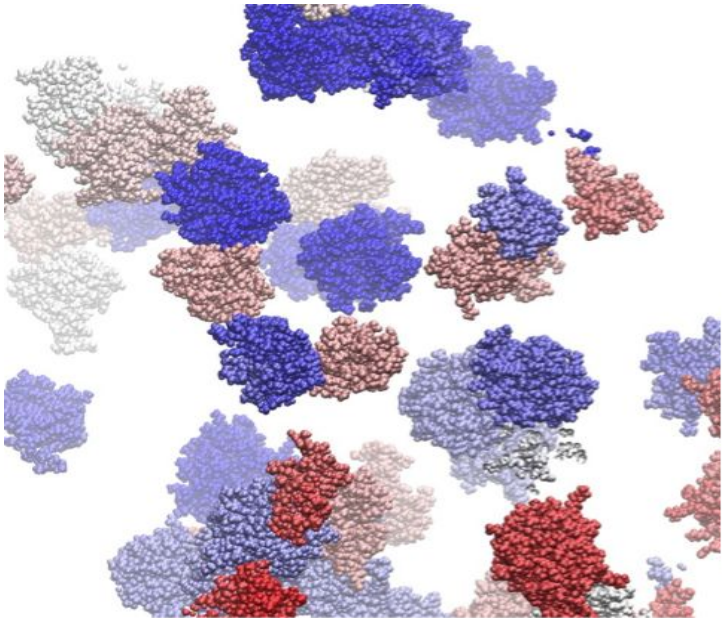
Лекция 6

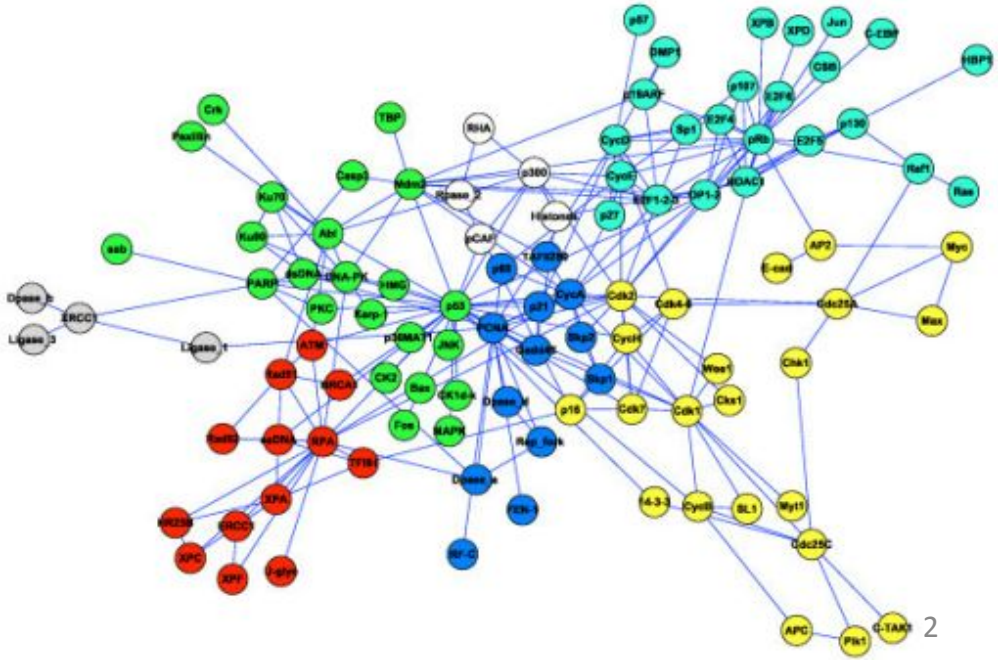
Методы исследования взаимодействий с участием белков (Co-IP, equilibrium microdialysis, ITC, MST, SPR, BLI, QCM). Примеры.

Случанко Н. Н.

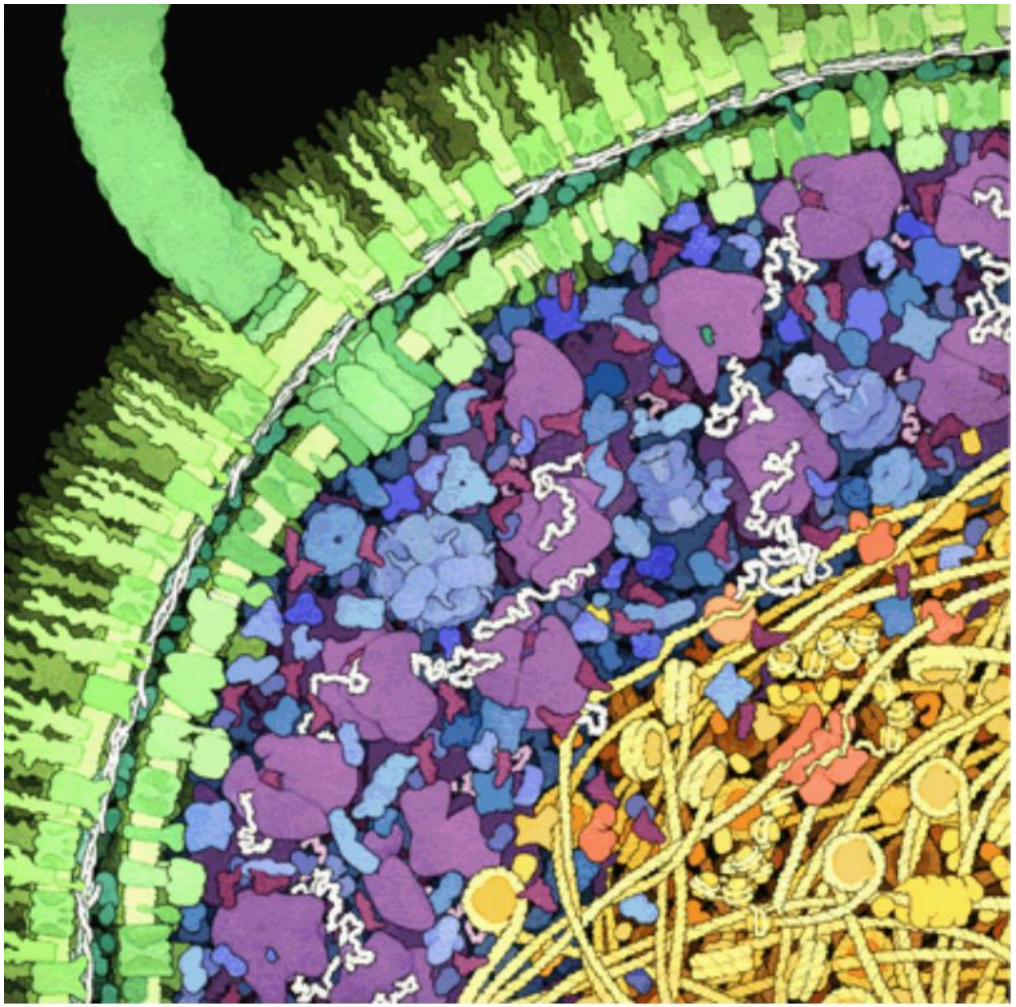
Protein-protein interactions (PPIs)

- >80% of proteins function via interaction with other proteins (PMID: 17640003)
- For each protein ~10 protein partners (interactome)
- Human "interactome" – 300–650 000 PPIs (PMID: 28968506)
- Mechanisms are in the core of the vital processes
- Data are deposited and systematized in databases – *MINT, iHOP, InAct*





Interactions of proteins control the life of the cell



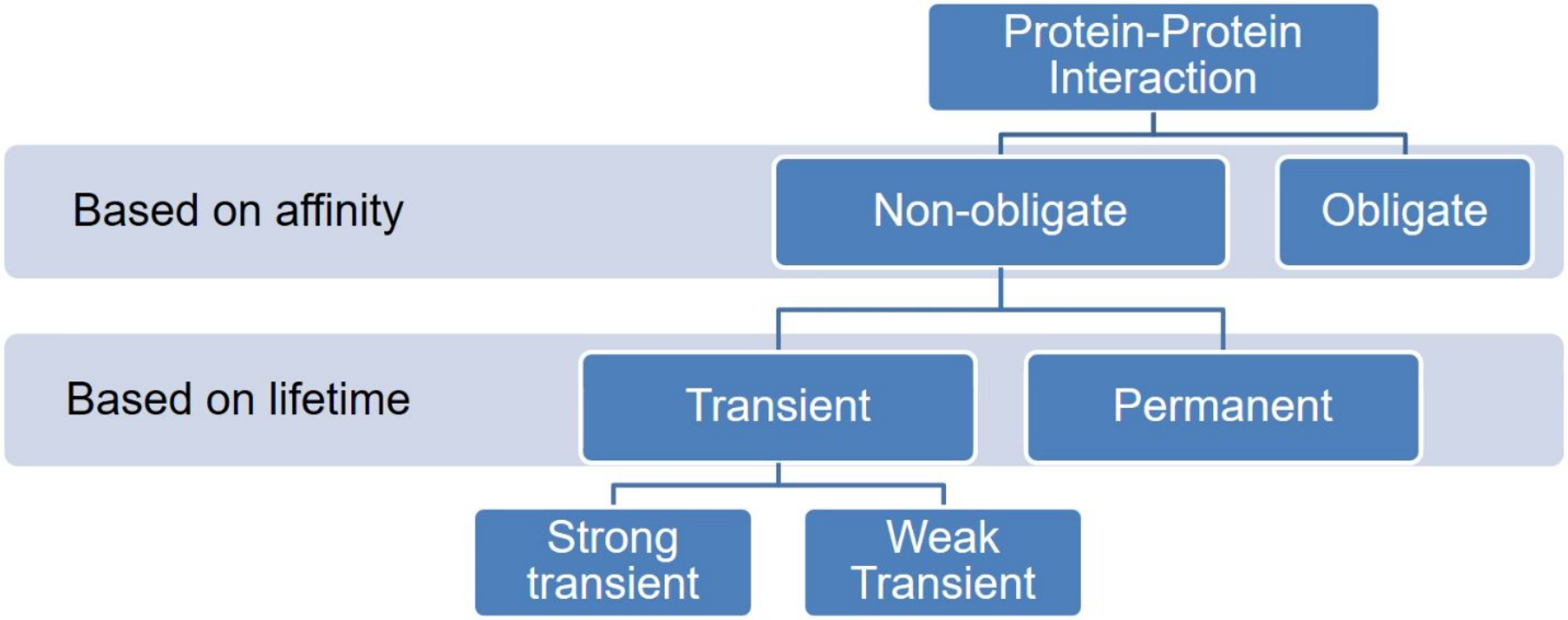
Escherichia coli drawn to molecular scale by David Goodsell

Interactions of proteins control the life of the cell

… cell biochemistry would appear to be largely run by a set of protein complexes, rather than proteins that act individually and exist in isolated species.

Cell 1992, Bruce Alberts & Miake-Lye

Types of PPIs



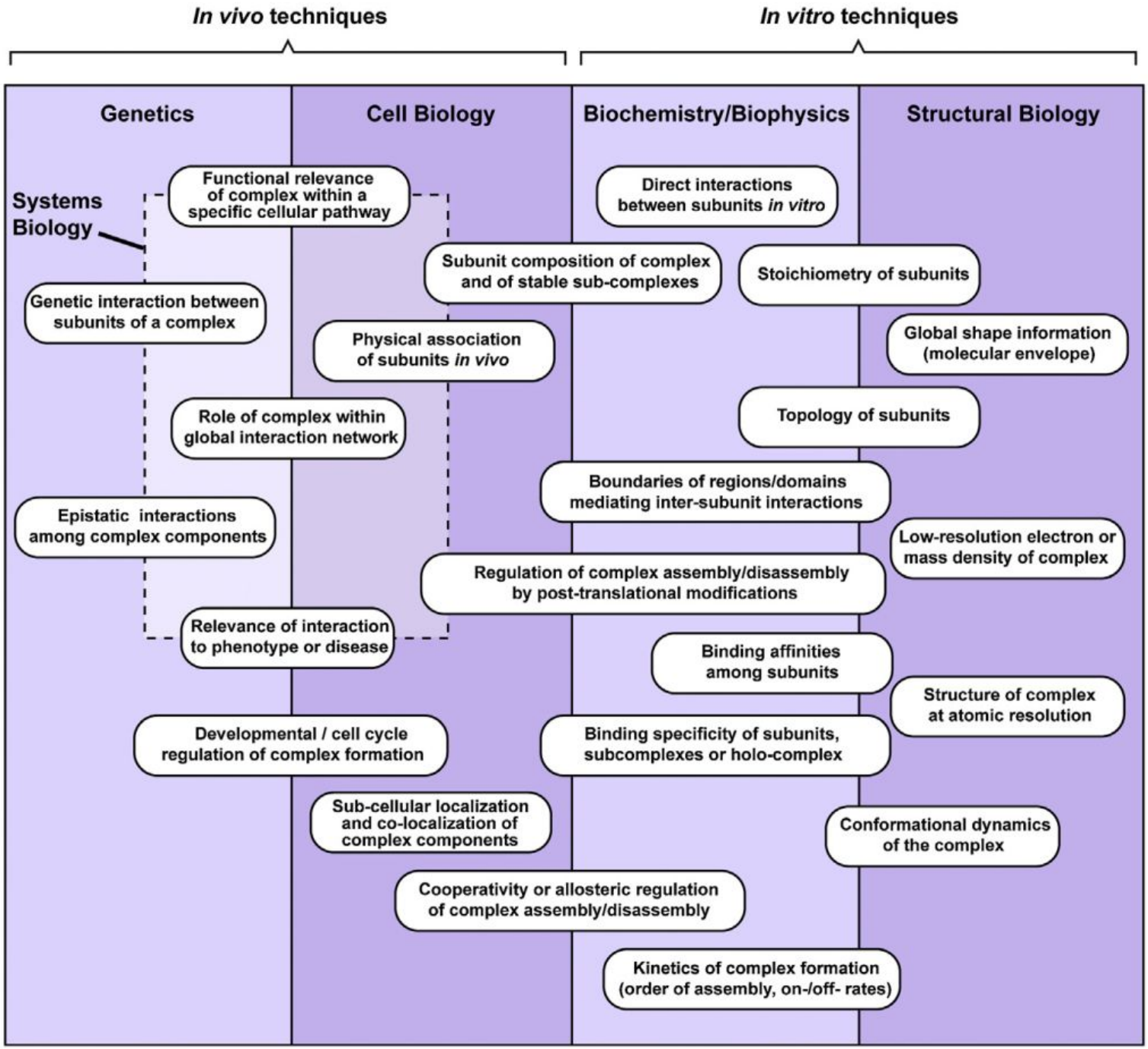
Types of PPIs

Homologous interactions:

- The same proteins
- Oligomers
- Coiled-coil
- Amyloids

Heterologous interactions:

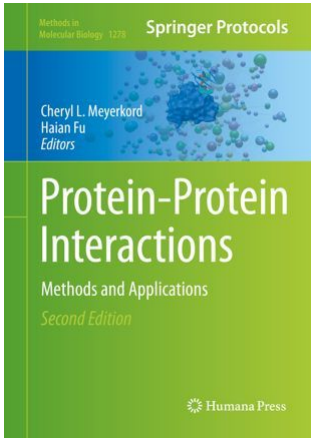
- Different proteins
- Enzyme – inhibitors
- Antibody – antigen
- Protein complexes


In vivo techniques
In vitro techniques
Genetics
Cell Biology
Biochemistry/Biophysics
Structural Biology
Systems Biology
Functional relevance of complex within a specific cellular pathway
Direct interactions between subunits *in vitro*
Subunit composition of complex and of stable sub-complexes
Stoichiometry of subunits
Genetic interaction between subunits of a complex
Physical association of subunits *in vivo*
Global shape information (molecular envelope)
Role of complex within global interaction network
Topology of subunits
Boundaries of regions/domains mediating inter-subunit interactions
Epistatic interactions among complex components
Low-resolution electron or mass density of complex
Regulation of complex assembly/disassembly by post-translational modifications
Relevance of interaction to phenotype or disease
Binding affinities among subunits
Structure of complex at atomic resolution
Developmental / cell cycle regulation of complex formation
Binding specificity of subunits, subcomplexes or holo-complex
Sub-cellular localization and co-localization of complex components
Conformational dynamics of the complex
Cooperativity or allosteric regulation of complex assembly/disassembly
Kinetics of complex formation (order of assembly, on-/off- rates)

Types of PPIs

Qualitative methods:

- Co-immunoprecipitation (Co-IP)
- Pull-down

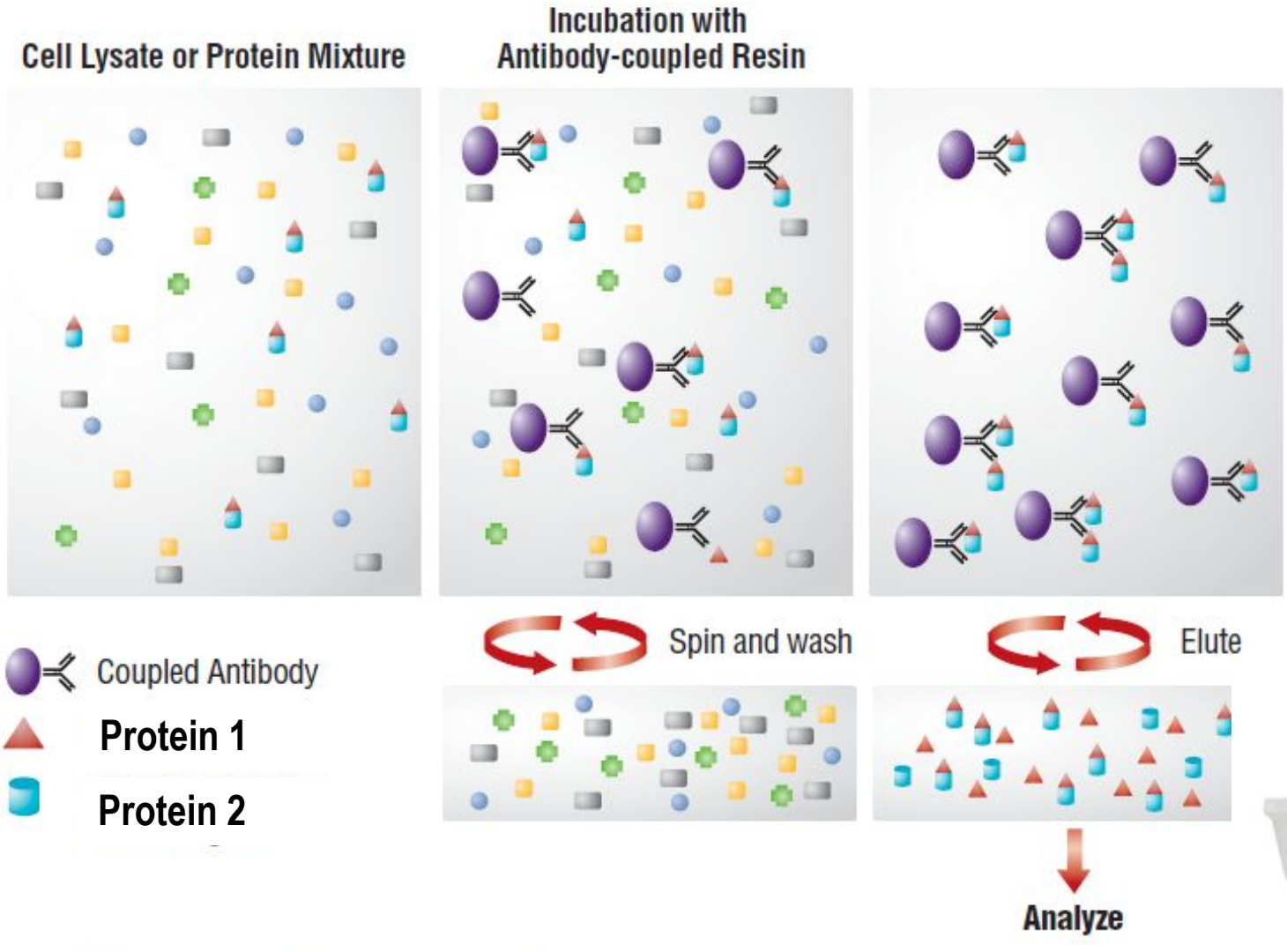


https://link.springer.com/book/10.1007%2F978-1-4939-2425-7

Quantitative methods:

- Isothermal titration calorimetry (ITC)
- Surface plasmon resonanse (SPR)
- Quartz microbalance (QMB)
- Fluorescence polarization (FP)
- others

Detecting PPI: co-immunoprecipitation (Co-IP)



http://www.piercenet.com

Detect with Ab to 2 (Immunoblot)

Reciprocal Co-IP in investigation of 14-3-3 interacting proteins

Direct

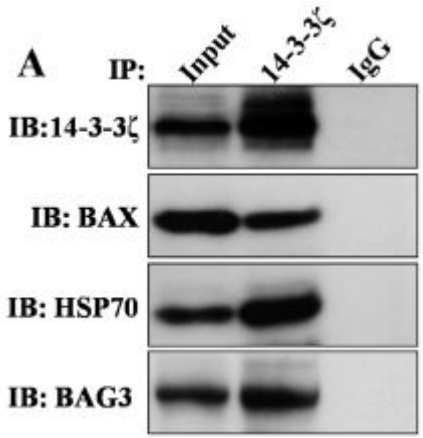
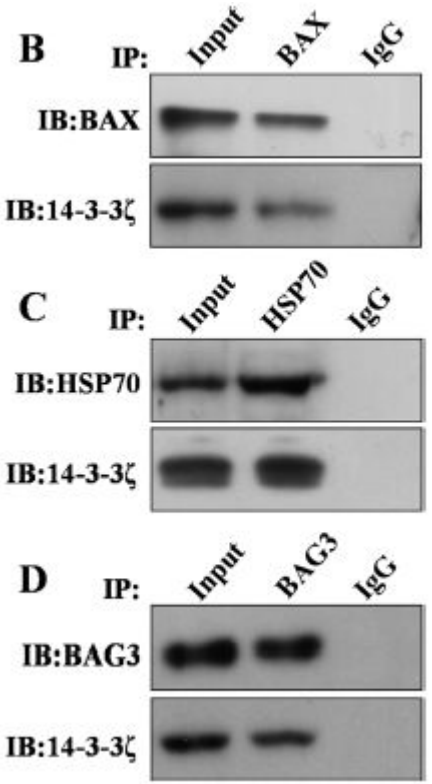


Figure 4. Immunoblot analysis after coimmunoprecipitation assay. Immunoprecipitation assays of 14-3-3ζ, BAX, HSP70, and BAG3 proteins were carried out in U266 cells as described in Experimental Procedures. (A) Immunoblot analysis demonstrated that 14-3-3ζ protein binds with BAX, HSP70, and BAG3 proteins. No band of 14-3-3ζ was observed in the negative control (IgG). Input stands for the total cell lysate extracted from U266 cells. Similarly, reverse immunoprecipitation assays confirmed the binding of (B) BAX, (C) HSP70, and (D) BAG3 protein with 14-3-3ζ.

Immunoprecipitation of 14-3-3 and detection of bound partner proteins

Reverse



Immunoprecipitation of partner proteins and detection of 14-3-3

Ge et al, J.Proteom.Res., 2010: 5848-5858

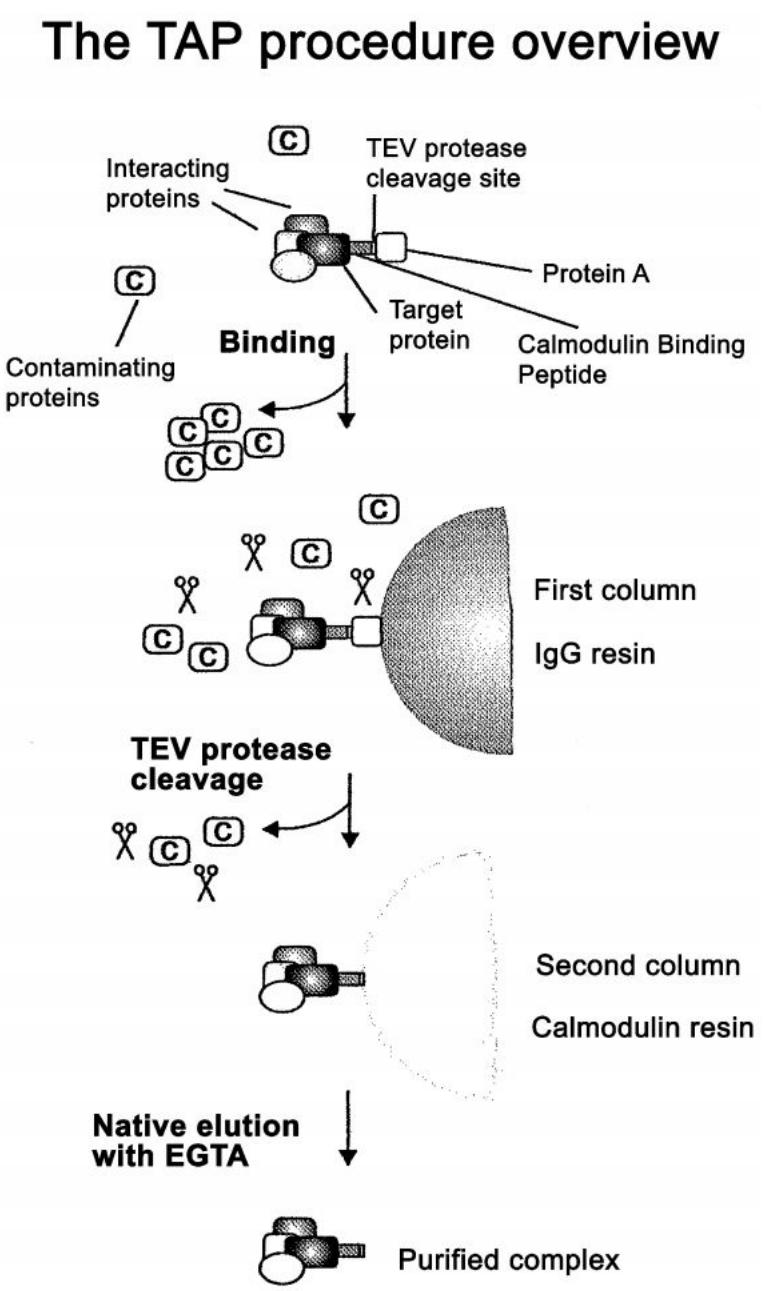
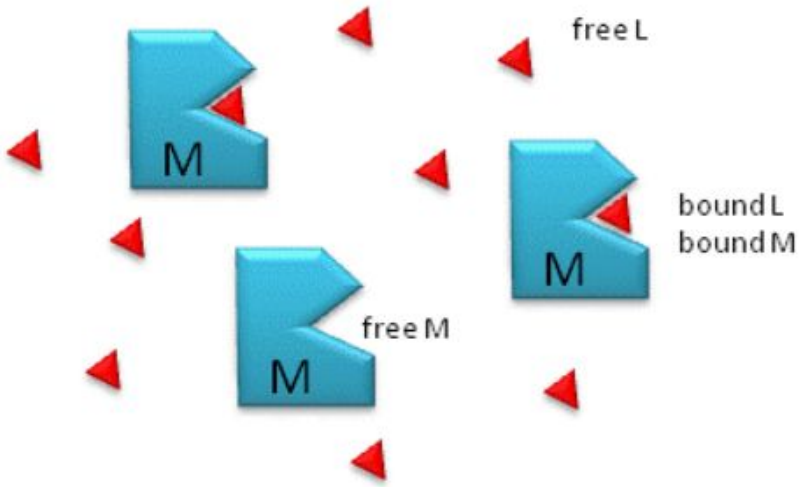


Fig. 1. Overview of the TAP strategy.

Tandem affinity purification (TAP)

M + L <--> ML



M is free macromolecule
L is free ligand
ML is complex

Case 1 (specific)

Lo >> Mo, Lo=L_{free} or you can measure L_{free}

hyperbola $$ML = \frac{Mo * L_{free}}{K_D + L_{free}}$$

Case 2 (general)

Lo $\not>>$ Mo, you can't measure L_{free}

parabola $$K_D = \frac{(Mo - ML) * (Lo - ML)}{ML}$$

$$ML = \{-(Lo+Mo+K_D) +/- \sqrt{(Lo+Mo+K_D)^2 - 4MoLo)}\}/2$$

https://employees.csbsju.edu/hjakubowski/classes/ch331/bind/olbindderveq.html

Simple binding A+B ↔ AB quadratic equation

$$A + B \rightleftharpoons AB$$

$$K = \frac{[A]*[B]}{[AB]} = \frac{\left([A]_0 - [AB]\right)*\left([B]_0 - [AB]\right)}{[AB]}$$, where $[A]_0$ and $[B]_0$ are total or initial concentrations, K is the K_d

$$[AB]*K = \left([A]_0 - [AB]\right)*\left([B]_0 - [AB]\right)$$

$$[AB]*K = \left([A]_0*[B]_0\right) - \left([AB]*[A]_0\right) - \left([AB]*[B]_0\right) + [AB]^2$$

$$[AB]^2 - \left([A]_0 + [B]_0 + K\right)*[AB] + [A]_0*[B]_0 = 0,$$ which is the form of

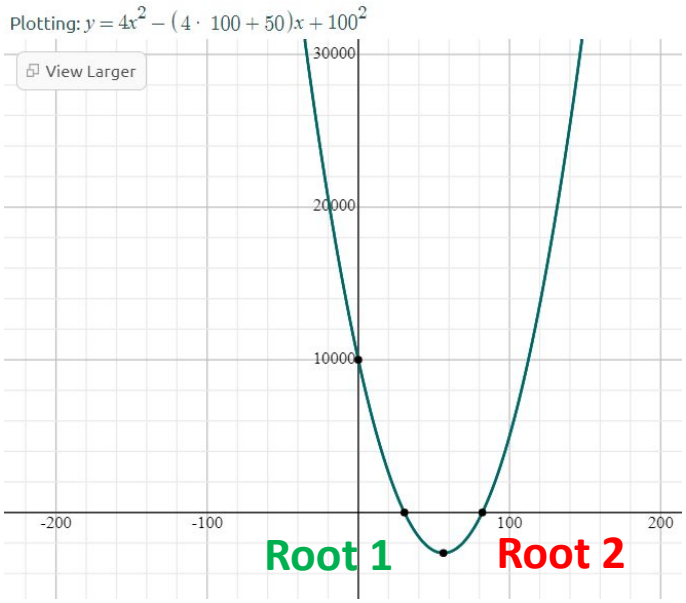
$$ax^2 + bx + c = 0$$ where $a = 1, b = -\left([A]_0 + [B]_0 + K\right), c = [A]_0*[B]_0$, thus

$$[AB] = \left(\left([A]_0 + [B]_0 + K\right) - \sqrt{\left([A]_0 + [B]_0 + K\right)^2 - 4*[A]_0*[B]_0}\right)/2$$

Online quadratic equation solver:
(just put your numbers for Ao, Bo, K_D and choose the right root)

https://www.symbolab.com/solver/equation-calculator/%5Cleft(100-x%5Cright)%5Ccdot%5Cleft(10-x%5Cright)-15%5Ccdot%20x%3D0

Simple binding A+B ↔ AB quadratic equation



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Dimerization process

$M + M <==> M_2$ or D

$Kd = [M][M]/[D] = [M]^2/[D]$

$[Mo] = [M] + 2[D]$

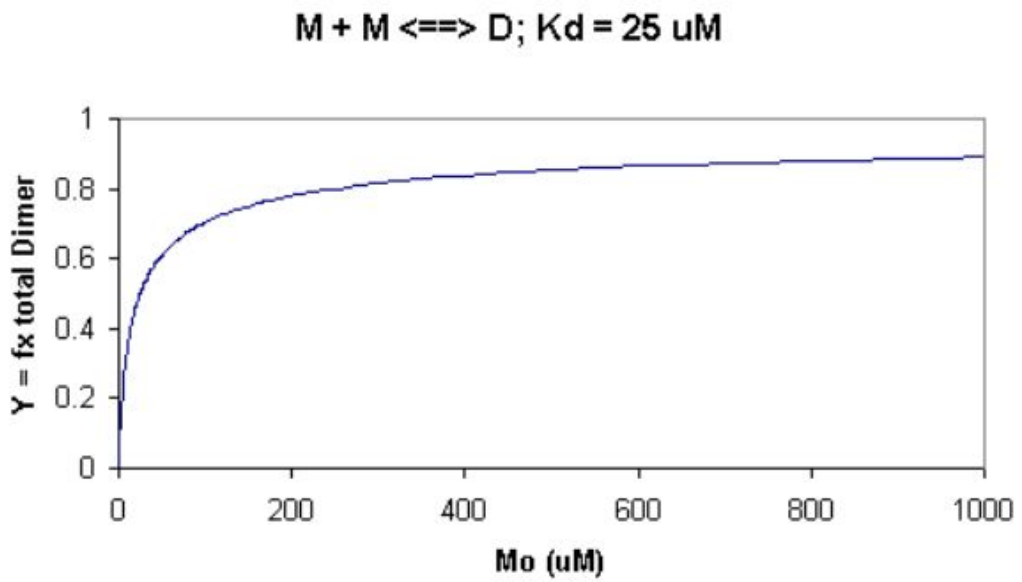
$[M] = [Mo] - 2[D]$

$Kd = (Mo-2D)(Mo-2D)/D$

$4D^2 - (4Mo+Kd)D + (Mo)^2 = 0$

$Y = 2[D]/[Mo]$

Lo $\not>>$ Mo => quadratic equation



For a reversible process, one can assess thermodynamics of binding

$$A + B \rightleftharpoons AB$$

$$Kd = 1/Keq$$

$$\Delta G^o = - R\ T\ \ln Keq = R\ T\ \ln Kd$$

@ 20 °C

25 µM = $25*10^{-6}$ M

$$\Delta G^o = R*T * (-10.6) = -6.2 \text{ kcal/mol}$$

2 cal/mol*K

For a reversible process, one can assess thermodynamics of binding

$$A + B \rightleftharpoons AB$$

$$Kd = 1/Keq$$

$$\Delta G^o = - R\ T\ \ln Keq = R\ T\ \ln Kd$$

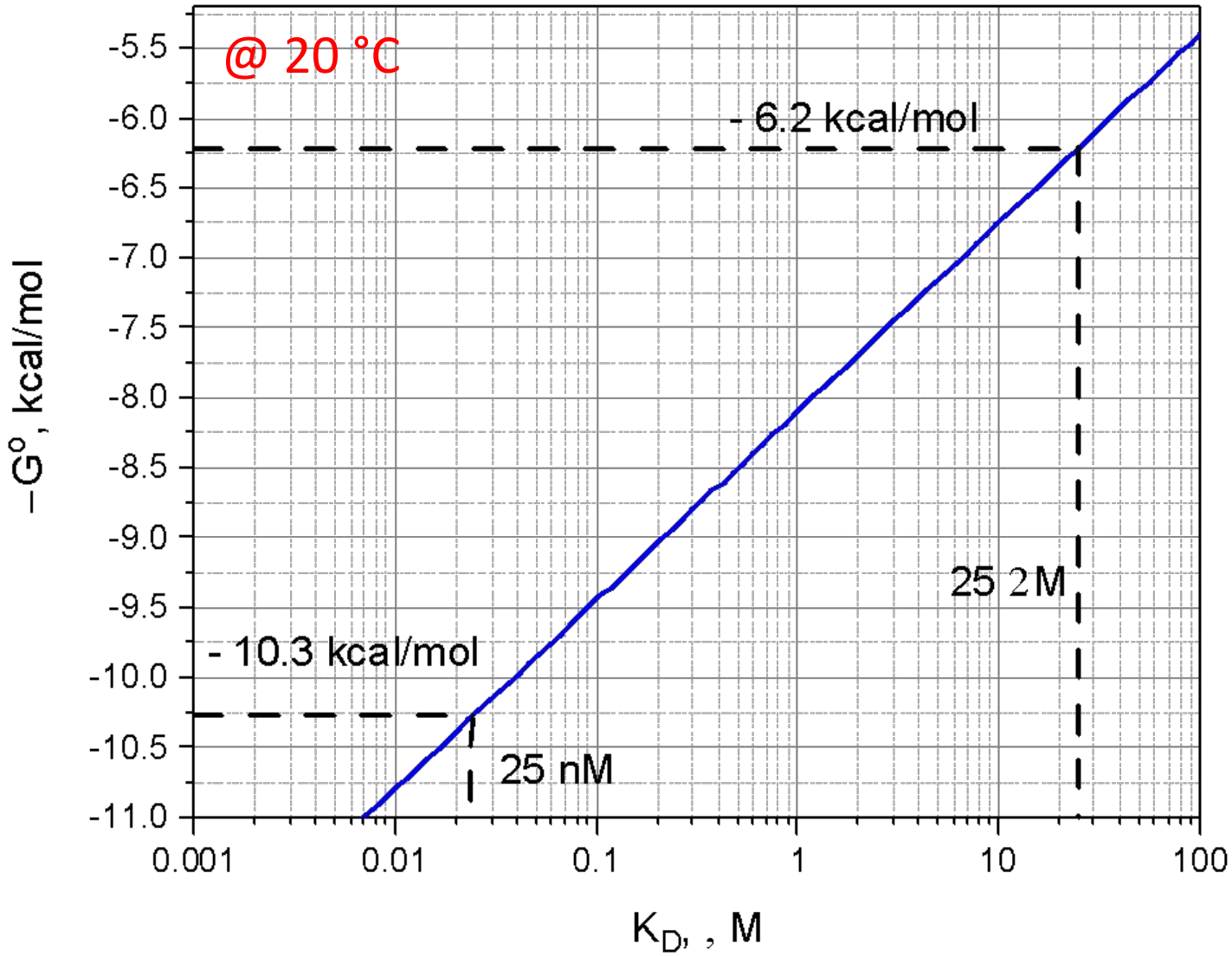
@ 20 °C

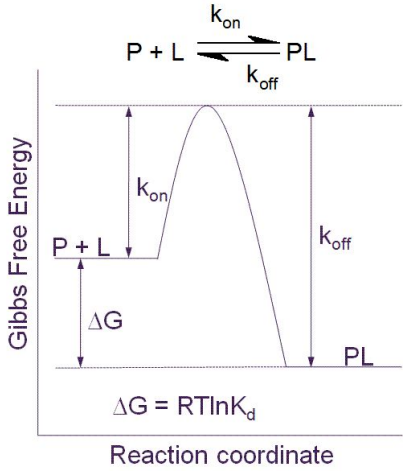
25 nM = $25*10^{-9}$ M

$$\Delta G^o = R*T * (-17.5) = -10.3 \text{ kcal/mol}$$

2 cal/mol*K

$\Delta G^o = R\,T\,\ln K_D$





At equilibrium, both forward and reverse reaction rates are equal

$V_{on} = V_{off}$

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

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

$k_{off} / k_{on} = [A][B] / [AB] =$ Kd = 1/Keq

Thermodynamics of interaction

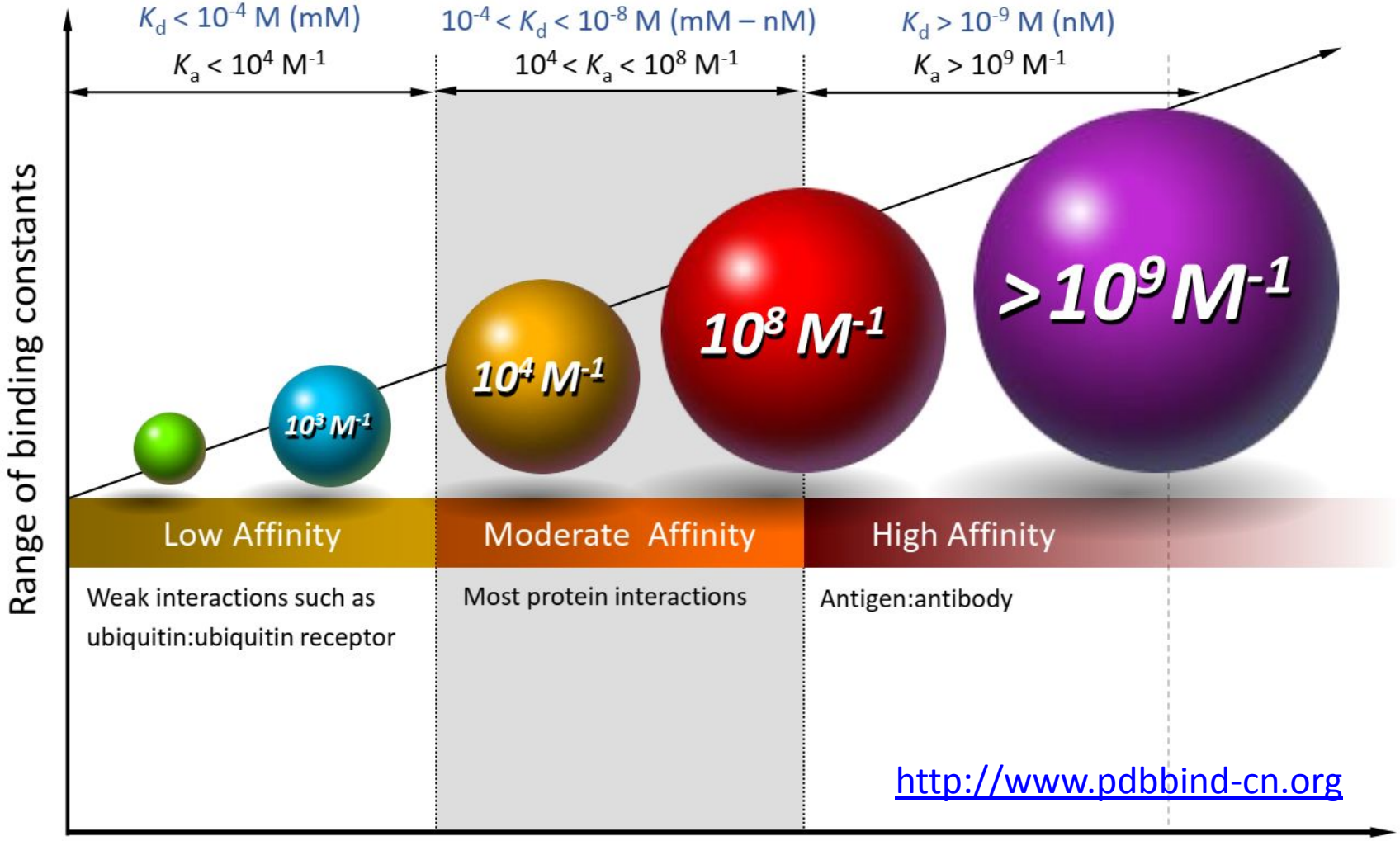
$R\,T\,\ln K_D = \Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$

Gibbs free energy — Enthalpy — Entropy

Summary of the Four Scenarios for Enthalpy and Entropy Changes

	$\Delta H > 0$ (endothermic)	$\Delta H < 0$ (exothermic)
$\Delta S > 0$ (increase in entropy)	$\Delta G < 0$ at high temperature $\Delta G > 0$ at low temperature Process is spontaneous at high temperature	$\Delta G < 0$ at any temperature Process is spontaneous at any temperature
$\Delta S < 0$ (decrease in entropy)	$\Delta G > 0$ at any temperature Process is nonspontaneous at any temperature	$\Delta G < 0$ at low temperature $\Delta G > 0$ at high temperature Process is spontaneous at low temperature

Binding affinity range



http://www.pdbbind-cn.org

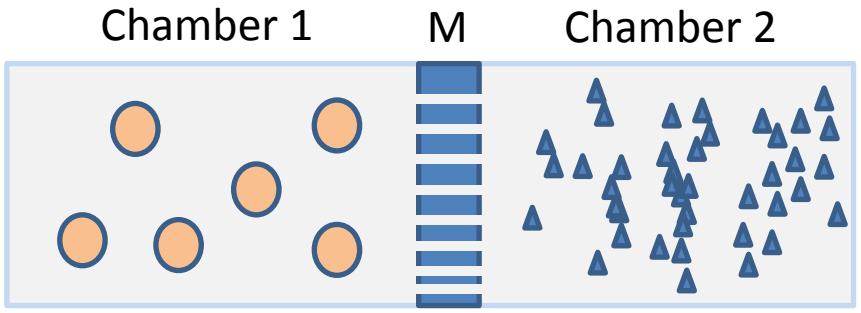
1,772,210 binding data : http://www.bindingdb.org/bind/index.jsp

Methods to study PPI
(and other interactions!)

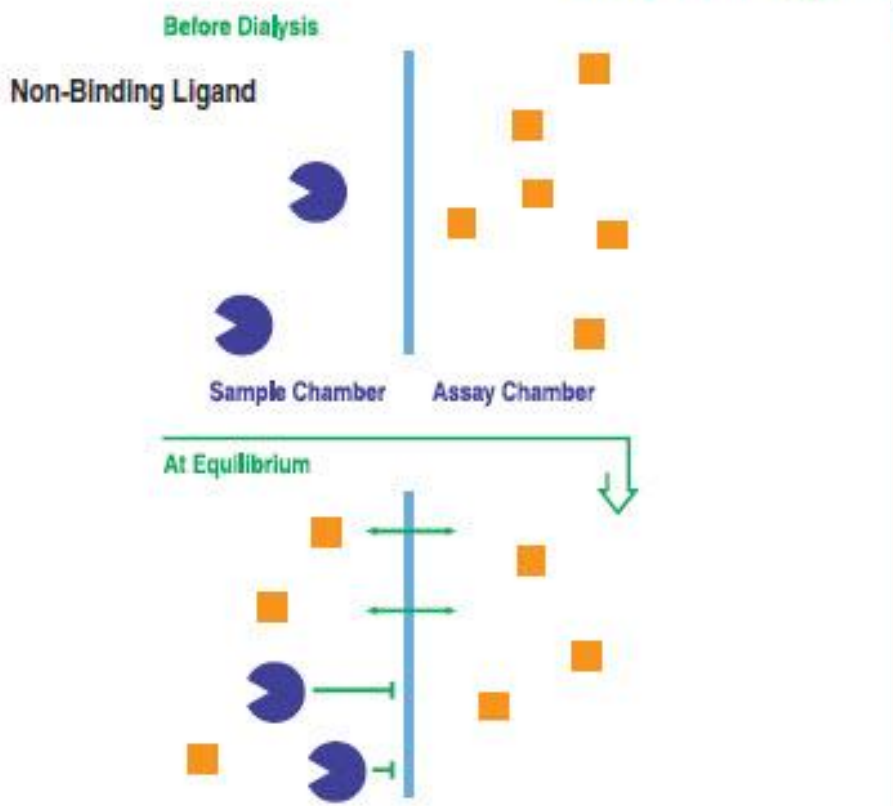
- Equilibrium microdialysis (EMD)
- Fluorescence polarization (FP)
- Isothermal titration calorimetry (ITC)
- Microscale thermophoresis (MST)
- Surface plasmon resonance (SPR)
- Biolayer interferometry (BLI)
- Quartz crystal microbalance (QCM)

Equilibrium microdialysis (EMD)

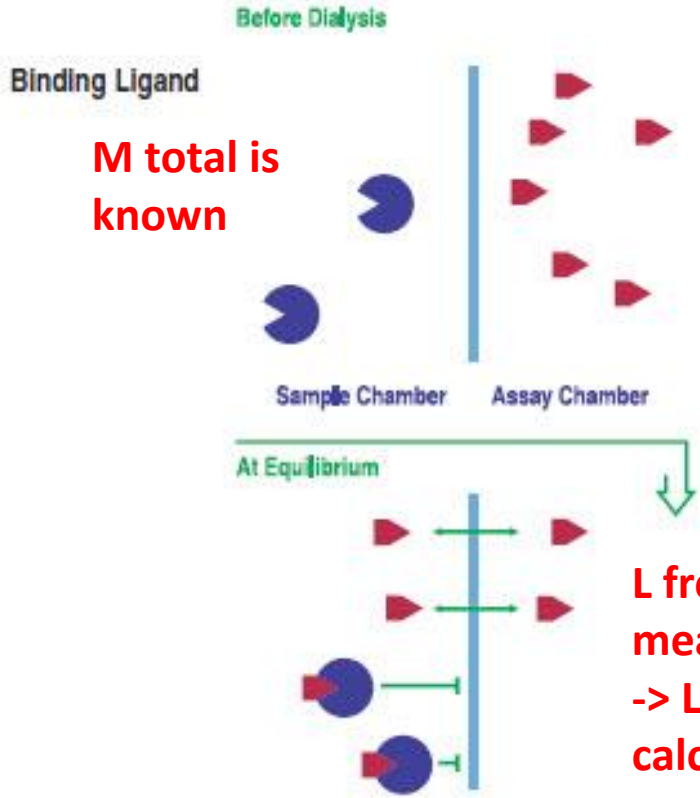
- Two chambers of equal volume facing each other
- Semipermeable membrane separates the two chambers
- MW cutoff of the membrane allows a ligand to pass through
- Macromolecule with MW higher than cutoff remains in its chamber
- The initial concentrations are known precisely
- The experiment runs till reaching an equilibrium
- At equilibrium, concentrations of L in both chambers are measured
- Parameters of interaction are determined



Equilibrium microdialysis (EMD)



If the ligand and protein do not bind to each other the ligand is free to cross the membrane. At equilibrium, the concentration of the ligand in the assay chamber will be exactly half that initially placed in the sample chamber.



If the ligand and protein form a complex, the bound ligand will be unable to diffuse across the membrane and will remain in the sample chamber. The concentration of the ligand will still be equivalent on either side of the membrane upon reaching equilibrium. In this case, however, the ligand concentration in the assay chamber is reduced by the total amount of ligand bound to the protein divided by two.

Equilibrium microdialysis (EMD)

$$M + L \leftrightarrow ML$$

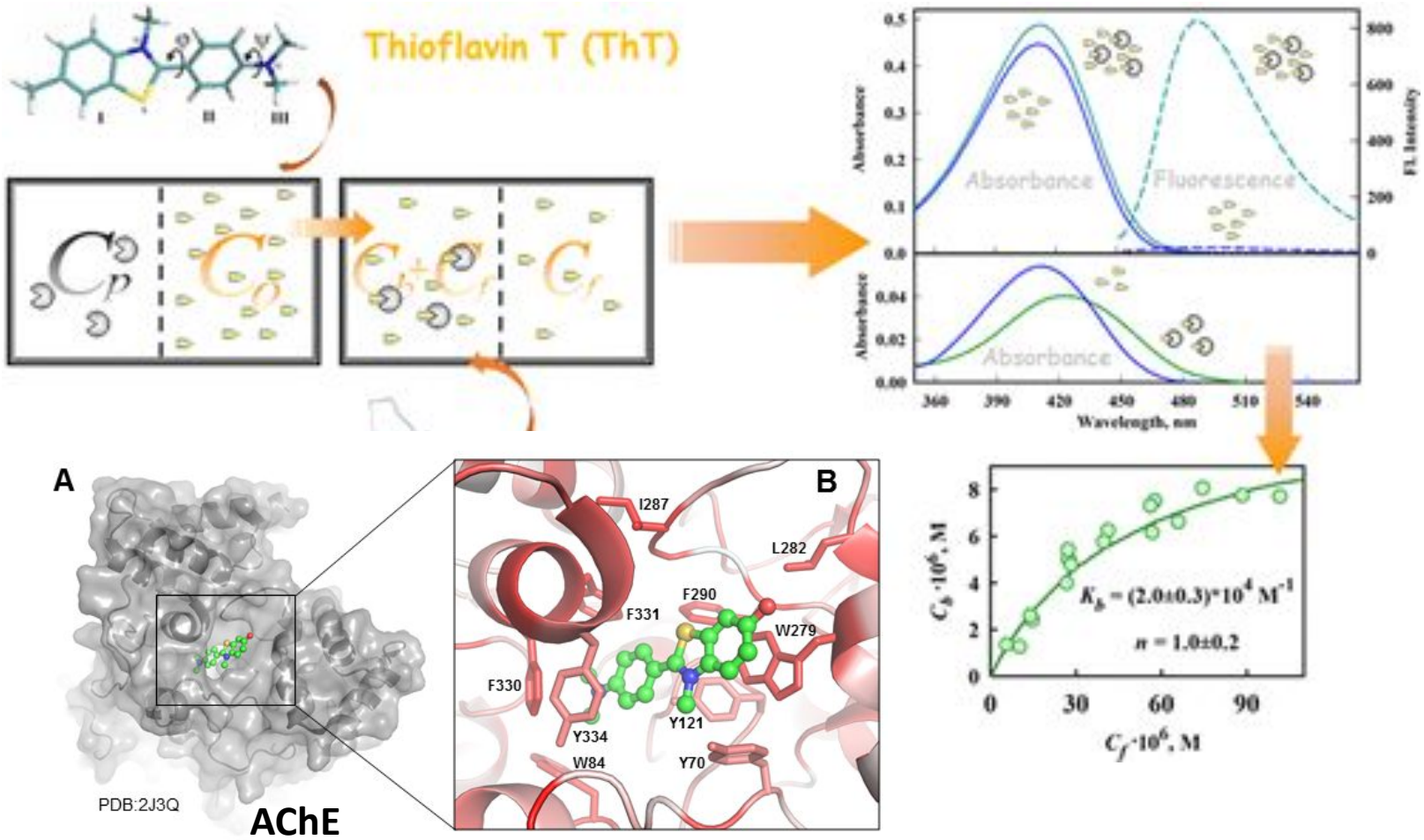
$$K_D = \frac{[M] * [L]}{[ML]}$$

Features

- Fast
- Easy
- Inexpensive
- Accurate determination of affinity (KD) and stoichiometry of interaction
- Membrane type (pore sizes) determines the applicability to a certain M and L

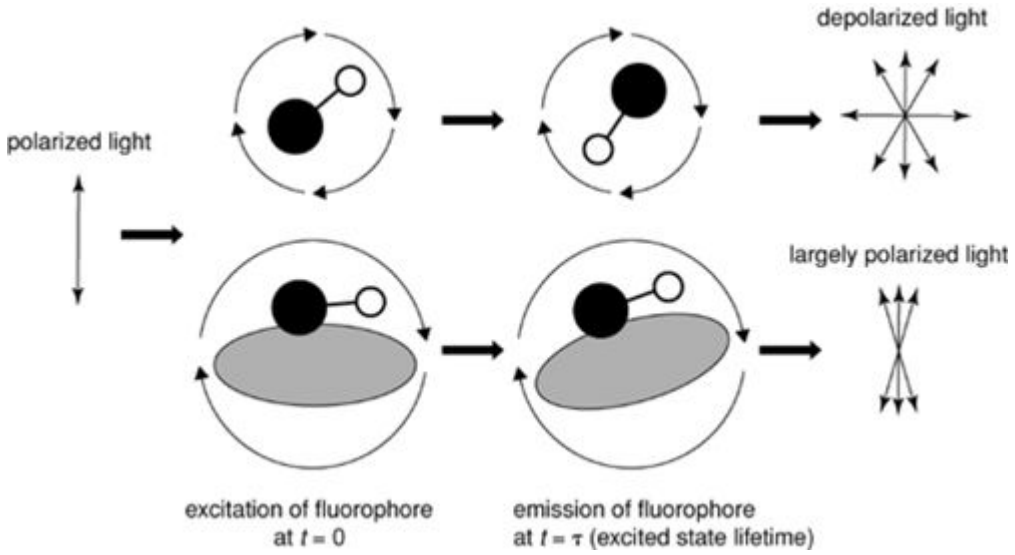
Equilibrium microdialysis (EMD)

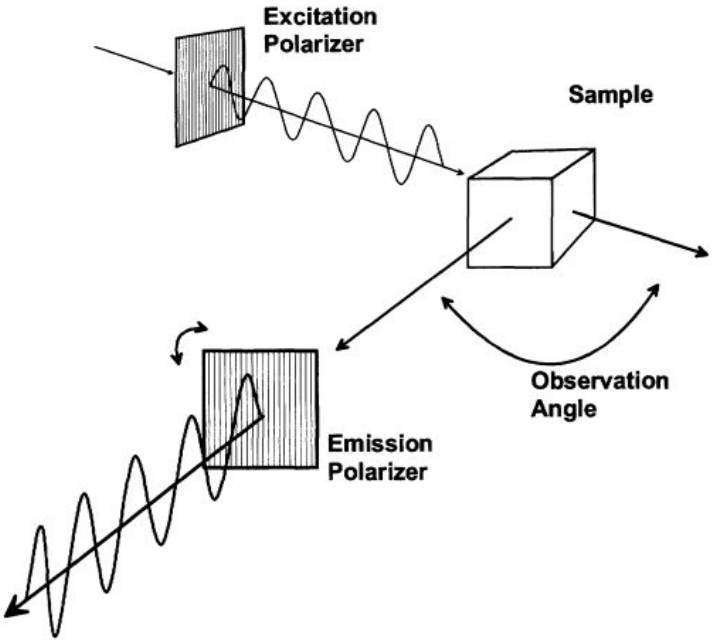
Thioflavin T (ThT) binding to acetylcholinesterase (AChE)



DOI: 10.1021/acschemneuro.8b00111

Fluorescence polarization (FP)





The degree of polarization is associated with the size of the particle bearing a fluorophore

Fluorescence polarization (P) or anisotropy (r):

$$P = \frac{(F_{\parallel} - F_{\perp})}{(F_{\parallel} + F_{\perp})} \qquad r = \frac{(F_{\parallel} - F_{\perp})}{(F_{\parallel} + 2F_{\perp})} = \frac{2P}{3 - P}$$

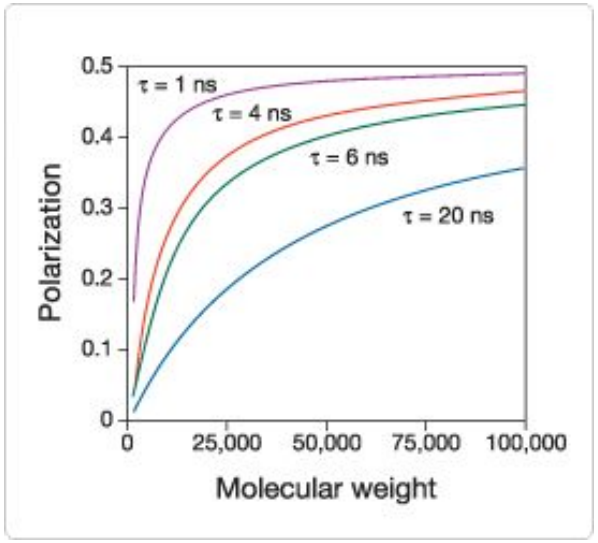
where $F_{\parallel}$ = fluorescence intensity parallel to the excitation plane

$F_{\perp}$ = fluorescence intensity perpendicular to the excitation plane

- no nominal dependence on dye concentration
- P has physically possible values ranging from –0.33 to 0.5 (never achieved)
- Typical range 0.01-0.3 or 10-300 mP (P/1000)
- Precision is normally 2 mP

Fluorescence polarization and molecular size

dyes with various fluorescence lifetimes (τ)



Simulation of the relationship between molecular weight (MW) and fluorescence polarization (P)

Φ is found to increase by ~1 ns per 2400 Da increase of MW

Perrin equation (1926):

$$\left(\frac{1}{P}-\frac{1}{3}\right)=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{\tau}{\phi}\right)$$

Fundamental P (Po) ~0.5 (max)

rotational correlation time of the dye:

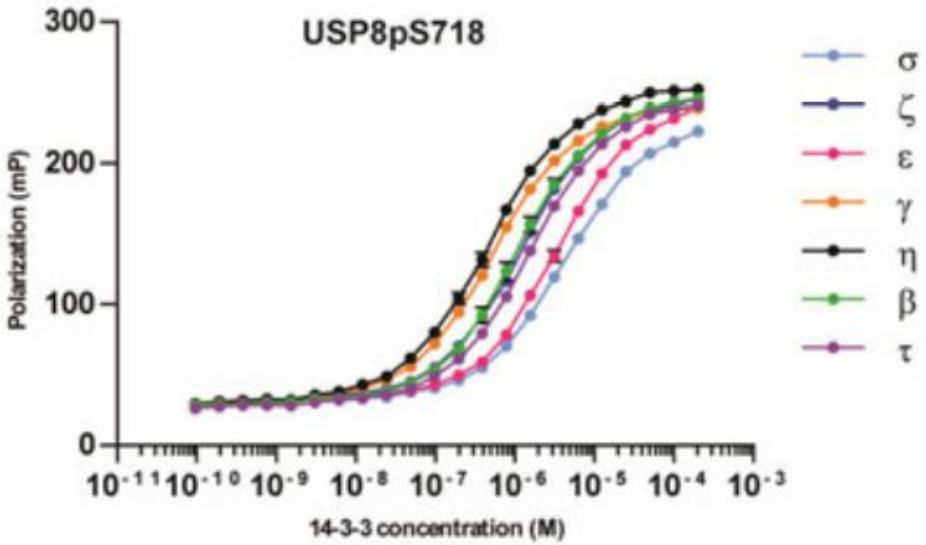
$$\phi=\frac{\eta V}{RT}$$

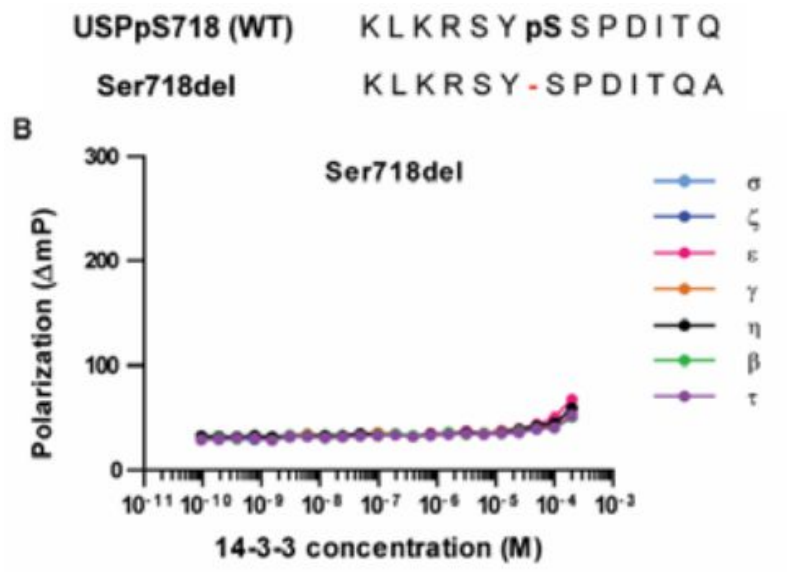
η = solvent viscosity, T = temperature, R = gas constant and V = molecular volume of the fluorescent dye (or its conjugate)

FP features

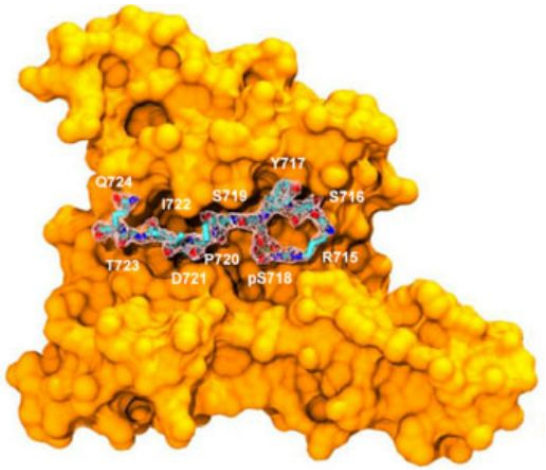
- Great tool to study interactions
- Small sample consumption
- Low limit of detection
- Rapid response
- Real-time (not only equilibrium studies)
- Kinetic analysis (association/dissociation) is possible
- Separation of bound and free species not needed
- Good for high-throughput studies

FP is very good for high-throughput studies



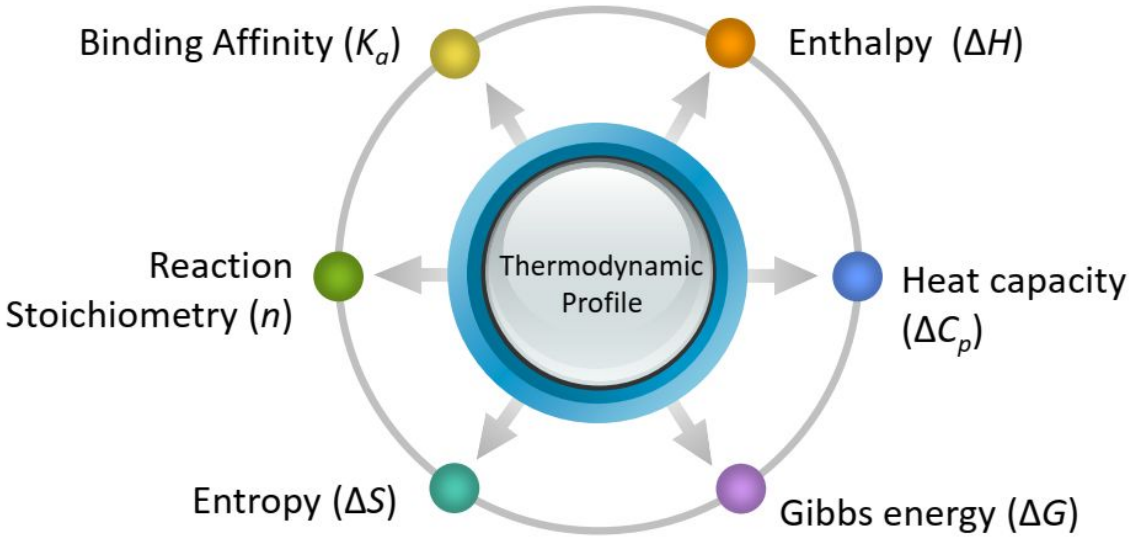


Isoform	Kd FP μM
14-3-3η	0.42 ± 0.02
14-3-3γ	0.48 ± 0.02
14-3-3β	0.96 ± 0.03
14-3-3ζ	1.07 ± 0.03
14-3-3τ	1.34 ± 0.04
14-3-3ε	2.77 ± 0.08
14-3-3σ	3.27 ± 0.11



DOI: 10.1002/1873-3468.13017

Isothermal titration calorimetry (ITC)

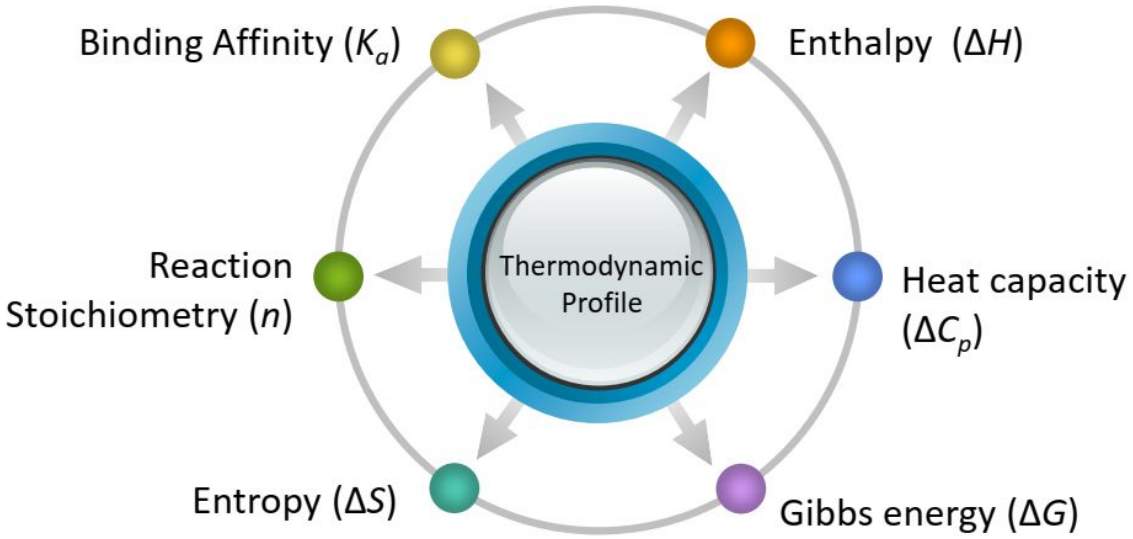


- *Calor* (Latin, *heat*) + *metry* (Greek, *measure*)
- Direct measurement of heat *q* either released or absorbed in molecular binding during gradual titration
- Label-free measurement
- Microcalorimeters: as low as 100 µl

https://www.youtube.com/watch?v=o_IpWcWKNXI

Sangho Lee (c)

Isothermal titration calorimetry (ITC)



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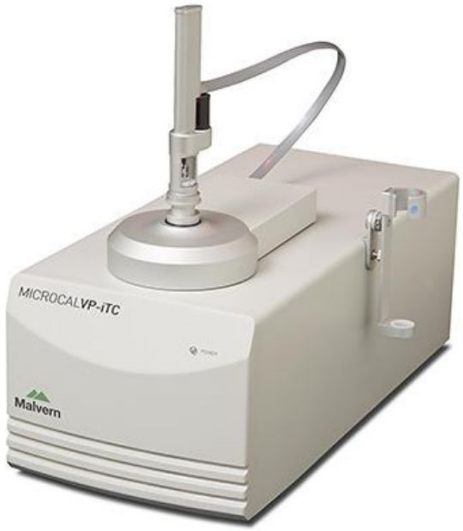
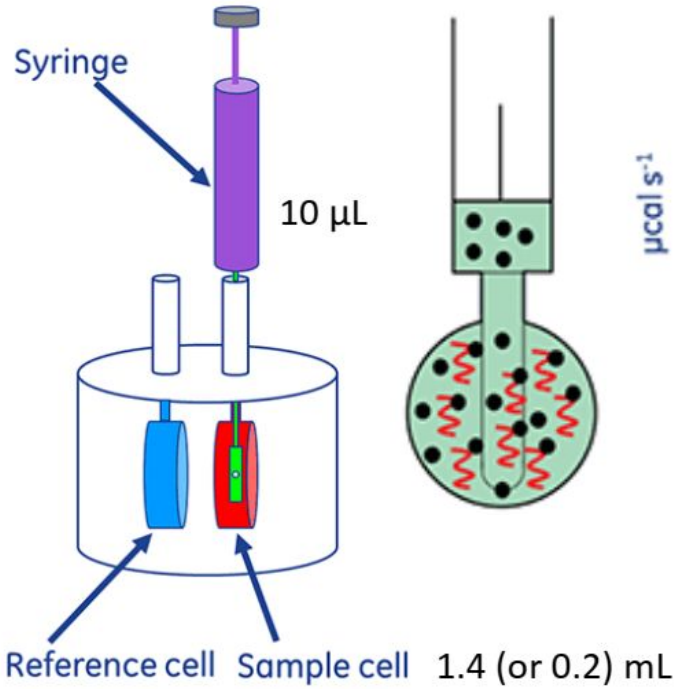
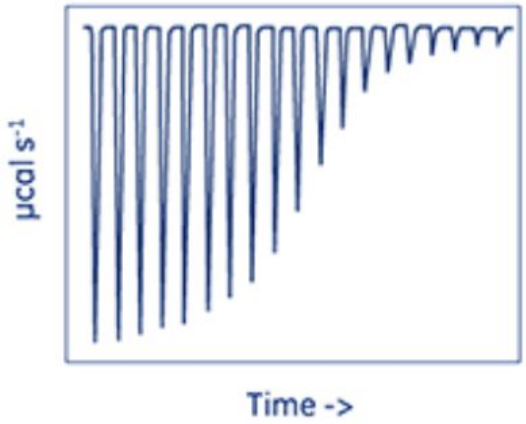
Sangho Lee (c)

ITC experiment

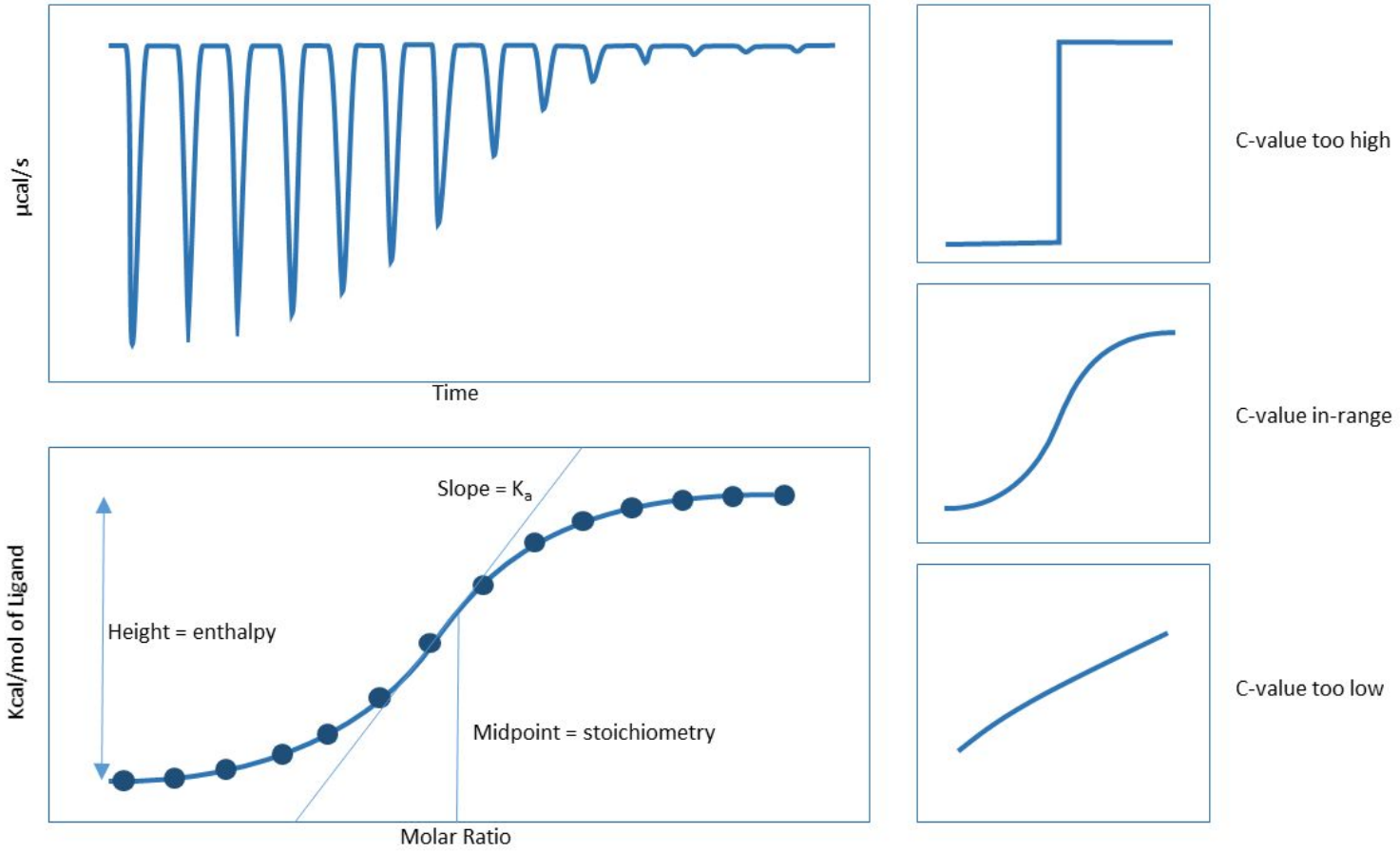






- Exothermic reaction (common for PPI)
- The sample cell becomes warmer than the reference cell
- Binding causes a downward peak in the signal
- Heat released is calculated by integration under each peak

ITC thermogram



Is calculated

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S$$

Determined in the experiment

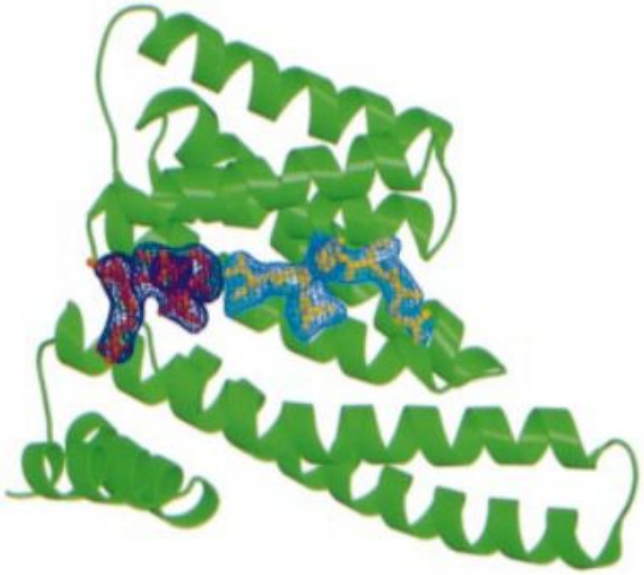
$1/K_D$

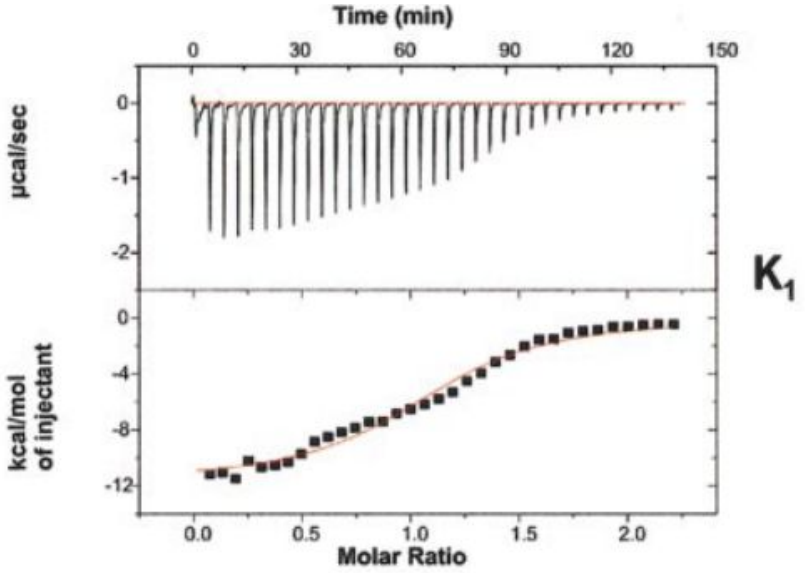
$$c = n * Ka * M$$

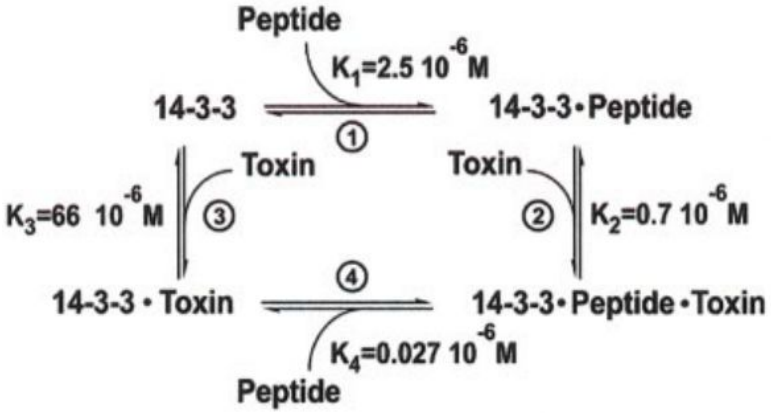
stoichiometry

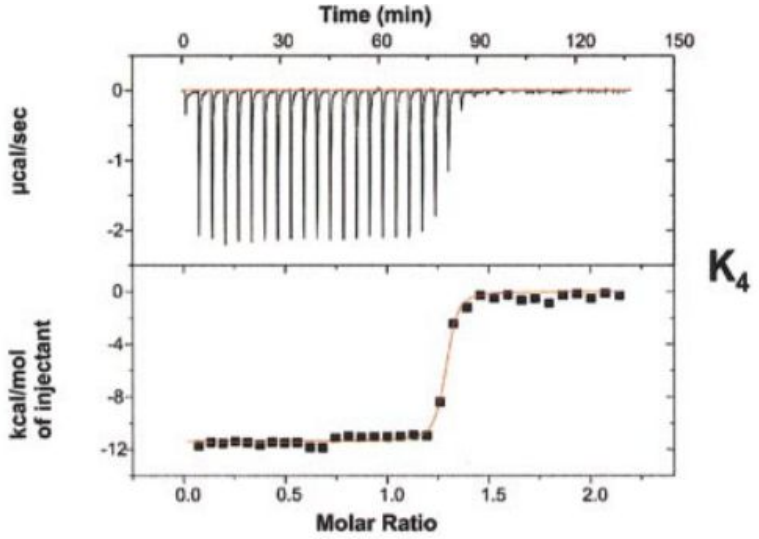
C of macromolecule in the cell

Small-molecule stabilizer of protein-peptide interaction









The EMBO Journal Vol. 22 No. 5 pp. 987–994, 2003

ITC pros and cons

Advantages:

- Ability to determine thermodynamic binding parameters (i.e. stoichiometry, association constant, and binding enthalpy) in a single experiment
- Modification of binding partners are not required

Disadvantages:

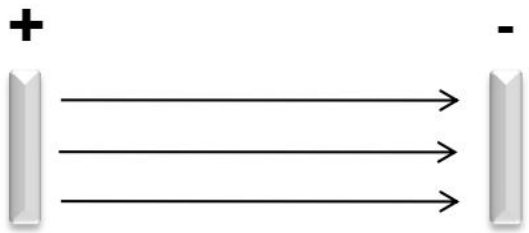
- Large sample quantity needed
- Kinetics (i.e. association and dissociation rate constants) cannot be determined
- Limited range for consistently measured binding affinities
- Non-covalent complexes may exhibit rather small binding enthalpies since signal is proportional to the binding enthalpy
- Slow with a low throughput (0.25 – 2 h/assay), not suitable for HTS

Thermophoresis

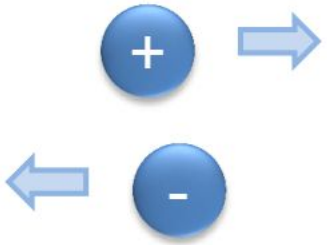
- The movement of molecules in a temperature gradient

Electrophoresis

Electric Field

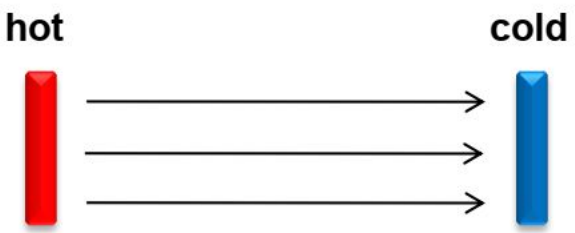


charge, (size)

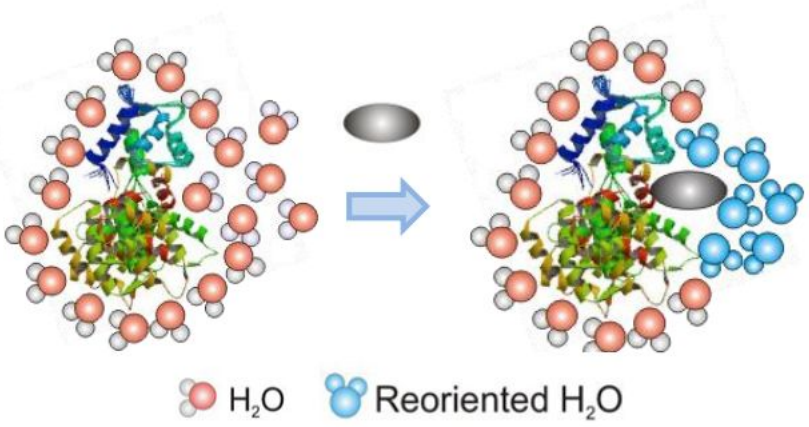


Thermophoresis

Temperature-Gradient



charge , **size** and **hydration shell**



nano TEMPER technologies

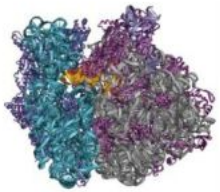
$$c_{hot}/c_{cold} = \exp(-S_T \Delta T)$$

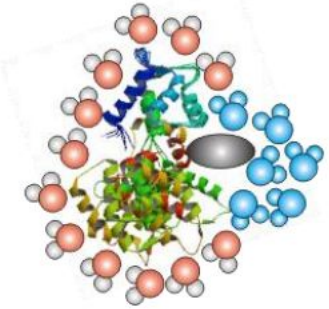
$$S_T = \frac{A}{kT}\left(-\Delta s_{hyd}(T) + \frac{\beta\sigma_{eff}^2}{4\pi\varepsilon\varepsilon_0 T} \times \lambda_{DH}\right)$$

size

hydration shell

charge ^{2}

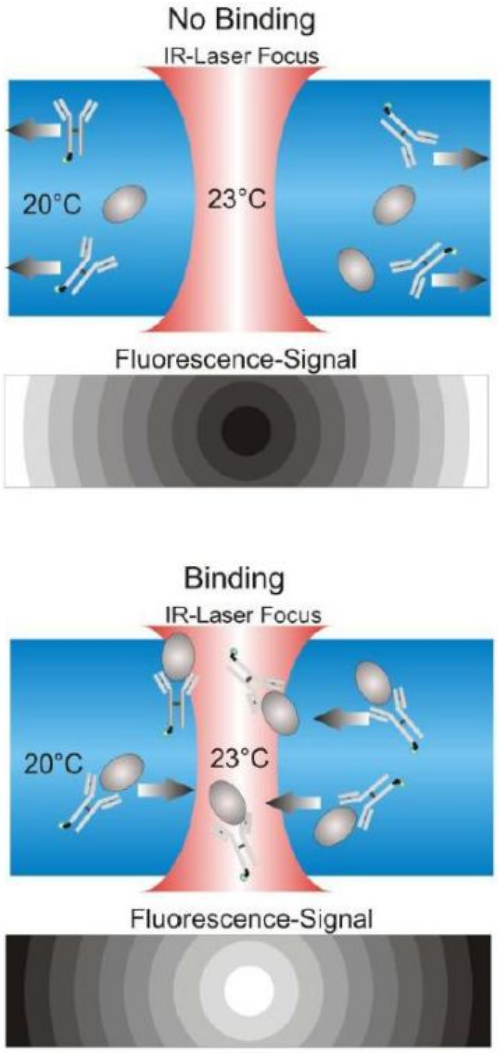


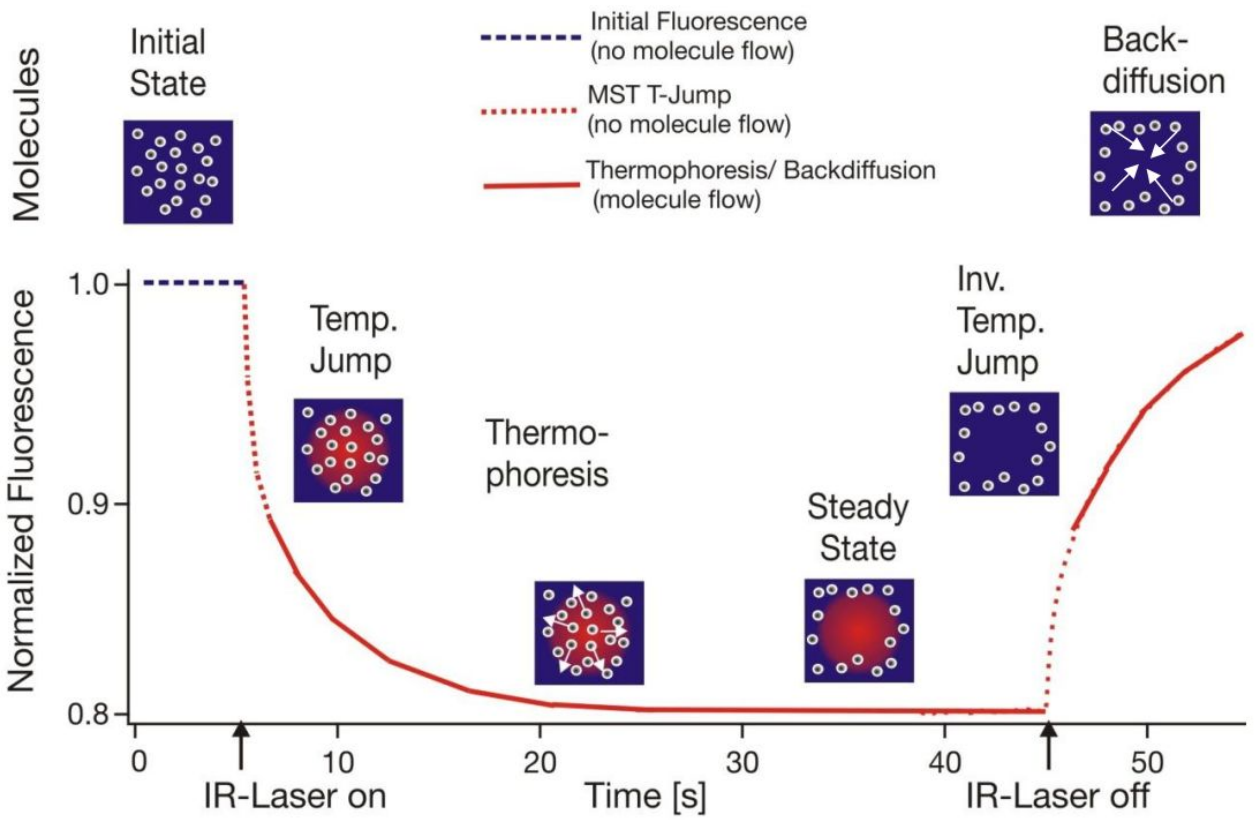


Duhr and Braun PNAS 103, 19678–19682 (2006)
Duhr and Braun PRL 96, 168301 (2006)

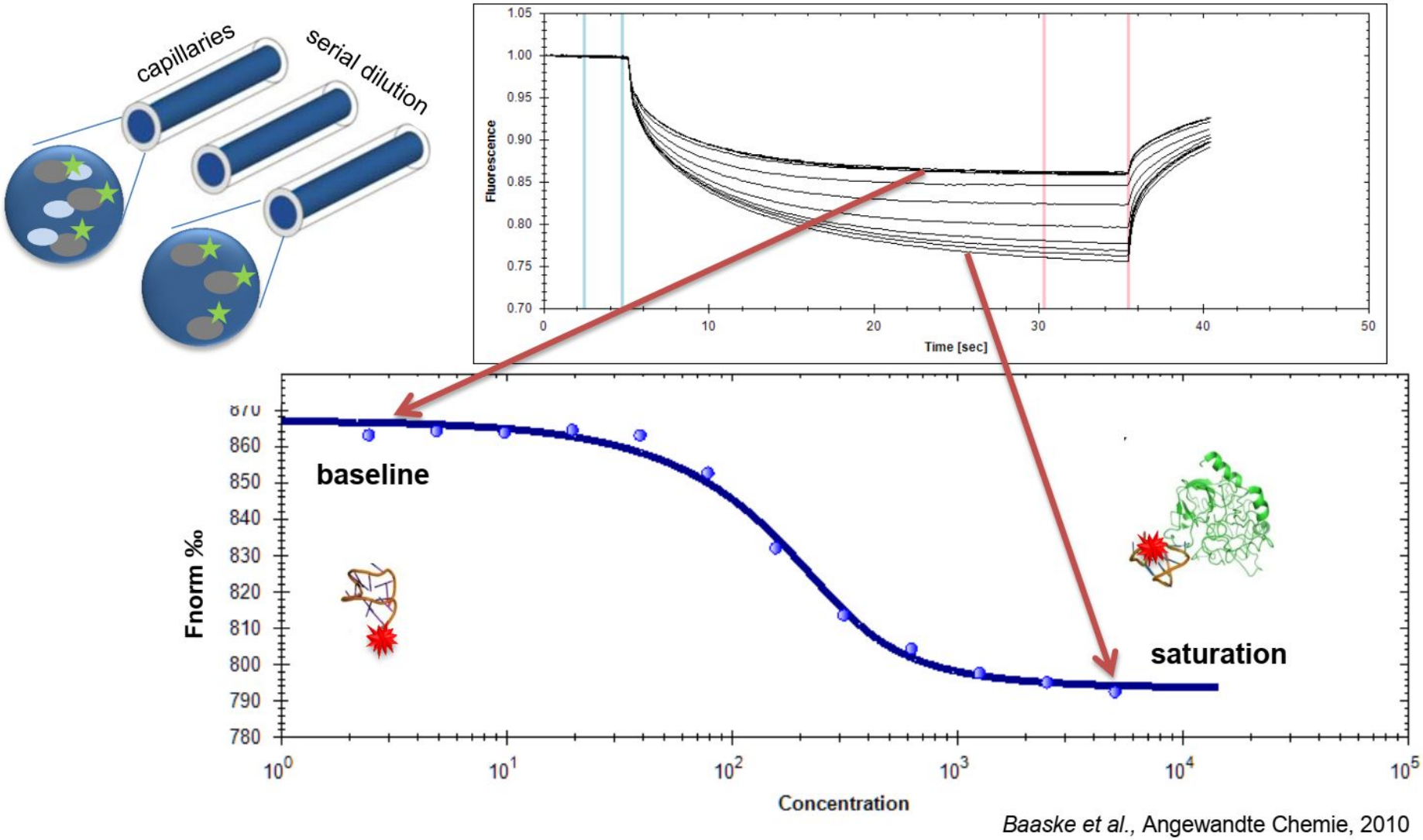
nano TEMPER technologies

Phases of MST experiment



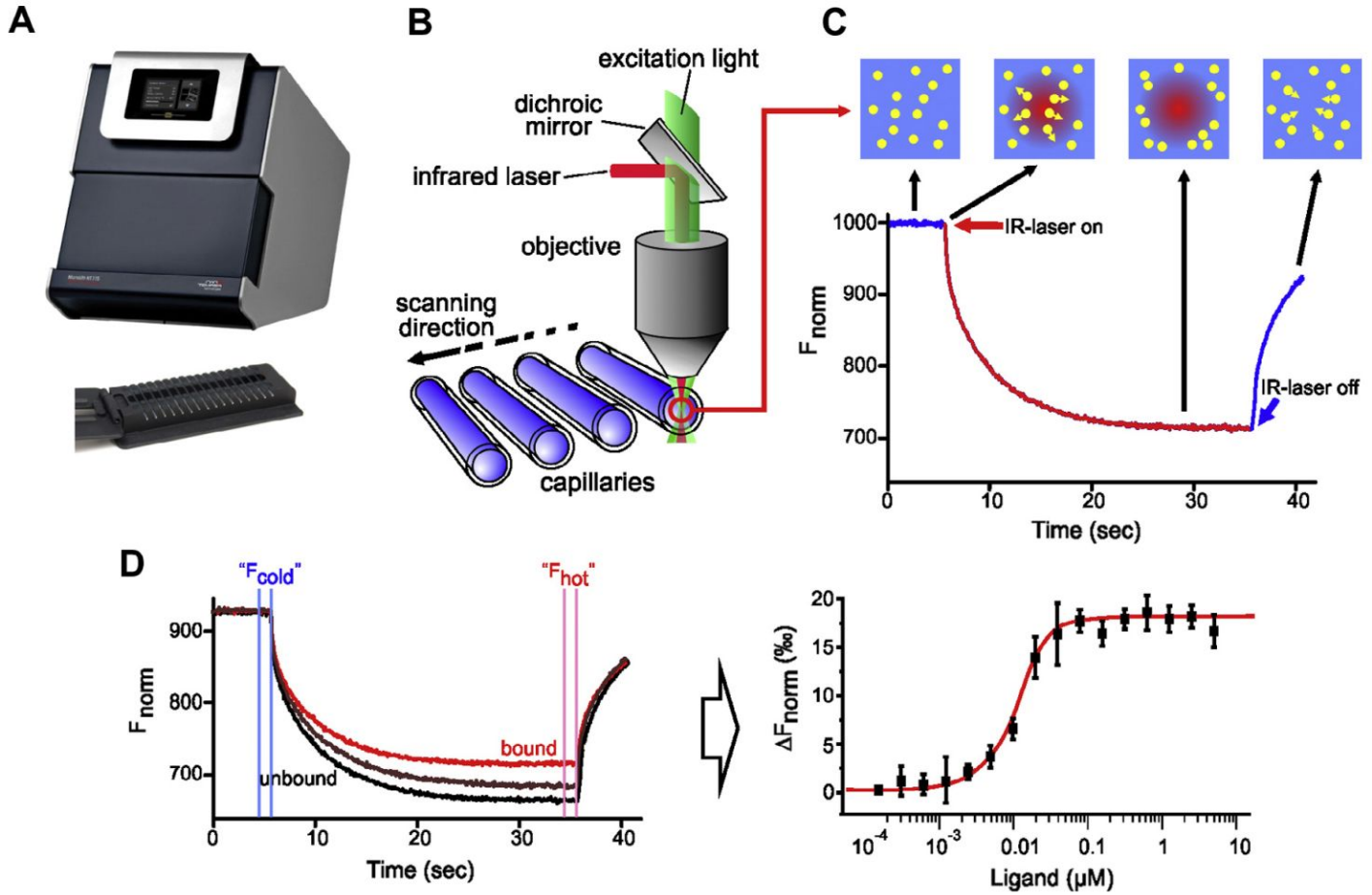


Typical MST binding curve



Baaske et al., Angewandte Chemie, 2010

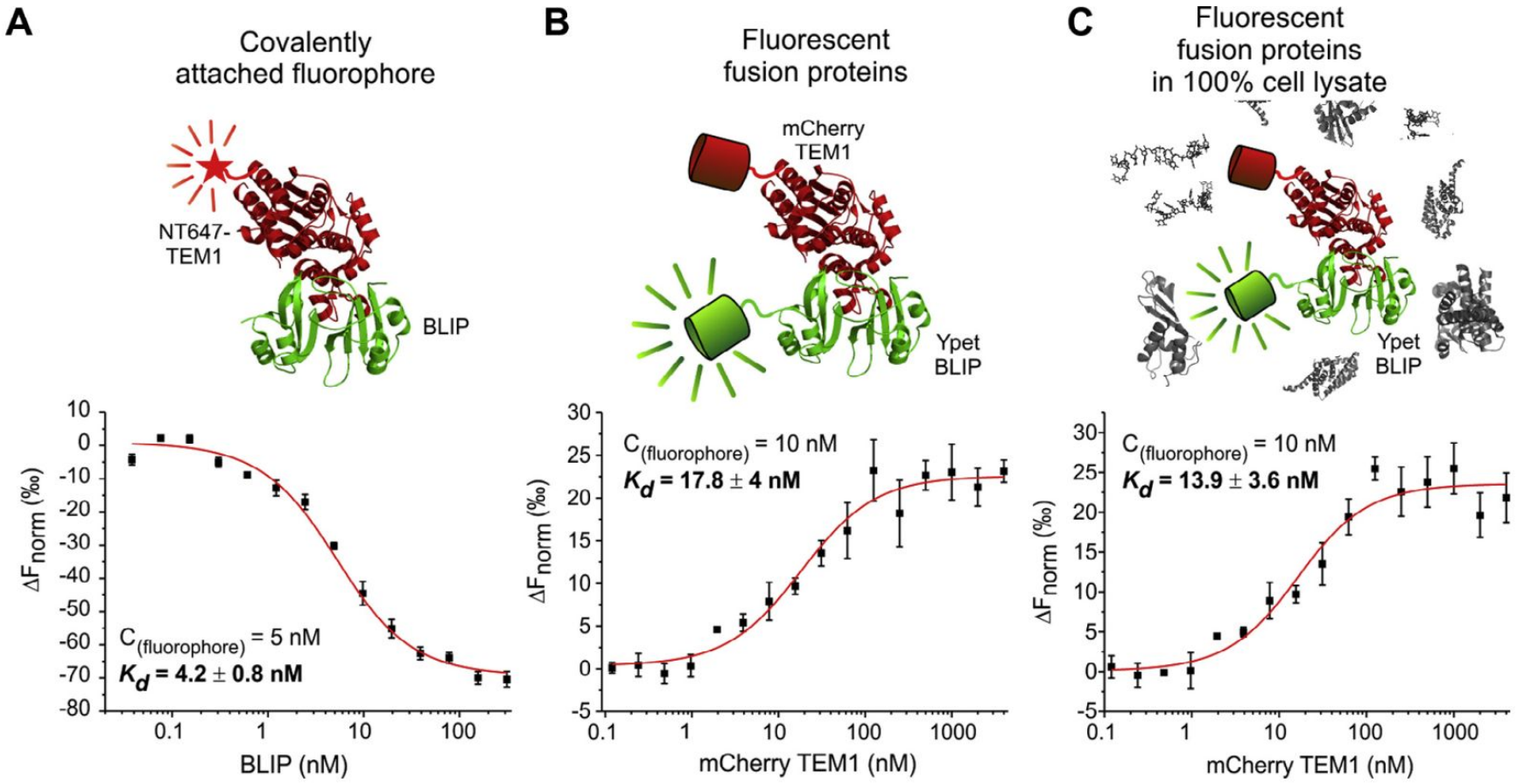
Microscale thermophoresis (MST)



https://www.youtube.com/watch?v=rCot5Nfi_Og
https://www.youtube.com/watch?v=4U-0lyHQ0wg

M. Jerabek-Willemsen et al./Journal of Molecular Structure 1077 (2014) 101–113

MST data examples



M. Jerabek-Willemsen et al./Journal of Molecular Structure 1077 (2014) 101–113

ARTICLE

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DOI: 10.1038/ncomms1093

Protein-binding assays in biological liquids using microscale thermophoresis

Christoph J. Wienken[1], Philipp Baaske[1,2], Ulrich Rothbauer[3], Dieter Braun[1] & Stefan Duhr[1,2]

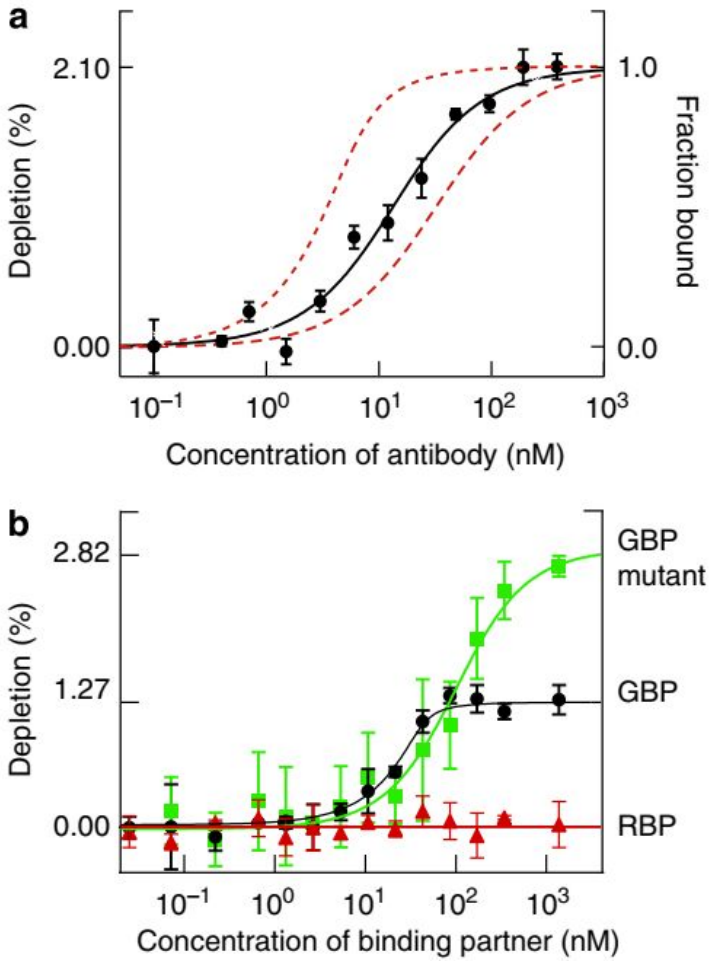


Figure 2 | Protein–protein interactions measured with MST. To determine the affinity of a binding reaction, a titration series of one binding partner is performed while the fluorescent binding partner is kept at a constant concentration. (**a**) The binding of fluorescently labelled hIFN-γ to a specific antibody is analysed with MST (black circles). The antibody is titrated from 100 pM to 700 nM. The change in the thermophoretic signal leads to a $K_d = 10 \pm 2$ nM. For comparison, the dashed lines show binding curves for 1 and 30 nM. (**b**) The interaction of the intrinsic fluorescent protein GFP with binders of varying affinities is measured. The GBP shows a high affinity of 2.3 ± 2.1 nM (black circles), which is confirmed by a reference experiment on an Attana quartz crystal microbalance device. To ensure that the measured interaction truly represents specific binding, a GBP mutant (R37A) and non-binding RBP are further analysed with MST. The exchange of the arginine at the binding interface reduces the affinity to 80 ± 38 nM (green squares), while the RBP shows the expected baseline (red triangles). The error bars represent the s.d. of each data point calculated from three independent thermophoresis measurements.

MST pros and cons

Advantages:

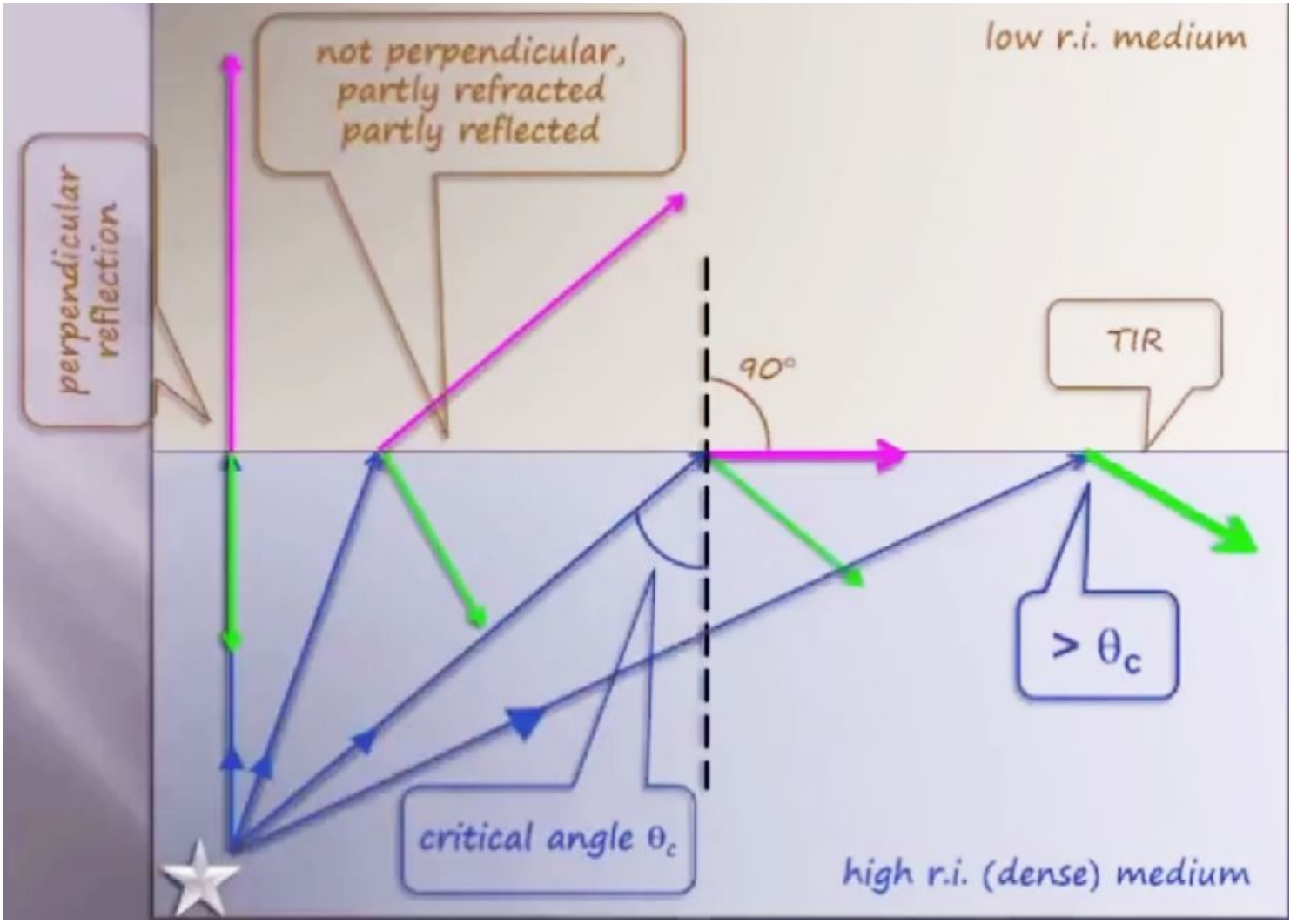
- Small sample size
- Immobilization free
- Minimal contamination of the sample (method is entirely optical and contact-free)
- Ability to measure complex mixtures (i.e. cell lysates, serum, detergents, liposomes)
- Wide size range for interactants (ions to MDa complexes)

Disadvantages:

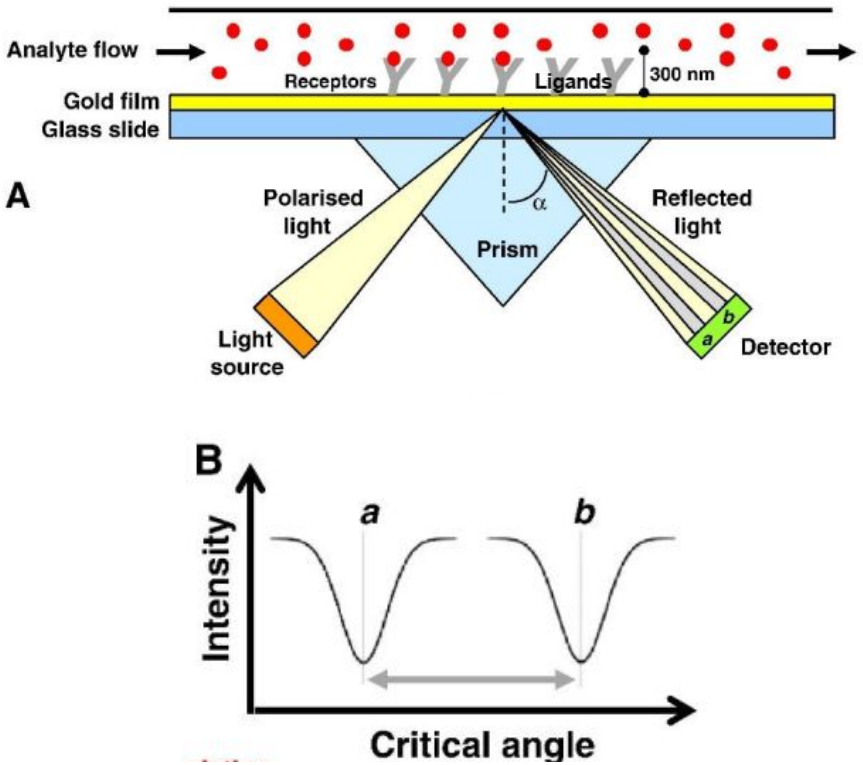
- Hydrophobic fluorescent labelling required, may cause non-specific binding
- No kinetic information (i.e. association and dissociation rates)
- Highly sensitive to any change in molecular properties

Surface plasmon resonance (SPR)

Reflection and refraction at different angles



Surface plasmon resonance (SPR)



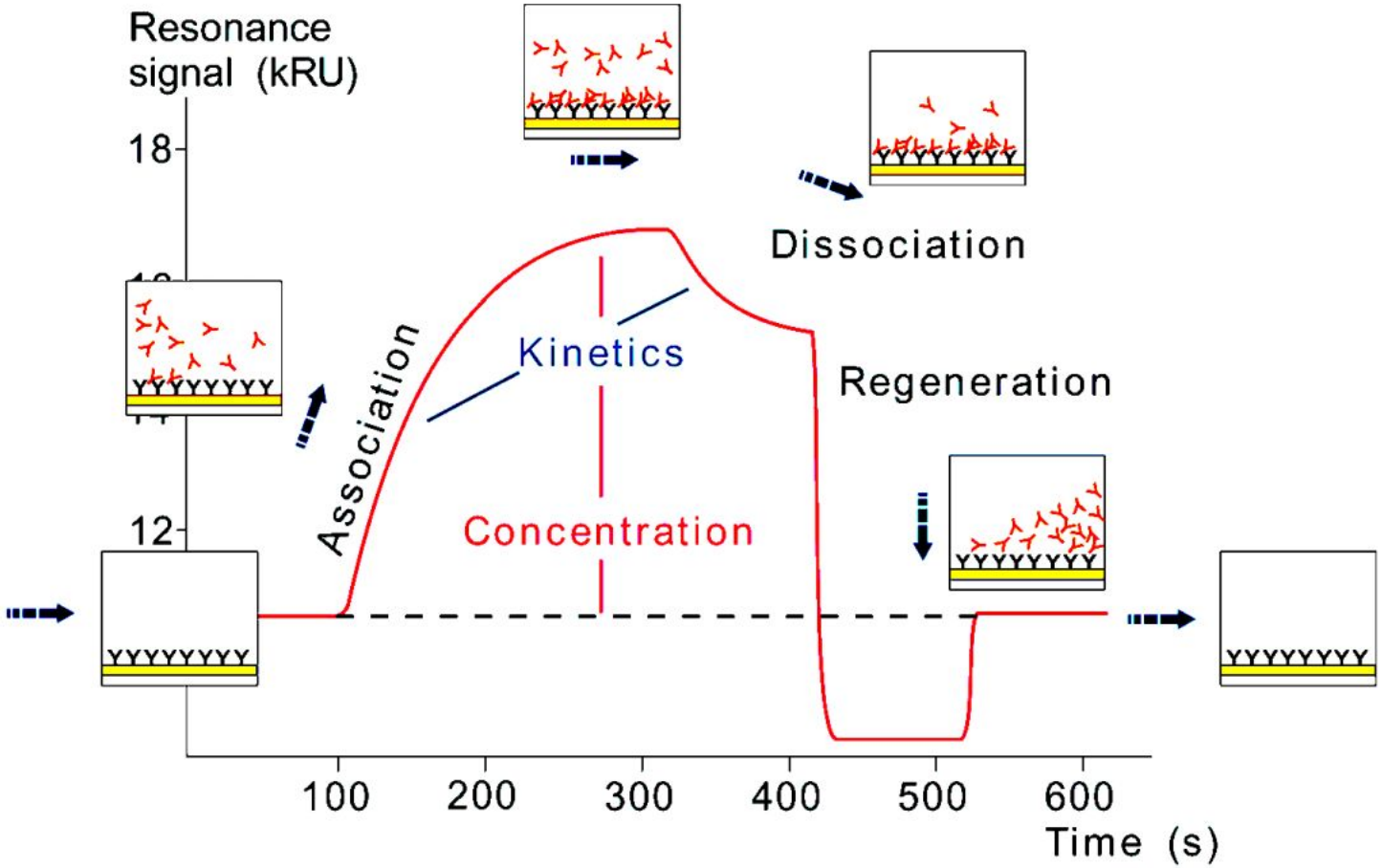
- To measure the refractive index near to a sensor surface
- Polarised light is directed through a prism to the under surface of the gold film where surface plasmons are generated at a critical angle of the incident light.
- This absorption of light is seen as a decrease in intensity of the reflected light. Resonance or response units (RU) are used to describe the increase in the signal, where 1 RU is equal to a critical angle shift of 10^{-4} deg or 10^{-12} g mm^{-2}.
- When a steady-state is achieved (all binding sites occupied), the maximum RU is determined (*n*: No. of binding sites in Ligand)

$$RU_{max} = nRU_L\left(\frac{MW_A}{MW_L}\right)$$

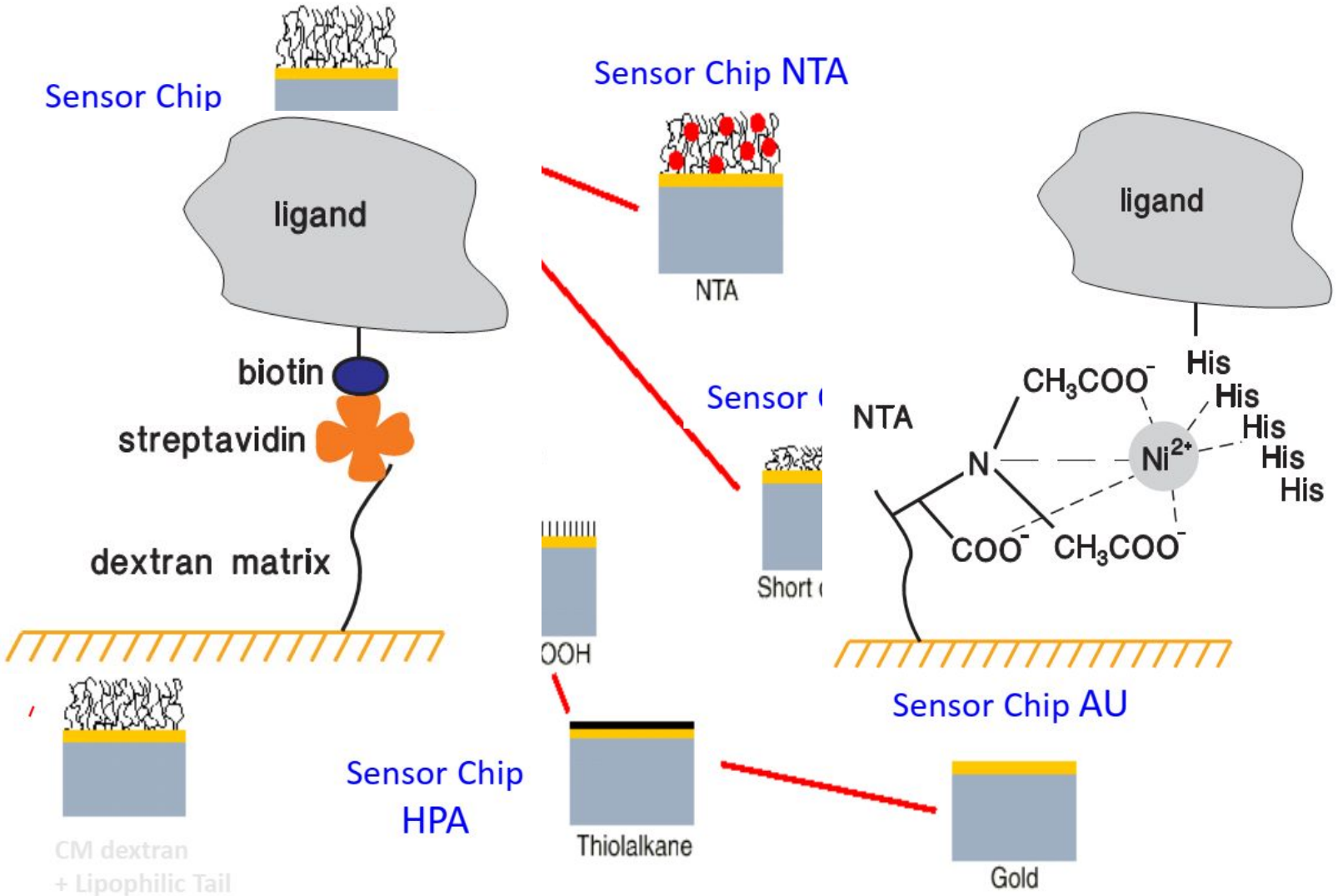
https://www.youtube.com/watch?v=sM-VI3alvAI
https://www.youtube.com/watch?v=oUwuCymvyKc
https://youtu.be/o8d46ueAwXI

Patching, *Biochim. Biophys. Acta* (2014)

SPR sensorgram



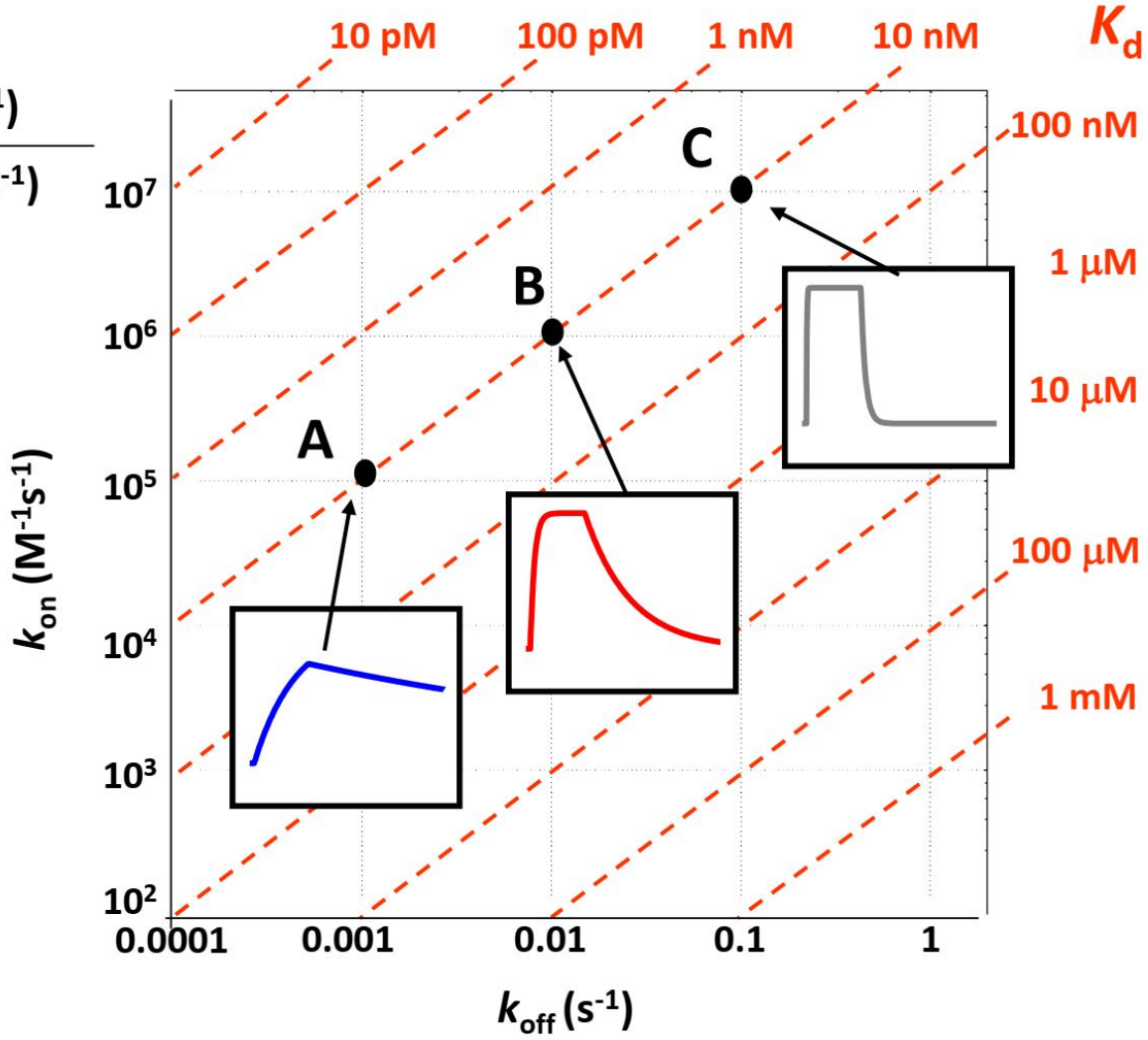
Chips



Biacore

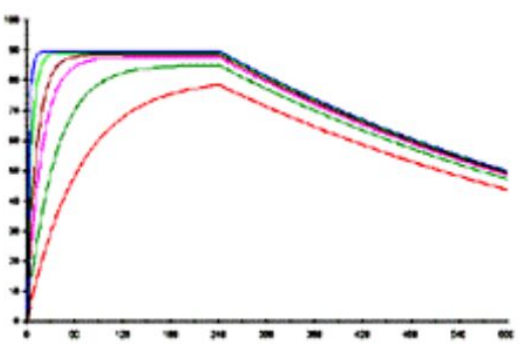
Why is kinetic analysis important?

$$K_d = \frac{k_{off}\,(s^{-1})}{k_{on}\,(M^{-1}s^{-1})}$$

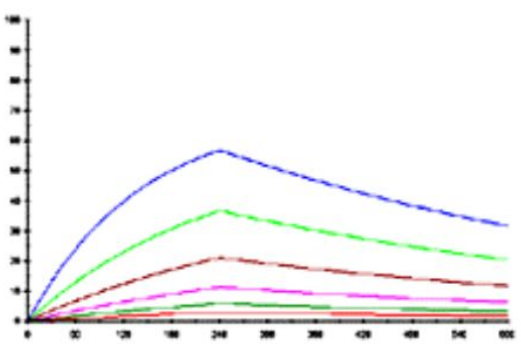


Practical considerations

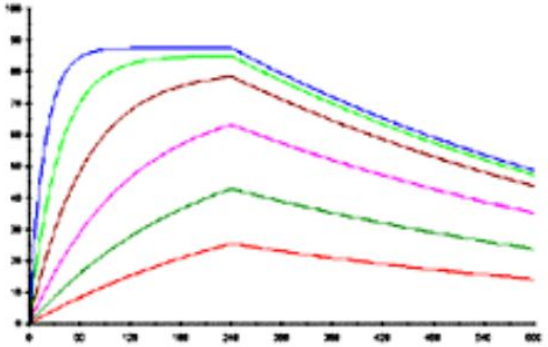
- Use several concentrations (ideally, 10 times below till 10 times above K_D)
- Accurate protein concentration must be determined
- Zero concentration should also be included



Too high concentration



Too low concentration



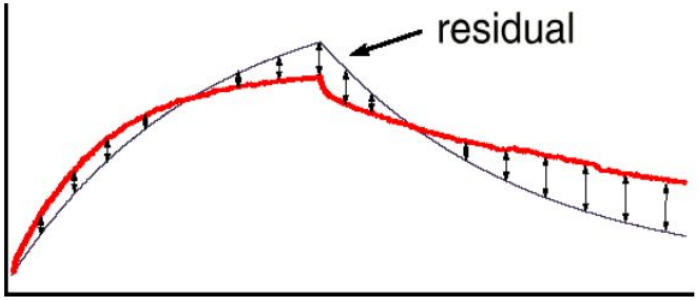
Optimized

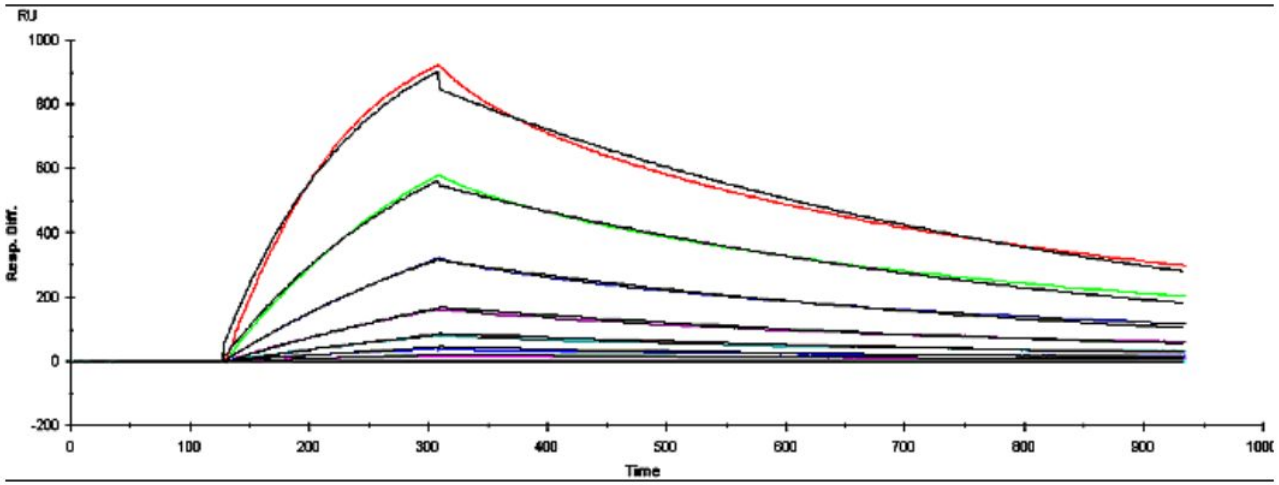
https://www.youtube.com/watch?v=e_tNkxbE2kY

Data analysis by simultaneous fitting of all curves using a binding model

k_{on}, k_{off}, and RU_{max} are calculated by global curve fitting

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





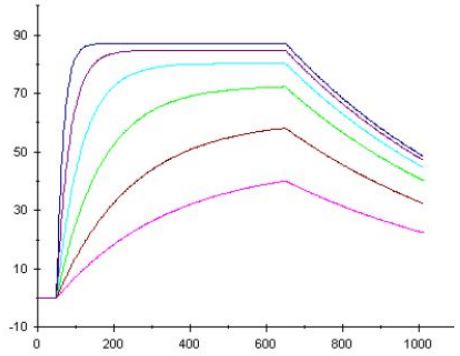
Biacore

Steady-state and kinetic ways to determine affinity (K_D)

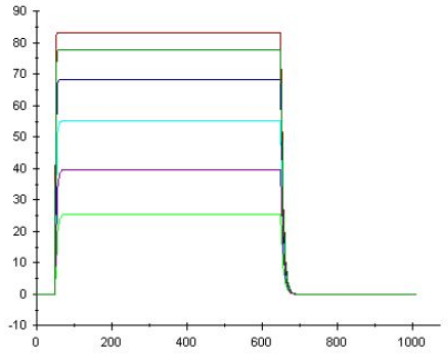
- Kinetic determinations give an independent value

$$K_a = \frac{k_{on}}{k_{off}} \qquad K_d = \frac{k_{off}}{k_{on}}$$

- Steady-state response levels give one value for affinity constants
- Steady-state can be used for fast interactions where kinetics are not available



Kinetics and affinity

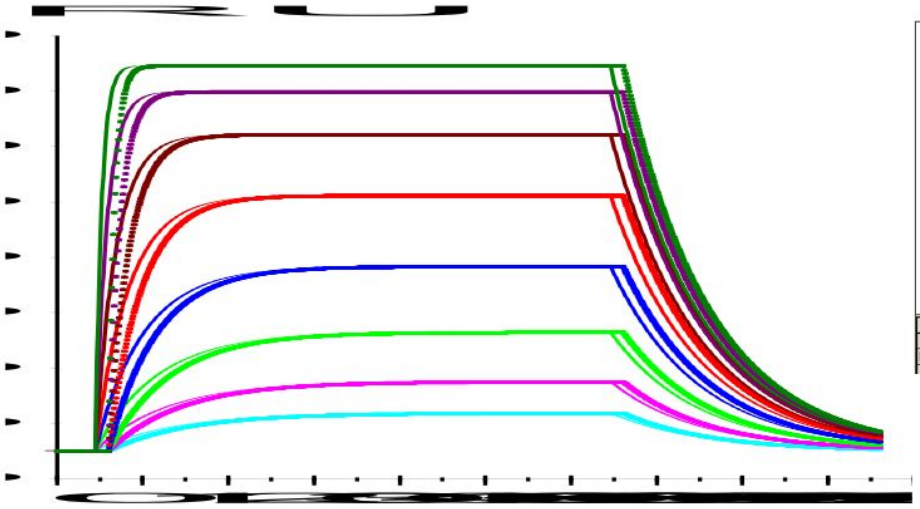


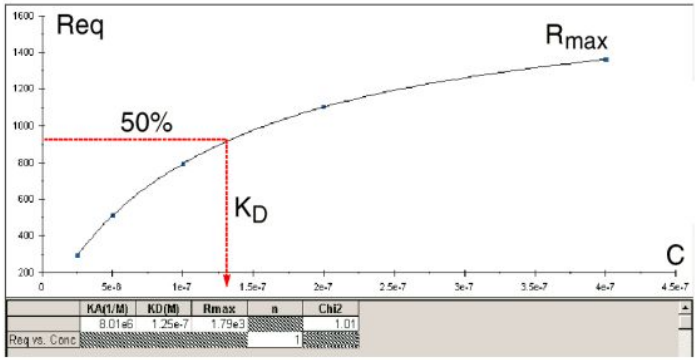
Affinity only

Biacore

Steady-state and kinetic ways to determine affinity (K_D)

- **Response at equilibrium can be plotted against the concentration to determine the affinity**
  - Response should be at or close to equilibrium at all concentrations for a reliable measurement





Biacore

SPR pros and cons

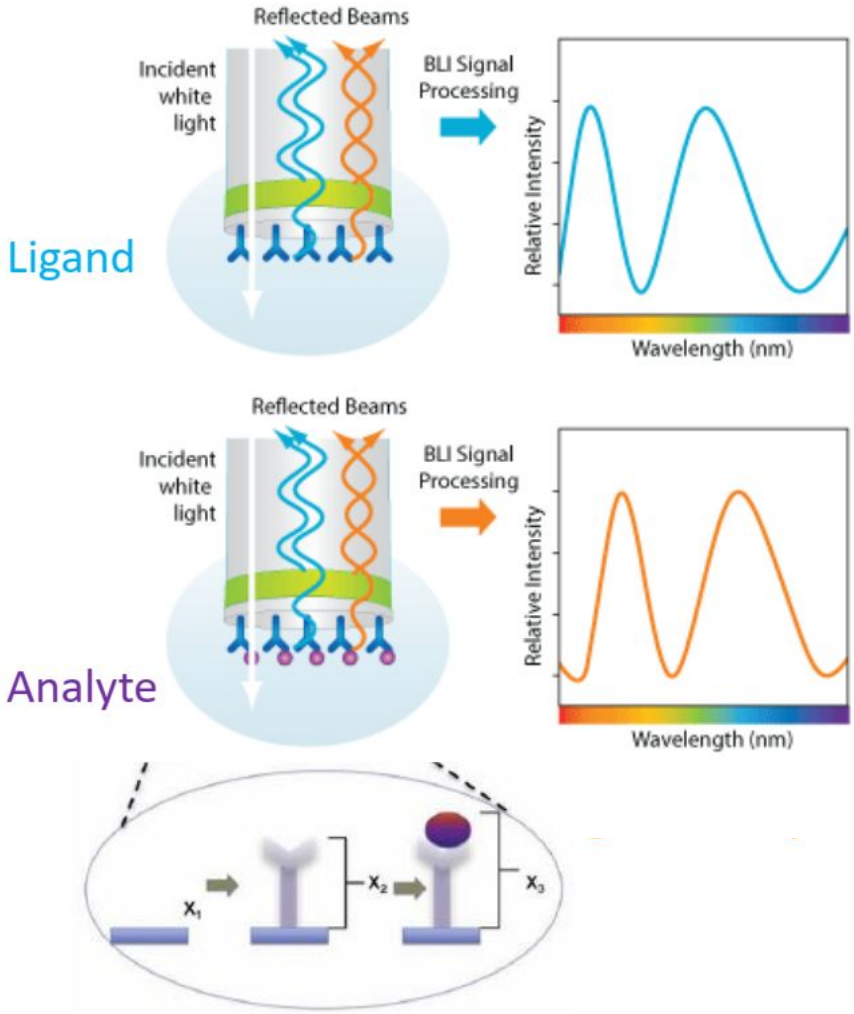
Advantages:

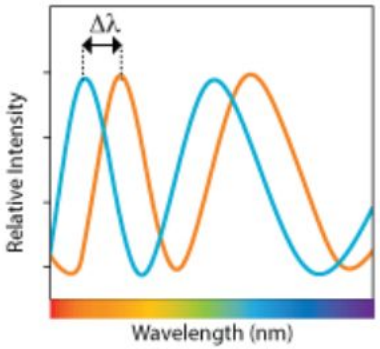
- Label-free detection
- Real-time data (i.e. quantitative binding affinities, kinetics and thermodynamics)
- Medium throughput
- Sensitivity and accuracy
- Measures over a very wide range of on rates, off rates and affinities
- Small sample quantity

Disadvantages:

- Expensive instrument and sensors
- Expensive maintenance
- Steep learning curve
- Specialized technician or senior researcher required to run experiments
- Immobilization of one of the binding partners required

Biolayer interferometry (BLI)





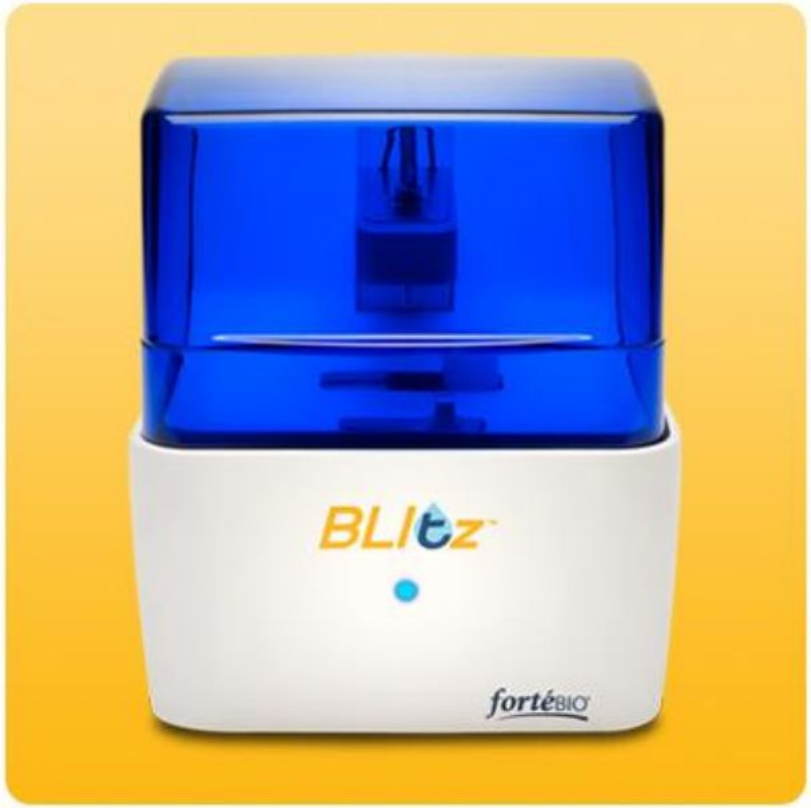
Optical thickness change at the sensor tip due to binding causes wavelength shift $\Delta\lambda$

https://www.moleculardevices.com/applications/biologics/bli-technology#gref

ForteBio; Citartan et al. Analyst (2013)

Instruments

8 channels



1 channel

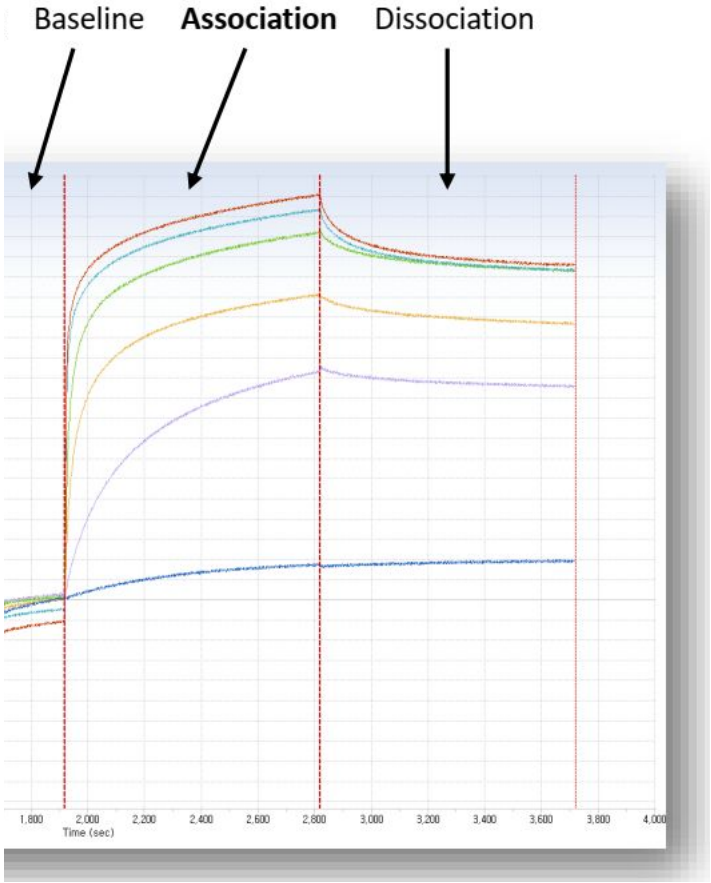
Instruments

8 channels

BLI sensorgrams

Key Benefits of BLI

- Label-free detection
- Real-time results
- Simple and fast
- Improves efficiency
- Crude sample compatibility



Exemplary studies:

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0106882
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4089413/

BLI pros and cons

Advantages:

- Label-free detection
- Real-time data
- No reference channel required
- Crude sample compatibility
- Fluidic-free system so less maintenance needed

Disadvantages:

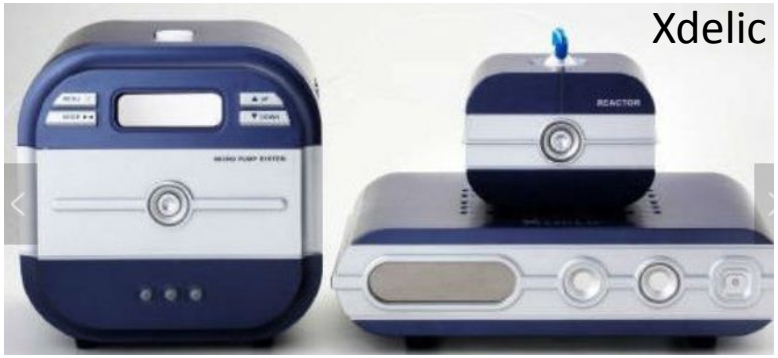
- Immobilization of ligand to surface of tip required
- No temperature control
- Low sensitivity (100-fold lower sensitivity of detection compared to SPR)

ITC vs SPR and BLI comparison

	ITC	SPR, BLI
Affinity range (K_d)	nM to sub-mM (pM with competition)	nM to low mM
Pros	• Thermodynamic parameters (ΔG, ΔH, ΔS) • No immobilization	• Kinetic parameters (k_{on}, k_{off}) • "Dirty" samples possible • "Less" sample required High throughput
Cons	• "More" sample required • Lows to medium throughput	• Mass transfer limitation • Immobilization artifacts

Quartz crystal microbalance (QCM)

- High frequent oscillations of the quartz crystal (5-10 MHz) with the Au chip
- Mass detection with super accuracy – quartz crystal resonator senses ~1 Hz
- Upon mass deposition on the QCM sensor, the frequency decreases
- Sensitivity can be ~ 20 ng/cm^2 per Hz
- Low throughtput, rather rare method
- Sample volume 50-200 ul
- Label-free



Sauerbrey equation:

freq change mass change

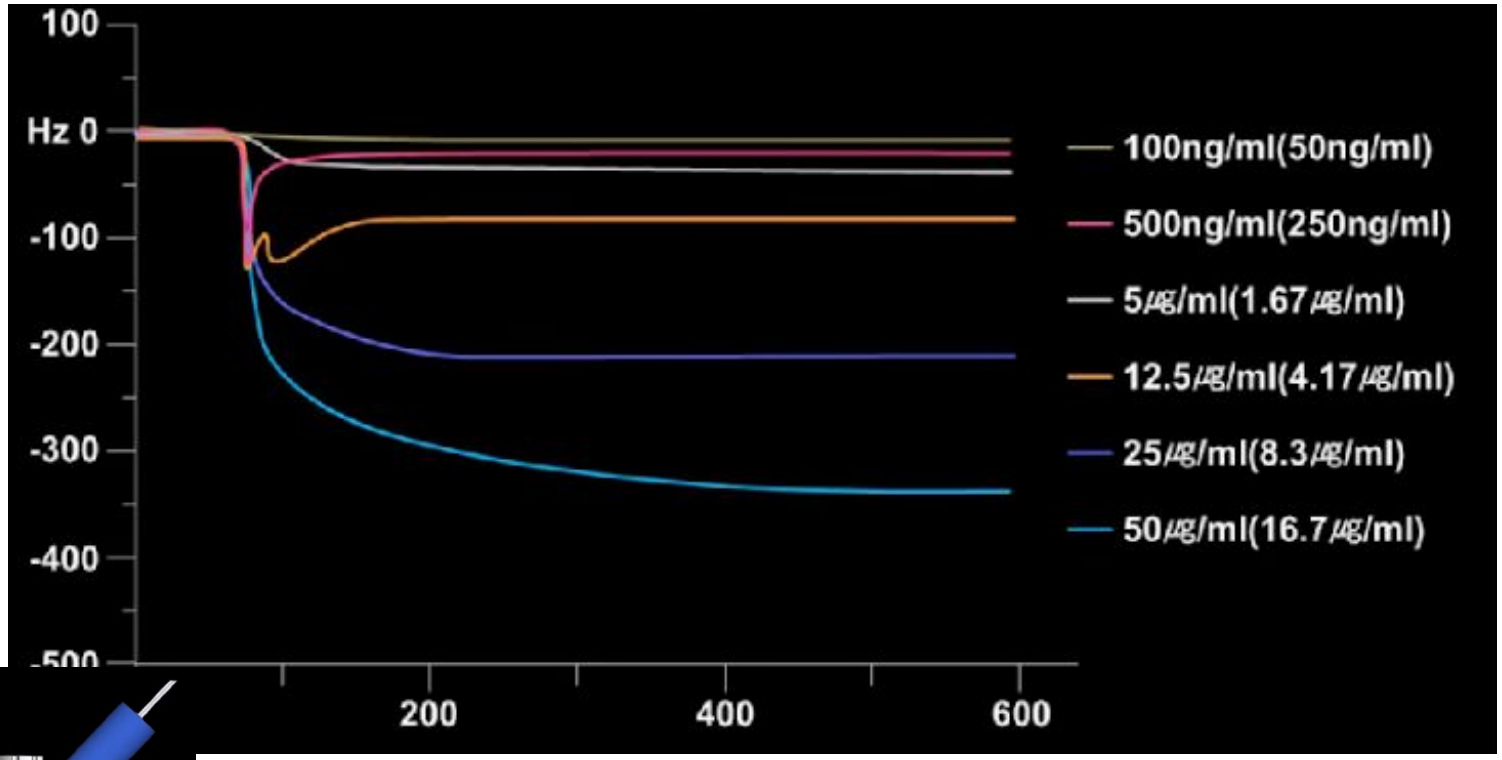
$$\Delta f = -\frac{1}{C_{QCM}}\Delta m$$

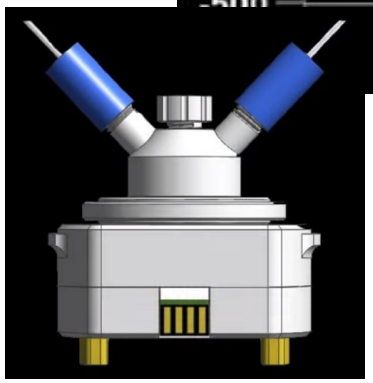
sensitivity

https://www.youtube.com/watch?v=xDKOUpSR3EQ

https://openqcm.com/openqcm

Microfluidics delivers the sample and the deposited mass fraction is measured





https://www.youtube.com/watch?v=xDKOUpSR3EQ

Overview of the course

- Proteins: size and hydrodynamic parameters
- Identification of proteins by their sequence
- Spectroscopy methods
- Stability of proteins
- Protein structure – high resolution methods
- Protein structure – low resolution methods
- Interactions involving proteins