Approches complémentaires pour l'analyse des interactions moléculaires.

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Ile d'Oléron


How to characterize a macromolecular complex?

Stoichiometry

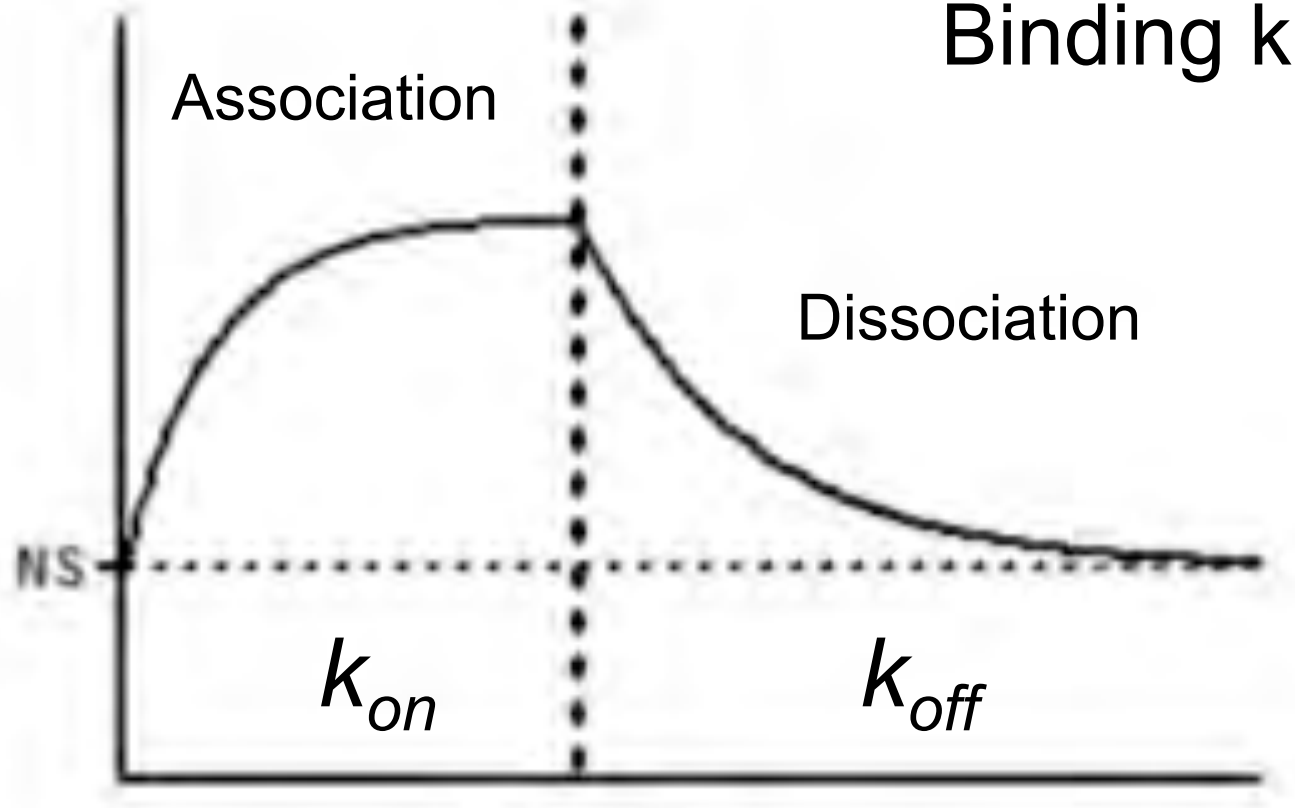
Stability

Affinity

Kinetics

Thermodynamic parameters

Binding kinetics

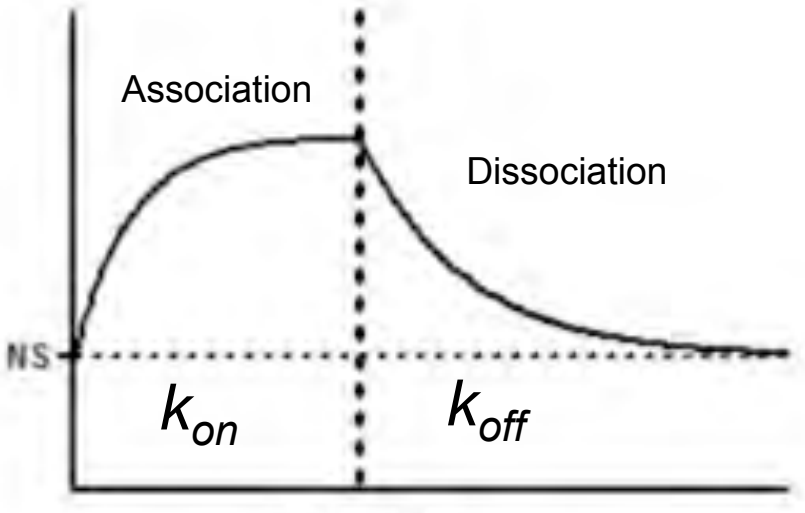


Association: how fast molecules bind = k_{on} ($M^{-1}s^{-1}$)

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$

Dissociation: how fast complexes fall apart = k_{off} (s^{-1})

Binding kinetics



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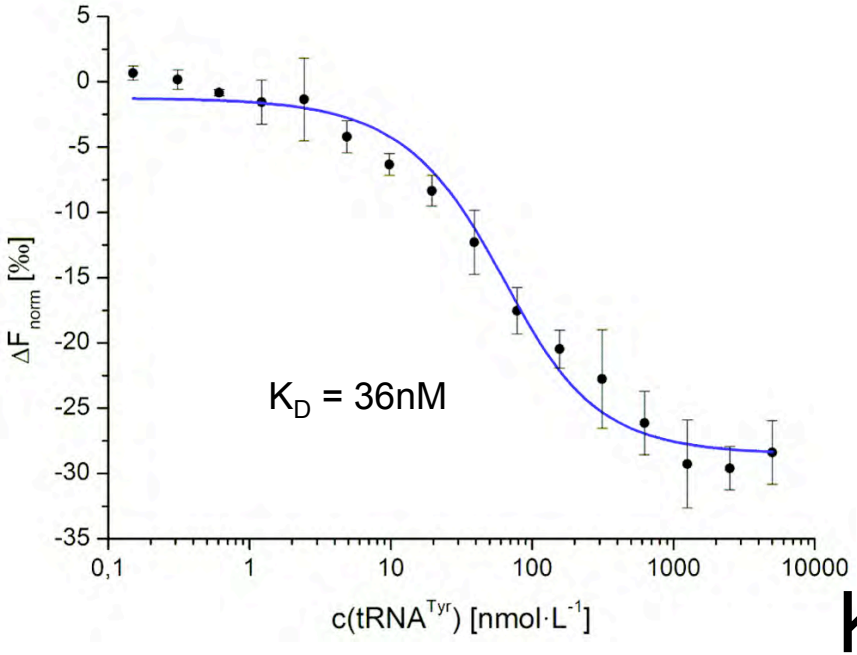


Equilibrium

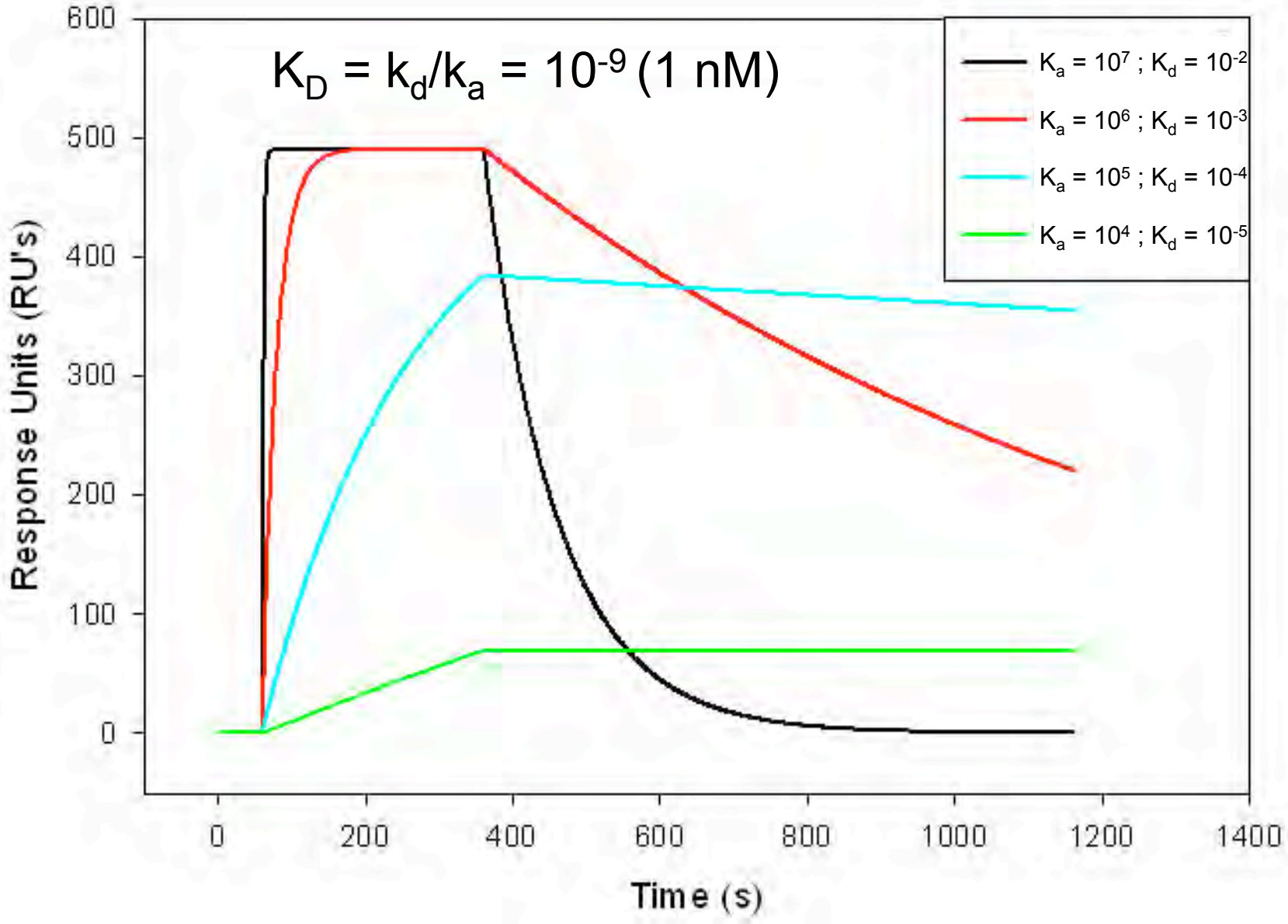
forward binding = backward unbinding

$$k_{on}\,[A][B] = k_{off}\,[AB]$$

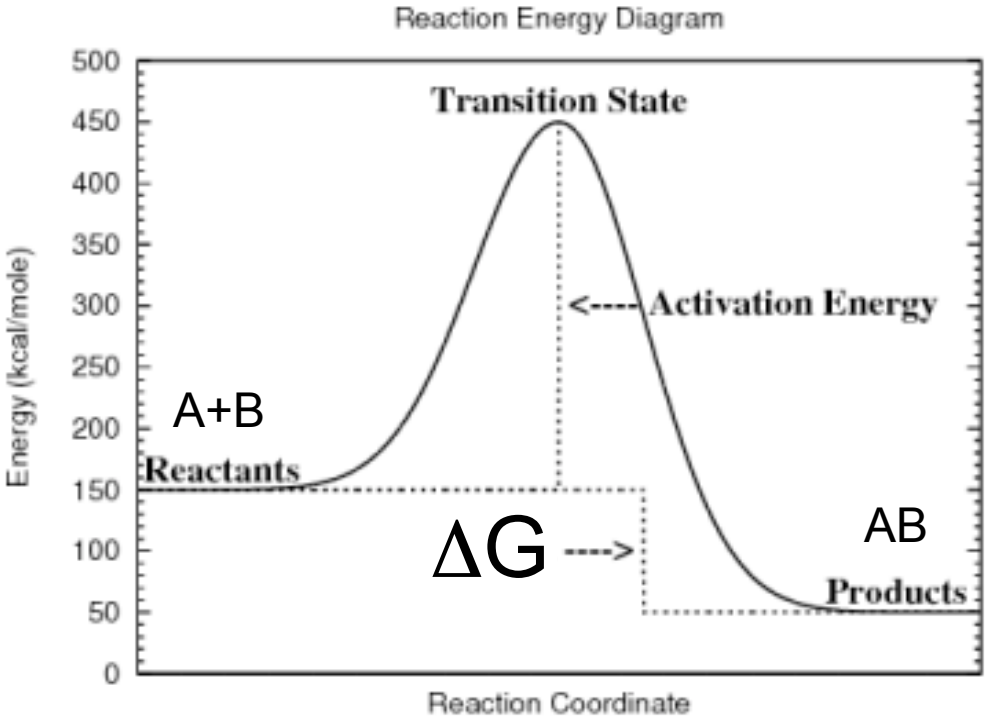
Equilibrium dissociation constant

$$K_D = [A][B] / [AB] = k_{off} / k_{on}$$

Same Affinity … Different Kinetics



Thermodynamic parameters



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

ΔG: Change in free (Gibbs) energy

ΔH: Change in enthalpy.
It is a measure of the hydrogen bonds and van der Waals contacts involved in the interaction.
Can be measured as heat exchange

ΔS: Change in entropy.
Change in 'chaos': Change in mobility/rigidity conformational changes, solvation (hydrophobic sites)

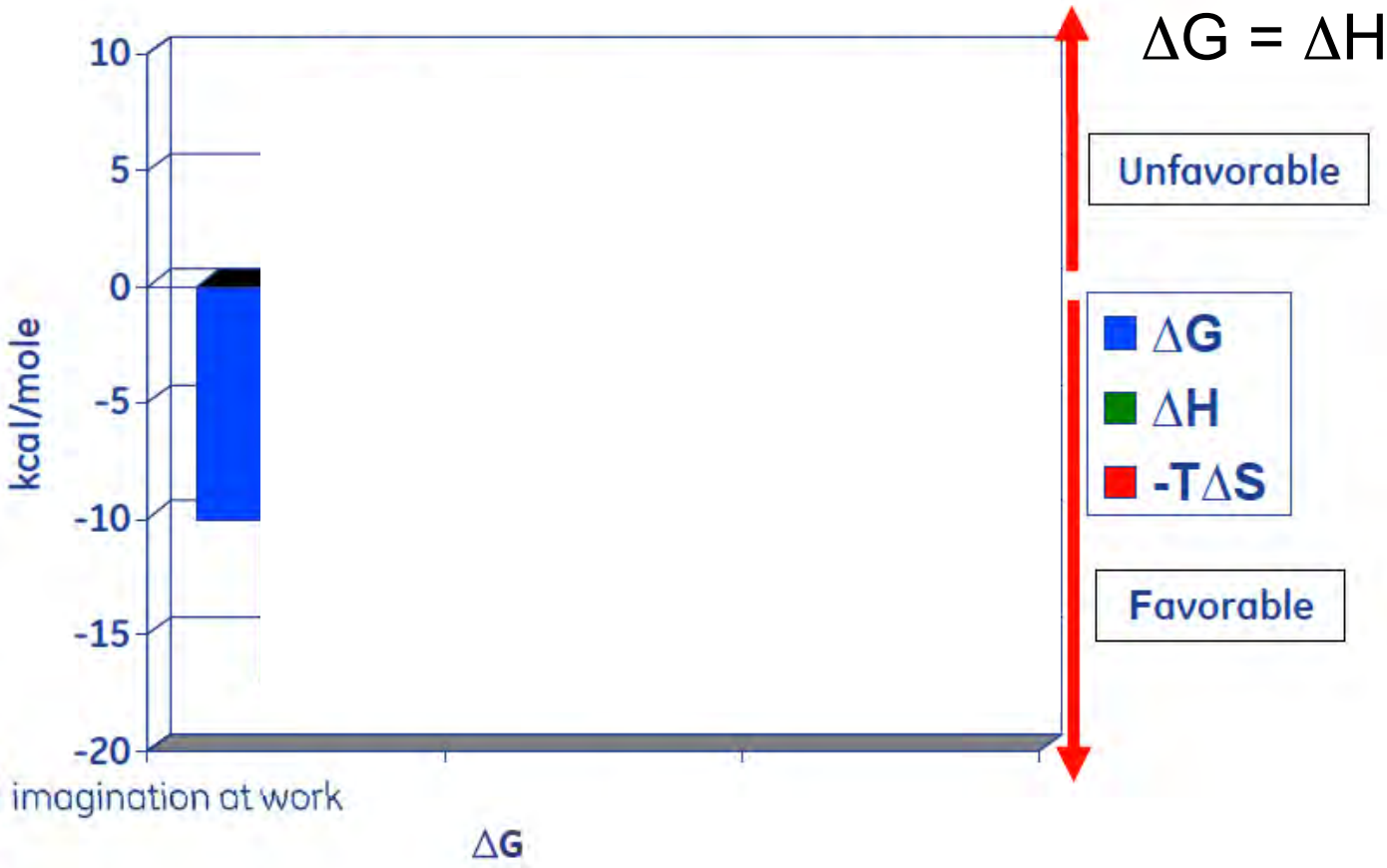
$$\Delta G = RT\ \ln(K_D)$$

R is the ideal gas constant, T is the temperature in the Kelvin scale

Affinity is just part of the picture

MicroCal
Part of GE Healthcare

All three interactions have the same binding energy (ΔG)



imagination at work

Binding equilibria and free energy

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

Biologically relevant interactions generally have ΔG values in the range of -5 to -10 kcal/mol.

Common language	K_d (M)	K_a (M^{-1})	ΔG° (kJ/mol)	ΔG° (kcal/mol)
No affinity (high millimolar)	> 10^{-1}	< 10^{1}	> -5.9	> -1.4
Very weak affinity (low millimolar)	10^{-3} to 10^{-1}	10^{1} to 10^{3}	-18 to –5.9	-4.3 to -1.4
Low affinity (high micromolar)	10^{-5} to 10^{-3}	10^{3} to 10^{5}	-30 to –18	-7.1 to –4.3
Moderate affinity (low micromolar)	10^{-6} to 10^{-5}	10^{5} to 10^{6}	-36 to –30	-8.5 to –7.1
High affinity (nanomolar)	10^{-9} to 10^{-6}	10^{6} to 10^{9}	-53 to -36	-13 to –8.5
Very high affinity (pico/femtomolar)	10^{-14} to 10^{-9}	10^{9} to 10^{14}	-83 to -53	-20 to -13
Effectively irreversible (low femtomolar)	< 10^{-14}	> 10^{14}	< -83	< -20

Biologically relevant interactions use multiple non-covalent interactions to obtain the required affinities.

Strength (kcal/mole)*

Bond Type	Length (nm)	In Vacuum	In Water
Covalent	0.15	90	90
Ionic	0.25	80	3
Hydrogen	0.30	4	1
van der Waals attraction (per atom)	0.35	0.1	0.1

Molecular Biology of the Cell (NCBI bookshelf)

afmb
architecture et fonction
des macromolécules biologiques

FRISBI
Integrated Structural Biology

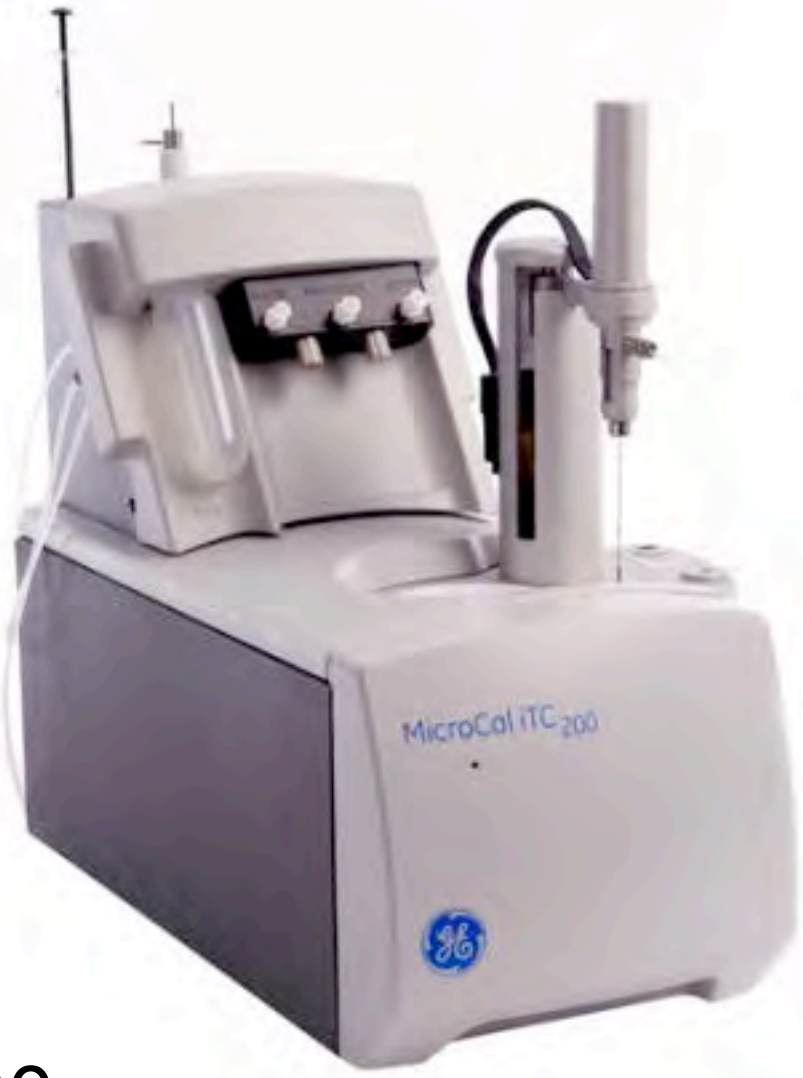
Techniques available at the AFMB

Techniques (in order of apparition at the AFMB)	N:M	$\Delta H/\Delta S$	K_D	k_{on}/k_{off}	Stability
FP (Fluorecence)	**YES**	no	**YES**	no	yes
TSA (Thermal Shift Assay)	no	no	yes?	no	**YES**
SPR (Surface Plasmon Resonance)	yes	yes	**YES**	**YES**	-
MALS (Multi-Angle Light Scattering)	**YES**	no	no	no	-
ITC (Isothermal Titration Calorimetry)	**YES**	**YES**	**YES**	yes	-
MST (Microscale Thermophoresis)	YES	yes	**YES**	no	-
BLI (Bio-Layer Interferometry)	YES	no	**YES**	**YES**	-

Other: gel shift, native gel, chromatography, DLS, CD, AUC…

GE Healthcare

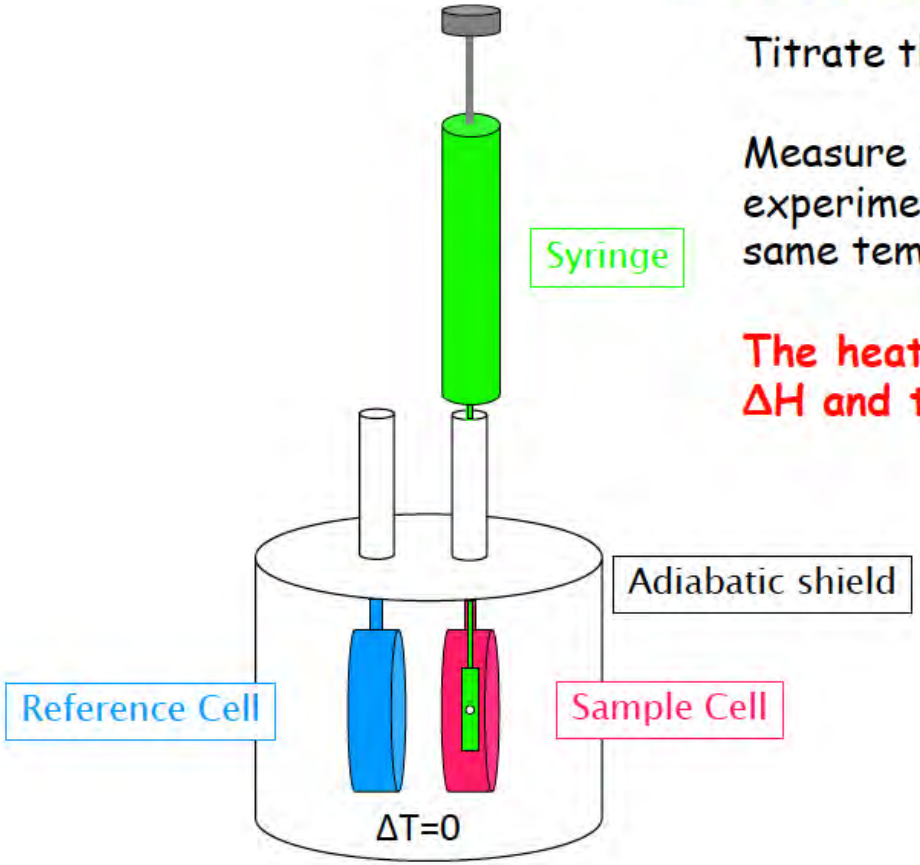
ITC



Isothermal
Titration
Calorimetry

MicroCal ITC 200

What happens during an ITC experiment?



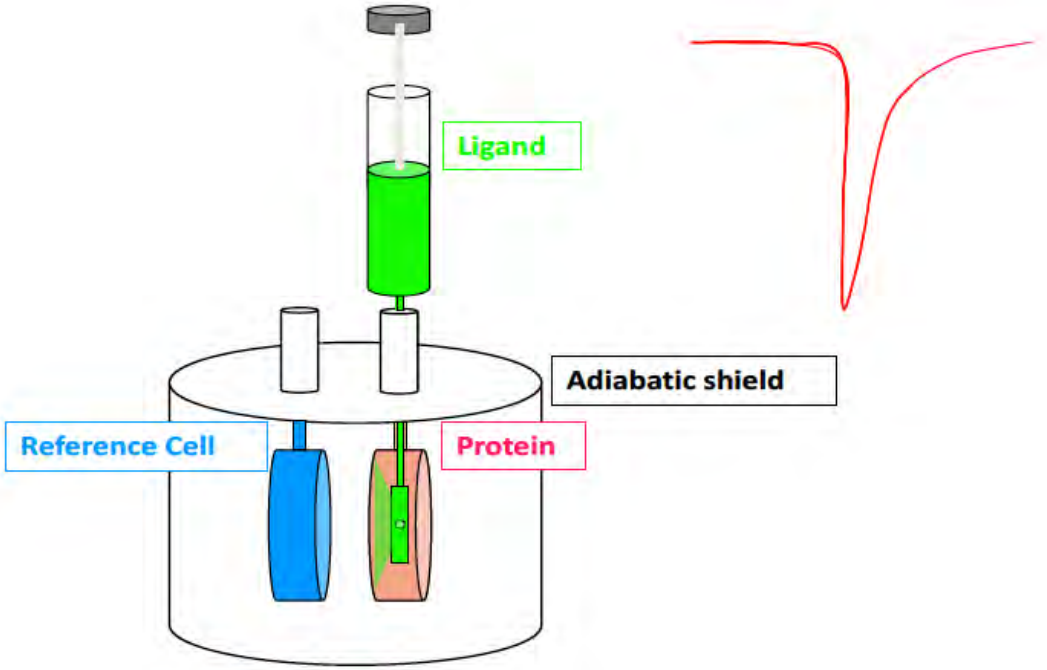
Protein target in sample cell

Compound (binding partner) in syringe

Titrate the compound into the protein

Measure the power needed to keep the experiment and reference cells at the same temperature (DP)

The heat provided is proportional to the ΔH and to the amount of complex formed



Endothermique reaction

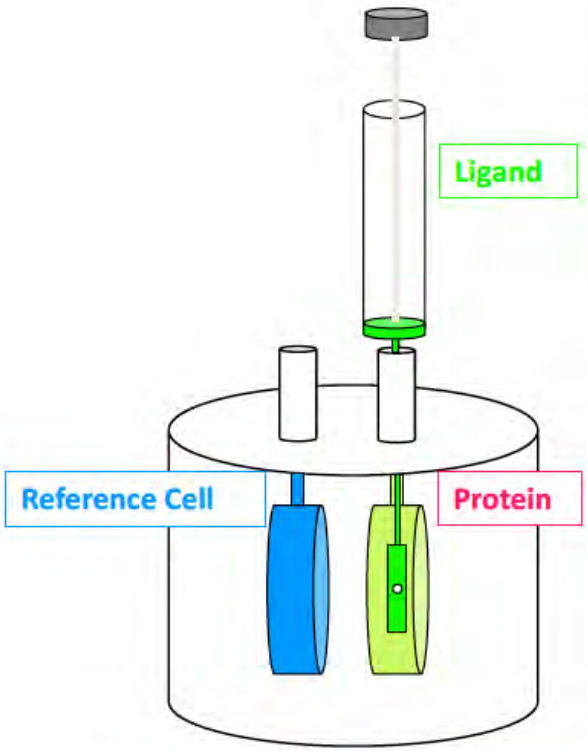
➔ Heat absorbed in sample cell

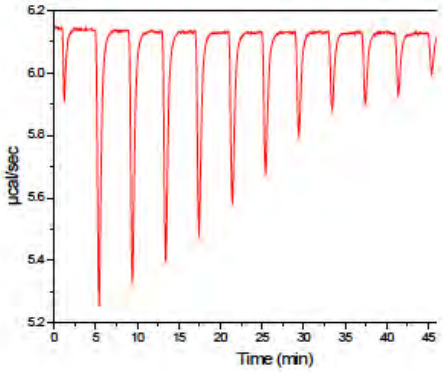
➔ extra energy will be provided in order to keep $\Delta T=0$

Exothermique reaction

➔ Heat released in sample cell

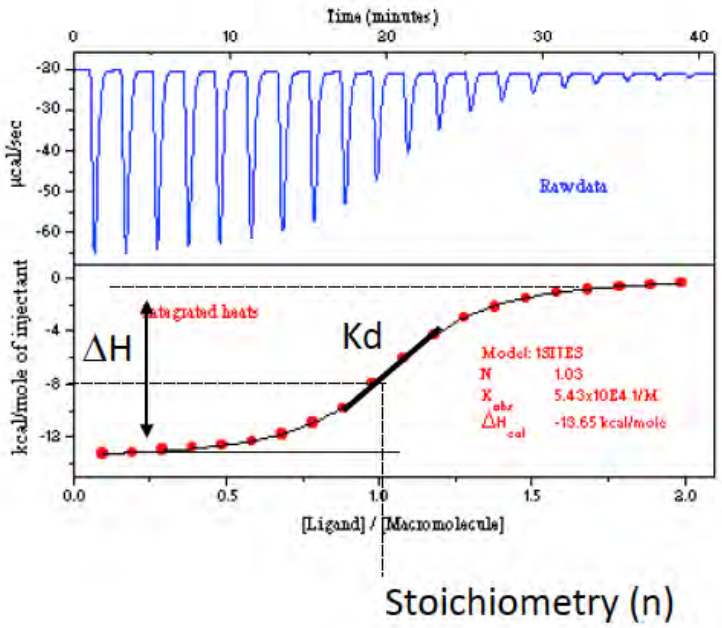
➔ less energy will be provided in order to keep $\Delta T=0$





- Each peak corresponds to one injection
- During the titration the signal gets smaller because there is less protein available for binding to the ligand
- The area under the peaks is calculated (heat in µcal) and converted to kcal/mol of injectant (ΔH)

Each enthalpy point is plotted against the ratio of [ligand]:[protein]



Data points are fitted according to binding models

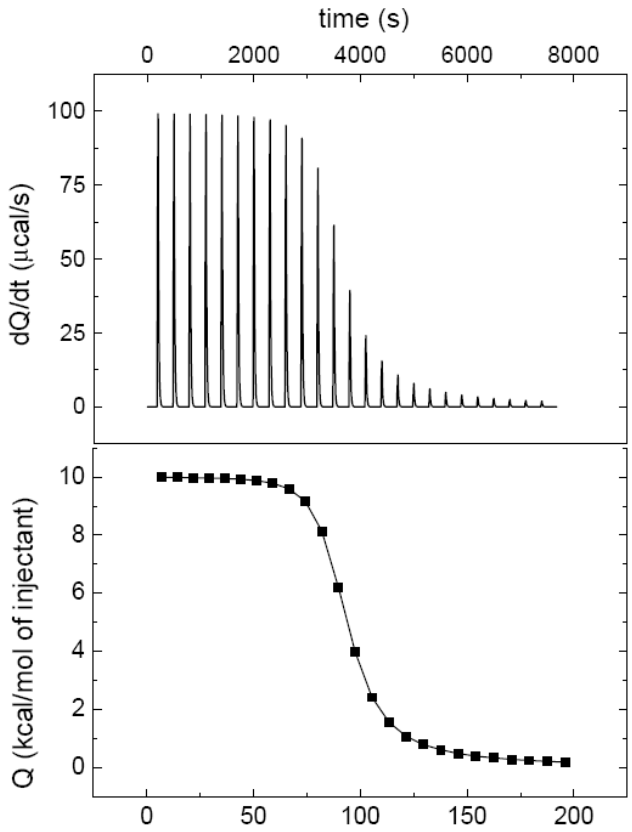
ΔH, n and K_D are calculated from the fitting

ΔG and ΔS are derived from the equations:

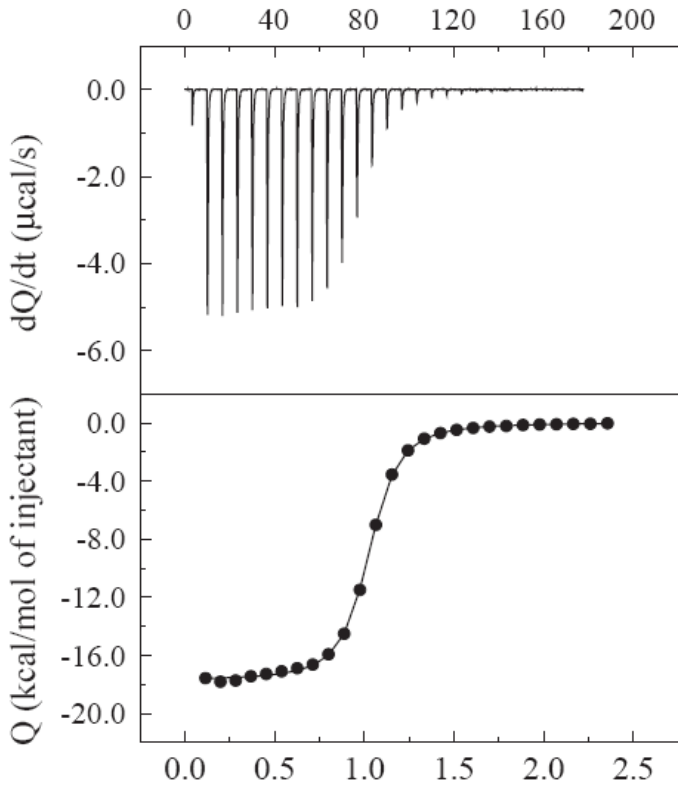
$$\Delta G = RT \ln K_D$$

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

Endothermique



Exothermique



NanoTemper technologies

MST

Micro Scale Thermophoresis

Monolith NT.115

What is Thermophoresis?

Electrophoresis:

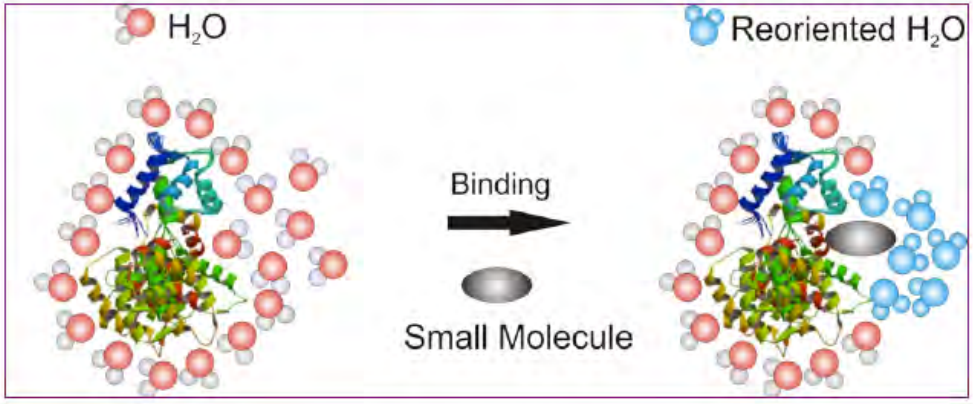
We apply an electric field
We separate the molecules by charge (and also size)

Thermophoresis:

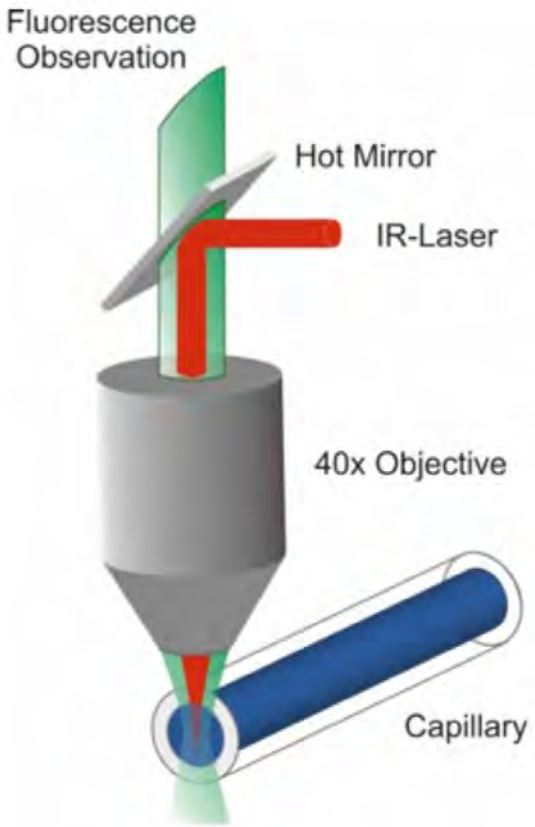
Sample is in solution in a capillary
An infrared laser generates a temperature gradient (between 1-6 K)
Thermophoresis is the motion of molecules in temperature gradients.

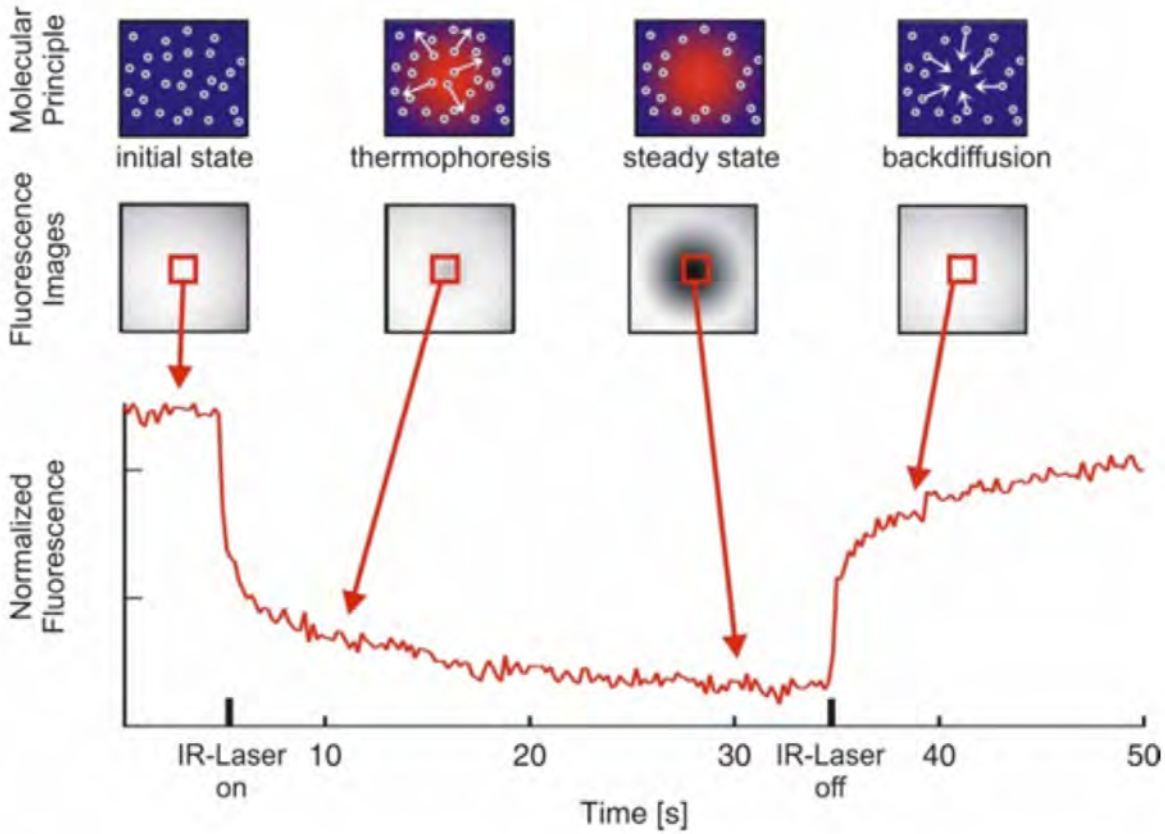
Movement is detected by through fluorescence of one of the binding partners.

Thermophoresis depends on size, charge, solvation entropy and conformation of the molecule.



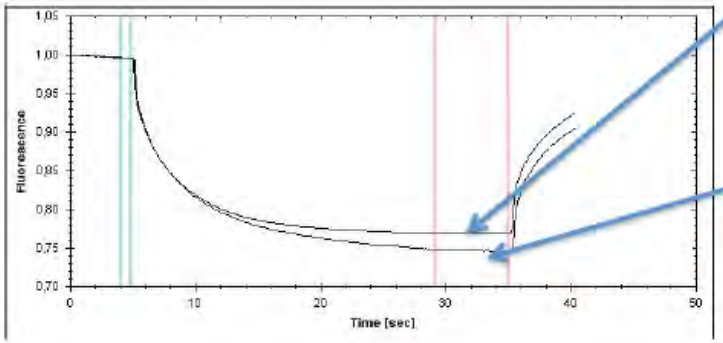
Thermophoresis experiment





- 16 capillaries
- Concentration of the fluorescent partner kept constant
- Titration of the non-fluorescent partner

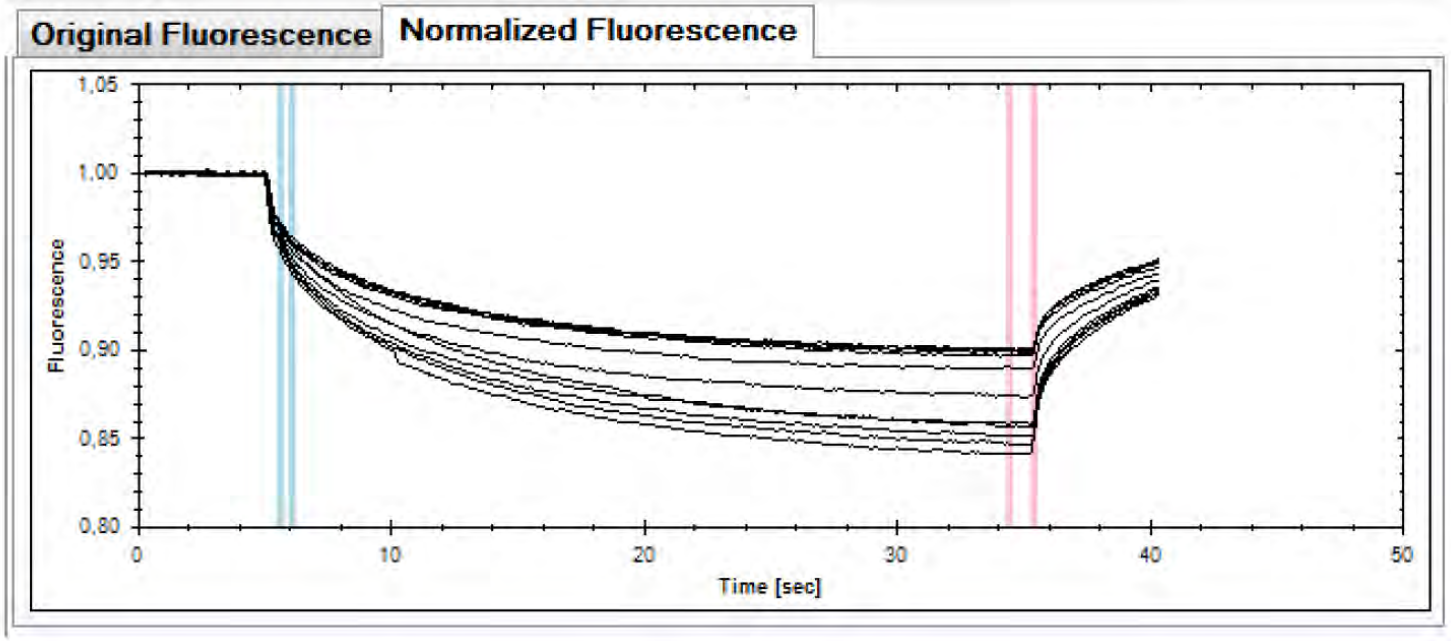
Thermophoresis mesurement (MST) on first capillary (highest concentration of ligand)



MST on second capillary

Depending on the level of complexation, the labelled molecules will move differently on the temperature gradient.

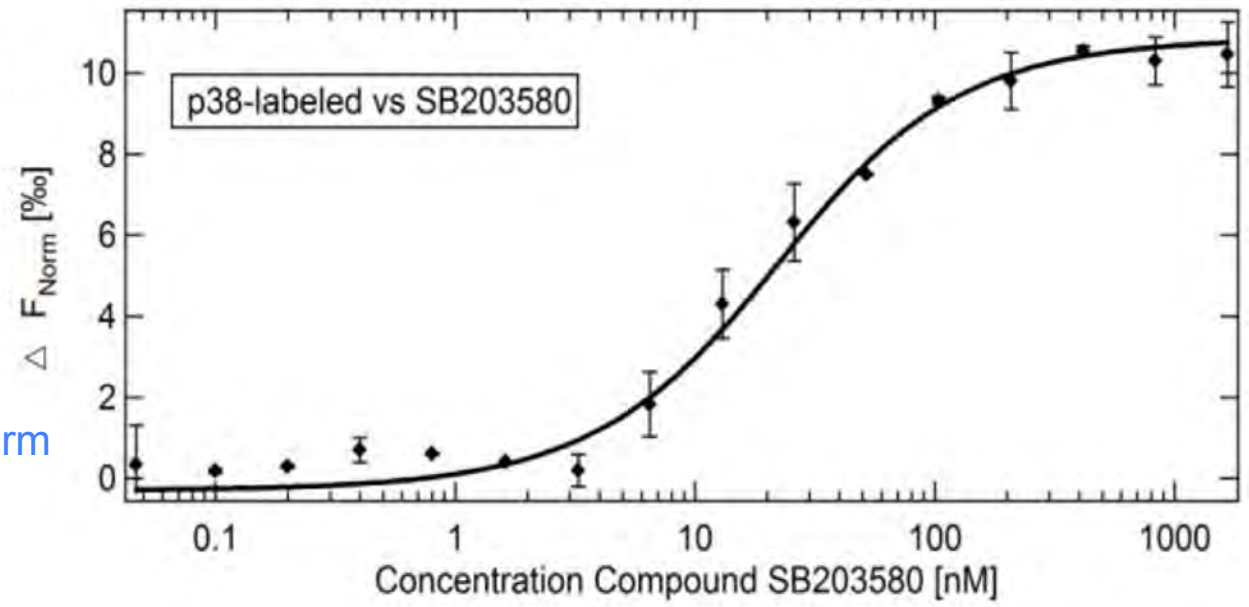
Thermophoresis will depend on size + charge + hydration shell + conformation



Measurement over 16 capillaries

$F_{norm} = F_{conc} - F_0$

$\Delta F_{norm} = F_{norm} - F_{norm}$



The fluorescent partner must be monodisperse and should not stick to the capillaries

In most cases you will need to add additives to the buffer: detergent (0.05% Tween 20) BSA, L-arginine, glycerol…

Ionic force and pH must be adapted to the interaction

Chose the right type of capillary: standard, coated (hydrophilic, hydrophobic)

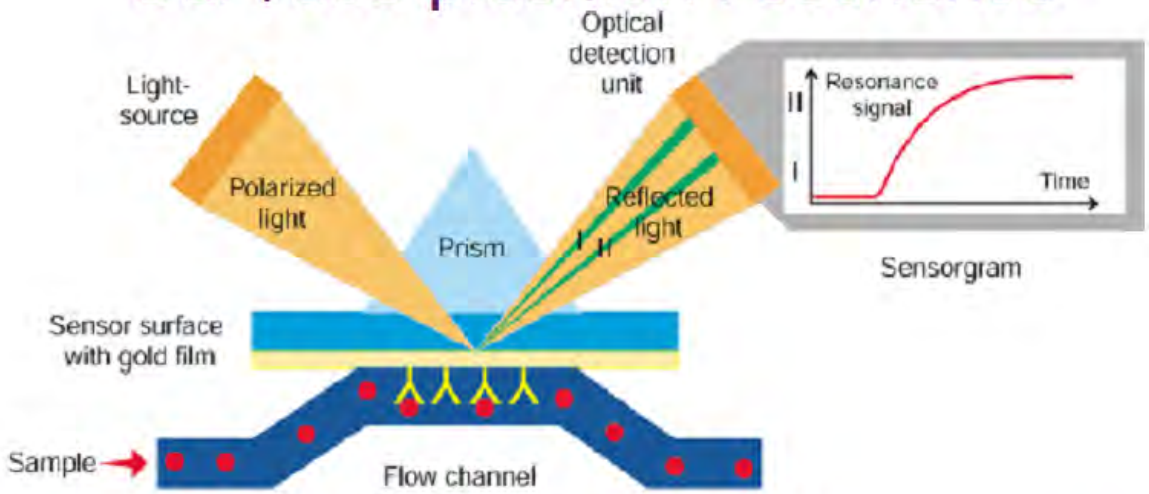
GE Healthcare

SPR

Surface
Plasmon
Resonance

Biacore T200

Surface plasmon resonance



Physical phenomenon: Surface plasmon resonance

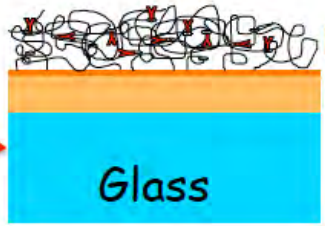
SPR machine detects refractive index changes close to the sensor surface

On the sensor surface we will immobilize one of the components (LIGAND)
The other component (ANALYTE) will be injected in a continuous flow.

Binding of the analyte will change the refractive index near the surface, this change will generate a resonance signal that will be measured in real time and represented in a sensorgram

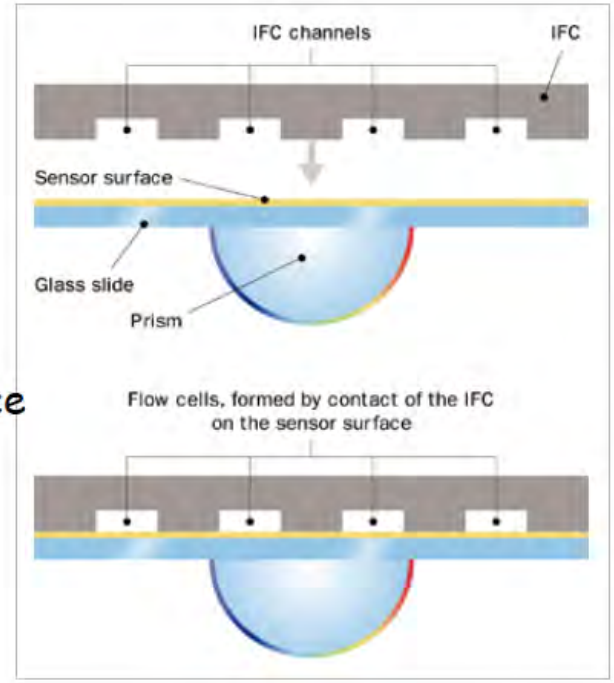
A sensorgram represents the resonance signal (in resonance units RU) as a function of time. **This response is proportional to the mass retained near the surface**

Sensor Chips and flow cells



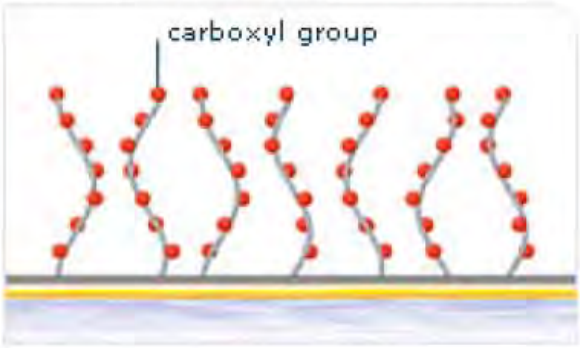
Dextran matrix
Gold 50 nm

Flow cells are formed by pressing an integrated microfluidic cartridge (IFC) against a sensor surface



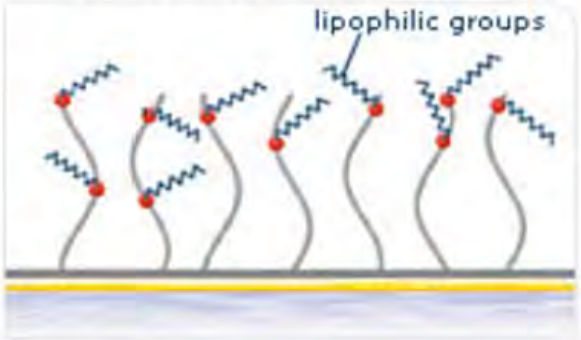
Different types of sensor chips

CM5 (carboxymethyl-dextran) Coupling via -NH2, -SH, -CHO, -OH or -COOH

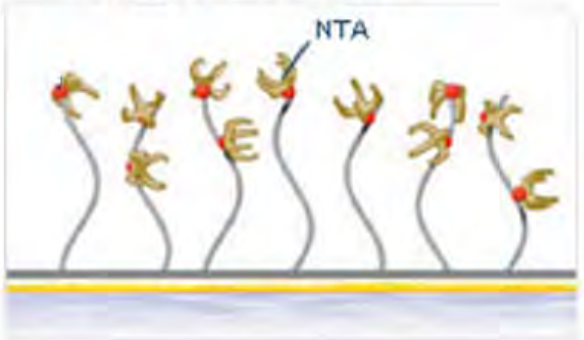


SA (immobilization of biotylilated molecules)

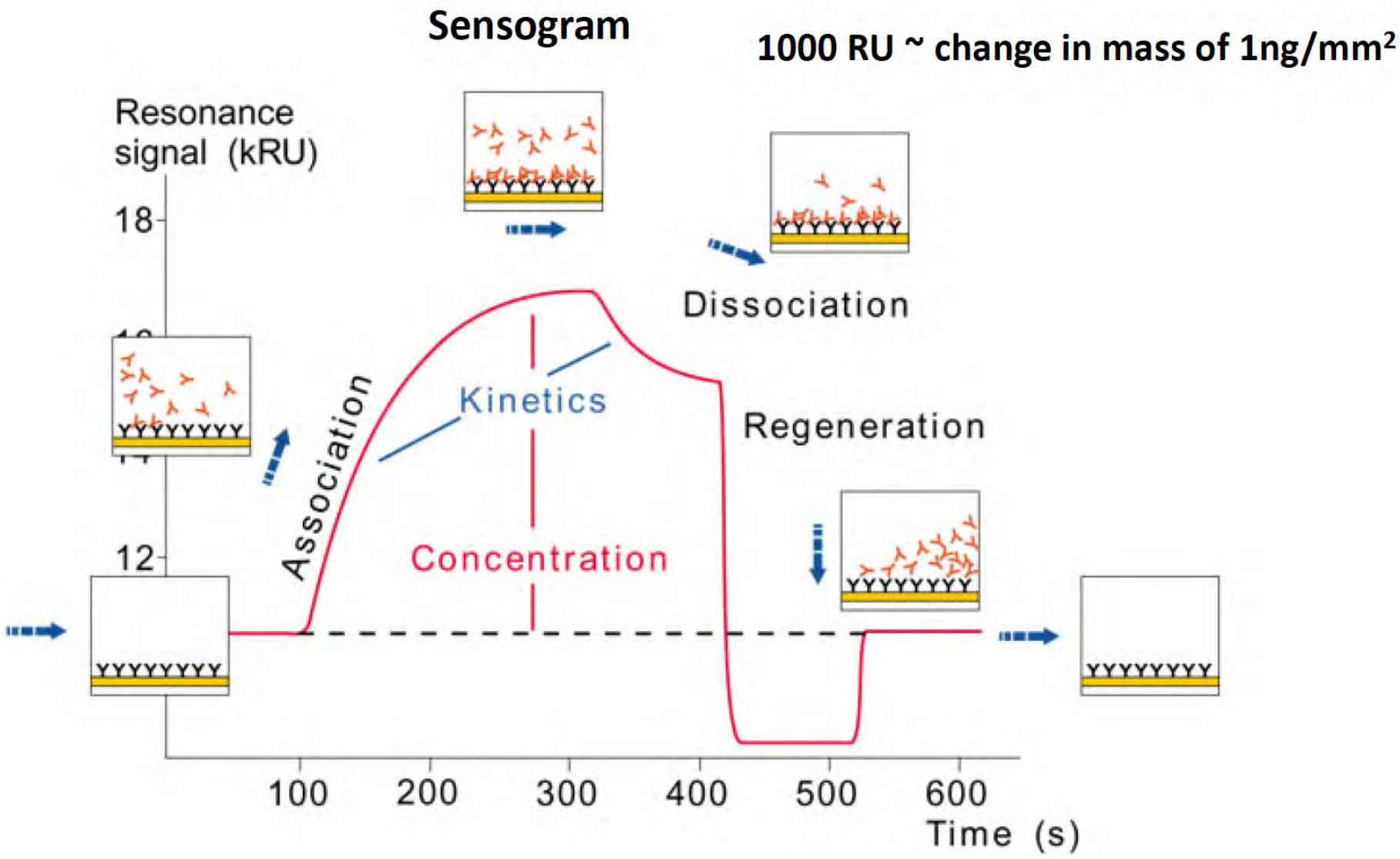
L1 (immobilization of membrane structures)



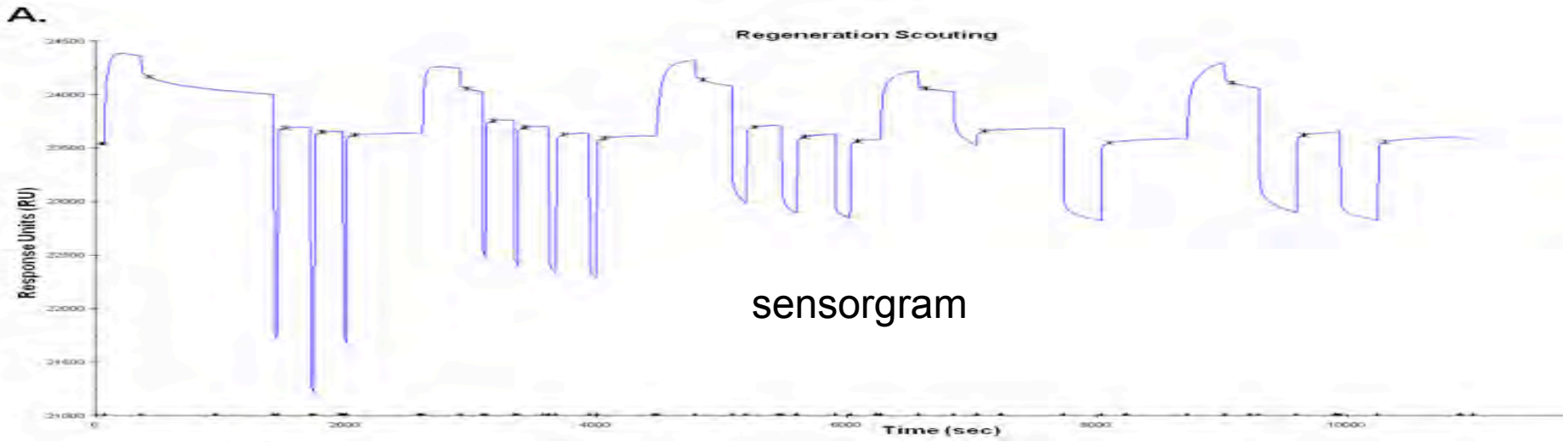
NTA (binding of Ni^{2+} / his-tagged proteins)



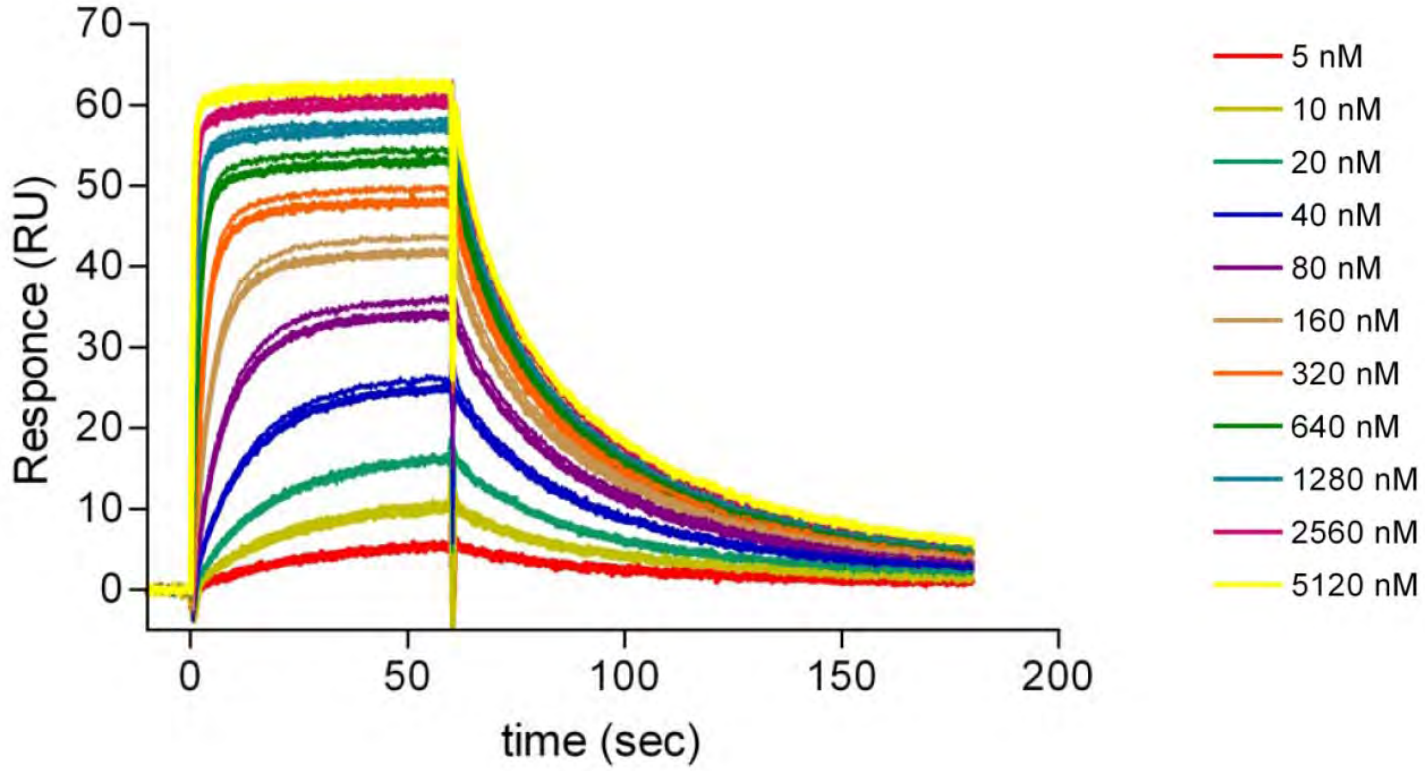
Typical binding kinetic's experiment



Example of binding



sensorgram



How to set up an SPR experiment?

Ligand Immobilization

- Choice of Immobilization chemistry (covalent, via a His-tag, biotin/ streptavidine)
- Stability of ligand
- Level of immobilisation (enough but not too much…)

  Kinetic analysis: immobilize low amount of ligand to avoid rebinding of analyte during dissociation

Analyte Binding

- Choice of running buffer (pH, ionic strength, detergent…)
- MW of the analyte (small molecules may be difficult to analyse)
- Availability of the analyte

Regeneration

- Regeneration solution (ligand/analyte-dependent): detergent, pH, salt…
- Stability of the ligand

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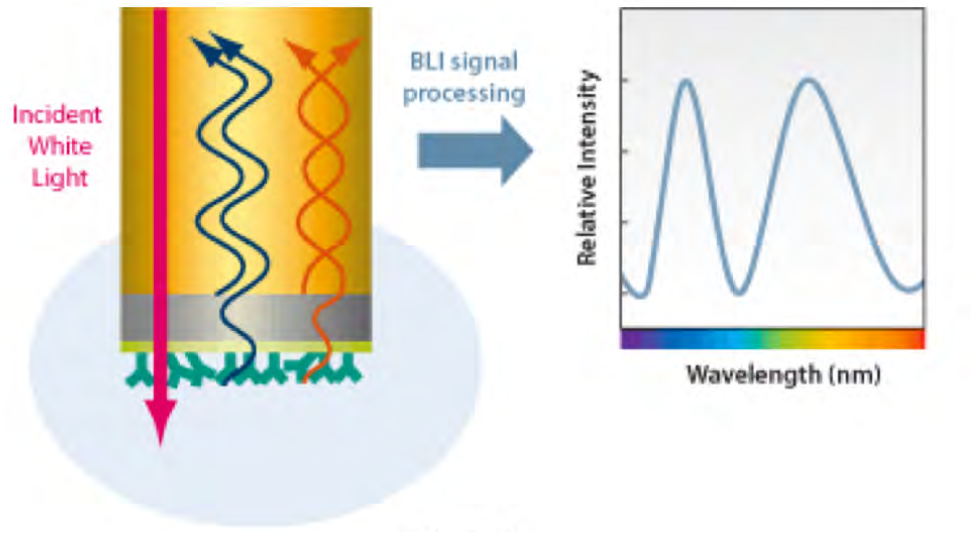
A Division of **Pall Life Sciences**

BLI

Bio-Layer
Interferometry

Octet Red 96

Bio Layer Interferometry



Bio-Layer Interferometry (BLI) is an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer.

Bio Layer Interferometry

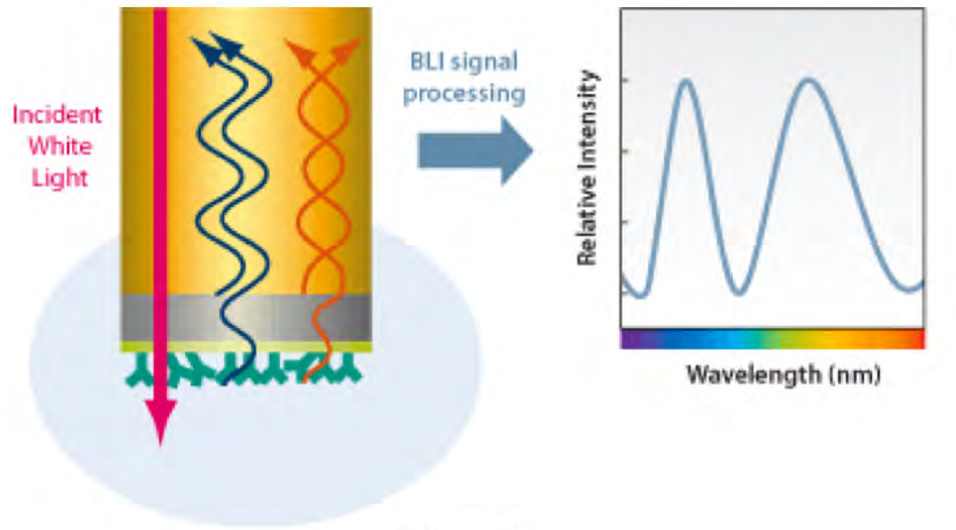
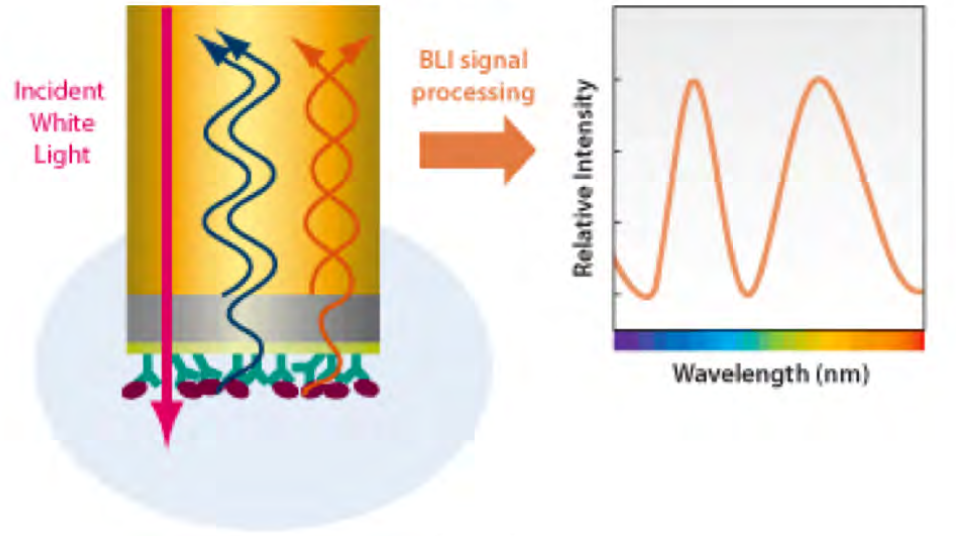


Figure 1

Bio-Layer Interferometry (BLI) is an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer.



Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured in real-time

Bio Layer Interferometry

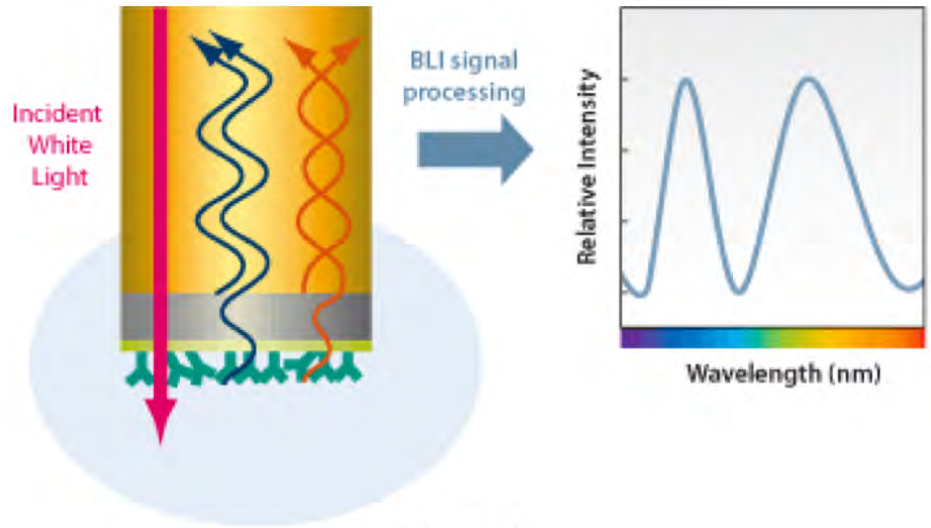
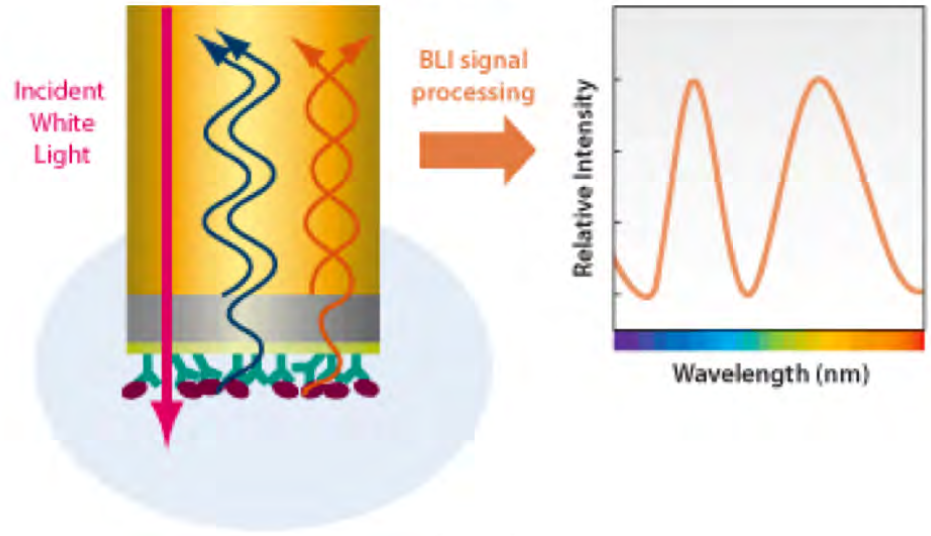
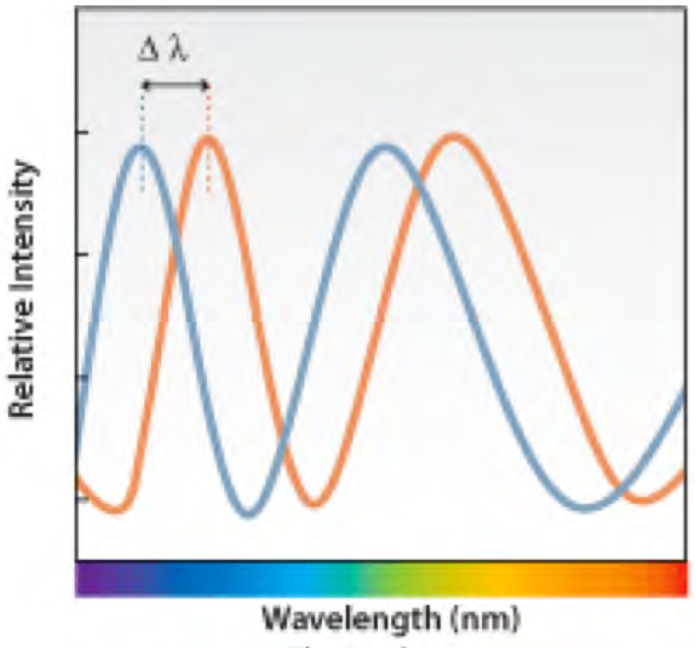


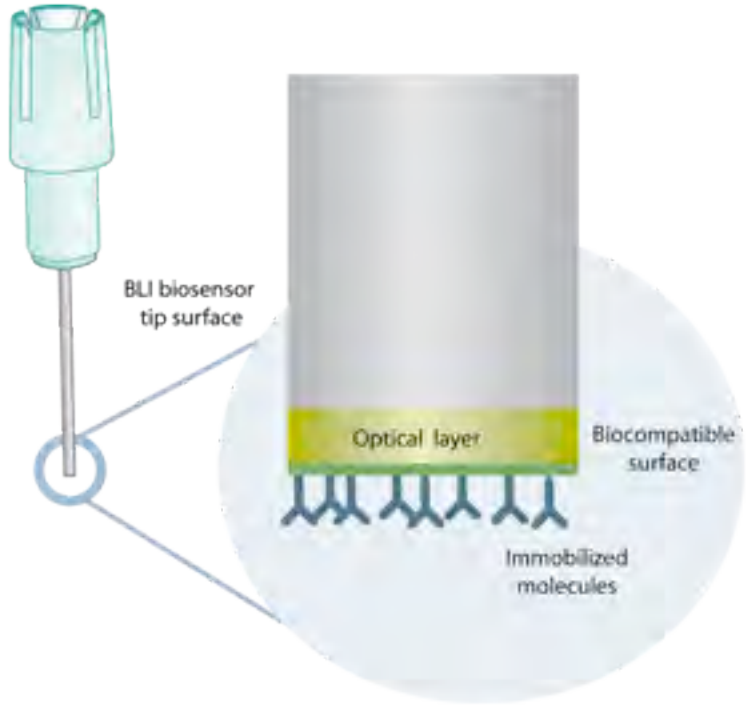
Figure 1





The binding between a ligand immobilized on the biosensor tip surface and an analyte in solution produces an increase in optical thickness at the biosensor tip, which results in a wavelength shift, Δλ, which is a direct measure of the change in thickness of the biological layer.

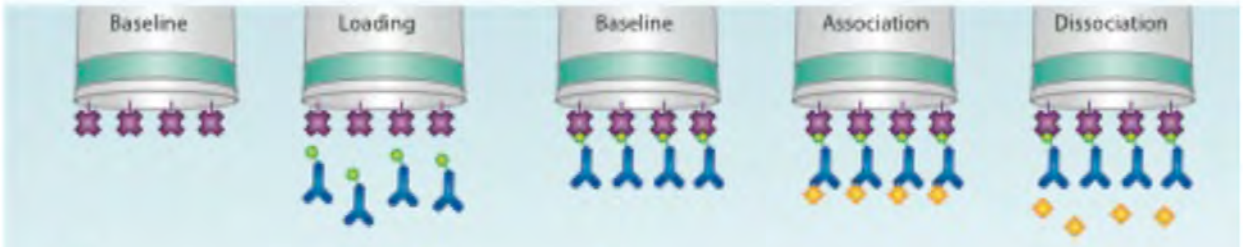
Dip and Read™ Biosensors

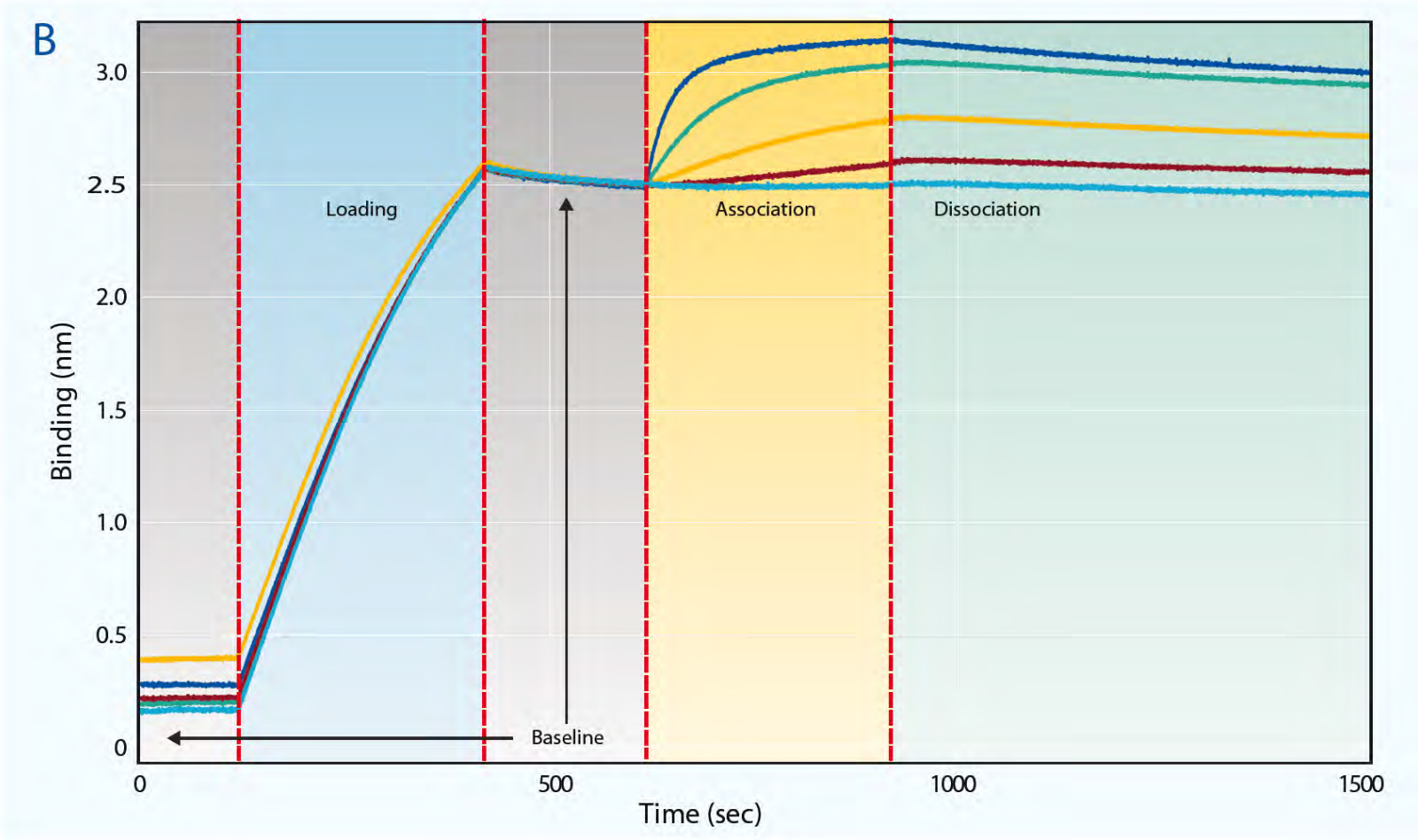


Direct immobilisation: amine coupling, biotin intercation with streptavidin

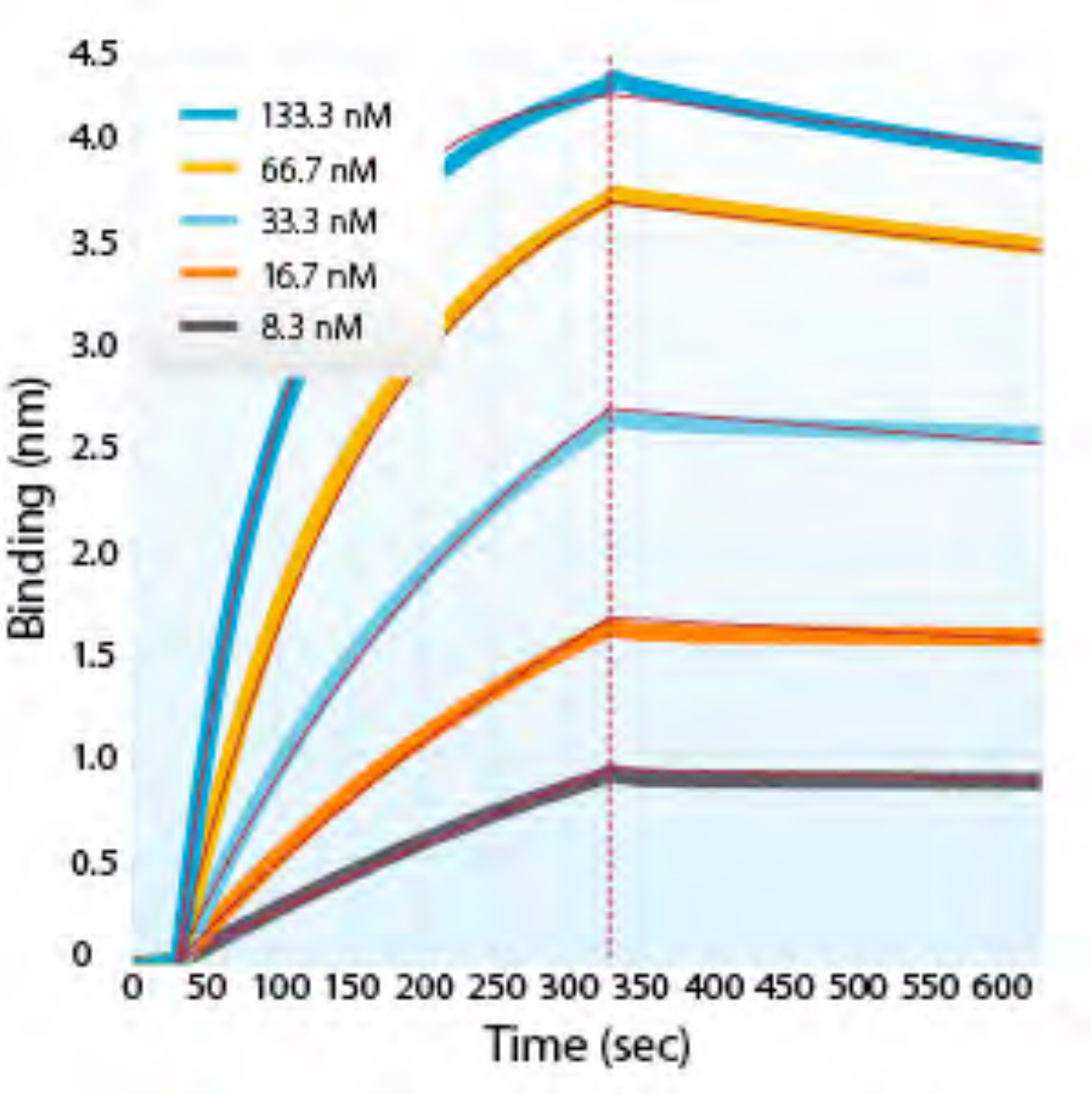
Capture-based approach: high affinity capture antibody (anti-FC, anti-His) or use of known motif or tag (protein A, Ni NTA)

Typical binding kinetic's experiment





Example of binding



ITC	MST	SPR	BLI
Affinity Thermodynamics	Affinity	Affinity Kinetics	Affinity Kinetics
Interaction in solution No labeling is required No limit on molecular weight	Easy to use No limit on molecular weight	Low sample amount Measure in any buffer (No) limit on molecular weight	Fast and Easy to use Measure in any buffer (culture media) (No) limit on molecular weight
Big amount of protein High sample concentration Buffers must match excatly	Interaction capillaries The labelled protein must be really monodisperse	Microfluidics Ligand immobilisation Regeneration Non specific interaction with sensor chip	Ligand immobilisation (Regeneration) Non specific interaction with bio sensor surface
Cost 80k€ No maintenance	Cost 90k€ No maintenance	Cost 300k€ Expensive maintenance	Cost 150k€ No maintenance
Fragile syringe	Capillaries	Sensor chips	Bio-sensors

Which one is the best?

It depends on:

- The question you want to answer
- The amount of material you have
- Time…

They all have advantages et disadvantages

Very important

be aware of the limitations

never try to over-interpret your results

How much protein do I need?

For all techniques

Purity is crucial for obtaining quantitative reliable results.

« Real » concentration must be measure as accurately as possible

ITC Protein 300µl concentration= 10 x K_D
Ligand 60 µl concentration = 100 x K_D

Biacore Ligand, depends on immobilisation 50-400 nM, 100µl
Analyte, titration between 0.1 and 10 x K_D
Quantity depends on contact time

Thermophorèse Labeled protein 100 µl 20 µM
Ligand 20 µl concentration = 40-50 x K_D

BLI Ligand, depends on immobilisation 50-400 nM, 200µl
Analyte, titration between 0.1 and 10 x K_D, 200µl

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Survey of the year 2007 commercial optical biosensor literature

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Abstract:
In 2007, 1179 papers were published that involved the application of optical biosensors. …. We found a disappointingly low percentage of well-executed experiments and thoughtful data interpretation. We are alarmed by the high frequency of suboptimal data and over-interpreted results in the literature….

In fact, a problem in most of the published data we see is that the authors apparently did only one experiment; it looks like they walked up to the machine, chucked in their samples, and **published whatever data came out**.

Many users who generate poor-quality data are either **too ignorant** to recognize the problem **or too lazy** to want to fix it.