


Simon Davis, Ed Evans: ☎21336

t-cell biology group



Our activities are directed at understanding the molecular basis of leukocyte recognition, and engineering these through site-directed and systematic models of T cell activation. The scope of our work extends from the molecular to the cellular scale, drawing on X-ray crystallography, interaction studies, single molecule fluorescence detection methods, global gene expression and bioinformatic analysis.

The Wellcome Trust

www.t-cellbiology.org/teaching

Methods Course, 2008

- ~~1. Expression cloning~~
2. Protein expression (overview)
 - i. Why express proteins at all?
 - ii. How to decide on an expression strategy
 - ~~iii. The methods~~
 - ~~bacterial expression~~
 - ~~mammalian expression (transient, stable)~~
 - iv. Judging protein quality
3. The BIAcore (Surface Plasmon Resonance)

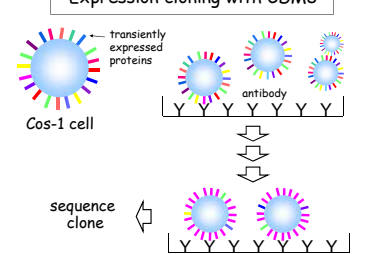
Expression cloning

Cloning a molecule based only on a test of protein expression e.g. antibody detection, ligand binding or enzymatic activity

i.e. don't need to know sequence

⇒ Ideal in the pre-PCR lab!
⇒ Still useful in some cases...

Expression cloning with CDM8



transiently expressed proteins

Cos-1 cell

antibody

sequence clone

Why express and study proteins?

1. Proteins are of fundamental interest: biological systems are all about protein recognition
2. An understanding of immunological phenomena increasingly depends on understanding how proteins behave
3. Can expect hard answers to scientific questions: is this how my protein looks?
4. Modern immunology is reagent-driven so the choice of protein can set the research agenda
5. This can provide many opportunities for collaboration (i.e. lots of papers)

The expression strategy

Don't just do what your lab has always done – think about your protein!

Your protein → Cytosolic?

Bacterial expression (e.g. pET vectors)

- fast
- often very large amounts

The expression strategy

Your protein → Secreted or membrane bound?

Bacterial re-folds

- yields can be low (~1%)
- refold conditions generally differ for each protein
- sparse-matrix screens are available to help

The expression strategy

Your protein → Secreted or membrane bound?

Bacterial secretion systems

- e.g. pET-12a,b,c vectors
- yields often very low

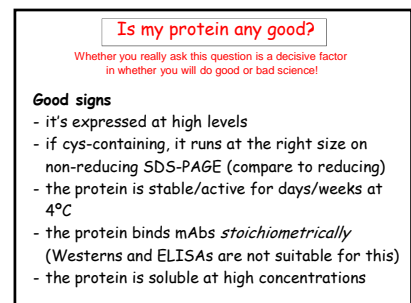
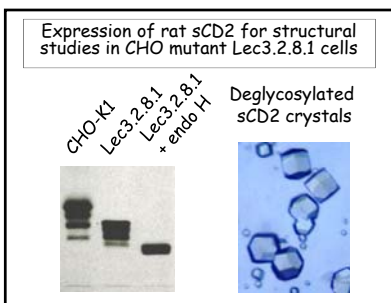
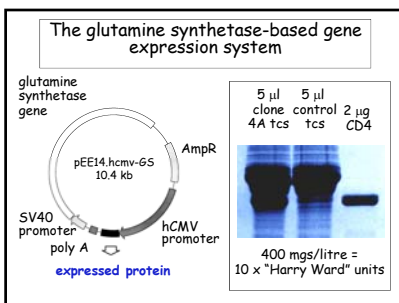
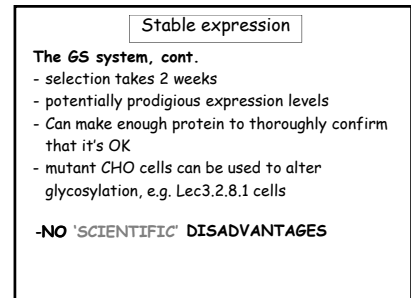
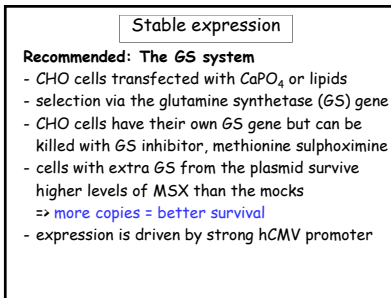
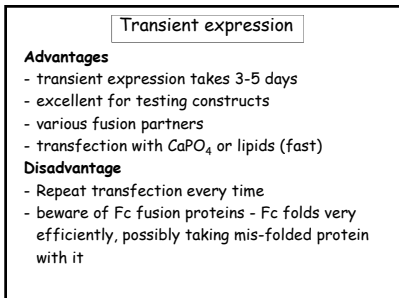
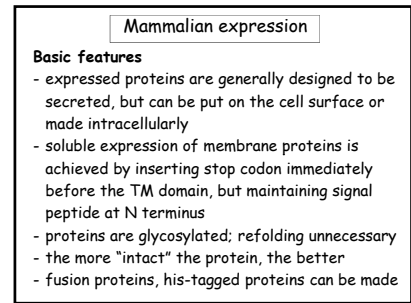
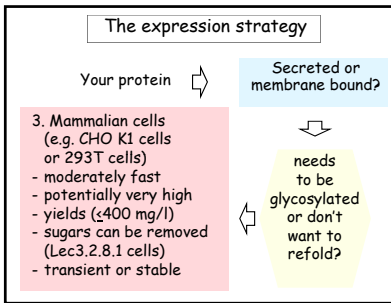
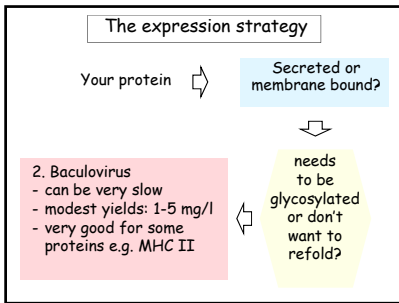
The expression strategy

Your protein → Secreted or membrane bound?

1. Yeast (e.g. *Pichia*)

- fast
- very high yields
- metabolic labelling (NMR)
- deglycosylation possible
- poor folding of e.g. IgSF proteins

needs to be glycosylated or don't want to refold?



Is my protein any good?

Whether you really ask this question is a decisive factor in whether you will do good or bad science!

Good signs, cont.

- the protein is non-aggregated according to gel filtration - the absolute key for doing BIAcore experiments and structural studies properly

- NB Gels do not tell you this!

Nuffield Dept. Clinical Medicine
D.Phil. Students techniques course
Ed Evans
Surface Plasmon Resonance
edward.evans@ndm.ox.ac.uk

What is Surface Plasmon Resonance for?

The accurate measurement of the properties of inter-molecular interactions.
(Contrast with interaction screens and crude measurements of bond strength e.g. AUC)

Why use Surface Plasmon Resonance?

A full understanding of the function of proteins requires accurate knowledge of the nature of their interactions.

Why use Surface Plasmon Resonance?

A full understanding of the function of proteins requires accurate knowledge of the nature of their interactions.

Example: Costimulation vs. Inhibition
B7.1 and B7.2 both bind to CD28 and CTLA-4.

BUT

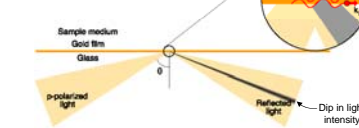
B7.2 & CD28 are constitutively expressed, others on activation
B7.1 is dimeric, B7.2 is not

CD28, although dimeric, is monovalent
CTLA-4 binds its ligands much more strongly than CD28
B7.1 binds its ligands more strongly than B7.2

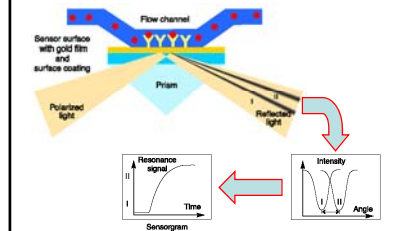
RESULT: The inhibitory B7.1:CTLA-4 complex is ~1000 times more stable than the costimulatory B7.2:CD28 complex.

Principle of Surface Plasmon Resonance

Angle of 'dip' affected by:
1) Wavelength of light
2) Temperature
3) Refractive index n_2



Surface Plasmon Resonance in the BIAcore



Uses of the BIAcore

- Equilibrium measurements:
 - Affinity (K_D or K_D)
 - Enthalpy (van't Hoff analysis)
- Kinetics: determination of k_{on} and k_{off}
- Testing valency
- Analysis of specificity e.g. drug screening
- In combination with mutagenesis:
 - Epitope mapping
 - Contribution of residues to binding
- Isolation of binding components from a mixture (unknowns can be identified by linked MS)
- Binding of protein, DNA, RNA...
 - BUT not for very small analytes (unless some amplification is used)

BIAcore machines (a selection)

- BIAcore 2000 – now obsolete but will many results you read about will be on this machine
- BIAcore 3000 – replaced 2000 with more automation etc. WHAT WE HAVE. No longer made.
- T100 – modern replacement of above machine. Much more sensitive and accurate (and expensive)
- All of above have 4 flow cells i.e. Control plus up to three samples can be tested simultaneously
- A100 – none in Oxford, can immobilise 4 proteins in each flow cell => test 16 proteins inc. control. Very, very, very expensive!
- Flexchip – no longer made – for large experiments – can immobilise 400 proteins using a spotter and test all of them against one binding partner at a time. However, much cheaper as far less precise.

Step 1: Immobilisation

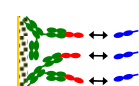
2 Main options:

- Direct:
 - Covalently bind your molecule to the chip
- Indirect:
 - First immobilise something that binds your molecule with high affinity e.g. streptavidin / antibodies

Direct:



Indirect:

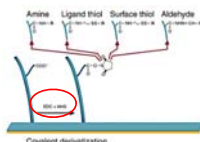


Immobilisation: sensor chip technology

- CM5 chips (most common):
Ligand capture via native groups (see next)
- SA: Coated in streptavidin for capture of biotinylated molecules. NB Can produce your own from CM5!
- NTA: Capture of ligands by metal chelation e.g. His tagged proteins
- HPA: Flat hydrophobic surface, adding liposomes forms lipid monolayers, containing any molecules you inserted into the liposome.
- C1: Like CM5 but without dextran matrix => more space to bind large particles e.g. cells and viruses, but far fewer molecules bound.
- L1: Surface contains lipophilic substances that will insert into and hence immobilise intact liposomes allowing complete bilayers to be immobilised.

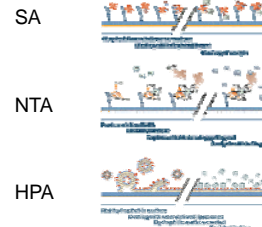
Immobilisation: Carboxymethyl binding

CM5 Sensor Chip

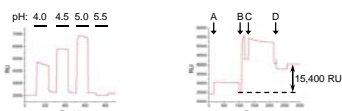


N.B. Carboxymethyl groups are on a dextran matrix:
This is negatively charged =>
Need to do a "preconcentration" test to determine optimum pH for binding (molecule needs to be +ve)

Immobilisation: Other sensor chips



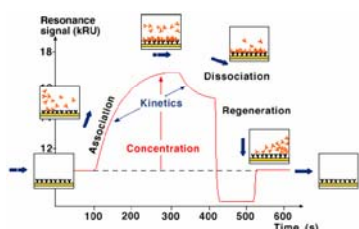
Sensorgrams (raw data)



Pre-concentration:
An antibody was diluted in buffers of different pH and injected over a non-activated chip. Maximum electrostatic attraction occurs at pH 5

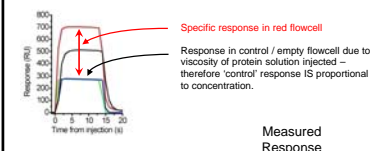
Immobilisation:
A. Inject 70µl 1:1 EDC:NHS
B. Inject 7µl mAb in pH5 buffer (in this case @370µg/ml)
C. Inject 70µl Ethanolamine
D. Inject 30µl 10mM Glycine pH2.5

Step 2: ligand binding The ideal sensorgram



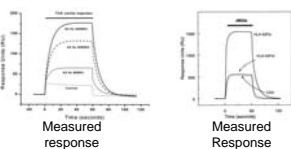
"Specific" Binding

- Each chip has four 'flow-cells'
- Immobilise different molecules in each flow-cell
- Must have a 'control' flowcell
- 'Specific binding' is the response in flow-cell of interest minus response in the control flowcell



"Specific" Binding

- Each chip has four 'flow-cells'
- Immobilise different molecules in each flow-cell
- Must have a 'control' flowcell
- 'Specific binding' is the response in flow-cell of interest minus response in the control flowcell



In theory: Affinity

1. Measures how favourable an interaction is
2. Best expressed as affinity constant: K_A
3. For $A + B \leftrightarrow AB$ $K_A = \frac{[AB]_{eq}}{[A]_{eq}[B]_{eq}} = \frac{1}{K_D}$
 - Best thought of as the ratio of [products] vs. [reactants] at **equilibrium**
 - Note the units (M^{-1})
 - Higher affinity = higher K_A

In theory: Affinity

4. Also expressed as dissociation constant: K_D
 - The inverse of K_A
 - Usually thought of as concentration of A at which half of B is bound ($[B]=[AB]$) at equilibrium
$$K_D = \frac{[A]_{eq}[B]_{eq}}{[AB]_{eq}}$$
 - Units are M
 - **Higher affinity = lower K_D**

Measuring the affinity constant

- One could simply measure [A], [B] and [AB] at equilibrium and calculate K_D
- In practice this is difficult and the following approach is used.
- Increasing fixed concentrations of one molecules A ([A]) are added to a fixed small amount of its ligand B and you measure the amount of bound A (Bound)
- Plot the results and fit the 1:1 Langmuir equation to the data to determine K_D and $Bound_{max}$

DERIVATION

$$K_D = \frac{[A][B]}{[AB]} \quad (1)$$

and

$$[B] = [B_{total}] - [AB] \quad (2)$$

By substitution of (2) into (1) and rearranging, we get

$$[AB] = \frac{[A][B_{total}]}{[A] + K_D}$$

or

$$Bound = \frac{[A]Bound_{max}}{[A] + K_D}$$

Edward Evans @ndm.ox.ac.uk www.1cellbiology.org/teaching

Measuring affinity constant

$$Bound = \frac{[A]Bound_{max}}{[A] + K_D}$$

- Data are circles
- Line is non-linear fit of the equation performed by a computer (e.g. Origin, R)
- Gives the indicated values for K_D and $Bound_{max}$
- If the fit is good it indicates that binding follows the simple 1:1 model
- Difficult to see if fit is poor in this plot

Edward Evans @ndm.ox.ac.uk www.1cellbiology.org/teaching

Scatchard plot

- A plot of $\frac{Bound}{[A]}$ versus [A]
- Linear for a 1:1 interaction
- If curved it indicates wrong model and possible problem with the experiment
- Most commonly concave up Usually caused by experimental error (often heterogeneity) Sometimes due to negative cooperativity
- Far less common is to see concave down Usually caused by positive cooperativity

DERIVATION

$$Bound = \frac{[A]Bound_{max}}{[A] + K_D}$$

$$Bound/[A] = \frac{Bound_{max}}{K_D} - \frac{Bound_{max}Bound}{[A](K_D + [A])}$$

Divide both sides by $[A]K_D$ and rearrange, giving

$$\frac{Bound}{[A]} = \frac{Bound_{max}}{K_D} - \frac{Bound_{max}Bound}{[A](K_D + [A])}$$

Plot of $\frac{Bound}{[A]}$ versus $\frac{Bound}{[A]}$

Edward Evans @ndm.ox.ac.uk www.1cellbiology.org/teaching

In practice: Equilibrium Binding Analysis

N.B. Measurement of affinities etc. should usually be done at physiological temperature (i.e. 37°C), although this is more difficult. Sometimes 25°C data can be used to compare fold differences in binding or to test for any binding at all (i.e. specificity studies).

Edward Evans @ndm.ox.ac.uk www.1cellbiology.org/teaching

In practice: Equilibrium Binding Analysis

N.B. Measurement of affinities etc. should usually be done at physiological temperature (i.e. 37°C), although this is more difficult. Sometimes 25°C data can be used to compare fold differences in binding or to test for any binding at all (i.e. specificity studies).

Edward Evans @ndm.ox.ac.uk www.1cellbiology.org/teaching

Equilibrium Binding Analysis - continued

Scatchard plot: rearrangement of binding isotherm to give a linear plot. Not so good for calculating K_D , as gives undue weight to least reliable points (low concentration)

$$\frac{Bound}{[A]} = \frac{K_{off}[A]}{K_{on} + K_{off}[A]}$$

Plot Bound/Free against Bound
Gradient = $1/K_D$

Edward Evans @ndm.ox.ac.uk www.1cellbiology.org/teaching

In theory: Kinetics

Since biological systems are not at equilibrium, the rate of binding and dissociation is critical

For a simple 1:1 interaction ($A + B \leftrightarrow AB$)...

- Rate of dissociation
 - $d[AB]/dt = -k_{diss}[AB]$
 - where k_{diss} is the dissociation rate constant (k_{off})
- Rate of association
 - $d[AB]/dt = k_{ass}[A][B]$
 - where k_{ass} is the association rate constant (k_{on})
- At equilibrium the rate of association must equal the rate of dissociation

$$k_{diss}[AB] = k_{ass}[A][B] \Rightarrow k_{diss}/k_{ass} = [A][B]/[AB] = K_D$$

Edward Evans @ndm.ox.ac.uk www.1cellbiology.org/teaching

Dissociation

- Any reaction of the form $d[AB]/dt \propto [AB]$ will be exponential so
 - i.e. $[AB]_t = [AB]_0 e^{-k_{diss}t}$
 - k_{diss} determined directly by curve fitting
- The half life ($t_{1/2}$) can be calculate as follows:

Since at $t = t_{1/2}$
 $[AB]_t/[AB]_0 = 0.5 = e^{-k_{diss}t_{1/2}}$
 It follows that
 $-k_{diss}t_{1/2} = \ln(0.5) = 0.693$
 Thus $t_{1/2} = 0.693/k_{off}$

Edward Evans @ndm.ox.ac.uk www.1cellbiology.org/teaching

Association

- In most experimental system it is impossible to follow association alone in the absence of simultaneous dissociation
- For the simple interaction $A + B \leftrightarrow AB$
 $d[AB]/dt = k_{ass}[A][B] - k_{diss}[AB]$
 It follows that $[AB]_t = [AB]_{final} (1 - e^{-k_{obs}t})$
 where $k_{obs} = k_{ass}[A] + k_{off}$
 Thus one needs to know k_{off} and [A] as well as measuring [AB] to calculate the k_{on}

Edward Evans @ndm.ox.ac.uk www.1cellbiology.org/teaching

Factors affecting kinetics

- The association rate constant does not vary that much
 - Association requires two proteins to collide in the correct orientation and in the correct conformation
 - Depends on diffusion so will be similar for most proteins
 - The basic rate is about $10^5 \text{ M}^{-1} \text{ s}^{-1}$
 - Can be accelerated by long range electrostatic forces
 - Increased rate of collision
 - Steer binding sites into correct orientation
 - E.g. barnase/barnstar interaction
- The dissociation rate constant varies considerably and is responsible for most variation in affinity constants
 - It is determined by the number and strength of bonds in the contact interface
 - Depends on size of interface and the degree of surface-shape and electrostatic complementarity

edward.evans@ndm.ox.ac.uk www.t-cellbiology.org/teaching

In practice: Kinetics

edward.evans@ndm.ox.ac.uk www.t-cellbiology.org/teaching

Potential pitfalls

- Protein Problems: **Aggregates** (common)
Concentration errors
Artefacts of construct
- Importance of controls: Bulk refractive index issues
Control analyte
Different levels of immobilisation
Use both orientations (if pos.)
- Mass Transport: Rate of binding limited by rate of injection: k_{on} will be underestimated
- Rebinding: Analyte rebinds before leaving chip
 k_{off} will be underestimated

Last two can be spotted if measured k_{on} and k_{off} vary with immobilisation level (hence importance of controls)

Less common applications

- Temperature dependence of binding

van't Hoff analysis: $\Delta G = -RT \ln(K_e) = \Delta H - T\Delta S$
 $\ln(K_e) = \frac{-\Delta H}{R} \left(\frac{1}{T} \right) + \frac{\Delta S}{R}$
 Gradient: $\frac{-\Delta H}{R}$
 Intercept: $\frac{\Delta S}{R}$

edward.evans@ndm.ox.ac.uk www.t-cellbiology.org/teaching

In theory: Thermodynamics

- Binding is favoured if it leads to a net increase in disorder or **entropy**.
- This includes entropy of...
 - the system (interacting molecules and solvent)
 - represented as change in entropy or ΔS
 - the environment (everything else)
 - as the system releases or absorbs heat it changes the entropy of the surroundings
 - heat release is measure as change in enthalpy or ΔH

edward.evans@ndm.ox.ac.uk www.t-cellbiology.org/teaching

Gibbs free energy change

- The change in Gibbs free energy (ΔG) is a measure of the net change in universal entropy - i.e. the extent to which binding is favoured.
 $\Delta G = \Delta H - T \Delta S$
 If $\Delta G < 0$ then binding is favoured.
- ΔG depends on concentration. At equilibrium $\Delta G = 0$
- ΔG° is the standard state ΔG which assumes all components are at the standard state concentration of 1 M ($\text{mol} \cdot \text{L}^{-1}$)
- It can be calculated from the affinity constant
 $\Delta G^\circ = RT \ln K_D$
 $R = \text{Gas Constant } (2 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1})$ $T = \text{absolute temp. in Kelvin } (^\circ\text{C} + 273.18)$ and K_D is expressed in units M

edward.evans@ndm.ox.ac.uk www.t-cellbiology.org/teaching

Origins of enthalpy and entropy changes

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

- Change in enthalpy (ΔH)
 - Release of heat ($\Delta H < 0$) favours binding
 - This happens when bonds are formed
 - e.g. hydrogen bonds, salt bridges, van der Waals contacts
 - However bonds are also broken upon binding
 - displacement of water and ions (always)
 - conformational change (sometimes)
- Change in entropy ($T\Delta S$)
 - Increase in entropy ($\Delta S > 0$) favours binding
 - Protein/protein interactions leads to decrease in entropy
 - Stabilise conformation at the binding interface
 - Decreased rotation/translation of proteins
 - However displacement of water from the binding interface leads to an increase in entropy (the hydrophobic effect)

edward.evans@ndm.ox.ac.uk www.t-cellbiology.org/teaching

The key role of water

- Water is present at very high concentrations (55 M) and interacts with protein surfaces
- Thus, many water bonds need to be broken, which has an unfavourable enthalpic effect
- Water can also act as glue filling in gaps between surfaces that lack surface shape complementarity

- Water is believed to form an organised shell over hydrophobic surfaces. Ejection of water from these surfaces into free solution has favourable entropic effect. This is the 'hydrophobic effect'.
- Note that there is a weak unfavourable enthalpic effect as well since the water molecules in the shell interact weakly

edward.evans@ndm.ox.ac.uk www.t-cellbiology.org/teaching

Measuring thermodynamic parameters

- ΔS can't be measured directly
- ΔG and ΔH are measured and $\Delta G = \Delta H - T\Delta S$
- ΔH can be measured in 2 ways
 - calorimetry (see later) or
 - van't Hoff analysis

Van't Hoff analysis

- ΔG is measured over a range of temperature and plotted
- The non-linear van't Hoff equation* is fitted to the data to determine ΔH , ΔS and ΔC
- The slope represents ΔH
- This plot is curved for macromolecular interactions as ΔH varies with temperature
- The curvature represents ΔC

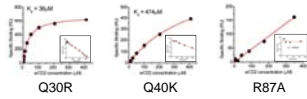
* Non-linear van't Hoff equation
 $\Delta G = \Delta H_{T_0} - T\Delta S_{T_0} + \Delta C(T - T_0) - T\Delta C \ln \left(\frac{T}{T_0} \right)$
 where T_0 is an arbitrary reference temperature

edward.evans@ndm.ox.ac.uk www.t-cellbiology.org/teaching

Less common applications

2. Combination with mutagenesis

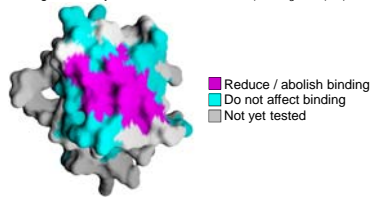
Binding of CD2 by CD48 mutants at 25°C (WT $K_D = 40\mu\text{M}$)



Less common applications

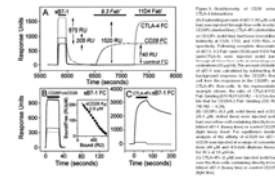
2. Combination with mutagenesis

Binding of CD2 by CD48 mutants at 25°C (WT $K_D = 40\mu\text{M}$)



Less common applications

3. Estimation of valency



Less common applications

3. Screening

Newer BIAcore machines are capable of high throughput injection. With target immobilised, many potential partners / drugs can be tested for binding.

4. Identification of unknown ligands

Mixtures e.g. cell lysates, tcs, food samples etc. can be injected over a target and bound molecules can then be eluted into tandem mass spectroscopy for identification.

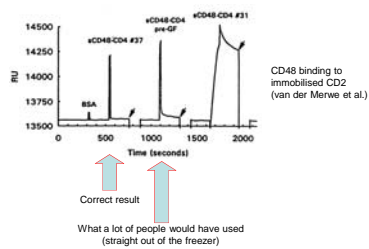
BIAcore availability

- T-cell Biology Group (us) – BIAcore 3000
 - NDM machine so if in the department, only charge for consumables.
 - Must have BIAcore experience / training already (except if a formal collaboration)
 - Book at www.t-cellbiology.org/contact.htm
- Dunn School – contact van der Merwe lab BIAcore 3000 x 2 - ~£300 a day inc chips
- Chris O'Callaghan: T100 (talk to him about details)

Techniques in our lab

- First choice for probing molecular interactions: SPR (BIAcore)
- Probing homodimeric interactions: AUC
- Probing size & shape of complexes: AUC
- Probing detailed thermodynamics: ITC
- Probing oligomerisation state or associations in cell or on their surface: FRET / BRET
- Probing for longer range associations or associations between things you can't make: single molecule microscopy

One last warning: take care



Newton, Isaac (1642-1727)

On how he made discoveries:

By always thinking unto them. I keep the subject constantly before me and wait till the first dawnsings open little by little into the full light.

SIMON'S FREE ADVICE

- science is the best job in the world !
- don't fret about being famous in 3 years
- if your project isn't as flash as you thought it was going to be, it probably doesn't matter
- just get enough data for your D.Phil !
- it doesn't really matter what you do your D.Phil on
- what is important is what you do your post-doc on
- as a post-doc you have to be working on something important, so if you get a result it will be important !
- so try to decide during your D.Phil years what is going to be important
- there's a world shortage of good young post-docs, so from Oxford you can go pretty much anywhere