Approches complémentaires pour l'analyse des interactions moléculaires.

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Juin 2019
Ile d'Oléron


How to characterize a macromolecular complex?

Stoichiometry

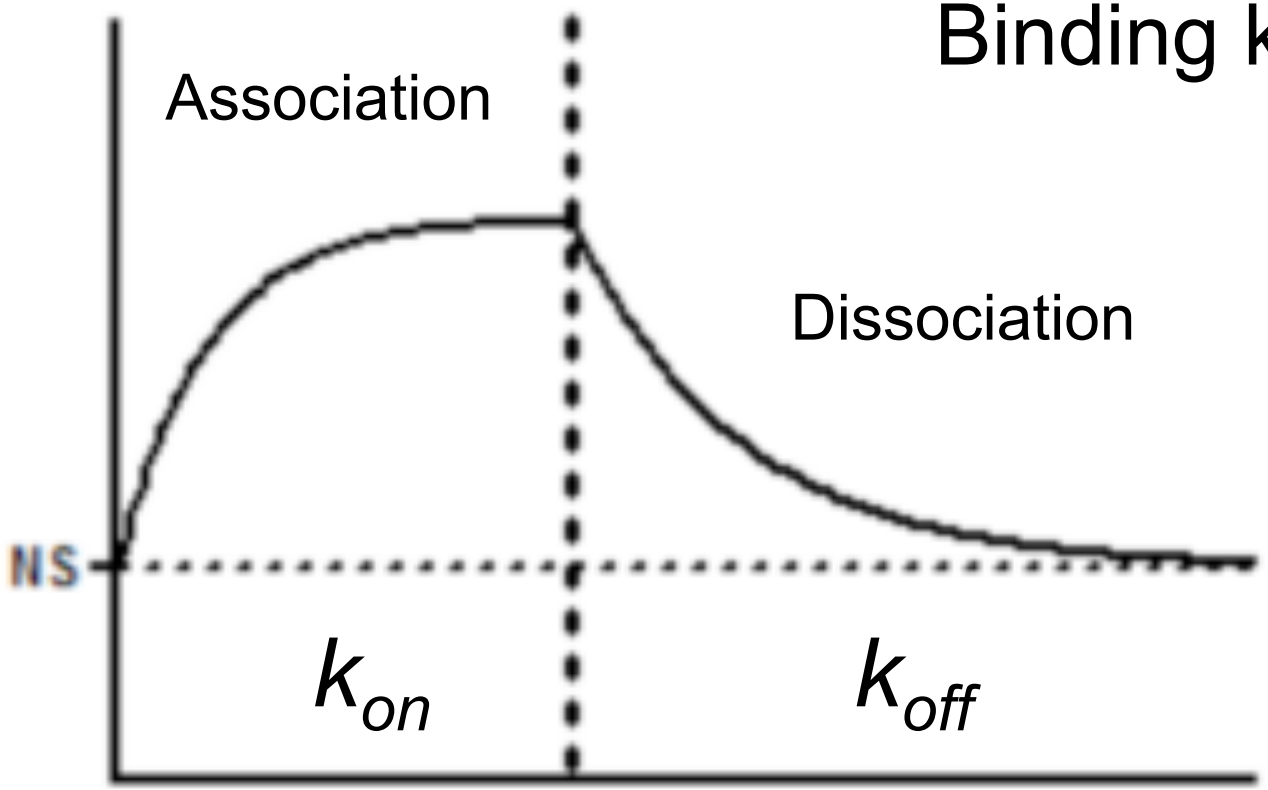
Stability

Affinity

Kinetics

Thermodynamic parameters

Binding kinetics

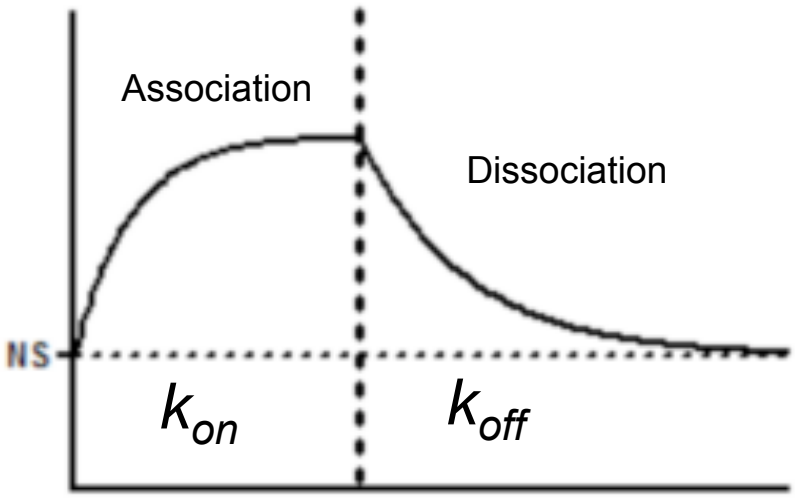


Association: how fast molecules bind = k_{on} ($M^{-1}s^{-1}$)

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

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

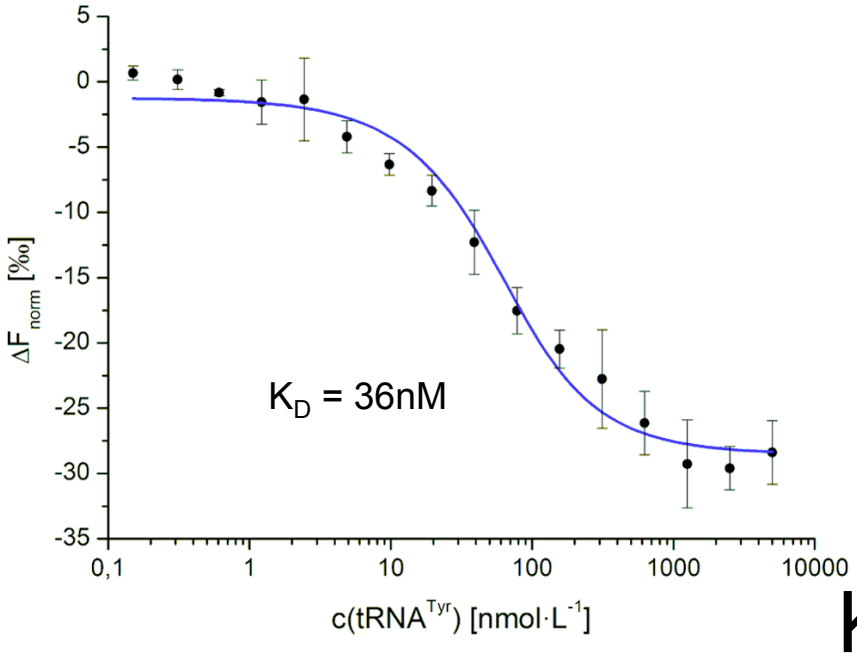
Binding kinetics



Association: how fast molecules bind = $M^{-1}s^{-1}$

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

Dissociation: how fast complexes fall apart = s^{-1}



Equilibrium

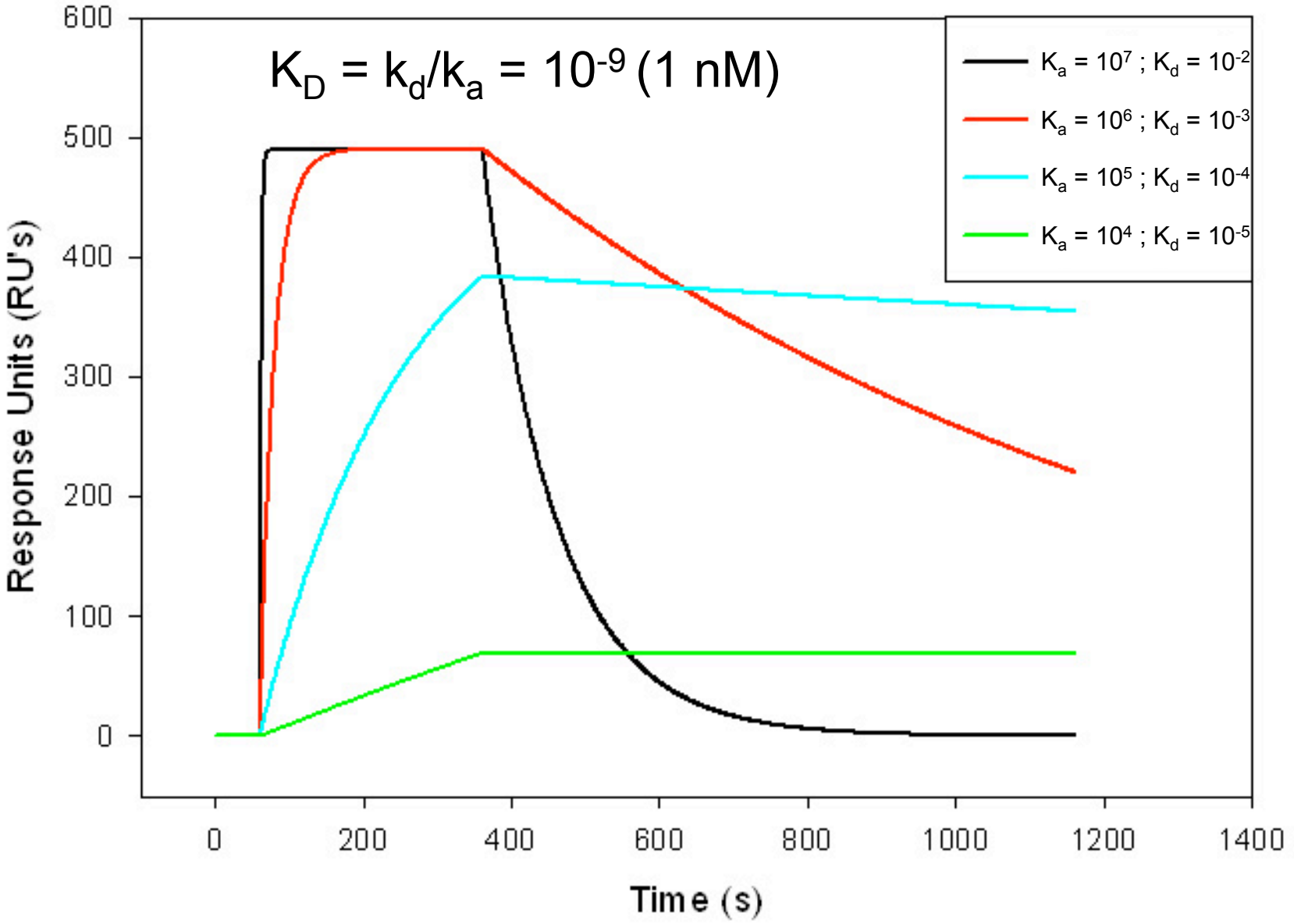
forward binding = backward unbinding

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

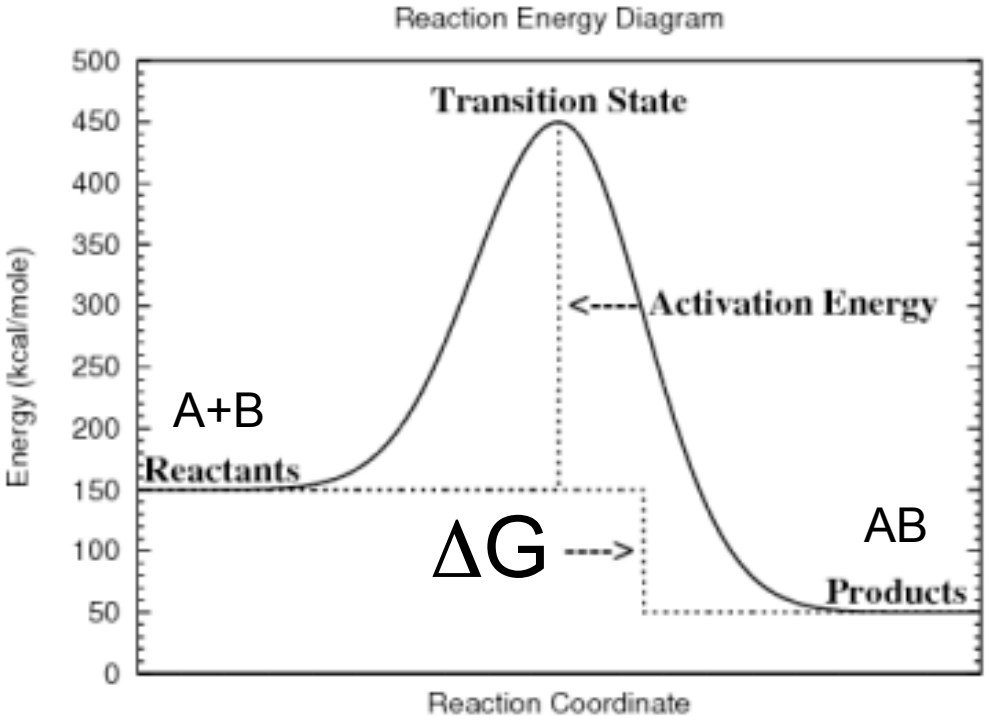
Equilibrium dissociation constant

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

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)

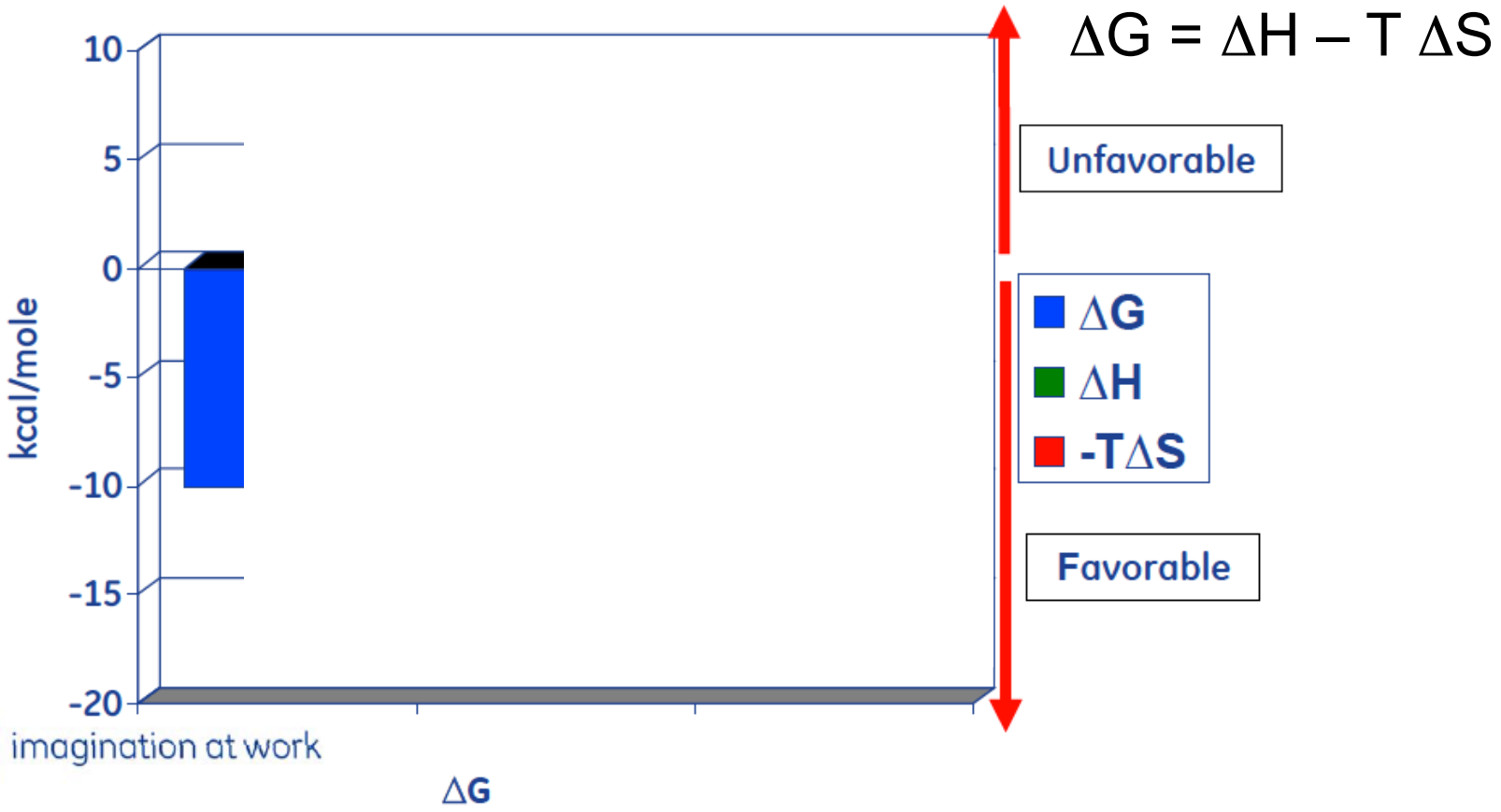
$$\Delta G = RT\ \ln(K_D)$$

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

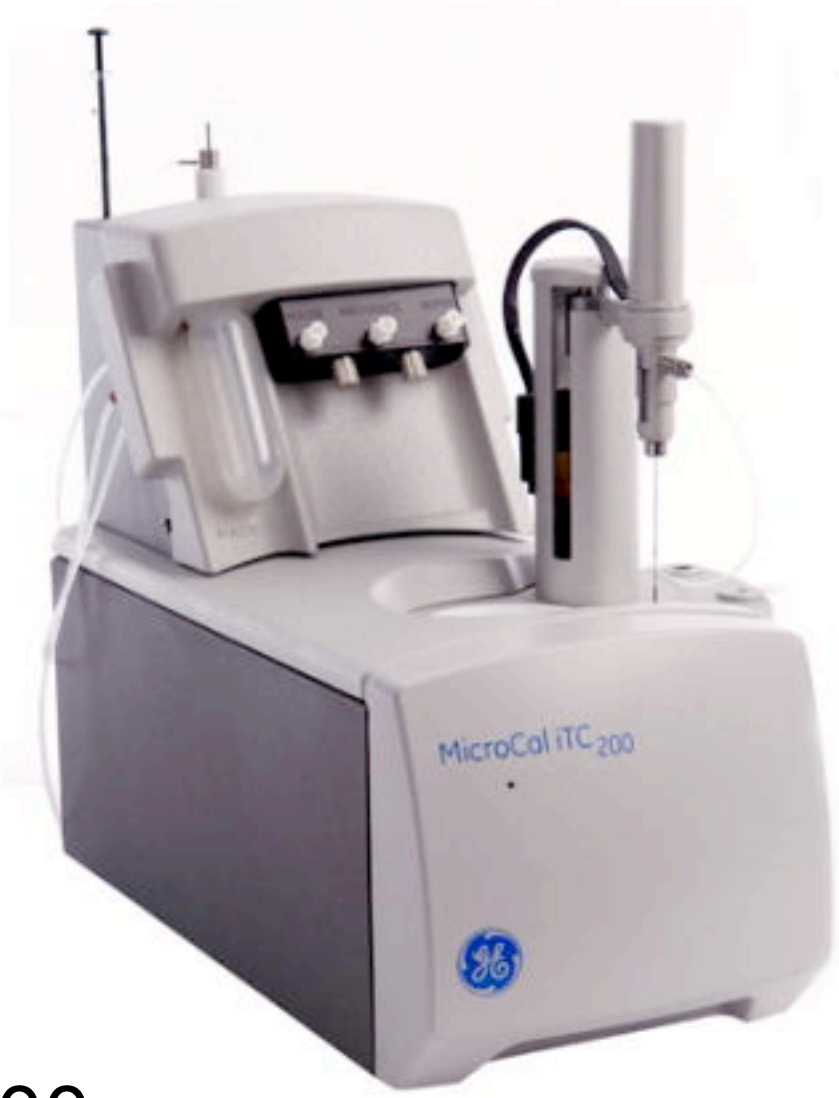
Techniques available at the AFMB

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, Hold-up…

GE Healthcare

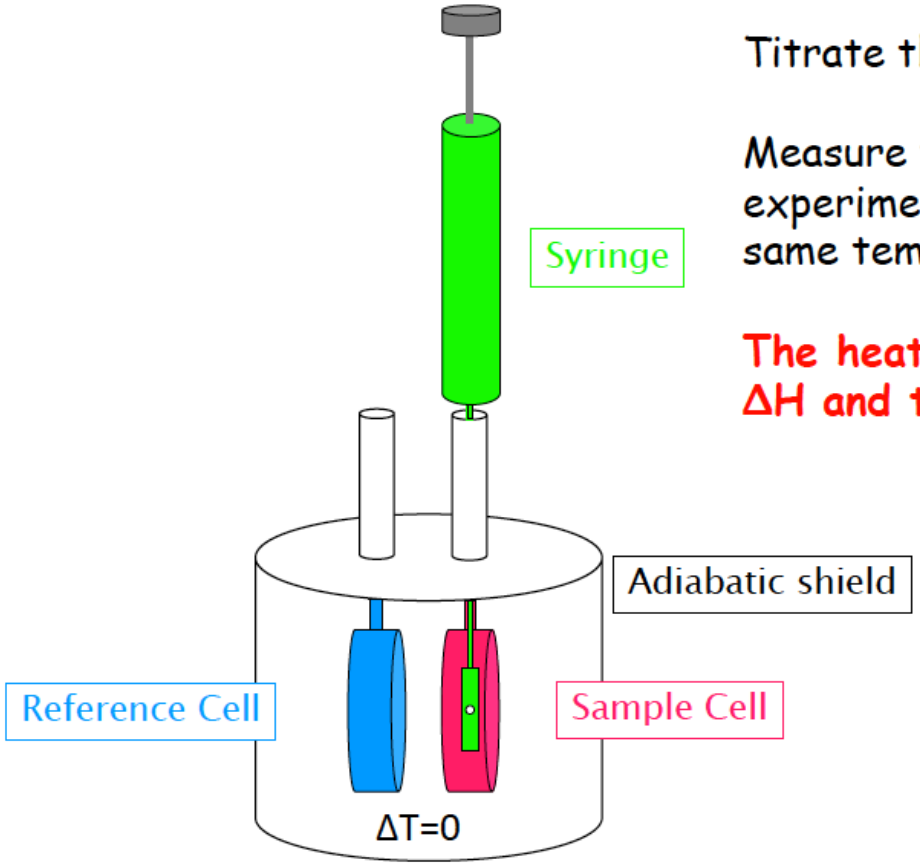
ITC



Isothermal
Titration
Calorimetry

MicroCal ITC 200

What happens during an ITC experiment?



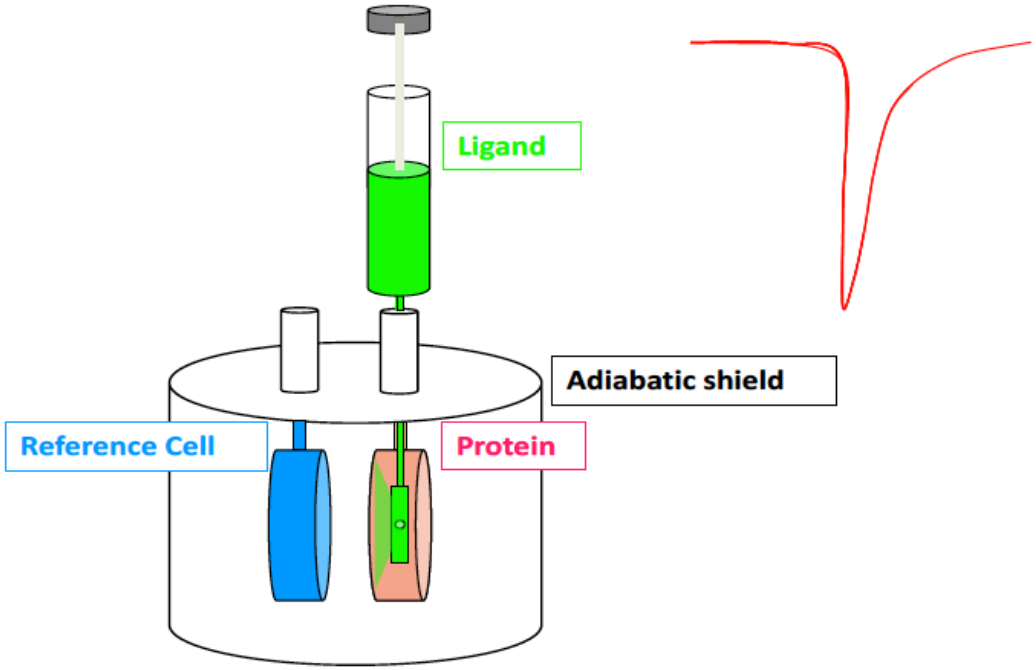
Protein target in sample cell

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

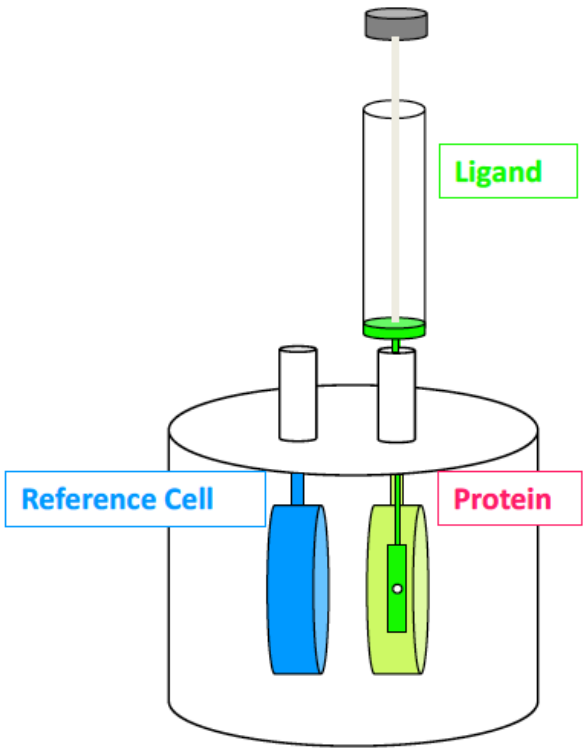
➔ Heat absorbed in sample cell

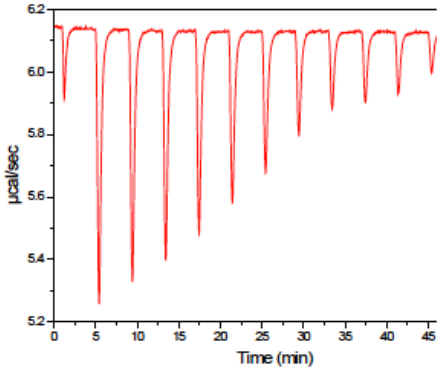
➔ extra energy will be provided in order to keep $\Delta T=0$

Exothermique reaction

➔ Heat released in sample cell

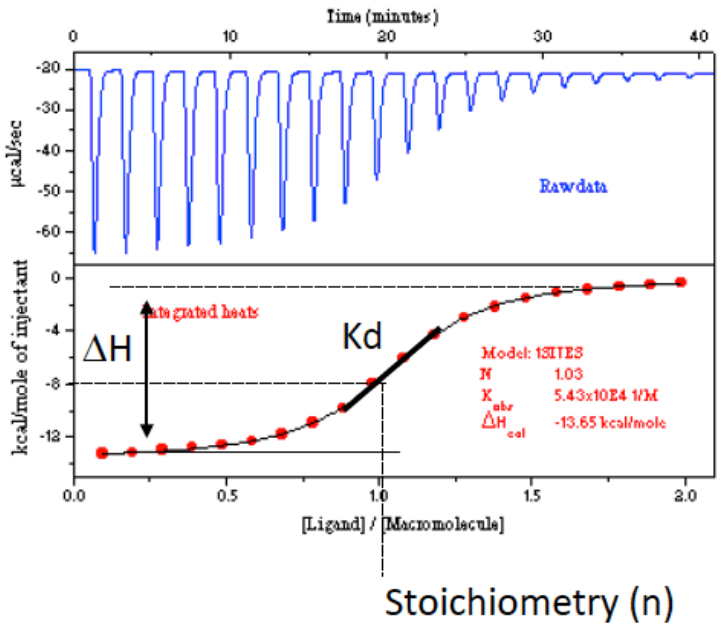
➔ less energy will be provided in order to keep $\Delta T=0$





- 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

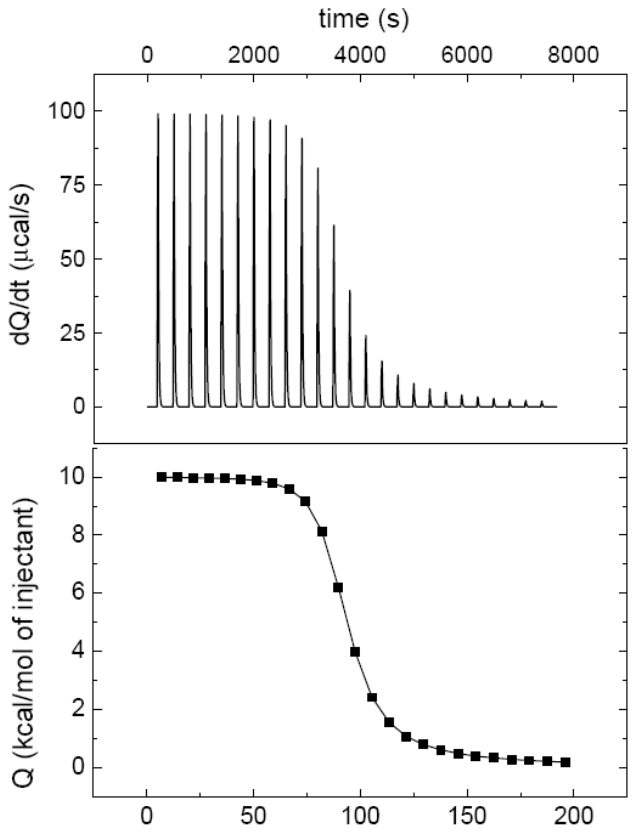
ΔH, n and K_D are calculated from the fitting

ΔG and ΔS are derived from the equations:

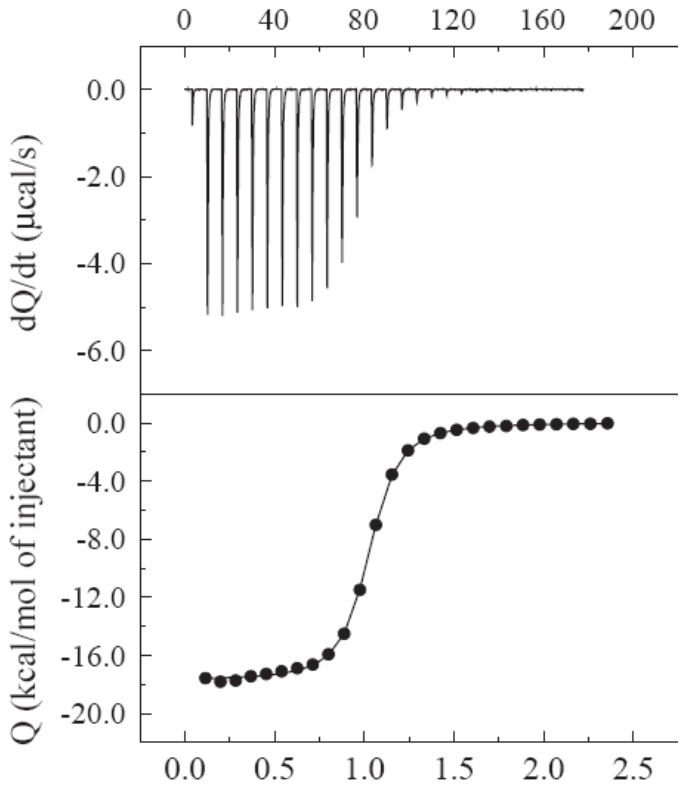
$$\Delta G = RT \ln K_D$$

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

Endothermique



Exothermique



NanoTemper technologies

MST

Micro Scale Thermophoresis

Monolith NT.115

What is Thermophoresis?

Electrophoresis:

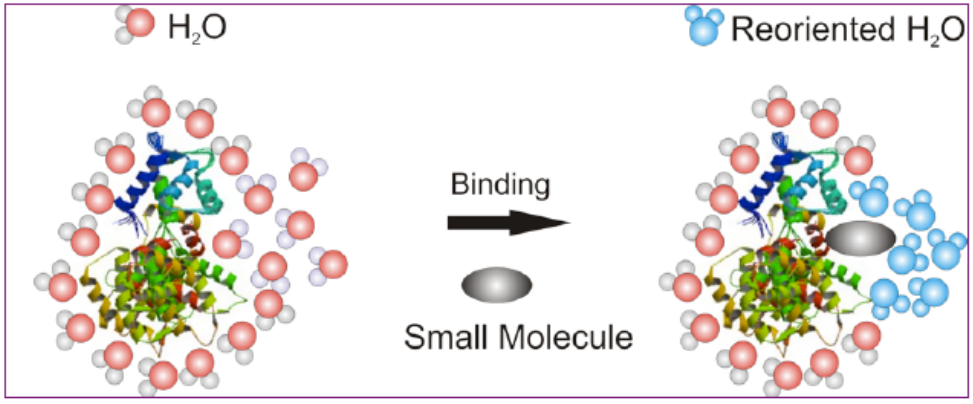
We apply an electric field
We separate the molecules by charge (and also size)

Thermophoresis:

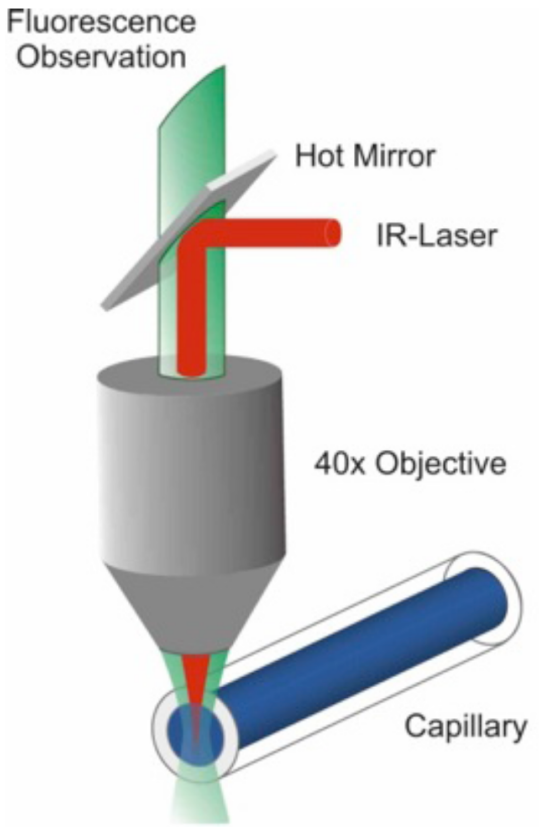
Sample is in solution in a capillary
An infrared laser generates a temperature gradient (between 1-6 K)
Thermophoresis is the motion of molecules in temperature gradients.

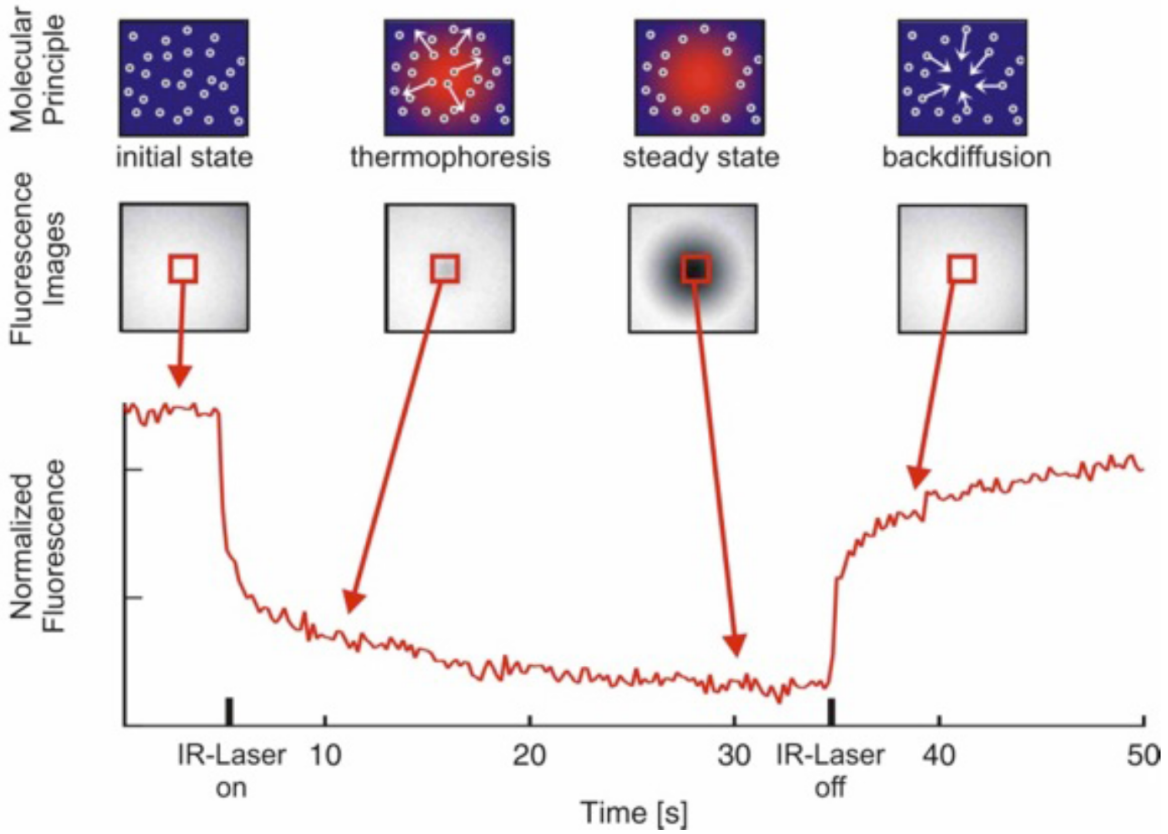
Movement is detected by through fluorescence of one of the binding partners.

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



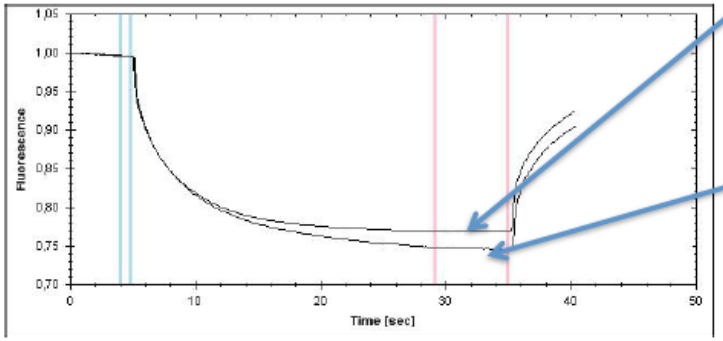
Thermophoresis experiment





- 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

Coming out: Nanopedia 2018, page 42

IR-laser induced temperatures

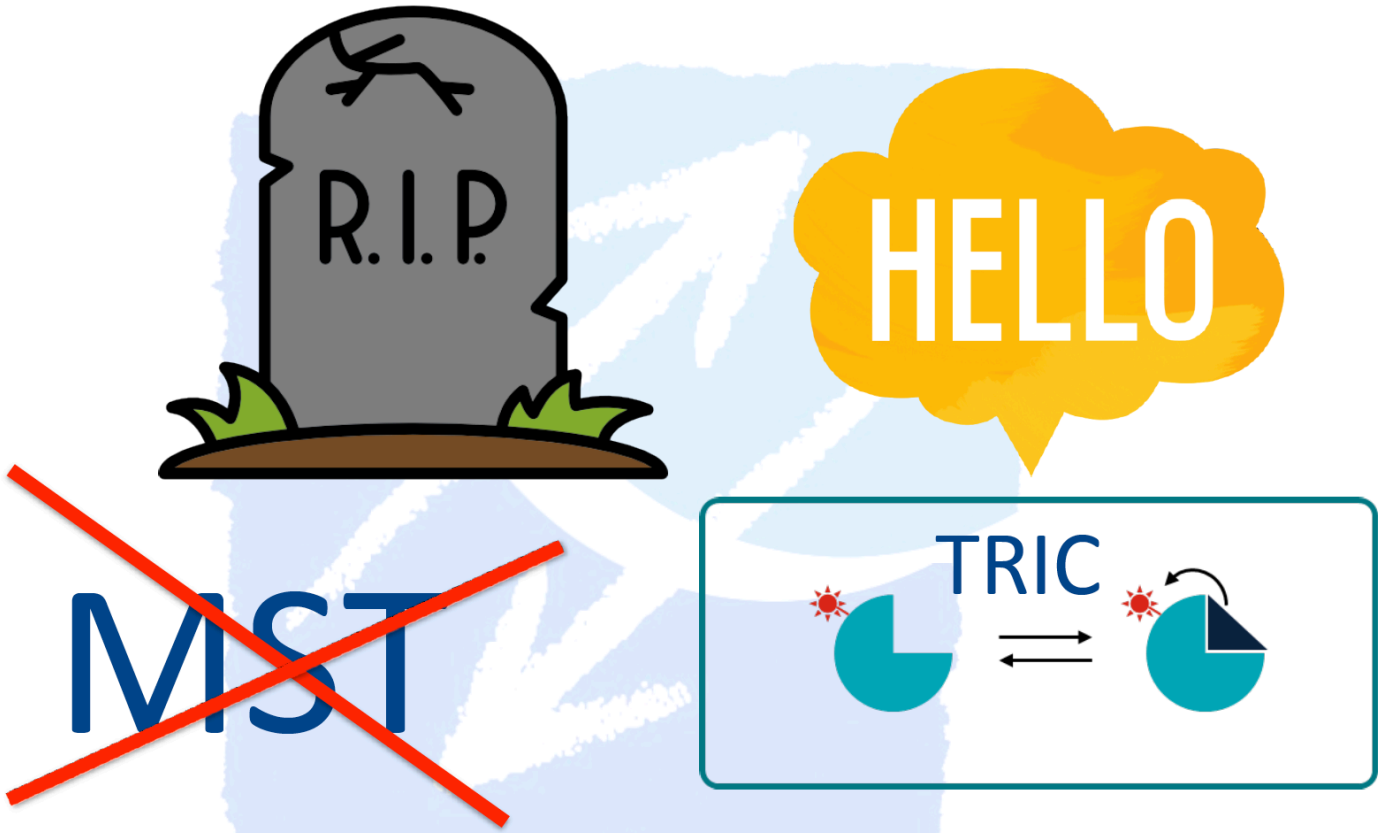
This table summarizes the MST-on times at different MST powers at which a temperature increase ΔT of 10 K is exceeded. At timepoints earlier than the ones noted in the table, the temperature increase is time-dependent but always lower than 10 K. Therefore an MST-on time of 1.5 seconds usually means a temperature increase of only few K. Note that the temperature increase is always less than 10 K at low MST power.

MST power	Low	Medium	High
MST-on time (ΔT<10 K)	-	5 sec	2.5 sec

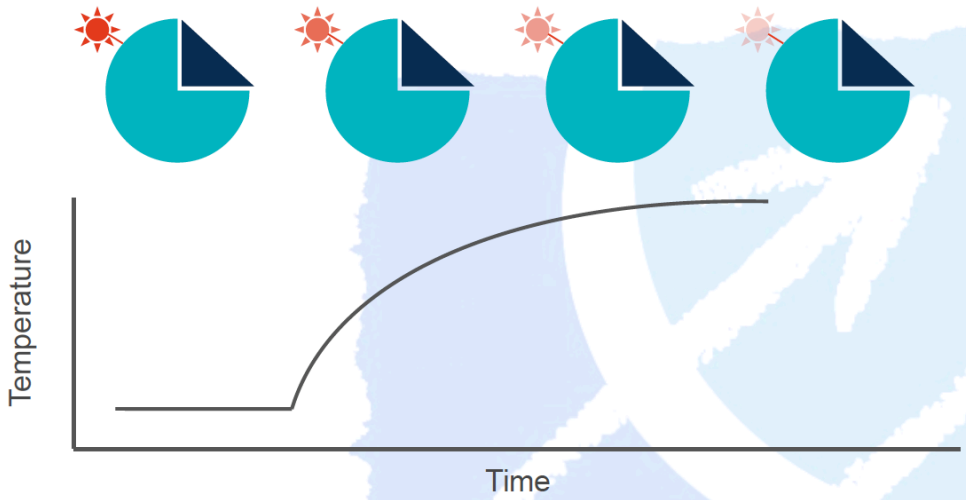
- This means a **massive temperature change** with higher MST powers and longer on-times!
  - K_D is a temperature-dependent parameter
  - Protein structure will be affected
- In addition, ligand, buffer ions, salt ions etc. also perform thermophoresis: pH, ionic strength etc. change!

Dr. Stephan Uebel, Max-Planck-Institute of Biochemistry, Martinsried, Germany

Rest in Peace, Thermophoresis



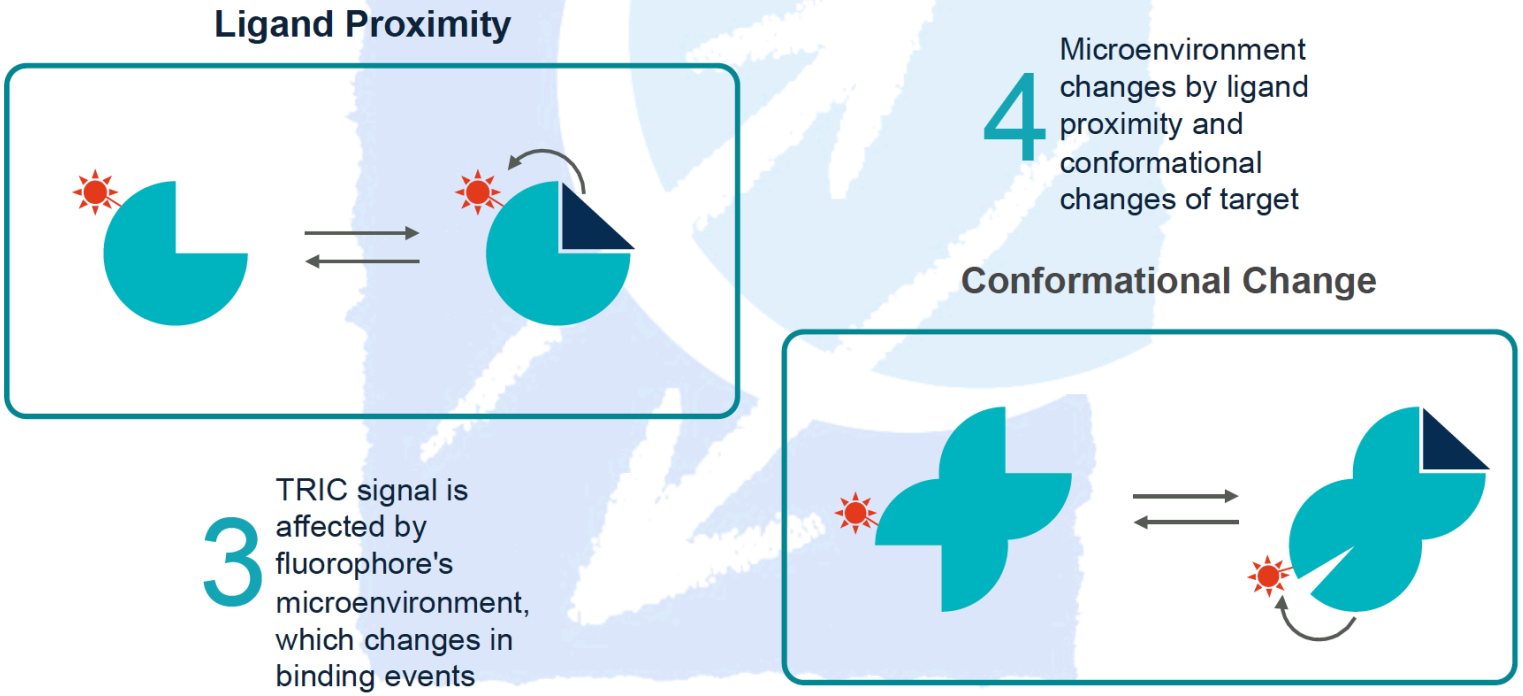
Temperature Related Intensity Change TRIC



- An effect where the fluorescence intensity of a fluorophore is temperature dependent
- Extent of temperature dependence is strongly related to the chemical environment of the fluorophore, which can be affected by binding of a ligand to the target

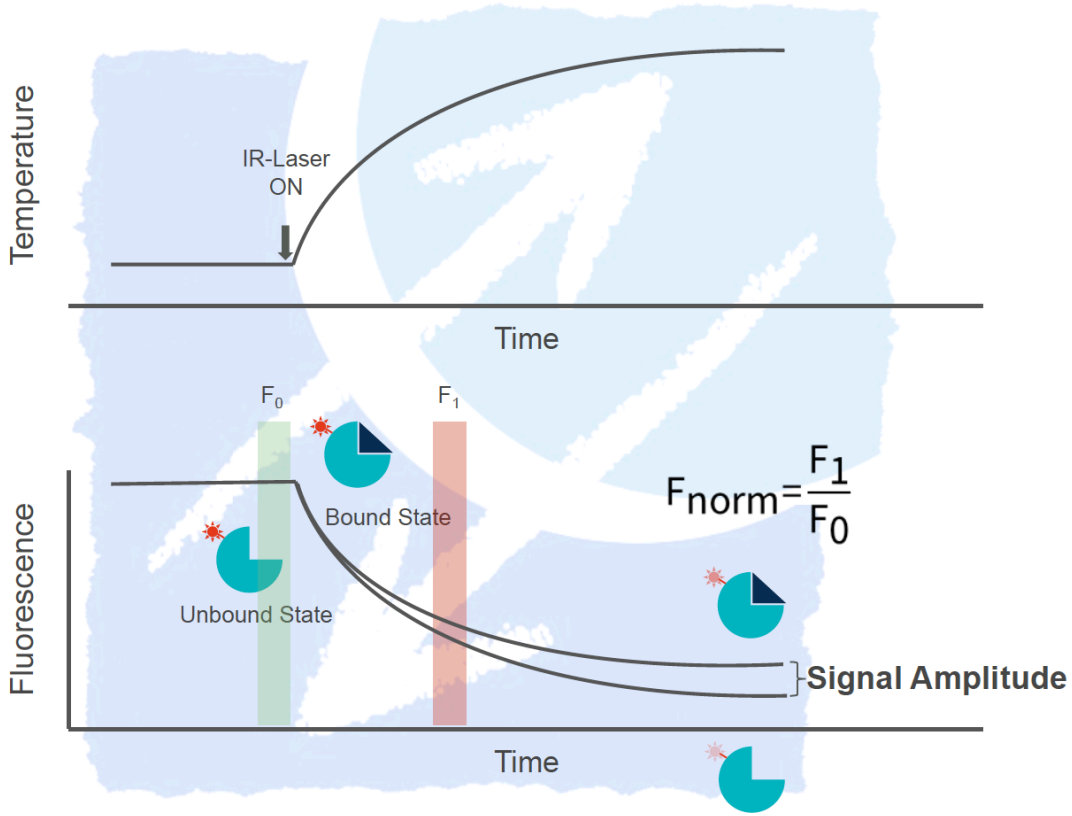
Temperature Related Intensity Change TRIC

- Changes in chemical environment are caused by proximity of ligand or conformational change



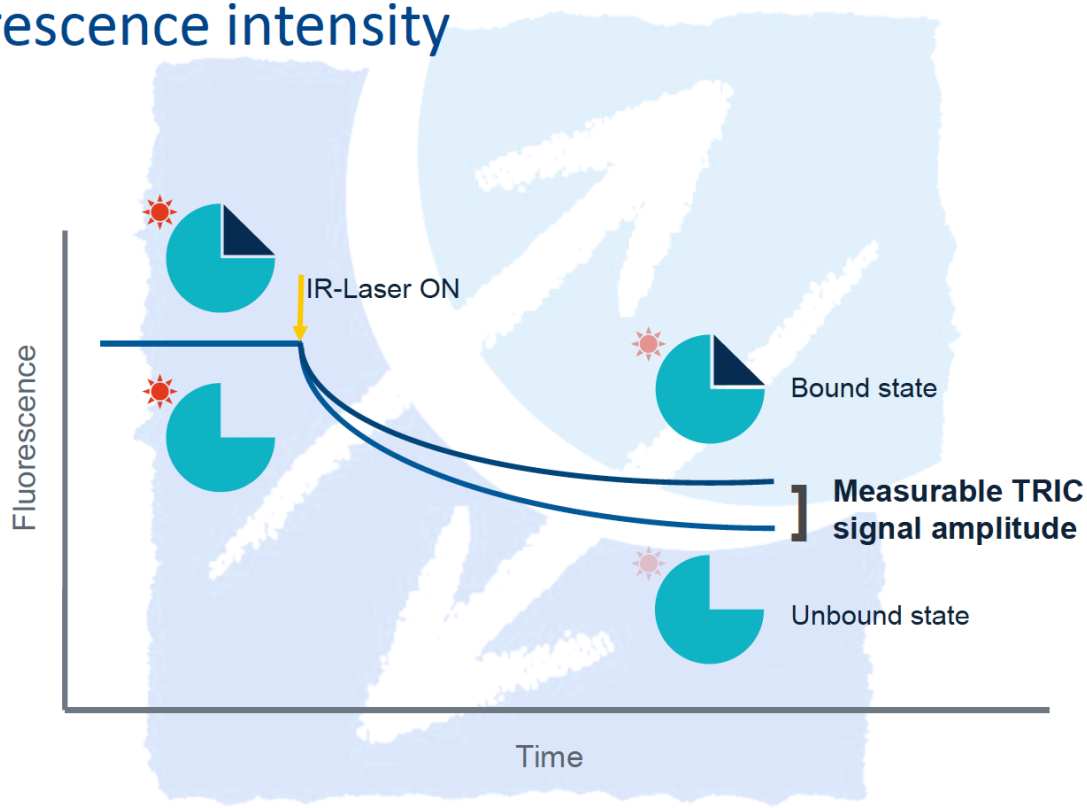
Temperature Related Intensity Change TRIC

- Change in fluorescence intensity is plotted over time



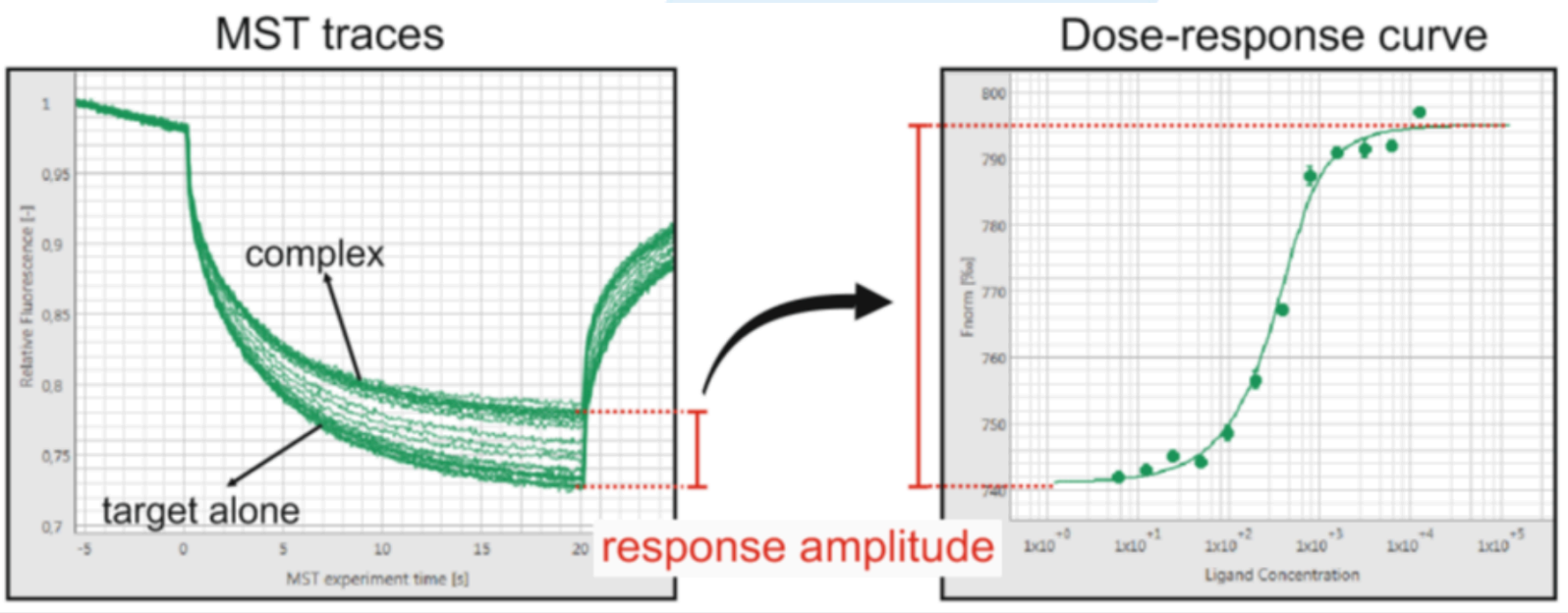
TRIC - Summary

- Binding events are detected by measuring changes in fluorescence intensity



TRIC - Binding Isotherm

- Response amplitude is used to build binding isotherm



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)

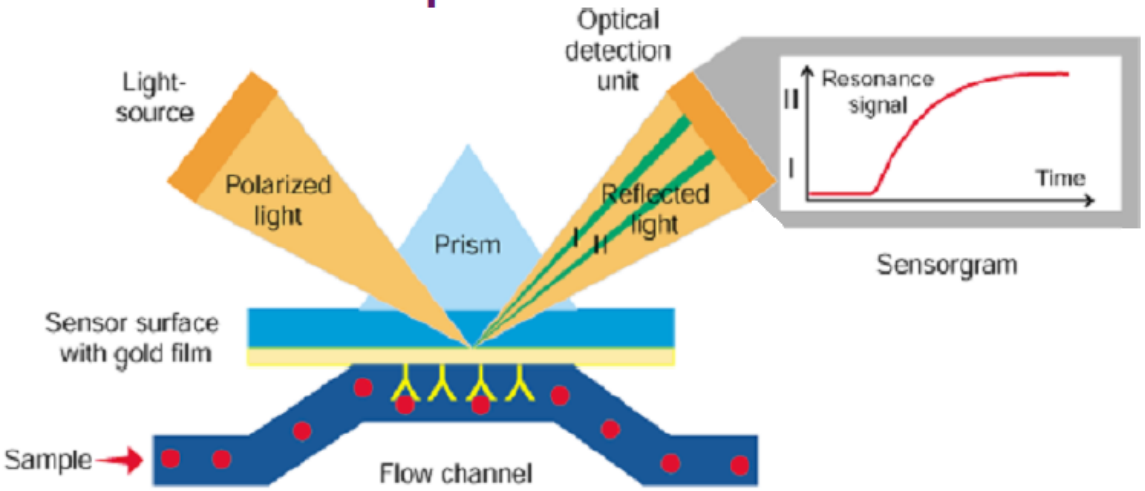
GE Healthcare

SPR

Surface
Plasmon
Resonance

Biacore T200

Surface plasmon resonance



Physical phenomenon: Surface plasmon resonance

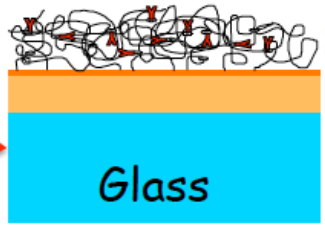
SPR machine detects refractive index changes close to the sensor surface

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

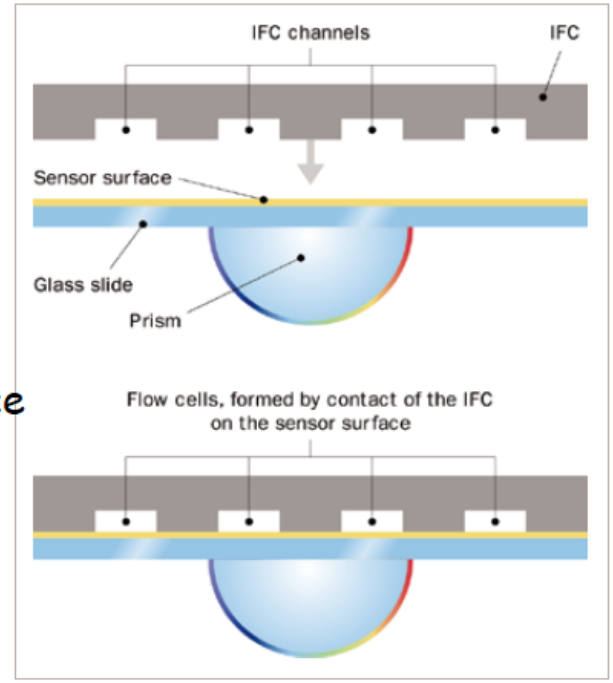
A sensorgram represents the resonance signal (in resonance units RU) as a function of time. **This response is proportional to the mass retained near the surface**

Sensor Chips and flow cells



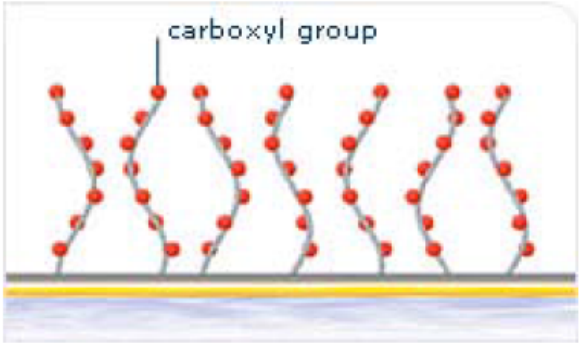
Dextran matrix
Gold 50 nm

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

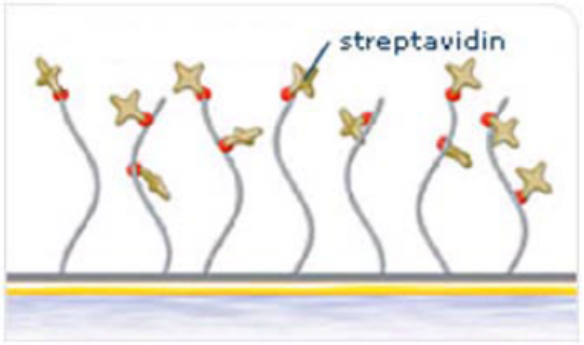


Different types of sensor chips

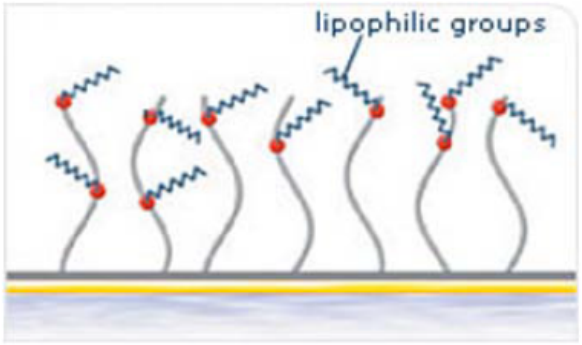
CM5 (carboxymethyl-dextran) Coupling via -NH2, -SH, -CHO, -OH or -COOH



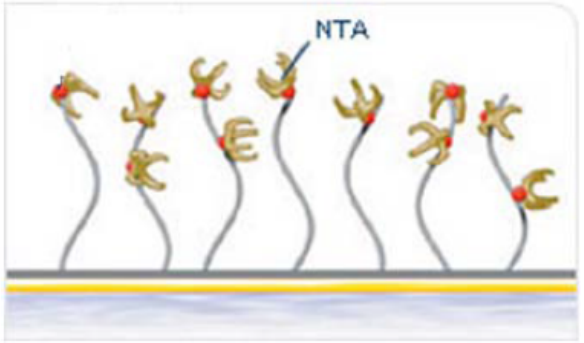
SA (immobilization of biotylilated molecules)



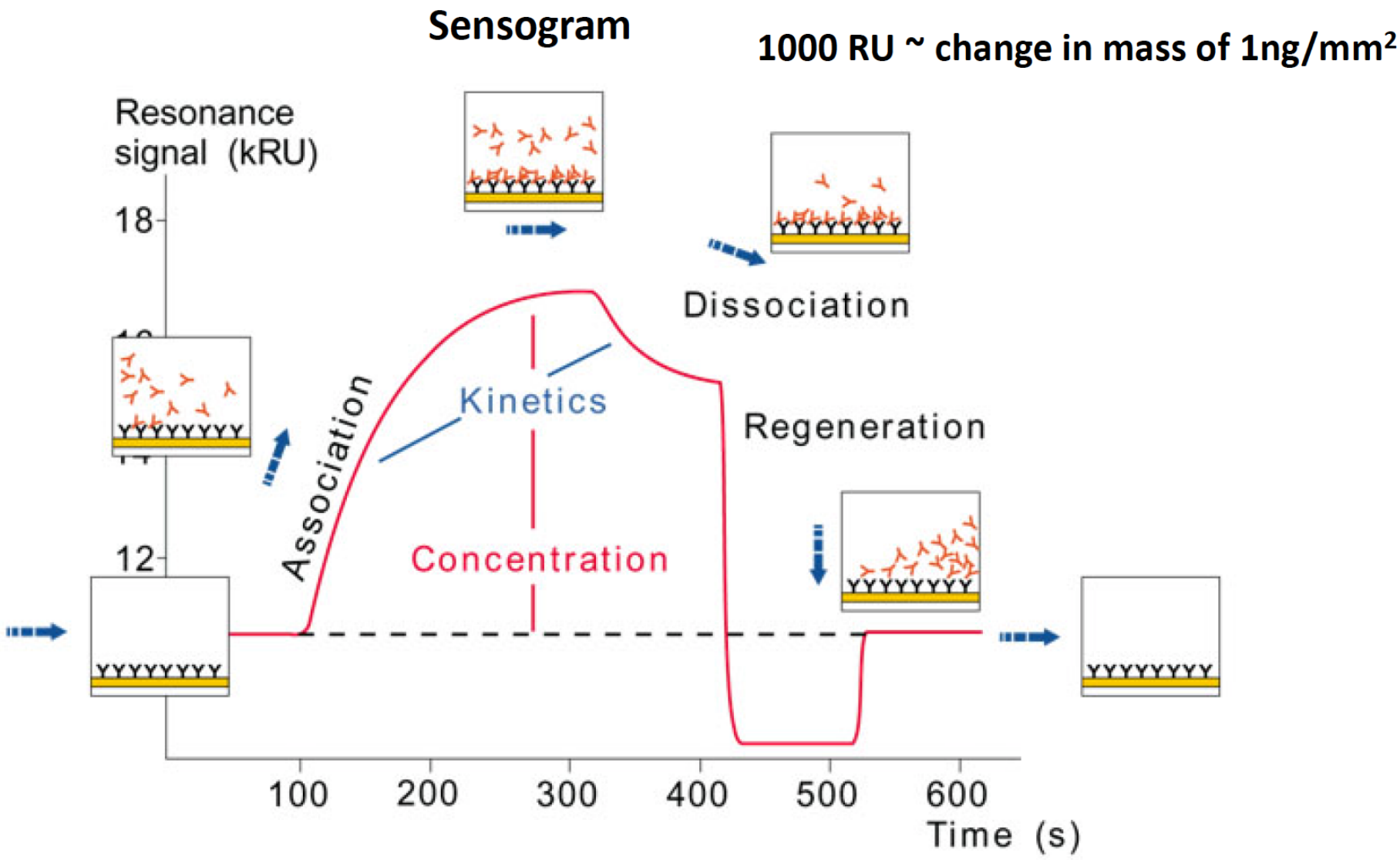
L1 (immobilization of membrane structures)



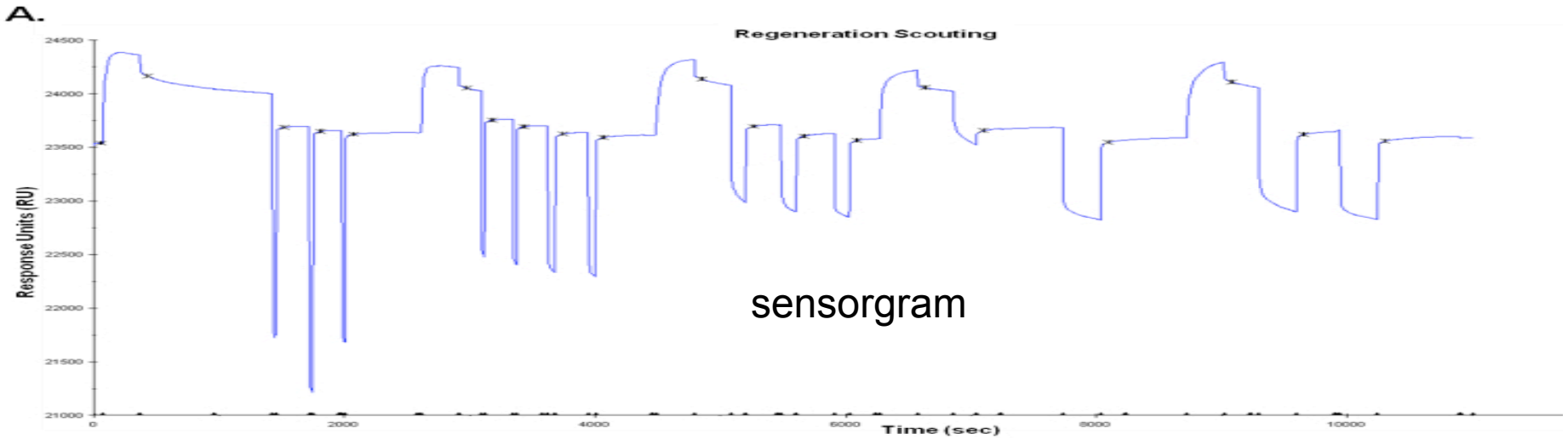
NTA (binding of Ni^{2+} / his-tagged proteins)



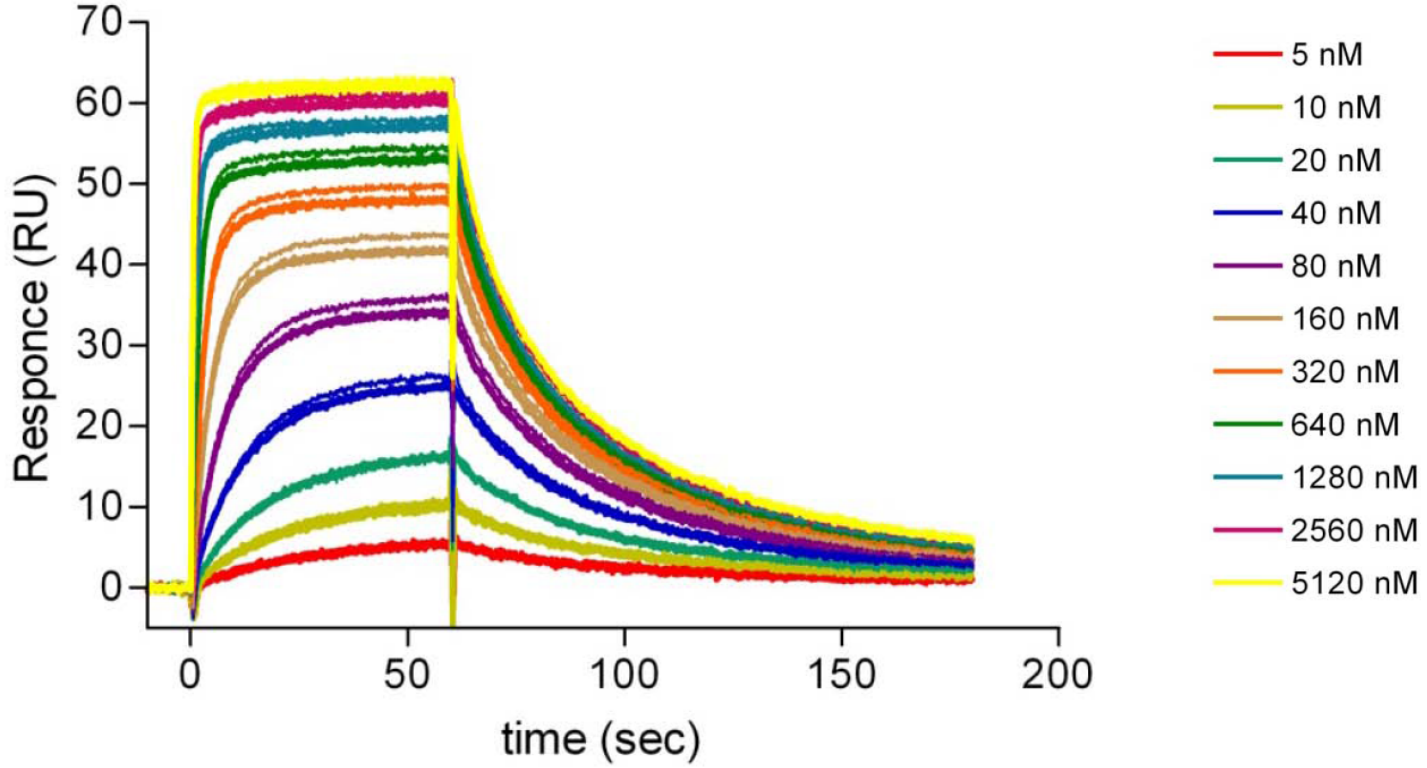
Typical binding kinetic's experiment



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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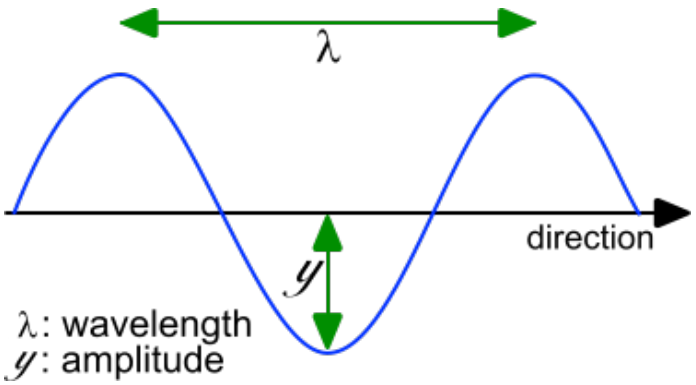
BLI

Bio-Layer
Interferometry

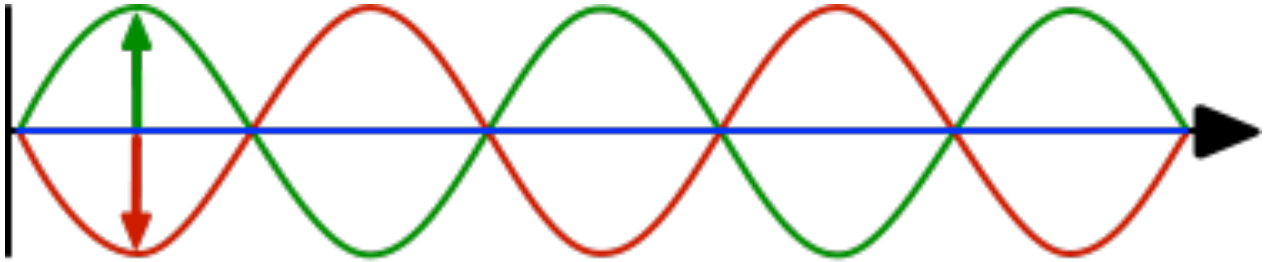
Octet Red 96

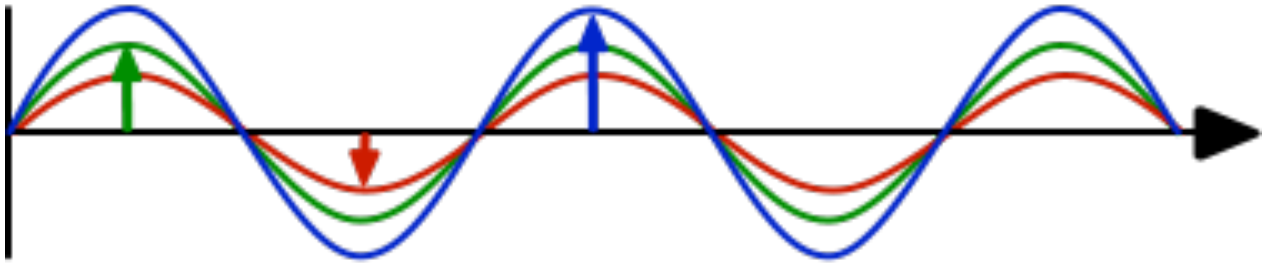
Optical interferometry



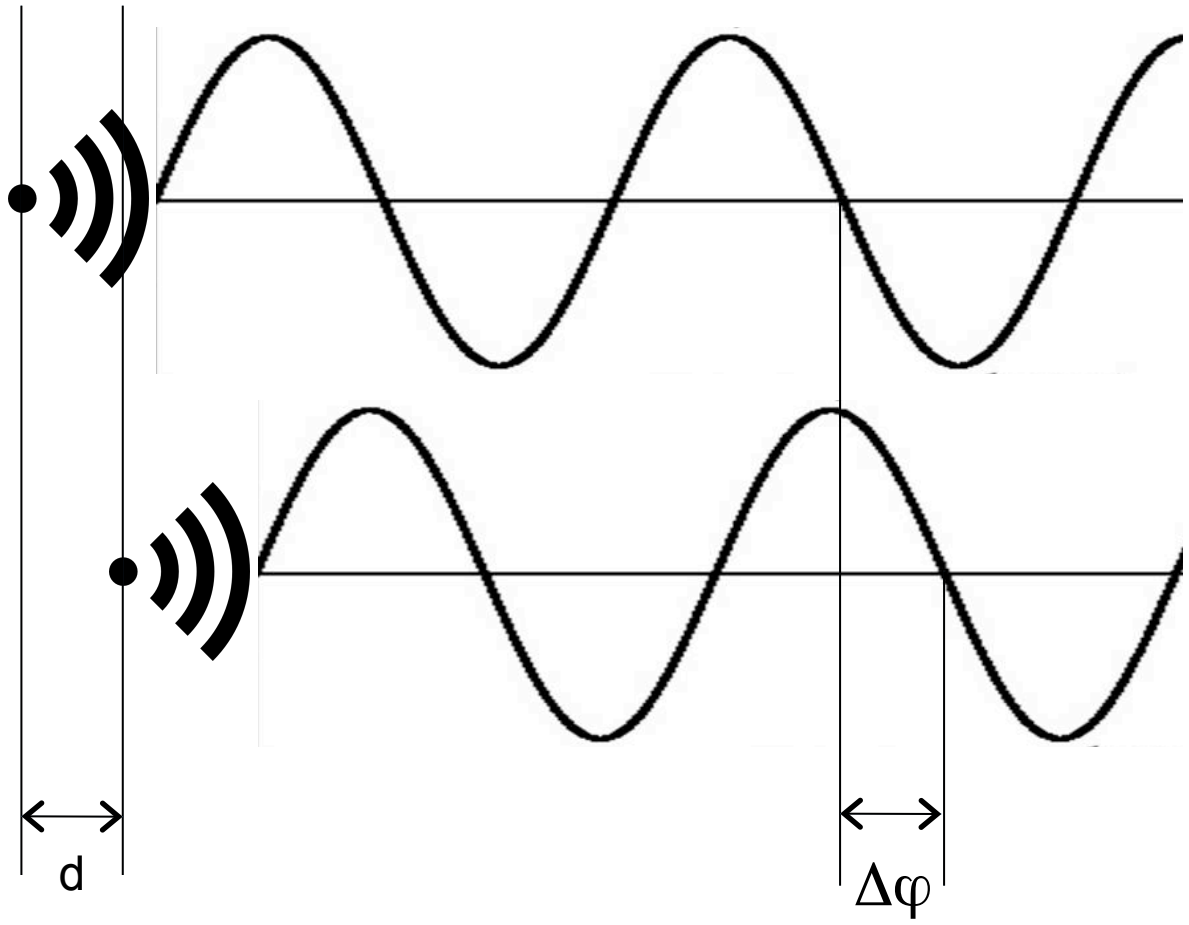
A light wave has a wavelength, amplitude and direction.

When two light waves interact the result of the interaction depends on the phase and amplitude of the waves.


Destructive interference


Constructive interference

Phase difference and optical path difference

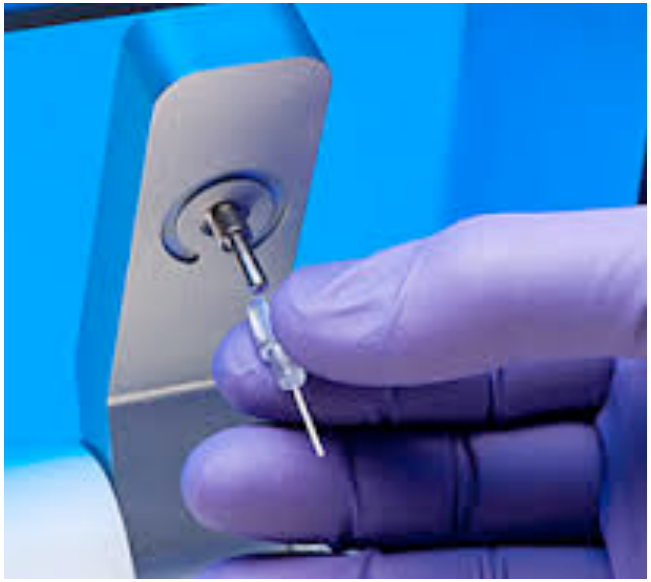


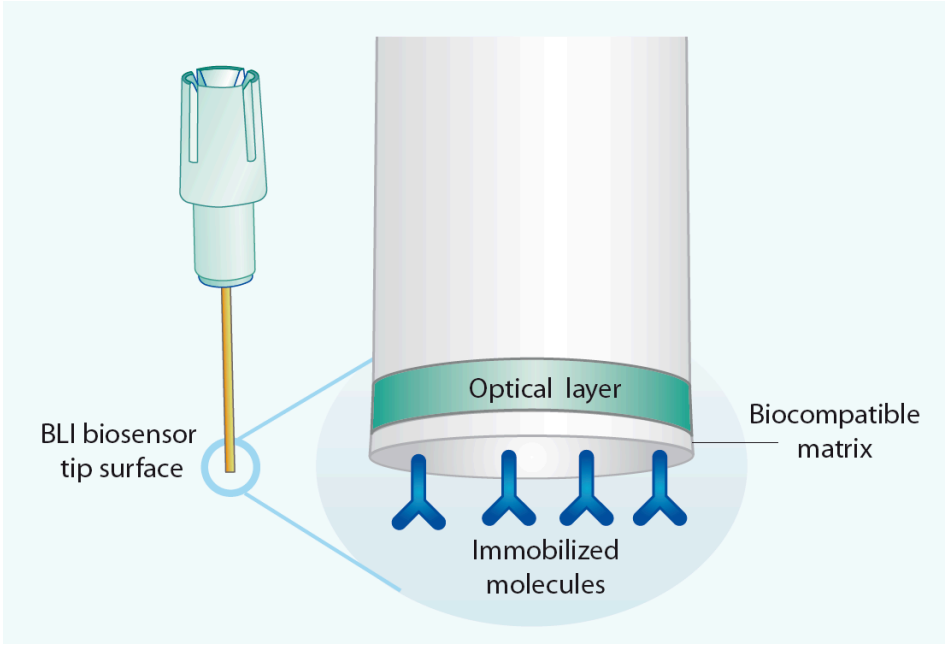
$$\Delta\varphi = 2\pi d/\lambda$$

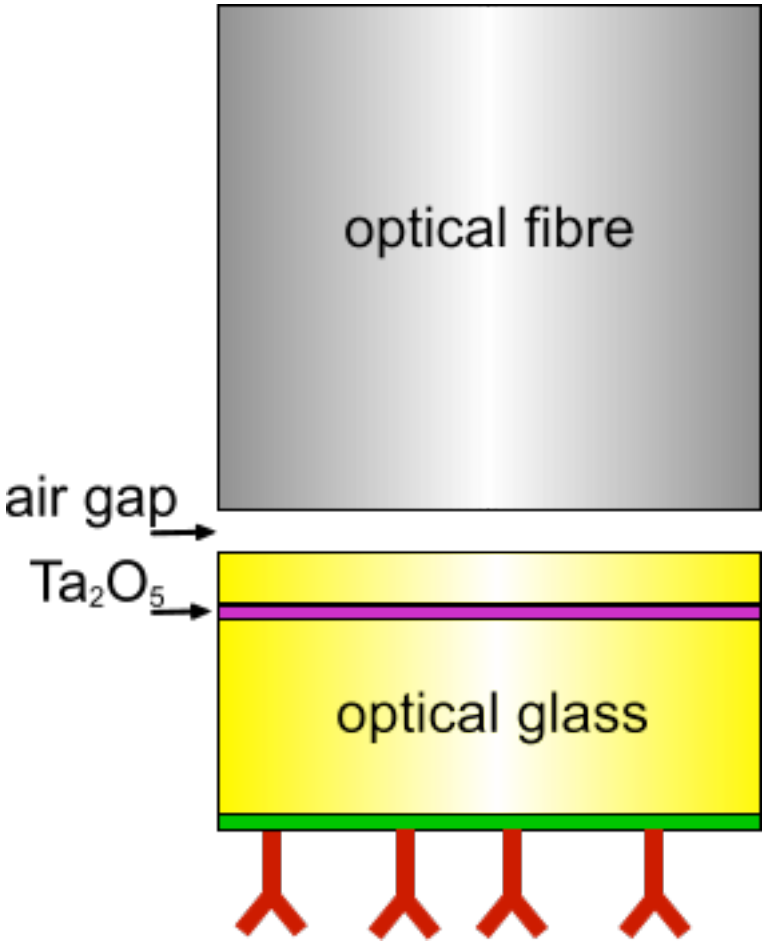
Optical path difference = distance

Phase difference

Sensor construction

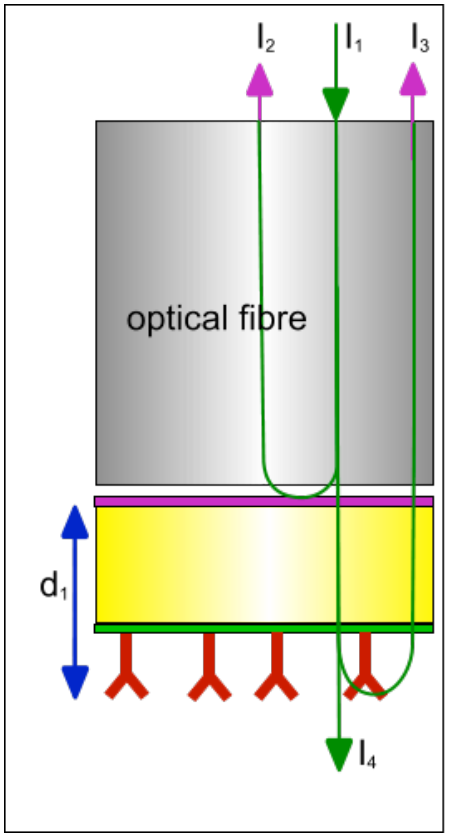


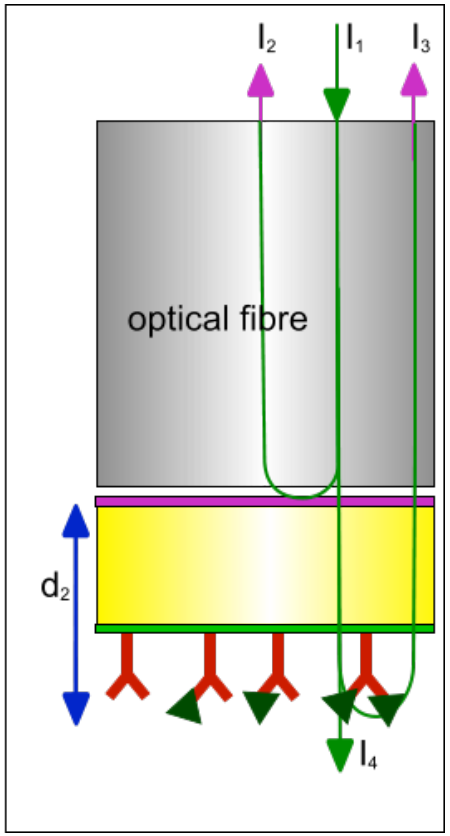




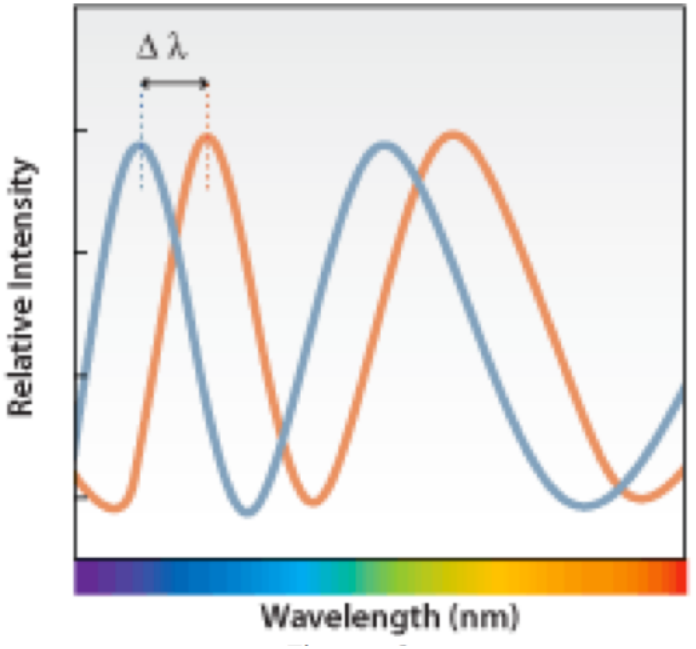
http://www.biapages.nl

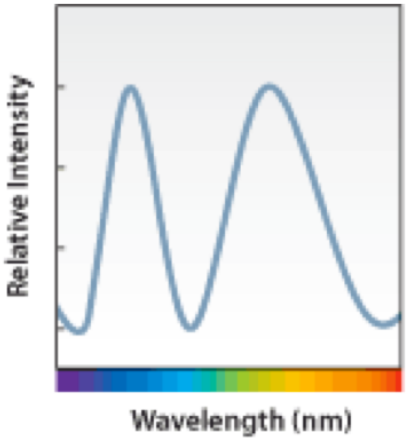
Bio Layer Interferometry

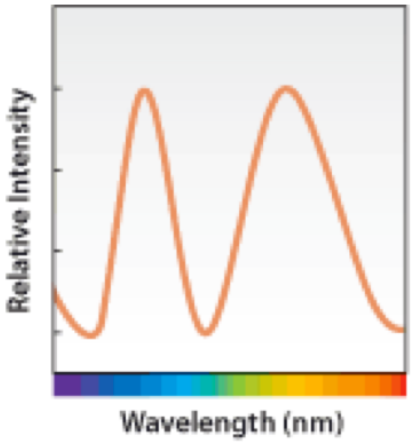




$$I = I_2 + I_3 + 2\sqrt{I_2\ I_3}\ \cos\left(\frac{2\pi\ \Delta}{\lambda}\right)$$



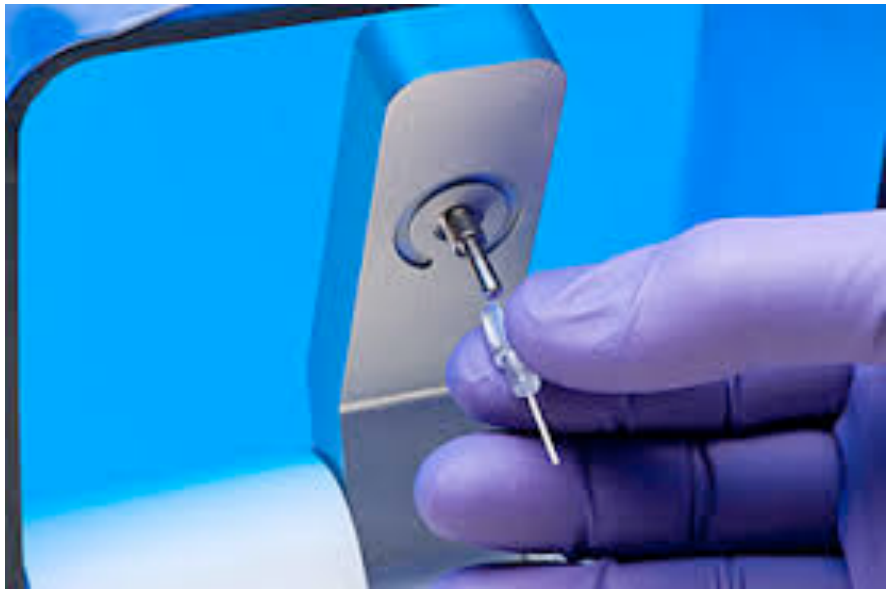


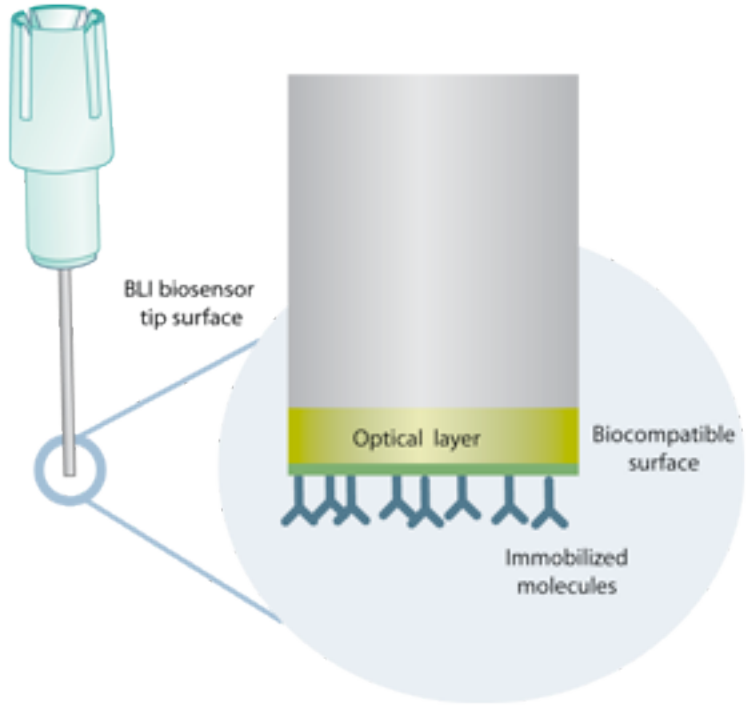


When molecules bind to the sensor the interferometric pattern shifts to the right and when molecules dissociate the interferometric pattern shifts to the left.

http://www.biapages.nl

Dip and Read™ Biosensors

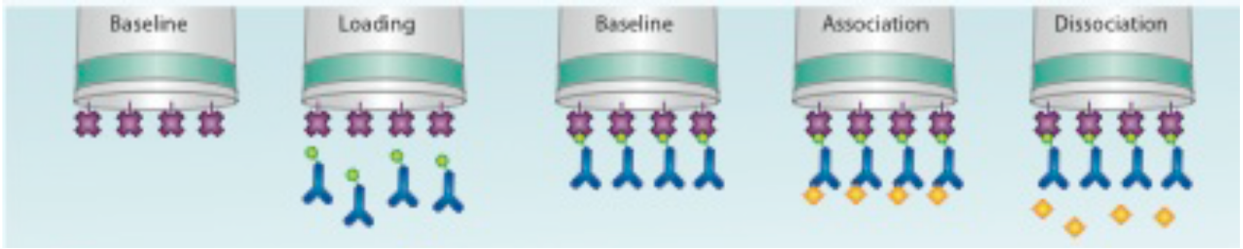


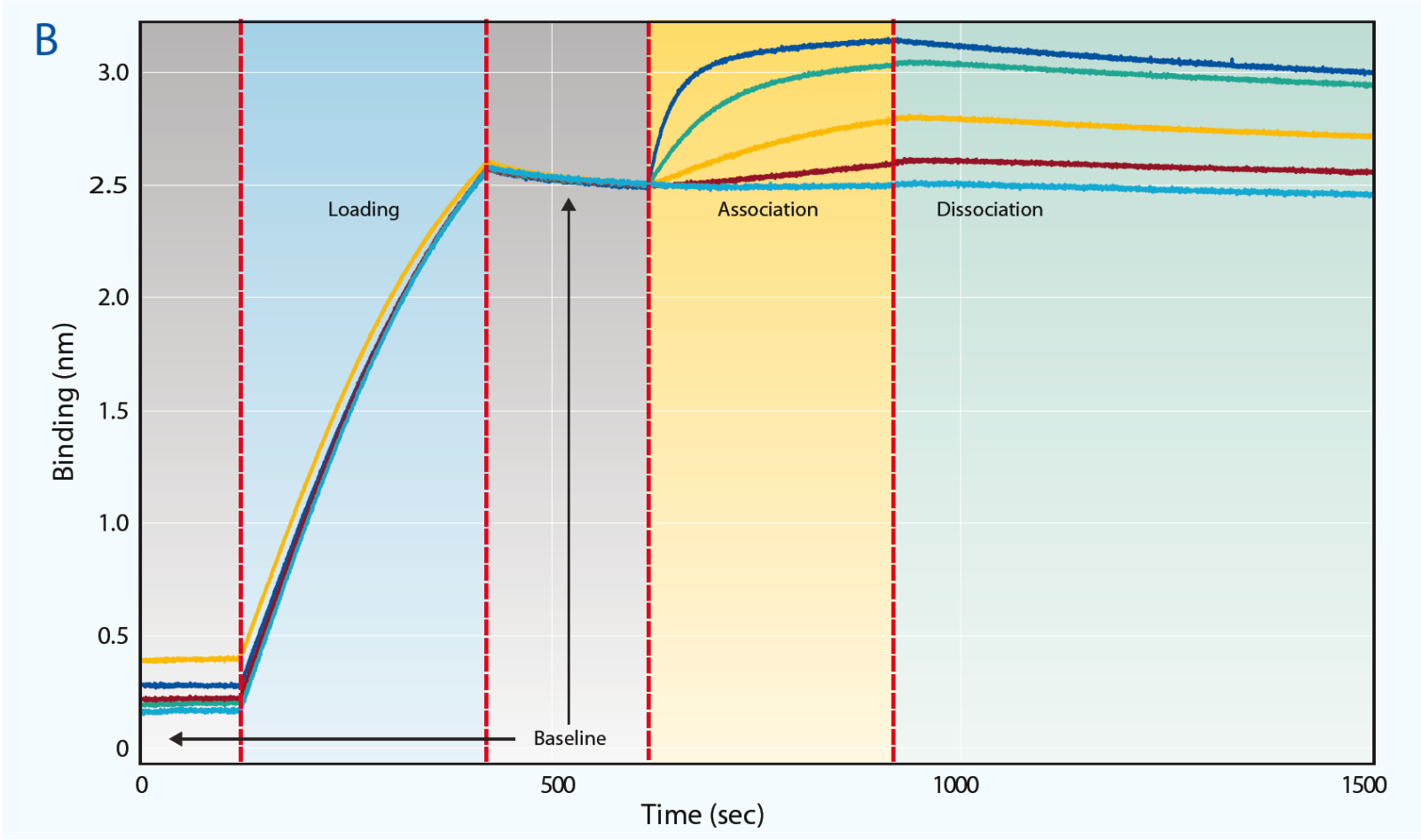


Direct immobilisation: amine coupling, biotin intercation with streptavidin

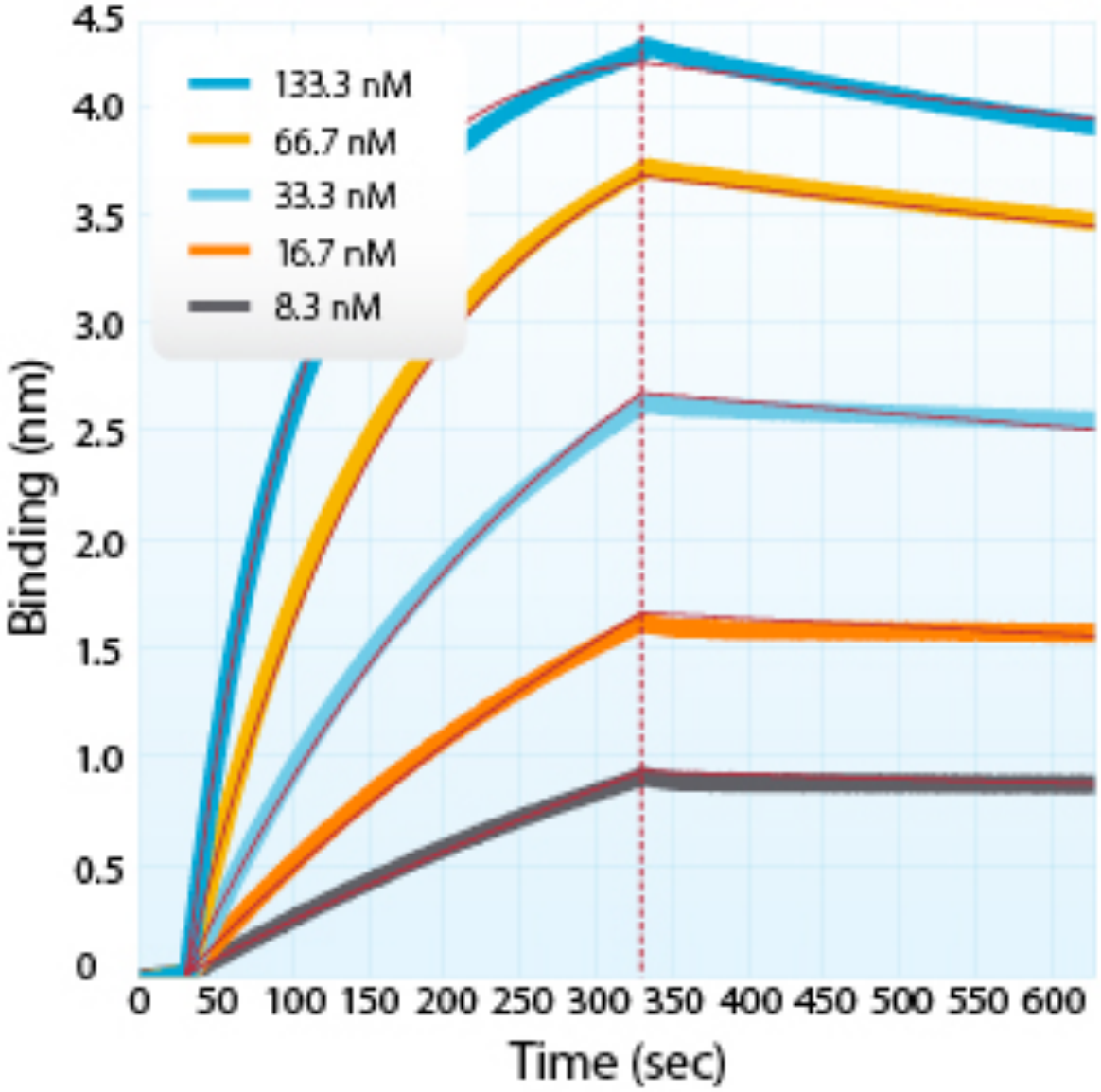
Capture-based approach: high affinity capture antibody (anti-FC, anti-His) or use of known motif or tag (protein A, Ni NTA)

Typical binding kinetic's experiment



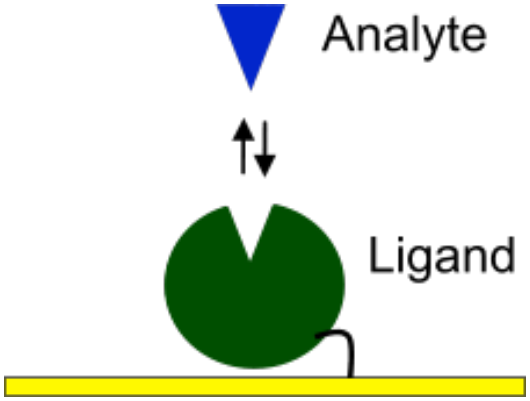


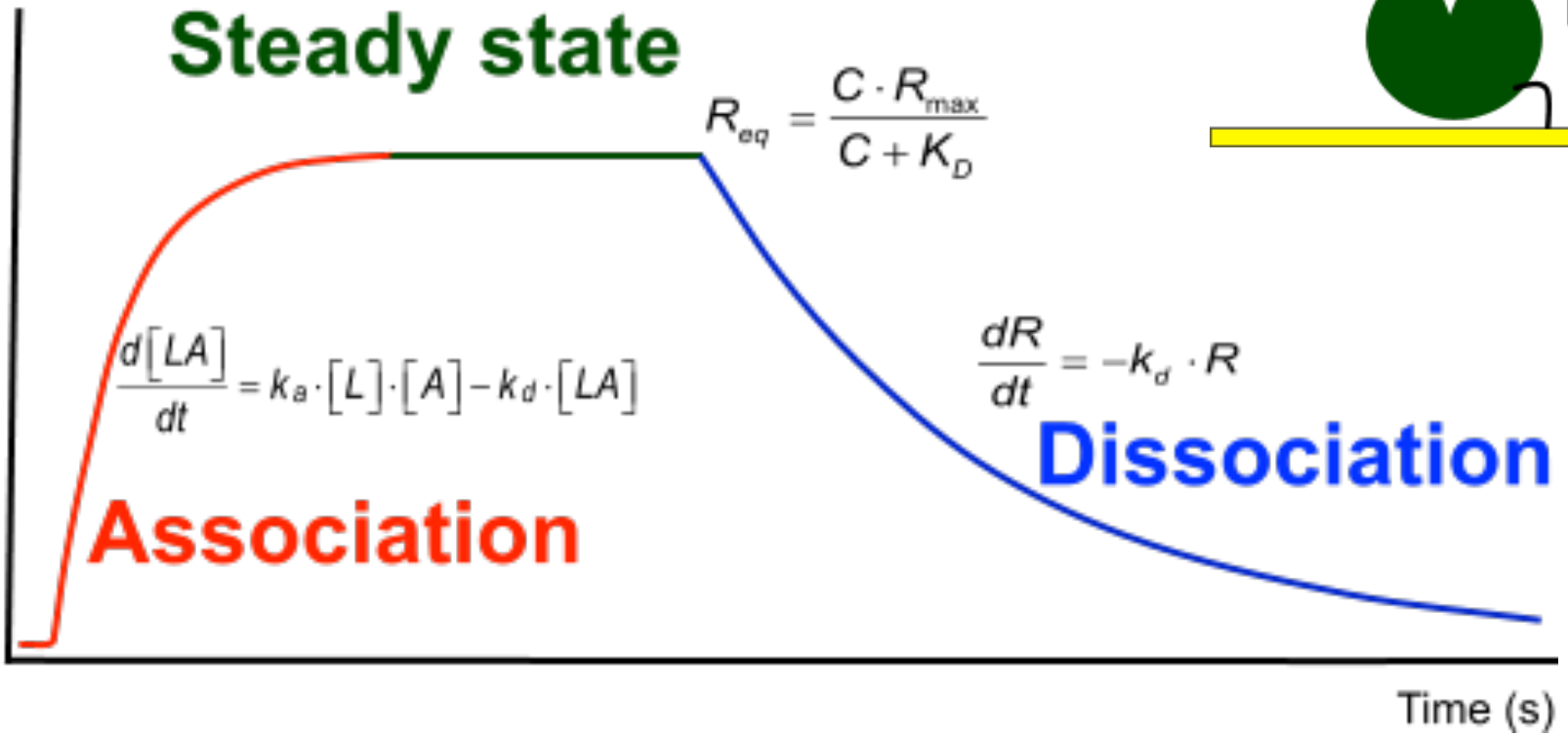
Example of binding



Data analysis

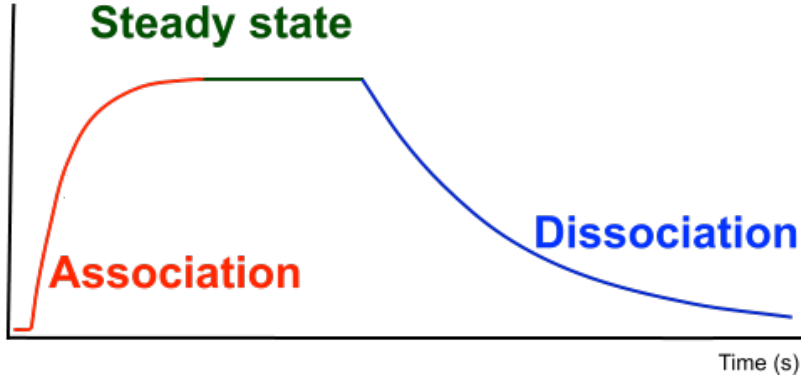
In the case of a simple 1:1 interaction





L = Ligand, A = Analyte, R = Response
k_a = association rate constant, k_d= dissociation rate constant
K_D = affinity constant

Data analysis: Association



Association rate equation

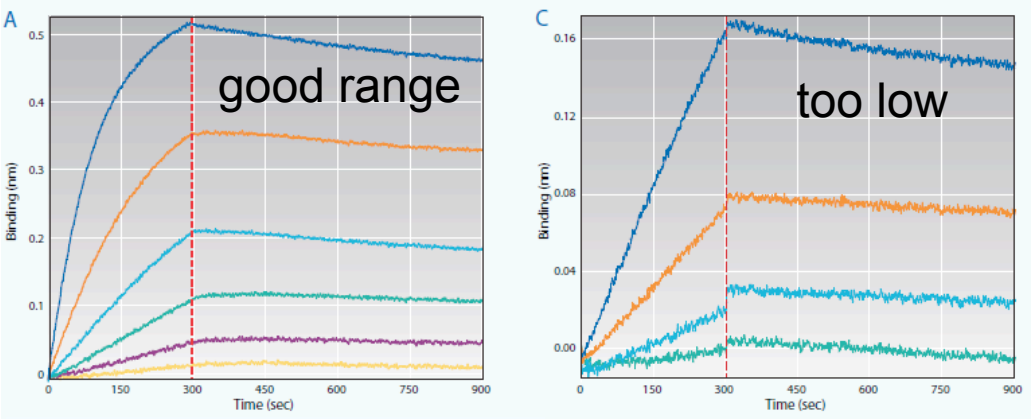
$$\frac{d[LA]}{dt} = k_a \cdot [L] \cdot [A] - k_d [LA]$$

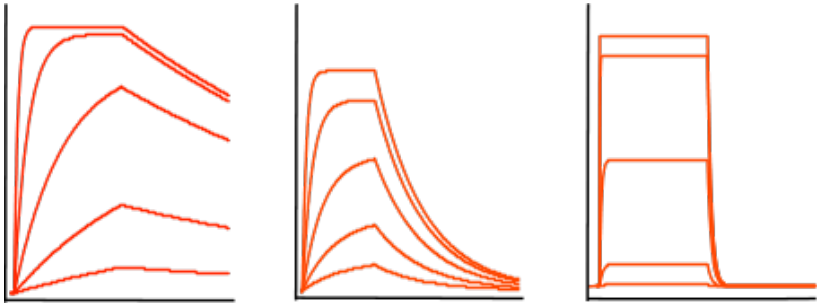
At the same time LA complex is forming, it also decays back to L and A.

$$Y = Y_0 + A(1 - e^{-k_{obs} * t})$$

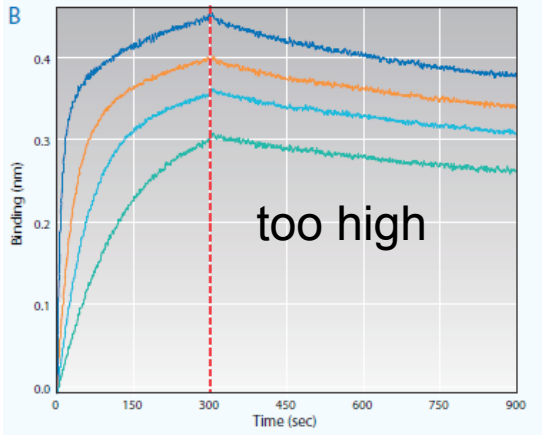
equation used to fit

Y = level of binding, A is an asymptote
k_{obs} is the observed rate constant.





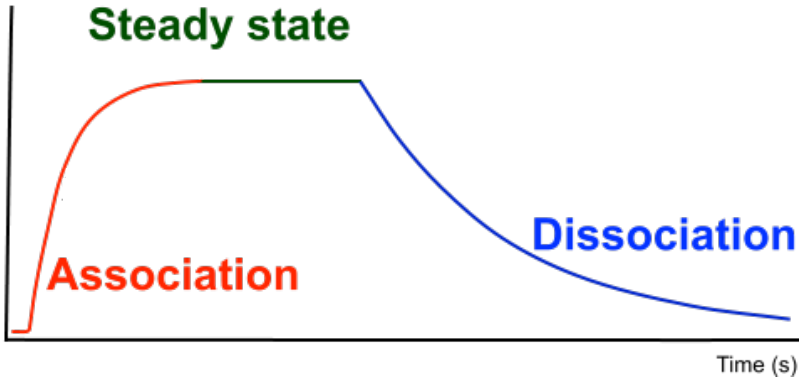
Simple exponentional curves.



Analyte concentration

$0.1\ K_D < [A] < 10\ K_D$

Data analysis: Dissociation



Dissociation rate equation

$$\frac{dR}{dt} = -k_d \cdot R$$

$$Y = Y_0 + Ae^{-k_d * t}$$

equation used to fit

Y = level of binding, A is an asymptote

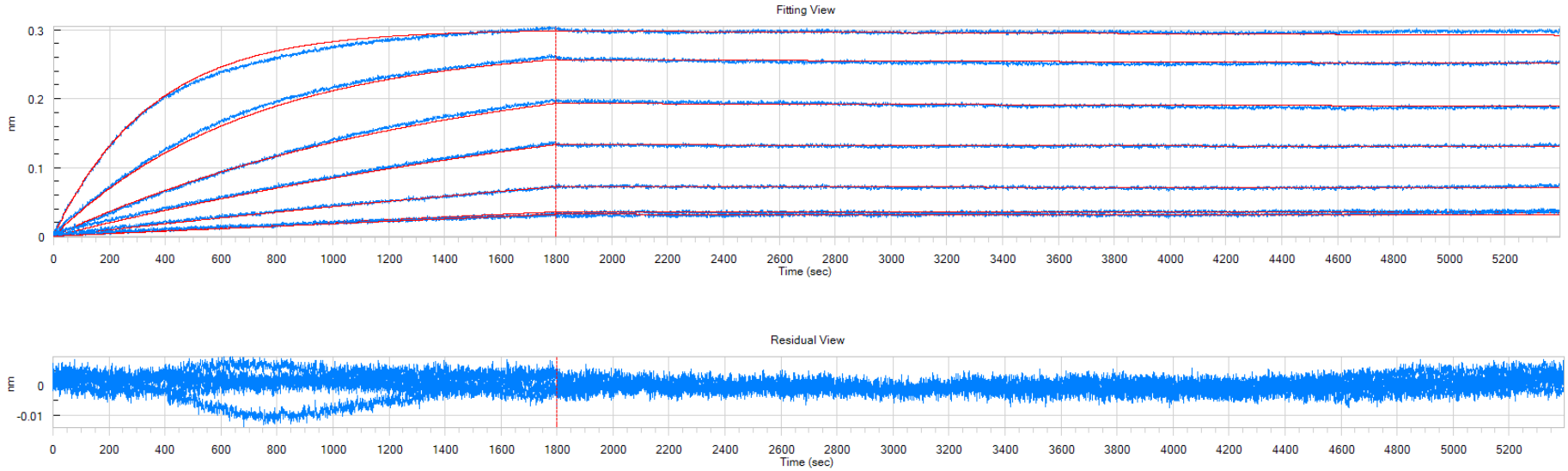
Time to 5% dissociation

k_d (s^{-1})	Time (min)	Time (hour)
$1.\ 10^{-1}$	0.0	0.0
$1.\ 10^{-2}$	0.1	0.0
$1.\ 10^{-3}$	0.9	0.0
$1.\ 10^{-4}$	8.5	0.1
$1.\ 10^{-5}$	85.5	1.4
$1.\ 10^{-6}$	854.9	14.2

The dissociation curve should decrease at least 5% before analysis is attempted.

Example of tight binding model 1:1

Ag concentration 0.23 nM to 15 nM Ag

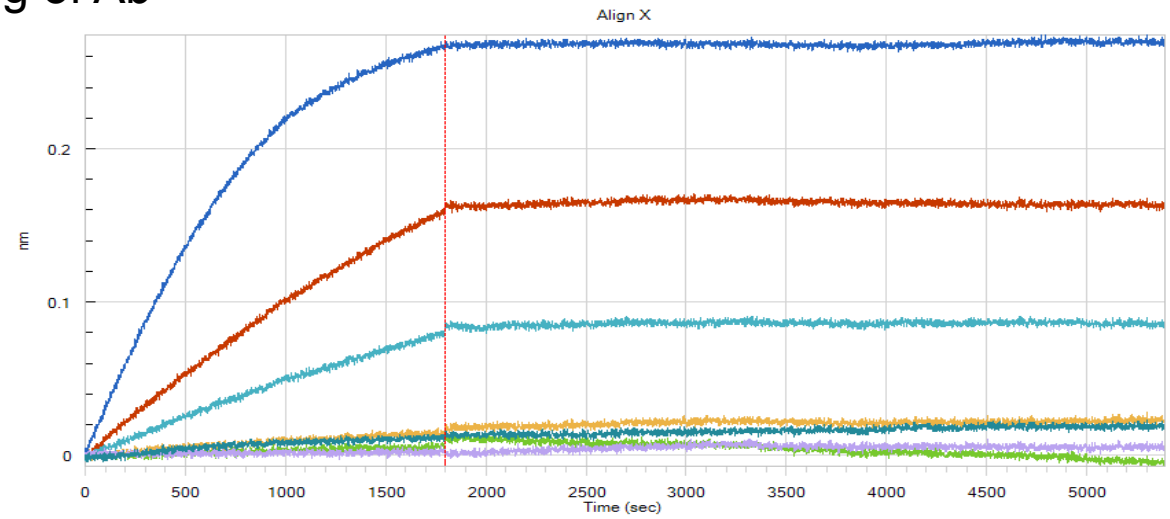


Dissoc. Loc.	Conc. (nM)	Response	KD (M)	KD Error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Rmax	Rmax Error	kobs(1/s)	Req	Req/Rmax(%)	Full X^2	Full R^2
A4	15	0.3026	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.3008	0.0001	2.847E-03	0.3002	99.8	0.271485	0.999259
B4	7.5	0.2608	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2792	0.0001	1.427E-03	0.2781	99.6	0.271485	0.999259
C4	3.75	0.1975	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2688	0.0002	7.163E-04	0.2666	99.2	0.271485	0.999259
D4	1.87	0.1355	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2839	0.0002	3.602E-04	0.2792	98.3	0.271485	0.999259
E4	0.93	0.0719	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2667	0.0003	1.821E-04	0.258	96.7	0.271485	0.999259
F4	0.46	0.0327	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.2451	0.0004	9.307E-05	0.2294	93.6	0.271485	0.999259
G4	0.23	0.0312	3.133E-11	<1.0E-12	1.894E05	1.823E02	5.935E-06	7.896E-08	0.4267	0.0007	4.950E-05	0.3755	88.0	0.271485	0.999259

KD (nM)	Kass($M^{-1}s^{-1}$)	Kdiss(s^{-1})
0.031	1.89×10^{5}	5.9×10^{-6}

Example of very tight binding model 1:1

loading of Ab



5 nM

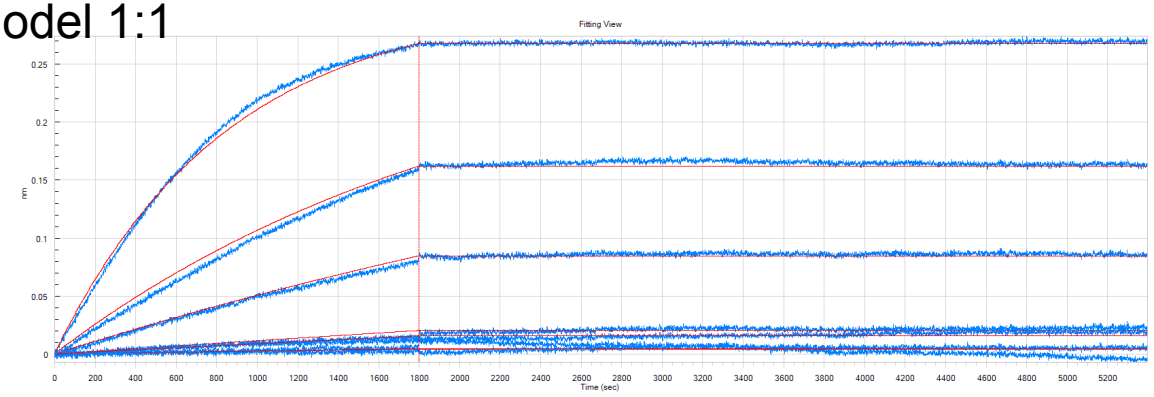
2 nM

0.8 nM

0.32 nM, 0.128 nM
0.051 nM, 0.02 nM

assoc 1800s
dissoc 3600s

Fit model 1:1

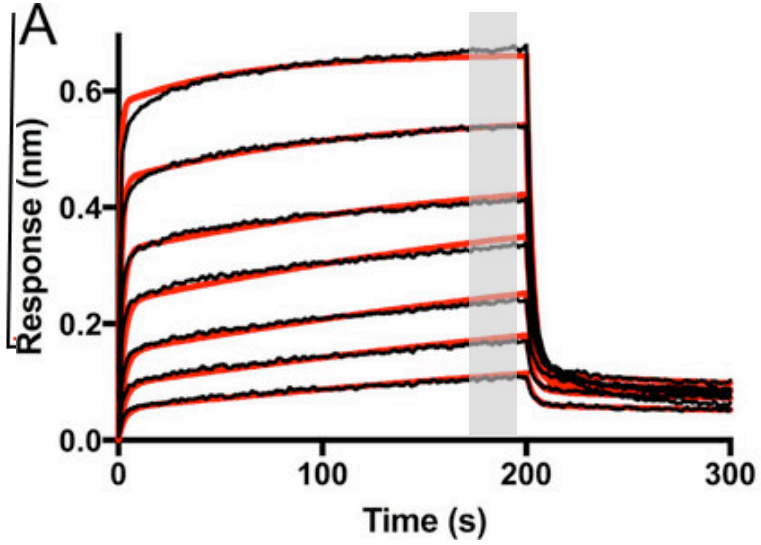


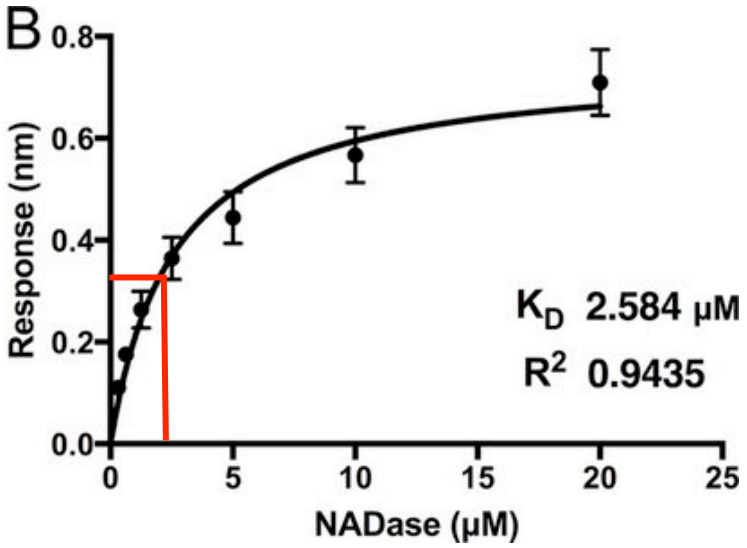
increase dissociation time ?

KD (nM)	Kass($M^{-1}s^{-1}$)	Kdiss(s^{-1})
< 0.001	2.35 x 10^5	< 10^{-7}

Octet RED96
10^2 – 10^7
10^{-6} – 10^{-1}
1 mM – 10 pM
10^2 – 10^7
10^{-6} – 10^{-1}
1 mM – 10 pM

Data analysis: Steady state





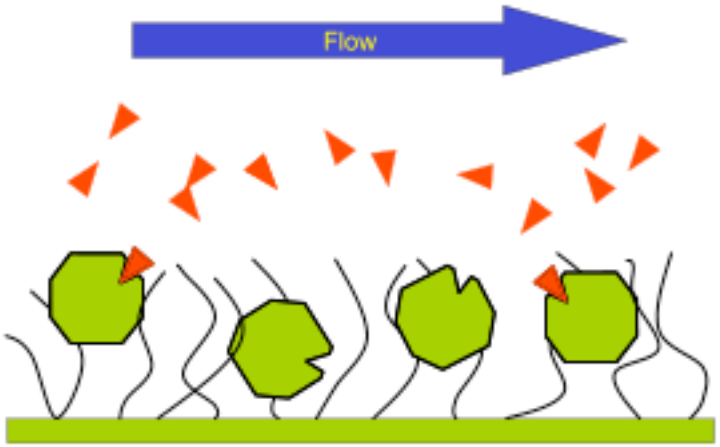
Steady state equation

$$\frac{k_d}{k_a} = \frac{[L][A]}{[LA]} = K_D$$

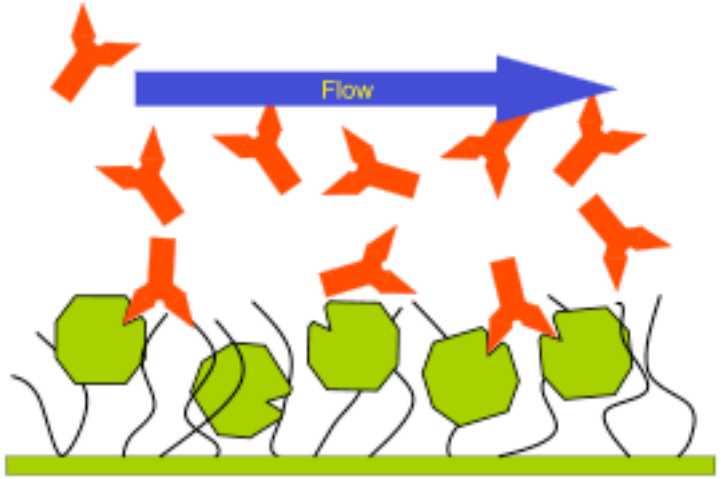
Approximate calculated time required to reach 99.9% steady state at analyte concentrations ranging from 0.01 to 100 times KD)

concentration analyte	k_d (s^{-1})			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
0.01 x K_D	68 s	11.5 min	115 min	1140 min
0.1 x K_D	63 s	10.5 min	105 min	1047 min
1 x K_D	34 s	5.8 min	57 min	576 min
10 x K_D	6 s	1 min	10.5 min	1105 min
100 x K_D	1 s	0.1 min	1 min	11 min
$k_a = 1.10^5$ $M^{-1}s^{-1}$				

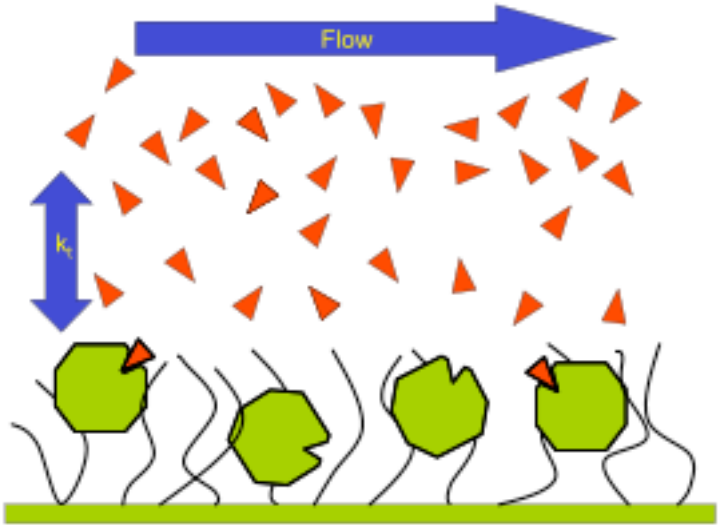
Data analysis: models



one to one

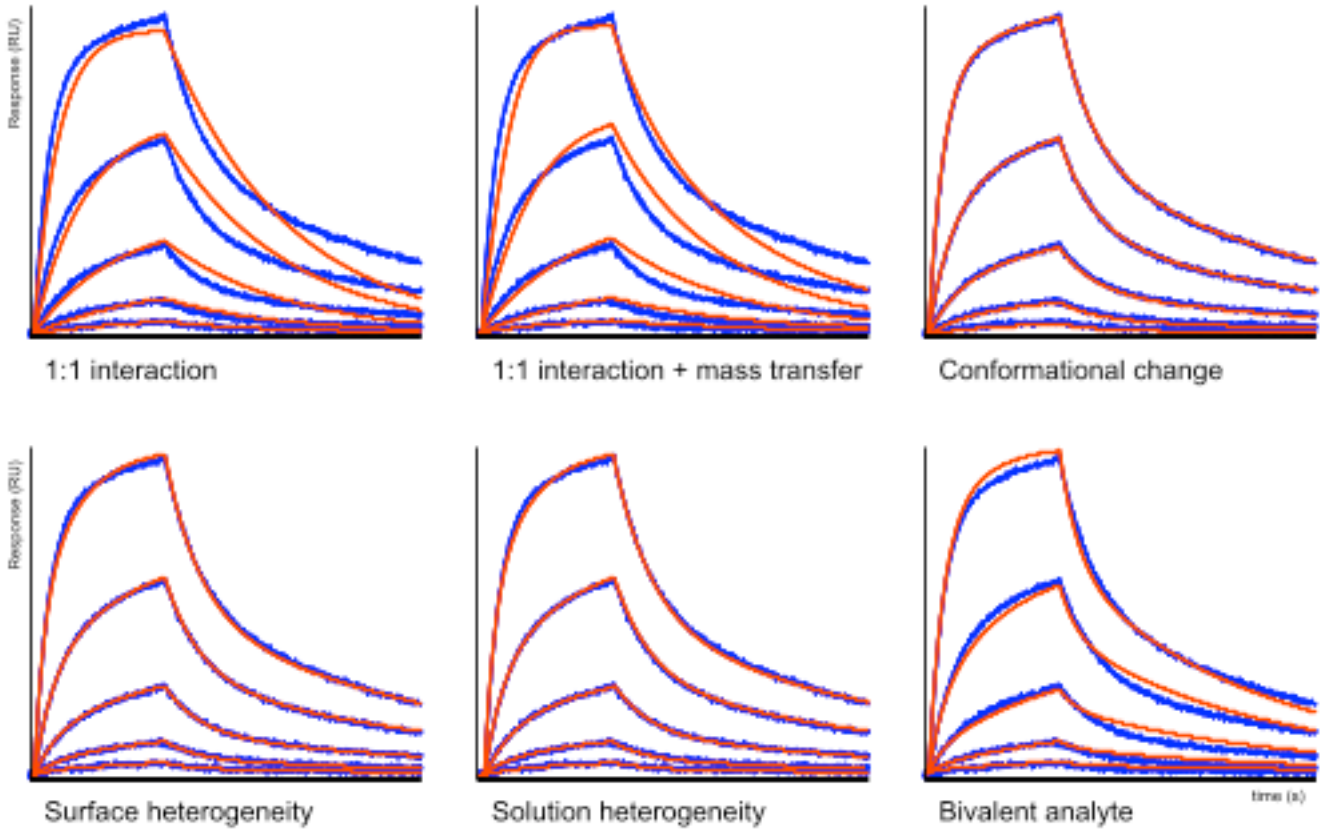


bivalent analyte



mass transfer

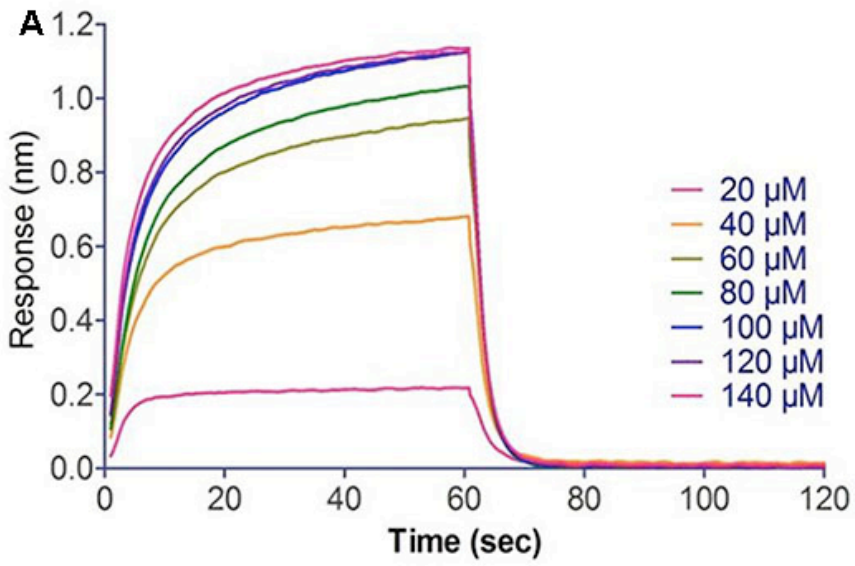
Data analysis: models

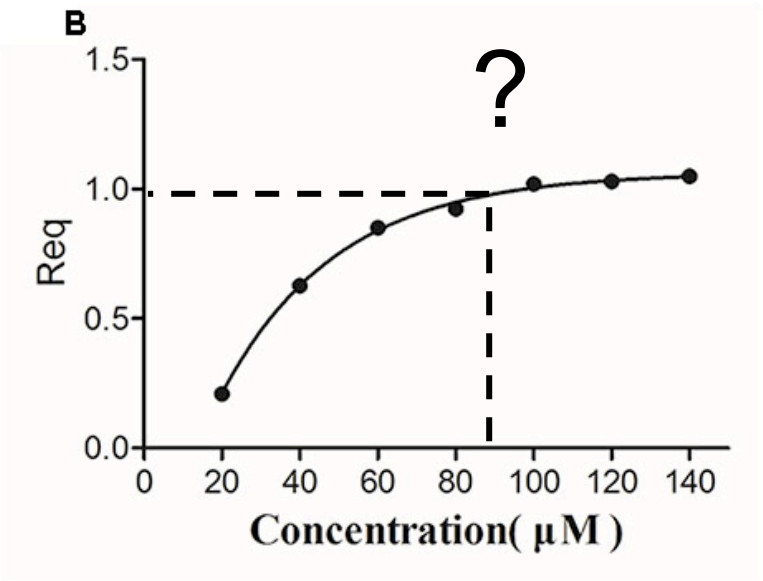


Model shopping is not the proper way to fit the data.

https://www.sprpages.nl/

Example from the literature





C

Kinetics of the interaction between KRAS and 0375-0604 by BLI

K_D (μM)	k_{on} (1/Ms)	k_{dis} (1/s)
92	$2.9\times10^{+03}$	2.6×10^{-01}

Xie et al, FrontPharma, 2017

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 ≈ 100k€ No maintenance	Cost ≈ 90k€ No maintenance	Cost ≈ 300k€ Expensive maintenance	Cost ≈ 200k€ 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

Review Article

Journal of Molecular Recognition

Received: 16 September 2008, Accepted: 17 September 2008, Published online in Wiley InterScience: 2008

(www.interscience.wiley.com) DOI:10.1002/jmr.928

Survey of the year 2007 commercial optical biosensor literature

Rebecca L. Rich[a] and David G. Myszka[a]*

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.