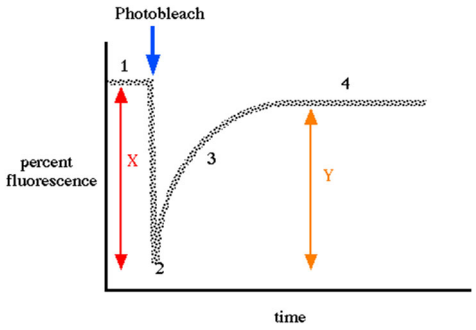
Biophysics 210: Biological Light Microscopy
Discussion Section 7: Measuring Cellular Processes/Fluorescent Biosensors
Tuesdays 1-2:30pm
Location: Genentech Hall Rm N114

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 table of photoactivatable fluorescent proteins on FPbase (use the Switch Type to filter the table). How do their brightnesses compare with standard fluorescent proteins?

    b. If you only were going to be 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?

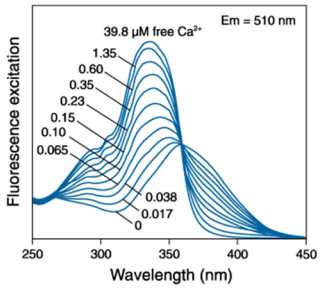
1. For of the following scenarios, pick whether you'd attempt to measure the given interaction via FRET or a split-GFP (BiFC). Why?
    a) Monitoring assembly of nuclear pore components into a stable complex.

    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

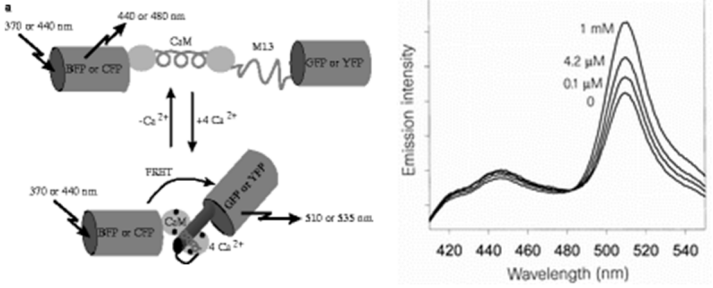
2. What two potential confounders do you need to consider when you're initially setting up a FRET experiment with two proteins, and what measurements do you take to correct for them? Why aren't these necessary for a FRET sensor made up of a single polypeptide?



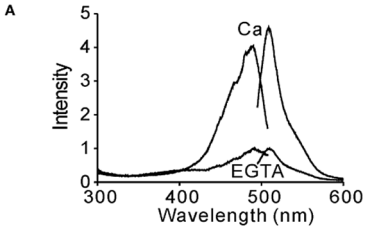
3. Sensors for Ca^{2+} concentrations in cells have been important tools in neuroscience, cell biology, and other fields for 30+ years.

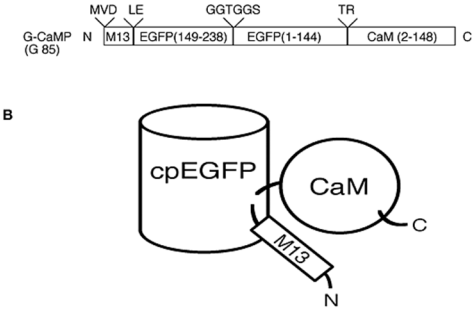
-Fura 2 is a dye that can be added to cells; it allows measurement of Ca2+ by comparing the ability of 340nm vs 380nm light to excite 510nm emitted light.

-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? How do Halo-tag based fluorophores address some of the dye based methods issues?

c. Which of these systems are easiest to use on “standard” microscopes, and which may require special objectives/filters?

4. FLIM (Fluorescence Lifetime Imaging) measures the relaxation time of a fluorophore to its ground state, thus providing an additional parameter to resolve molecular species and dynamics. For a single molecular species, the emitted fluorescence intensity at time t, I(t), is:

$$I(t) = I_0 e^{-t/\tau}$$

where I_0 is the initial fluorescence intensity at time 0 and τ is the fluorescence lifetime.

a) What is one advantage of using FLIM over fluorescence intensity measurements?

In time resolved Fluorescence Lifetime measurements, a pulsed laser is used to excite the fluorophores, and photon counting PMTs and specialized electronics are used to record the arrival time of the emitted photons with respect to the excitation pulse and build a time resolved photon histogram. The histogram is then fitted to a single or multiexponential decay to determine τ. (Another method of data analysis involves Fourier transforming the data and plotting it on a two-dimensional phasor plot). For Fluorescence Lifetime Imaging, the laser is scanned over the sample with a point scanner confocal or two photon microscope, and the intensity histogram is calculated and analyzed at each pixel.

b) The accuracy of the exponential fit is strongly dependent on the number of photons collected per each pixel/decay histogram. Assuming that you are using a bright fluorophore, generally photon counts are kept at around 200,000 to 500,000 counts per second max to avoid artifacts due to detector and electronics saturation (pile up). What would your pixel dwell time have to be in order to acquire a minimum of 100 photons per pixel? And what would be your frame rate for a 512X512 image? (in actual life images are acquired integrating ~100 frames at ~10us pixel dwell time). What is one of the major drawbacks of FLIM? What could you do to increase your time resolution?

The relaxation time of a fluorophore is inherently sensitive to the physical parameters of its environment such as membrane tension, temperature, pH, hydration, ionic strength etc., making FLIM based sensors particularly appealing to investigate these properties. The papers by Okabe et al, 2012 and Inada et al, 2019 describe the design and use of a fluorescent polymeric thermometer (FPT) to measure temperature in cells with subcellular resolution.

c) What is the principle behind the temperature sensitivity of these types of probes (Okabe et al 2012 figure 1b and text)?

d) The FPT responds to temperature increases with a higher brightness and longer lifetime. Why do the authors choose to use changes in lifetime over brightness to quantify temperature changes? How is this useful when building a calibration curve?

e) Looking at the calibration curve of this FTP, in which temperature ranges these indicators are more sensitive? Could they be used to look at metabolic changes between 35C and 40C?

f) In the Okabe et al 2012 paper, the Fluorescent Temperature Sensors (FTP) are injected into the cells via a microneedle (a rather cumbersome procedure). What changes were made to the FTPs in the Inada et al 2019 paper that allowed the FTPs to enter the cells through simple incubation?

7. Compare GCaMP and VoltageFluor dyes for measuring membrane potential. What does each sensor measure, and what are the key differences in temporal resolution and accuracy?

8. For each of the following biological questions, decide whether PLA or FRET is the more appropriate method. Justify your choice based on spatial resolution, temporal resolution, and experimental constraints.

a) Case 1 Transient signaling interaction: You want to determine whether two signaling proteins interact transiently within seconds after receptor activation in live cells.

b) Case 2 Rare protein interaction in tissue: You want to detect whether two proteins are in close proximity in a fixed tissue sample, where expression levels are low and interactions are rare.

c) Case 3 Testing direct binding vs co-localization: You observe two proteins co-localize in microscopy and want to determine whether they are directly interacting or just near each other in live cells.