

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

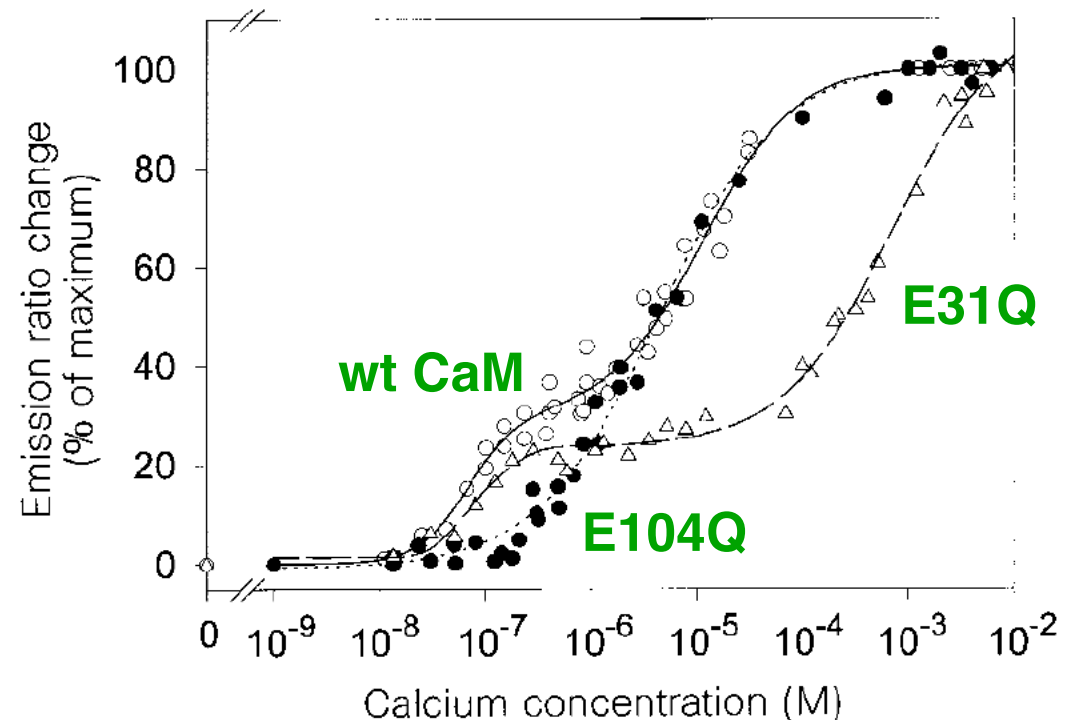
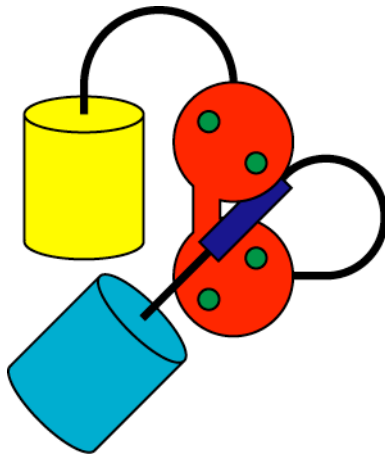
Lecture 7: Binding and affinity measurements

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

- II. Techniques for studying binding

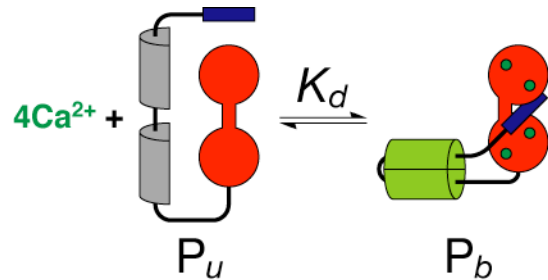
Q: Our design task is to alter calcium sensitivity of pericam derivatives. We chose mutants to make, but **how will we determine the results** of our perturbations?

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.



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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 Θ . Θ can be expressed as a function of calcium concentration as follows:

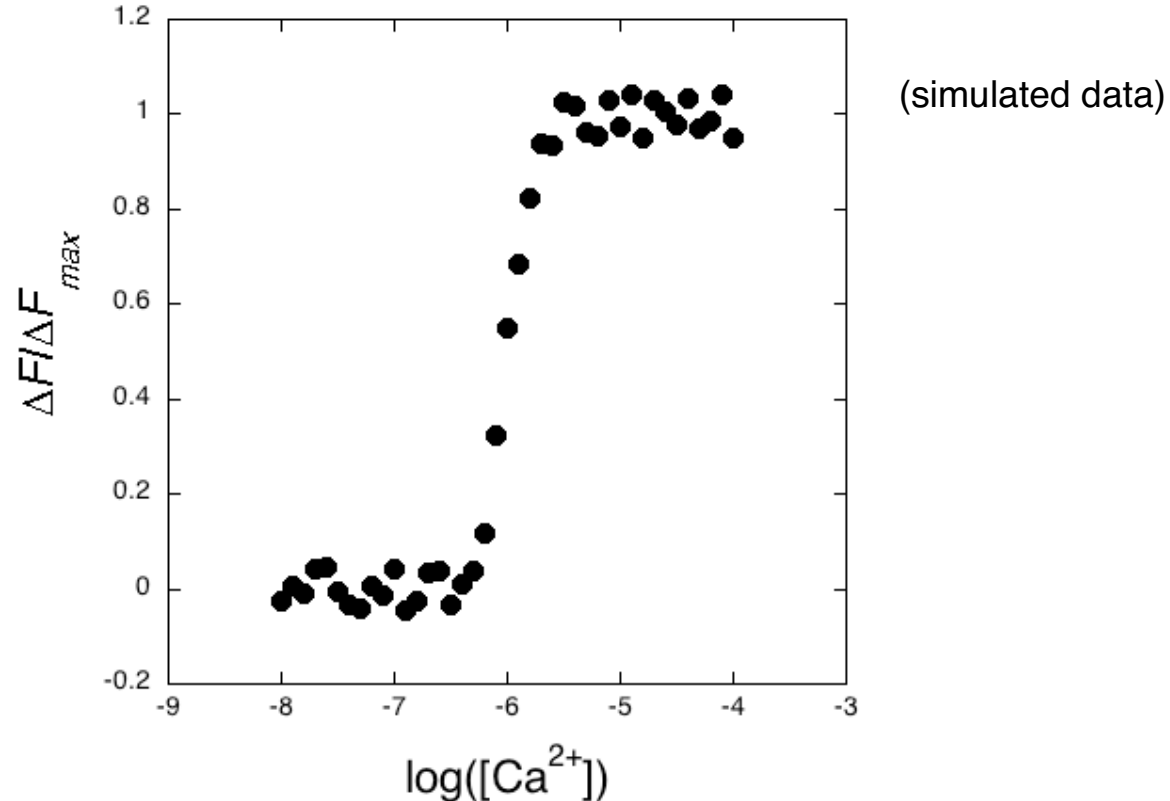
$$\begin{aligned}\Theta &= \frac{[P_b]}{[P]_{tot}} = \frac{[P_b]}{[P_b]K_d/[Ca^{2+}]^4 + [P_b]} \\ &= \frac{[Ca^{2+}]^4}{K_d + [Ca^{2+}]^4} \\ &= \frac{[Ca^{2+}]^4}{(EC_{50})^4 + [Ca^{2+}]^4}\end{aligned}$$

$EC_{50} = (K_d)^{1/4}$ 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.

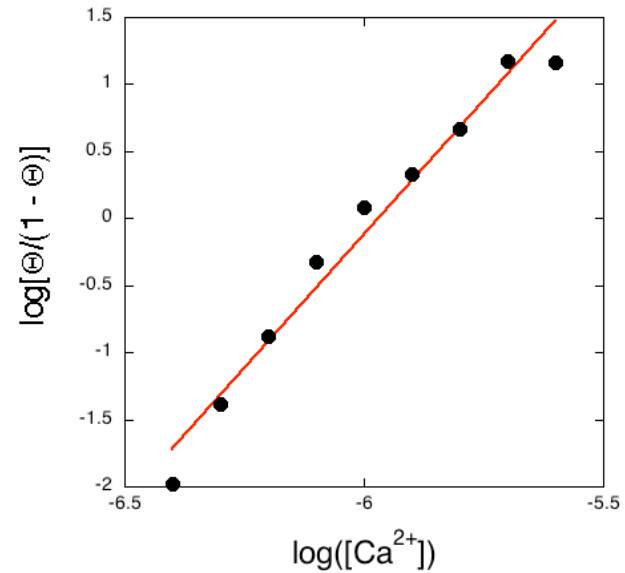
If we had fluorescence data over a range of calcium concentrations, and assuming these equations accurately describe calcium binding to pericam, we could determine the EC_{50} and K_d using a variety of methods:

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 $\log(EC_{50})$

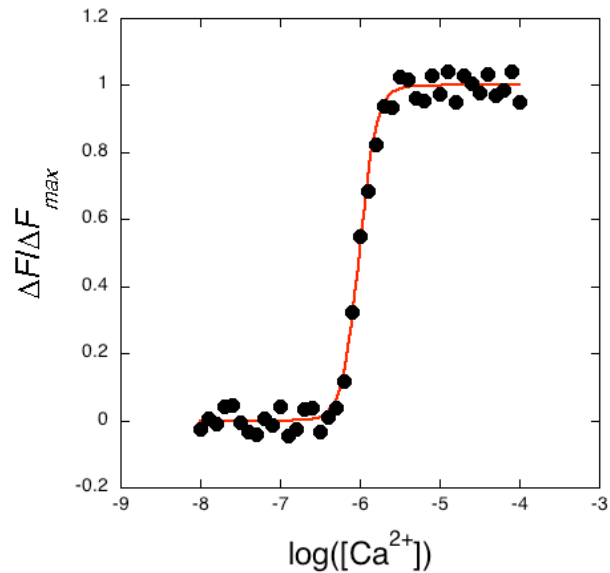


3. Curve fitting

- consider the equation we derived:

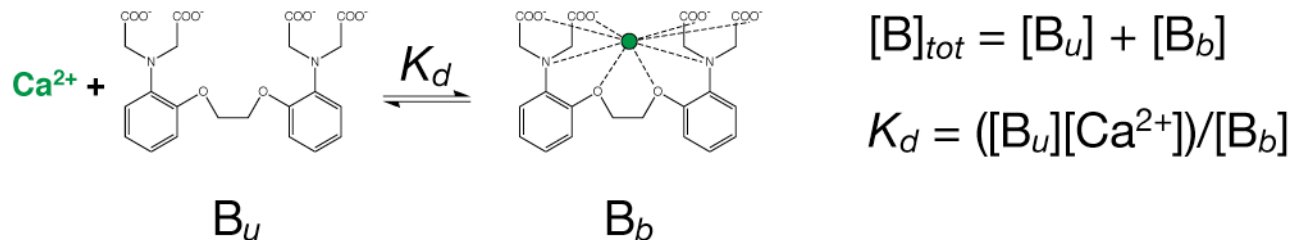
$$\frac{\Delta F}{\Delta F_{max}} = \frac{[Ca^{2+}]^4}{(EC_{50})^4 + [Ca^{2+}]^4}$$

- 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.

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:

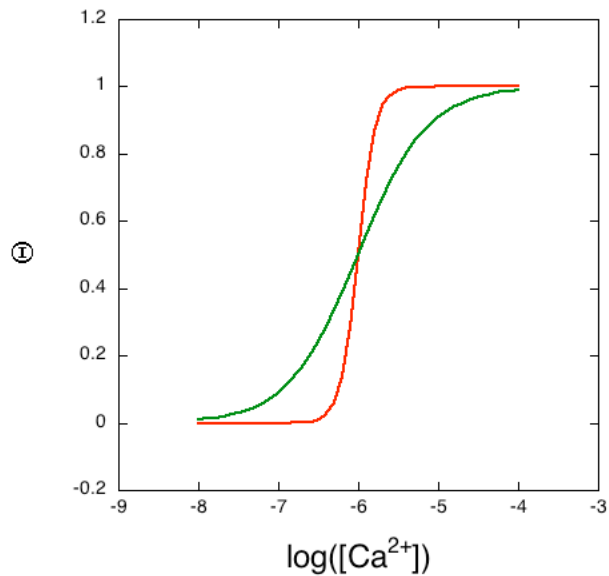
$$\Theta = \frac{[B_b]}{[B]_{tot}} = \frac{[B_b]}{[B_b]K_d/[Ca^{2+}] + [B_b]}$$

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

Hill coeff. = 1

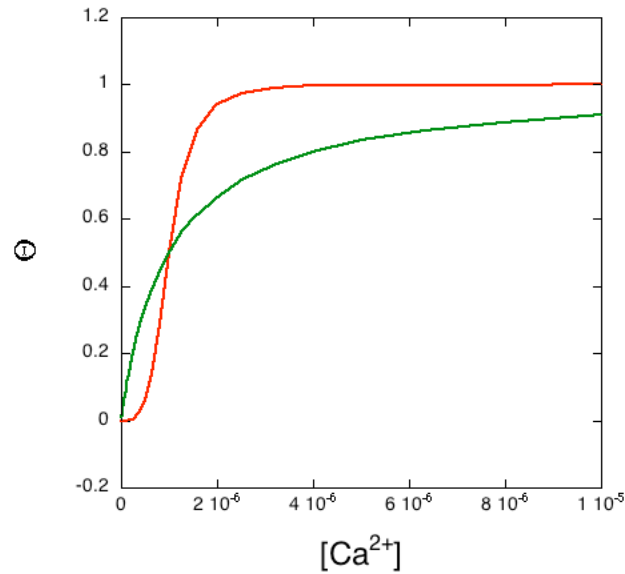
Simulated binding curves for **BAPTA** and **pericam** compare as follows:

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



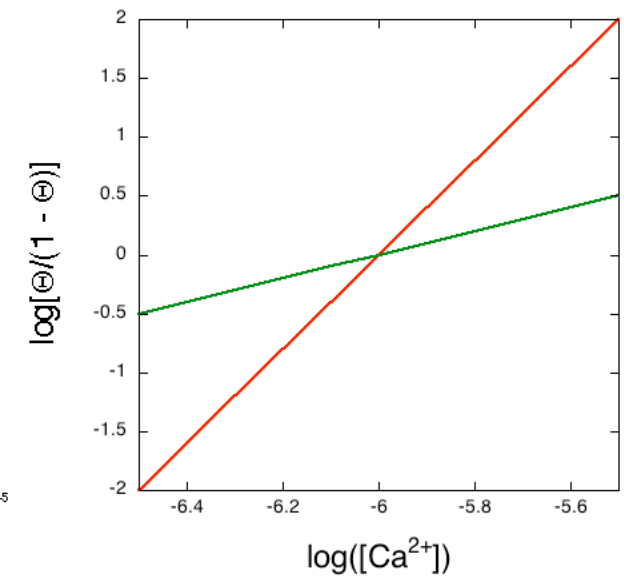
pericam: sharper transition
BAPTA: broader transition

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



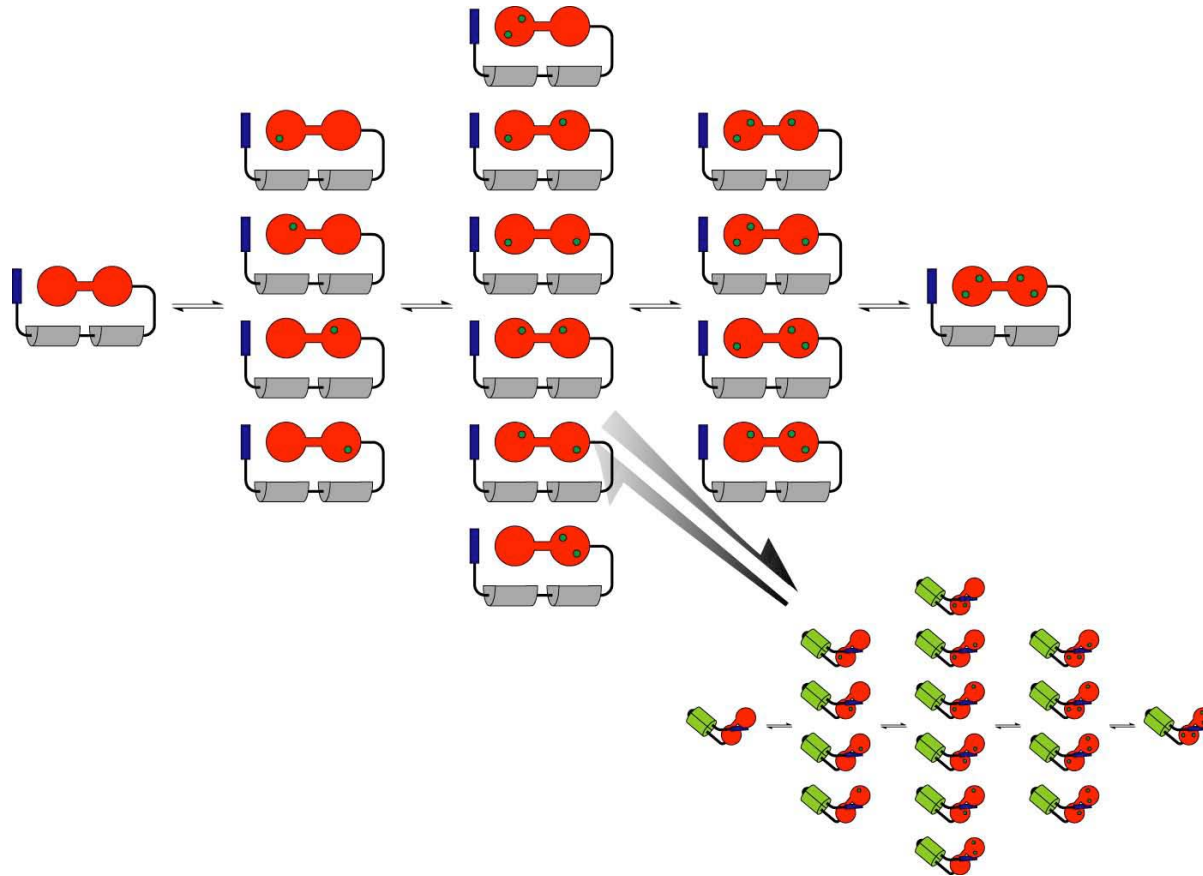
pericam: sigmoidal transition
BAPTA: smooth transition

Hill plot

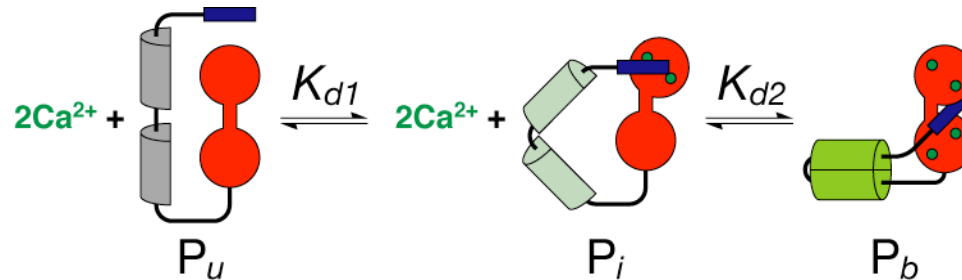


pericam: greater slope (Hill coefficient)
BAPTA: lesser slope

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 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:

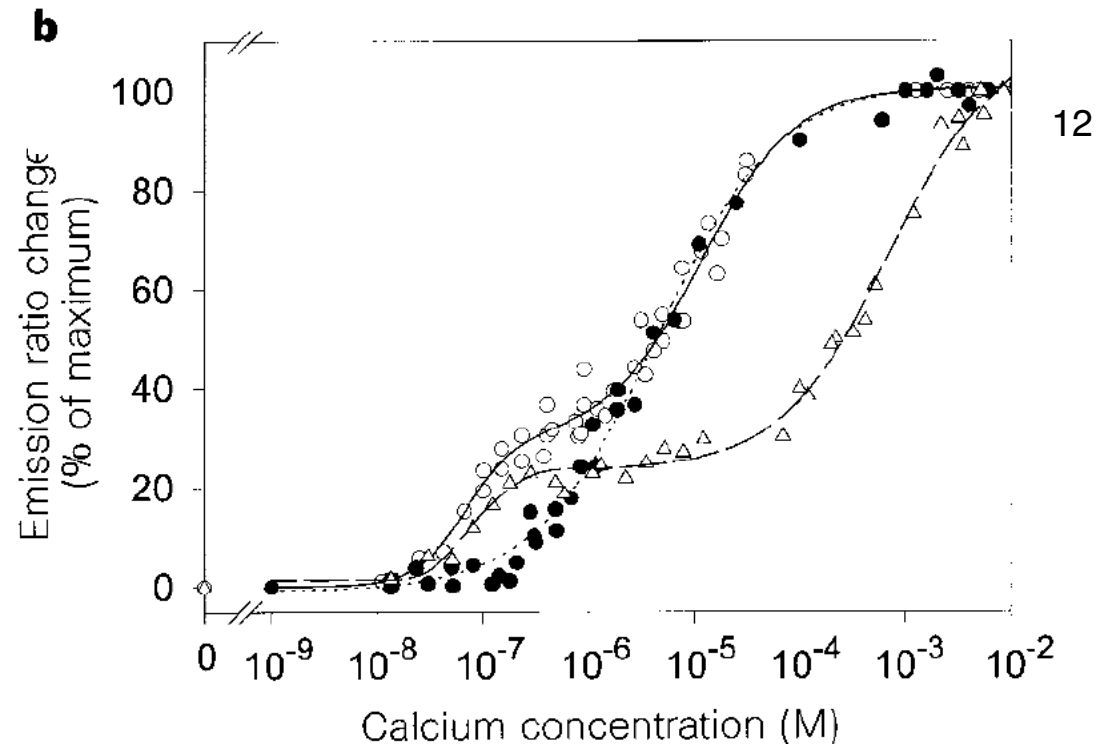


$$\frac{\Delta F}{\Delta F_{max}} = f_1 \times \frac{[Ca^{2+}]^{n_1}}{K_{d1} + [Ca^{2+}]^{n_1}} + f_2 \times \frac{[Ca^{2+}]^{n_2}}{K_{d2} + [Ca^{2+}]^{n_2}}$$

n_1 and n_2 are the Hill coefficients associated with the transitions from P_u to P_i and from P_i to P_b , respectively. f_1 and f_2 are the fractional fluorescence changes associated with these two steps.

Why is modeling pericam with **two calcium-dependent** transitions particularly appropriate?

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



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- your mutations in pericam may affect the apparent K_d s (or EC_{50} 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 $p\text{Ca}^{2+}$ determined by affinity of the buffer (in our case EGTA) and absolute amount of Ca^{2+} present

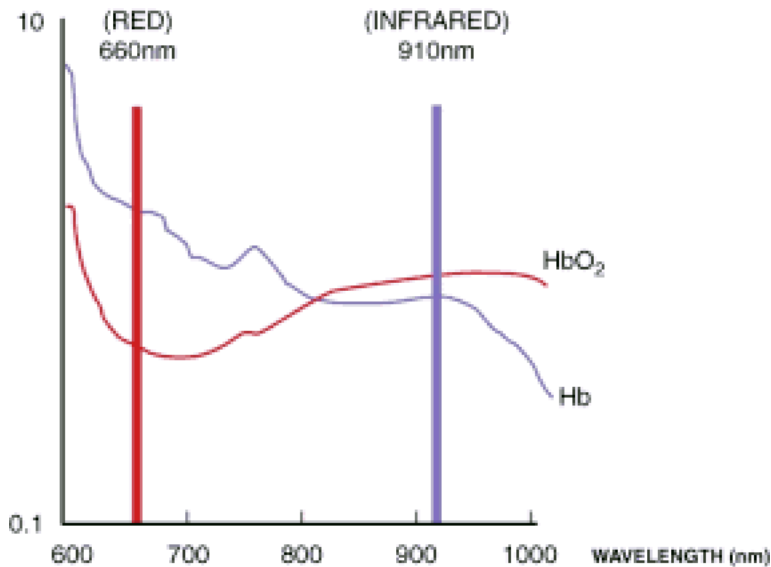
$$[\text{Ca}^{2+}] = \frac{[\text{Ca}^{2+} - \text{EGTA}]}{[\text{EGTA}] \cdot K_a}$$

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

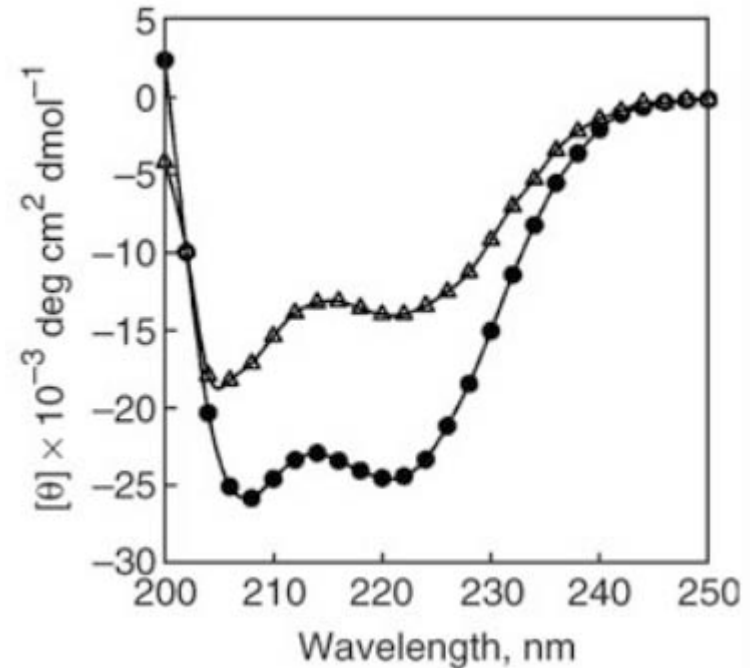
Binding may be quantified using methods other than fluorescence

Other techniques for titration curve measurement:

- absorbance spectroscopy
e.g. O₂ binding to Hb,



- circular dichroism, e.g. Ca²⁺ binding to TrC fragments

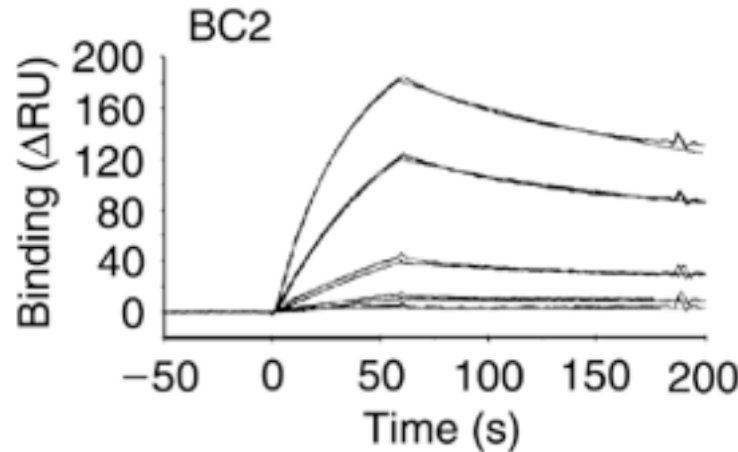


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Greenfield (2007) *Nat. Protoc.* 1: 2733-41

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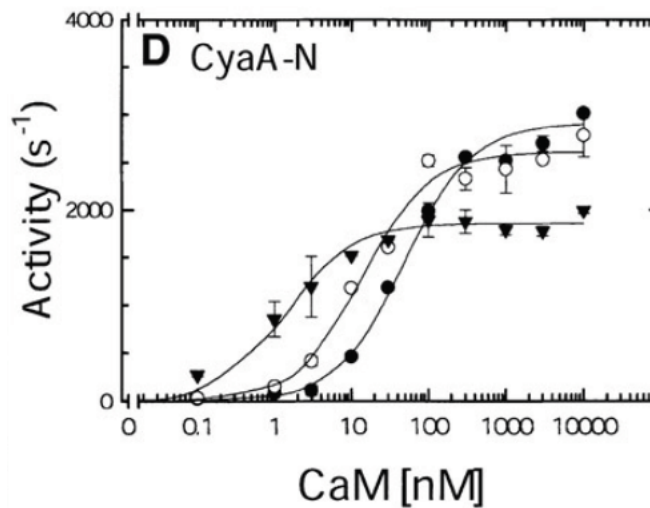
- surface plasmon resonance, *e.g.* antibody binding to a target



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

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 Source: De Santis, R., et al. "Novel Antitenascin Antibody with Increased Tumour Localisation for Pretargeted Antibody-Guided RadiolimmunoTherapy (PAGRIT)." *British Journal of Cancer* 88 (2003): 996-1003. © 2003.

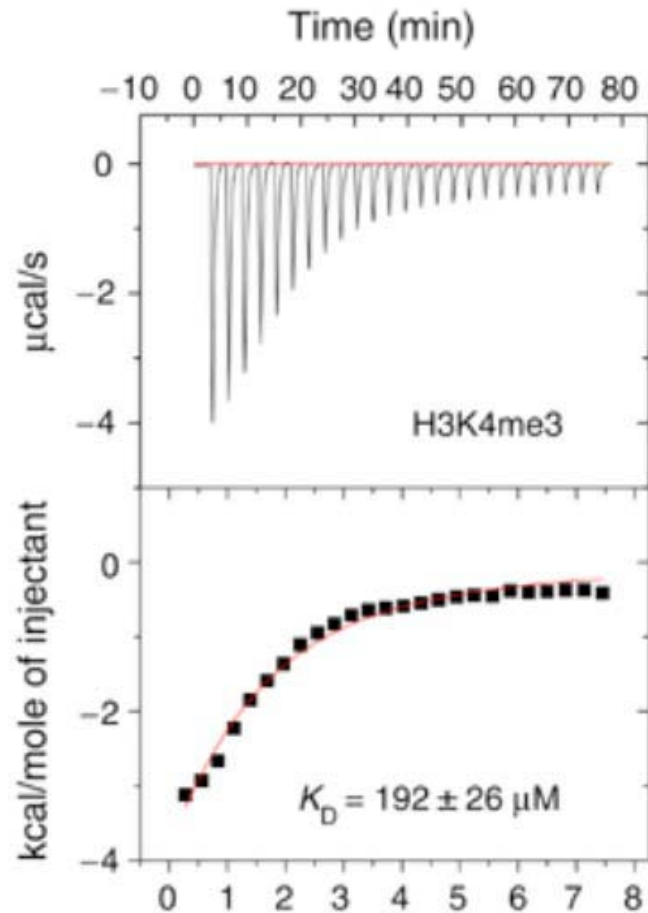
- enzymatic activity, *e.g.* CaM-dependent enzyme activation



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

Reprinted by permission from Macmillan Publishers Ltd: The EMBO Journal. Source: Shen, Y., et al. "Physiological Calcium Concentrations Regulate Calmodulin Binding and Catalysis of Adenylyl Cyclase Exotoxins." *EMBO J* 21 (2002): 6721-6732. © 2002

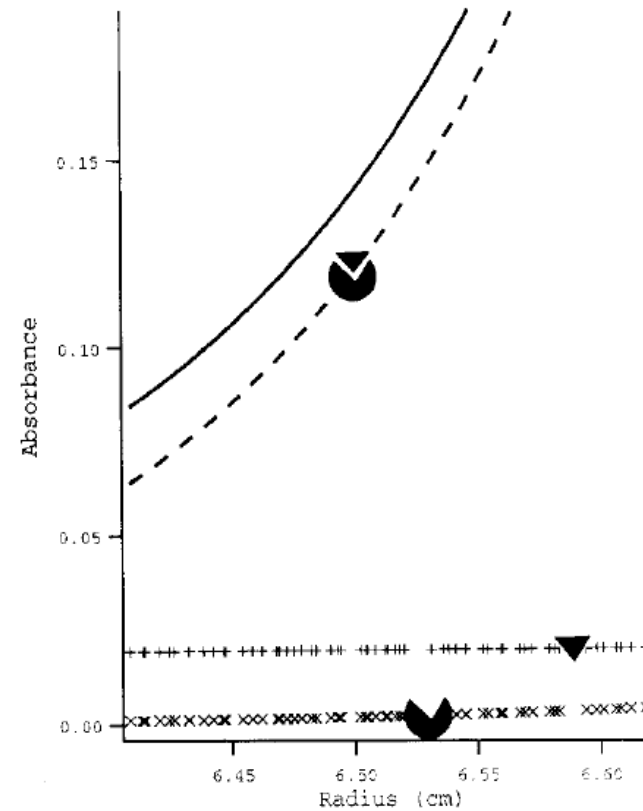
- calorimetry, *e.g.* peptide-protein interaction



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

Reprinted by permission from Macmillan Publishers Ltd: The EMBO Journal. "Structural Basis for Molecular Recognition and Presentation of Histone H3 By WDR5." *EMBO J* 25 (2006): 4245-4252. © 2006

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



Arkin & Lear (2001) *Anal. Biochem.* 299: 98-107

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