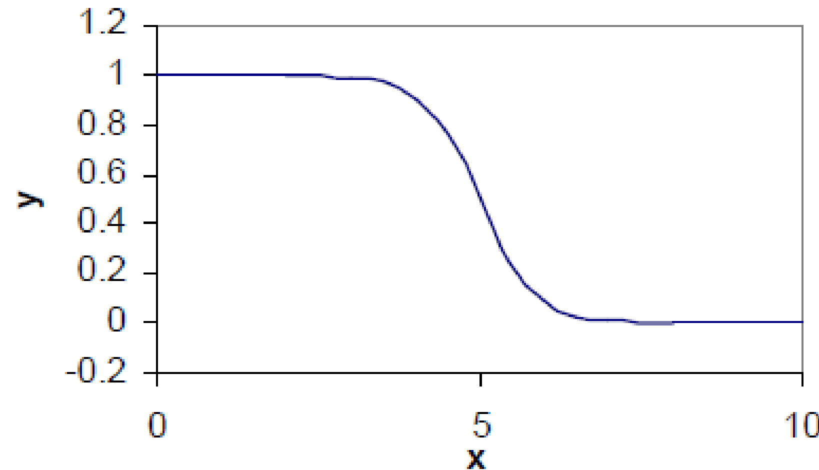
More biosensors, optical actuators

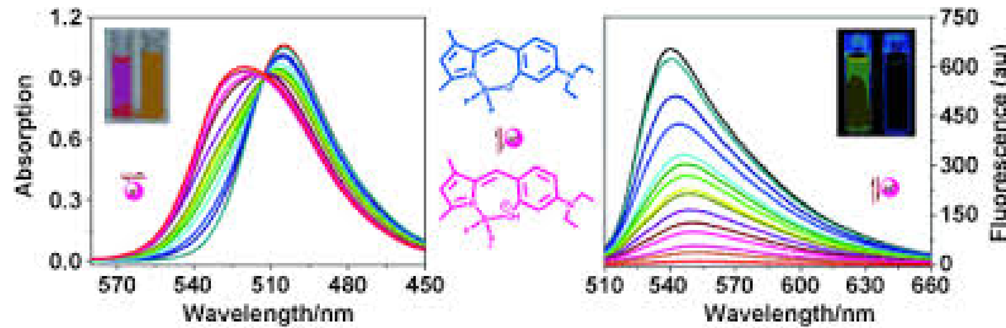
- Last class
  - Watershed
  - Intro to biosensors
- This class
  - Dye based sensors
  - Protein based sensors
  - Actuators

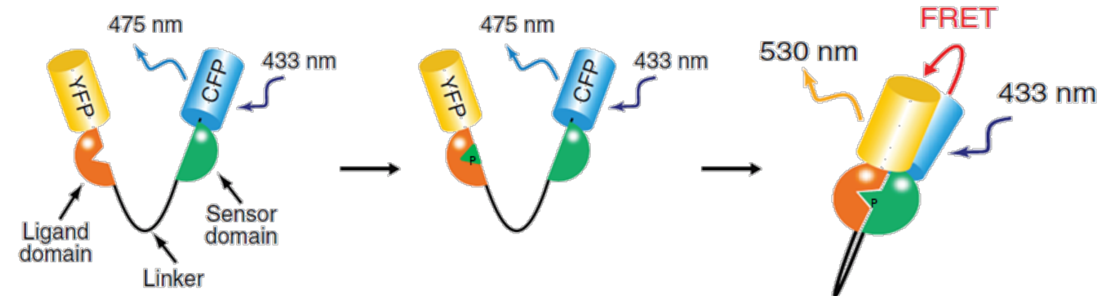
Biosensors at large

- Anything that changes fluorescence in response to physiological change
- Difficult to optimize across every parameter
- Check pH sensitivity



Sensor	K_d (nM)	k_{off} (s^{-1})
GCaMP3	345±17	2.57
GCaMP5G	447±10	2.52
GCaMP6s	144±4	1.12
GCaMP6m	167±3	2.06
GCaMP6f	375±14	3.93





Measuring signals

- Easy thing to think about is change in fluorescence divided by original fluorescence (dF/F)
- Unfortunately it's useless
- Signal to noise ratio = signal – background/std
- Smallest feature you can resolve is when SNR = 1
- What is the smallest amount of signal you can resolve in some time measurement

dF/F = (12-2)/2 = 5

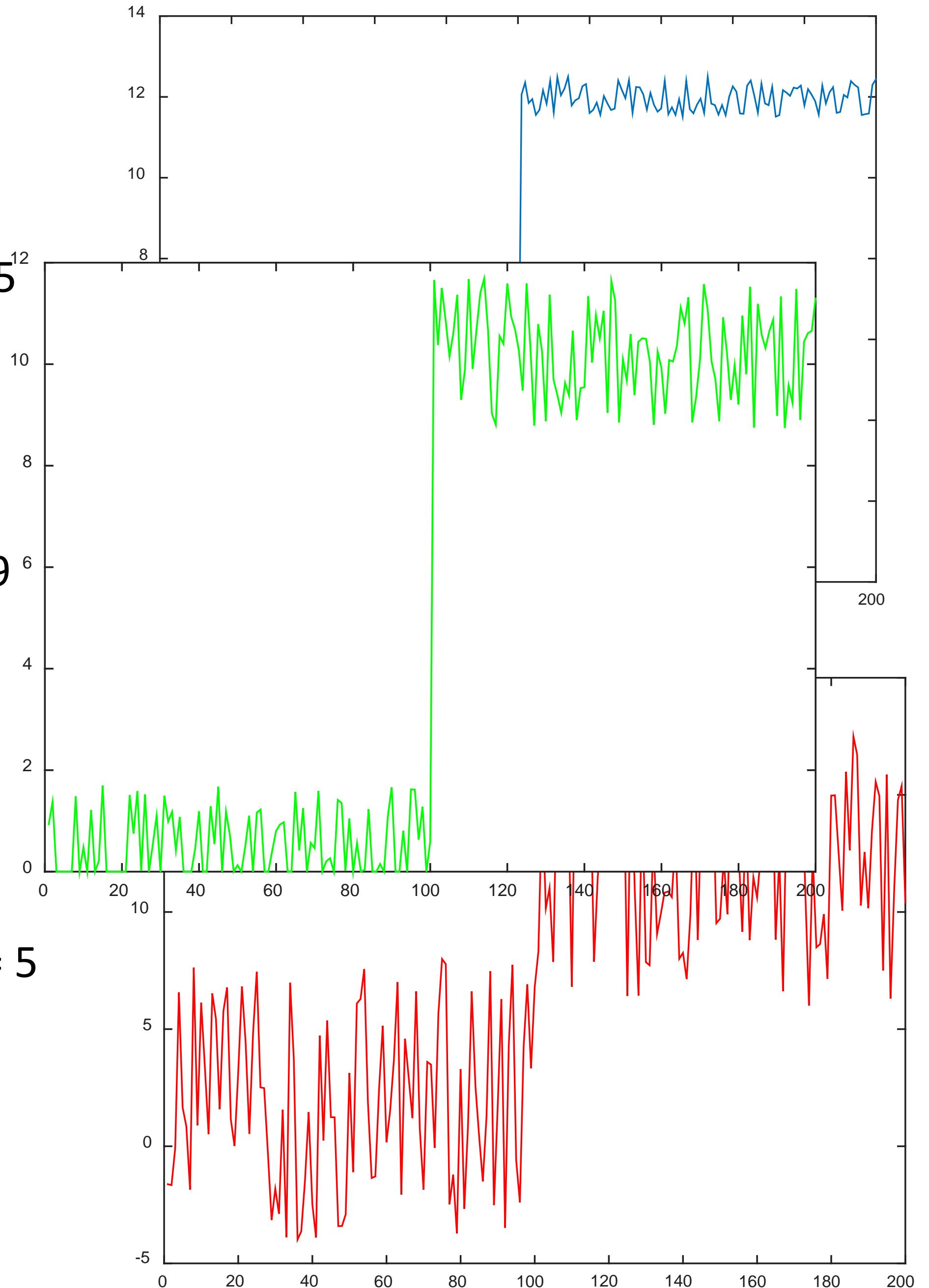
dF/F = 500%
Hooray!

dF/F = (10-.1)/.1 = 99

dF/F = 9900%
Hooray?

dF/F = (12-2)/2 = 5

dF/F = 500%
Hooray?

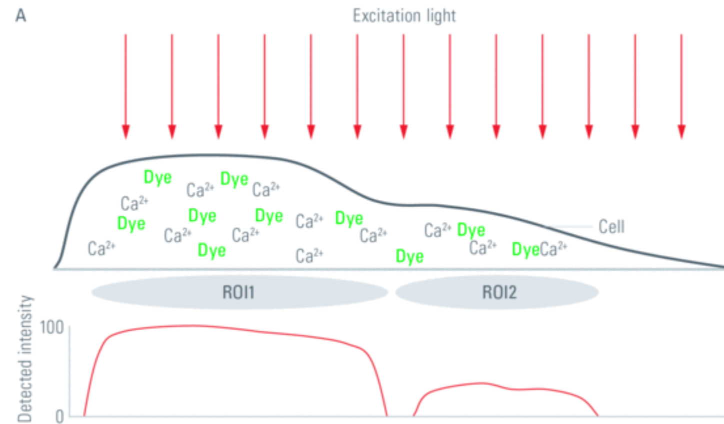


This is what we're interested in

Ratiometric imaging

$$Total\ Flourescence = \#Flourophores\ * QY(env)\ * absorption + \text{background}$$

- Ratiometric imaging is by far the easiest way to get absolute units on a measurement
- Measuring a single color makes it impossible unless you know the number of the fluorophores present (usually impossible)
- Having two colors *at a defined stoichiometry* can correct for this issue
- Doesn't affect localization based sensors

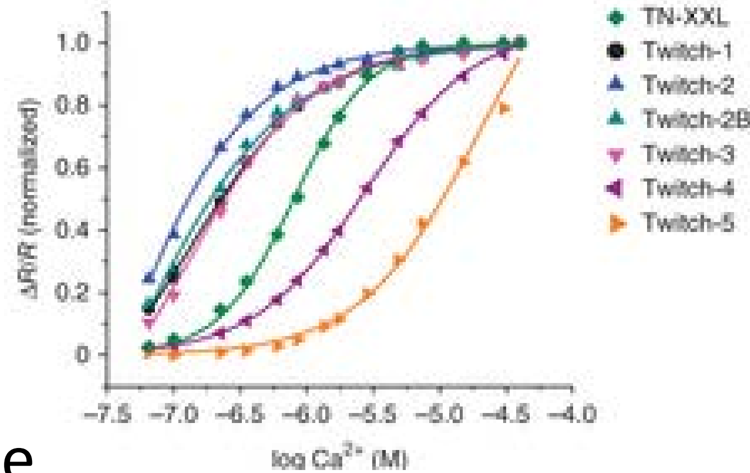


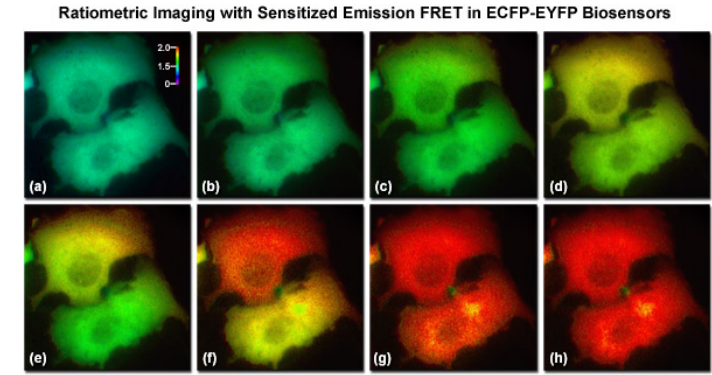
$$Ratio = \frac{N_1 * QY_1 * \varepsilon_1}{N_2 * QY_2 * \varepsilon_2}$$

$$= \frac{QY_1 * \varepsilon_1}{QY_2 * \varepsilon_2}$$

Calibrating ratiometric sensor

- Use either two excitations, or two emissions (or both)
- First have to map each color at known concentrations of analyte
- Generate, and fit ratio to hill curve, with known concentrations on the x axis
- Use fit as look-up table for future experiments
- You can NOT change laser intensities or alignment after determining fit





1. Subtract background

$$F_1 = I_1 \left(N_{Fl} * QY_1 * \varepsilon_1 + QY_2 * N_{Fl} * \varepsilon_2 + QY_{AF1} * \varepsilon_{AF1}\right) + bg$$

$$F_2 = I_2 \left(QY_1 * N_{Fl} * \varepsilon_1 + N_{Fl} * QY_2 * \varepsilon_2 + QY_{AF2} * \varepsilon_{AF2}\right) + bg$$

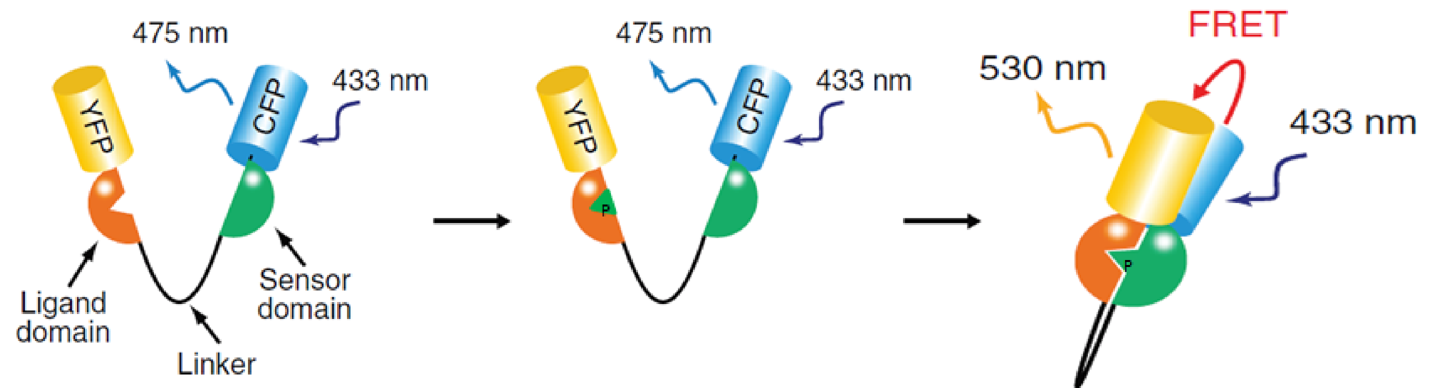
$$Ratio = \frac{QY_1 * \varepsilon_1}{QY_2 * \varepsilon_2}$$

1. Determine laser intensities to use
2. Determine non-expressing autofluorescence from each laser
3. Look at bleed through from each excitation – should be 0
4. Subtract background from non-cell region
5. Subtract bleed through equivalent from each channel
6. Divide to get ratio

Principles of sensor design

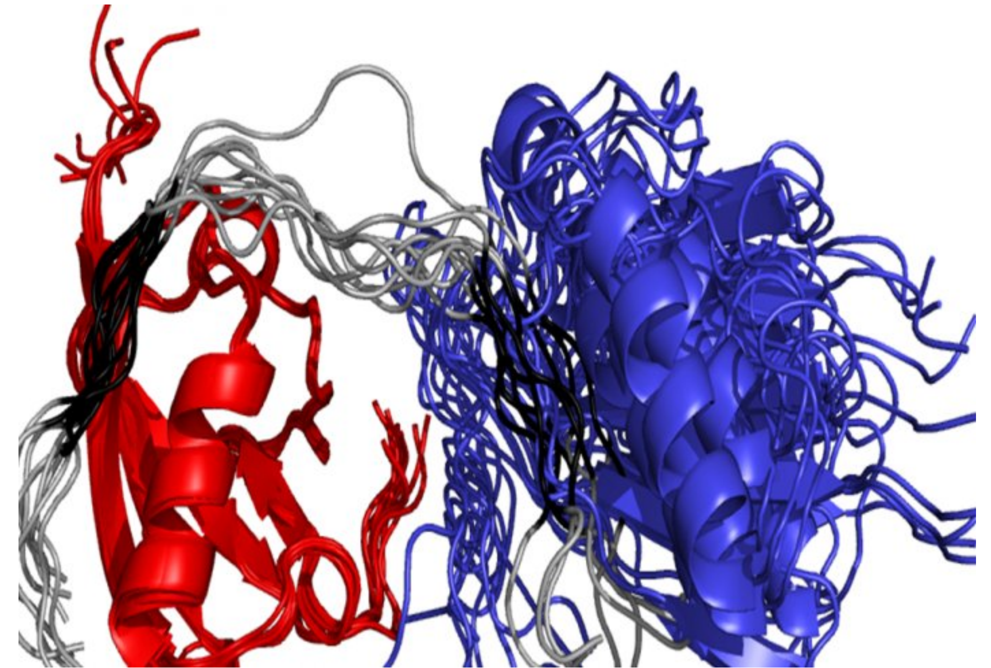
- Imagine a FRET pair – highly sensitive to distance between fluorophores
- Find a protein, somewhere in nature, that binds to the ligand you're interested in measuring
- Attach FPs to either side of protein that undergoes large conformational change
- Try to maximize conformational distance

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



Issues with this process

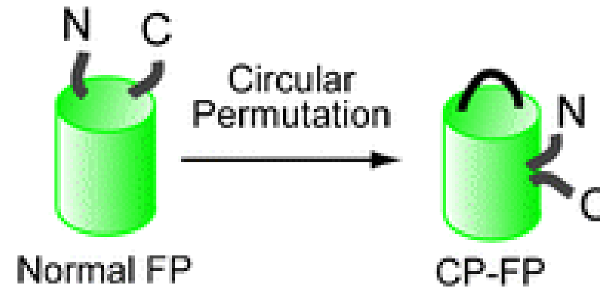
- Nearly every prototype sensor had the same range of 0.25
- Crazy given the large conformational changes
- Remember that proteins are flexible beasts, not static crystal structures
- Even if the FPs are on the same lobe, still see same FRET changes
- Knowledge: Large conformational changes are NOT needed

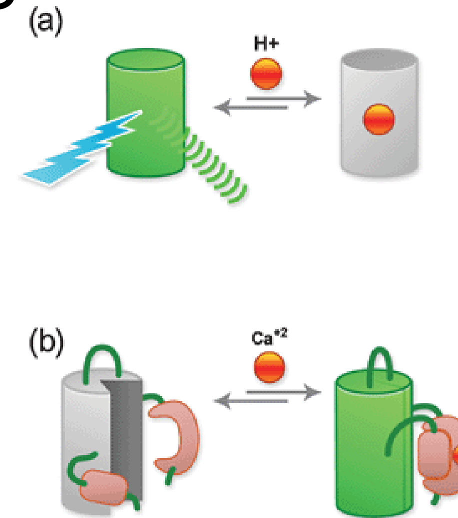


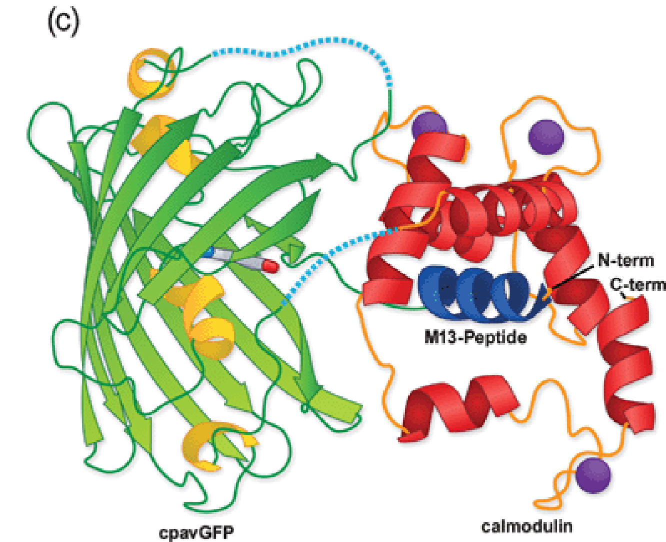
NMR structure of DNA repair protein

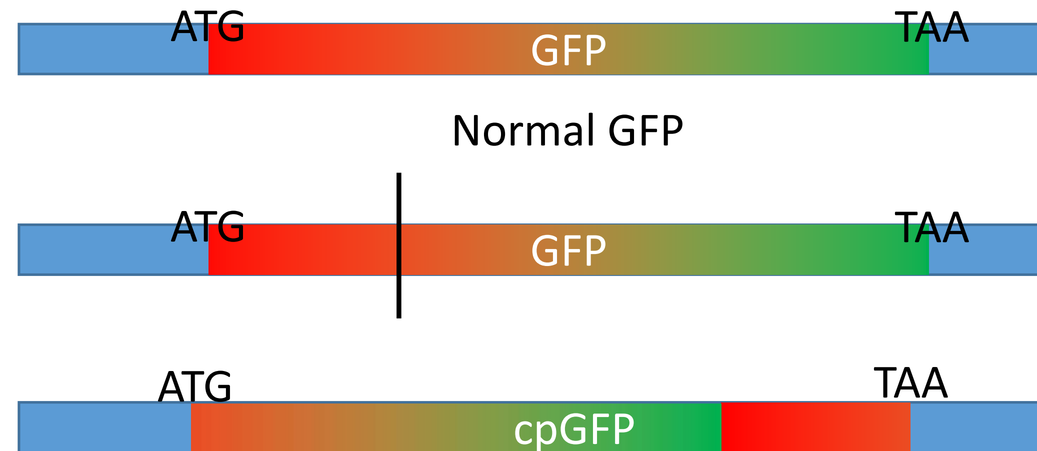
Circularly permuted proteins

- GFP folding is robust, so you can change where the C and N termini live
- Circularly permuted GFP has a very high sensitivity to pH
- Sensors can be made that modulate chromophore stability (pK_a)



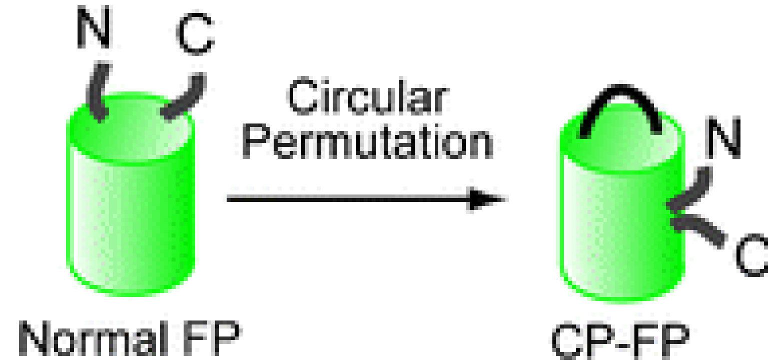


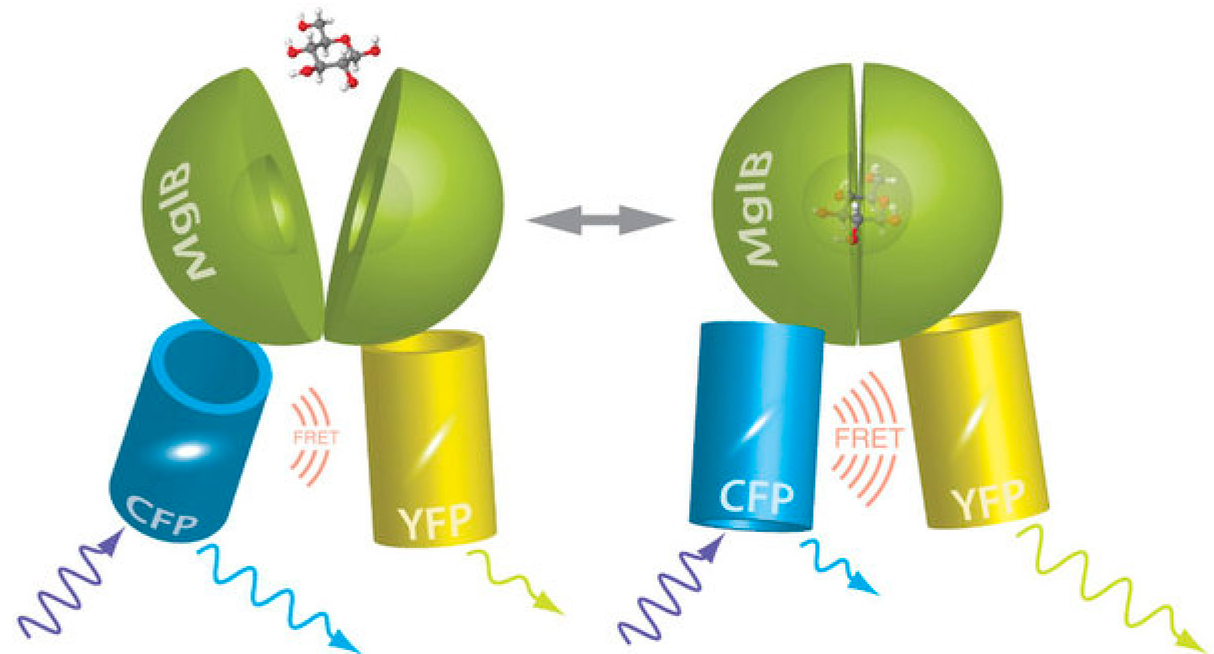




Two main schemes for detection

- Find active domain
- Attach FRET pairs
- Attach to cpGFP
- For both schemes, you need a protein domain that will change conformation in the presence of what you want to detect

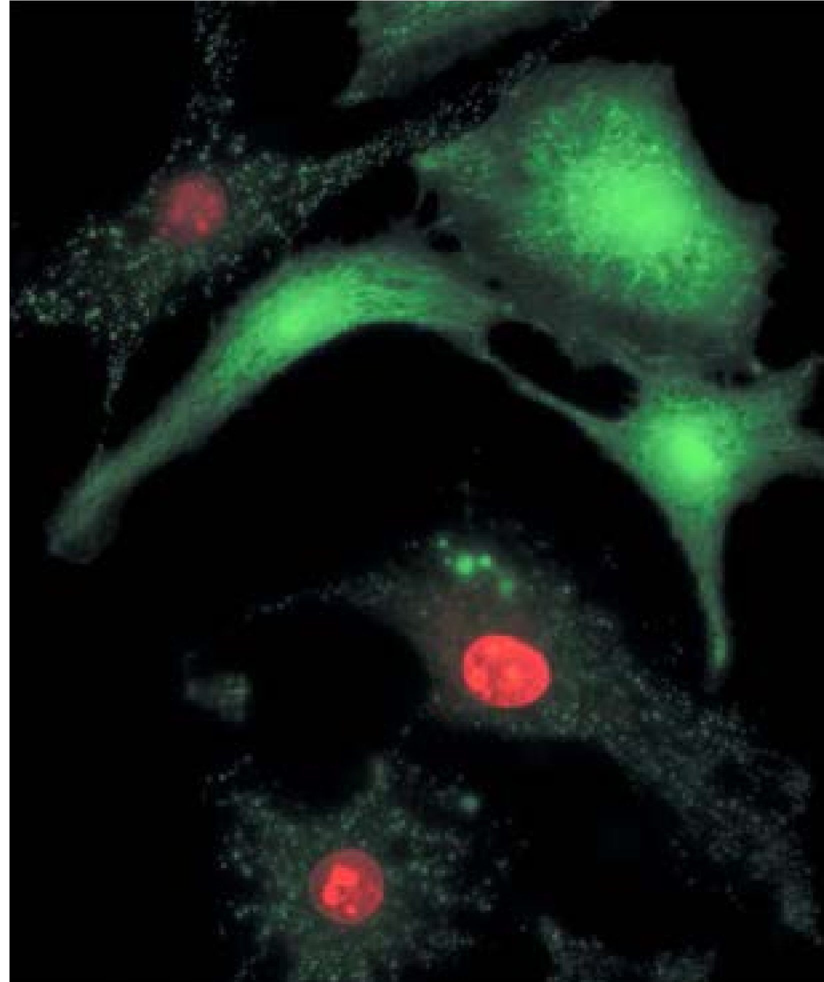




Dye based sensors

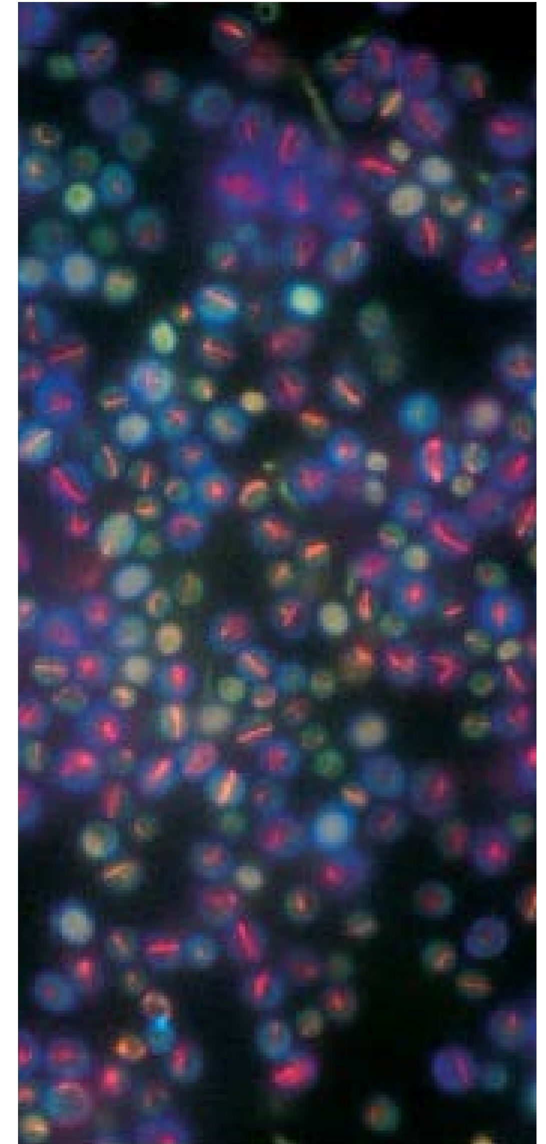
LiveDead stains

- Important thing to test both live and dead stains, hopefully all cells fall into one category
- Kits come with a dye to mark living cells and a second dye that will only stain dead cells
- Can be counted by microscopy or FACS



Calcein AM – used to detect funcinoal esterases
Ethidium Homodimer – Loss of membrane integrity

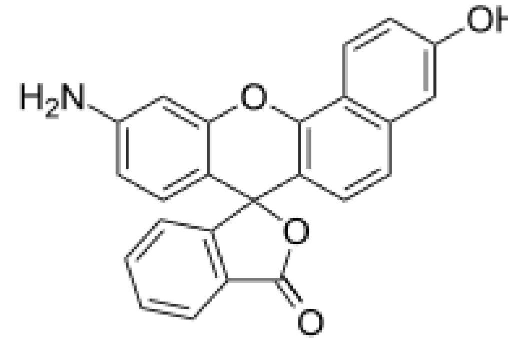
FUN1 – Starts green, live cells convert it to red
Counterstain all cells with Calcofluor White (blue)

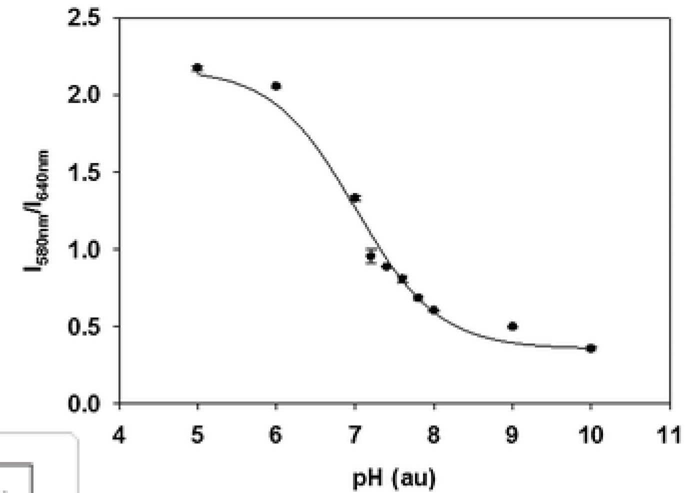


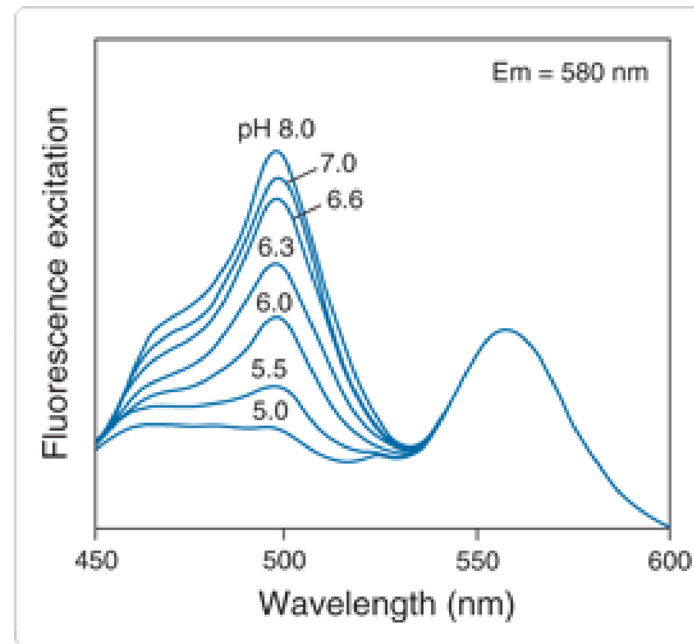
pH sensors

- pH changes everything, so it's very easy to generate pH sensitive fluorophores
- Numerous dyes and FPs, variety of colors and sensitivities
- Want to worry about color, photostability, solubility, pK_a

SNARF





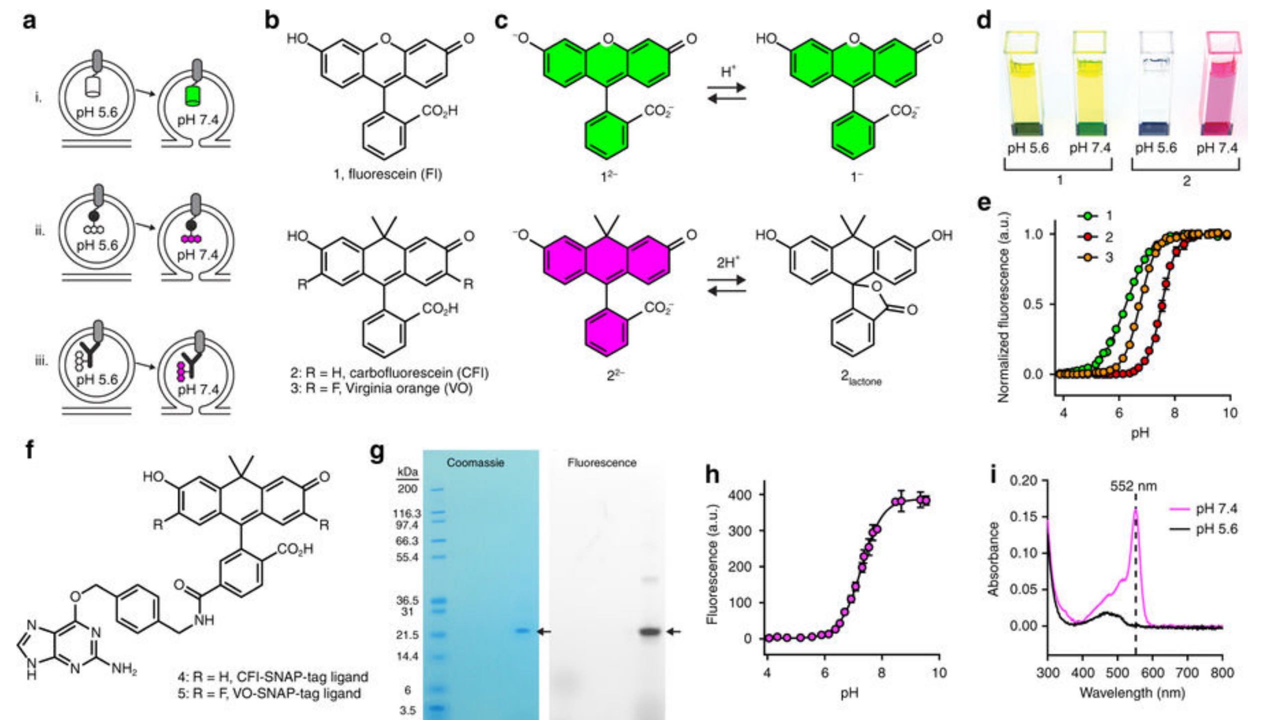


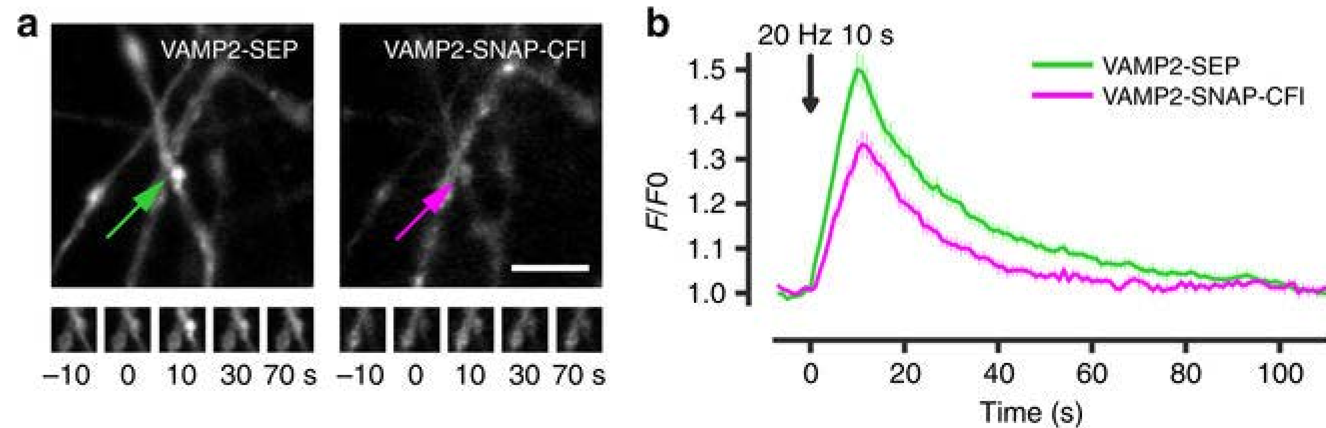
New red pH sensor

Semisynthetic fluorescent pH sensors for imaging exocytosis and endocytosis

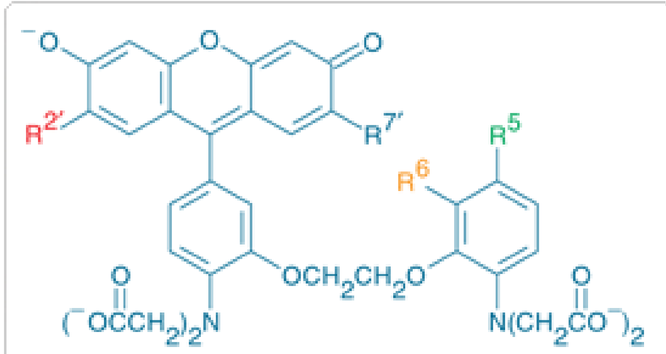
Magalie Martineau, Agila Somasundaram, Jonathan B. Grimm, Todd D. Gruber, Daniel Choquet, Justin W. Taraska, Luke D. Lavis & David Perrais

Received:
23 March 2017
Accepted:
12 October 2017
Published online:
10 November 2017



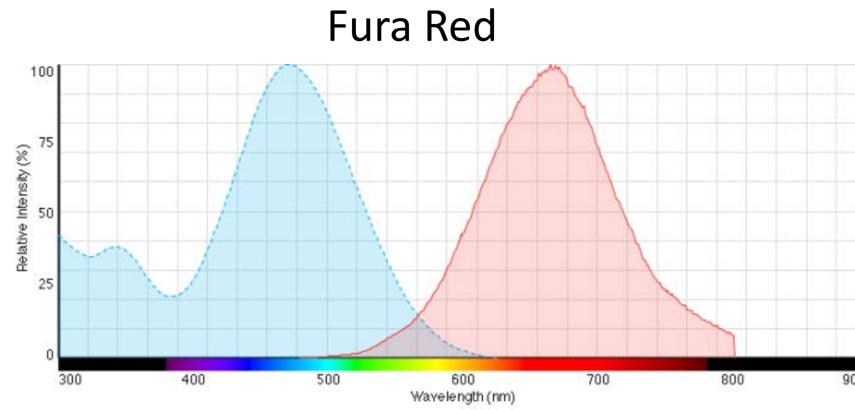


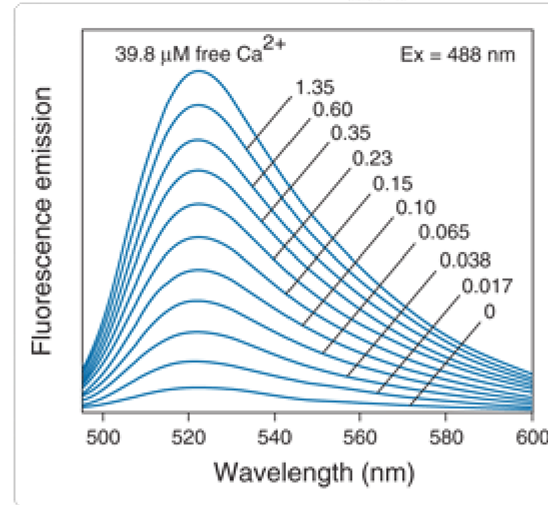
Calcium sensors



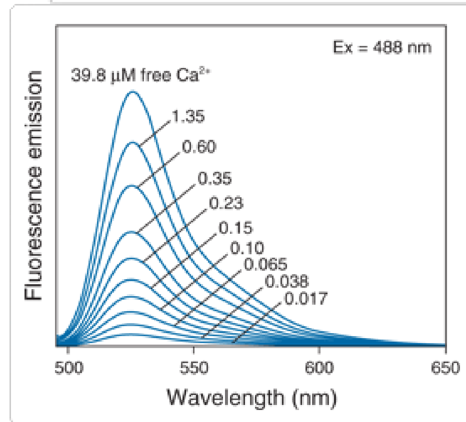
Indicator	$K_d(Ca^{2+})$	$R^{2'}$	$R^{7'}$	R^5	R^6
Fluo-3	0.39 μM	Cl	Cl	CH_3	H
Fluo-4	0.35 μM	F	F	CH_3	H
Fluo-5F	2.3 μM	F	F	F	H
Fluo-5N	90 μM	F	F	NO_2	H
Fluo-4FF	9.7 μM	F	F	F	F

Fura Red

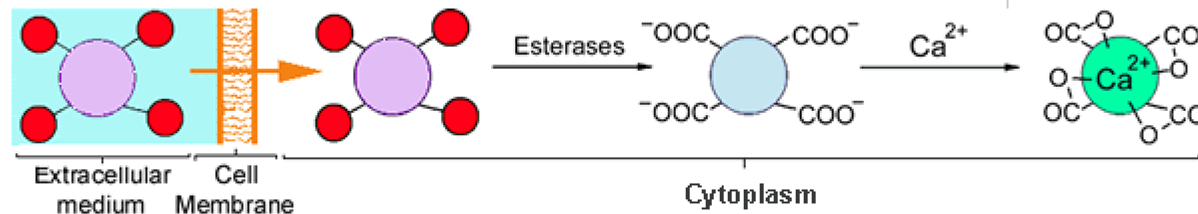




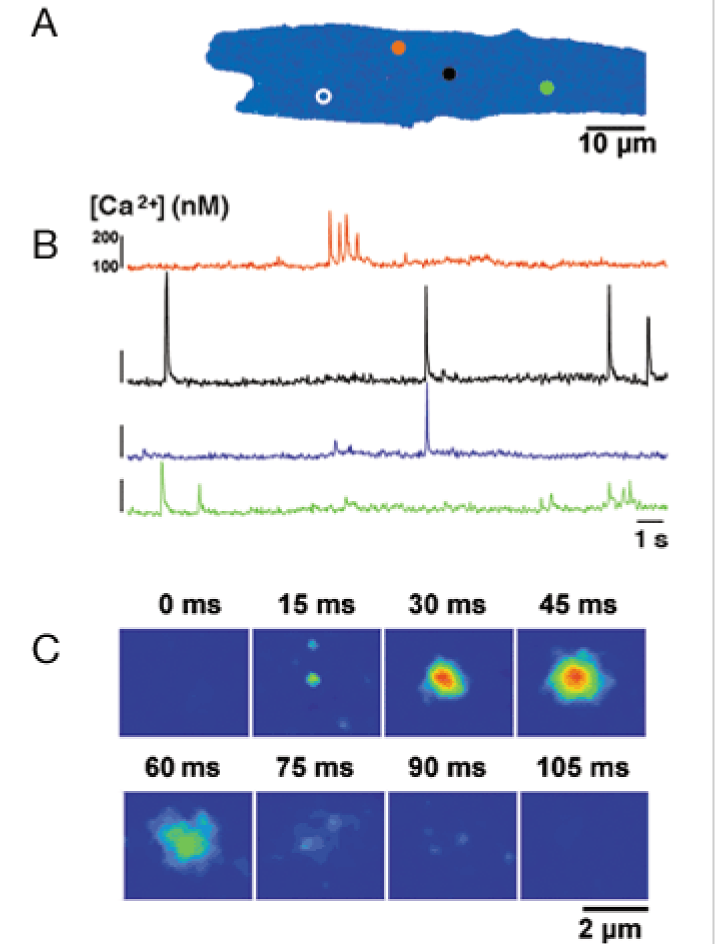
Oregon Green BAPTA



Fluo3

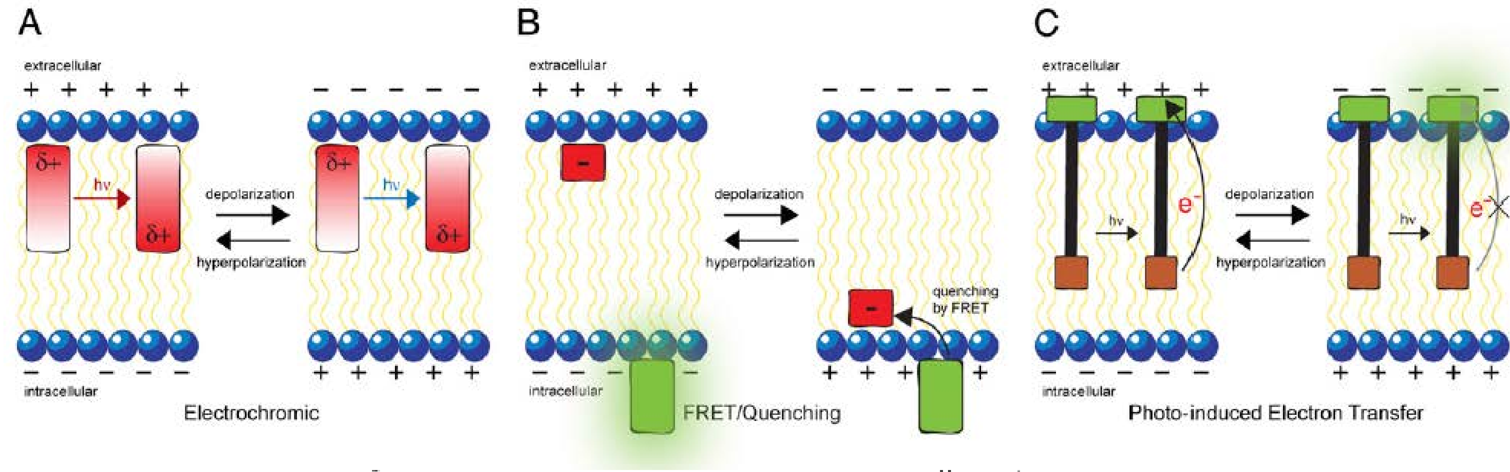


Fluo-4 AM
Acetoxymethlester



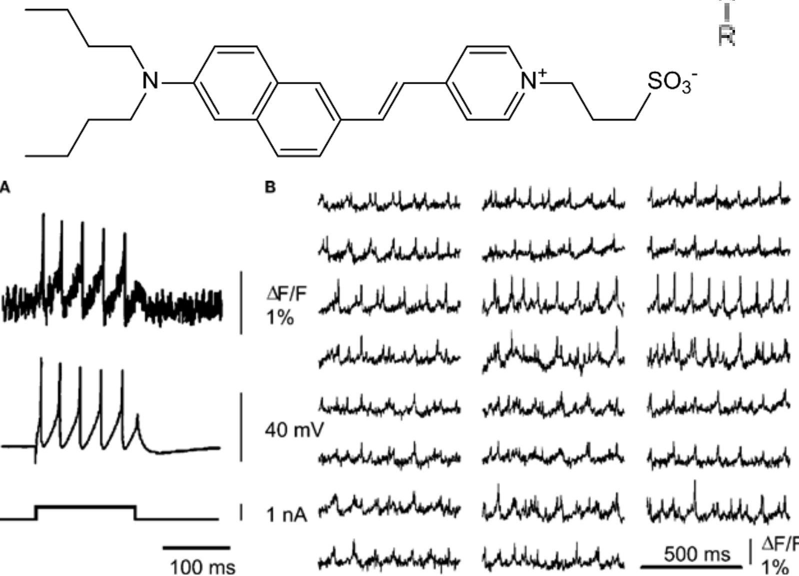
Voltage sensors

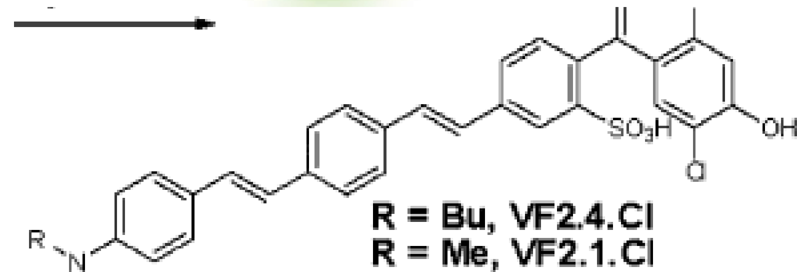
- Two types, those that localize to charged region of space (slow), and those that live in the membrane and sense the local electric field (can be fast)
- Dianepps is the most common. Phototoxic, low signal to noise ratio
- New dye by Tsien group is the best, Fluuovolt

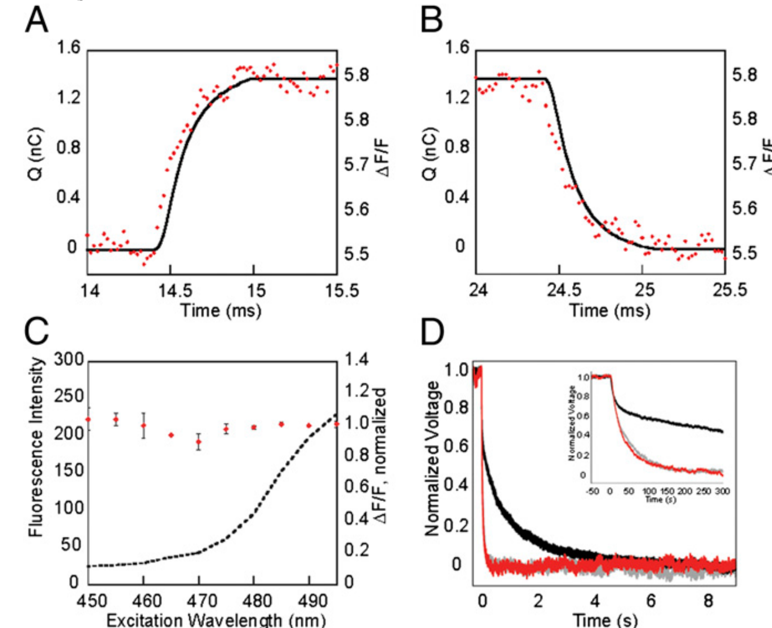


Di-4-anepps

Membrane localized



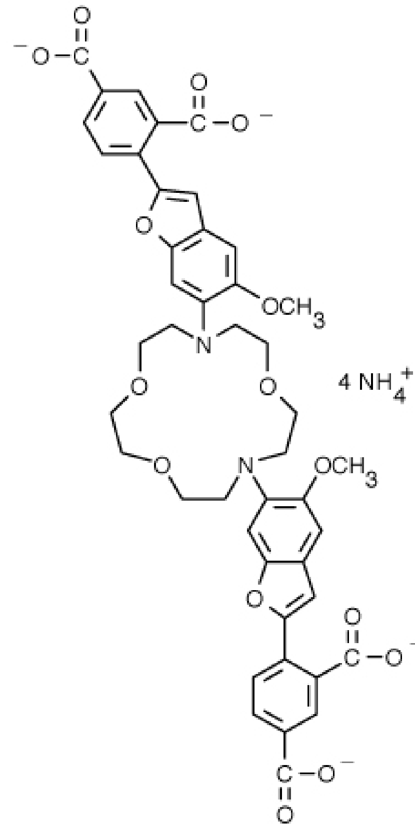




Sodium an potassium ions

- No exceptional fluorescent dyes (or FPs)
- Usually extremely sensitive to changes in pH
- Hard to get dyes that are selective for one ion over another

SFBI – Na sensitive salt



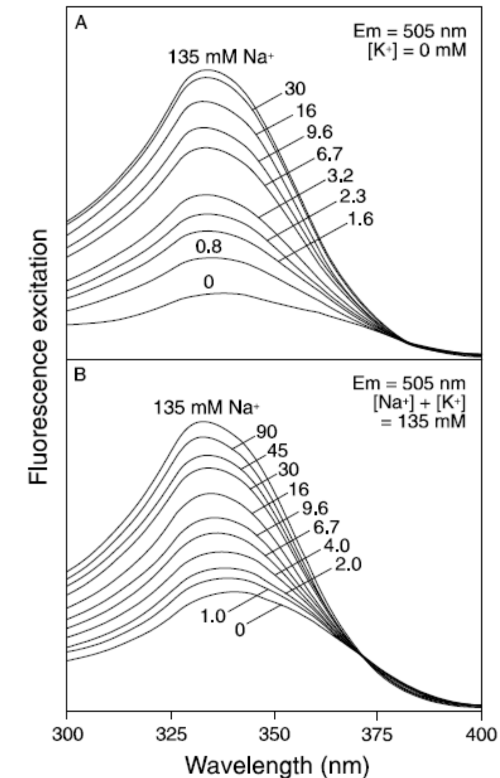


Figure 2. SBFI's excitation spectral response to Na^+: A) in K^+-free solution and B) in solutions containing K^+ with the combined Na^+ and K^+ concentration equal to 135 mM. The scale on the vertical axis is the same for both panels.

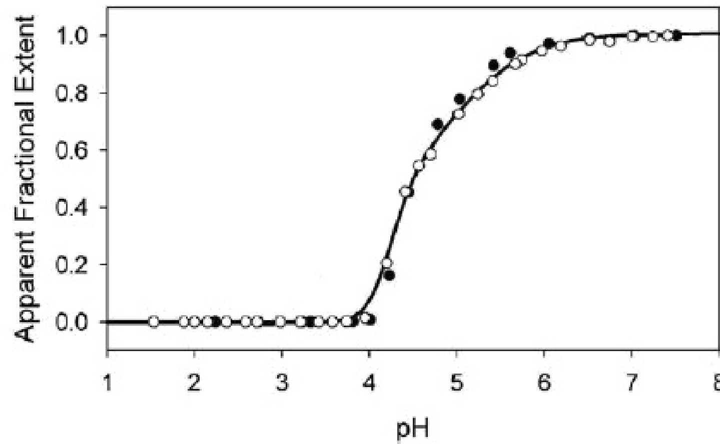
Additional dye based sensors

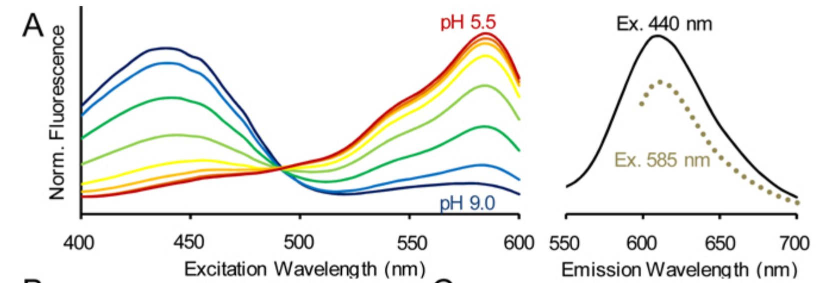
- Other metals (Zn, Mg) – Similar to Ca sensitive domains – come in a variety of colors
- Reactive oxygen species – One or more reactive species modifies the dye to become fluorescent
- Na, K, Cl – Crown ether chelators
- Endocytosis – OxyBURST, oxidated endosomes turn on fluorescence, fluorescent growth factors
- Ion channels – Fluorescently labeled toxins

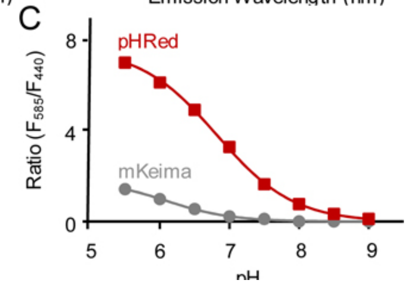
Fluorescent protein sensors

FP based sensors

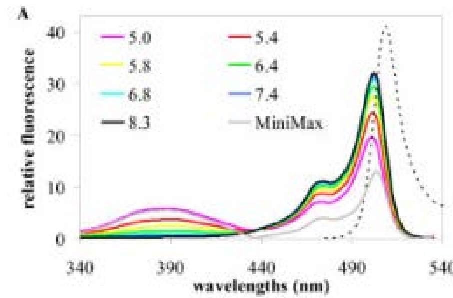
- GFP is pH sensitive
- pH directly modifies chromophore
- pHluorin, ratiometric pHluorin, and super ecliptic pHluorin are all mutations of GFP that move the pK_a into the physiological range
- Measuring the emission at 488 is a measure of cellular pH

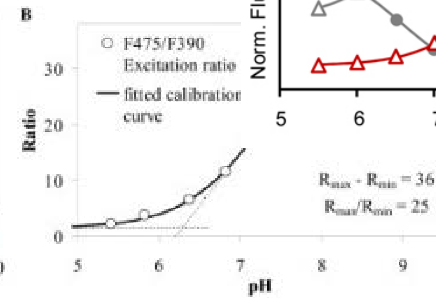




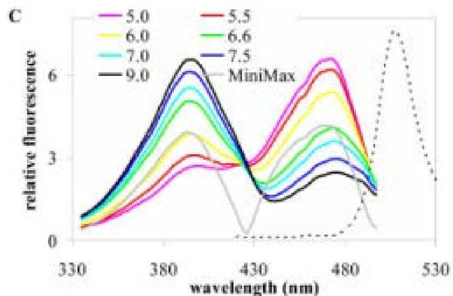


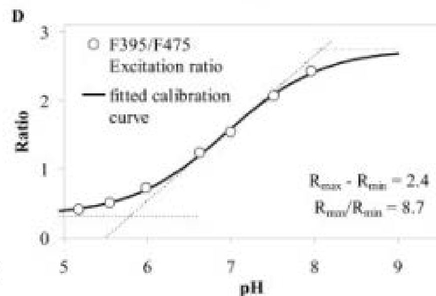
pHluorin



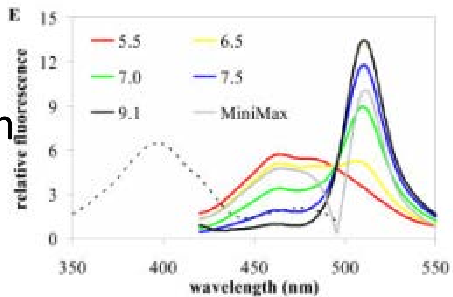


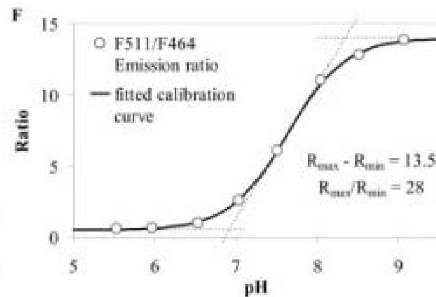
R pHluorin





SE pHluorin

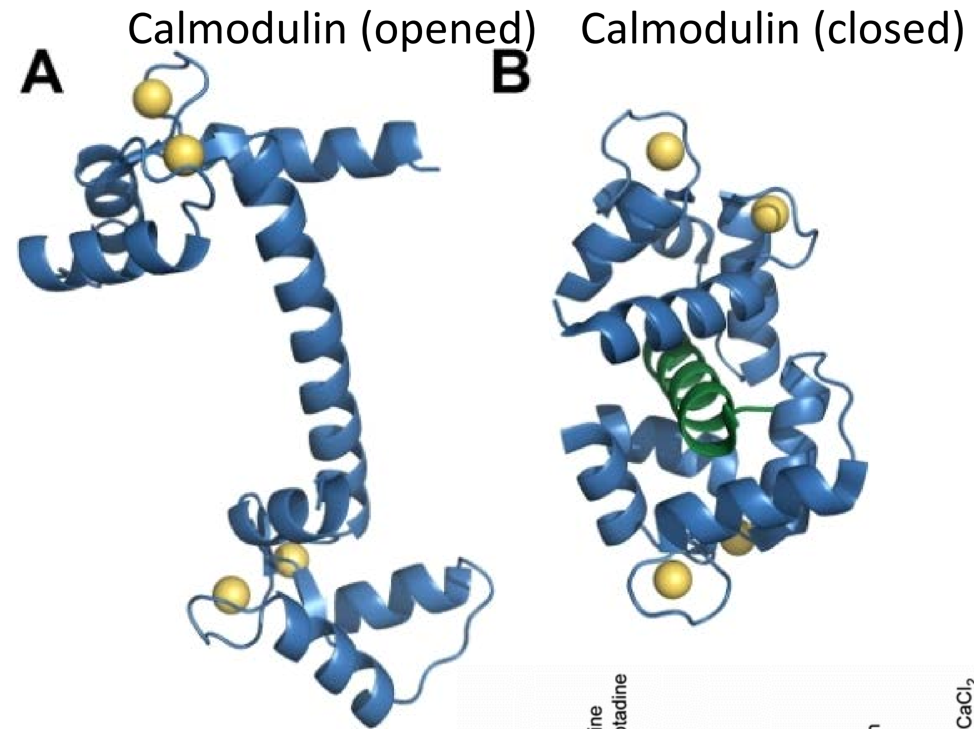


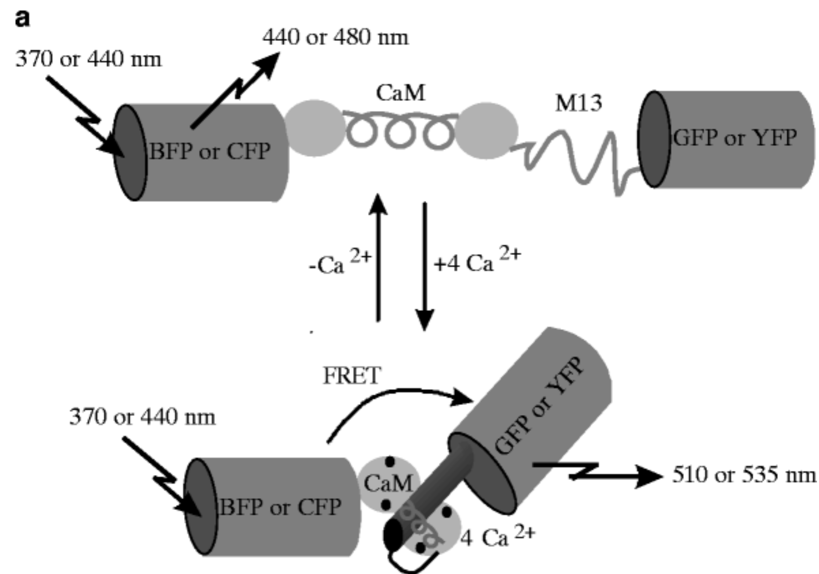


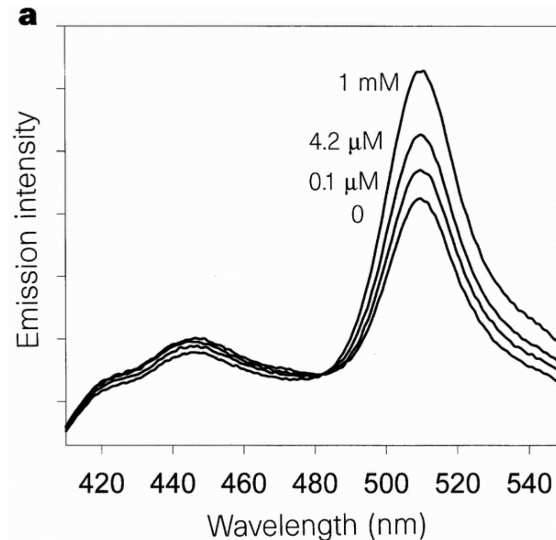
- http://biosensor.dpb.carnegiescience.edu/

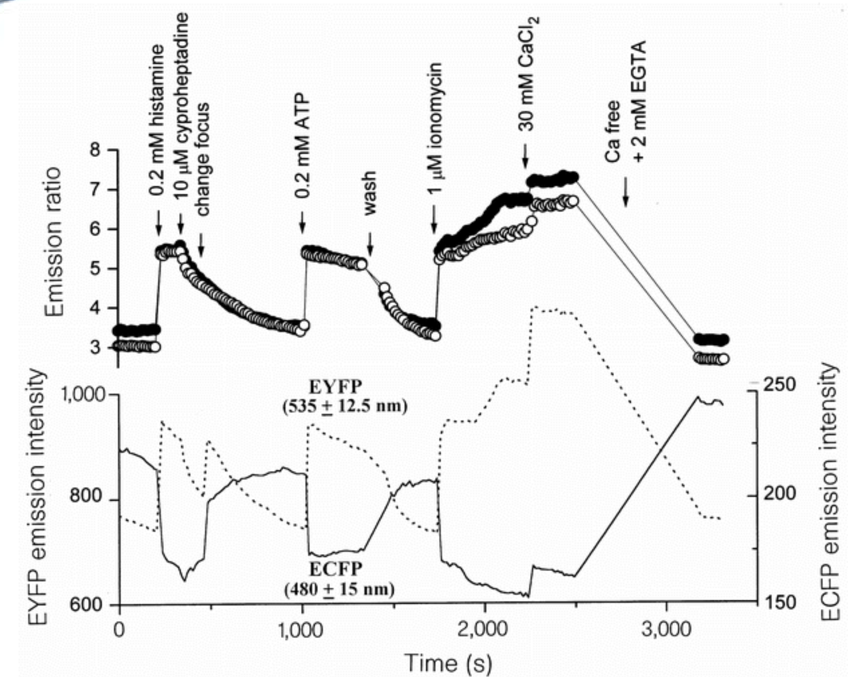
Calcium FP sensors

- First one was based on FRET Cameleon
- Calmodulin M13 domain
- Emerged from Tsien lab in 2000



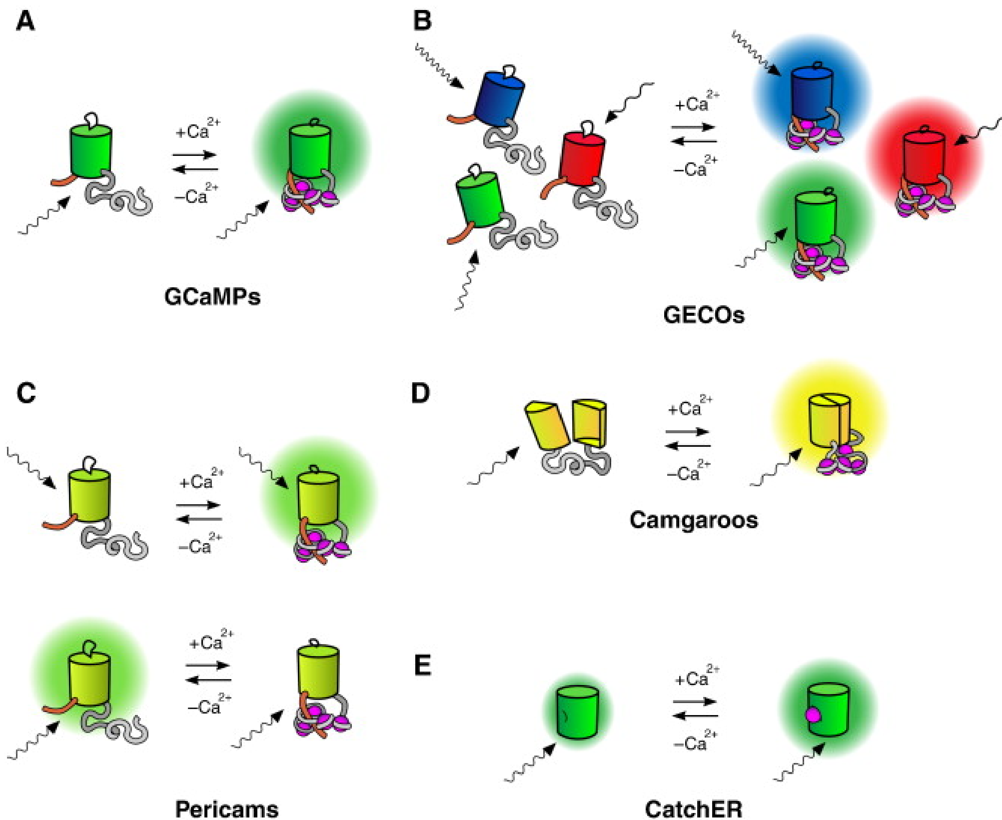


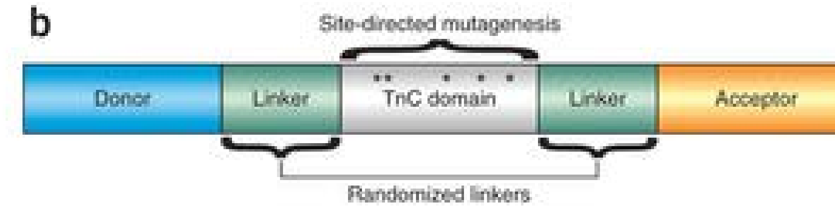


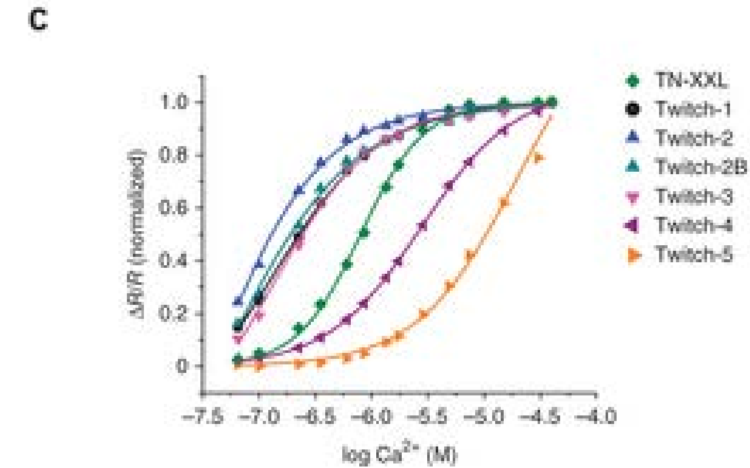


Newer Ca^{++} sensors

- GCaMP, Twitch, Pericams, CatchER







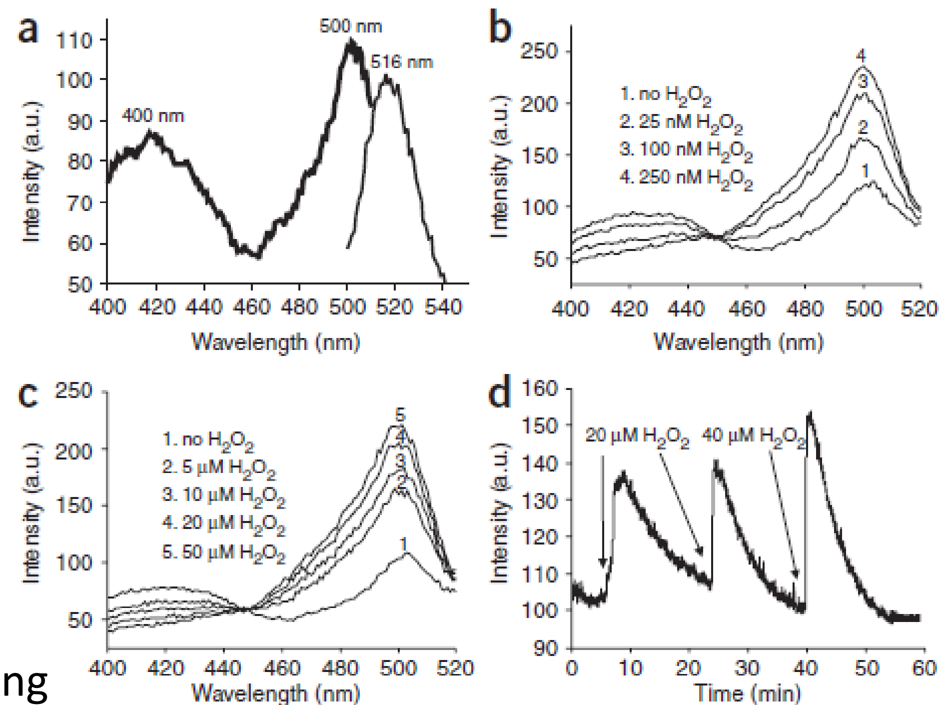
Calcium sensors are EXTREMELY good. Researchers have optimized all the properties – brightness, sensitivity, kinetics, folding

Still lacking a really good red single color calcium sensor

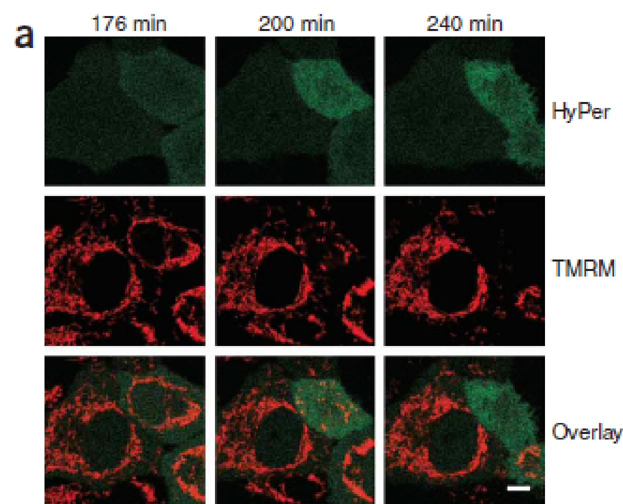
Each of the main sensors (GCaMP6, TWITCH, etc.) have > 1000 citations. Good to go.

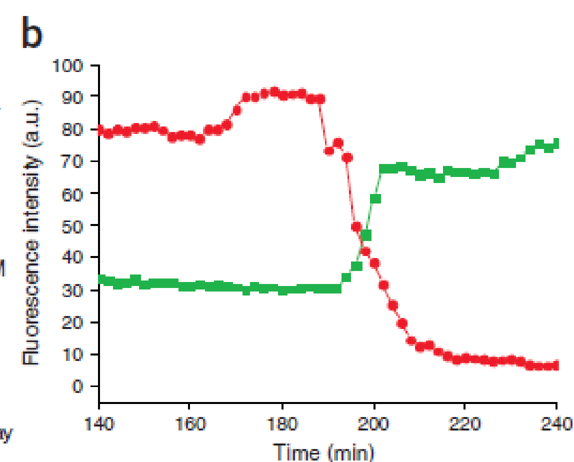
HyPer – FP to detect H_2O_2

- Fuse cpYFP to hydrogen peroxide sensing domain from prokaryotes, OxyR
- Increased H_2O_2 will cause an increase in fluorescence
- Based on modification of cysteine (sulfur) groups
- Bright enough to get sub-cellular resolution



HeLa mitochondria undergoing apoptosis

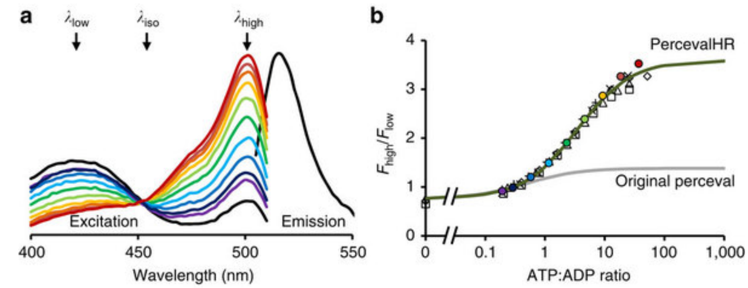


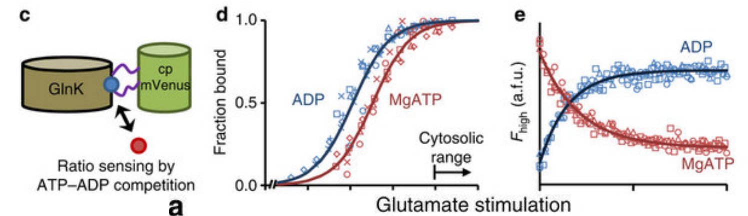


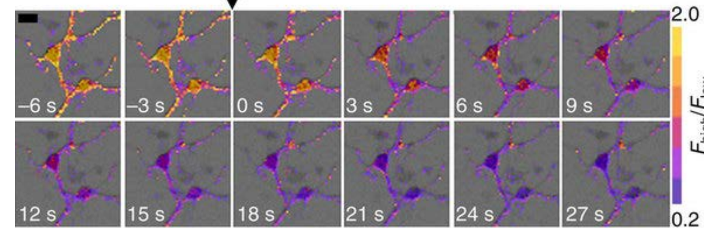
Original paper has > 500 citations, probably OK to use or an updated version

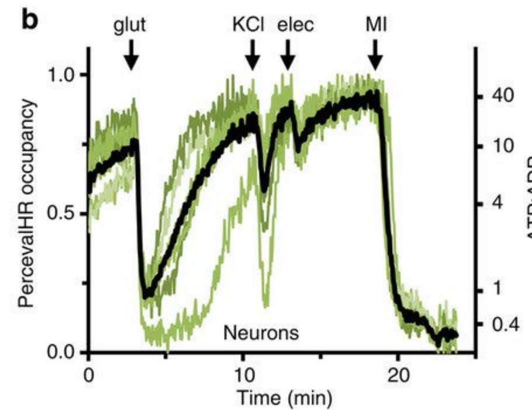
Perceval – ATP sensing

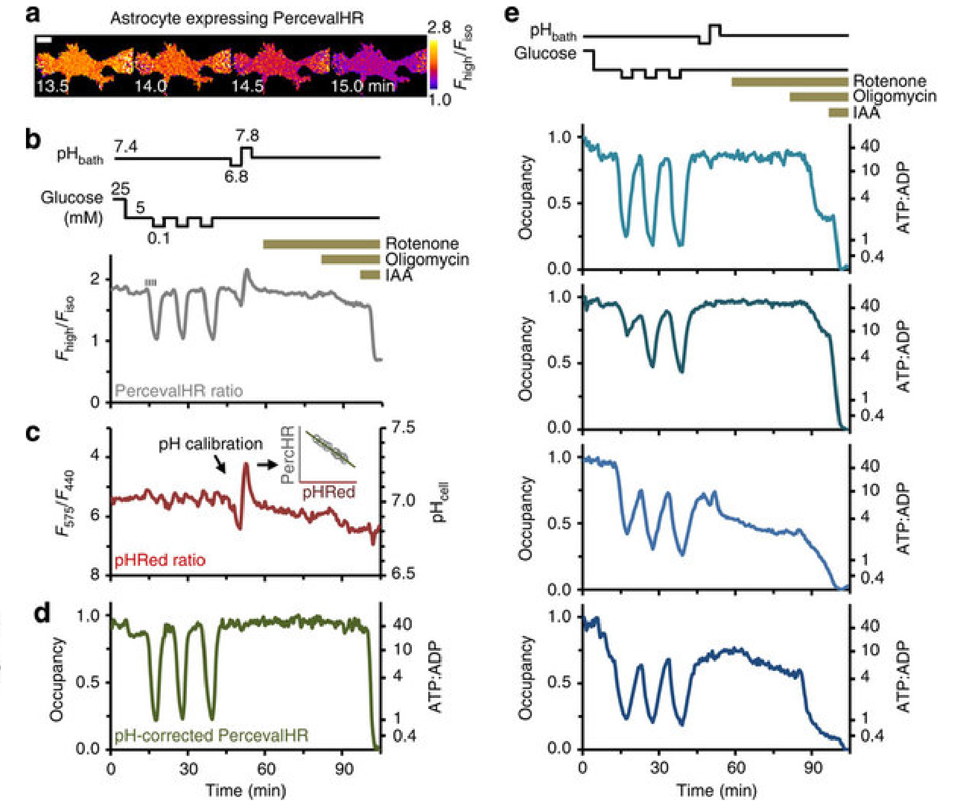
- Start with GlnK domain from *E. coli*
- Attach a circularly permuted Venus
- PercevalHR – sensor of ATP:ADP ratio
- Senses in the range of mammalian cytoplasmic ATP levels
- Ratiometric sensor, dual excitation
- Needs pHRed correction, have to monitor changes in pH











On to Matlab…