UNIVERSITY OF CAMBRIDGE

Biophysics of protein interactions: Measuring affinities

Day 7: Tuesday 28th March

This talk

- Protein interaction affinities
  - Equilibrium and kinetic models
- Kinetic measurement
  - Surface Plasmon Resonance (SPR, Biacore)
- Equilibrium measurement
  - SPR
  - Isothermal Titration Calorimetry (ITC)
  - Fluorescence polarisation

Protein association

$$A + B \rightleftharpoons AB$$

Protein association

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$$K_D = \frac{[A] \cdot [B]}{[AB]}$$

Protein association

- *K_D is the concentration at which the binding is half-saturated*
- If [A] << K_D then [A] = [AB] when [B] = K_D
- K_D is units of concentration (M)

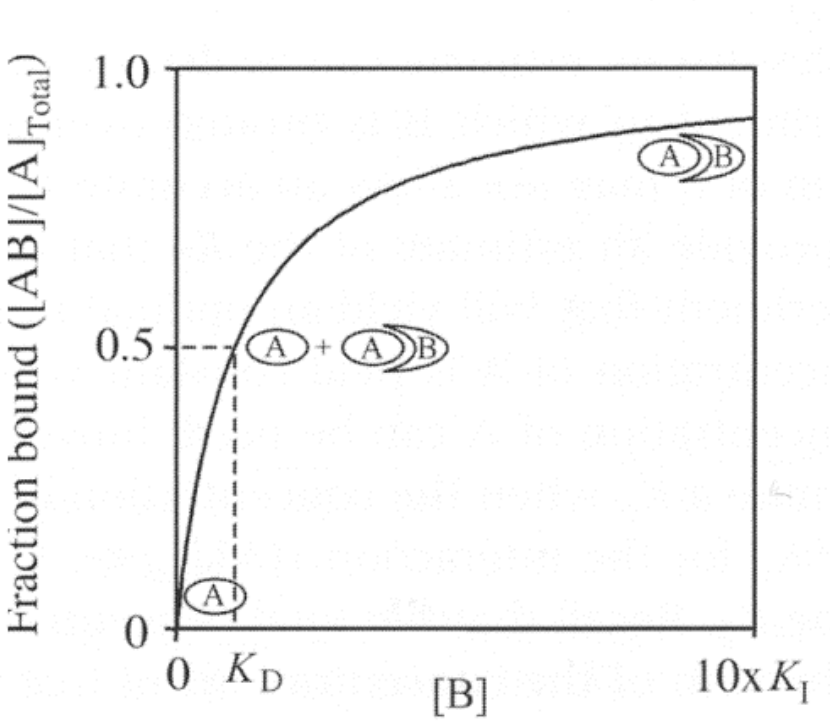

Image: Goodrich and Kugel (2006) Binding and Kinetics for Molecular Biologists, CSHP

Protein association

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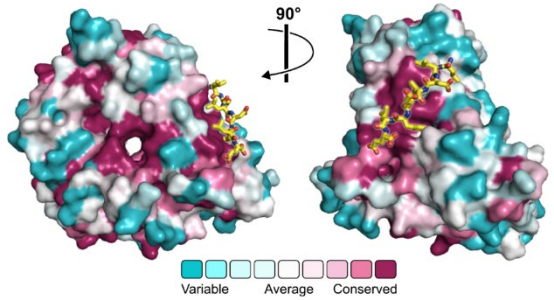
$$K_D = \frac{k_{off}}{k_{on}} \frac{(s^{-1})}{(M^{-1}s^{-1})}$$

What does this mean?

Interaction affinity	Interaction half-life
1 mM	0.7 ms
1 uM	0.7 s
1 nM	11.5 min
1 pM	8 days

Assuming k_{on} is approximately diffusion limited ($\sim 10^6$–10^7 $M^{-1}s^{-1}$)

What does this mean?



Clathrin:peptide

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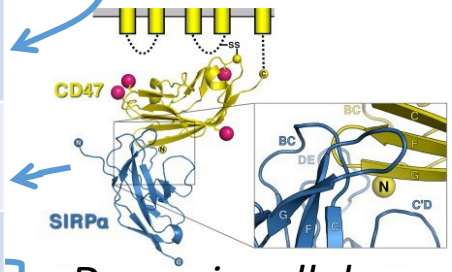
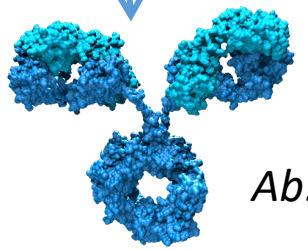


Dynamic cellular interactions



Ab:antigen

Why measure affinities?

- To understand the dynamics of the interaction
- To compare interactions under different conditions
  - Post-translational modifications
  - Different physiological conditions
  - Cooperativity
- To prove mutations disrupt interaction

How do you measure affinities?

- Kinetic measurements
  - Surface plasmon resonance
  - Bio-layer interferometry
- Thermodynamic (equilibrium) measurements
  - Surface plasmon resonance
  - Fluorescence polarisation
  - Isothermal titration calorimetry
  - Microscale thermophoresis
  - Composition-gradient multi-angle light scattering
  - …and more!

$$K_D = \frac{k_{off}}{k_{on}}$$

$$K_D = \frac{[A] \cdot [B]}{[AB]}$$

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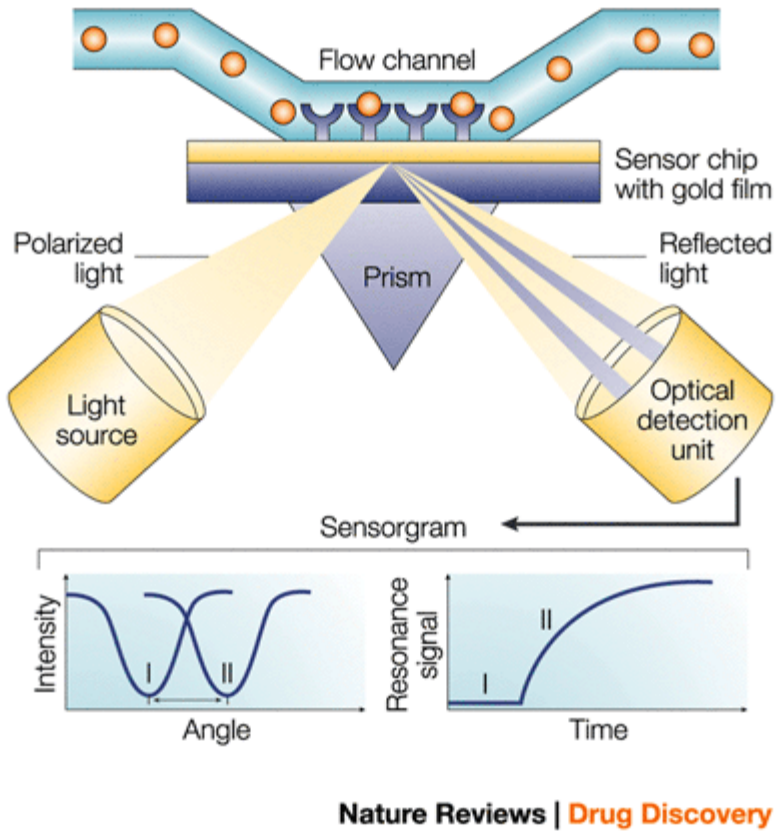
Surface plasmon resonance (SPR)

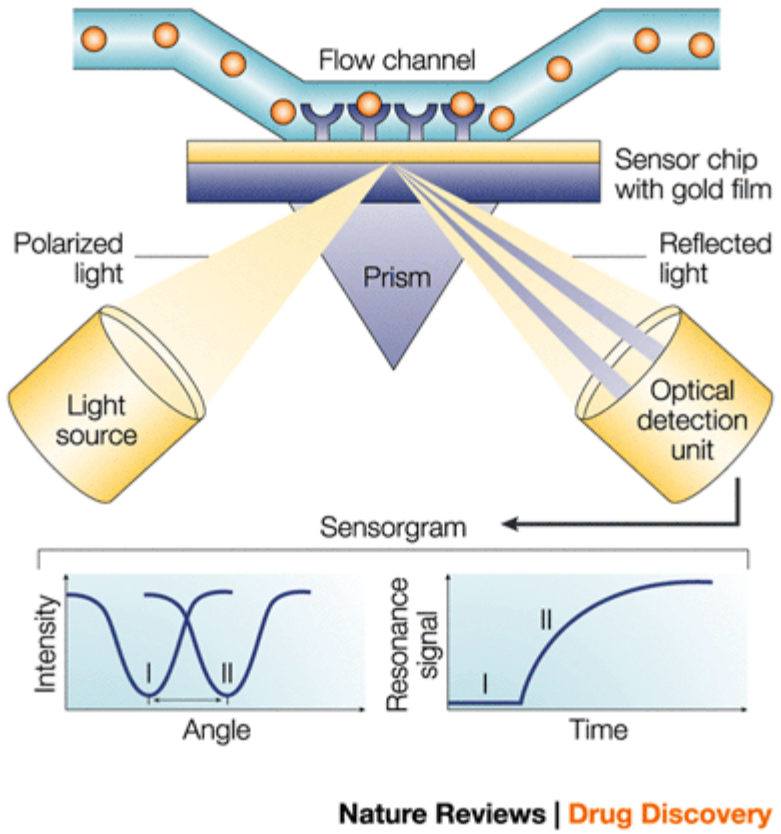
- Evanescent wave excites surface plasmon of gold-coated surface
  - Disperses energy, yielding ‘dip’ in reflected light



Cooper (2002) *Nature Reviews Drug Discovery* 1: 515-28

Surface plasmon resonance (SPR)

- Evanescent wave excites surface plasmon of gold-coated surface
  - Disperses energy, yielding ‘dip’ in reflected light
  - Change in mass within evanescent field changes resonance (‘dip’ angle)
  - This is reported at the SPR response (in RU)



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SPR surfaces

- SPR surface is a dextran hydrogel on a thin gold layer
- The dextran is ***functionalised*** to enable the capture of the ligand
- Molecules that are captured on the SPR surface are called ***ligands***
- Molecules that interact with the ligands during the experiment are called ***analytes***

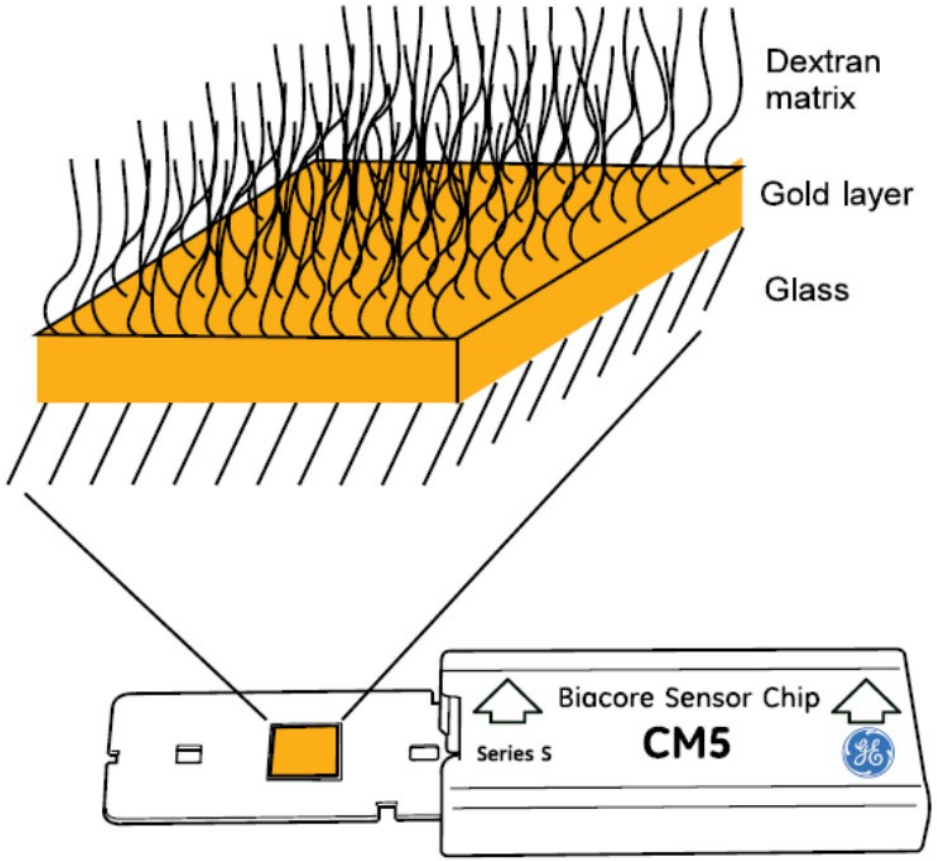


Image: Cytiva

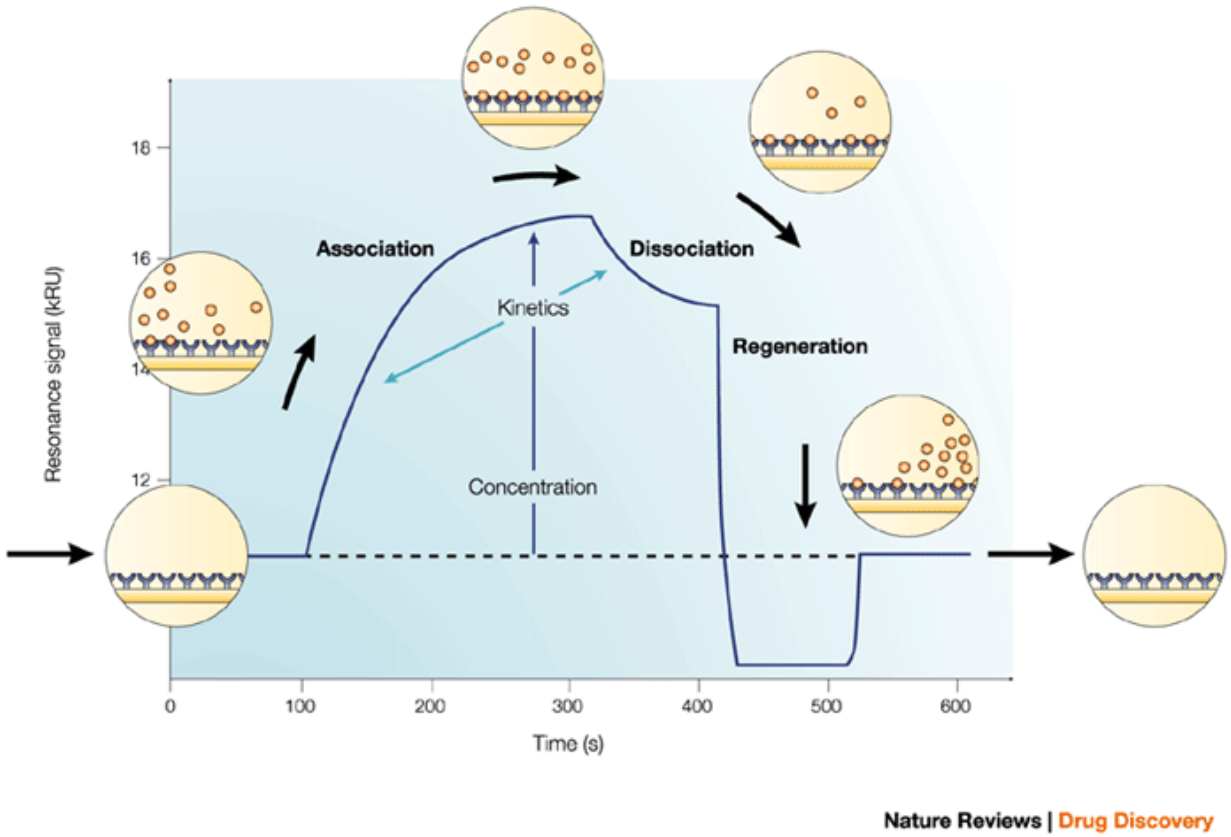
SPR experiments

- SPR instruments use computer controlled pumps and microfluidics to control the flow of ligand, analyte and buffer onto the surface
- T200 instrument (shown) has four flow cells on the one surface
  - Can capture ***different ligands*** on the four surfaces
  - Can flow the ***same analyte*** over all surfaces, to measure relative changes in SPR response (binding)

Image: Cytiva

SPR sensorgrams

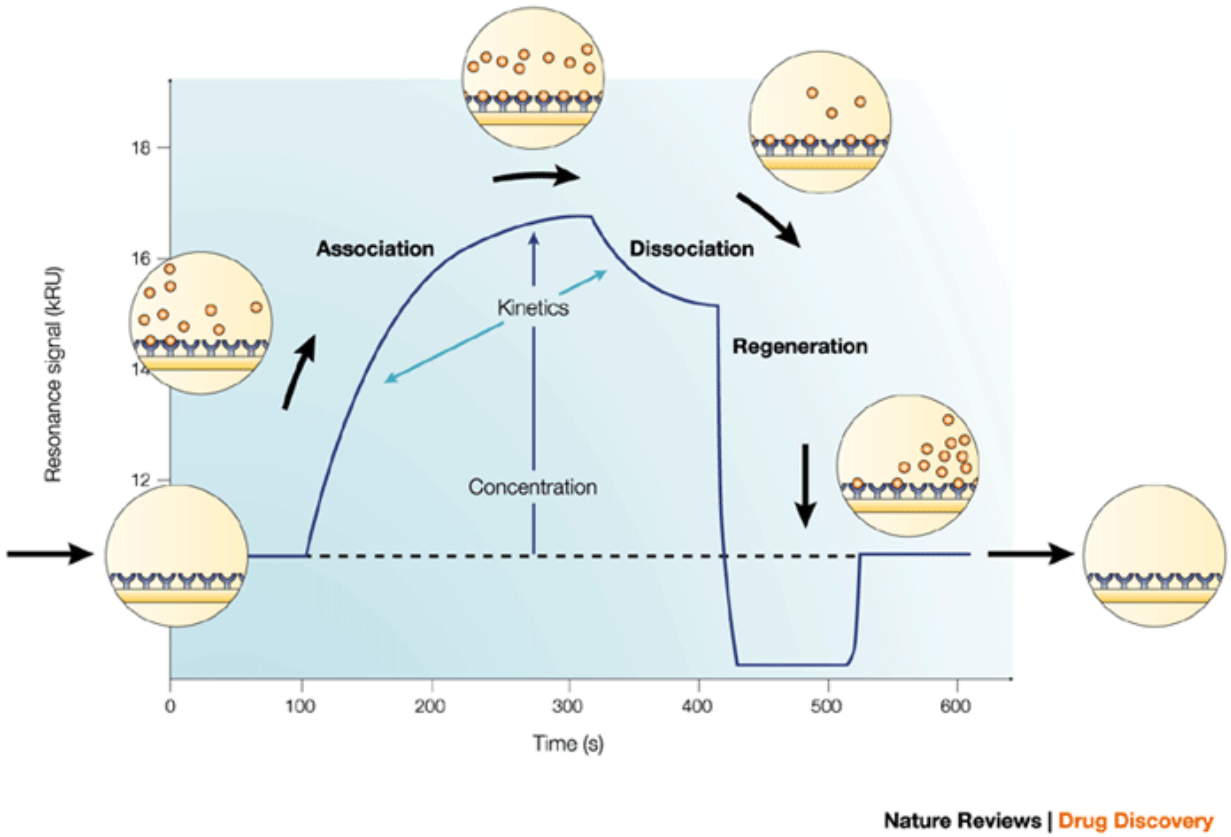
- Capture ***ligand*** on the surface
- Flow ***analyte*** over the surface and measure change in SPR signal (RU)
  - ***Association phase***
- Flow buffer over the surface and measure change in SPR signal (RU)
  - ***Dissociation phase***
- ***Regenerate*** surface to remove residual analyte
  - low pH, high pH, salt, detergent



Cooper (2002) *Nature Reviews Drug Discovery* 1: 515-28

SPR sensorgrams

- The height of the sensorgram is related to the mass of analyte that associates with the surface
  - 1000 RU = 1 ng/mm^2 = 10 mg/mL



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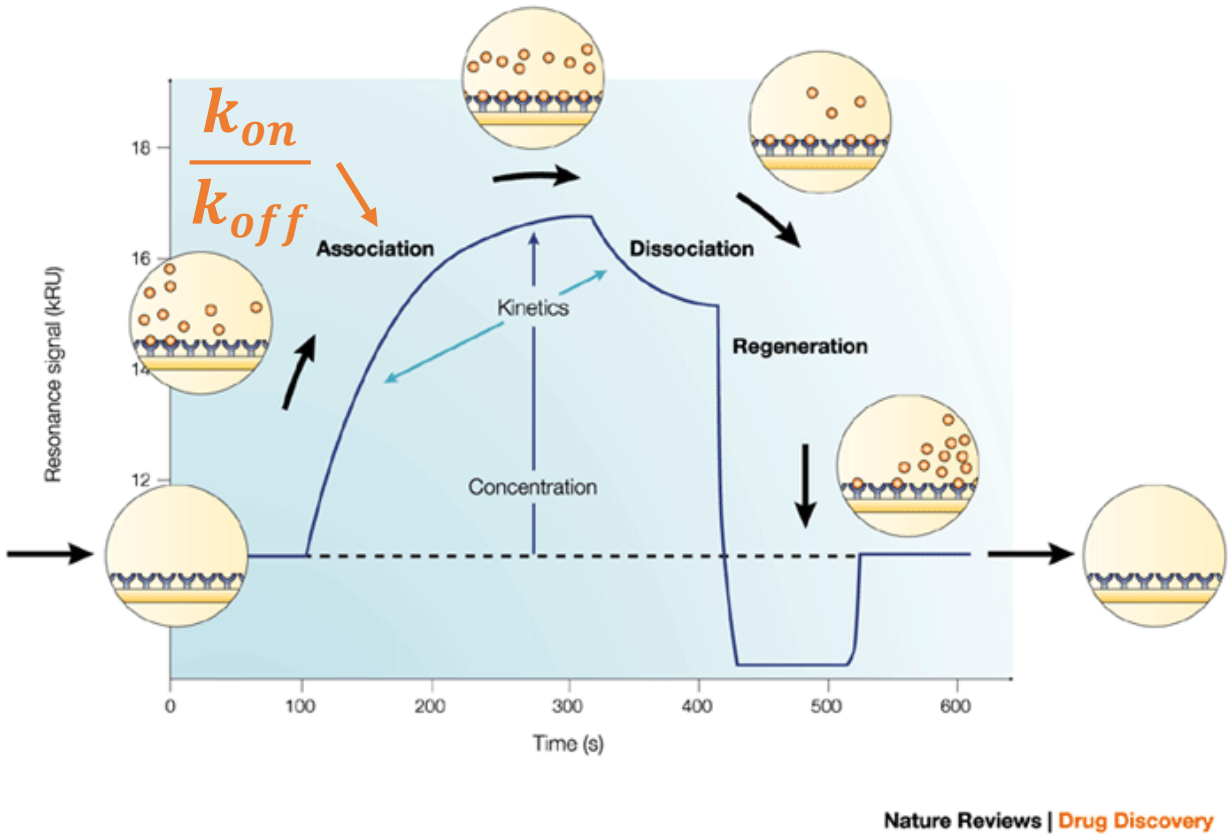

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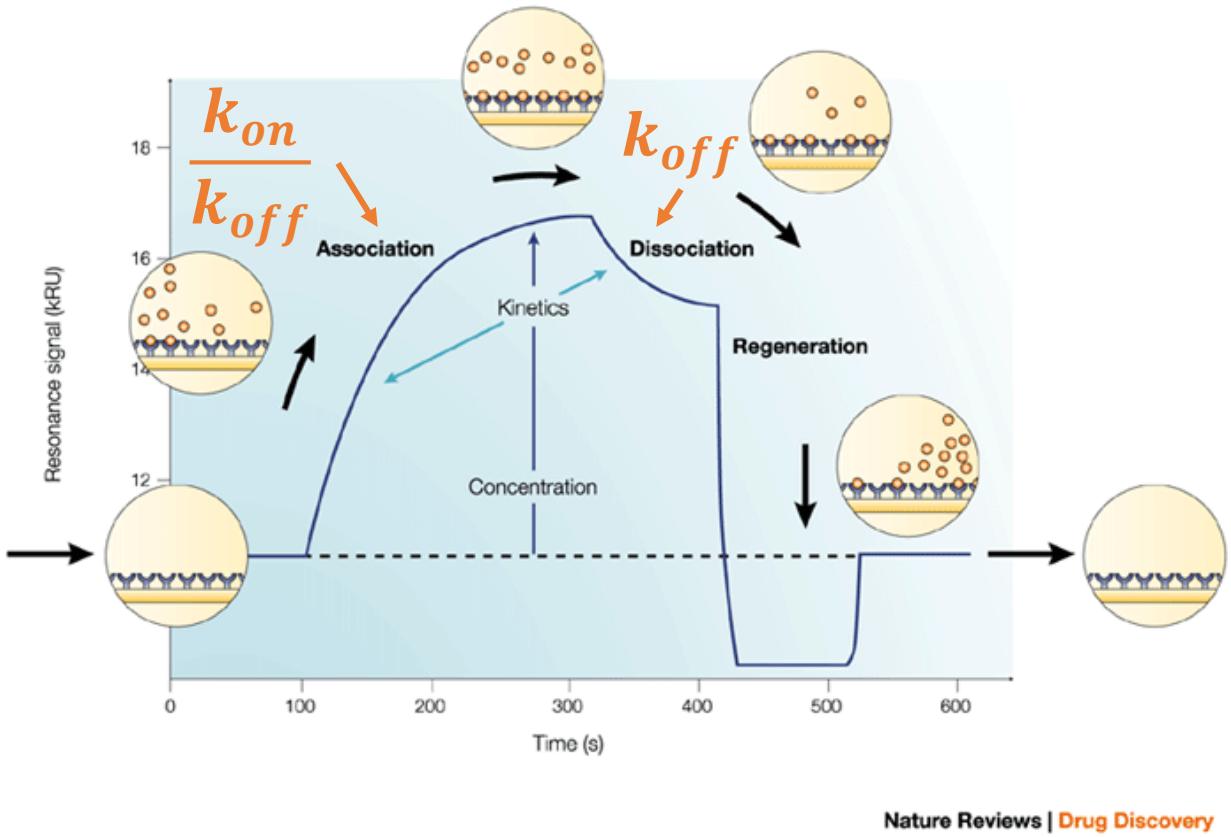
- Shape during the dissociation phase is determined by the dissociation rate (k_{off})



Cooper (2002) *Nature Reviews Drug Discovery* 1: 515-28

Ligand capture chemistry – Carboxymethyldextran (CM)

- Chemicals EDC and NHS activate the carboxyl groups on CM surface
- Amine groups on the ligand (lysine side chains and N terminus) form covalently bonds with CM
- Ligand is captured in random orientation
- Can get surfaces with different CM chain lengths
  - CM7 > CM5 > CM3
- Can capture with other chemistries (e.g. cysteine residues via thiols)

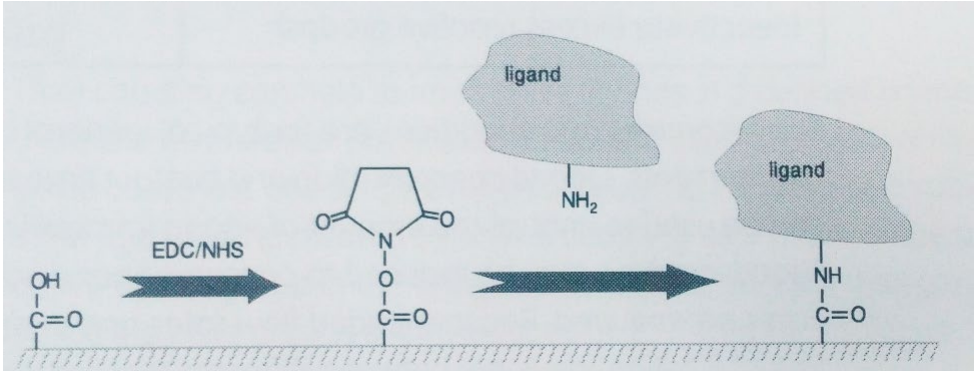
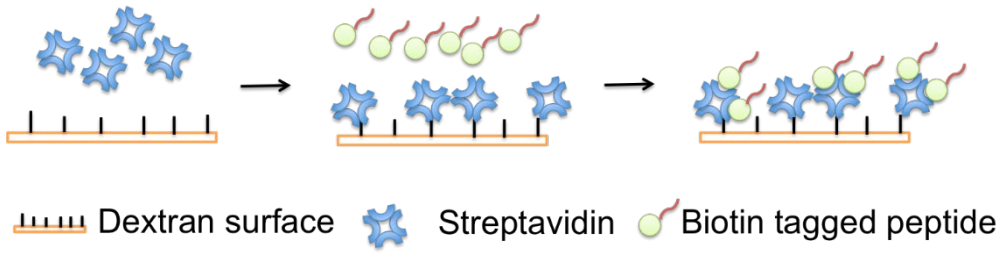


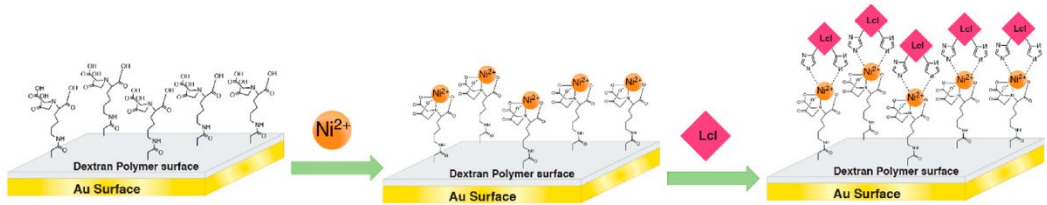
Figure 4-5. Amine coupling of ligands to the sensor surface.

Image: Cytiva

Directional capture – streptavidin and NiNTA

- When ligands are captured chemically:
  - Randomly orientated, so a proportion may have their analyte binding surface blocked
  - Coupling process is harsh and can damage ligands
- Ligands can be capture using tags:
  - Highly specific
  - Defined orientations may prevent blocking of analyte-binding sites
- SA surfaces have covalently associated streptavidin, capture biotinylated ligands
  - You can make your own using CM5 surfaces and purified streptavidin
- NTA surfaces can bind His-tagged proteins





Images: Biosensing instruments USA; Su et al. (2018) *Sensors* 18: 2668

Other capture technologies

- Lipophilic surfaces (L1) allow the study of membrane (liposome) binding

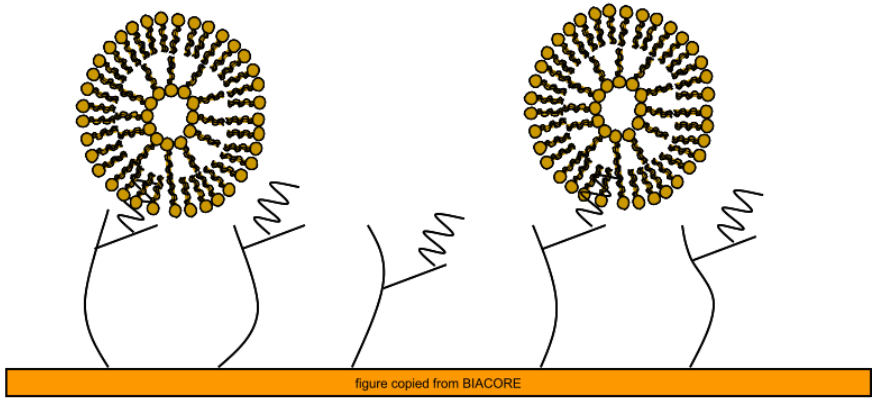


Image: sprpages.nl

Other capture technologies

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- Antibodies for directional capture
  - Capture antibody on CM5 surface then capture antigen as ligand and flow over analyte
  - Useful if regeneration conditions prove difficult to optimise

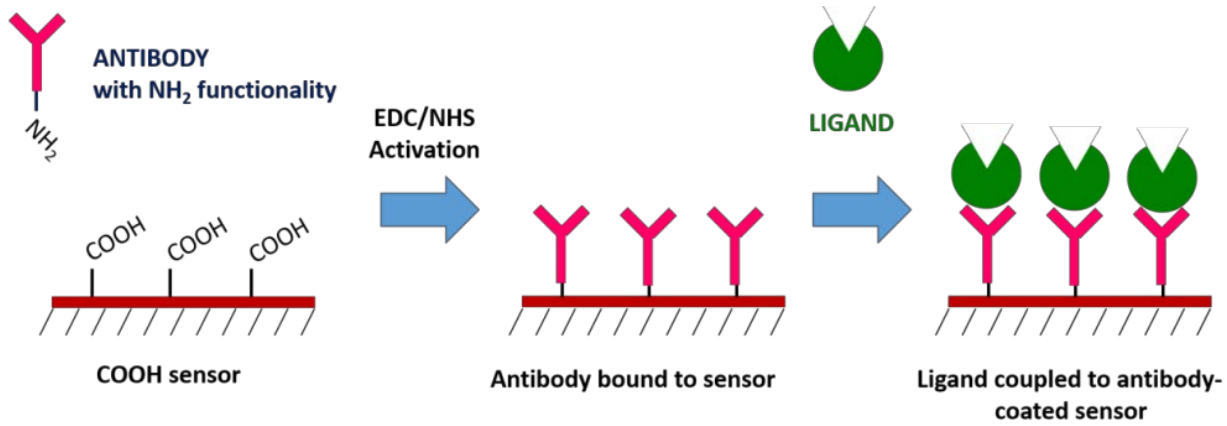

Image: https://nicoyalife.com/blog/spr-sensor-guide/

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  - Useful if regeneration conditions prove difficult to optimise
- Biotin CAPture
  - Streptavidin binds surface via DNA hybridisation, binds biotinylated ligand
- And many more…

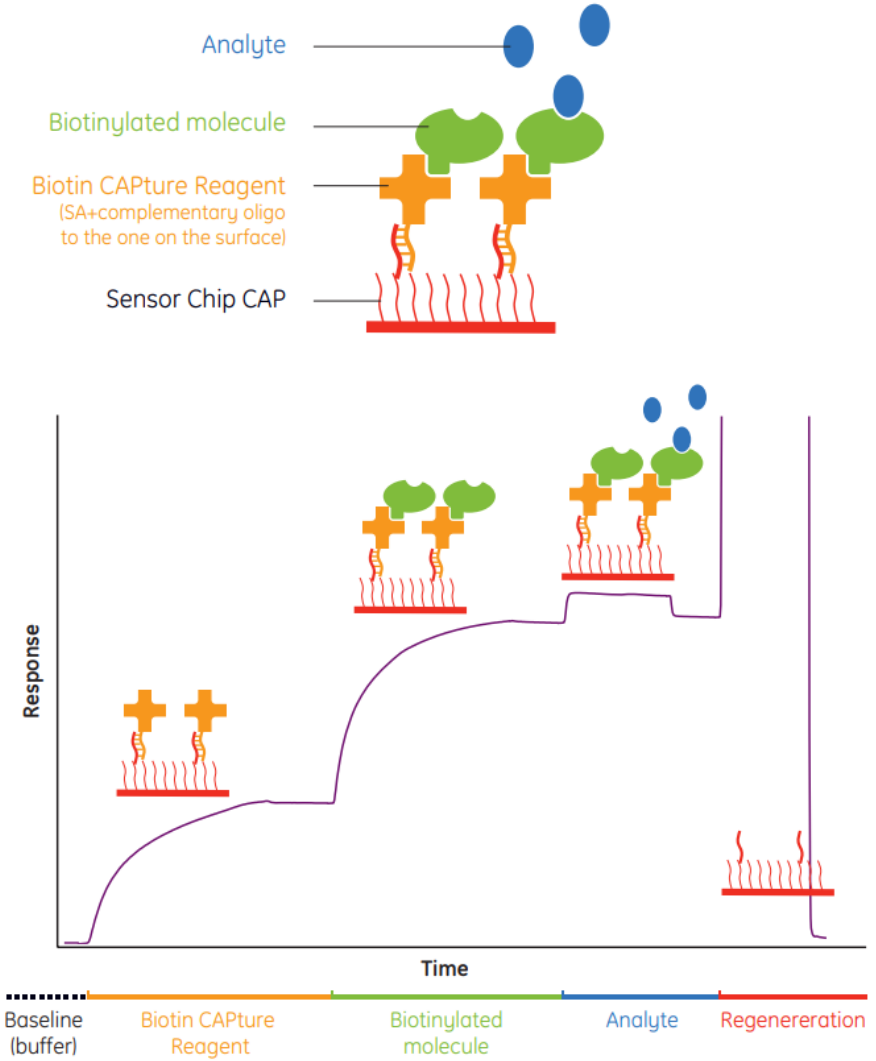
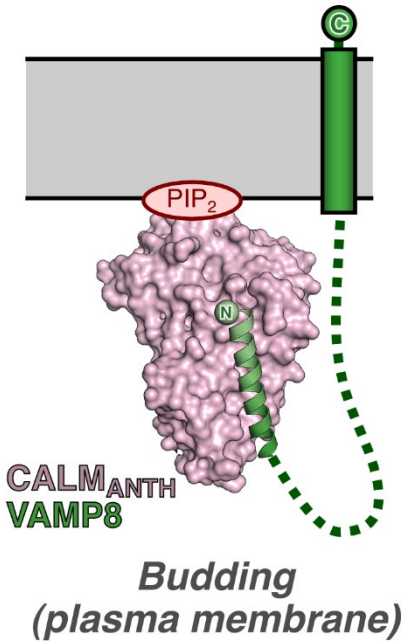
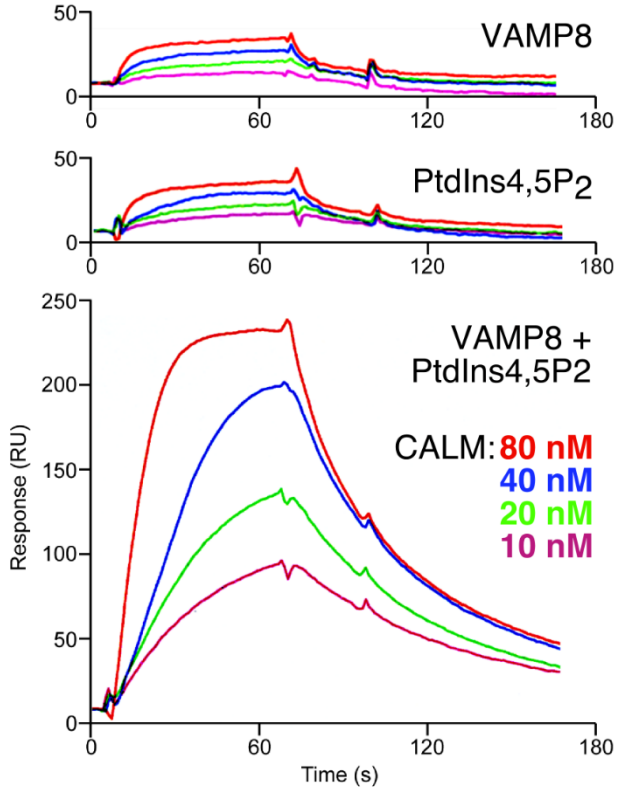


Image: Cytiva

Coincident detection of proteins and phosphoinositides on membranes





Miller et al. (2011) *Cell*, 147: 1118–31

The challenges with SPR

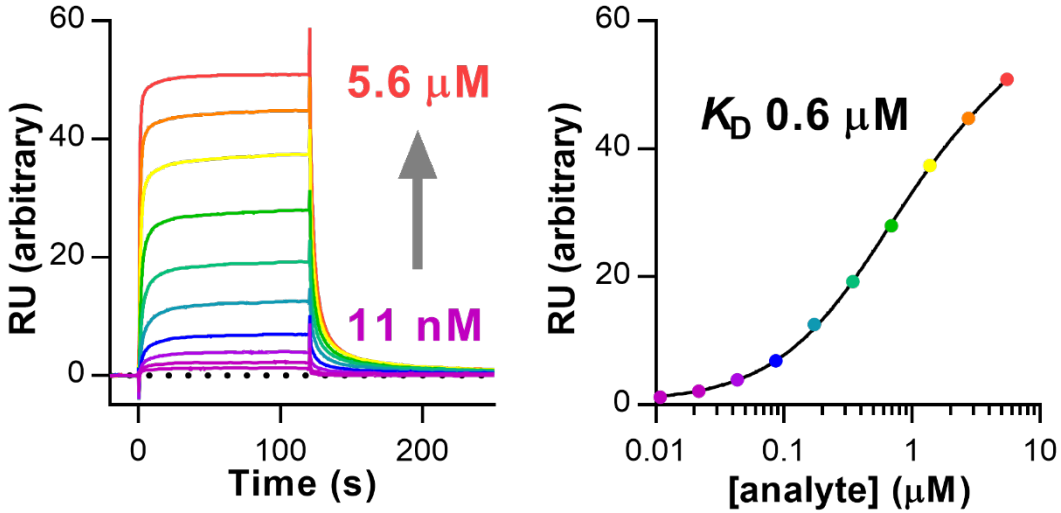
- Optimising capture
  - Capture conditions can denature ligand
  - Need low capture to minimise rebinding and mass transport
- Optimising regeneration
  - Incomplete regeneration means analyte stays bound, lowers effective [ligand]
  - Too harsh regeneration denatures ligand, lowering effective [ligand]
- Works great for antibody:antigen interactions, but optimising conditions for other assays can be challenging

This talk

- Protein interaction affinities
  - Equilibrium and kinetic models
- Kinetic measurement
  - Surface Plasmon Resonance (SPR, Biacore)
- Equilibrium measurement
  - SPR
  - Isothermal Titration Calorimetry (ITC)
  - Fluorescence polarisation

SPR can also be used for equilibrium measurements

- Measure increase in total response (RU) with increasing [analyte]
- Plot increase in RU as function of the [analyte] to determine K_D
  - Don't measure k_{on} or k_{off}
- Essential that injections reach equilibrium
  - Easiest to achieve for lower affinity (high nM to µM affinity)



Isothermal titration calorimetry (ITC)

- Label-free analysis
- Tells you enthalpy, stoichiometry and affinity of interactions
  - Can work out free energy
- Gold standard for protein:protein interactions

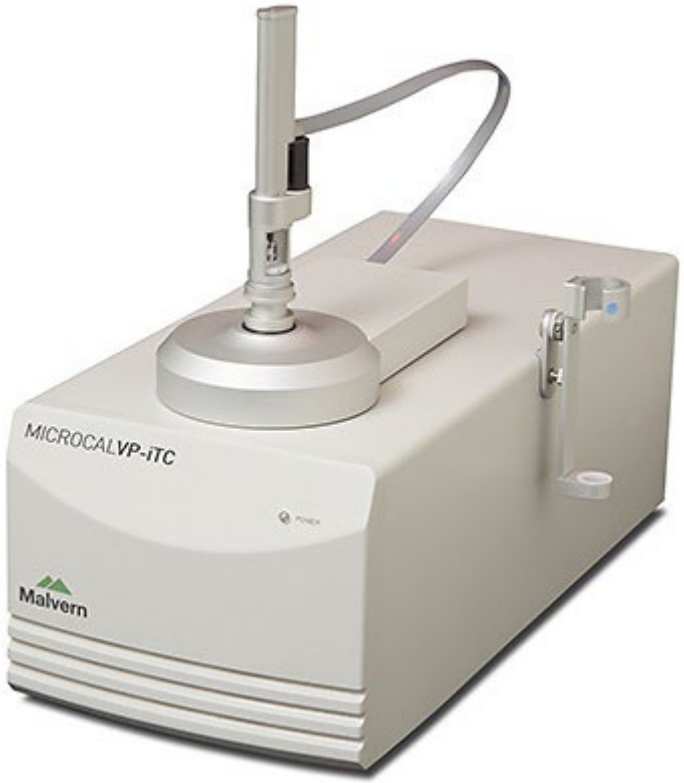


Image: Malvern Panalytical

Gibbs free energy (G)

- A measure of the thermodynamic potential of a system
- The thermodynamic potential (G) is minimized when a system reaches chemical equilibrium
- A process will be **favourable** (**spontaneous**) if it decreases the Gibbs free energy of the system (**if $\Delta G < 0$**)

Gibbs free energy (G)

- The Gibbs free energy of at any moment is defined as the enthalpy of the system minus the temperature times the entropy of the system

$$\Delta G = \Delta H - T\Delta S$$

ΔG = change in Gibbs free energy

ΔH = change in Enthalpy (Heat)

ΔS = change in Entropy (Disorder)

Enthalpy (ΔH) is heat

- Caused by formation or breaking of bonds
- If a reaction is **exothermic** then ΔH is negative*
- If a reaction is **endothermic** then ΔH is positive*

*Assuming constant pressure

Photograph: kennardphillipps/Reuters

Entropy (ΔS) is disorder

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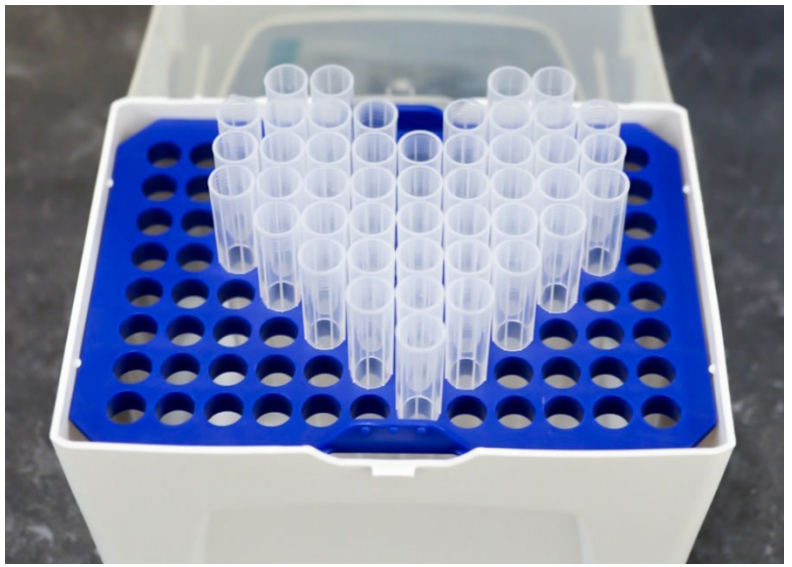


Going from disorder to order costs energy…

Change in Gibbs free energy (ΔG)

$$\Delta G = \Delta H - T\Delta S$$

- Making things more ordered ($\Delta S < 0$) cost energy, making things more disordered ($\Delta S > 0$) releases energy
- Exothermic reactions release energy ($\Delta H < 0$), endothermic reaction store energy ($\Delta H > 0$)

Gibbs free energy (G)

- The change in Gibbs free energy conferred by a binding interaction is related to the K_D of the interaction

$$\Delta G = RT \ln(K_D)$$

R = Gas constant, T = Temperature

ITC characterises thermodynamic properties of interactions

- Isothermal Titration Calorimetry
- Measure power required to keep a reference cell at the same temperature as the sample cell as we inject (titrate) an analyte into the sample cell
- Ligand is in the sample cell
- Analyte is injected into the cell as a series of short injections
- System returns to equilibrium between each injection

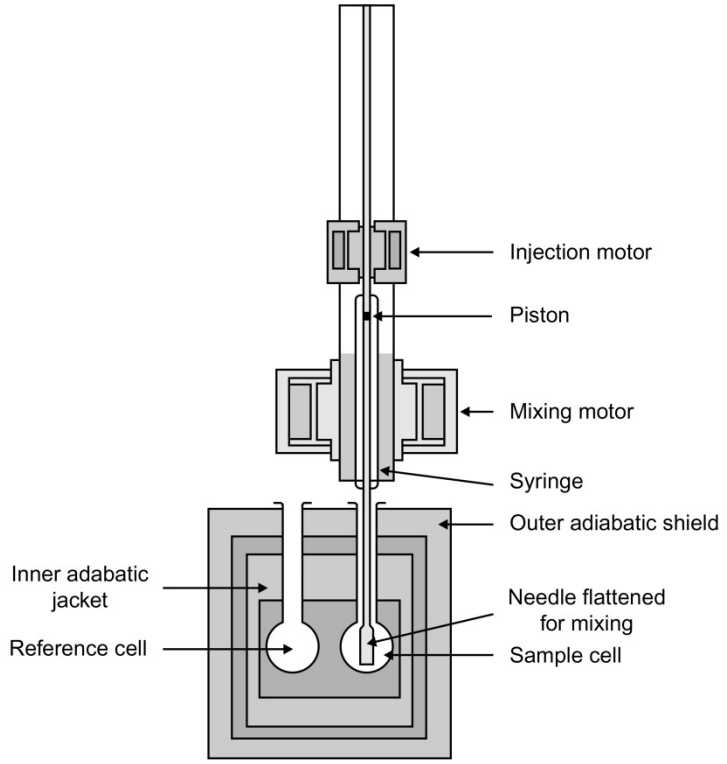


Image: Malvern Panalytical (?)

ITC measures heat evolved during binding interactions

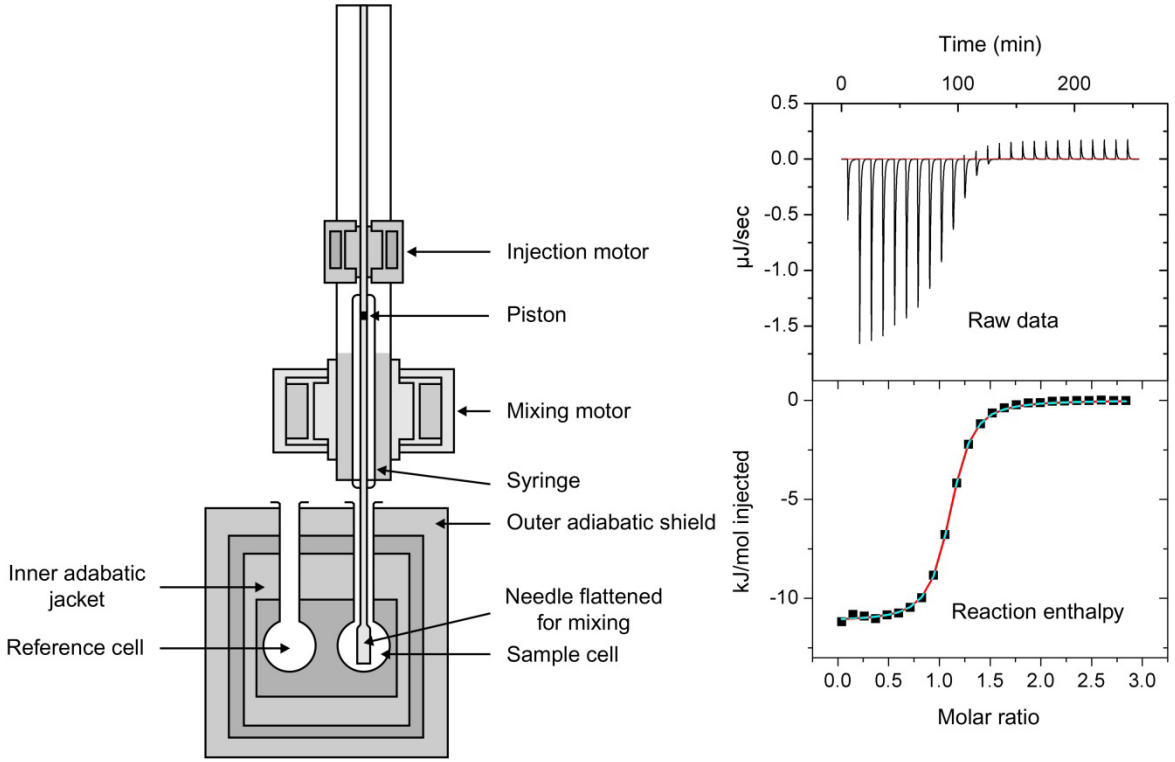


Image: Malvern Panalytical (?)

Results of ITC measurement

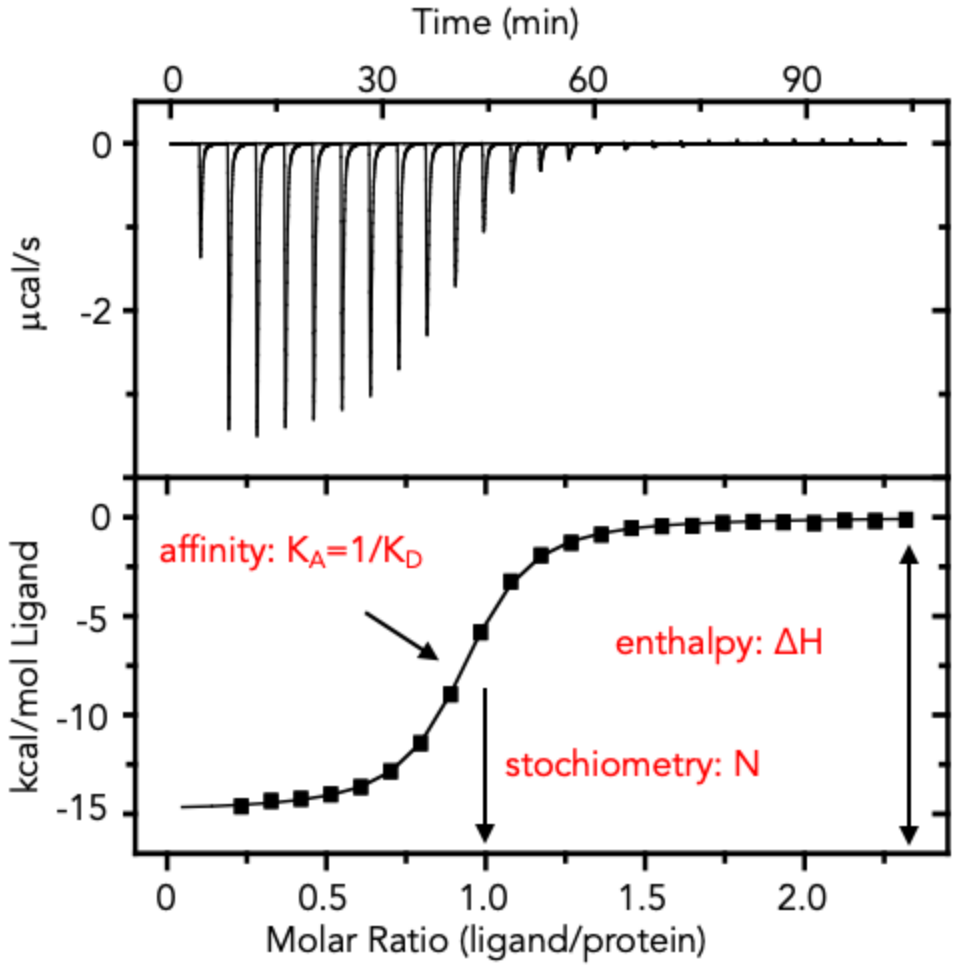


Image: https://cmi.hms.harvard.edu/isothermal-titration-calorimetry

The energetics (thermodynamics)

$$\Delta G = RT \ln K_D$$

$$\Delta G = \Delta H - T\Delta S$$

ΔG	Gibbs free energy
ΔH	Enthalpy
ΔS	Entropy
R	Gas constant = 1.985 cal K^{-1} mol^{-1}
T	Temperature in Kelvin
K_D	Equilibrium dissociation constant

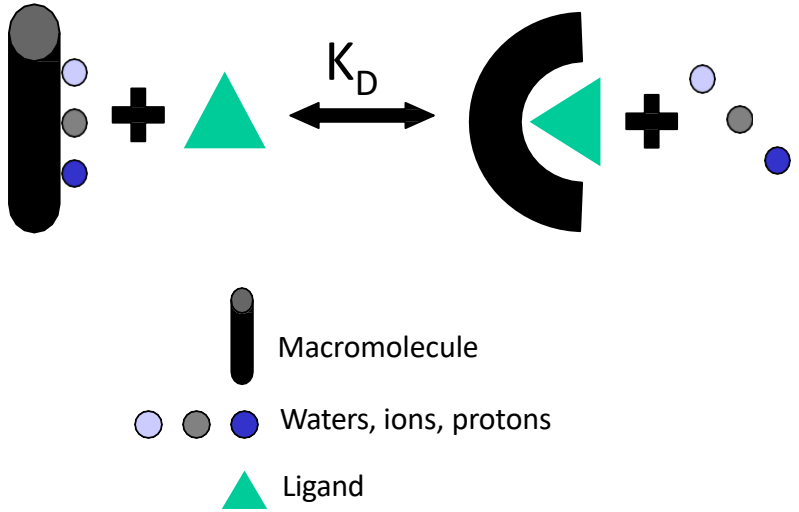
ΔH, ***change in enthalpy*** is indication of changes in hydrogen and van der Waals bonding

$-T\Delta S$, ***change in entropy*** is indication of changes in hydrophobic interaction and/or comformational changes

We measure ΔH and K_D directly in an ITC experiment

Elucidation of binding mechanism

- Enthalpic (ΔH) Contributions
  - Hydrogen bonding and van der Waals interactions
    - Solvent also plays a role
  - ΔH is negative for enthalpically driven reactions
- Entropic (ΔS) Contributions
  - Hydrophobic effect, water release (favourable)
  - Conformational changes and reduction in degrees of freedom (unfavourable)
  - ΔS is positive for entropically driven reactions (-TΔS)



Freire (2007) *Eur. Pharm. Rev.* 5: 73-78

Measuring the thermodynamics

A. Good hydrogen bonding with unfavourable conformational change
B. Binding dominated by hydrophobic interaction
C. Favourable hydrogen bonds and hydrophobic interaction

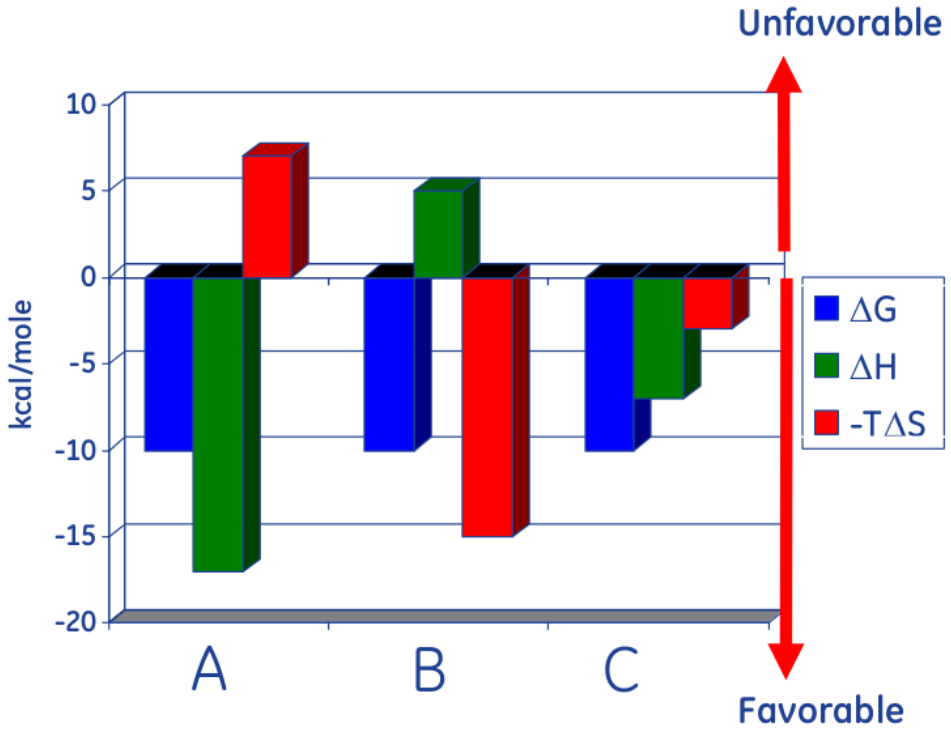


Image: Malvern Panalytical

ITC challenges

- Non-specific heat generation
  - Buffers must match absolutely
- Relatively large amounts of protein required
  - Less of an issue with new machines
- Low enthalpies evolved
  - Try changing the temperature of experiment
- Difficult/Labour intensive
  - Not with automated machines

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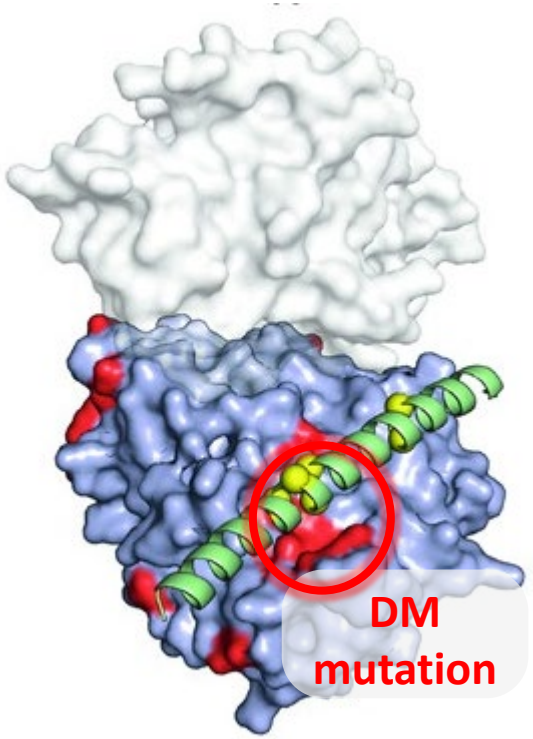
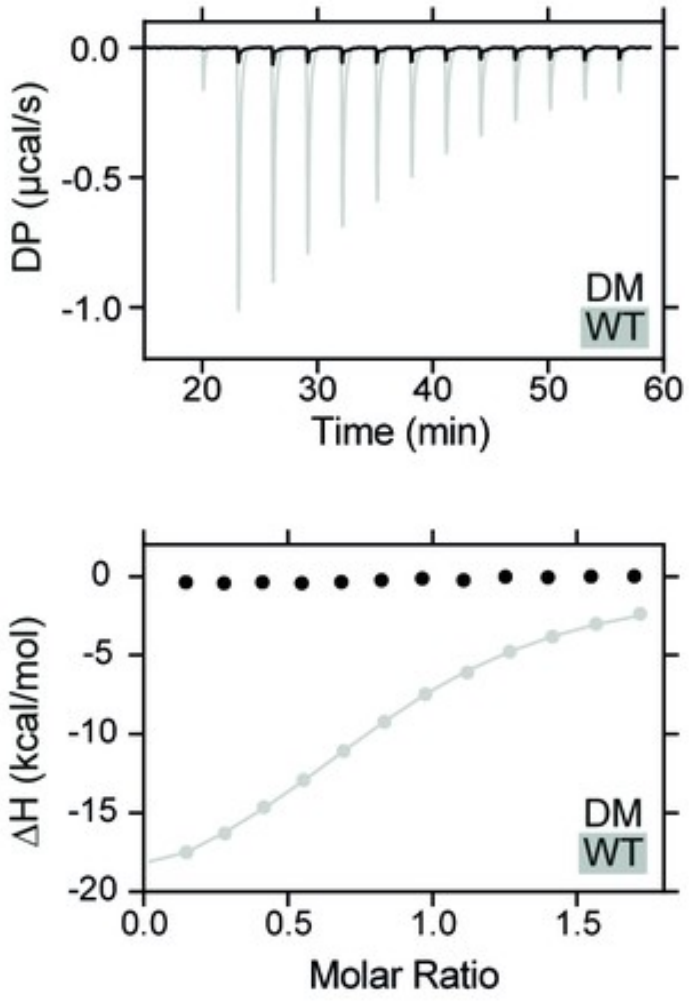
Image: Malvern Panalytical

ITC of mutant confirms PTPRK:Afadin interaction interface

- Afadin is an important cell adhesion molecule that is phosphorylated by PTPRK
- We mapped the interaction interface using biochemistry (pull-downs and IPs) and AlphaFold
- Mutations at the predicted interface abolished the interaction





Hay et al. (2022) *eLife*, 11: e79855

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Fluorescent molecules absorb and emit light

- Absorb light at excitation wavelength
- Emit light at emission wavelength
- Time delay between excitation and emission is called the fluorescence lifetime (τ)

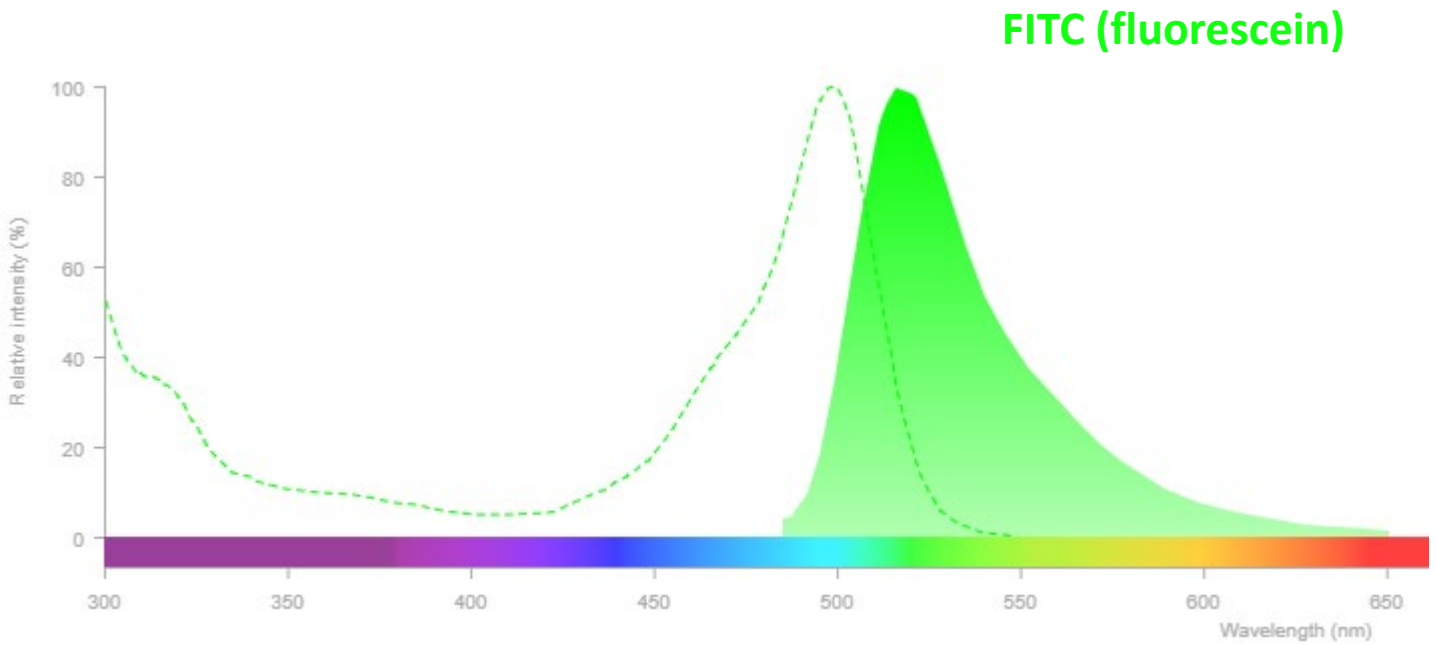
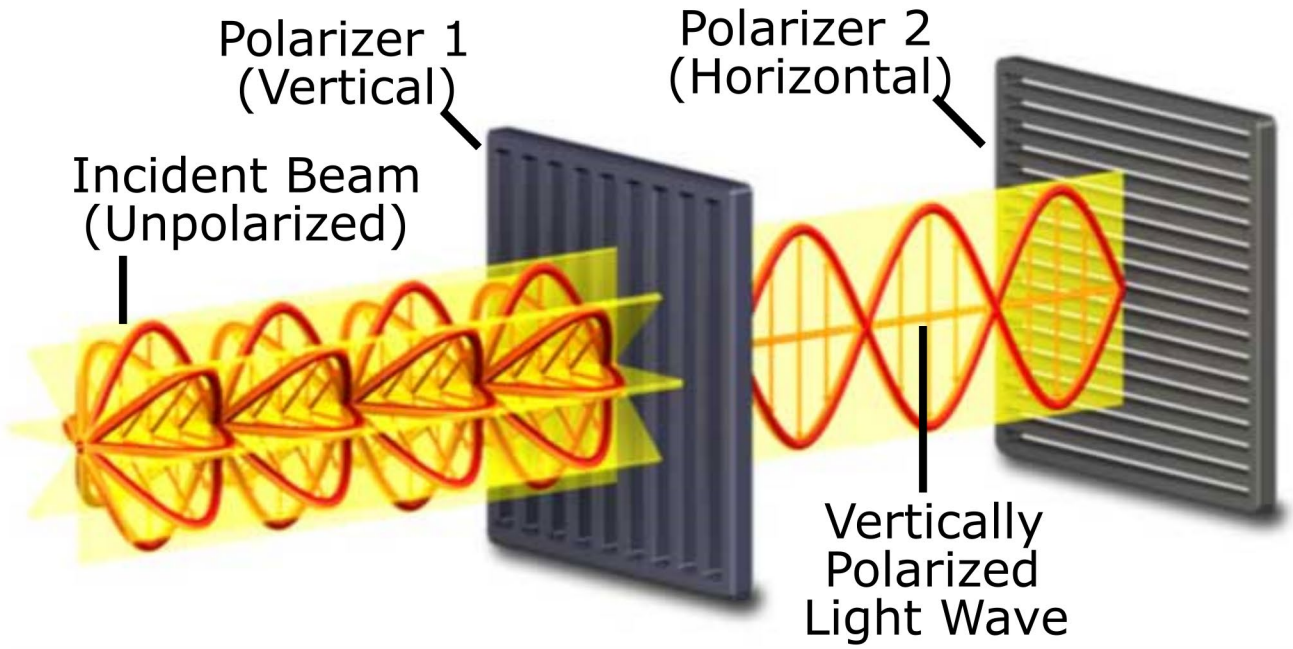


Image: https://www.thermofisher.com/order/fluorescence-spectraviewer#!/

Light can be polarised



‘Plane’ polarisation

Image: EVIDENT / Olympus lifescience

Fluorescence polarisation

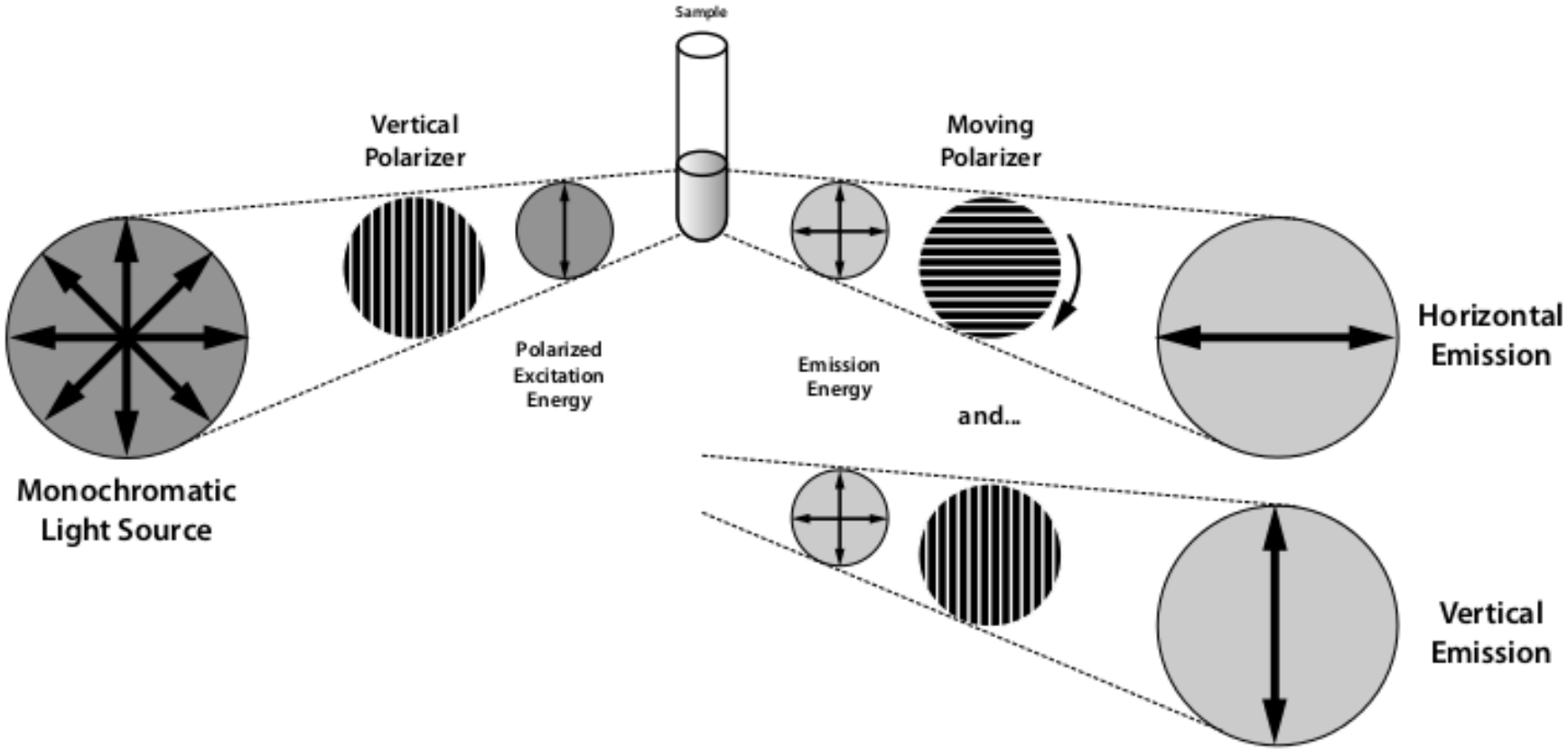
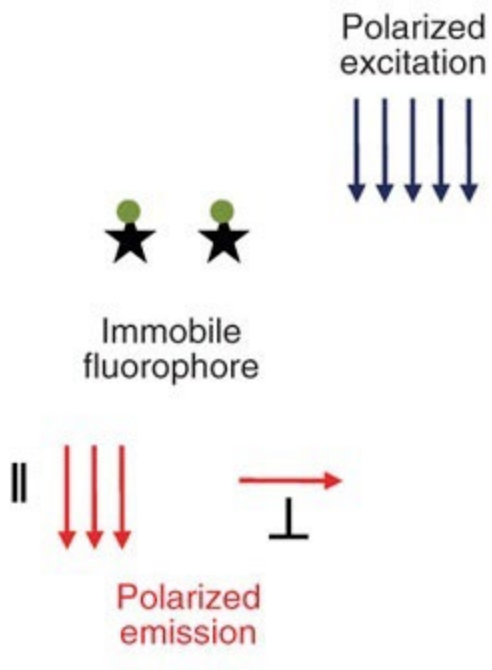


Image: Invitrogen

Fluorescence polarisation

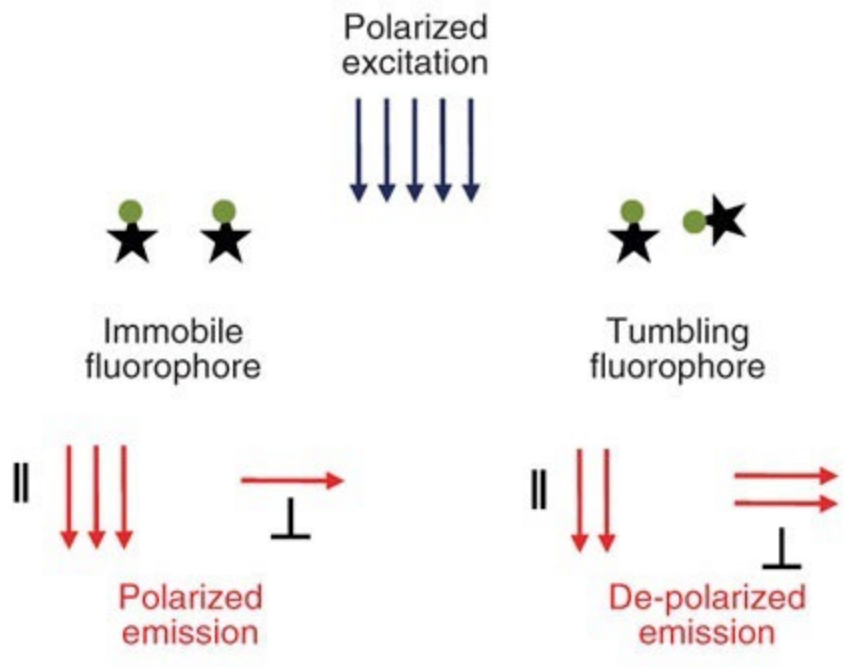
- If a fluorophore is excited with polarised light and remains immobile, most of the emitted light will have the same polarisation



Rossi and Taylor (2011) *Nat. Protoc.* 6:365-87

Fluorescence polarisation

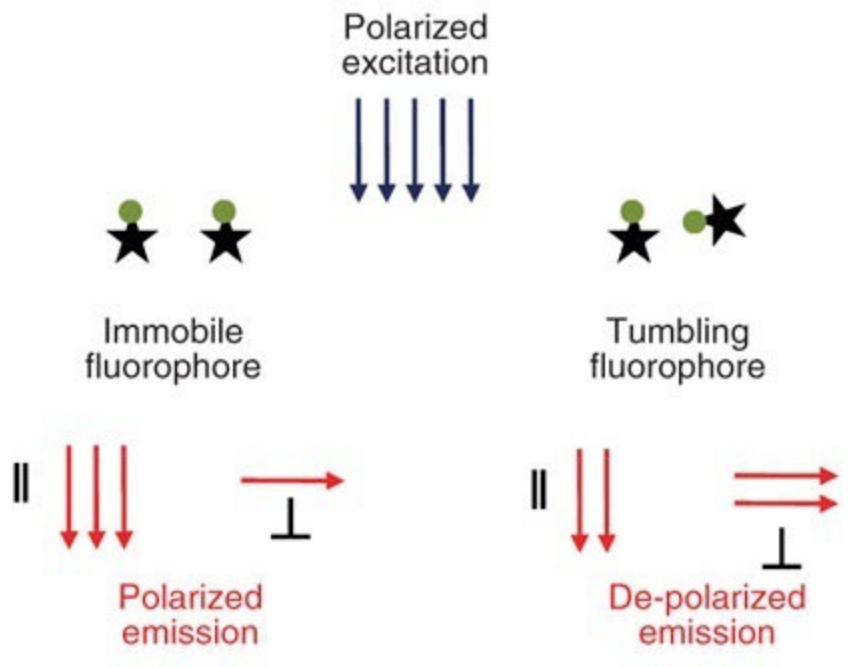
- If the fluorophore **rotates** during its fluorescence lifetime, **less** of the emitted light will have the same polarisation



Rossi and Taylor (2011) *Nat. Protoc.* 6:365-87

Fluorescence polarisation correlates with tumbling time

- By measuring the **ratio of fluorescence** polarisation, we can measure how rapidly a molecule is tumbling



Rossi and Taylor (2011) *Nat. Protoc.* 6:365-87

Fluorescence polarisation correlates with tumbling time

$$P \propto \frac{3\eta V}{RT}$$

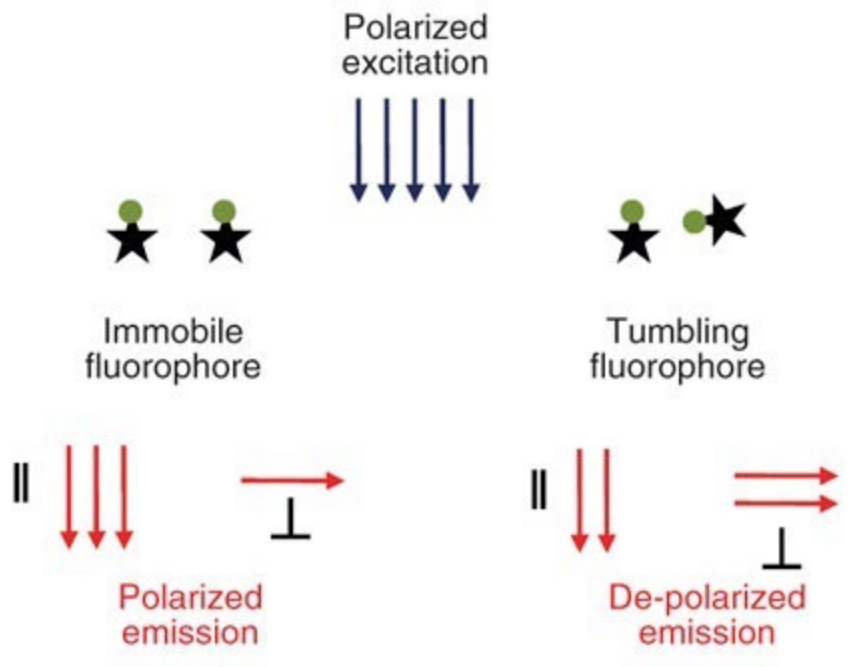
P = Polarisation value

V = molecular volume

η = viscosity

R = gas constant

T = Temperature (K)



Rossi and Taylor (2011) *Nat. Protoc.* 6:365-87

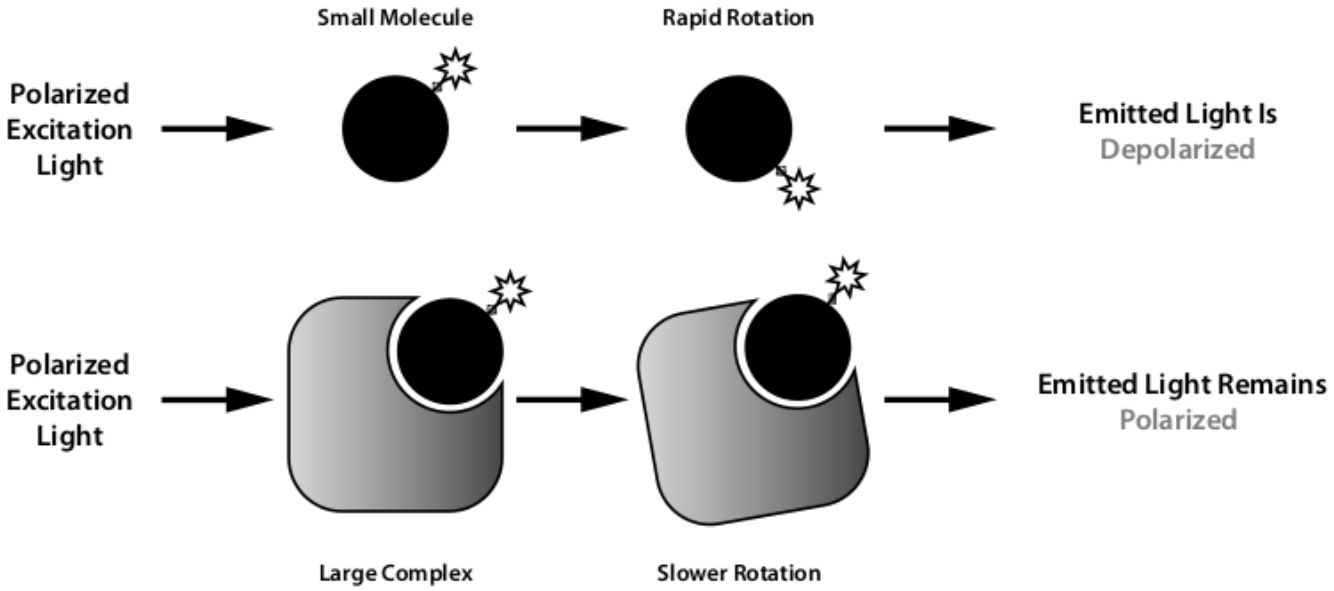
Big molecules tumble slower than small ones

$$P \propto \frac{3\eta V}{RT}$$

Bigger molecules have a larger molecular volume (V)

Image: Invitrogen

Fluorescence polarisation measures protein:ligand binding



…if binding event causes a significant change in the tumbling of the ligand

Image: Invitrogen

Fluorescence polarisation

- Titrate unlabelled protein into solution containing constant amount of labelled ligand (peptide)
- Look at fluorescence polarisation of labelled ligand
- Uses nM concentrations of fluorescent ligand
- Can measure interactions with nM to sub-mM affinities

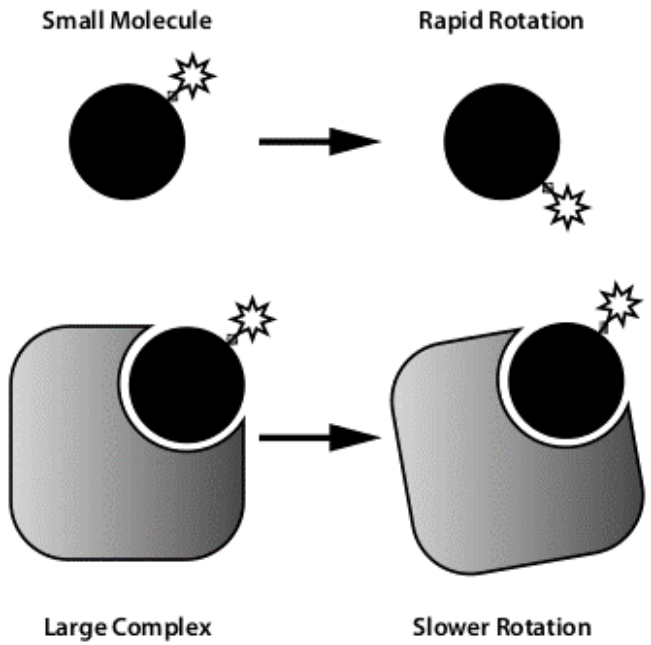


Image: Invitrogen

Measuring fluorescence polarisation

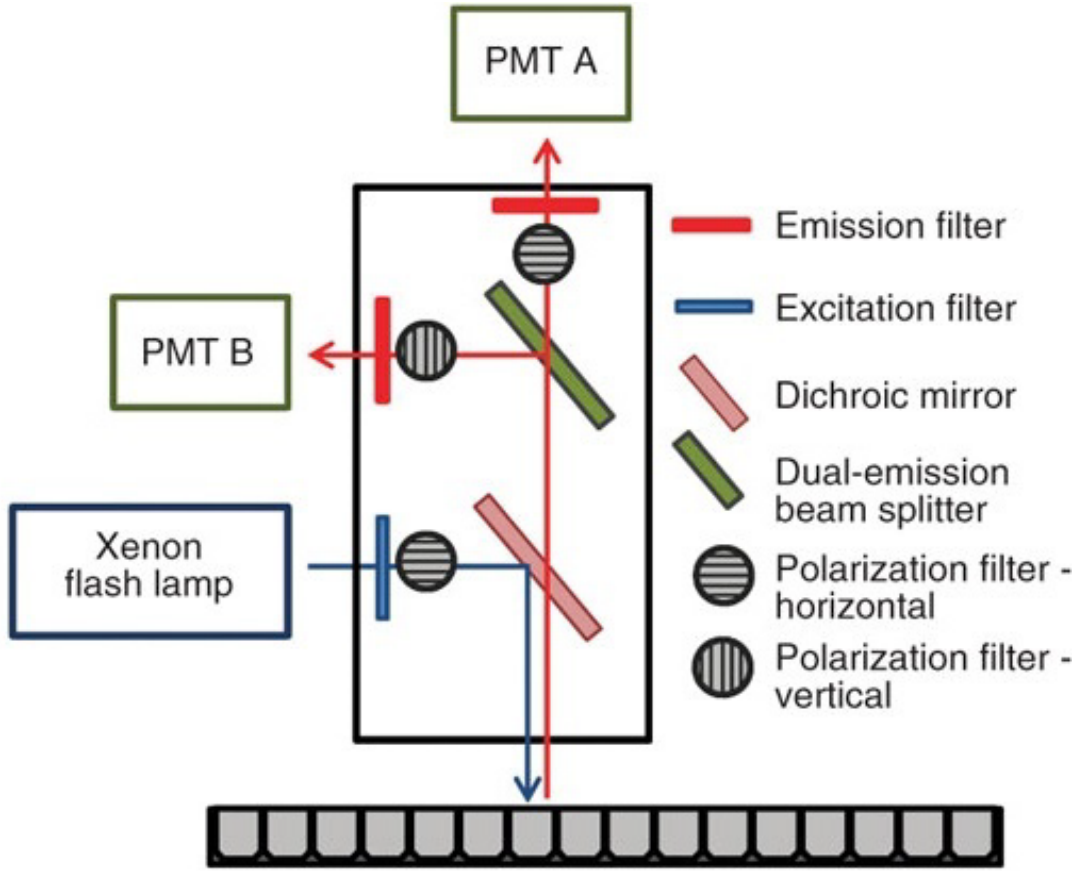
Full-size

Half volume

Plate images: Perkin Elmer

Fluorescence polarisation

- Excite fluorophore with plane-polarised light
- Pass emitted light through polarising filters parallel or perpendicular to the polarisation of the exciting light



Rossi and Taylor (2011) Nat. Protoc. 6:365-87

Fluorescence polarisation (FP) vs fluorescence anisotropy (FA)

- Two different ways of expressing the data:

Fluorescence Polarisation (P)

$$P = I^{\parallel} - I^{\perp} / I^{\parallel} + I^{\perp}$$

Fluorescence Anisotropy (A)

$$A = I^{\parallel} - I^{\perp} / I^{\parallel} + 2 \times I^{\perp}$$

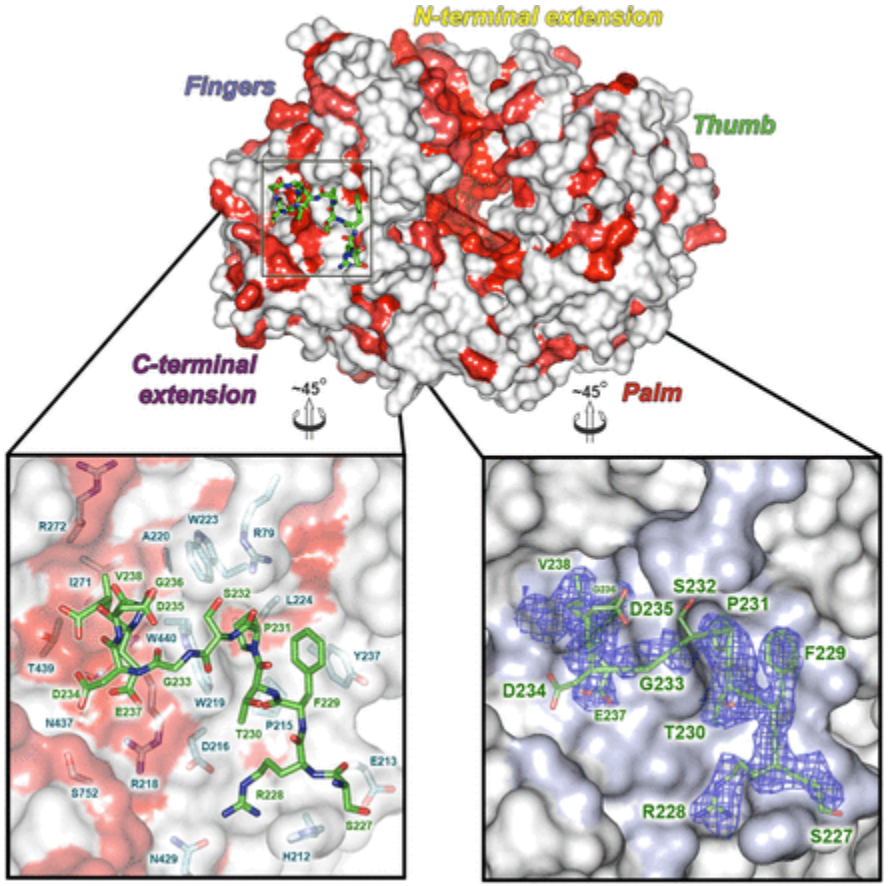
Where $I^{\parallel}$ = Intensity of emitted light with polarisation **parallel** to incident light,
$I^{\perp}$= Intensity of emitted light **perpendicular** to incident light

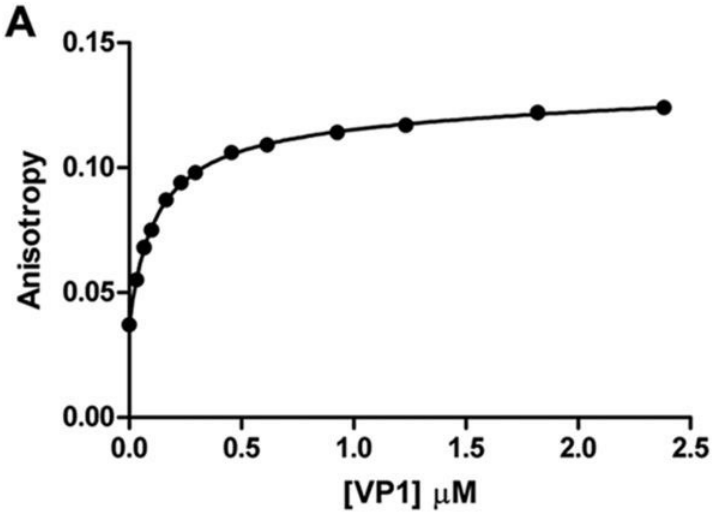
Fluorescence anisotropy

- We generally use **anisotropy**
  - Maths is slightly more convenient, but information content is the same
- Fluorescence anisotropy is a ratio, thus unit-less
- Immobile molecules will have A=0.4
- Highly mobile molecules will have A=0
  - Polarisation of excited light is randomly oriented, so $I^{\parallel} - I^{\perp} = 0$

$$A = \sum_{i=1}^{n} f_i \times A_i$$

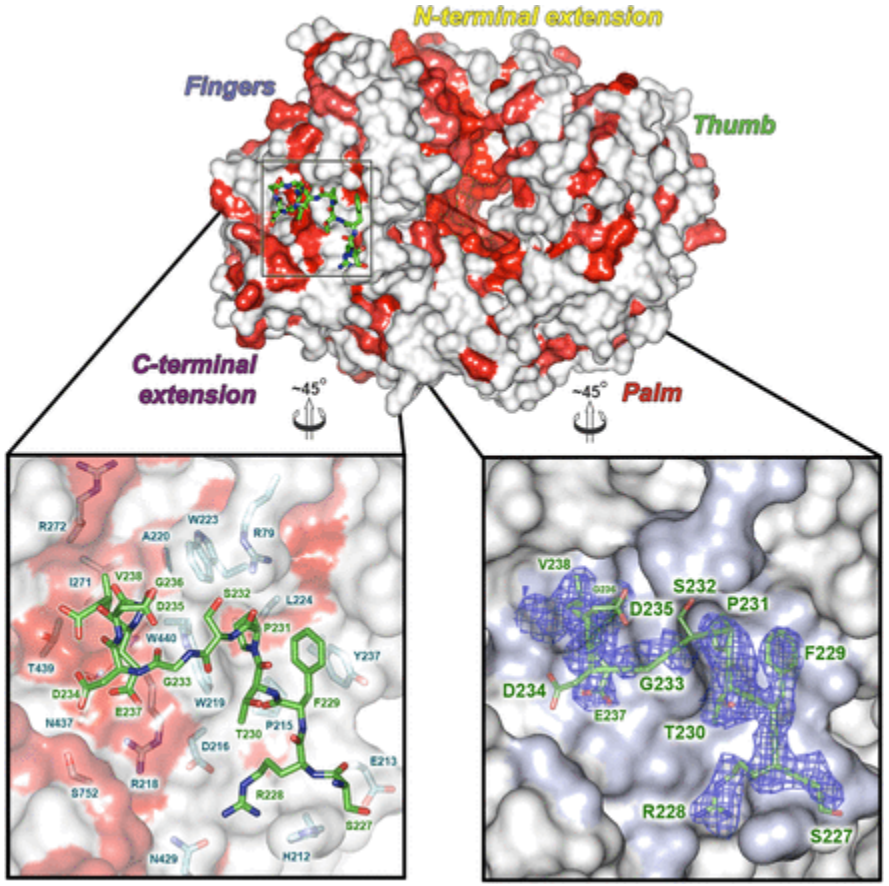
Fluorescence anisotropy experiment

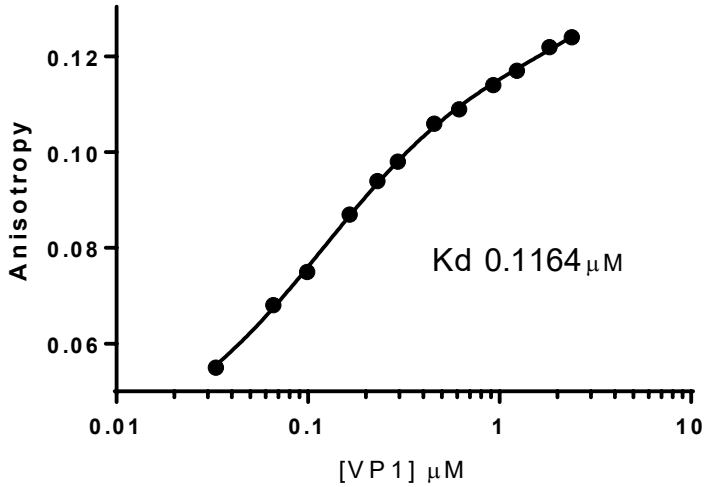




Bahar et al. (2013) *Journal of Virology*, 87: 3229–3236

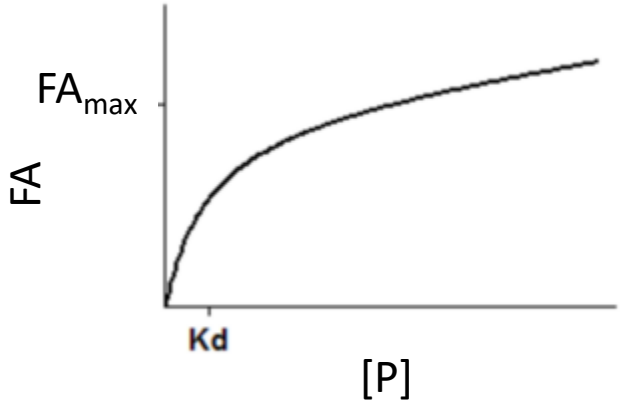
Fluorescence anisotropy experiment





Bahar et al. (2013) *Journal of Virology*, 87: 3229–3236

One-site binding model



[(Fluorescent)Ligand] is constant

$$FA = FA_{max}*[P]/(K_D+[P]) + NS*[P] + Background$$

- Assumptions:
  - No depletion of protein by the ligand
  - Linear increase in anisotropy due to viscosity
  - Constant ligand fluorescence (no quenching)

One-site binding

$$K_D = \frac{[A] \cdot [B]}{[AB]}$$

- [A] and [B] are amount of *free* A and B
- If $[A]_{Total} << K_D$, $[B]_{Total} \approx [B]$

$[B]_{Total} \approx [B]$

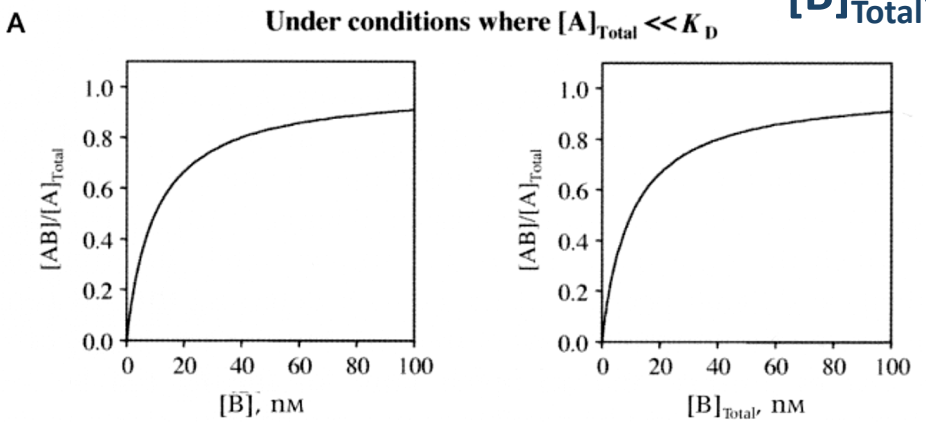


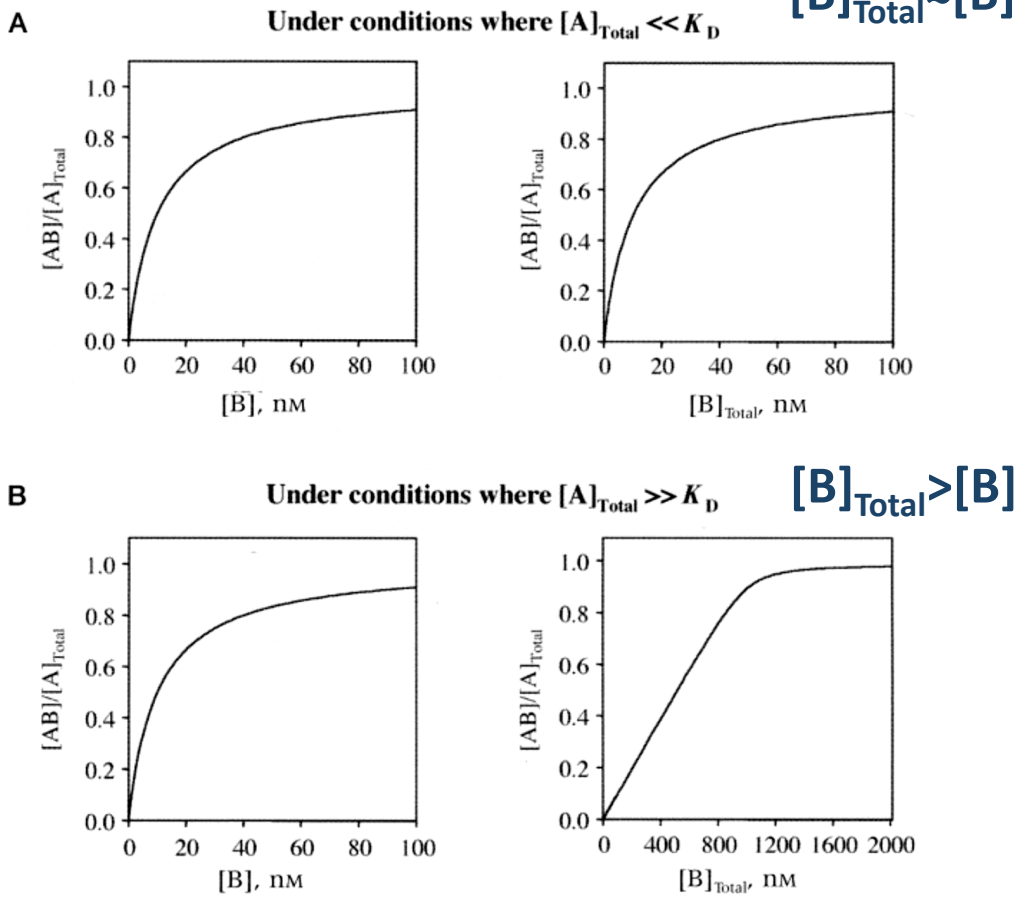
Image: Goodrich and Kugel (2006) Binding and Kinetics for Molecular Biologists, CSHP

One-site binding

$$K_D = \frac{[A] \cdot [B]}{[AB]}$$

- [A] and [B] are amount of *free* A and B
- If $[A]_{Total} << K_D$, $[B]_{Total} \approx [B]$
- If $[A]_{Total} \approx K_D$, $[B]_{Total} > [B]$

$[B]_{Total} \approx [B]$



$[B]_{Total} > [B]$

Image: Goodrich and Kugel (2006) Binding and Kinetics for Molecular Biologists, CSHP

When won't fluorescence anisotropy work

- When tumbling of ligand doesn't change significantly upon binding protein
  - Protein must be much larger than ligand
- When K_D is too low (tight binding)
  - Need [ligand]<<K_D, but limit of reliable detection on standard plate readers for FA is ~1 nM
  - This invalidates assumption that ligand binding doesn't decrease $[P]_{free}$
    …but you can work around this by doing clever competition assays
- When protein is fluorescent or quenches the fluorophore!

Fluorescence competition assays

- Measure increase in anisotropy as ligand is liberated from protein by a competitor

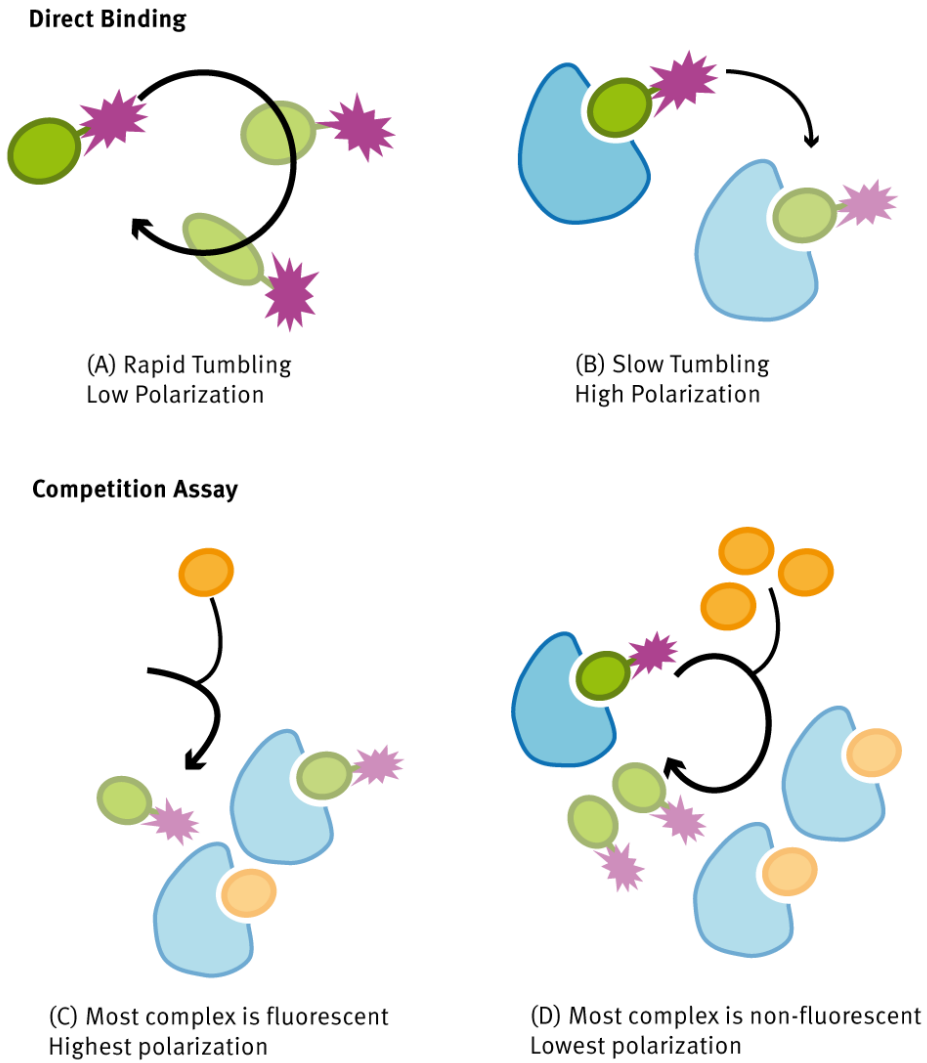
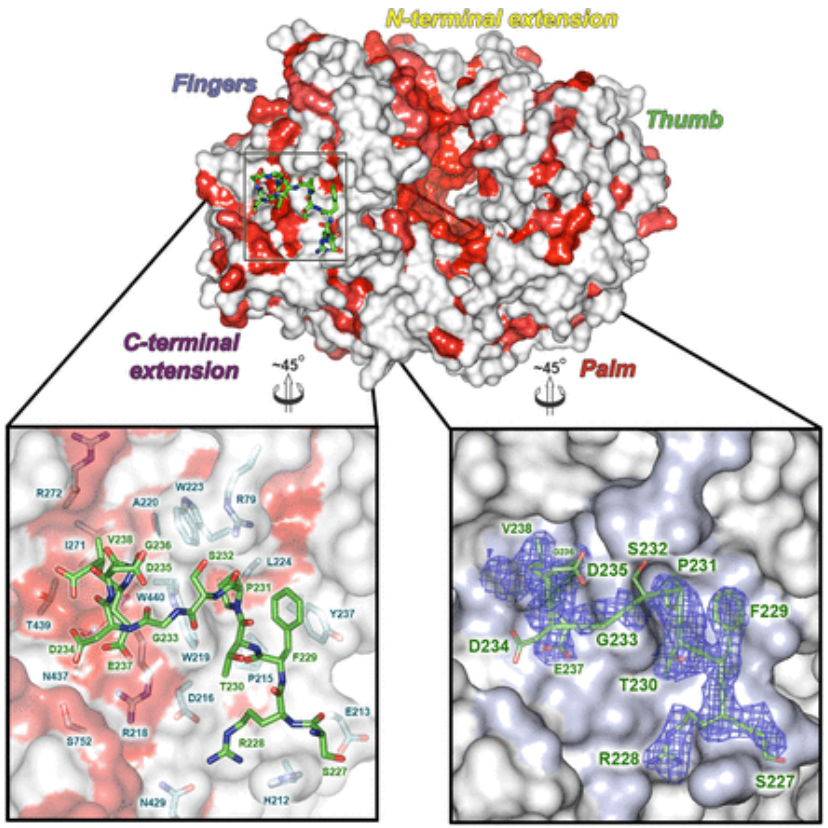
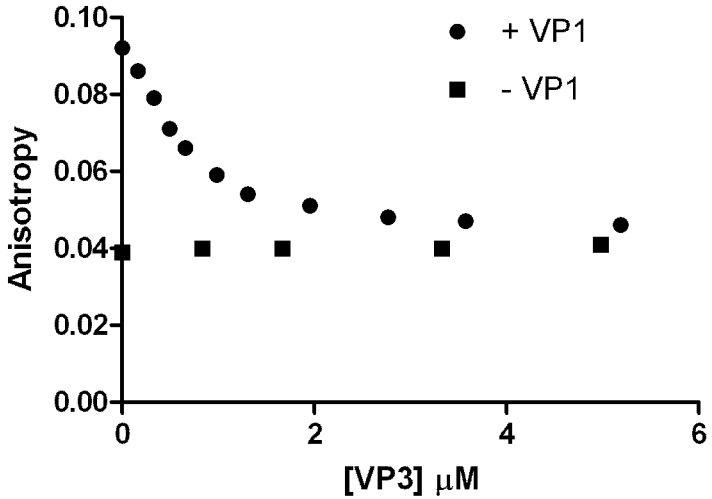


Image: BMG labtech

Fluorescence competition assays

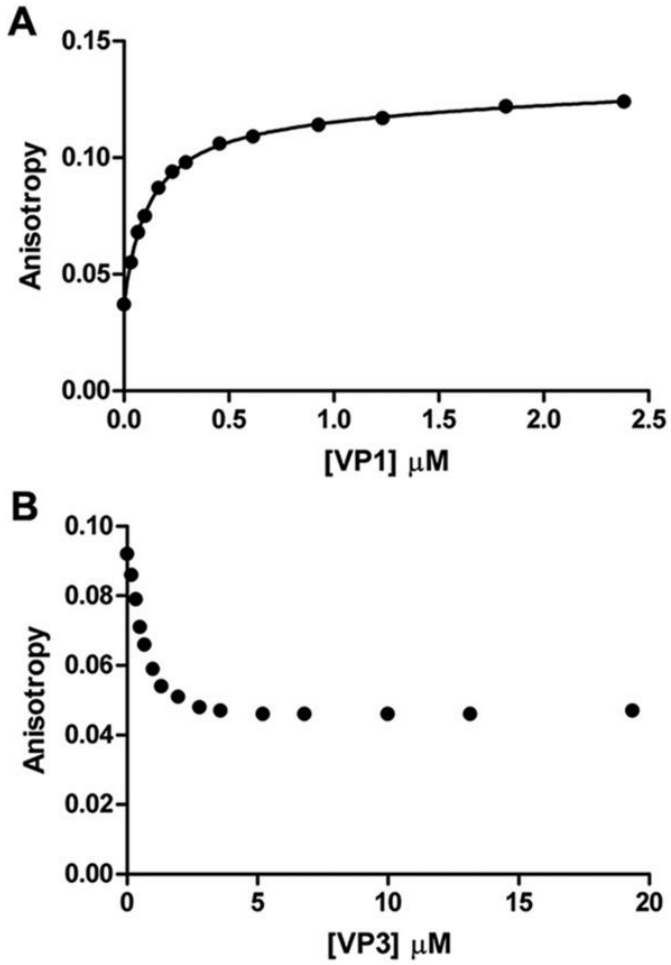




Bahar et al. (2013) *Journal of Virology*, 87: 3229–3236

Fluorescence competition assays

- Can measure K_D of unlabelled ligands (so long as you know K_D of labelled ligand)
- Can measure very tight binding affinities ($K_D \approx$ [ligand])
- Use [Protein] that is ≤ 2 × KD (~75% binding) then titrate in unlabelled competitor



Bahar et al. (2013) *Journal of Virology*, 87: 3229–3236

Affinity measurements - summary

Technique	Solution based?	Label free?	Thirst for protein	Cost per experiment	K_D range	Other
Pull-downs	Yes	No	Low – Medium	Cheap	<nM to µM	Quick and easy, but only semi-quantitative
SPR	No	No	Low	Expensive	nM to µM	Kinetics or equilibrium
ITC	Yes	Yes	High	Cheap	~µM to mM	Stoichiometry, enthalpy
Fluorescence anisotropy	Yes	No	Low	Cheap	nM-µM	Easy, can use competition
MST	Yes	No*	Low	Cheap	nM–µM	Expensive machine, Can do competition
CG-MALS	Yes	Yes	Huge	Cheap	nM-µM	Stoichiometry

This talk

- Protein interaction affinities
  - Equilibrium and kinetic models
- Kinetic measurement
  - Surface Plasmon Resonance (SPR, Biacore)
- Equilibrium measurement
  - SPR
  - Isothermal Titration Calorimetry (ITC)
  - Fluorescence polarisation

Tomorrow: Looking at protein structures