Microscale thermophoresis: warming up to a new biomolecular interaction technique

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Biomolecules, such as RNA, DNA, proteins and polysaccharides, are at the heart of fundamental cellular processes. These molecules differ greatly with each other in terms of their structures and functions. However, in the midst of the diversity of biomolecules is the unifying feature that they interact with each other to execute a viable biological system. Interactions of biomolecules are critical for cells to survive and replicate, for food metabolism to produce energy, for antibiotics and vaccines to function, for spreading of diseases and for every other biological process. An improved understanding of these interactions is crucial for studying how cells and organs function, to appreciate how diseases are caused and how infections occur, with infinite implications in medicine and therapy. Many biochemical and biophysical techniques are currently being employed to study biomolecular interactions. Microscale thermophoresis (MST) is a relatively new biophysical technique that can provide powerful insight into the interactions of biomolecules and is quickly being adopted by an increasing number of researchers worldwide. This article provides a brief description of principles underpinning the MST process, in addition to benefits and limitations.

What is MST? And how does it work?

MST is a biophysical technique that measures how strongly biomolecules interact based on their degree of dispersion upon heating ('*thermo*' = heat; '*phoresis*' = migration). Essentially, one of the biomolecules (a protein, for example) is labelled with a fluorophore designated the 'target', while the other molecule (RNA, for example) is unlabelled and is mixed with the target in varying concentrations acting as the 'ligand' (Figure 1). Next, the baseline fluorescence is measured, followed by a short exposure with an IR laser (heat source) on each sample; the change in fluorescence between the 'hot' and 'cold' state of each sample is detected. If the ligand is interacting with the fluorescently labelled target molecule, the complexes will diffuse at a different rate when heated up relative to the individual components alone. These micro-movements (Figure 1) are detected by the machine as a drop in fluorescence and are plotted as such.

What kind of information can we obtain from MST?

MST has a variety of applications. Focusing on the features of greatest interest to the biochemist, MST can be used to determine dissociation constants (K_d), an equilibrium measurement of the likelihood of a complex to dissociate at a certain concentration, in reference to interacting biomolecules. This can be useful to study affinity between different systems such as protein–protein (e.g. antigen–antibody interactions), RNA–protein complexes (e.g. ribosomal studies) and small molecule protein (e.g. drug-binding studies). For example, using MST, researchers probed an interaction between a small molecule drug (cetylpyridinium chloride) with the hepatitis B viral dimeric nucleocapsid protein HBcAg to use this novel drug as a potential inhibitor for viral replication. MST can also probe nucleic acid–protein interactions. For example, researchers investigated the binding of G-quadruplex RNA aptamer AIR-3 with the human interleukin-6 receptor,
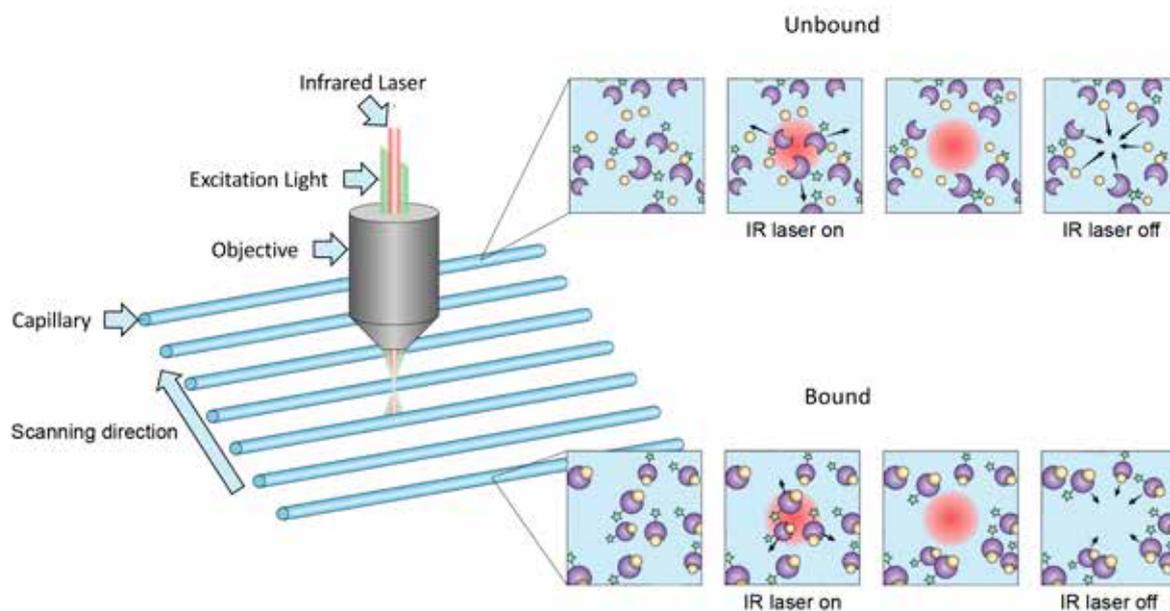
Figure 1. Microscale thermophoresis mechanism of action. Diagram of the general mechanism of MST. An IR laser is used to heat the sample which will either contain molecules in a bound or unbound state. Upon stimulation with the IR laser (shown in the *IR laser on* panels), the molecules will diffuse away. Once the IR laser is turned off, the molecules will diffuse back to the IR stimulated area (shown in the *IR laser off* panels). If the biomolecules bind, there will be a change in the diffusion pattern once the IR laser is turned on, compared with the unbound state. The biomolecular diffusion is measured as a change in fluorescence output of a fluorophore (represented by the green star) attached to the target molecule.

which plays a role in cancer and inflammatory diseases. With the assistance of MST, the researchers aimed to understand the RNA structure, shape, and RNA interaction site and binding stoichiometry. Enzyme kinetics and competition studies can also be conducted. Importantly, MST experiments are carried out in solution and are able to mimic biologically relevant environments. Buffer choice is essentially unlimited (as long as the target and ligand molecules are 'happy' with the buffer conditions) and samples could even be run in serum or cell lysates to detect a biomolecular interaction, which can provide unique information, only otherwise available via *in vivo* assays.

What are the advantages and disadvantages of MST?

One of the important advantages of MST over other techniques is the range of biomolecules that can be studied: from sugars or small molecule drugs up to large proteins, enzymes and even more complex structures such as ribosomes. The versatility of samples that can be studied makes this a highly attractive technique in the field. MST also has advantages over other fluorescence-based techniques, including the simplicity of measurement, the low sample volume required and the infinite variety of buffer formulations supported. The technique can also be used to provide insight into hydration shell dynamics, molecular charge interactions and changes in the size of biomolecules during interaction. The subtleties of such specific applications will not be described here, but those

interested can refer to the additional reading section. Furthermore, should fluorescent labelling not be optimal or desired for the system, there are adaptations that can be made for non-fluorescent approaches.

As with any technique, there are negative aspects that users should consider. The initial cost of the machine and upfront expense of dyes can be a hurdle, however, given the smaller sample volume required, some of these costs can be offset by the reduced amount of high-quality starting sample needed. Fluorescent labelling, in general, can be a negative aspect because of the potentially time-consuming process of efficiently labelling your target. This may be partially circumvented by using fluorophores previously shown to provide reliable results (see *Fluorophores* below). MST does not produce detailed information regarding the shape or location of the binding. To do this, one would need to consider incorporating complementary studies such as X-ray crystallography, nuclear magnetic resonance or small-angle X-ray scattering.

Pioneer voyage

Sample quality. As with other biophysical techniques, one of the most critical things is to start with high-quality, monodispersed samples (i.e. with particles of uniform size). This often requires multiple different methods such as: gel electrophoresis, static light scattering and more recently, using the Nanotemper Tycho NT.6 which employs differential scanning fluorimetry to measure protein foldedness.

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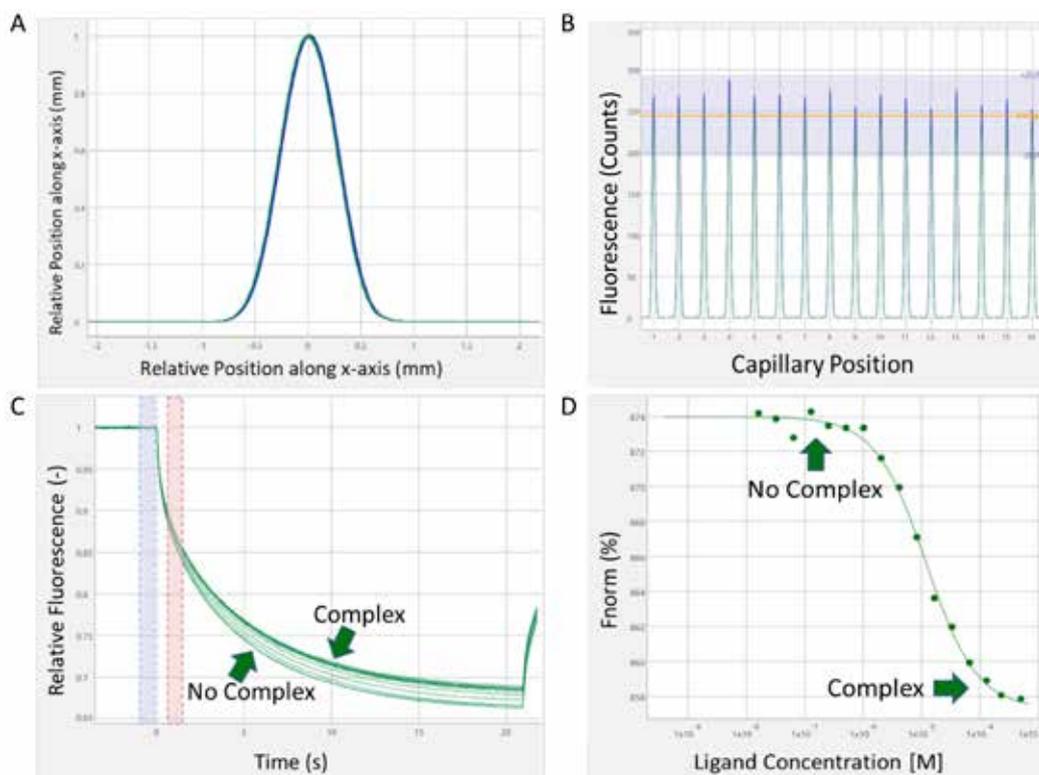
Fluorophore. After sample quality is confirmed, the next step is to determine which fluorophore will best suit the experiment. MST utilizes differences in thermal shift by detecting fluorescence to evaluate biomolecular interaction, therefore only one of your interacting molecules needs to be fluorescently labelled. For nucleic acids, Cy5 fluorescent labelling is reliably detected by the MST sensor. Companies that synthesize nucleic acid sequences will provide Cy5 labelling as an option, and thus would be MST-ready. For proteins, using the RED-tris-NTA red fluorescent dye sold by NanotemperTM is a simple way to label Histidine-tag reconstituted proteins. Dyes that can efficiently label primary amines like those found in lysines or dyes specific to labelling of cysteine residues are also options. It is also extremely important to thoroughly evaluate the fluorescent labelling procedure used, to ensure that your target molecule is sufficiently labelled. An unlabelled target will still interact with the ligand but will not be measured by the MST instrument resulting in an inaccurate K_d value. Determining the labelling efficiency will also help verify whether the experimental concentrations will be sufficiently above the dissociation constant so the target will stay fluorescently labelled.

Buffer. An important consideration when choosing a biophysical technique is the buffer conditions of the experiment. MST is extremely versatile, supporting almost any buffer condition. There are, however, a few modifications to the experimental buffer that may improve the MST results. The first is adding Tween-20 (0.05% final concentration) to help mitigate protein adsorption and aggregation. The second is using bovine serum albumin (BSA; 0.4 mg/ml) in the buffer to prevent protein adsorption to microcentrifuge tubes, where the BSA will coat the tube, allowing free diffusion of the target and ligand molecules.

Starting concentrations. Following labelling and buffer selection, the next step is to determine the concentrations of the target and ligand to be used. The target concentration must be high enough with the attached fluorophore to be detectable by the MST machine. This is performed in the 'pretest' option of the software, in which the machine will scan two capillary tubes containing only target solution, to determine if the fluorescence is sufficient to continue (Figure 2A). Performing the pretest is highly recommended, as identification of insufficient fluorescence can alert the user to make adjustments to the labelling or concentrations, minimizing wasted materials from a failed or indeterminant binding affinity experiment. After the pretest, if binding has been previously shown (i.e. via an alternate biophysical method), one can skip the binding check step. If binding is not known, it is advisable to perform the software's binding check. This will provide a 'yes-no' answer as to whether there is detectable binding and requires less sample than a full binding affinity. Binding checks require four sample capillaries of target only, and four sample capillaries of target mixed with a high concentration of ligand. After all the above parameters have been investigated, the binding affinity test can be performed.
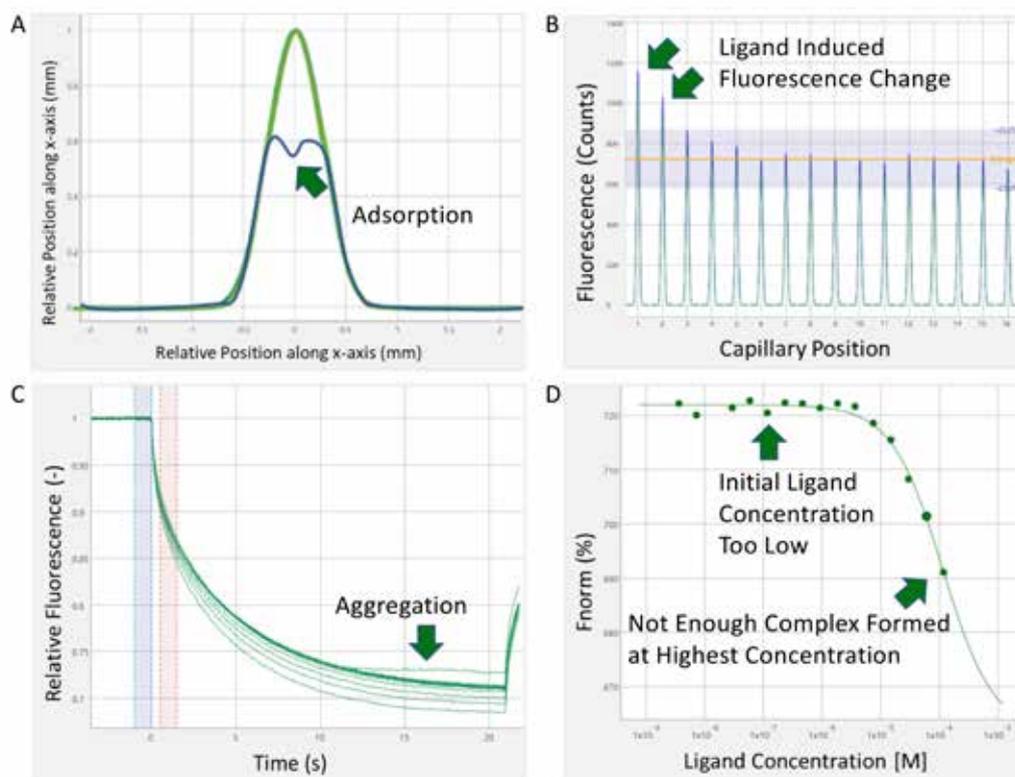
Figure 3. Visualizing common problems. Plots representing some common errors that can arise during a complete MST experiment. **A**) Adsorption during a pretest experiment. **B**) Fluorescent inhomogeneity during a binding affinity test. **C**) Aggregation of sample during a binding affinity test. **D**) Incomplete binding curve after a binding affinity test.

Binding affinity measurement. To get an accurate binding curve, it is advisable to begin with an initial ligand concentration that is 20× the K_d. However, the K_d is often unknown, therefore it is generally a good idea to start with a ligand concentration that is several-fold higher than the target (20–30 times) so as to observe the full range of the interaction. A 16-tube serial dilution of ligand is performed and then a fixed amount of target is added to each tube. Pipetting accuracy in the previous steps is generally not an issue, but to gain an accurate binding curve one must be fully confident in the volumes pipetted. This can be visualized by reviewing the capillary scans output provided by the machine and ensuring there are no fluctuations (Figure 2B). Although only 10 µl of sample is required per capillary, as a pipetting error can be more prominent with the smaller volumes used, we recommend mixing samples to final volumes of 20 µl using low-bind pipette tips. A raw MST trace plot should ideally show a change in relative fluorescence as ligand concentration increases (Figure 2C). An accurate binding curve should have a few points at high concentration representing complete binding, a few points at concentrations with no binding, and ideally, the remaining points defining the slope of the binding curve (Figure 2D).

Diagnosis of common errors. It is important to be able to understand what potential problems will present visually in MST results. Target adsorption can present itself similarly to a warped Gaussian peak in initial pretest scans (Figure 3A). As mentioned above, BSA addition to the buffer is often ideal in preventing this. Fluorescence inhomogeneity in capillary scans is harder to diagnose (Figure 3B), but the most common issue is improper fluorescence binding to the target. This is especially common when ethylenediaminetetraacetic acid (EDTA) is present in the buffer, and a Histidine-tag binding dye or other metal-ion dependent marker is being used. Changing the buffer to one without EDTA can help mitigate this. Aggregation is easily diagnosed (Figure 3C), but less easy to remedy. If possible, use a lower concentration of both ligand and target, and add detergent (e.g. Tween-20). Incomplete binding curves are identified easily, and the solution is to use higher (or lower) concentrations of the ligand (Figure 3D). If the problem persists after increasing ligand concentration, the efficiency of the labelling of fluorescent dye to the target should be re-evaluated.

Concluding remarks

While learning a new technique such an MST may come across as a challenging task, once the fundamentals are understood and the potential pitfalls are properly diagnosed, MST can be a powerful biophysical technique that an increasing number of researchers are turning towards. MST has proven to be an efficient, cost-effective and reliable technique that has the potential for continuous improvement as optics and fluorescence continue to progress. An exciting future for MST is the

continuing research into label-free MST experiments that can utilize intrinsic fluorescence, such as that generated by aromatic amino acids. Given the astounding amount of biomolecular interactions that a single cell performs every second, researchers need every tool possible to dissect the infinite complexity that nature has evolved to generate and sustain life. MST is an invaluable asset in the continuing search for this knowledge. ■

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Further reading

- Seo, H.W., Seo, J.P., Cho, Y., Eunkyong, K., Kim, Y.J. and Jung, G. (2019) Cetylpyridinium chloride interaction with the hepatitis B virus core protein inhibits capsid assembly. Virus Res. **263**, 102–111
- Szameit, K., Berg, K., Kruspe, S. et al. (2016) Structure and target interaction of a G-quadruplex RNA-aptamer. RNA Biol. **13**, 973–987
- Ascher, D.B., Wielens, J., Nero, T.L., Doughty, L., Morton, C.J. and Parker, M.W. (2014) Potent hepatitis C inhibitors bind directly to NS5A and reduce its affinity for RNA. Sci. Rep. **4**, 4765
- Asmari, M., Ratih, R., Alhazmi, H.A. and Deeb, S.E. (2018) Thermophoresis for characterizing biomolecular interaction. Methods **146**, 107–119
- Dijkman, P.M. and Watts, A. (2015) Lipid modulation of early G protein-coupled receptor signalling events. Biochim. Biophys. Acta Biomembr. **1848**, 2889–2897
- Moon, M.H., Hilimire, T.A., Sanders, A.M and Schneekloth, J.S. Jr. (2018) Measuring RNA–ligand interactions with microscale thermophoresis. Biochemistry **57**, 4638–4643
- Mueller, A.M., Breitsprecher, D., Duhr, S., Baaske, P., Schubert, T. and Langst, G. (2017) Microscale thermophoresis: a rapid and precise method to quantify protein–nucleic acid interactions in solution. Methods Mol. Biol. **1654**, 151–164
- Rodrigo-Unzueta, A., Martinez, M.A., Comino, N., Alzari, P.M., Cenal, A. and Guerin, M.E. (2016) Molecular basis of membrane association by the phosphatidylinositol mannosyltransferase PimA enzyme from Mycobacteria. J. Biol. Chem. **291**, 13955–13963
- Sparks, R.P. and Fratti, R. (2019) Use of microscale thermophoresis (MST) to measure binding affinities of components of the fusion machinery. Methods Mol. Biol. **1860**, 191–198
- Wienken, C.J., Baaske, P., Rothbauer, U., Braun, D. and Duhr, S. (2010) Protein-binding assays in biological liquids using microscale thermophoresis. Nat. Commun. **1**, 100
- Seidel, S.A., Wienken, C.J., Geissler, S., et al. (2012) Label-free microscale thermophoresis discriminates sites and affinity of protein-ligand binding. Angew. Chem. Int. Ed. Engl. **51**, 10656–10659
- Patel, T.R., Chojnowski, G., Astha, Koul, A., McKenna, S.A. and Bujnicki, M.A. (2017) Structural studies of RNA-protein complexes: a hybrid approach involving hydrodynamics, scattering, and computational methods. Methods **118–119**, 146–162