**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