



## Module 2 overview

### *lecture*

1. Introduction to the module
2. Rational protein design
3. Fluorescence and sensors
4. Protein expression

### *lab*

1. Start-up protein eng.
2. Site-directed mutagenesis
3. DNA amplification
4. Prepare expression system

## **SPRING BREAK**

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

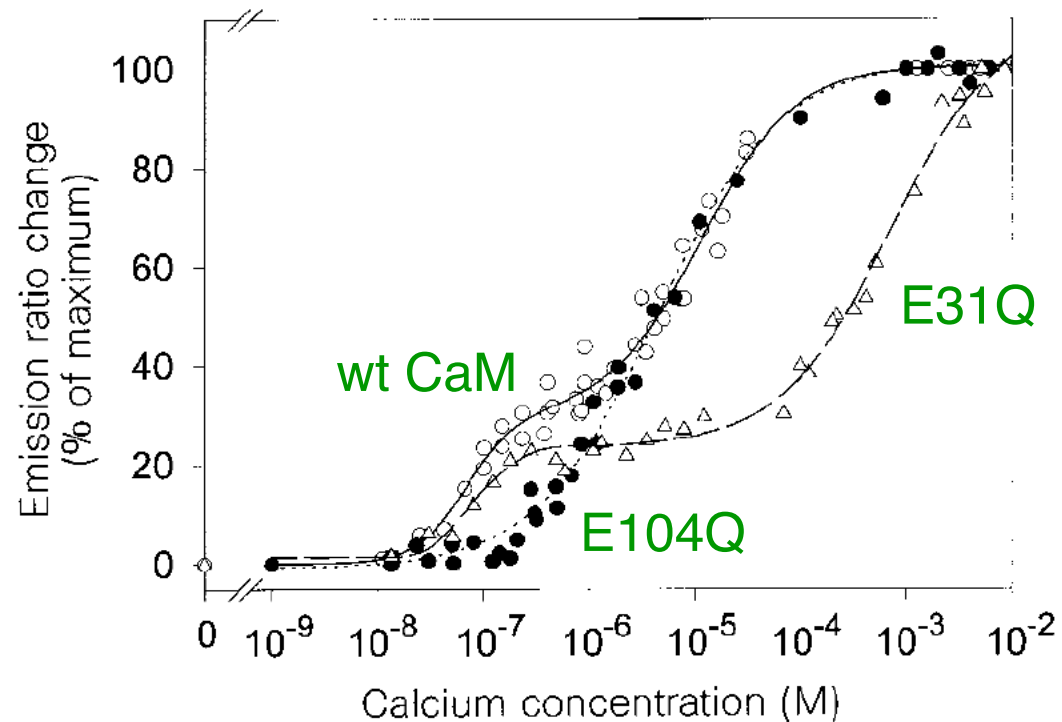
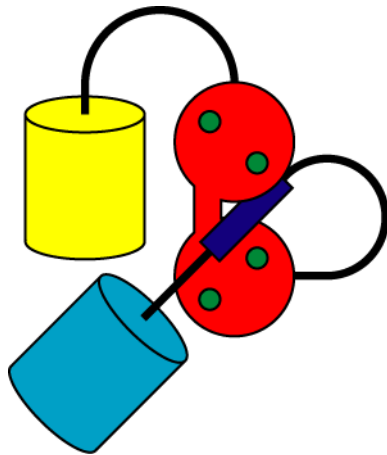
5. Gene analysis & induction
6. Characterize expression
7. Assay protein behavior
8. Data analysis

## Lecture 6: Binding and affinity measurements

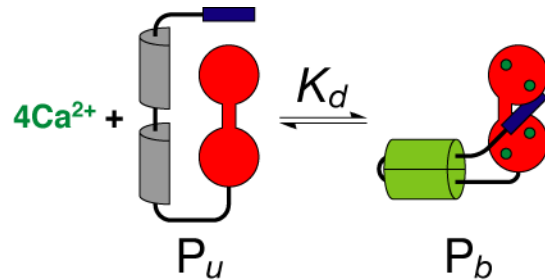
- I. Titration analysis
  - A. Estimating  $K_d$  &  $EC_{50}$  from fluorescence data
  - B. Multisite binding and cooperativity
  
- II. Techniques for studying binding

Q: Our design task is to alter calcium sensitivity of pericam derivatives. We chose mutants to make, but **how will we determine the results** of our perturbations?

A: We will measure **titration curves**, by adding fixed amounts of calcium to samples of mutant pericam and measuring an indicator of binding (in this case fluorescence). From these data, we will extract estimates of binding parameters.



What is going on during a titration measurement?



$$[P]_{tot} = [P_u] + [P_b]$$

$$K_d = ([P_u][Ca^{2+}]^4)/[P_b]$$

We want to measure  $K_d$  from fluorescence.  $P_u$  and  $P_b$  are fluorescent to different degrees, so that the total fluorescence is equal to the sum of contributions from the two species:

$$F = F_u [P_u] + F_b [P_b]$$

Fluorescence measurements can be converted to **fractional saturation**:

$$\frac{F_{max} - F}{F_{max} - F_{min}} = \frac{\Delta F}{\Delta F_{max}} = \frac{[P_b]}{[P]_{tot}}$$

$\Delta F/\Delta F_{max}$  is equal to the fractional saturation of pericam calcium binding sites, often abbreviated  $\Theta$ .  $\Theta$  can be expressed as a function of calcium concentration as follows:

$$\Theta = \frac{[P_b]}{[P]_{tot}} = \frac{[P_b]}{[P_b]K_d/[Ca^{2+}]^4 + [P_b]}$$

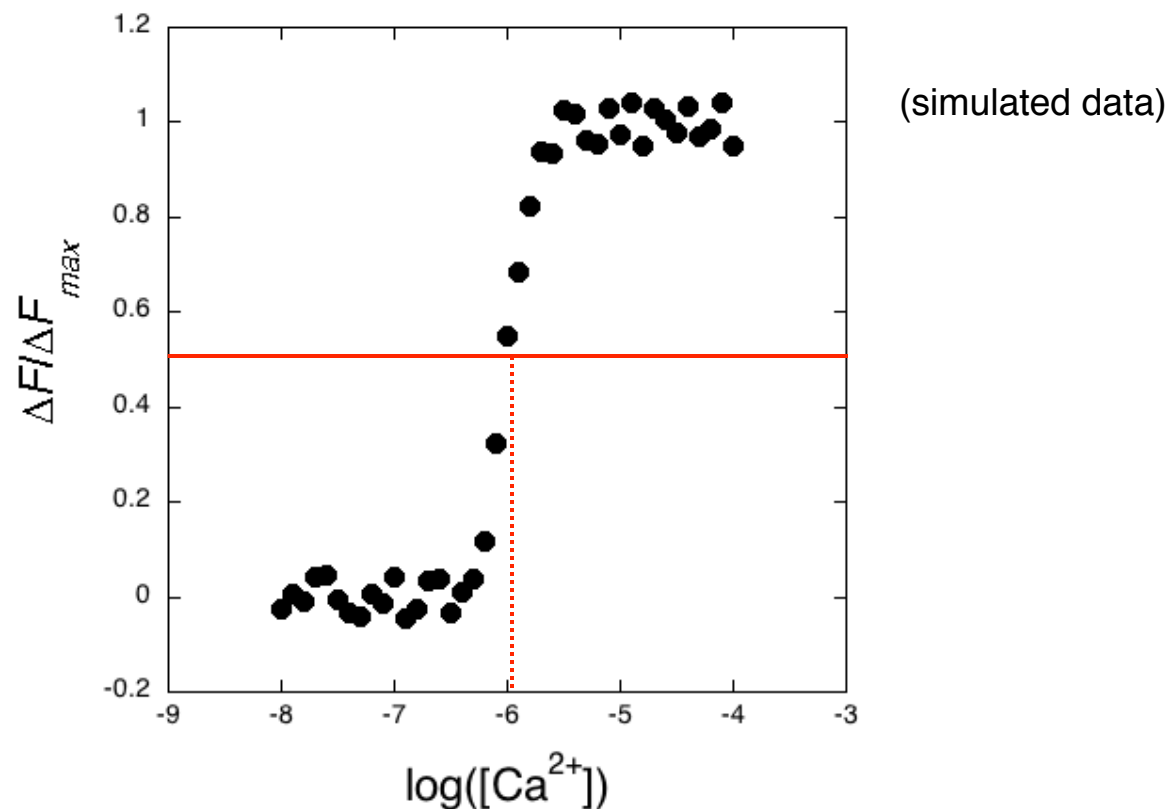
note:  
 $[P_u] = [P_b]K_d/[Ca^{2+}]^4$

$EC_{50} = (K_d)^{1/4}$  is the calcium concentration at which **half-maximal** binding and fluorescence change occurs.

Calcium sensing could realistically be performed for calcium concentrations near the  $EC_{50}$ , and our design goal is equivalent to **shifting the  $EC_{50}$**  of the pericam derivatives.

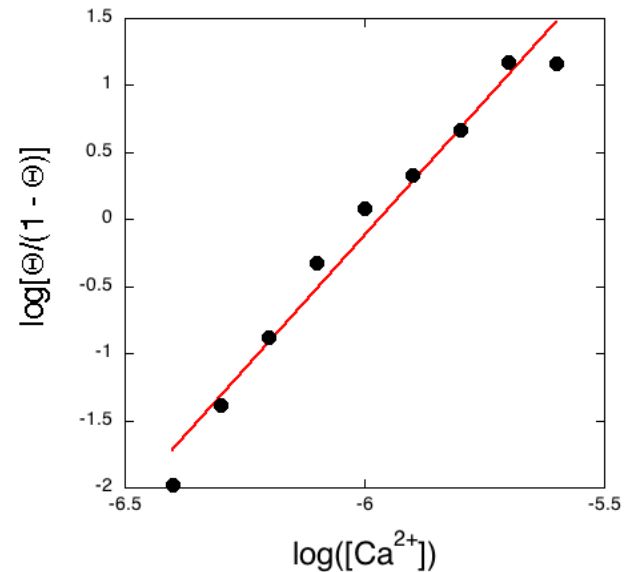
If we had fluorescence data over a range of calcium concentrations, and assuming these equations accurately describe calcium binding to pericam, we could determine the  $EC_{50}$  and  $K_d$  using a variety of methods:

1. Look for **midpoint** of the fluorescence change



## 2. Hill analysis

- substitute  $\Theta = \Delta F / \Delta F_{max}$
- set  $y = \log[\Theta / (1 - \Theta)]$
- plot  $y$  vs.  $x = \log([Ca^{2+}])$  for transition region
- slope tells number of cooperative binding sites, “Hill coefficient”
- x intercept is  $\log(EC_{50})$

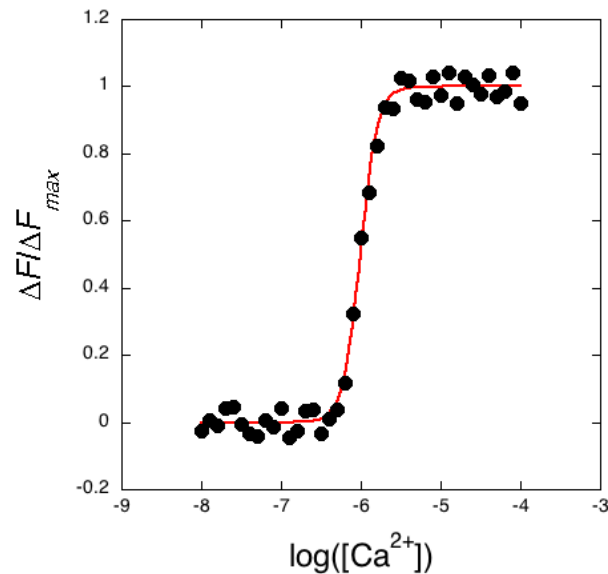


## 3. Curve fitting

- consider the equation we derived:

$$\frac{\Delta F}{\Delta F_{max}} = \frac{[Ca^{2+}]^4}{(EC_{50})^4 + [Ca^{2+}]^4}$$

- express as  $y = x^4 / (c^4 + x^4)$
- use Matlab or related to determine value of  $c$  that best fits the data
- could leave the exponent as a free parameter  $n$ , equal to Hill coeff.



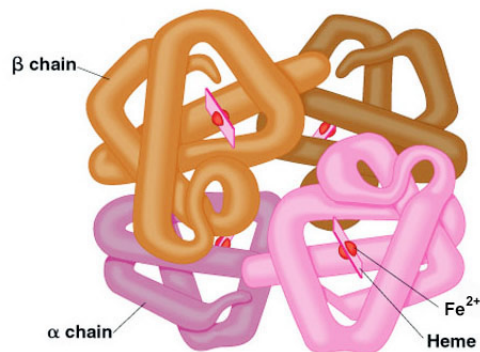


Hill coefficient reflects **cooperativity**, a phenomenon of binding to multiple binding sites on an individual target: **positive cooperativity** means that binding to one site promotes binding to other sites; **negative cooperativity** means that binding to one site depresses binding to other sites.

Literary description of positive cooperativity:

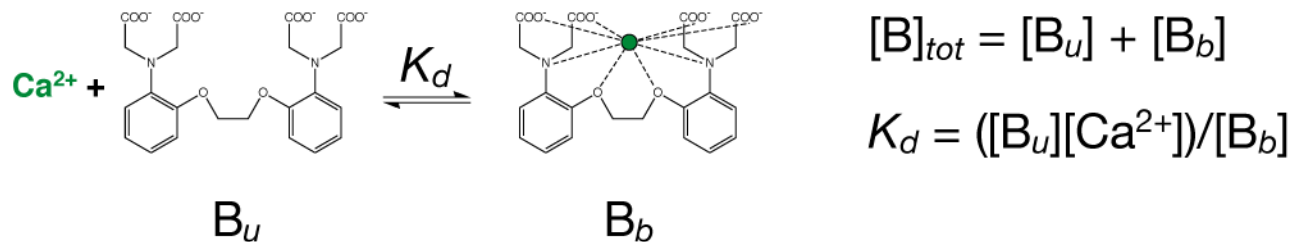
*“For whosoever hath, to him shall be given, and he shall have more abundance: but whosoever hath not, from him shall be taken away even that he hath.”*

*Matthew 13:12 (King James Bible)*



**Hemoglobin (Hill coef. ~3)**

For comparison with pericam, consider (noncooperative) calcium binding to BAPTA, a commonly used calcium-specific chelator:



In this case, the equation that describes a titration curve (fraction of bound sites vs. calcium concentration) is:

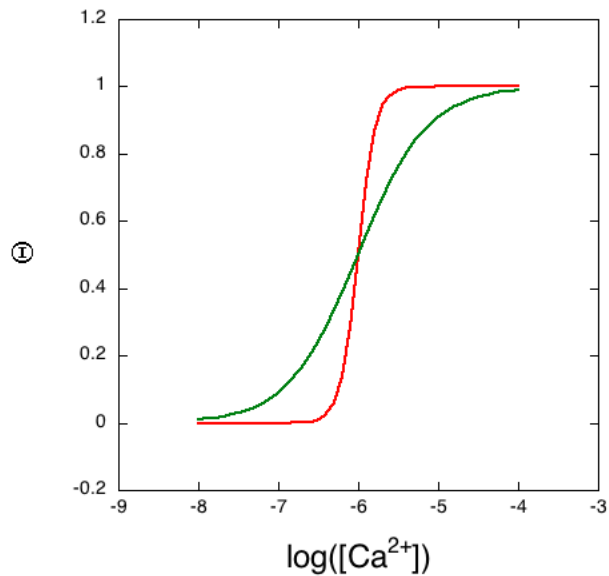
$$\Theta = \frac{[B_b]}{[B]_{tot}} = \frac{[B_b]}{[B_b]K_d/[Ca^{2+}] + [B_b]}$$

$$= \frac{[Ca^{2+}]}{K_d + [Ca^{2+}]}$$

Hill coeff. = 1

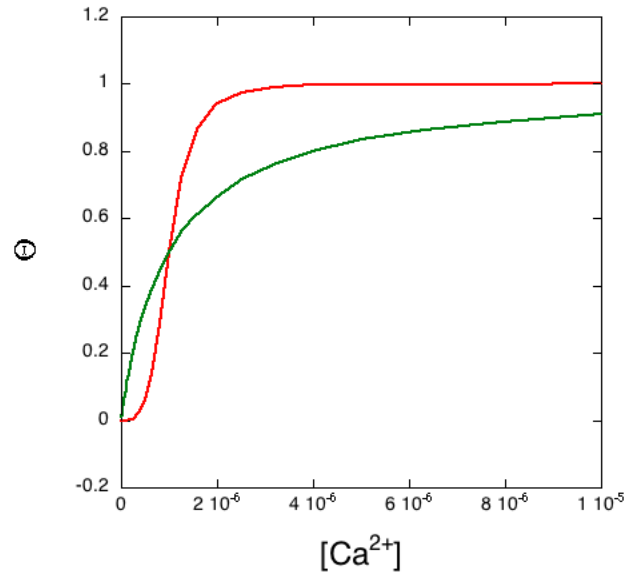
Simulated binding curves for **BAPTA** and **pericam** compare as follows:

saturation vs.  $\log([Ca^{2+}])$



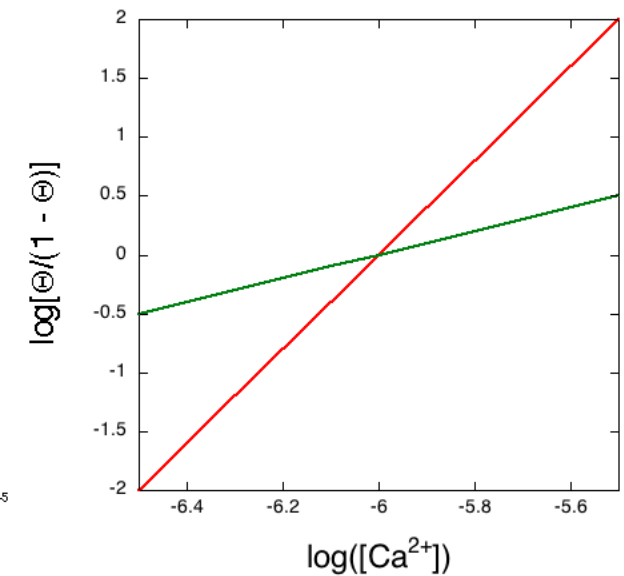
**pericam: sharper transition**  
**BAPTA: broader transition**

saturation vs.  $[Ca^{2+}]$



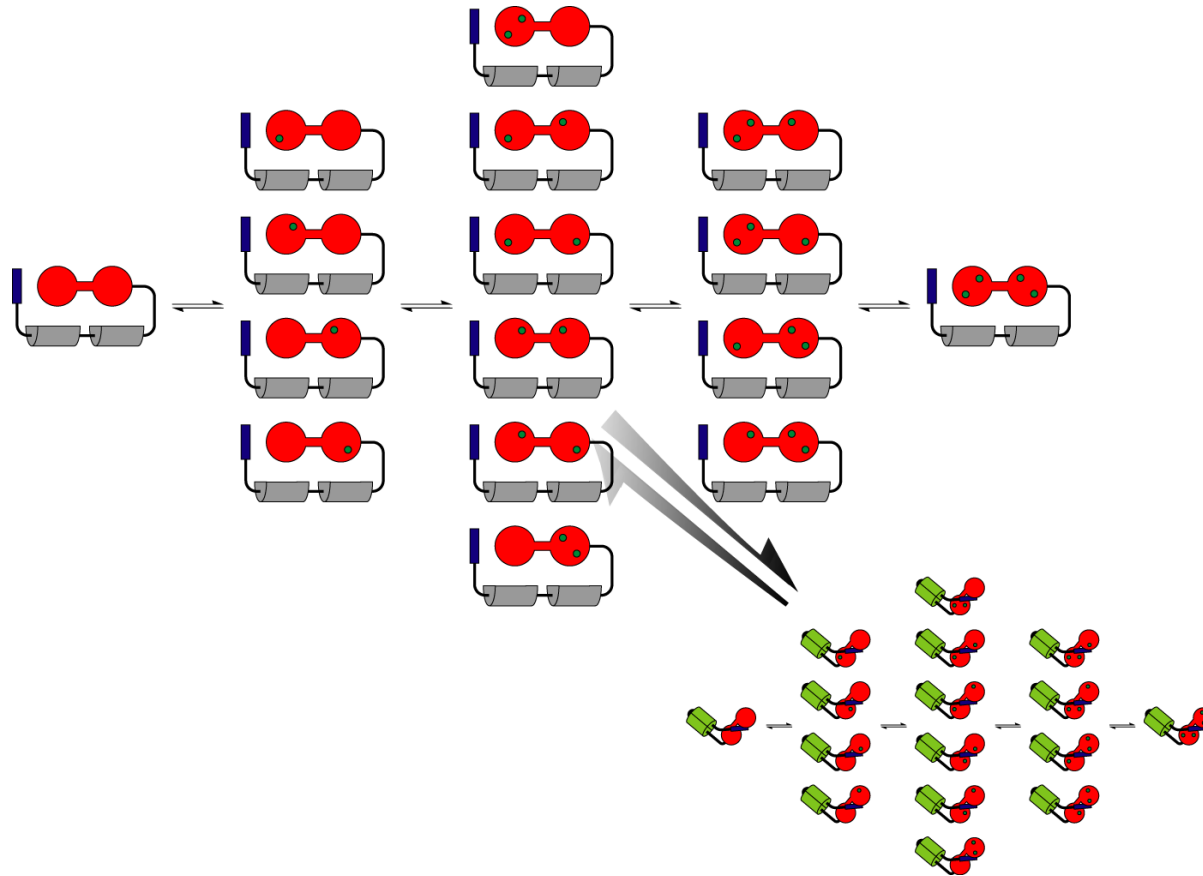
**pericam: sigmoidal transition**  
**BAPTA: smooth transition**

Hill plot

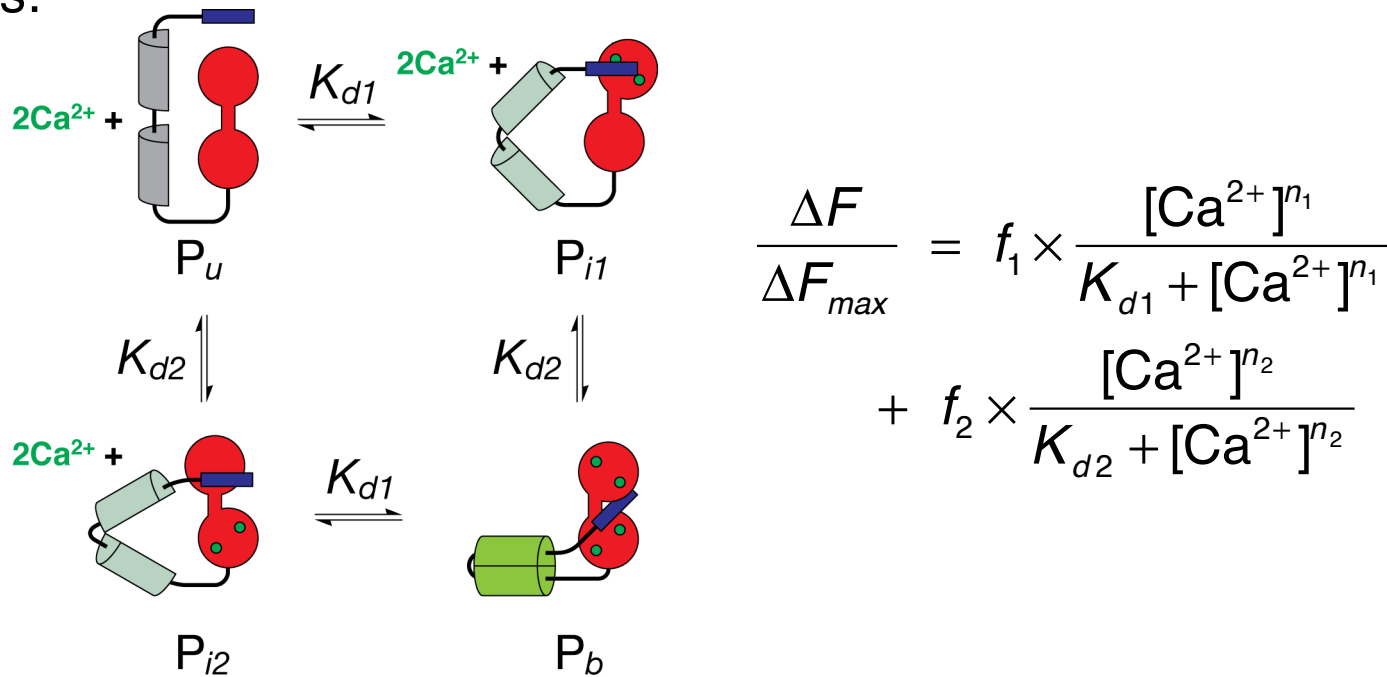


**pericam: greater slope (Hill coefficient)**  
**BAPTA: lesser slope**

The titration behavior we've been discussing for pericam is idealized, because we have been assuming **complete cooperativity** among the calcium binding sites (all four  $\text{Ca}^{2+}$  ions bind at once). In fact, binding to individual sites *can occur independently*, but each site's apparent affinity depends on whether the other sites are occupied.



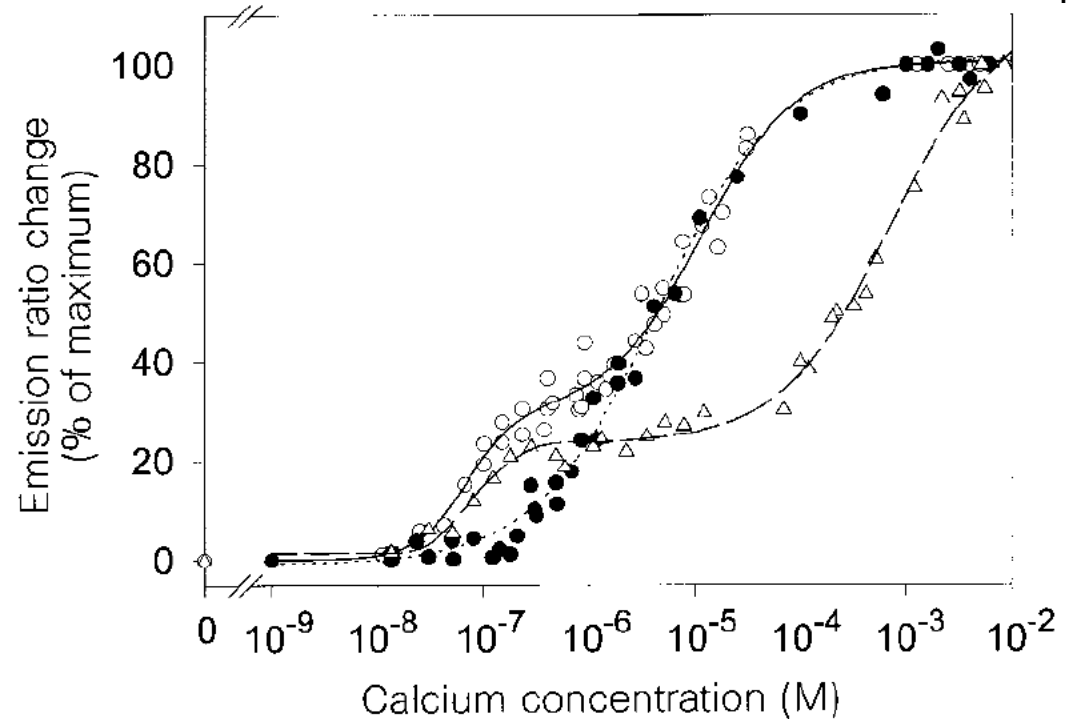
No titration curve provides enough detail (features) to accurately fit all the independent equilibrium constants in the “full picture.” One possibility is to settle for a compromise that fits data from CaM-based sensors: 13



$n_1$  and  $n_2$  are the Hill coefficients associated with independent pairwise calcium binding events, and  $f_1$  and  $f_2$  are the **fractional fluorescence changes** associated with these two steps.

For what *structural reasons* is modeling pericam with **two calcium-dependent transitions** particularly appropriate?

One- and two-step transition behavior of CaMeleons  
(Miyawaki *et al.*):



- your mutations in pericam may affect the apparent  $K_d$ s (or EC<sub>50</sub>s) and Hill coefficients for two transition steps
- the “resolution” of the two steps may be affected—note that the parent pericam appears to have a single transition, in part due to the E104Q mutation (*cf.* curve with black circles above)
- in some cases, your mutations may affect the relative fluorescence changes of the two transition steps

## Note on calcium concentrations

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How is a range of known calcium concentrations be produced?

- note that  $EC_{50}$ s are typically around  $1 \mu\text{M}$ ; the most useful calcium concentrations will be **near the  $EC_{50}$**
- you may need  $> 1 \mu\text{M}$  protein to make robust measurements
- need to make sure that we know the concentration of **unbound calcium**, as opposed to total calcium concentration

$$\frac{\Delta F}{\Delta F_{max}} = f_1 \times \frac{[\text{Ca}^{2+}]^{n_1}}{K_{d1} + [\text{Ca}^{2+}]^{n_1}} + f_2 \times \frac{[\text{Ca}^{2+}]^{n_2}}{K_{d2} + [\text{Ca}^{2+}]^{n_2}}$$

We will solve this problem by using a **calcium buffer**

- analogous to a pH buffer, with **pCa<sup>2+</sup>** determined by affinity of the buffer (in our case EGTA) and absolute amount of  $\text{Ca}^{2+}$  present

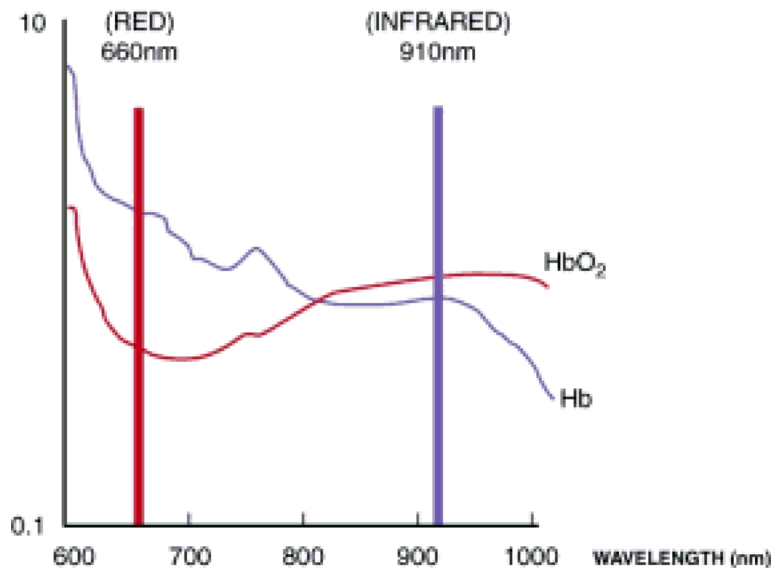
$$[\text{Ca}^{2+}] = \frac{[\text{Ca}^{2+} - \text{EGTA}]}{[\text{EGTA}] \cdot K_a}$$

- with  $10 \text{ mM}$   $\text{Ca}^{2+}$ -EGTA/EGTA mixtures, the amount of pericam present is unlikely to have much effect

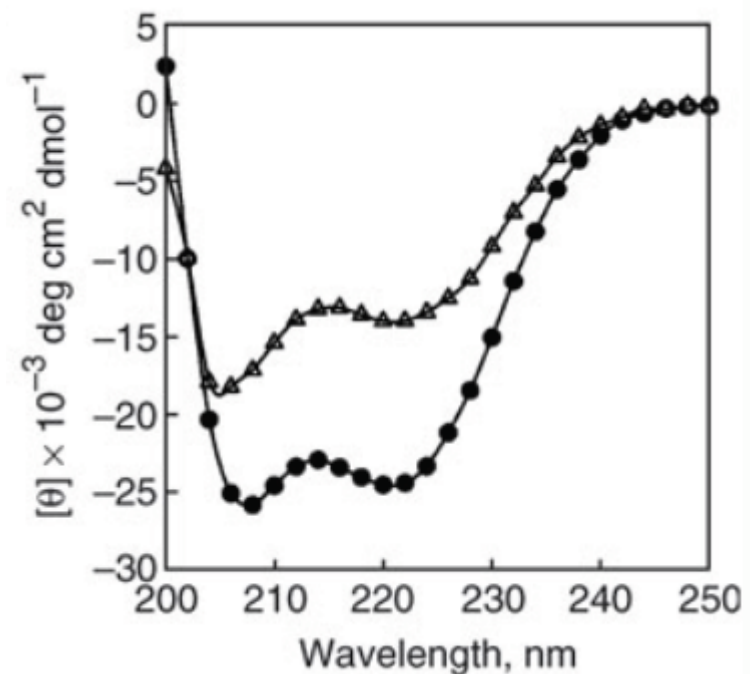
## Binding may be quantified using methods other than fluorescence

Other techniques for titration curve measurement:

- absorbance spectroscopy, *e.g.* O<sub>2</sub> binding to Hb
- circular dichroism, *e.g.* Ca<sup>2+</sup> binding to TrC fragments



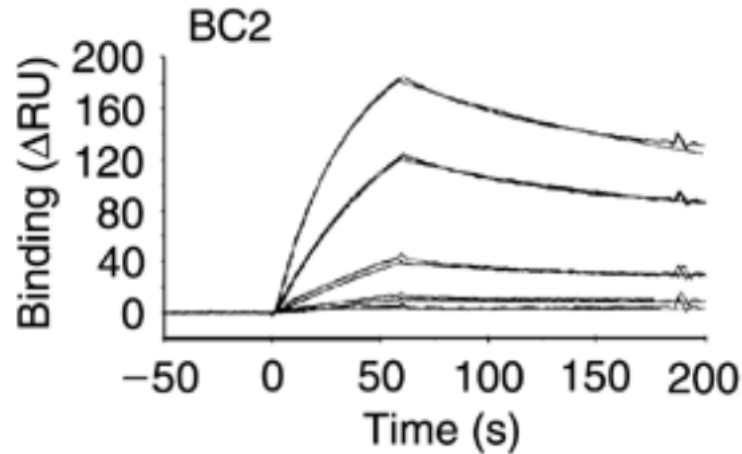
[www.oximetry.org/pulseox/principles.htm](http://www.oximetry.org/pulseox/principles.htm)



Greenfield (2007) *Nat. Protoc.* 1: 2733-41

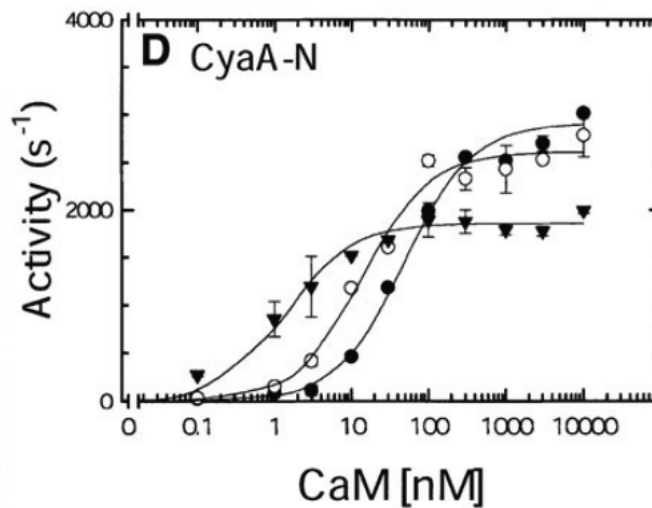


- surface plasmon resonance, *e.g.* antibody binding to a target



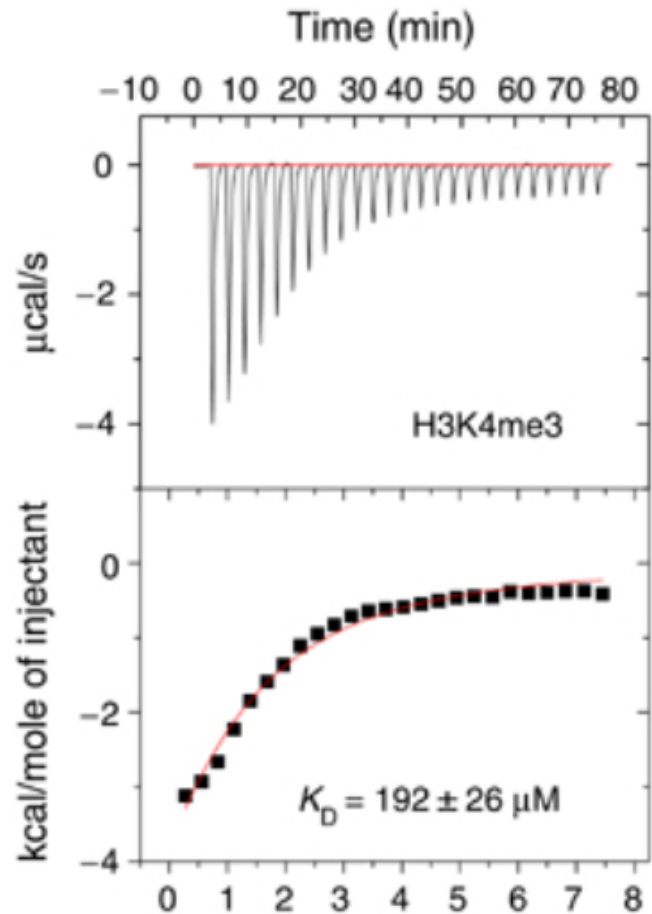
De Santis *et al.* (2003)  
*Br. J. Cancer* 88: 996-1003

- enzymatic activity, *e.g.* CaM-dependent enzyme activation



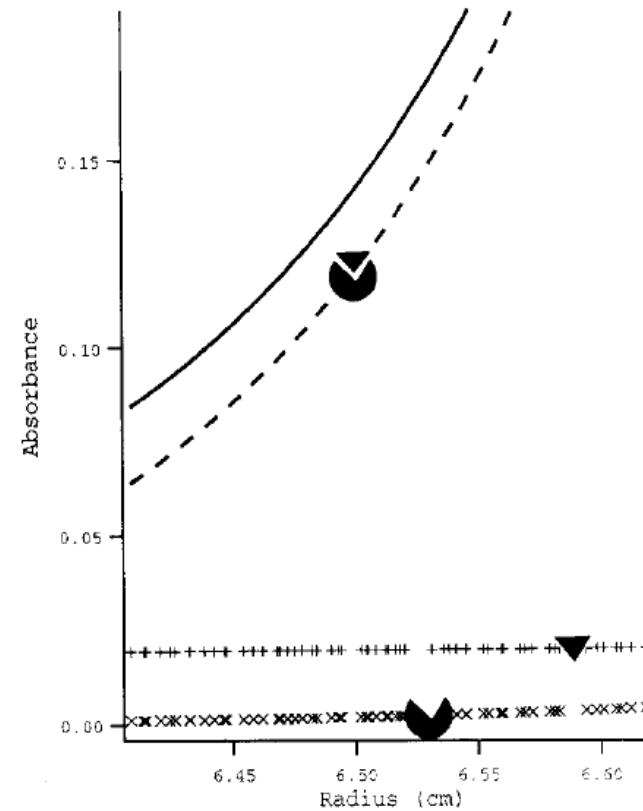
Shen *et al.* (2002)  
*EMBO J.* 21: 6721-32

- calorimetry, *e.g.* peptide-protein interaction



Schütz *et al.* (2006) *EMBO J.* 25: 4245-52

- analytical ultracentrifugation, *e.g.* ligand-protein binding



Arkin & Lear (2001) *Anal. Biochem.* 29: 98-107