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
SPRING BREAK	
4. Protein expression	4. Prepare expression system
5. Purification and protein analysis	5. Induce protein
6. Binding & affinity measurements	6. Characterize expression
7. High throughput engineering	7. Assess protein function

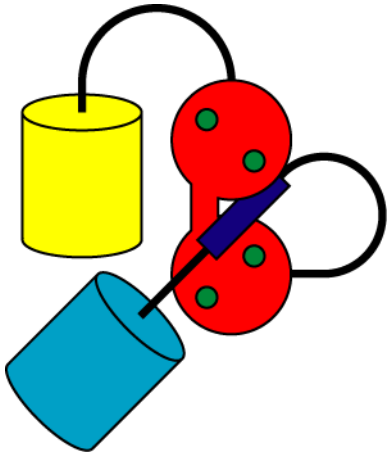
http://www.youtube.com/watch?v=SHX0pv8_JOE

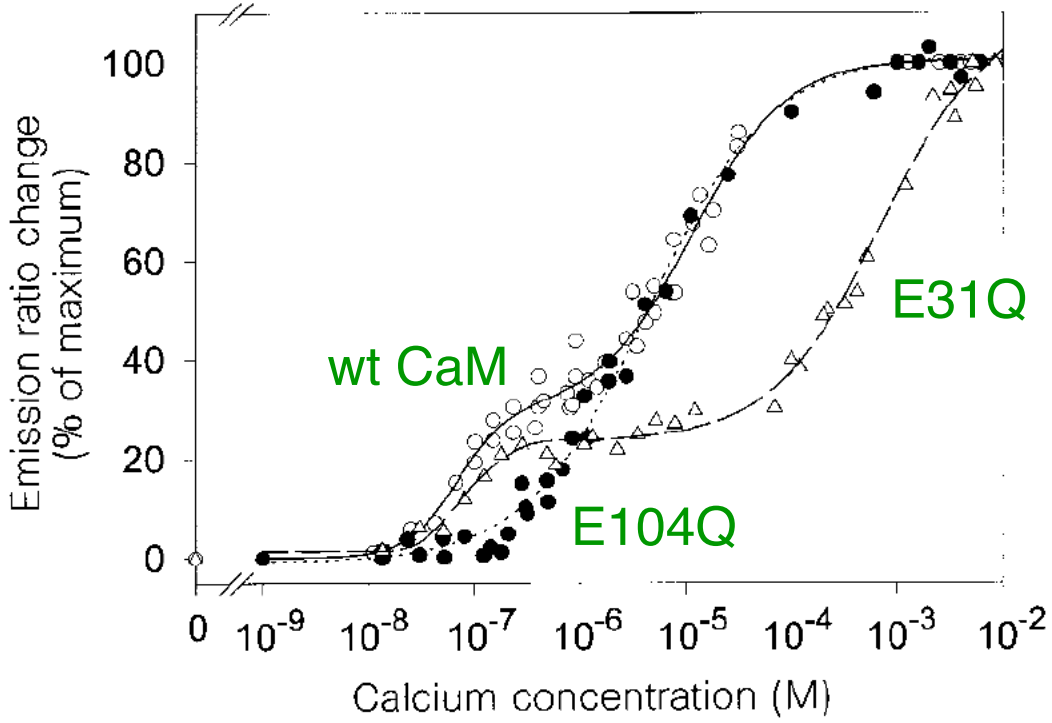
Lecture 6: Binding and affinity measurements

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

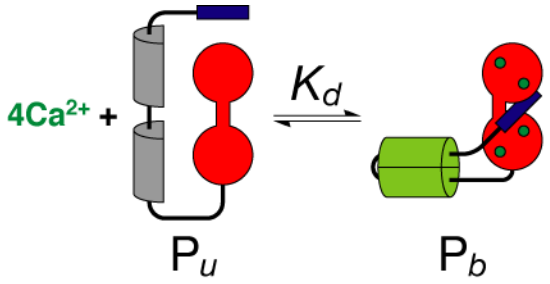
II. Techniques for studying binding

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?





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:

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

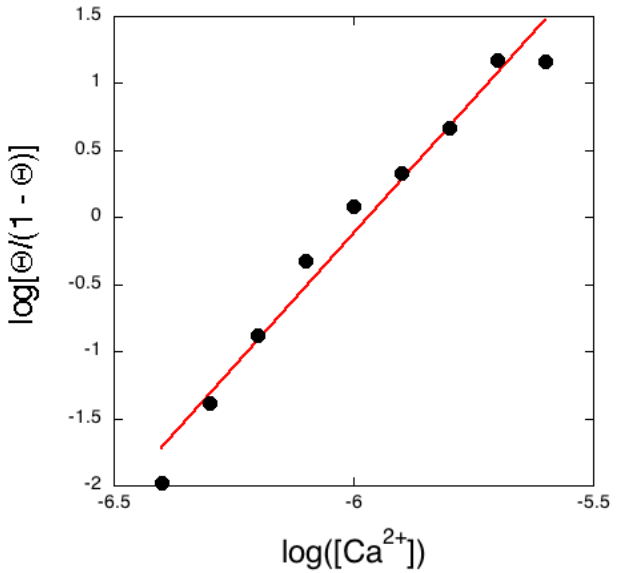
note:
$[P_u] = [P_b]K_d/[Ca^{2+}]^4$

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

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 $\log(EC_{50})$

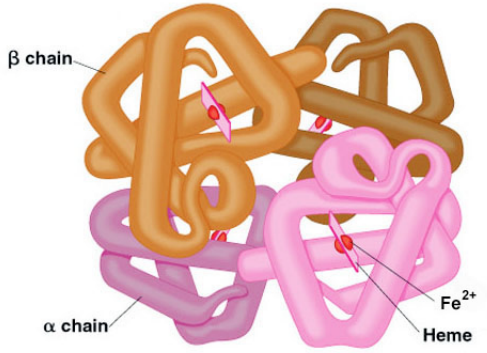


Hill coefficient reflects cooperativity, a phenomenon of binding to multiple binding sites on an individual target: positive cooperativity means that binding to one site promotes binding to other sites; negative cooperativity means that binding to one site depresses binding to other sites.

Literary description of positive cooperativity:

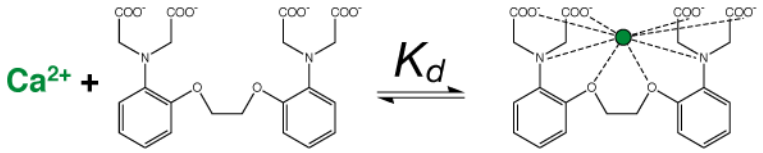
"For whosoever hath, to him shall be given, and he shall have more abundance: but whosoever hath not, from him shall be taken away even that he hath."

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:



B_u B_b

$$[B]_{tot} = [B_u] + [B_b]$$

$$K_d = ([B_u][Ca^{2+}])/[B_b]$$

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

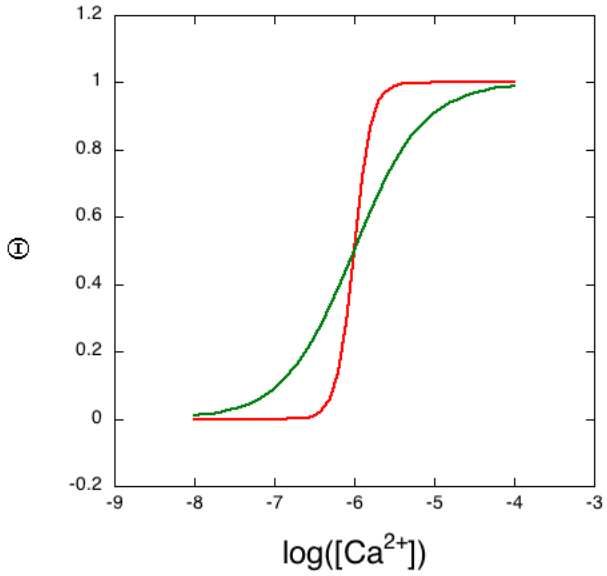
$$\Theta = \frac{[B_b]}{[B]_{tot}} = \frac{[B_b]}{[B_b]K_d/[Ca^{2+}] + [B_b]}$$

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

Hill coeff. = 1

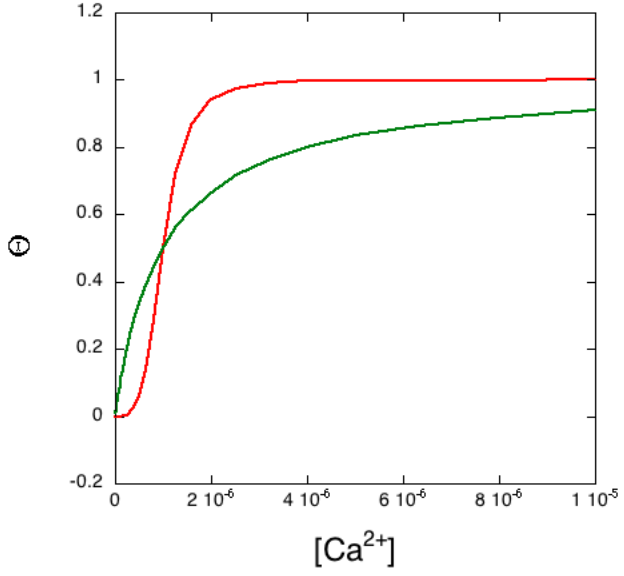
Simulated binding curves for BAPTA and pericam compare as follows:

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



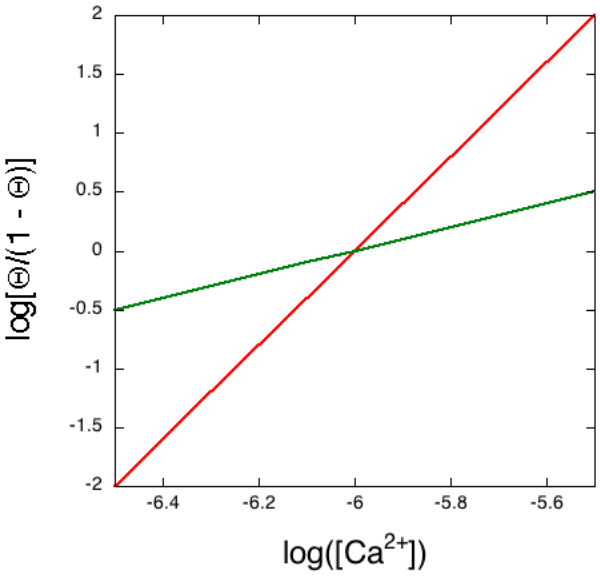
pericam: sharper transition
BAPTA: broader transition

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



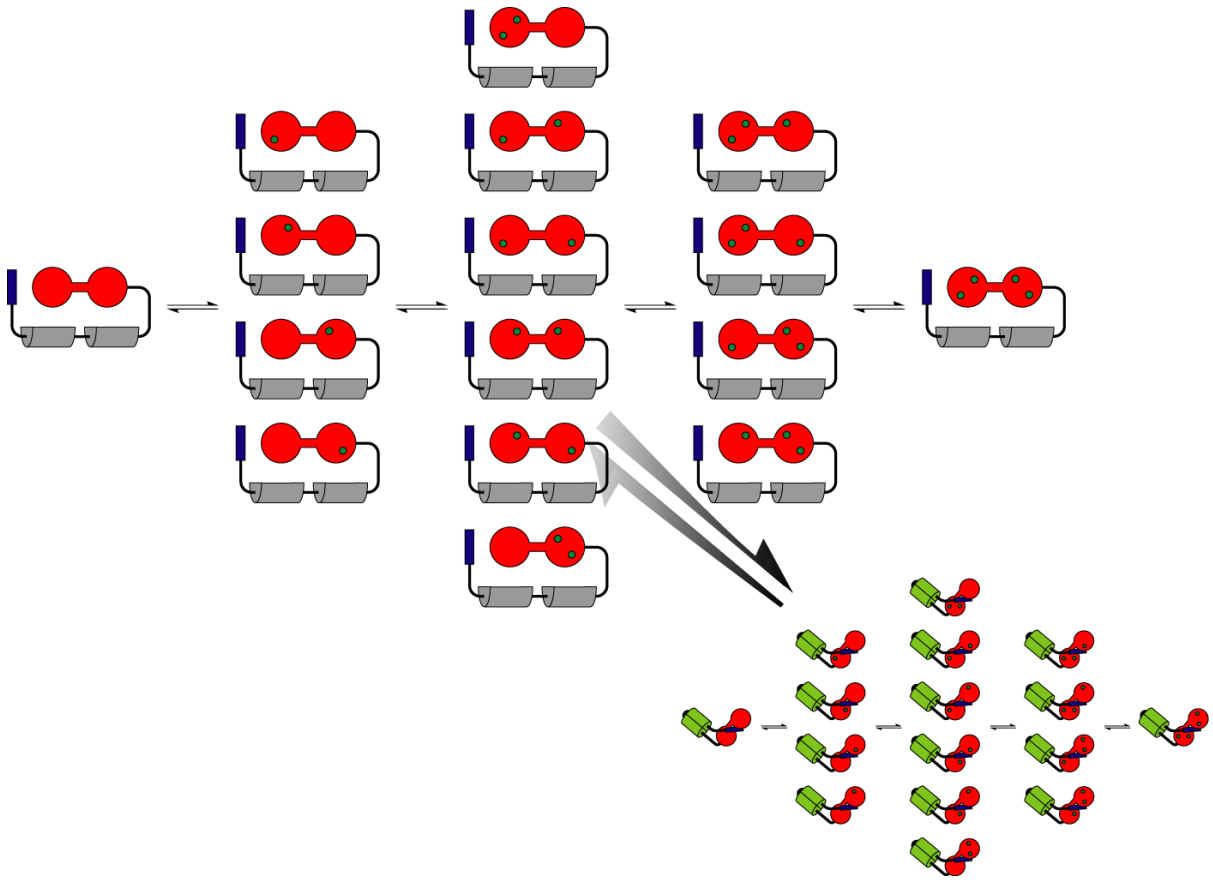
pericam: sigmoidal transition
BAPTA: smooth transition

Hill plot

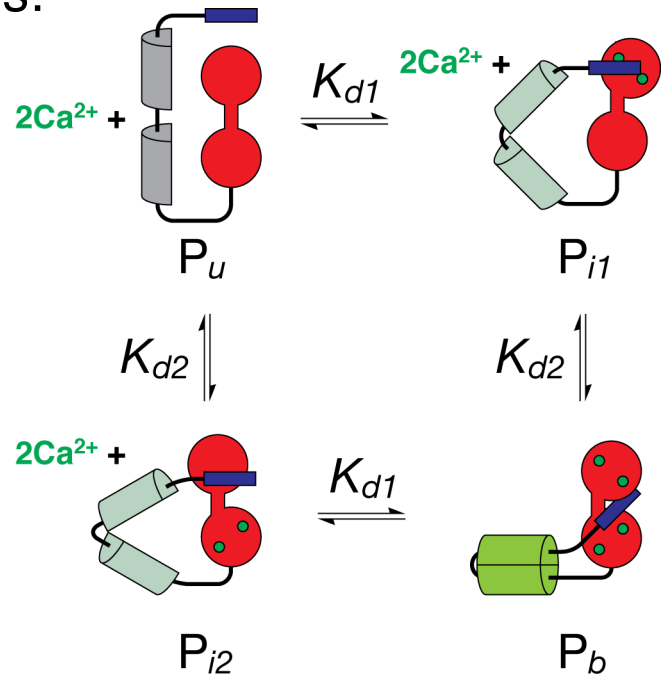


pericam: greater slope (Hill coefficient)
BAPTA: lesser slope

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:

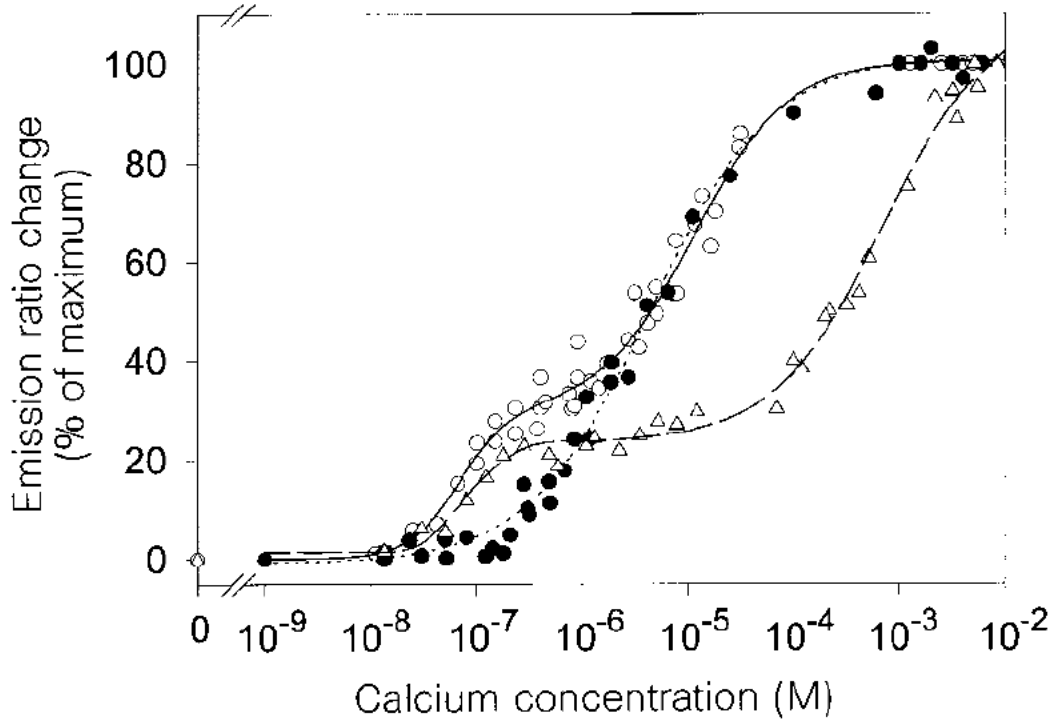


$$\frac{\Delta F}{\Delta F_{max}} = f_1 \times \frac{[Ca^{2+}]^{n_1}}{K_{d1} + [Ca^{2+}]^{n_1}} + f_2 \times \frac{[Ca^{2+}]^{n_2}}{K_{d2} + [Ca^{2+}]^{n_2}}$$

n_1 and n_2 are the Hill coefficients associated with 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_ds (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

How is a range of known calcium concentrations be produced?

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

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

We will solve this problem by using a calcium buffer

- analogous to a pH buffer, with pCa^{2+} determined by affinity of the buffer (in our case EGTA) and absolute amount of Ca^{2+} present

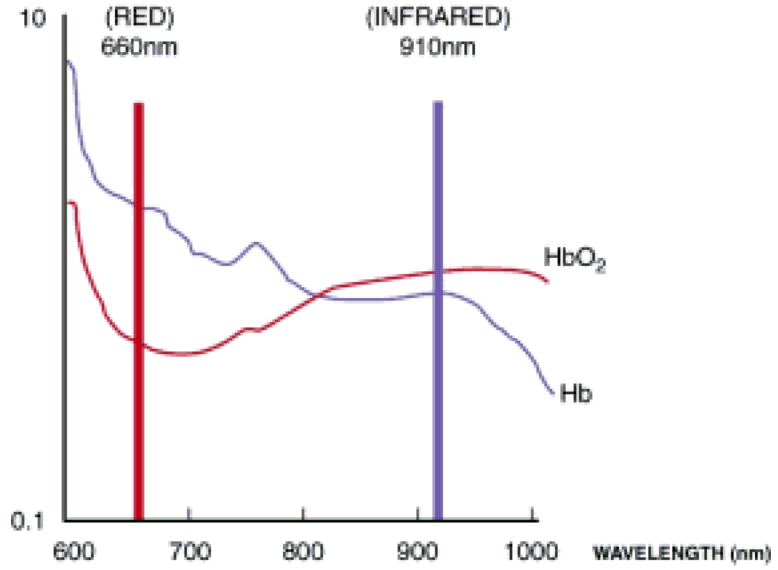
$$[Ca^{2+}] = \frac{[Ca^{2+} - EGTA]}{[EGTA] \cdot K_a}$$

- 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

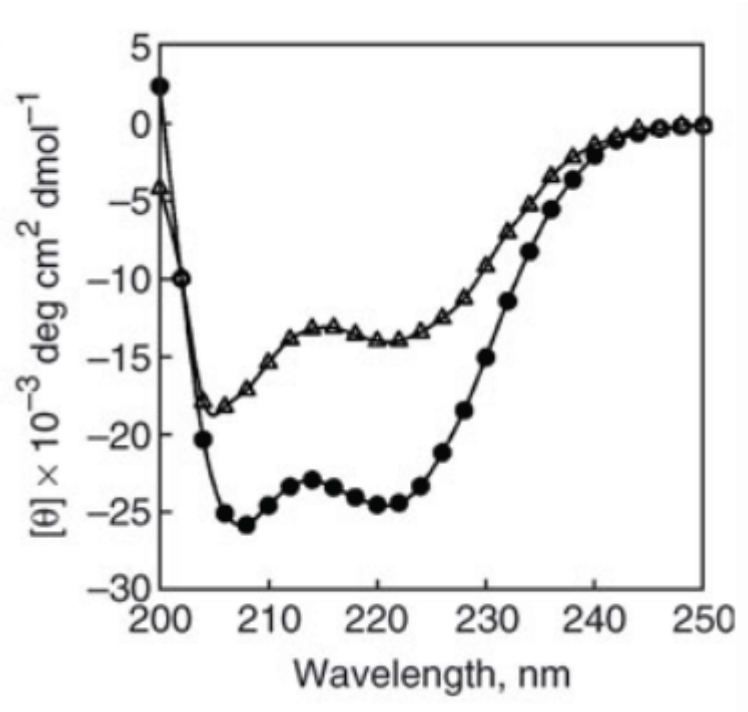
Other techniques for titration curve measurement:

- absorbance spectroscopy, *e.g.* O_2 binding to Hb



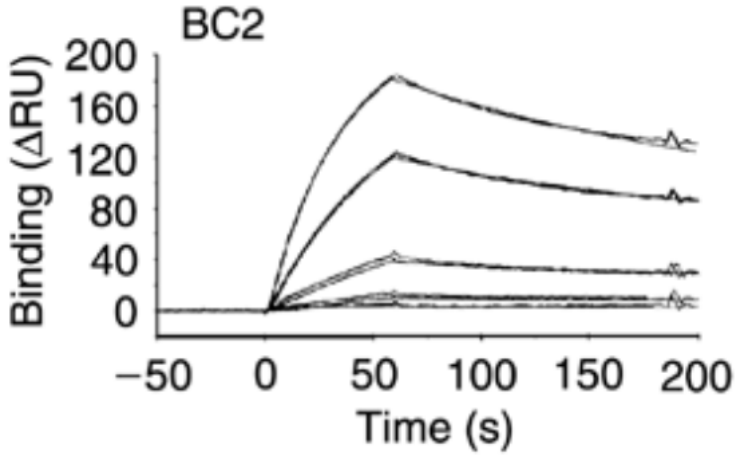
www.oximetry.org/pulseox/principles.htm

- circular dichroism, *e.g.* Ca^{2+} binding to TrC fragments



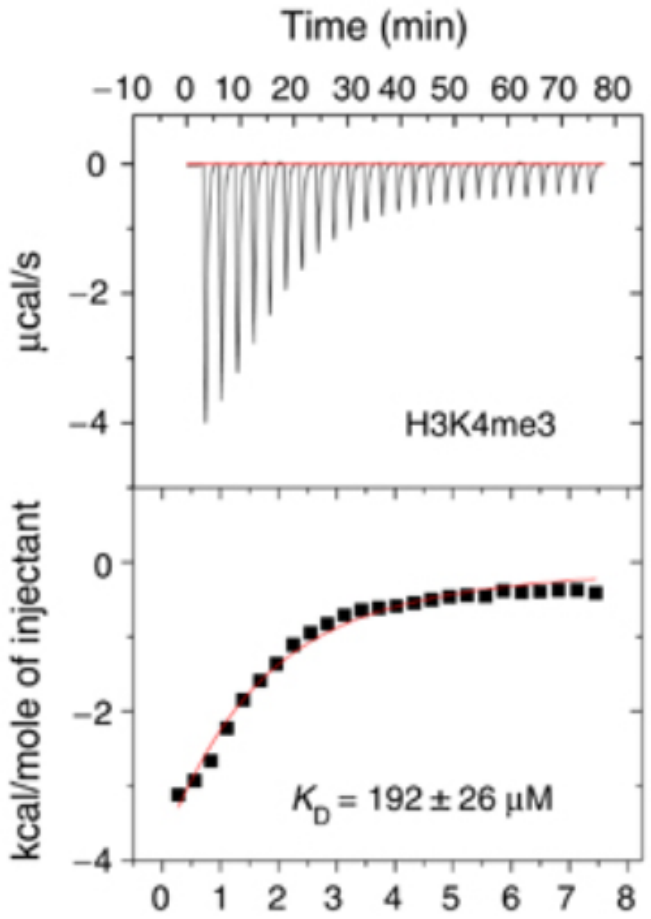
Greenfield (2007) *Nat. Protoc. 1:* 2733-41

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



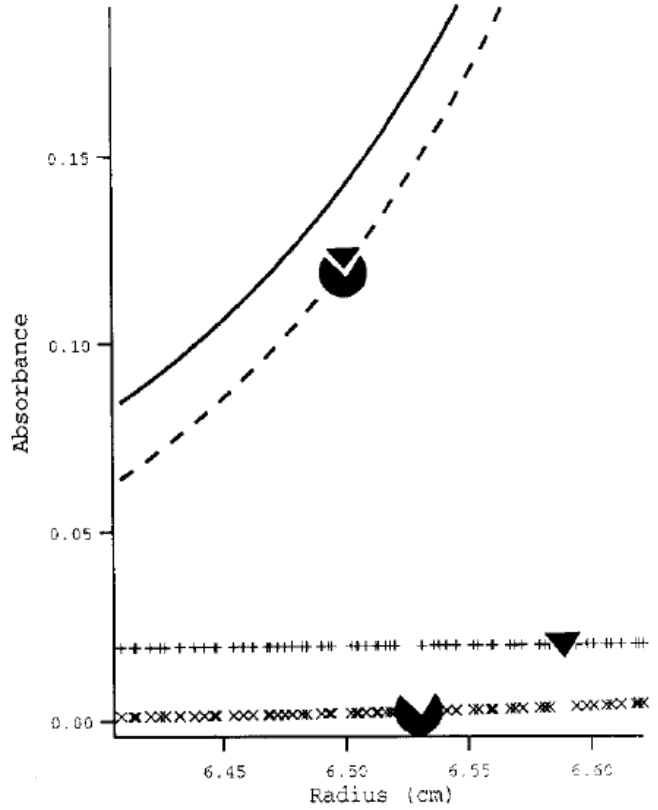
De Santis *et al.* (2003)
Br. J. Cancer 88: 996-1003

• 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