Microscale thermophoresis (MST)

Josef Houser

Biomolecular Interactions and Crystallography Core Facility

CEITEC MU

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101004806

BIC Core Facility

Biomolecular Interactions and Crystallography

- One of 12 CEITEC MU Core Facilities
- CF founded 2012, fully operational since 2015
- Open for internal & external users

- 19 techniques, 30 instruments

- **Biophysical techniques to characterize biomacromolecules and their interactions**
- **Advanced instrumentation for X-ray crystallography**

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Thermophoresis

- Particle movement in **thermal gradient**
- Unlike diffusion does not depend on concentration gradient
- Frequently used term „**Microscale thermophoresis (MST)**“ to highlight small gradient (several Kelvins) on the level of single molecules

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Theory

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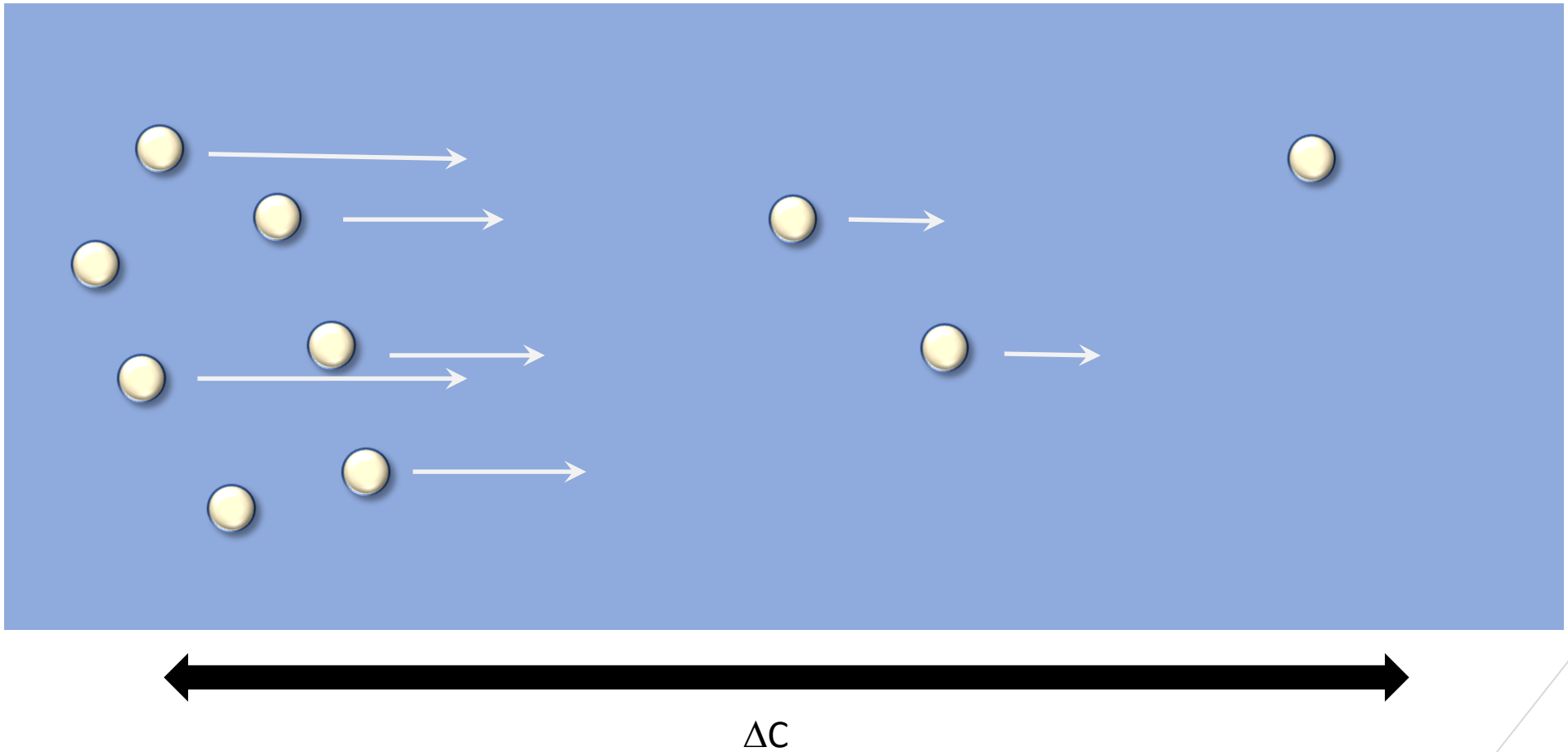
Diffusion

= movement of particles in concentration gradient

T = const.



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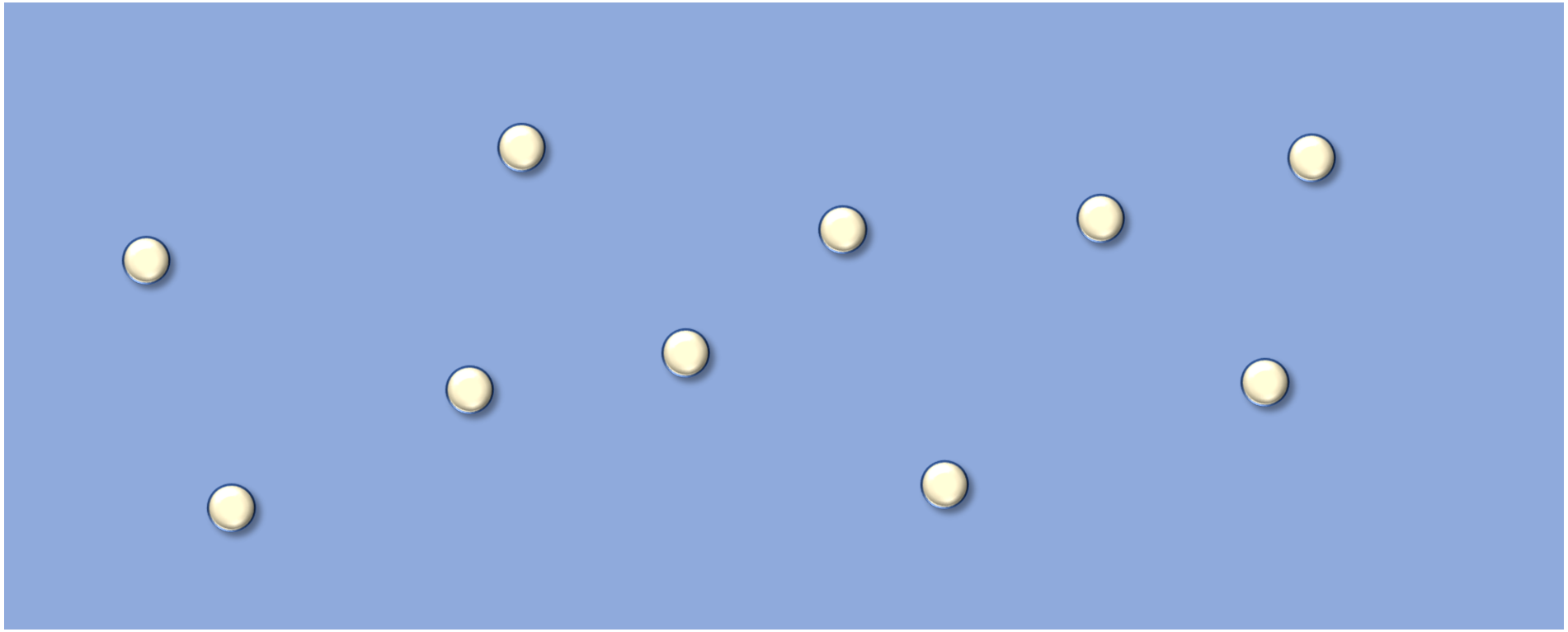
Diffusion

= movement of particles in concentration gradient

T = const.

End point



$\Delta C = 0$

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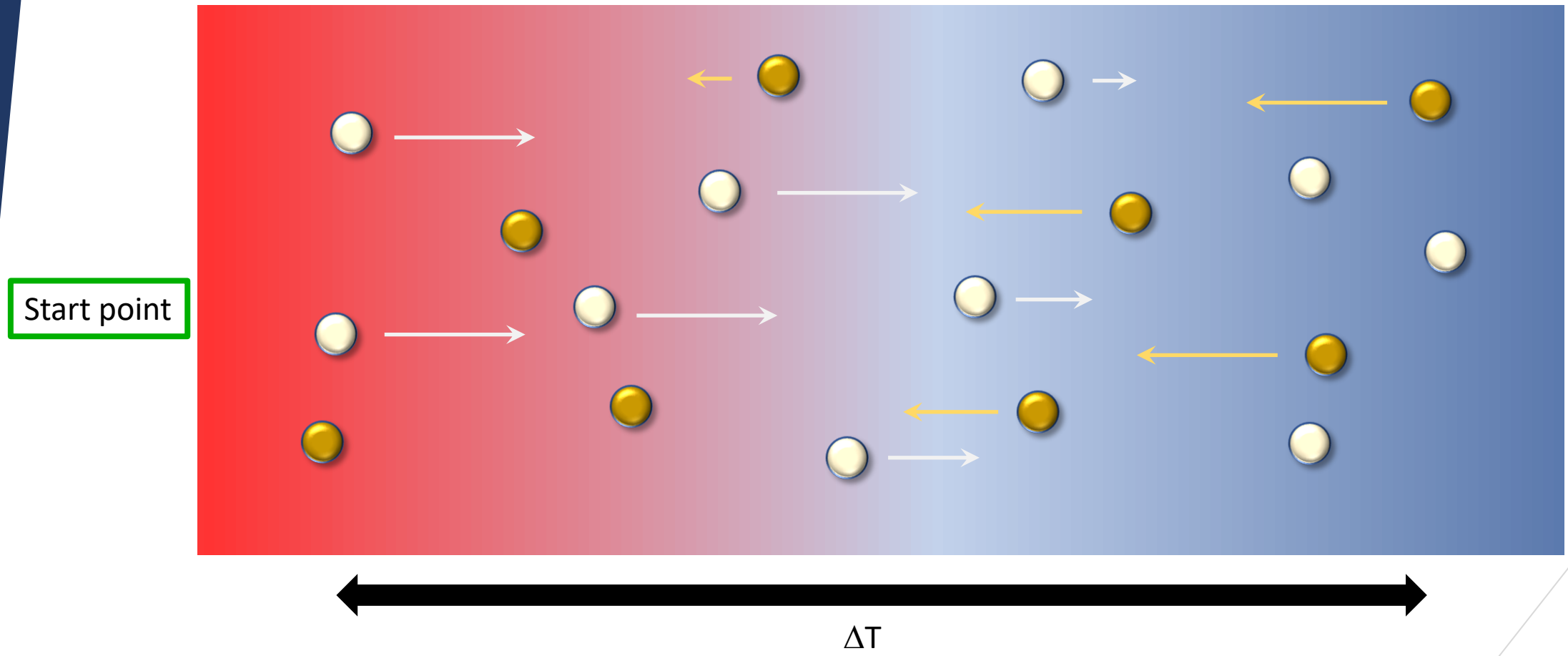
Thermophoresis

= movement of particles in temperature gradient

C = const.



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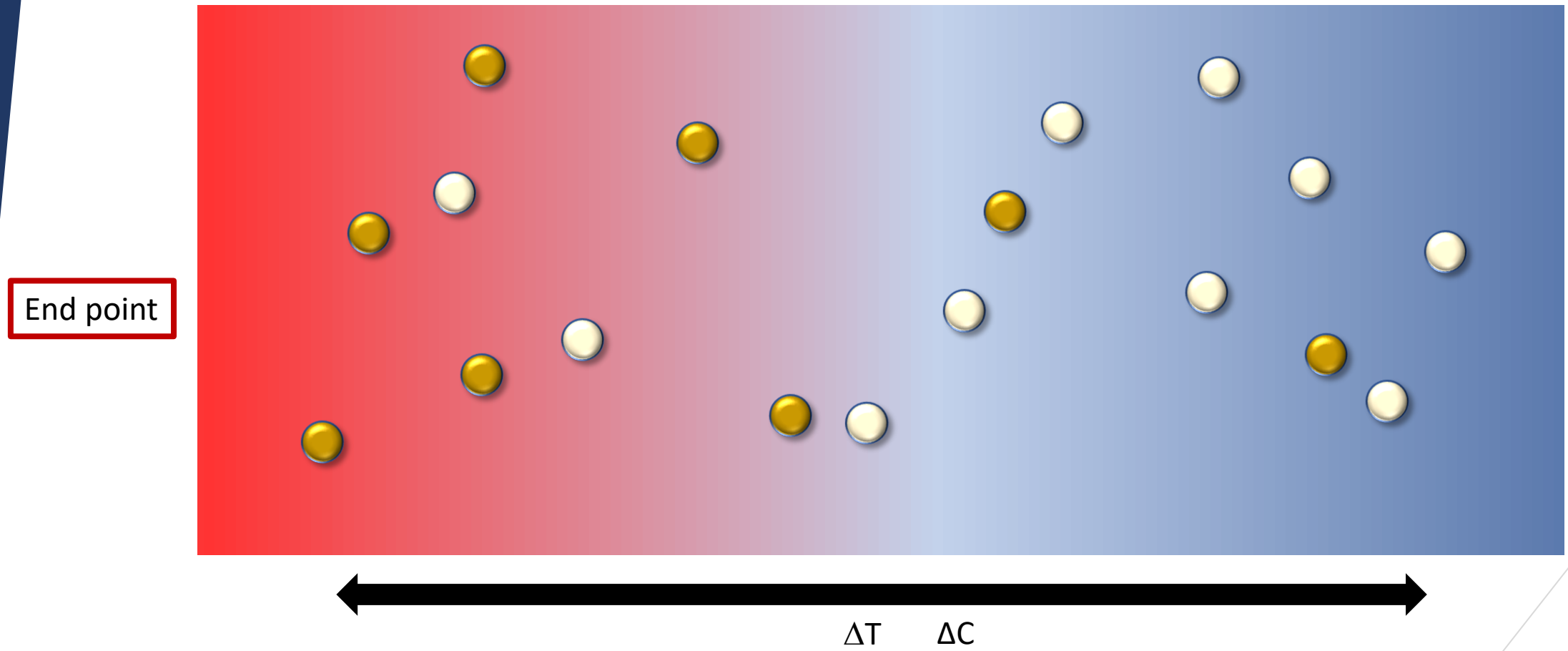
Thermophoresis

= movement of particles in temperature gradient



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History

Thermophoresis in liquids (*Carl Ludwig*)

Thermophoresis in liquids (*Charles Soret*)

1856

1870

1879

2006

2008

Thermophoresis in gas (*John Tyndall*)

Thermophoresis in solids (*Phillip Schoen*)

Thermophoresis in solids (*Amelia Barreiro*)

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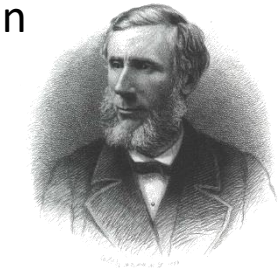


2006

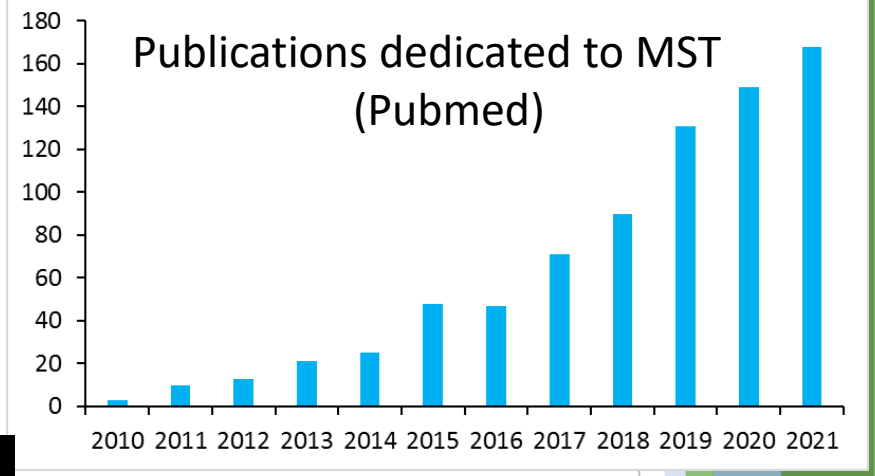
Thermophoresis in solids (*Phillip Schoen*)

2008

Thermophoresis in solids (*Amelia Barreiro*)

2010

First papers on thermophoresis application to affinity of biomacromolecules



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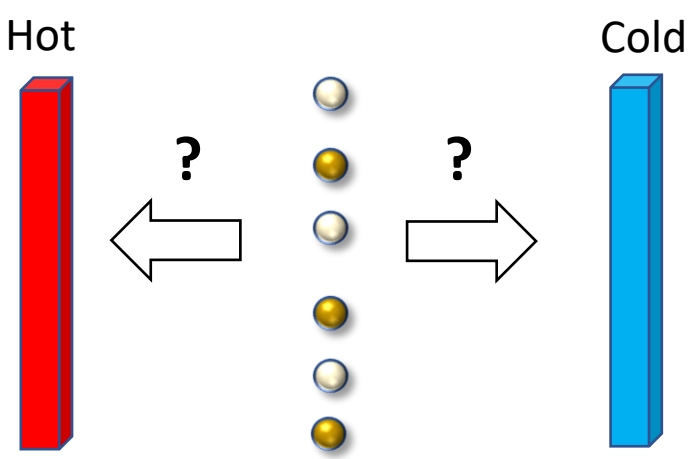
Movements of particles

Thermophoresis

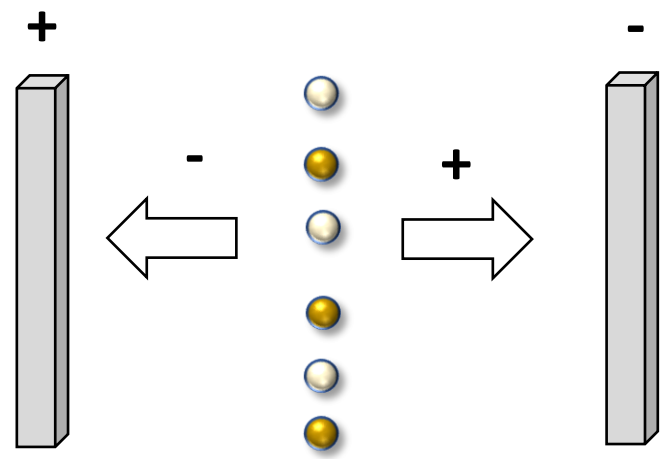
in temperature gradient



Thermophoretic parameters
(Sorret coefficient)

Electrophoresis

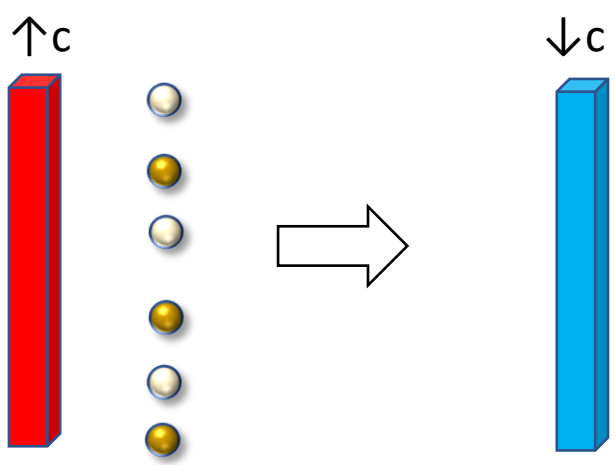
in electric field



Charge, (size)

Mass Diffusion

in concentration gradient



Particle radius,
solvent viscosity

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A bit of theory…

Particle flux j in solution (modified Fick's law)

$$j = -D\Delta\rho - \rho D_T \Delta T$$

mass diffusion ($\overrightarrow{j_m}$)

thermal diffusion ($\overrightarrow{j_T}$)

D … diffusion coefficient
ρ … particle density
D_T … thermal diffusion coefficient
T … temperature (Kelvins)
Δ … differencial value (delta)

Duhr and Braun, PNAS, 2006

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A bit of theory…

At steady state (“equilibrium”), the flux $j = 0$

thermal diffusion + mass diffusion = 0

$$\Delta\rho = \rho \frac{D_T}{D} \Delta T$$

The difference in molecular density (concentration) depends on:

- Initial concentration
- The temperature gradient
- Thermal and mass diffusion coefficitents

Duhr and Braun, PNAS, 2006

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A bit of theory…

At steady state ("equilibrium"), the flux $j = 0$

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$$\Delta\rho = \rho \frac{D_T}{D} \Delta T$$

Soret coefficient (S_T)

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

$$\frac{\text{Thermal diffusion coefficient}}{\text{Mass diffusion coefficient}}$$

Duhr and Braun, PNAS, 2006

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

$$\frac{\text{Thermal diffusion coefficient}}{\text{Mass diffusion coefficient}}$$

$$\frac{c_{hot}}{c_{cold}} = e^{-S_T.\Delta T}$$

Duhr and Braun, PNAS, 2006

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Soret coefficient… for proteins not so easy

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

A … surface area of the molecule
T … temperature (Kelvins)
s_{hyd} … hydratation enthropy of the molecule – solution interface
σ_{eff} … the effective charge
ε … dielectric constant
β … temperature derivative
λ_{DH} … Debey-Hueckel length

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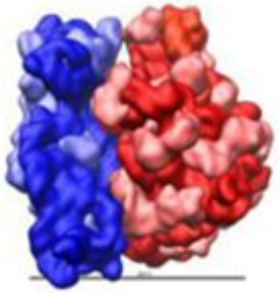
Soret coefficient… for proteins

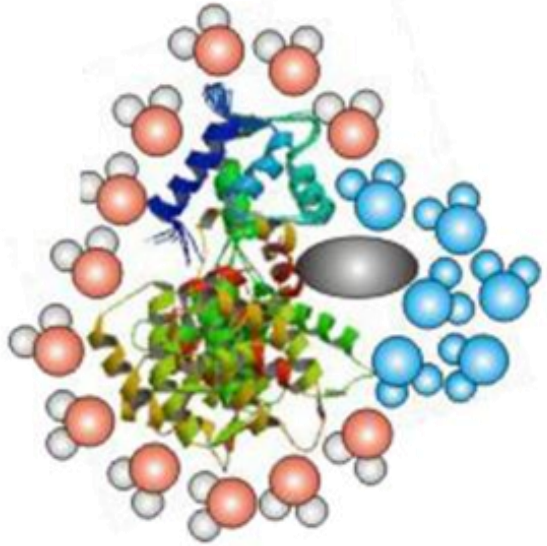
$$S_T = \frac{A}{kT}\left(-\Delta s_{hyd}(T) + \frac{\beta\sigma_{eff}^{2}}{4\varepsilon\varepsilon_0 T} \times \lambda_{DH}\right)$$

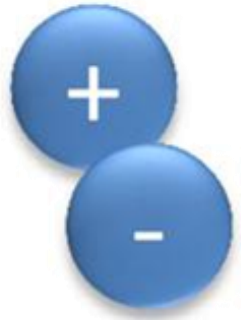
size

charge ²

hydration shell







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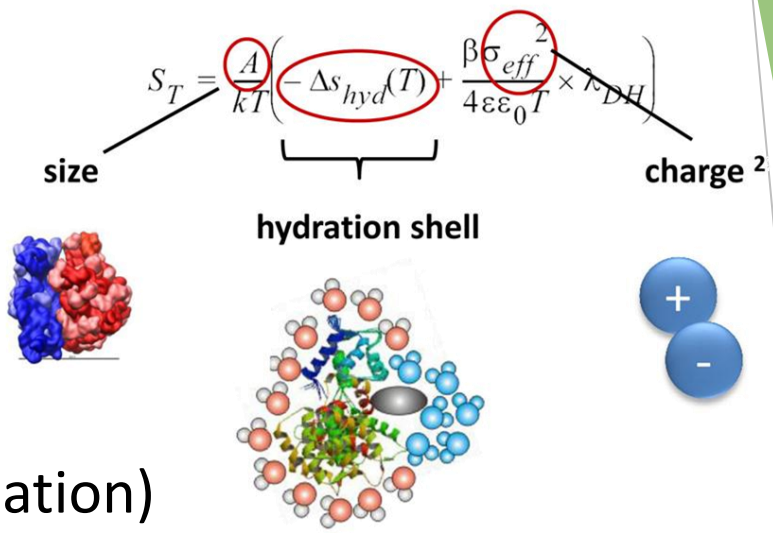
Soret coefficient… for proteins



Soret coefficient S_T depends on:

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

Strength of MST – almost every interaction causes changes in one of these parameters (not in mean temperature)

→ is measurable by MST

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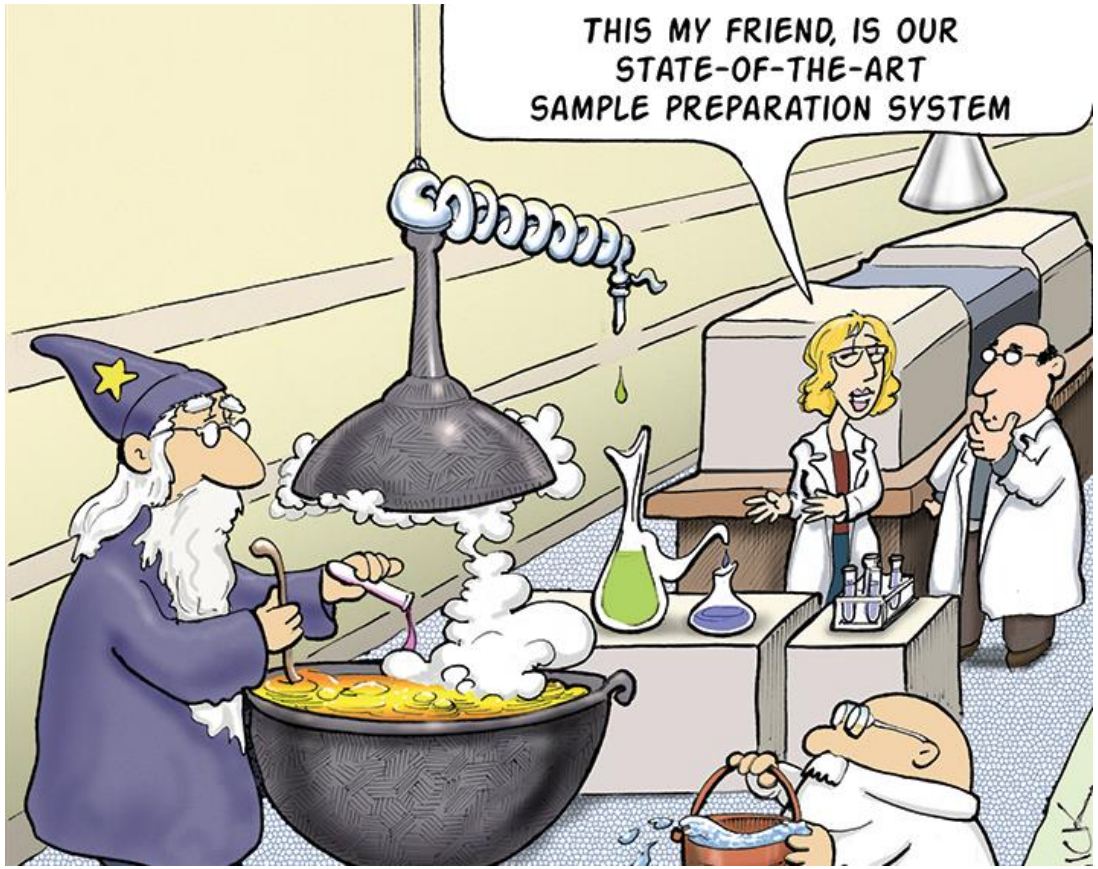
Experiment



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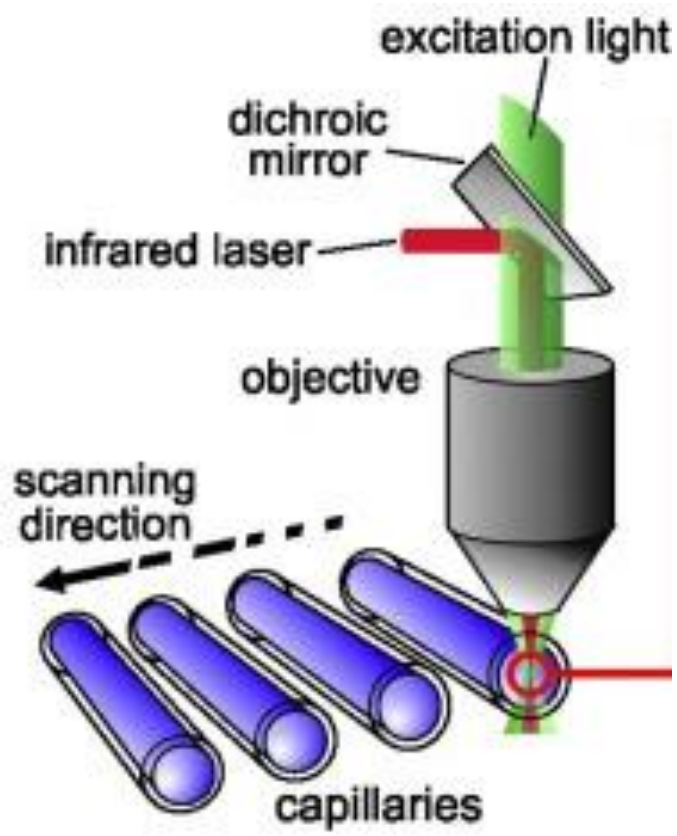
MST measurement – laser

- **Infrared laser**
  - Creates temperature gradient
  - ΔT depends on the laser power and time (>10 K after 5 s at 40% laser power)
- **Excitation laser**
  - Excites fluorescence
  - Red, blue, green or UV laser
  - Suitable fluorescent dye needed



Jerabek-Willemsen, 2014

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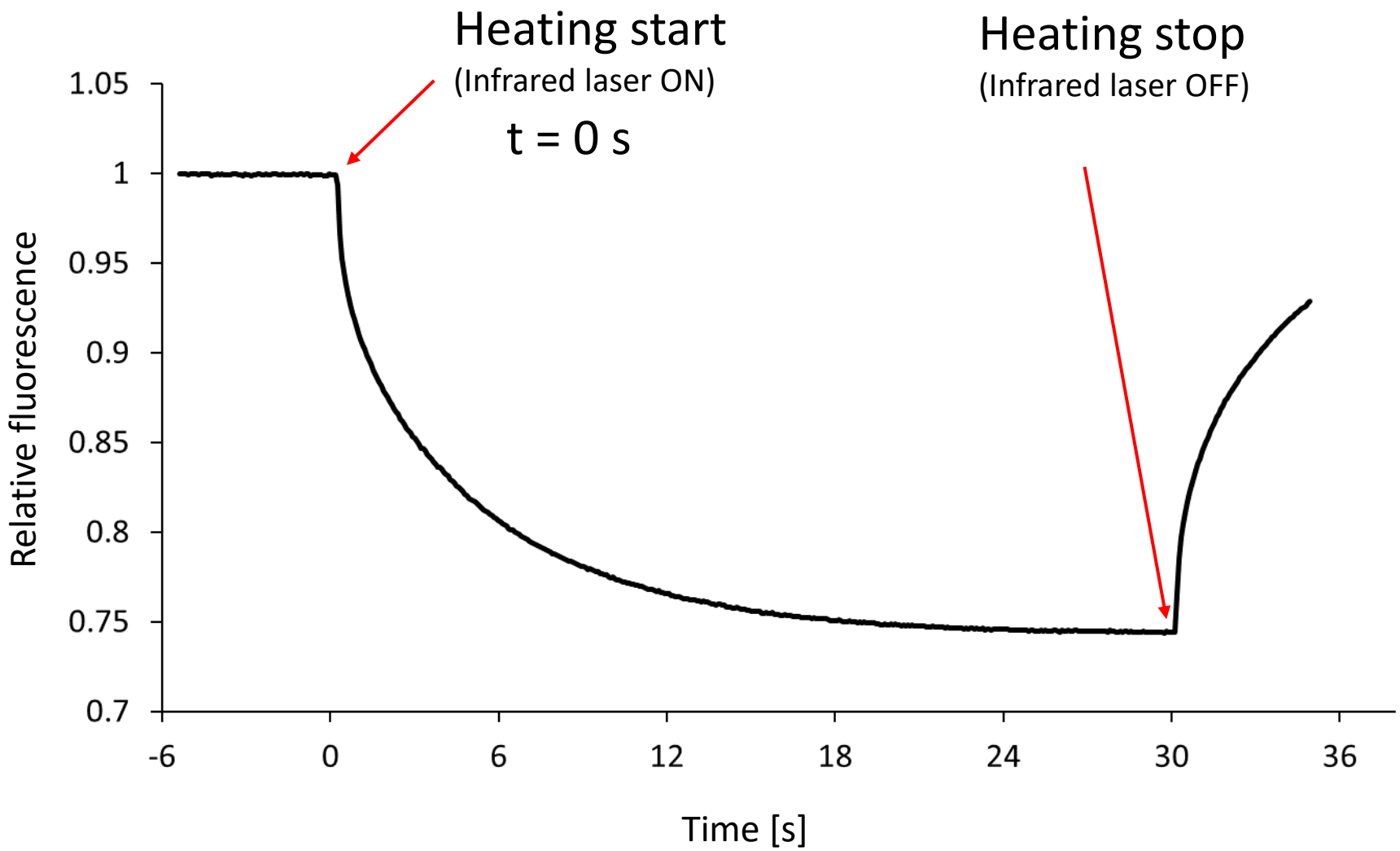
MST curve



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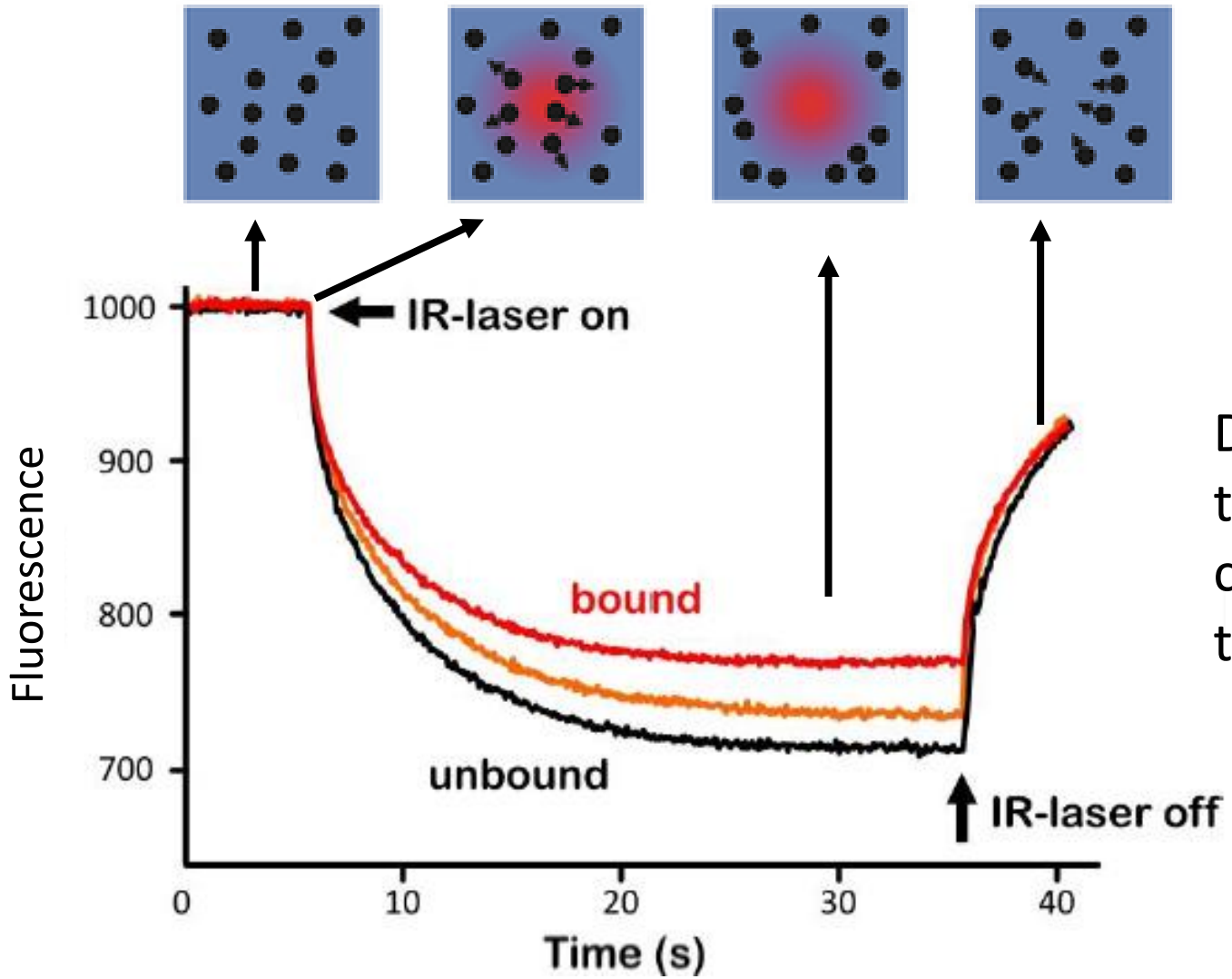
MST measurement



Different Soret coefficient for target alone and target in complex with ligand causes the differences in MST curves

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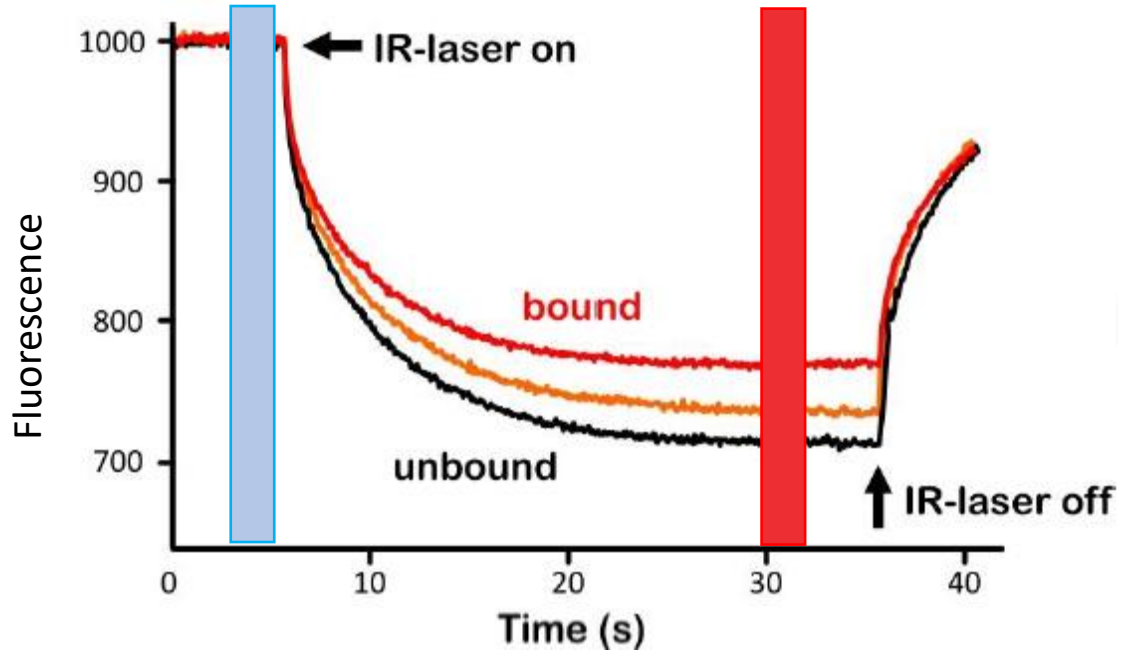
MST measurement

- Data analyses



Normalized fluorescence F_N , F_{norm}

$$F_N = \frac{\langle \mathbf{F_{hot}} \rangle}{\langle \mathbf{F_{cold}} \rangle}$$

$$\frac{c_{hot}}{c_{cold}} = e^{-S_T.\Delta T}$$

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A bit more theory…

If the fluorescence $F_{norm,free}$ of free molecules differs from fluorescence $F_{norm,bound}$ of bound molecules then for the bound fraction *FB* of binding partner B:

$$F_{norm} = (1 - FB)\, F_{norm,free} + (FB)\, F_{norm,bound}$$

Equilibrium constant K_D is calculated as:

$$K_D = \frac{1}{K_A} = \frac{[A]_{free}[B]_{free}}{[AB]} = \frac{([A]-[AB])([B]-[AB])}{[AB]}$$

Leading to quadratic equation with a single unknown variable K_D:

$$FB = \frac{[AB]}{[B]} = \frac{[A]+[B]+K_D - \sqrt{([A]+[B]+K_D)^2 - 4[A][B]}}{2[B]}$$

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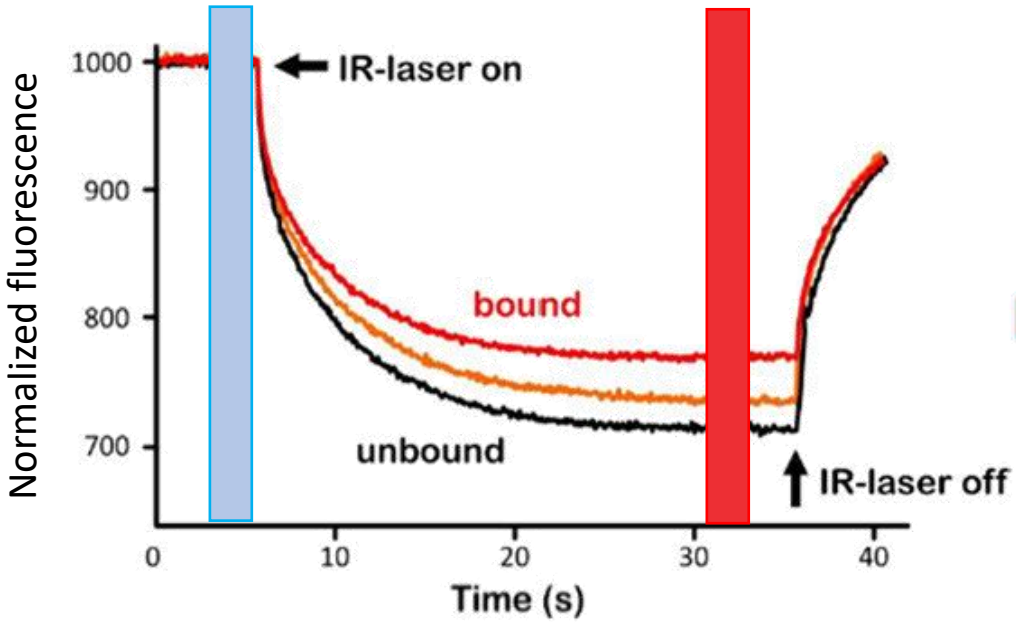
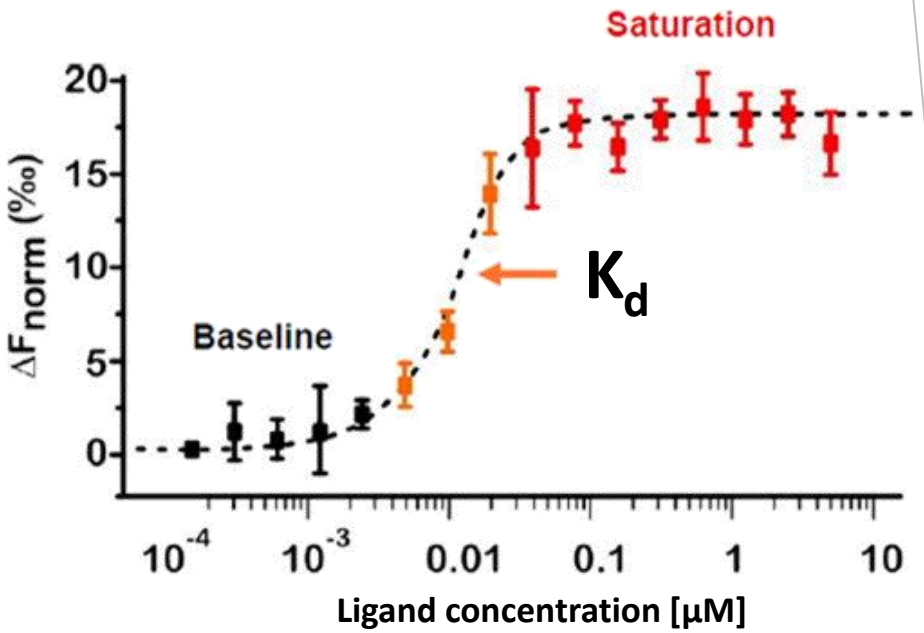
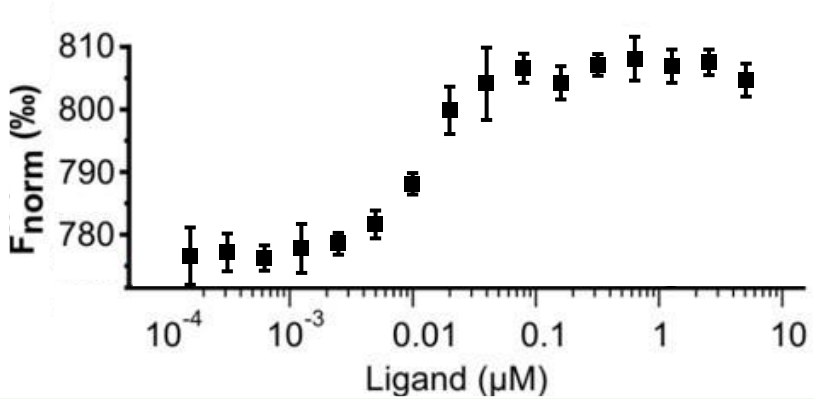
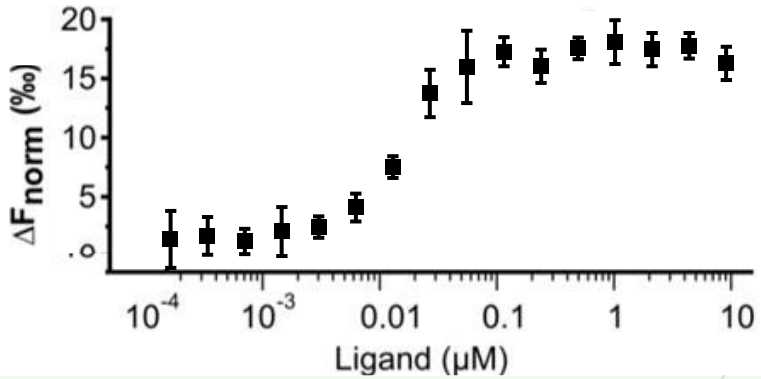
Experiment evaluation









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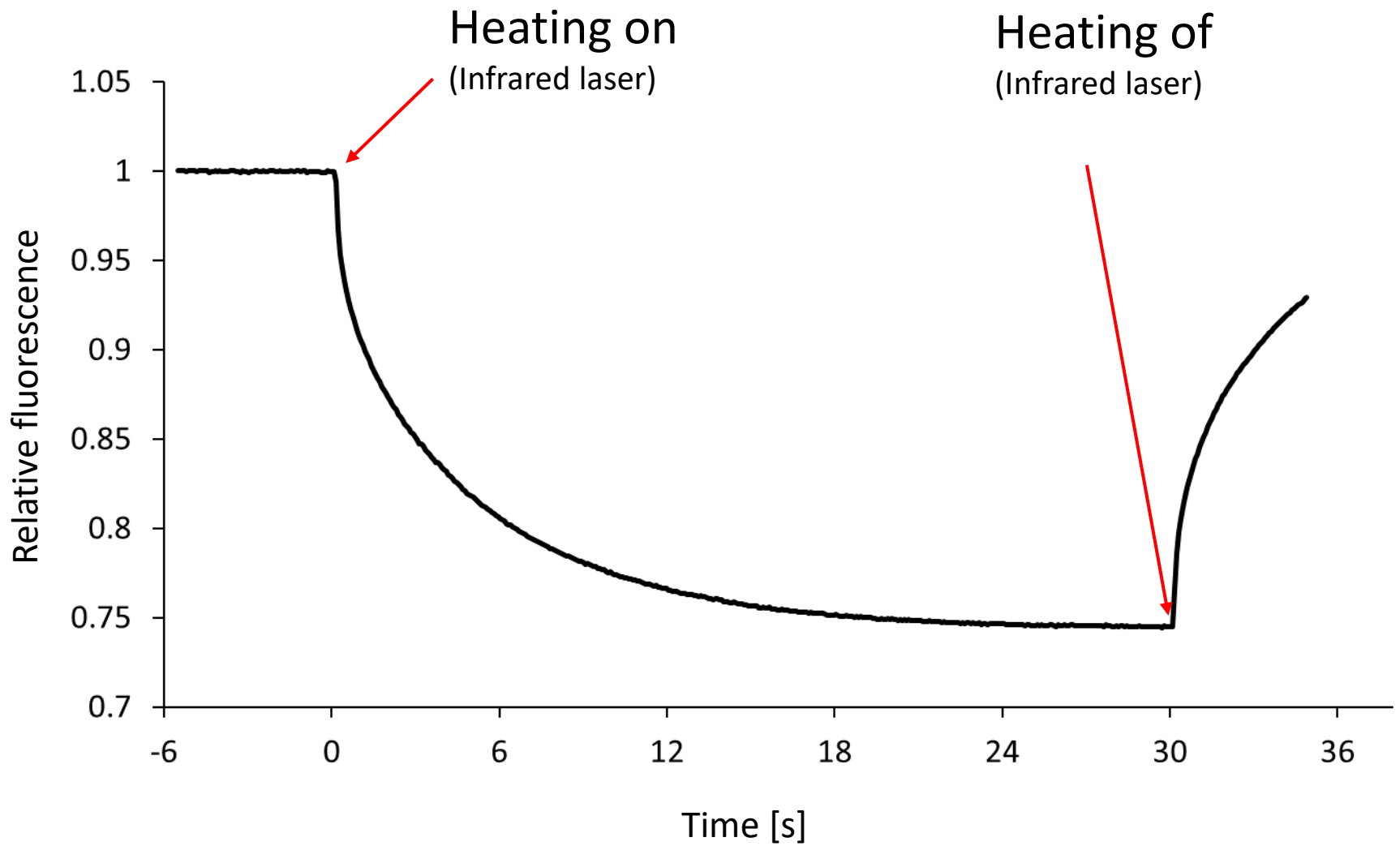
Dissecting the MST timetrace



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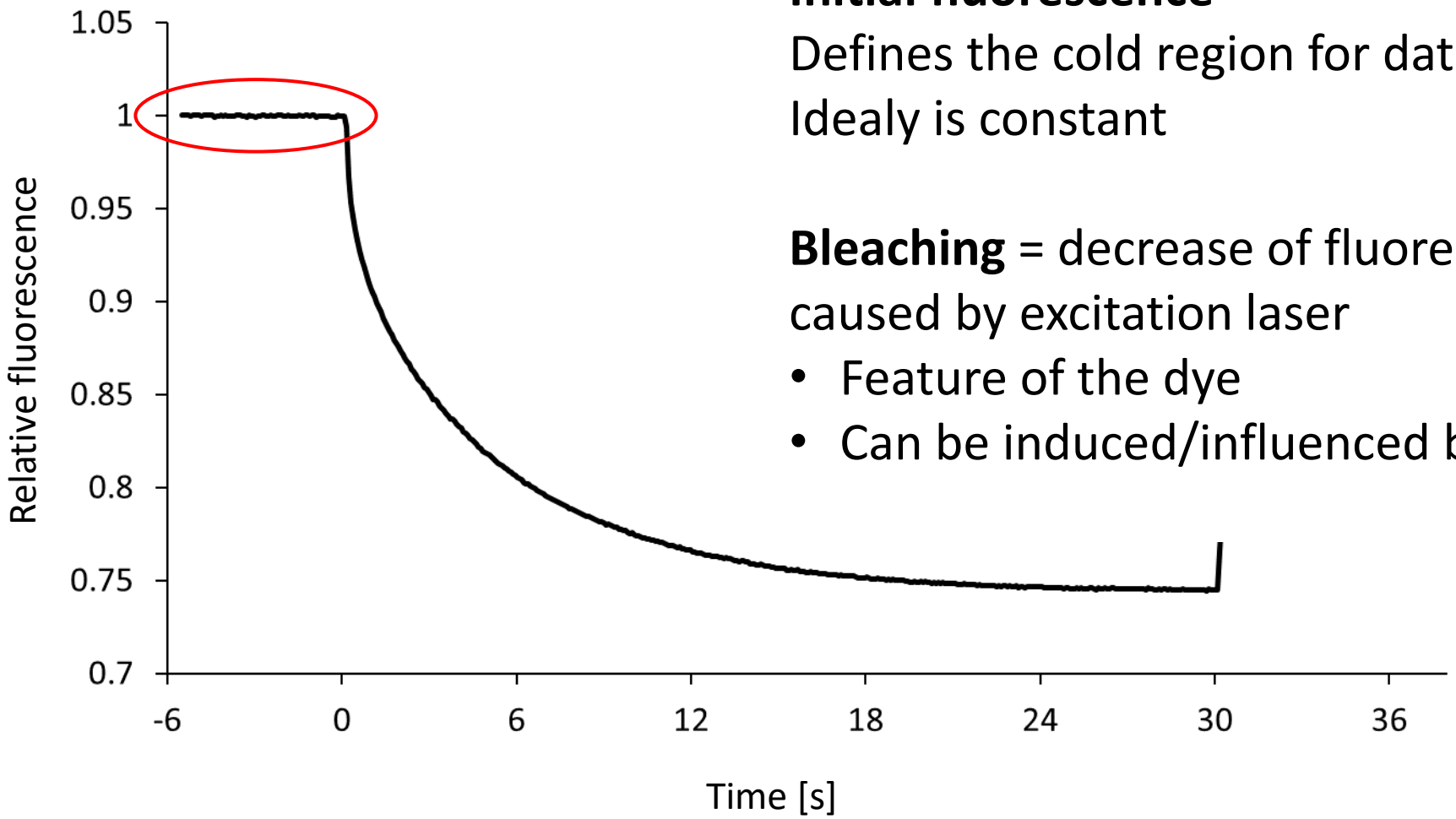

Initial fluorescence
Defines the cold region for data analyses
Idealy is constant

Bleaching = decrease of fluorescence signal caused by excitation laser

- Feature of the dye
- Can be induced/influenced by ligand

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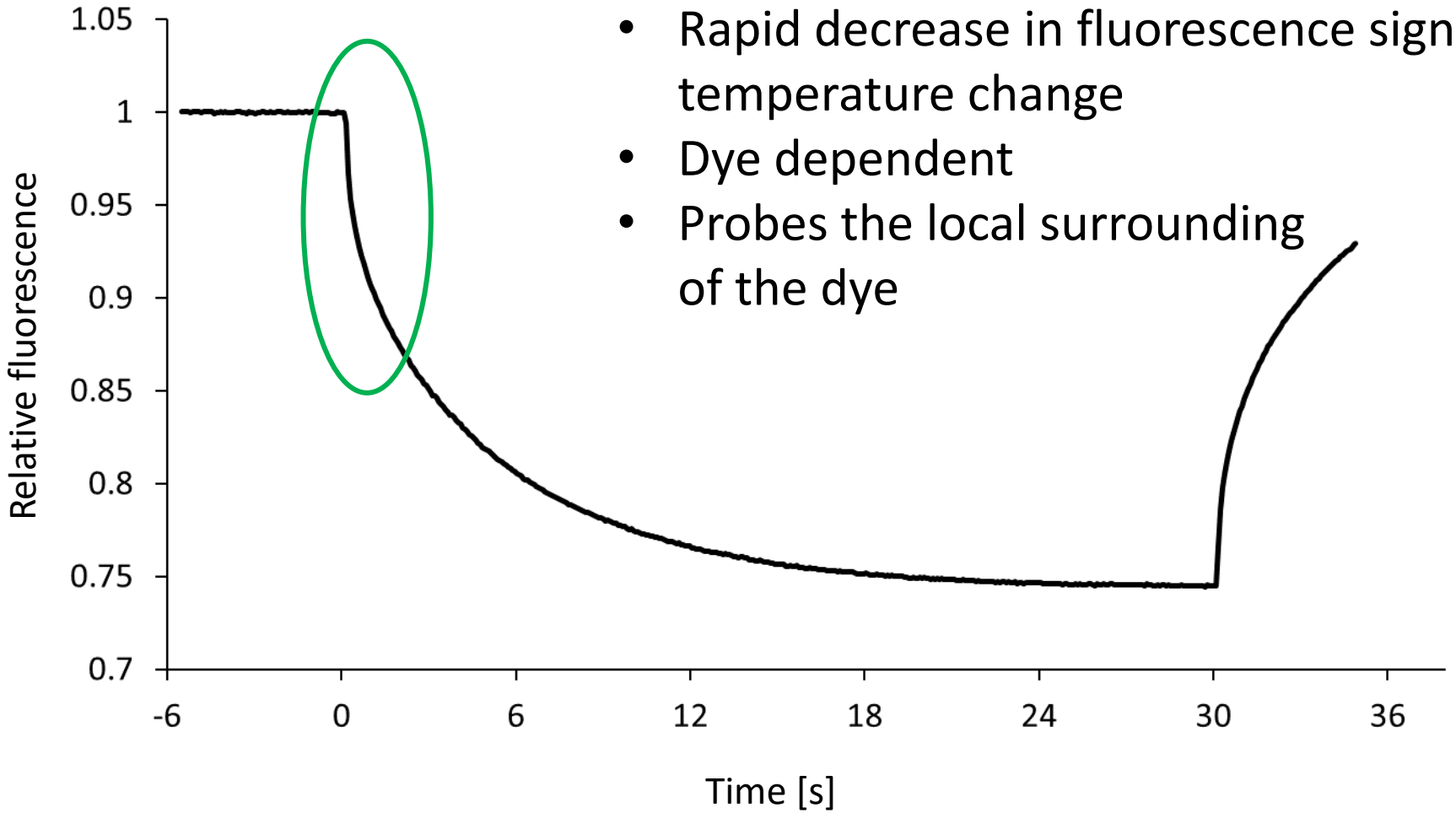
Dissecting the MST timetrace



T-jump

- Rapid decrease in fluorescence signal caused by temperature change
- Dye dependent
- Probes the local surrounding of the dye

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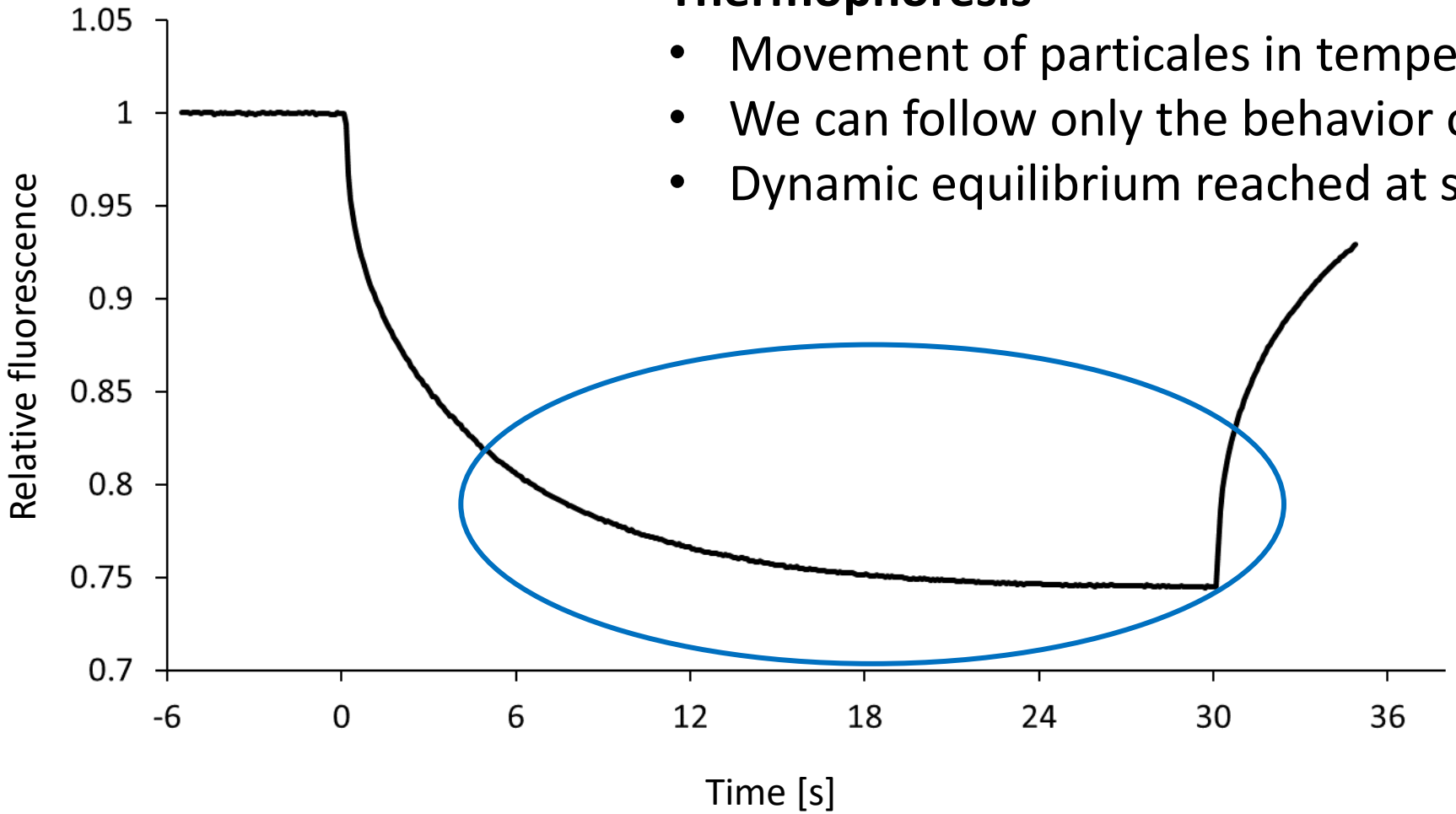
Dissecting the MST timetrace

Thermophoresis

- Movement of particales in temperature gradient
- We can follow only the behavior of the target
- Dynamic equilibrium reached at steady state



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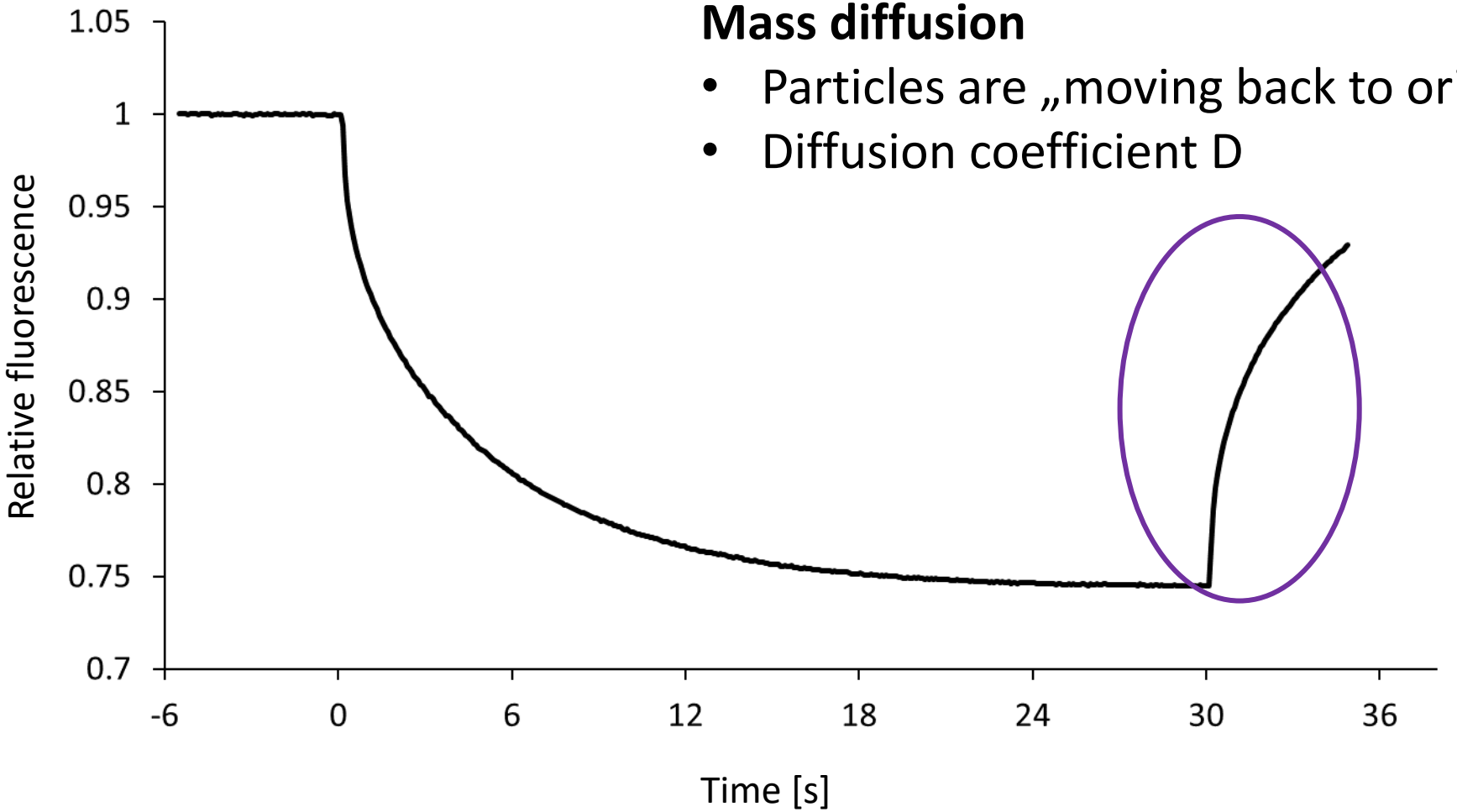
Dissecting the MST timetrace



Mass diffusion

- Particles are „moving back to original position“
- Diffusion coefficient D

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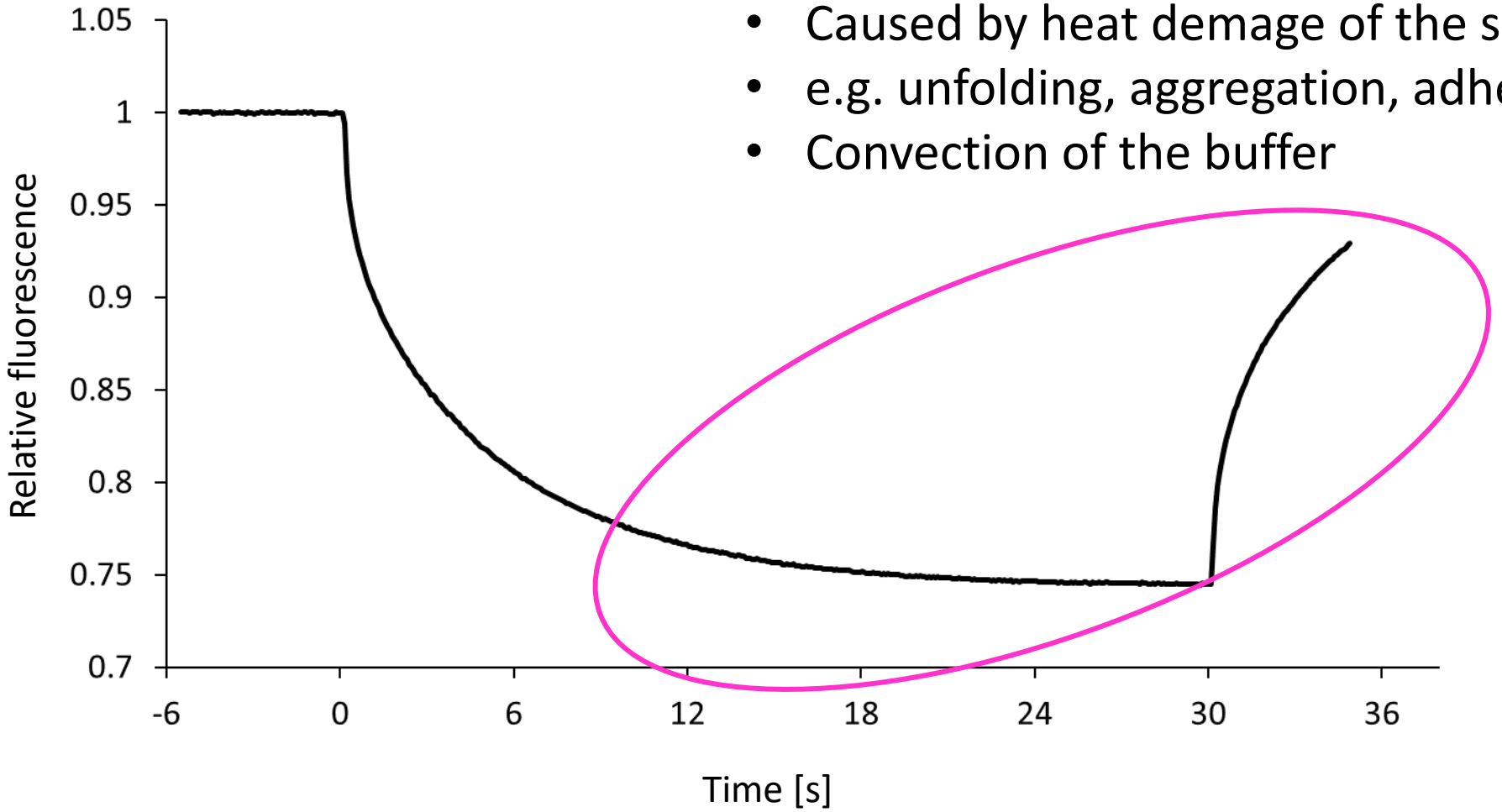
Dissecting the MST timetrace

Irreversible effects

- Caused by heat demage of the sample
- e.g. unfolding, aggregation, adhesion to capillary
- Convection of the buffer



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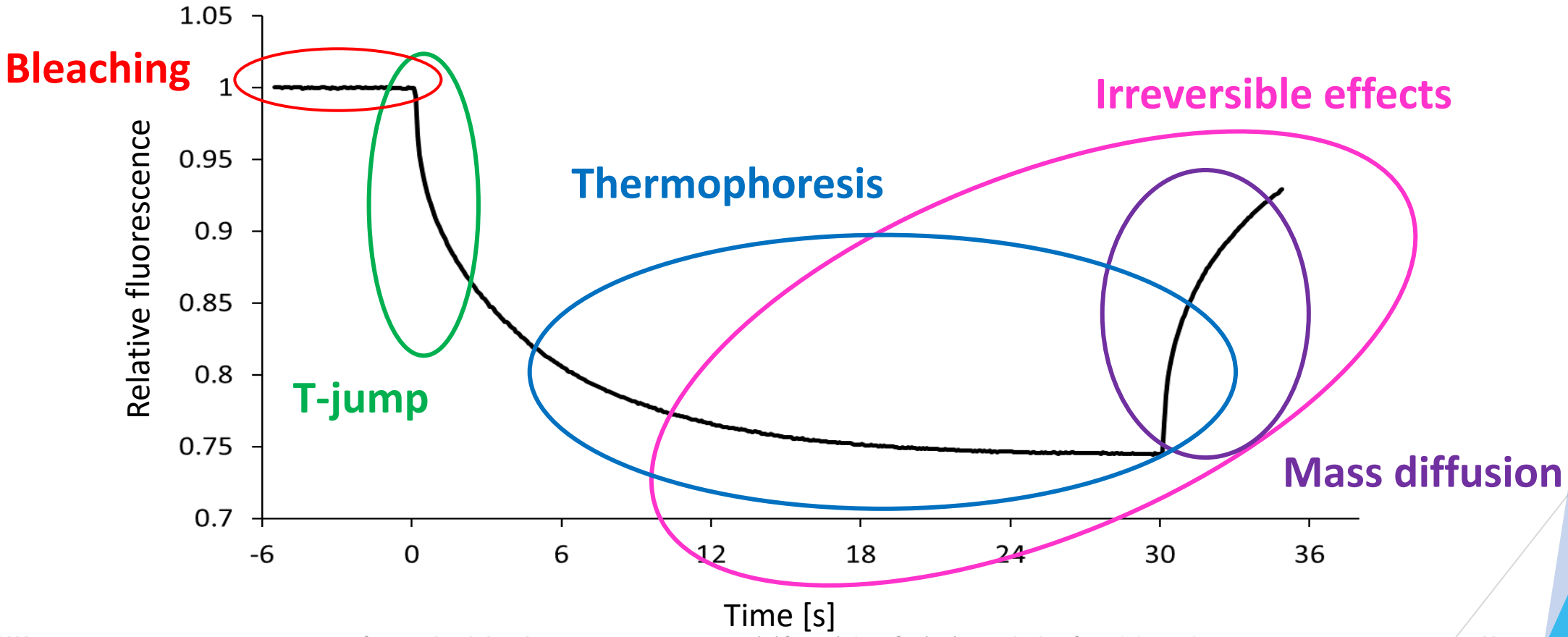
Dissecting the MST timetrace ?

- MST do not measure thermophoresis but „fluorescence under thermal perturbation“
- **TRIC – temperature related intensity change** (NanoTemper, 2018)



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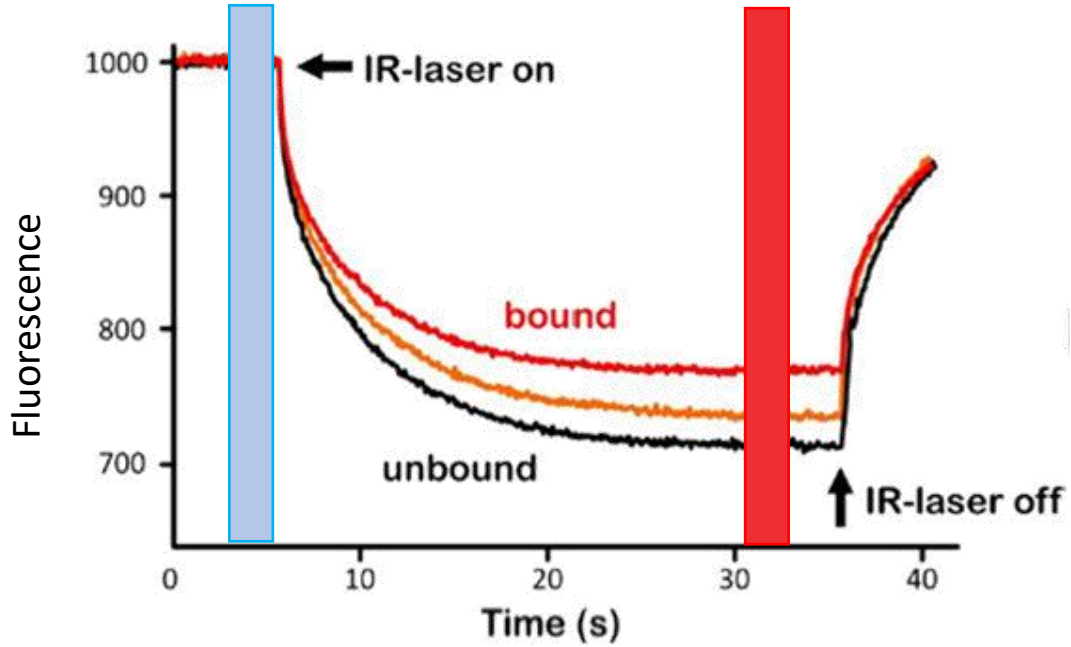
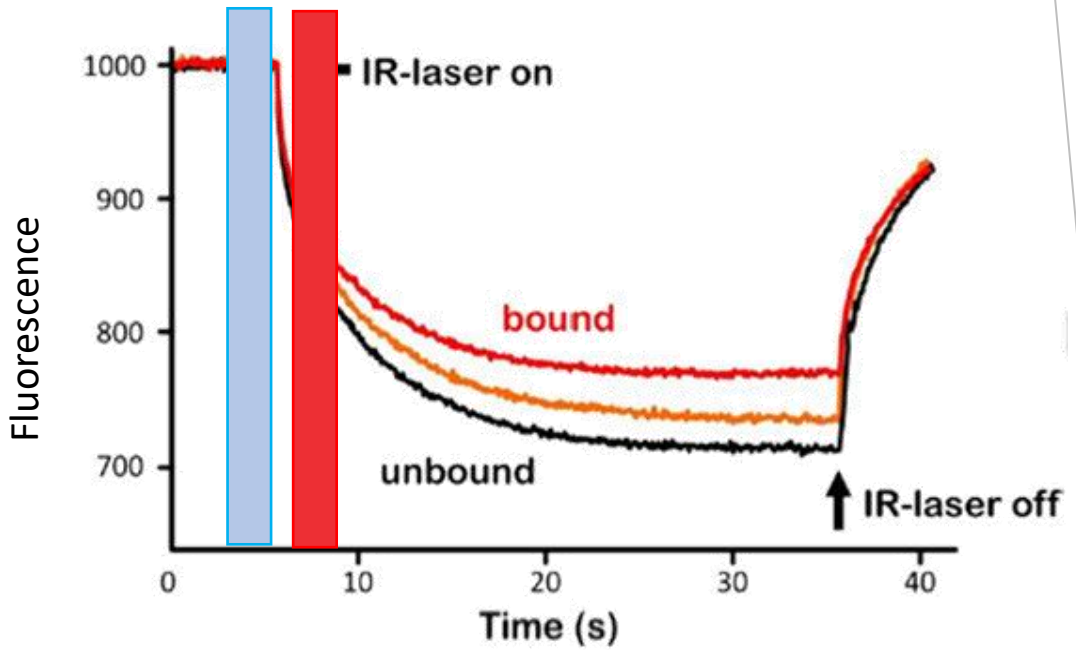
MST data analysis



Original approach
"Hot region" at the maximum difference of signal

MST timetrace



Current approach
"Hot region" at the minimal effect of sample heating

TRIC timetrace

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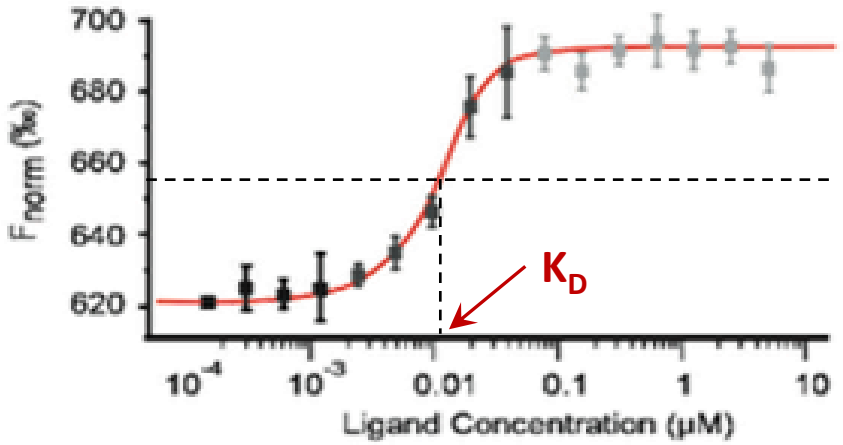
What can be measured by MST?

Affinity

- What is the strength of interaction?
- Labelled partner (target) at constant $c \leq K_D$
- Serial dilution of second partner (ligand) in range of expected K_D



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More than affinity (special cases)

- **Multiple binding events** within one experiment
- **Stoichiometry** determination
- **Inhibition assay**
- **Thermodynamics** measured by MST

- Interaction with **liposomes**
- Measurement in **crowdy samples** (blood, cell lysate)

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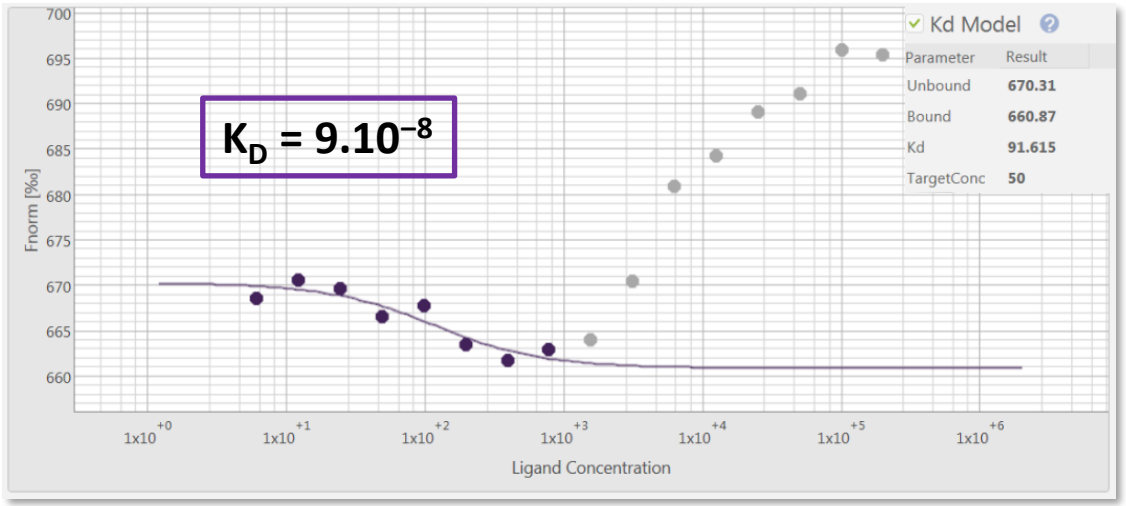
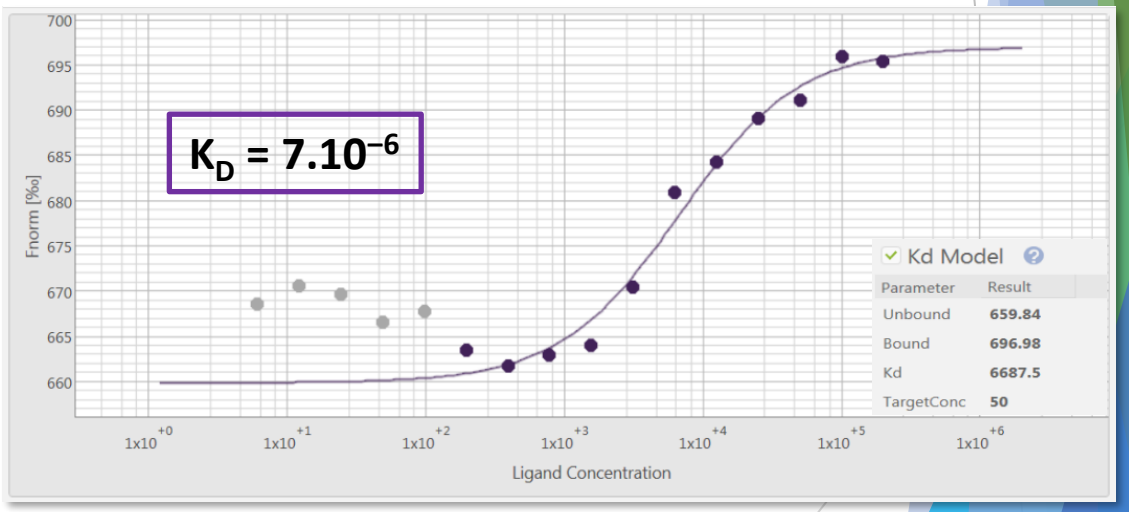
Multiple binding events

Two independent binding events in one measurement

- Target 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





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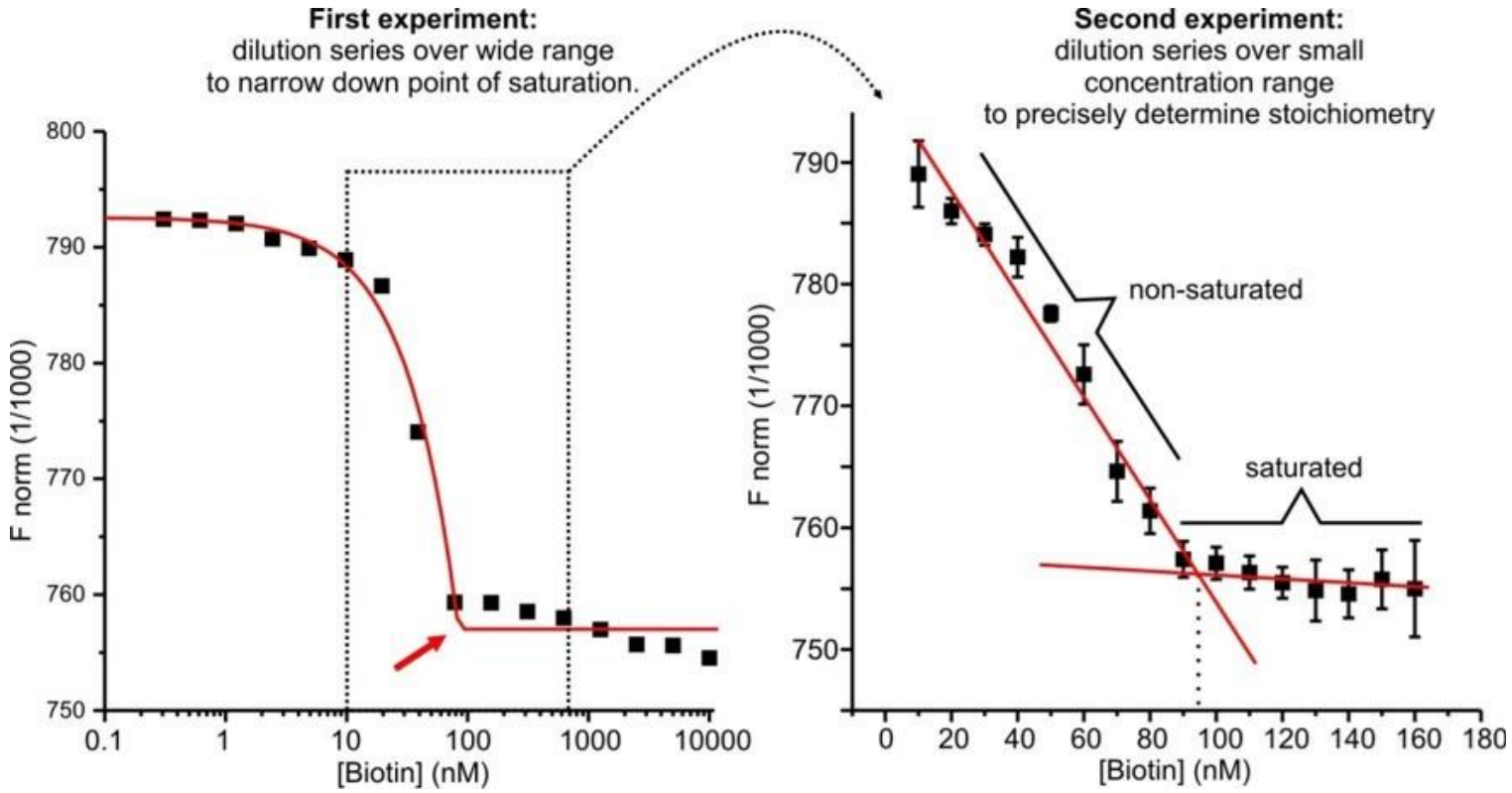
Stochiometry

- Target (labelled) at constant c > K_D
- Several dilution of ligand in range of expected molecular ratio



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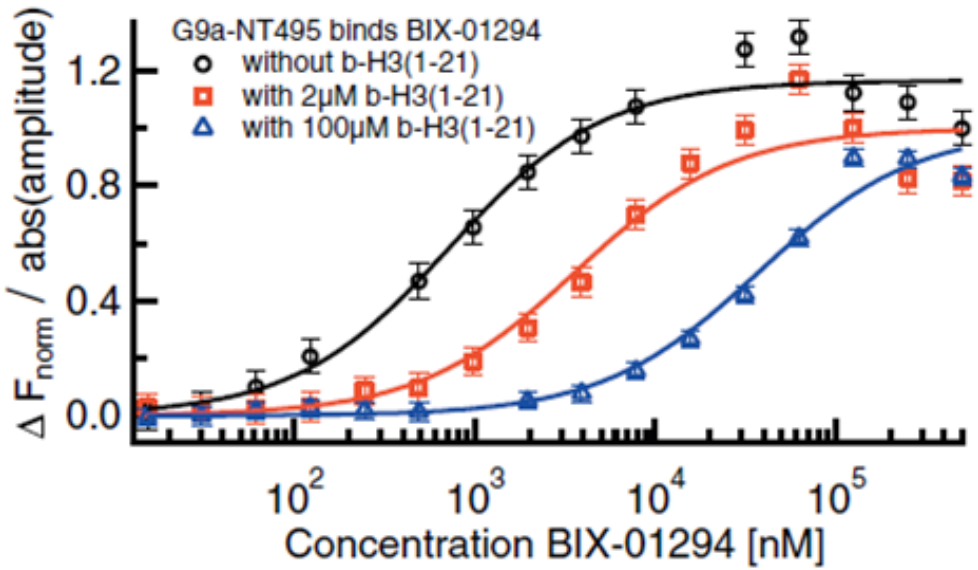
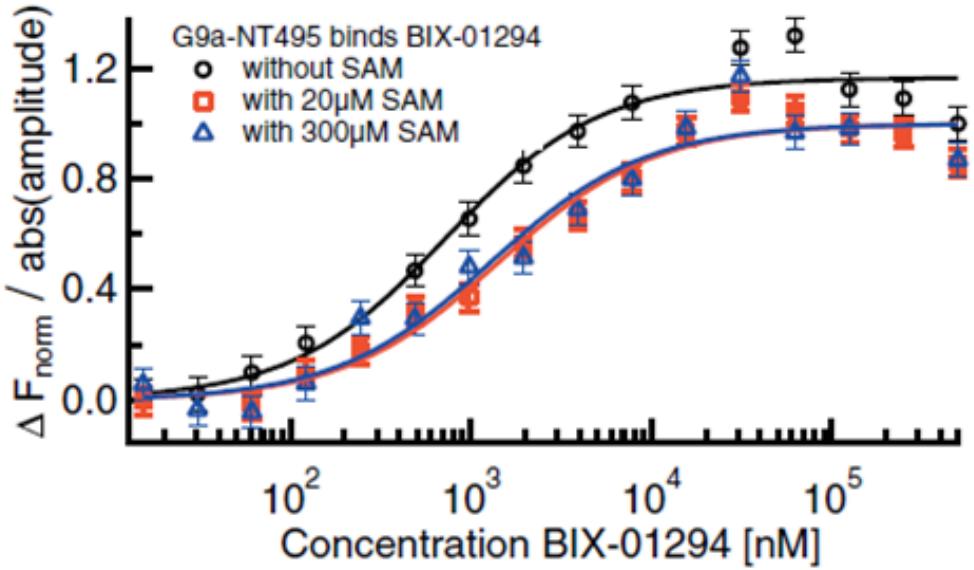
Inhibition assay

- Standard affinity measurement in **presence** and **absence** of inhibitor
- Comparison of curves / calculated K_D





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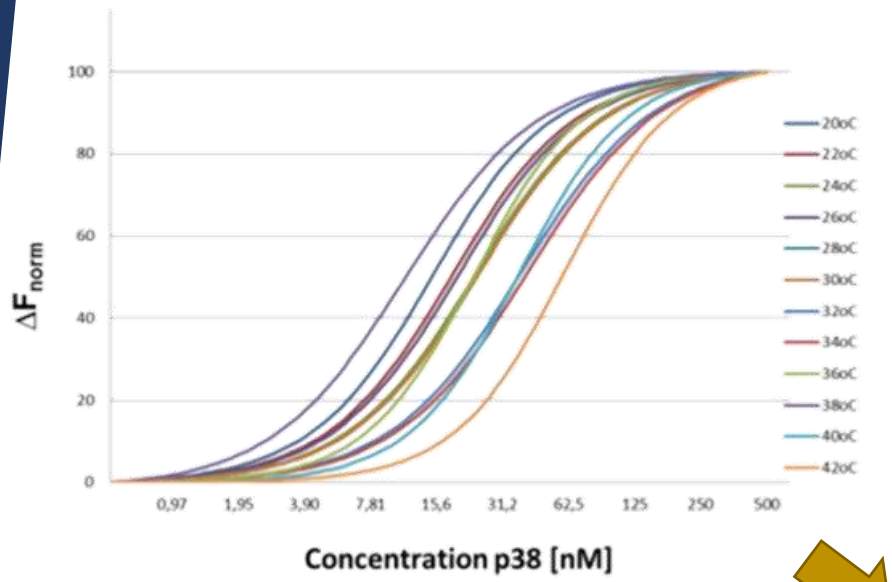
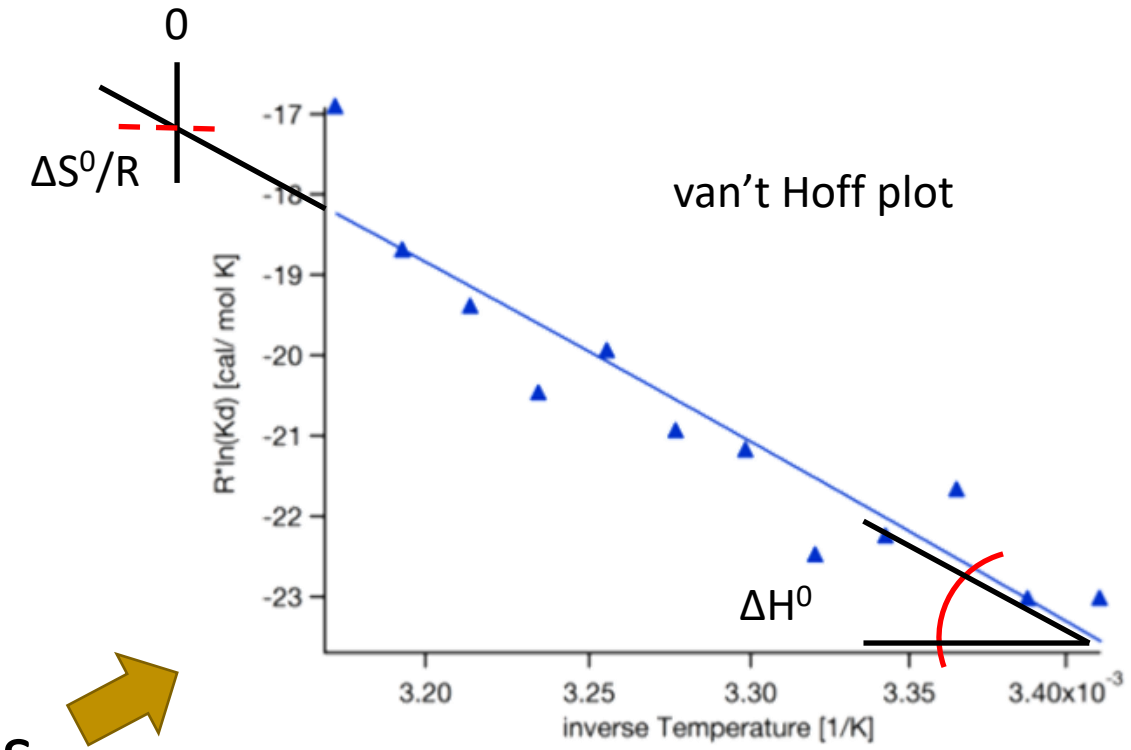
Thermodynamics

- K_D determination at various temperatures
- Calculation of thermodynamic parameters



K_D's



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Practical aspects

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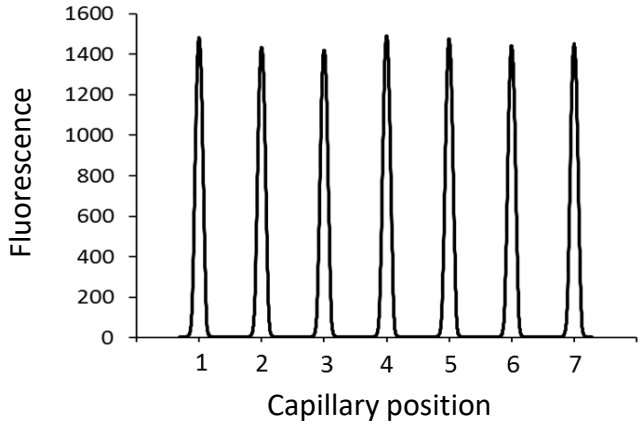
Experiment

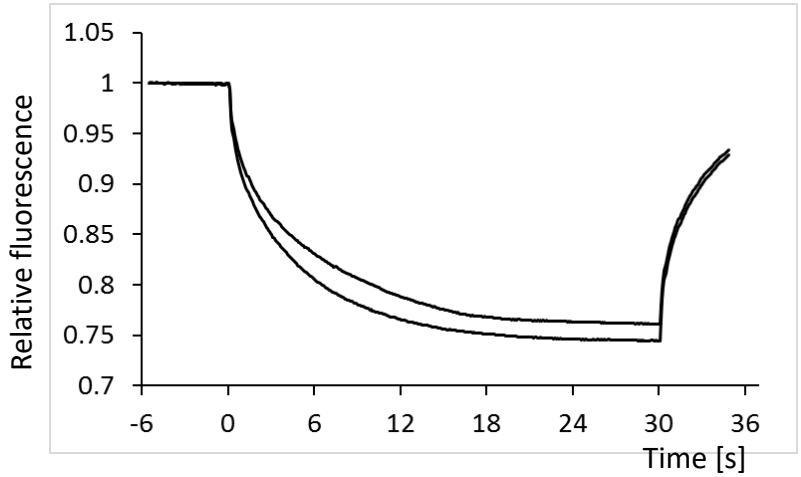
1. Sample preparation

2. Capillary filling

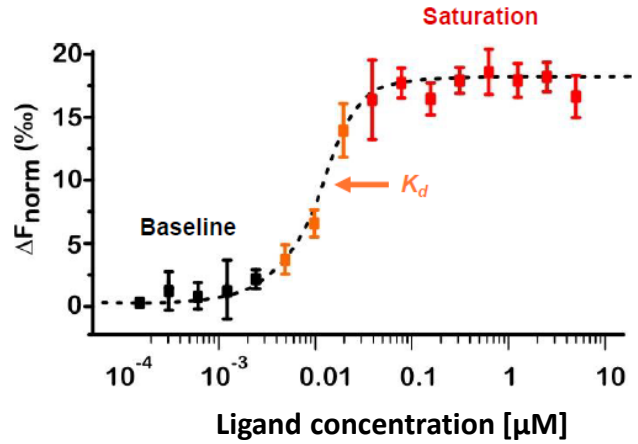
3. Capillary scan



4. MST measurement



5. Data analysis



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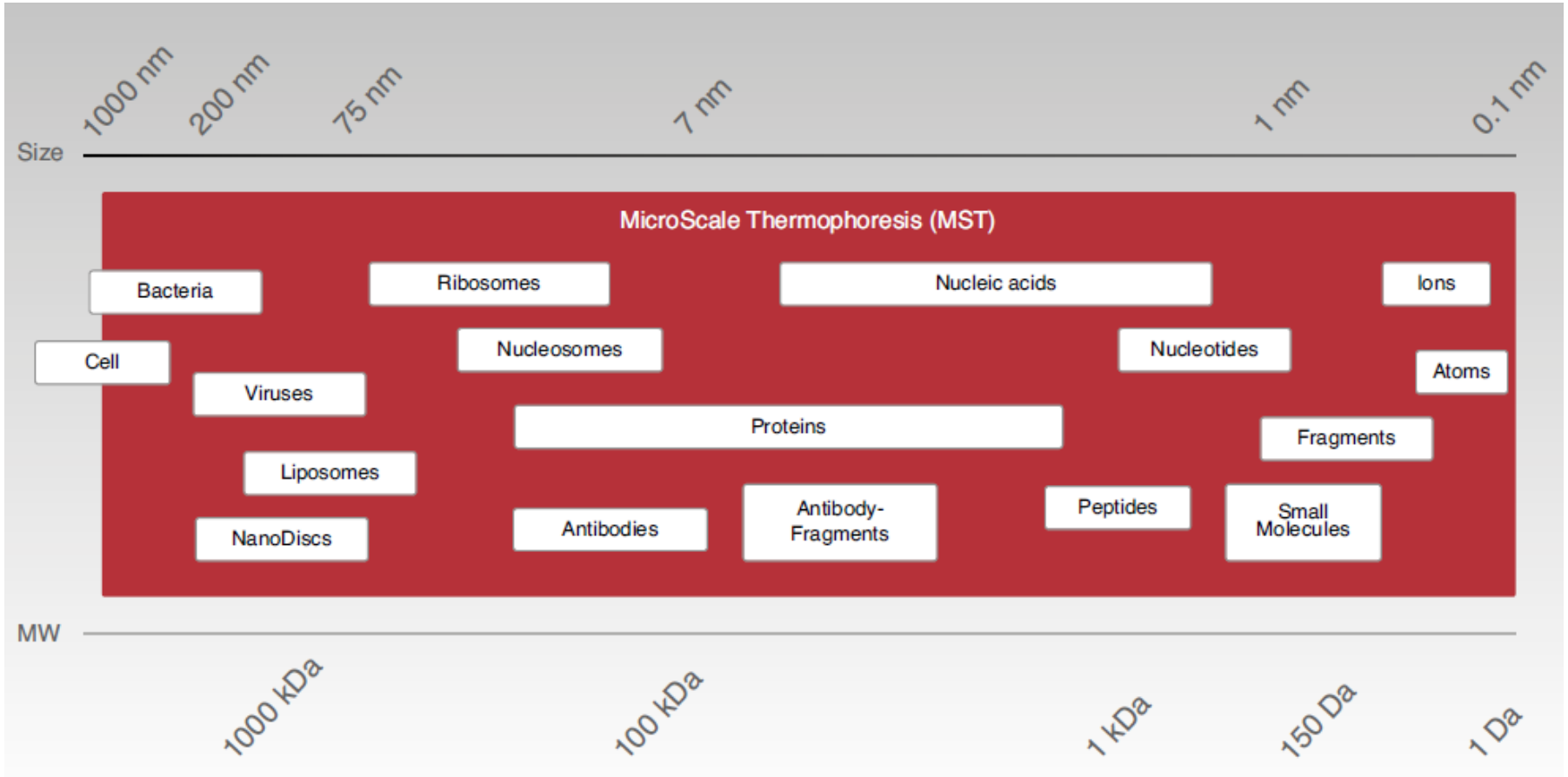
Sample

Size range



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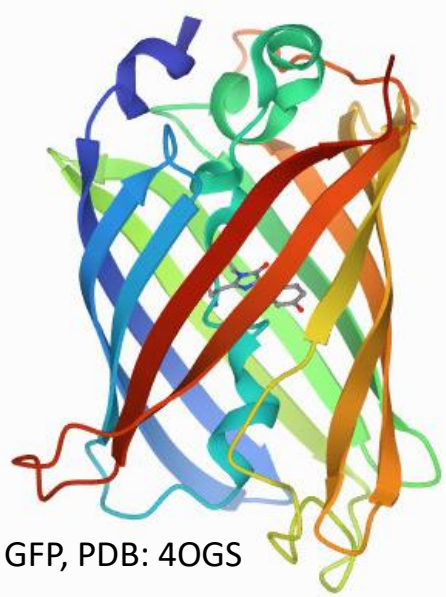
Sample

Labelling

- Instrument measures fluorescence signal
  - Dyes compatible with blue, green or red laser
  - Commercial dyes or specialised dyes from MST manufacturer (NanoTemper)

Monolith NT.115	LED 1 /nm	LED 2 /nm	Blue Dyes	Green Dyes	Red Dyes
NT.115 Blue/Green	Ex:470 Em:520	Ex:550 Em:600	FITC/FAM/GFP/YFP	Cy3/RFP/mCherry	no detection
NT.115 Blue/Red	Ex:470 Em:520	Ex:625 Em:680	FITC/FAM/GFP/YFP	no detection	Cy5/Alexa647
NT.115 Green/Red	Ex:520 Em:570	Ex:625 Em:680	YFP	Cy3/RFP	Cy5/Alexa647

- Labelling is necessary unless
  - You work with **fluorescent proteins**
    - GFP (green)
    - YFP (yellow)
  - You have "**label free" instrument**
    - Uses intrinsic flourescence of tryptophanes



GFP, PDB: 4OGS

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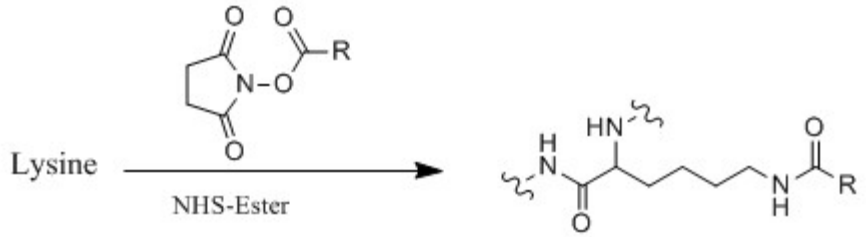
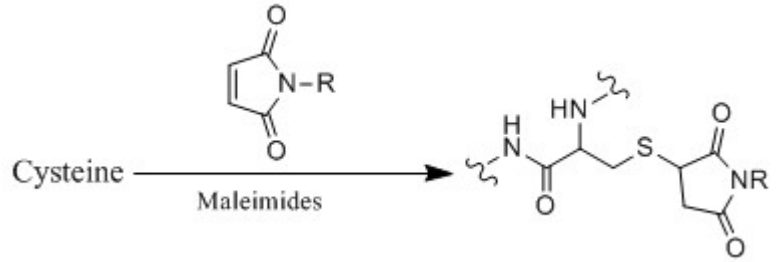
Sample labelling

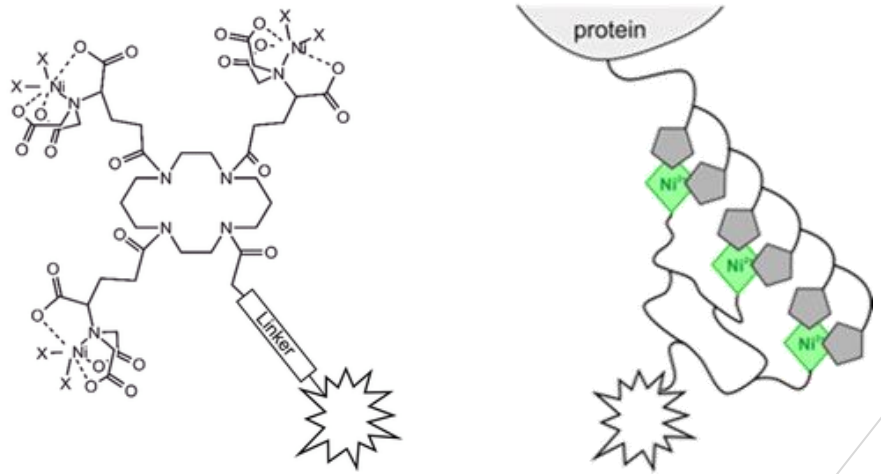
Reactive groups availability:



Amino group – Lysine, N-terminus



Thiol (sulfhydryl) group – Cystein



His-tag

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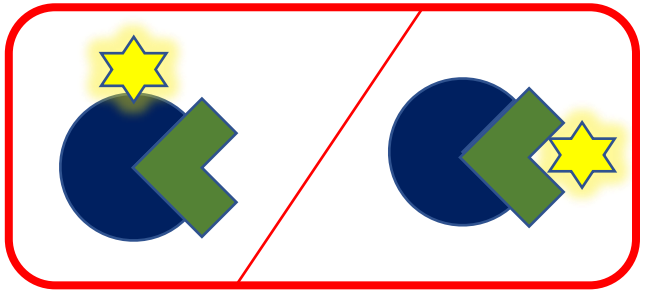
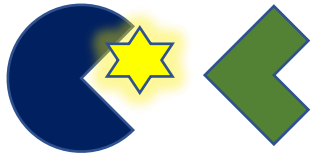
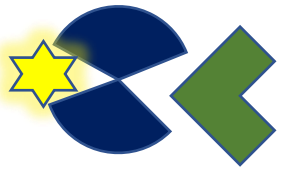
Risks of labelling



Interference with interaction

1. 

2. 

3. 

1. Sterical hindrance
2. Conformation changes
3. Non-specific interaction
4. Adhesion to labware
5. Solubility change, aggregation

4. 

5. 

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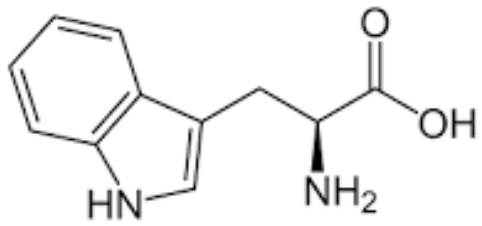
LabelFree MST

Fluorescence in **UV region**

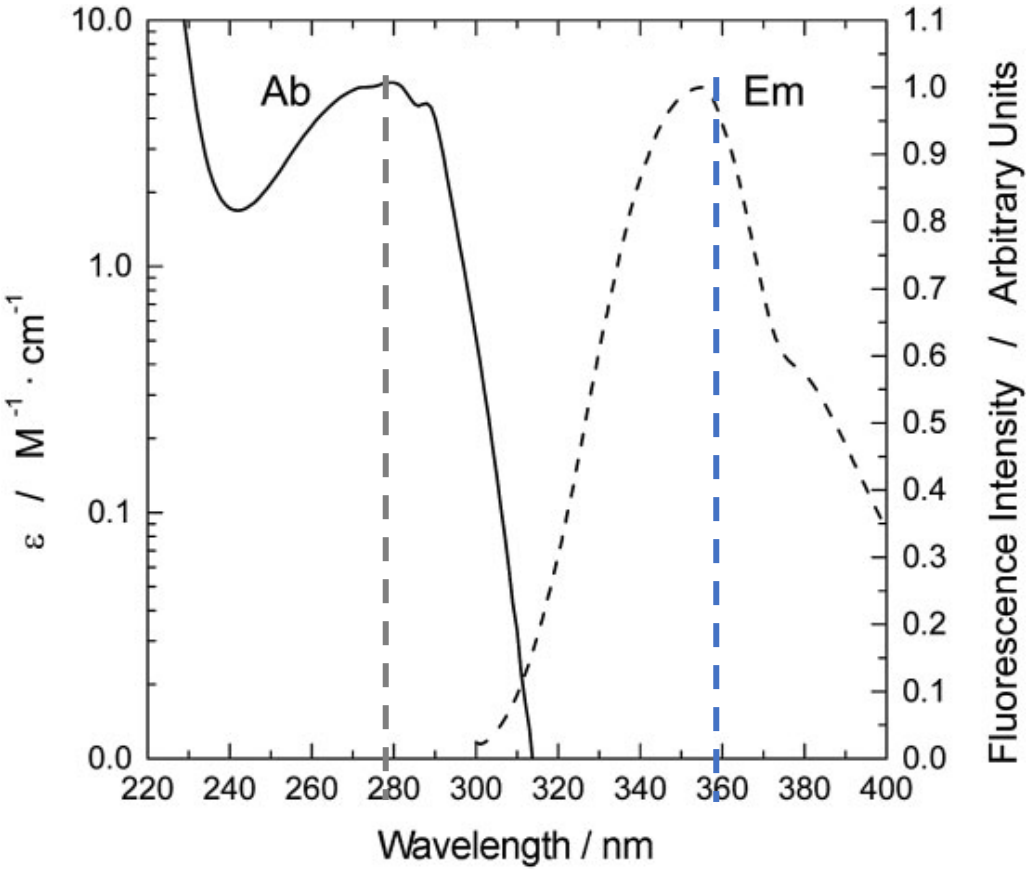
- Excitation: 280 nm
- Emission: 360 nm

- **Tryptophan**



tryptophan

Absorption and emission spectrum of tryptophan



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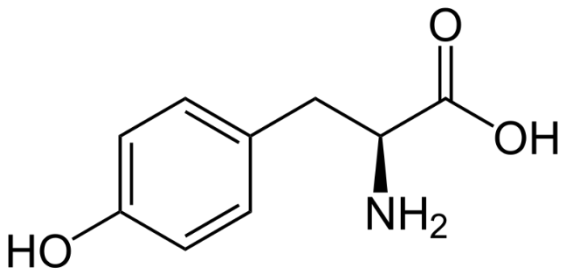
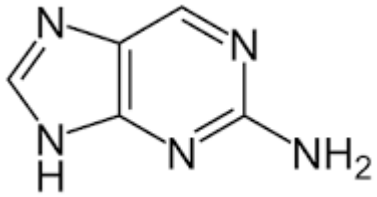
LabelFree MST

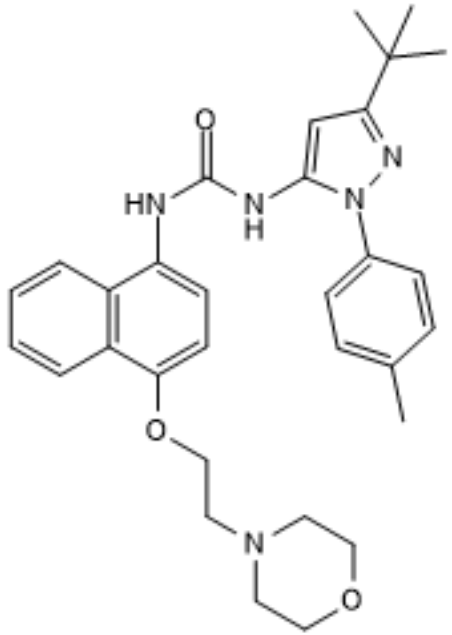
- Other molecules compatible with “LabelFree” instrumentation
  - Tyrosine (?)
  - Nucleic bases analogues
  - Specific organic molecules



tyrosine



2-aminopurine



BIRB-796 (kinase inhibitor)

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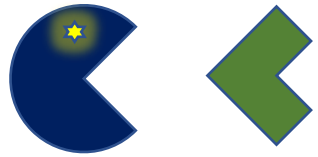
Risks of not labelling

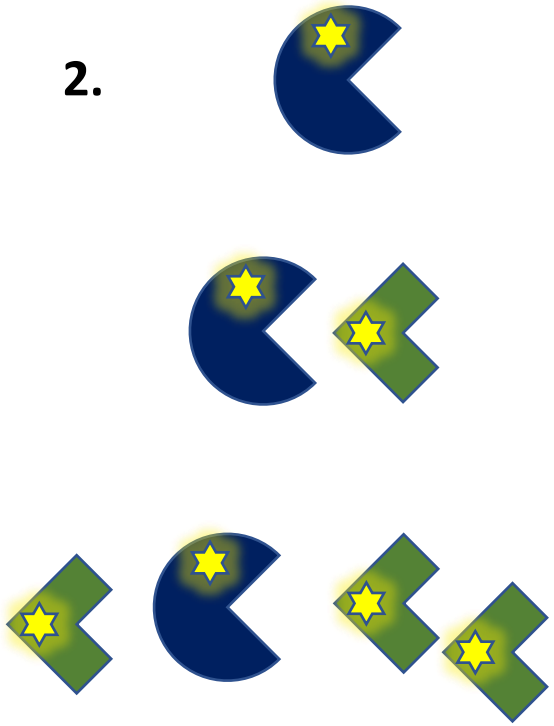
1. Weak signal
   - High concentration needed
   - Unsuitable for low Kd's

2. Signal from both binding partners
   - Mainly for protein-protein interactins
   - Signal dependent on ligand concentration
   - Not suitable for MST experiment

1.



2.



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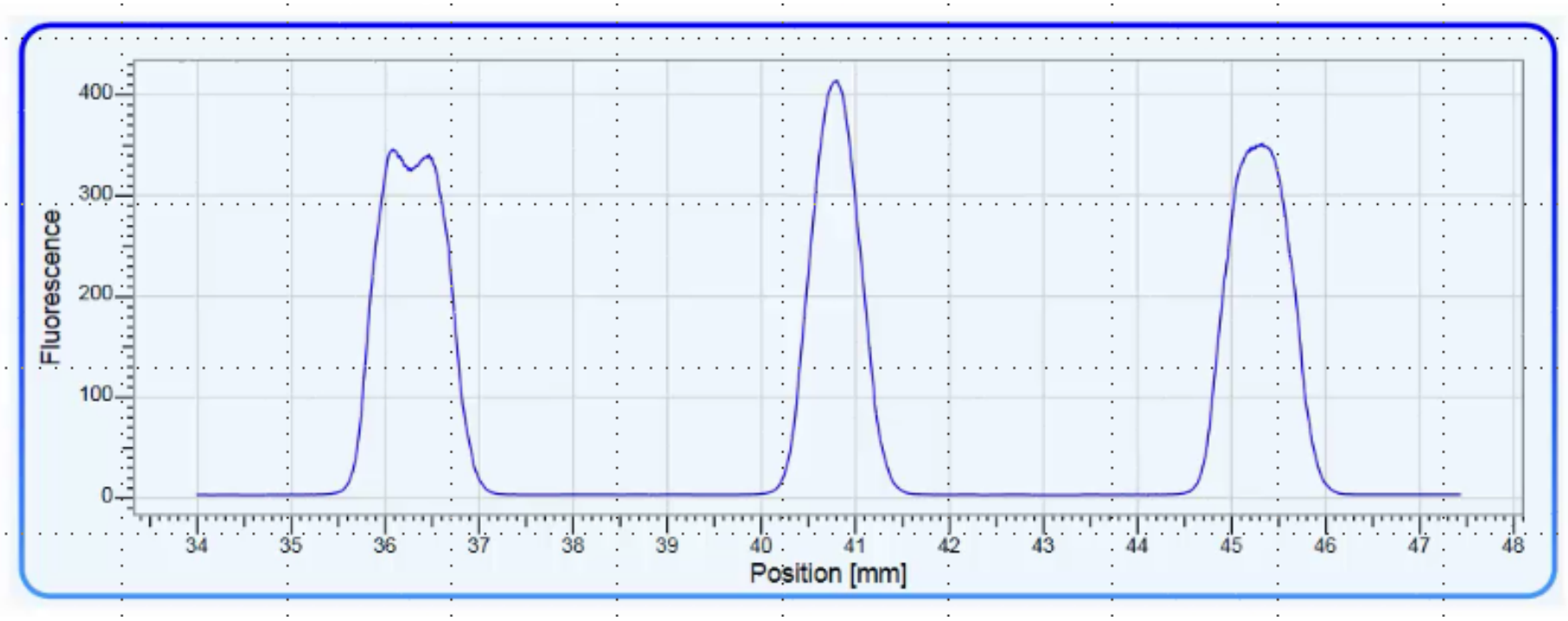
Which capillary to use?

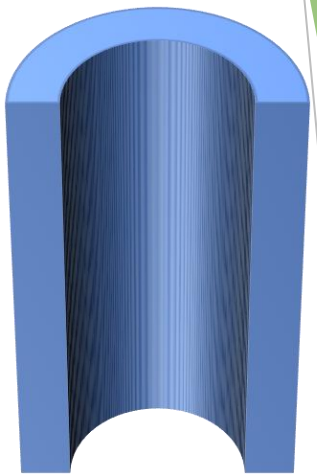
Sample sticking to the capillary wall



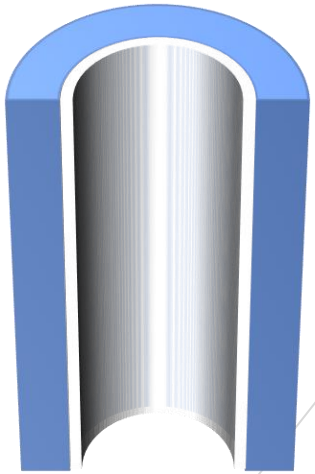
A lot of sticking

No sticking

A bit of sticking



Standard



Coated (premium)

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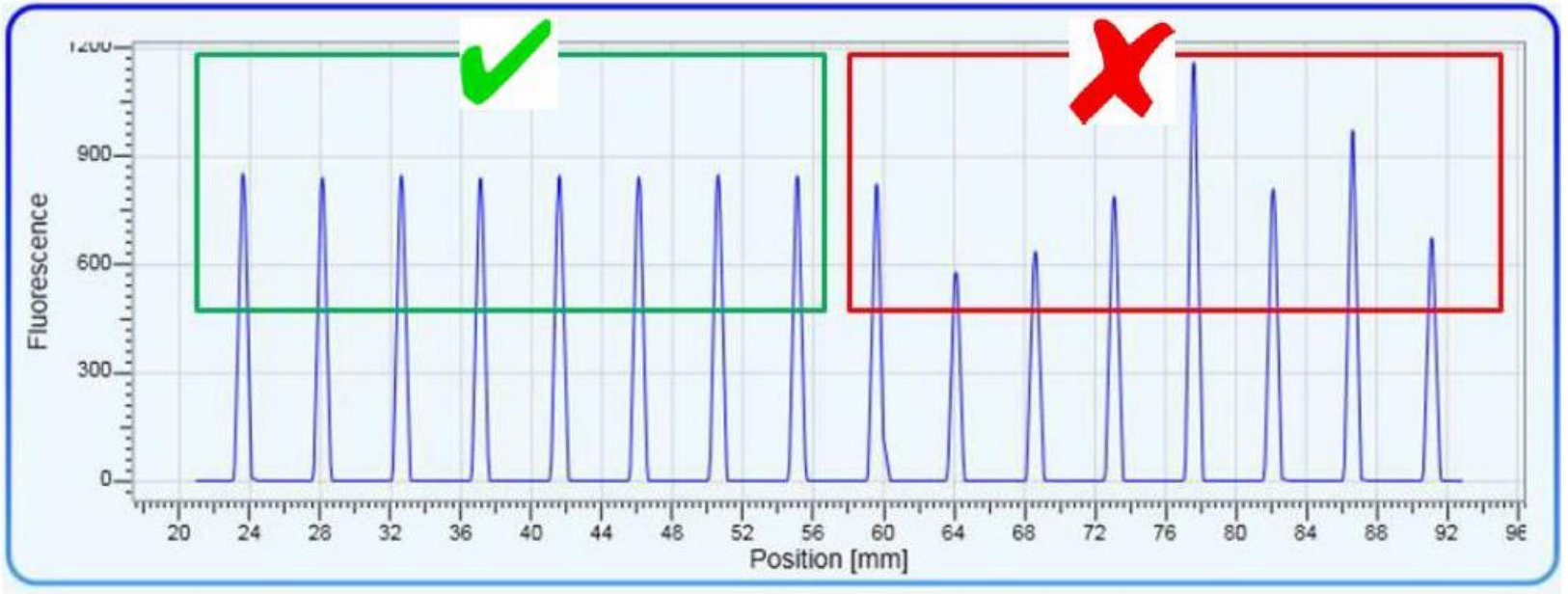
Initial fluorescence



- 10% deviation from average is acceptable
- Optimize sample quality, buffer composition
- Sample homogeneity
- Pipet more accurately – MST is highly sensitive

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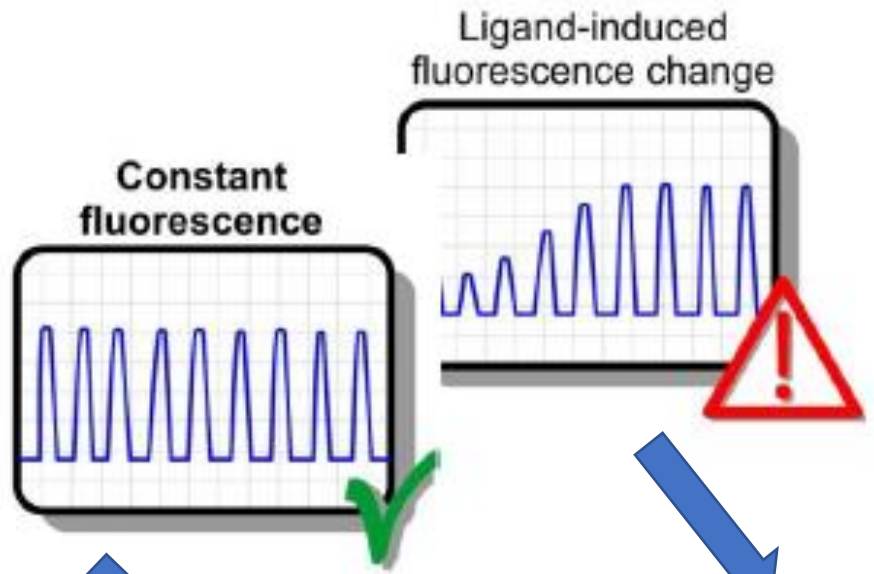
Ligand-dependent fluorescence?



Standard measurement

Direct evaluation of fluorescence

or

Optimization of conditions (additives, buffer, labelling)

?

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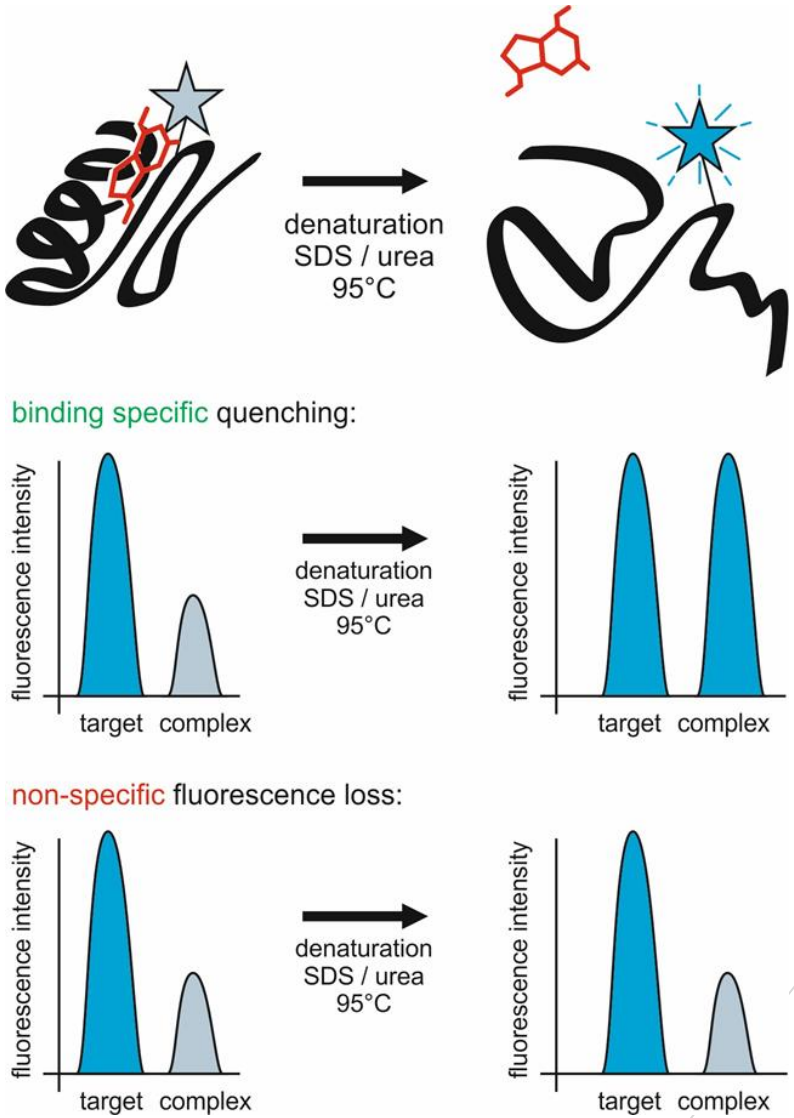
Troubleshooting: fluorescence

SD test

- In case of ligand-induced fluorescence change
- add SDS + DTT mix to first and last sample of the dilution series

  (lowest and highest ligand concentration)
- denature (95°C, 5 min)
- check fluorescence



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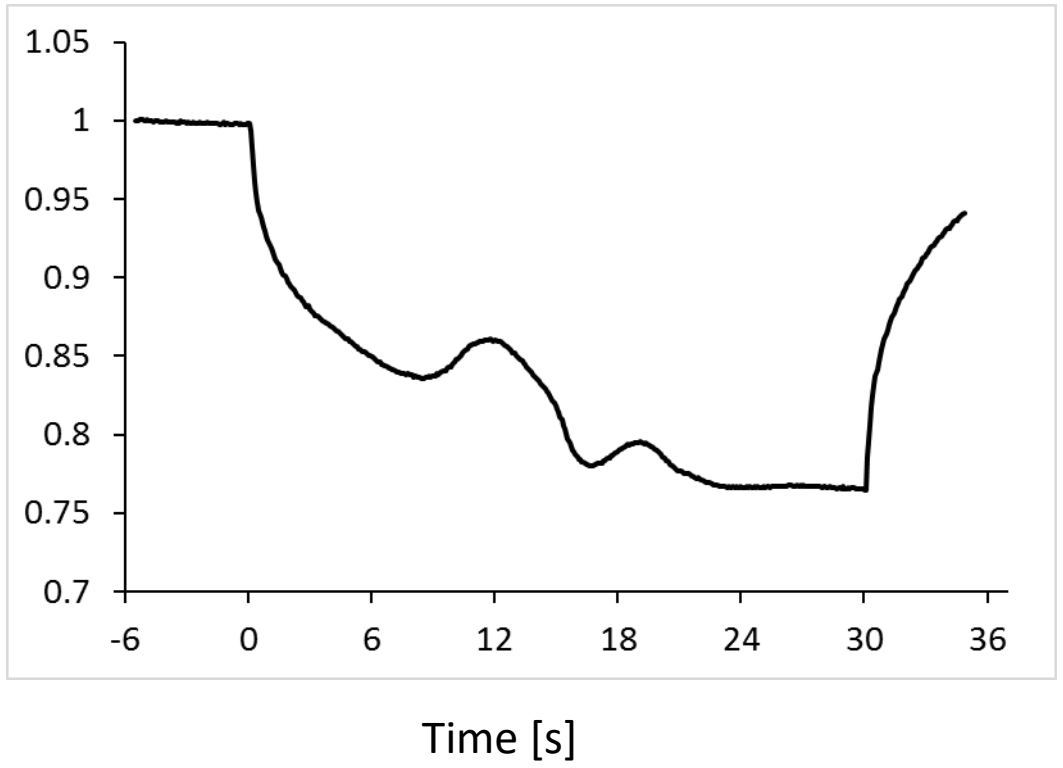
Troubleshooting: aggregation



Optimization is necessary:

- Centrifuge sample before loading capillary
- Add detergents (0.05% TWEEN20, pluronic F-12, BSA)
- Optimize buffer composition (pH, salt, additives)

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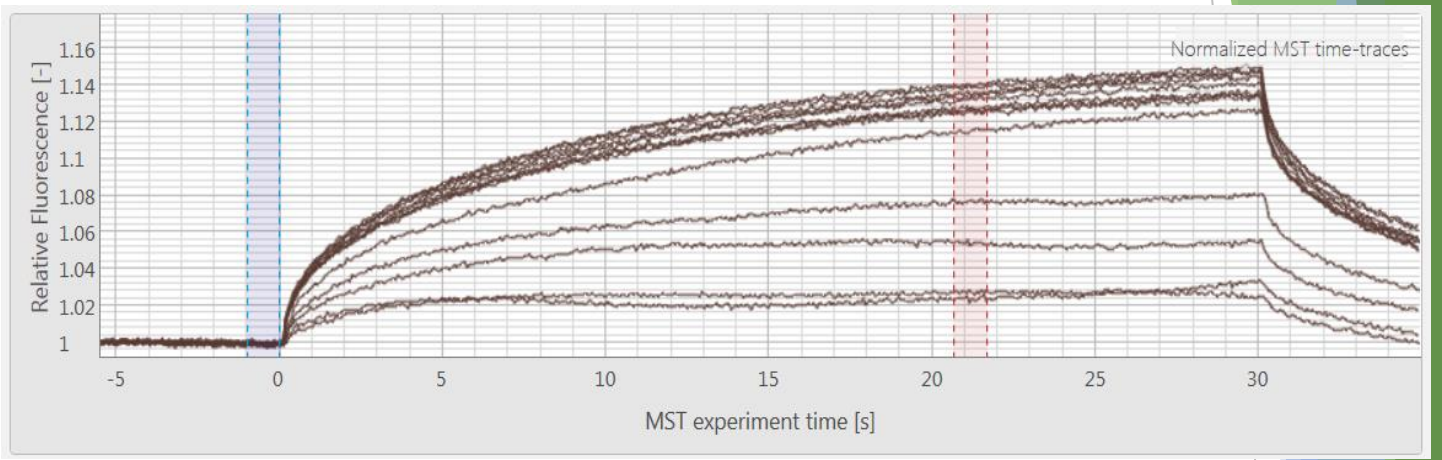
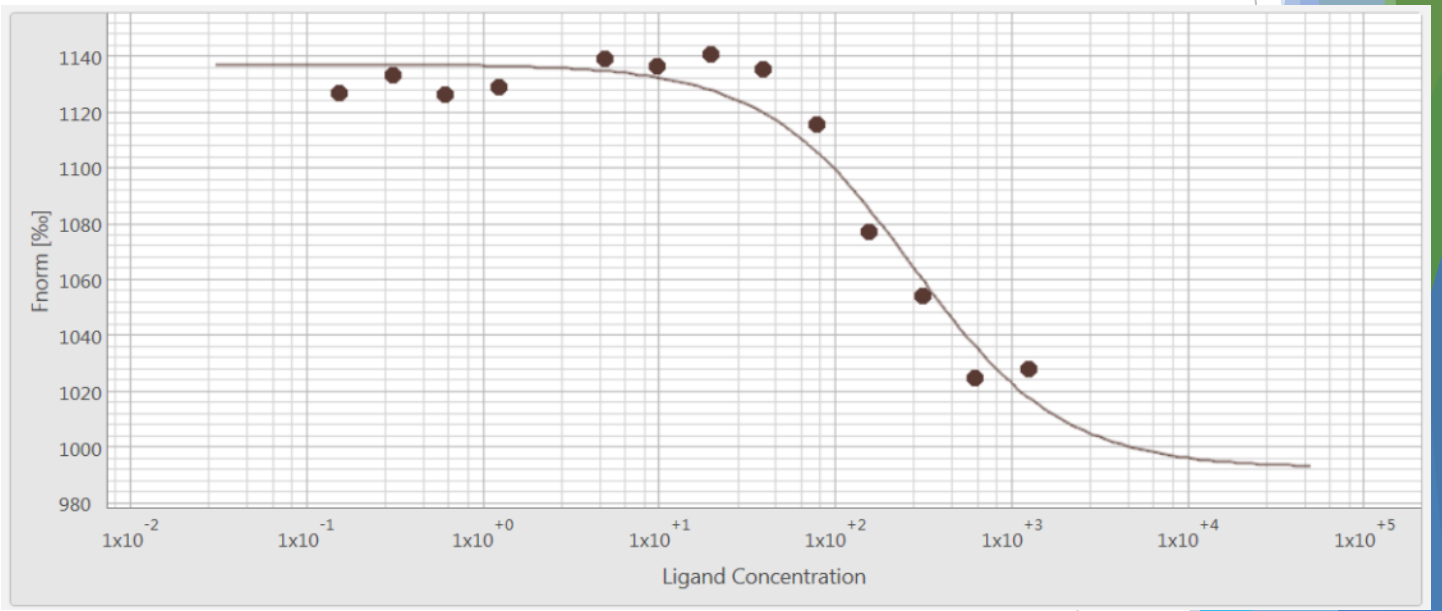
Negative thermophoresis

- Relatively rare
- Evaluation identical as in case of “normal” MST





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Real hardware

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MST machines

Currently only by company NanoTemper Technologies

- Monolith X
- Monolith NT.115
- Monolith Automated
- Dianthus

Dianthus

Monolith X

Monolith NT.115

Monolith Automated

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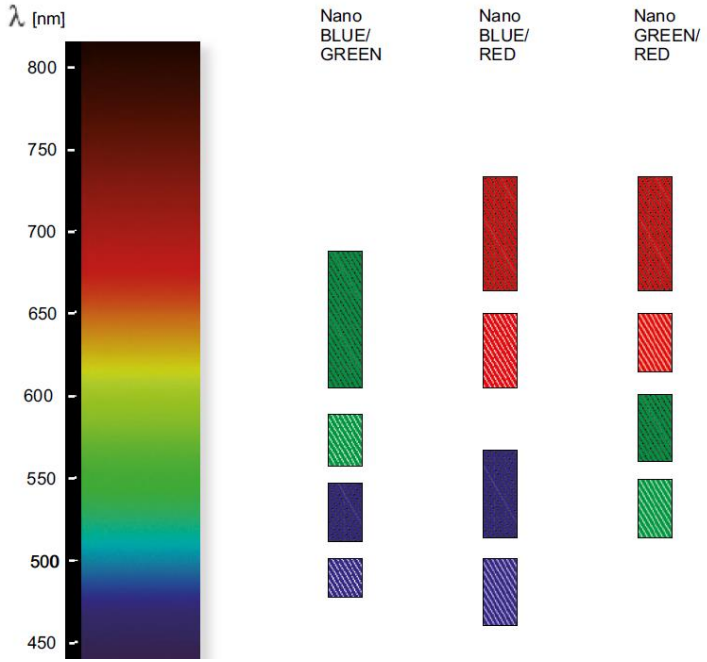
Monolith (Monolith NT.115)

- nM to mM K_D range
- 24 capillaries (16 in the old version)
- Two fluorescence channels (BLUE, GREEN, RED)



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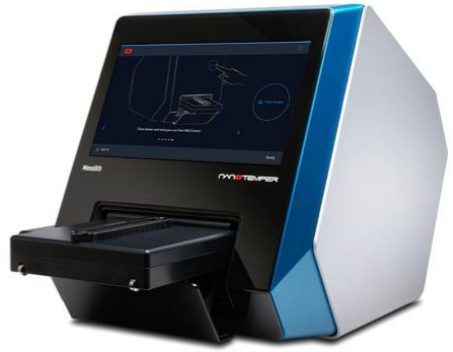
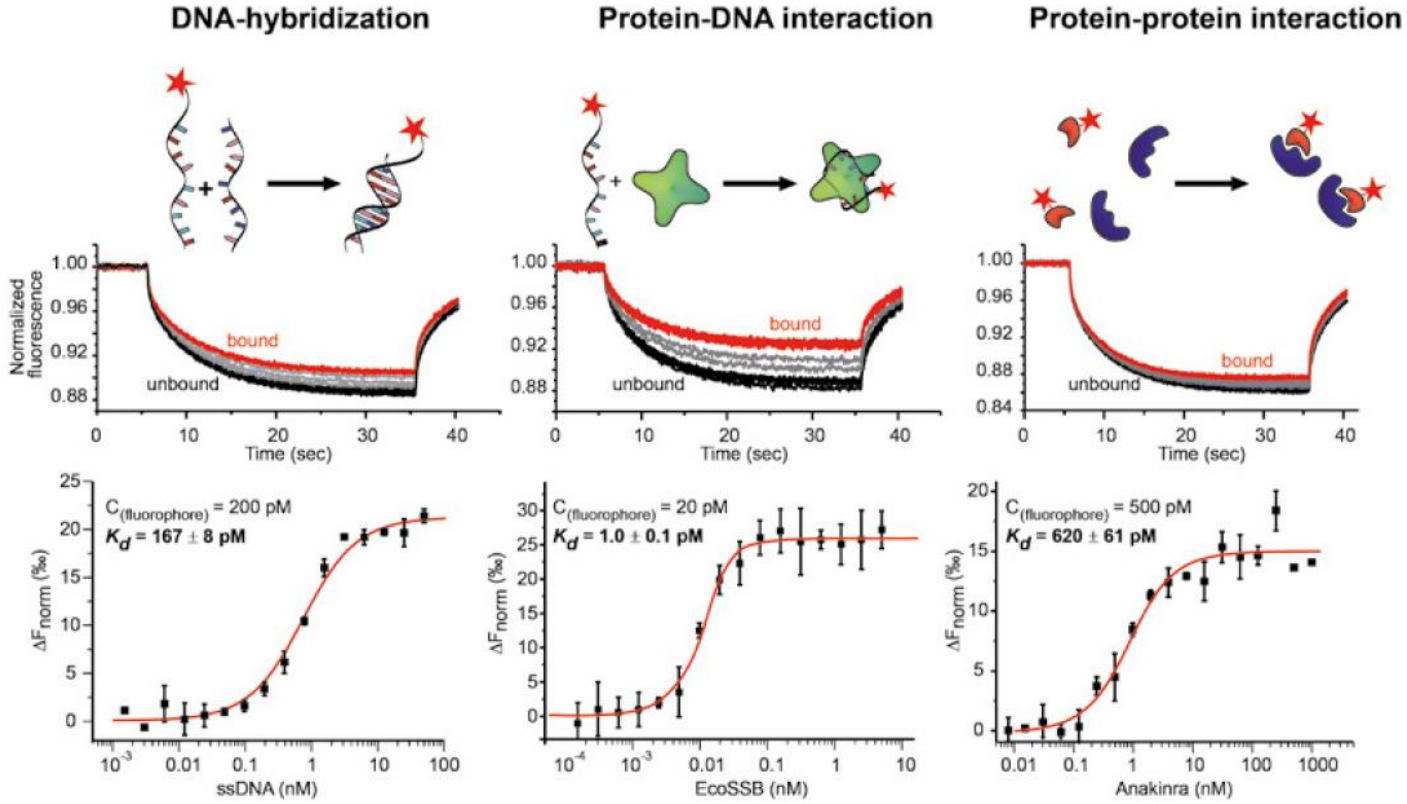
Monolith Pico



- pM to mM K_D range
- Only RED fluorescence channel



Jerabek-Willemsen, 2014

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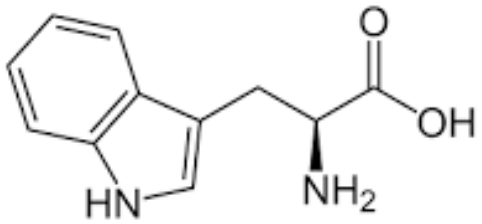
Monolith LabelFree

Fluorescence in **UV region**

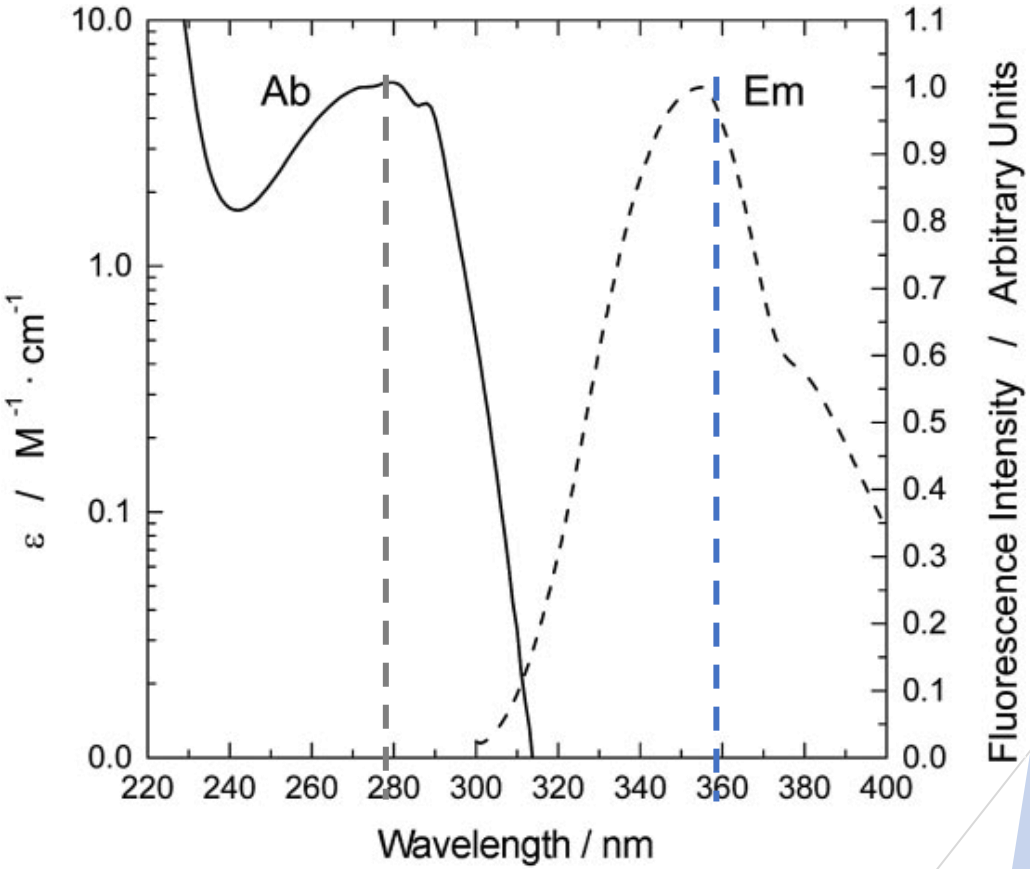
- Excitation: 280 nm
- Emission: 360 nm

- **Tryptophan**, ev. other molecules



tryptophan

Absorption and emission spectrum of tryptophan



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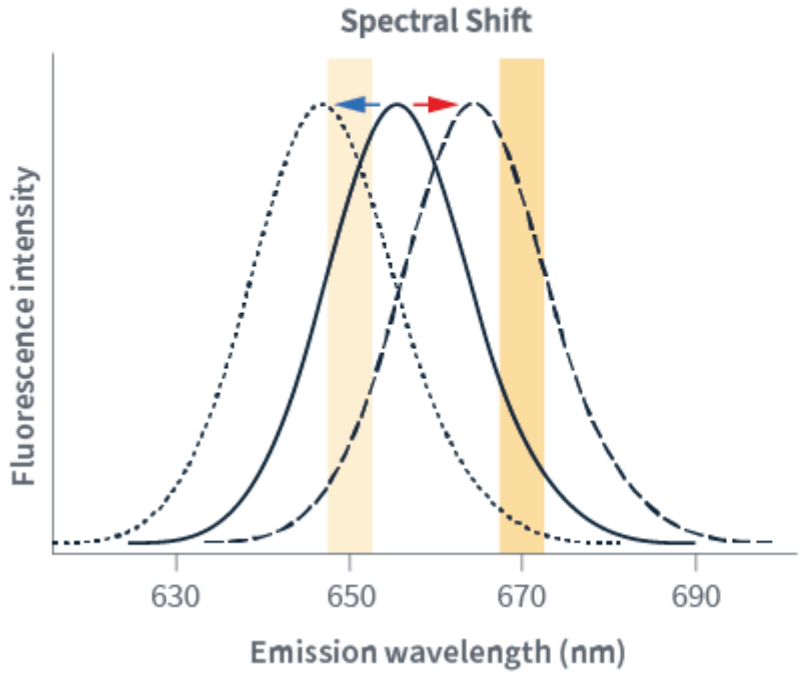
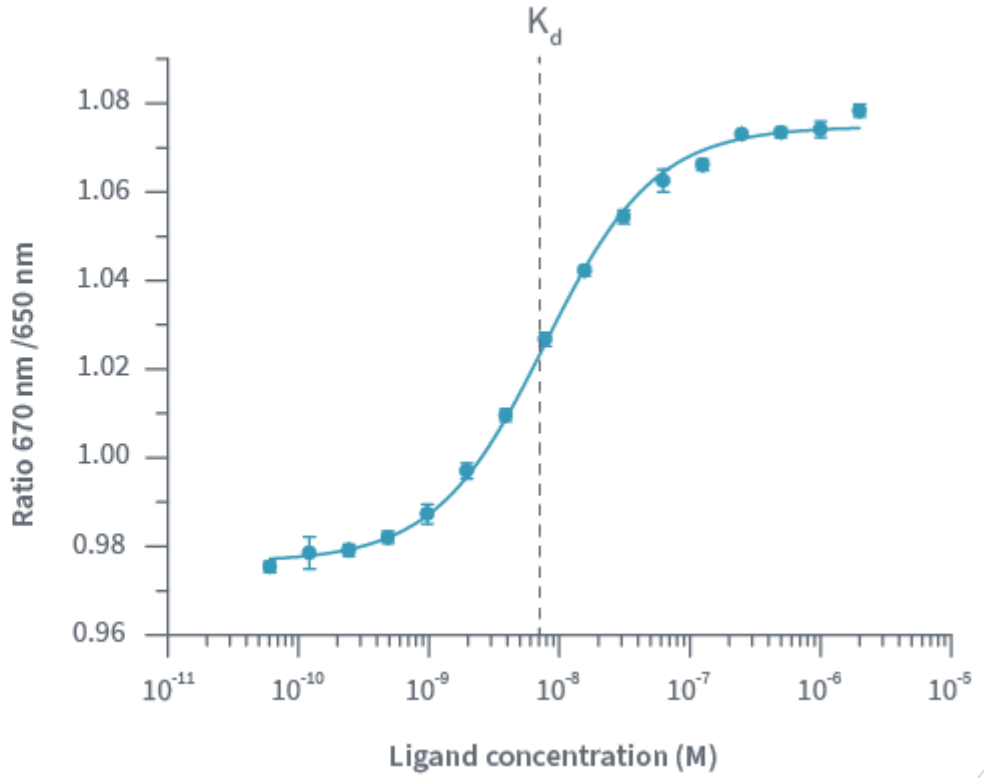
Monolith X

- In addition to MST utilizes technology of „Spectral shift“
- Analysis of fluorescence profile change induced by ligand presence





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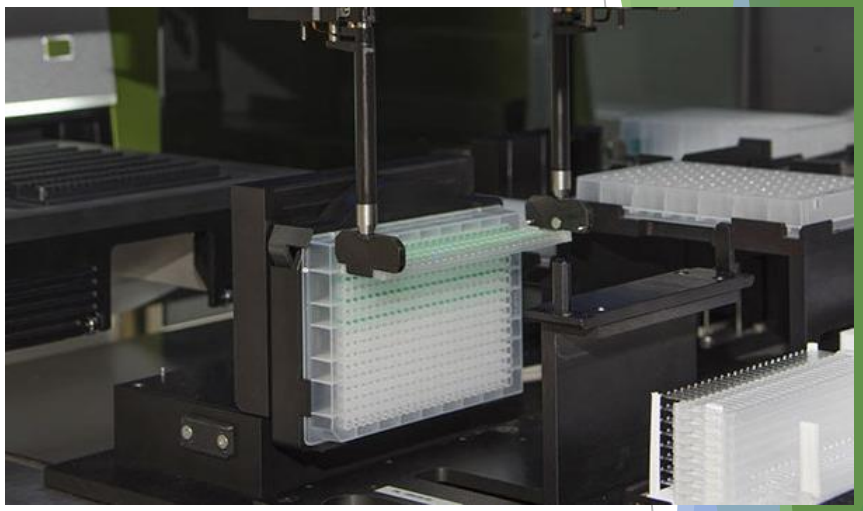
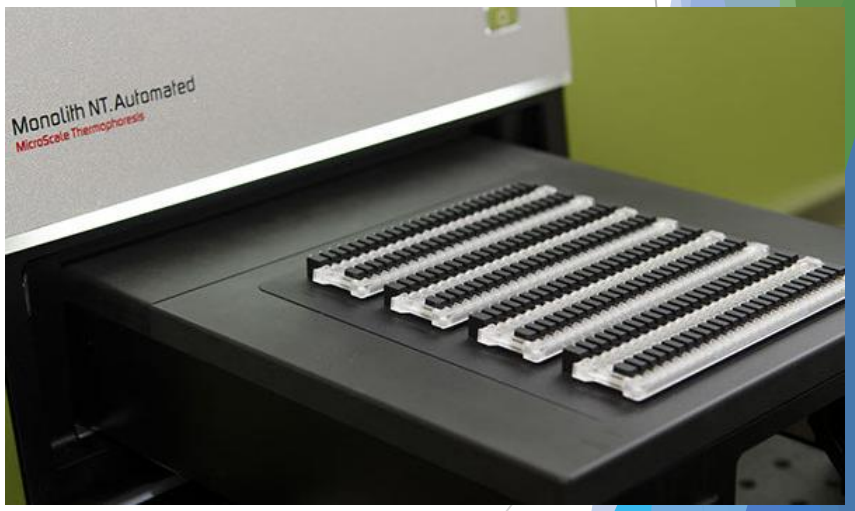
Monolith Automated

- Two channels possible
- **96 samples** in a run
- High-throughput applications: Fragment screening





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Dianthus

- 384-well plate-based instrument (no capillaries)
- High-throughput screening of binding affinities
- Using MST/TRIC and Spectral shift
- Compatible with automation
- Up-to 1350 K_d's determined per day

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MST Summary

- Thermophoresis is sensitive to subtle changes – **almost every interaction** will give a signal
- In real system, several processes happen concurrently – **MST > TRIC**
- Method is suitable for a **broad range of samples** (from ions to viruses) with relatively low sample consumption
- **Careful data analysis** is necessary – only relevant curves should be evaluated (pipetting accuracy, bleaching, denaturation, aggregation)

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Biomolecular
I nteractions and
Crystallography Core Facility

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MUNI | CEITEC

instruct Integrating Biology

ARBRE Association of Resources for Biophysical Research in Europe

CIISB Czech Infrastructure for Integrative Structural Biology

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