Module 2 overview

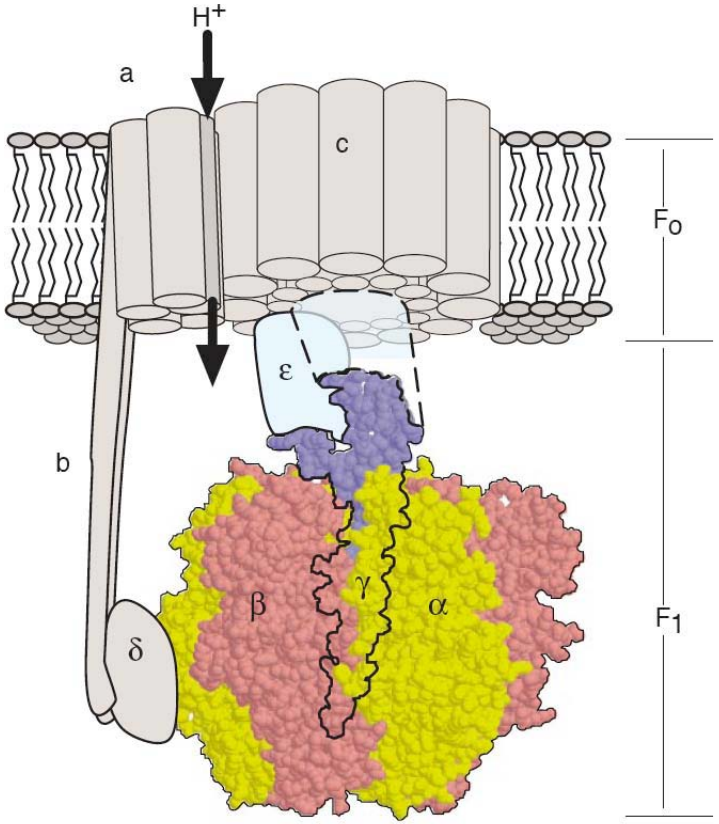
lecture	*lab*
1. Introduction to the module	1. Start-up protein eng.
2. Rational protein design	2. Site-directed mutagenesis
3. Fluorescence and sensors	3. DNA amplification
4. Protein expression	4. Prepare expression system
SPRING BREAK	
5. Review & gene analysis	5. Gene analysis & induction
6. Purification and protein analysis	6. Characterize expression
7. Binding & affinity measurements	7. Assay protein behavior
8. High throughput engineering	8. Data analysis

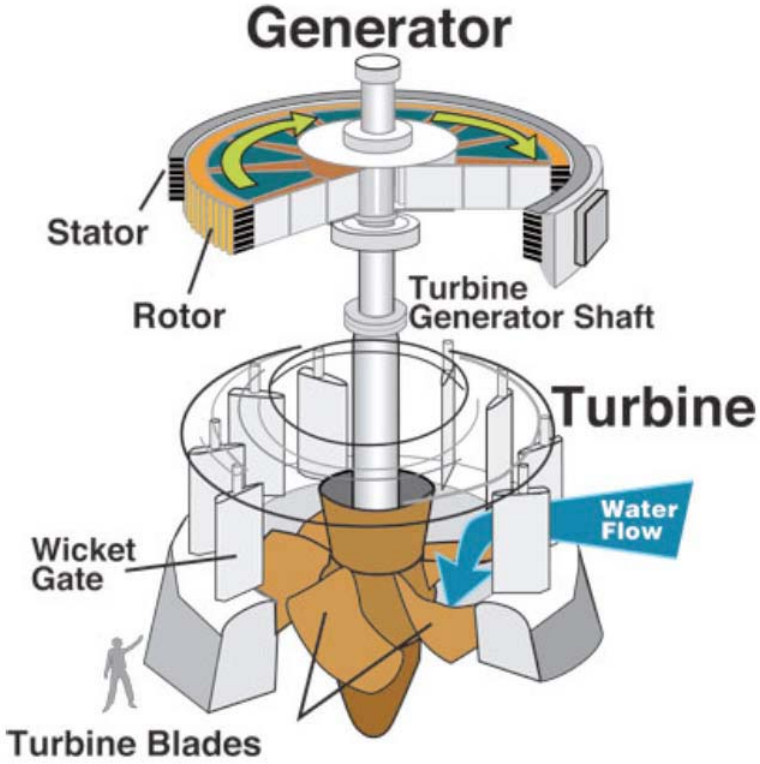
Lecture 1: Introduction to the module

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

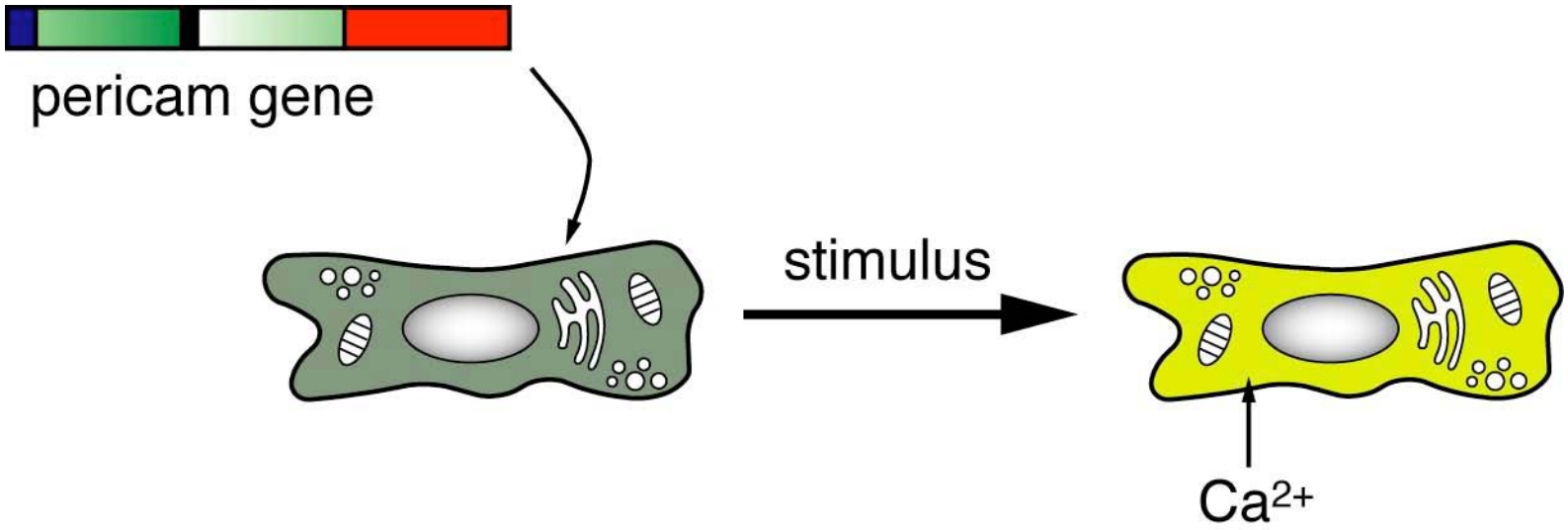




Wang & Oster (1998) *Nature 396:* 279-82

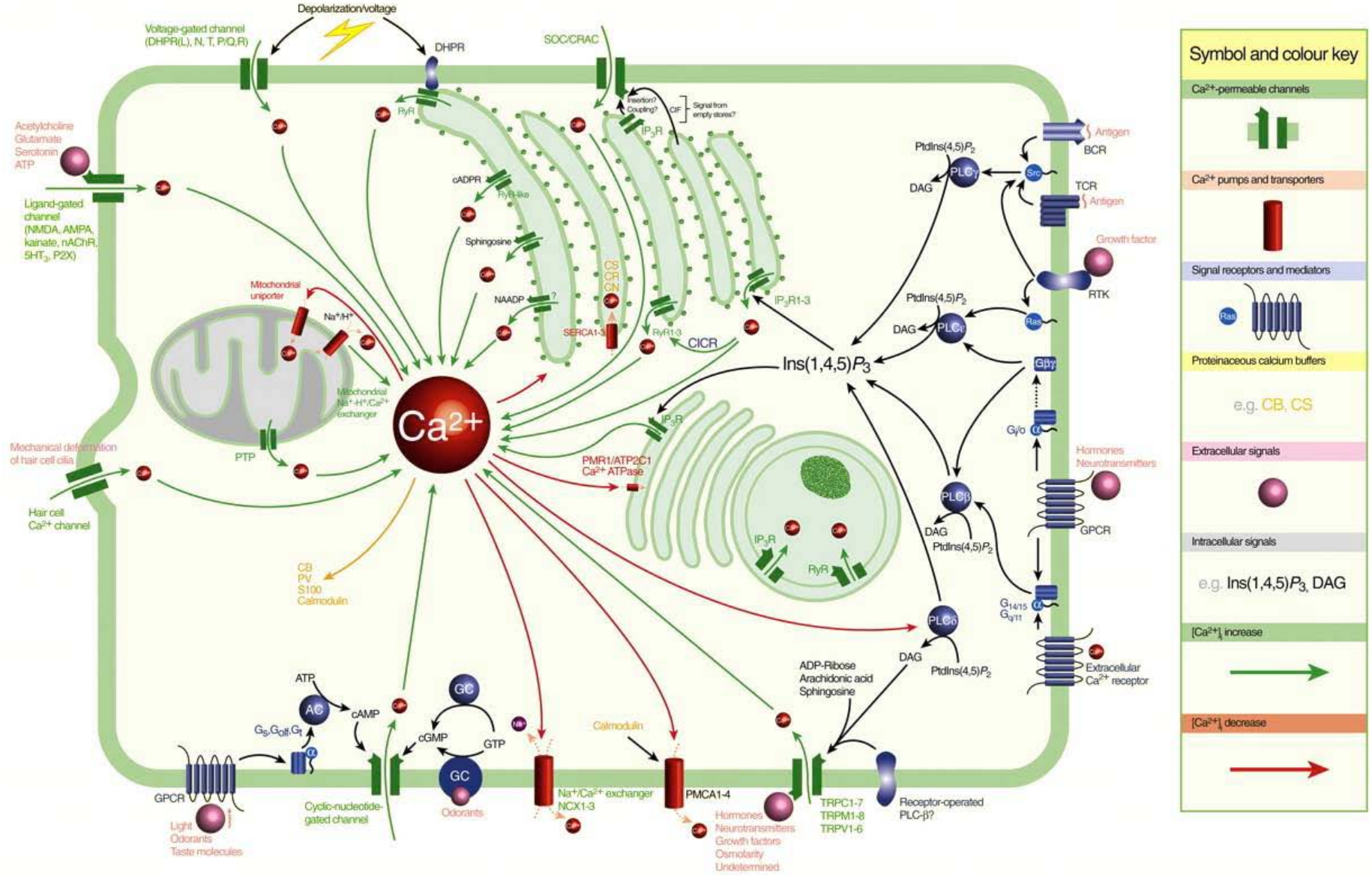
www.symscape.com/node/420

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



See Nagai, T., et al. “Circularly Permuted Green Fluorescent Proteins Engineered to Sense Ca2+.” *PNAS* 98, no. 6 (March 6, 2001): 3197-3202.

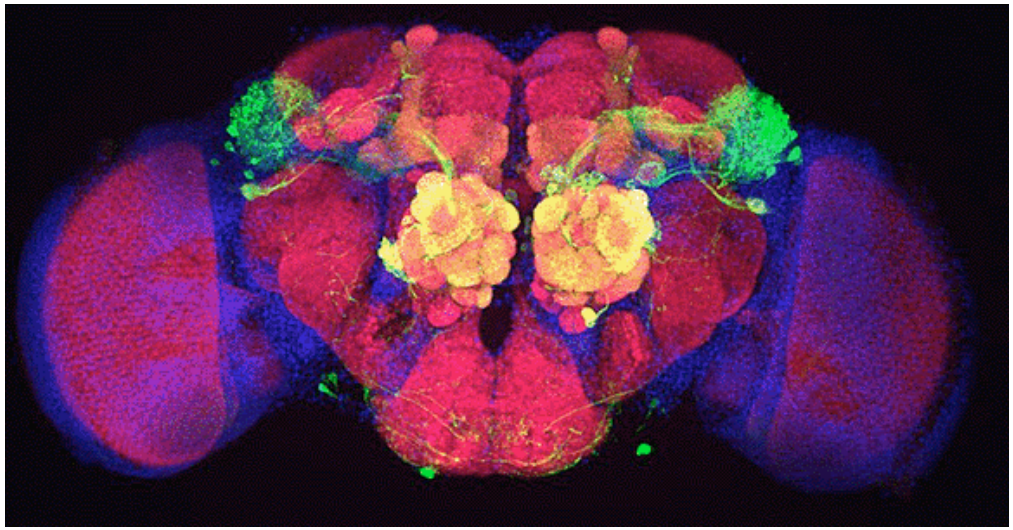
Nagai *et al.* (2001) *Proc. Natl. Acad. Sci. USA 98:* 3197-202



From Nowycky, M. C., and A. P. Thomas. Fig 1 in "Intracellular calcium signaling." *Journal of Cell Science* 115 (2002): 3715-3716. Reproduced with permission. Journal of Cell Science: http://jcs.biologists.org

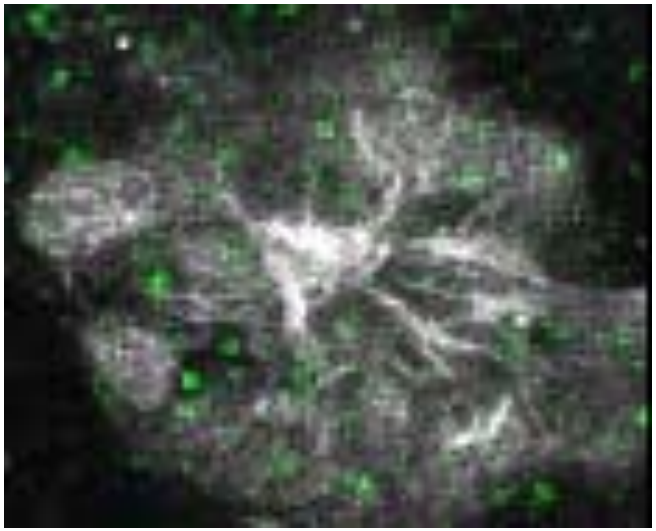
Calcium indicators can be used to detect signaling in individual cells and multicellular ensembles. Two purposes:

- 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)



Courtesy of The Axel Lab at Columbia University.
Used with permission.

caproic acid stimulus



J. W. Wang *et al*. (2003) *Cell 112:* 271-82.

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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*).

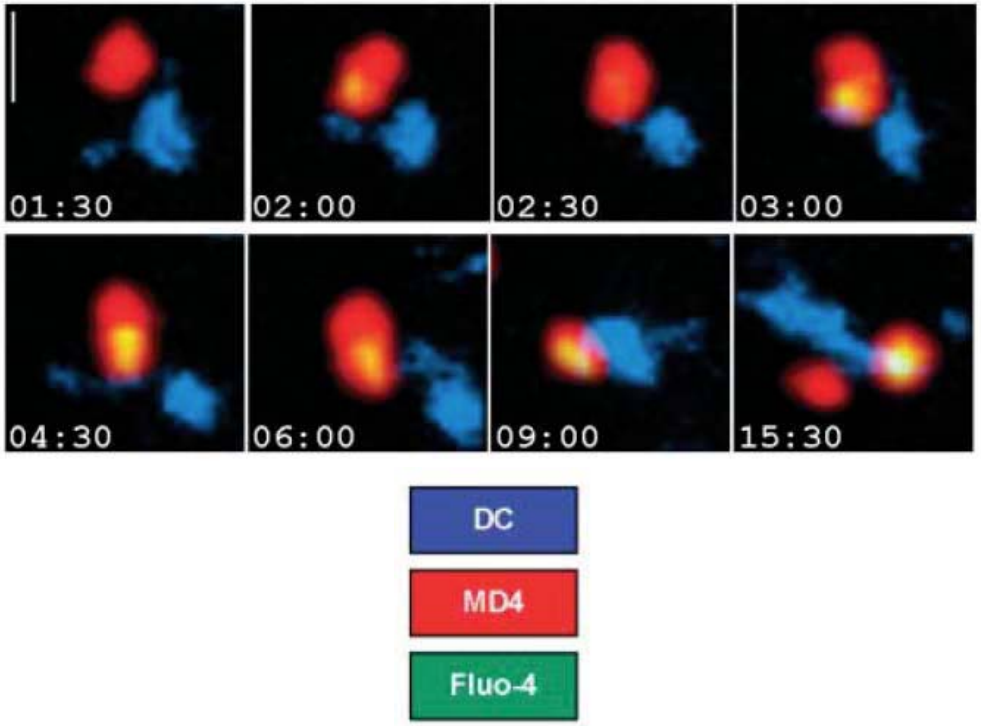
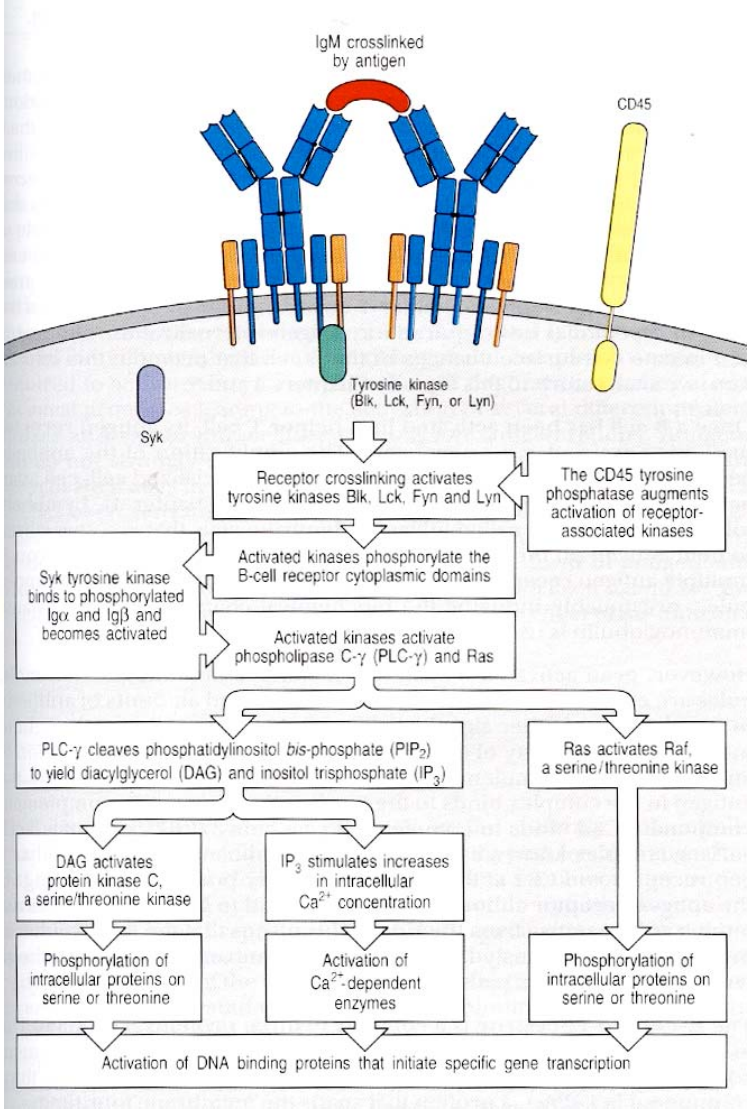
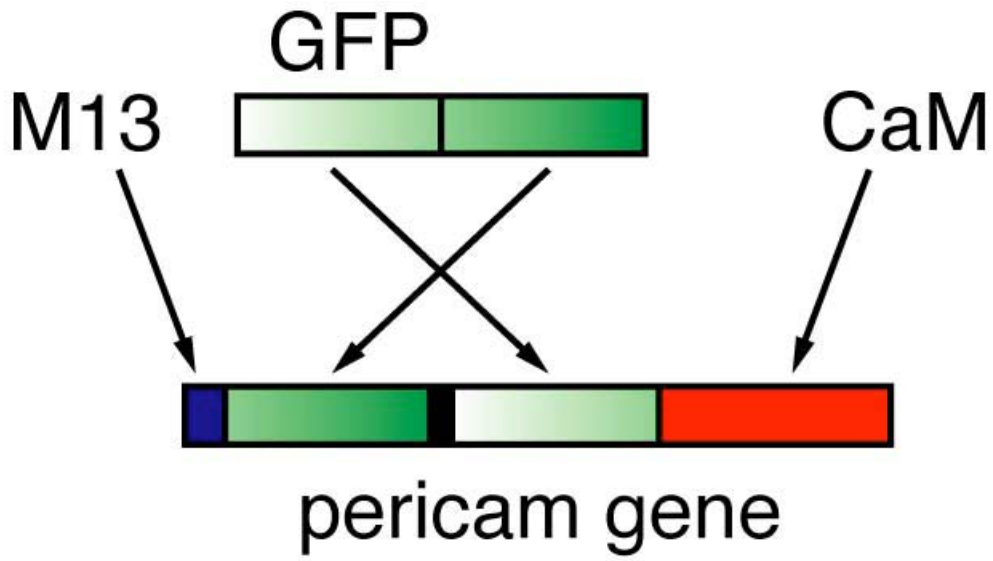


Image from Qi, H. et al. "Extrafollicular Activation of Lymph Node B Cells by Antigen-Bearing Dendritic Cells." *Science* 312, no. 5780 (June 16, 2006): 1672-1676. DOI: 10.1126/science.1125703.



Source: Figure 3.35 in Janeway, C. A., and P. Travers. *Immunobiology*. 2nd ed. New York, NY: Garland Science, 1996.



GFP = green fluorescent protein
CaM = calmodulin, a calcium-sensing protein
M13 is a CaM-binding fragment of a cellular kinase

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 Ca^{2+} ions using EF hand amino acid sequence motifs
- Ca^{2+}-CaM binds short segments of target proteins, modulates activity

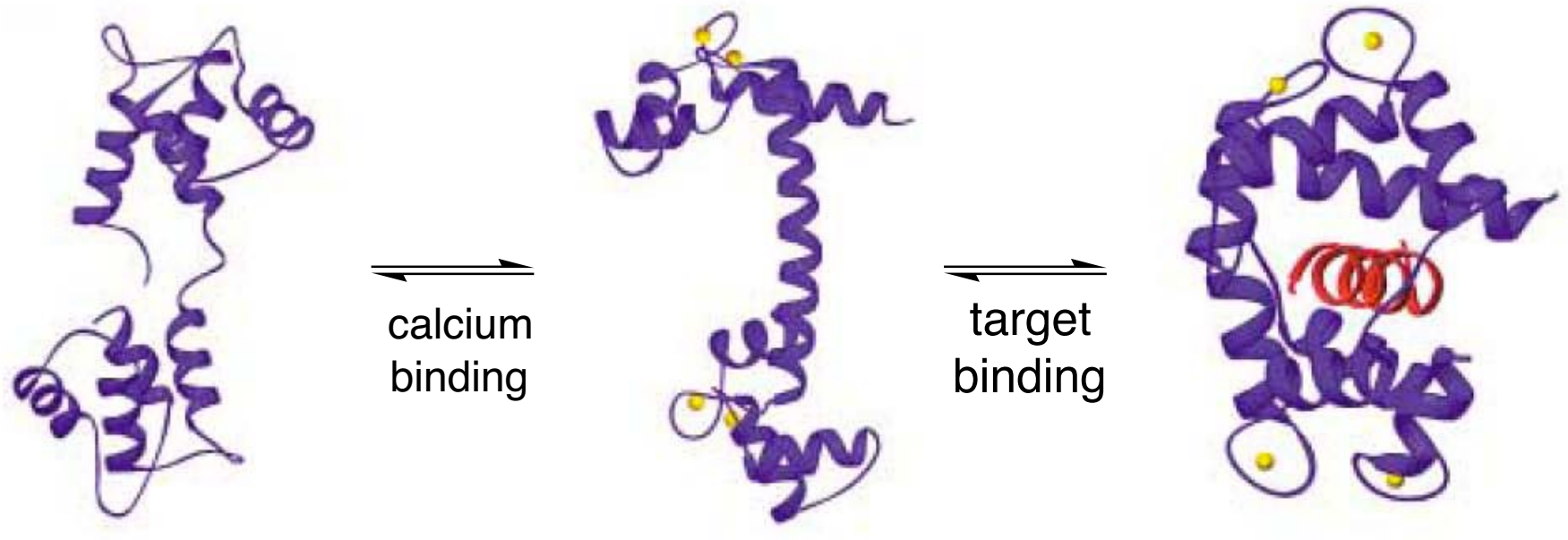
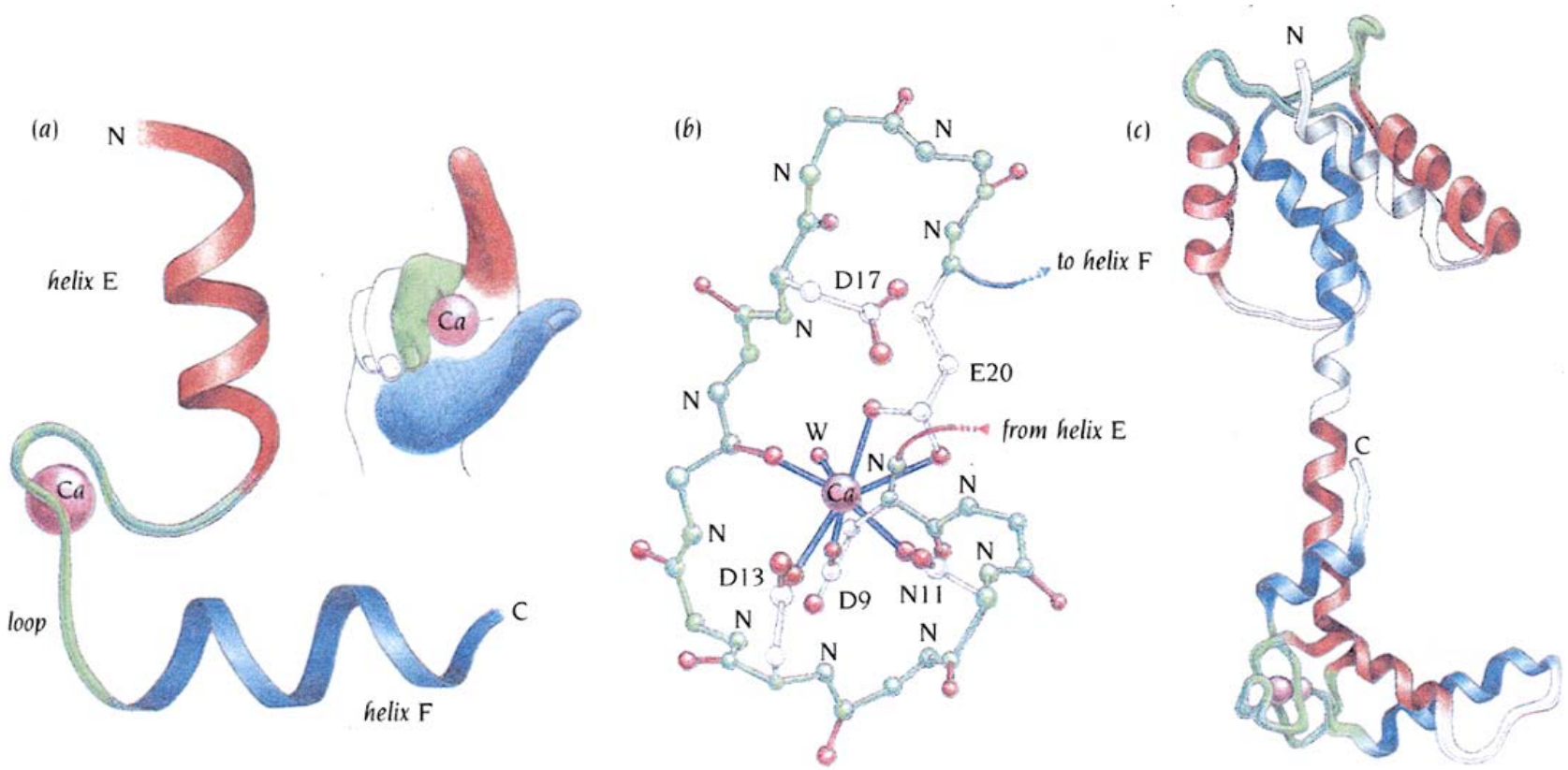


Image from Vetter, S. W., and E. Leclerc. "Novel Aspects of Calmodulin target Recognition and Activation." *Eur J Biochem* 270, no. 3 (2003): 404-414.

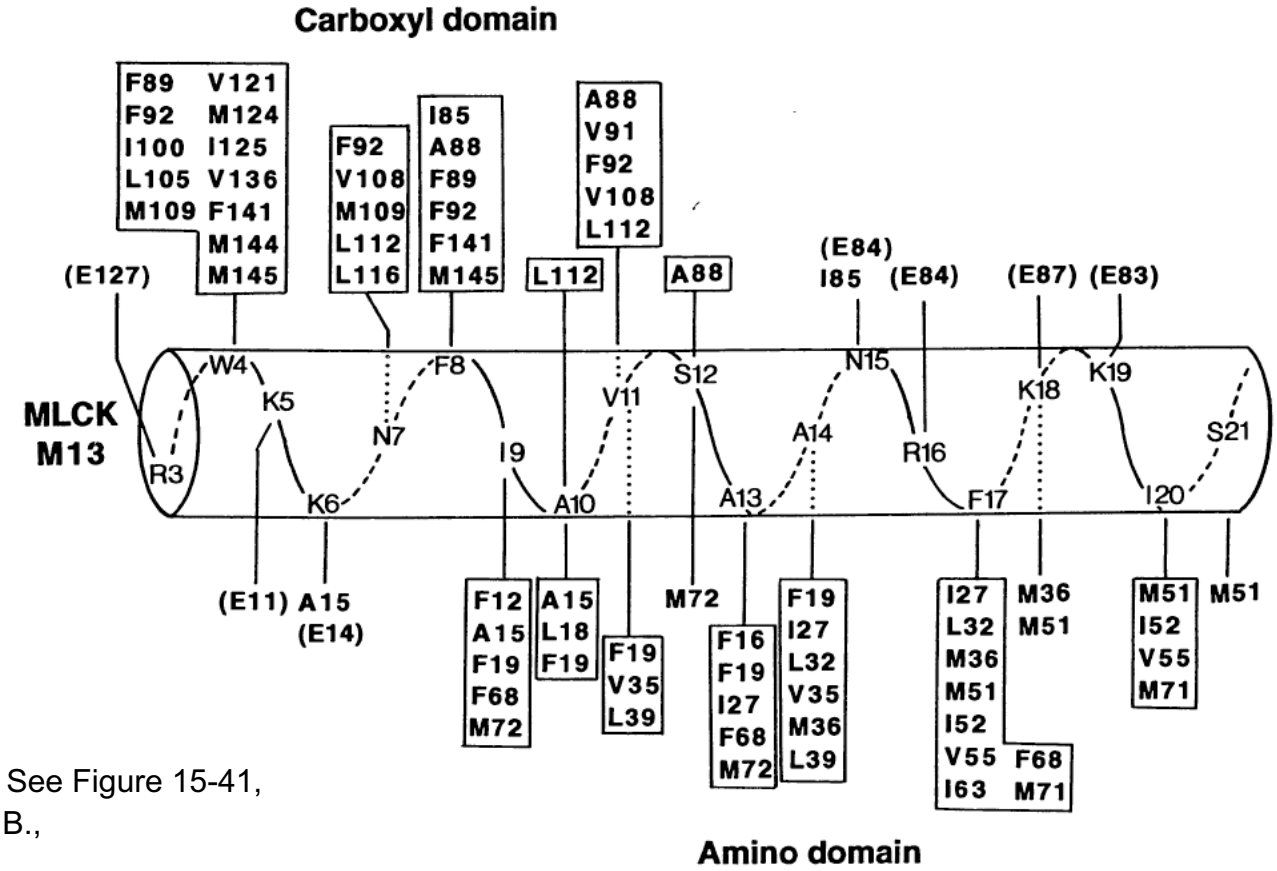


EF hand binding motif named for E & F helices of the calcium-binding protein parvalbumin; example of helix-loop-helix structure, with calcium bound in the loop

N- and C-terminal domains of CaM both contain two EF hand motifs

Image: Figure 2.13 in Branden, C., and J. Tooze. *Introduction to Protein Structure*. 2nd ed. New York, NY: Garland Science, 1999.

- Ca^{2+}-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.

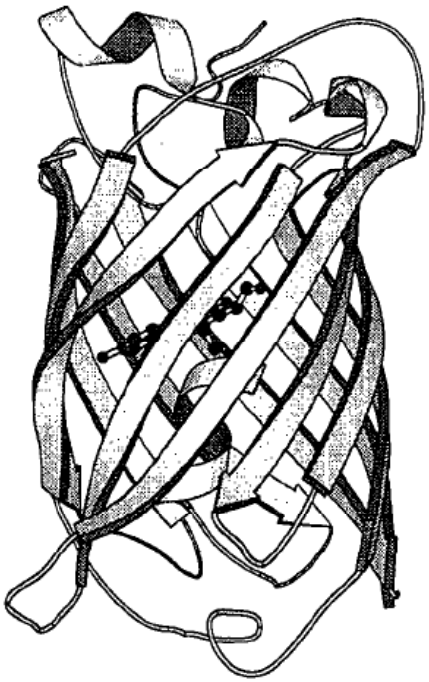


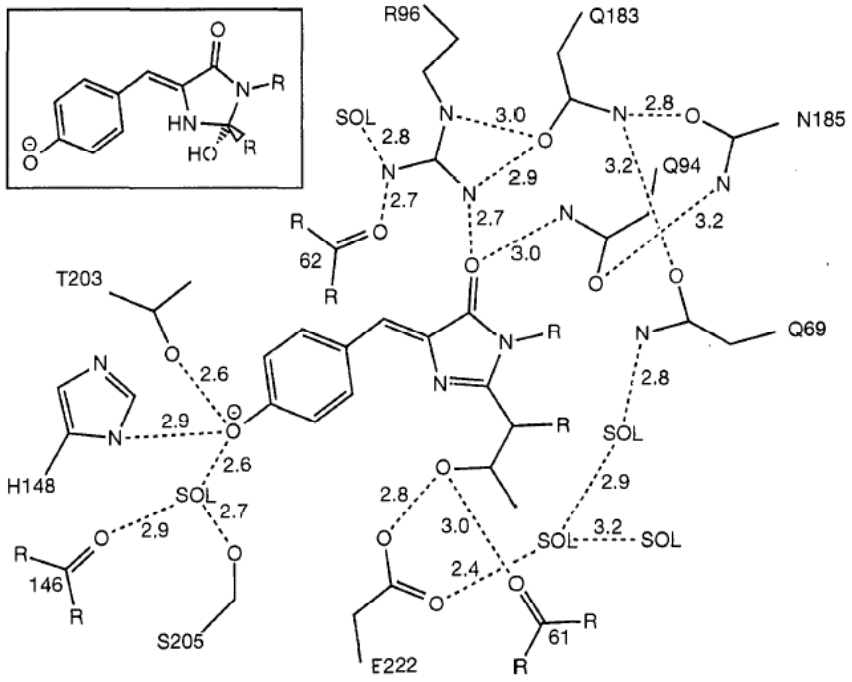
Ikura *et al.* (1992) *Science 256:* 632-8.

Image removed due to copyright restrictions. Still image from YouTube video about jellyfish fluorescence. Alternative: see this video from the public TV series "Secrets of the Sequence." Vculifesciences. "A Green Light for Biology -- Making the Invisible Visible." YouTube. June 24, 2008. Accessed August 25, 2010. http://www.youtube.com/watch?v=Sl2PRHGpYuU.

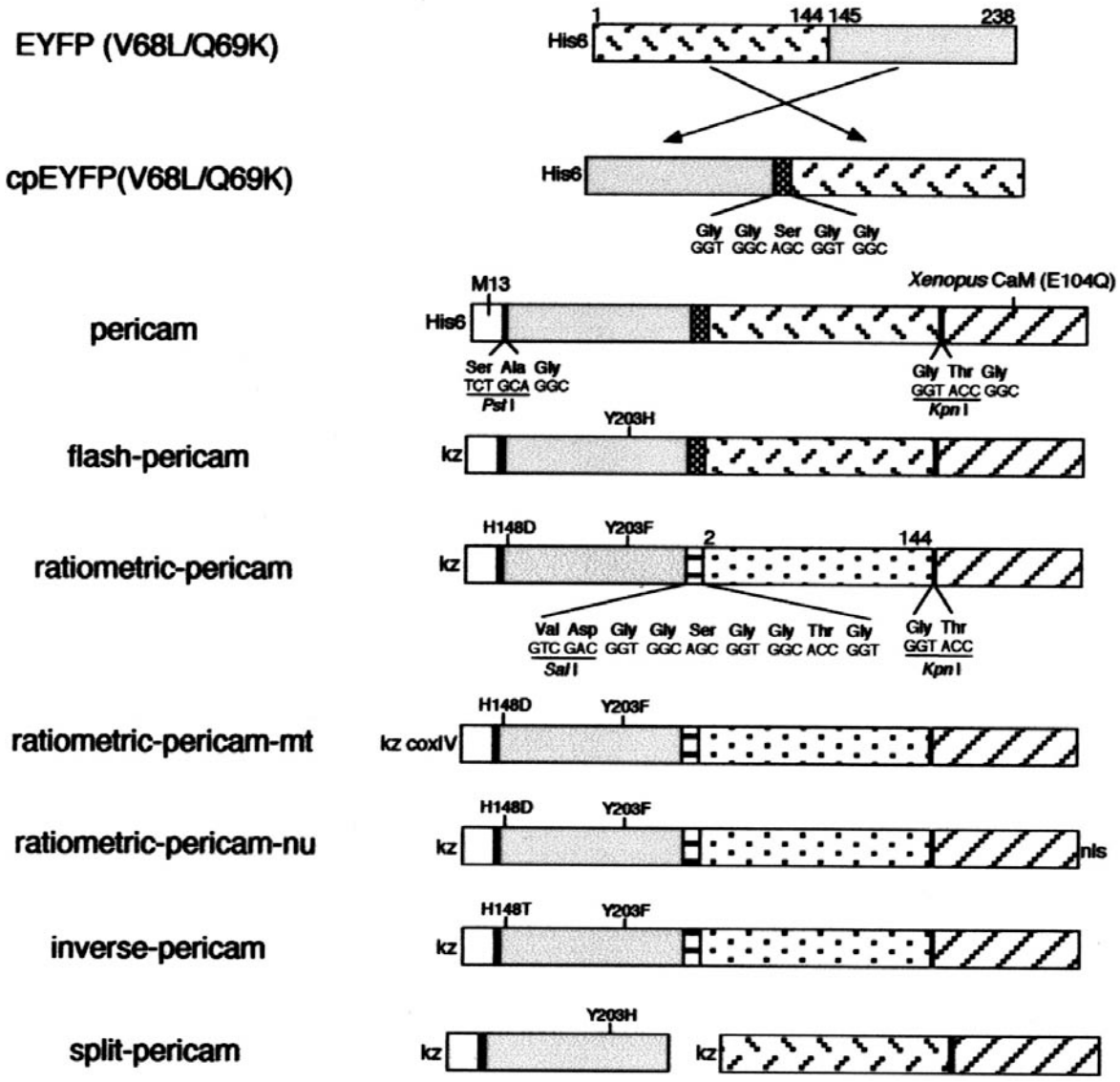
Green Fluorescent Protein (GFP)

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.



parent construct

dynamic range

ratiometric ΔF

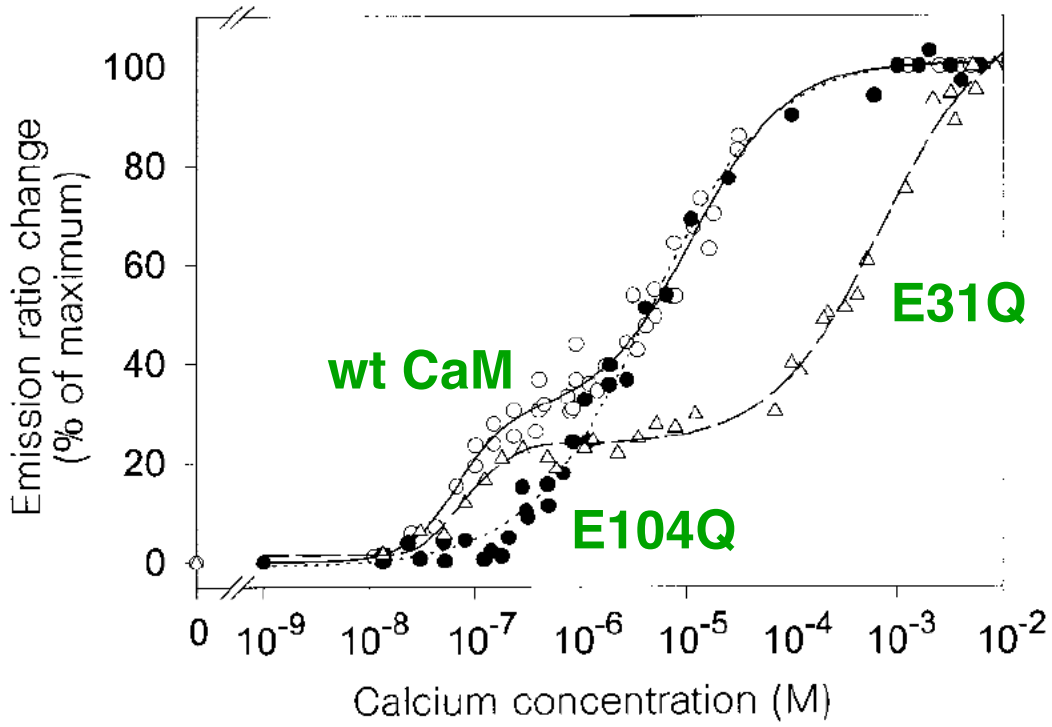
mitoch. localization

nuclear localization

inverse ΔF

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:

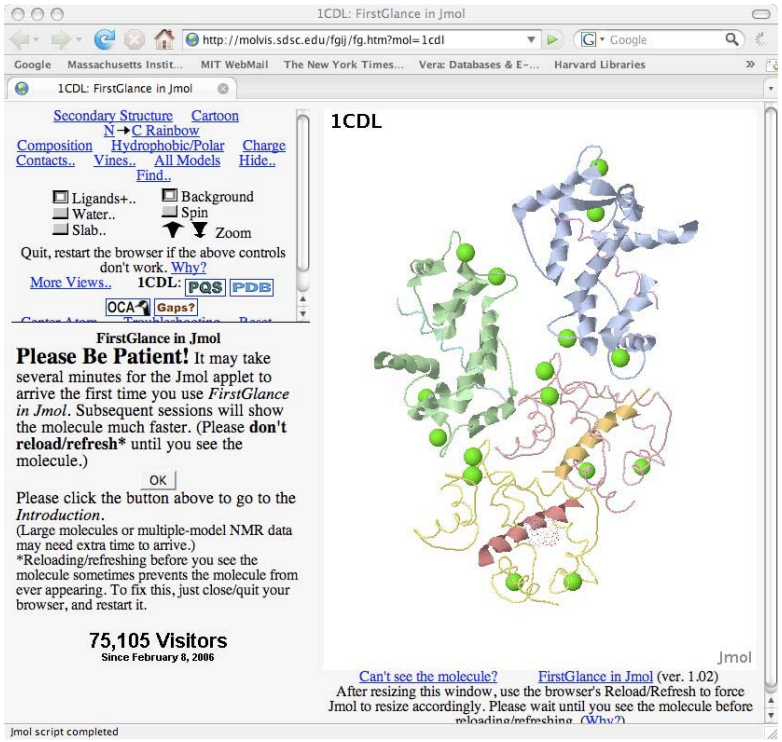


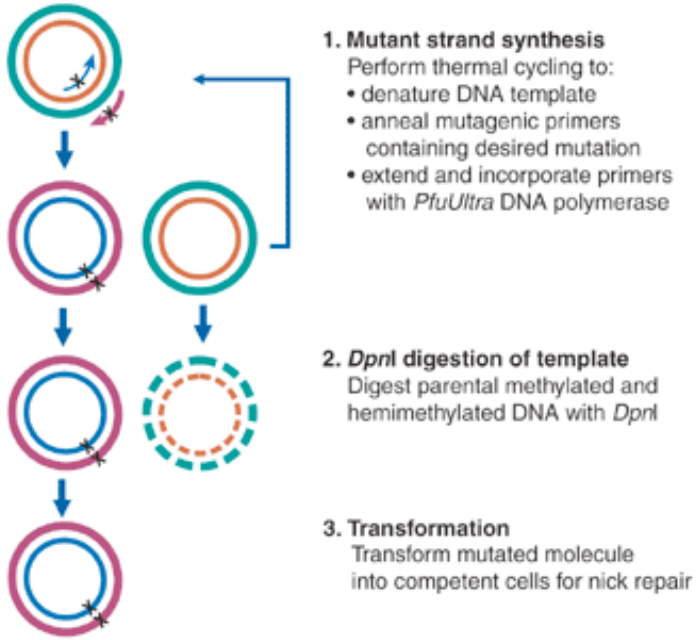
In this module, our goal will be to influence the calcium sensitivity of "inverse pericam."

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



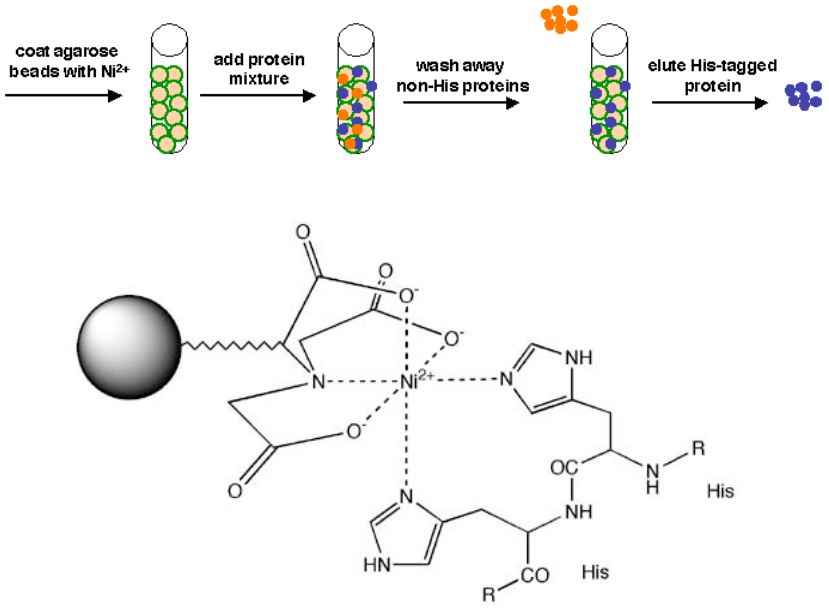


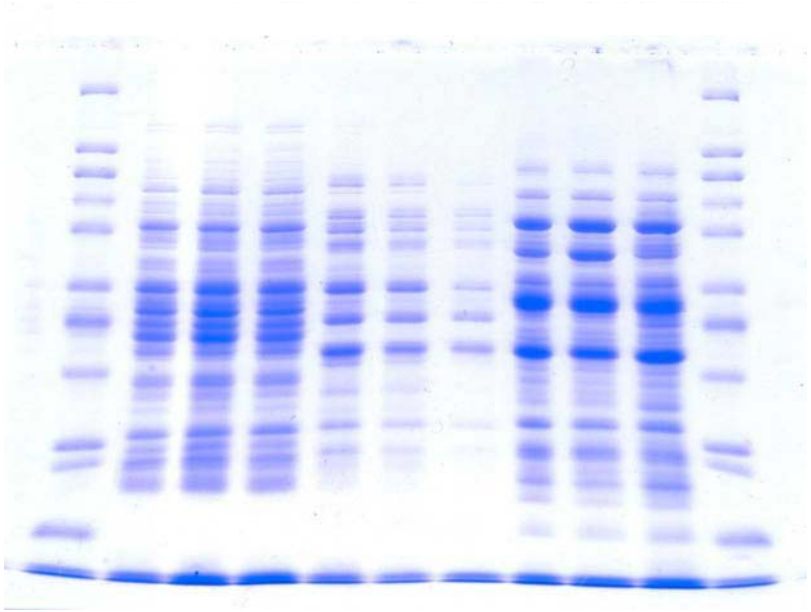
Courtesy of Agilent Technologies.
Used with permission.

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



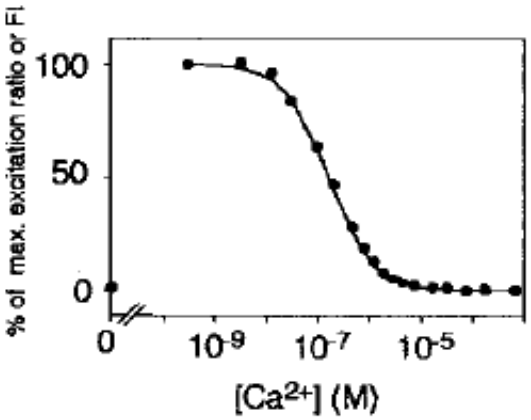


Step 3: Analyze calcium titration behavior of mutant pericams.

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

Courtesy of Thermo Fisher Scientific. Used with permission.

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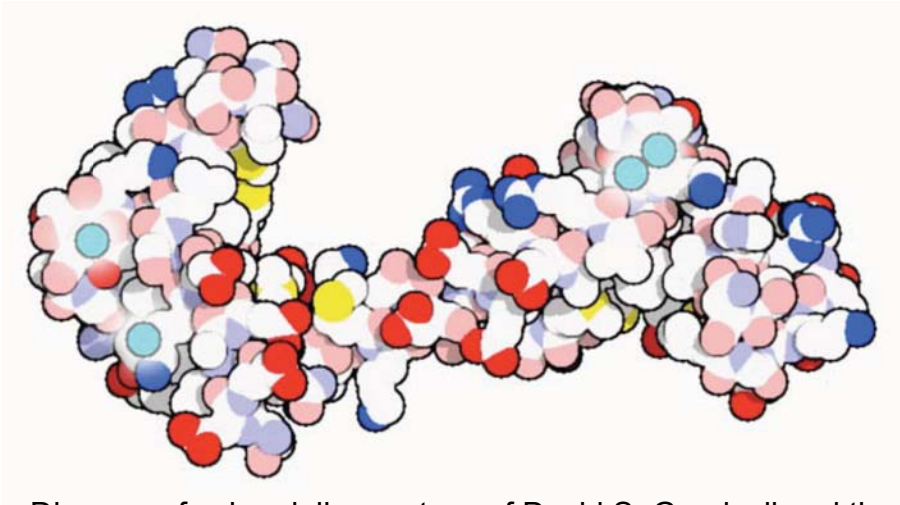

Diagram of calmodulin courtesy of David S. Goodsell and the RCSB PDB.

20.109 Laboratory Fundamentals in Biological Engineering

Spring 2010