Journal of Visualized Experiments

Mapping the binding site of an aptamer on ATP using MicroScale Thermophoresis

--Manuscript Draft--

Manuscript Number:	JoVE55070R3
Full Title:	Mapping the binding site of an aptamer on ATP using MicroScale Thermophoresis
Article Type:	Methods Article - JoVE Produced Video
Keywords:	MicroScale Thermophoresis; interaction; Binding affinity; Aptamer-small molecule; Binding site mapping
Manuscript Classifications:	4.13: Nucleic Acids, Nucleotides, and Nucleosides; 95.51: Life Sciences (General)
Corresponding Author:	Thomas Schubert 2bind GmbH Regensburg, Bavaria GERMANY
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	schubert@2bind.de
Corresponding Author's Institution:	2bind GmbH
Corresponding Author's Secondary Institution:	
First Author:	Clemens Entzian
First Author Secondary Information:	
Other Authors:	Clemens Entzian
Order of Authors Secondary Information:	
Abstract:	Characterization of molecular interactions in terms of basic binding parameters such as binding affinity, stoichiometry, and thermodynamics is an essential step in basic and applied science. MicroScale Thermophoresis (MST) is a sensitive biophysical method to obtain this important information. Relying on a physical effect called thermophoresis, which describes the movement of molecules through temperature gradients, this technology allows for fast and precise determination of binding parameters in solution with freedom of buffer conditions (from buffer to lysates/sera). MST uses the fact, that an unbound molecule displays a different thermophoretic movement than a molecule, which is in complex with a binding partner. The thermophoretic movement is altered in the moment of molecular interaction due to changes in size, charge, and hydration shell. By comparing the movement profiles of different molecular ratios of the two binding partners, quantitative information such as binding affinity (pM to mM) can be obtained. Even challenging interactions between molecules of small size, such as aptamers and small compounds can be studied by MST. Using the well-studied model-interaction between the DH25.42 DNA-aptamer and ATP, this manuscript provides a protocol to characterize aptamer-small molecule interactions. This study demonstrates, that MST is highly sensitive and permits to map down the binding site of the 7.9 kDa DNA-aptamer to the adenine of ATP.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

2bind GmbH
BioPark III
Josef Engert Str. 13
93053 Regensburg
Tel: +49 941 9432849
Fax: +49 941 9432474
Email: info@2bind.de

Teena Mehta, Ph.D.
Science Editor

27. May 2016

Cover letter:

Dear Dr. Mehta;

please find enclosed our article entitled: "Mapping the binding site of an aptamer on ATP using MicroScale Thermophoresis" by Entzian et. al. for consideration as a publication in JoVE.
Nucleic Acid Aptamers are very interesting and versatile binding molecules with various applications from therapeutics to diagnostics as they have the potential to bind more or less any kind of target molecule. Even challenging molecules like small molecules such as drugs, metabolites or antibiotics can be bound by aptamers. For the later assay design it is absolutely essential to understand the basic binding parameters of the aptamer-small molecule interactions. However, the lack of sophisticated methods to study small molecule-aptamer interactions remains an issue in the field.
The presented article demonstrates the potential of the innovative MicroScale Thermophoresis technology to overcome this issue. The article shows a detailed protocol how to use the MicroScale Thermophoresis (MST) to characterize aptamer-small molecule interactions. The technology is described in detail and the strengths are shown. Using an example study the user is guided step by step through the process. Footnotes help the user to find solutions for problems. Mapping down the binding site of the well known ATP aptamer of Huizenga and Szostak on the ATP molecule demonstrates the power of the MicroScale Thermophoresis.

Suggested reviewers:

Prof. Dr. Gernot Längst
Biochemie III
Universität Regensburg
Universitätsstraße 31
93053 Regensburg
Germany

Prof. Dr. Ciara O´Sullivan
Nanobiotechnology & Bioanalysis Group,
Department of Chemical Engineering,
Universitat Rovira i Virgili,
Avinguda Països Catalans, 26
Tarragona 43007
Spain

Dr. Philipp Baaske
NanoTemper Technologies GmbH
Flößergasse 4
81369 Munich
Germany

Prof. Beatrix Süss
Technische Universität Darmstadt
Fachbereich Biologie, B2|03
Schnittspahnstraße 10
64287 Darmstadt

Prof. Ulrich Hahn
Universität Hamburg
Fachbereich Chemie
Institut für Biochemie und Molekularbiologie
Martin-Luther-King-Platz 6
20146 Hamburg

Prof. Jean-Jacques Toulmé
ARNA Laboratory - Inserm U869
Institut Européen de Chimie et Biologie
2 rue Robert Escarpit
33607 Pessac,
France

Thank you in advance for your consideration

Yours sincerely

Dr. Thomas Schubert, CEO. 2bind GmbH
Corresponding Author, schubert@2bind.com

TITLE:
Mapping the binding site of an aptamer on ATP using MicroScale Thermophoresis

AUTHORS:
Entzian, Clemens[1]; Schubert, Thomas[1]

AFFILIATION:
[1]2bind GmbH, Am Biopark 13, 93053 Regensburg, Germany

(email)
schubert@2bind.de
clemens.entzian@vkl.uni-regensburg.de

CORRESPONDING AUTHOR:
Schubert, Thomas

KEYWORDS:
Small molecule-aptamer interaction, binding parameters, MicroScale Thermophoresis, binding affinity, stoichiometry, thermodynamics, nucleic acids, binding site mapping

SHORT ABSTRACT:
MicroScale Thermophoresis (MST) is a sensitive technology to characterize aptamer-target interactions. This manuscript describes an MST protocol to characterize aptamer-small molecule interactions.

LONG ABSTRACT:
Characterization of molecular interactions in terms of basic binding parameters such as binding affinity, stoichiometry, and thermodynamics is an essential step in basic and applied science. MicroScale Thermophoresis (MST) is a sensitive biophysical method to obtain this important information. Relying on a physical effect called thermophoresis, which describes the movement of molecules through temperature gradients, this technology allows for the fast and precise determination of binding parameters in solution and allows the free choice of buffer conditions (from buffer to lysates/sera). MST uses the fact that an unbound molecule displays a different thermophoretic movement than a molecule that is in complex with a binding partner. The thermophoretic movement is altered in the moment of molecular interaction due to changes in size, charge, and hydration shell. By comparing the movement profiles of different molecular ratios of the two binding partners, quantitative information such as binding affinity (pM to mM) can be determined. Even challenging interactions between molecules of small sizes, such as aptamers and small compounds, can be studied by MST. Using the well-studied model interaction between the DH25.42 DNA aptamer and ATP, this manuscript provides a protocol to characterize aptamer-small molecule interactions. This study demonstrates that MST is highly sensitive and permits the mapping of the binding site of the 7.9 kDa DNA aptamer to the adenine of ATP.

INTRODUCTION:

Interaction between molecules is the basis of nature. Hence, scientists in many fields of basic and applied research try to understand the fundamental principles of molecular interactions of different kinds. MicroScale Thermophoresis (MST) enables scientists to perform the fast, precise, cost-efficient, and quality-controlled characterization of molecular interactions in solution, with a free choice of buffers. There are already more than 1,000 publications using MST, from 2016 alone, describing different kinds of analyses, including library screenings, binding event validations, competition assays, and experiments with multiple binding partners[1-8]. In general, MST permits the study of the classical binding parameters, such as binding affinity (pM to mM), stoichiometry, and thermodynamics, of any kind of molecular interaction. A great advantage of MST is the ability to study binding events independent of the size of the interaction partners. Even challenging interactions between small nucleic acid aptamers (15-30 nt) and targets such as small molecules, drugs, antibiotics, or metabolites can be quantified.

Current state-of-the-art technologies to characterize aptamer-target interactions are either lab-intense and highly complex or fail to quantify aptamer-small molecule interactions[9,10]. Surface Plasmon Resonance (SPR)-based assays[11,12] and truly label-free calorimetric approaches, such as Isothermal Titration Calorimetry (ITC)[13-15], isocratic elution[16], equilibrium filtration[17,18], in-line probing[19], gel-shift assays, stopped-flow fluorescence spectroscopy[20,21], fluorescence anisotropy (FA)[22,23], single-molecule fluorescence imaging[24,25], and Bio-layer interferometry (BLI)[26] are also either imprecise or incompatible with aptamer-small molecule interactions. Other principal issues of these methods are low sensitivity, high sample consumption, immobilization, mass transport limitations on surfaces, and/or buffer restrictions. Only a few of these technologies provide integrated controls for aggregation and adsorption effects.

MST represents a powerful tool for scientists to overcome this limitation to study the interactions between aptamers and small molecules[27-29], as well as other targets such as proteins[30-33]. The technology relies on the movement of molecules through temperature gradients. This directed movement, called “thermophoresis,” depends on the size, charge, and hydration shell of the molecule[34,35]. The binding of a ligand to the molecule will directly alter at least one of these parameters, resulting in a changed thermophoretic mobility. Ligands with small sizes may not have considerable impact in terms of size change from the unbound to the bound state, but they can have dramatic effects on the hydration shell and/or charge. The changes in the thermophoretic movement of molecules after interactions with the binding partner enables the quantification of basic binding parameters[2,7,34,36,37].

As depicted in **Figure 1A**, the MST device consists of an infrared laser focused onto the sample within the glass capillaries using the same optics as for fluorescence detection. The thermophoretic movement of proteins via the intrinsic fluorescence of tryptophans[6] or of a fluorescently-labeled interaction partner[3,8] can be monitored while the laser establishes a temperature gradient (ΔT of 2-6 °C). The resulting temperature difference in space, ΔT, leads to the depletion or accumulation of molecules in the area of elevated temperature, which can be quantified by the Soret coefficient (S_T):

$$S_T : \frac{c_{hot}}{c_{cold}} = exp(-S_T\,\Delta T)$$

c_{hot} represents the concentration in the heated region, and c_{cold} is the concentration in the initial cold region.

As shown in **Figure 1B**, a typical MST experiment results in an MST movement profile (time trace), consisting of different phases, which can be separated by their respective timescales. The initial fluorescence is measured in the first 5 s in absence of the temperature gradient to define the precise starting fluorescence and to check for photobleaching or photoenhancement. The Temperature Jump (T-Jump) represents the phase in which the fluorescence changes before thermophoretic movement. This initial decrease in fluorescence depends on heat-dependent changes of fluorophore quantum yield. The thermophoresis phase follows, in which the fluorescence decreases (or increases) due to the thermophoretic movement of the molecules until the steady-state distribution is reached. The reverse T-Jump and concomitant back diffusion of fluorescent molecules can be observed as indicated in **Figure 1B** after the laser is switched off. In order to access basic binding parameters, different molar ratios of the interaction partners are analyzed and compared. Typically, 16 different ratios are studied in one MST experiment, whereas the optical visible molecule is kept constant and is supplied with an increasing amount of the unlabeled ligand. The interaction between the two binding partners induces changes in the thermophoresis, and thus in the normalized fluorescence, F_{norm}, which is calculated as following:

$$F_{norm} = \frac{F_{(hot)}}{F_{(cold)}}$$

F_{hot} and F_{cold} represent averaged fluorescence intensities at defined time points of the MST traces (**Figure 1C**). Binding affinities (K_d or EC_{50} values) can be calculated by curve fitting (**Figure 1D**).

Overall, MST is a powerful tool to study molecular interactions of any kind. This manuscript offers a protocol to characterize the challenging interaction between the small molecule adenosine triphosphate (ATP; 0.5 kDa) and the 25-nt short ssDNA aptamer DH25.42 (7.9 kDa). Over the course of the manuscript, the binding site of the aptamer on the ATP molecule is mapped down to the adenine group of the ATP.

PROTOCOL:

1. Preparation of the aptamer working stock

1.1. Follow the manufacturer's instructions and dissolve the oligonucleotide (5-Cy5-CCTG GGGGAGTATTGCGGAGGAAGG-3, sequence from Reference 18) in water, reaching a 100-µM final concentration.

1.2. Prepare the aptamer working solution by diluting the oligonucleotide stock to 200 nM with binding buffer (20 mM Tris, pH 7.6; 300 mM NaCl; 5 mM $MgCl_2$; 0.01% Tween-20). NOTE: Centrifugation of molecule stocks for 5 min at 13,000 x g may help to remove aggregates.

1.3. Incubate the mixture for 2 min at 90 °C, let the sample immediately cool down on ice, and use the sample at room temperature.

2. Preparation of the ligand dilution series

2.1. For each ligand (adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), guanosine triphosphate (GTP), cytosine triphosphate (CTP), deoxyadenosine triphosphate (dATP), and S-adenosyl methionine (SAM); 1 mM stock each), prepare a 16-step serial dilution in 200-µL micro reaction tubes. NOTE: Centrifugation of ligand stocks for 5 min at 14,000 x g may help to remove aggregates. Low volume, low binding reaction tubes are recommended to avoid adsorption of molecules to the tube walls.

2.2. Start with a maximum concentration of at least 50 times higher than the estimated affinity and reduce the ligand concentration by 50% in each dilution step. NOTE: The concentration finder tool implemented in the control software simulates binding data and helps with finding the right concentration range for the dilution series

2.3. Fill 20 µL of the ligand stock (1 mM) in tube 1. Add 10 µL of aptamer binding buffer into micro reaction tubes 2 to 16.

2.4. Transfer 10 µL of tube 1 to tube 2 and mix properly by pipetting up and down several times. Transfer 10 µL to the next tube and repeat this dilution for the remaining tubes.

2.5. Discard the 10-µL excess from the last tube. Avoid any buffer dilution effects. The buffer in tube 1 and in tubes 2-16 must be identical.

3. Preparation of the final reaction mix

3.1. Prepare the individual binding reactions with a volume of 20 µL (10 µL of aptamer working solution + 10 µL of the respective ligand dilution) to minimize pipetting errors. A volume of only 4 µL is sufficient to fill the capillary.

3.2. Add 10 µL of the 200-nM aptamer working solution to 10 µL of each ligand dilution and mix properly by pipetting up and down several times.

3.3. Incubate the samples for 5 min at room temperature and fill the samples into standard capillaries by dipping the capillaries into the sample. Longer incubation times may be necessary for some interactions; however, 5 min is adequate for most. Touch the capillaries only on the sides, **NOT** on the middle part, where the optical measurement will be taken.

3.4. Place the capillaries onto the capillary tray and start the MST device.

4. Starting the MST device

NOTE: The device provides two pre-installed software packages, the ‘‘control” software for the technical setup of the experimental conditions and the ‘‘analysis” software for the interpretation of the produced data.

4.1. Before placing the capillary tray into the MST device, start the control software and adjust the overall desired temperature by selecting ‘‘enable manual temperature control” in the ‘‘temperature control” dropdown menu. Adjust the temperature to 25 °C in this way. NOTE: The MST instruments can be temperature-controlled from 22 to 45 °C.

4.2. Wait for the temperature to reach the expected level and then place the capillary tray into the MST device.

4.3. Set the LED channel to ‘‘red” for Cy5 dyes and adjust the LED power to gain a fluorescence signal of 300 to 1,000 fluorescence units at the MST device with a standard sensor. 10% LED power is used in this study. NOTE: 6,000 to 18,000 fluorescence units are recommended for the MST with a high-sensitivity sensor.

5. Capillary scan

5.1. Carry out a capillary scan to check different quality aspects of the sample by choosing the capillary position on the “control” software and clicking on “start cap scan” **before** starting the MST measurement.

5.2. Inspect the capillary scan for fluorescence enhancement/quenching and sticking effects (U-shaped or flattened peaks) in the software. NOTE: More details on the detection and handling of fluorescence and sticking effects can be found in the discussion.

6. MST measurement

NOTE: Before starting the MST measurement, make sure to exclude sticking effects, enhancement/quenching effects, or pipetting errors, and ensure that the capillary scan indicates that the fluorescence signal is sufficient. For more details, see the discussion.

6.1. Assign the ligand concentrations from the dilution series to the respective capillary position in the ‘‘control” software. Consider the dilution step of mixing the aptamer and ligand (1:1).

6.2. Enter the highest concentration of ligand (500 µM) for capillary #1, select the correct dilution type (here, 1:1), click on the maximum concentration, and use the drag function to automatically assign the remaining concentrations in capillaries #2-16. The lowest concentration is 15.26 nM.

6.3. Enter the concentration of the fluorescent aptamer (here, 100 nM) in the respective section of the control software.

6.4. Use the default settings, which detect the fluorescence for 5 s, record the MST for 30 s, and record the fluorescence for a further 5 s after the inactivation of the laser to monitor the back diffusion of molecules.

6.5. Adjust the laser power to 20% in the respective section of the control software. NOTE: In order to receive the best signal-to-noise ratio and to avoid unspecific effects, a laser power of 20-40% is recommended. In specific cases, a higher laser power may be required to get a good separation of unbound and bound molecules.

6.6. Save the experiment after selecting the destination folder and start the MST measurement by pressing the ‘‘Start MST measurement” button. NOTE: The .ntp file will be generated in the destination folder. Using this setup, one measurement lasts 10-15 min.

6.7. Repeat the experimental procedure at least twice for a more accurate determination of the EC_{50} value. NOTE: In order to test the technical reproducibility, the same capillaries can be scanned several times (technical repeats).

7. MST data analysis

NOTE: The analysis software enables the analysis of data on the fly during the measurement. The analysis software plots the MST time traces and changes in the normalized fluorescence (F_{norm}) versus the ligand concentration[37].

7.1. Start the MST analysis software (MO.Affinity Analysis) and load the .ntp file from the destination folder. Select “MST” as the analysis type in the data selection menu. NOTE: In case of ligand-dependent fluorescence effects, the initial fluorescence can be chosen for analysis.

7.2. Add the respective technical or biological run(s) to a new analysis by drag-and-drop or by pressing the “+” button below the respective experimental run.

7.3. Press the information button below the respective experimental run to obtain information on the properties of the experiment, MST traces, capillary scan, capillary shape, initial fluorescence, and bleaching rate. NOTE: These raw data can also be inspected in later steps of the analysis.

7.4. Visually inspect the MST traces for aggregation and precipitation effects, visible as bumps and spikes. NOTE: For more information on the detection and handling of aggregation effects, read the discussion.

7.5. Visually inspect the capillary scan and the capillary shape overlay for adsorption effects, visible as flattened or U-shaped peaks. Visually inspect the capillary scan and the initial fluorescence for fluorescence effects. Visually inspect the bleaching rate for photobleaching effects.

7.6. Switch to the dose-response mode and change the analysis setting to “expert” mode by pressing the respective button. Select “T-Jump” as the MST evaluation strategy.

7.7. Select the “Hill” model for curve fitting. The binding parameters will automatically be calculated. Normalize the data by choosing the respective type of normalization in the

"compare results" menu. Export the data either as an .xls or .pdf. NOTE: The table below the binding graph summarizes the calculated binding parameters.

REPRESENTATIVE RESULTS:

In this study, MST was applied to characterize the binding site of the DH25.42 DNA aptamer[18] on ATP. In contrast to other studies characterizing the interaction of ATP or ATP-mimicking small molecules with proteins randomly-labeled with one or more fluorophores[38-40], this study includes a labeled version of the 7.9-kDa ssDNA aptamer with one Cy5 molecule on the 5´end. Different ATP derivatives and related molecules, all differing from ATP in various positions, were used to map the binding site on the ATP molecule. Dilution series (in 16 steps, reducing the ligand concentration by 50% each) of the different ligands were prepared and mixed with a constant amount of Cy5-labeled aptamer. Samples were analyzed in standard capillaries at 10% LED and 20% laser power. Movement profiles (MST time traces) were recorded and fluorescence units, derived from the T-Jump phase, were plotted versus the ligand concentration (compare **Figure 1C**). Curve fitting was performed applying the Hill equation, resulting in EC_{50} values. In 5 independent biological repeats (4 different operators, 2 different aptamer stocks, 2 different ATP stocks), the ATP-aptamer interaction shows a negative binding amplitude of 6 to 13 units and an average EC_{50} value of 52.3 ± 5.0 µM (**Figure 2A**). Error bars in the binding graphs and "±" presented in the affinity data represent the standard deviation of 5 biological repeats (5 independent measurements in a different capillary). Previously-reported affinities for the interaction of the DH25.42 aptamer with [2,8,5'-^{3}H]-adenosine and ATP agarose were comparable. The interaction of radiolabeled adenosine with the aptamer was quantified by a centrifugal filter assay, and an affinity (K_d) of 6 ± 3 µM was determined. Isocratic elution experiments with ATP immobilized to agarose resulted in an affinity (K_d) of 13 µM[18,41].

For a better side-by-side comparison, the data can be normalized to the fraction of complexed molecules (fraction bound, FB) using the following equation:

$$FB = \frac{value(c) - free}{complexed - free}$$

where *value(c)* is the MST value measured for the concentration c, *free* is the MST value for the unbound state (lowest concentration), and *complexed* is the MST value for the fully-bound state (**Figure 2B**). This normalization is ideal to compare the results of different ligands in one graph, as shown in **Figure 2C**. The detected affinities of ADP (63.6 ± 5.9 µM in biological duplicates), AMP (91.6 ± 9.1 µM in biological duplicates), and SAM (44.4 ± 3.2 µM in biological duplicates), which differ from ATP in the number of phosphate groups, imply that this position has no or only minor influence on the binding behavior of the aptamer (**Figure 2C and D**). The OH group at the C2 carbon of the ribose of ATP could also be excluded from being the major binding site, as dATP was also bound by the aptamer with a slightly reduced affinity (64.4 ± 6.1 µM in biological duplicates). Changing the purine group of ATP to the pyrimidine group CTP resulted in non-binding of the aptamer, demonstrating the importance of this group for the interaction. The aptamer bound to adenine with an affinity of 141.7 ± 9.4 µM (in biological duplicates), showing that the binding site must be in this part of the ATP molecule. The purine

molecules GTP and ATP differ in the green shaded area represented in **Figure 2D**, which represents the main binding site of the aptamer on ATP. Another study used non-quantitative elution experiments with different ATP derivatives to elute a radiolabeled aptamer from ATP agarose, which showed comparable results to this MST study[18].

FIGURE LEGENDS:

Figure 1: MicroScale Thermophoresis. (A) The technical setup of the MST technology is shown. The optics focus on the center of glass capillaries, thereby detecting the fluorescence signal of the optically-visible molecule. An IR laser is utilized to establish a temperature gradient in the observation window of the optical system. Changes in fluorescence can be utilized to monitor the thermophoretic movement of the molecules in solution (B) MST time trace-movement profile of molecules in a temperature gradient. The initial fluorescence is measured for 5 s while the laser is off. Switching on the laser generates a temperature gradient. After the immediate T-Jump phase, in which the fluorescent dye decreases its signal yield upon heat induction, the thermophoretic movement takes place and is observed for 30 s. After the laser is turned off, the molecules diffuse back. (C) Results of a typical MST experiment: (Left) 16 capillaries containing the same concentration of fluorescent molecule and an increasing concentration of the unlabeled ligand; the MST time traces are recorded and normalized to their initial fluorescence. (Right) The normalized fluorescence; the difference between F_{cold} and F_{hot} is plotted against the concentration of the ligand. A curve fit of this data yields binding parameters, such as the binding affinity. Re-print with permission from Elsevier, Methods[28]; license number 3890230800113.

Figure 2: MST data analysis. (A) The baseline corrected normalized fluorescence ΔF_{norm} (‰), derived from the MST T-Jump signal, is plotted against the ATP concentration (in µM). The Hill equation (EC_{50}) was applied for curve fitting. The error bars represent the standard deviation from five biological repeats. (B) Fraction-bound plot of the data shown in **Figure 2A**. The respective data sets were normalized to the fraction bound, and the average of these normalized data is presented in the binding graph. The error bars indicate the standard deviation of 5 biological repeats. (C) The fraction-bound graph shows a quantitative comparison (Hill fit) of the different ligands to the aptamer. The error bars represent the standard deviation of two biological repeats. (D) Binding affinities (EC_{50}) of different ligands to the aptamer. The green shaded area indicates the binding site of the aptamer on the adenine group of ATP. This figure is modified from Elsevier, Methods[28]; license number 3890230800113. Data sets from the previous study are reanalyzed and expanded within this study.

Figure 3: Assay optimization for MST experiments. (A) Protein adsorption and sticking effects can be detected in the capillary scan. Irregular peak shapes, such as flattened or U-shaped peaks, indicate the sticking of the sample material to the glass surface. Changing the capillary type (standard, premium, or hydrophobic) can prevent molecule adsorption, resulting in a regular peak shape. (B) The MST time traces also serve as a quality control, since aggregates can be detected there as bumps and spikes. The experimental conditions can be optimized by improving the solubility of the molecules (*e.g.,* including detergents such as Pluronic F-127 or varying pH values or salt concentrations). Centrifugation may help to remove larger aggregates.

To ensure optimal data quality, the MST time traces should resemble the given example.

DISCUSSION:

Quality controls:

Unspecific sticking/adsorption of sample material to surfaces, as well as aggregation effects, have a dramatic influence on the quality of the affinity data. However, only a few state-of-the-art technologies offer accurate and rapid options to monitor and avoid these effects. MST offers integrated quality controls that detect and help to overcome these issues, allowing for the stepwise optimization of the technical setup. Important information on sticking and fluorescence effects can be extracted from the capillary scan and capillary shape (**steps 5.2, 7.6 and 7.7**), whereas aggregation/precipitation effects (**step 7.5**) can be monitored in the movement profiles. These controls enable for the constant improvement of data quality and represent critical steps in the protocol.

Detection of adsorption effects and troubleshooting:

The capillary scan is primarily conducted as an initial step to determine the position of the capillaries on the tray holder by scanning the fluorescence over it. Each peak represents one capillary, indicating the starting point for the MST measurement. Visual inspection of the peak shape enables the determination of the adsorption of molecules to the glass surface, which is an essential quality control that should be performed before starting the MST experiment. The capillary shape overlay allows for the easy detection of flattened, bumpy peak shapes, or even of peaks that resemble a U-form, indicating the adsorption/sticking of molecules to the inner glass surface of the capillary (**Figure 3A**, upper panel). Differently-coated capillary types (standard, premium, and hydrophobic) help to ensure the solubility and minimal adsorption of the molecules to the capillaries. Capillaries should be tested for their suitability prior to the binding experiments. Detergents such as Tween (0.005-0.1%) or Pluronic F127 (0.01-0.1%) may also prevent unspecific adsorption effects. Optimization at this stage of the experiment is essential for high data quality (compare **Figure 3A**, lower panel).

Detection of fluorescence effects and troubleshooting:

Variations in the peak height of the scanned capillaries offer another layer of information on sample characteristics. Random differences in capillary fluorescence may, on the one hand, be due to pipetting errors or, on the other hand, originate from large sample aggregates in the scanning region that may increase the fluorescence drastically. High pipetting accuracy is mandatory to avoid dilution effects. Mixing all solutions by pipetting up and down increases the accuracy further. In addition, it is essential to keep the buffer consistent in each capillary. Strategies to handle aggregation effects are presented in a later paragraph.

Systemic changes of fluorescence with increasing ligand concentration often indicate ligand dependent quenching/enhancement effects. The “SD Test” was carried out in order to discriminate binding-induced fluorescence changes from unspecific fluorescence loss; this test monitors fluorescence intensities under denaturing conditions. In case of ligand-dependent fluorescence effects, the fluorescence intensities under denaturing condition should be identical, independent of the concentration of titrant. If the difference in fluorescence

intensities is still present under these conditions, material was either lost due to unspecific adsorption to tube walls or due to aggregation and subsequent centrifugation.

In order to perform the SD Test, 10 µL of the first and 10 µL of the last ligand dilution step were each carefully transferred to a fresh tube containing 10 µL of a 2 x SD Mix (4% SDS and 40 mM DTT). The samples were mixed and the molecules were denatured for 5 min at 95 °C. After filling the samples into capillaries, the fluorescence intensities were measured. If a ligand-dependent fluorescence effect is detected, the data can be analyzed directly by the binding information deduced from the fluorescence intensity changes.

Detection of aggregation effects and troubleshooting:
Bumpy, uneven MST time traces indicate aggregation and/or precipitation effects (**Figure 3B**, upper panel). Non-aggregated sample material displays a clean and smooth thermophoretic movement profile (**Figure 3B**, lower panel). The centrifugation of the sample material prior to use (10 min at max speed), the addition of detergents (0.005-0.1% Tween-20, 0.01-0.1% Pluronic F-127, or similar), the use of BSA (>0.5 mg/mL), or the change of buffer conditions (pH and ionic strength) are recommended to minimize the aggregation effects and to optimize the data quality. In order to obtain high-quality data, it is essential to minimize the aggregation effects.

Data analysis and curve fitting:
A thermophoretic movement profile is divided into different phases, which can be inspected either separately or concomitantly. The T-Jump describes the sudden change in fluorescence yield upon temperature change, representing an intrinsic characteristic of fluorophores. Direct binding in the close surroundings of the fluorophore or conformation changes upon binding highly affect the T-Jump. The slower thermophoresis refers to the movement of molecules in the temperature field, offering information on the overall structure of the formed complex. The default type of data analysis employs the “Thermophoresis + T-Jump” setting, exploiting both phenomena to determine the binding parameters. In case the two phases have opposite directions, it is recommended to analyze the phases separately. Please note that time-effects and different species of molecules might lead to differences in the affinity of the thermophoresis and T-Jump. Another type of analysis option is the manual setting, which enables the investigation of specific regions of the movement profile. Disturbances due to aggregation or convection can be excluded in this way. In order to improve data quality, cursors may be set before aggregation signals. Examining the early thermophoresis commonly produces data with less noise, since convection is a time-dependent process. This early manual setting is highly recommended for experiments with high laser power (80%). Upon choosing the data analysis setting, two fitting models integrated in the analysis software can be applied to fit the binding curve. The fit function for K_d from the law of mass action is suited for 1:1 binding modes or for multiple binding sites possessing the same affinity. The concentration-independent dissociation constant K_d describes the equilibrium between the bound and unbound state; thus, the affinity of a binding site to a ligand is measured. The concentration-dependent EC_{50} value derived from the Hill equation represents the effective dose of a ligand at which half of the labeled molecules are in the bound state. The Hill fit should be applied to

multivalent binding models, especially if they are cooperative.

The integrated quality controls represent one great advantage of MST over technologies such as SPR or ITC. These controls allow for the fast and easy optimization of the technical setup to ensure optimal data quality. In addition to the time-efficient measurements (K_d in 15 min) and the immobilization-free setup, MST offers the free choice of buffers, permitting the assessment of molecular interactions, even in lysates and sera[5,42,43]. Due to the fact that molecular thermophoresis depends on the size, charge, and hydration shell of molecules, there are no limitations in the size of the measured interaction partners during MST measurements. The dynamic affinity range, from pM to mM, together with the low sample consumption, completes the strengths of the MST technology. It is worth mentioning that MST generates precise data on basic binding parameters, such as binding affinity, stoichiometry, and thermodynamics. However, MST does not allow for the measurement of k_{on} and k_{off} rates. In addition, one has to consider that, for most MST experiments, a fluorescent modification must be added to one of the interaction partners. The generated data are in good agreement with state-of-the-art technologies, such as SPR and ITC[32,43-46]. However, these data are quality-controlled, the experiments are faster, and they consume less material. Overall, MST represents a powerful technology orthogonal to state-of-the-art technologies, with some additional advantages.

ACKNOWLEDGMENTS:
The authors have no acknowledgements.

DISCLOSURES:
C.E. and T.S. are employees of 2bind GmbH, which provides biophysical analytical services.

REFERENCES:
1 Linke, P. *et al.* An Automated Microscale Thermophoresis Screening Approach for Fragment-Based Lead Discovery. *J Biomol Screen.* **21** (4), 414-421.
2 Jerabek-Willemsen, M. *et al.* MicroScale Thermophoresis: Interaction analysis and beyond *Journal of Molecular Structure.* **1077** 101-113, doi:doi:10.1016/j.molstruc.2014.03.009, (2014).
3 Zillner, K. *et al.* Microscale thermophoresis as a sensitive method to quantify protein: nucleic acid interactions in solution. *Methods Mol Biol.* **815** 241-252.
4 Zhang, W., Duhr, S., Baaske, P. & Laue, E. Microscale thermophoresis for the assessment of nuclear protein-binding affinities. *Methods Mol Biol.* **1094** 269-276.
5 Wienken, C. J., Baaske, P., Rothbauer, U., Braun, D. & Duhr, S. Protein-binding assays in biological liquids using microscale thermophoresis. *Nat Commun.* **1** 100.
6 Seidel, S. A. *et al.* Label-free microscale thermophoresis discriminates sites and affinity of protein-ligand binding. *Angew Chem Int Ed Engl.* **51** (42), 10656-10659.
7 Seidel, S. A. *et al.* Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions. *Methods.* **59** (3), 301-315.
8 Schubert, T. *et al.* Df31 protein and snoRNAs maintain accessible higher-order structures of chromatin. *Mol Cell.* **48** (3), 434-444.
9 McKeague, M. & Derosa, M. C. Challenges and opportunities for small molecule aptamer

development. *J Nucleic Acids.* **2012** 748913, doi:10.1155/2012/748913, (2012).
10 Ruscito, A. & DeRosa, M. C. Small-Molecule Binding Aptamers: Selection Strategies, Characterization, and Applications. *Front Chem.* **4** 14, doi:10.3389/fchem.2016.00014, (2016).
11 Chang, A. L., McKeague, M., Liang, J. C. & Smolke, C. D. Kinetic and equilibrium binding characterization of aptamers to small molecules using a label-free, sensitive, and scalable platform. *Anal Chem.* **86** (7), 3273-3278.
12 Chang, A. L., McKeague, M. & Smolke, C. D. Facile characterization of aptamer kinetic and equilibrium binding properties using surface plasmon resonance. *Methods Enzymol.* **549** 451-466.
13 Jing, M. & Bowser, M. T. Methods for measuring aptamer-protein equilibria: a review. *Anal Chim Acta.* **686** (1-2), 9-18, doi:S0003-2670(10)01342-5 [pii]
10.1016/j.aca.2010.10.032, (2011).
14 Sokoloski, J. E., Dombrowski, S. E. & Bevilacqua, P. C. Thermodynamics of ligand binding to a heterogeneous RNA population in the malachite green aptamer. *Biochemistry.* **51** (1), 565-572, doi:10.1021/bi201642p, (2012).
15 Burnouf, D. *et al.* kinITC: a new method for obtaining joint thermodynamic and kinetic data by isothermal titration calorimetry. *J Am Chem Soc.* **134** (1), 559-565, doi:10.1021/ja209057d, (2012).
16 Mannironi, C., Scerch, C., Fruscoloni, P. & Tocchini-Valentini, G. P. Molecular recognition of amino acids by RNA aptamers: the evolution into an L-tyrosine binder of a dopamine-binding RNA motif. *RNA.* **6** (4), 520-527 (2000).
17 Jenison, R. D., Gill, S. C., Pardi, A. & Polisky, B. High-resolution molecular discrimination by RNA. *Science.* **263** (5152), 1425-1429 (1994).
18 Huizenga, D. E. & Szostak, J. W. A DNA aptamer that binds adenosine and ATP. *Biochemistry.* **34** (2), 656-665 (1995).
19 Lee, E. R., Baker, J. L., Weinberg, Z., Sudarsan, N. & Breaker, R. R. An allosteric self-splicing ribozyme triggered by a bacterial second messenger. *Science.* **329** (5993), 845-848, doi:329/5993/845 [pii]
10.1126/science.1190713, (2010).
20 Wickiser, J. K., Cheah, M. T., Breaker, R. R. & Crothers, D. M. The kinetics of ligand binding by an adenine-sensing riboswitch. *Biochemistry.* **44** (40), 13404-13414, doi:10.1021/bi051008u, (2005).
21 Jucker, F. M., Phillips, R. M., McCallum, S. A. & Pardi, A. Role of a heterogeneous free state in the formation of a specific RNA-theophylline complex. *Biochemistry.* **42** (9), 2560-2567, doi:10.1021/bi027103+, (2003).
22 Zhao, Q., Lv, Q. & Wang, H. Aptamer fluorescence anisotropy sensors for adenosine triphosphate by comprehensive screening tetramethylrhodamine labeled nucleotides. *Biosens Bioelectron.* **70** 188-193, doi:S0956-5663(15)00175-X [pii]
10.1016/j.bios.2015.03.031, (2015).
23 Zhang, D. *et al.* A sensitive fluorescence anisotropy method for detection of lead (II) ion by a G-quadruplex-inducible DNA aptamer. *Anal Chim Acta.* **812** 161-167, doi:S0003-2670(13)01578-X [pii]
10.1016/j.aca.2013.12.029, (2014).

24 Elenko, M. P., Szostak, J. W. & van Oijen, A. M. Single-molecule imaging of an in vitro-evolved RNA aptamer reveals homogeneous ligand binding kinetics. *J Am Chem Soc.* **131** (29), 9866-9867, doi:10.1021/ja901880v, (2009).
25 Elenko, M. P., Szostak, J. W. & van Oijen, A. M. Single-molecule binding experiments on long time scales. *Rev Sci Instrum.* **81** (8), 083705, doi:10.1063/1.3473936, (2010).
26 Zichel, R., Chearwae, W., Pandey, G. S., Golding, B. & Sauna, Z. E. Aptamers as a sensitive tool to detect subtle modifications in therapeutic proteins. *PLoS One.* **7** (2), e31948, doi:10.1371/journal.pone.0031948
PONE-D-11-17676 [pii], (2012).
27 Baaske, P., Wienken, C. J., Reineck, P., Duhr, S. & Braun, D. Optical thermophoresis for quantifying the buffer dependence of aptamer binding. *Angew Chem Int Ed Engl.* **49** (12), 2238-2241.
28 Entzian, C. & Schubert, T. Studying small molecule-aptamer interactions using MicroScale Thermophoresis (MST). *Methods.* **97** 27-34, doi:S1046-2023(15)30068-2 [pii]
10.1016/j.ymeth.2015.08.023, (2016).
29 Valenzano, S. *et al.* Screening and Identification of DNA Aptamers to Tyramine Using in Vitro Selection and High-Throughput Sequencing. *ACS Comb Sci.* **18** (6), 302-313, doi:10.1021/acscombsci.5b00163, (2016).
30 Jauset Rubio, M. *et al.* beta-Conglutin dual aptamers binding distinct aptatopes. *Anal Bioanal Chem.* **408** (3), 875-884, doi:10.1007/s00216-015-9179-z
10.1007/s00216-015-9179-z [pii], (2016).
31 Breitsprecher, D. *et al.* Aptamer Binding Studies Using MicroScale Thermophoresis. *Methods Mol Biol.* **1380** 99-111.
32 Stoltenburg, R., Schubert, T. & Strehlitz, B. In vitro Selection and Interaction Studies of a DNA Aptamer Targeting Protein A. *PLoS One.* **10** (7), e0134403, doi:10.1371/journal.pone.0134403
PONE-D-15-17612 [pii], (2015).
33 Kinghorn, A. B. *et al.* Aptamer Affinity Maturation by Resampling and Microarray Selection. *Anal Chem.* **88** (14), 6981-6985, doi:10.1021/acs.analchem.6b01635, (2016).
34 Duhr, S. & Braun, D. Why molecules move along a temperature gradient. *Proc Natl Acad Sci U S A.* **103** (52), 19678-19682 (2006).
35 Braun, D. & Libchaber, A. Trapping of DNA by thermophoretic depletion and convection. *Phys Rev Lett.* **89** (18), 188103 (2002).
36 Duhr, S., Arduini, S. & Braun, D. Thermophoresis of DNA determined by microfluidic fluorescence. *Eur Phys J E Soft Matter.* **15** (3), 277-286 (2004).
37 Jerabek-Willemsen, M., Wienken, C. J., Braun, D., Baaske, P. & Duhr, S. Molecular interaction studies using microscale thermophoresis. *Assay Drug Dev Technol.* **9** (4), 342-353.
38 He, K., Dragnea, V. & Bauer, C. E. Adenylate Charge Regulates Sensor Kinase CheS3 To Control Cyst Formation in Rhodospirillum centenum. *MBio.* **6** (3), e00546-00515.
39 Brvar, M. *et al.* Structure-based discovery of substituted 4,5'-bithiazoles as novel DNA gyrase inhibitors. *J Med Chem.* **55** (14), 6413-6426.
40 Pogorelcnik, B. *et al.* 4,6-Substituted-1,3,5-triazin-2(1H)-ones as monocyclic catalytic inhibitors of human DNA topoisomerase IIalpha targeting the ATP binding site. *Bioorg*

Med Chem. **23** (15), 4218-4229.
41 Jhaveri, S., Rajendran, M. & Ellington, A. D. In vitro selection of signaling aptamers. *Nat Biotechnol.* **18** (12), 1293-1297, doi:10.1038/82414, (2000).
42 Khavrutskii, L. *et al.* Protein purification-free method of binding affinity determination by microscale thermophoresis. *J Vis Exp.* (78), doi:10.3791/50541, (2013).
43 Ramakrishnan, M. *et al.* Probing cocaine-antibody interactions in buffer and human serum. *PLoS One.* **7** (7), e40518, doi:10.1371/journal.pone.0040518
PONE-D-12-07093 [pii], (2012).
44 Chen, M. *et al.* Antiviral activity and interaction mechanisms study of novel glucopyranoside derivatives. *Bioorg Med Chem Lett.* **25** (18), 3840-3844, doi:S0960-894X(15)00772-6 [pii]
10.1016/j.bmcl.2015.07.068, (2015).
45 Wan, C. *et al.* Insights into the molecular recognition of the granuphilin C2A domain with PI(4,5)P2. *Chem Phys Lipids.* **186** 61-67, doi:S0009-3084(15)00004-3 [pii]
10.1016/j.chemphyslip.2015.01.003, (2015).
46 Harazi, A. *et al.* The Interaction of UDP-N-Acetylglucosamine 2-Epimerase/N-Acetylmannosamine Kinase (GNE) and Alpha-Actinin 2 Is Altered in GNE Myopathy M743T Mutant. *Mol Neurobiol.* doi:10.1007/s12035-016-9862-x
10.1007/s12035-016-9862-x [pii], (2016).

Figure 1

Click here to download Figure Figure 1.jpg

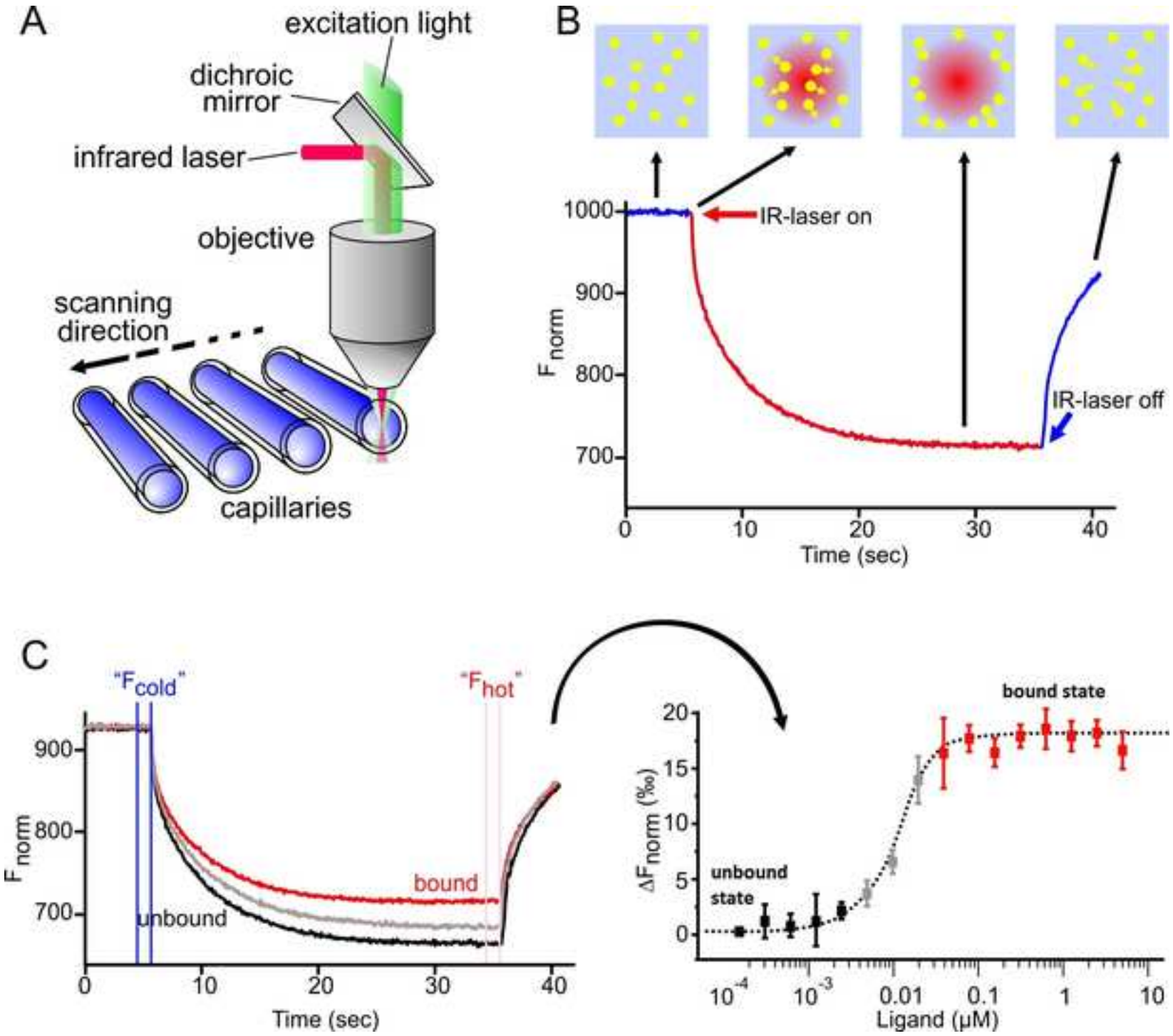


Figure 2

Click here to download Figure Figure 2.png

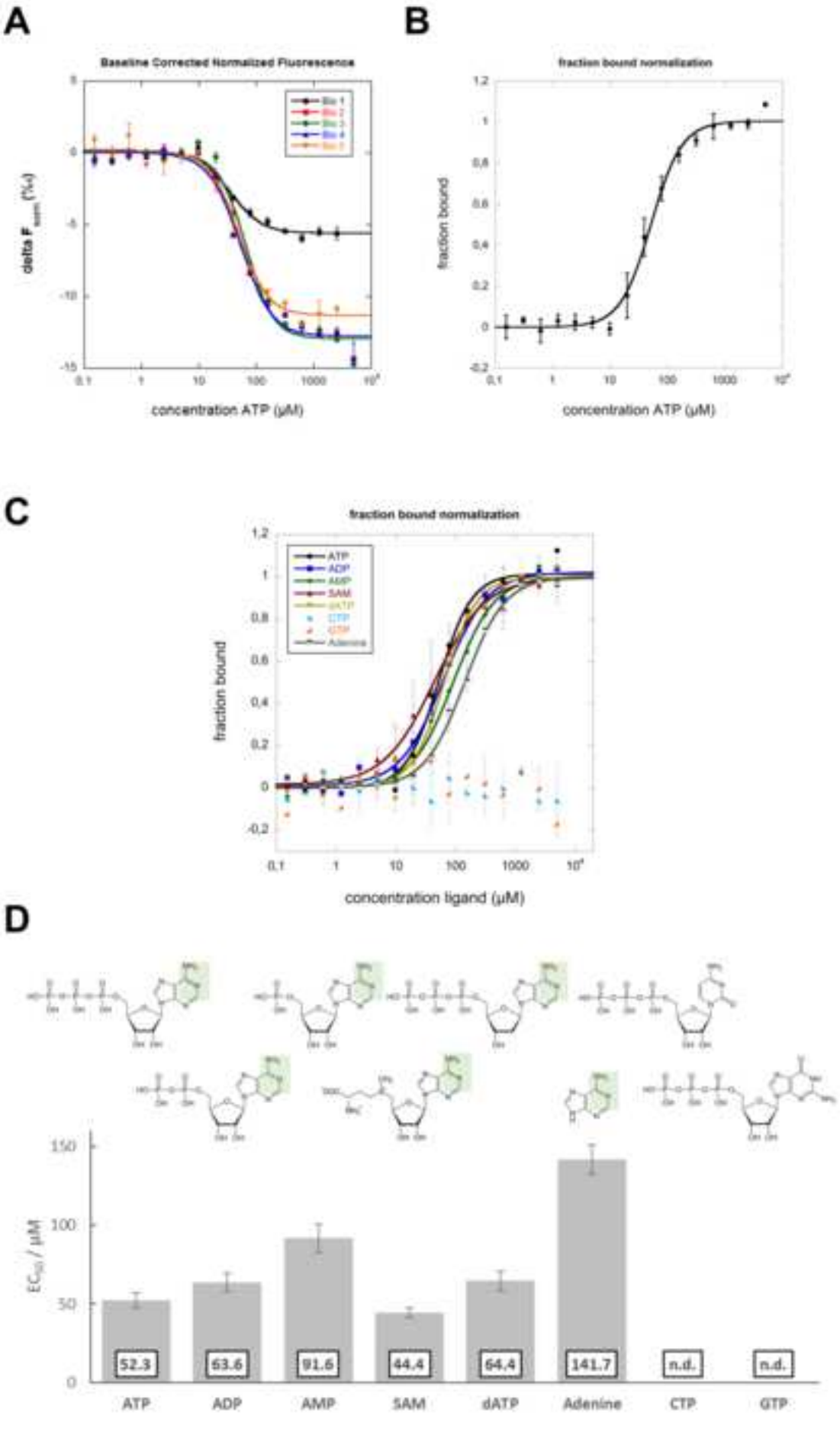
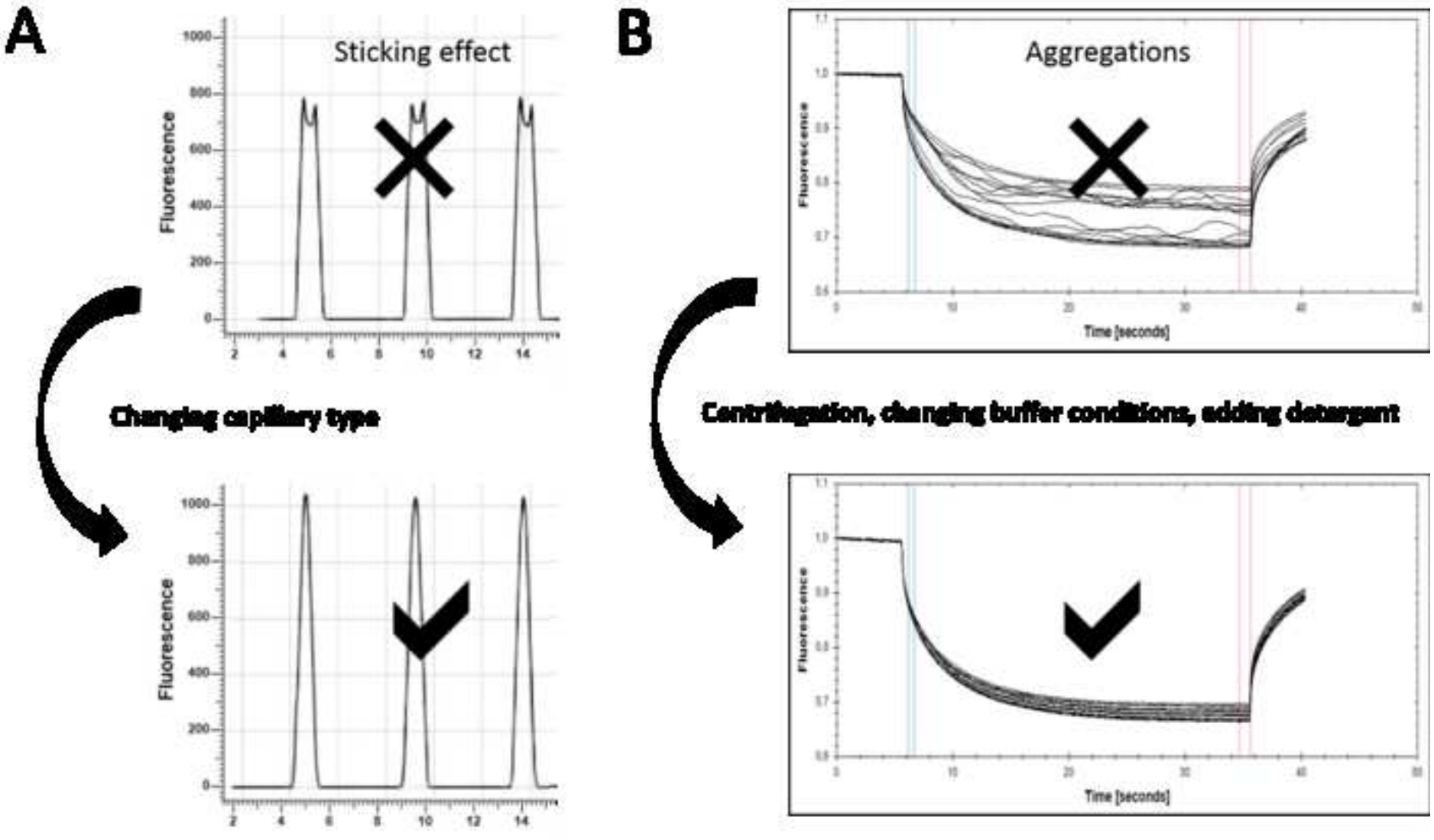


Figure 3



Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment JoVE Materials 2bind.xls

Name of Reagent/ Equipment	Company
Aptamer binding buffer	
Fluorescently labeled ATP aptamer	IDT, Leuven, Belgium
ATP	Sigma Aldrich, Germany
ADP	Sigma Aldrich, Germany
AMP	Sigma Aldrich, Germany
SAM	Sigma Aldrich, Germany
dATP	Sigma Aldrich, Germany
CTP	Sigma Aldrich, Germany
GTP	Sigma Aldrich, Germany
Monolith NT.115	NanoTemper Technologies, Munich, Germany
Monolith NT.115 capillaries Standard	NanoTemper Technologies, Munich, Germany
Eppendorf PCR tubes	Eppendorf, Germany
Monolith control software. 2.1.33, pre-installed on the device	NanoTemper Technologies, Munich, Germany
MO.affinity analysis v2.1.1	NanoTemper Technologies, Munich, Germany
Kaleidagraph 4.5.2	Synergy Software

Catalog Number	Comments/Description	Term in Manuscript
	20 mM Tris pH7.6; 300 mM NaCl; 5 mM MgCl2; 0.01%Tween-20	
	sequence: DH25.42 50-Cy5-CCTG GGGGAGTATTGCGGAGGAAGG-3	
A2383	1 mM stock solutions stored at - 20 °C	
A2754	1 mM stock solutions stored at - 20 °C	
A2252	1 mM stock solutions stored at - 20 °C	
A7007	1 mM stock solutions stored at - 20 °C	
11934511001	1 mM stock solutions stored at - 20 °C	
C1506	1 mM stock solutions stored at - 20 °C	
G8877	1 mM stock solutions stored at - 20 °C	
MO-G008	Blue/Red Channel	MST device with standard sensor, Mon
MO-K002		
30124537		

olith NT115 pico is MST device with high sensitivity sensor

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Mapping the binding site of an aptamer on ATP using MicroScale Thermophoresis

Author(s): Clemens Entzian, Thomas Schubert

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

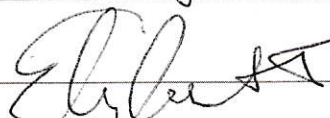
CORRESPONDING AUTHOR:

Name: Dr. Thomas Schubert

Department:

Institution: 2bind GmbH

Article Title: Mapping the binding site of an aptamer on ATP using MicroScale Thermophoresis

Signature: 

Date: 1. June 2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Dear Editor,

Dear Reviewers,

please find the line-by-line response letter in the following:

Editorial comments:
•NOTE: Please download this version of the Microsoft word document (File name: 55070_R2_070616) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

•Formatting:
-1.2 – Please use subscript numbers in chemical names. The spelling is corrected.
-Please define all abbreviations at first occurrence (ie SAM, etc.). The abbreviations are defined.
-Please format references using JoVE style. References are formatted in JoVE style.
-6.2 – "micro M" – Please use the Greek symbol Mu for micro, and do not spell out "micro". The spelling is corrected.
-Please include spaces between paragraphs. The spaces are included.
•Please copyedit the manuscript for numerous grammatical errors, some of which are indicated below. Such editing is required prior to acceptance and should be performed by a native English speaker.
-Line 23, Line 40– "This section" – This should be "manuscript" or "article" instead of "section". The wording is optimized.
-Line 29 – "these important information" The wording is optimized.
-Line 38 – "are obtained" The wording is corrected.
-Line 92 – "in MST movement profile" The wording is optimized.
-Line 113 – "However, this section describes a protocol, how MicroScale Thermophoresis can be used to characterize challenging interactions such as small molecule binding to an aptamer." – incorrect sentence structure The sentence is restructured.
-1.3 – Please correct the run-on sentence. The sentence is restructured.
-Please copyedit the manuscript for correct comma usage. Many commas are misplaced. The manuscript is copyedited.
-4.3 – "power are used" The wording is corrected.
-Please remove "Please" from all instructions and notes. This is not appropriate for a formal manuscript. Please is removed from all instructions and notes.
-6.6 – "A ntp" The sentence is restructured.
-7.5 – "data can be processes" The wording is corrected.
-Line 381 – "used by scientist" The sentence is restructured.
•Additional detail is required:
-3.2 – What should be done if precipitation occurs? The comment is excluded at that position of the manuscript. Information on precipitation / aggregation can be found in the discussion. Crosslinks from the protocol to the discussion are set.

-3.3 – Which part of the capillaries should be touched? The information is included in step 3.3.
-5.2 – What is done with the scan information? What is expected? A crosslink to the discussion is set.
-6.7 – Are the repeat measurements performed with the same capillaries? This aspect is clarified.
-7.3 – What is done if there are bumps and spikes? A crosslink to the discussion is set.
•Branding:
-Monolith – 3.4, Section 4 heading, Section 4 note, 4.1 (3 uses), 4.2, Discussion Monolith is excluded from the text.
-Section 4 note – Please remove the software versions, which should appear in the materials table only. The software versions are excluded.
-Line 261 – Kaleidagraph 4.5.2 (Synergy Software): The name of the software is excluded.
•Commercial language: Please use objective terms when describing the technology rather than using terms like "optimal" (Line 22), and "innovative" (Line 29, 47, 71). The wording is changed.
•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes. Figures/legends are marked

•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.
For two citations only a PMCID or PMID could be found. The remaining citations are supplemented with DOIs.

•IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In the manuscript „Mapping the binding site of an aptamer on ATP using MicroScale Thermophoresis", the authors provide a protocol for the characterization of aptamer-small molecule interaction studies using MicroScale Thermophoresis (MST). Moreover, they show how this technique can be used to map the binding site of the aptamer - ATP interaction, the adenine moiety and how MST can be used for sample quality control. The manuscript provides detailed and repeatable instructions on how to analyze the interaction between aptamers and small molecules, which is a challenging interaction for many other technologies, as stated by the authors. Despite an overall good data presentation and a mostly clear

explanation of the experimental procedures, there are several points that should be addressed. Especially the writing at times needs improvement, maybe the authors should have a pair of fresh eyes have a look on the manuscript.
Since most of my (quite numerous) recommendations are only minor corrections, I nevertheless recommend publication in the Journal of Visualized Experiments after these corrections are implemented.

Major Concerns:
N/A

Minor Concerns:
The authors state multiple times that MST is a novel technology. However, the technology is around for more than 7 years now and quite established - as stated by the authors when they refer to ~1000 publications using MST. The wording should be changed accordingly. The wording is optimized.
-In line 47 the abbreviation for MicroScale Thermophoresis is established as MST (except of the abstract). However, the following text is a mixture of both - MicroScale Thermophoresis (line: 50, 71, 82,…, 367, 373…), and MST (line: 53, 92, …, 372, 374…). MicroScale Thermophoresis should be replaced by MST throughout the remainder of the manuscript. MicroScale Thermophoresis was replaced by MST in the text.
-In line 51 - 53 the word "assay" is used six times in one sentence. It would be better to use synonyms instead or to re-structure the sentence. The wording is optimized.
-The variables used in the equations (line 90 and 107) need to be explained either in the text or next to the equations itself. The variables are defined in the text.
-In line 93-95 the authors explain, that "The initial fluorescence is measured in the first 5 sec in absence of the temperature gradient to ensure homogeneity of the sample". In fact, one cannot see whether the molecules in the sample are homogeneously distributed in the first 5 sec of the measurement. The 5 sec are more important to check for fluorescence changes due to excitation, such as photobleaching or photoenhancement. It is also required to determine the precise fluorescence intensity prior to laser activation. This aspect is corrected.
In line 113, - the word "however" makes no sense here. “However” is excluded.
-In line 156-157, how does one "make sure that no precipitation occurs"? Is it meant to check for precipitation, and if it occurs, to take measures to prevent it? What would a strategy for preventing precipitation be? The comment is excluded at that position of the manuscript. Information on precipitation / aggregation can be found in the discussion.
-In line 169 (and in general): Why don't the authors use the newest analysis software? To my knowledge, there is an "affinity analysis" software with more functions available for two years now. Describing the use of the old software would make an outdated protocol. The new affinity analysis software is now described.
-In line 188, "observe" should be replaced by "inspect" or similar. Wording is changed.
-In line 192 - 194 it would be helpful to include the troubleshooting strategies mentioned in figure legend 3 in case users observe sticking at this point during the experiment. Also, the aforementioned affinity analysis software helps by providing a "cap shape overlay", which is very helpful in identifying adsorption effects. This should be mentioned. A crosslink to the discussion is set here. The capillary shape is integrated in the text.
-In line 218: "Repeat the measurement". Do the authors mean "repeat sample preparation and measurement"? Measuring the same sample multiple times does not yield information about the reproducibility of the experiment, but just about reproducibility of the MST signals… of identical samples. This point is clarified.
-In line 233-235, the authors list the different analysis settings. They however do not explain which settings should be used. A good general recommendation would be to analyze the

earliest timepoints at the lowest MST power that result in a sufficient binding signal. At step 6.5. a recommendation is included.
- In line 237 and onward: I am confused as to why the Hill equation was used for fitting? Is this a multivalent interaction? It is assumed that two ATP molecules bind to the G-quadruplex DNA structure. It is not known, if the two binding sites possess the same binding affinity. As a consequence of this the Hill equation was used for curve fitting. A short paragraph on curve fitting is included in the discussion.
-In line 246: Do the authors refer to MS word? Maybe the correct abbreviations (.docx, .pdf etc) should be used. The correct abbreviations are now included.
-The term "technical repeats" should be explained. This term is now explained
-General comment on affinity data: what does the +/- values represent? Is this the standard deviation from multiple experiments? The explanation is now included in the results part.
-In line 307 it is mentioned that also biological repeats were done but data are not shown. However, those data might be more interesting than technical repeats as it is an experimental publication. The biological repeats are shown in the figures.
-In line 314 one could mention the type of capillary that might be useful in case one observe sticking of the molecules. Capillary types are now mentioned in the discussion.
-In line 317, "aggregations" should be changed to "aggregates". "Reaction" should be changed to "experiment". The wording is optimized. Also, aggregates can be detected in any MST trace presentation, not only in the normalized traces. This aspect is corrected
-In line 319: "exclude" should be changed to "remove" The wording is optimized.
-In line 327, can the authors provide references for their claims that aggregation events remain undetected by most other technologies? This text part is restructured, highlighting that MST is offering rapid and easy detection of both - unspecific adsorption and aggregation effects.
-In line 337: There must be a better word for "telling". The sentence is restructured.
-In line 351: Should be "By testing the fluorescence intensities under denaturing conditions, binding-induced fluorescence changes can be discriminated from unspecific fluorescence loss" or similar. The sentence is restructured.
-In line 361 - 363 it is written twice, that the "buffers have to be kept constant". So the sentence in line 363 "Buffers have to be kept constant within an experiment" can be left out. The sentence is restructured.
-In line 302 the Fnorm unit has a double "o". The unit is corrected.
-In line 368 - 369 and in line 380 it is written, that MST can be performed in any bio-liquid, lysate etc. There is no necessity to mention it twice in the same paragraph. The wording is optimized.
-In line 380-382: I wonder what the outlook about bacteria and cells adds to the protocol, especially since MST is known to be best suited for smaller particles ("from viruses to ions"; http://www.nanotemper-technologies.com/technologies/mst-technology/)
The outlook is excluded from the text. Nevertheless, expanding the application range of MST towards “from cells to ions” would be highly interesting. MST would more or less become a universal tool. But of course, this is currently just a dream, due to many issues that arise with cells in these assays.

Additional Comments to Authors:
The authors should check whether their "conflict of interest" statements are correct.
Conflict of interest is updated.

Reviewer #2:
Manuscript Summary:
In their manuscript Entzian and Schubert describe a new MicroScale Thermophoresis (MST) protocol to characterize aptamer - small molecule interaction. More precisely, using the

model-interaction between the DH25.42 DNA-aptamer and ATP the authors provided a MST protocol to characterize aptamer-ATP interactions. This study demonstrated that MST was a sensitive method and could be used to map down the binding site of the DNA-aptamer on the adenine of ATP.
Overall, the manuscript sheds a light on a useful new MST method to determine interactions between macromolecules and various ligands mostly small molecules and the authors also provided all the necessary know-how about the technical details for carrying out the experiment in this particular case. Some small revisions are only necessary before publication

Major Concerns:
N/A

Minor Concerns:
1. The authors should expand the critical comparison of their results with results available in the literature (e.g. ref 10). They could use even other studies that are taking advantage of alternative binding methods on this system. A short comparison is included in the results part.
2. The authors need to include all relevant current reviews dealing with MST methodology and outline how their manuscript brings novelty to the methodologies presented in these reviews. More citations are included
3. Binding of small molecules that mimic ATP to the ATP binding sites on various proteins has been investigated before with MST, for example for DNA Gyrase (J Med Chem. 2012 Jul 26;55(14):6413-26) and human DNA topoisomerase IIalpha (Bioorg Med Chem. 2015, 23(15):4218-29). Could the authors compare their protocol with some of protocols used herein? The studies are mentioned in the results.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
This manuscript describes how microscale thermophoresis can be used to characterize aptamer-small molecule interactions. Specifically, different ATP derivatives and related molecules were used to map the binding site of DH25.42 DNA aptamer on the ATP molecule. The work submitted is based on a paper the authors published in Methods in 2015.
MST is a relatively new but powerful technique used to measure binding affinity between biomolecules and has number of advantages over other more traditional approaches. The MST methodology described here is not novel but it is relatively well explained and easy to follow. The manuscript lacks detail at times, especially in the experimental procedure (see comments below).

Major Concerns:
1. An important step in the set-up is to always spin down the stocks of labeled or unlabeled molecules for 5min at 13,000 rpm to remove big aggregates, which is one of the main sources for noise. This step should be included in the protocol as it is only mentioned in the discussion. This step is now included. However, for this specific study centrifugation was not necessary.
2. In step 2.1, emphasize that the importance of using micro reaction tubes with low binding (or low volume microwell plates). This step is now included. However, for this specific study centrifugation was not necessary.
3. The capillaries only need 4 µL of sample, not 6 as stated in step 3.1, line 153. Air should be

left at both ends of the capillary. The aspect is corrected.
4. For step 3.3, add a note stating that incubation can be done for longer if required but 5 min should suffice for most instances. This information is now included.
5. In step 4.3, mention the optimal fluorescence signal range you need to run the experiment. Also make a comment on what you should do if the levels are outside the recommended range. This information is now included.
6. If having issues with non-specific sticking to glass surfaces in step 5.2, mention that you can test different types of covalently coated capillaries e.g. hydrophilic/hydrophobic. A crosslink to discussion is set here.
7. In step 6.2, a tube with no ligand should be included as a control. In the authors´ opinion including this control is not essential.
8. In step 6.5, mention that you can run the experiment using different IR laser powers to find optimum temperature gradient for system you are using. This information is now included.
9. Based on the fluorescence signal intensity you can adjust LED power to get to the correct range. This information is included in step 4.3
10. Mention that the analysis software contains a concentration finder function that can be used to determine the optimal concentration range of the ligand. The concentration finder is now described in step 2.2.
11. In the analysis software you should fix the labelled protein concentration before fitting curve. In step 6.3 the conc. of the fluorescent molecule is fixed. In the later analysis this fixed conc. will reappear. Please note, that this specific study uses the Hill equation which is anyway independent of the concentration of the labelled molecule.
12. In step 6.7, mention that you can average the three repeats to get a standard deviation before fitting the data. We decided to exchange the "older" version of the analysis software by the newest one, which allows to average the repeats and get a standard deviation automatically. See Step 7.12
13. Formatting of the references in the reference list is not consistent. Formatting is updated.

Minor Concerns:
14. In line 97, change 'is following' to 'follows'. The wording is optimized
15. In line 113, remove 'however'. Also add 'of' between 'protocol' and 'how'. The wording is optimized
16. In line 126, the '2' in MgCl2 should be in subscript. The wording is optimized
17. In line 161, remove 'may falsify results'. Change it to 'optical measurement will be taken from this position'. The wording is optimized
18. In step 7.5, line 238, change 'processes' to 'processed'. The wording is optimized
19. In step 7.5, you should enter the concentration of fluorescent molecule before fitting data. In step 6.3 the conc. of the fluorescent molecule is fixed. In the later analysis this fixed conc. will reappear. Please note, that this specific study uses the Hill equation which is anyway independent of the concentration of the labelled molecule.
20. In line 85, change 'tryptophanes' to 'tryptophans'. The spelling is corrected
21. In lines 275 and 278, change 'purin' to 'purine'. The spelling is corrected
22. In line 275, change 'pyrimidin' to 'pyrimidine'. The spelling is corrected
23. In line 304, 'hill' should be in capital. The spelling is corrected
24. 'State-of-the-art' is written 3 times in the last paragraph. The wording is optimized
25. The K in KD, kon and koff should be italicised. The k in kon and koff should not be in capital as they are rate constants. The spelling is corrected
26. Use micromolar as the unit for concentration in all the axes for the panels in Figure 2. The axes are changed to µM.
27. Check position of commas throughout manuscript. Commas are checked.

Additional Comments to Authors:
N/A