MicroScale Thermophoresis  
Precisely Revealing any kind of Molecular Interaction

2bind: The new Service Provider to study Molecular Interactions
Purpose

Molecular interactions are of key importance to many chemical and all biological processes, affecting enzymatic activity, targeting and structural dynamics. The quantification of interaction strengths and specificity are of fundamental importance in the development of pharmaceutical compounds revealing their potential efficacy.

This booklet describes the rather novel method of MicroScale thermophoresis in studying molecular interactions and structural dynamics.
MicroScale Thermophoresis by 2bind
A very sensitive and fast method to detect and to quantify molecular interactions

Our high quality bioanalytical services uses the changes of molecular thermophoretic mobility, when a molecule alters its shape or size. The system allows the detection of labelled or non-labelled molecules to determine the binding affinities of any pair of interacting molecules. Binding affinities are recorded in the time frame of minutes, with the binding partners in solution. Therefore the method allows the fast sampling of many different reaction conditions.
MicroScale thermophoresis (MST) is a new method that enables the quantitative analysis of molecular interactions in solution at the microliter scale and high sensitivity.

The technique is based on the effect that molecules move in temperature gradients, a physical effect called thermophoresis.

The thermophoresis of molecules depends on the molecular size, charge, solvation entropy and hydration shell.

Since at least one of these parameters is typically affected upon binding, the method can be used for the analysis of each kind of molecular interaction or modification of small molecules, proteins/peptides, DNA, sugars or molecular complexes.

Setup of the MST instrument. The aqueous solution inside the capillary is locally heated with a focused IR-laser, which is coupled into an epifluorescence setup using an IR mirror.

Picture provided by NanoTemper
MicroScale thermophoresis allows to study in any kind of reaction condition. The free choice of buffers allows to perform the measurements at optimal reaction conditions.

MST can be used to study the activity of molecules including enzymatic modifications of substrates or competitive binding inhibitors and substrates to an enzyme.

Furthermore, the reaction setups are temperature controlled, allowing to monitor structural changes of molecules of the denaturation of nucleic acids.
Applications

Any kind of molecules can be measured that either have a fluorescent tag (chemically labelled with a fluorophor, or GFP) emitting red, green or blue light, or the molecules exhibit intrinsic fluorescence (for example tryptophan). The reaction partner should either not be fluorescent, or emit light in a different spectral range.

» protein: protein, RNA, DNA, small molecule, ions
» DNA: DNA, RNA, protein
» Aptamer: small molecules, proteins and protein complexes
» modified proteins/peptides: proteins, antibodies
» sugar: protein

Applications to monitor the conformational change or denaturation of proteins and DNA can also be followed. Structural changes do dramatically affect the solvation entropy and hydration shell of the molecules leading to large changes in thermophoresis.

The independent detection of two fluorophores allows the observation of two competing compounds with a substrate.

The technology is useful to detect weak binding events which are typically difficult to access. For example the binding of small molecules or single ions to proteins.

Parameters that can be studied with MST: $K_D$, $V_{max}$, $K_{cat}$, $K_p$, $\Delta H$, $\Delta G$, $\Delta S$.

Key Advantages of MST by 2bind

1. High assay diversity, flexibility and robustness
2. Unmatched sensitivity – Measurement of interactions between any molecules – from ion to ribosome
3. Dynamic affinity range (pM to mM)
4. Fast measurements (within 10 mins)
5. Works in solution - in any buffer system – no surface immobilisation necessary
6. High information content – straightforward detection of aggregates and sample related artefacts
7. Measurement of fluoresently labelled and label free probes. Independent detection of two different fluorophores
8. Large controlled temperature range (20 – 45°C)
9. Low sample consumption (4 μl of a 10 nM solution)
10. Small reaction volumes (4 μl)
11. Measurements with crude fractions, extracts and in serum are possible
Fluorescent labelled molecules

Simultaneous multicolor detection and competition assays

Allows the direct comparison of the strength of binding affinities.

An IR-Laser is used to generate a precise local temperature gradient within a thin glass capillary, filled with the reaction sample. The temperature of the aqueous solution in the laser spot is raised by up to $\Delta T=5$ K.

The gradient in temperature induces the thermophoretic movement of the molecules in the capillaries. Molecules in the reaction, being located in the IR-Laser focus, are monitored due to their fluorescence and their motion along this temperature gradient is recorded.
The thermophoretic mobility of molecules is very sensitive and depends on molecules, sequences, structures and also the kind of interacting compound. Therefore any of the different parameters in the MST measurement cycle may be altered upon binding and can be used for the identification and quantification of interactions.

The IR-Laser, heating the sample, is switched on only for a short time resulting in the instant warming of the laser focus and due to the small volume to an instant cooling after switching the laser off. The mobility of the molecules is determined during the whole process from the initial state (laser off), the temperature jump (the moment when the laser is switched on), the thermophoresis state (when the molecules diffuse out of the focus), the steady state (equilibrium phase; similar diffusion rates in- and outside of the focus) and the back diffusion (the time frame after switching of the laser, allowing the back diffusion of the molecules into the observation window).

**Measurement Parameters**

MicroScale thermophoresis. The fluorescence inside a capillary is measured and the fluorescence in the IR-laser heated spot is plotted against time. The IR-laser is switched on at $t=5\ s$ and the fluorescence changes as the temperature increases. There are two effects, separated by their time-scales, contributing to the new fluorescence distribution: the fast temperature jump (time scale $\approx 3\ s$) and the thermophoretic movement (time scale $\approx 30\ s$). Once the IR-laser is switched off ($t=35\ s$) the molecules diffuse back.

*Picture provided by NanoTemper*
Depending on the size, charge, structure and hydration shell molecules exhibit distinct kinetics in the temperature field and can be discriminated. The measurement of binding affinities now combines the serial dilution of a compound with the fluorescent substrate and the quantification of its mobility at different substrate:compound ratios.

The change of thermophoresis from the unbound to the fully bound substrate in the concentration gradient of the compound allows the determination of binding affinities.

The NanoTemper MST systems allow the analysis of 16 capillaries in one round of measurement, allowing to study a broad concentration range.

The principles of a MicroScale Thermophoresis measurement. An IR-Laser heats the reaction solution in a small area of the capillary that is at the same time the focus for fluorescence detection. Changes in the thermophoretic mobility of a fluorescent molecule is monitored in several capillaries with increasing concentrations of an interacting compound.

Picture provided by NanoTemper
Determining binding affinities

A serial dilution of the compounds in solution is sufficient

Normalized fluorescence of serial dilutions reveals even the smallest change in thermophoresis

Molecular interactions are studied after a serial dilution of a potential interactor. The fluorescence of up to 16 capillaries is recorded and normalized.

The kinetics of the fluorescent substrate during the temperature jump, the thermophoresis and in the steady state phase is quantified. Usually one of these parameters changes upon binding to the interacting molecule and serves to determine binding affinities.
Determining Binding Affinities

As an example the binding of a fluorescently labeled DNA to a protein is shown in this section. The interaction of the protein with the DNA shows clear changes of thermophoresis from the free to the fully bound form.

Differences can be observed in the temperature jump kinetics and as well for the steady state phase. A curve in between the unbound and fully bound state reflects the partially bound DNA (Figure 4).

The partially bound DNA gives rise to curve plot that exhibits an intermediate kinetics in between the plot of the bound and unbound protein. The reason is that the kinetics of an ensemble is measured, integrating the thermophoretic mobility of bound and unbound states.

DNA binding to GST-AT1. The concentration of the fluorescently labelled DNA is kept constant and GST-AT1 is titrated. The fluorescence inside the capillary is measured for each different GST-AT1 concentration and the normalized fluorescence in the heated spot is plotted against time. The IR-laser is switched on at $t = 5$ s and the fluorescence changes as the temperature increases. There are two effects, separated by their time scales, contributing to the new fluorescence distribution: the fast temperature jump (time scale $\sim 1$ s) and the thermophoretic movement (time scale $\sim 10$ s).

Both effects show the binding of the labeled DNA to its target: the temperature jump signal increases upon binding of GST-AT1, whereas the thermophoresis decreases upon binding. Once the IR-laser is switched off ($t = 45$ s) the molecules diffuse back.
Quantifying The Interaction

The thermophoresis curves are recorded for a series of capillaries containing a serial dilution of one compound. The aim of the titration is to cover substrate: compound ratios that reveal the non-bound and the fully bound state. Next the data is interpreted and the gates for quantifying temperature jump, fluorescence, steady state thermophoresis and others are set.

Not all of the parameters have to change upon binding. The parameters are quantified, compared in plots and significant changes are determined.

Data points serve for curve fitting and calculation of the binding affinity. The fitted data points are then normalized to visualize binding affinities.

The example shows the data and quantification of the binding affinity for two DNA binding proteins.

**Binding analysis.** The concentration of the fluorescently labelled DNA is kept constant and the DNA binding proteins are titrated. The temperature jump data for different targets is plotted against the DNA concentrations. The proteins show a sigmoidal binding curve with different affinities. The measured values are fitted with the Hill-equation to determine the “fully bound” state. For a better comparison, the binding curves are normalized to the fraction of bound molecules. The value 1 denotes that 100% of the fluorescently labelled DNA is bound to its target.
Combinatorial Aspects

MST is as simple as to prepare a serial dilution and to record changes of at least one of the parameters in the measurement cycle. However, those differences may not always pop up using standard conditions and therefore optimisation of the conditions may be required.

Fluorescence
Fluorescence intensity varies dramatically with regard of to the fluorophor, the sequence of DNA/protein and the location of the fluorophor within the molecule.

Fluorescence intensity may be also a good indicator of an interaction event, if the interaction results in a net change of intensity.

Placing a fluorophor close to the interaction site may result in a very sensitive interaction assay. In some cases different fluorophores have to be tested in order to avoid substrate dependent non-specific effects.

Capillaries
Substrates and compounds may stick to the glass capillaries and therefore initial experiments have to be performed to identify the best capillary type (standard, hydrophobic, hydrophilic). In addition blocking reagents like BSA and detergent should be tested in the assay, as they can change the sensitivity of the assay.
Combinatorial Aspects

Buffers
The buffer may also affect the ability to detect an interaction. Low salt buffers do generally result in higher differences in molecular thermophoresis. It is also recommended to try different buffer systems to reveal clear interaction signals.

Laser intensity
Changes in the hydration shell and combinatorial effects may result in very small changes in thermophoresis kinetics that can be hidden in the signal to noise ratio. The temperature difference between the hot and cold area generated by the IR-Laser could have an effect on differential thermophoresis. Especially with small compounds compared to the substrate, better results may be obtained at specific temperature gradients. These temperature gradients are regulated by the laser intensity that can be tuned from 10 to 100%.

Additionals
The reaction can be performed in a temperature controlled fashion. Assays running at different temperatures may be informative on the specificity of the assay.

It is always advisable to run positive and/or negative controls in parallel.
Appendix

What is the best way to find your interaction partner?

Comparing molecular interaction assay methods

The different methods to study molecular interactions have specific requirements regarding substance concentration, reaction conditions, compound immobilisation and setup labor.

MicroScale thermophoresis performed by 2bind is the best choice in almost every respect.
### Facts and Features

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<th>Method</th>
<th>Affinity Range (K&lt;sub&gt;d&lt;/sub&gt;)</th>
<th>Advantages</th>
<th>Draw Backs</th>
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<tr>
<td>2bind MicroScale Thermophoresis</td>
<td>pM to mM</td>
<td>Measurements in solution&lt;br&gt;Free choice of reaction conditions&lt;br&gt;Low material consumption&lt;br&gt;Temperature controlled&lt;br&gt;Fast measurements&lt;br&gt;Interaction between small and big molecules detectable&lt;br&gt;Fluorescence and label-free assays&lt;br&gt;Multiplex assays</td>
<td>K&lt;sub&gt;on&lt;/sub&gt; and K&lt;sub&gt;off&lt;/sub&gt; rates are not accessible</td>
</tr>
<tr>
<td>Surface Plasmon Resonance (SPR)</td>
<td>sub nM to low mM</td>
<td>High sensitivity&lt;br&gt;Method of choice measuring K&lt;sub&gt;on&lt;/sub&gt; and K&lt;sub&gt;off&lt;/sub&gt; rates</td>
<td>Lab-intense establishment of new assays&lt;br&gt;Covalent coupling might interfere binding event&lt;br&gt;Artefacts stemming from mass transport limitations close to an interface</td>
</tr>
<tr>
<td>Isothermal Titration Calorimetry (ITC)</td>
<td>nM to sub mM</td>
<td>Method of choice to measure thermodynamic parameters of an interaction</td>
<td>High material consumption&lt;br&gt;High preparation time&lt;br&gt;Buffer limitations&lt;br&gt;Low to medium high throughput&lt;br&gt;Interactions with no or only a small change in enthalpy not measurable</td>
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<tr>
<td>Fluorescence Correlation Spectroscopy (FCS)</td>
<td>low nM to mM</td>
<td>High sensitivity&lt;br&gt;In vivo measurements possible</td>
<td>Low throughput&lt;br&gt;High preparation time&lt;br&gt;Large changes in molecular masses required for detection</td>
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2bind molecular interactions
Be smart
By Researchers for Researchers
Advancement through knowledge

2bind: We are the new Service Provider to study Molecular Interactions
Dr. Thomas Schubert with Prof. Gernot Längst (University of Regensburg)