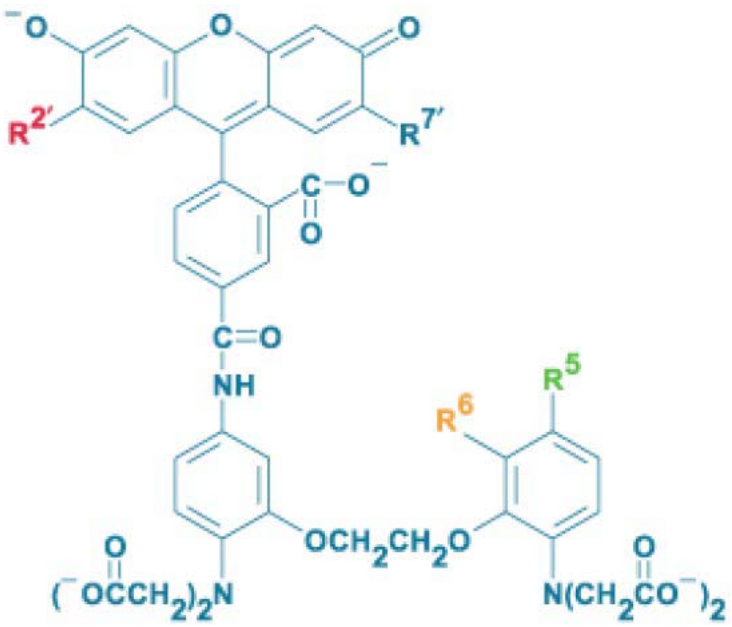
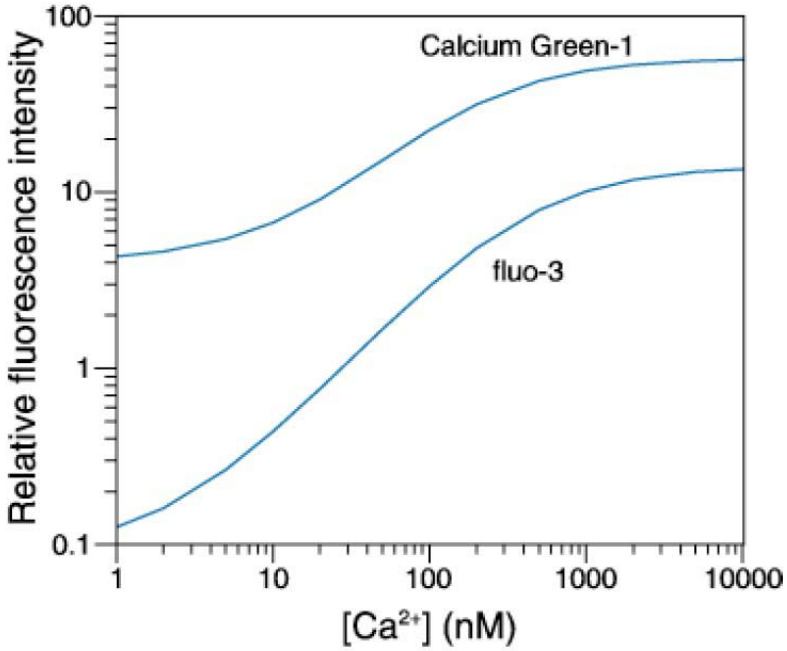
Calcium Green & related dyes

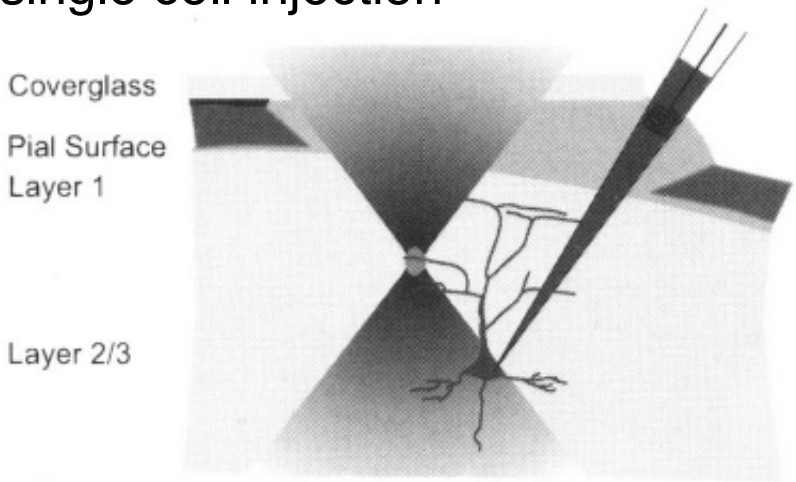




What is the significance of having a dye with high fluorescence **intensity** or **dynamic range**? **SNR**

How are calcium dyes applied to cells?

single cell injection



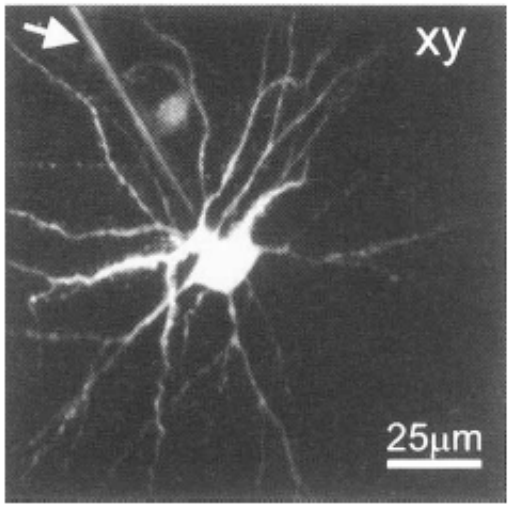
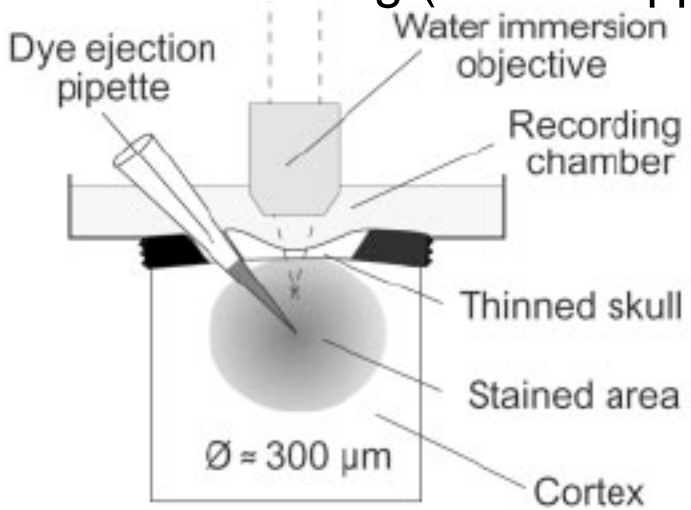
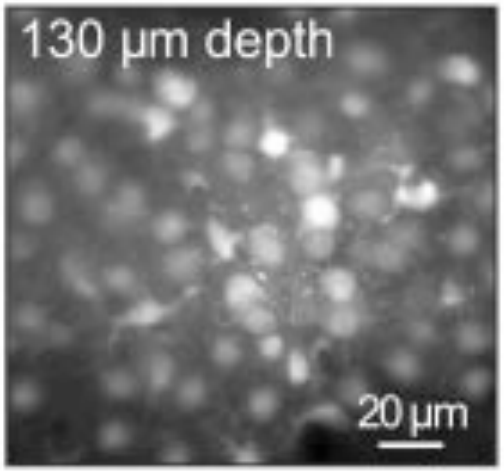


Image from Svoboda, K., et al. "*In vivo* Dendritic Calcium Dynamics in Neocortical Pyramidal Neurons." *Nature* 385, no. 6612 (1997): 161-165.

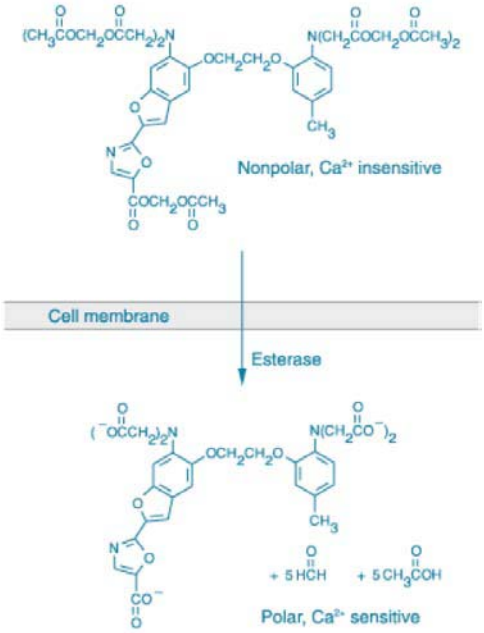
multicellular loading (or bath application)





Courtesy of National Academy of Sciences, U. S. A. Used with permission.
Source: Stosiek, C., et al. "*In vivo* Two-Photon Calcium Imaging of Neuronal Networks." *PNAS* 100, no. 12 (2003): 7319-7324.

AM-esters



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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 measurements

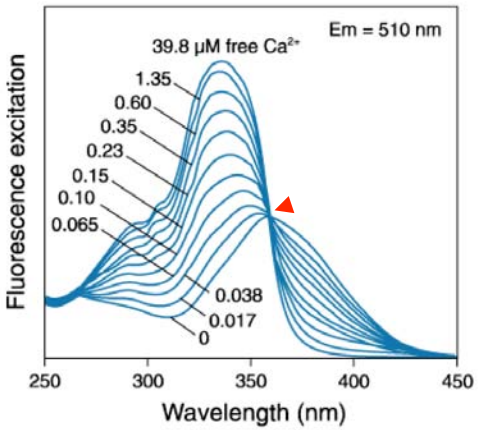
Suppose you measure fluorescence intensity from a cell, but you don't know either how much dye is present or what the calcium concentration is; you have one equation in two unknowns ($[L]_{tot}$ and $[Ca^{2+}]$):

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

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

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**

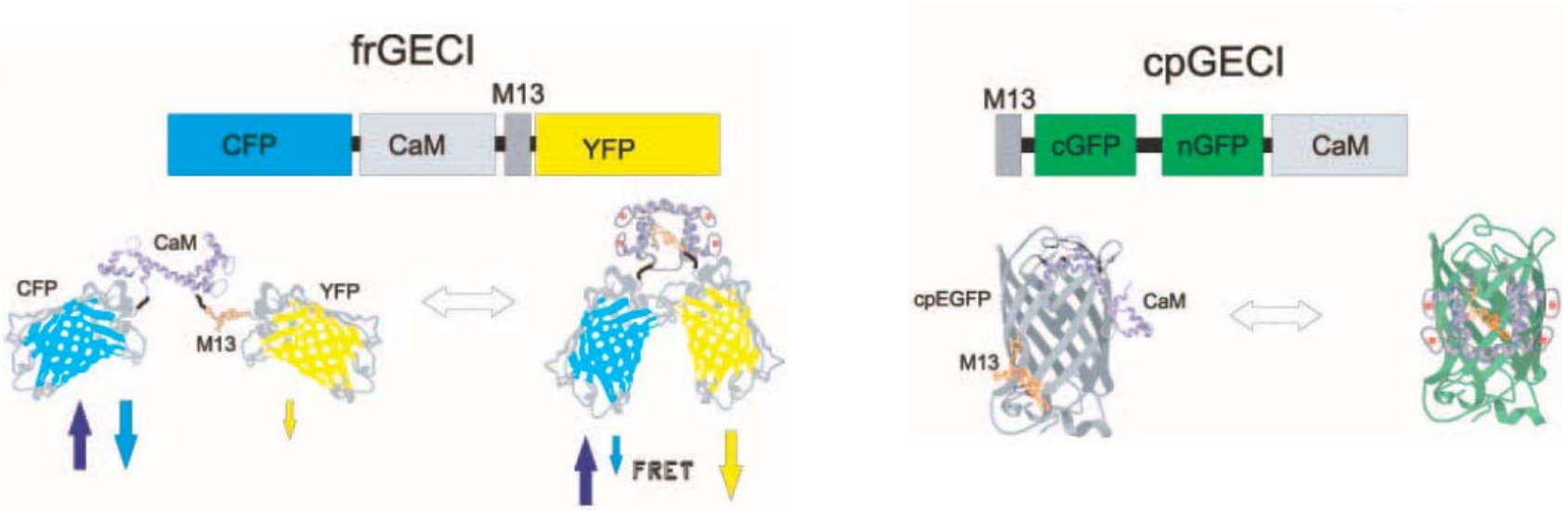


Image from Kotlikoff, M. I. "Genetically Encoded Ca2+ indicators: Using Genetics and Molecular Design to Understand Complex Physiology." *J Physiol* 578 (2007): 55-67. Copyright © 2003 John Wiley & Sons. Reprinted with permission.

Genetically-encoded calcium sensors:

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

20.109 Laboratory Fundamentals in Biological Engineering

Spring 2010