

## 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

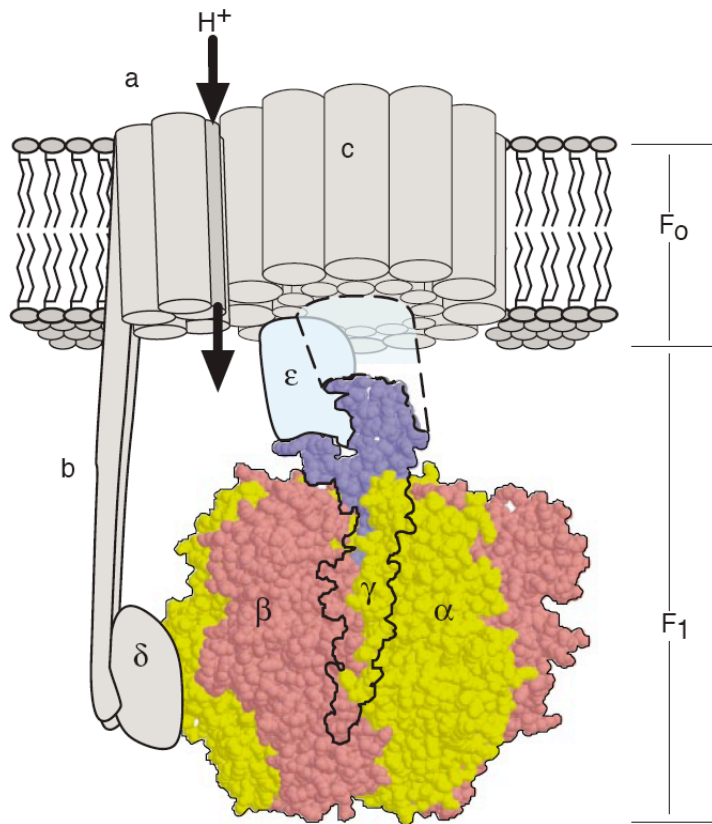
## **PRESIDENT'S DAY**

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

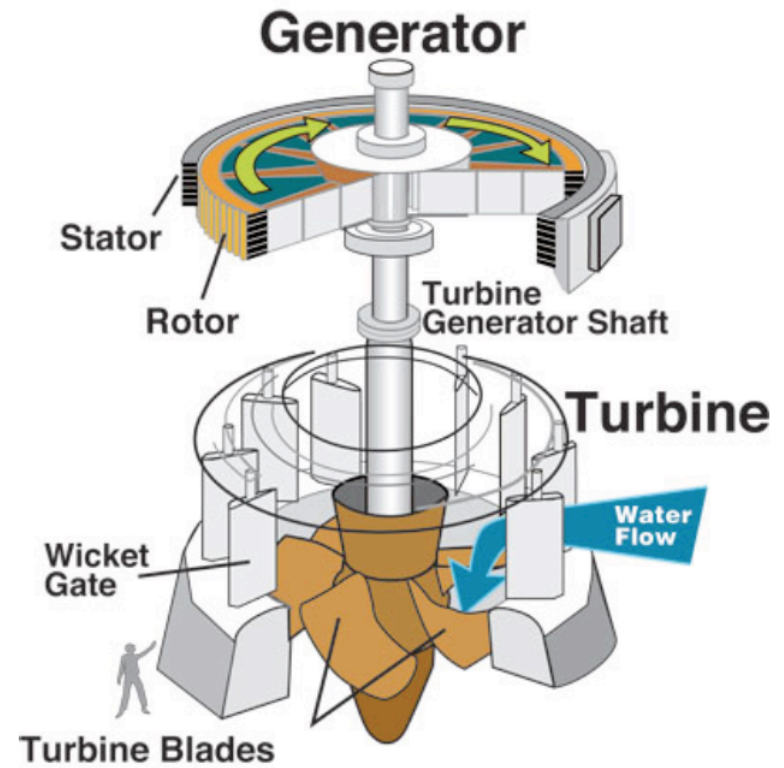
4. Prepare expression system
5. gene analysis & induction
6. Characterize expression
7. Assay protein behavior
8. Data analysis

## **Lecture 1: Introduction to the module**

- I. Engineering proteins
  
- II. Pericam: an engineered protein sensor
  - A. Imaging calcium signaling
  - B. Calmodulin and GFP
  - C. Pericam variants
  
- III. Reengineering Pericam: experimental overview
  - A. Structure-based design
  - B. Protein expression and purification
  - C. Measurements and analysis

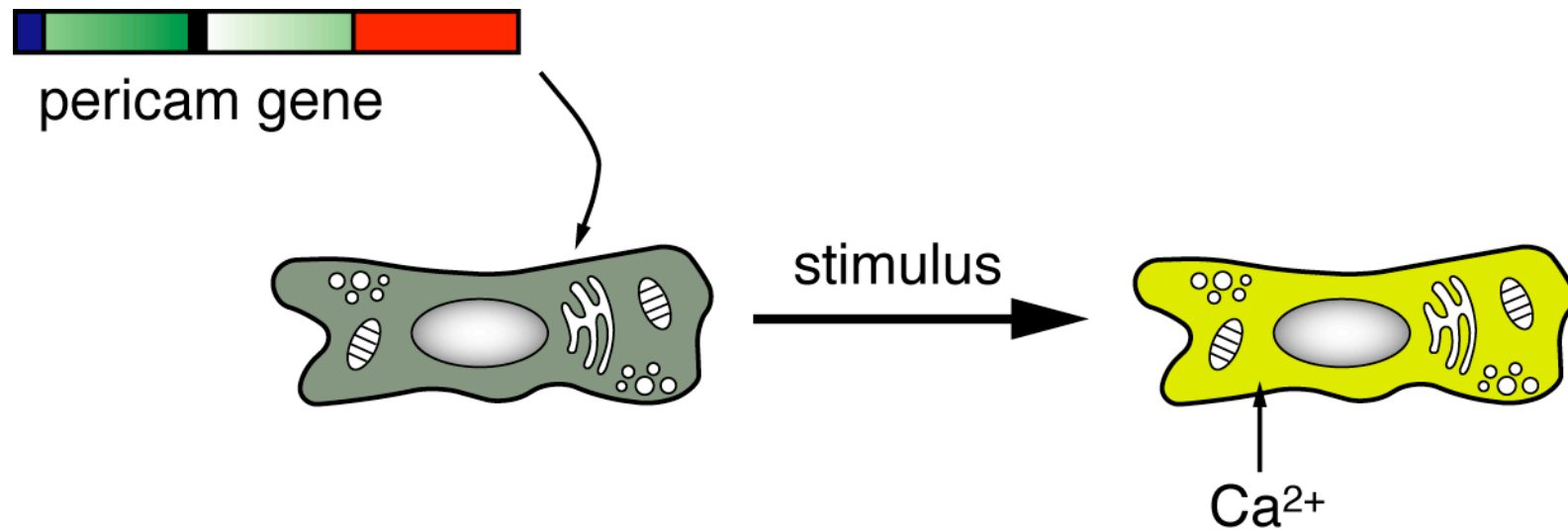


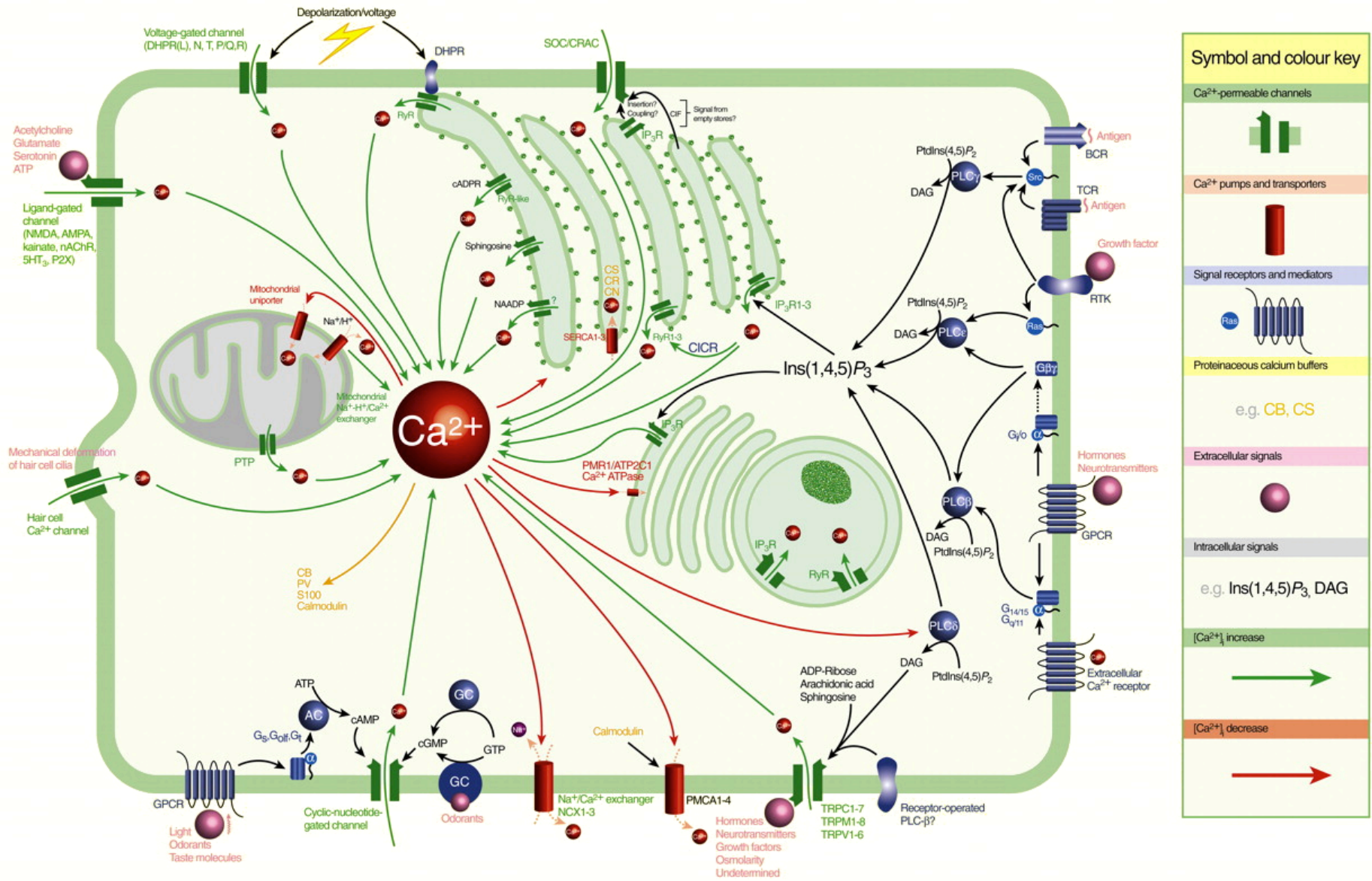
Wang & Oster (1998) *Nature* 396: 279-82



[www.symscape.com/node/420](http://www.symscape.com/node/420)

## Pericam: a protein-based machine for measuring $[Ca^{2+}]$

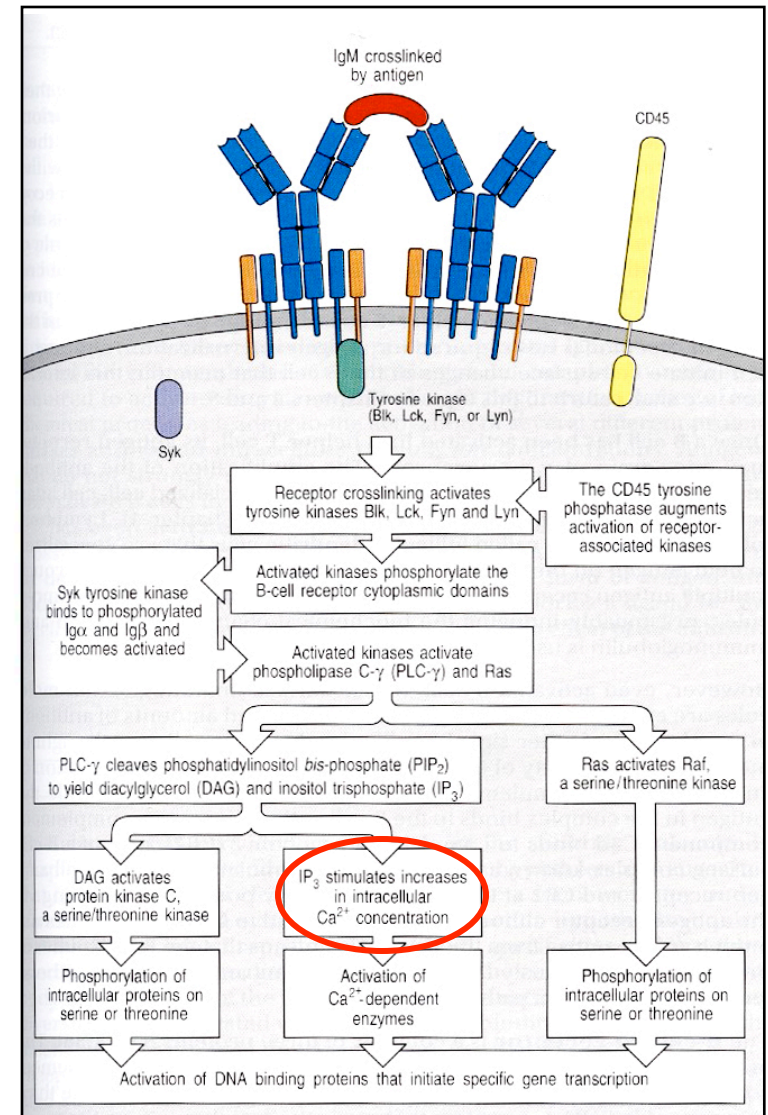
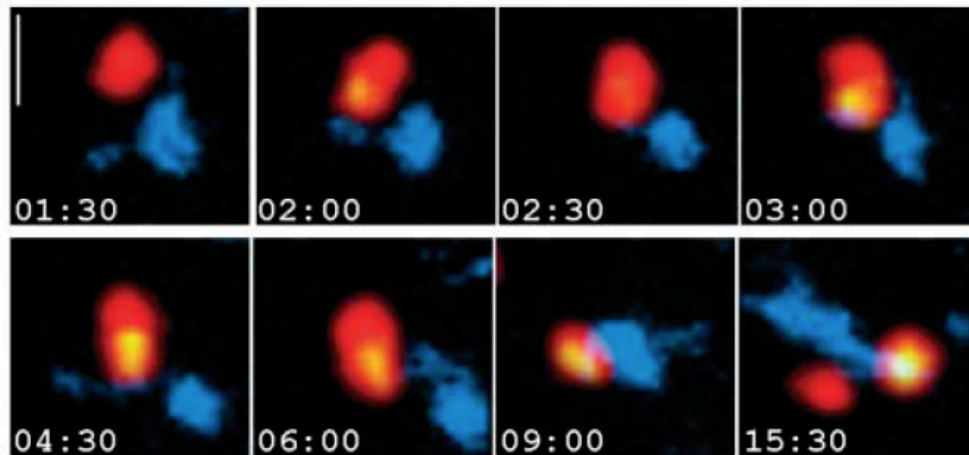




Calcium indicators can be used to detect signaling in individual cells and multicellular ensembles. Two purposes:

- learn what stimuli trigger calcium fluctuations and how calcium behaves in context of an organism or system
- use calcium as a “handle” on cell-cell interaction (*e.g.* neural activity)

Calcium is important to cellular signaling in the immune system. Activation of B-cells can be detected by calcium imaging in lymph nodes (Qi *et al.*, 2006, *Science*).





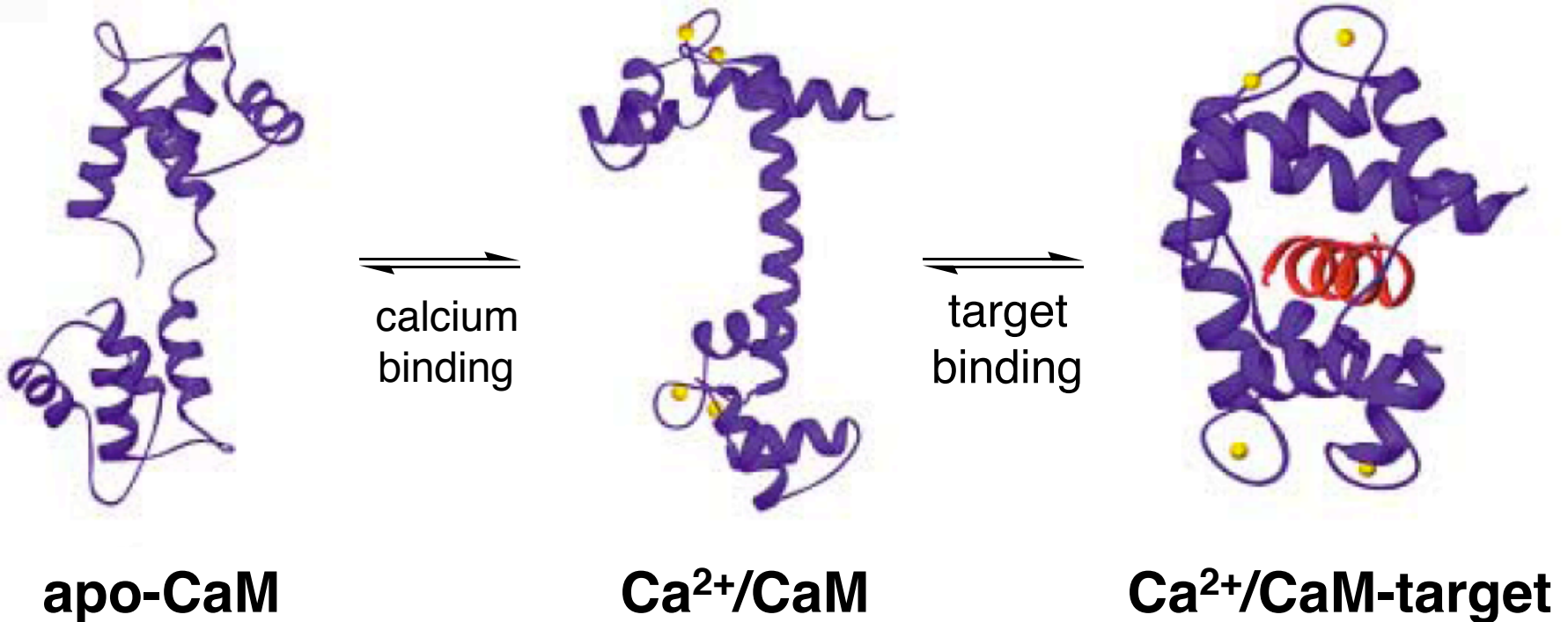
pericam gene

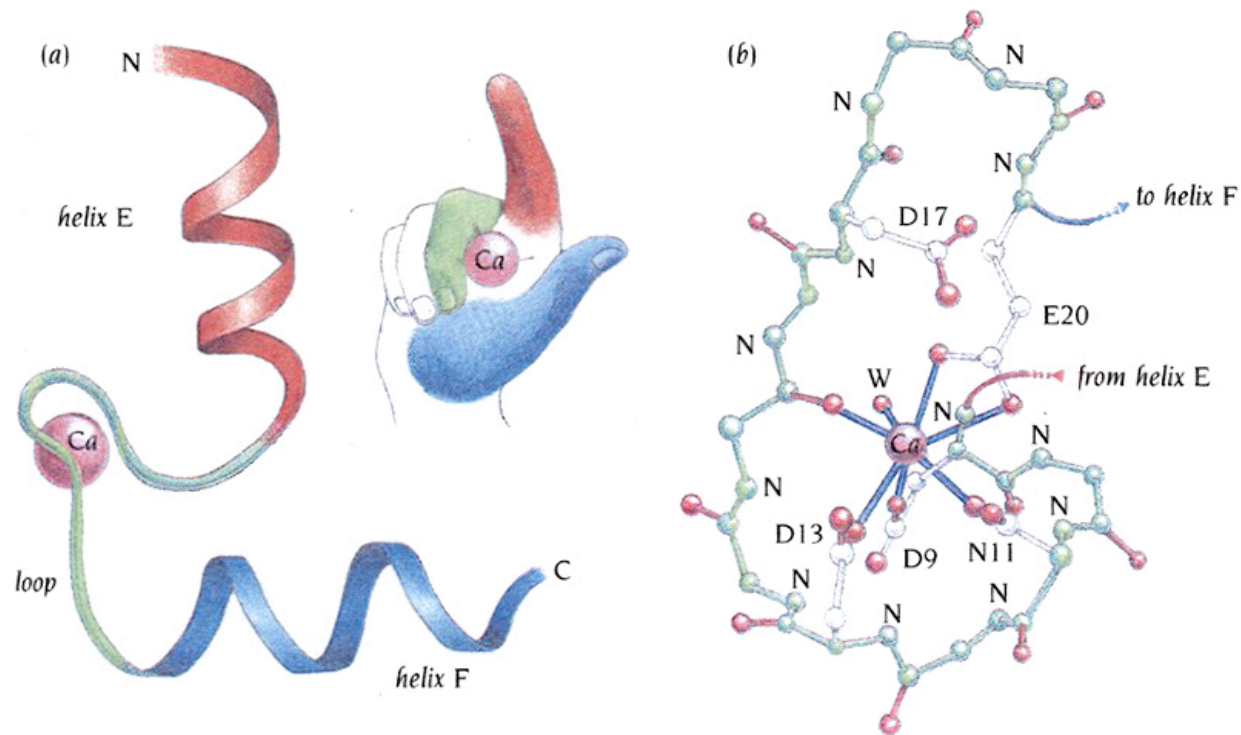
Pericam is a second generation calcium sensor, based on design strategies originally developed by Roger Tsien and colleagues. Tsien won a 2008 Nobel Prize for engineering novel forms of GFP.



## Calmodulin (CaM) facts and figures

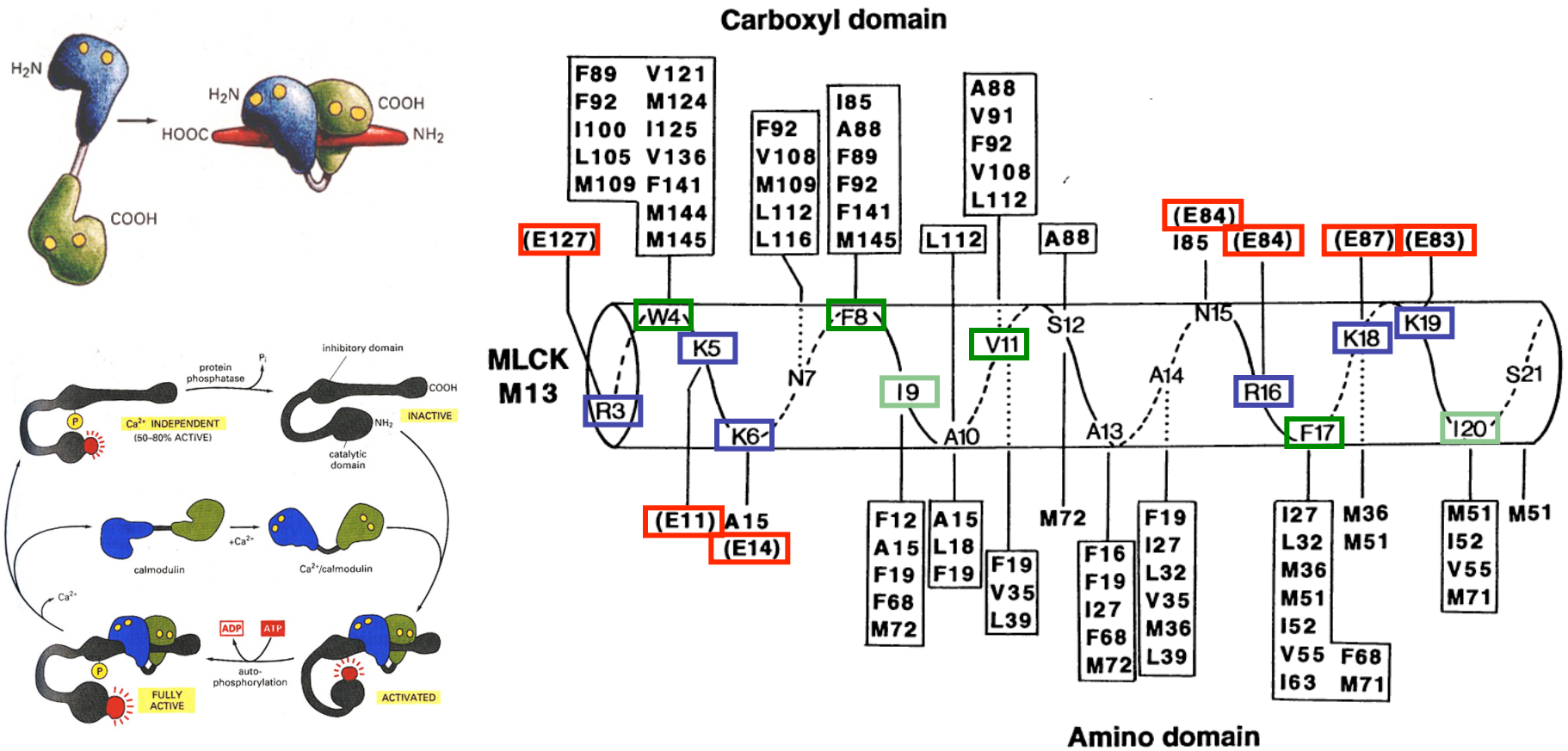
- 16-18 kD (depending on species), ~20 x 40 Å protein
- highly conserved among eukaryotes (vertebrate and yeast calmodulin are functionally interchangeable)
- binds four  $\text{Ca}^{2+}$  ions using EF hand amino acid sequence motifs
- $\text{Ca}^{2+}$ -CaM binds short segments of target proteins, modulates activity





EF hand binding motif named for E & F helices of the calcium-binding protein parvalbumin; example of helix-loop-helix structure, with calcium bound in the loop

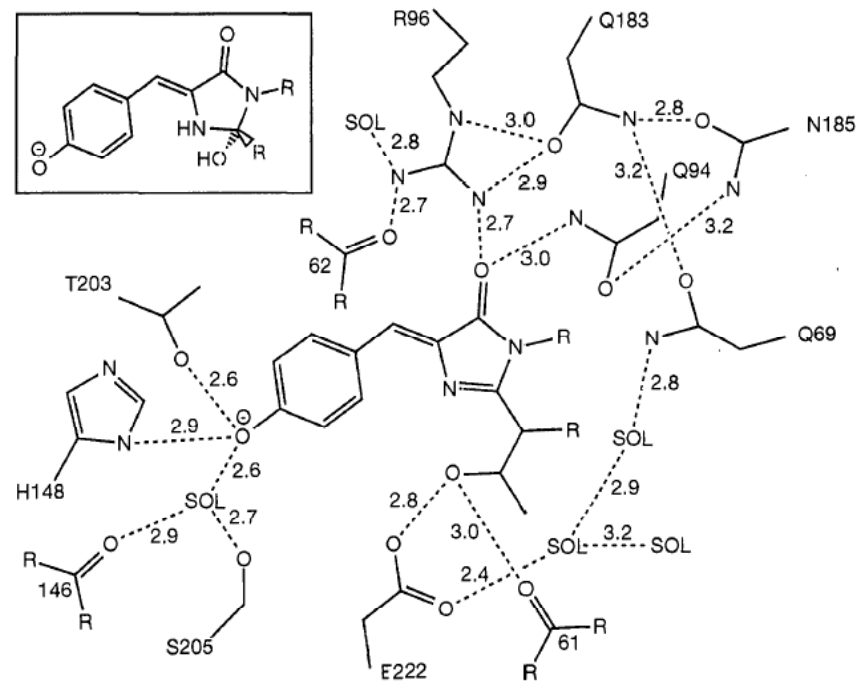
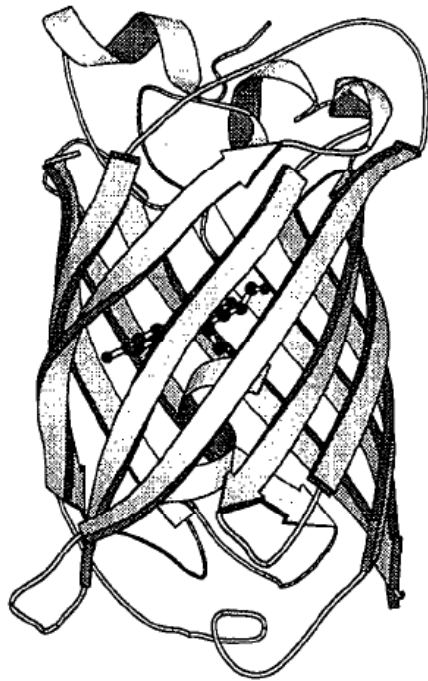
Ca<sup>2+</sup>-saturated CaM binds to peptides by “grasping” target sequences, in helical conformation, between N- and C-terminal domains. In many cases, this activates an enzyme by sequestering an inhibitory domain (e.g. M13 from MLCK). Interactions between CaM and targets involve hydrophobic contact area and charge-charge interactions.





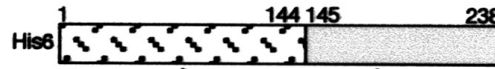
## Green Fluorescent Protein (GFP)

from the jellyfish *Aequoria victoria* is a protein fluorophore and component of genetically-encoded calcium indicators. The molecular structure (1996) shows a chromophore formed by spontaneous cyclization and oxidation of three amino acids (Ser/Thr65, Tyr66, and Gly67).

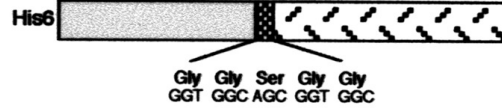


Ormo *et al.* (1996) *Science* 273: 1392-5.

EYFP (V68L/Q69K)



cpEYFP(V68L/Q69K)

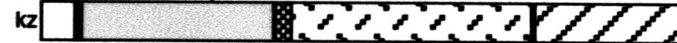


pericam



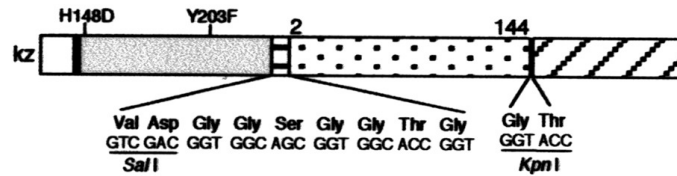
parent construct

flash-pericam



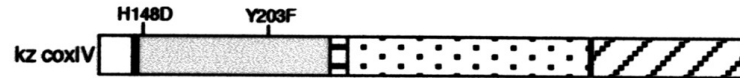
dynamic range

ratiometric-pericam



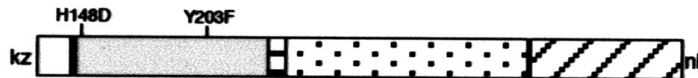
ratiometric  $\Delta F$

ratiometric-pericam-mt



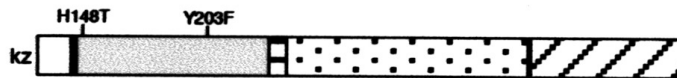
mitoch. localization

ratiometric-pericam-nu



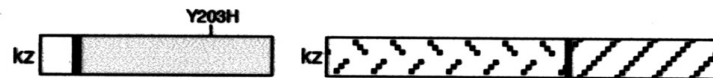
nuclear localization

inverse-pericam



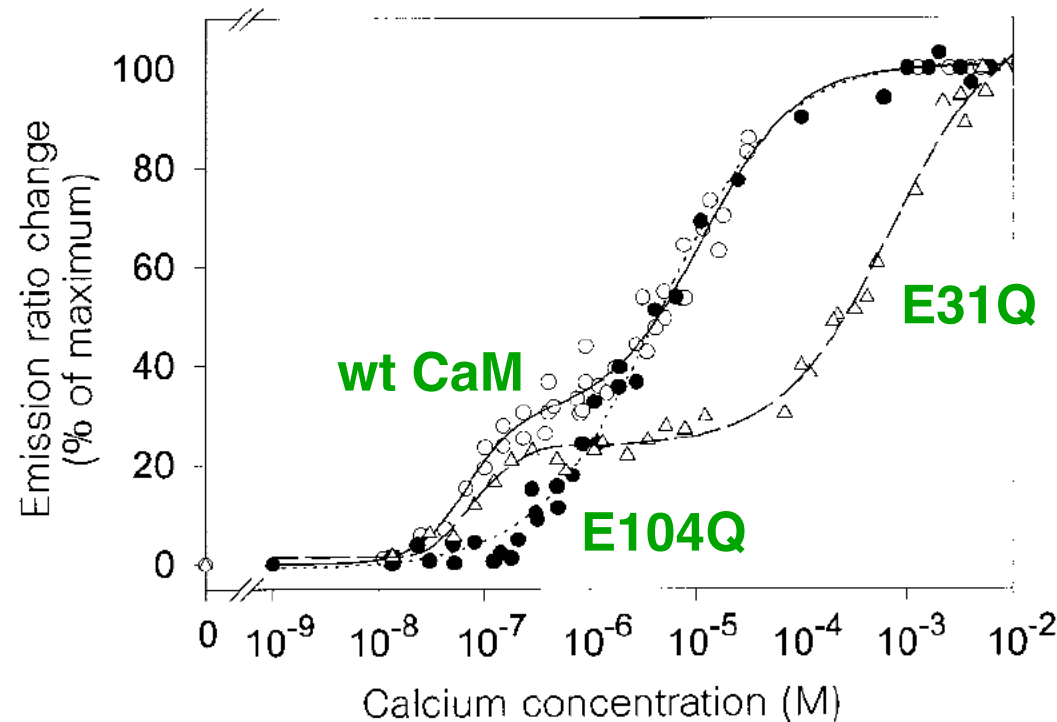
inverse  $\Delta F$

split-pericam



fun

Mutations can also affect calcium sensitivity; both  $K_d$  (affinity) and cooperativity (slope/shape of transition) can be affected. Miyawaki *et al.* engineered calcium sensitivity of CaMeleons, a related type of engineered protein calcium sensor:

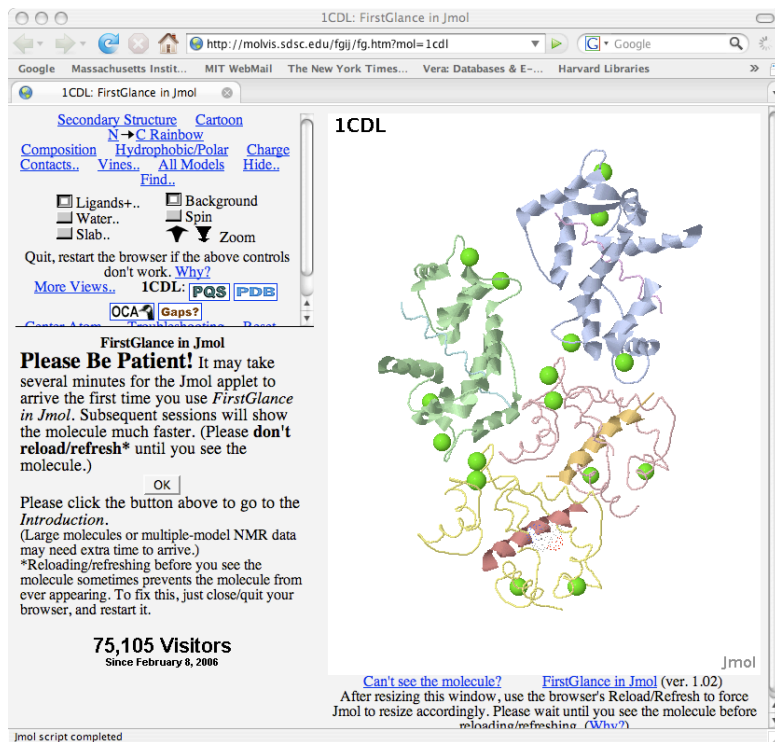


**In this module, our goal will be to influence the calcium sensitivity of “inverse pericam.”**

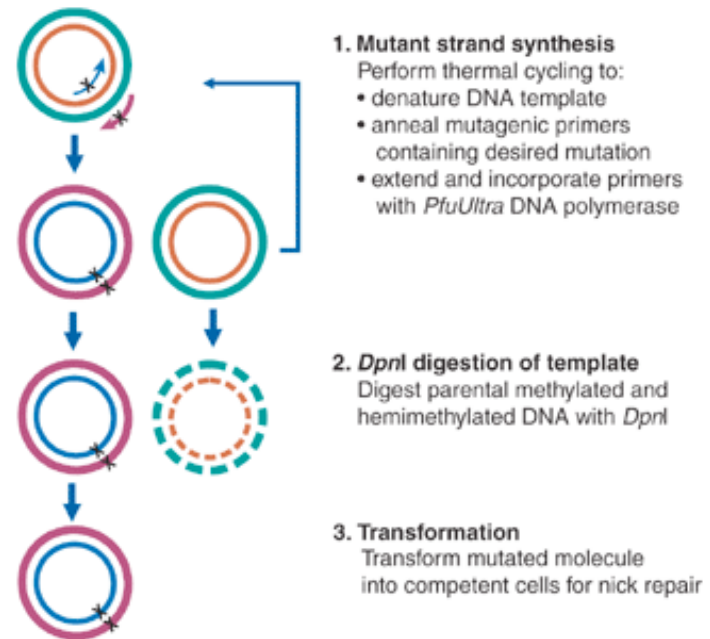
# Step 1: Design and implement mutations to affect inverse pericam's calcium sensitivity.

## Skills:

- Use computational tool to look closely at protein structures
- Design primers to make site mutations in the pericam gene
- Perform mutagenesis using PCR



The screenshot shows a web browser window displaying the FirstGlance in Jmol interface for the protein 1CDL. The interface includes a navigation menu with options like 'Secondary Structure', 'Cartoon', 'Composition', 'Hydrophobic/Polar', 'Charge', 'Contacts...', 'Vines...', and 'All Models'. There are also controls for 'Ligands+', 'Water..', 'Slab..', 'Background', 'Spin', and 'Zoom'. A message box says 'Please Be Patient! It may take several minutes for the Jmol applet to arrive the first time you use FirstGlance in Jmol. Subsequent sessions will show the molecule much faster. (Please don't reload/refresh\* until you see the molecule.)'. At the bottom, it says '75,105 Visitors Since February 8, 2006' and 'Jmol script completed'.

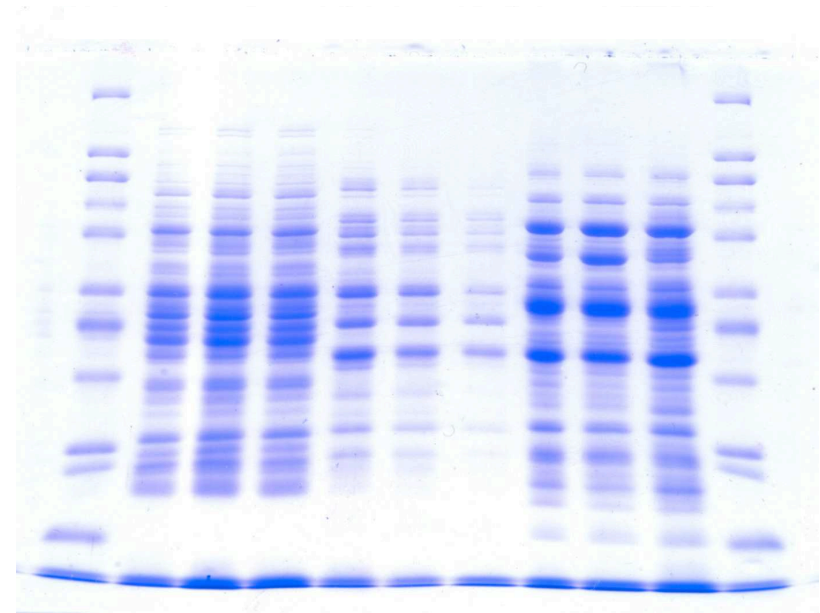
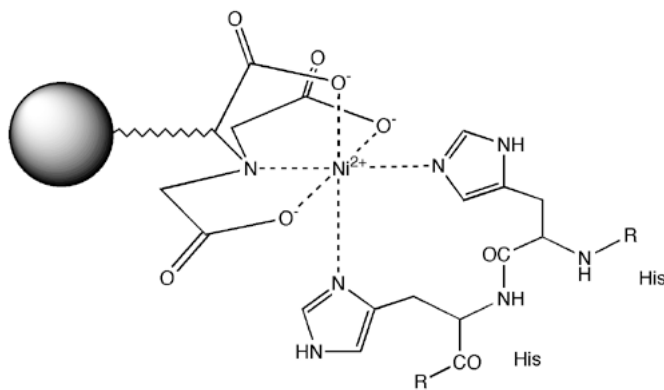
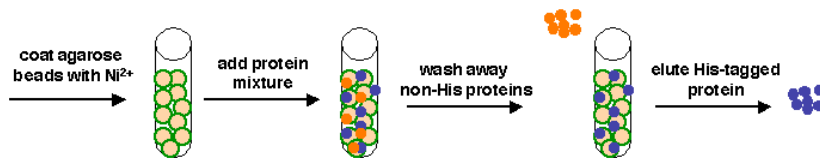




**Step 2:** Express and purify mutant inverse pericams for analysis.

**Skills:**

- Transform plasmid DNA into *E. coli*
- Induce protein expression using IPTG
- Purify mutant pericams using affinity-based separation
- Assay protein expression and purity using SDS-PAGE



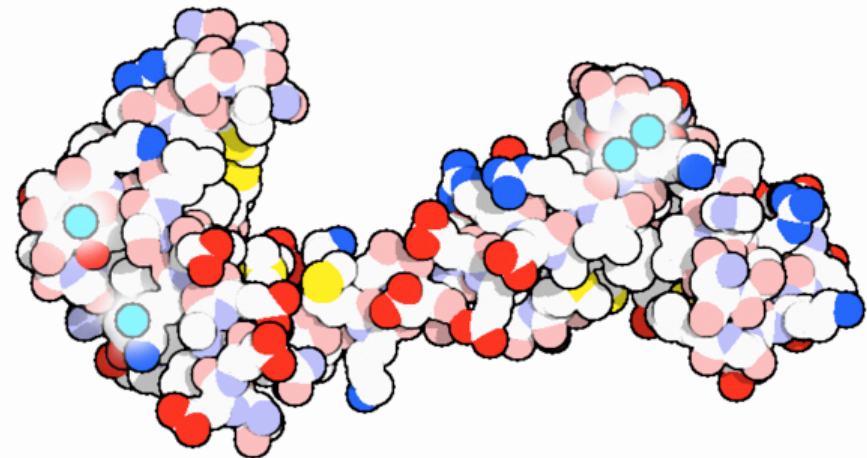
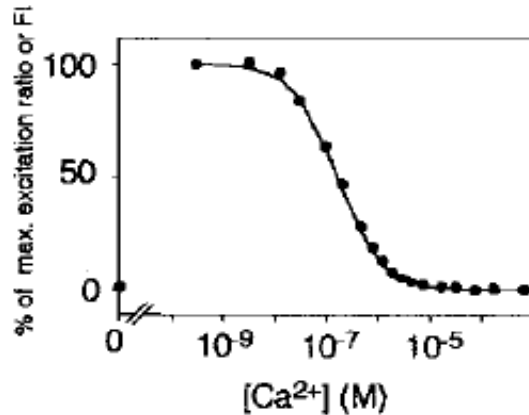
### Step 3: Analyze calcium titration behavior of mutant pericams.

#### Skills:

- Perform fluorescence assays to measure calcium binding
- Use software to extract binding parameters from the data
- Pool data from across the class to observe patterns



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* * * * * * * * *
MADQLTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRS LGQNPTEAELQDMINEVDAD
* * * * * * * * * * *
GNGTIDFPEFLTMMARKMKD TDSEEEIREAFRVFDKGN GYISAAELRHVMTNLGEKLT
* * * * *
DEEVDEMIREADIDGGQVNYEEFVQMMTAK
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