DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

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August 24, 2020

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Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Subject: Submission of revisions of JoVE61784

Dear Dr. Bajaj,

Thank you for considering our manuscript and for the reviewer's comments. The comments were helpful and showed ways to improve the paper.

We have provided a response to each of the points raised by the reviewers and the editor, and we detailed the changes made in the revised version. We hope that this version will be acceptable for publication in JoVE.

Thank you very much for your consideration.

Sincerely,

Grzegorz P. Piszczek -S

Digitally signed by Grzegorz P. Piszczek -S
Date: 2020.08.24 20:20:32 -04'00'

Grzegorz Piszczek, Ph.D.
Director, Biophysics Core Facility
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EDITORIAL COMMENTS:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

The manuscript was carefully proofread.

• Protocol Language:
1) Split up long steps into 2-3 steps (e.g., 4.2)

Protocol step 4.2 and other long steps were divided into smaller parts.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc.) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:
1) 2.1: Which buffer? Which antibodies and antigens? Please list them in the table of materials with RRIDs and concentrations.

Protocol steps describing software operations were revised and all necessary details have been provided. Additional experimental details were included when applicable.
Regarding the antibody and antigen sample: This protocol describes a procedure useful for the characterization of any antigen-antibody interaction, and for this reason we would like to use the generic "antigen" and "antibody" terms. The particular antigen-antibody system used to generate the representative results is listed in the Materials table. We would like to avoid confusing readers by suggesting that this protocol works only for a particular antigen or a particular antibody.

• Protocol Numbering: All steps should be lined up at the left margin with no indentations.

The indentation has been corrected.

• Protocol Highlight:
1) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
2) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
3) Notes cannot be filmed and should be excluded from highlighting.
4) Highlight a total of < ~2.75 pages including line spaces.

Protocol highlighting has been revised. Several additional critical points were included, and the highlighting of notes was removed. The size of the highlights is within the acceptable limits.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The Discussion has been revised to make sure that all required points are included. Point one, modifications and troubleshooting, is described in paragraphs five and three of the Discussion, respectively. Point two is covered in the first paragraph and point three in the second paragraph. Future applications are described in the fifth paragraph and the critical steps in the third paragraph.

• References: Please spell out journal names.

Full journal names were provided.

• Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Mili-Q, Milli-Q, DiscoverMP, Origin, Microsoft Office Excel, etc 1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

All names of commercial products were removed throughout the body of the manuscript.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All figures and tables in the manuscript are original.

COMMENTS FROM PEER-REVIEWERS:

Reviewer #1:

Manuscript Summary:

Mass photometry (MP) is a recent invention for label free determination of the antibody-antigen affinity. Different from SPR, the most common label free optical technique for studying binding reactions, MP is a label- and immobilization-free technique that detects and quantifies molecular weights and populations of antibodies and antigen-antibody complexes on a single-molecule level. MP analyzes the antigen-antibody sample within minutes.

We would like to thank the reviewer for their time and comments. We think that their suggestions improved the clarity of the protocol and make it easier to follow by the readers. We have addressed each individual point below.

Major Concerns:

1. The manuscript needs to include the fundamental principles of MP instead all the claimed benefits. This is very important to connect to the protocols described in this work as well as the data processing equations used in data analysis software .

We agree that since the MP is a new method, an expanded description of its fundamental principles will be adequate and useful for the readers. The introduction has been expanded accordingly, and now includes description of the basic principles of MP with a particular focus on the explanation of the MP signal generation and its interpretation. The connection between the MP experimental data and the calculations of the equilibrium constants was included in the same paragraph. The formalism used in the calculations is now explained in the introduction, starting with the principles of the mass action law and affinity constants definitions. All derivation steps starting from basic definition and leading to the equations used in fitting the experimental data are provided in the introduction and in the supplementary information. We also included additional references for readers interested in the technical details of the MP optical system.

2. What kinds of factors affect the accuracy of the measurements and the global drift for MP techniques to determine the antibody-antigen binding? What are the differences of MP technique compared to those early mass based methods including SPR and QCM. What are the best biological binding systems that MP technique is most suitable for?

We agree with the reviewer that the direct comparison of the MP with a well-established method such as the SPR will help with understanding strengths and weaknesses of this new technique. Accordingly, a discussion section paragraph has been added to describe differences and similarities of the MP and the SPR. We did not include the QCM in this comparison to keep the discussion concise and relevant for the largest possible group of readers. Although it has been shown that QCM coupled biosensors can detect antibody binding, to the best of our knowledge, this method is not routinely used to obtain binding affinities for protein-protein interactions.

The most important factors affecting the MP accuracy (protein purity, particulate-free buffer and the flow cell preparation) are listed in the protocol and again in the discussion.

The best biological system for MP studies is antigen-antibody binding. This is related to three important factors - MP is not able to detect proteins with small molecular mass, and antibodies molecular mass of 150 kDa fits perfectly into the MP mass detection range. Antigen-antibody binding affinity is usually strong, typically in the nanomolar range, and without special modifications the MP can directly measure sub-micromolar affinities. And lastly, antibodies have two antigen binding sites, and MP is particularly suited for the studies of multivalent interactions. For all those reasons the antigen-antibody interactions are the sole focus of this protocol. All of the limitations of the MP technique mentioned above are also listed in the manuscript.

3. The protocols need to have a brief thesis statement for the criteria for each procedure that ensure the most reliable and accurate measurements. The concentration (lower and upper limits) required for antibody and antigen needs to be provided.

We thank the reviewer for pointing out that providing simplified directions for the concentration selection would make the protocol easier to follow. Point 2.3 of the protocol has been modified accordingly, and now includes a suggested antigen and antibody concentrations suitable for most systems, together with the acceptable concentration ranges and the criteria for potential concentration optimization. The procedure to assess the quality of the chamber preparation has also now been provided.

4. Protocol:

(1) 1.1.2 The requirement for the Coverslip optical quality needs to be discussed and explained

The coverslip testing procedure is described in the note to this protocol step (1.1.2), and the example images of sufficient and unacceptable optical glass quality are shown in Fig. 1 A-C. We thank the reviewer for pointing out that we did not clarify that the same testing procedure is used to identify the working side of the coverslips and to confirm the efficiency of cleaning. This is now explicitly stated in the protocol.

(2) 1.2 flow chamber picture needs to be provided.

We agree with the reviewer that this may be useful for understanding how the flow chamber is assembled. We now include the picture of the assembled chamber in Fig. 2.

(3) 2.1 why the buffer and protein solution needs to be filtered with 0.22 um syringe filters

We thank the reviewer for pointing out that filtration may not be feasible for all proteins. In the protocol step 2.1 we now explain the purpose of buffer filtration and provide an alternative method of protein stock preparation by the use of centrifugation.

Minor Concerns:
Implementation of the equilibrium constants fitting procedure in Excel: The basis for the fitting procedure needs to be discussed to help readers to understand those equations used. Please also compare it with those used in SPR to help readers to understand this new MP technique.

All equation derivation steps are now provided in the introduction and in the supplementary information starting with the basic definitions. The comparison of SPR and MP techniques were also added in the discussion. We were not able, however, to include a direct comparison of formalisms used in the MP and the SPR data analysis. Calculations used by MP are based on equations defining the equilibrium conditions in the binding mixture. On the other hand, the principal technique of the SPR analysis is based on the measurements of the reaction rates. In a typical SPR experiment, the equilibrium constants are calculated from the ratios of the on- and off-rates of binding. This fundamental difference is now explained in the new paragraph comparing both techniques.

Reviewer #2:
Manuscript Summary:
In the manuscript Wu and Piszczek describe a protocol for determining the affinity of a purified antigen to a bivalent antibody using mass photometry. The protocol is supported by a description of how to fit three Gaussians to the obtained histogram, and an Excel sheet to find the dissociation constants.

We would like to thank the reviewer for careful reading of the manuscript and for their comments. We think that their suggestions will make the protocol easier to follow for the readers. We have addressed each individual point below.

Major Concerns:
None.

Minor Concerns:
1. Mass photometry makes the determination of the concentration of antibodies with singly- and doubly-occupied binding sites possible. While antibodies with one and two bound antigens are incorporated into the formalism, no mention is made of the potential interaction, i.e. cooperativity, between the binding sites. The theory of successive binding of two ligands, i.e. the difference between microscopic and macroscopic (apparent) dissociation constants, is described in e.g.
http://www.pdg.cnb.uam.es/cursos/BioInfo2002/pages/Farmac/Comput_Lab/LecF00/Lec12/LigBind.pdf

or in the following book: Introduction to Macromolecular Binding Equilibria, 4.3.1. Statistical Effects in Multisite Binding (author: C. P. Woodbury, CRC Press, 2008)
According to these principles, if there is no cooperativity between the two binding sites, the apparent dissociation constant of the second binding site should be 4-times larger than that of the first one. If Kd2<4*Kd1, then positive cooperativity is present, if Kd2>4*Kd1, negative cooperativity is present. The described technology is perfectly suitable for revealing such cooperativity.

We agree with the reviewer that one of the advantages of MP is its ability to characterize multivalent binding, which includes an estimation of cooperativity. In our original research paper (reference 11 in the manuscript), we included the analysis the reviewer recommends and stated that the MP results for the antigen-antibody pair studied here suggest a positive cooperativity between the two binding sites. In the interest of keeping the protocol relatively simple and concise, we initially omitted the discussion of the relation between the micro- and macroscopic binding constants. We agree however, that this might be of interest to some readers, and we now included it in the introduction.

2. Regarding the Excel sheet:

- It would be useful to highlight those cells in the Excel sheet, which need to be modified by the user. In addition, it would also be helpful to protect other cells in order to prevent accidentally overwriting formulas.

We thank the reviewers for those suggestions. The Excel worksheet was reorganized to combine all fields that have to be edited and they are now highlighted in yellow. Unfortunately, in the Excel versions we tested, the Solver function will not run in a protected worksheet, and consequently we had to leave the spreadsheet fields unprotected.

- As far as I know, Solver is not enabled in Excel by default. A short description of how to enable it would be useful for less tech-savvy users

We thank the reviewer for this excellent suggestion. We now included a Note after step 5 of the protocol describing the steps required to enable the Excel Solver.

- Ka2 is the reciprocal of Kd1, and Ka1 is the reciprocal of Kd2 (cells B4, B5).

Thank you very much for pointing this out. The correction has been made.

3. Regarding the reliability of the estimation:

- A short description of how to obtain the confidence intervals in Excel is required.

We agree that many readers would appreciate a detailed explanation of this procedure. Fortunately, the reference 14 in the protocol (Kemmer and Keller, Nature Protocols 2010) contains a thorough, comprehensive and easy to follow description of the confidence intervals calculation procedure in Excel. In the interest of the manuscript brevity we did not include the

full description in the protocol, but a new sentence was now added, directing interested readers to this excellent reference.

- While the confidence interval of the estimated parameters is available in the manuscript, the authors should also provide some information about the reproducibility of the mass photometric measurement itself, e.g. by showing error bars in Fig. 4.

We thank the reviewer for this suggestion. The error bars based on the standard deviations of the Gaussian fits are now included in Fig. 4. To help readers further assess the reproducibility of the MP measurements, we also included an additional figure (Fig. 5) showing replicates of the MP molecular mass distributions measurements.

Reviewer #3:

Minor Concerns:

in the discussion include a pro/con comparison with spr-based binding assays which are considered gold standard

We thank the reviewer for their time and the suggestion. We included a new paragraph in the discussion section describing pros and cons of both techniques.