Biophysical techniques for HTS II

InnoCore IO2.4 HTS for Drug Discovery

Josef Houser
houser@mail.muni.cz

MUNI

CEITEC BIC

CEITEC MASARYK UNIVERSITY

Outline

- Selected biophysical techniques
  - Sufrace plasmon resonance
  - Bio-layer interferometry
  - Microscale thermophoresis
  - Isothermal titration calorimetry

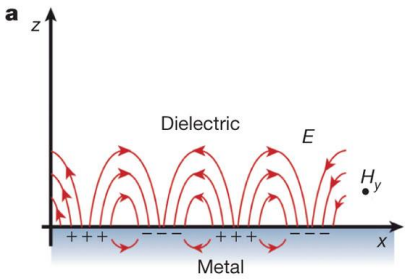
- High-throughput and biophysical techniques

Surface plasmon resonance (SPR)

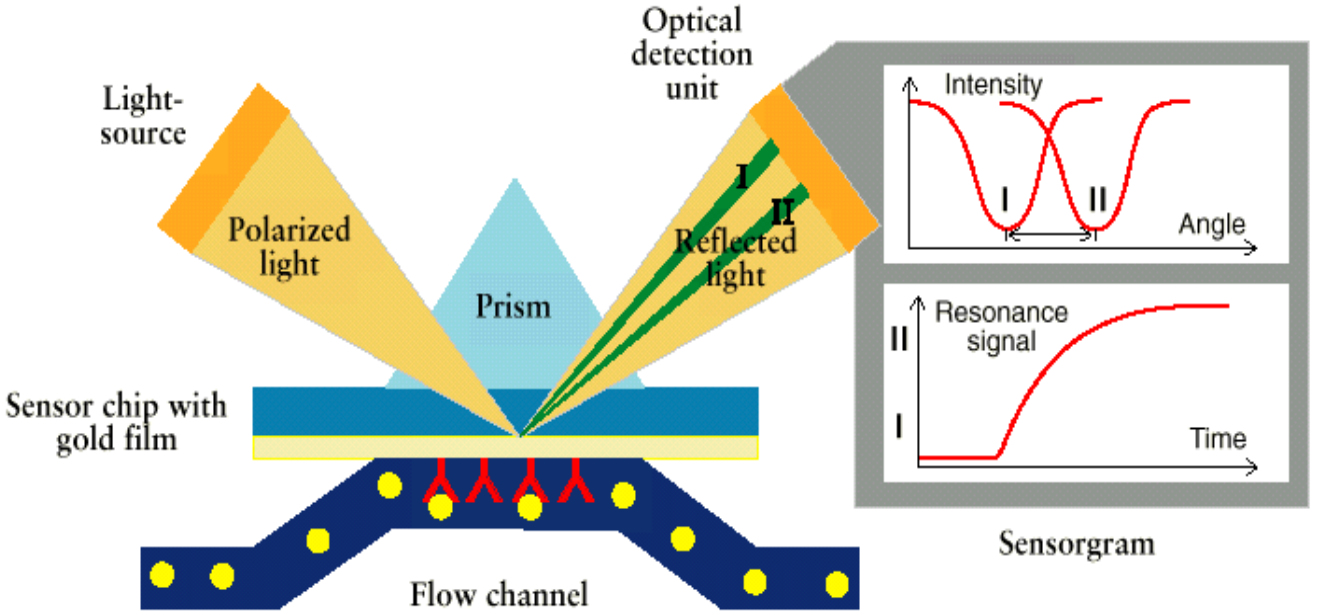
Bio-layer Interferometry (BLI)

SPR – Basic principles

One binding partner immobilized on chip surface (ligand), second partner is free in solution (analyte).



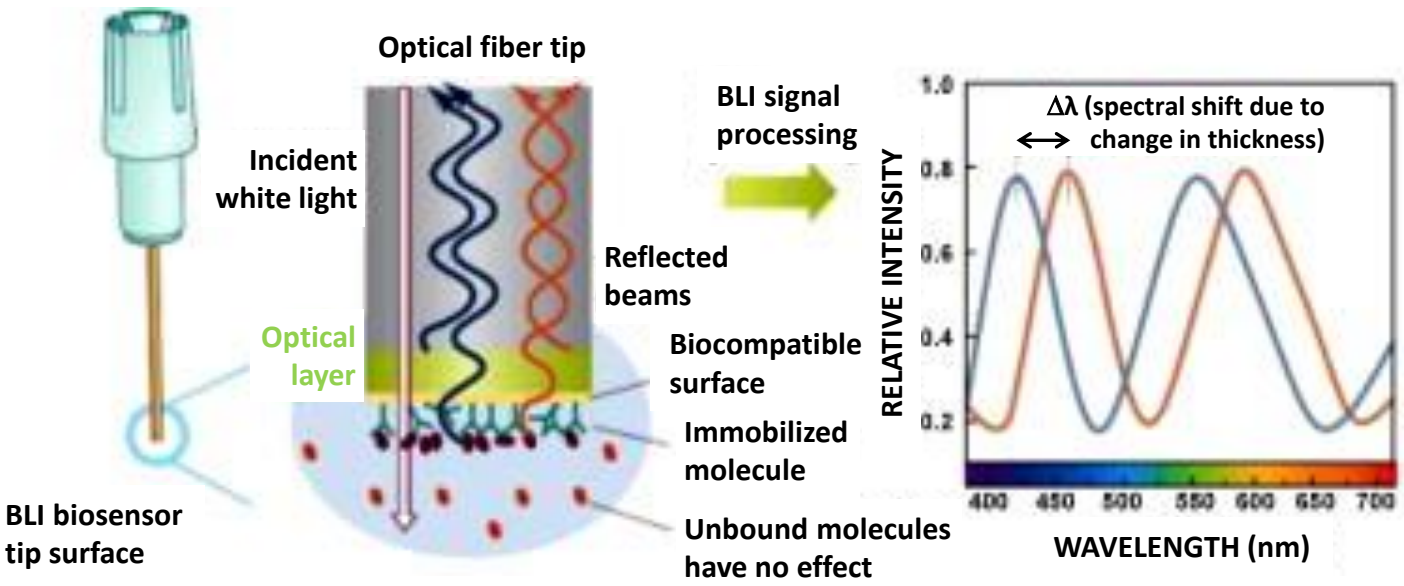
Plasmon – collective oscillation of electrons at surface



- At certain combination of incident angle and wavelength the free electrons on the metal surface are excited -> decrease in reflected light intensity.
- This effect depends on **refractive index** that varies with the **analyte binding** to the surface-bound ligand.

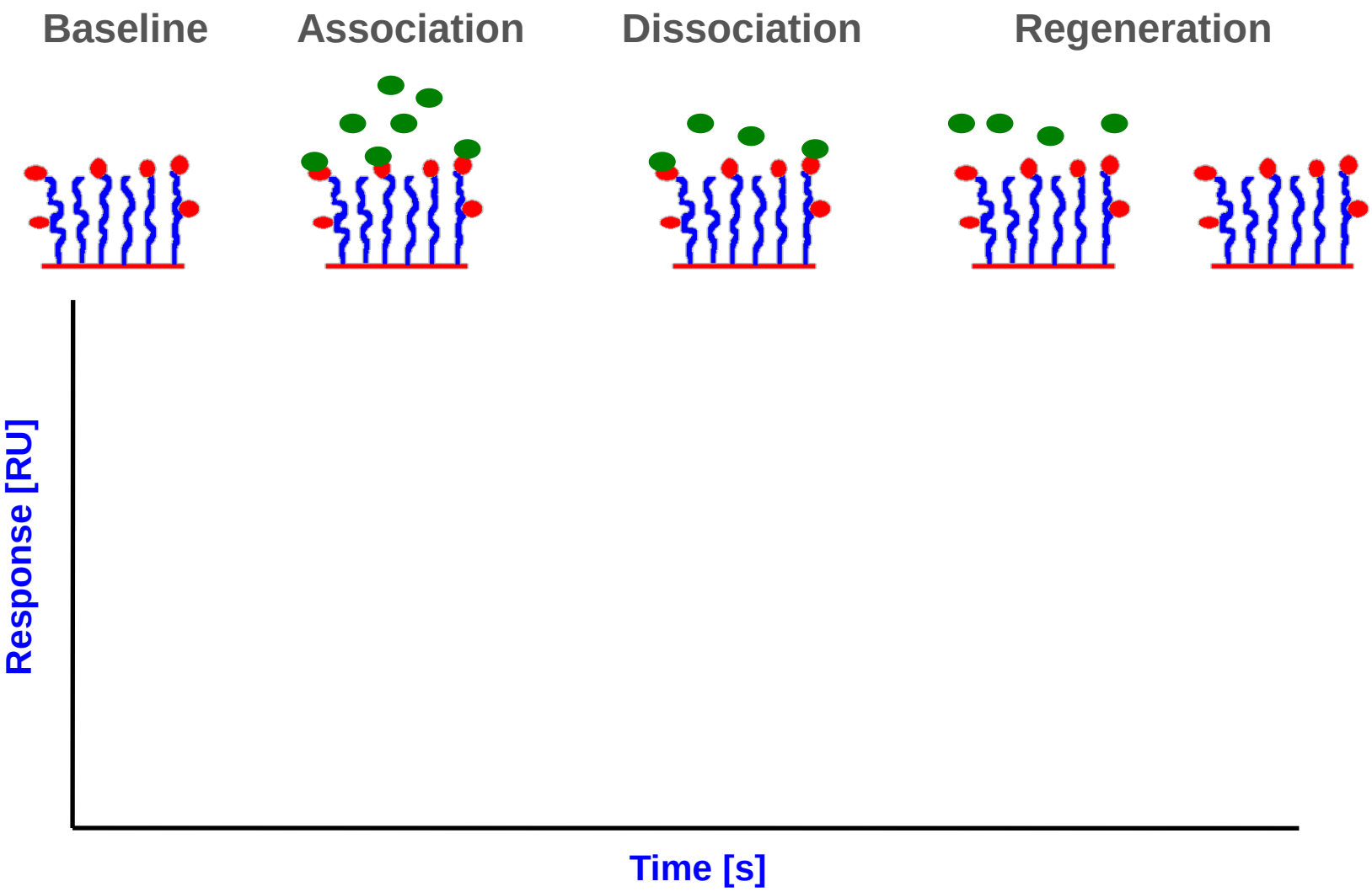
BLI – Basic principles

One binding partner immobilized on sensor surface (ligand), second partner is free in solution (analyte).



- Light reflects from the inner tip surface and outer tip surface resulting in formation of **interference pattern**.
- Binding of analyte on the sensor tip results in **change of the thickness** of the optical layer -> shift in the interference pattern.

Simple binding



Individual phases of experiment

$$v_{(association)} = k_a * [analyte]_{(solution)}$$
$$v_{(dissociation)} = k_d * [analyte]_{(bound)}$$

$[analyte]_{(solution)} >> [analyte]_{(bound)}$

$v_{(association)} >> v_{(dissociation)}$ **association phase**

$v_{(association)} = v_{(dissociation)}$ **steady state**

-> response is proportional to K_D and R_{max}

$[analyte]_{(solution)} << [analyte]_{(bound)}$

$v_{(association)} << v_{(dissociation)}$ **dissociation phase**

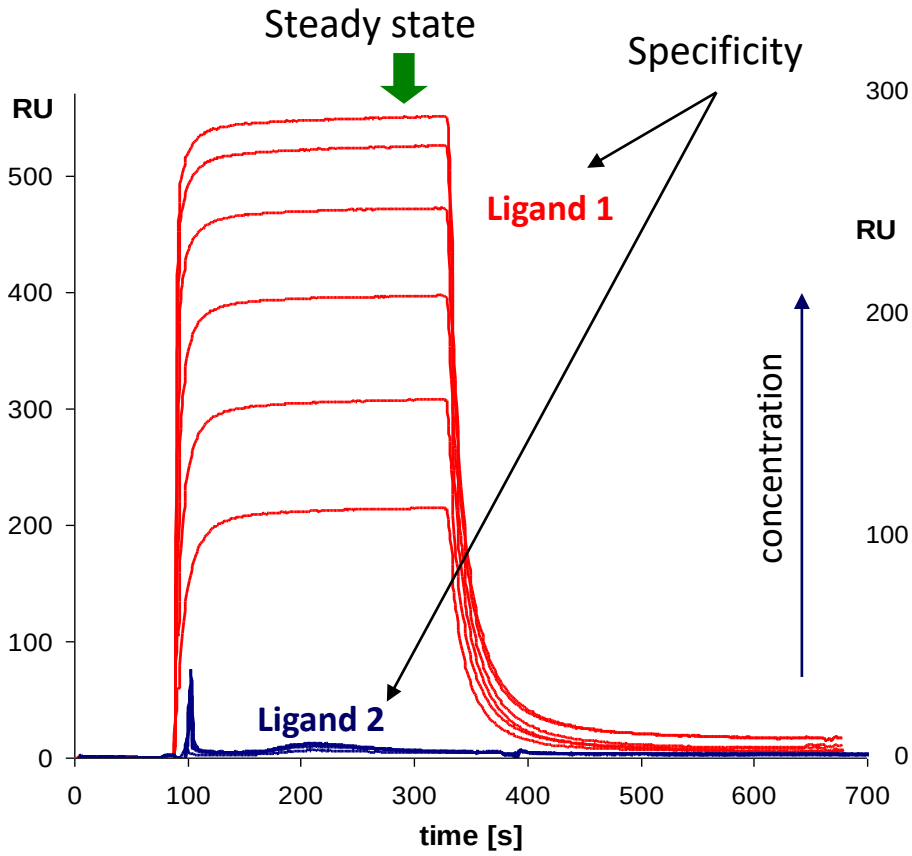
Fast vs. slow binding

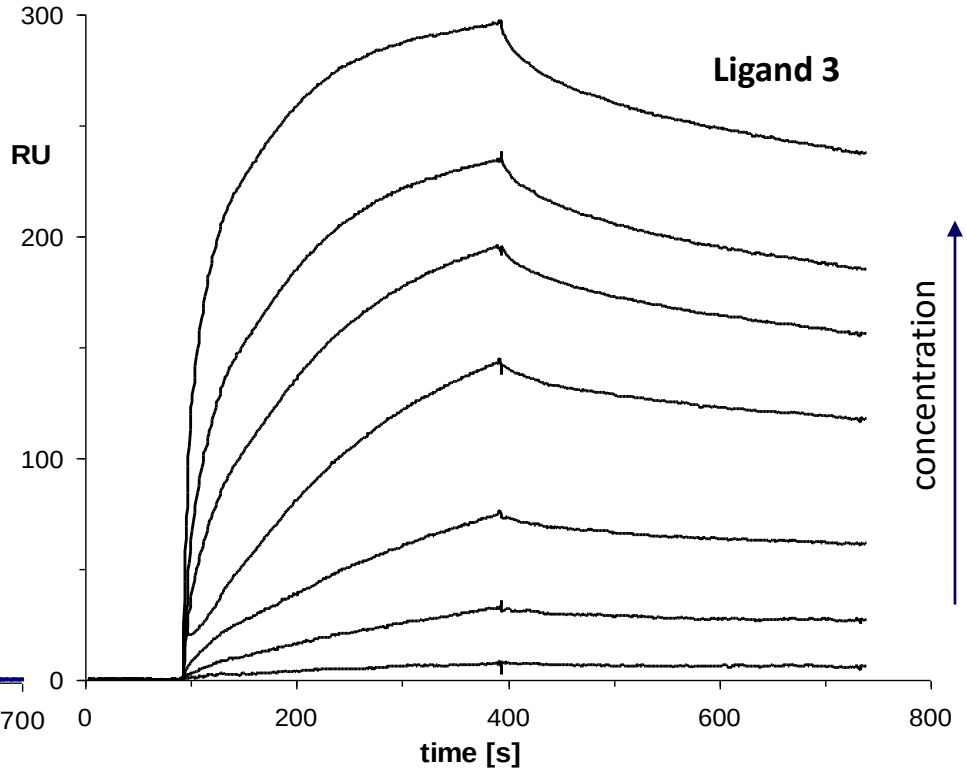
Fast complex association and dissociation

Fast equilibrium ⇨ K_A, K_D

Slow complex association and dissociation

Kinetic constants k_a, k_d ⇨ K_A, K_D

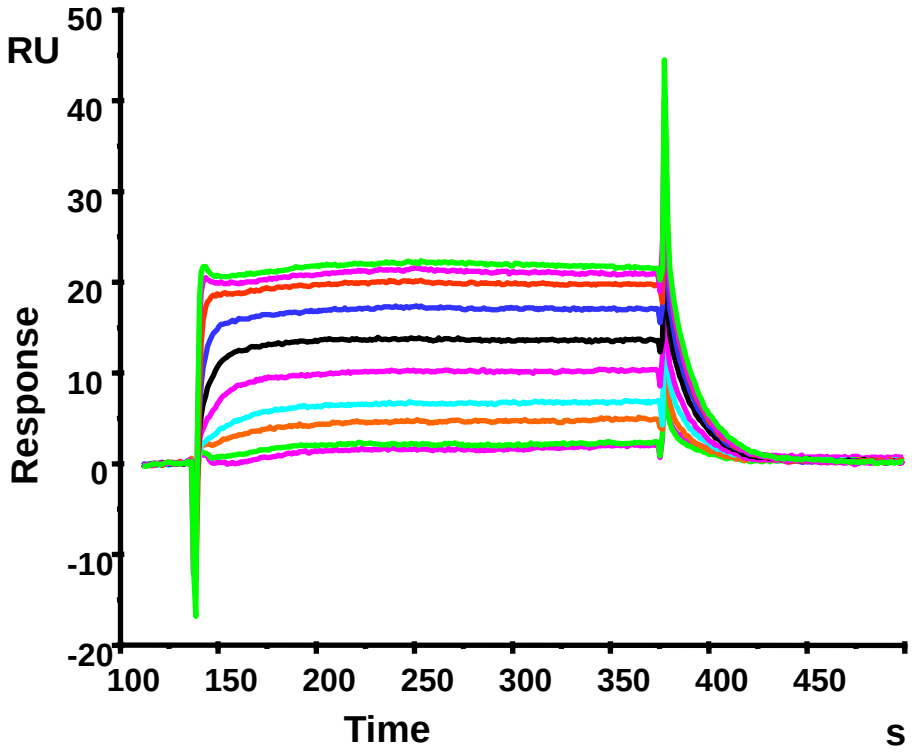


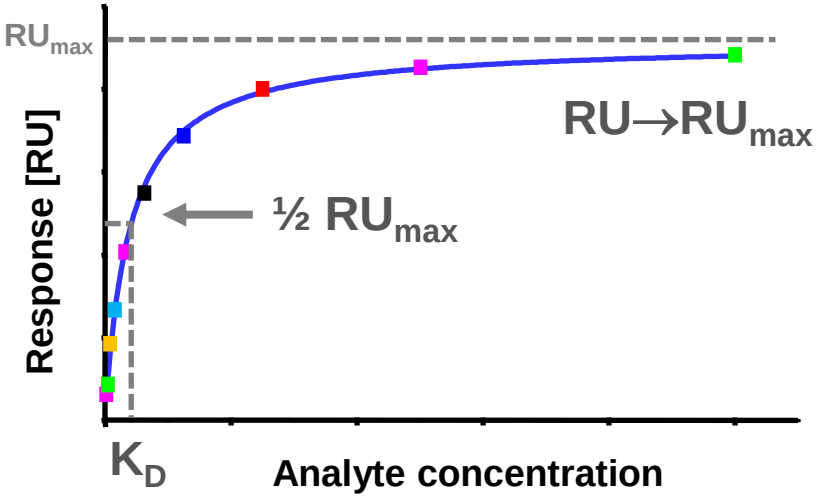


Direct binding assay

Fast association and dissociation

K_A, K_D





$$K_D = \frac{[\text{Protein}]\ [\text{Analyte}]}{[\text{Protein-Analyte}]}$$

$RU = ½\ RU_{max}$

↓

$[\text{Protein}] = [\text{Protein-Analyte}]$

↓

$K_D = [\text{Analyte}]$

Factors influencing binding and response

- **Density** of the molecules **on chip**
- **Concentration** of molecules **in solution**
- **Strength of interaction** between both molecules
- Total **mass** of interacting partner (for SPR)
- Portion of **active molecules** present –
  proper sample characterization needed, changes upon immobilization – site accessibility restriction, conformational changes, intermolecular distance

Which binding partner to immobilize?

- Stability – minutes-hours-days-months
- Availability
- Molecular mass

$$Response_{max} = Response_{ligand} \times \frac{M_r(analyte)}{M_r(ligand)} \times \frac{\left(\frac{\partial n}{\partial c}\right)_{analyte}}{\left(\frac{\partial n}{\partial c}\right)_{ligand}}$$

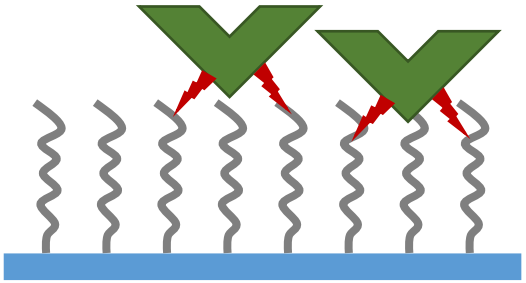
- Immobilization technique
- Multivalency – avidity effect

Immobilization techniques

High flexibility in creating biospecific surfaces

Direct covalent coupling

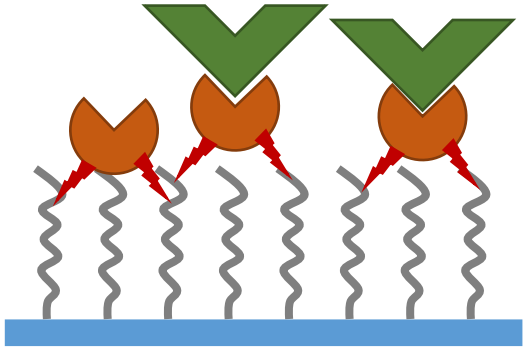
- Stable
- Suitable regeneration needed



- Amine (Lys, N-term)
- Thiol (Cys)
- Aldehyde
- Carboxyl

Capture

- Multi-step process
- Less stable binding
- Easier regeneration (not for SA)



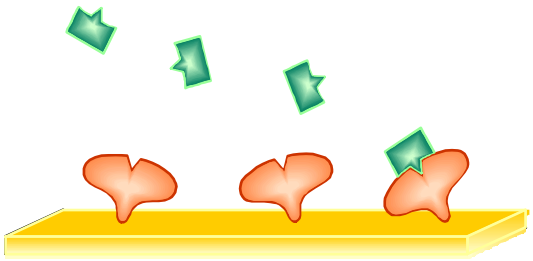
- Streptavidin – Biotin
- NTA-Ni^{2+} – His_6
- Anti-His_6 – His_6
- ProteinA – mAb
- Anti-GST – GST

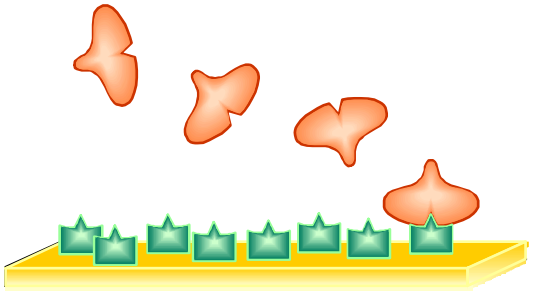
Flexibility in Assay Design

Multiple assay formats providing complementary data

Direct measurement

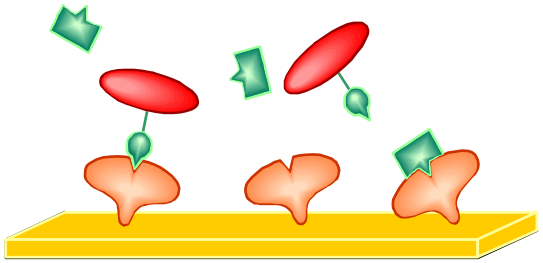
Direct Binding Assay (DBA)



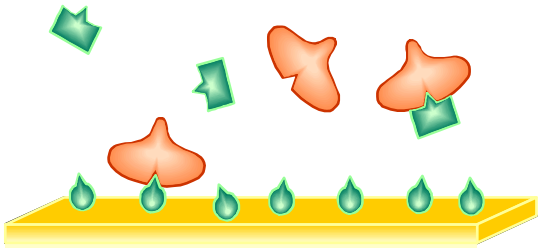


Indirect measurement

Surface competition assay (SCA)



Inhibition in solution assay (ISA)

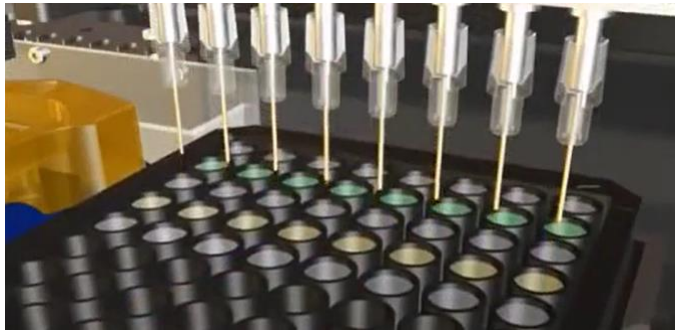


Two channels necessary – reference

- “Non-interacting” surface serves as a blank
- Elimination of non-specific interactions
- Enhancement of weak interaction resolution

- **Possible reference surfaces**:
  - Unmodified surface – gold, dextran layer,…
  - Activated and blocked surface without immobilized ligand/protein
  - Inactivated/non-functional protein

Multichannel set-up

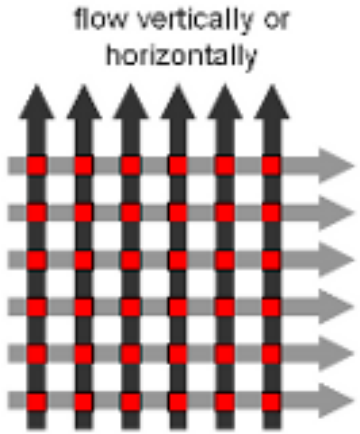


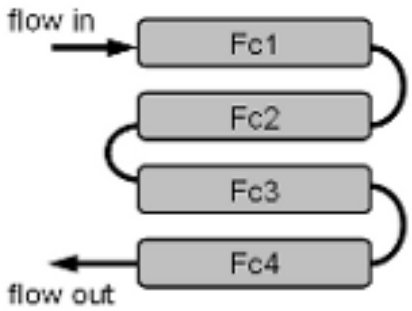
gfycat.com

- One or more references
- Multiple channels (SPR) – 2, 4, 6, 36, …
- Multiple detection spots
- Multiple sensors (BLI) – 2, 8, 16, ...

➢High throughput

➢Parallel reference





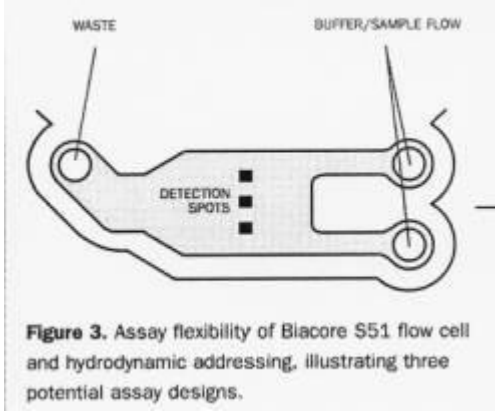


Figure 3. Assay flexibility of Biacore S51 flow cell and hydrodynamic addressing, illustrating three potential assay designs.

Specialized techniques

- **Membrane** proteins
- **Multi-layer** approaches – antibodies, protein complexes
- **Whole cell** immobilization
- **Thermodynamics** measured by SPR
- Ligand **recovery** – coupling to MS

Main SPR biosensors

- *GE Healtcare* – Biacore T200, Biacore 4000, Biacore 3000, etc.
- *Reichert* – SR7000DC
- *BioRad* – ProteOn™ XPR36
- *Biosensing Instrument* – Bi4000, Bi3000, etc.
- *Nicoya* – OpenSPR

Biacore T200

OpenSPR

SR7000DC

Bi4000

ProteOn™ XPR36

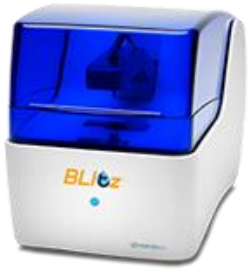
Main BLI biosensors

- *Fortebio* – Octet HTX, Octet 384, Octet RED96e, BLItz, etc.
- *Gator Bio* – Gator

Gator

Octet HTX

Octet RED96e

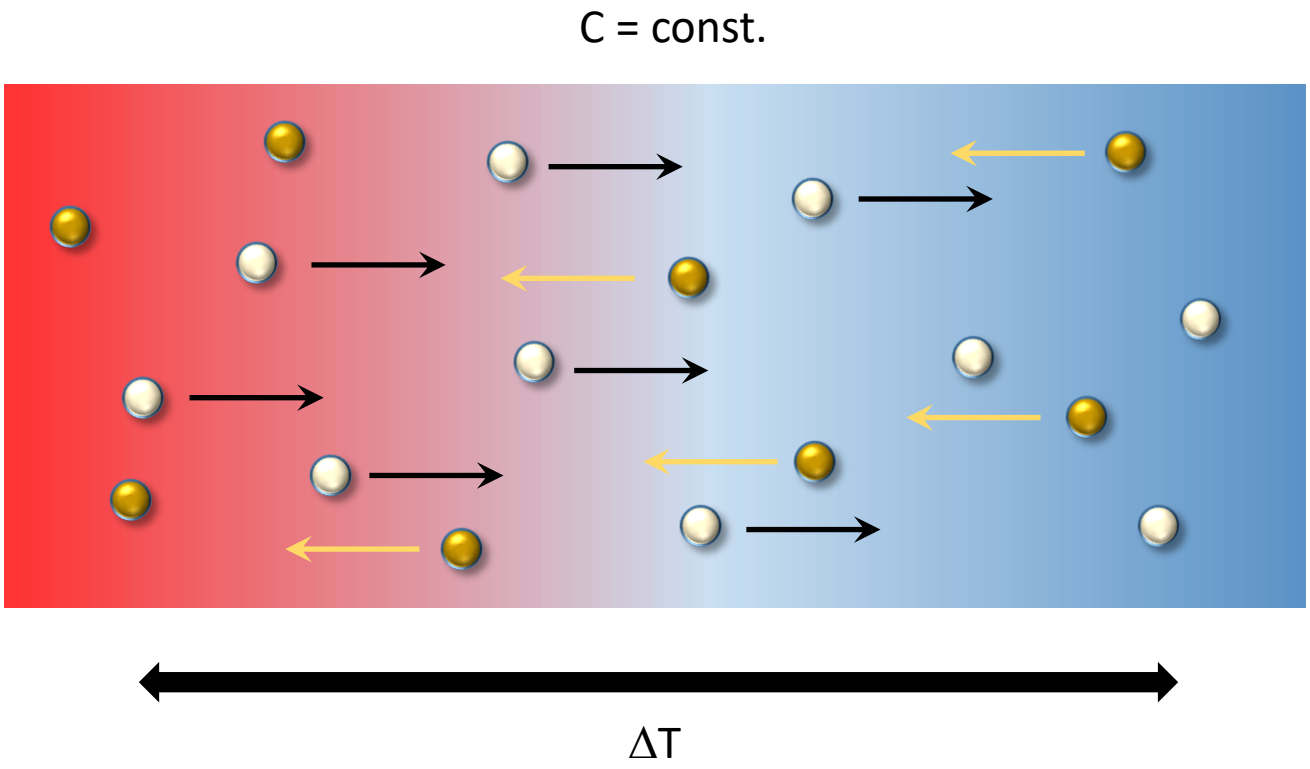


BLItz

Microscale Thermophoresis (MST)

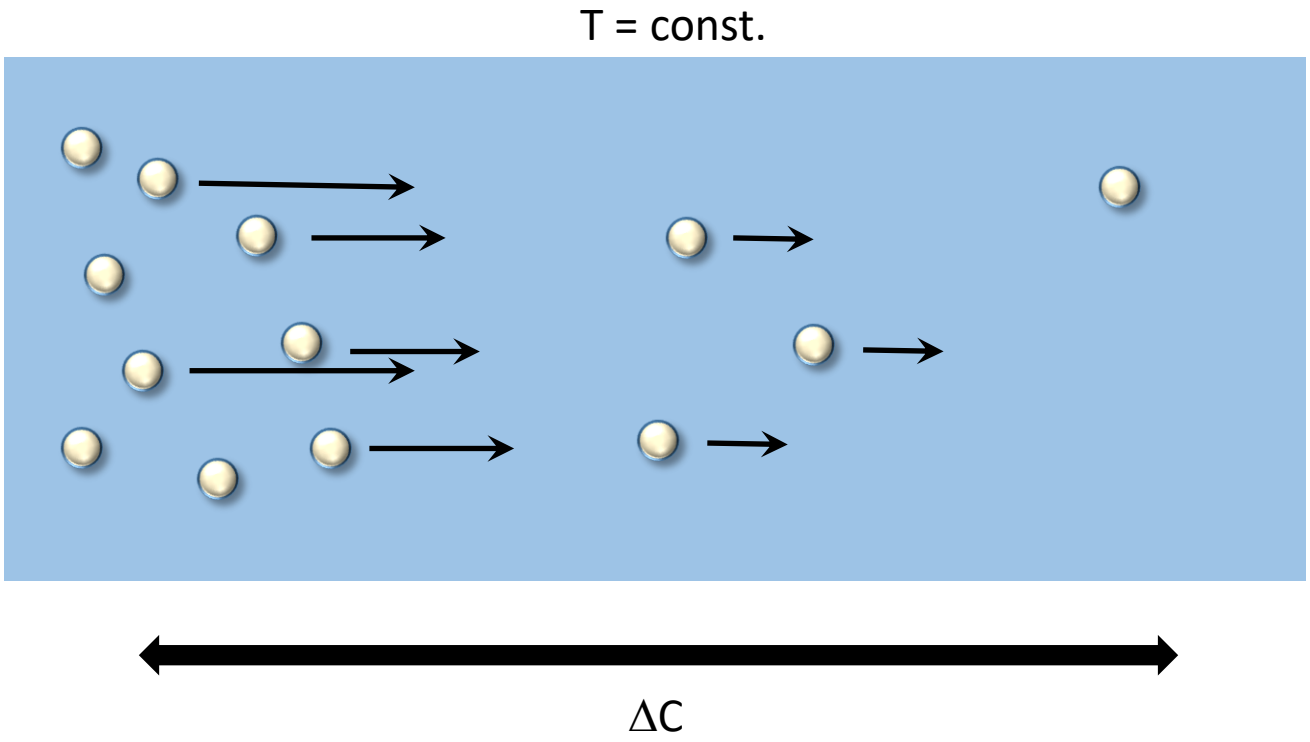
Microscale thermoforesis (MST)

= movement of particles in temperature gradient

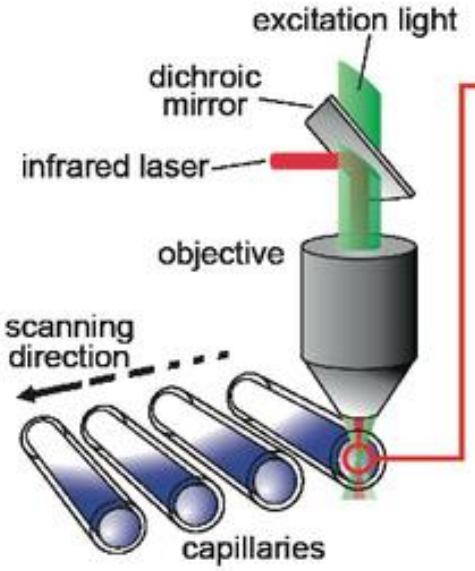


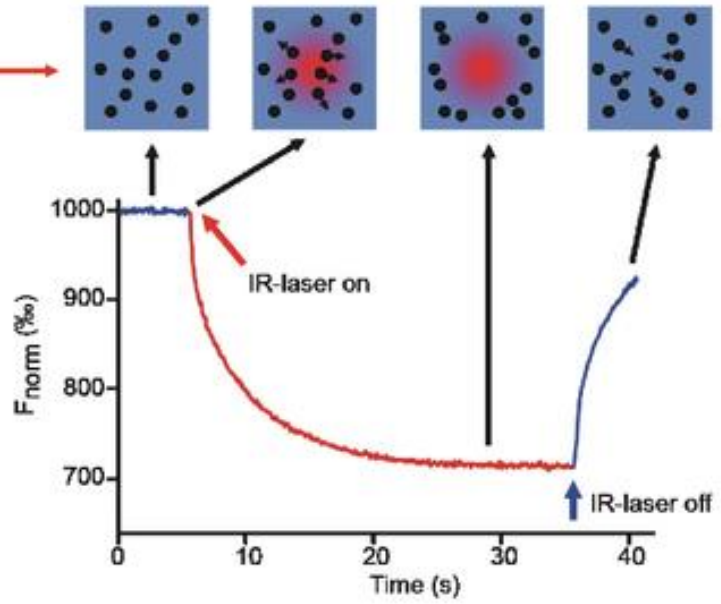
Diffusion

= movement of particles in concentration gradient



MST – Basic principles





A bit of theory…

$c_{hot}/c_{cold} = \exp(-S_T \Delta T)$

Soret coefficient S_T is defined as

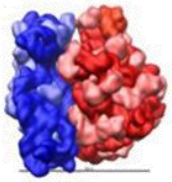
$$S_T = \frac{D_T}{D}$$

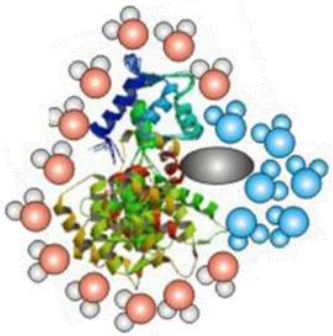
D… diffusion coefficient
D_T…thermal diffusion coefficient

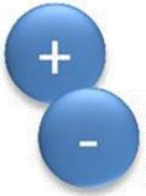
$$S_T = \frac{A}{kT}\left(-\Delta s_{hyd}(T) + \frac{\beta\sigma_{eff}^2}{4\varepsilon\varepsilon_0 T} \times \lambda_{DH}\right)$$

size — charge ^{2}

hydration shell





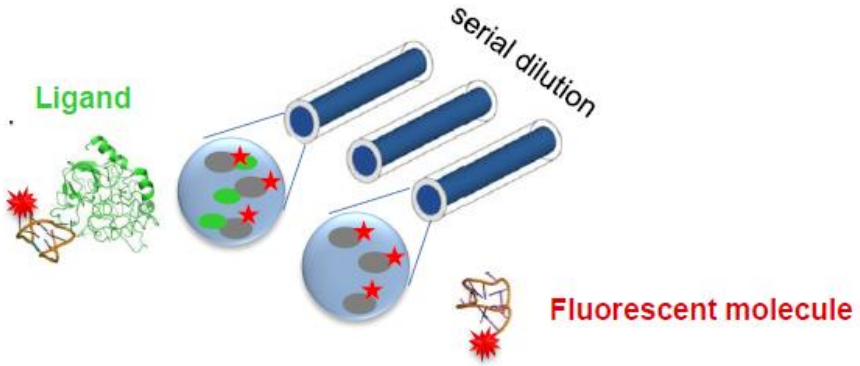


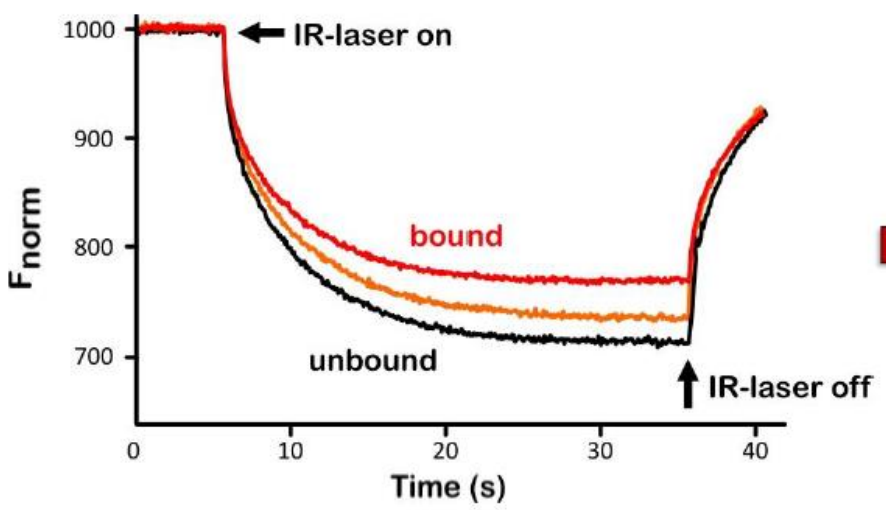
Soret coefficient S_T depends on:

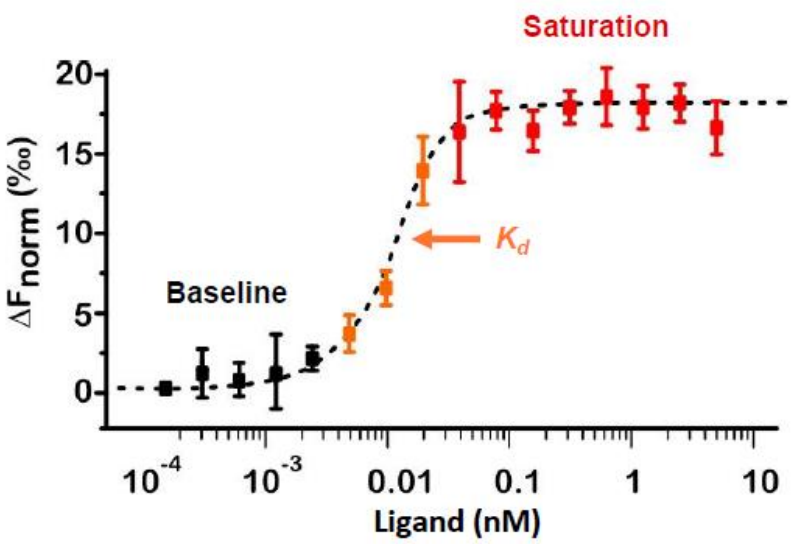
- mean temperature
- particle **size**
- surface area (~ **hydration** shell, solvation, conformation)
- electrostatic potential (~ **charge**)

Instead of exact calculation,
only difference in fluorescence upon binding is considered

MST – Basic principles







Assumptions

- Only **ONE** binding partner gives signal
- Both partners **INTERACT**
- Affinity is within applicable **RANGE**
- Sample is in sufficient
  CONCENTRATION and **QUALITY**

Fluorescent labeling

- **Fluorescent dyes**
  - Blue – FITC, FAM, ...
  - Green – Cy3, mCherry
  - Red – Cy5, Alexa647

- Amino coupling
- Thiol coupling
- His-tag binding dye
- etc.

- **Intrinsic fluorescence** – Tryptophan

- **Fluorescent proteins** – GFP (green)
  YFP (yellow)

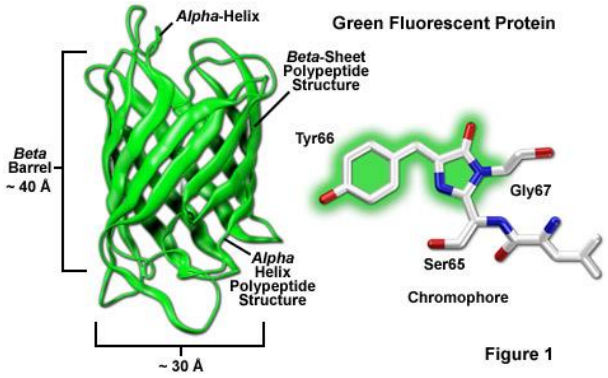
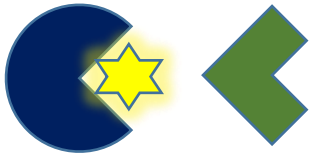


Figure 1

Labeling issues

Interference with interaction

1. 

1. Sterical hindrance

2. Conformation changes

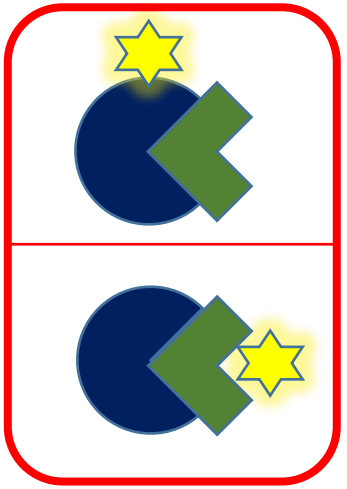
3. 

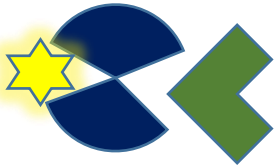
3. Non-specific interaction

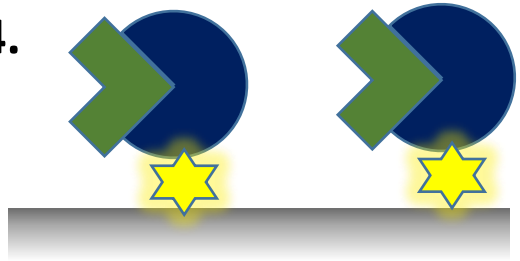
4. Adhesion to labware

5. 

5. Solubility change, aggregation



2. 

4. 

What can we get by MST measurement?

- **Affinity**

  What is the strength of interaction?

  K_D, K_A

- **Stochiometry**

  How many molecules interact?

  N

- **Thermodynamics**

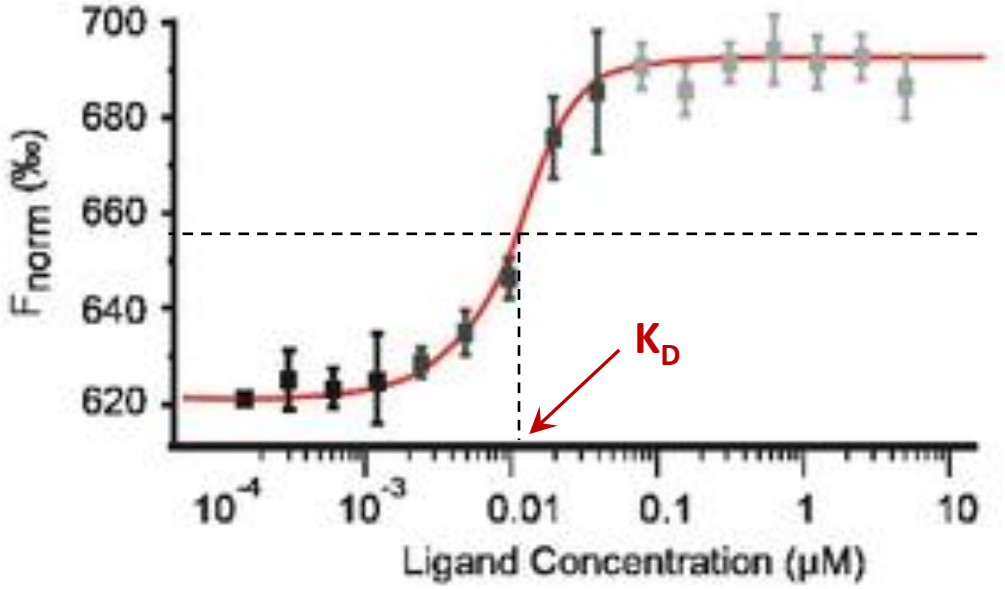
  What energetics is behind interaction?

  ΔG, ΔH, ΔS

Affinity

Labeled partner at constant $c \leq K_D$

Serial dilution (2-fold) of second partner in range of expected K_D



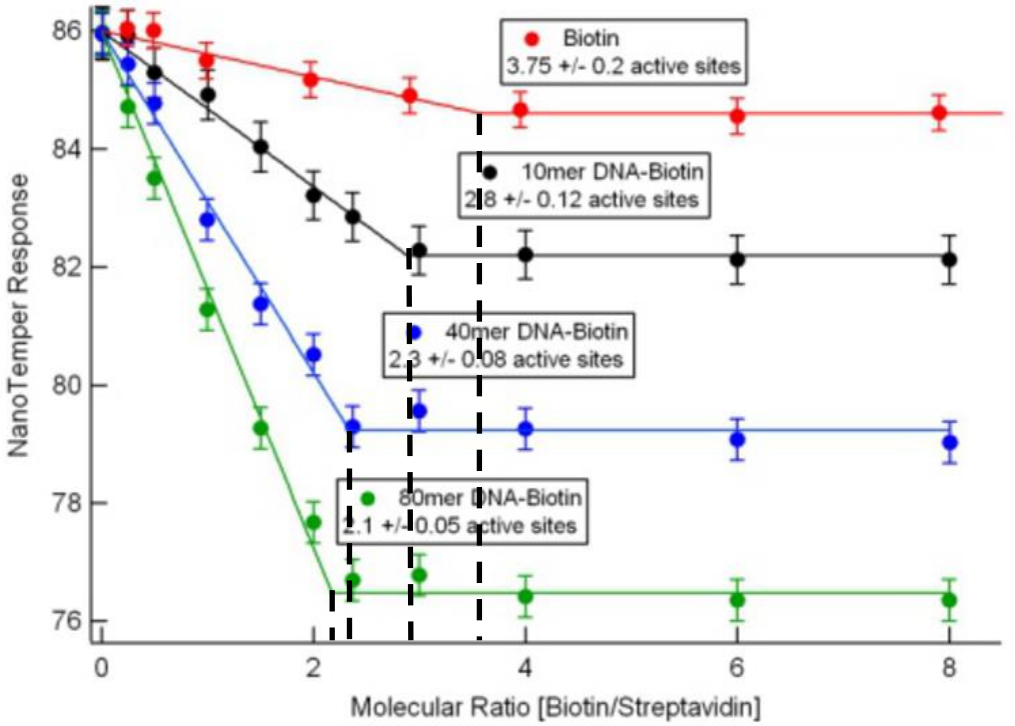
More than affinity (special cases)

- **Stoichiometry** determination
- **Multiple binding events** within one experiment
- **Inhibition assay**
- **Thermodynamics** measured by MST
- Interaction with **liposomes**
- Measurement in **crowdy samples** (blood, cell lysate)

Stochiometry

Labeled partner at constant c > K_D

Several dilution of second partner
in range of expected molecular ratios



Streptavidin – Biotin interaction

4 sites / tetramer

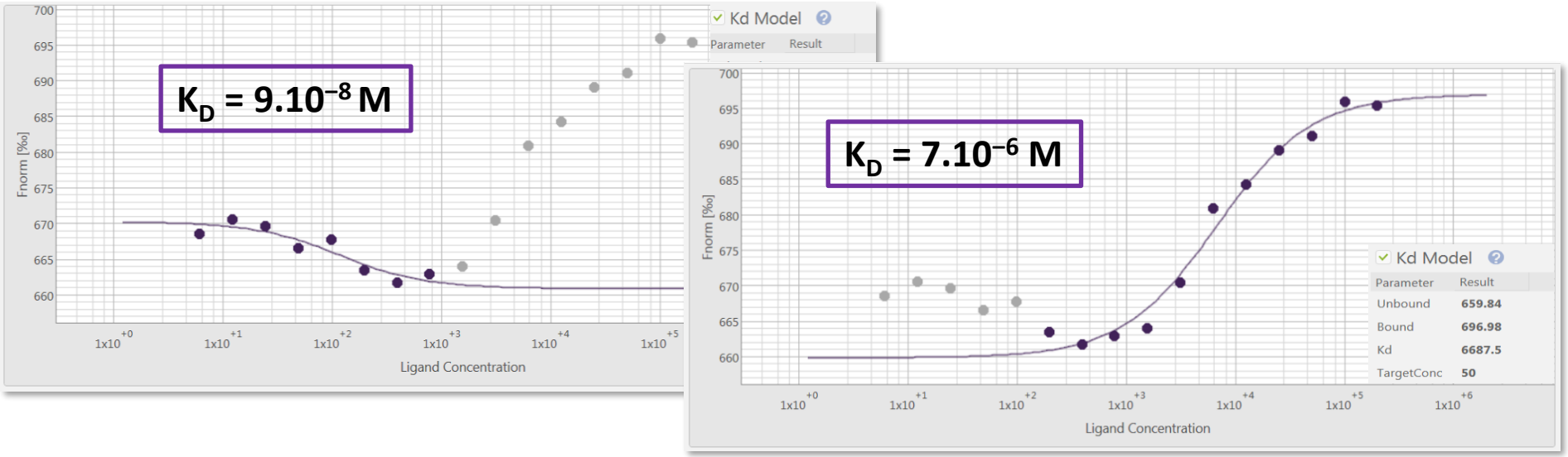
DNA-modified biotin
–> sterical hindrances

Multiple binding events

Two independent binding events in one measurement

Labeled partner at constant $c \leq K_{D,(stronger)}$

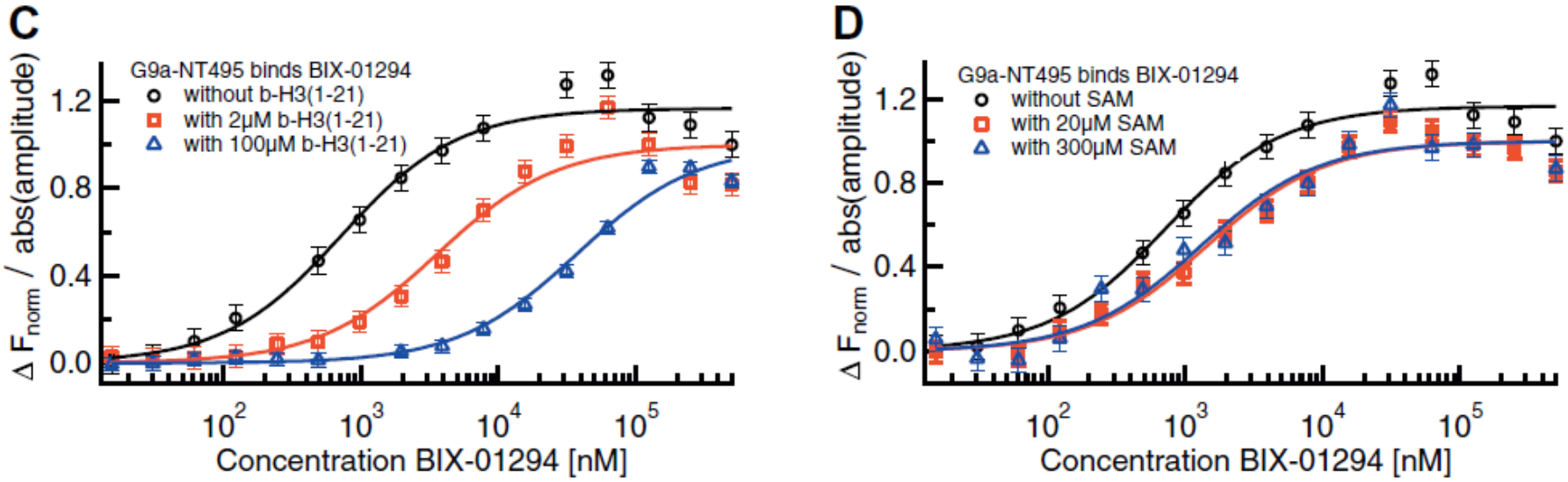
Both K_D's far enough to be distinguishable
but close enough to be covered within one dilution row



Inhibition assay

Standard affinity measurement
in **presence** and **absence** of inhibitor

Comparison of curves / calculated K_D



MST machines

- Monolith NT.115
- Monolith NT.115^{Pico}
- Monolith LabelFree
- Monolith Automated

– all by Nanotemper

Monolith NT.115

Monolith LabelFree

Intrinsic Trp fluorescence

Monolith NT.115^{Pico}

High sensitivity

Monolith Automated

High throughput

Isothermal Titration Calorimetry (ITC)

Calorimetry

- **Calorimetry** – technique based on measurement of heat that is generated (exothermic) or consumed (endothermic) in physical or chemical process

Early calorimeters

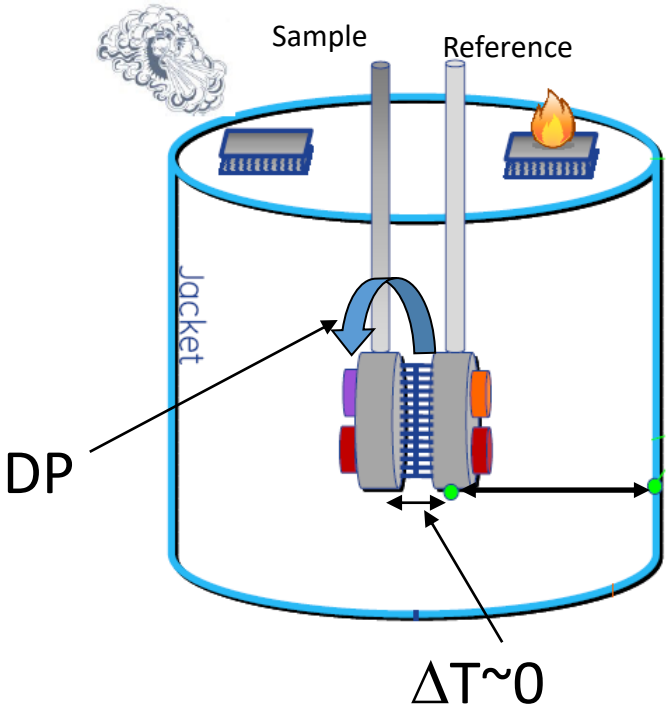
Inside of whole-body calorimeter

warvick.ac.uk

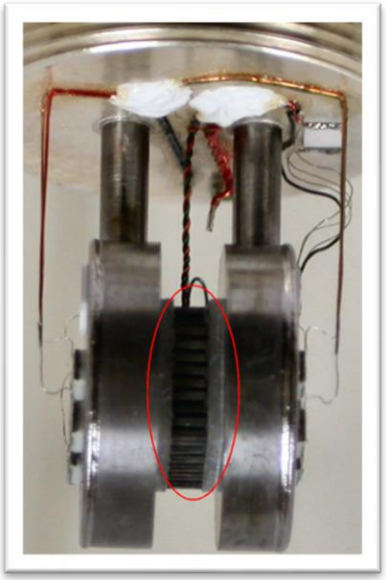
Microcalorimeter

Microcalorimeter – principle

- We measure difference in heat exchange between sample and reference cell while keeping both at the same temperature



DP = Differential power
ΔT = Temperature difference

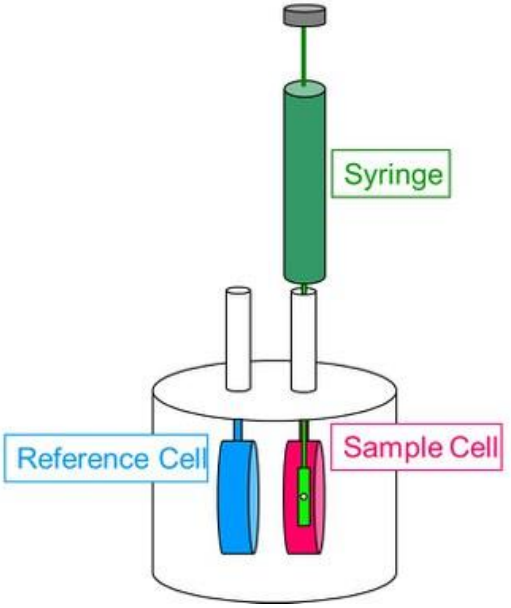


The DP is a measured power differential between the reference and sample cells to maintain a zero temperature difference between the cells

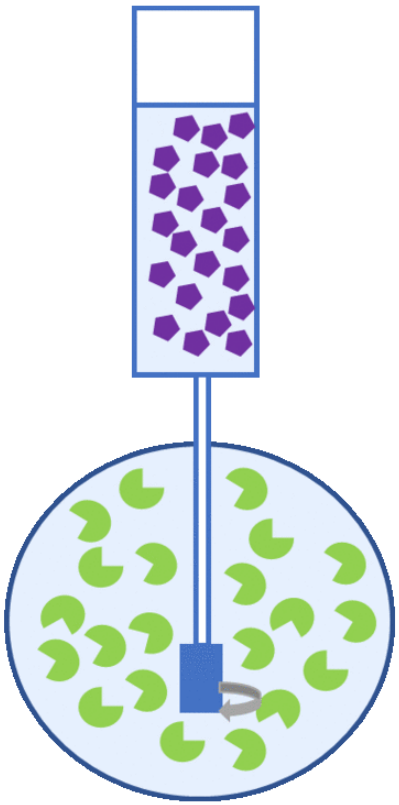
Reference Calibration Heater
Sample Calibration Heater
Cell Main Heater

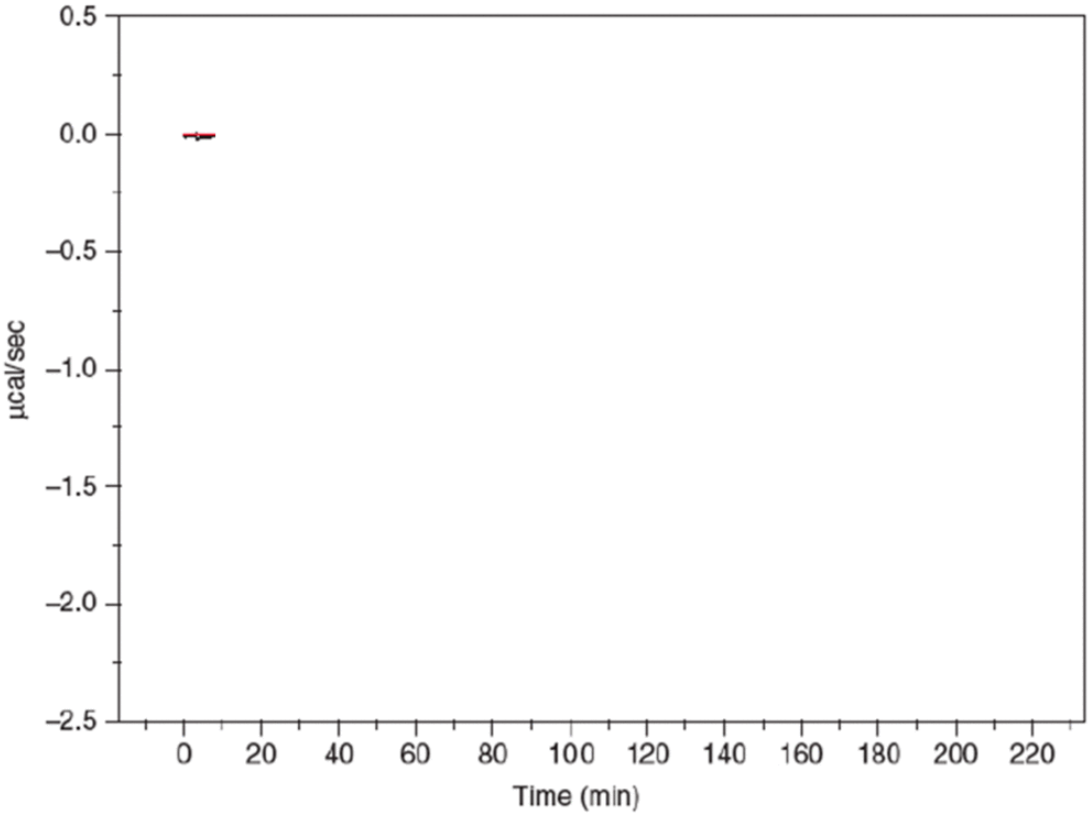
Isothermal titration calorimetry (ITC)

- All processes (including protein-ligand interaction) are associated with heat absorption or release
- ITC detects the heat change caused by ligand binding
- Measurement **in solution**, **no labeling**
- Determines:
  - affinity (K_D/K_A)
  - thermodynamics (ΔH, ΔG, ΔS)
  - stoichiometry (n)

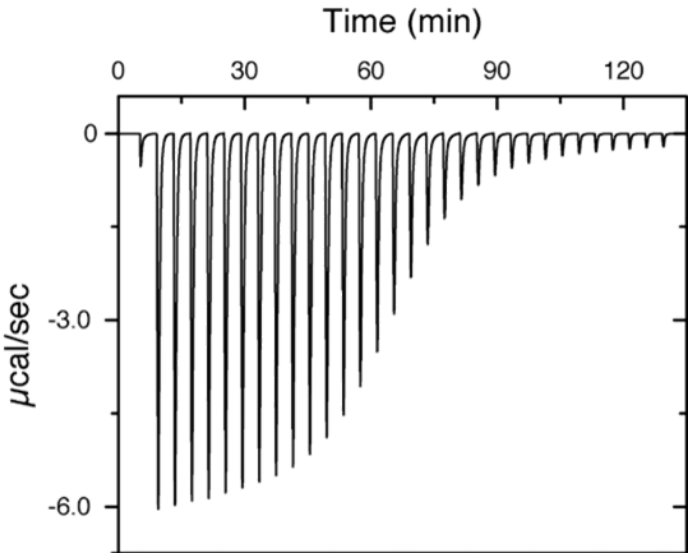


ITC experiment

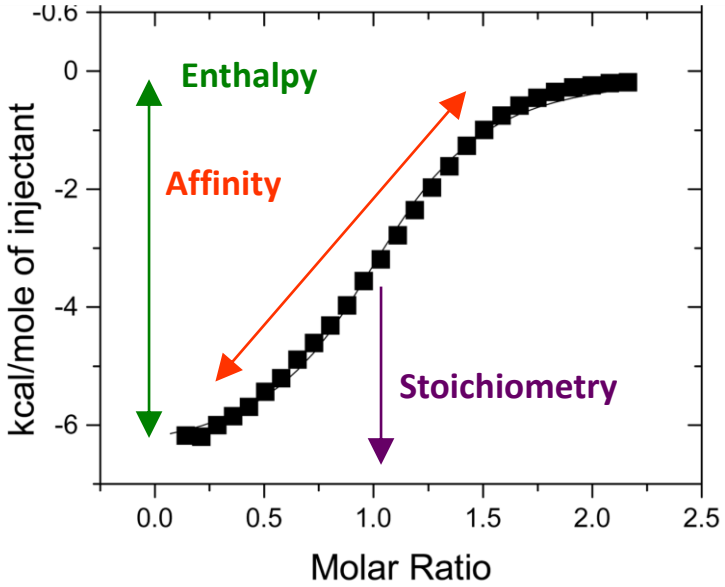




ITC experiment



Peak integration



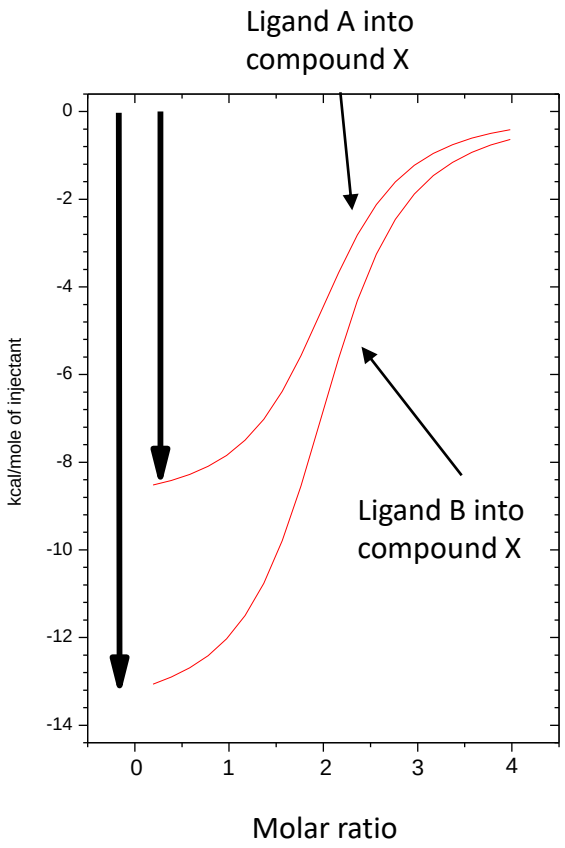
$$P + L \underset{K_D}{\overset{K_A}{\rightleftharpoons}} PL$$

$$\Delta G^\circ = -RT \ln K_A$$

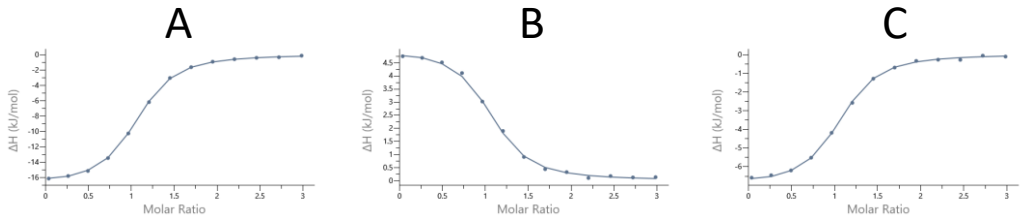
$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

Integration of heats are used to extract affinity (K_D), stoichiometry (N) and binding enthalpy (ΔH) using appropriate binding model

Thermodynamics



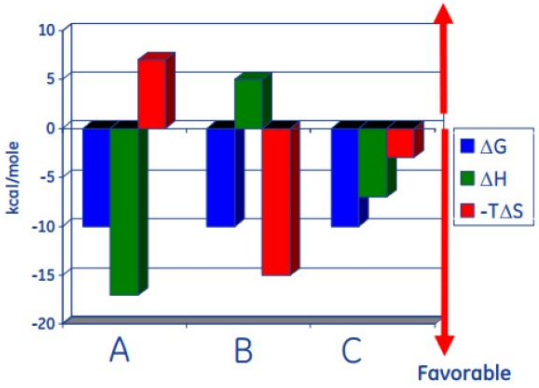
- The same affinity and stoichiometry but different enthalpy (heat)
- ITC tells us we have different binding mechanisms



A. Good hydrogen bonding with unfavorable conformational change

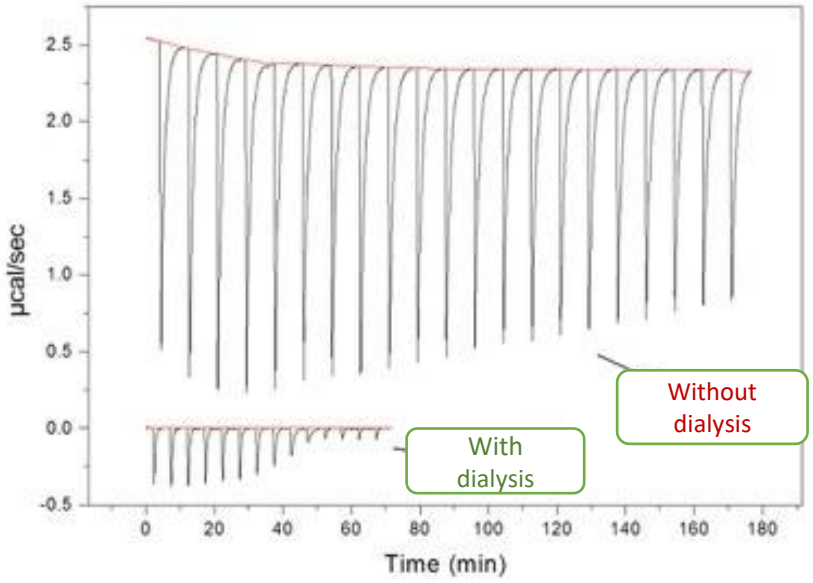
B. Binding dominated by hydrophobic interaction

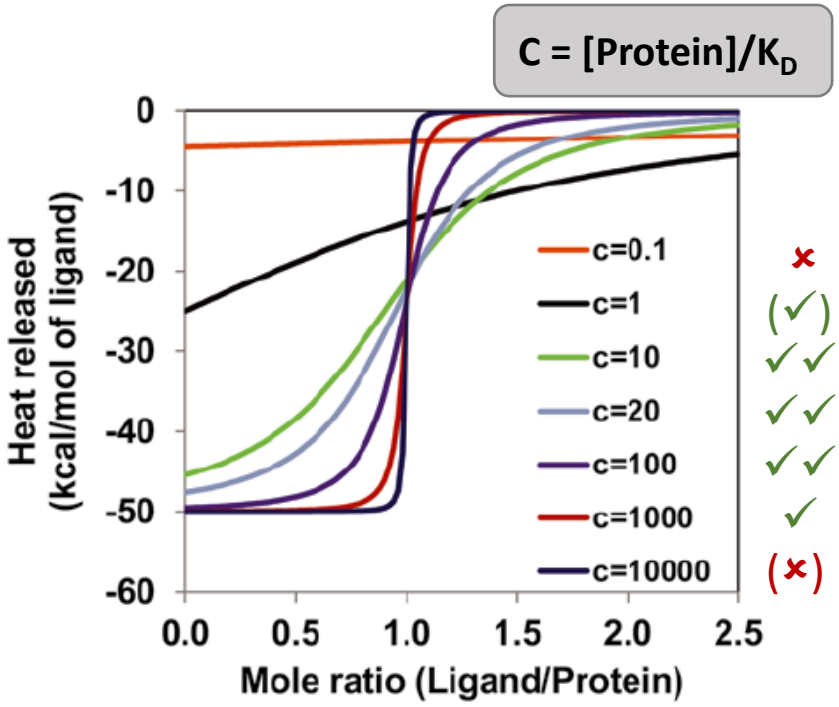
C. Favorable hydrogen bonds and hydrophobic interaction



Key points for ITC measurement

- **Buffer match** between interacting partners (dialysis, buffer exchange)
- **Accurate** protein and ligand **concentration**
- **Adjusted concentration** with respect to K_D
- Sample **homogeneity**

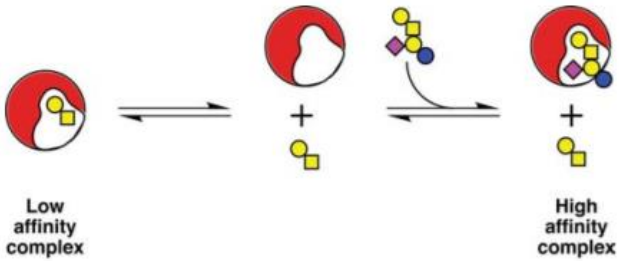




Experimental variability

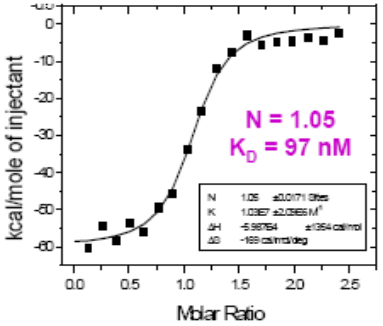
Competition titration

Displacement of low affinity ligand with high affinity ligand.
Change in apparent affinity and enthalpy

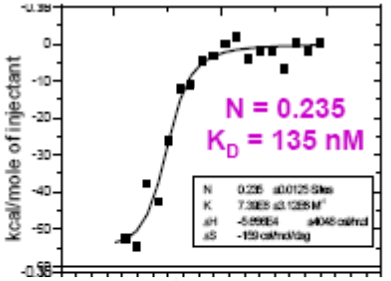


Sample quality assessment

Portion of active protein derived from N value



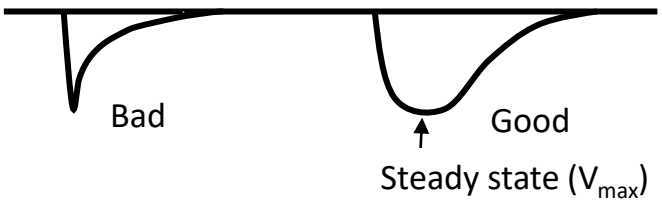
100% of Batch 1 protein active based on stoichiometry



23% of Batch 2 protein active based on stoichiometry

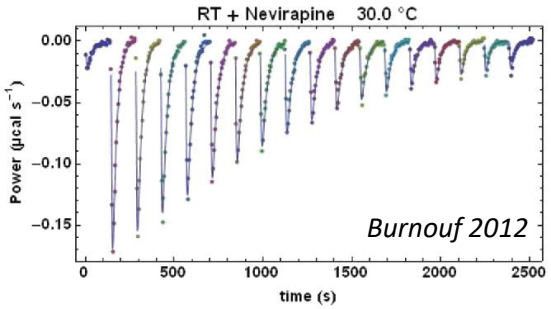
Single injection method

One injection of substrate into enzyme with the aim to reach V_{max} as fast as possible and observe the signal decay associated with substrate depletion and product formation.



Kinetic analysis

Fitting of binding curves with kinetic model



ITC calorimeters

- *Malvern* – PEAQ-ITC, ITC_{200}, VP-ITC
- *TA Instruments* – Nano ITC, Affinity ITC

Nano ITC

PEAQ-ITC

ITC_{200}

VP-ITC

Affinity ITC

Method comparison

Basic comparison

	SPR	BLI	MST	ITC
Parameters	K_D/K_A, k_{on}, k_{off}	K_D/K_A, k_{on}, k_{off}	K_D/K_A, N	K_D/K_A, N, ΔG, ΔH, ΔS
K_D range [M]	$10^{-13} – 10^{-3}$	$10^{-11} – 10^{-3}$	$10^{-11} – 10^{-1}$	$10^{-12} – 10^{-2}$
Speed (per K_D)	15 – 120 min	15 – 60 min	15 – 30 min	30 – 120 min
Sample modification	Immobilization	Immobilization	Labeling	None
Complex samples	✓	✓	✓	✗
High throughput	✓	✓	✓	✓

Kinetics vs. Affinity in Drug design

High affinity – first aim in drug discovery

BUT

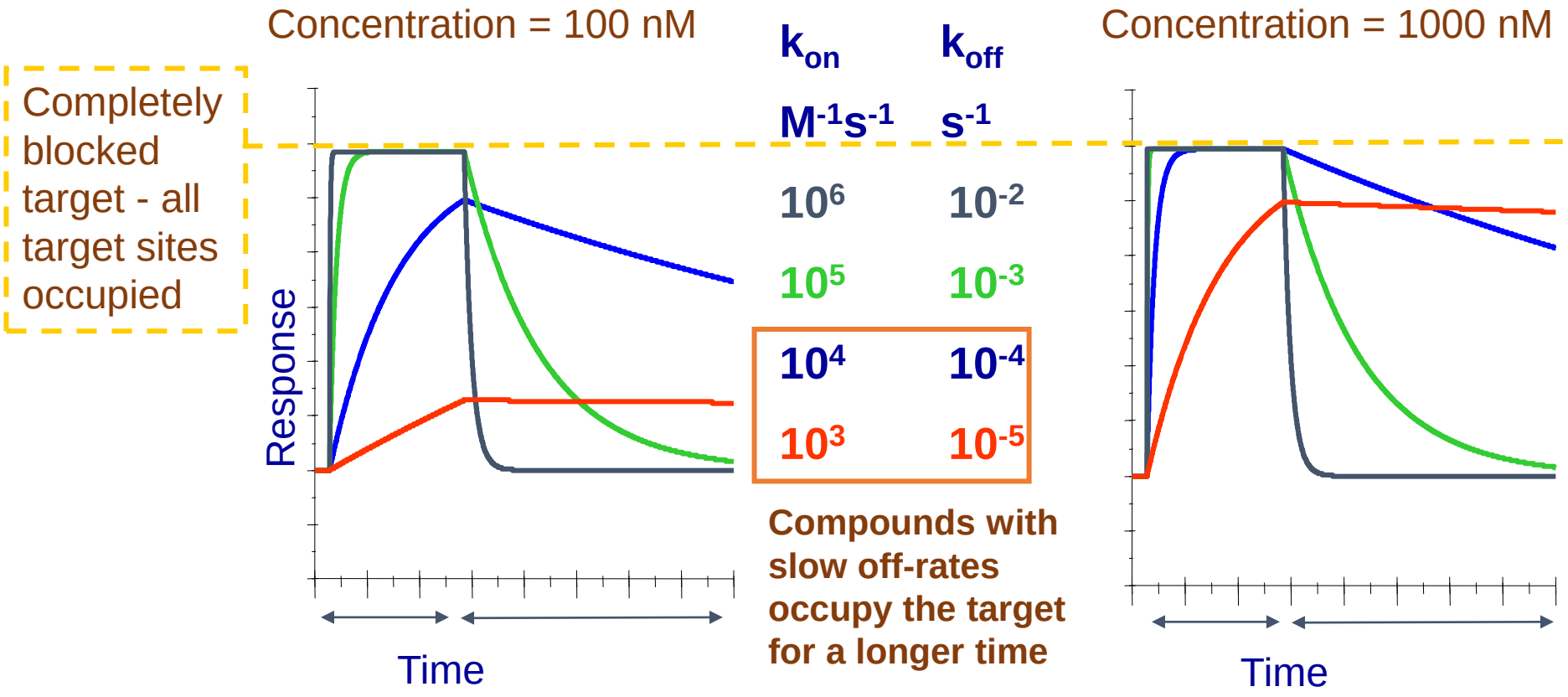
May be caused by high k_a and k_d = fast dissociation (!)

Kinetics – lower k_a AND k_d may mean longer effect

This fact is known but usually not considered !

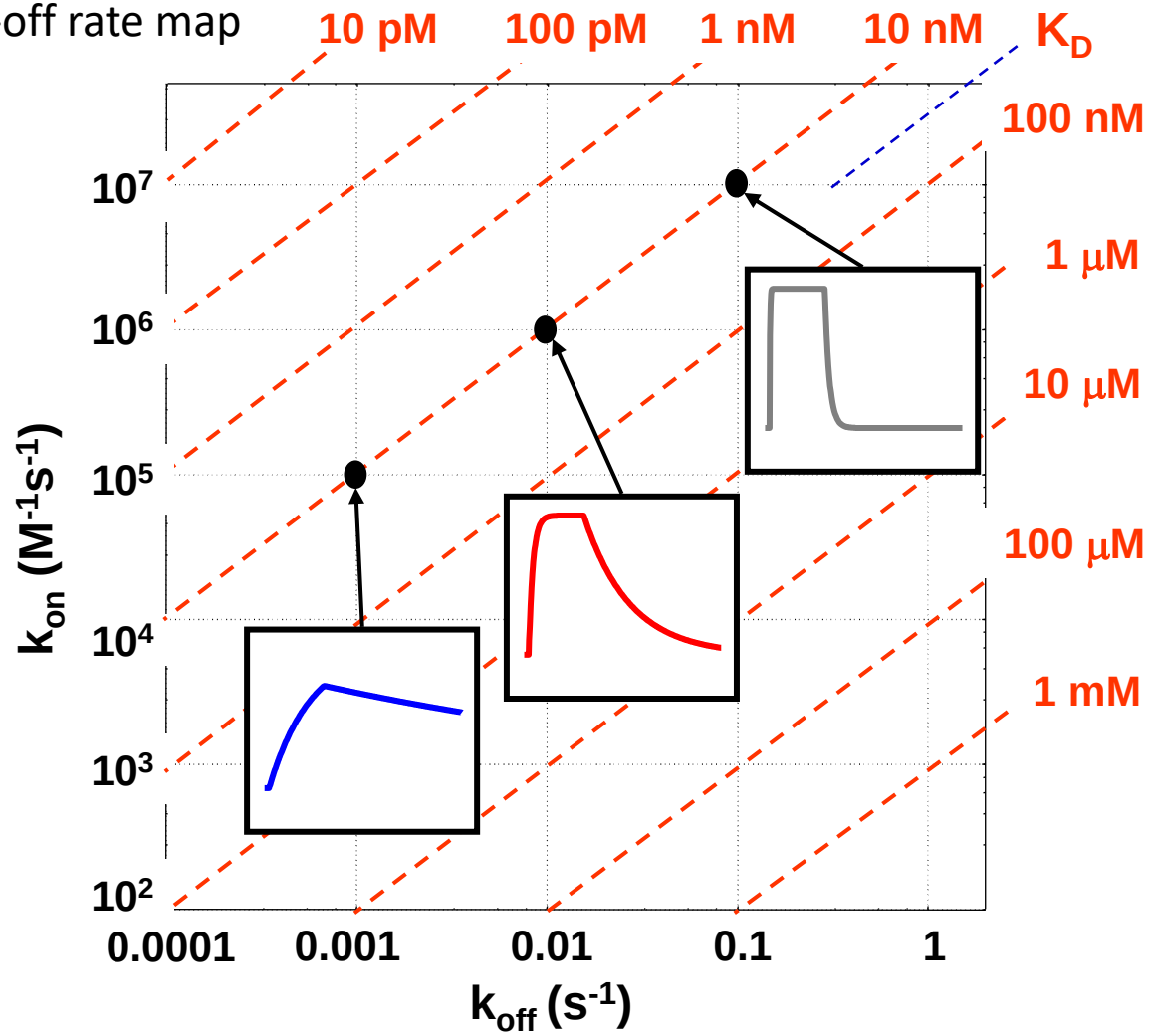
Same affinity but different kinetics

- All 4 compounds have the same affinity K_D = 10 nM = 10^{-8} M
- The binding kinetic constants vary by 4 orders of magnitude



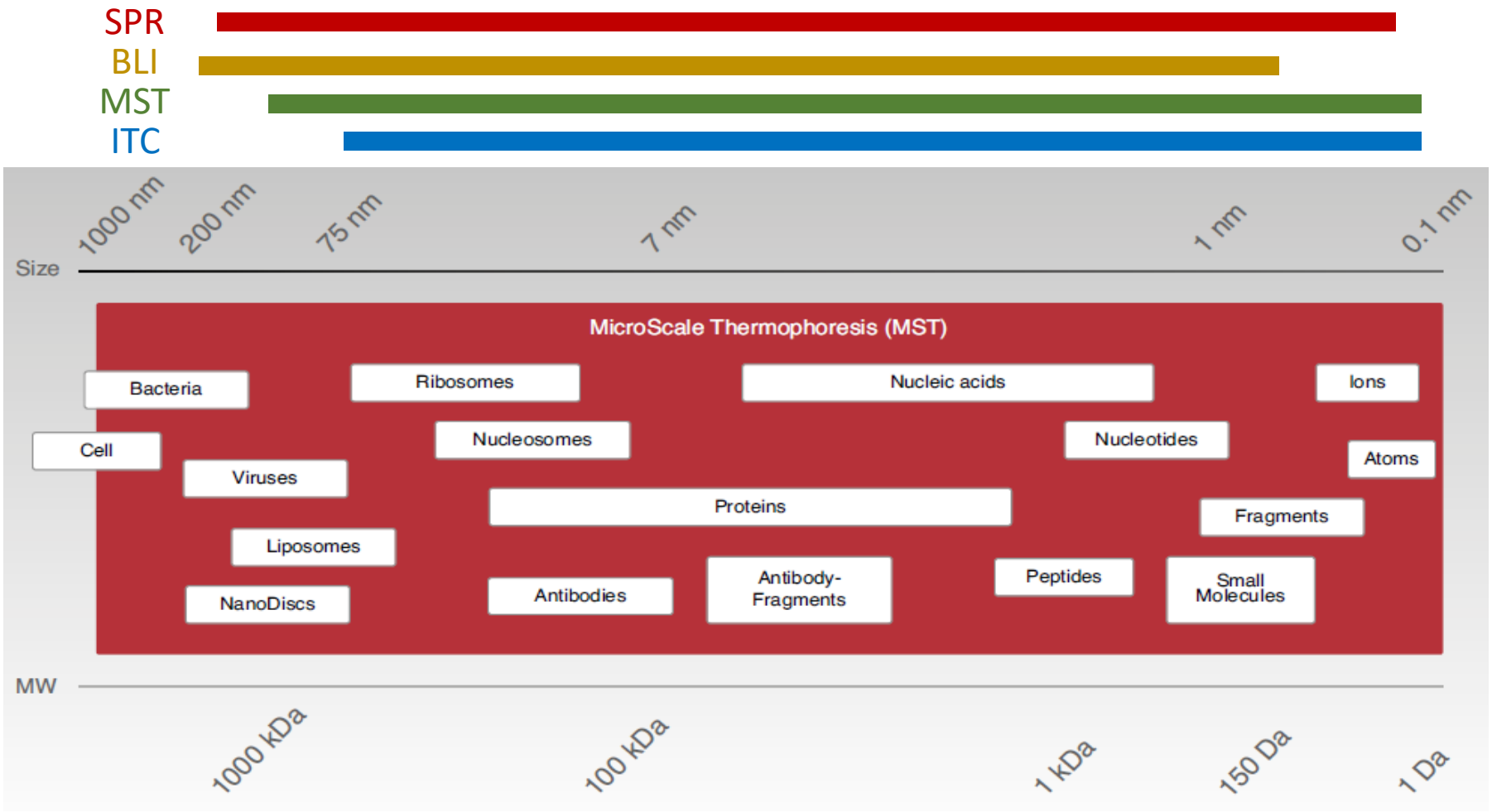
Same affinity but different kinetics

HIV-p inhibitors: on-off rate map



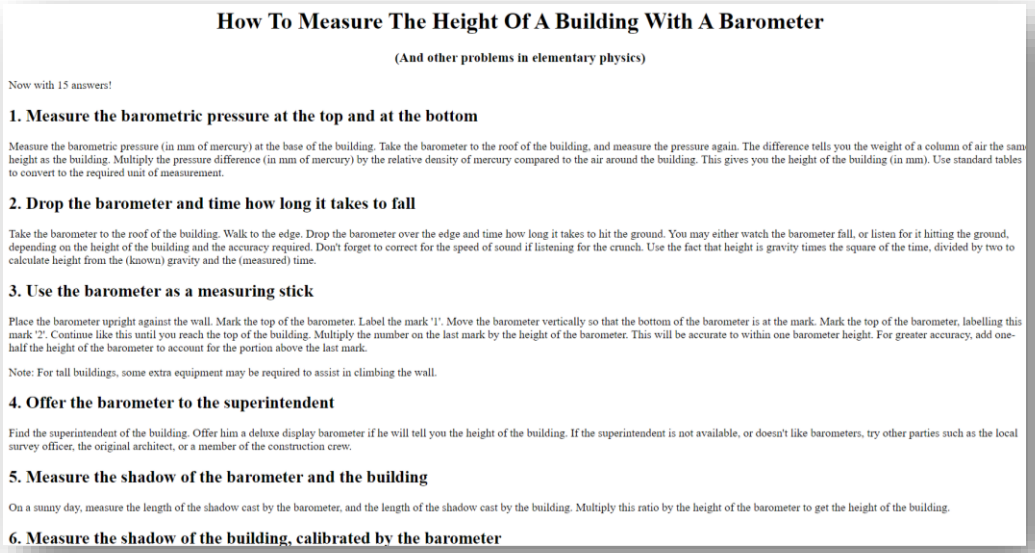
Size range

Method specific, Instrument specific, Case specific



Non-typical applications

- Many techniques were developed with one clear aim
- Study of processes and application of physics lead to alternative applications



How To Measure The Height Of A Building With A Barometer

(And other problems in elementary physics)

Now with 15 answers!

1. Measure the barometric pressure at the top and at the bottom

Measure the barometric pressure (in mm of mercury) at the base of the building. Take the barometer to the roof of the building, and measure the pressure again. The difference tells you the weight of a column of air the same height as the building. Multiply the pressure difference (in mm of mercury) by the relative density of mercury compared to the air around the building. This gives you the height of the building (in mm). Use standard tables to convert to the required unit of measurement.

2. Drop the barometer and time how long it takes to fall

Take the barometer to the roof of the building. Walk to the edge. Drop the barometer over the edge and time how long it takes to hit the ground. You may either watch the barometer fall, or listen for it hitting the ground, depending on the height of the building and the accuracy required. Don't forget to correct for the speed of sound if listening for the crunch. Use the fact that height is gravity times the square of the time, divided by two to calculate height from the (known) gravity and the (measured) time.

3. Use the barometer as a measuring stick

Place the barometer upright against the wall. Mark the top of the barometer. Label the mark '1'. Move the barometer vertically so that the bottom of the barometer is at the mark. Mark the top of the barometer, labelling this mark '2'. Continue like this until you reach the top of the building. Multiply the number on the last mark by the height of the barometer. This will be accurate to within one barometer height. For greater accuracy, add one-half the height of the barometer to account for the portion above the last mark.

Note: For tall buildings, some extra equipment may be required to assist in climbing the wall.

4. Offer the barometer to the superintendent

Find the superintendent of the building. Offer him a deluxe display barometer if he will tell you the height of the building. If the superintendent is not available, or doesn't like barometers, try other parties such as the local survey officer, the original architect, or a member of the construction crew.

5. Measure the shadow of the barometer and the building

On a sunny day, measure the length of the shadow cast by the barometer, and the length of the shadow cast by the building. Multiply this ratio by the height of the barometer to get the height of the building.

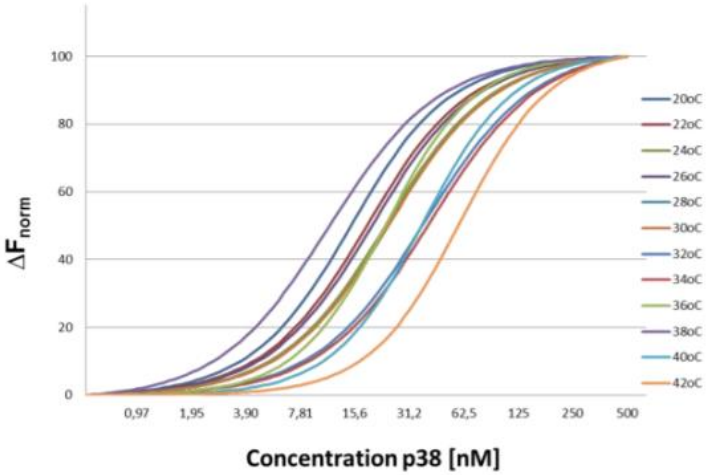
6. Measure the shadow of the building, calibrated by the barometer

esmerel.com

Thermodynamics from K_D

K_D determination at various temperatures

Calculation of thermodynamic parameters

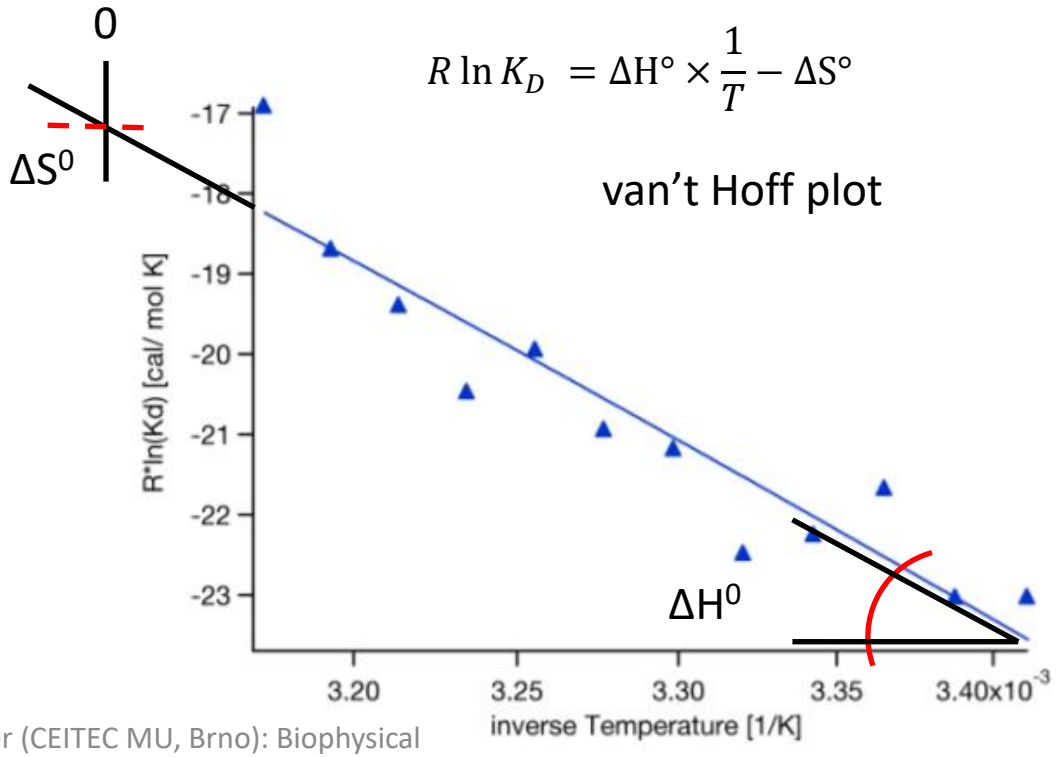


K_D's

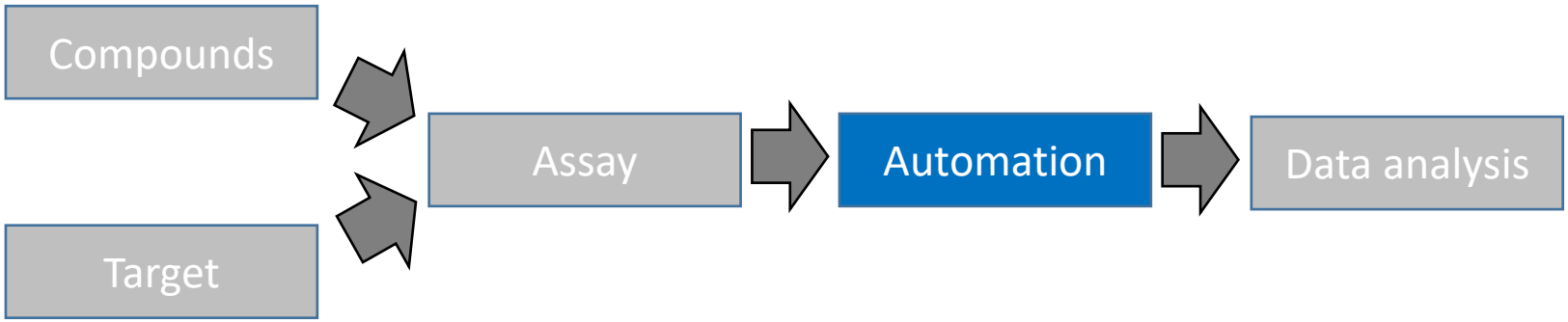
$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K_A = RT \ln K_D$$

$$R \ln K_D = \Delta H^\circ \times \frac{1}{T} - \Delta S^\circ$$

van't Hoff plot

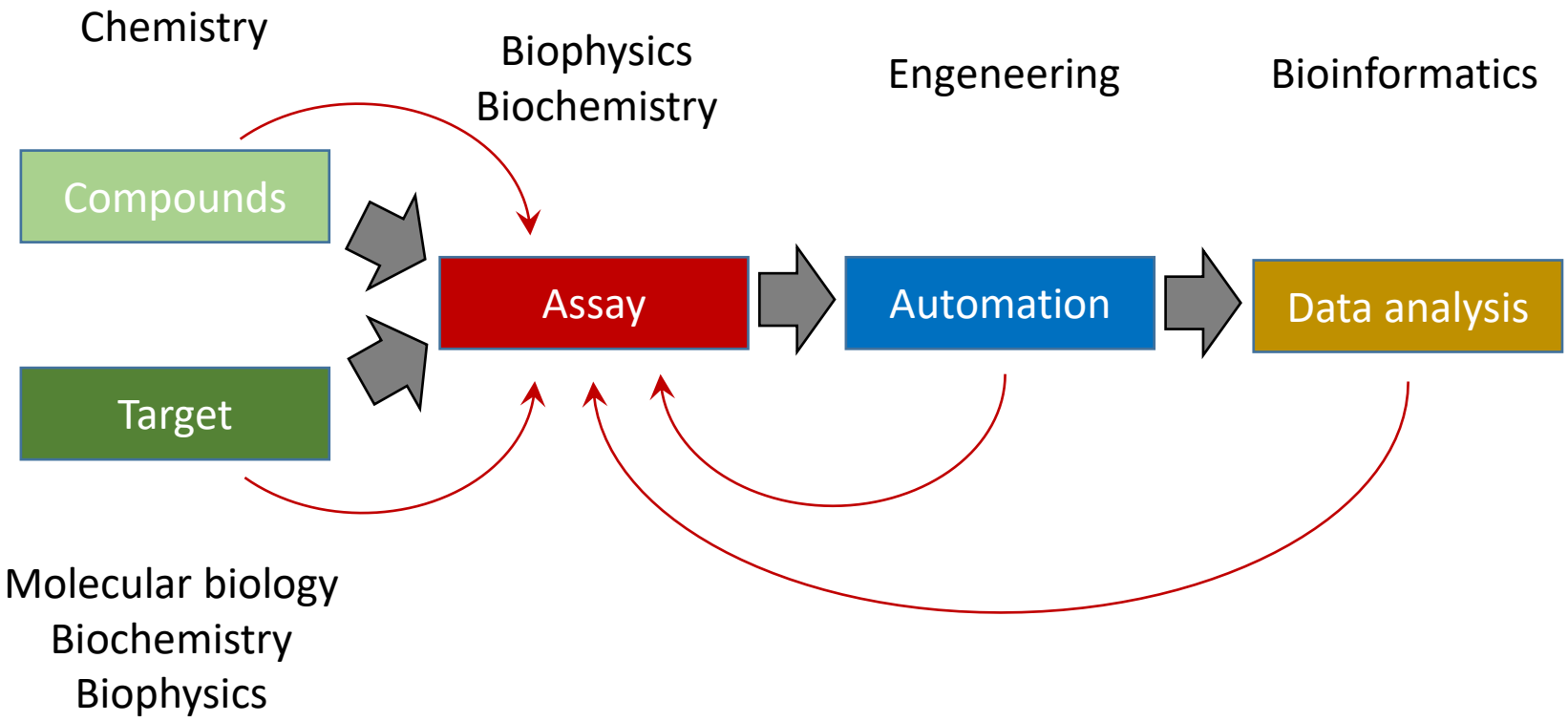


High throughput
&
Biophysical techniques



HTS scheme

If you want to develop HTS process, you need to **know as much as possible** about all given parts.



HTS assay

Assay is chosen based on several criteria:

- Target and compounds **compatibility**
- **Feature** of interest (affinity, kinetics, inhibition)
- **Speed** (high-throughput)
- **Accessibility** (unique x routine)
- **Price**
- **Fashion** (?)

HTS assays – fashion

"Old-fashioned" vs "Cool" methods

FASHION ?

PURPOSE

What is old?

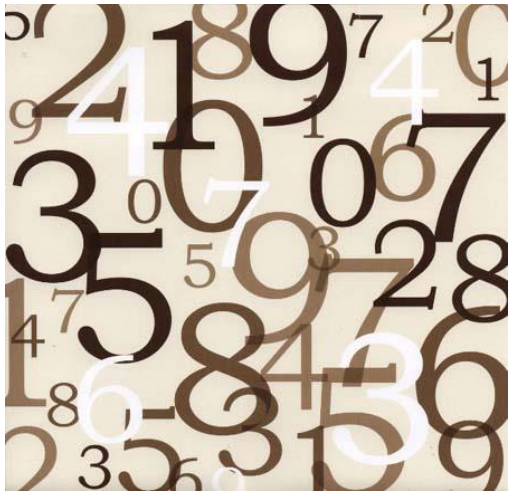
What is modern?

Two informational levels of methods

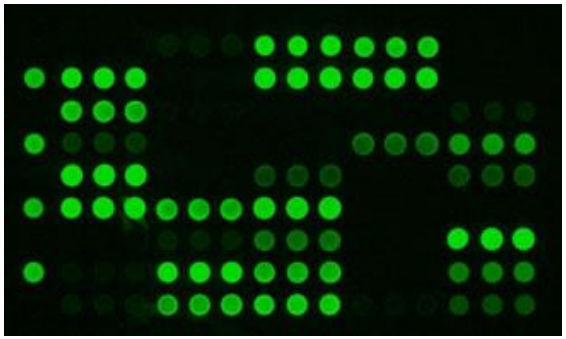
Qualitative

Semi-quantitative

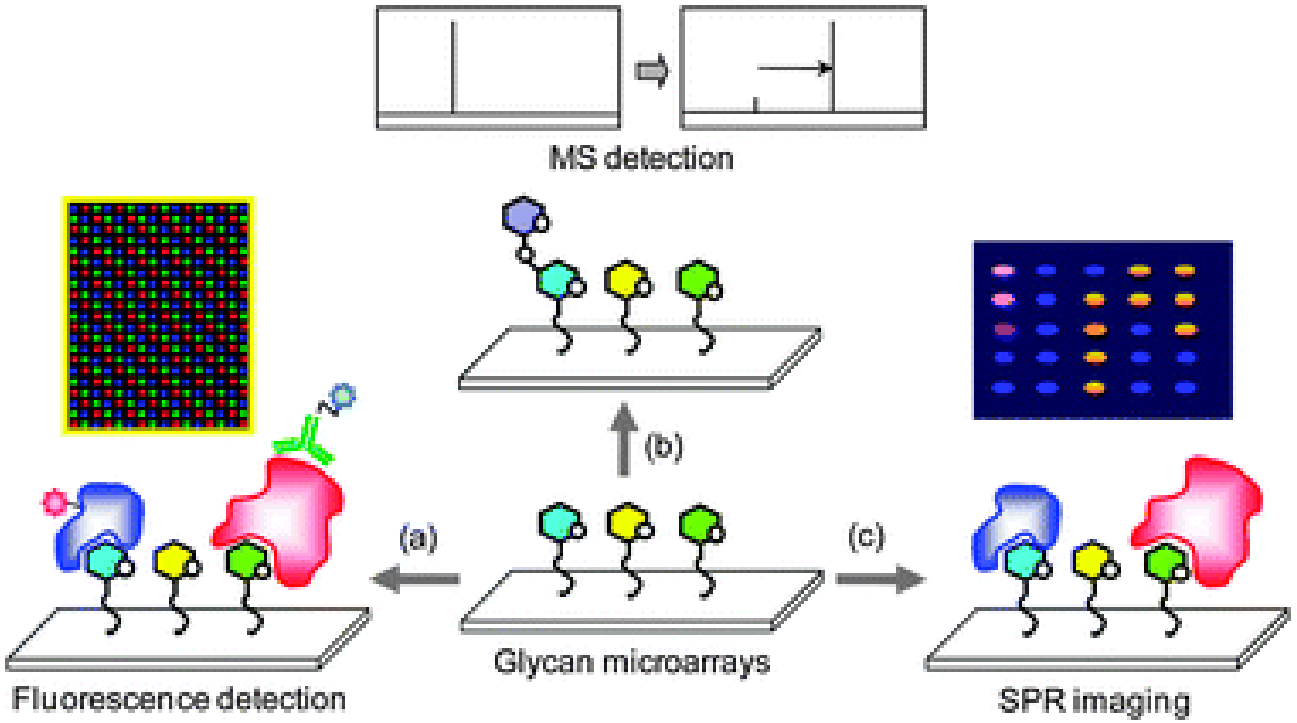
Quantitative



Microarrays



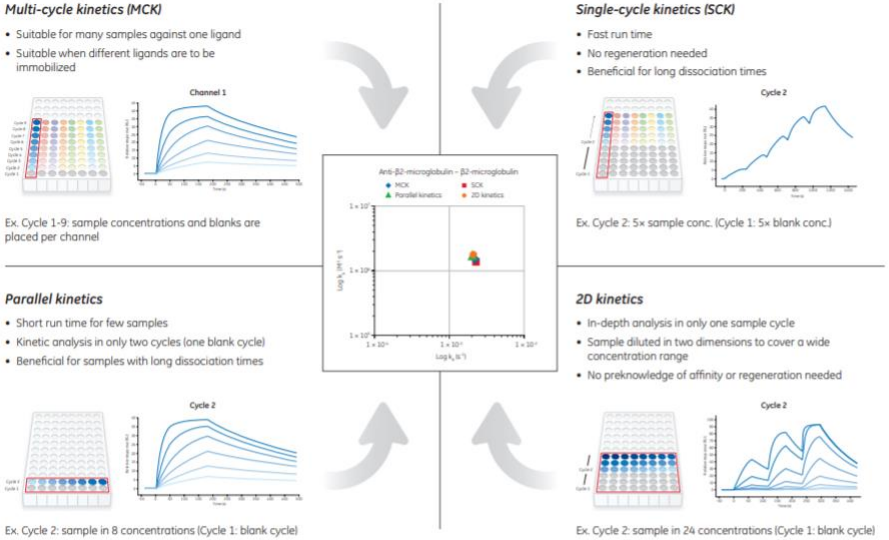
- High screening capacity possible
- Semi-quantitative



SPR Automated

Biacore 8K (highest model)

- 16 channels
- **Up to 4x384 samples** in a run
- 2300 interacting molecules/day
- 64 kinetic characterizations/4 hrs



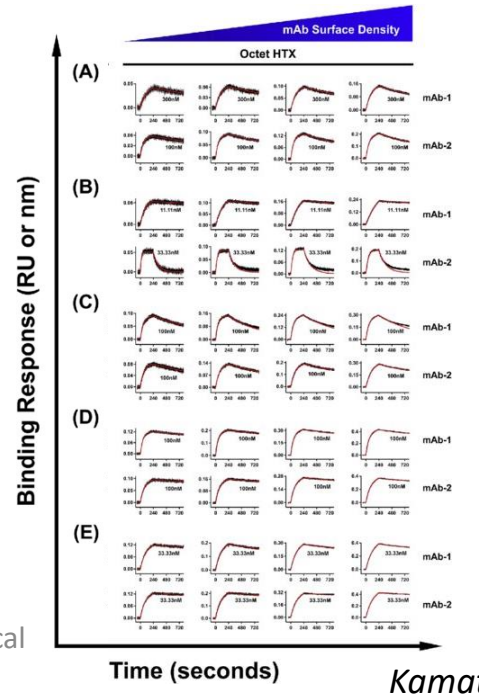
www.cytivalifesciences.com

BLI Automated

Octet HTX

- **Up to 96 samples** simultaneously
- 96 samples quantitation/2 mins
- Up to 32x32 epitope binning/8 hrs

analytica-world.com

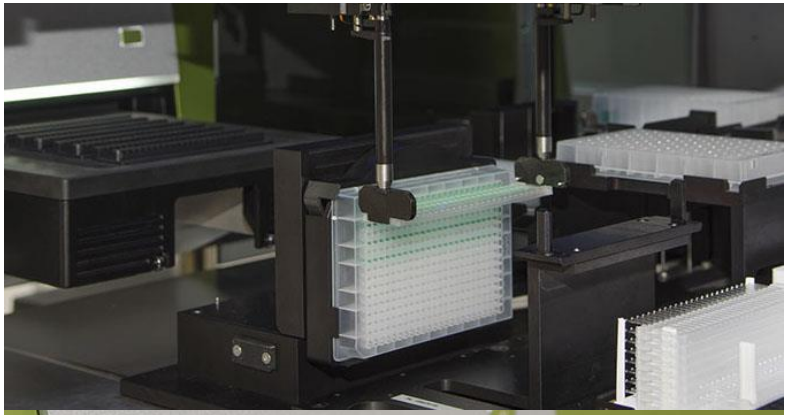


Kamat 2017

MST Automated

Monolith.NT Automated

- Two channels possible
- **96 samples** in a run
- 8 affinities/30 mins



ITC Automated

Auto PEAQ-ITC
Auto ITC200

- **Up to 4x96 samples** in a run
- >24 titrations/day
- High reproducibility

Everything Automated ?

Existing strategies:

1) High number of cheap workers
2) Processing lines (combination of multiple instruments and robotics)
3) Miniaturization – lab on chip
4) *In silico* approaches

Take home message

- Many techniques available with various principles, sample requirements, detection limits,…
- There is no single ideal method – combination needed
- Method knowledge is crucial to get the best results
- Sample quality is equally (if not more) important