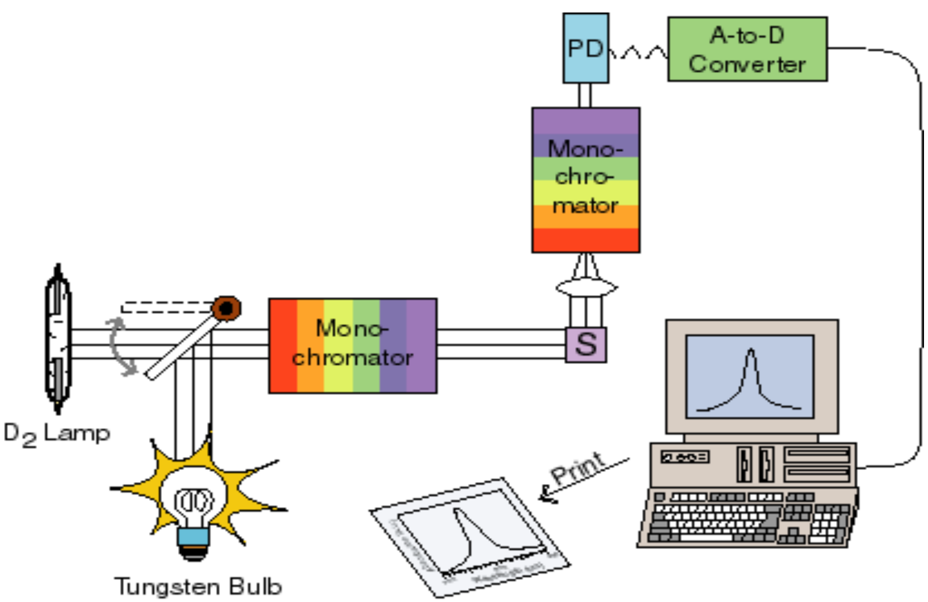
Experimental determination of K_D

1. ***Equilibrium Dialysis*** – a direct measurement of the partitioning of a ligand between the bound and free states.

2. ***Centrifugation***

3. ***Spectroscopic Measurements*** (Δfluorescence, ΔCD, ΔAbs, etc.)



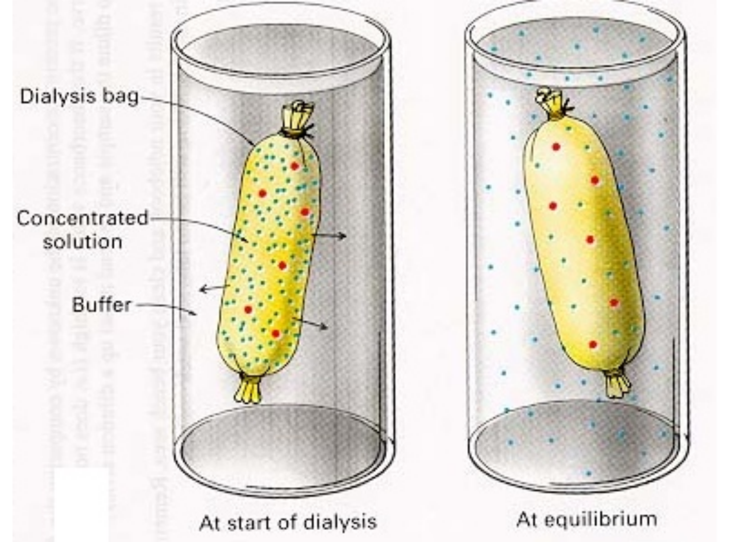
Fluorescence Spectrophotometer

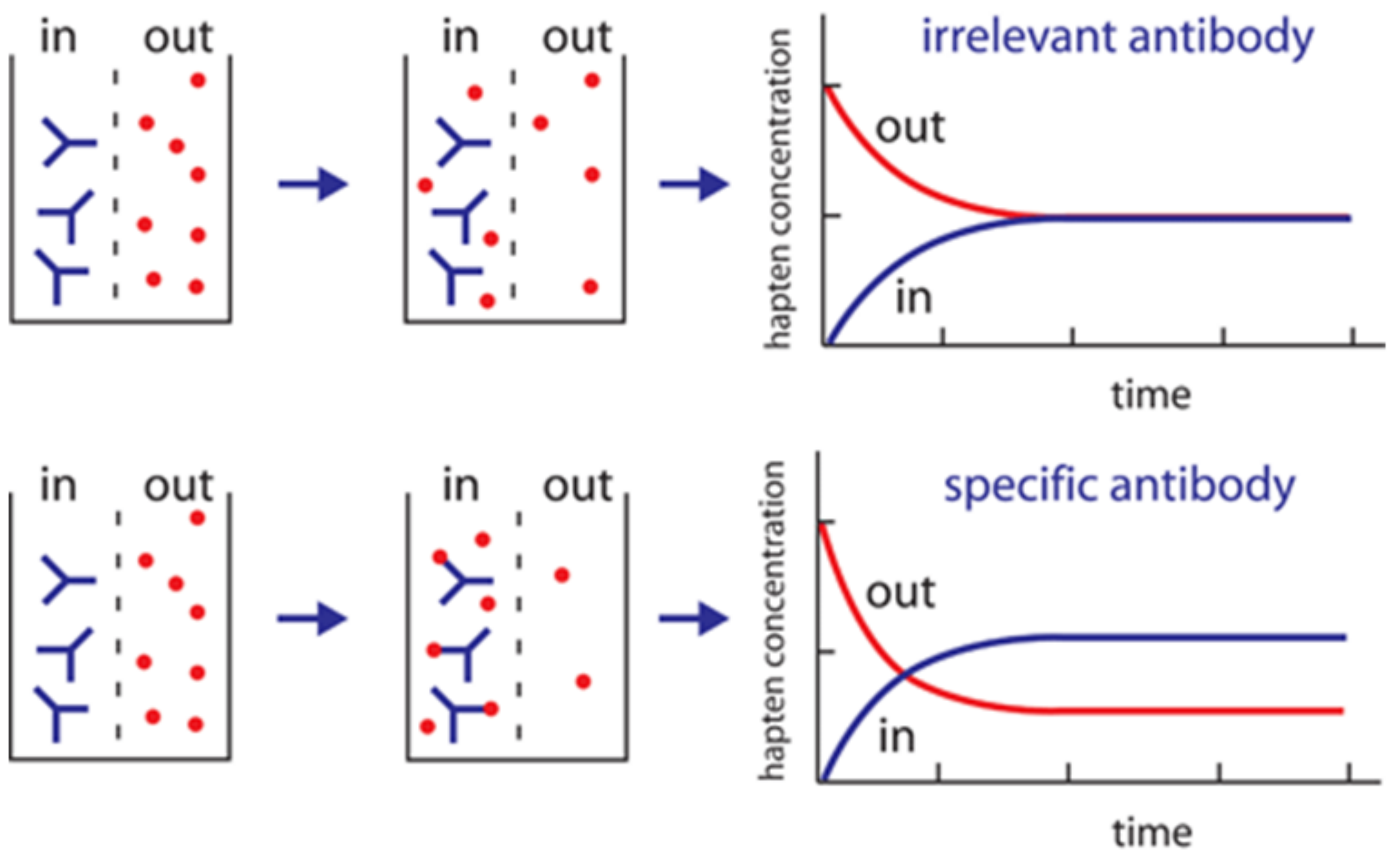
4. ***Calorimetry***

5. ***BIACore***

Equilibrium Dialysis

This technique exploits one of the most beautiful idea in thermodynamics and statistical mechanics - equality of the chemical potential.

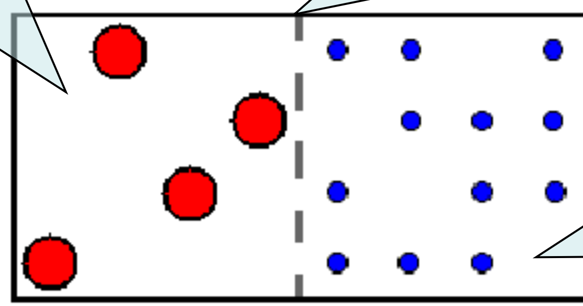




The equilibrium dialysis experiment

The protein (M) is present only in the left cell of the dialysis chamber.

The cells are separated by a semipermeable membrane, through which only the ligand can pass.



The small molecule (L) is present only in the right cell.

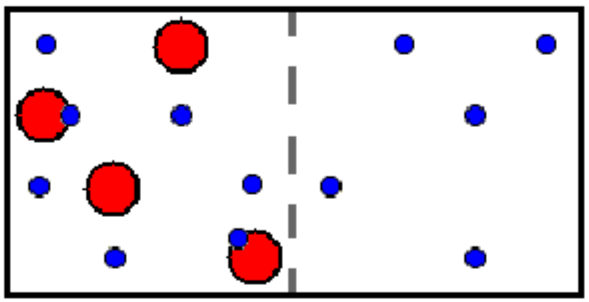
Starting concentrations:

Right cell: *[ML] = 0; [L] = 12; [M] = 0.*

Left cell: *[ML] = 0; [L] = 0; [M] = 4.*

- When equilibrium is reached, the concentration of free ligand will be the same in both cells.

- However, because the protein can bind the ligand, the concentration of total ligand will be higher in the left cell.



Equilibrium concentrations:

Left cell: [ML] = 2; [L] = 5; [M] = 2.

Right cell: [ML] = 0; [L] = 5; [M] = 0.

Now we can calculate the K_D:

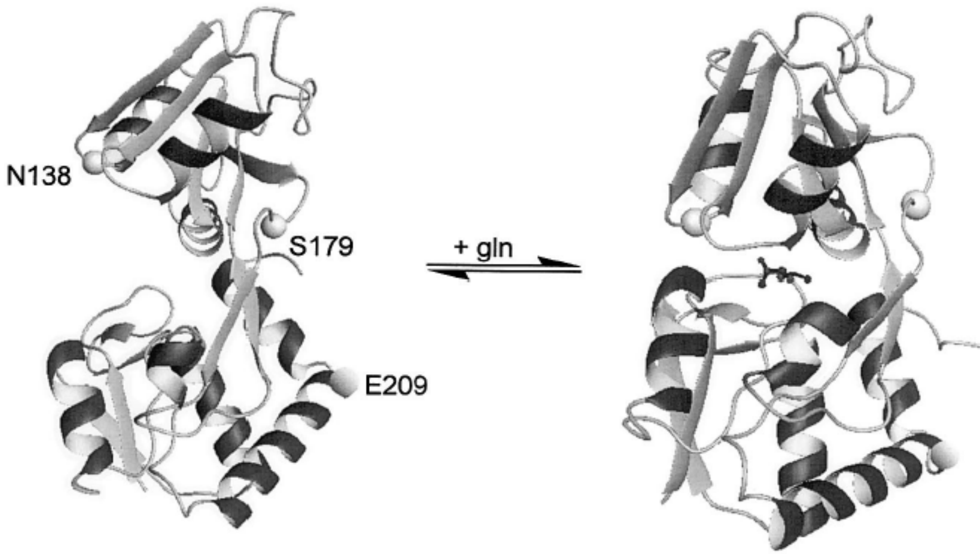
$$K_D = \frac{[M][L]}{[ML]} = \frac{2\times 5}{2} = 5$$

Features of this binding equilibrium:

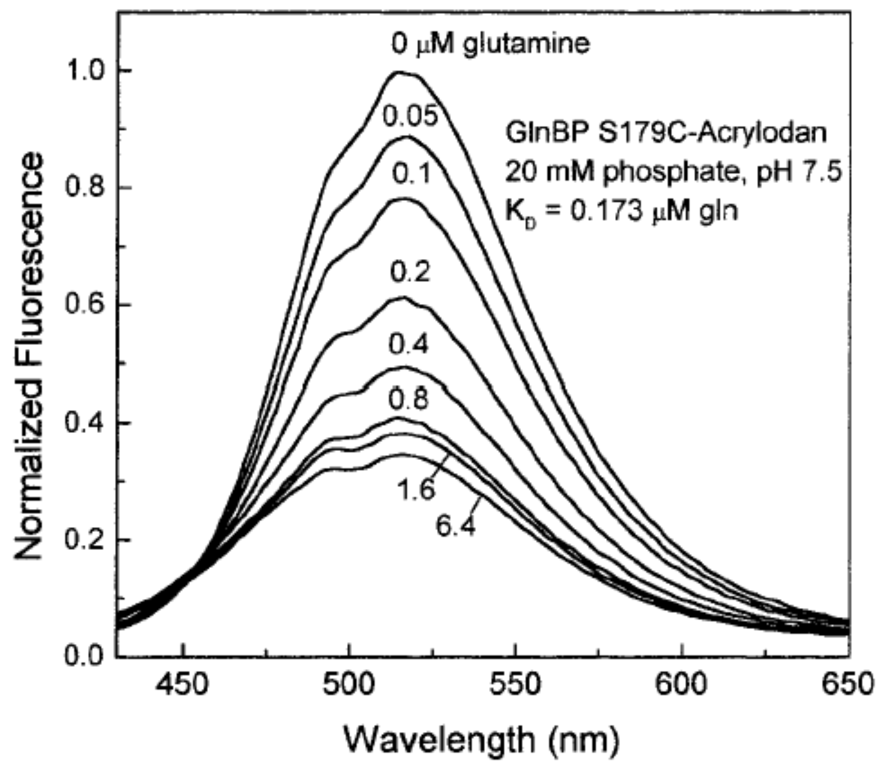
[L] = 5 in both cells $\qquad$ $[L]_{total}$ = 7 in the left cell

$[L]_{total}$ - [L] = 2 = [ML] in the left cell

Molecular biosensor

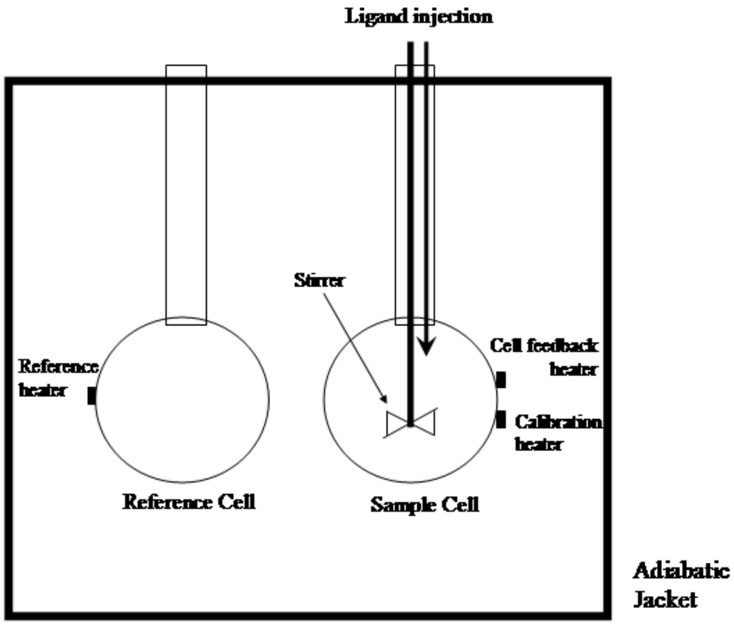


Structure of E. coli GlnBP with and without glutamine. The amino acids which were mutated to cysteine residues are indicated.

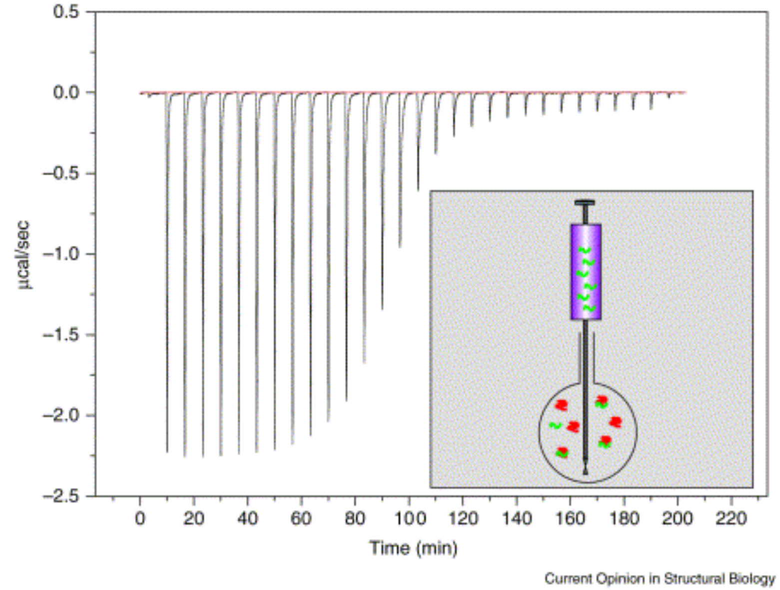


The fluorescence intensity response of the GlnBP mutant S179C labeled with acrylodan.

Isothermal Titration Calorimetry

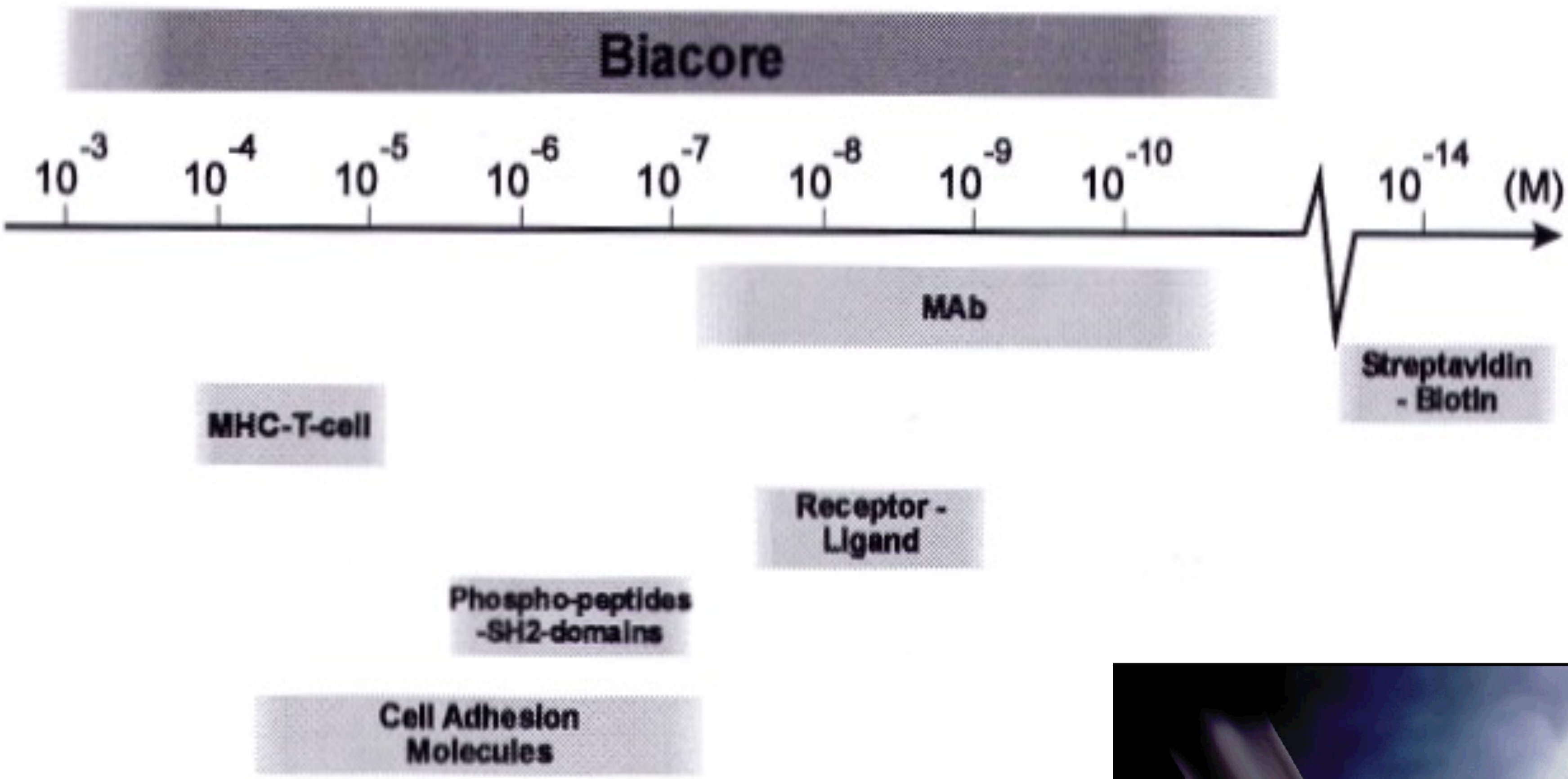


Leavitt and Freire, 2001

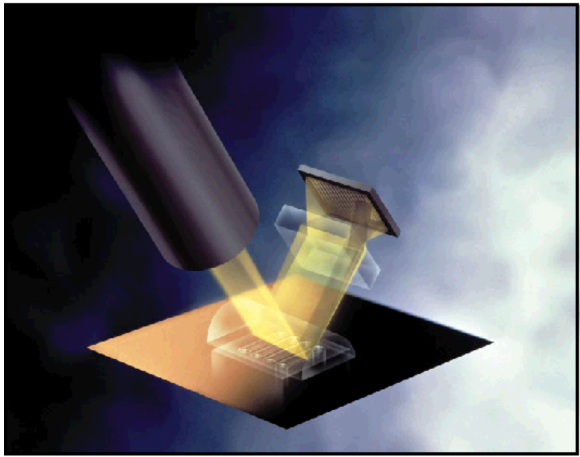


- *Measure the heat released during binding reaction.*
- *Done by comparing how much energy needed to keep the temperature constant in the reactive chamber and in a reference chamber.*

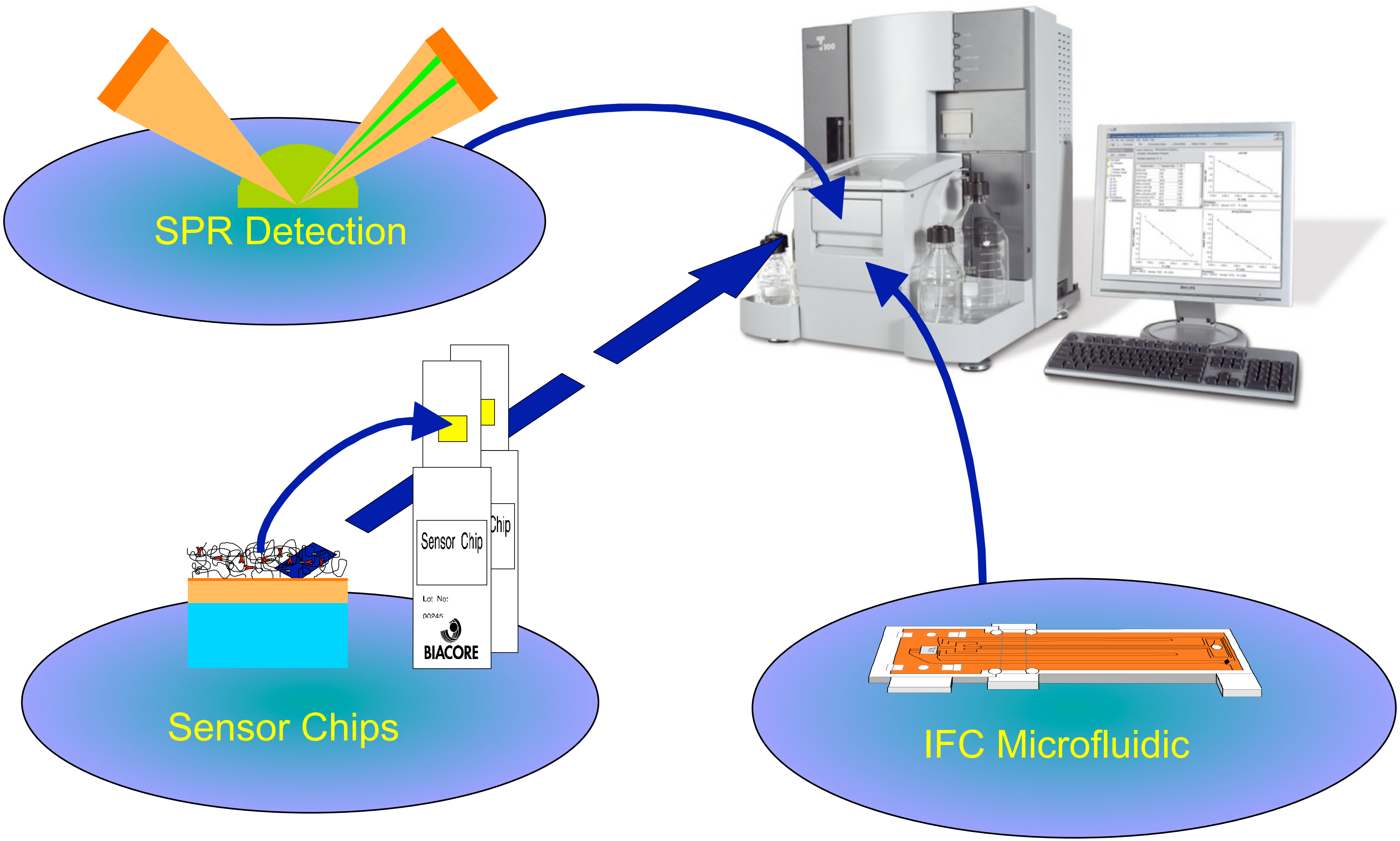
Probing Biological Affinities



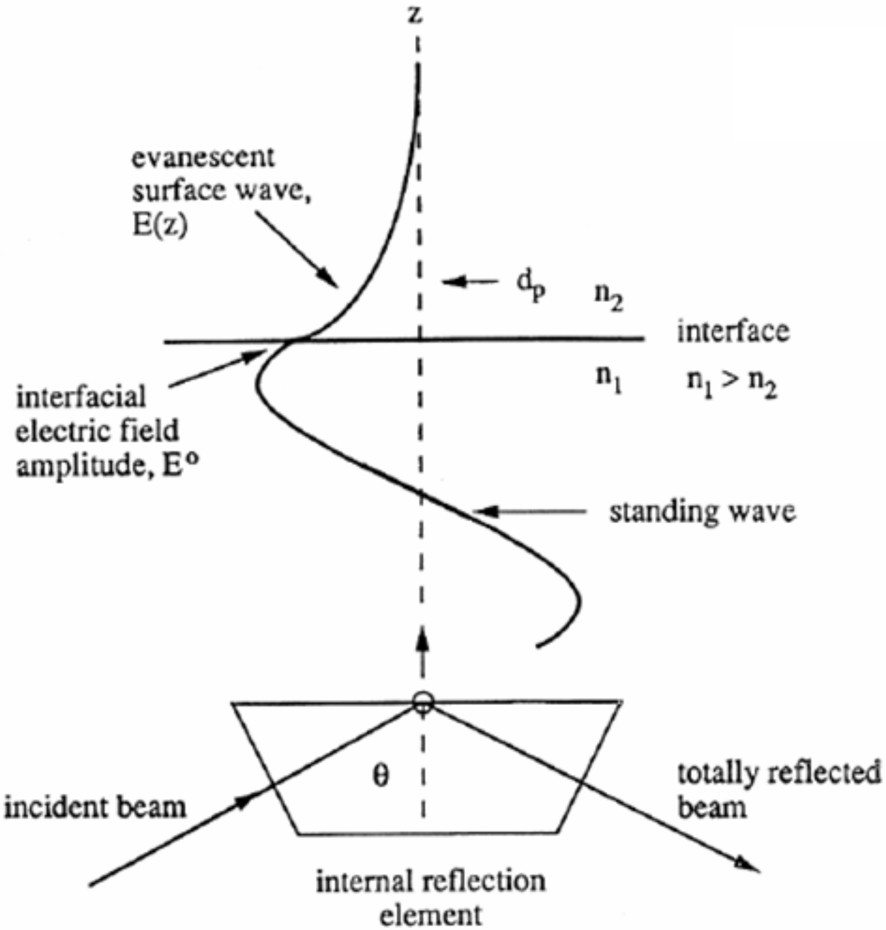
***B**iomolecular **I**nteraction*
***A**nalysis*
Biacore



The Corner-stones of the Technology



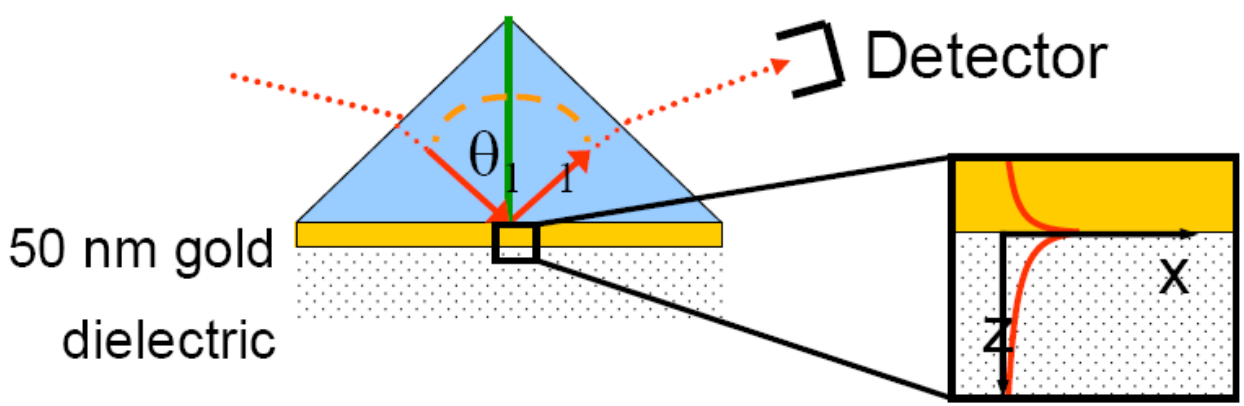
Evanescent Field



Depth of penetration

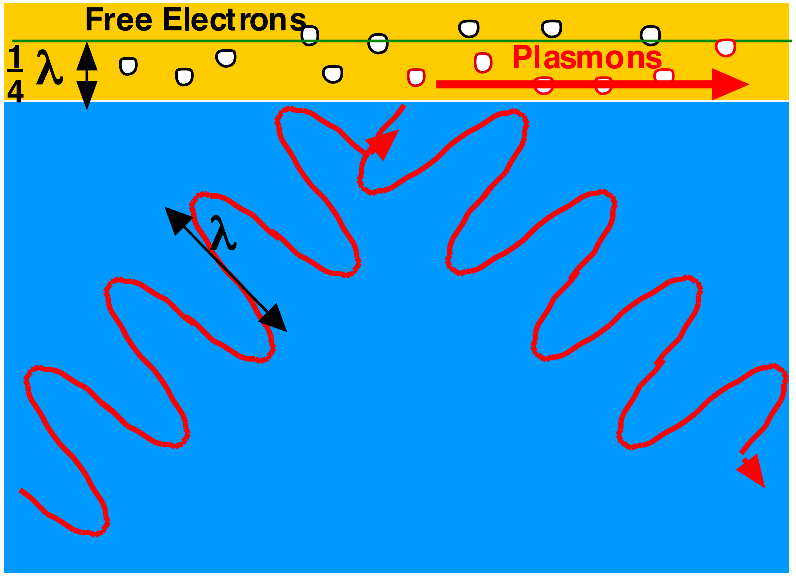
$$E(z) = E_0 e^{z/d_p}$$

$$d_p = \frac{\lambda}{4\pi\sqrt{n_1^2 \sin\theta - n_2^2}}$$



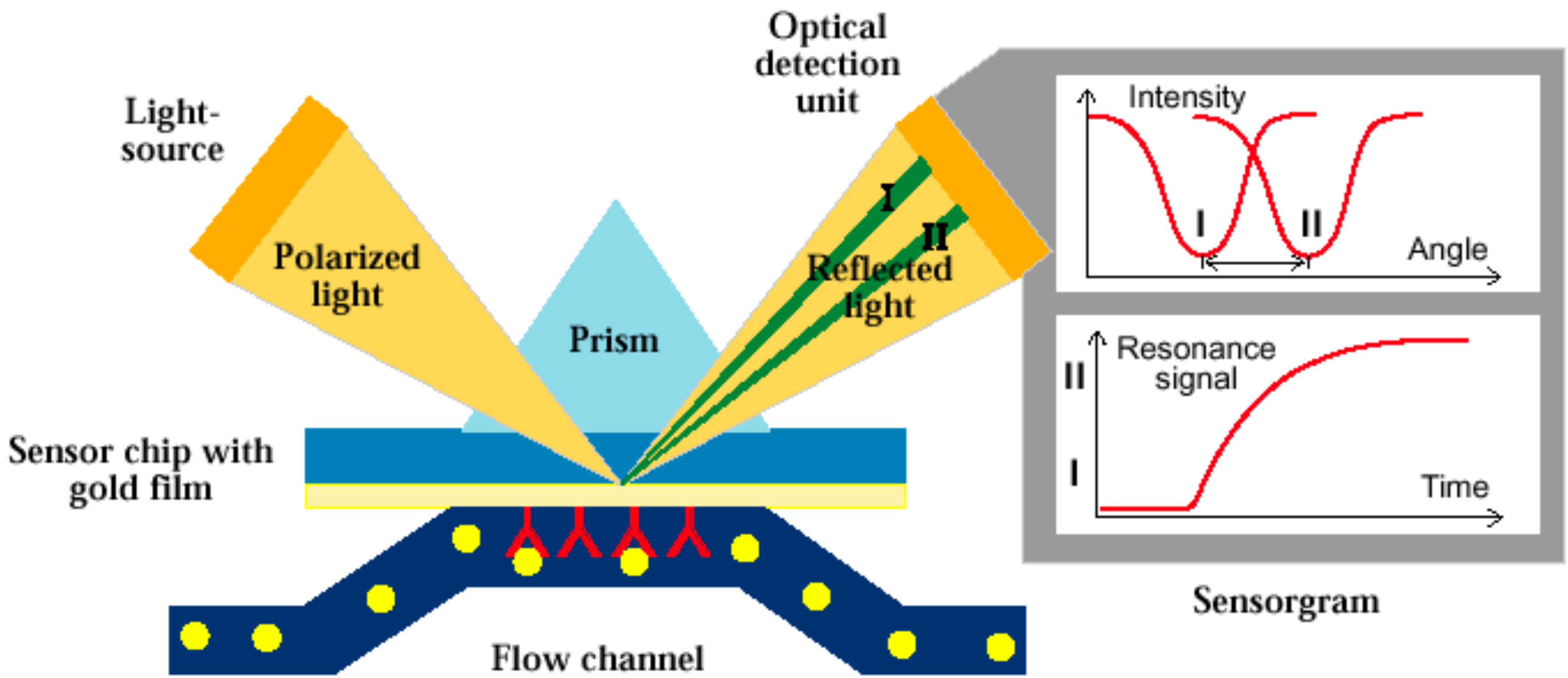
Surface Plasmon Resonance

If a thin gold film is placed on the reflecting surface, the photons can interact with free electrons in the gold surface.



Under the right conditions, this causes the photons to be converted into plasmons and the light is no longer reflected.

Fixed wavelength light, in a fan-shaped form, is directed at the sensor surface and binding events are detected as changes in the particular angle where SPR creates extinction of light.

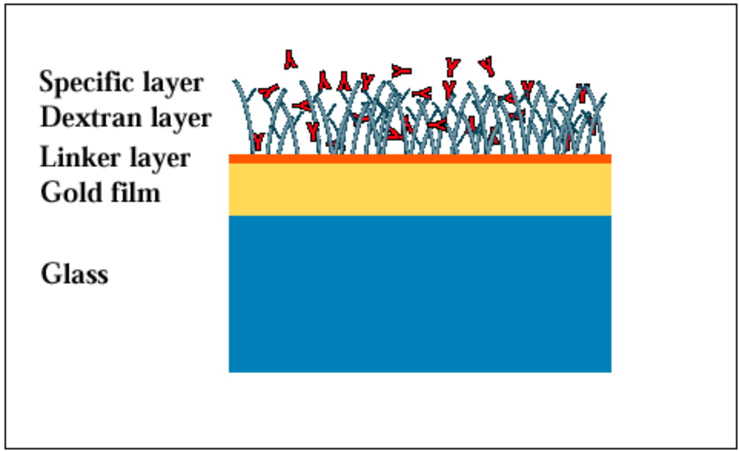


Effect of binding on SPR.

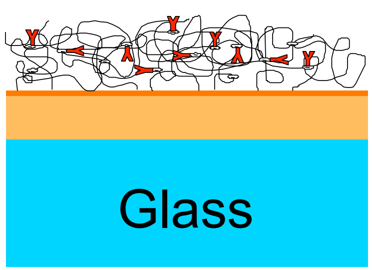
Plasmons create an electric field (evanescant) that extends into the medium surrounding the film.

This is affected by changes in the medium (eg. binding of analyte), and results in a change in the velocity of the plasmons.

This change in velocity alters the incident light vector required for SPR and minimum reflection.



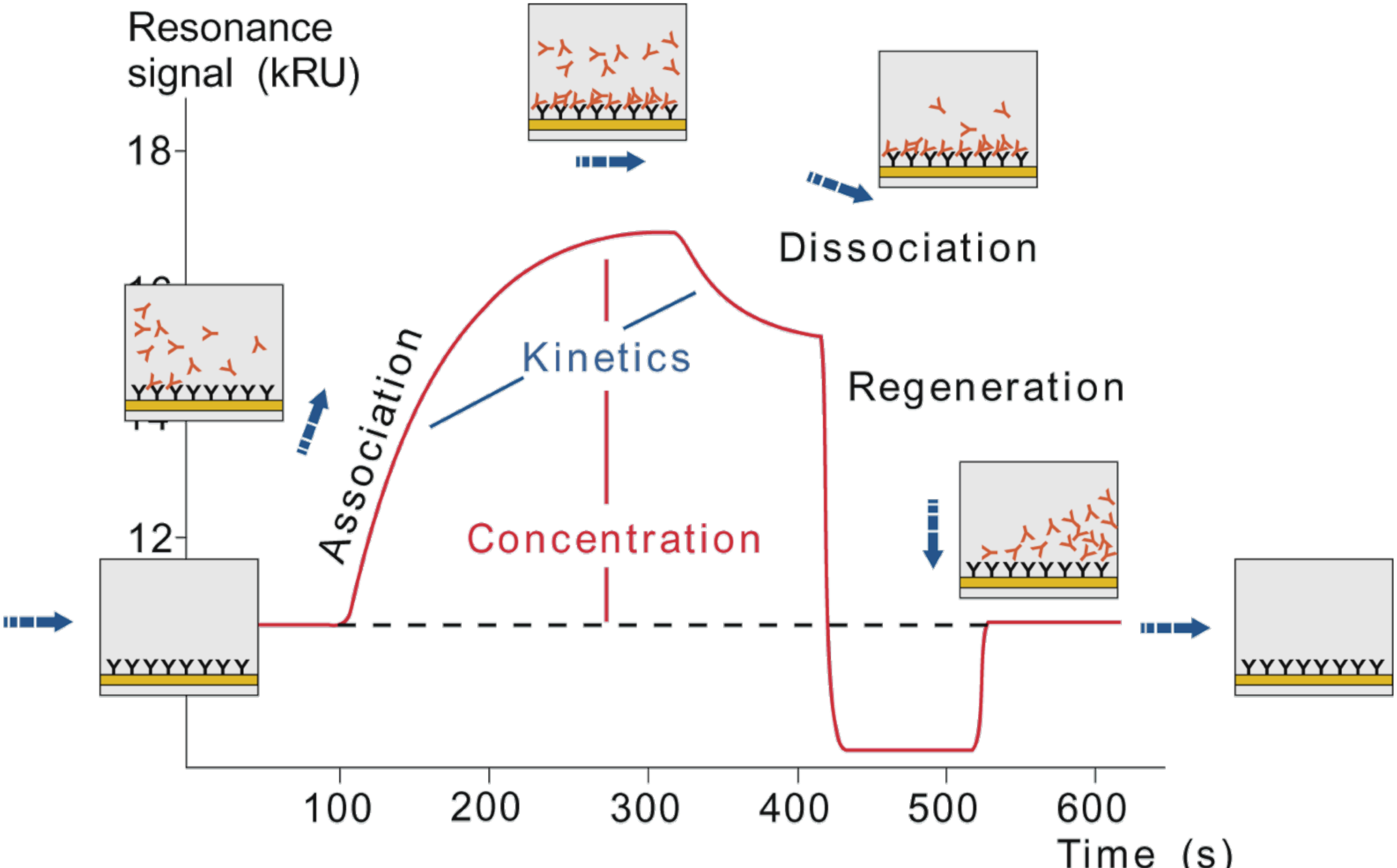
Sensor Chips



Sensor Chip specific matrix

Gold 50 nm

The Sensorgram



Extracting Rate Constants from Sensograms

- Measure binding curves
- Decide on a model to describe the interaction
- Fit the curve to a mathematical rate equation describing the model

$$\boxed{\frac{dR}{dt} = k_a C\left(R_{\max} - R\right) - k_d R}$$

R_{max} - maximum binding capacity
(R_{max} – R) - Free concen

- Obtain values for the constants k_a, k_d, R_{max}
- Assess the fit
  - overlay pots, residual plots
  - acceptable statistics e.g. chi^2 – curve fidelity
  - Biological and experimental relevance of the calculated parameters