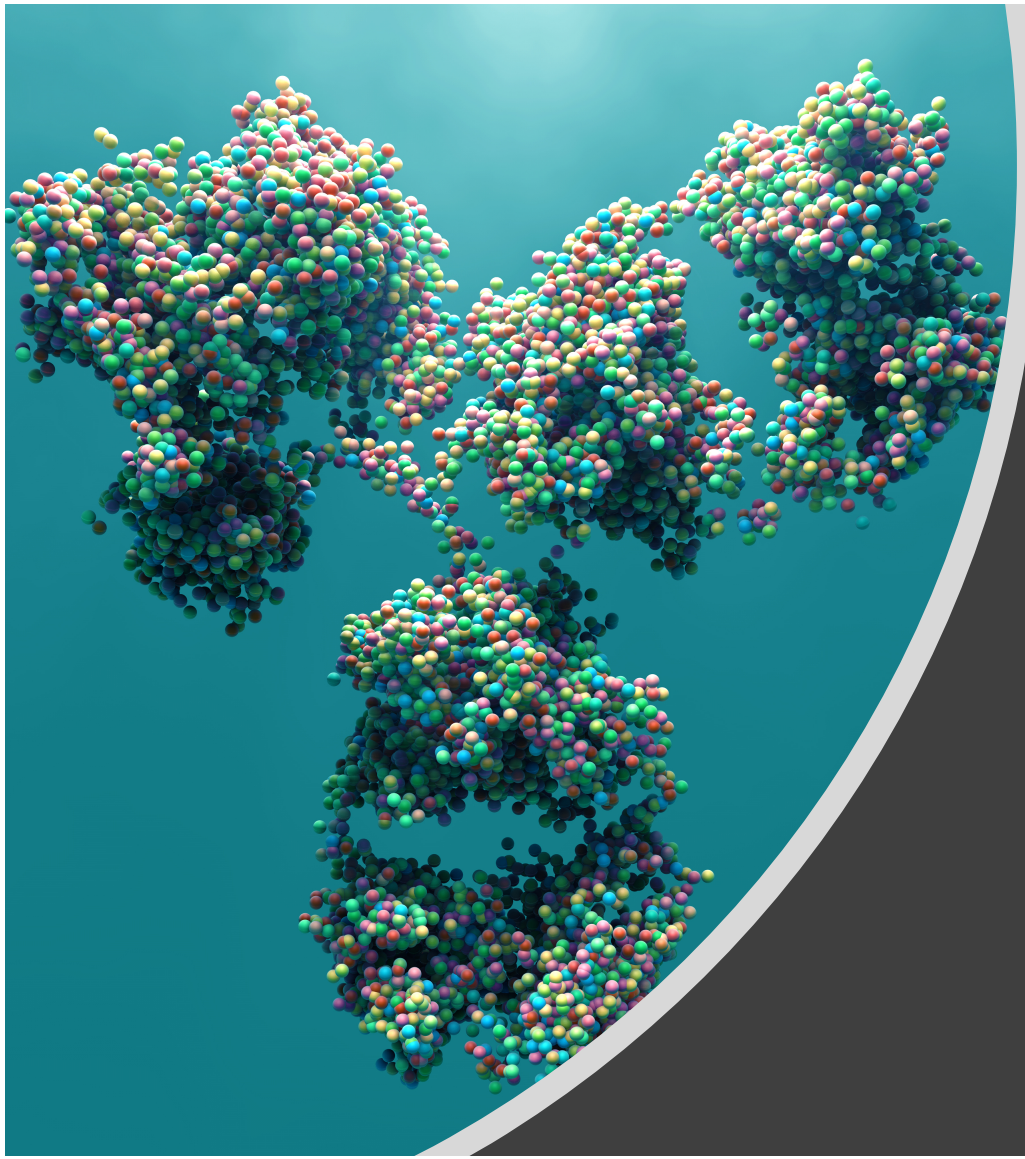
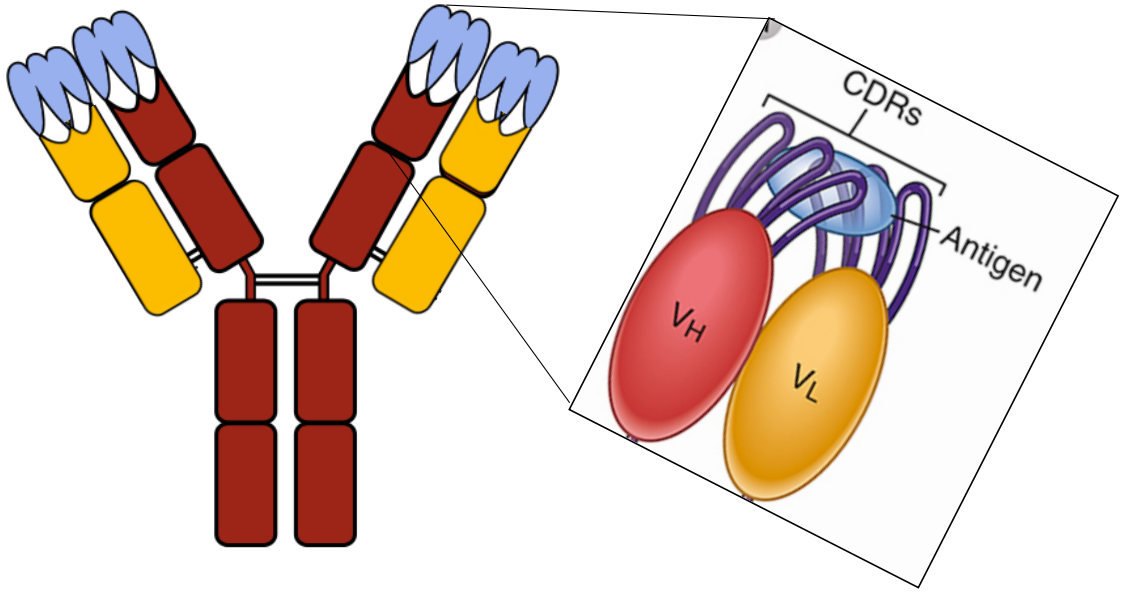


The antigen-antibody interaction

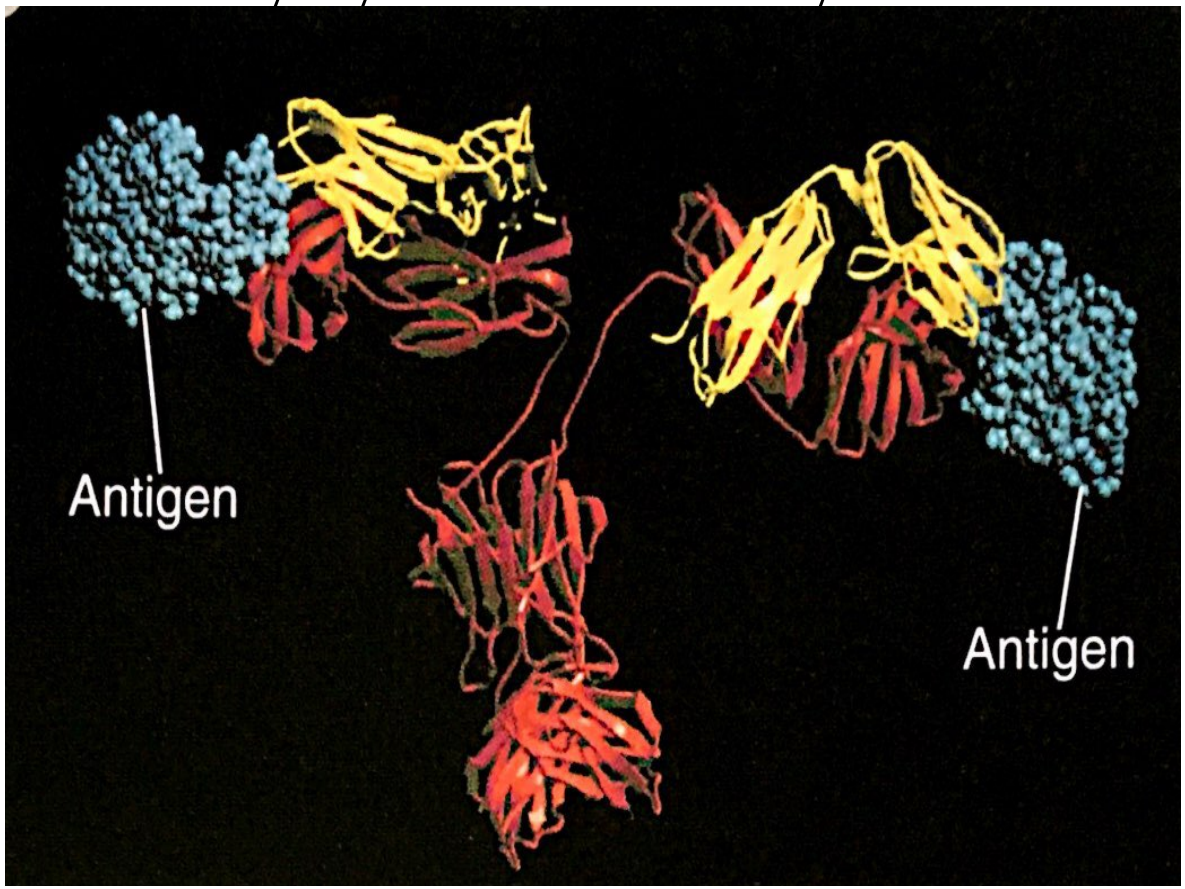
Characterization of scFvs that bind lysozyme



- The goal of this screen is to find a scFv clone with improved binding to lysozyme
- Antibody with a lower K_d for its antigen means a more stable interaction and a higher affinity
- We sorted a library of scFv yeast that bind to lysozyme
- Today will determine the dissociation constant of a single clone scFv with lysozyme

CDRs generate antigen binding site specificity

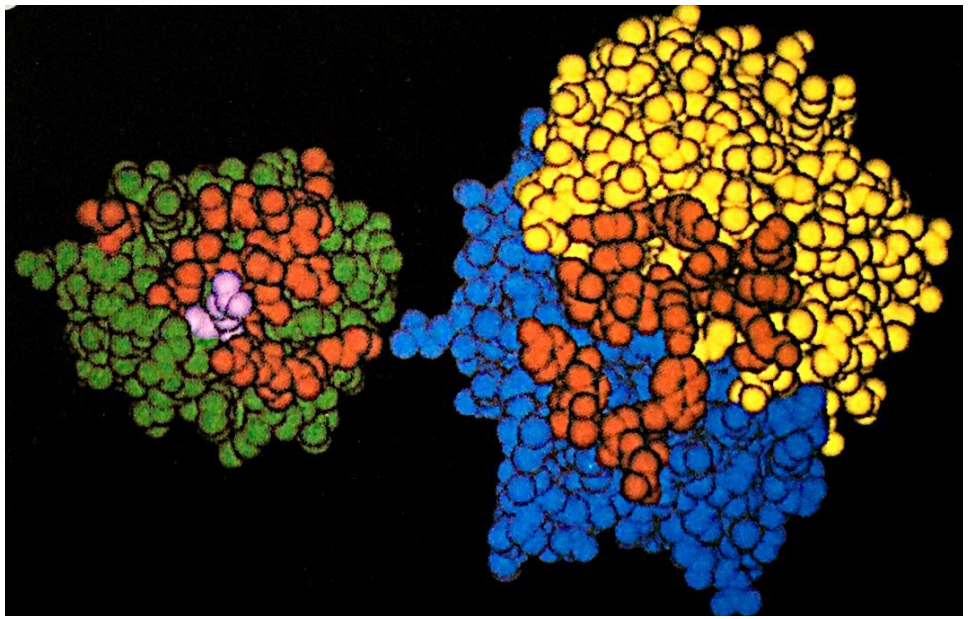
Lysozyme bound to antibody



- Specificity, degree to which an antibody differentiates between different antigens
- Finger-like CDRs usually recognize 15-22 amino acids
- Basic antibody structure maintained (β strands) when variability confined to CDR loops

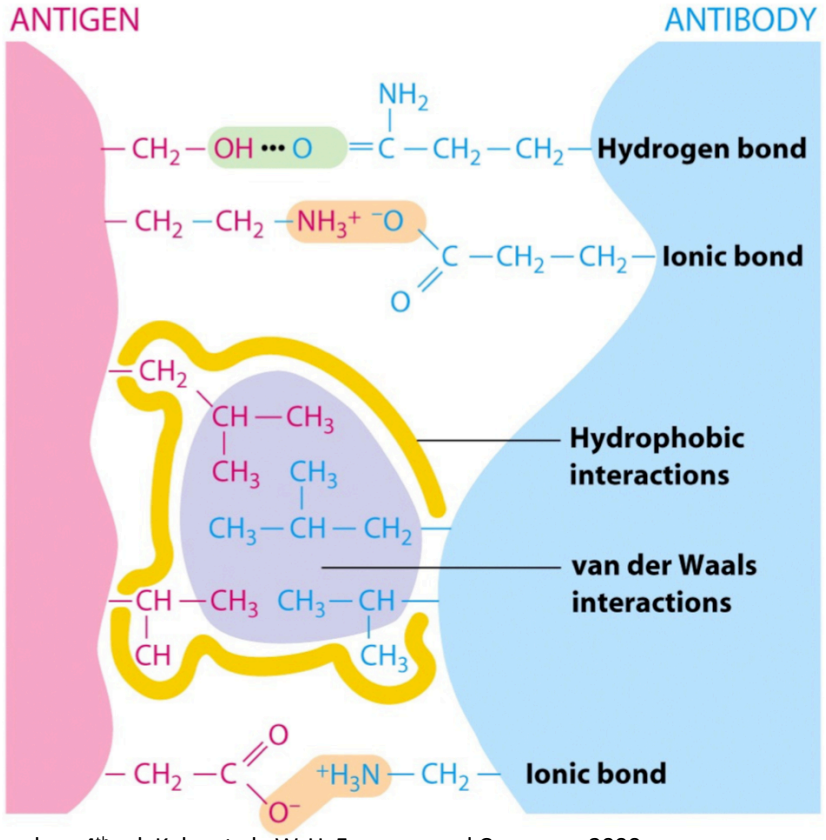
The Antigen - Antibody interaction forms multiple contacts

3D: Lysozyme bound to variable region



- Green: lysozyme
- Blue/Yellow: V_L and V_H
- Red amino acids that interact
- Pink critical glutamine reside fits into cleft of CDR

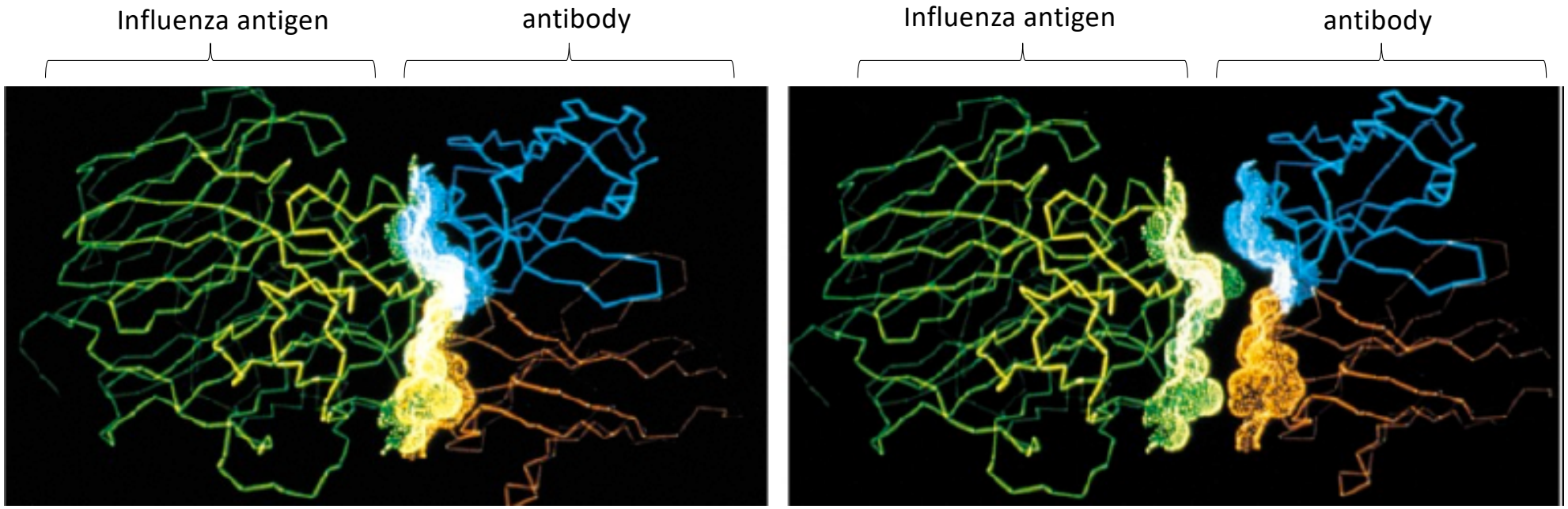
Noncovalent bonds form the basis of the antibody binding site



Immunology 4th ed. Kuby et al. W. H. Freeman and Company; 2000.

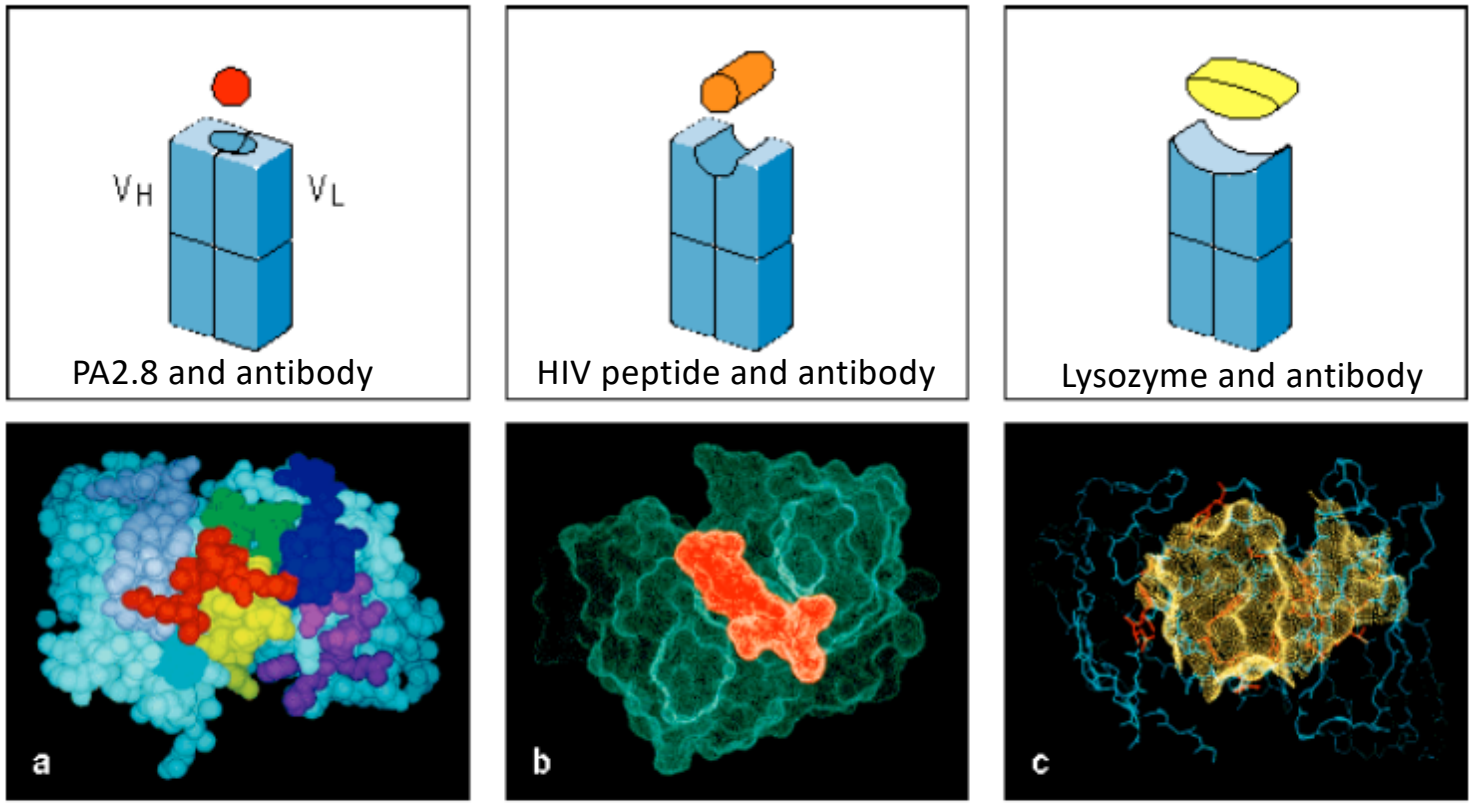
- Strength of each of these noncovalent interactions is weak
  - Many noncovalent bonds are required to form a strong interaction
- Each of these interactions operates over a very small distance (~1 Å)
- This requires a high degree of complementarity between the CDR of the antibody and the antigen

Influenza antigen and antibody binding illustrates complementary when separated by 8 Å



Immunology 5th ed. Kuby et al. W. H. Freeman and Company; 2000.

Large variation in antibody binding pockets



Immunobiology: The Immune System in Health and Disease 5th ed. Janeway CA Jr, Travers P, Walport M, et al. New York: Garland Science; 2001.

Binding a monovalent antigen by an antibody can be described by a bimolecular equation

$$\text{Antigen} + \text{Antibody} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Antigen-Antibody}$$

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB$$

K_1=rate of association
K_{-1}=rate of disassociation

The equilibrium association constant (K_a) is a good indicator for antibody affinity

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB$$

$$K_a = \frac{[AB]}{[A][B]}$$

- Ratio of products to reactants
- Affinity, the strength of the total noncovalent interactions between one antigen and antibody
- Units of K_a are concentration^{-1}
- Example: nM^{-1}

Equilibrium dissociation constant (K_d) is an indicator of the stability of a complex

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB$$

$$K_d = \frac{[A][B]}{[AB]}$$

- Ratio of reactants to products
- Antibodies produced in a typical immune response usually varied from $K_d = 10^{-7}$ (~100nM) to 10^{-9} (~1nM)
- Units of K_d are concentration
- The smaller the K_d the more stable the interaction

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Range of biologically important interactions

Type of Interaction	K_D (molar)	ΔG^0_{bind} (at 300K) kcal/mol
Enzyme:ATP	~1×10^{-3} to ~1×10^{-6} (millimolar to micromolar)	**-4 to -8 kcal/mol**
signaling protein binding to a target	~1×10^{-6} (micromolar)	**-8 kcal/mol**
Sequence-specific recognition of DNA by a transcription factor	~1×10^{-9} (nanomolar)	**-12 kcal/mol**
small molecule inhibitors of proteins (drugs)	~1×10^{-9} to ~1×10^{-12} (nanomolar to picomolar)	**-12 to -17 kcal/mol**
biotin binding to avidin protein (strongest known non-covalent interaction)	~1×10^{-15} (femtomolar)	**-21 kcal/mol**

Antibody-antigen interactions

higher K_D value
weaker interaction

lower K_D value
stronger interaction

Adapted from Kuriyan, The Molecules of Life, Chapter 12, Molecular Recognition

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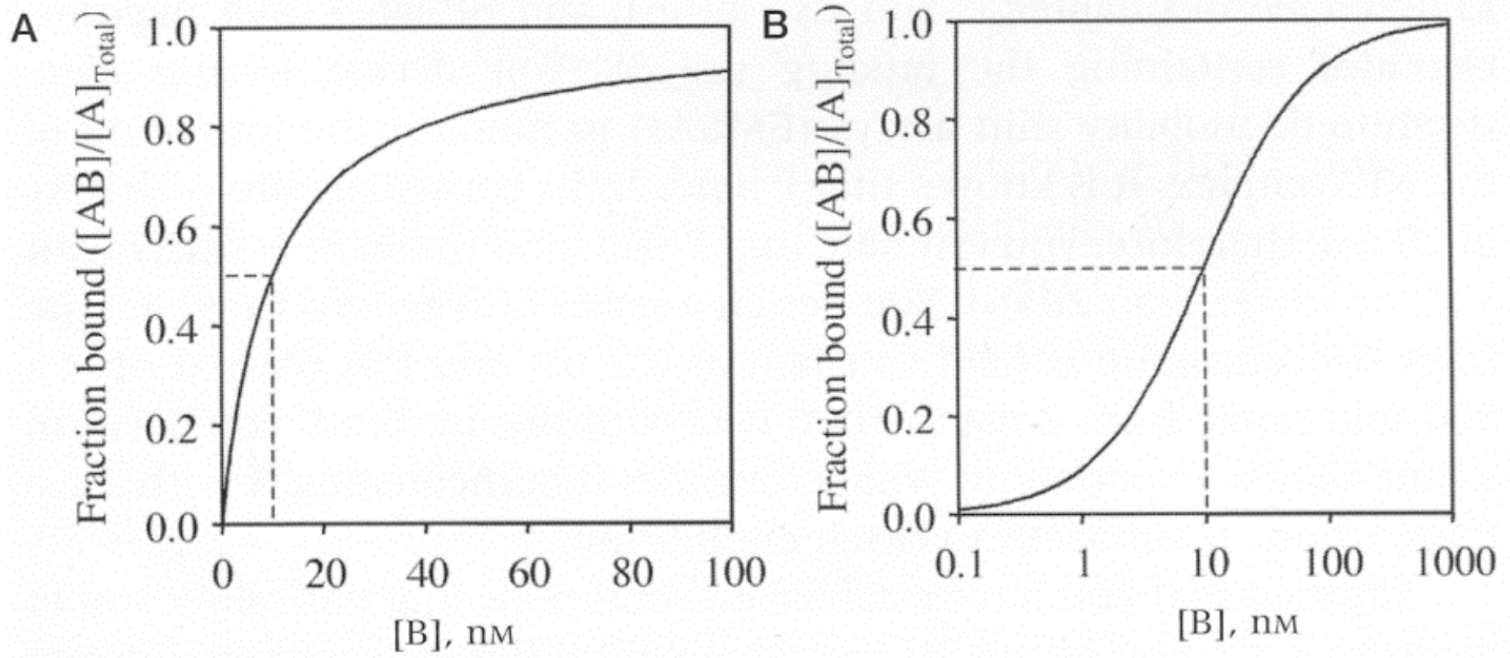
Logarithmic vs. Linear display of data

historic convention *current convention*



Biomolecular binding interaction at equilibrium:

Why is antibody dissociation constant (K_d) equal to the antigen concentration at which 50% antibody is bound to antigen?

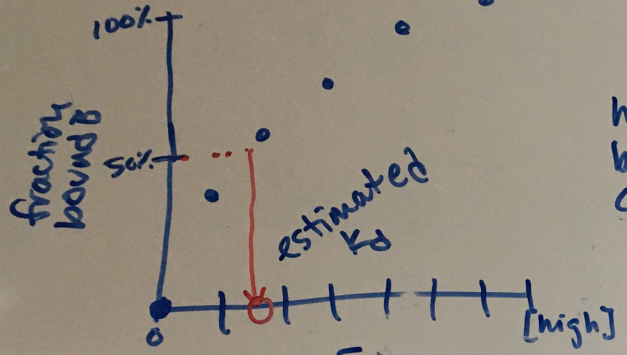
Lysozyme

Lysozyme-ScFv

$A + B \rightleftharpoons AB$

ScFv

$K_d = \frac{[A][B]}{[AB]}$



hyperbolic binding curve

$$\text{fraction bound B} = \frac{\frac{[AB]}{[AB]}}{\frac{[B]}{[AB]} + \frac{[AB]}{[AB]}} = \frac{1}{\frac{[B]}{[AB]} + 1} = \frac{1}{\left(\frac{K_d}{[A]} + 1\right)\frac{[A]}{[A]}} = \frac{[A]}{K_d + [A]}$$

$$\frac{[B]}{[AB]} \qquad K_d = \frac{[A][B]}{[AB]} \div [A] = \frac{[B]}{[AB]}$$

$$\text{fraction bound B} = \frac{[A]}{K_d + [A]} = \frac{1}{2} \text{ or } 50\%$$

$$K_d = [A]$$

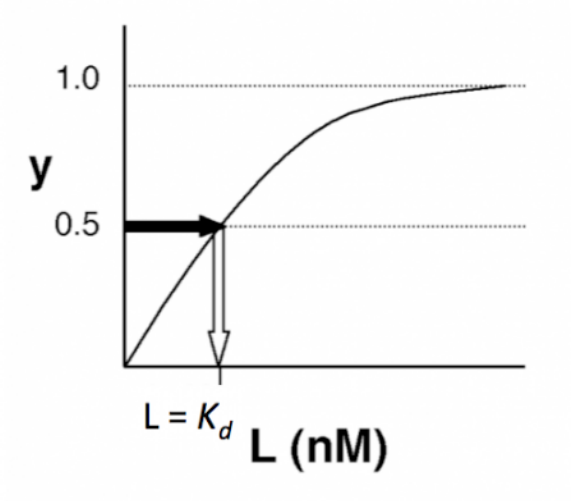
Mathematical relationship between fraction bound and free reactant makes estimations easy

$$L + Ab \rightleftharpoons_{k_r}^{k_f} C$$

$$y = \frac{[L]}{[L] + K_d}$$

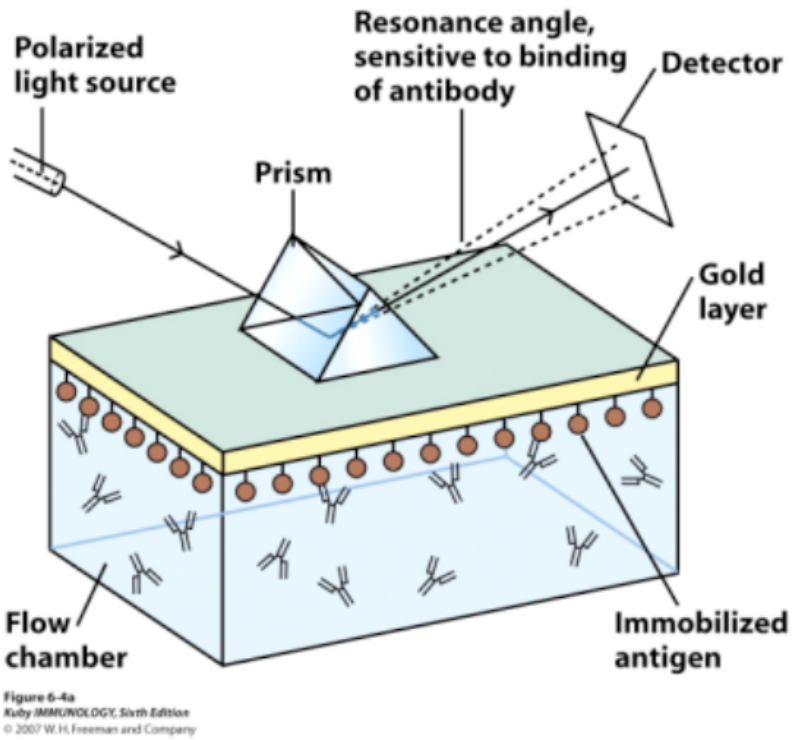
If L in excess (in solution), and $[L] = L$ constant

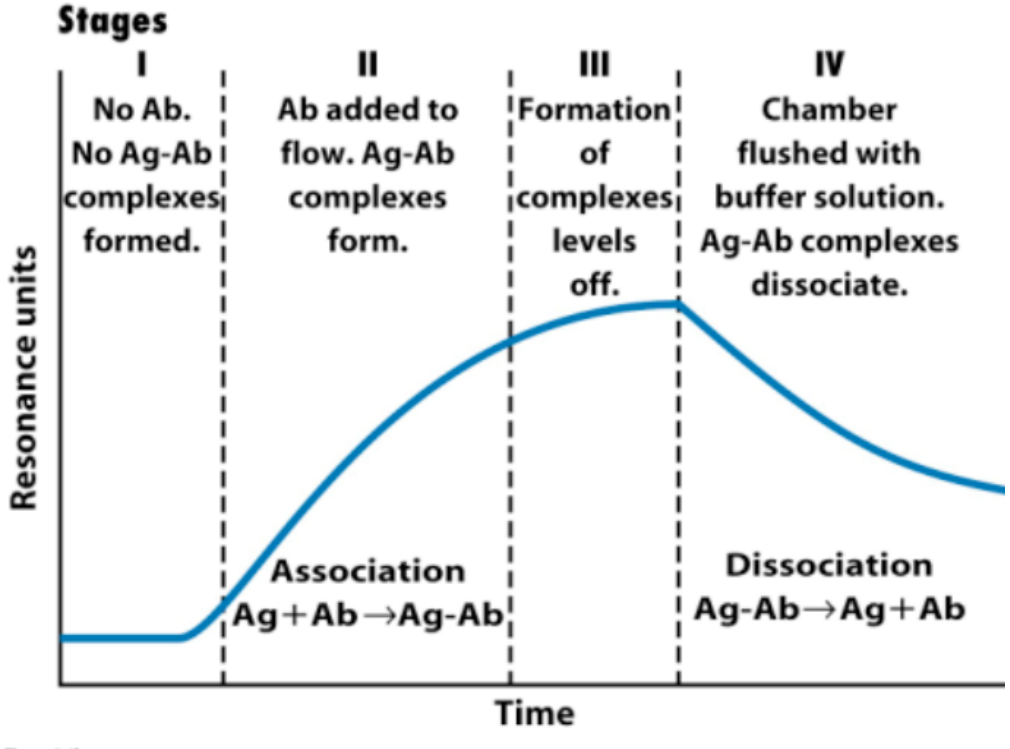
- at $L = K_d$ $\quad y = 0.5$
- if $L << K_d$ then $\quad y \approx \frac{[L]}{K_d}$ (linear relationship)
- if $L >> K_d$ then $\quad y \approx 1$ (at saturation)



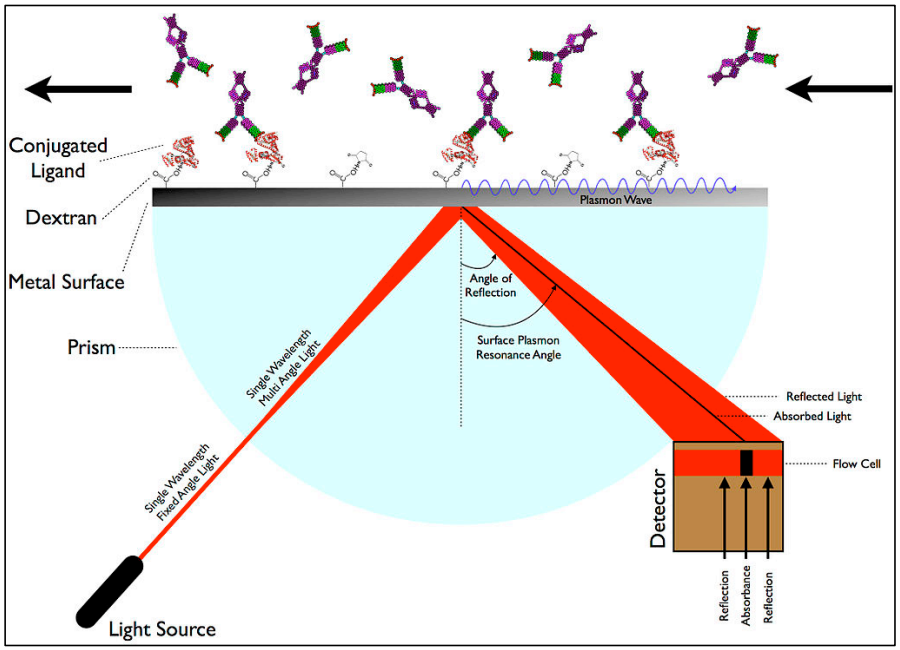
Alternative methods to measure binding dynamics without necessitating equilibrium

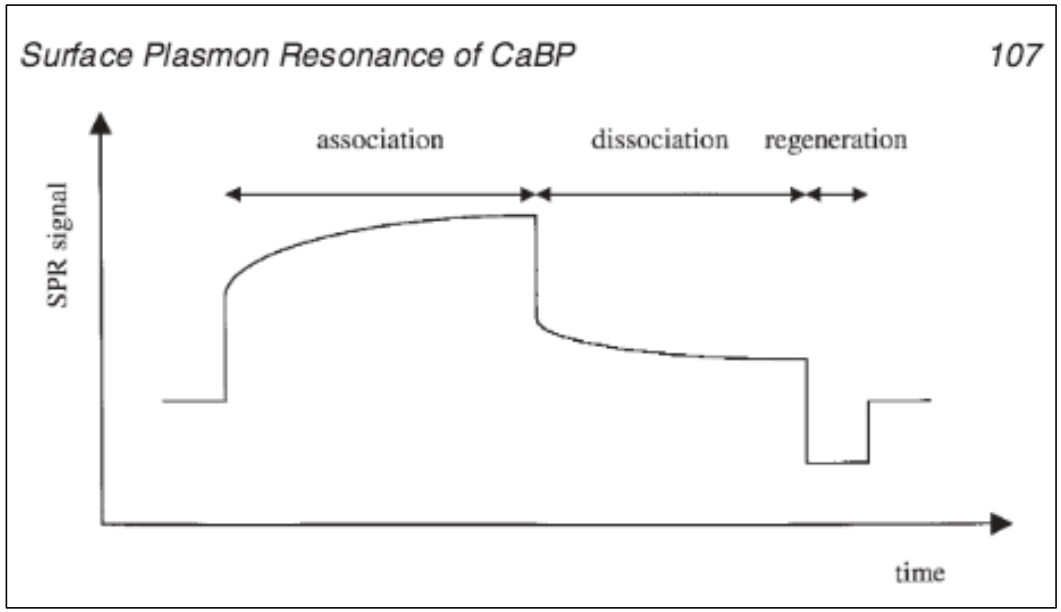
Binding of the antibody to the antigen alters the resonance readout and can translate to affinity





Surface plasmon resonance (Biacore)



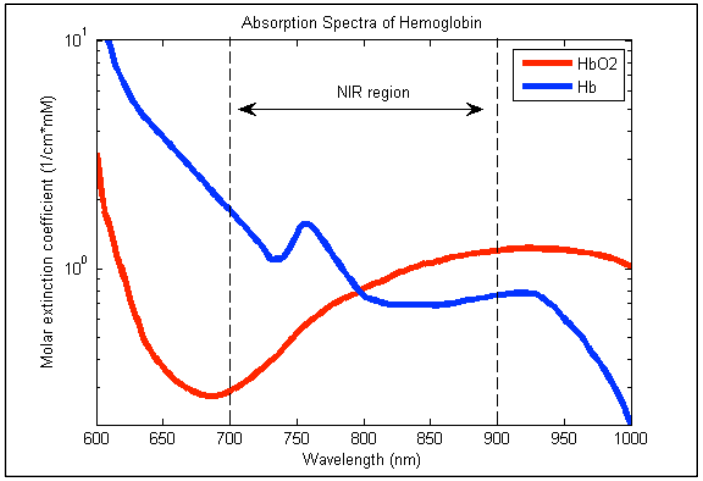


Julenius (2002) *Methods Mol Biol* **173**: 103-111

Binding may be quantified using methods other than fluorescence

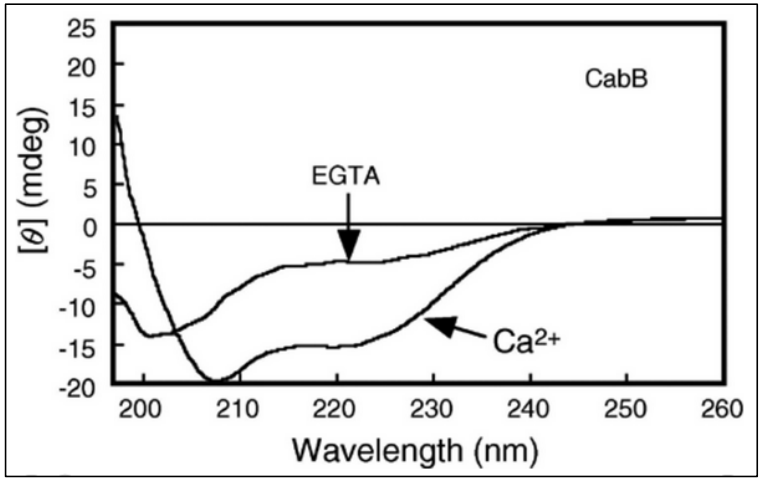
- absorbance spectroscopy

  e.g. hemoglobin binding to O_2



- circular dichroism

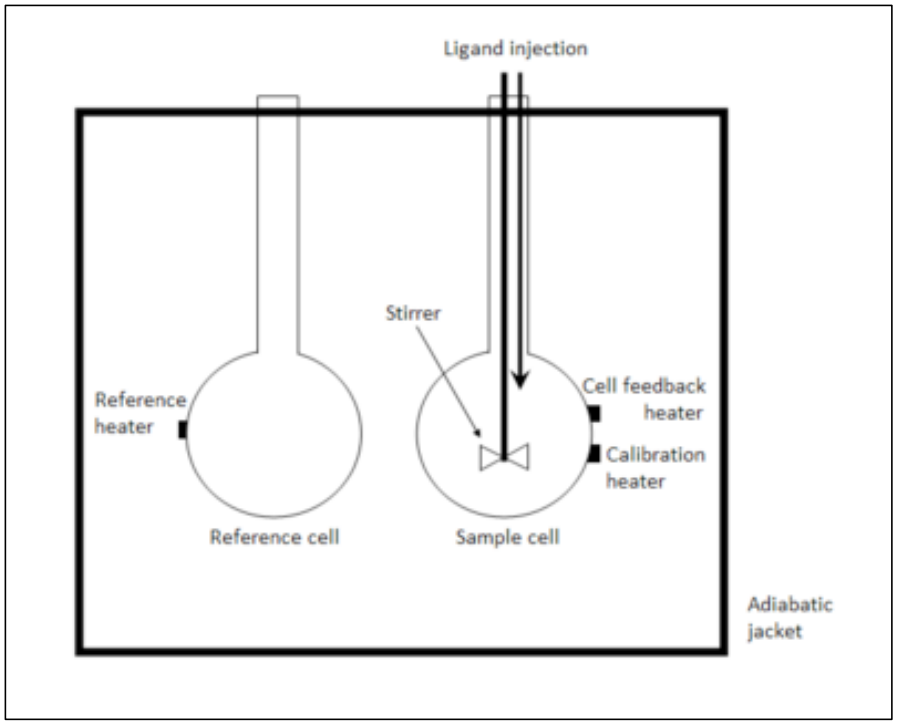
  e.g. Ca^{2+} binding to CabB



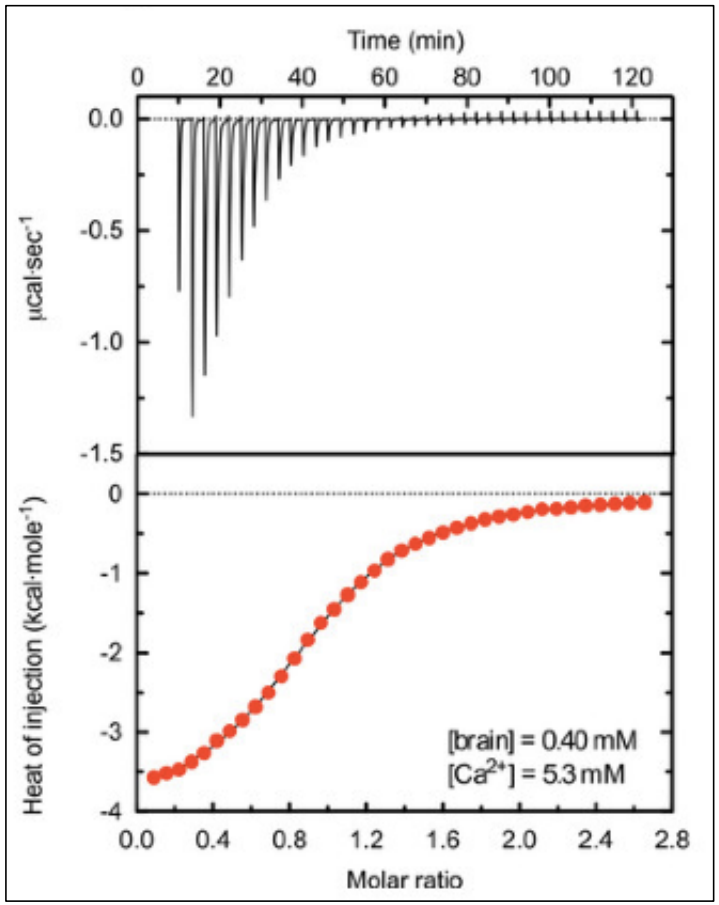
oxymetry.org

Yonekawa *et al*.
(2005) *FEMS Microbio Lett* **244**: 315-321

Isothermal titration calorimetry measures thermodynamic parameters of interactions



$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S$$



Backman (2015) *PeerJ* **3**: e944

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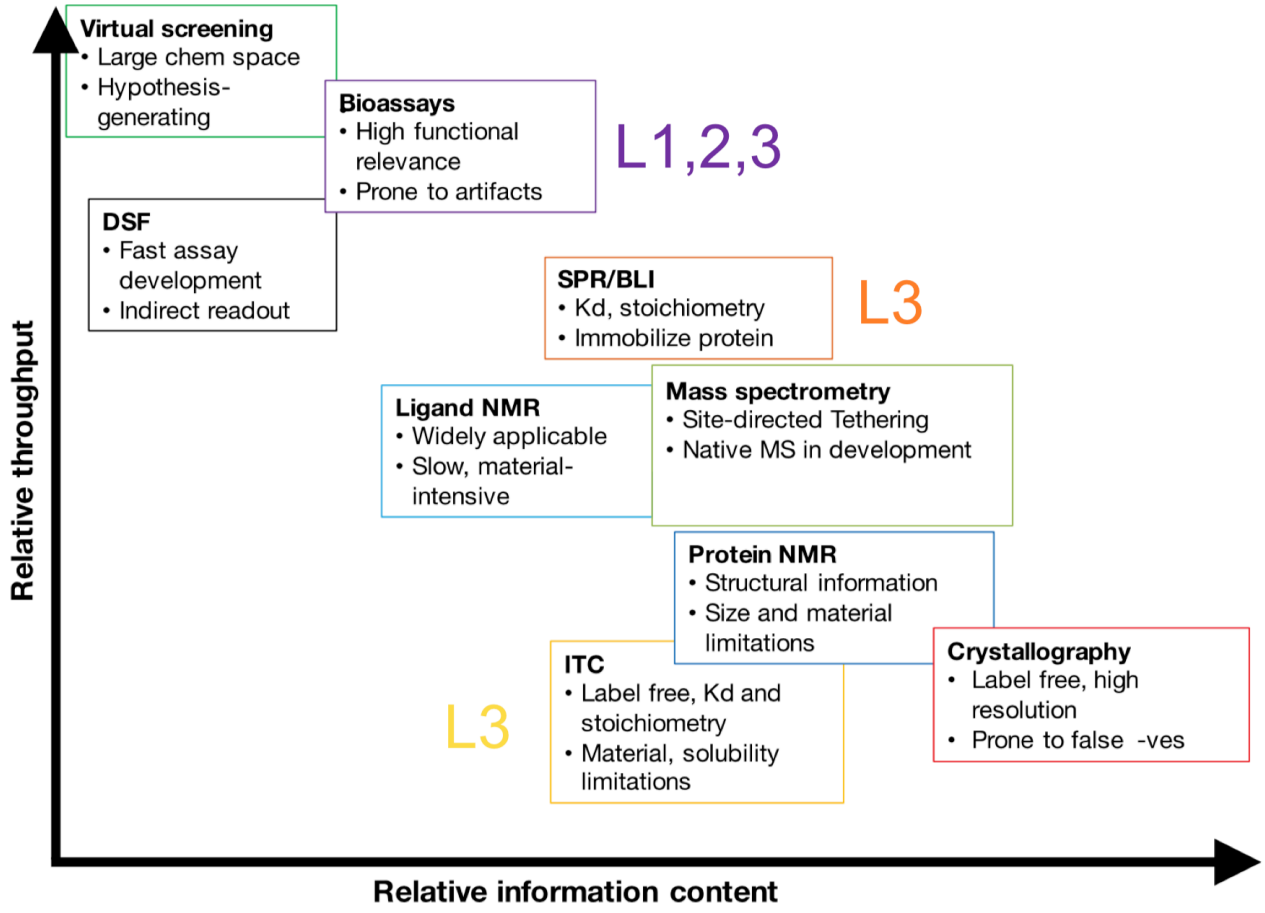
Methods to evaluate binding interactions

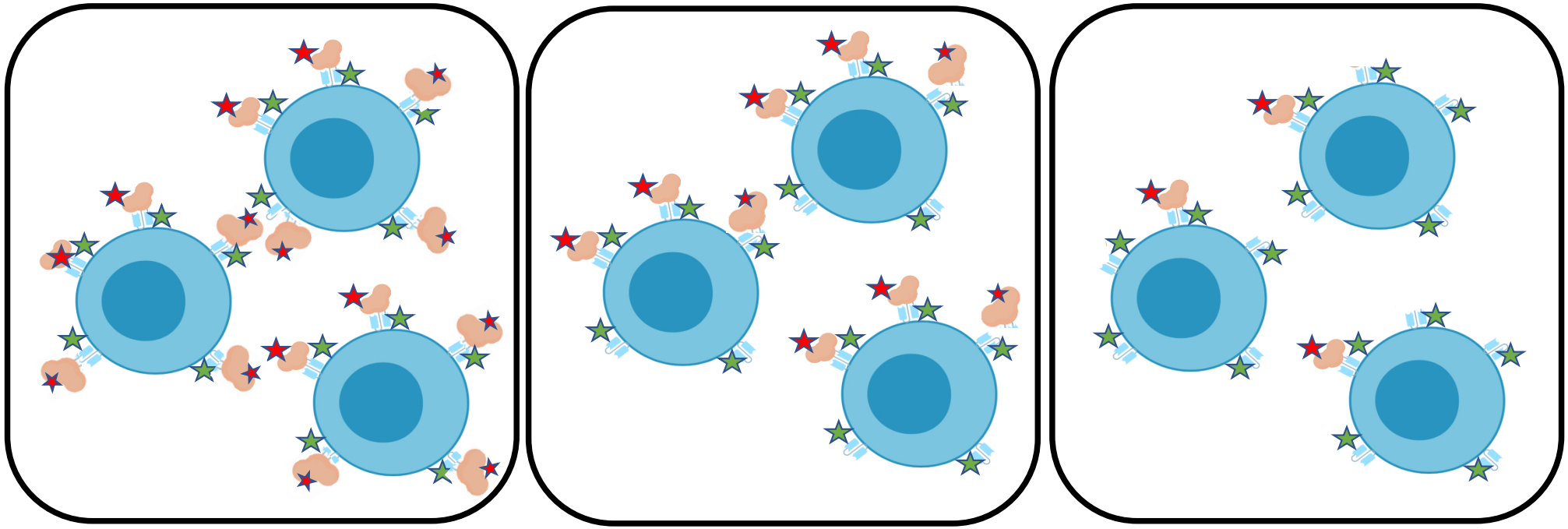


Practically: how will we measure equilibrium binding with different antigen concentrations?

Tube #1

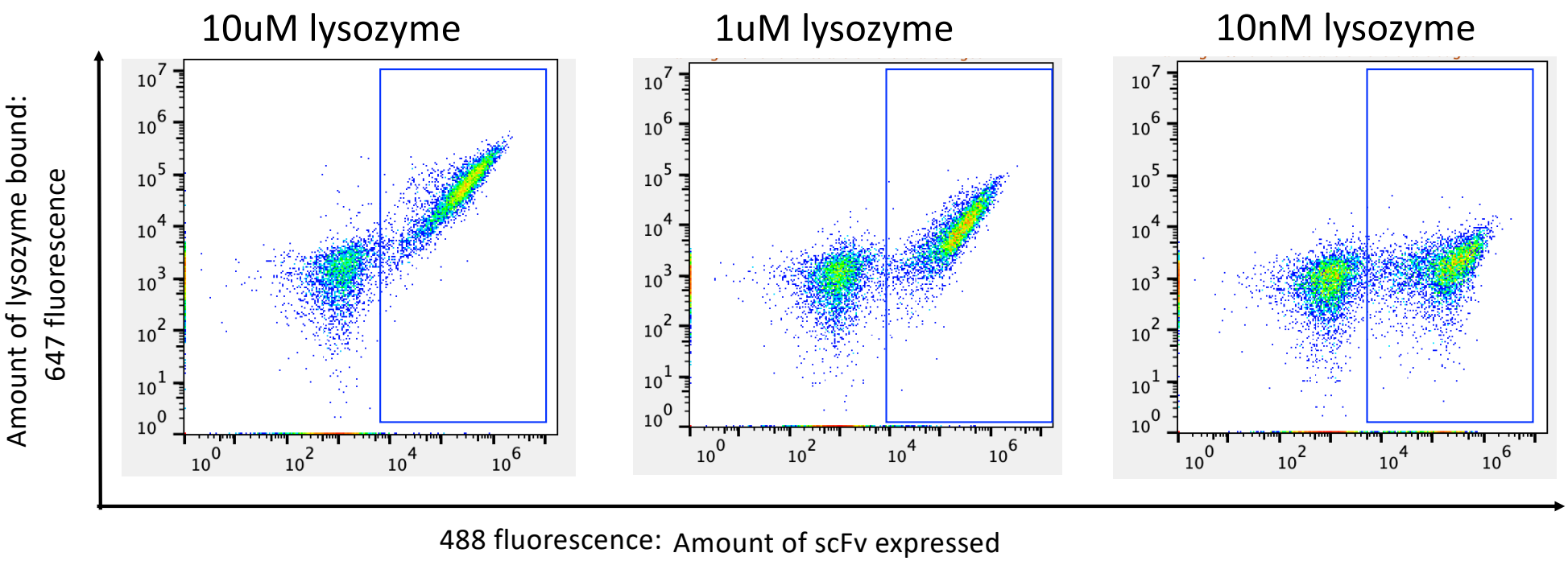
Tube #2

Tube #3

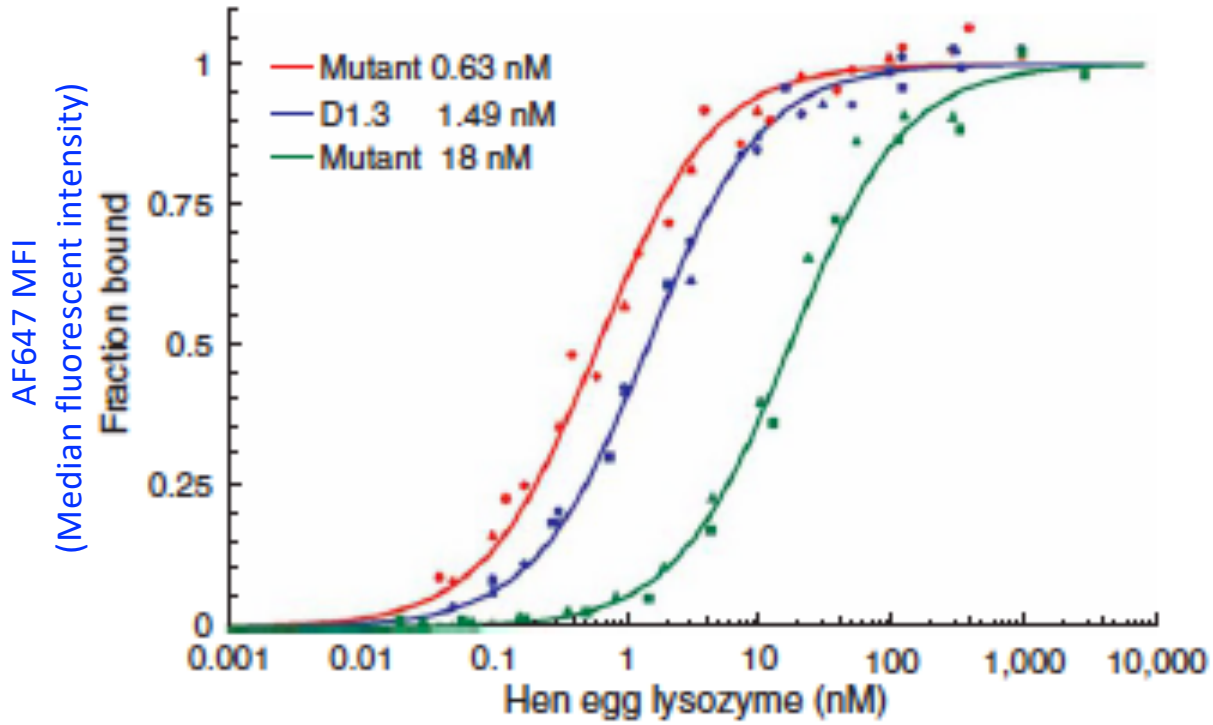


Median fluorescent intensity of gated scatterplot is to fraction bound

scFv Clone 14989 (650nM K_d) incubated with:



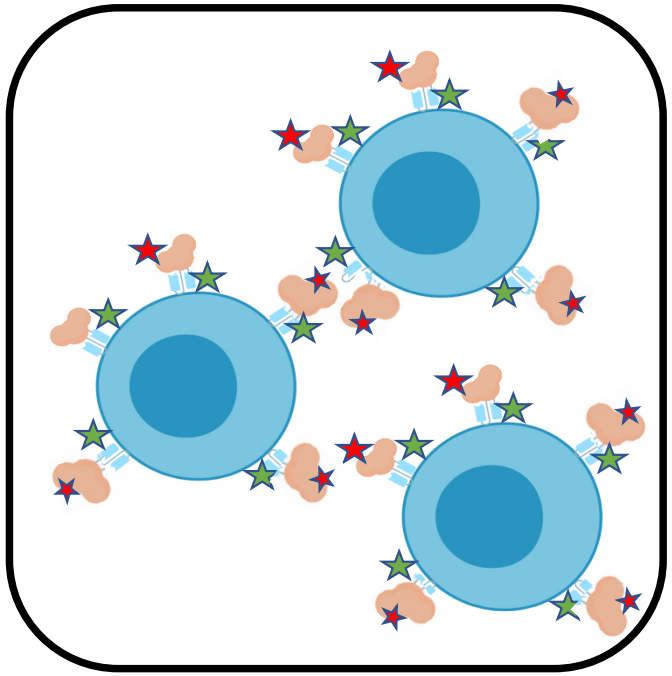
Plotted MFI illustrates fraction of antigen bound to antibody



Do you agree with the K_d?

Today in “lab”

1) Set up titration of equilibrium binding reactions



2) Analyze flow cytometry data