METODS IN PROTEOMICS
C G 9 9 0

Quantitative analysis of protein-protein interactions

Ctirad Hofr

LifeB – Laboratory of interaction and function of essential Biomolecules

Functional Genomics and Proteomics

National Centre of Biomolecular Research

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Overview of quantitative methods for analysis of protein-protein interactions

1

Theory = basis for practice

Binding curve, equilibrium dissociation constant, linear range of the detector.

2

Fluorescence leads in numbers, but you can do without it

Determination of binding affinity of fluorescently labeled proteins - fluorescence anisotropy, microscale thermophoresis, detection of binding of molecules immobilized on the surface - surface plasmon resonance; study of binding of unmodified proteins directly in solution - isothermal titration calorimetry.

3

Which is best - comparison of methods

Summary of the practical advantages and disadvantages of quantitative methods for protein-protein interaction analysis.

Binding curve 1 isotherm

To bind two proteins A,B and form an A.B complex at constant temperature

we define **equilibrium constants association and dissociation**

$$K_A = \frac{[AB]}{[A][B]} \quad K_D = \frac{[A][B]}{[A \cdot B]}$$

If protein B is added sequentially to protein A, the binding curve can be expressed by the equation

$$Y = \% \text{ bound} = \frac{[B]}{[B]+KD} \cdot 100\%$$

Dependence of the binding rate on the total concentration of added protein B

% bound molecules

100
75
50
25
0

0 20 40 60 80 100

K_D

Protein concentration [B]

K_D dissociation constant - the protein concentration at which exactly half of the molecules are bound

Binding curve 2
origin of the equation

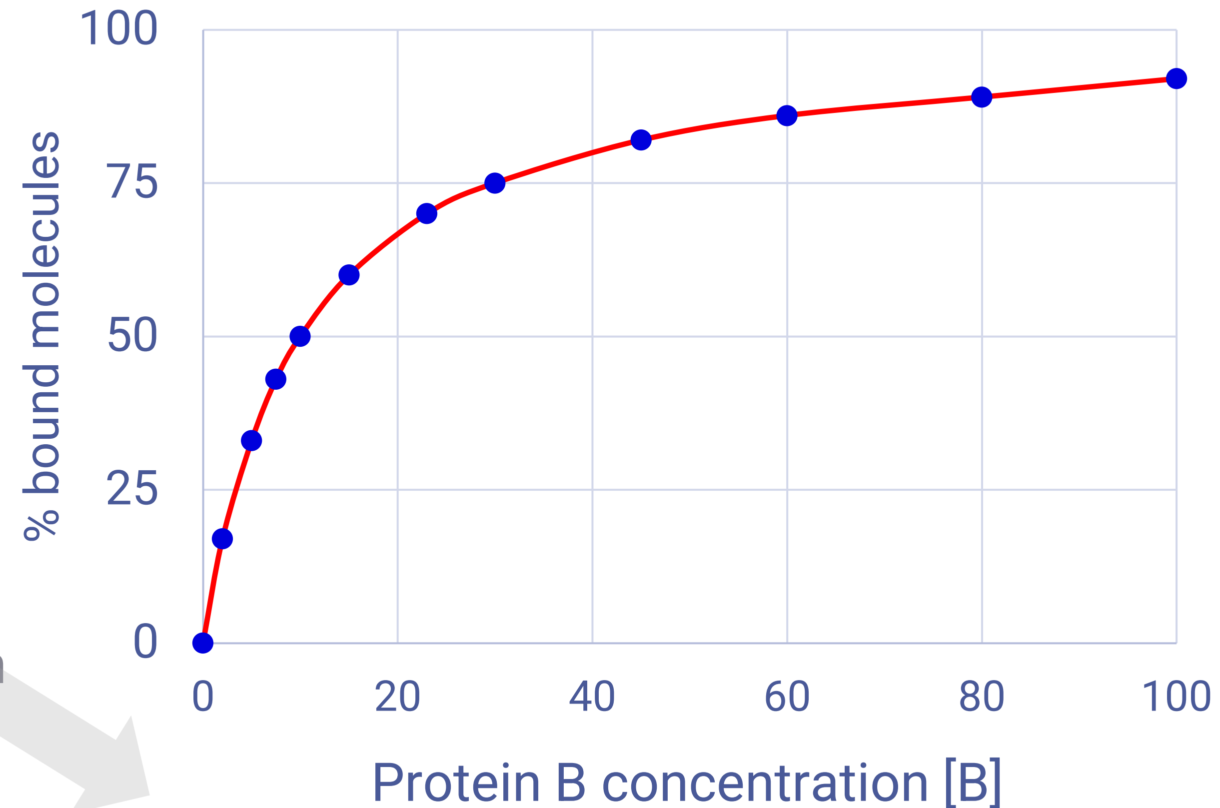
The binding rate is expressed as the ratio of the concentration of complex A.B to the total concentration of protein A multiplied by 100%.

$$Y = \%\,bound = \frac{[A \cdot B]}{A_{TOT}} \cdot 100\%$$

$$K_A = \frac{1}{K_D}$$

After substituting **A.B** to **A_{TOT}** we get the equation for the binding curve

$$[A \cdot B] = K_A[A][B] \quad A_{TOT} = [A \cdot B] + [A]$$



$$\%\,bound = \frac{[B]}{[B] + KD} \cdot 100\%$$

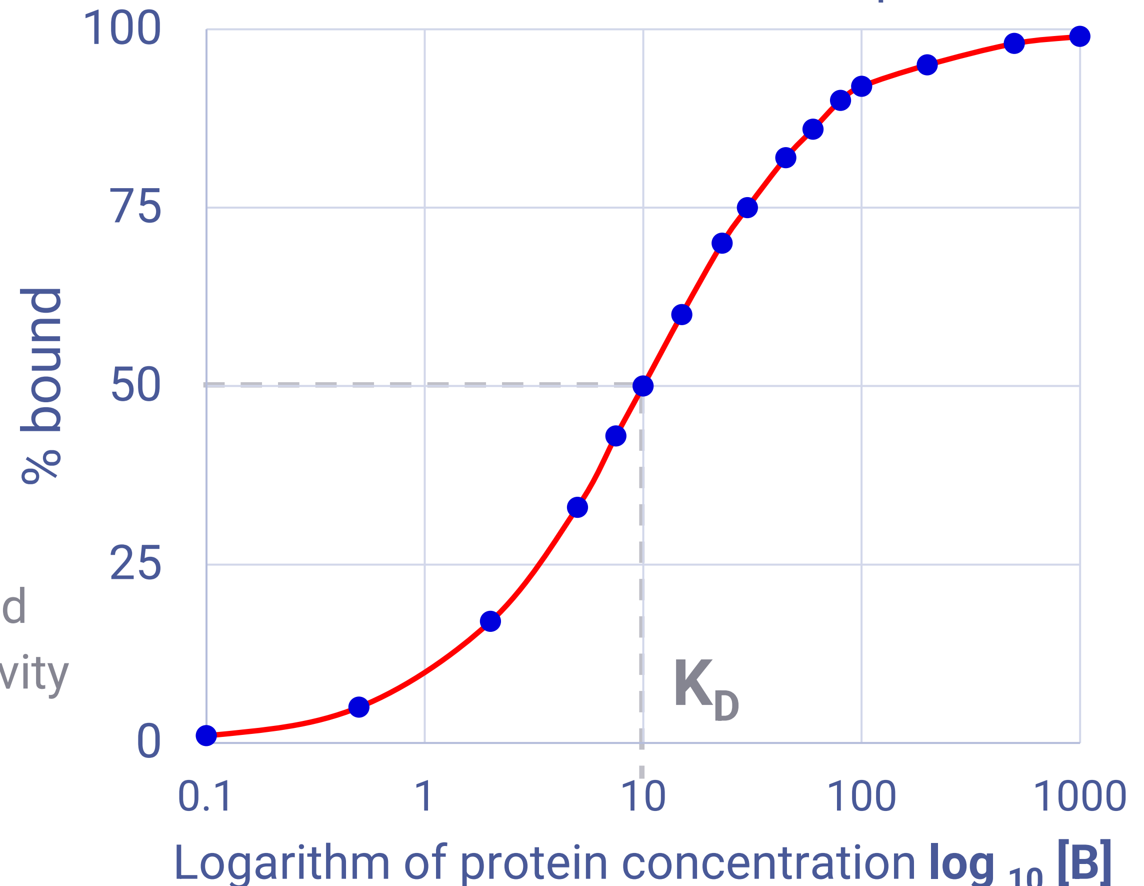
The binding curve is a hyperbola with the value of the dissociation constant K_D in the denominator.

Binding curve 3 –
logarithm of concentration = sigmoid

If protein A is added to protein B over a sufficiently **wide concentration range**, the binding rate versus **logarithm of the concentration of B** is a **sigmoid**.

$$\% \text{ bound} = \frac{[B]}{[B] + K_D} \cdot 100\%$$

K_D dissociation constant - inflection point of sigmoid
Slope of the sigmoid - a measure binding cooperativity when multiple proteins B bind to a single protein A.



How does binding cooperativity change the shape of the sigmoid?

https://en.wikipedia.org/wiki/Hill_equation_(biochemistry)

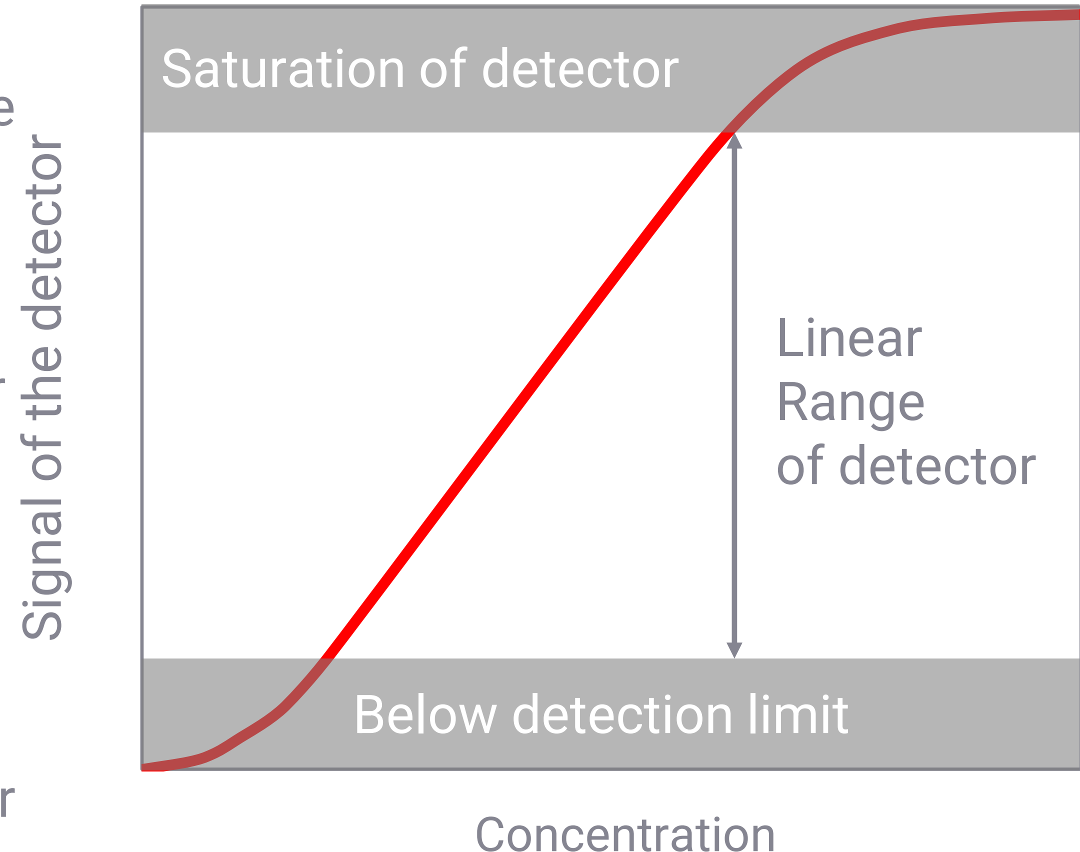
Know your detector - linear detector range = accurate quantitative measurements

Overall, the detection curve has a **sigmoid shape**.

For accurate quantitative measurements, it is essential that the increment in signal is directly proportional to the increment in concentration of the protein-protein complexes.

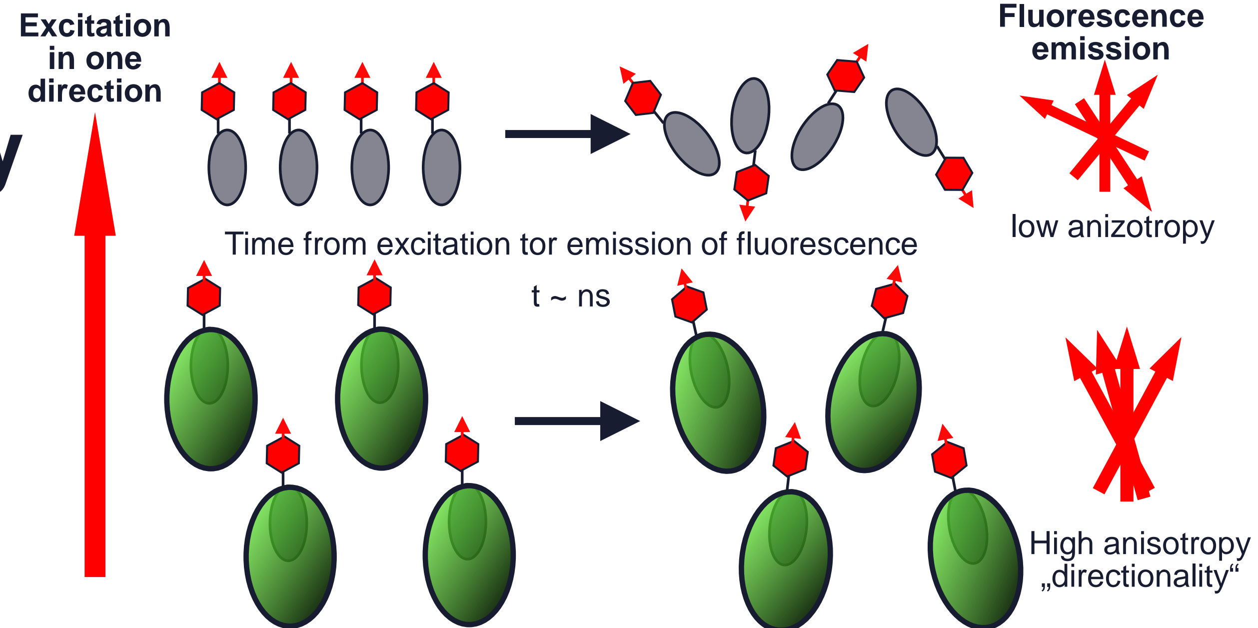
This is fulfilled for the **linear range of the detector** l- the detection region, where an increase in concentration, for example, doubles the signal value.

Area **below the linear range** - we are close to the minimum detection limit = non-linear response.
Area **above the linear range** - the detector is overwhelmed with signal - **saturated**, a large change in concentration will cause a relatively small and non-linear increase in signal.



Always find out the linear range of the detector. For quantitative measurements it is essential that the measurement values are within the linear range of the detector.

Fluorescence anisotropy-based determination of binding affinity

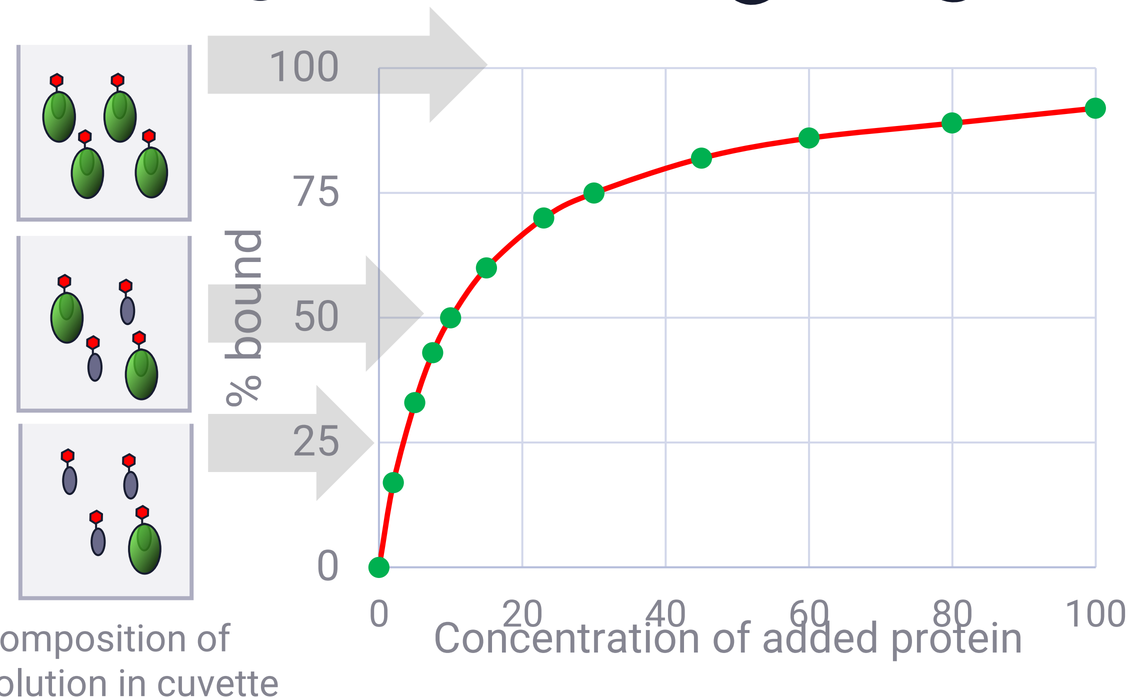


Fluorescence anisotropy - principle

The "directionality" of the emitted light increases after the formation of a protein-protein complex; after excitation by linearly polarized light, the fluorescence of the labelled protein-protein complex is emitted predominantly in one direction.

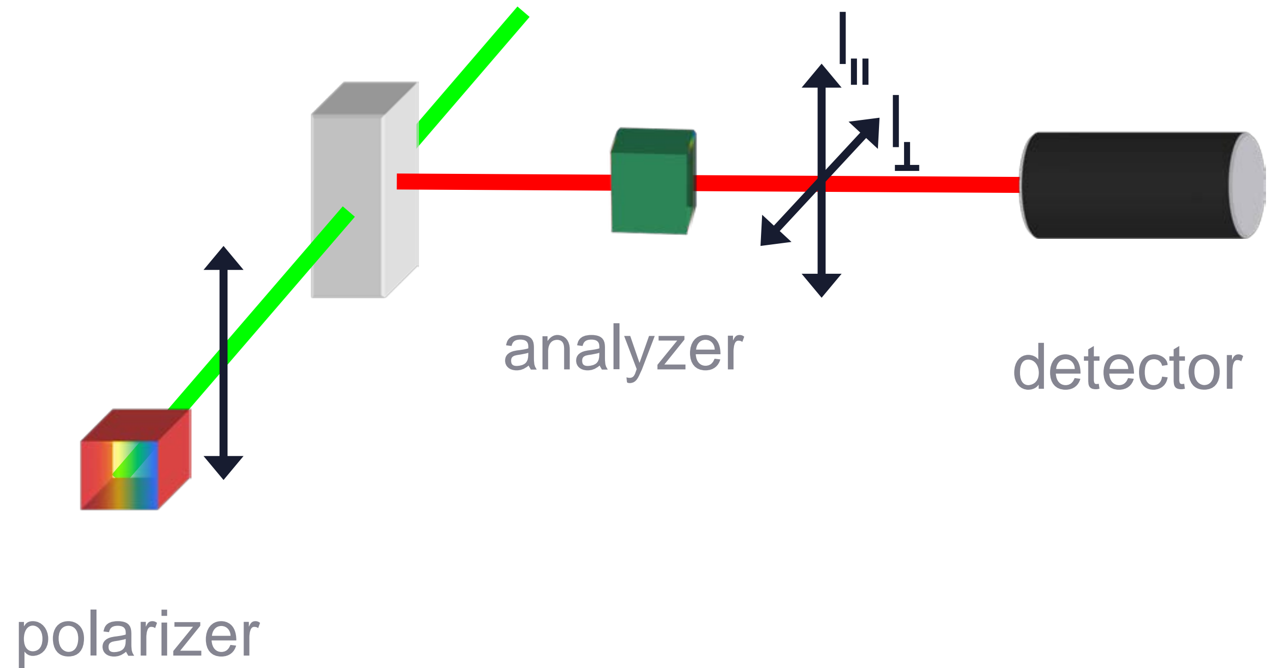
Practically

- we label the smaller of the proteins.
- It is sufficient to label 100 mg of protein in a cuvette.
- added larger protein is not labelled.
- total concentration of the added protein is at least 10 times greater than the concentration of the labeled protein.



Fluorescence anisotropy measurement setup

$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}} = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$$



The value of anisotropy r is the ratio of the **difference of I_{VV} - I_{VH} fluorescence intensity** at parallel (vertical) and perpendicular (horizontal) rotation of the emission analyzer to the excitation polarizer and the **total fluorescence intensity I_{VV}+2I_{VH}** in 3D - all three directions of fluorescence propagation.

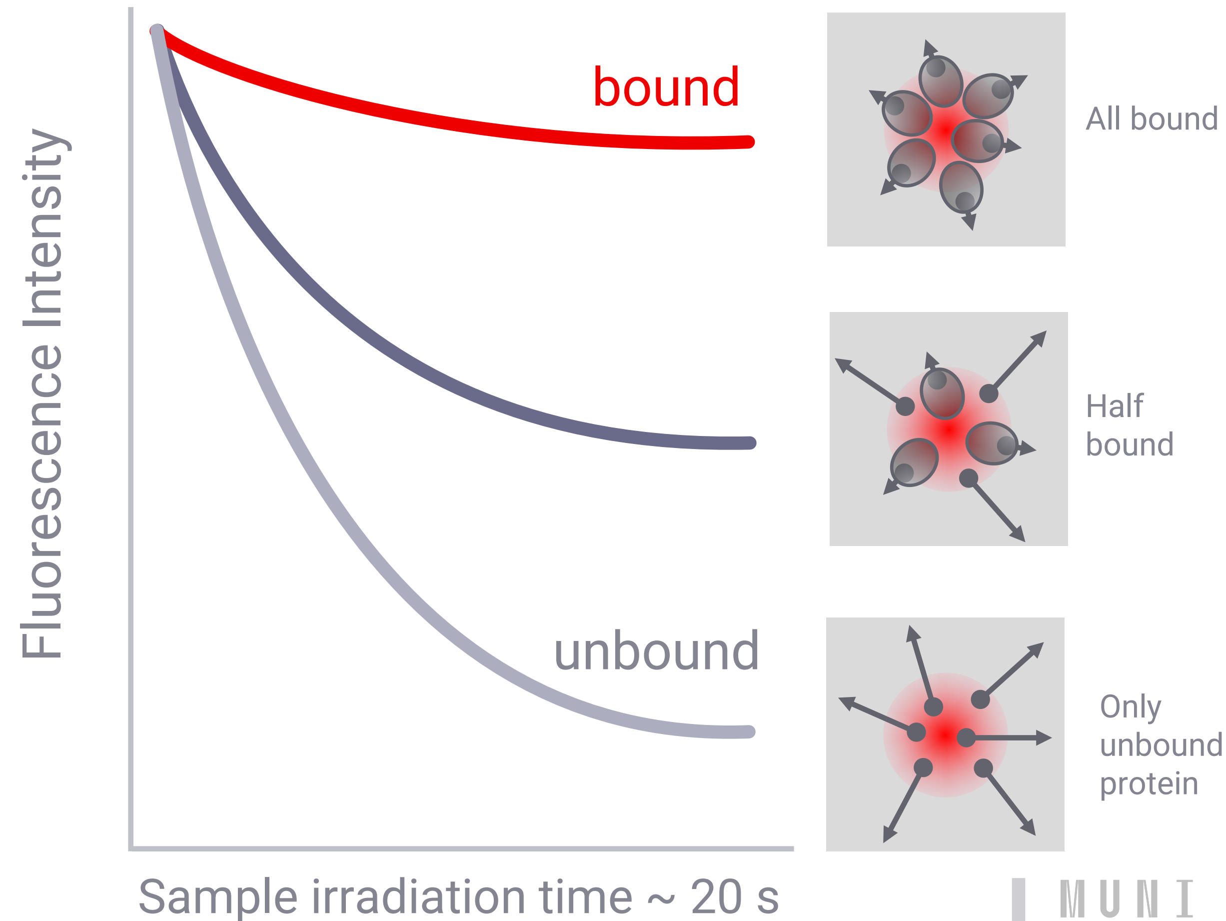
Practically

- Instrument - fluorometer with polarizer of excitation light and rotatable polarizer = analyzer of emitted fluorescence to detect intensity in different directions.
- Required ~10x higher concentration than normally used for measuring conventional fluorescence - polarizers transmit 10x less light.
- Anisotropy **r** is a dimensionless without unit (ratio of numbers).

Microscale thermophoresis MST

Principle of MST

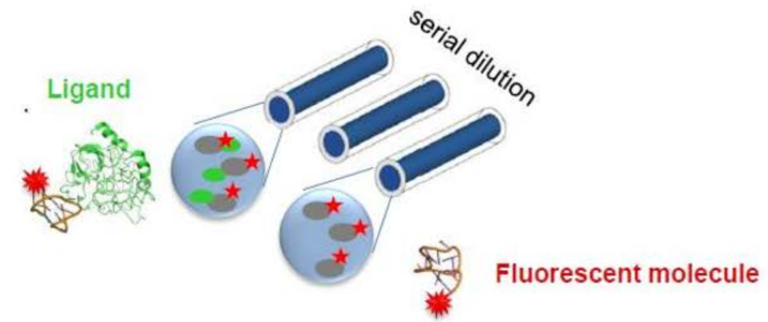
- We generate a local temperature gradient - irradiate the sample in the capillary with an infrared (IR) laser.
- At the same time, we illuminate the sample with excitation light for the fluorophore that labels the smaller protein.
- We detect the movement of the fluorescently labelled molecules as a change in fluorescence in the micro-region illuminated by the IR laser.
- At a constant concentration of the fluorescently labelled protein, we increase the concentration of the added unlabeled protein = ligand.
- We observe a decrease in the rate of fluorescence decrease over time with increasing ligand concentration.

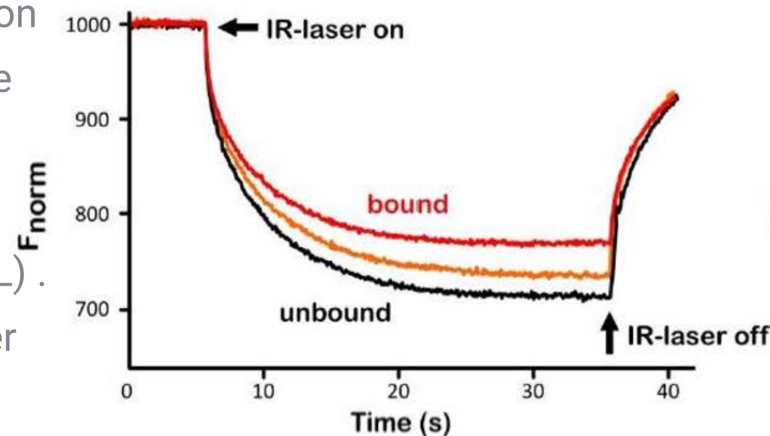


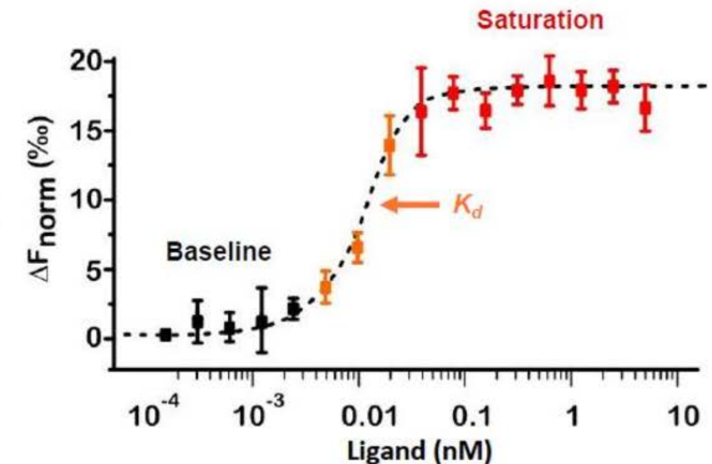
https://en.wikipedia.org/wiki/Microscale_thermophoresis

Practical MST measurement of protein-protein interactions

- Label fluorescently a smaller protein-analyte.
- Create a dilution series of the second protein = ligand by diluting it 2 times.
- Mix the solutions so that the concentration of the labeled protein is the same, but the concentration of the ligand varies by 5 orders of magnitude.
- Aspirate the samples into capillaries (5uL) .
- Measure the change in fluorescence after switching on the IR laser.
- Plot the change in fluorescence versus the logarithm of the ligand concentration.
- From the inflection point of the sigmoid, determine the dissociation constant of the protein-ligand complex formation.

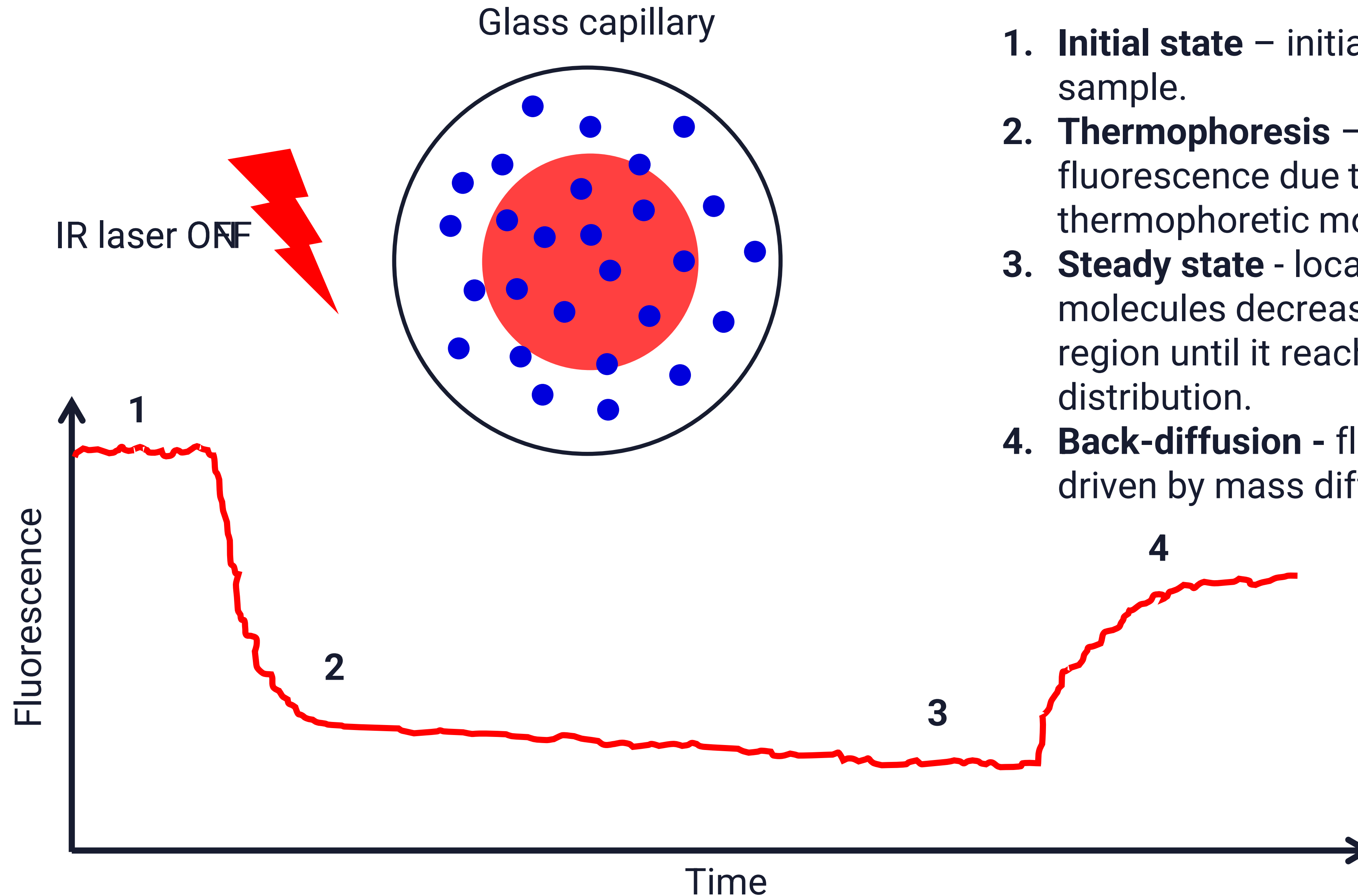






Kindly provided by Dr. Josef Houser,
CORE FACILITY Biomolecular Interactions and Crystallization
http://bic.ceitec.cz/cs

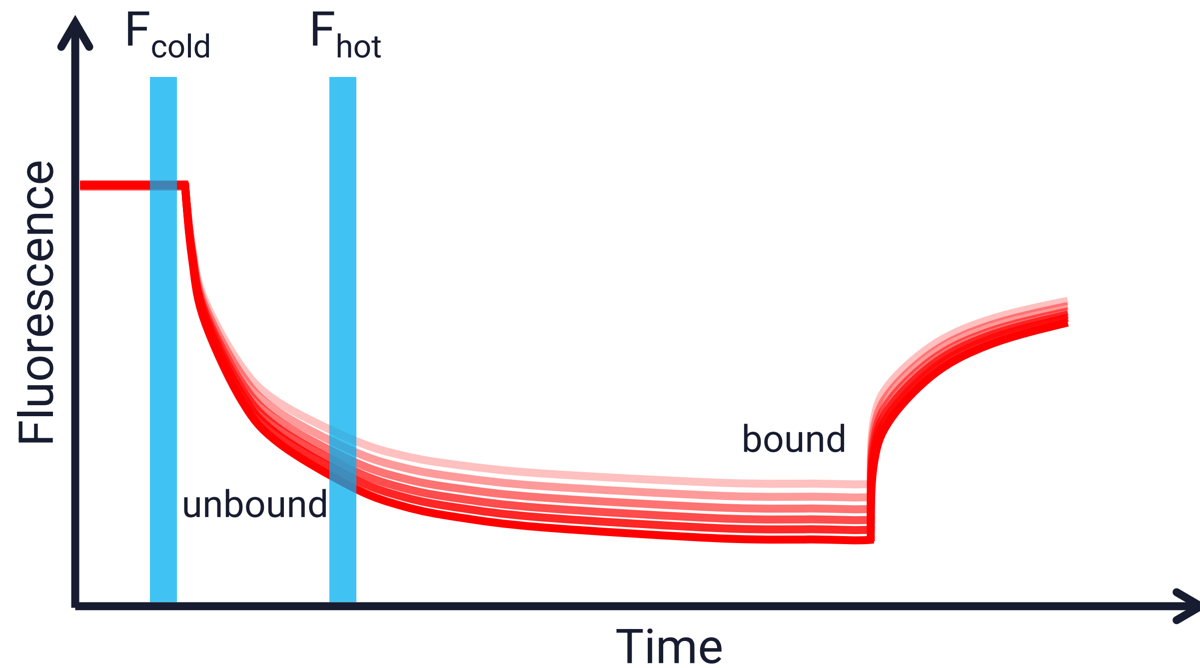
MST – Microscale Thermophoresis

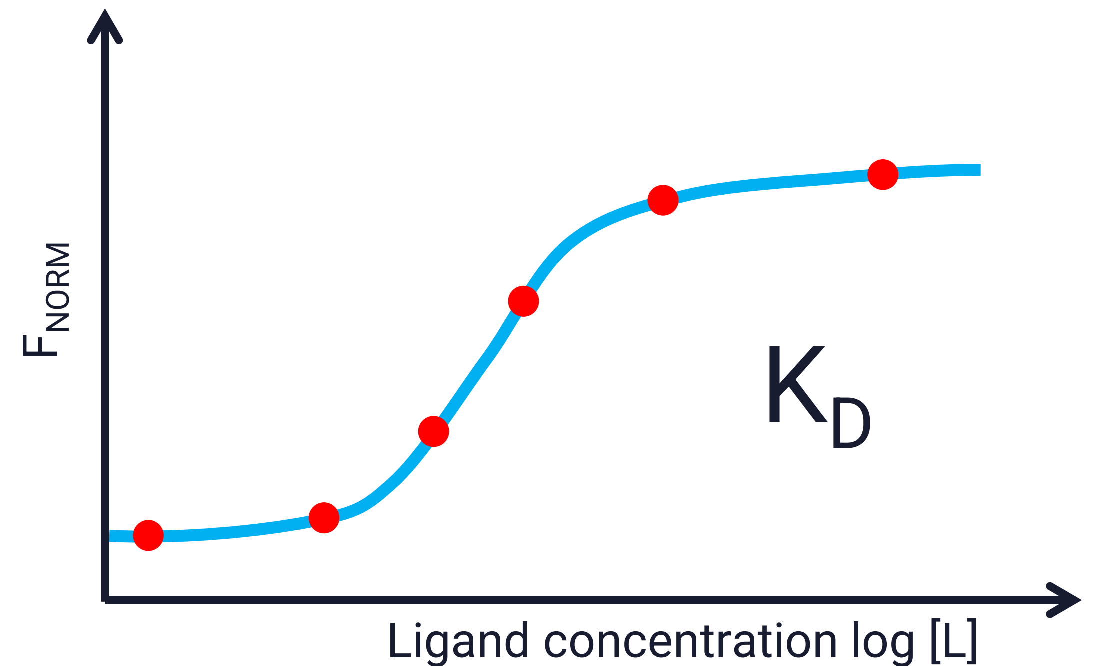


1. **Initial state** – initial fluorescence of sample.
2. **Thermophoresis** – change in fluorescence due to the thermophoretic motion of molecules.
3. **Steady state** - local concentration of molecules decreases in the heated region until it reaches a steady-state distribution.
4. **Back-diffusion -** fluorescence recovery driven by mass diffusion of molecules.

Prepared by Tomáš Janovič

Analyzing protein-protein interaction with MST





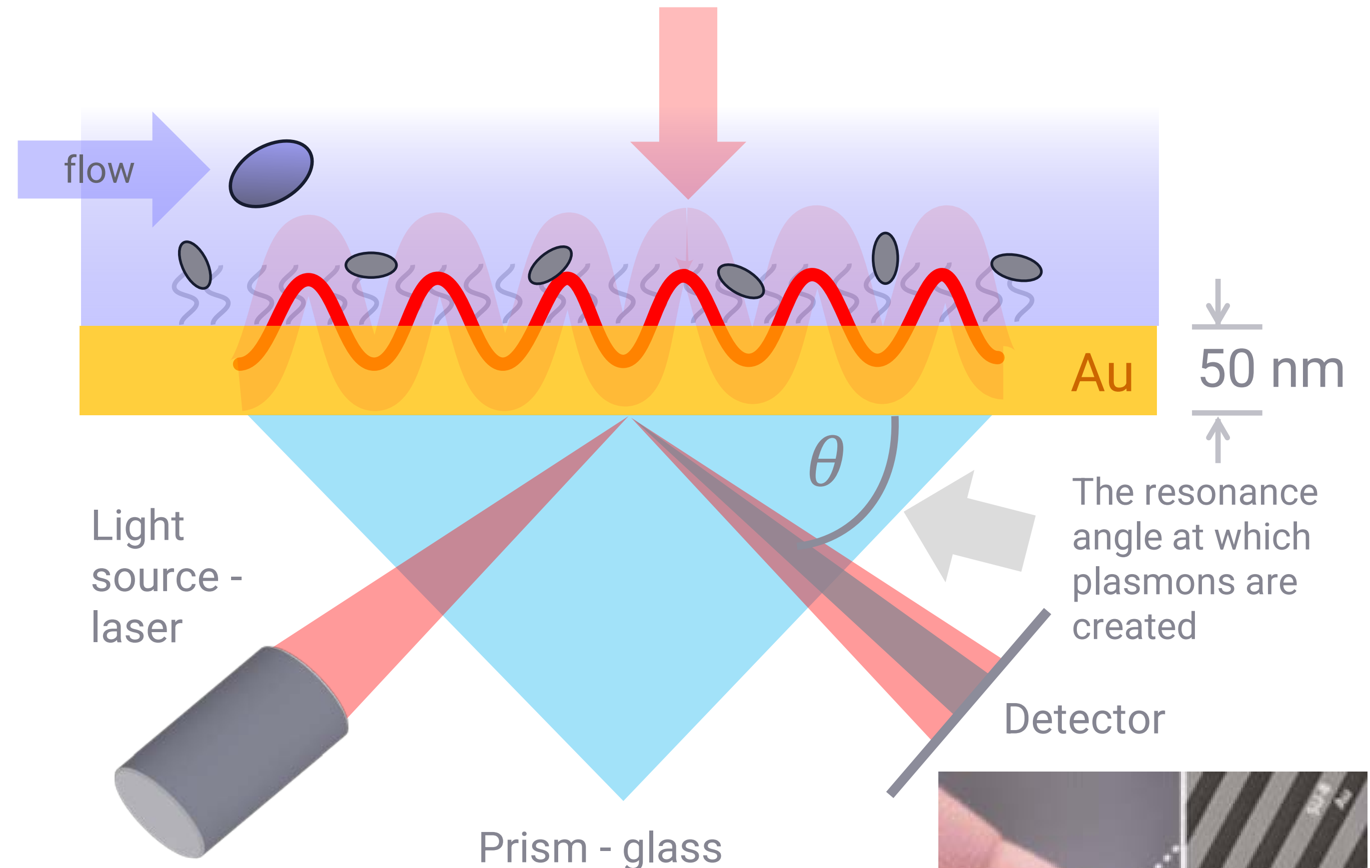
Prepared by Tomáš Janovič

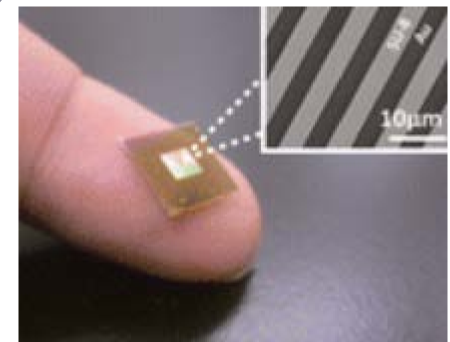
Protein-protein interaction on the surface
Surface plasmon resonance - SPR

Principle

At the transition between the glass and the gold layer, ideally 50 nm thick, the light - laser is reflected. At the resonance angle θ, an increased absorption occurs, which is detected by the detector. An evanescent (disappearing) wave of resonant electrons = plasmons is produced, which gradually decays with distance from the surface. The range of the evanescent wave is approximately 100 nm into the solution space.
Plasmons are very sensitive to changes in the environment in which they move. Thus, SPR allows the **detection of changes due to the binding of proteins on the surface** of the gold layer.
The gold is coated with dextran, on which one interacting protein is **immobilized**.
A second protein is added to a buffer that washes the surface with the immobilized protein.
Once the protein-protein complex is formed on the surface, a change in the resonance angle θ is detected.
We observe the **kinetics of complex formation in real time**.

Evanescent wave of plasmons - resonating electrons on the surface = surface resonance plasmons that absorb part of the light



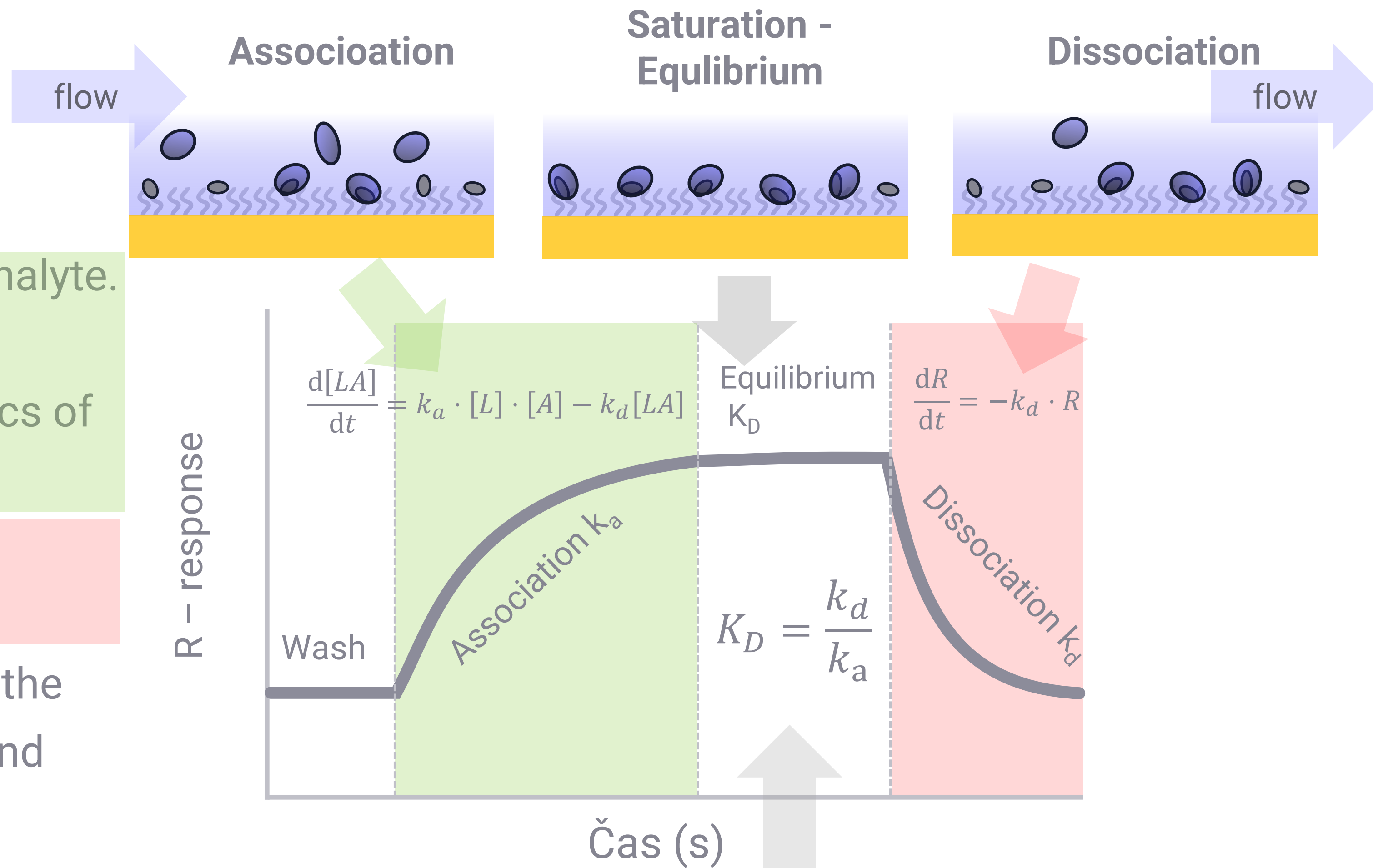


SPR measurement protocol

- Immobilize protein L - ligand on SPR chip.
- Saturate the binding sites on the surface without immobilized protein L.
- Wash the chip with buffer.
- Wash the chip with the second protein A - analyte.
- Detect signal change.
- The initial part of the curve shows the kinetics of the association of the two proteins.
- The final part of the curve describes the dissociation kinetics of both proteins.
- By fitting the binding models, we determine the values of the **association rate constant k_a** and **dissociation rate constant k_d**.

The **association rate constant** k_a describes the rate of complex formation, i.e. the number of LA complexes formed per second in a one molar solution of L and A. The units of k_a are $\mathbf{M^{-1}s^{-1}}$ and are typically between 1.10^3 and 1.10^7 in biological systems.

The **dissociation rate constant** k_d describes the stability of the complex, i.e. the fraction of complexes that decays per second. The unit of k_d is $\mathbf{s^{-1}}$ and is typically between 1.10^{-1} and 1.10^{-6} in biological systems. A k_d of $1.10^{-2}s^{-1} = 0.01\ s^{-1}$. This means that 1 percent of the complexes decay per second.



We determine **equilibrium dissociation constant K_D** from the ratio **k_d** and **k_a**.

www.sprpages.nl

ITC - Isothermal Titration Calorimetry
Description of the interaction by measuring the heat exchange

Principle

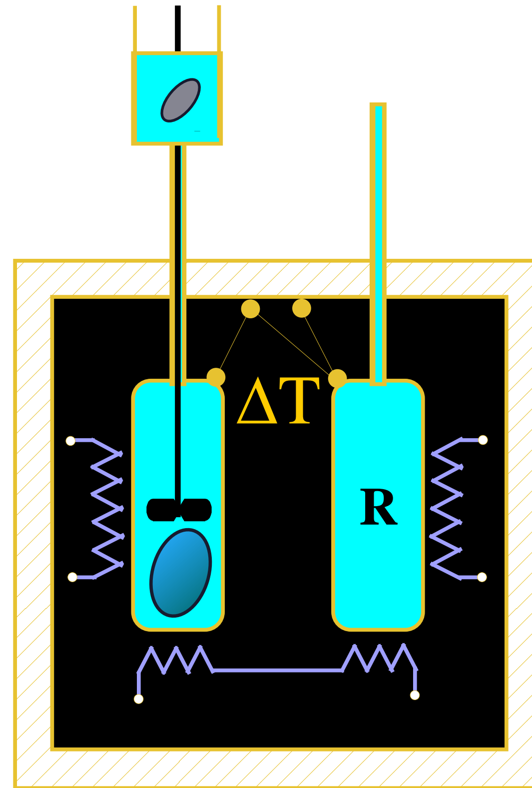
-We measure the **reaction heat – binding enthalpy** that is released or consumed when a protein is added by an injector to a protein solution in a measuring cell. The injector is in the shape of a propeller that stirs the mixture in the measuring cell.

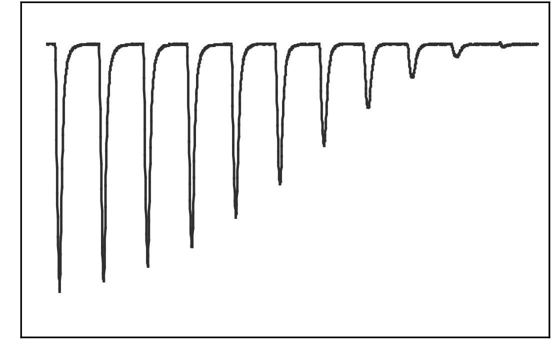
-The temperature of the sample cell is compared with that of a reference cell containing only buffer.

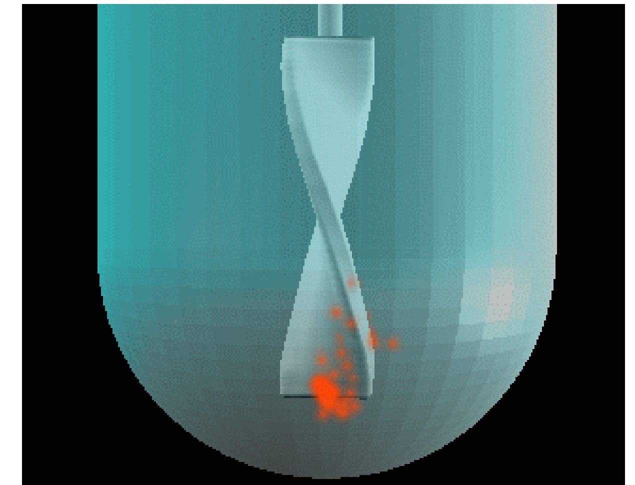
-When the temperature changes between cells, the sample cell is heated or cooled. The heat that is exchanged to equalize the temperatures is recorded after each addition of protein from the injector.

The result is a **titration binding curve**.

-From the known molar concentrations and heat change, we determine the **molar binding enthalpy** - the heat of binding of the protein.





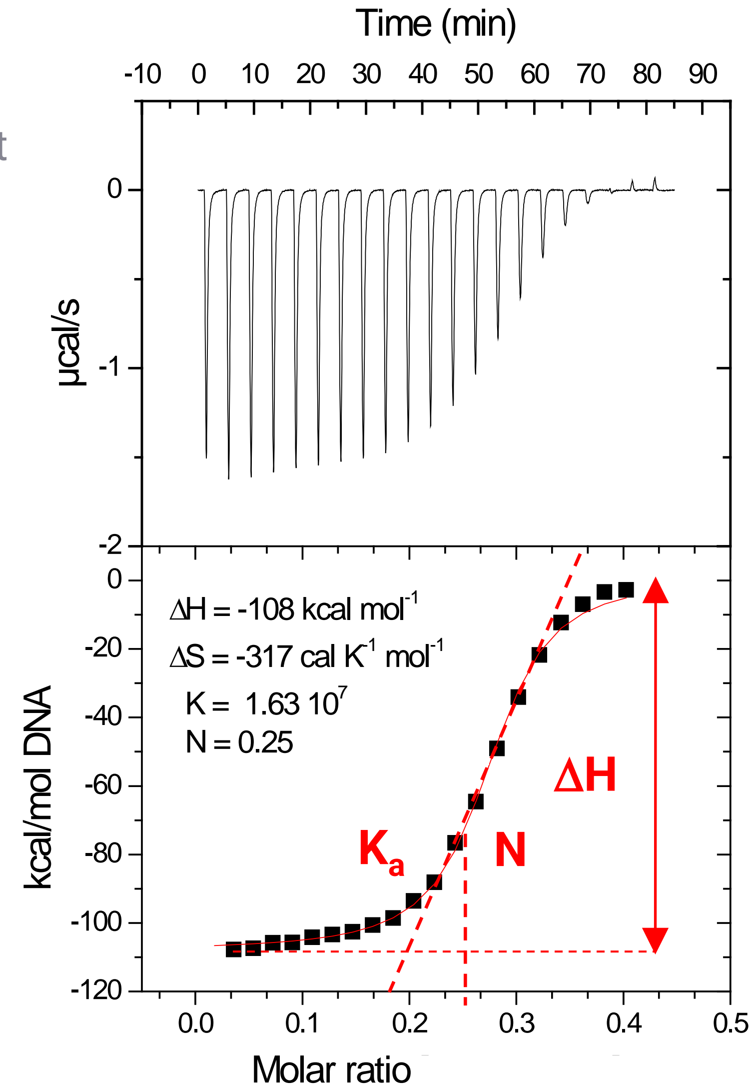


http://www.youtube.com/watch?v=cYj5IOELaVI

https://youtu.be/o_IpWcWKNXI?t=44

ITC measurement practically

- Determine protein concentrations as accurately as possible.
- Measure a "blank" titration - buffer in injector, protein in cell at 25°C.
- Place a second protein in the injector at 10x higher concentration than of the first protein in the cell.
- Measure the titration curve.
- Subtract the blank titration from the titration curve.
- Normalize to the protein concentrations in the cell and injector.
- Fit the resulting sigmoid with a suitable binding model.
- The **height** of the curve indicates the **binding enthalpy**, the **slope** corresponds to the **equilibrium association constant**, and the **inflection point** corresponds to **stoichiometry** - the molar ratio of protein binding.
- A complete thermodynamic description of $\Delta H, \Delta S, \Delta G, K_A$ binding and N-binding stoichiometry can be obtained from a single measurement.



Binding enthalpy ΔH

Stoichiometry **N**

Binding constant K_A

Free energy ΔG

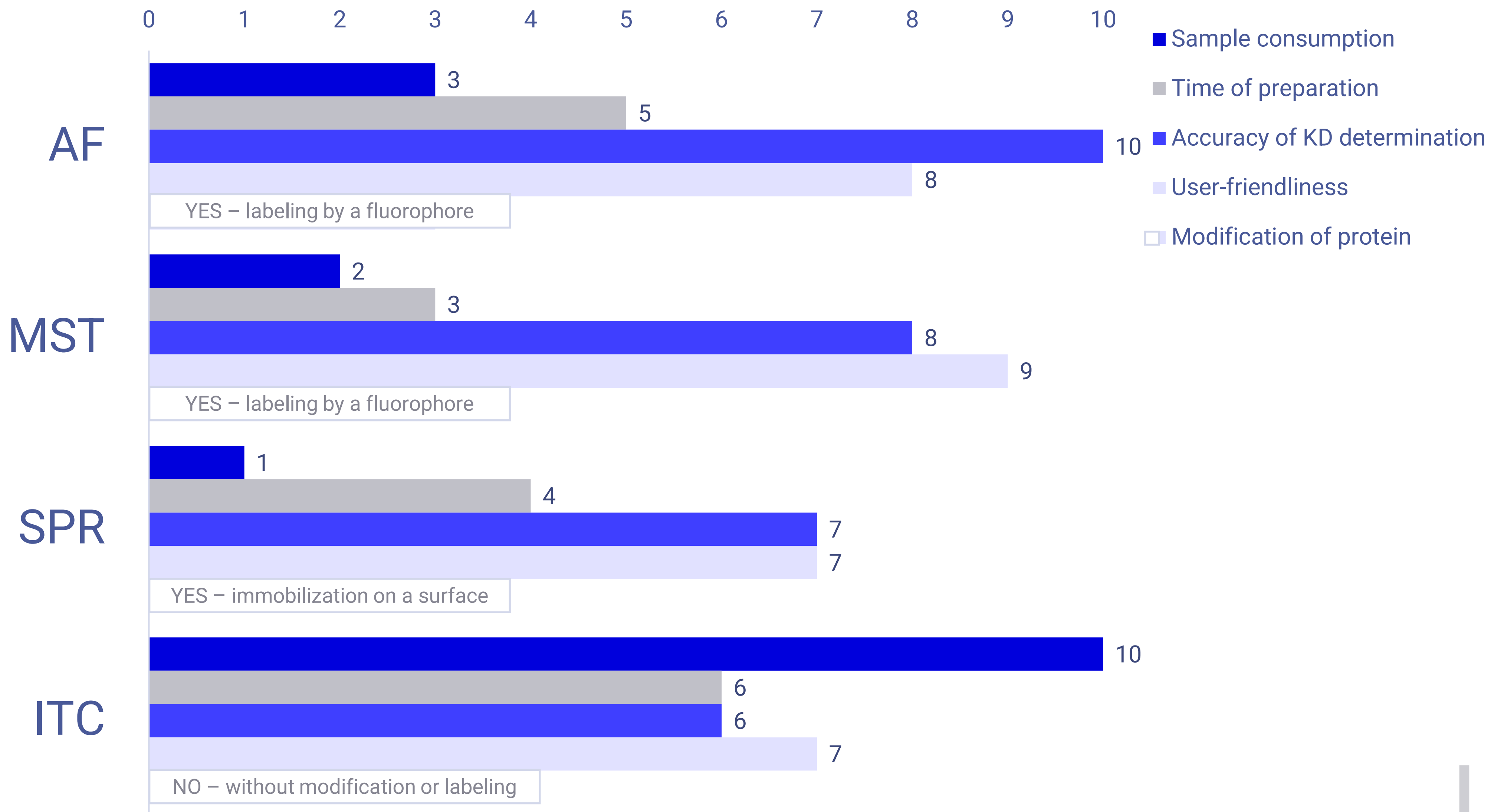
calculated $\Delta G = -RT\ln K_a$

Entropy ΔS

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

3

Comparison of quantitative methods



Where can you find out more?

C7230 Fluorescence methods in the life sciences - the journey from molecule to cell

C7235 practicals

Theoretical explanations of fluorescence principles and hands-on training in the application of fluorescence approaches

Autumn 2022

FE010 Experimental Methods in Biophysics - life science laboratory approaches and excursions

Lectures by experts and excursions to research laboratories of internationally renowned companies
Autumn 2022

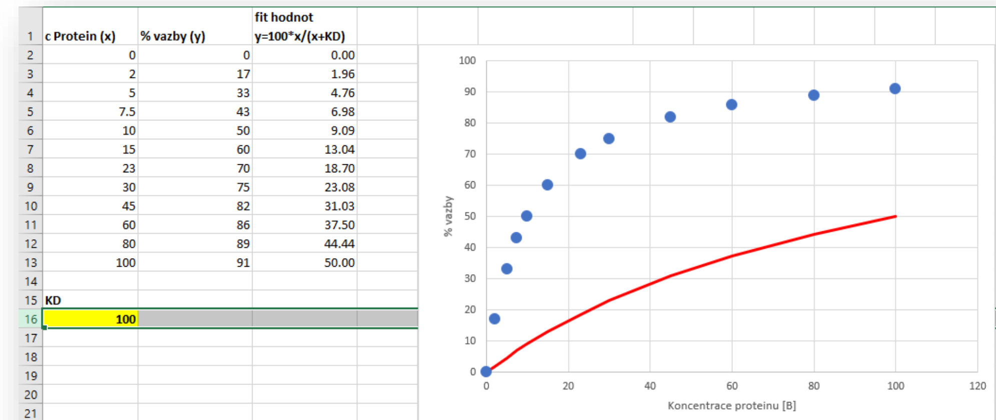
Binding curve fitting for smart minds

http://www.lablifeb.org/courses/

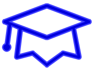
You can test how the **shape of the binding curve** changes depending on the KD in the file

KD_vliv_na_tvar_krivky.xlsx

when you manually change the K_D value on line 16.



	c Protein (x)	% vazby (y)	fit hodnot y=100*x/(x+KD)
2	0	0	0.00
3	2	17	1.96
4	5	33	4.76
5	7.5	43	6.98
6	10	50	9.09
7	15	60	13.04
8	23	70	18.70
9	30	75	23.08
10	45	82	31.03
11	60	86	37.50
12	80	89	44.44
13	100	91	50.00
14			
15	KD		
16	100		



You can try **automatic** data fitting in Excel by minimizing the sum of squared deviations of measured data and fit values using **Solver** add-in in the file

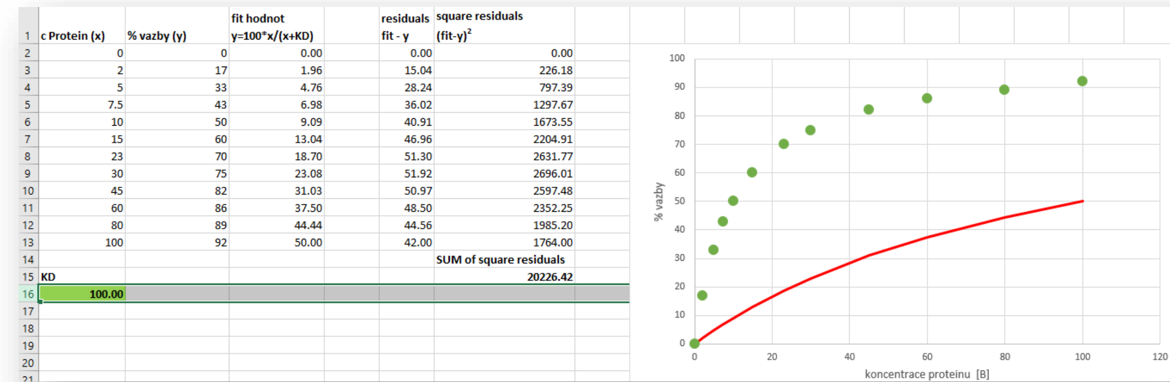
Automat_KD_fit.xlsx

A video tutorial on how to activate the Solver add-in in Excel is in the file

Resitel_Solver_ON.mp4

The tutorial is based on a video by Karl Zuvela

https://youtu.be/4jpoCGWmfeM



	c Protein (x)	% vazby (y)	fit hodnot y=100*x/(x+KD)	residuals fit - y	square residuals $(fit-y)^2$
2	0	0	0.00	0.00	0.00
3	2	17	1.96	15.04	226.18
4	5	33	4.76	28.24	797.39
5	7.5	43	6.98	36.02	1297.67
6	10	50	9.09	40.91	1673.55
7	15	60	13.04	46.96	2204.91
8	23	70	18.70	51.30	2631.77
9	30	75	23.08	51.92	2696.01
10	45	82	31.03	50.97	2597.48
11	60	86	37.50	48.50	2352.25
12	80	89	44.44	44.56	1985.20
13	100	92	50.00	42.00	1764.00
14					SUM of square residuals
15	KD				20226.42
16	100.00				