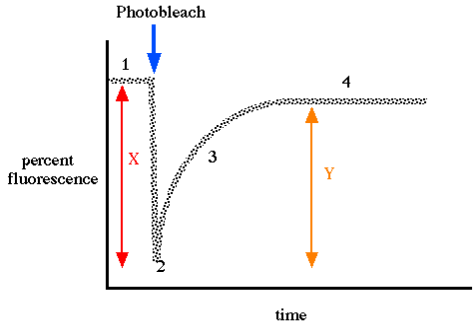
Biophysics 210: Biological Light Microscopy

Discussion Section 8: Measuring Cellular Processes/Fluorescent Biosensors

1) FRAP (fluorescence recovery after photobleaching) and FLIP (Fluorescence loss in photobleaching) were some of the earliest microscopy tools available for measuring protein dynamics.

a. Below is an idealized FRAP curve. What information about your protein of interest can you gain from the difference between 1 and 4? What information can you gain from the shape of curve (3)?



b. Which method would you use to compare the relative stability of actin structures in the cytoplasm vs. actin structures in the nucleus- FRAP or FLIP? Which would you use to see if there was trafficking between your nuclear and cytoplasmic actin pools?

c. Commonly, collection of data for FRAP involves measuring 3 ROIs at each time point- the ROI of your bleached area, the ROI of the whole cell, and an ROI outside the cell. What is the purpose of each of these ROIs?

d. Name an advantage of FRAP over some of the more specialized methods we learned about this week.

2) Many protein dynamic and trafficking applications that once would have been done with FRAP or FLIP are now done with photoactivatable and photo-switchable fluorescent proteins.

a. Look at the properties of photoactivatable GFP (PA-GFP), PA-TagRFP, and mEos 2 and 3.2 at the NIC's list of photoactivatable fluorescent proteins here. How do their brightnesses compare with standard fluorescent proteins here?

b. If you only were going to tagging and measuring the dynamics of one protein in your cells, should you use a photoactivatable or switchable protein? Why?

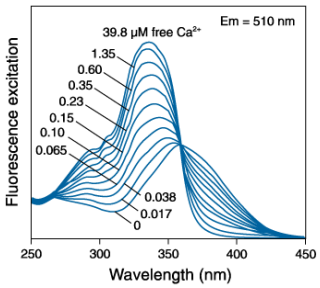
c. Would you use a photoactivatable or photoswitchable protein if you wanted to watch the trafficking of Your Favorite Protein between two differently labeled compartments in the cell? Why?

3) For of the following scenarios, pick whether you'd attempt to measure the given interaction via FRET or a split-GFP (BiFC). Why?

a. Monitoring assembly of nuclear pore components into a stable complex.
b. Watching transient interactions between vesicle coat proteins and integral membrane proteins during exocytosis.
c. Examining if a tubulin mutant can co-assemble and disassemble with normal subunits into microtubules (bonus question- is there another technique you might use for this?)
d. Looking for interaction of two different low-abundance transcription factors on the promoter of a target gene

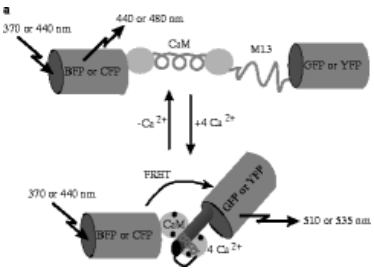
4) What two potential confounders do you need to consider when you're initially setting up a FRET experiment with two proteins, and what measurements do you take to correct for them? Why aren't these necessary for a FRET sensor made up of a single polypeptide?

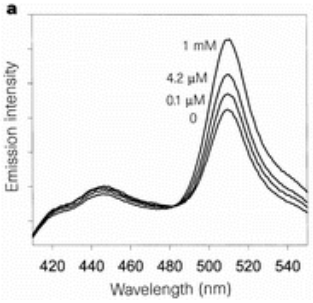
5) Sensors for Ca^{2+} concentrations in cells have been important tools in neuroscience, cell biology, and other fields for 30+ years.



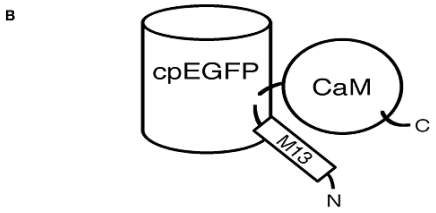
-Fura 2 is a dye that can be added to cells; it allows measurement of Ca2+ by comparing the ability of 340nm vs 380nm light to excite 510nm emitted light.

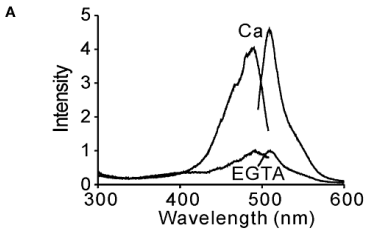
-Cameleons are FRET sensors based on the calmodulin (CaM) and myosin 13 (M13) protein domains, which bind to each other in response to calcium. Mutations in the domains can tune the sensitivity of various Cameleons to different ranges of calcium concentration.





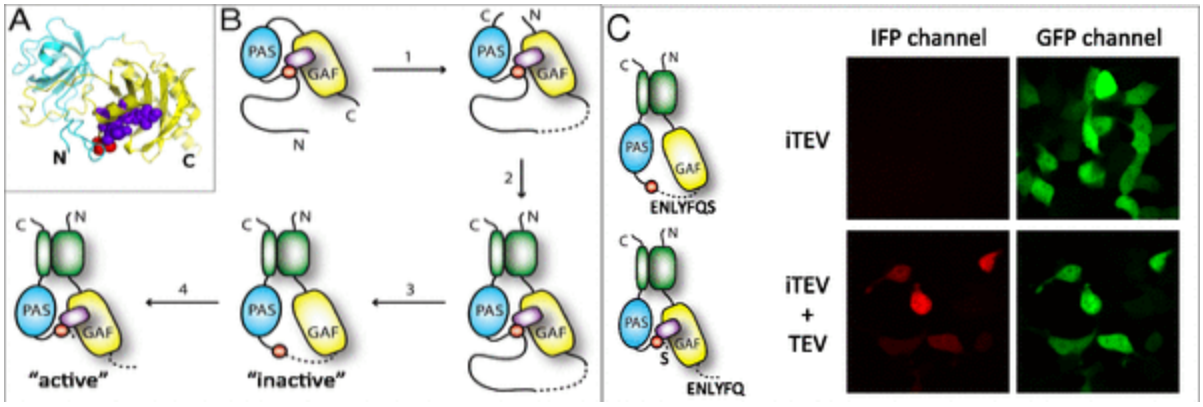
-GCamPs are sensors based on circularly permuting GFP, which allows fusion of the CaM and M13 domains close to the GFP chromophore. The binding interactions of CaM and M13 in response to local calcium concentrations will then significantly affect the brightness of the GFP.





a. What are the advantages and disadvantages of the ratiometric vs “switchlike” methods?
b. What are the advantages and disadvantages of using fluorescent proteins vs dye based methods?
c. Which of these systems are easiest to use on “standard” microscopes, and which may require special objectives/filters?

6) A recent Shu lab publication (To et al, PNAS 2015) describes a system where a circularly permuted IFP (yellow and blue ovals) is fused to split GFP (green ovals). After removal of some of the protein sequence (step 3 below), a critical residue of the chromophore (red circle) is unable to assume its normal position in the permuted protein unless the linker between the two halves of IFP is cut; by engineering different cut sites into the linker you can therefore use this system (called iProtease) to test for activity of specific proteases (such as TEV below) simply by looking for infrared fluorescence.



a. What two roles does the split GFP serve in iProtease? Are there other proteins that could replace one or both functions?
b. Why was step 3 (the shortening of the linker) necessary to make iProtease a functional reporter? What is the evidence for this?
c. Is biliverdin required for iProtease as it is for other IFPs? Is it transiently or covalently attached?
d. Would iProtease work for calpain, which has an 11aa cleavage recognition sequence? What about an enzyme that has a 20aa recognition sequence?
e. The paper introduces a version of iProtease called iCasper that works as an *in vivo* caspase reporter. What advantages might this system have over dye based or

FRET based reporters? (Hint: how is caspase activation VERY different from the calcium release that used FRET sensors in question 5?)

f. Bonus: what new directions or modifications can you imagine for iProtease?