Gideon Schreiber

- **Bio-molecular interactions**
- Purification Table
- Analytical gel filtration
- SPR – Surface plasmon resonance
- ITC – Isothermal Titration Calorimetry
- Elisa
- Proximity Ligation
- Microscale Thermophoresis
- Split DHFR

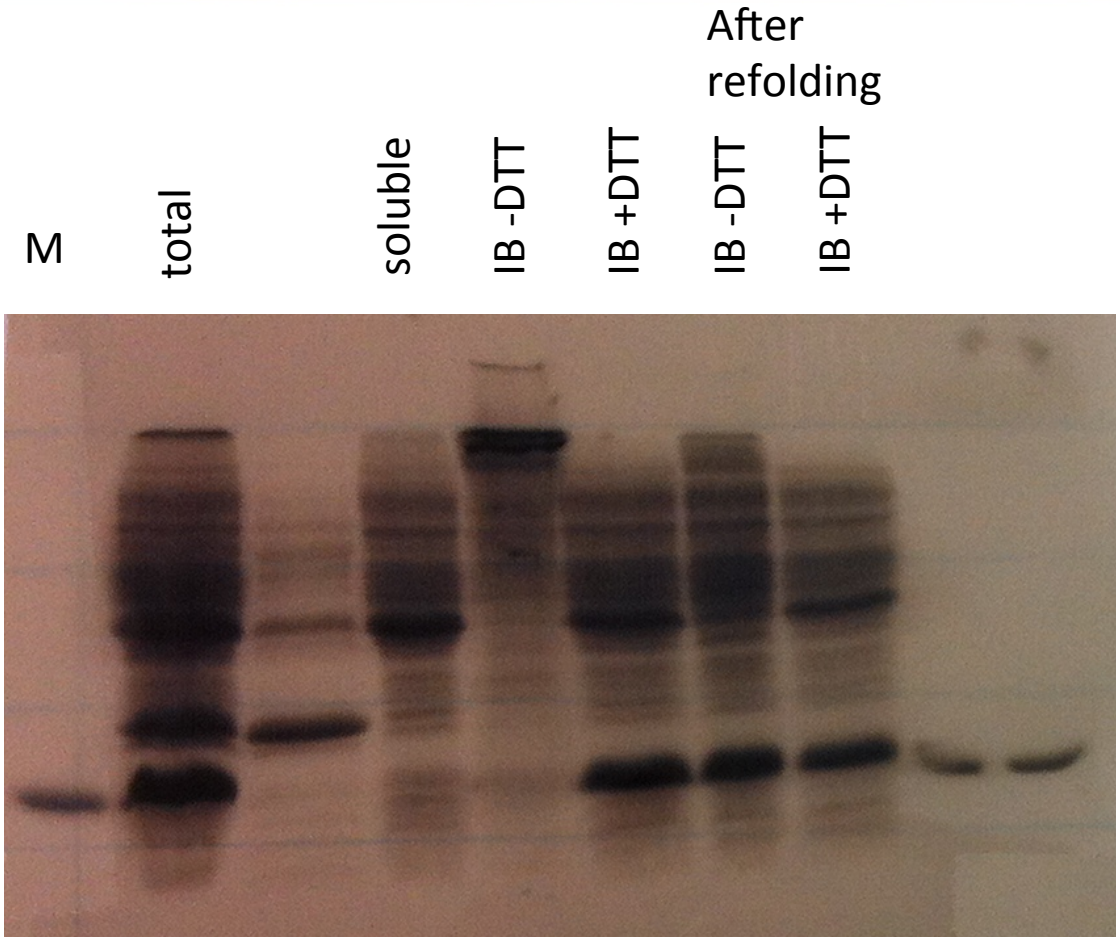
Purification table (example)

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
CE (1)	500	3,000	15,000	0.2	100	--
AS (2)	100	2,400	4,000	0.6	80	3.0
IEC (3)	45	1,440	500	2.9	48	14.5
GF (4)	50	1,000	125	8.0	33	40.0

Steps: (1) Crude cell extract; (2) ammonium sulfate fractionation;
(3) ion exchange chromatography; (4) gel filtration.

Purity-check: SDS-PAGE, analytical gel filtration, activity versus standard etc.

The importance of DTT



Analytical gel filtration: separation by size

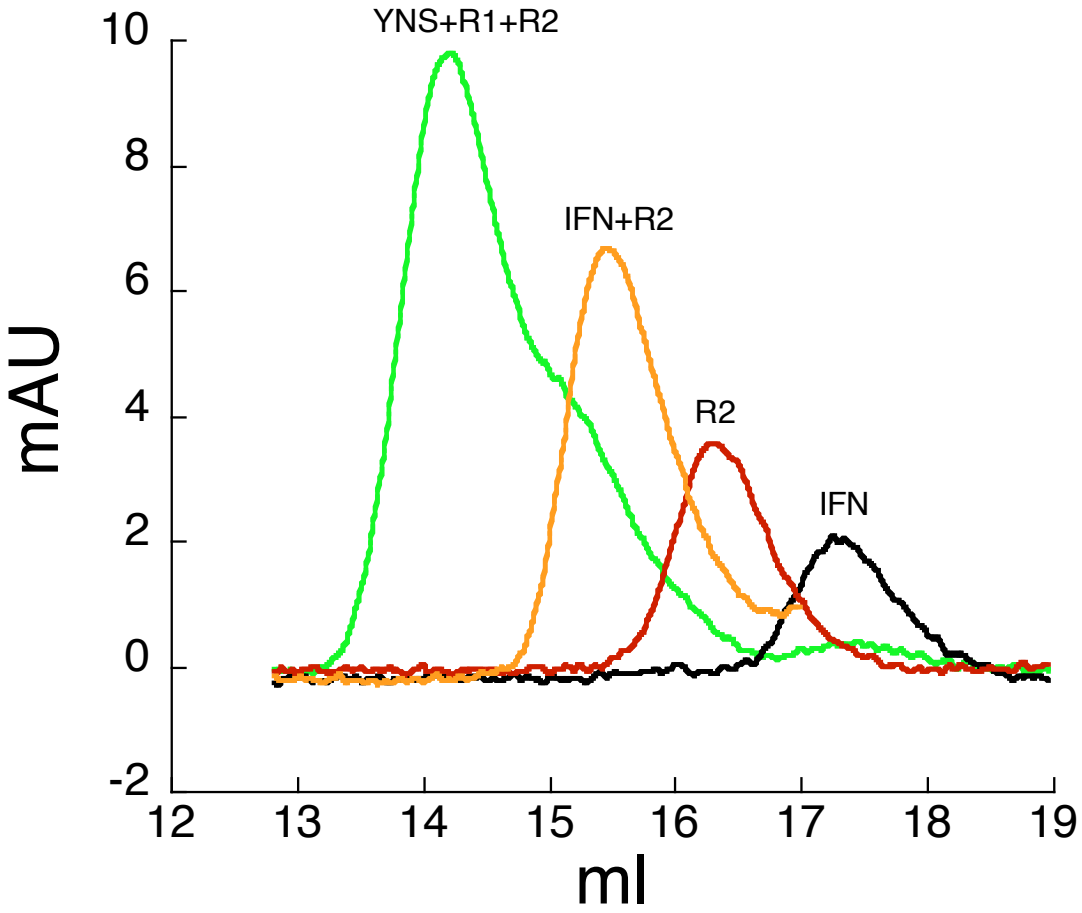
Advantages:

All the materials are in liquid phase.
Can handle some types of solvents (pH, ionic strength).
Measures the size of the molecules.
Measures percent complex formation and stoichiometry.
Few artifacts, robust and reliable results.

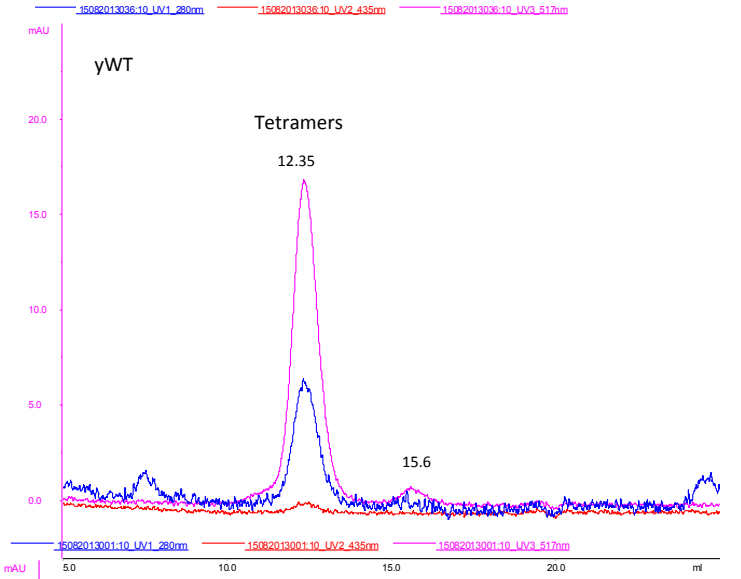
Disadvantages:

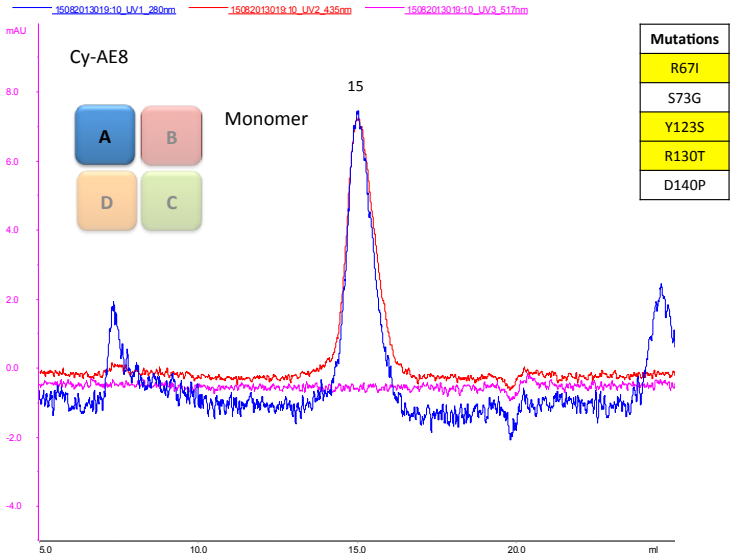
Requires a probe to view the proteins, either intrinsic or extrinsic.
Requires relative high amounts of materials.
Very low throughput.
Does not measure affinity.
Complexes binding at sub 0.1 µM affinity will disintegrate during the run.

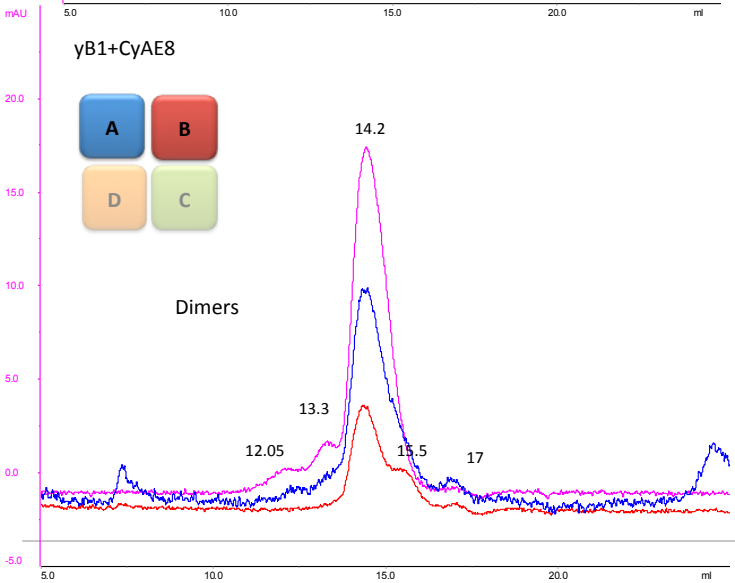
Three proteins forming a complex

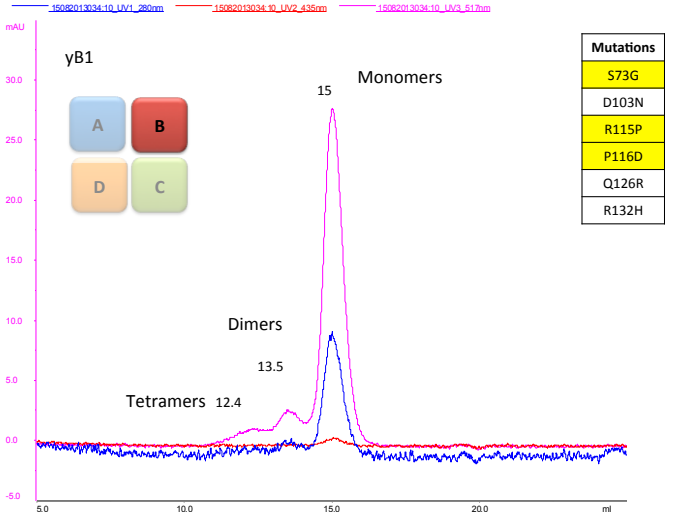


Monitoring fluorescence labeled proteins by GF, how a homotetramer is transformed into a hetero-dimer.



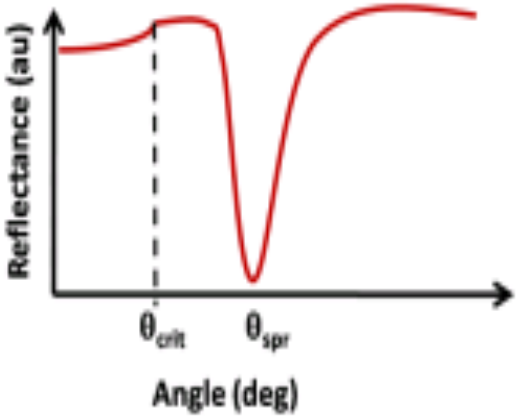






SPR: ProteOn™ XPR36, BIAcore Protein Interaction Array Systems

High Throughput Real Time Label Free Biomolecular Interactions



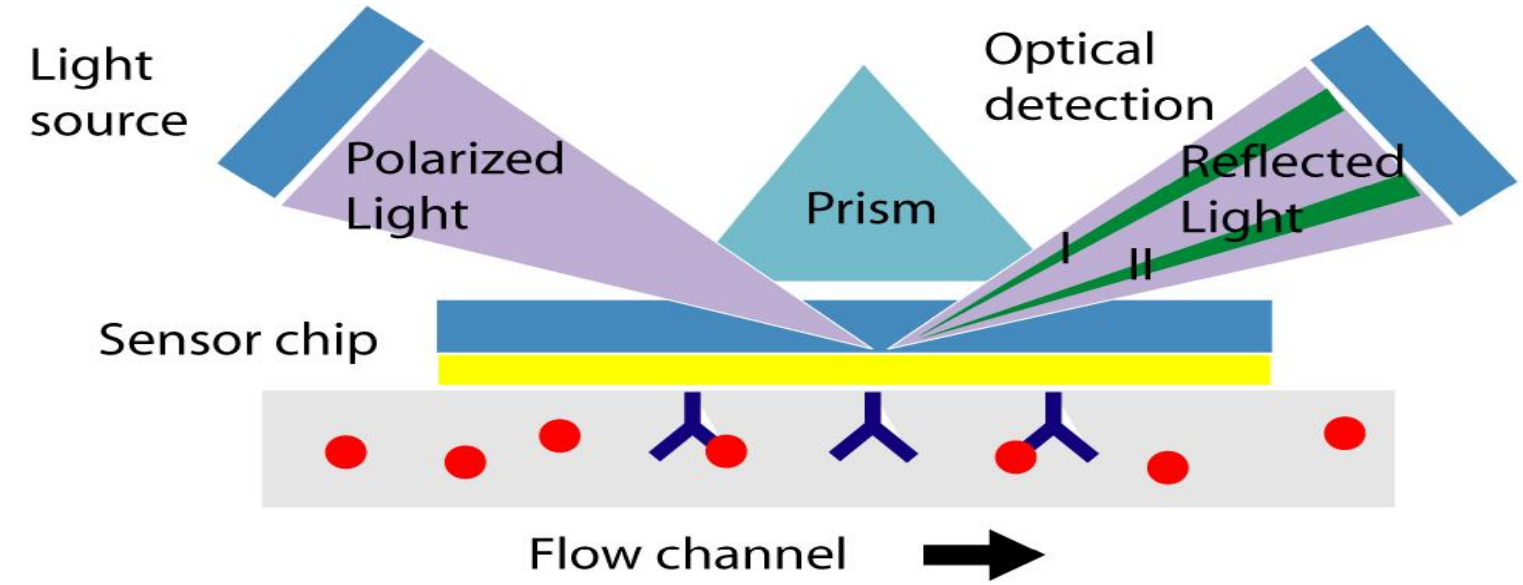
- SPR occurs when a thin conducting film (50 nm) is placed at the interface between the two optical media.

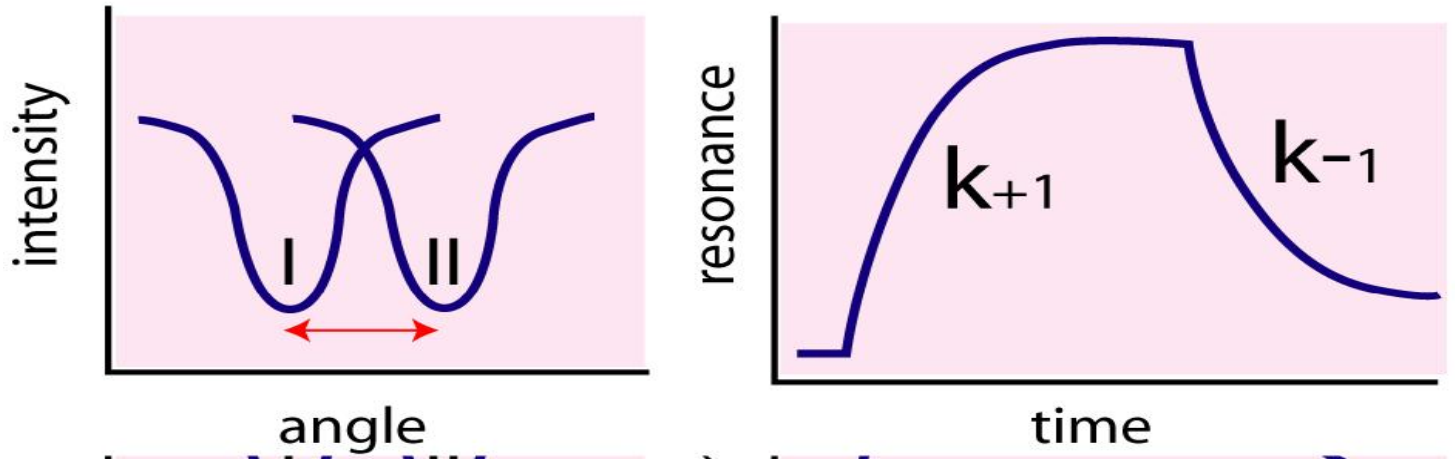
- At a specific incident angle, the surface plasmon in the conducting film resonantly couple with the light - this is our RESONANCE !

- Since energy is absorbed in this resonance, the reflected intensity, shows a drop at the angle where SPR is occurring.

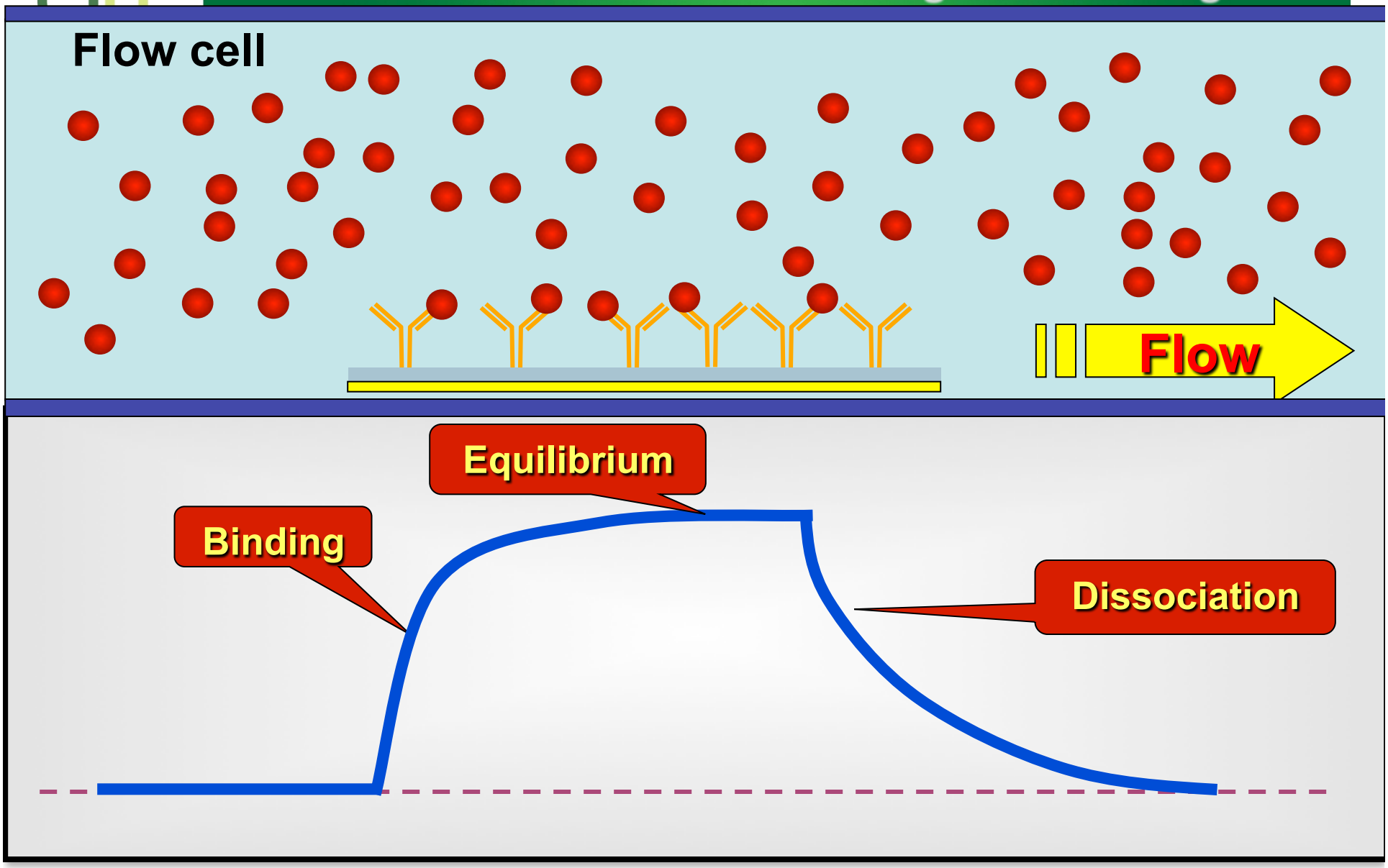
Change in mass of complex bound on surface is proportional to change in angle of totally reflected polarized light.
(0.1 deg =~ 1000 RU =~ 1 ng/mm^2)

Typical SPR Signal





From binding to SPR sensogram



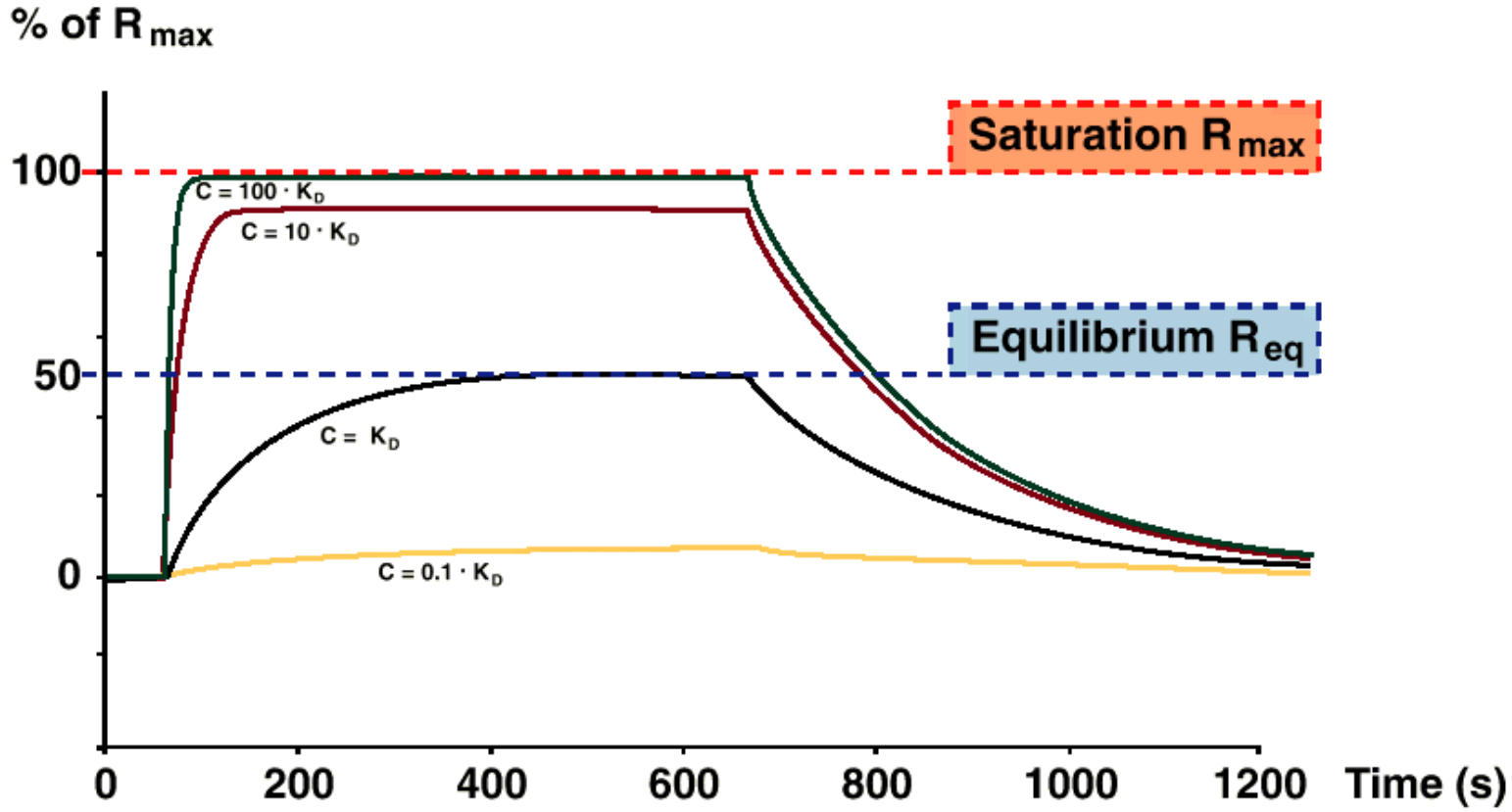
Types of Data Analysis Using SPR

- **Yes/No** – binding partners.
- **Kinetics** – k_a and k_d association and dissociation rate constants, respectively.
- **Equilibrium** – K_D affinity constant.
- **Concentration Determination** – concentration of active analyte.
- **Thermodynamics** – free energy, enthalpy and entropy. Explains the magnitudes of the binding kinetic constants.

Bio-molecules Interactions:

- Protein-Protein
- Protein-Peptide
- Antibody-Antigen
- Protein-Small Molecules
- DNA-Protein
- DNA-DNA
- Carbohydrate-Protein
- Lipids

Information is plotted as a Sensorgram



Important considerations

- What to consider when preparing for an SPR experiment
- What is R_{max} and why is it important
- Optimizing your experiment
  - Ligand Immobilization Strategies
  - Optimizing analyte Injection for good kinetic analysis
  - Mass Transport and Re-binding
- Data Analysis - choosing the correct references
- Preparing data for publication

What you need to know before you start…

Interacting compounds info:

- Molecular Weights
- pI
- Tags
- Concentration
- In which buffer (pH, salt, additives)
- Sample purity
- Binding observed with other methods?
- Will it be a 1:1 interaction?

Experiment design:

- Which molecule is the ligand and which is the analyte
- Ligand immobilization strategy and density
- Analyte interaction strategy (flow rate, time, conc.)
- Assess non-specific binding (high pI proteins)
- Are there any known regeneration conditions?
- Positive/negative controls

Seems like a lot but these are the factors you consider before most experiments!

The relationship between ligand and analyte and the importance of R_{max}

$$R_{\max} = n \frac{M_A}{M_L} R_L$$

R_{max}	Maximum theoretical response of analyte for a given ligand level
n	Stoichiometric number of analyte-ligand interaction
M_A	Analyte molecular weight
M_L	Ligand molecular weight
R_L	Surface density of ligand

Why is it theoretical? Assuming that:

- All of the ligand is active
- Ligand is 100% pure
- All binding sites are available

Experimentally you can measure how active the ligand is using:

% active ligand = (actual R_{max} / theoretical R_{max})*100

Ligand Immobilization – Decisions.. Decisions….

Golden Rule: for good and reliable kinetic analysis, we want Rmax of ~200 RU

What is the ligand density that will give us Rmax = 200 RU ?

$$R_{max} = n \frac{M_A}{M_L} R_L \; ? \longrightarrow 200 \text{ RU} = 1 \text{ x } \frac{1000 \text{ Da}}{10{,}000 \text{ Da}} \text{ x } 2000 \text{ RU}$$

BUT this is using theoretical Rmax: assuming that the ligand is 100% active, pure an available…
In practice, we need to immobilize more, taking into account the lower activities.

Typical ligand activity values

Covalent coupling: 40-70%, but could be much lower

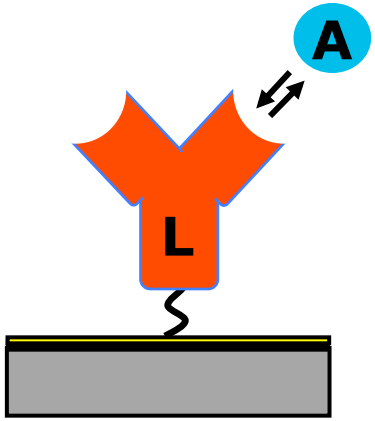
Capturing (Ab, His-tag, etc.): 60-90%

Ligand Immobilization – Decisions.. Decisions….

- Which molecule should be ligand, which should be analyte?
  - Purity, tag, pI, non-specific binding, ratio of MWt, number of samples to be screened (1 target molecule against 30 new drugs), etc
- Which method to immobilize the ligand to the sensor chip surface
  - Coupling or Capturing
- Experimental parameters to use
  - pH, salt, detergent, flow rate, injection volume, temperature & concentration
- Confirm ligand activity using control analytes if possible

Experimental Optimization takes time as there are many different options to try.
ProteOn SPR system allows you to screen 6 different parameters in one experiment

Coupling using Covalent Attachment of Ligand

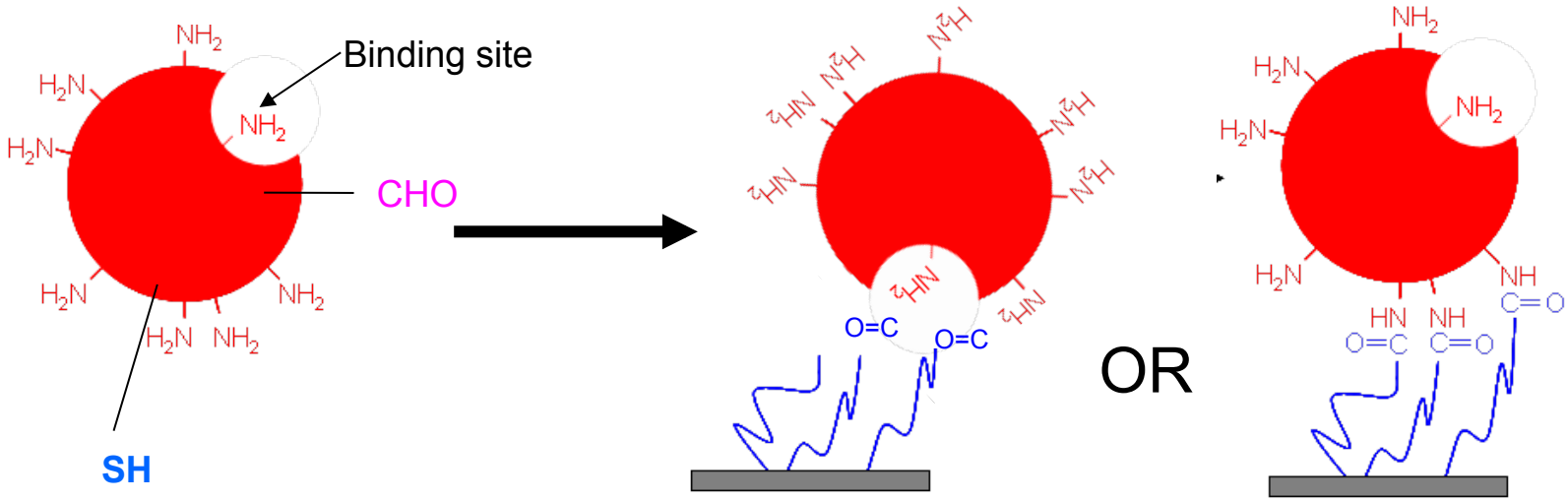


Advantages

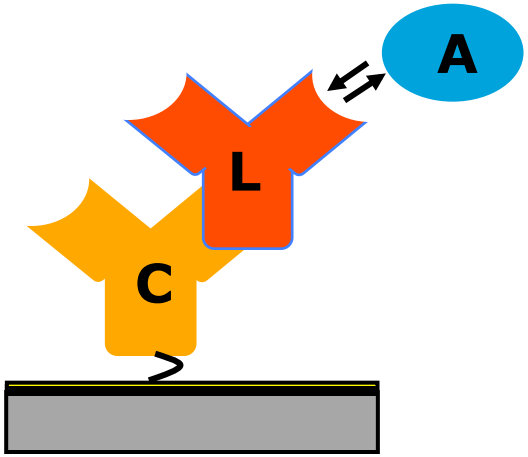
1. EDC/NHS - universal & simple
2. Thiol or Aldehyde – location specific (SH, CHO)

Disadvantages

1. Coupled heterogeneously
2. Can inactivate ligand



Capture using non-covalent attachment of Ligand

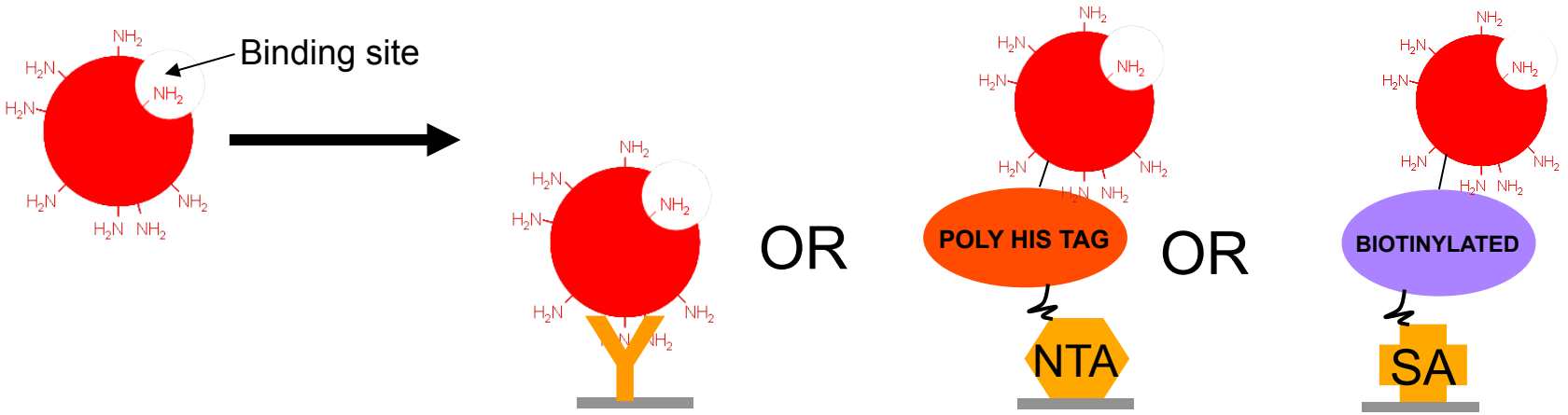


Advantages

- Specific Orientation
- Ligand Remains Active, free binding site
- Used with Crude Samples
- Allows easier regeneration

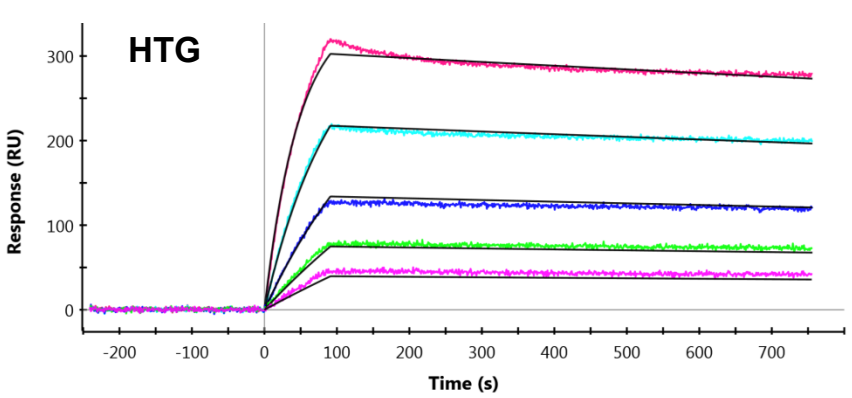
Disadvantages

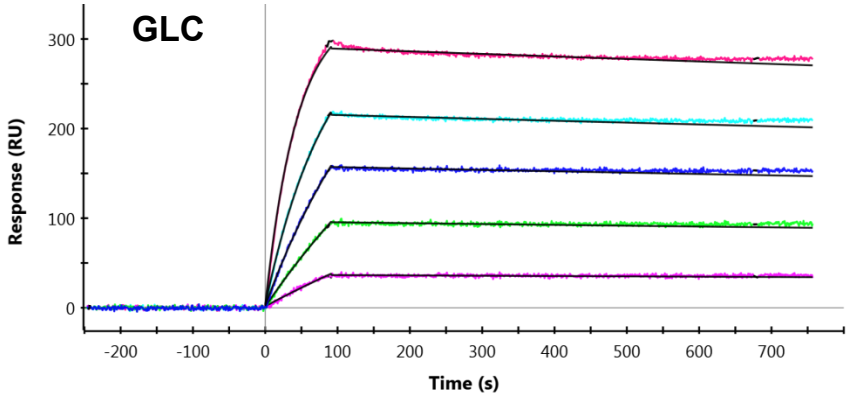
- Must have a Tag! Eg. His tag, Biotin
- Or have an antibody
- May increase assay time – extra steps



To couple or not to couple…
Capture leads to higher activity

His-tagged Protein A amine coupled or captured to sensor surface with Human IgG as analyte





	Ligand density	k_a	k_d	K_D	R_{max}	Activity
	RU	1/Ms	1/s	nM	RU	%
HTG chip	**58**	2.1×10^5	1.5×10^{-4}	0.74	358	**32%**
GLC (amine coupled)	**101**	2.3×10^5	1.0×10^{-4}	0.45	355	**18%**

$$\%Activity = \frac{Fitted\ R_{max}}{Theoretical\ R_{max}}$$

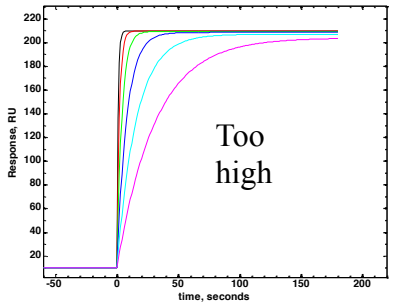
Interaction Step - Analyte Preparation

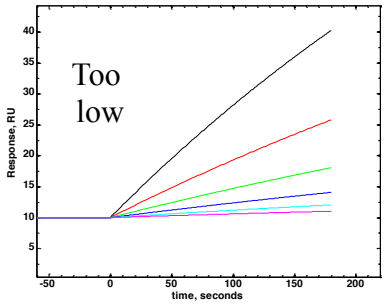
- Knowing the analyte concentration is CRUICIAL!!! Directly affects k_a and K_D
- Dilute your analyte with the running buffer to minimize bulk effects
- Short spin before injecting – the online degasser is only for the running buffer
- Be sure signal is stable before injecting
- Prepare five or six concentrations (serial dilutions) covering 0.1 to 10 times the K_D

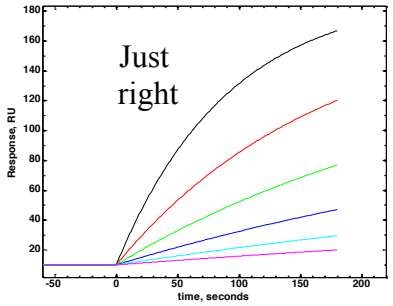
K_D = 10 nM → Use 1 nM to 100 nM

Interaction Step
Association Phase

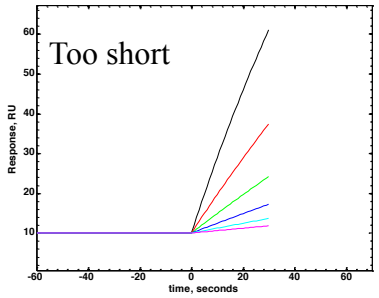
Concentration Effect

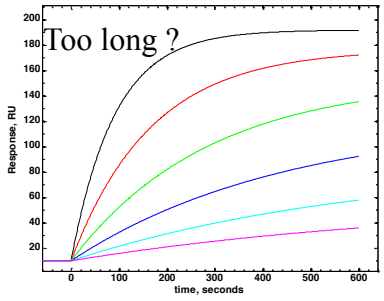


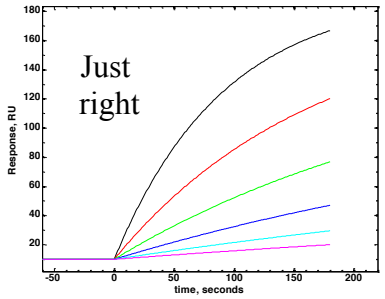




Contact time







The binding and unbinding events are first order – exponential. A curvature is required for good data fitting.

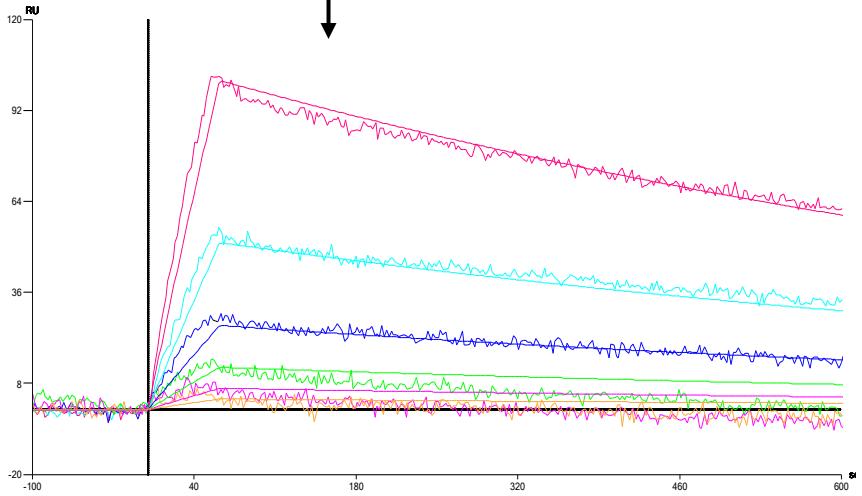
Models having higher k_a and k_d will reach equilibrium faster and will display earlier curvature in sensorgrams

Mass Transport

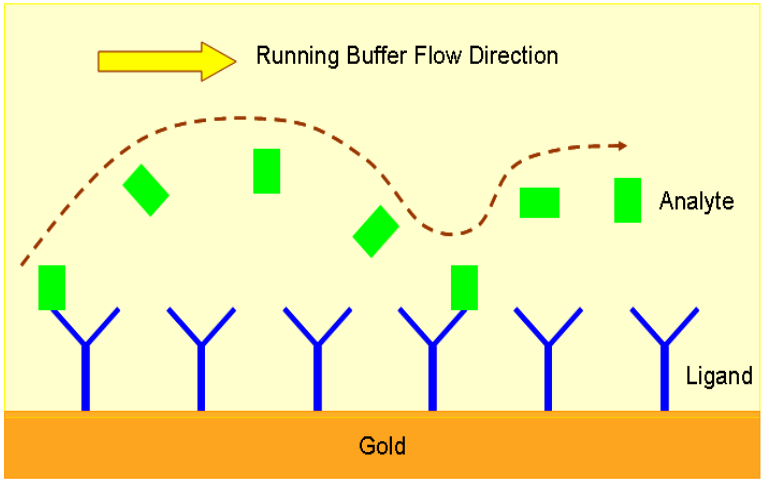
When diffusion of the analyte to the ligand surface is slower than the rate of binding, mass transport (MT) effect creates a shortage of the analyte at the ligand surface.

MT effect: (1) Straight binding curves (2) Kinetics affected by flow rate

Inconsistency in fitting



MT effect in dissociation - rebinding effect

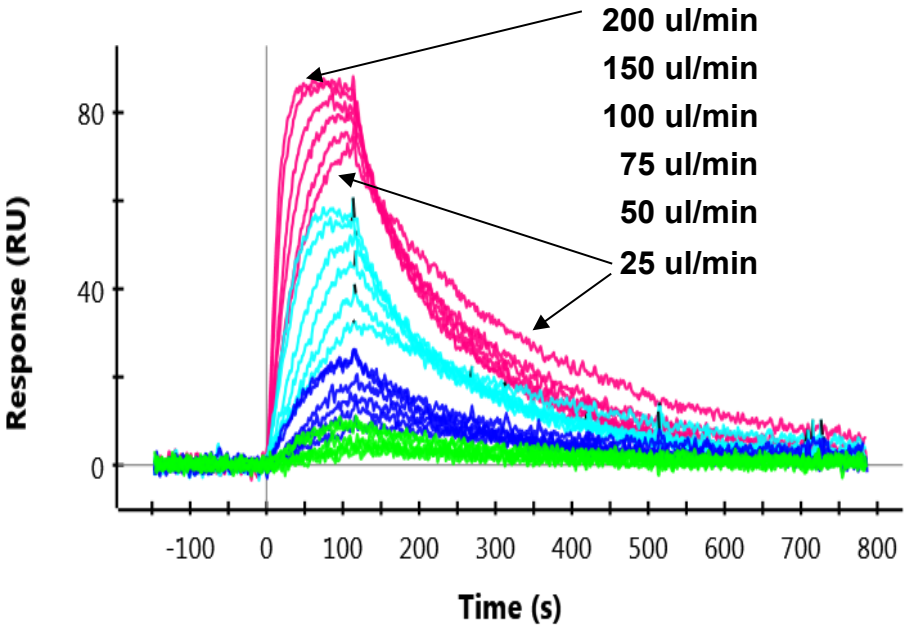


Mass Transport – how to avoid ?

There are two easy ways to avoid being in mass transfer limitation conditions

1. Decrease ligand density
   - Rule of thumb – work at Rmax < 200 RU.

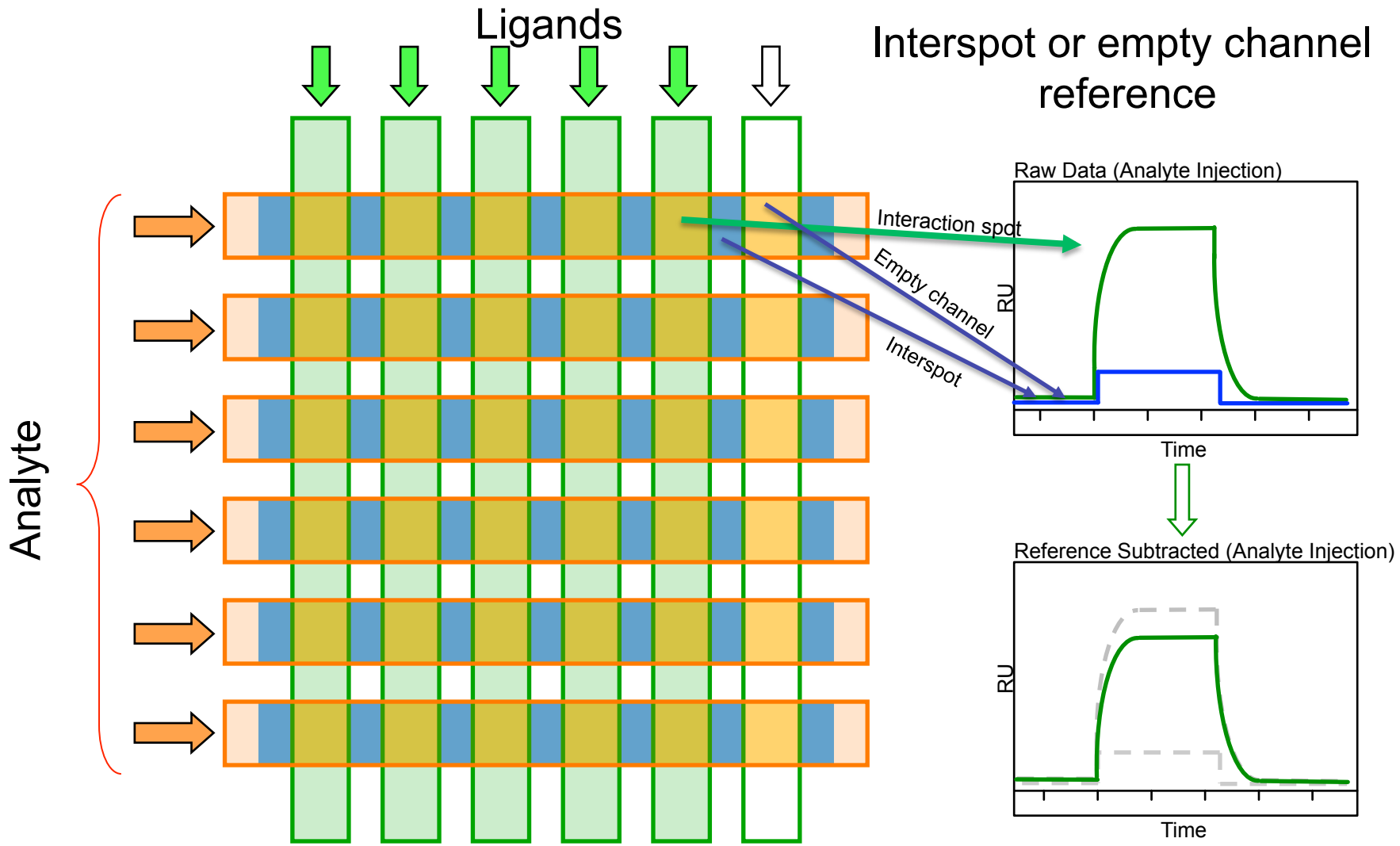
2. Increase analyte flow rate:



Fitting to 1:1 Langmuir model

Parameter / Flow rate	k_a (1/Ms)	k_d (1/s)	K_D (M)
25 ul/min	1.6×10^{4}	3.6×10^{-3}	2.2×10^{-7}
50 ul/min	2.0×10^{4}	4.9×10^{-3}	2.5×10^{-7}
75 ul/min	2.6×10^{4}	5.5×10^{-3}	2.1×10^{-7}
100 ul/min	3.2×10^{4}	6.1×10^{-3}	1.9×10^{-7}
150 ul/min	3.9×10^{4}	6.5×10^{-3}	1.7×10^{-7}
200 ul/min	4.2×10^{4}	7.0×10^{-3}	1.6×10^{-7}

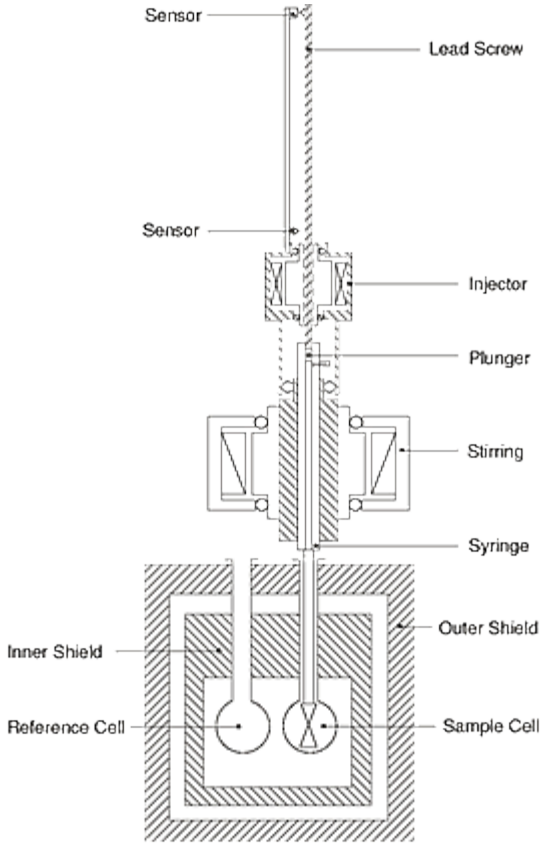
Blank Surface Reference



Kinetic Models

- **Langmuir:** Simple 1:1 bimolecular interaction ← Start here!
- **Langmuir + Drift:** Calculates linear drift /loss of mass from the surface of the chip
- **Mass Transport**: The diffusion of the analyte to the surface is slower than the interaction itself.
- **Heterogeneous Ligand**: One analyte is binding two separate ligand species.
- **Heterogeneous Analyte**: Two analytes compete for binding to one ligand site.
- **Bivalent Analyte**: The injected analyte has two binding sites to the ligand.
- **Two State**: Accounts for a complex that changes shape after interacting with the analyte

Isothermal Titration Calorimetry (ITC)



Taken from Micro Cal website

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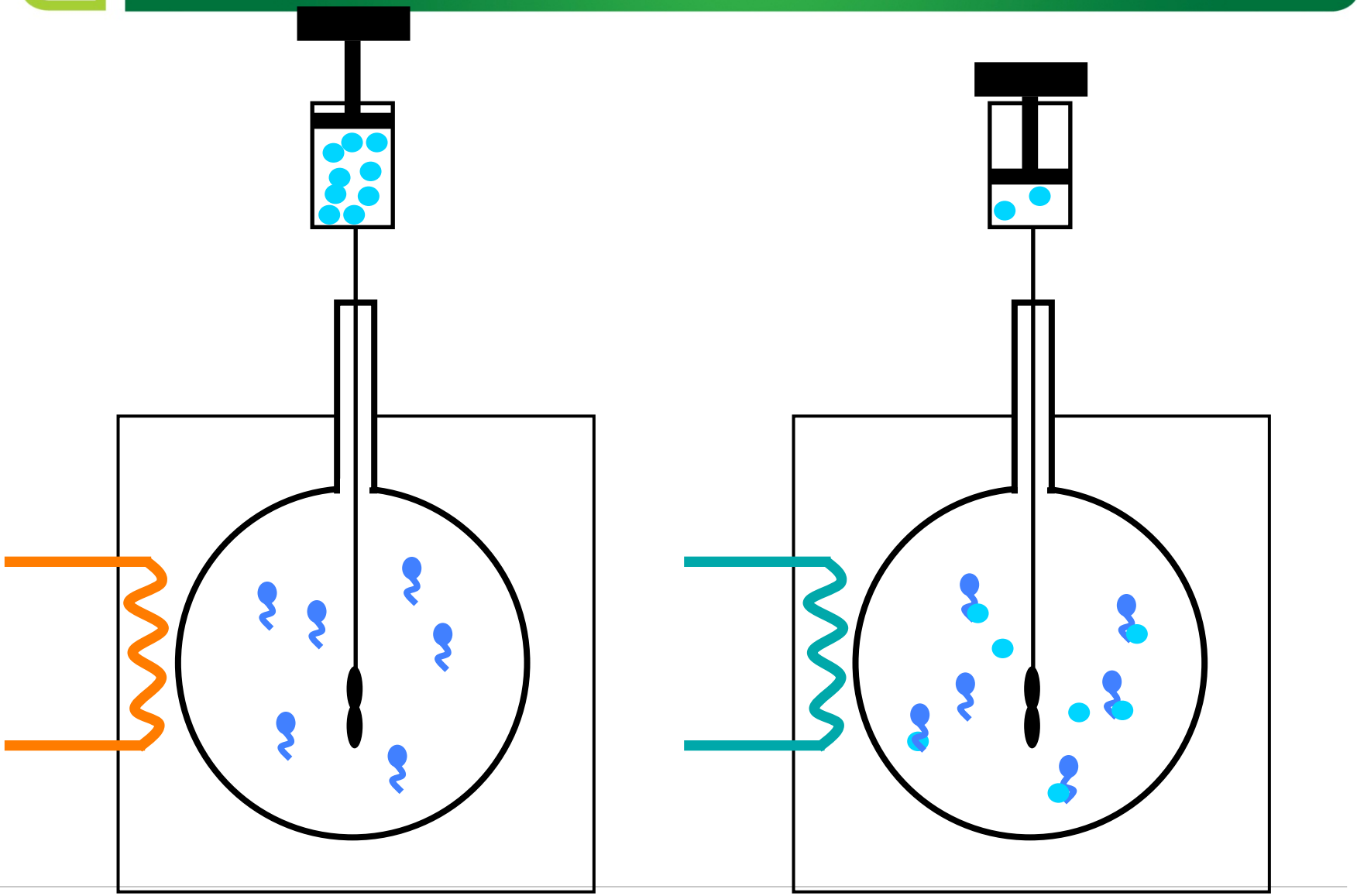

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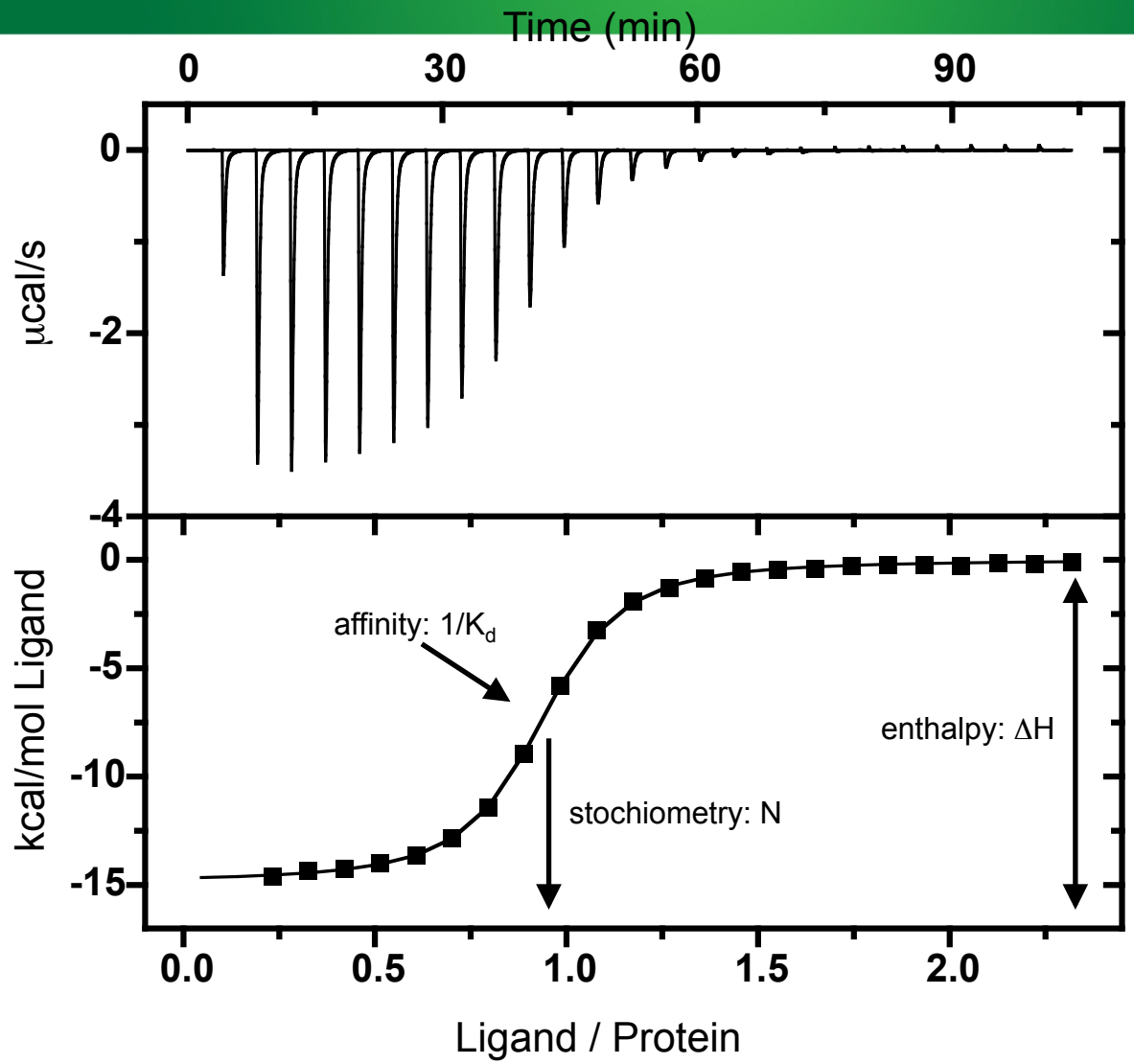

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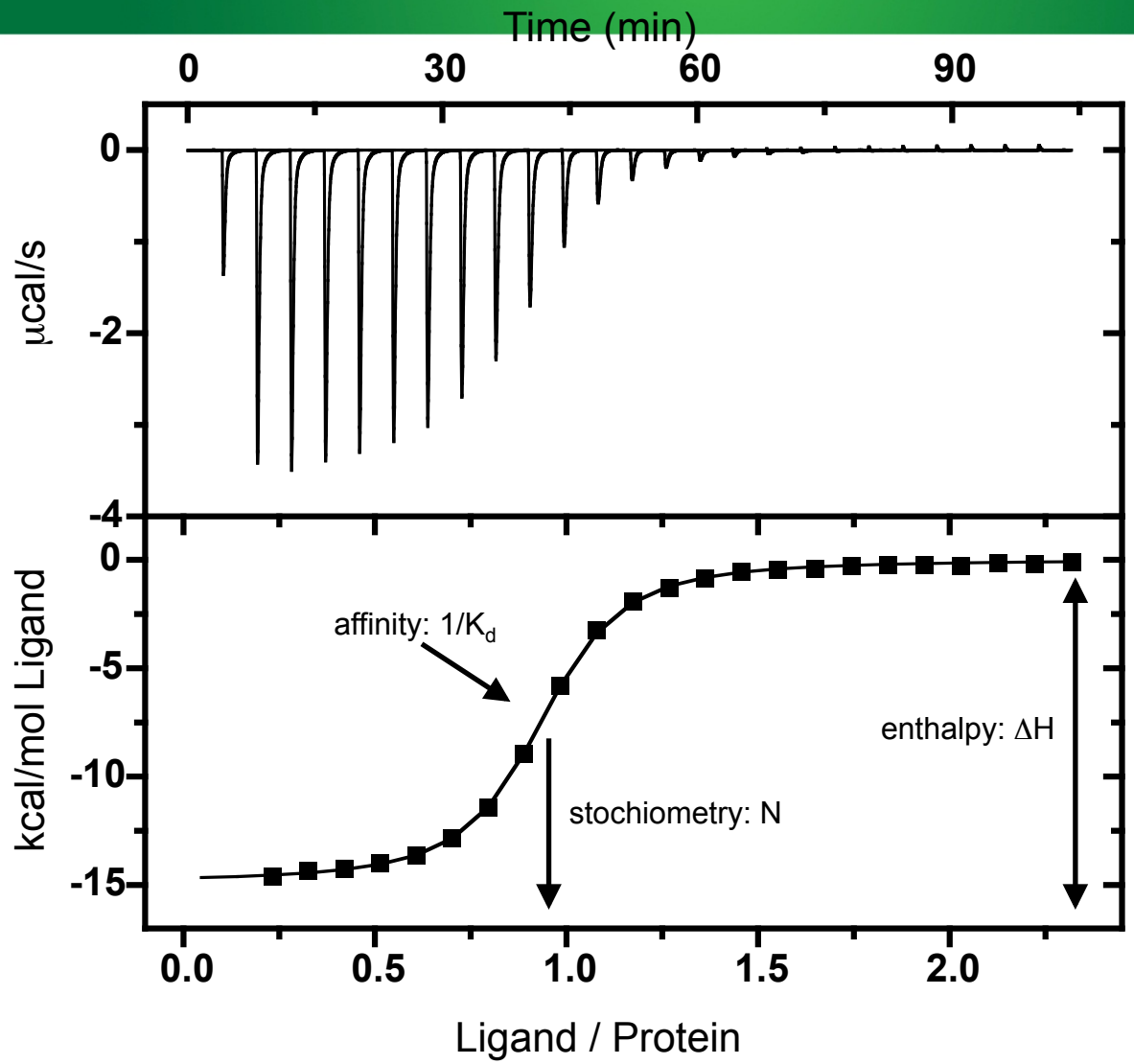

$\Delta G = -14.1$

$\Delta H = -19.9$

$T\Delta S = -5.8$

Isothermal Titration Calorimetry (ITC)

Review of Free Energies, Enthalpies, and Entropies of Binding

$\Delta G°bind = RT \ln K_D$ (where R= 1.98 cal mol^{-1} K^{-1}; T= 273.2 K, and RT =0.62 kcal/mol at 37°C)
Note log relationship between free energy and binding constants

Recall that $\Delta G°bind$ is relative to standard conditions (typically 1M reactants, 25 °C, standard salt)

A convenient rule of thumb is that a 10-fold change in binding constant corresponds to 1.4 kcal / mol.

$\Delta\Delta G°A1\text{-}A2 = RT \ln(K_DA1 / K_DA2) = (0.62 \text{ kcal / mol}) \ln(10^{-8} \text{ M} / 10^{-7} \text{M}) = -1.4 \text{ kcal / mol}$

How many kcal / mol change in free energy do you need to change K_D 100-fold?

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Recall also that free energy has enthalpy and entropy components:

$\Delta G° = \Delta H° - T\Delta S°$ (and therefore) $-RT\ln K_A = \Delta H° - T\Delta S°$

When is an interaction strong?

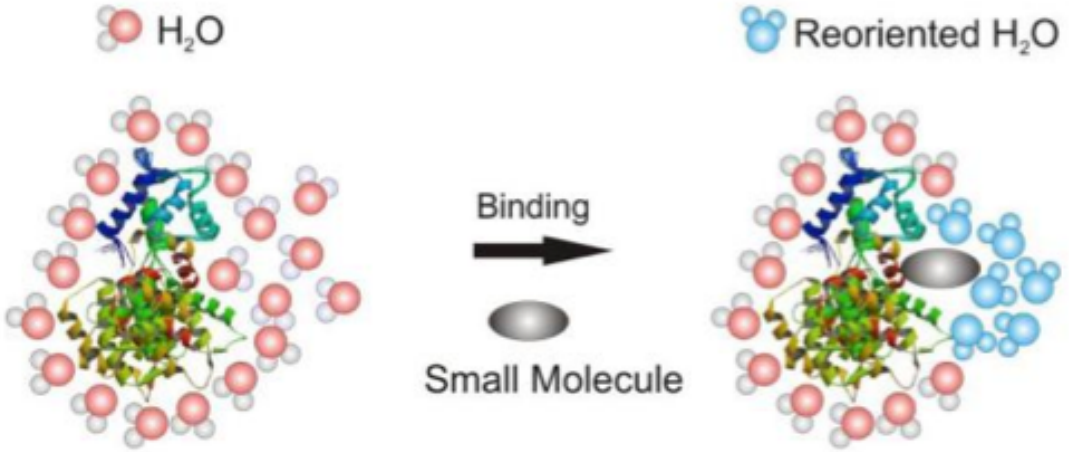
$\Delta G°$ must be large and negative
$\Rightarrow \Delta H°$ must be large and negative (gain new bonds)
$\Rightarrow \Delta S°$ must be large and positive (gain more entropy)

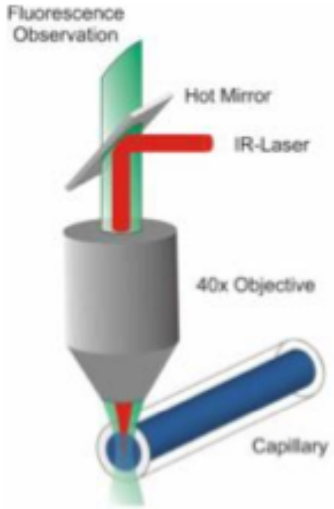
Microscale Thermophoresis

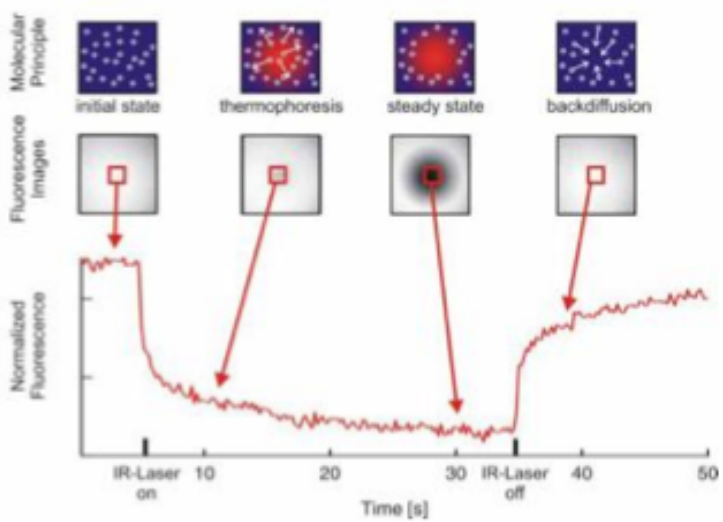
MST is a technique that detects changes in the hydration shell of molecules.

Infrared lasers are used to achieve precise temperature gradients within thin glass capillaries that are filled with a solution of choice.

Molecules move along these temperature gradients. Any change of the hydration shell affects the thermophoretic movement, and is used to determine the binding constant







Application of the method

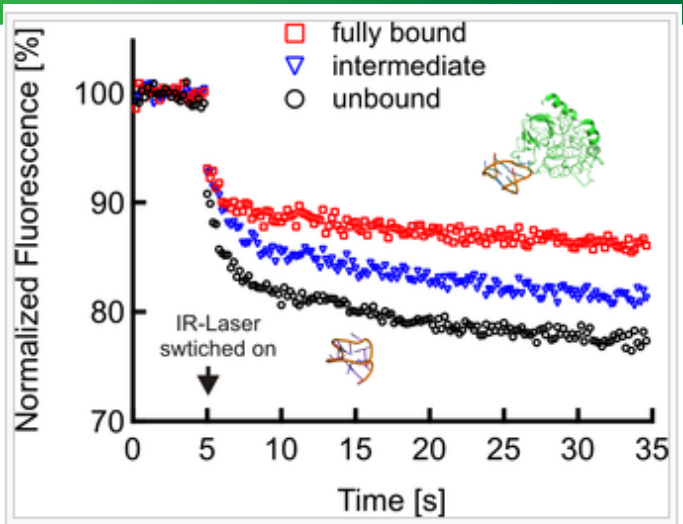
Requires only very small amounts of proteins.

Proteins do not have to be pure, particularly if the protein is fused to GFP (or similar).

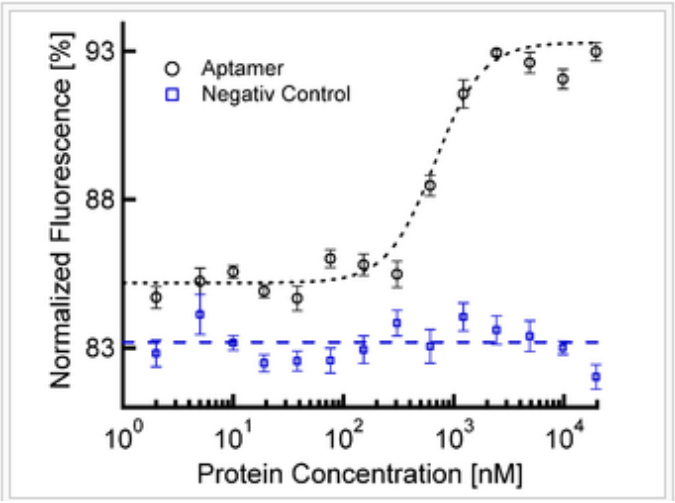
The protein at constant concentration is labeled (either by a dye or co-expressed) while the second protein (ligand) is titrated

Provides equilibrium binding data, but not real time kinetic data.

The concentration of the titrating protein has to be known.



Microscale thermophoresis aptamer-protein binding assay. The thermophoretic movement of the fluorescently labeled aptamer changes upon binding to the protein



Stopped-Flow

Stopped-flow is a spectroscopic technique used for studying fast reaction mechanisms in solution over timescales of about 1ms up to 100's seconds. In general, two reagents are rapidly mixed together and then 'stopped' in an observation cell. The sample cell is irradiated with (usually) monochromatic light and as the reaction proceeds the change in the recorded signal, usually a fluorescence signal or the absorbance at a specific wavelength, is recorded as a function of time.

A series of stopped-flow experiments can be used to show the effect of parameters such as temperature, pH and reagent concentration on the kinetics of the reaction.

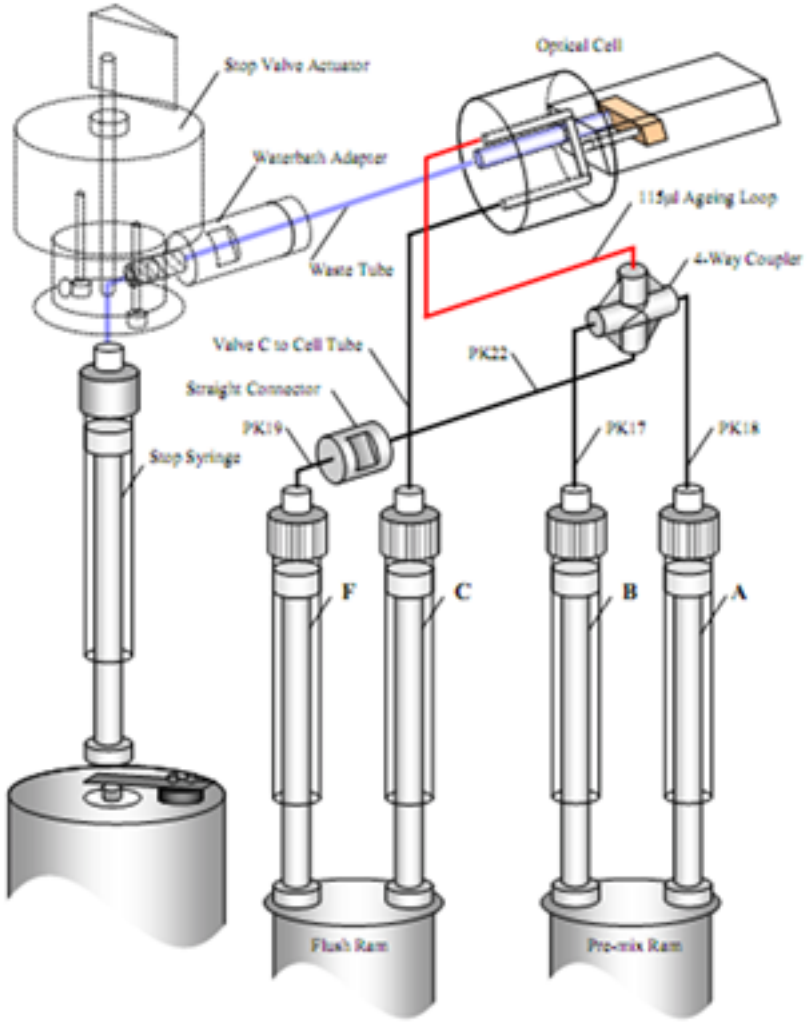
Normal and sequential mixing

Advantages:

Rapid mixing (1 ms).
All the materials are in liquid phase.
Can handle many types of solvents (pH, viscosity, ionic strength).
Not influenced by the size of the molecules.
Few artifacts, trusted rate constants.
Can monitor circular dichroism, absorbance and fluorescence.

Disadvantages:

Requires a probe to view the reaction (binding, folding, enzymatic rates), either intrinsic or extrinsic.
Requires relative high amounts of materials.
Low throughput.



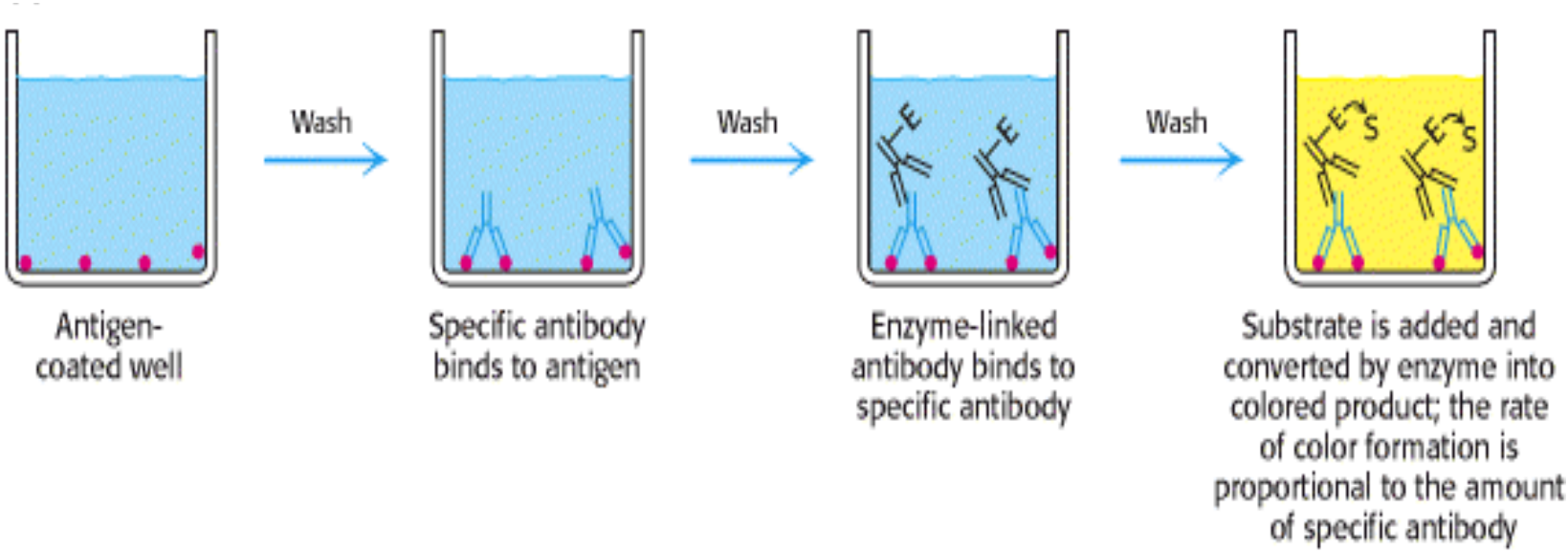
Exploring protein-protein interactions using ELISA

- Enzyme-linked immunosorbent assay
- Name suggests three components
  - Antibody (immuno)
    - Allows for specific detection of protein of interest
  - Solid phase (sorbent)
    - Allows one to wash away all the material that is not specifically captured
  - Enzymatic amplification
    - Allows you to turn a little capture into a visible color change that can be quantified using an absorbance plate reader

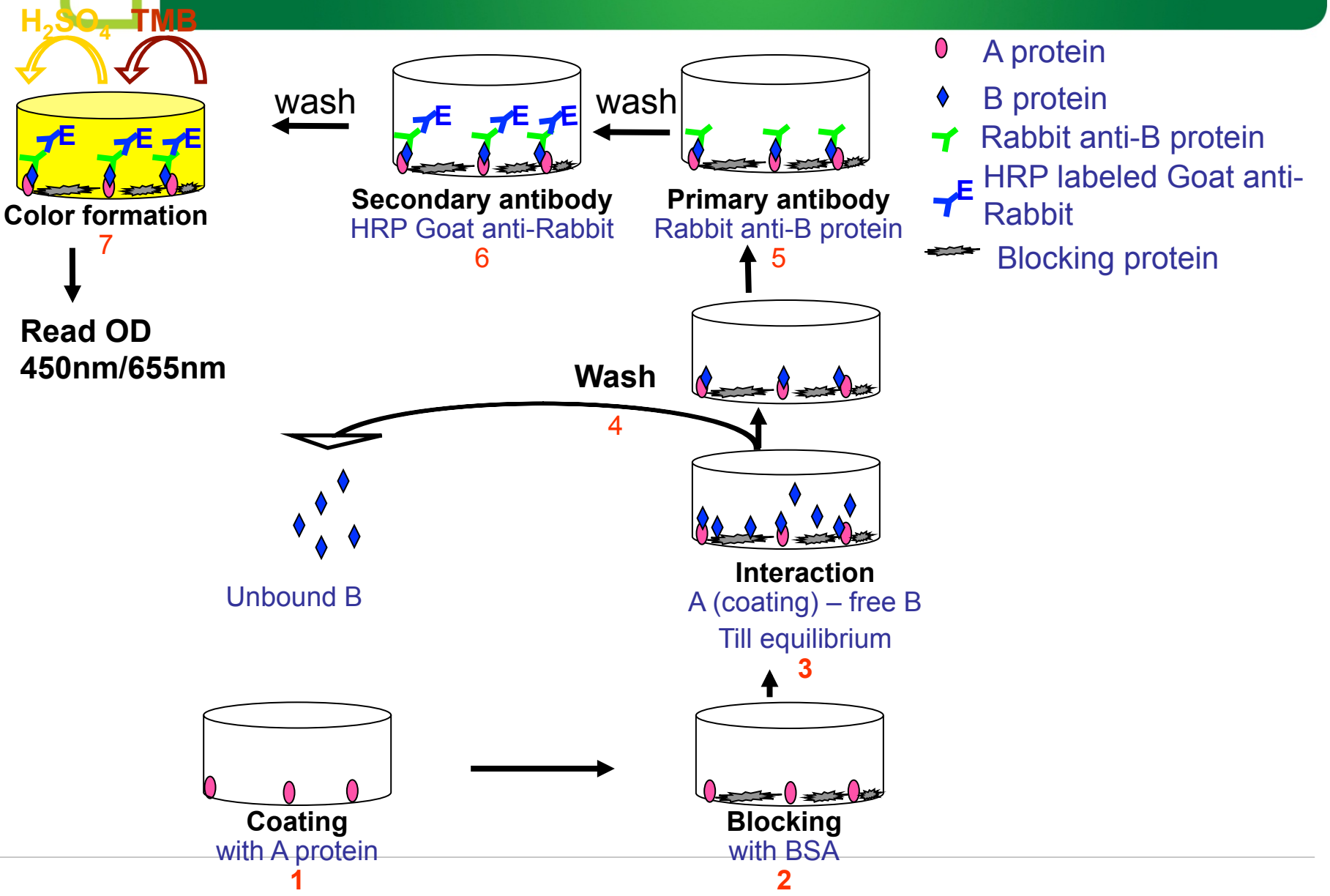
Concepts of ELISA

Widely use for diagnostics:

- Pregnancy (hormone)
- HIV (antibody)
- Hepatitis B (viral antigen+antibody)
- Food allergens



ELISA of protein-protein interaction



Consideration

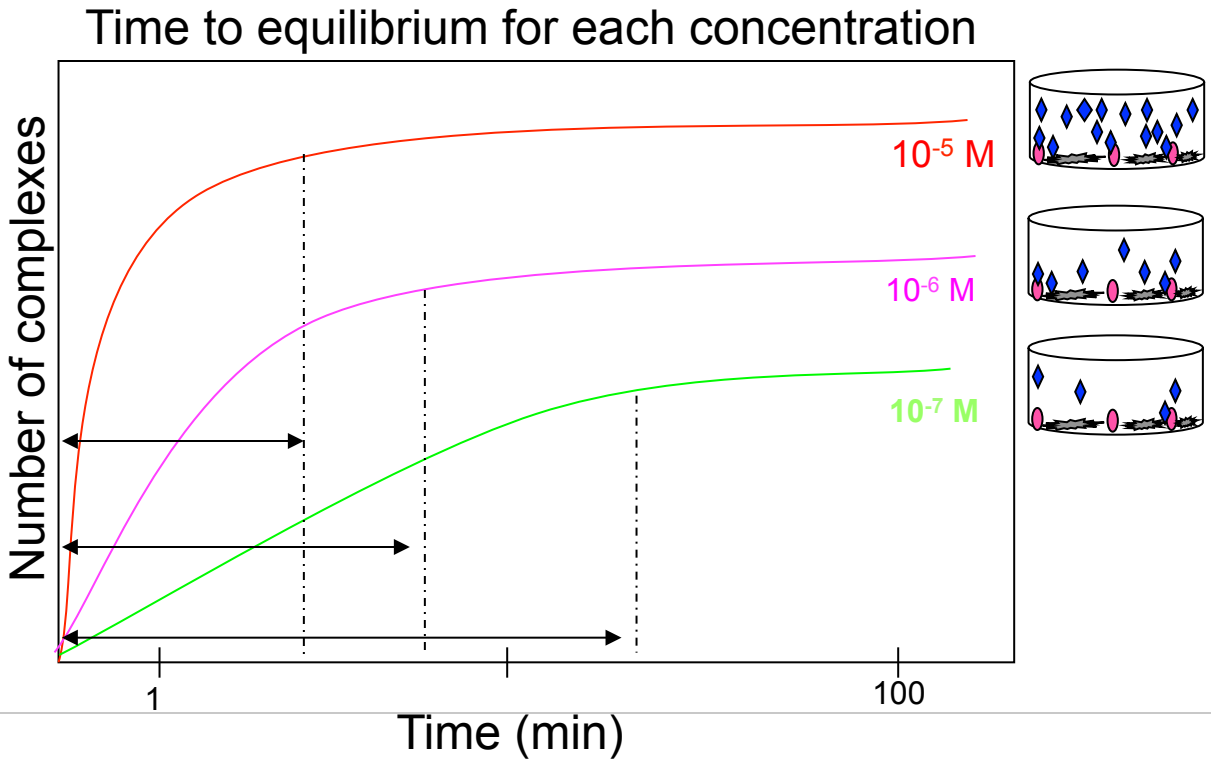
- The coated protein concentration can affect the false positive signal
- Non-specific adsorption of the coated protein to surface might affect the protein conformation
- The actual concentration of the absorbed protein available for interaction is smaller than the original concentration
- Blocking time should be sufficient to ensures no empty spaces are left

Consideration

- Binding – pH and temperature are very important
- The interaction takes place between the solid and liquid phases rather then in solution
- Binding – equilibrium time depends on the affinity of the interaction and the protein concentration (calibration needed)

Is time to equilibrium always the same?

- Washing step should be done only at equilibrium
- The association time is concentration depended
- Performing washes before all concentrations reach equilibrium will result in fouled measurements
- Calibration needed



Coating protein (A):

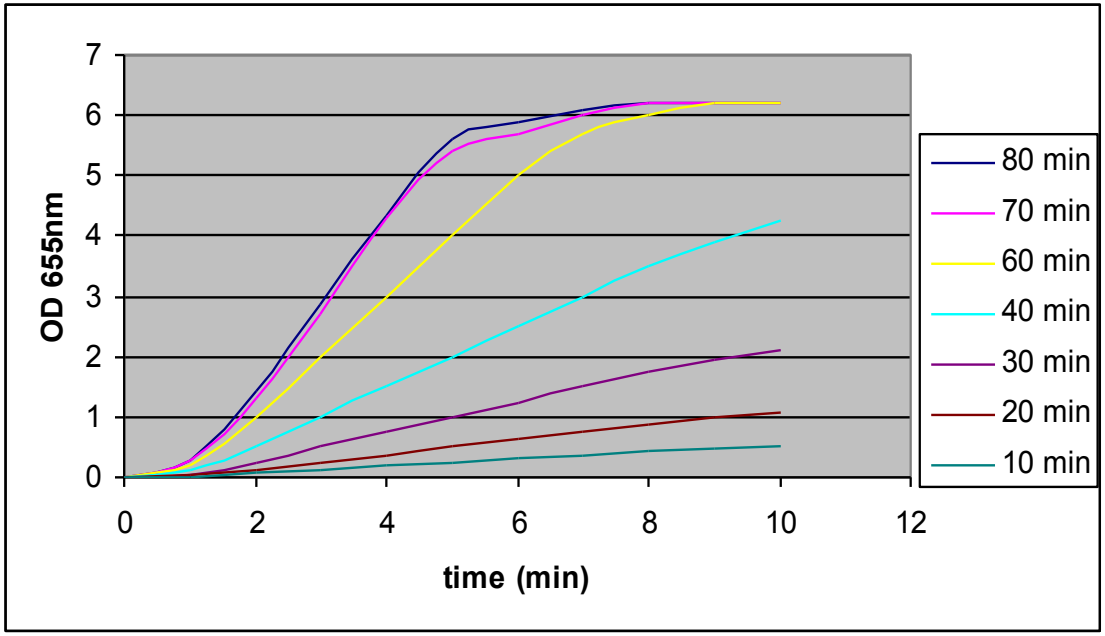
fixed concentration

interacting protein (B):

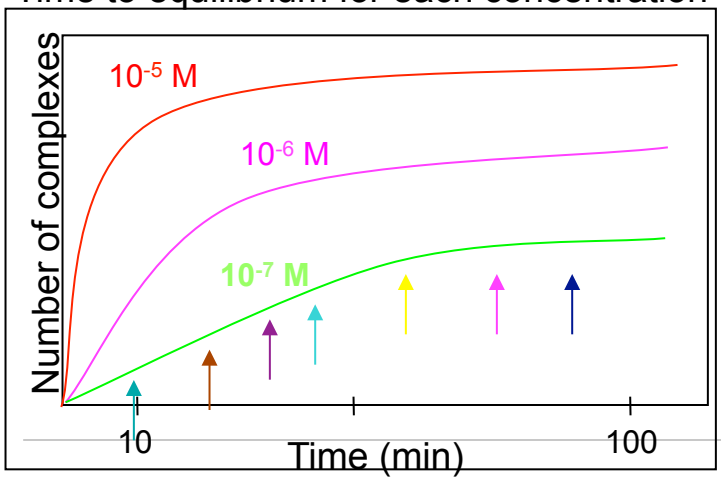
$10^{-5}/10^{-6}/10^{-7}$ concentrations

Calibrating the equilibrium time

OD Vs time for 10^{-7} M at different incubation times



Time to equilibrium for each concentration



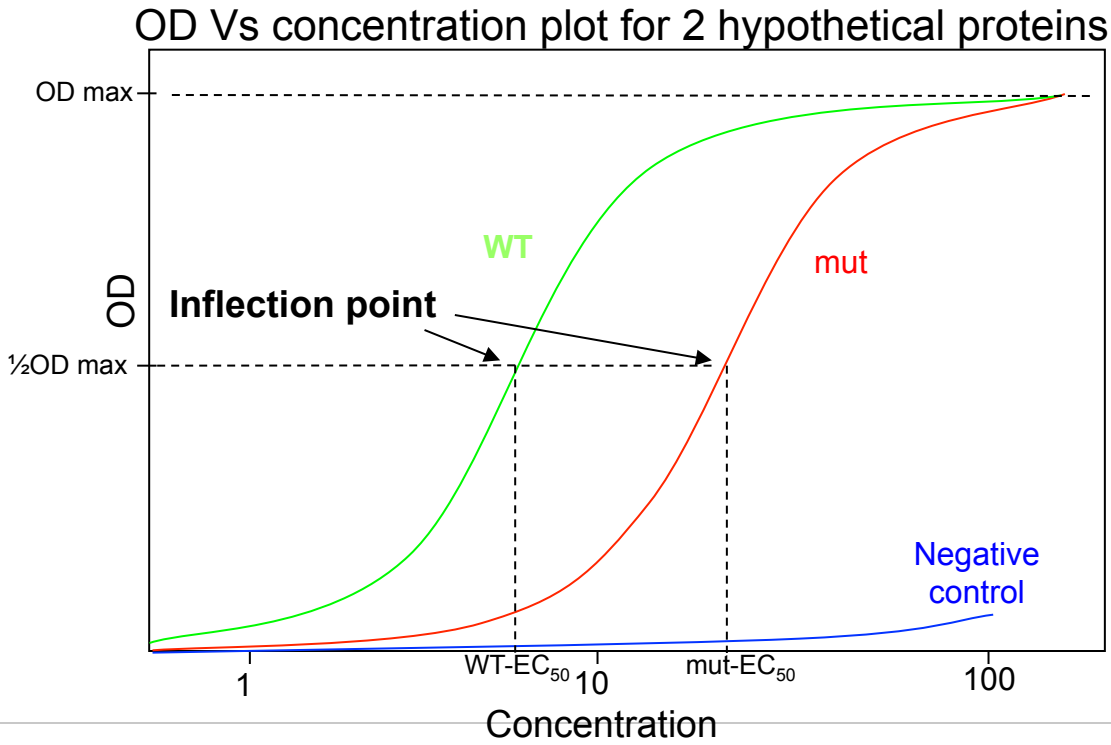
Consideration

- The number of washes is case specific and depend of the interaction affinity
- Each wash step reduce the non-specific interaction signal but also push the interaction out of equilibrium
- proper controls should be done for each step (96 wells are available)

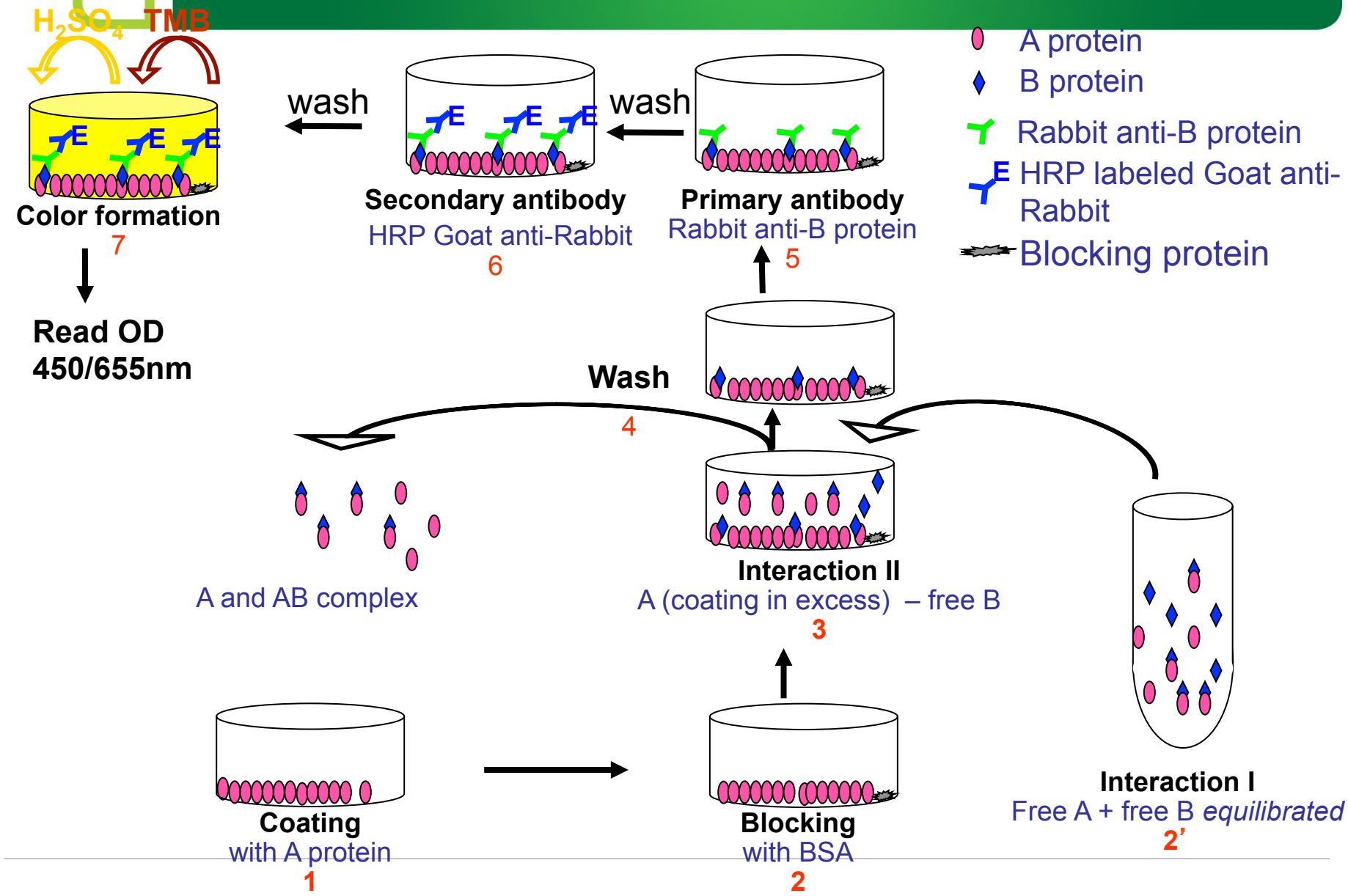
Plotting the data

- The plot by itself can give only a qualitative knowledge about the interaction
- Comparison between the curves of 2 interactions can give relative quantitative knowledge

$$\Delta\Delta G = \Delta G^{WT} - \Delta G^{mut} = -RT \ln\left(\frac{EC_{50}^{WT}}{EC_{50}^{mut}}\right)$$



semi competitive ELISA



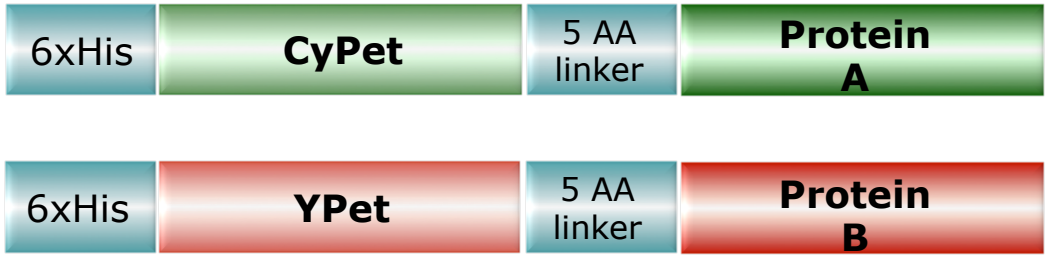
Method Advantages

- Sensitive – enzyme catalyst amplification
- Simple - passive attachment to solid phase, easy separation of bound/unbound proteins by washing steps
- Adjusted for screening large number of samples
- small amount of protein needed
- cheap

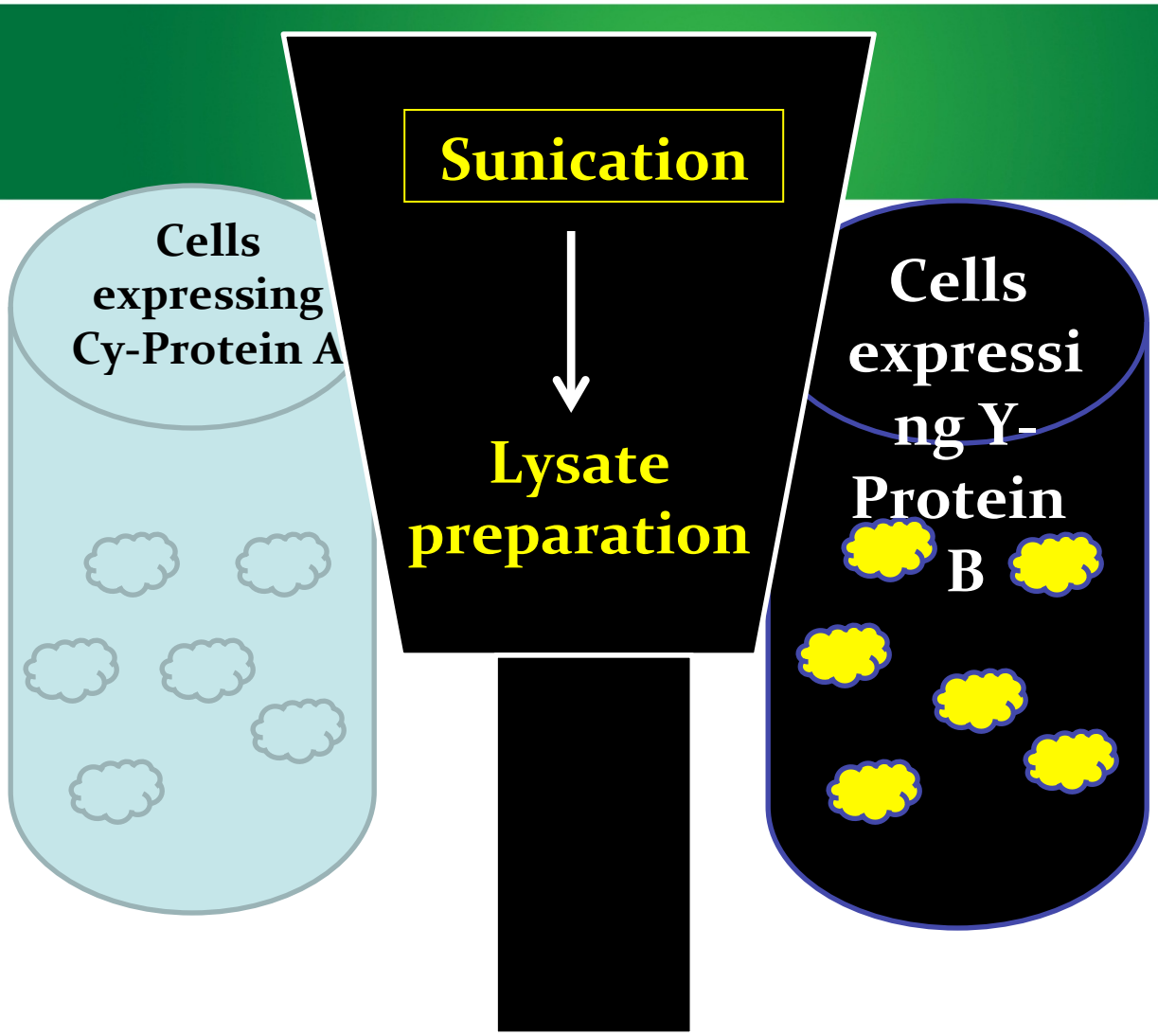
Method limitation

- Indirect measurement (washes, 2 antibody and enzyme involve)
- solid phase problems
- Can not measure kinetics of the interaction only the equilibrium
- Only relative affinity can be measure
- The measurement is done in-vitro

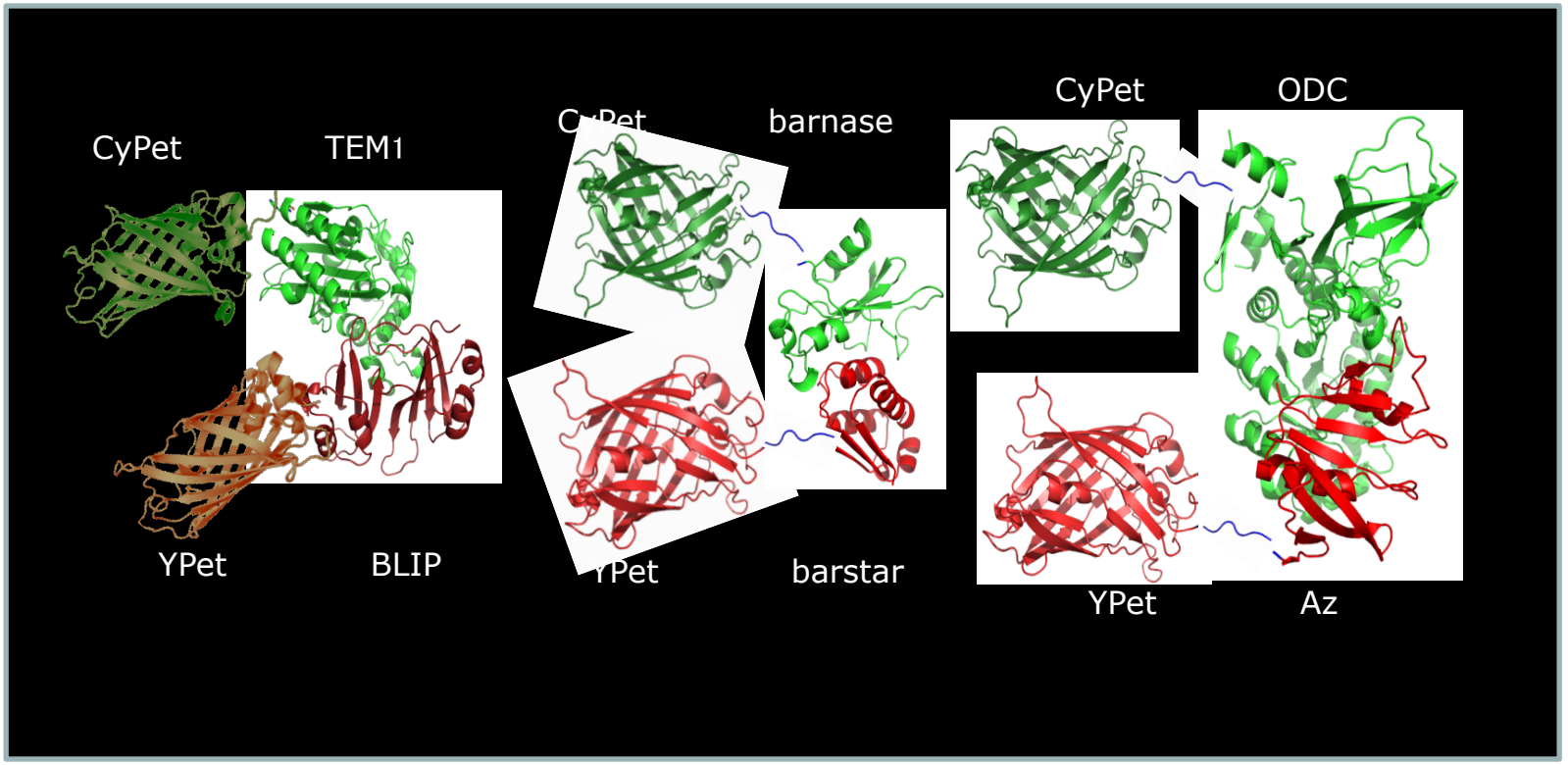
Measuring binding through FRET of binding partner



First – You design your DNA constructs

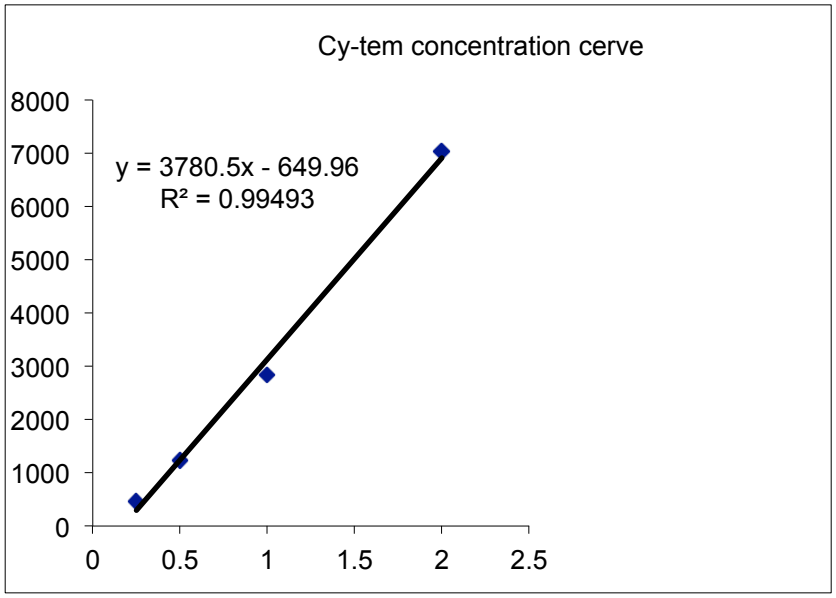

Sunication
Cells expressing Cy-Protein A
Lysate preparation
Cells expressi ng Y-Protein B

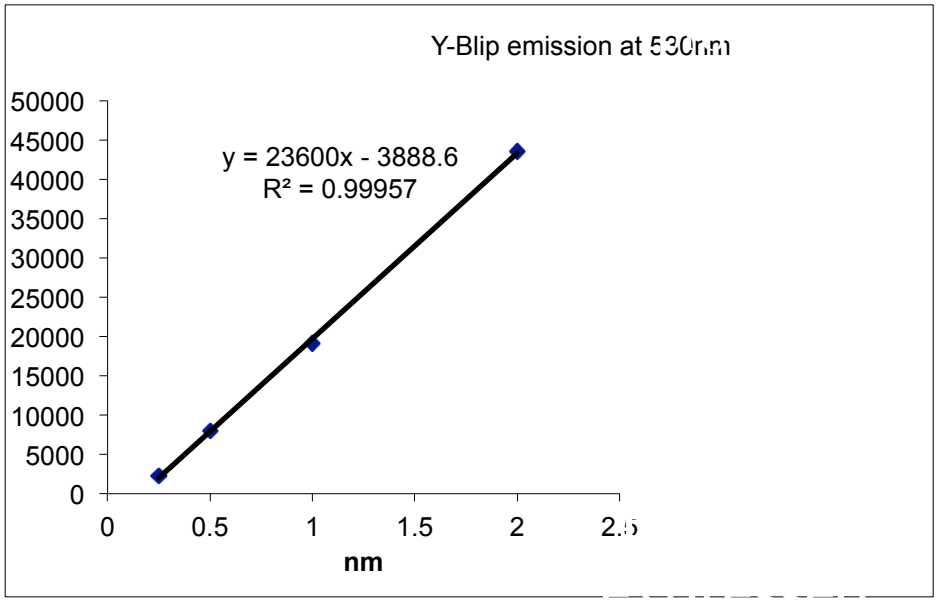
We used three protein pair systems for the method development



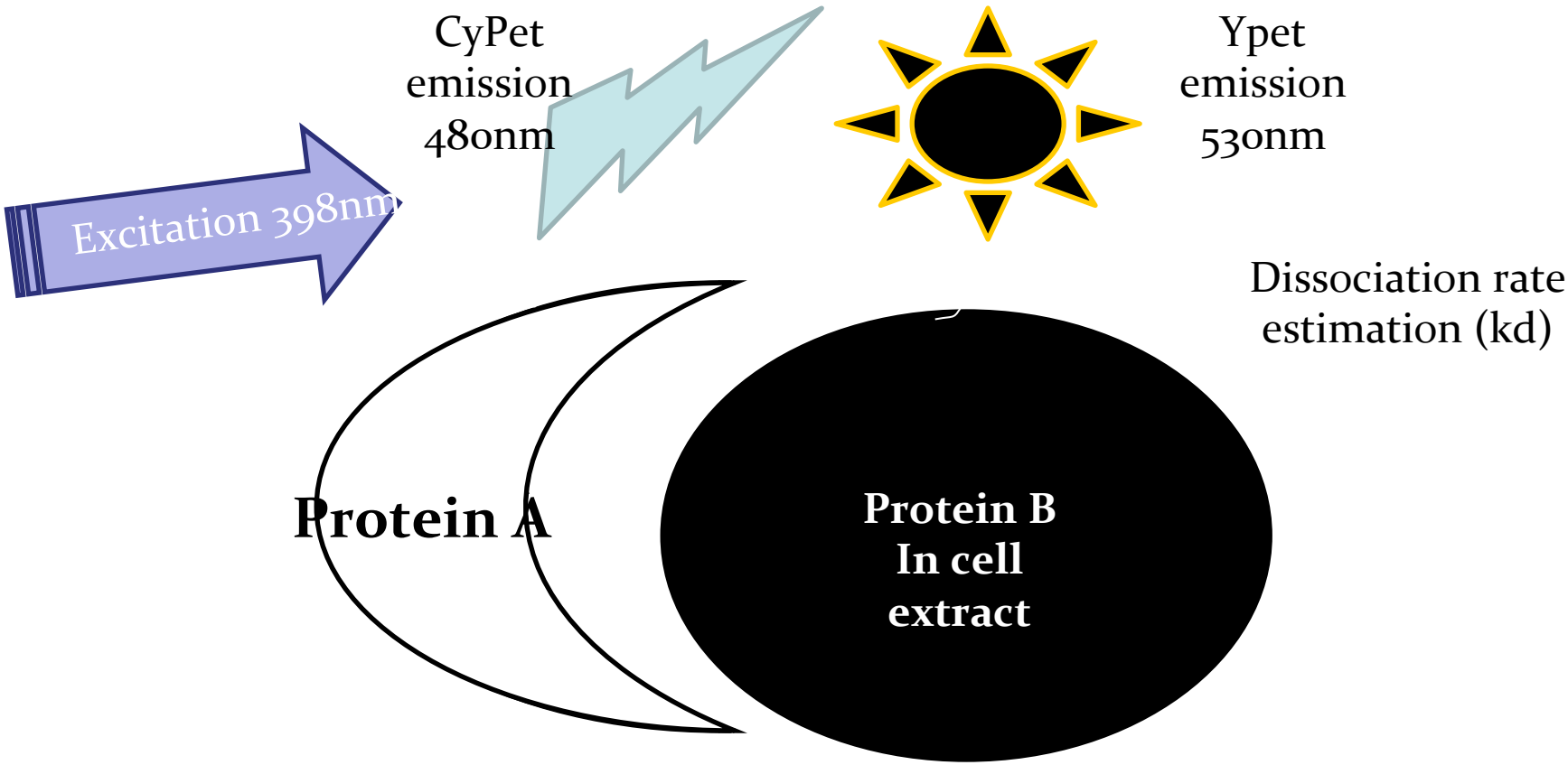
Then use the CyPet and Ypet fluorescence intensities to get an estimation of your protein concentration

These data are independent on the fusion partner

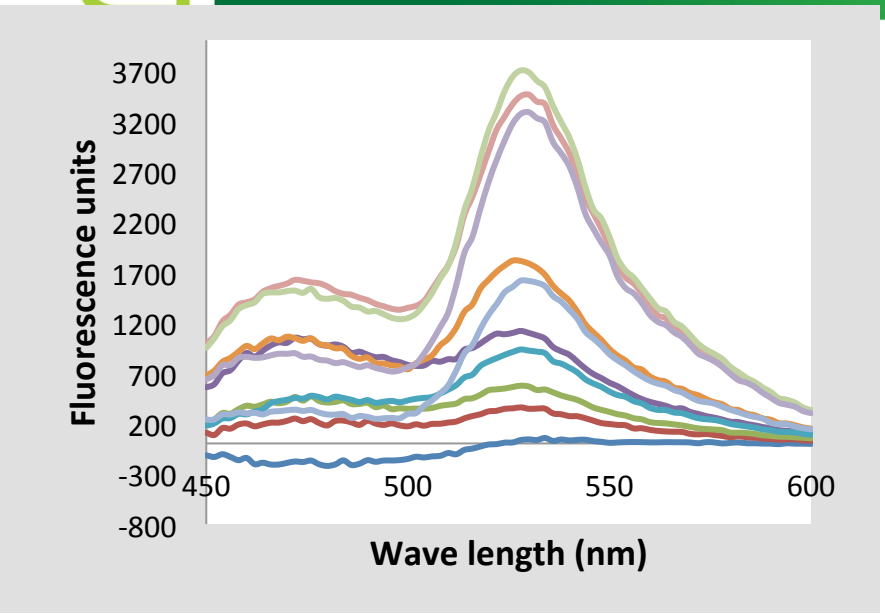


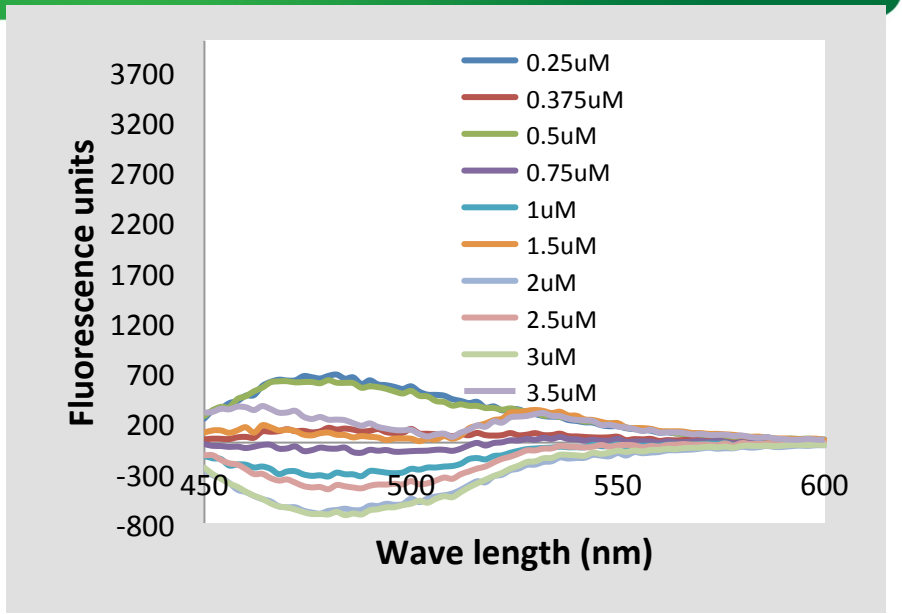


FRETex


CyPet
emission
480nm
Ypet
emission
530nm
Excitation 398nm
Dissociation rate
estimation (kd)
Protein A
Protein B
In cell
extract

What do you get?

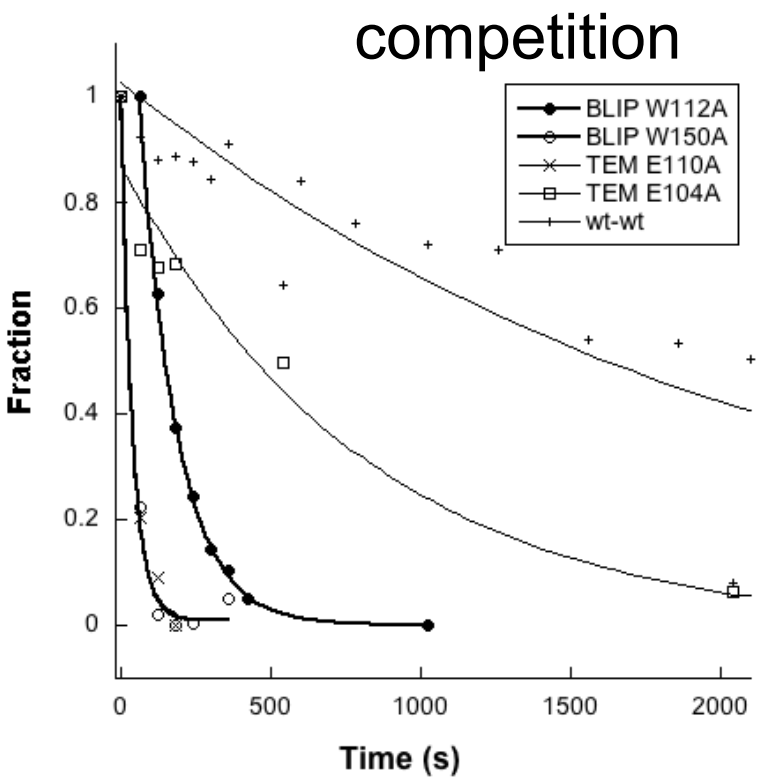


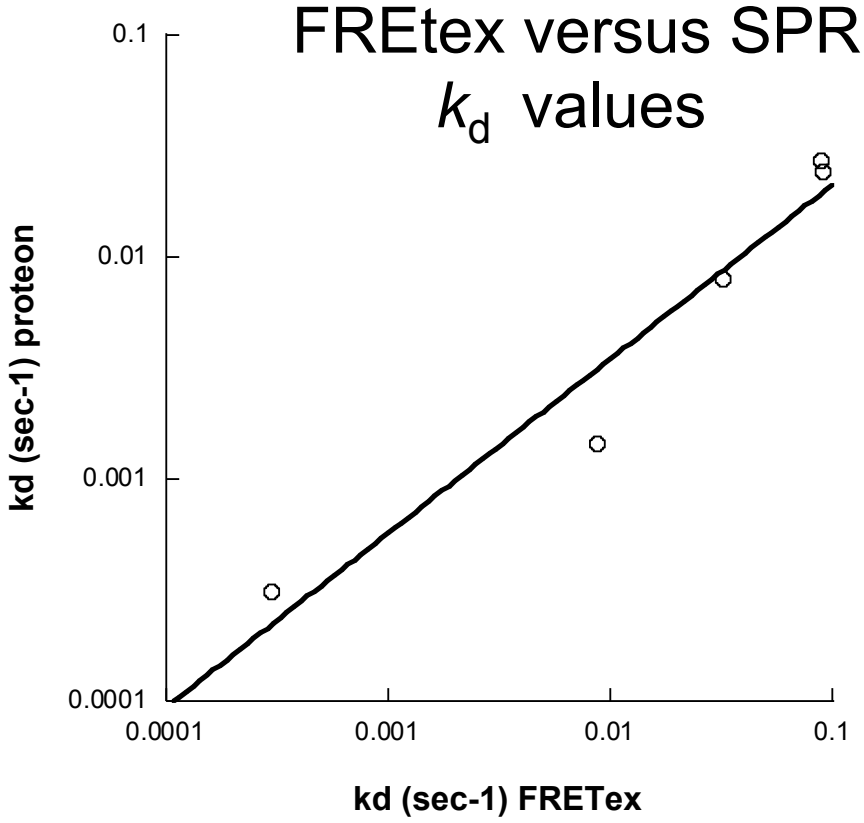


FRET – indication of interacting proteins (Tem Blip)

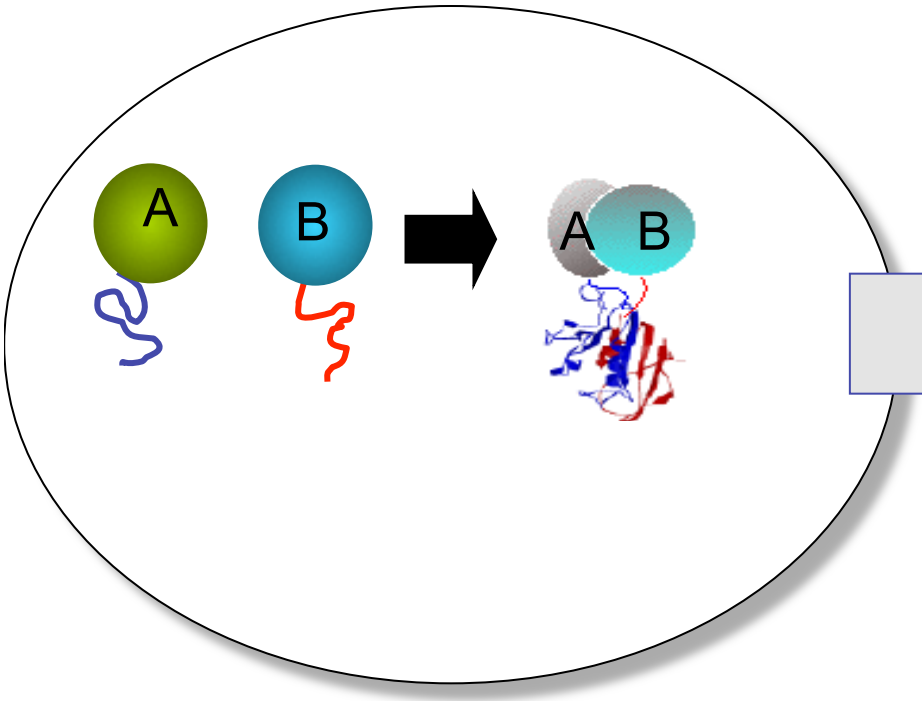
Negative control: no FRET when the proteins don't interact (Tem & Barstar)

Chase experiments measure the k_d, and are very precise

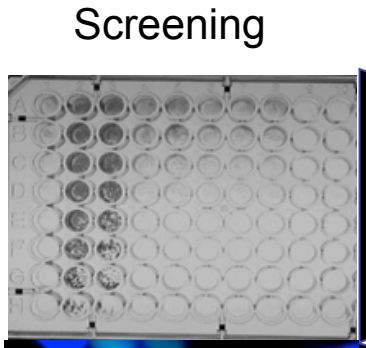


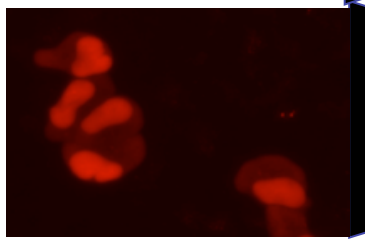


PCA
(Protein fragment Complementation Assays)

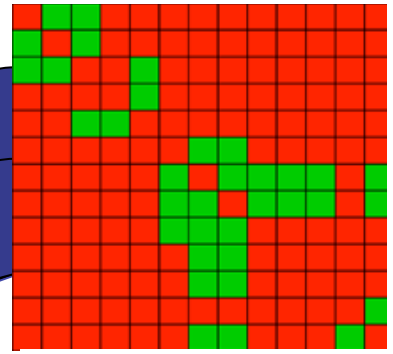


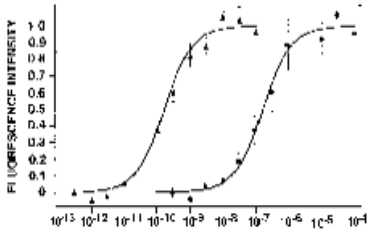
Screening

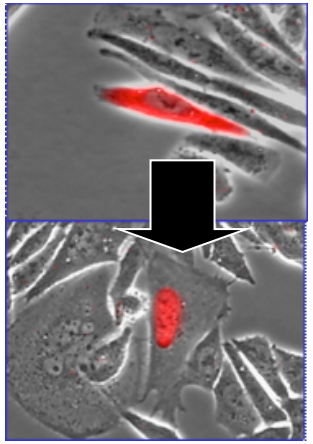




Localization
Trafficking







Genes are transfected into cells.

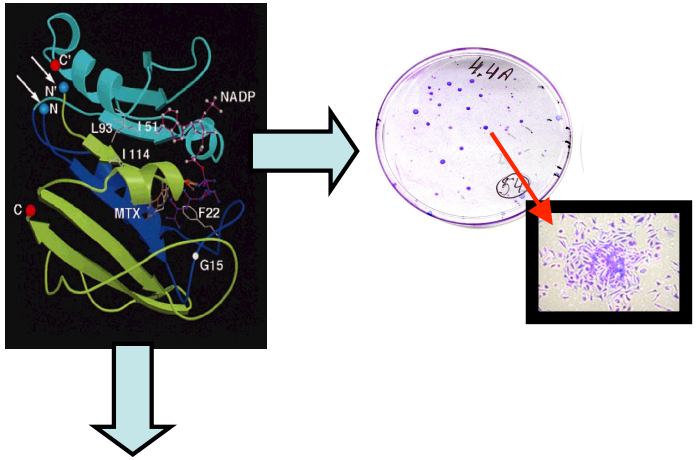
The cell makes proteins A and B which have reporter tags on either end (N- or C-terminal).

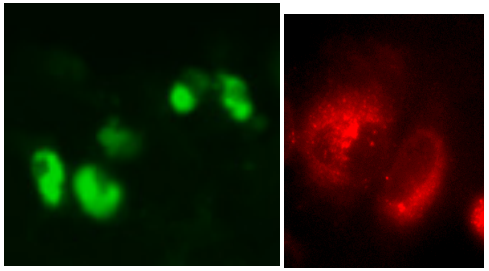
A/B complex formation reconstitutes the reporter and generates a signal.

Any gene, cell and reporter protein can be used.

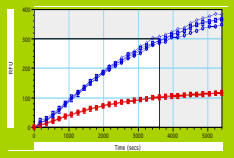
A world of PCA

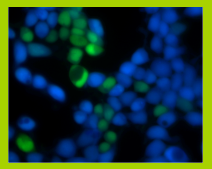
DHFR



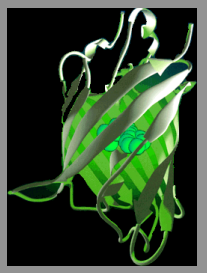


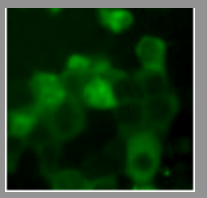
β-lactamase

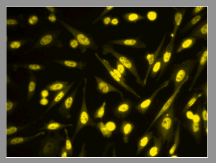


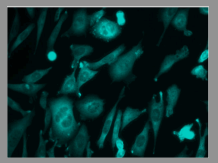


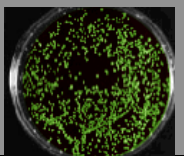
GFP



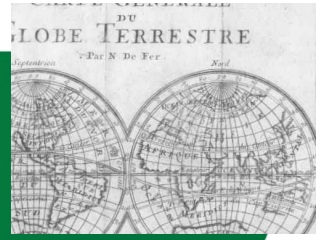








PCA in Network Mapping



- Molecular interactions are detected directly.

- Genes are expressed in a relevant cellular context, in which components of the underlying pathway exist.

- Events induced by any pathway purturbation can be detected, linking specific interactions to specific pathways.

- Subcellular locations of protein complexes can be determined unambiguously.

Direct binding measurements on cells

In principle similar to ELISA but on cells

One has to label to ligand to monitor it
Methods of labeling: 1. fluorescence, 2. Radioactive (I125 for proteins)

Labeled proteins (ligands that bind extracellular receptors) are incubated with the cells, then washed a few times and measured.

For fluorescence label: FACS, ELISA plate reader.
For I125 labeled – gamma counter

Doing a series of concentrations provides a binding curve
One can also use one labeled ligand, and chase it with different “cold” ligand. This provides an apparent binding affinity of the “cold” ligand. This is a fast and efficient method to monitor many different mutations, or different chemicals.

Advantages/disadvantages

Summary

We learned about many different methods

Each has its advantages

And non is perfect