Module 1 overview

lecture

1. Introduction to the module
2. Rational protein design
3. <mark>Fluorescence and sensors</mark>

lab

1. Start-up protein eng.
2. Site-directed mutagenesis
3. DNA amplification

PRESIDENT'S DAY

4. Review & gene analysis
5. Protein expression
6. Purification and protein analysis
7. Binding & affinity measurements
8. High throughput engineering

4. Prepare expression system
5. gene analysis & induction
6. Characterize expression
7. Assay protein behavior
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
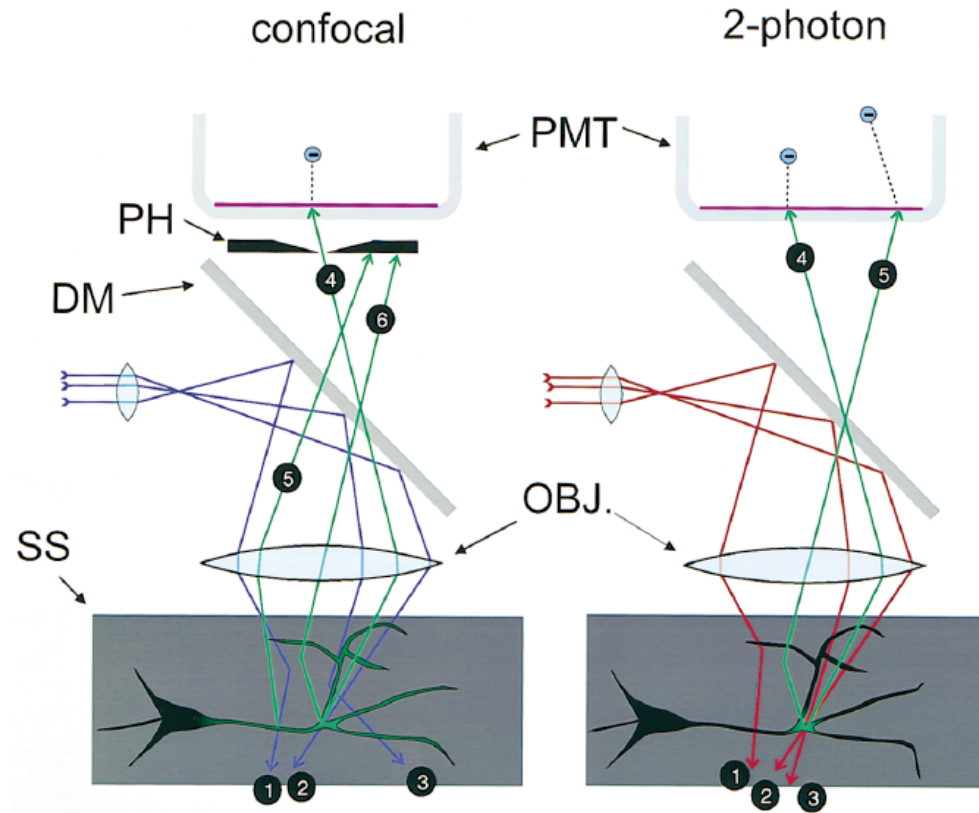   D. Advantages of genetically-encoded sensors

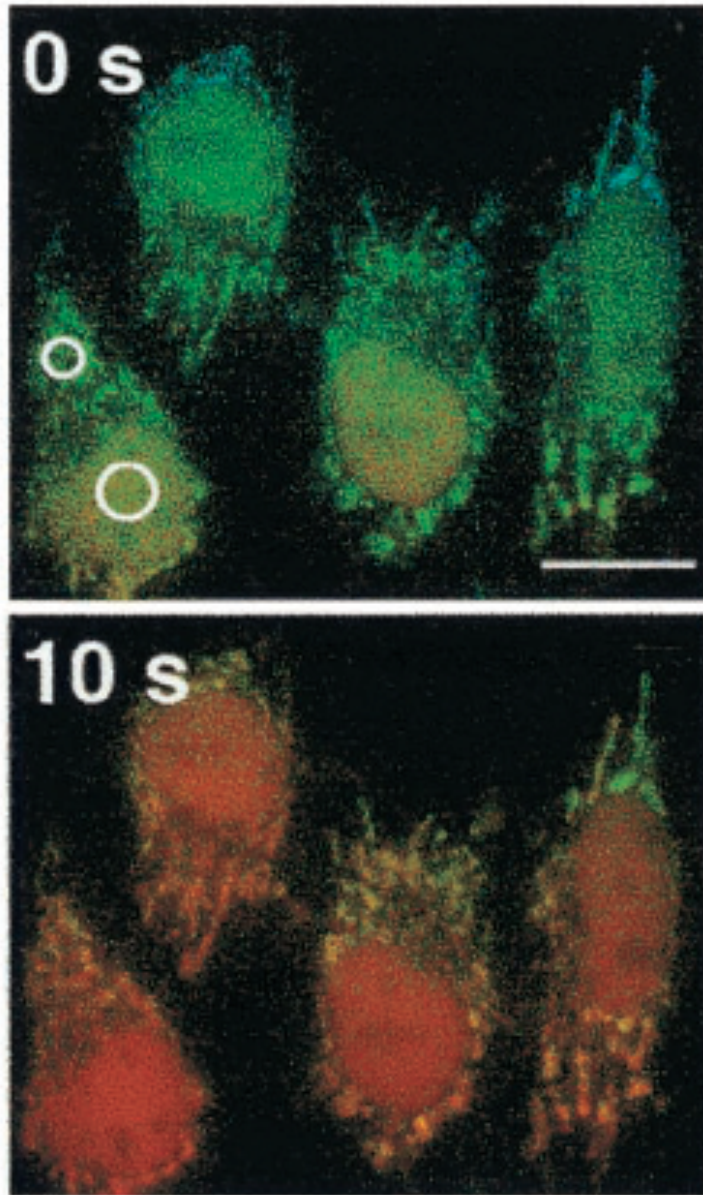# fluorescence/luminescence microscopy

H. Lehmann & S. von Prowazek (1913)
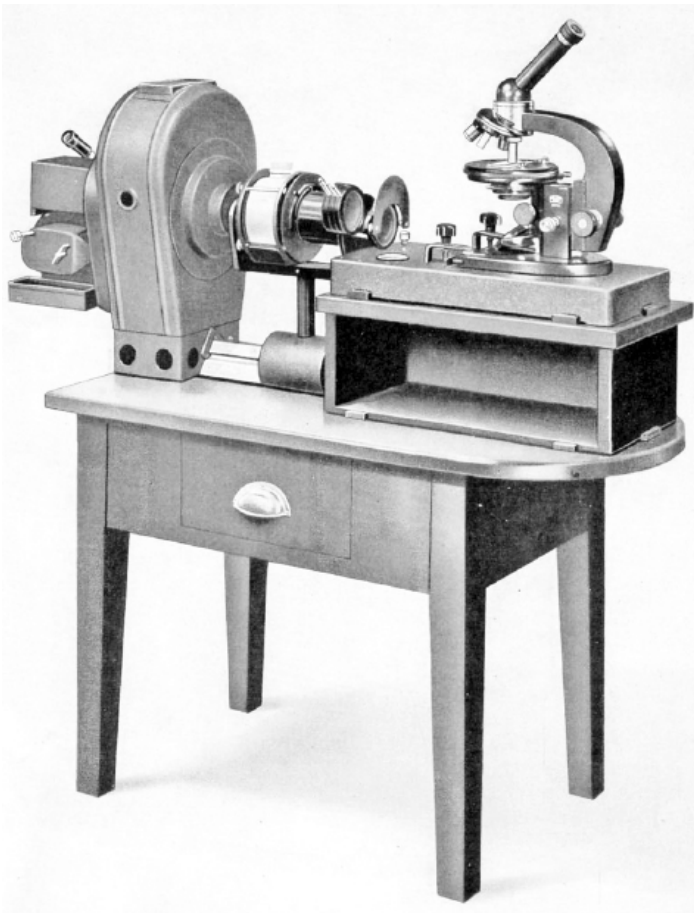
laser scanning microscopy

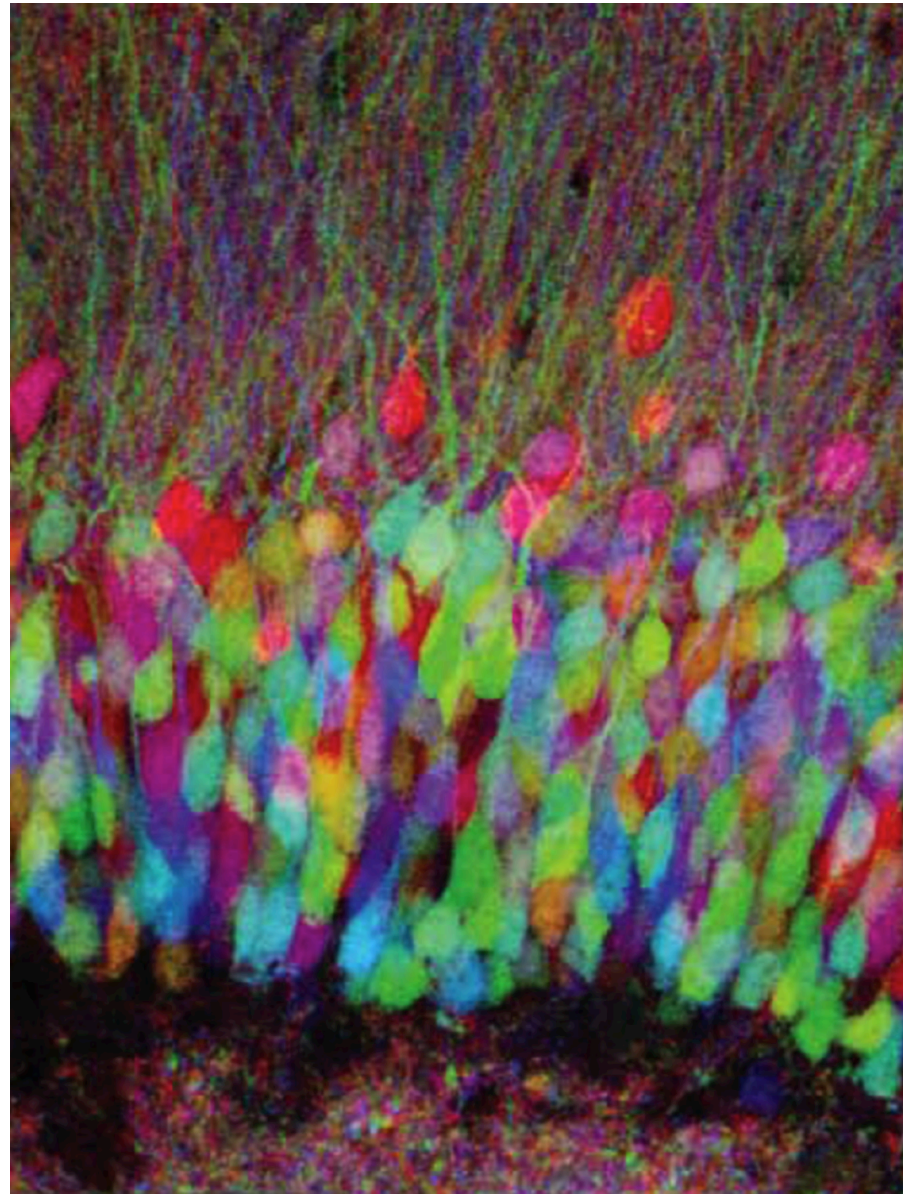www.zeiss.com

Denk & Svoboda (1997) *Neuron*

functional imaging

0 s

10 s

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

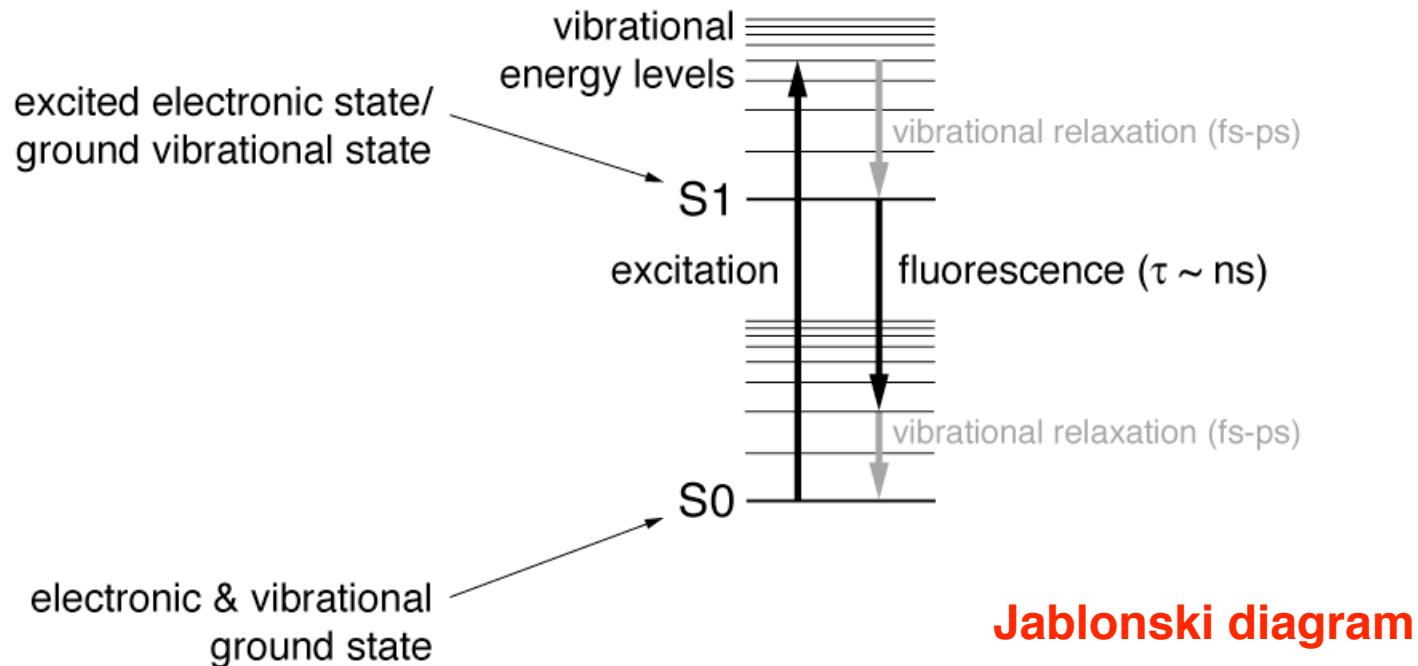## anatomical imaging & histology


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

in vitro assays

Introduction to fluorescence

Fluorescence arises from transitions among molecular energy levels:

vibrational energy levels

excited electronic state/ ground vibrational state

S1

excitation

vibrational relaxation (fs-ps)

fluorescence ($\tau \sim$ ns)

vibrational relaxation (fs-ps)

S0

electronic & vibrational ground state

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
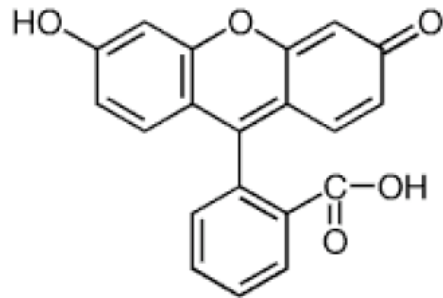
fluorescein
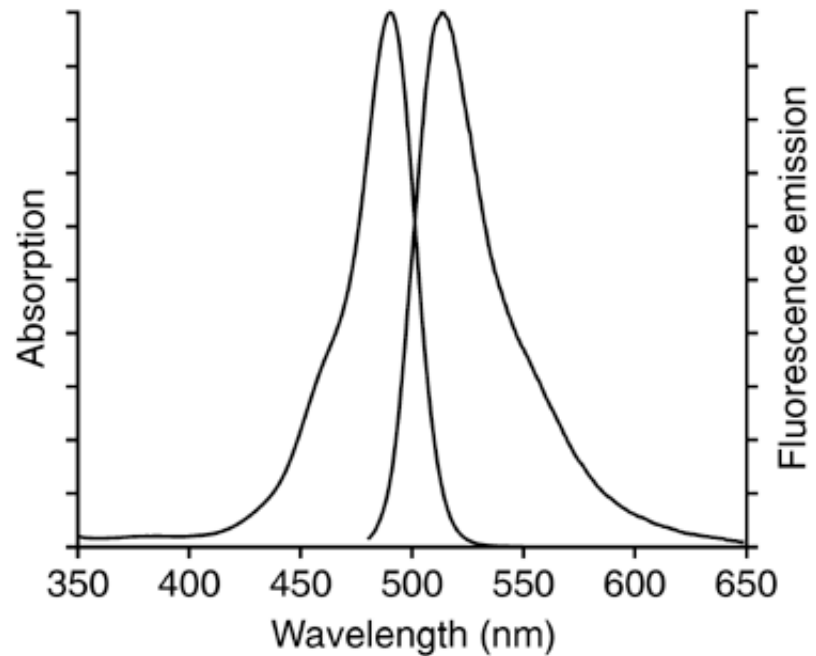
probes.invitrogen.com

- 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
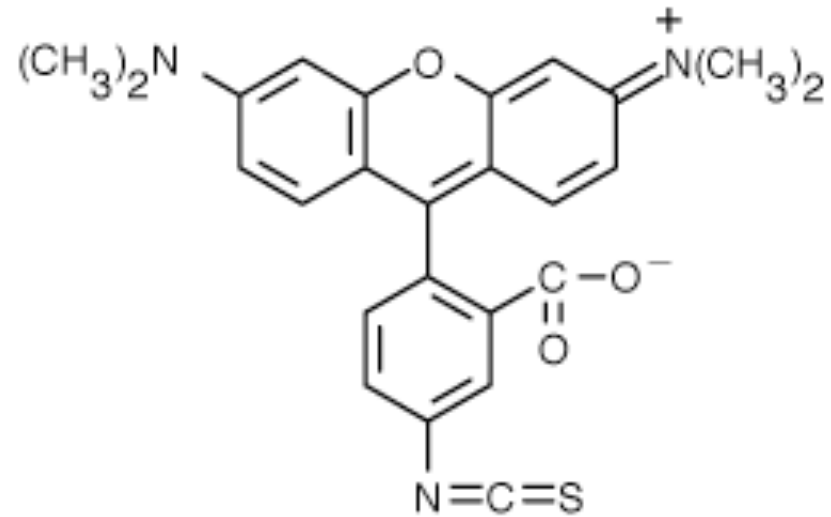
tetramethylrhodamine isothiocyanate
λ_{em} = 580 nm

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:

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

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.*
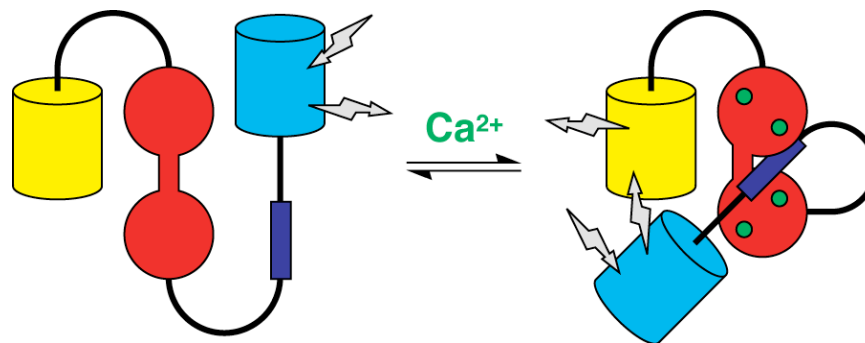

Donor fluorescence $J(\lambda)$ Acceptor absorption

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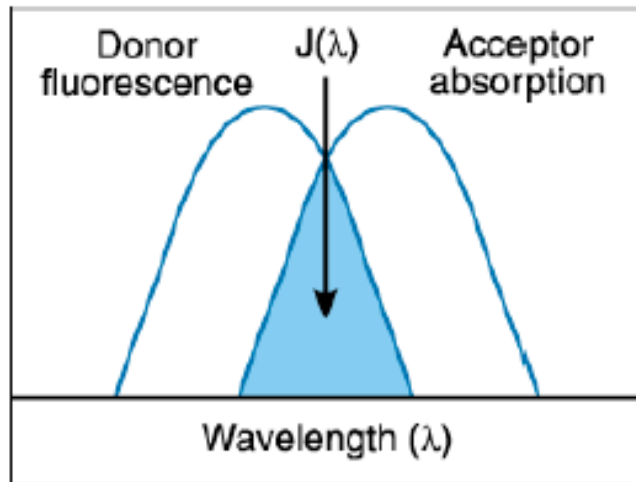
The distance at which 50% of excited donors are deexcited by the FRET mechanism is defined as the **Förster radius** (usu. 10-100 Å):

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

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


Ca²⁺

Fluorescent calcium sensors

A wide variety of fluorescent calcium dyes are available. They differ along several axes:
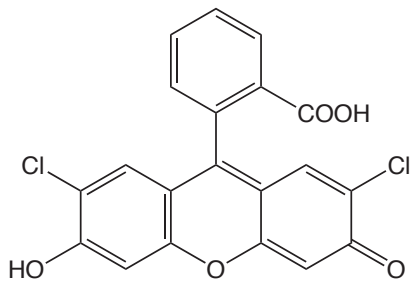- calcium affinity
- absorbance and emission properties
- structural properties (*e.g.* protein *vs.* small molecule, membrane permeability, binding and localization)

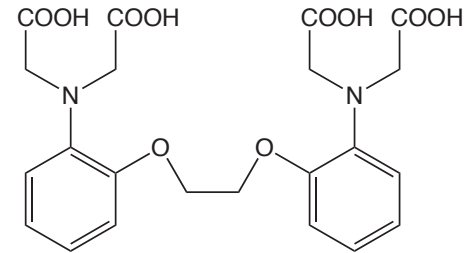Indicators with each set of properties may be suitable for specific experiments.

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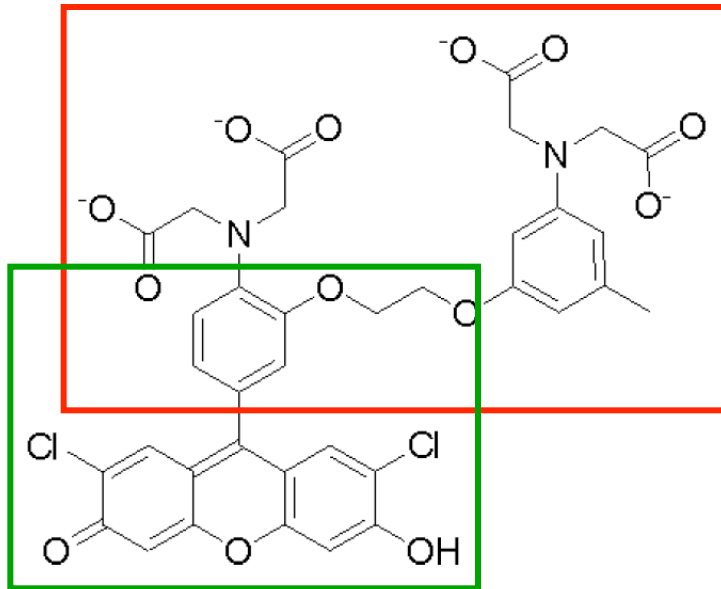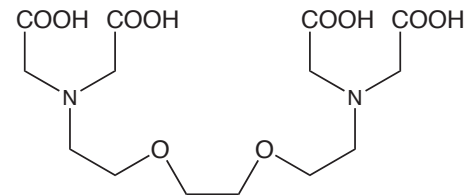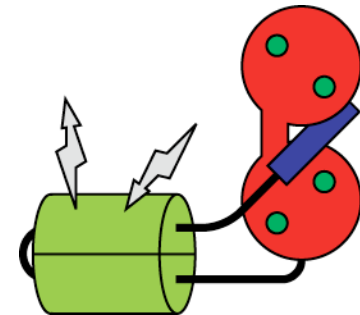

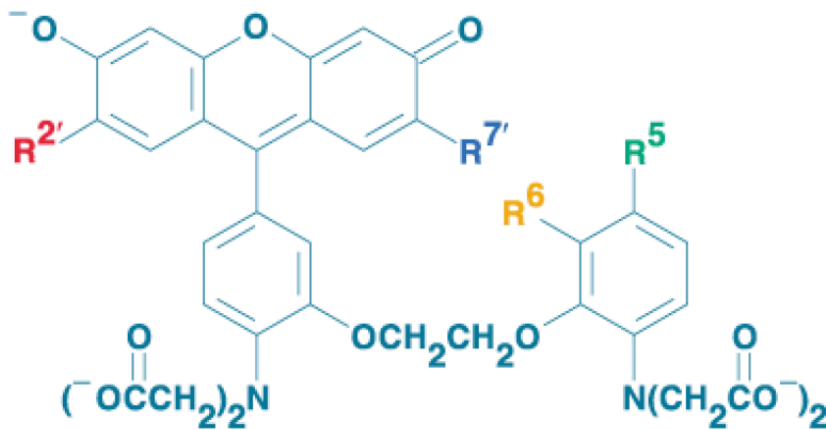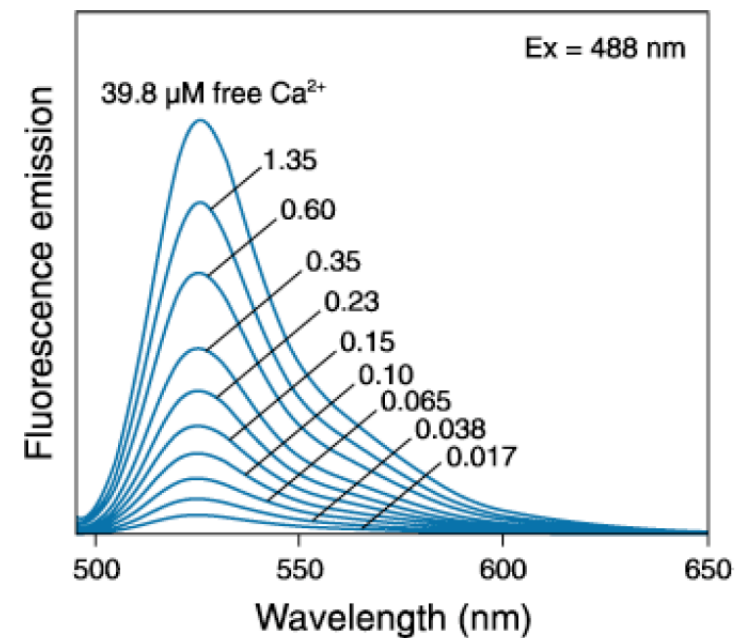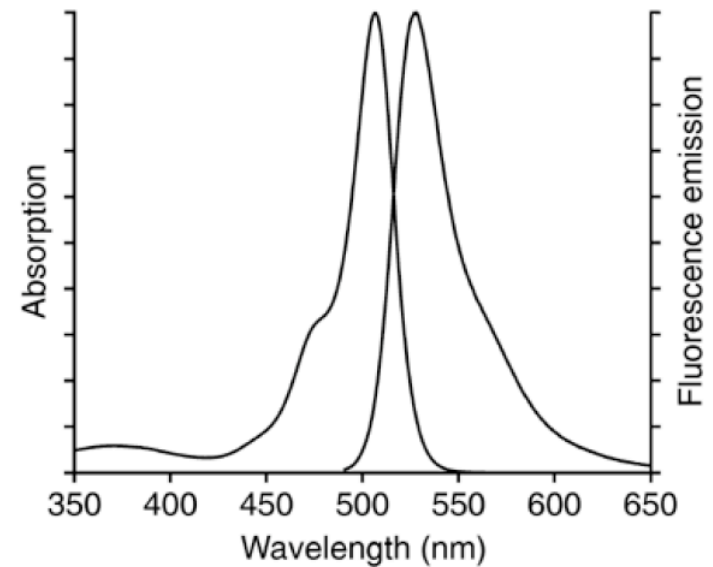
Fluo-3

BAPTA

EGTA

dichlorofluorescein

Fluo dyes:

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

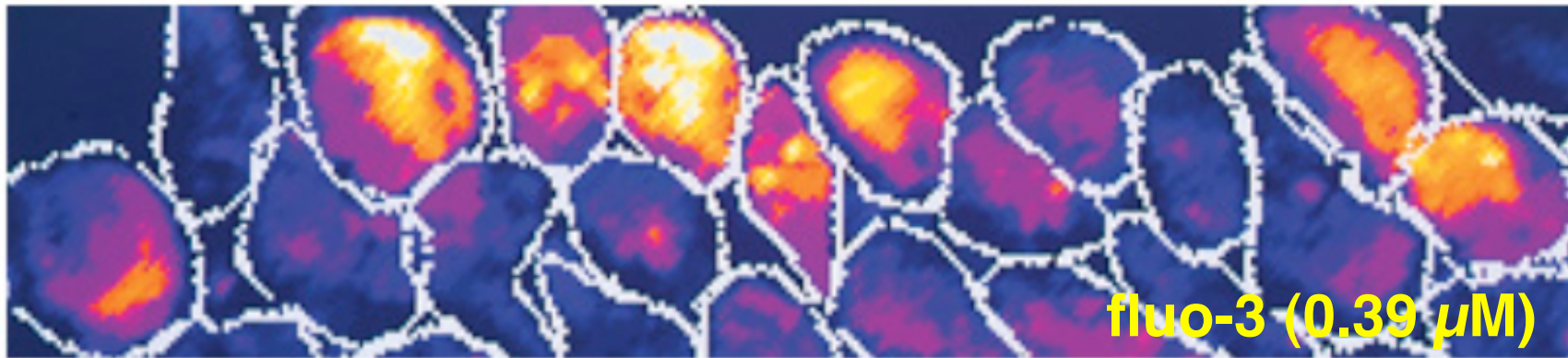**Fluo-3 Spectra**

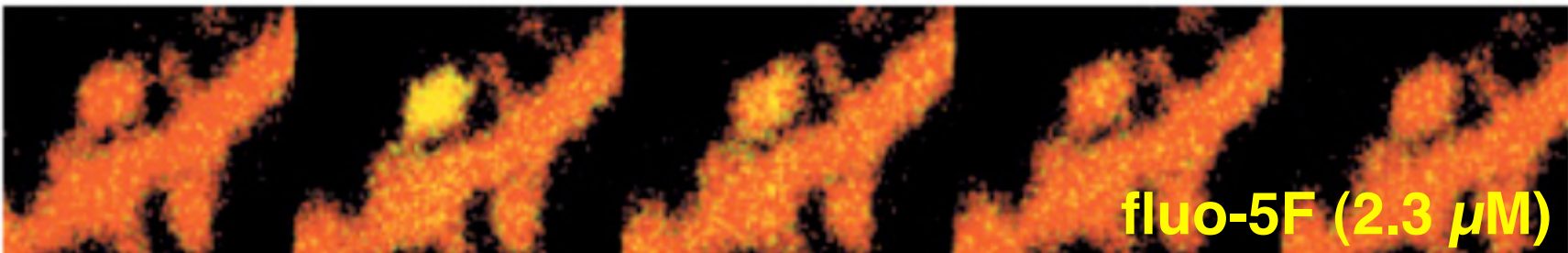| Indicator	$K_d(Ca^{2+})$	$R^{2'}$	$R^{7'}$	R^5	R^6
Fluo-3	0.39 µM	Cl	Cl	CH_3	H
Fluo-4	0.35 µM	F	F	CH_3	H
Fluo-5F	2.3 µM	F	F	F	H
Fluo-5N	90 µM	F	F	NO_2	H
Fluo-4FF	9.7 µM	F	F	F	F

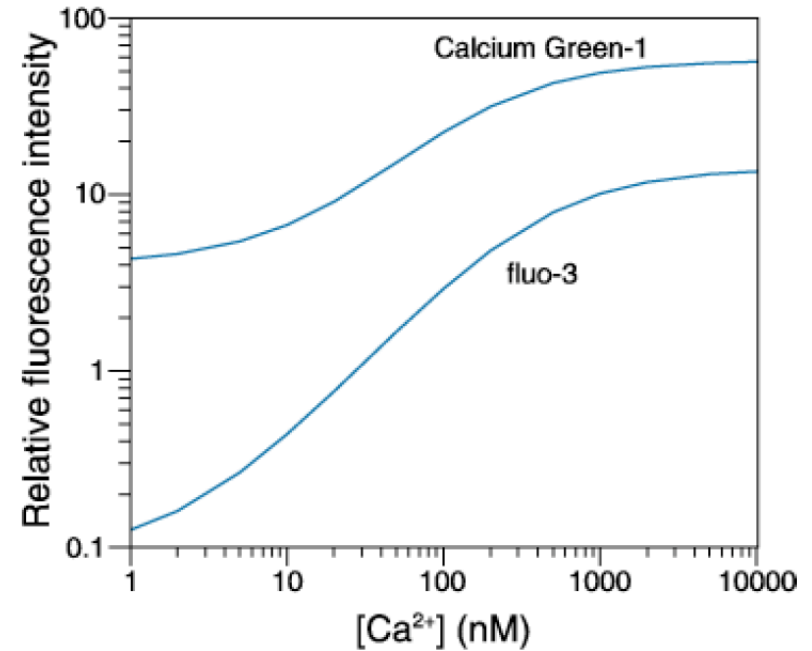Sensors with different calcium **affinities** (K_d values) may be appropriate for different applications:

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

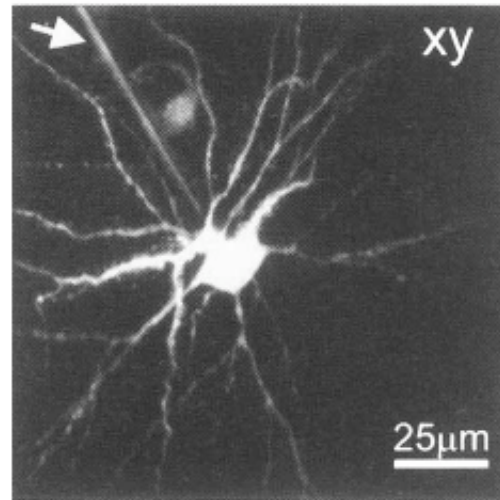fluo-3 (0.39 μM)

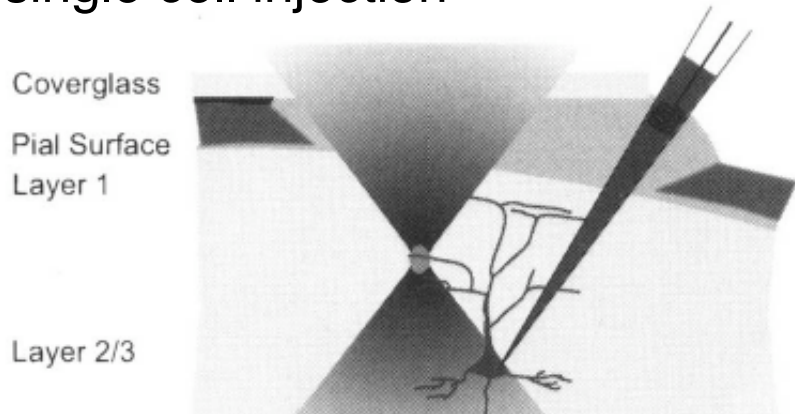calcium transients in dendritic spines

fluo-5F (2.3 μM)

Calcium Green & related dyes
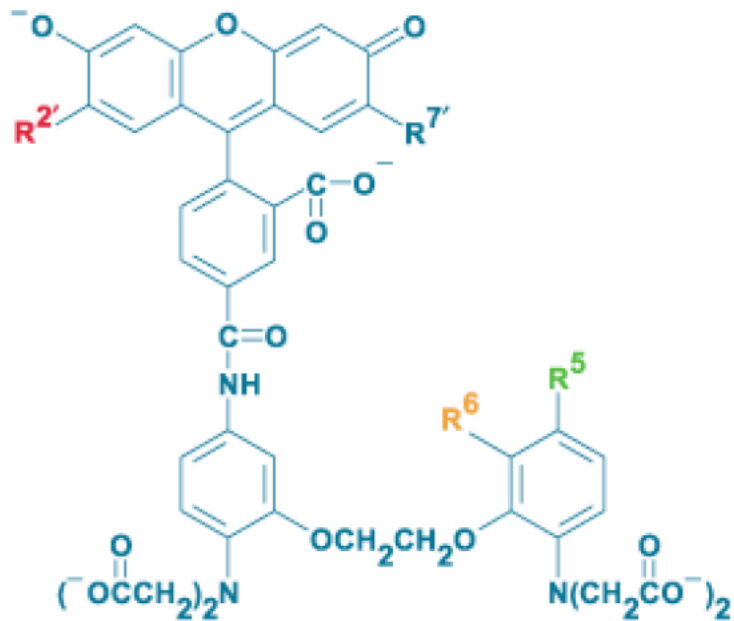
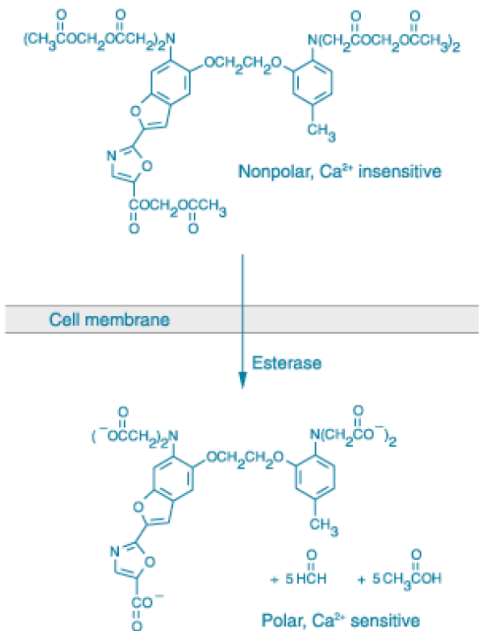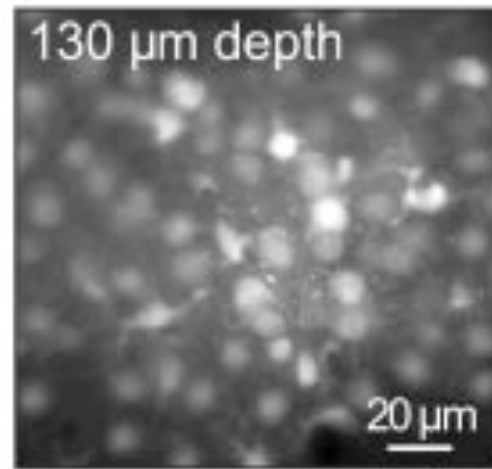What is the significance of having a dye with high fluorescence **intensity** or **dynamic range**?   SNR

How are calcium dyes applied to cells?

single cell injection

multicellular loading (or bath application)

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:

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

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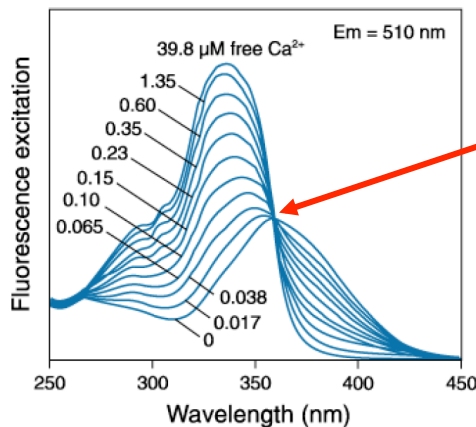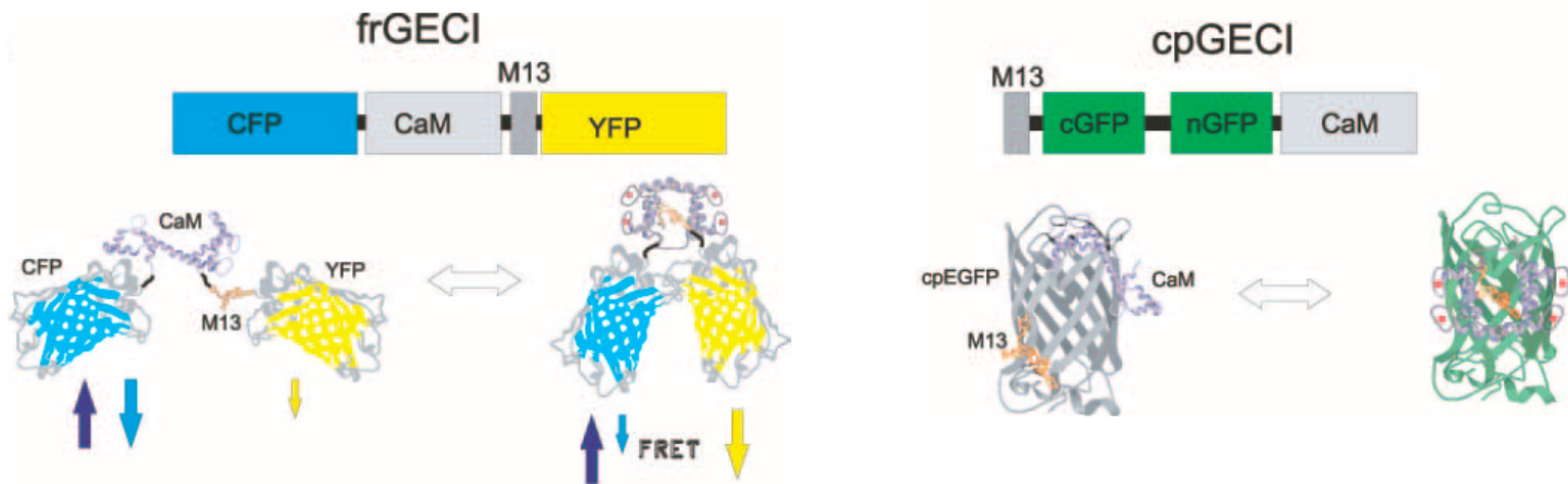
"isosbestic point"

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:
- 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.*).

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