

## Module 2 overview

### *lecture*

1. Introduction to the module
2. Rational protein design
3. Fluorescence and sensors

### *lab*

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

### **SPRING BREAK**

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

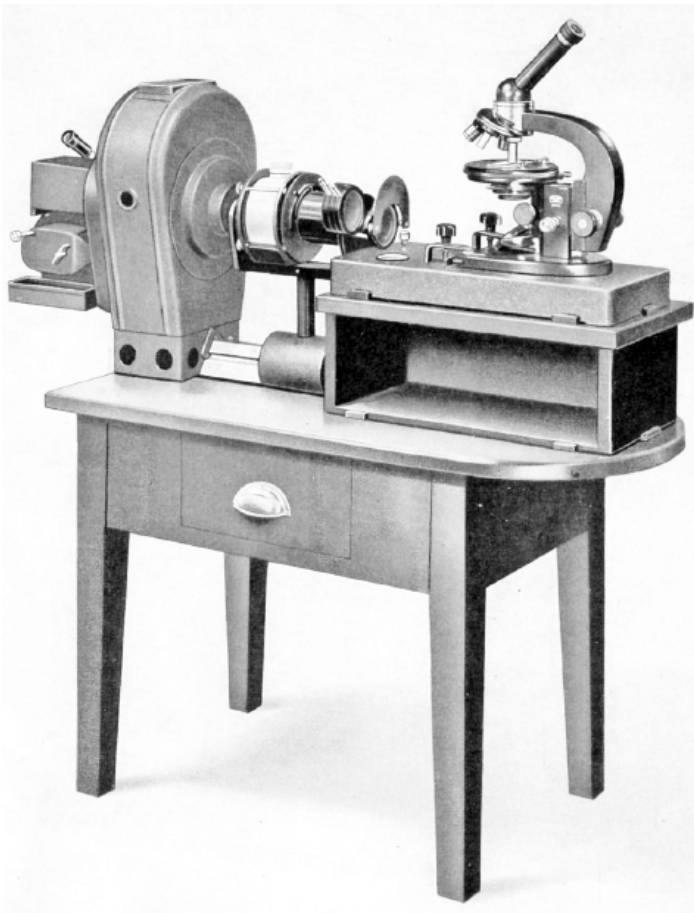
4. Prepare expression system
5. Induce protein
6. Characterize expression
7. Assess protein function

## 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  $\text{Ca}^{2+}$  sensors in cells
  - C. *In vivo* limitations and remedies
  - D. Advantages of genetically-encoded sensors

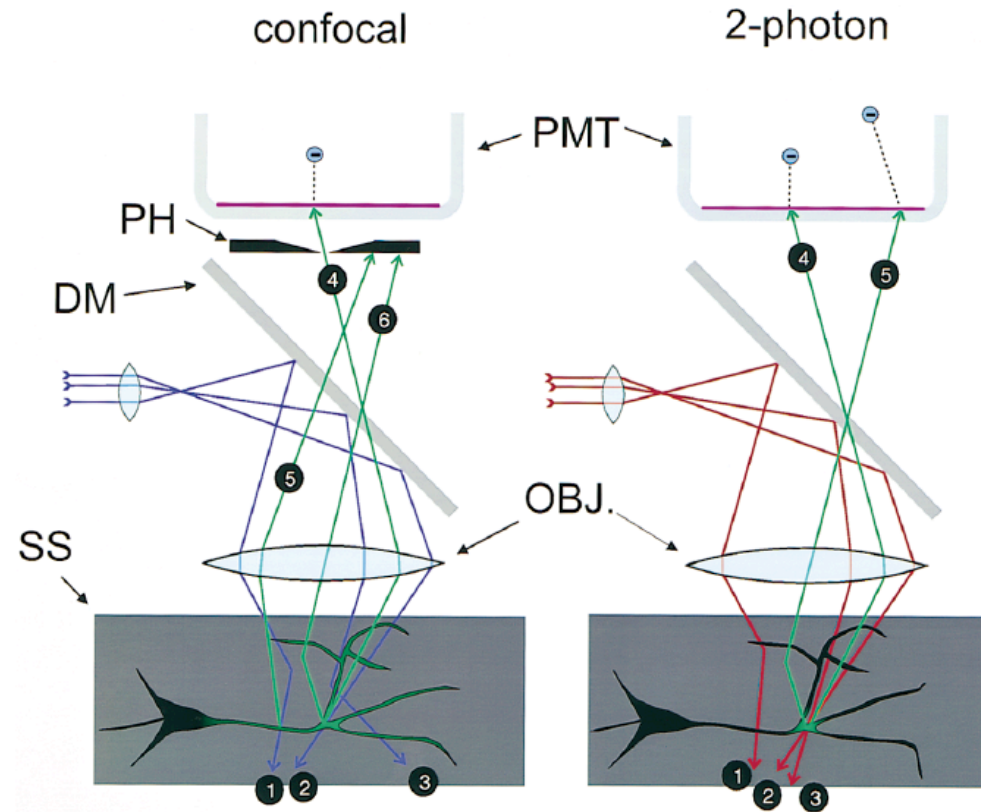
## fluorescence/luminescence microscopy

H. Lehmann & S. von Prowazek (1913)



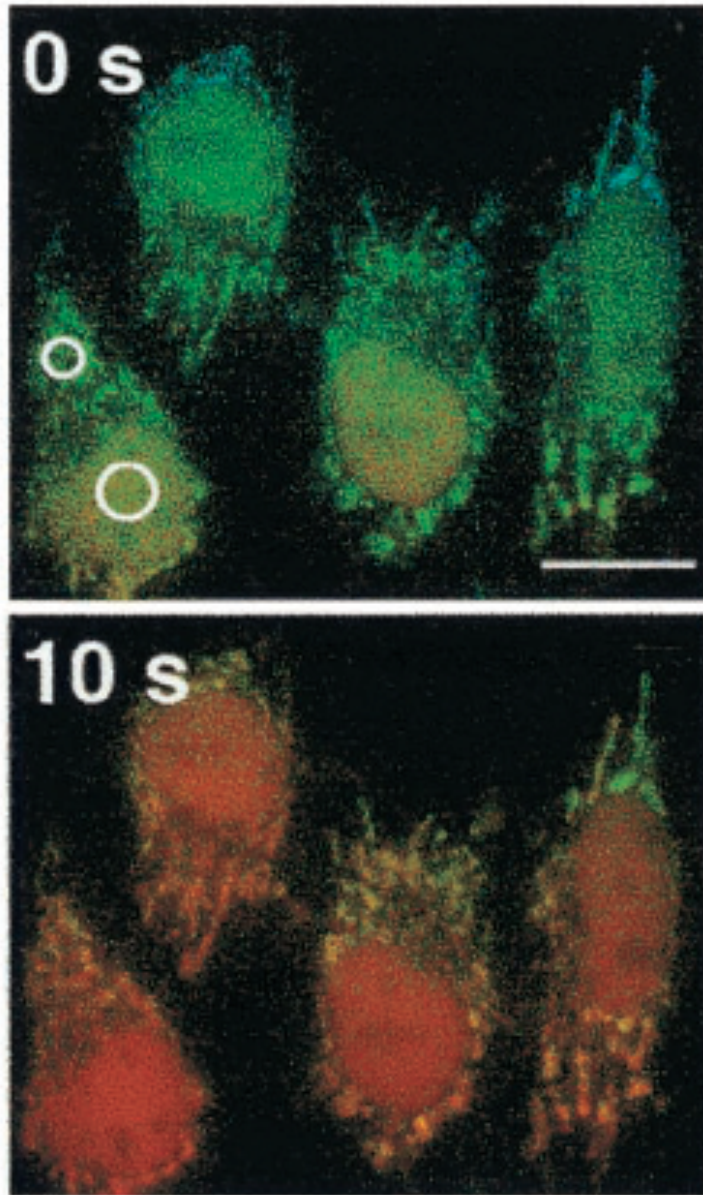
www.zeiss.com

## laser scanning microscopy



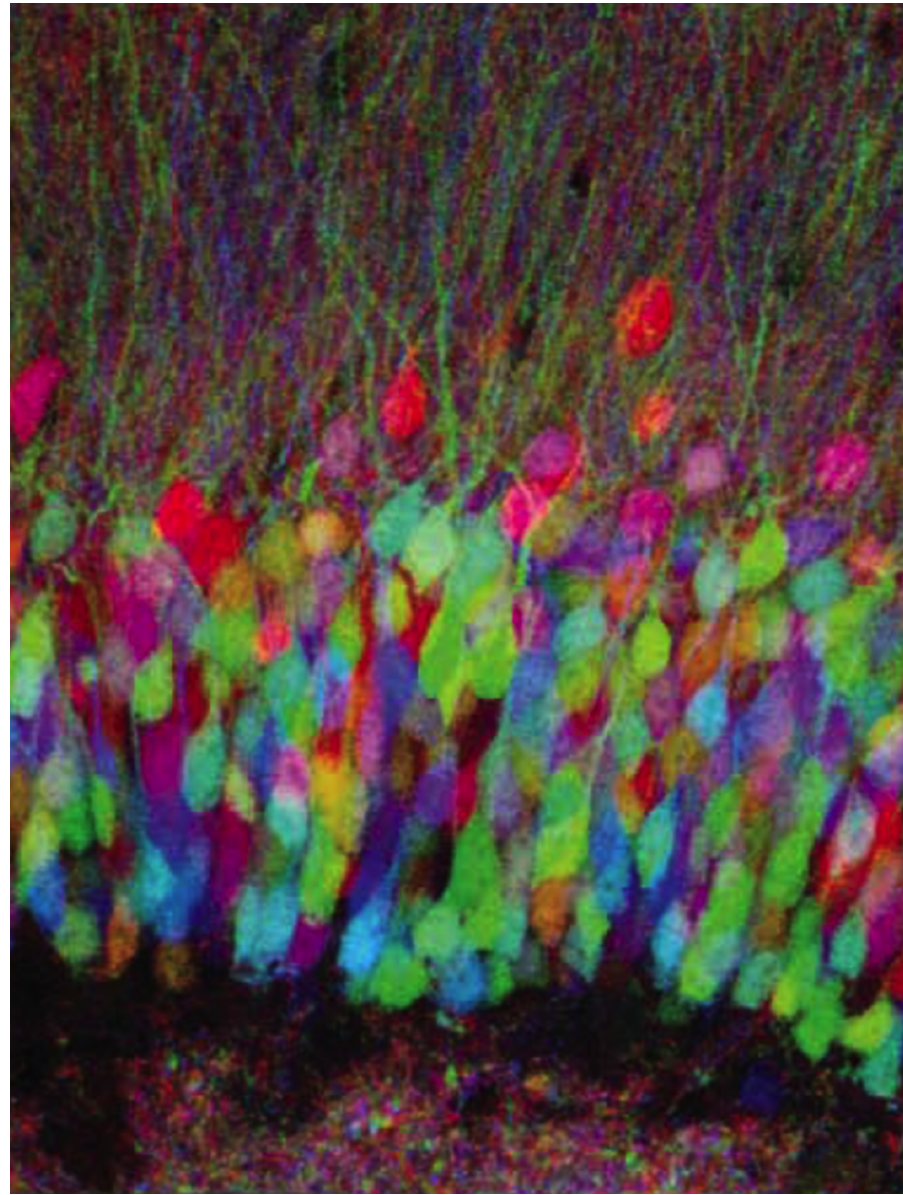
Denk & Svoboda (1997) *Neuron*

functional imaging



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

anatomical imaging & histology



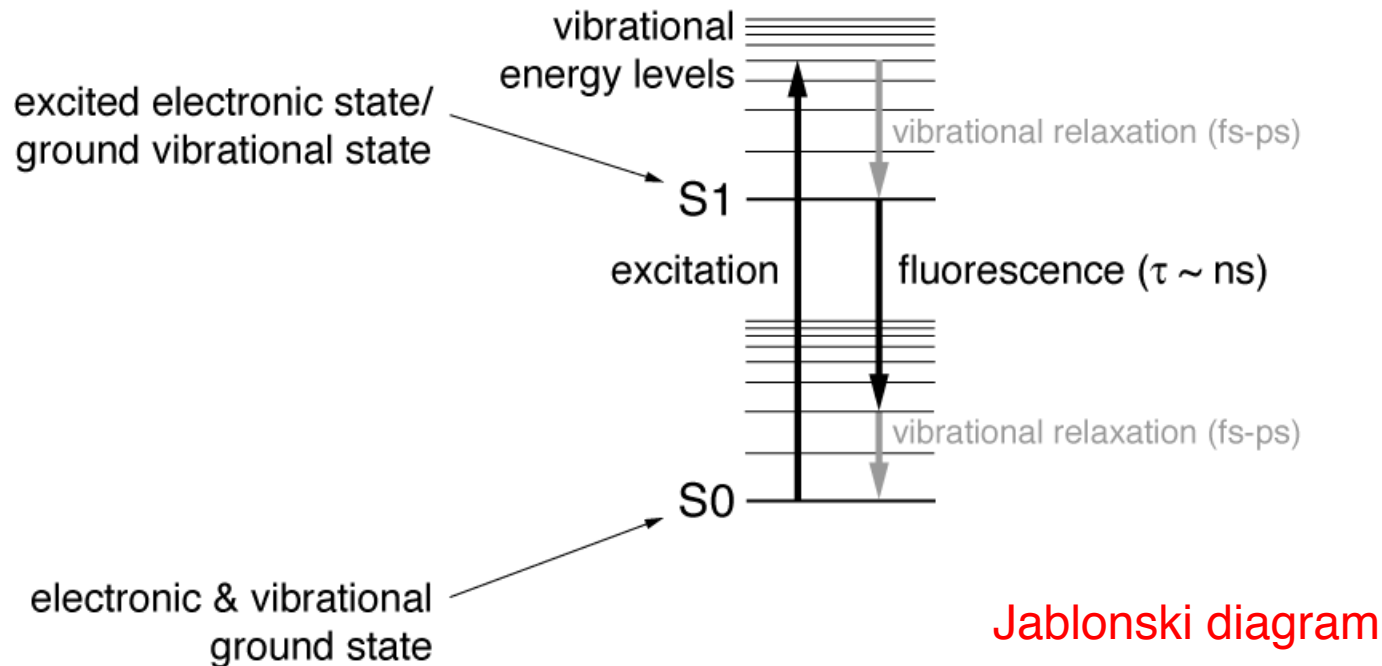
Livet *et al.* (2007) *Nature*



*in vitro assays*

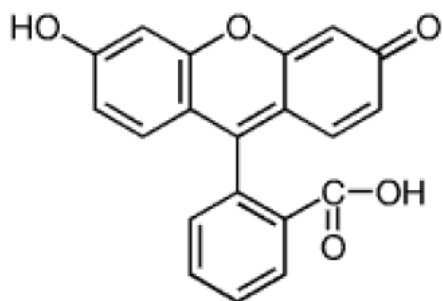
## Introduction to fluorescence

Fluorescence arises from transitions among molecular energy levels:

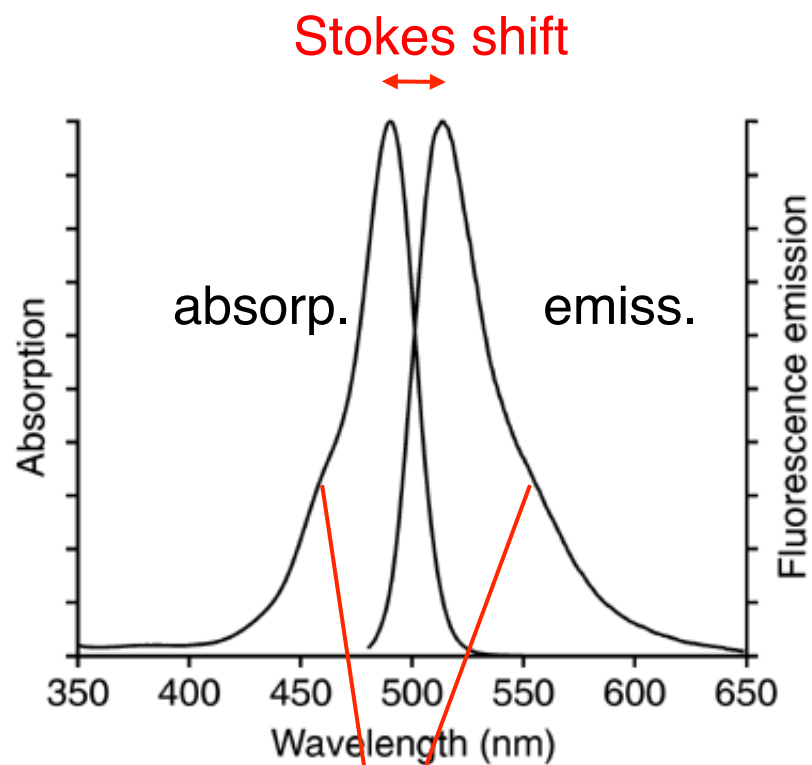


- electronic energy level transitions correspond to visible wavelengths
- vibrational energy level transitions correspond to infrared wavelengths
- rotational energy levels are coupled to vibrations and account for the smooth appearance of absorption/emission spectra

Fluorescence spectra for a typical fluorophore

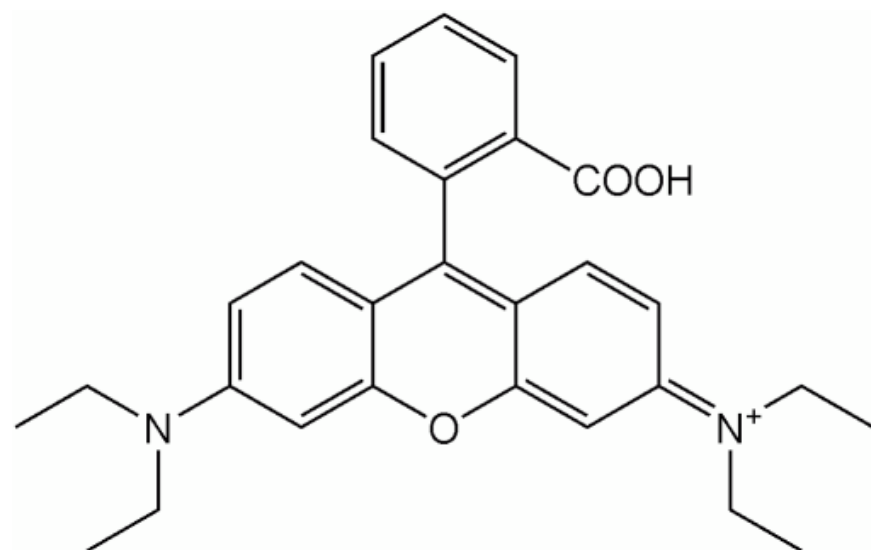


fluorescein



probes.invitrogen.com

“mirror image rule”



rhodamine B  
 $\lambda_{em} = 565 \text{ nm}$



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

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

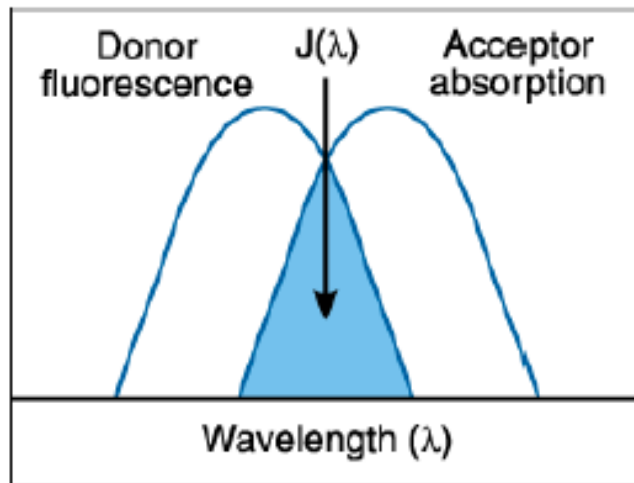
such that

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

where  $\tau$  is the **fluorescence lifetime**, incorporating both  $\Gamma$  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*.

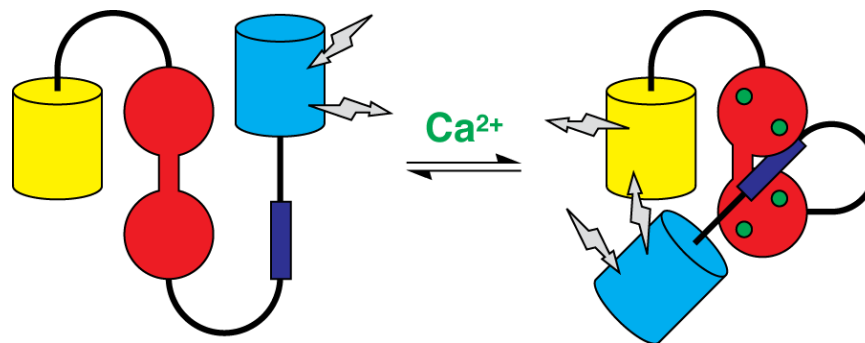


The distance at which 50% of excited donors are deexcited by the FRET mechanism is defined as the **Förster radius** (usu. 10-100 Å):

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

**FRET efficiency** is defined as:

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



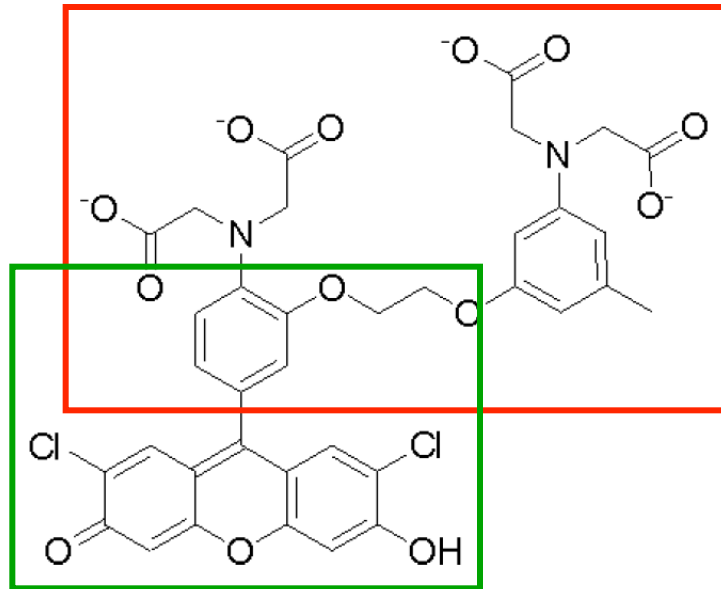
## Fluorescent calcium sensors

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

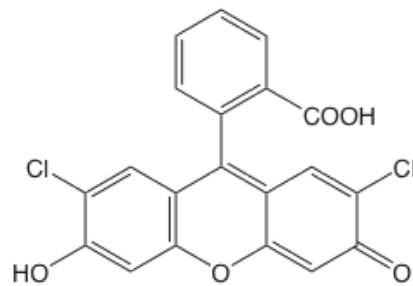
- calcium affinity
- absorbance and emission properties
- structural properties (*e.g.* protein vs. small molecule, membrane permeability, binding and localization)

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

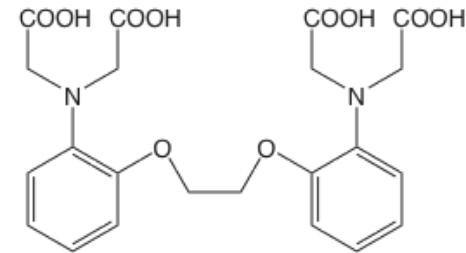
A typical calcium sensor consists of a calcium sensitive component attached to one or more fluorescent moieties:



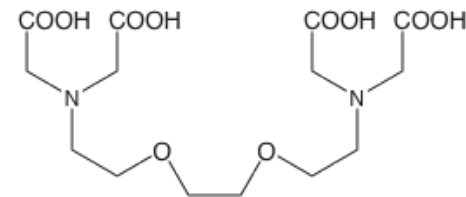
**Fluo-3**



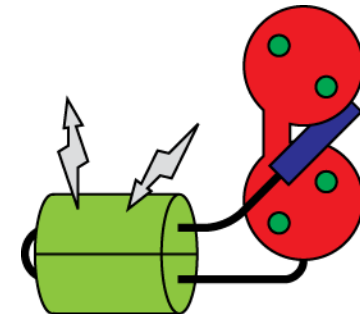
**dichlorofluorescein**



**BAPTA**

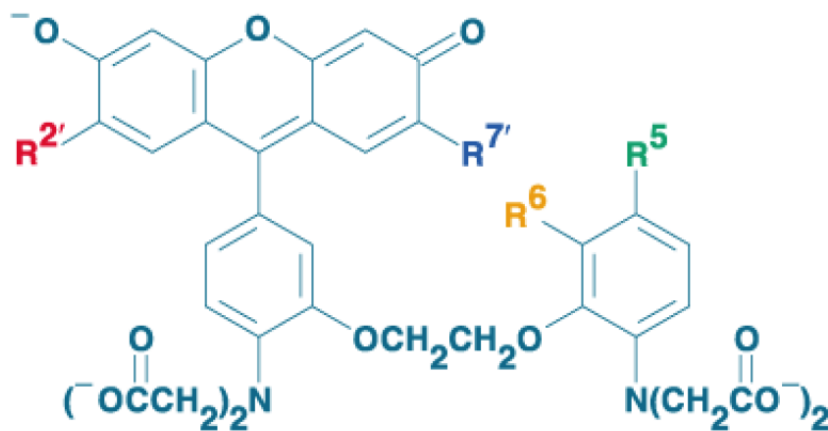


**EGTA**



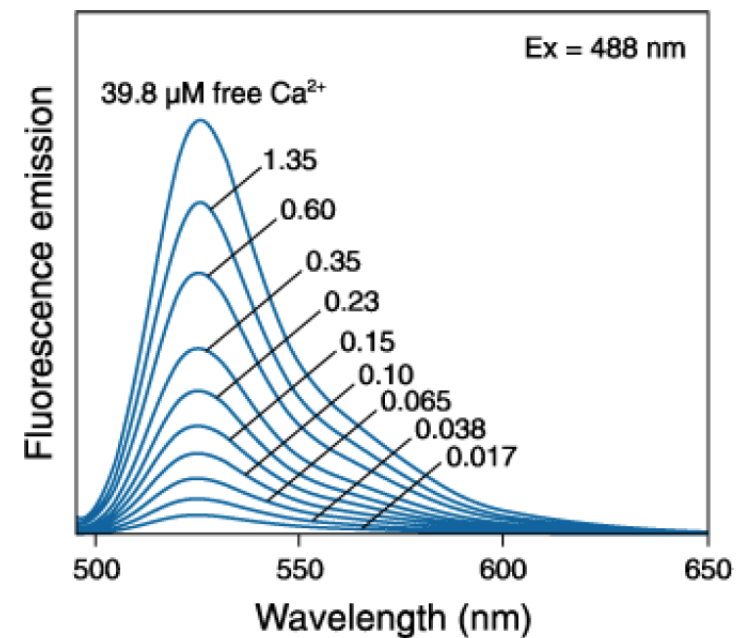
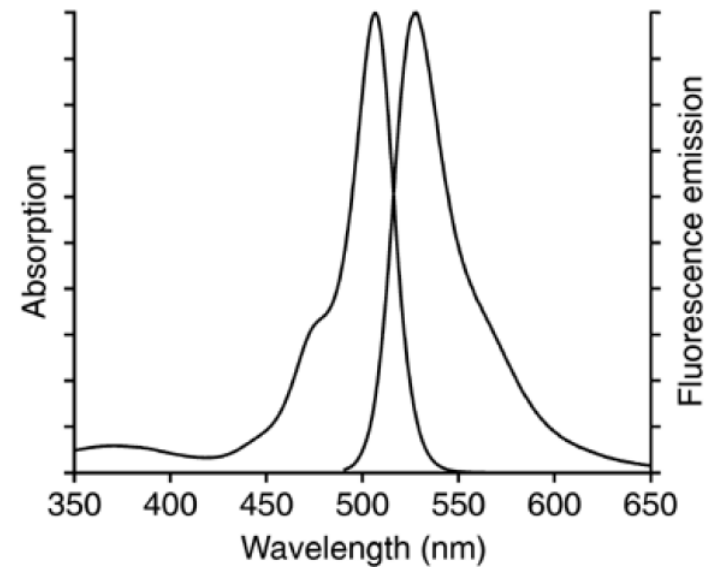
## Fluo dyes:

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



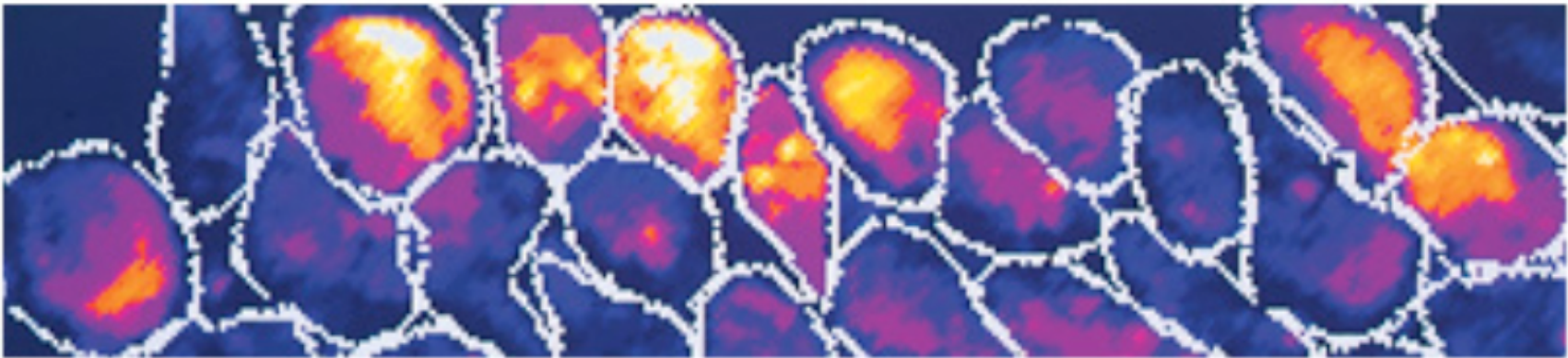
Indicator	$K_d(\text{Ca}^{2+})$	$\text{R}^{2'}$	$\text{R}^{7'}$	$\text{R}^5$	$\text{R}^6$
Fluo-3	0.39 $\mu\text{M}$	Cl	Cl	$\text{CH}_3$	H
Fluo-4	0.35 $\mu\text{M}$	F	F	$\text{CH}_3$	H
Fluo-5F	2.3 $\mu\text{M}$	F	F	F	H
Fluo-5N	90 $\mu\text{M}$	F	F	$\text{NO}_2$	H
Fluo-4FF	9.7 $\mu\text{M}$	F	F	F	F

## Fluo-3 Spectra



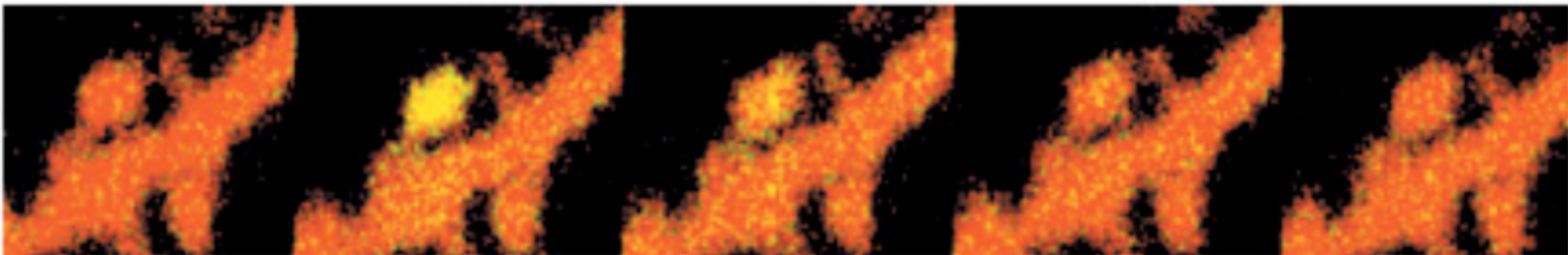
Sensors with different calcium **affinities** ( $K_d$  values) may be appropriate for different applications:

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

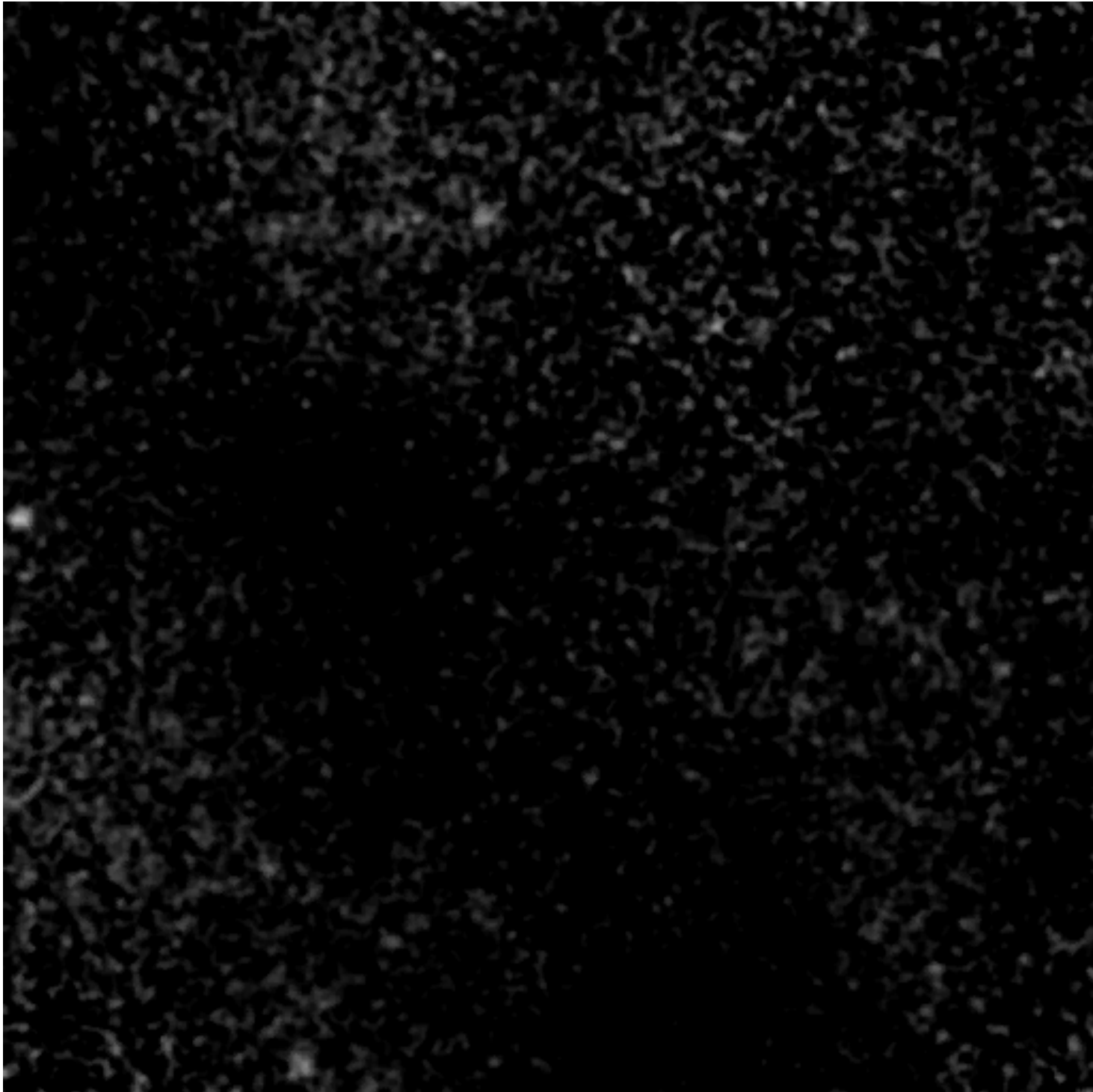


fluo-3 ( $0.39 \mu\text{M}$ )

calcium transients in dendritic spines



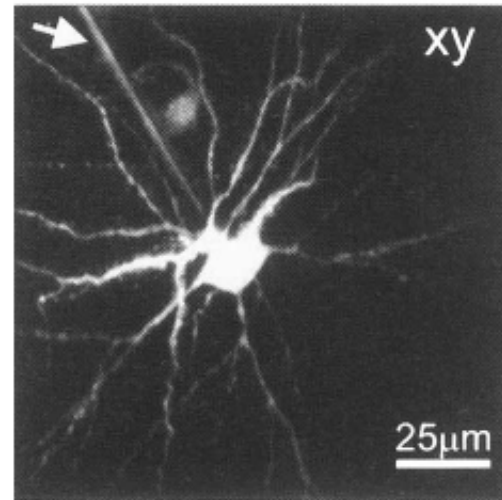
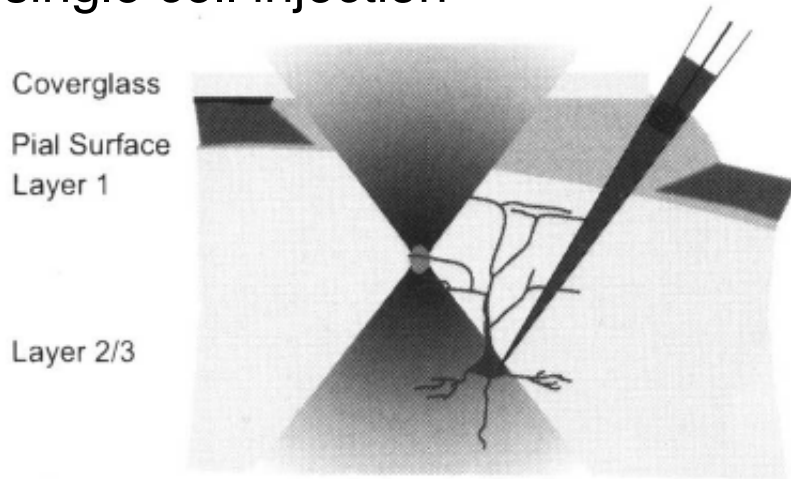
fluo-5F ( $2.3 \mu\text{M}$ )



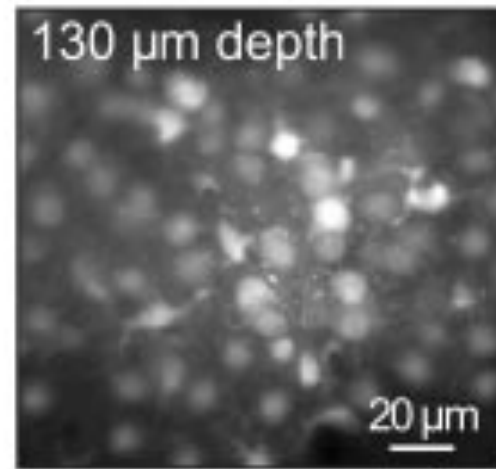
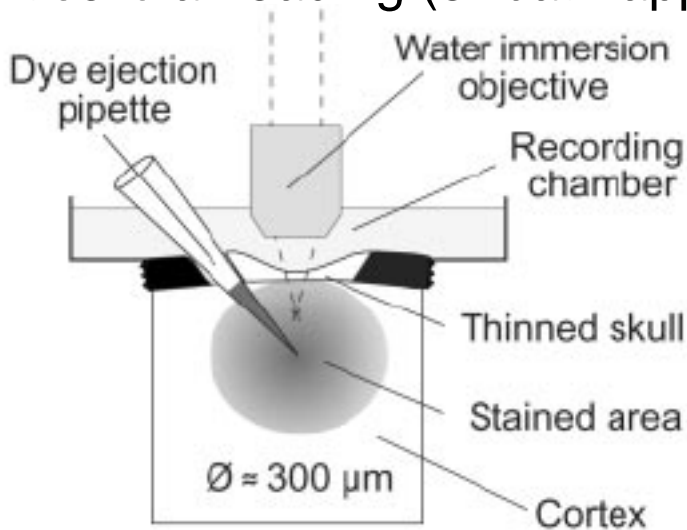
Ohki *et al.* (2006) *Nature*

# How are calcium dyes applied to cells?

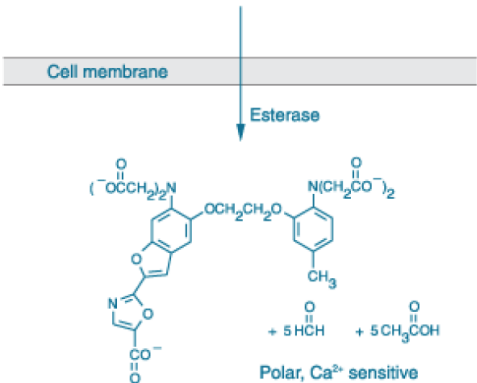
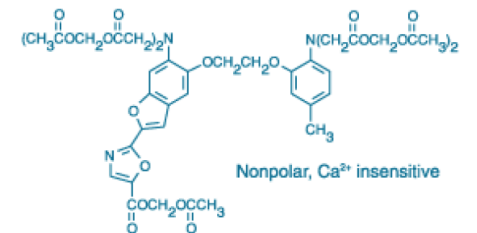
## single cell injection



## 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, <i>e.g.</i> using <u>dextran conjugate</u>
dye leaks from cell or is sequestered into inappropriate cellular compartments	loss of fluorescence responses, higher background fluorescence	use <u>dextran conjugates</u> or targeted indicators, <u>ratiometric imaging</u>
dye bleaches over the course of experiments	loss of fluorescence responses	lower imaging duty cycle, select dyes with low bleaching, <u>ratiometric imaging</u>

## Ratiometric imaging

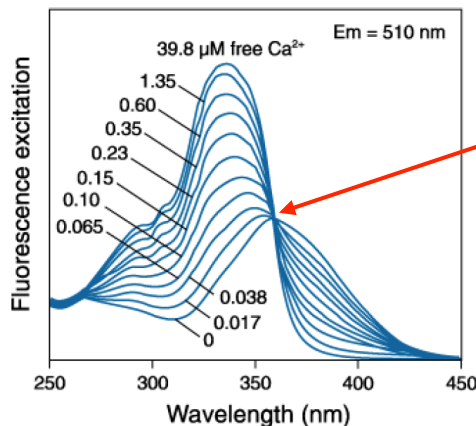
Suppose you measure fluorescence intensity from a cell, but you don't know either how much dye is present or what the calcium concentration is; you have **one equation in two unknowns** ( $[L]_{\text{tot}}$  and  $[Ca^{2+}]$ ):

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

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

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

$$\begin{aligned} F_{\text{tot}}^* &= F_{Ca^{2+}}^* [L \cdot Ca^{2+}] + F_{\text{free}}^* ([L]_{\text{tot}} - [L \cdot Ca^{2+}]) \\ &= F^* [L]_{\text{tot}} \quad (\text{if } F^* \text{ is independent of } [Ca^{2+}]) \end{aligned}$$



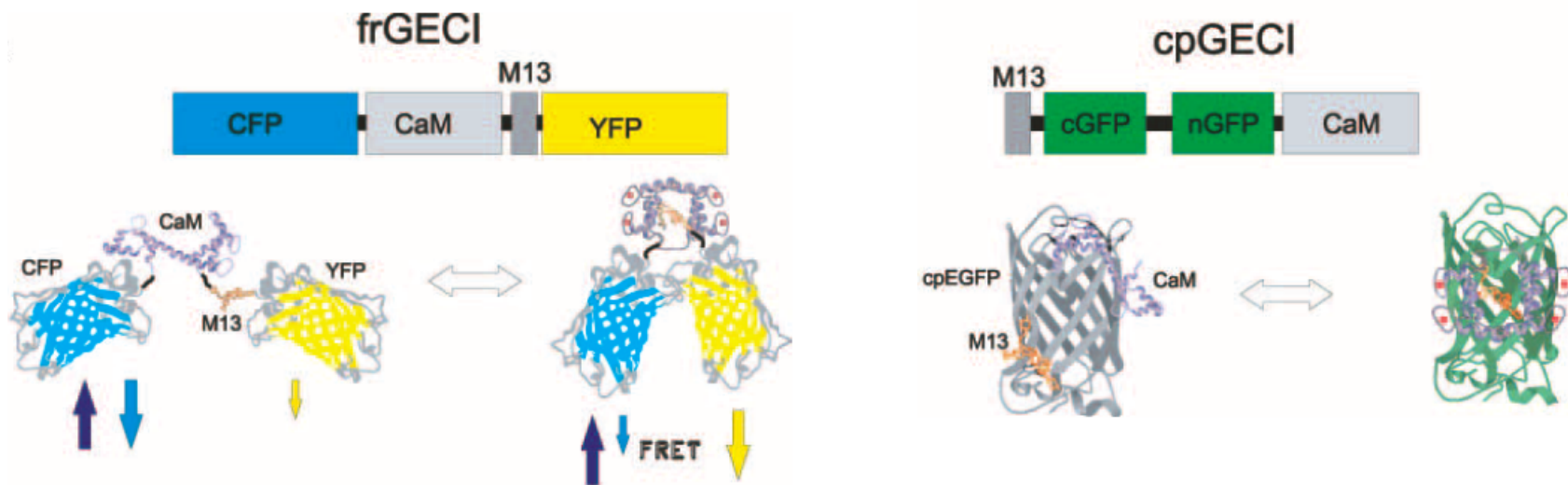
“isosbestic point”

The **ratio  $F/F^*$**  is independent of  $[L]_{\text{tot}}$  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, <i>e.g.</i> using <u>dextran conjugate</u>
dye leaks from cell or is sequestered into inappropriate cellular compartments	loss of fluorescence responses, higher background fluorescence	<del>use <u>dextran conjugates</u> or targeted indicators, <u>ratiometric imaging</u></del>
dye bleaches over the course of experiments	loss of fluorescence responses	<del>lower imaging duty cycle, select dyes with low bleaching, <u>ratiometric imaging</u></del>

**use proteins**

**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

