Quantitative assessment of molecular interactions

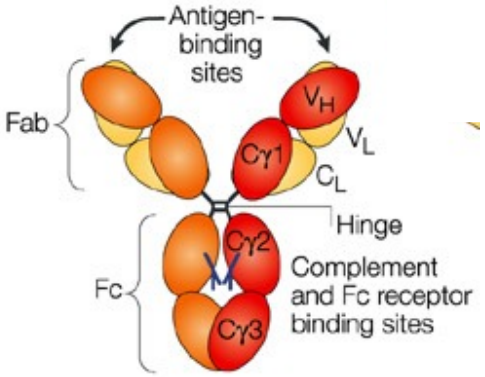
Technical Journal Club

12. 04. 2016

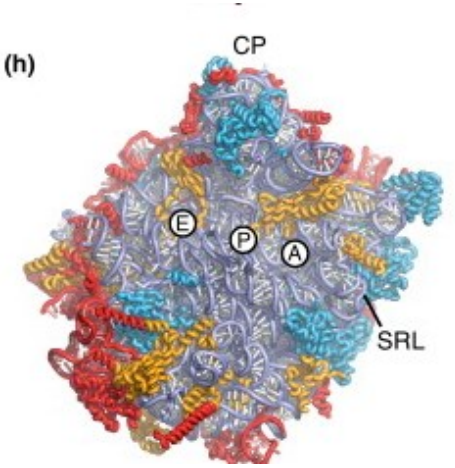
Marc Emmenegger

What are molecular interactions?

- Between antigen - antibody.
  - E.g. haemaglutinin (glycoprotein) – antibody interaction.
- Receptor – ligand.
  - E.g. Ca2+ - Calmodulin interaction.
  - Ach – AchR
  - Haemaglutinin – sialic acid R
- Between protein and RNA
  - E.g. in macromolecular assemblies such as ribosomes.
- Between protein and DNA
  - TF binding to DNA

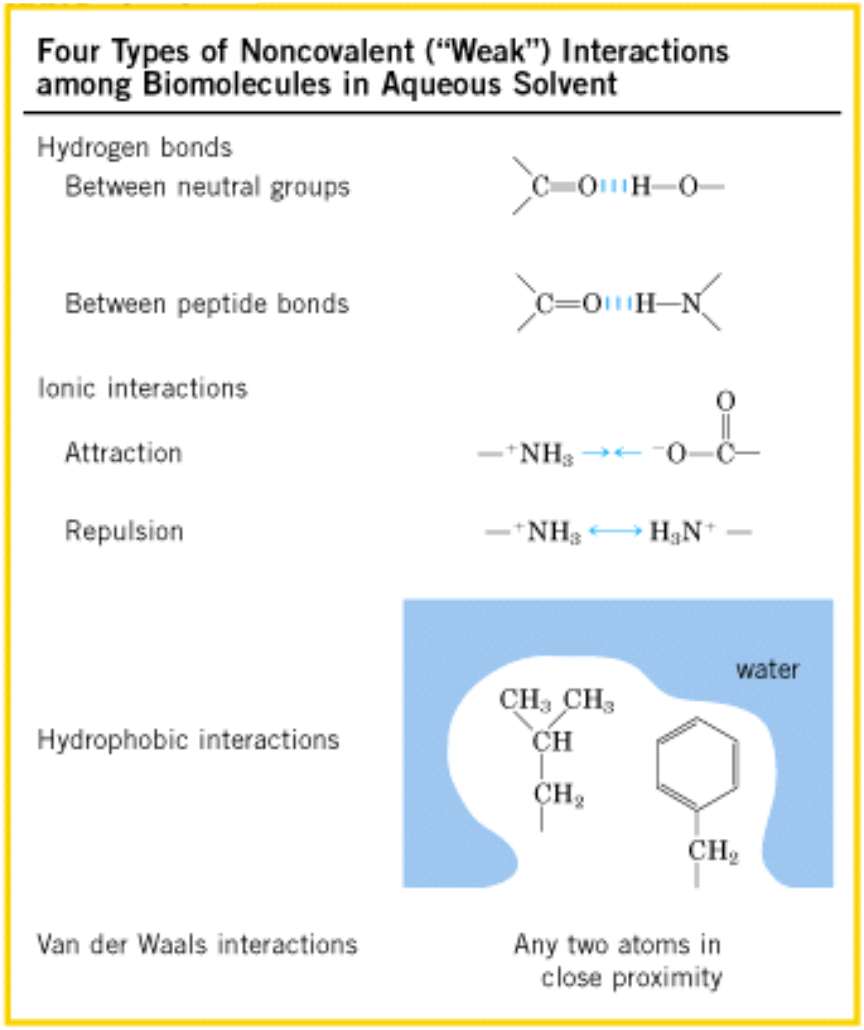


IgG1

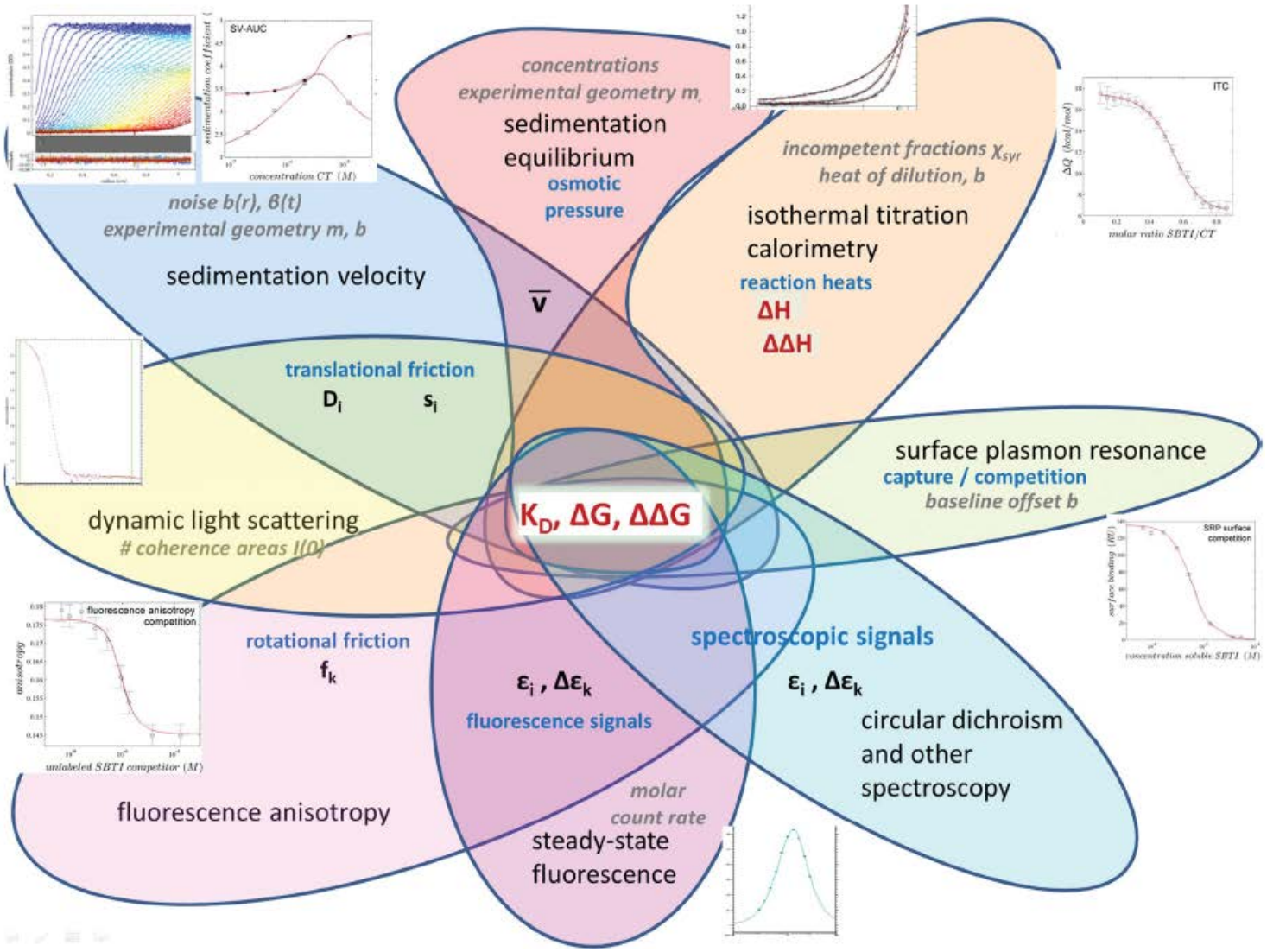


Woof, et al. (2004). Nature.
Klinge, et al. (2012). Tr in Bioch. Sc.

Intermolecular interactions are characterised by non-covalent bonds



Techniques available to quantitatively study interactions



Zhao, et al. (2015). Acta Cryst.

The quantitative determination of interactions is important in…

- … pre-crystallisation studies.
- … clinical diagnostics.
- … secondary screenings following high-throughput screenings.
- … rational drug design whereby high-affinity binders should be selected.

… but there are many pitfalls reported!

MBoC TECHNICAL PERSPECTIVE

A Guide to Simple and Informative Binding Assays

Thomas D. Pollard

Departments of Molecular Cellular and Developmental Biology, of Molecular Biophysics and Biochemistry and of Cell Biology, Yale University, New Haven, CT 06520-8103

- Many binding experiments reported to be poorly designed and fall short of extracting all of the useful information.
- Fail to measure affinity.
- Binding experiments are not digital, with black/white yes/no answers.
- Requirements: Reaction must be at equilibrium at time of measurement! Concentration of one reactant must be varied.

Studying binding affinities

- *Surface Plasmon Resonance* (**SPR**)

→ Kinetic measurement, derivation of K_D.

- *Isothermal titration calorimetry* (**ITC**)

→ Thermodynamic measurement, derivation of K_D.

- *Microscale thermophoresis* (**MST**)

→ Measurement of concentration change, derivation of S_T and K_D.

→ Assessment of basic thermodynamic/kinetic parameters.

Short reminder on basic thermodynamics

- $Ab + Ag \leftrightarrow Ab - Ag$ (1)
- $K_a = \frac{[Ab-Ag]}{[Ab][Ag]}$ (2)
- $[Ab - Ag] = K_a[Ab][Ag]$ (3)
- $K_d = \frac{1}{K_a} = \frac{[Ab][Ag]}{[Ab - Ag]}$ (4)
- $[Ab - Ag]K_d = [Ab][Ag]$ (5)
- At equilibrium: $[Ag] = [Ab - Ab] \rightarrow K_d = [Ab]$ (6)
- Kd < 10-7 M → High-affinity binder.

Short reminder on basic kinetics

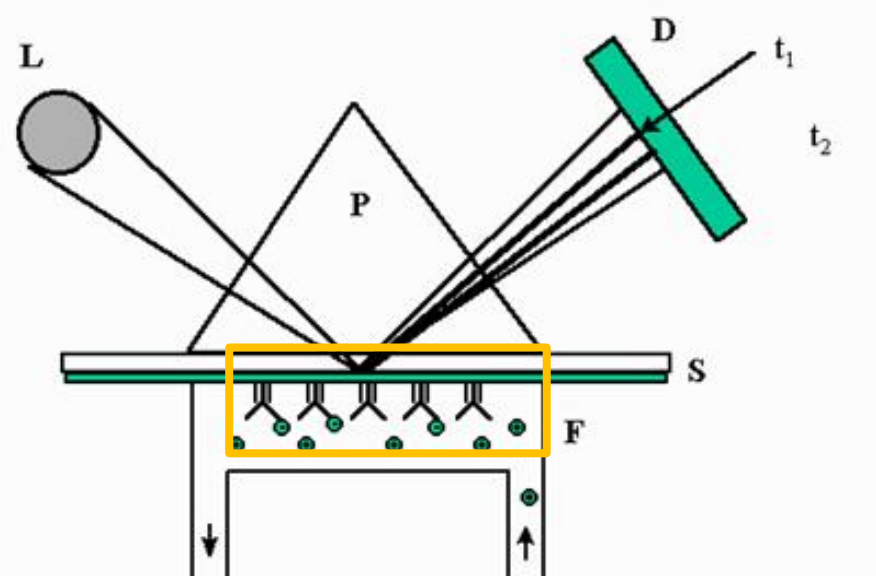
- $On = k_{on}\,[Ab][Ag]$ (7)
- $Off = k_{off}[Ab\ -Ag]$ (8)
- At equil.: $On = Off \rightarrow k_{on}[Ab][Ag] = k_{off}[Ab\ -Ag]$ (9)
- $\frac{k_{on}}{k_{off}} = \frac{[Ab-Ag]}{[Ab][Ag]} = K_a$ (10)
- $\frac{k_{off}}{k_{on}} = K_d$ (11)

→ K_d and K_a (thermodynamic constants) can be obtained by the ratio between $k_{on}$and k_{off}(kinetic *rate* constants).

- k_{on} is a second order rate constant.
- $k_{off}$is a first order rate constant.

Assessing molecular interactions: **Surface Plasmon Resonance**

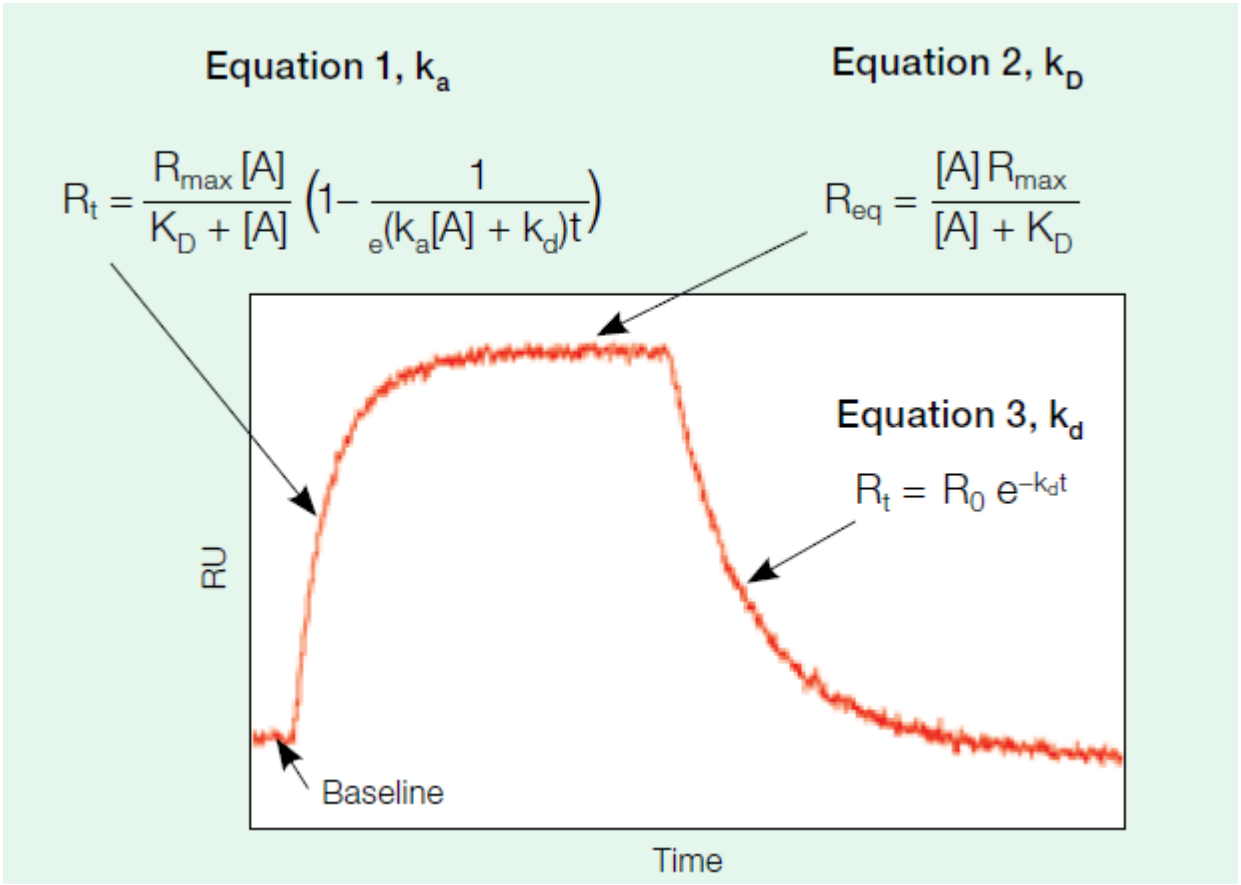
- Phenomenon which occurs when light is reflected off thin metal films.



- Change of local refractive index upon binding of antigen to antibody.
- Leads to change of SPR angle.
- Changes in intensity of reflected light detected by sensor.
- Rates of change of SPR signal (**change of refractive index over time**) yield rate constants for association/dissociation (Kon/Koff) of the reaction.

Assessing molecular interactions: **Surface Plasmon Resonance**

SPR sensogram



- Pre-steady state (kon)
- Steady state
- Post-steady state (koff)
- Kd = koff/kon

BioRad bulletin 6044A

Assessing molecular interactions: **Surface Plasmon Resonance**

Surface plasmon resonance measurements of plasma antibody avidity during primary and secondary responses to anthrax protective antigen

Heather E. Lynch [a,1], Shelley M. Stewart [a,1], Thomas B. Kepler [b], Gregory D. Sempowski [a], S. Munir Alam [a,*]

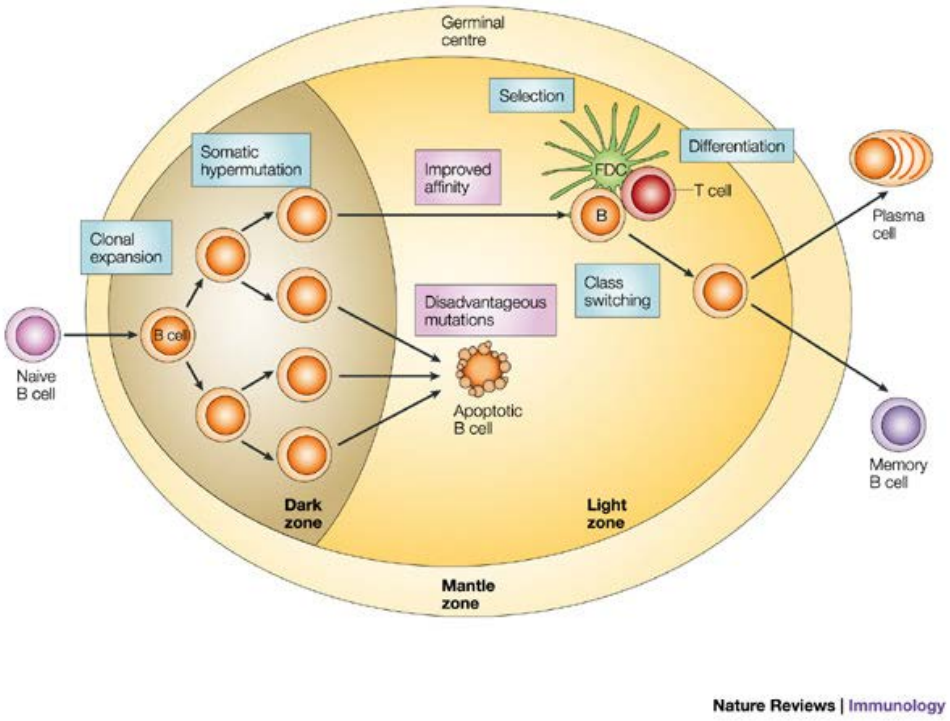
[a] *Duke Human Vaccine Institute and Departments of Medicine, Duke University Medical Center, Durham, NC 27710, United States*
[b] *Department of Microbiology, Boston University School of Medicine, Boston, MA 02118, United States*

- Generation of high-avidity antigen-specific antibody response crucial for efficacy of many vaccines.
- Success of vaccine depends on overall strength and duration of elicited protective immunity.
- Protective humoral immunity against many pathogens depends on establishment of long-lived plasma cells that secrete high-affinity antibodies as a result of B cell selection events that occur in germinal centres within B follicles of reactive lymphoid tissue.

Assessing molecular interactions: **Surface Plasmon Resonance**

- Within germinal centres, B cells undergo selection process that involves cloncal expansion, somatic hypermutation and class switching.

- Developments give rise to memory B cells and plasma cells that secrete high-avidity antibodies and maintain long-term antibody production.



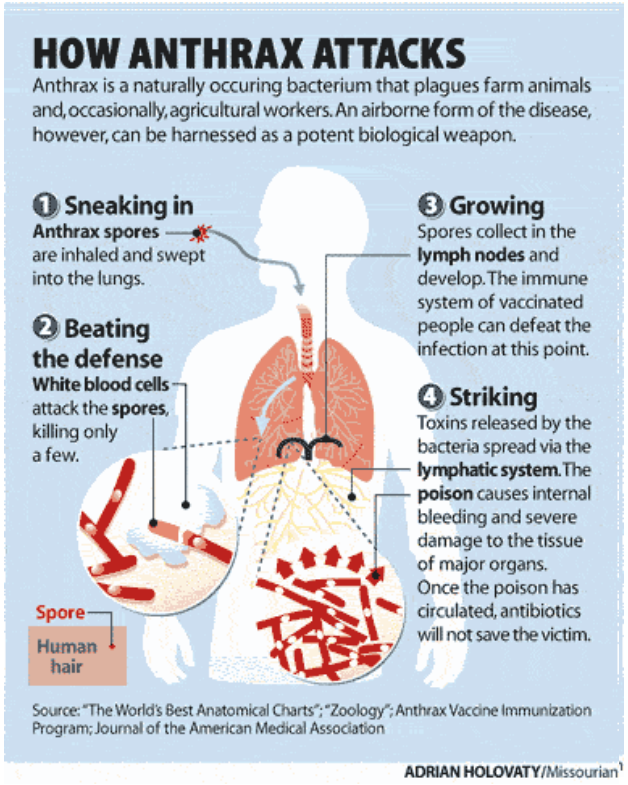
- Importance for host protection upon secondary challenge.

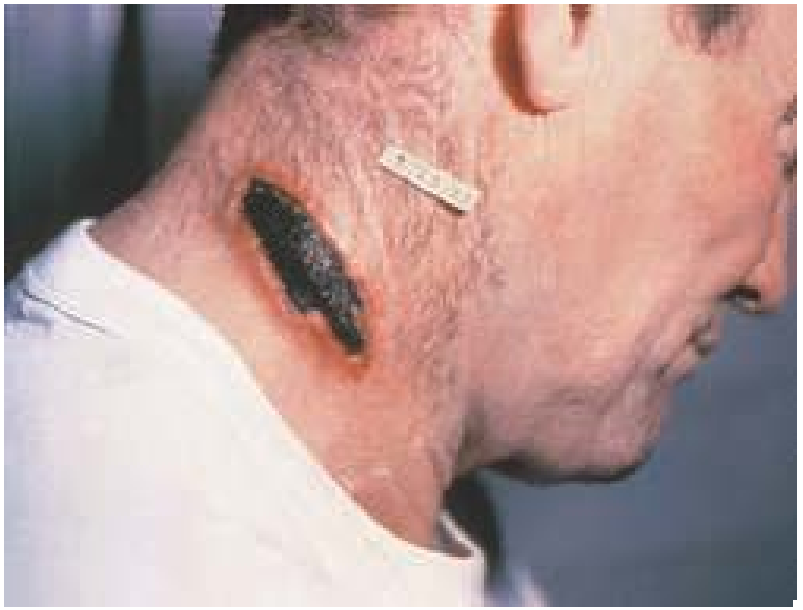
Küppers (2003). Nat Rev Immunol.

Assessing molecular interactions: **Surface Plasmon Resonance**

- **Antigen** in this study: recombinant Bacillus anthracis protective antigen (rPA).

- Anthrax has gained recent popularity with regards to its use as a bioweapon.

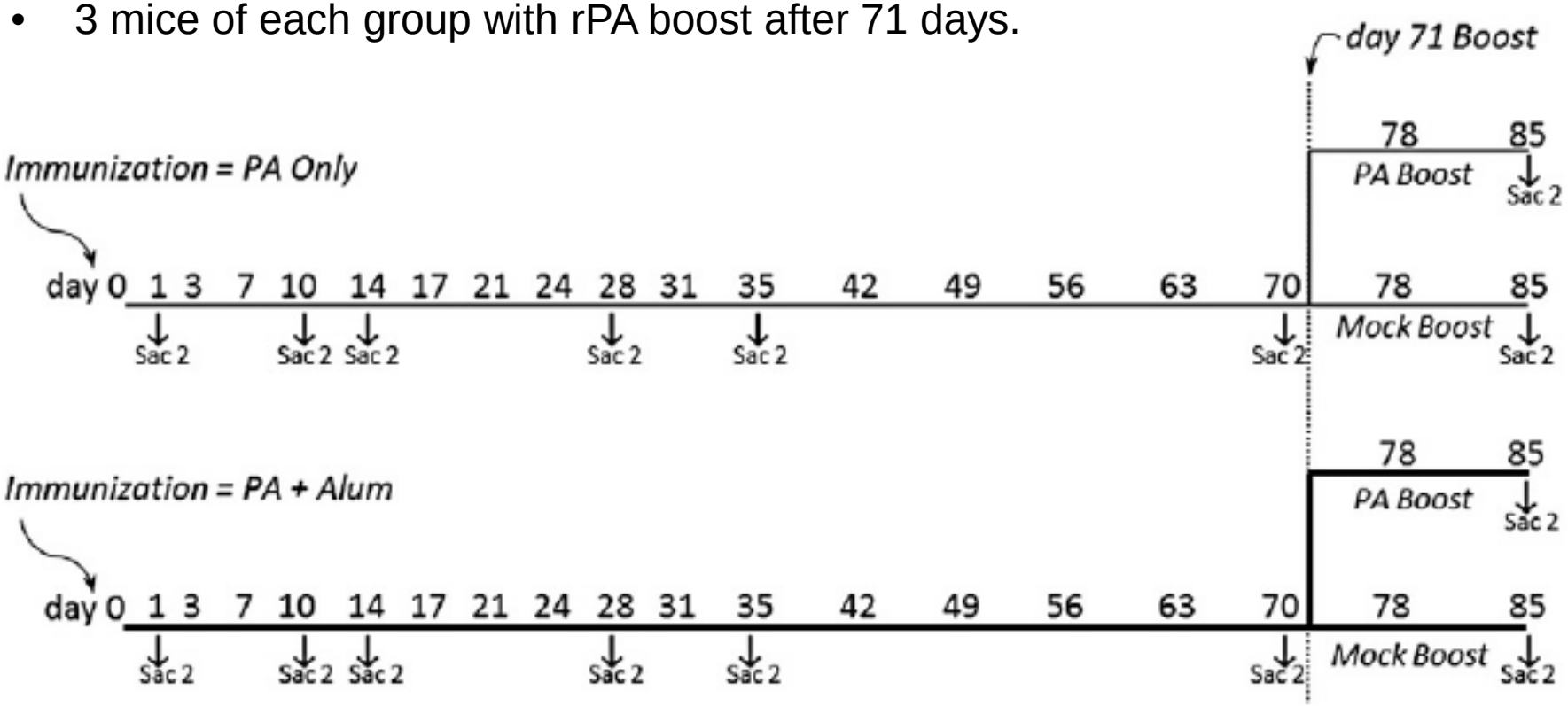




- Aim of study: Check antigen-specific antibody avidity following subcutaneous vaccination with saline, rPA, or Anthrax Vaccine Adsorbed (AVA), containing PA.

Assessing molecular interactions: **Surface Plasmon Resonance**

- Immunisation of 18 mice:
  - 6 with saline (not shown)
  - 6 with rPA
  - 6 with AVA
- 3 mice of each group with rPA boost after 71 days.



Assessing molecular interactions: **Surface Plasmon Resonance**

- Immobilisation of antigens in SPR:
  - Flow cell 1: Anthrax lethal factor (not included in vaccine mixture! → Background)
  - Flow cell 2: rPA (included in vaccine mixture!)

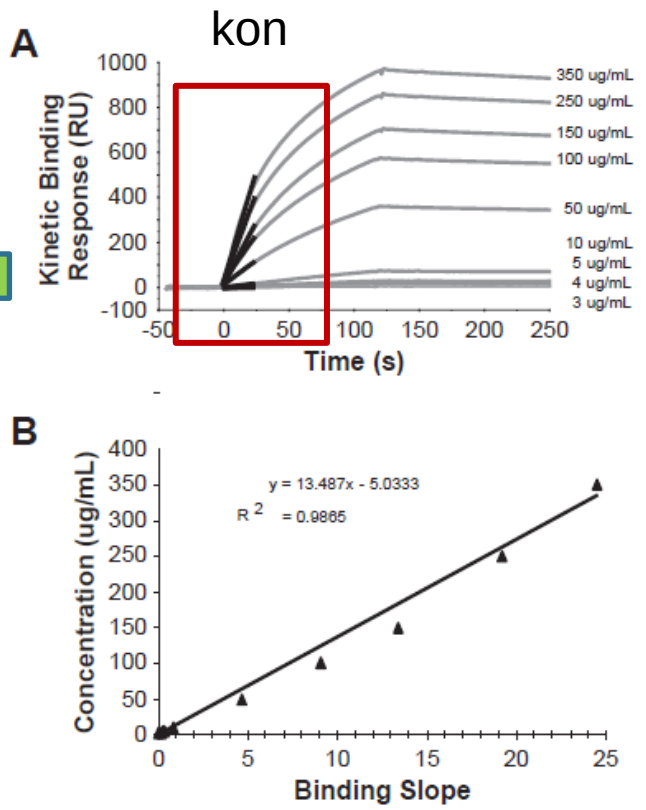
- **Positive controls**: Two previously tested **anti-PA monoclonal antibodies** were run at 100 ug/mL, followed by titration curve of one of these antibodies from 0.1 – 350 ug/mL for calibration.

- Sensogram (primary readout of SPR).

- Analyse 10 second window (slope).

  → pre-steady state: k_{on}

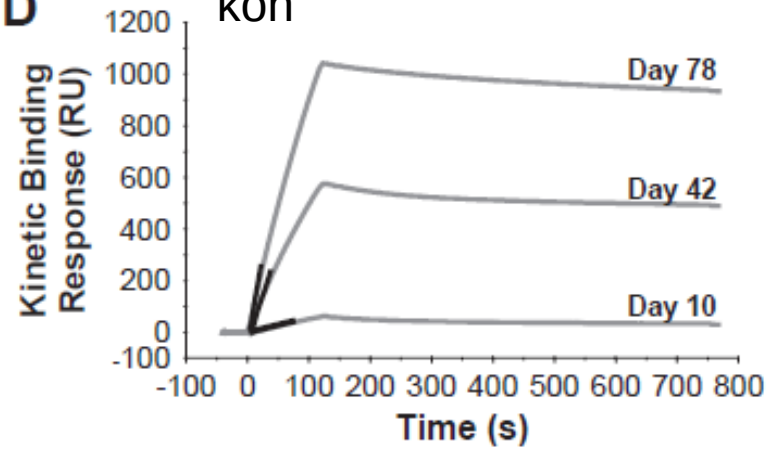
→ Obtain standard curve



Assessing molecular interactions: **Surface Plasmon Resonance**

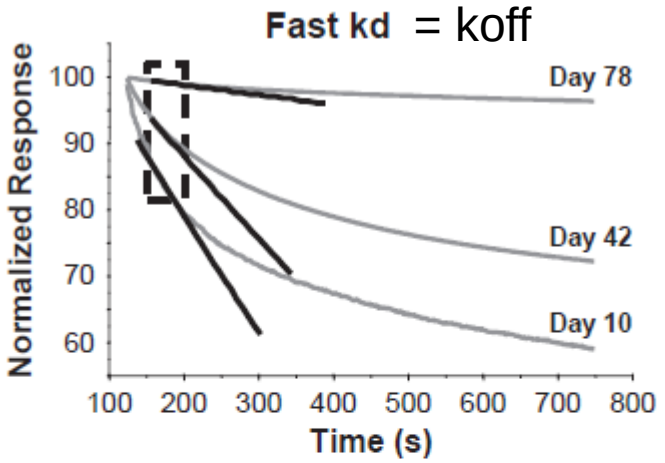
Collected serum from mice diluted 1:50.

D kon



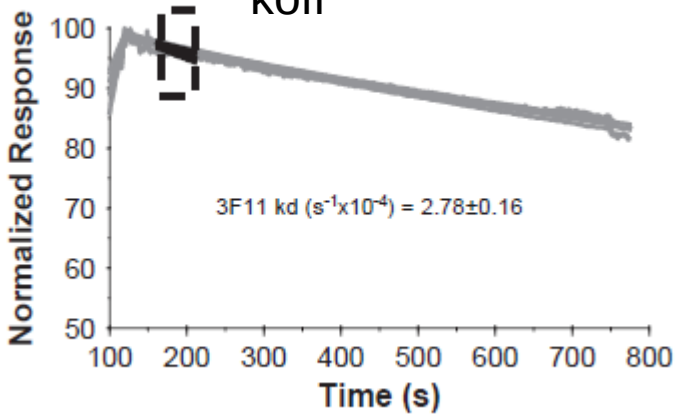
Biphasic koff of polyclonal serum antibodies

E

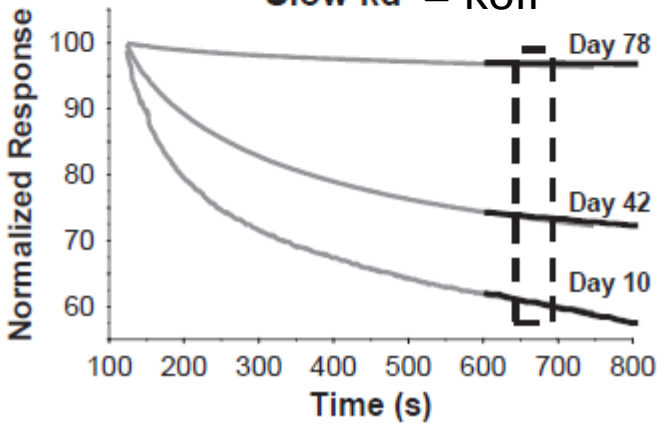


koff of monoclonal antibody

C koff

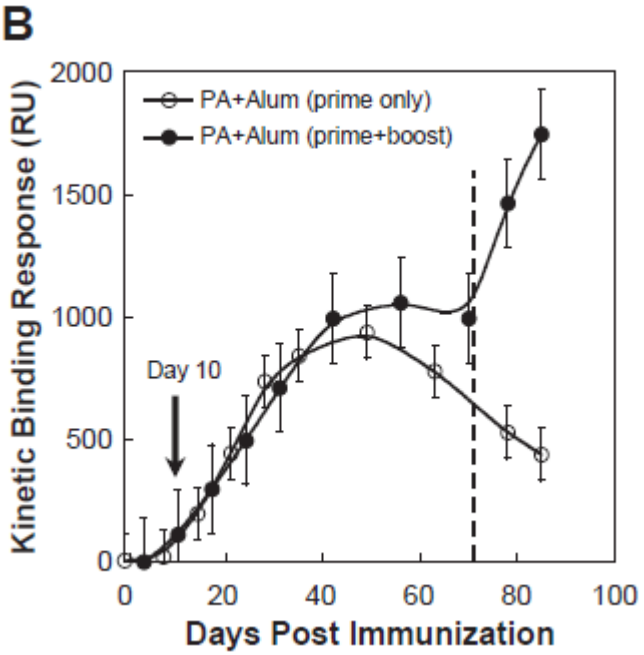


F Slow kd = koff



Assessing molecular interactions: **Surface Plasmon Resonance**

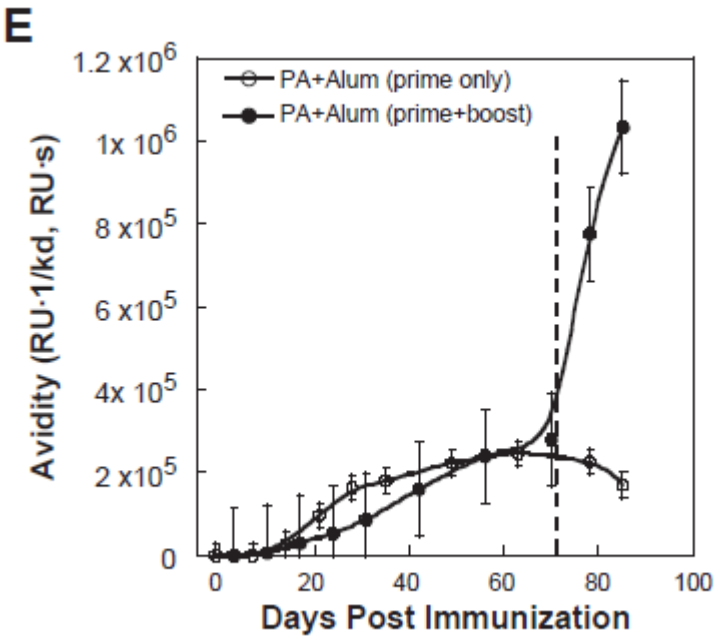
Importance of the boost



- Strong secondary humoral response to antigen upon application of boost.

koff/kon

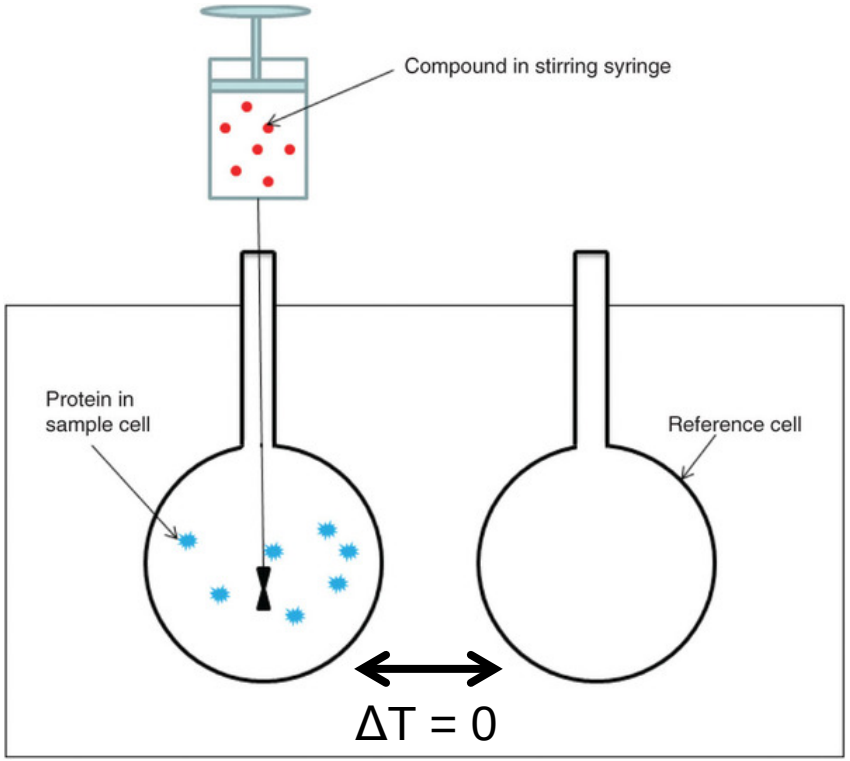
Avidity



- Weak avidity antibodies in the first 10 days!
- Subsequently increased.
- Best after boost! → Secondary response more specific (less broad).

Assessing molecular interactions: **Isothermal titration calorimetry**

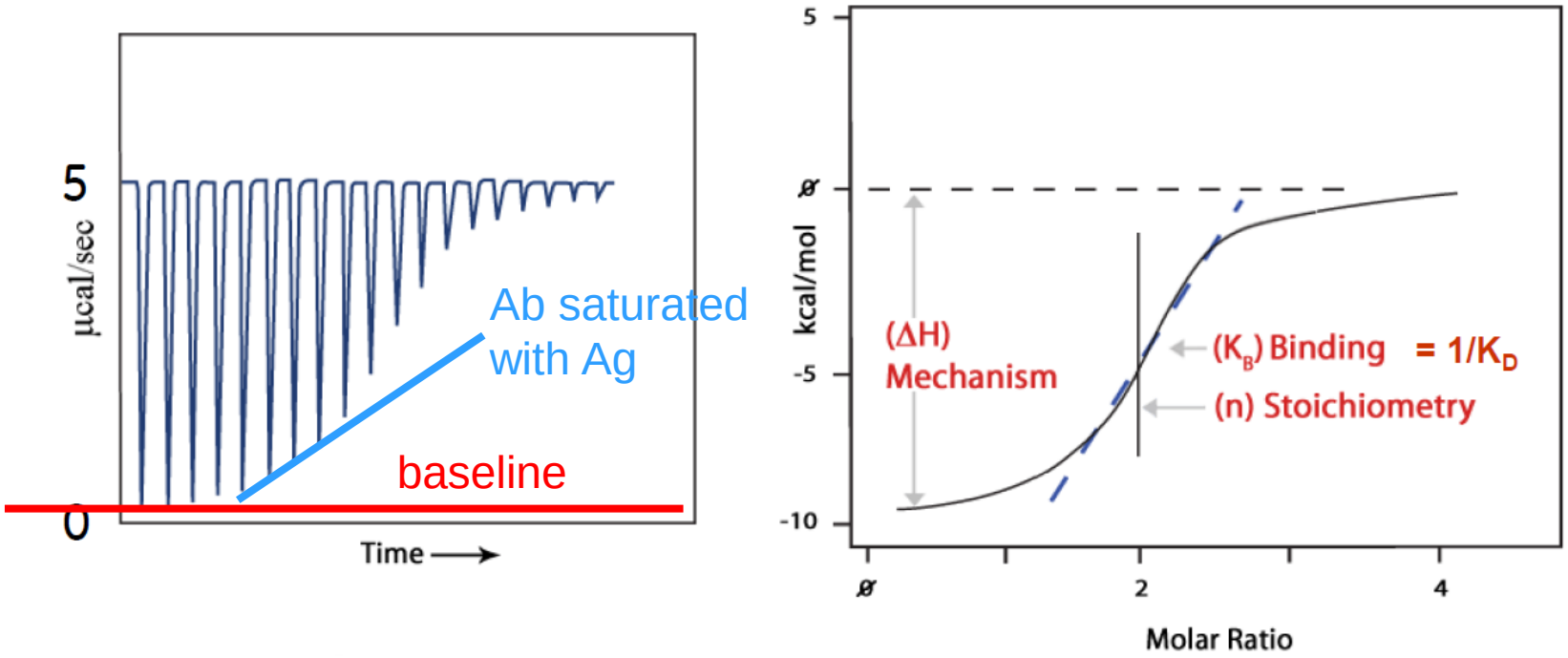
- Direct measurement of heat generated/absorbed when molecules interact.



- Ligand titrated into sample cell in precisely known aliquots.
- Differential Power between reference and sample necessary to maintain $\Delta T = 0$.
- Exothermic $\Delta Q < 0$, endothermic $\Delta Q > 0$.
- Plot power needed to maintain reference and sample at identical temperature against time.

Zhou, et. al. (2011). Nat Prot.

Assessing molecular interactions: **Isothermal titration calorimetry**



- Upon application of antibody, it binds to the antigen → Production of heat.
- Back to baseline.
- Next application, binds again → Production of heat.
- Until saturation is achieved.

Assessing molecular interactions: **Isothermal titration calorimetry**

- Release/absorption of certain amount of heat: $Q = V * \Delta H * \Delta L$ with Q = heat, V = volume of reaction cell, ΔL = increase in concentration of bound ligand.

- For a simple model, ΔL becomes $[P] * (K_a \frac{[L]_i}{1+K_a [L]_i} - \frac{K_a [L]_{i-1}}{1+K_a [L]_{i-1}})$ and allows the assessment of K_a and K_d.

- Since ΔH and, via –RT ln(K_a), ΔG are known, the entropy ΔS can be identified: $\Delta G = \Delta H - T\Delta S$

Assessing molecular interactions: **Summary**

- For long time, ITC had been the only technique capable of resolving enthalpic (ΔH) and entropic (ΔS) components of binding affinity.
- Knowledge whether reaction is enthalpy- or entropy-driven is important in drug development.
- ITC does not yield insight into kinetics! The formation of the product could take hours (but once it is formed, could be tightly bound).
- On the other hand, SPR does not inform about contributions of ΔH or ΔS or about stoichiometries.
- Both require rather high amount of reagents…

Assessing molecular interactions: **Microscale Thermophoresis**

- Recently, novel technique developed, novel approach to answer the same/similar questions.

Protein-binding assays in biological liquids using microscale thermophoresis

Christoph J. Wienken[1], Philipp Baaske[1,2], Ulrich Rothbauer[3], Dieter Braun[1] & Stefan Duhr[1,2]

- Sample-efficient, free-solution (vs. immobilised) method.

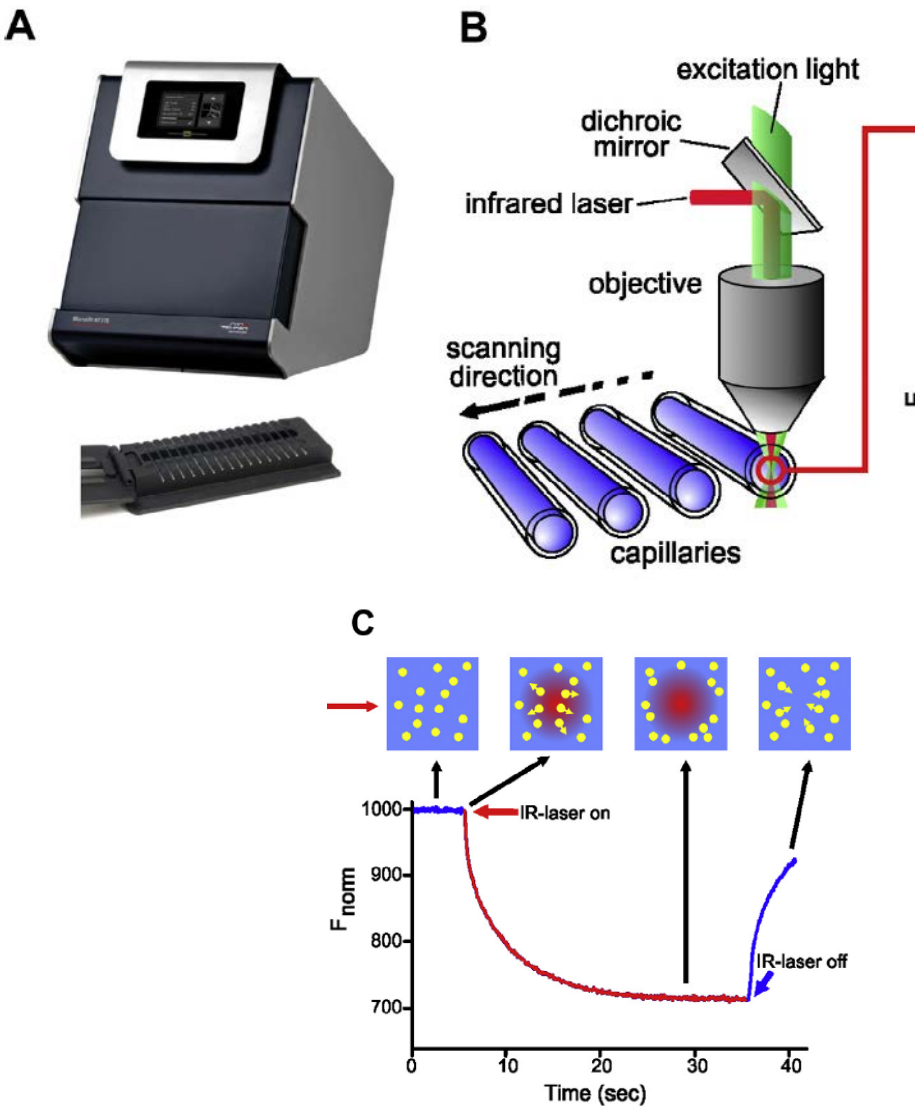
- Thermophoresis of molecule provides information about molecule size, charge and hydration shell.

What is **Microscale Thermophoresis**?

- Thermophoresis: Directed motion of molecules induced by temperature gradient.
- Tool to characterise protein and small-molecule interactions in buffer and biological liquids.
- Optical method (as is SPR).
- Assay development very simple.
- Preparation very simple.

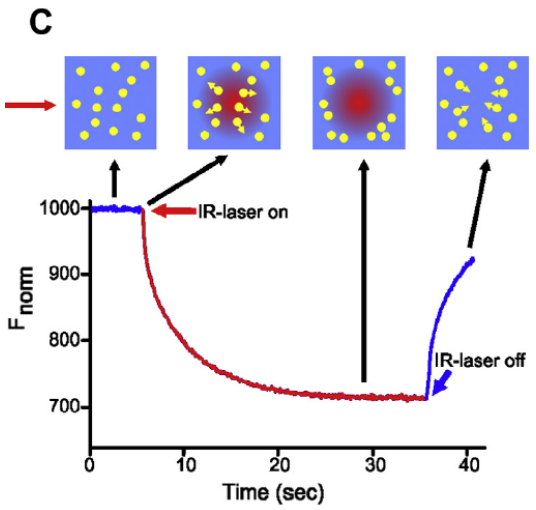
Microscale Thermophoresis: Experimental setup

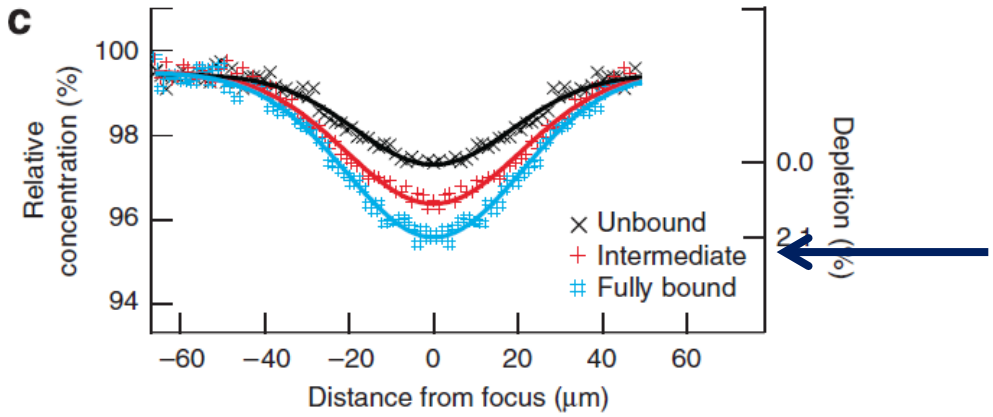
- Infrared laser coupled into path of fluorescent excitation/emission using infrared dichroic mirror.
- 100 um diameter glass capillaries require a maximum of 500 nL sample volume.
- Infrared laser creates spatial temperature distribution at the length of capillary.
- Temperature rise induces **spatial concentration distribution** visualised by fluorophore covalently linked to antigen via a primary amine on a lysine, e.g. (statistical distribution of dye).



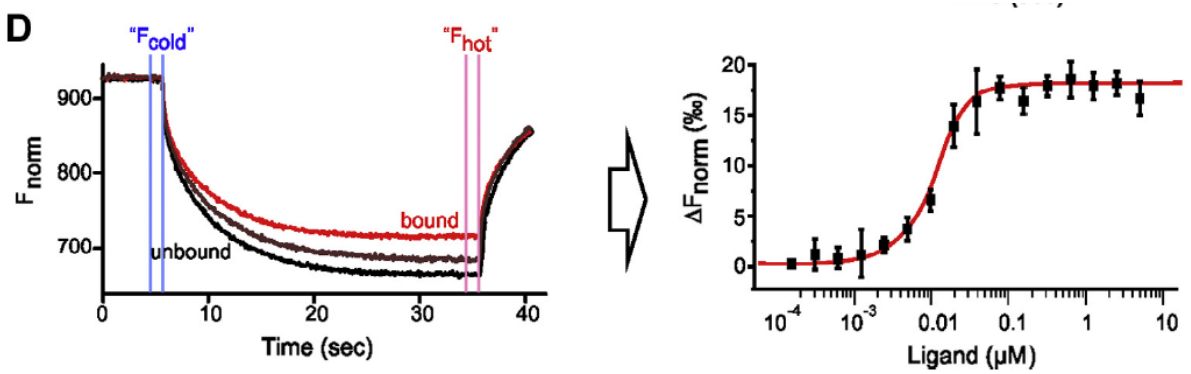
Microscale Thermophoresis: Experimental setup

- Measure change in concentration between initial state and steady state.
- Use various concentration ratios.





[antibody]



ΔFnorm = Fhot/Fcold

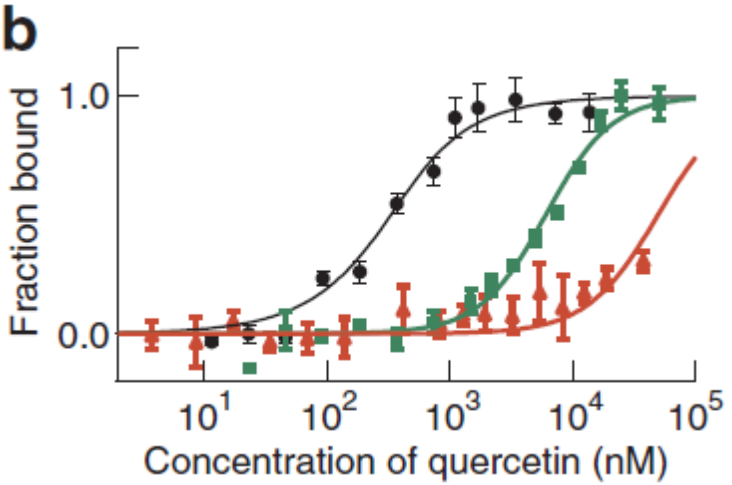
Microscale Thermophoresis: Competitive binding in serum

- Analyses of protein interactions typically performed in buffered salt solutions.

- However, lack components present in biological liquid (e.g. serum).

→ Test in serum might be important to take the right decision in drug development.

- Quercetin binding to cAMP-dependent PKA titrated in sample buffer (black) 5% serum (green), 30 % serum (red).
- Kd (black) = 0.13 (0.04) uM
- Kd (green) = 6 (0.4) uM
- Kd (red) = 50 (7) uM



Microscale Thermophoresis: Competitive binding in serum

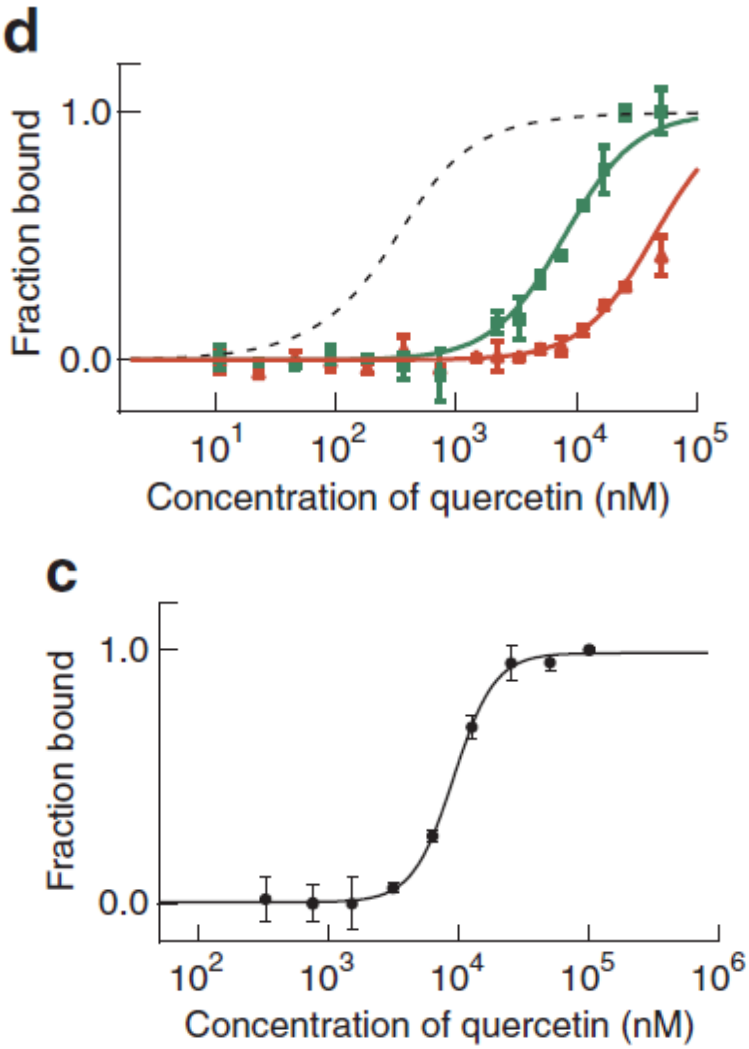
- Experiment was repeated in sample buffer with addition of human serum albumin (HSA) reflecting concentration in 5% or 30% serum.

- Kd (green) = 7.8 (0.6) uM
- Kd (red) = 43 (6) uM

→ Affinity in serum is lost due to binding to HSA.

- HSA was labelled and quercetin was titrated → Kd = 9.3 (0.5) uM.

→ The most abundant protein of serum influences the Kd of a seemingly unrelated molecule!



Microscale Thermophoresis: Interaction analysis and beyond

MicroScale Thermophoresis: Interaction analysis and beyond ☆

Moran Jerabek-Willemsen [a], Timon André [a,b], Randy Wanner [a,c], Heide Marie Roth [a], Stefan Duhr [a], Philipp Baaske [a], Dennis Breitsprecher [a,*]

- Determining binding affinities is not the only thing MST does.
- When acqueous solution is heated locally, molecules start moving along temperature gradient, opposing mass diffusion, resulting in stationary spatial concentration distribution:

$$c/c_0 = \exp[-S_T{}^*(T - T_0)]$$

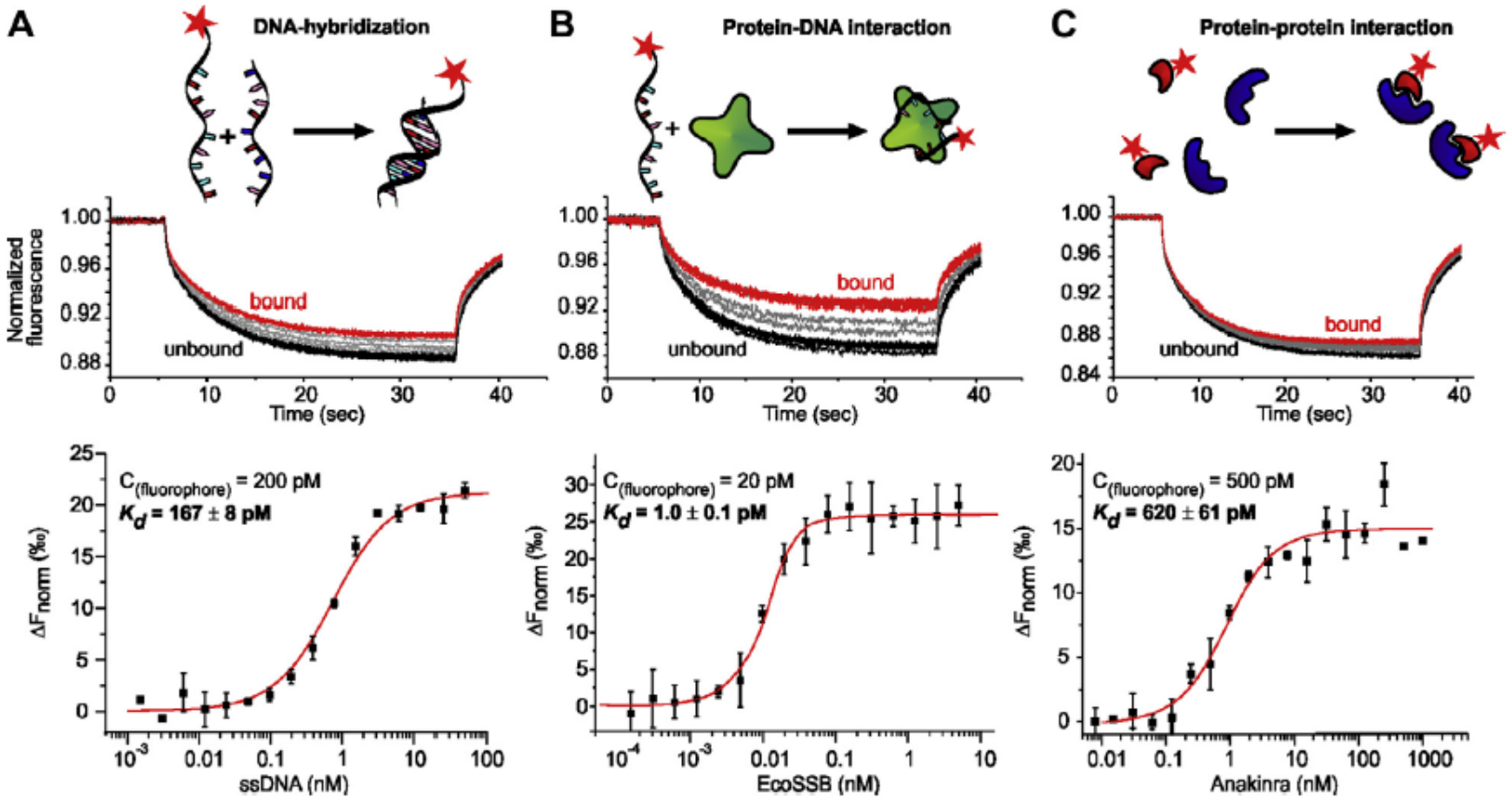
- Relative concentration depends solely on Soret coefficient S_T and ΔT.
- Soret coefficient:

$$S_T = \frac{A}{kT}\left(-\Delta s_{\mathrm{hyd}}(T) + \frac{\beta\sigma_{\mathrm{eff}}^2}{4\varepsilon\varepsilon_0 T} \times \lambda_{\mathrm{DH}}\right)$$

- This includes surface area, hydration entropy, effective charge, etc., all of which influence the Soret coefficient.

Microscale Thermophoresis: Interaction analysis and beyond

- A: Hybridisation of 16mer with complementary Cy5-labeled DNA strand.
- B: E. coli single-strand binding protein (EcoSSB) with Cy5-labeled oligo.
- C: Anakinra (IL-1R antagonist) with IL-1R labeled with NT-647.

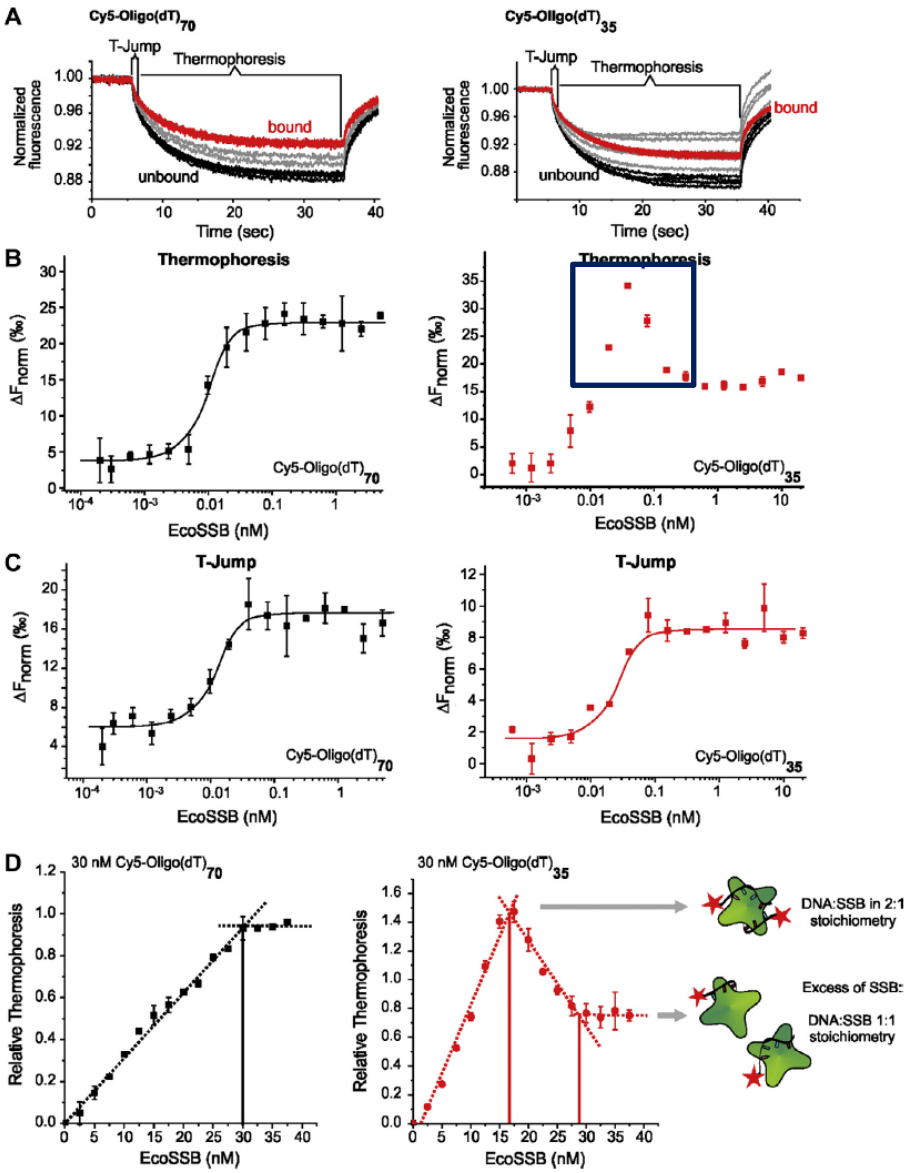


Microscale Thermophoresis: Interaction analysis and beyond

- Repeat EcoSSB to labelled oligo.
- Left: Oligo (dT)70, right: Oligo (dT)35.
- T-Jump: Local surroundings of fluorophore. Thermophoresis: Global properties of entire molecule/complex.
- Assess difference of thermophoretic behaviour: Saturation experiment from 0 – 40 nM.

→ SSB completely bound to Oligo until saturation occurs, linear increase.

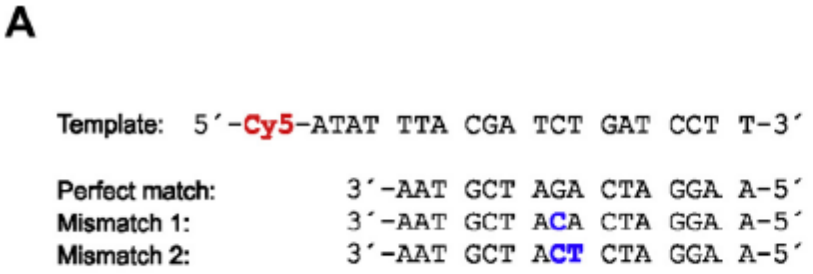
→ (dT)70 with 1:1 stoichiometry, (dT)35 with 2:1, at excess of SSB with 1:1 stiochiometry.

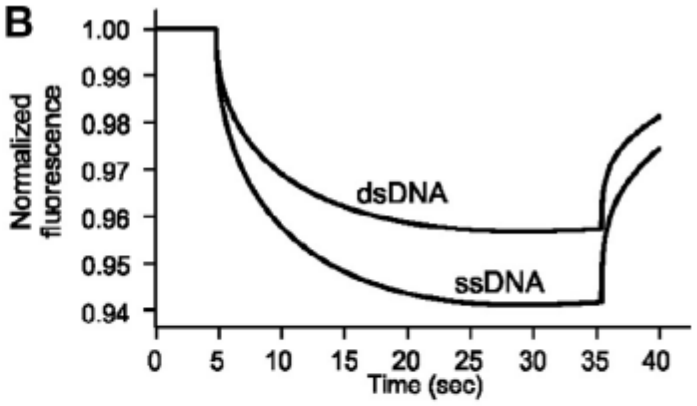


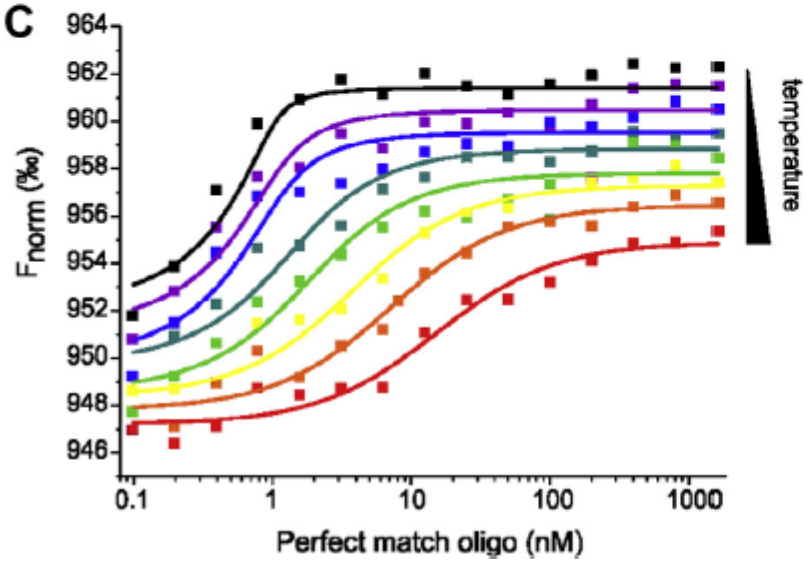
Microscale Thermophoresis: Interaction analysis and beyond

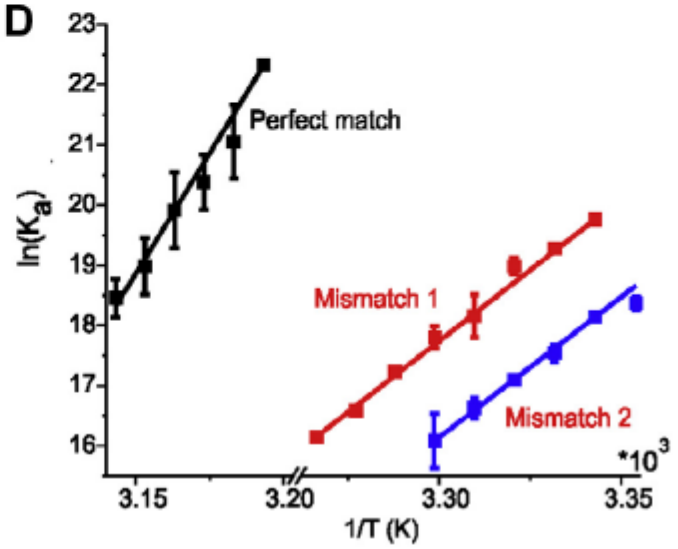
- MST can yield binding affinities and stoichiometries.
- What else?
- We saw that $\Delta G = RT \ln(K_D)$ and $\Delta G = \Delta H - T\Delta S$
- By measuring K_D over temperature range, ΔG, ΔH and ΔS can be calculated.

Microscale Thermophoresis: Interaction analysis and beyond









$$\ln(K_a) = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

Microscale Thermophoresis: Interaction analysis and beyond

E

	Experimental		Calculated (IDT biophysics software)	
	ΔH (kcal/mol)	ΔS (cal/K*mol)	ΔH (kcal/mol)	ΔS (cal/K*mol)
PM	-158.1	-460.6	-146.2	-421.9
MM1	-93.8	-274.1	-94.9	-276.4
MM2	-92.8	-274.3	-116.7	-333.4

Microscale Thermophoresis: Summary

- MST is an ultrasensitive method offering atomic resolution.
- It allows the detection of kinetic as well as thermodynamic parameters.
- MST might be an alternative to SPR and ITC.
- Advantages: Higher-throughput, less sample volume, unproblematic assay development, versatile.
- Will it replace SPR and ITC?

Summary of the three methods presented

	Surface Plasmon Resonance	Isothermal Titration Calorimetry	Microscale Thermophoresis
Detection system	Optical (alternative methods such as ultrasound exist)	Heat generated/absorbed	Fluorescence
Sample load	medium (ca. 200 uL)	high (mL range)	low (max 0.5 uL/capillary)
Sensitivity	lower nM	ca. 10 nM	lower pM
Kinetic measurement	Yes	No	Yes
Thermodydnamic measurement	No	Yes	No
Inference of kinetics	Yes	No	Yes
Inference of thermodynamics	No	Yes	Yes
Binding affinity	Yes	Yes	Yes
Inference of stoichiometry	No	Yes	Yes

Thank you for your attention!

Questions?