Journal of Visualized Experiments

Rapid Determination of Antibody-Antigen Affinity by Mass Photometry

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61784R1
Full Title:	Rapid Determination of Antibody-Antigen Affinity by Mass Photometry
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Additional Information:	
Question	**Response**
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Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Bethesda, MD, USA
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Please specify the section of the submitted manuscript.	Biochemistry
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Cover Letter

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June 24, 2020

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Benjamin Werth,
Sr. Science Editor - Chemistry | Biochemistry
JoVE

Dear Benjamin,

I would like to submit the manuscript “Rapid Determination of Antibody-Antigen Affinity by Mass Photometry”, by Di Wu and myself, for consideration in JoVE.

It describes the application of a recently developed single molecule technique, mass photometry, for antigen-antibody affinity measurements. Mass photometry is able to very quickly measure interaction affinities in solution using only a small amount of unmodified sample. We believe that the advantages of this new method, primarily its sensitivity, speed and accuracy, will make it extremely useful for the characterization of antibody binding properties. The manuscript describes both the experimental and data analysis procedures. Since in the case of antigens binding to two independent binding sites the data analysis will require fitting a cubic equation, we included a non-linear least squares fitting tool that we developed in Excel with the manuscript. We believe that this tool will be useful to JoVE readers who would like to apply this technique but lack access to a more sophisticated data analysis software.

As unbiased reviewers, none of whom have previously seen this manuscript, we suggest Dr. Sandro Keller, Dr. Joy Zhao, and Dr. Wojciech Kuban, who are all extremely experienced and recognized leaders in the field of biophysical interaction measurements.

To avoid a potential conflict of interest, we would prefer if the manuscript not be sent to colleagues associated with the mass photometer manufacturer, Refeyn, including Dr. Philipp Kukura.

Thank you very much for your consideration, and for the invitation to submit this manuscript,

Sincerely

Grzegorz P. Piszczek -S

Digitally signed by Grzegorz P. Piszczek -S
Date: 2020.06.24 19:51:58 -04'00'

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TITLE:

Rapid Determination of Antibody-Antigen Affinity by Mass Photometry

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KEYWORDS:

mass photometry, antibody affinity, protein-protein interactions, label-free, binding affinity measurements, multivalent protein interactions

SUMMARY:

We describe a single-molecule approach to antigen-antibody affinity measurements using mass photometry (MP). The MP-based protocol is fast, accurate, uses a very small amount of material, and does not require protein modification.

ABSTRACT:

Measurements of the specificity and affinity of antigen-antibody interactions are critically important for medical and research applications. In this protocol, we describe the implementation of a new single-molecule technique, mass photometry (MP), for this purpose. MP is a label- and immobilization-free technique that detects and quantifies molecular masses and populations of antibodies and antigen-antibody complexes on a single-molecule level. MP analyzes the antigen-antibody sample within minutes, allowing for the precise determination of the binding affinity and simultaneously providing information on the stoichiometry and the oligomeric state of the proteins. This is a simple and straightforward technique that requires only picomole quantities of protein and no expensive consumables. The same procedure can be used to study protein-protein binding for proteins with a molecular mass larger than 50 kDa. For multivalent protein interactions, the affinities of multiple binding sites can be obtained in a single measurement. However, the single-molecule mode of measurement and the lack of labeling imposes some experimental limitations. This method gives the best results when applied to measurements of sub-micromolar interaction affinities, antigens with a molecular mass of 20 kDa or larger, and relatively pure protein samples. We also describe the procedure for performing the required fitting and calculation steps using basic data analysis software.

INTRODUCTION:

Antibodies have become ubiquitous tools of molecular biology and are used extensively in both medical and research applications. In medicine, they are critically important in diagnostics, but their therapeutic applications are also expanding and new antibody-based therapies are constantly being developed[1-4]. The scientific applications of antibodies include many indispensable laboratory techniques such as immunofluorescence[5], immunoprecipitation[6], flow cytometry[7], ELISA, and western blotting. For each of these applications, obtaining accurate measurements of the antibody's binding properties, including binding affinity and specificity, is of crucial importance.

Since the first commercial surface plasmon resonance (SPR) instrument was introduced in 1990, optical biosensors have become the "gold standard" of antibody characterization, but other techniques, including ELISA, are also routinely used to measure antibody affinities[8,9]. These methods usually require immobilization or labeling of the analyzed molecules, which can potentially affect the interaction of interest. They are also relatively slow, involving multiple assay steps before the results can be collected for data analysis. A recently developed single-molecule method, mass photometry (MP), detects molecules directly in solution when they land on the surface of the microscope coverslip[10,11]. The light scattering-based optical detection that MP employs does not require protein labeling or modification. Individual protein molecules are recorded by the interferometric scattering microscope as dark spots appearing in the image (**Figure 1D**), and several thousand molecules can be detected during the one minute data acquisition[12]. The signal generated by each individual particle is quantified, and its contrast value (relative darkness) is calculated. The interferometric contrast values are proportional to the molecular masses of the proteins, which allows for the identification of bound and free species in the antigen-antibody mixture. At the same time, by counting molecular landing events, MP directly measures the species populations. This gives MP based methods a unique capability to independently quantify affinities of multiple binding sites.

Binding of the antigen (*Ag*) molecules to the two binding sites of the intact antibody (*Ab*) can be described as:

$$Ab + Ag \overset{K_{a1}}{\longleftrightarrow} Ab \cdot Ag$$
$$Ab \cdot Ag + Ag \overset{K_{a2}}{\longleftrightarrow} Ab \cdot Ag_2$$

with the equilibrium association constants K_{a1} and K_{a2} defined as:

$$K_{a1} = \frac{c_{Ab\cdot Ag}}{c_{Ab}\times c_{Ag}} = \frac{f_{Ab\cdot Ag}}{f_{Ab}\times c_{Ag}}, \quad K_{a2} = \frac{c_{Ab\cdot Ag_2}}{c_{Ab\cdot Ag}\times c_{Ag}} = \frac{f_{Ab\cdot Ag_2}}{f_{Ab\cdot Ag}\times c_{Ag}} \qquad (1)$$

`

where c_i and f_i represent concentration and fraction of the component *i*, respectively. The total antigen concentration $(c_{Ag})_{tot}$ can be expressed as:

$$\left(c_{Ag}\right)_{tot} = c_{Ag} + c_{Ab\cdot Ag} + 2c_{Ab\cdot Ag_2} = c_{Ag} + \frac{f_{Ab\cdot Ag}\times(c_{Ab})_{tot}}{f_{Ab}+f_{Ab\cdot Ag}+f_{Ab\cdot Ag_2}} + \frac{2f_{Ab\cdot Ag_2}\times(c_{Ab})_{tot}}{f_{Ab}+f_{Ab\cdot Ag}+f_{Ab\cdot Ag_2}} \qquad (2)$$

Since the total concentrations of the antibody $(c_{Ab})_{tot}$ and antigen $(c_{Ag})_{tot}$ are known, this equation can be used to directly fit the experimental component fractions obtained from the MP measurements and calculate the equilibrium association constants K_{a1} and K_{a2} (see **Supplementary Information**).

The MP data can also be used to estimate cooperativity between the two antibody binding sites[11]. For two antibody paratopes with identical microscopic binding constants, the statistical factors describing the process of population of the $Ab{\cdot}Ag$ and $Ab{\cdot}Ag_2$ complexes dictate that the apparent macroscopic equilibrium constants K_{a1} and K_{a2} will not be numerically equal, and $K_{a1} = 4K_{a2}$. Therefore, the experimental values of $K_{a1} < 4K_{a2}$ indicate positive cooperativity between the two antibody binding sites. Similarly, $K_{a1} > 4K_{a2}$ indicates negative cooperativity.

MP measurements of the antigen-antibody binding affinity are fast and require a small amount of material. The MP mass distributions used for equilibrium constant calculations provide additional information about the sample properties and enable the assessment of the sample purity, oligomerization, and aggregation in a single experiment. The same method can be used to measure high affinity protein-protein binding, and MP is particularly useful for studies of multi-valent protein interactions. Multi-protein complexes usually have large molecular masses, optimal for MP detection, and single-molecule data can be used to measure stoichiometry and calculate affinities of multiple binding sites simultaneously. This information is usually difficult to obtain using bulk-based methods.

Without modifications, the current protocol is suitable for measurements of relatively high-affinity, sub-micromolar interactions with antigens of a molecular mass of 20 kDa or larger. For optimal results, protein stocks should be of high purity, but there are no specific buffer requirements. By using MP, the antigen-antibody binding can be assessed in less than five minutes. The data collection and analysis required for accurate K_d calculations can be performed within 30 minutes.

PROTOCOL:

1. Prepare the flow chambers

1.1. Clean the glass coverslips

1.1.1. Using wash bottles with distilled water, ethanol, and isopropanol, rinse the 24 mm x 50 mm coverslips in the following order: water, ethanol, water, isopropanol, water. Dry the coverslips with a stream of clean nitrogen. It is important to rinse the coverslips from top to bottom, holding the bottom corner with soft-tipped forceps. Dry the coverslip in the same direction to avoid transferring contamination from the forceps (**Figure 2A**).

1.1.2. Similarly, rinse the 24 mm x 24 mm coverslips with distilled water, ethanol, and distilled water. Dry the coverslips with a stream of clean nitrogen.

1.1.3. Identify the working side of the coverslip, place a drop of distilled water on the surface of the clean coverslip and follow steps 3.1–3.2 of the protocol. Usually only one side of the 24 mm x 50 mm coverslip has the optical quality suitable for MP measurements.

NOTE: After focusing, no significant surface imperfections should be detectable, and the "signal" value shown in the data collection software should be less than 0.5% (**Figure 1A-C**). The working sides of all coverslips in the box are oriented in the same direction. The same procedure should be used to test the efficiency of the coverslip cleaning.

1.2. Assemble the flow chamber

1.2.1 Position the 24 mm x 24 mm coverslip on a piece of aluminum foil. Place strips of double-sided tape on top of the 24 mm x 24 mm coverslip as shown in **Figure 2B** and cut the tape along the edge of the glass. Separate the coverslip from the aluminum foil and attach it to the working side of the 24 mm x 50 mm coverslip (**Figure 2C**).

NOTE: Channel size can vary, but a width of 3 mm–5 mm is recommended. Wider channels require larger sample volumes and very narrow channels may be difficult to load. Usually, two parallel channels can easily be created on the 24 mm x 24 mm coverslip. Protocol can be paused here.

2. Prepare the antibody-antigen samples for the affinity measurements

2.1. Filter at least 2 mL of the PBS buffer using 0.22 µm syringe filters to remove dust particles or aggregates. Centrifuge the protein stock for 10 minutes at the maximum speed of the tabletop centrifuge (approximately 16,000 x *g*).

NOTE: PBS is the recommended buffer for this protocol, but MP has no particular buffer requirements, and other biological buffers are also acceptable. However, high glycerol concentrations (>10%) and very low ionic strengths (salt concentration <10 mM) may affect the image and data quality and are not recommended.

2.2. Determine the actual concentrations of the antibody and antigen stocks by measuring their 280 nm UV absorbance.

2.3. Calculate the measurement concentrations of the antigen-antibody mixture. If the estimated value of the antibody binding affinity is not known, plan to prepare a sample with 30 nM antigen and 20 nM antibody concentration. When the approximate affinity is known, the antibody to antigen ratio and their concentrations should be optimized according to the expected K_d values. Use the total antigen concentration in the mixture equal to the sum of the expected K_d and the total antibody concentration in the equation below. Assuming $K_{d1} = K_{d2}$ for the two paratopes of the antibody, this will result in comparable concentrations of the free antibody and the antibody-antigen complexes in the sample.

$$\left(c_{Ag}\right)_{tot} = (c_{Ab})_{tot} + K_d$$

2.3.1. Adjust the antibody concentration to keep the total protein concentration in the sample within the 10 nM and 50 nM range. Best results are obtained using mixtures with antibody concentrations between 5 nM and 25 nM.

NOTE: MP detects proteins with a molecular mass larger than 50 kDa. Consequently, sample concentrations of antigens with a molecular mass smaller than 50 kDa can exceed the typical 50 nM limit. However, at concentrations higher than approximately 100 nM, even low molecular mass antigens might affect the image quality and accuracy of the K_d determination.

2.4. Prepare 50 µL of the antibody-antigen mixture at its final measurement concentration calculated in step 2.3.

NOTE: Only one sample of the antigen-antibody mixture is required for the K_d determination. However, preparing several samples with different antigen to antibody ratios can help optimize sample concentration. If data from several samples are collected, they can be analyzed via a global fit.

2.5. Incubate the antigen-antibody mixture(s) for approximately 10 min at room temperature to allow the binding reaction to reach chemical equilibrium. Avoid unnecessarily long incubation times.

NOTE: Incubation time may vary depending on the binding kinetics. To confirm that the chemical equilibrium has been reached, sample measurements can be repeated at different incubation times. Time-invariant K_d values indicate sufficiently long incubation. Prolonged incubation may lead to significant protein adsorption to the surface of the plastic labware and, consequently, to significant errors in the protein concentration determination. For this reason, low-adhesion labware is strongly recommended for MP sample preparation[13].

3. Collect the Mass Photometry data

3.1. Apply a drop of microscope immersion oil on the MP instrument objective and place the assembled flow chamber on the microscope stage. Make sure the oil spans the gap between the coverslip and the objective.

3.2. Load the flow chamber and focus the mass photometer.

3.2.1. Deposit 10 µL of a clean, filtered buffer solution at one end of the flow chamber channel prepared in step 1. Liquid will enter the channel by capillary action.

3.2.2. Adjust the stage's Z-position to focus the microscope on the working surface of the 24 x 50 mm coverslip.

3.2.2.1 In the **Focus Control** tab of the data collection software, use the coarse stage movement **Up** and **Down** buttons to make the initial adjustments.

3.2.2.2 Click the **Sharpness** button to show the sharpness signal readout and use the fine **Up** and **Down** adjustment buttons to maximize the **Sharpness** value.

3.2.2.3 Click the **Set Focus** and **Lock Focus** buttons to activate the focus tracking function. A properly focused image (**Figure 1A,C**) should have the "signal" value below 0.5%.

NOTE: If the "signal" value at the maximum sharpness position is above 0.5%, this may indicate impurities on the glass surface or in the buffer.

3.3. Using the same channel, load 20 µL of the antibody-antigen sample by depositing it on one side of the channel and blotting the liquid from the other end with a small piece of blotting paper (**Figure 2D**).

NOTE: The volume of a 3–5 mm wide channel is approximately 10 µL. The additional sample volume is recommended to completely replace the buffer present in the channel and to avoid sample dilution.

3.4. After loading the sample, immediately click the **Record** button to start data collection, acquiring a 100 s video (**Figure 1D**).

3.5. At the end of the data collection enter the file name and click **OK** to save the data file.

3.6. Discard the coverslips and wipe the oil from the objective lens with cotton optical swabs wet with isopropanol.

NOTE: The protocol can be paused here.

4. Analyze the MP data

4.1. Process the collected video file using the MP data processing software to identify the landing events.

4.1.1. Use the **File/Open** menu option to load the file for the analysis and click **Analyze**.

4.1.2. Click the **Load** button to load the calibration function and save the analyzed data using the **File/Save Results As** menu option.

4.2. Fit the molecular mass distribution with Gaussian functions to obtain relative concentrations of each species in the sample. This analysis can be performed using a common scientific graphing software (see **Table of Materials**).

4.2.1. Import the "eventsFitted.csv" file into the software and plot the molecular mass distribution (column M in the .csv file) using the **Plot/Statistics/Histogram** function.

4.2.2. Double click on the histogram to open the **Plot Properties** window. Disable automatic binning and select a bin size of 2.5 kDa. Click the **Apply** and the **Go** buttons to create the **Bin Centers** and **Counts** data.

4.2.3. Select the **Bin Centers** and **Counts** columns and use the **Analysis/Peaks and Baseline/Multiple Peak Fit** menu function to fit the histogram with Gaussian functions. Double click to indicate the approximate peak positions on the distribution plot and then click the **Open NLFit** button.

4.2.4. Check the **Fixed** checkboxes for the "xc" peak centers and set their values to the expected molecular masses of the free antibody and the single and double antigen-antibody complexes. Check the **Share** option for the width parameters. Click the **Fit** button. The fitted peak height values of the Gaussian components represent the relative concentration of each species in the sample[11].

NOTE: Bin size may be adjusted to optimize the resolution of the mass distribution plot. The MP precision limit is approximately 1 kDa, and smaller bin sizes might amplify the noise of the distribution, while not revealing any additional information. Very large bin sizes will obscure the fine details of mass distributions.

4.3. Calculate the concentration fraction of each species using the following equation:

$$f_i = \frac{h_i}{h_{Ab}+h_{Ab\cdot Ag}+h_{Ab\cdot Ag_2}} \qquad (3)$$

where the h_i and f_i values represent peak heights and concentration fractions of the free antibody and the single- and double-bound antibody in the sample, respectively.

5. Calculate equilibrium constant values

5.1. Fit the concentration fractions of the interaction species calculated in step 4.3 with Eq. 1 and 2 using a suitable analytical software. Here we demonstrate a method to calculate equilibrium constants using a spreadsheet program[14] (see **Supplementary Information**).

5.1.1. Open the "Kd calculation.xlsx" worksheet. In this worksheet, the cell values in rows 1 to 10 highlighted in yellow can be modified to perform the equilibrium constants calculations.

5.1.2. Enter the estimated K_d values in nanomolar units into cells B1 and B2 in the table. Those starting values will be optimized in the fitting procedure. If the estimated K_d values are not known, leave the default values in cells B1 and B2 unchanged.

5.1.3. Enter the values of $(c_{Ab})_{tot}$ and $(c_{Ag})_{tot}$ in molar units into cells D2 and E2. Enter the fraction values calculated in step 4.3 into cells F2, G2, and H2. If multiple samples at different concentration ratios were measured, additional concentration values obtained for those samples can be entered in rows 2 to 10.

5.1.4. Select the **Data/Solver** menu function. Enter "$B$15" in the "Set Objective" box and "$B$1:$B$2" into the "By Changing Variable Cells:" box. Select the **Min** radio button for the **To:** option. Check the **Make Unconstrained Variables Non-Negative** checkbox and select **GRG Nonlinear** as the solving method. Click the **Solve** button. The best fit K_{d1} and K_{d2} values will be shown in cell B1 and B2 and the final sum of squared errors in cell B7.

NOTE: If the **Solver** function is not active, select **Options** under the **File** menu in the spreadsheet program. In the **Add-ins** category select the **Solver Add-in** under the **Inactive Application Add-ins** and click the **Go** button. Check the **Solver Add-in** checkbox and click **OK**.

[Place **Figure 1** here]

[Place **Figure 2** here]

REPRESENTATIVE RESULTS:

We have previously examined the interaction of human α-thrombin (HT) and mouse monoclonal anti-human thrombin antibody (AHT) using the MP based assay[11]. Since the molecular mass of the HT (37 kDa) is below the 50 kDa detection limit, the maximum sample concentration can exceed the 50 nM MP concentration limitation without negatively affecting the resolution of mass distributions. The experiment was planned as a titration series with the AHT antibody at a fixed 25 nM concentration, and the HT at concentrations of 7.5 nM, 15 nM, 30 nM, 60 nM and 120 nM. **Figure 3** shows the molecular mass distributions of the antigen-antibody mixtures and the antibody-only sample. Here we analyze the data using the method described in the protocol. A scientific graphing software was used to fit the mass distributions with three Gaussian components representing the free AHT, AHT·HT and AHT·HT_2. The known molecular mass values of the three components were fixed, and a single peak width parameter was fitted for the three species. Best-fit peak height parameters of the Gaussian components were normalized using Eq. 3 to obtain species concentration fractions (**Table 1**). Those values, together with the total antibody and antigen concentration for each sample were entered into the "Kd calculation.xlsx". The global fit in the spreadsheet yields K_{d1} = 40 nM (68.3% confidence interval: 28 nM, 68 nM) and K_{d2} = 28 nM (68.3% confidence interval: 17 nM, 45 nM). The experimental concentration fractions and the fit results are plotted in **Figure 4**. The dissociation constant values obtained here by fitting the integrated concentration fractions are in agreement with those obtained previously by directly fitting MP distributions, and with dissociation constant values obtained by Isothermal Titration Calorimetry (ITC)[11].

FIGURE AND TABLE LEGENDS:

Figure 1: Mass photometry images. (**A**) Representative native view image of the imaging buffer collected on a clean coverslip and (**B**) on a coverslip with surface imperfections. (**C**) Differential ratiometric image of the imaging buffer and (**D**) the AHT·HT solution.

Figure 2: MP flow chamber preparation and loading. (**A**) Coverslip holding position for the cleaning procedure. (**B**) Alignment of the 24 x 24 mm coverslip (middle layer) and the double-sided tape (top layer) on the surface of aluminum foil (bottom layer, not shown). Blue dashed lines show the location of cut lines. (**C**) Top and side view of the assembled flow chamber with two sample channels, and a picture of the assembled flow chamber. (**D**) Procedure for sample loading into a flow channel previously filled with buffer.

Figure 3: MP molecular mass distributions of the 25 nM AHT mixed with HT at 0, 7.5, 15, 30, 60 and 120 nM (A-F, respectively). Black dots show the experimental MP data plotted with 2.5 kDa bin size. Cyan, green, and blue lines represent the best-fit Gaussian distributions of the free antibody, single bound antibody, and double bound antibody species, respectively. Red lines show the sum of the three Gaussian components.

Figure 4: Fractions of the free AHT (blue), AHT·HT (red), and AHT·HT_2 (black) as a function of HT concentration. Points represent experimental values obtained from the Gaussian fitting of the MP distributions. Solid lines represent the best-fit using Eq. 1.

Figure 5: Technical replicates of the AHT molecular mass distribution measurements. Plots shows the reproducibility of the MP measurements and the purity of the antibody preparation.

Table 1: Normalized peak heights of Gaussian components obtained by fitting the MP distributions (**Figure 3**).

Supplementary Information: Implementation of the equilibrium constants fitting procedure in Excel and Affinity calculation worksheet.

DISCUSSION:

The Mass Photometry based protocol outlined here provides a fast and accurate method of measuring antigen-antibody binding affinities. MP analysis uses a very small amount of material, and additional information—including stoichiometry, oligomerization, and purity—can be assessed from the same data (**Figure. 5**). Without modifications, this method is applicable to the measurements of dissociation constants in the approximately 5 nM to 500 nM range, and for ligand molecules with molecular mass of approximately 20 kDa or larger. The same protocol can be used not only to analyze the antigen-antibody binding, but also to measure protein-protein interaction affinities when the molecular mass of at least one of the binding partners is larger than 50 kDa. Since the MP detection is non-specific, MP measurements can't be performed on samples containing a high concentration of carrier proteins or large molecular mass impurities.

SPR is commonly used for the characterization of the antigen-antibody binding, and direct comparison of both methods may help with the assay selection. In comparison with SPR, the MP

binding assay is faster and does not require protein immobilization. For a typical binding experiment, the MP requires less material than SPR. MP data reveal the stoichiometry of the complexes which is not readily available from the SPR[10,11]. Kinetic parameters of binding can be obtained from the MP data[13], but SPR measures the binding kinetics directly, and is able to measure a broader range of association and dissociation rates. The association constants of two separate binding sites can only be obtained from the SPR data when the association and dissociation rates of both sites are sufficiently different. On the other hand, the MP can precisely characterize molecular complexes with multiple binding sites[10,11]. SPR is able to measure binding affinity for small molecular mass ligands, and MP works best for ligands with molecular mass of approximately 20 kDa or larger.

Collecting good quality MP data is a critical step in obtaining accurate results from this protocol. Impurities in the buffer or on the surface of the coverslips will interfere with MP data collection. Improper focusing distorts the MP signal leading to errors in molecular mass estimates and equilibrium constants calculations. Both these factors should be examined when troubleshooting the protocol. An important consideration when working with low concentration protein solutions is that surface adsorption can lead to the loss of material and changes in the sample concentration. The resulting errors can be minimized by using low adsorption labware and by applying surface passivation. To obtain accurate results, it is important to allow all protein dilutions and mixtures to reach chemical equilibrium, but unnecessarily long incubation times should be avoided. For each protein system, it is recommended that the rates of the non-specific protein binding to the coverslip glass be assessed from the MP data. If significantly different rates are observed for different species, MP data can easily be corrected to avoid potential errors in the K_d calculations[10,11].

Several factors should be considered when planning sample preparation. Accurate results can be obtained by measuring a single sample, but the antigen and antibody concentrations have to be adjusted to obtain a comparable concentration of the free and bound species. Analysis of a single mass distribution dominated by one of the reaction species may provide acceptable K_d values estimates but usually yields relatively large fitting errors[11]. An alternative strategy involves a global analysis of titration data. The spreadsheet software based fitting tool provided with this protocol can be used for fitting both individual data and for global analysis. If a single experiment or data set is analyzed, the confidence intervals of best fit parameters can be estimated using the error projection method. A detailed description of the confidence intervals calculation procedure in the spreadsheet software is provided elsewhere[14]. When designing the assay, it is recommended to plan for replicate experiments. When data from replicate experiments are analyzed, standard deviation can be used to report errors of the K_d values.

The protocol described here can be modified to extend its applicability beyond the typical molecular mass and affinity range limitations. The range of measurable affinities is limited by the protein concentration range accessible by MP, typically from 10 nM to 50 nM. This range can be extended and protein samples at concentrations below 10 nM can be measured by using perfused flow cells and longer data acquisition times. Protein samples at higher concentrations can potentially be measured by using passivated coverslips for the MP measurements. For

antigens with a small molecular mass, the free antibody and the antigen-antibody complex peaks in the MP mass distributions will be unresolved. This will preclude the use of the Gaussian peak fitting analysis described in the protocol. In that case, the binding affinity can still be measured by titrating the antibody with the antigen and averaging the MP distributions to obtain the average molecular mass of all species for each sample. The binding equation can then be fit to this data to obtain the antigen-antibody binding affinity[11].

When accurate affinity information is not required, the protocol can be simplified and used for fast antibody interaction screening. In that case, the commercially available gasket wells can be used instead of sample channels to further simplify the experimental procedure.

ACKNOWLEDGMENTS:

We thank Keir Neuman for his critical reading of the manuscript. This work was supported by the intramural program of the NHLBI, NIH.

DISCLOSURES:

The authors have nothing to disclose.

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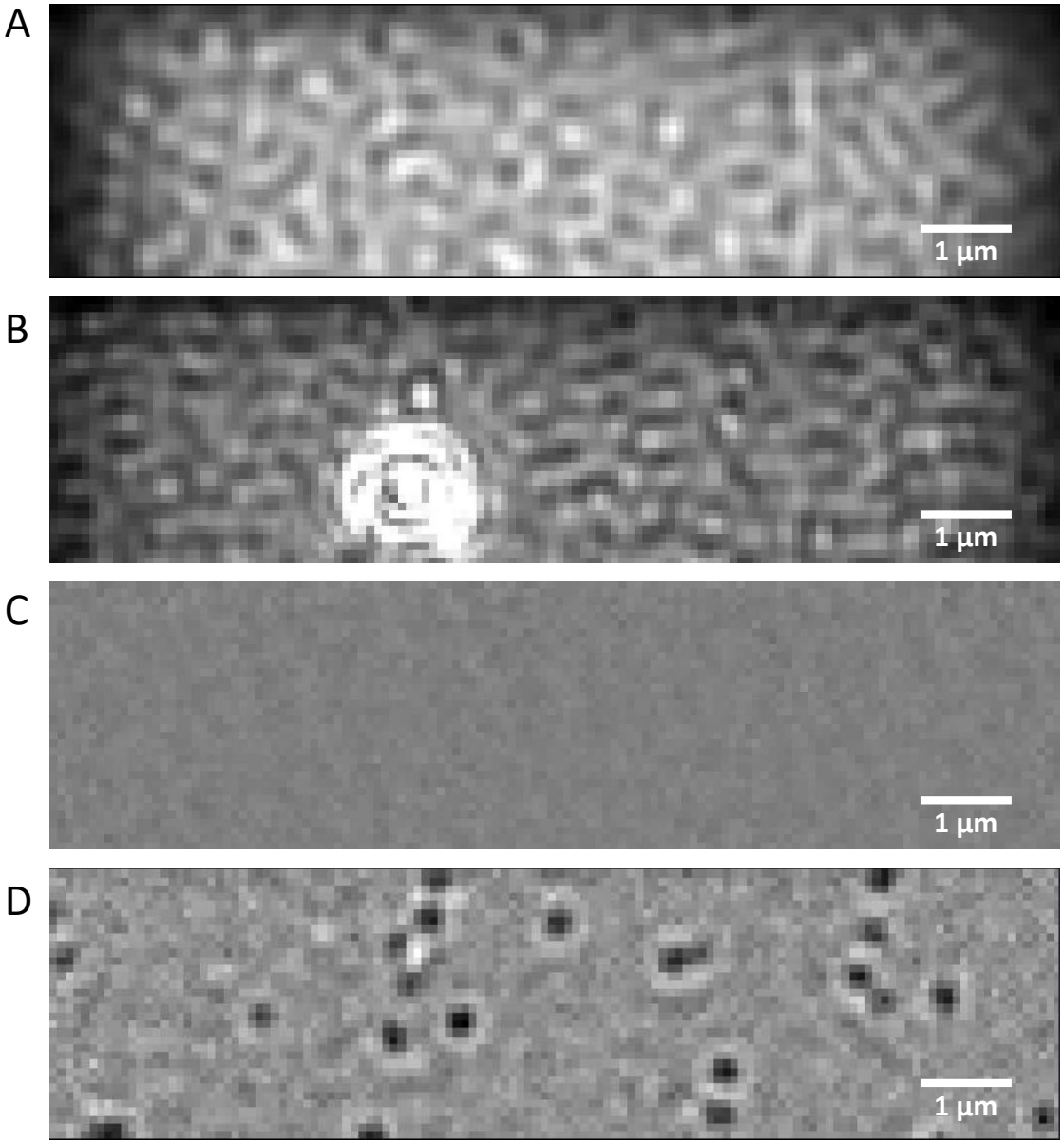
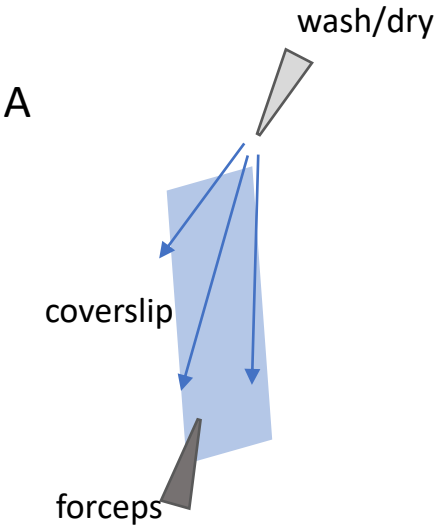
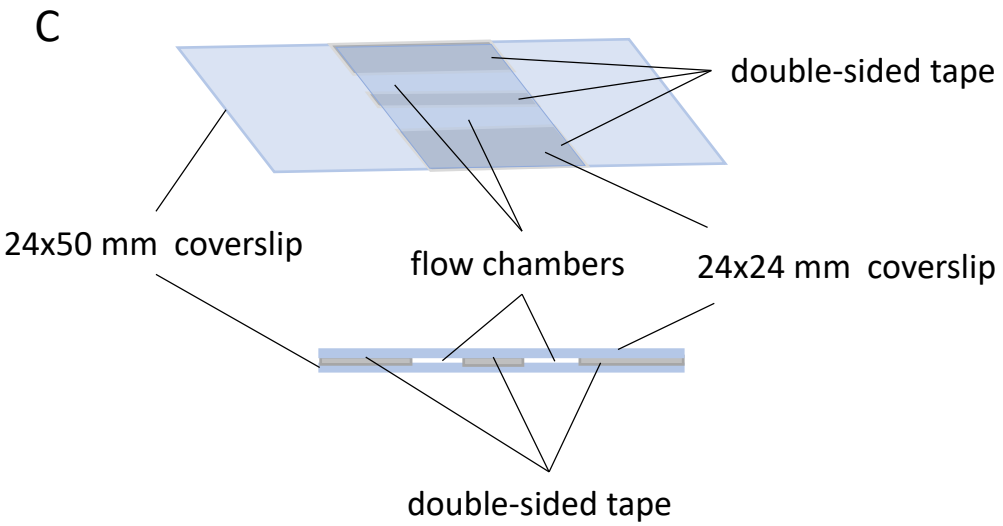
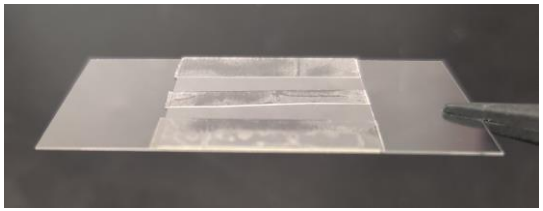
Figure 1

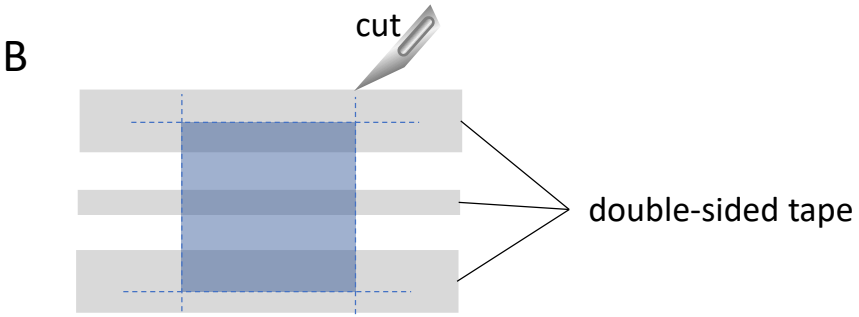
Click here to access/download;Figure;Fig1.pdf











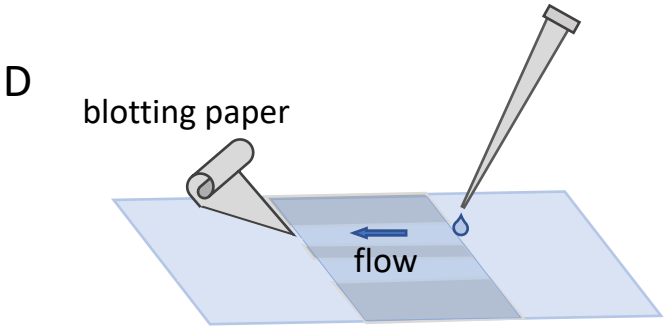


Figure 3

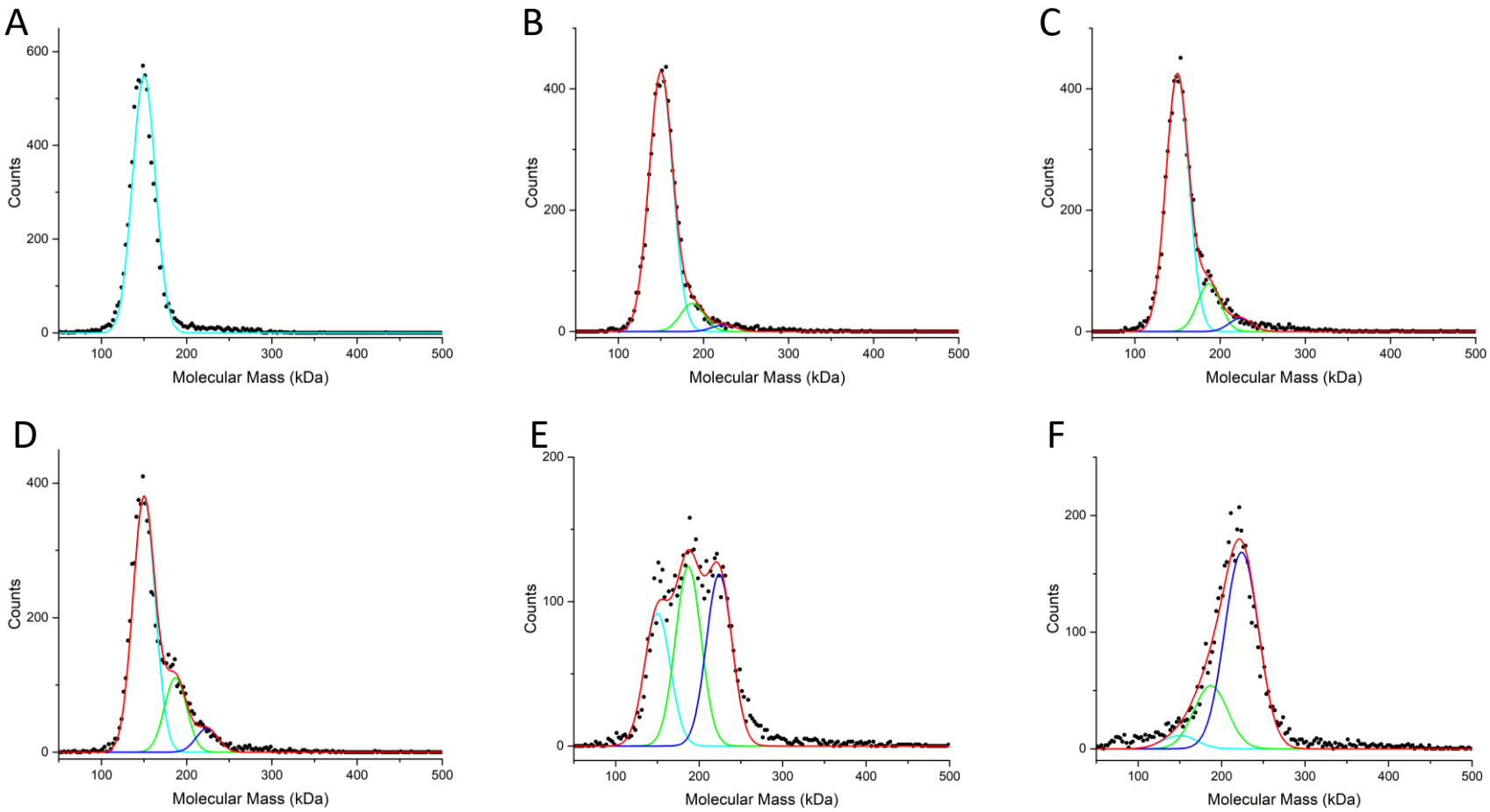


Figure 4

Click here to access/download;Figure;20200823 JoVE figure4.pdf

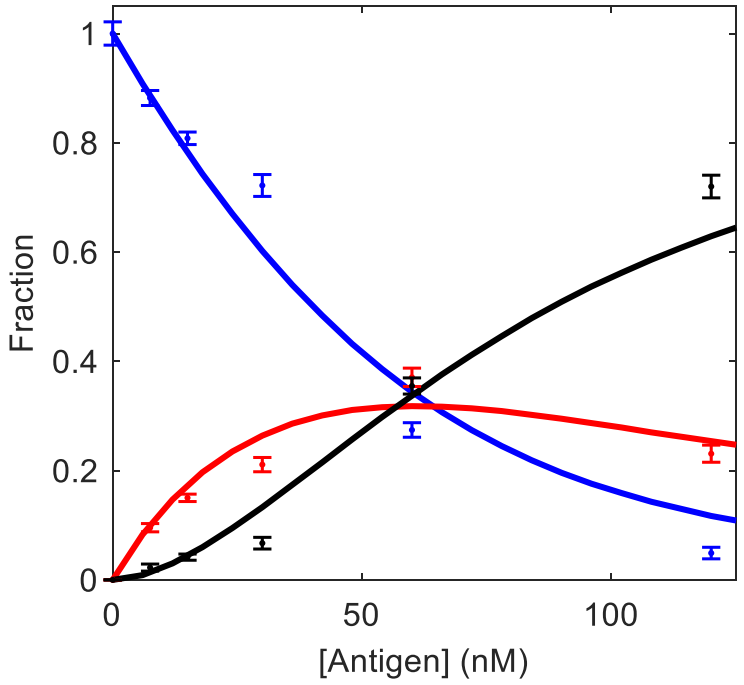


Figure 5

Click here to access/download;Figure;20200823 JoVE figure5.pdf

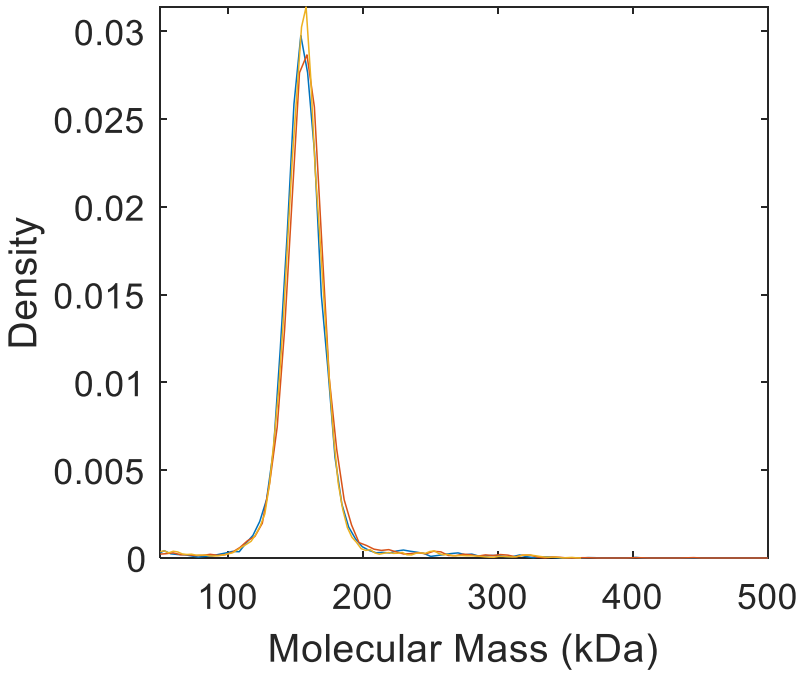


Table 1

Ab conc (M)	Ag conc (M)	f_Ab	f_AbAg	f_AbAg2
2.50E-08	7.50E-09	0.88196	0.09551	0.02254
2.50E-08	1.50E-08	0.80819	0.15018	0.04163
2.50E-08	3.00E-08	0.72185	0.21097	0.06719
2.50E-08	6.00E-08	0.27439	0.37072	0.35489
2.50E-08	1.20E-07	0.0491	0.23097	0.71993

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
AcquireMP	Refeyn		MP data collection software
Anti-human thrombin	Haematologic Technologies	AHT-5020	RRID: AB_2864302
Cotton-tipped applicators	Thorlabs	CTA10	cotton optical swabs for lens cleaning
Coverslips 24x24 mm	Globe Scientific	1405-10	
Coverslips 24x50 mm	Fisher Scientific	12-544-EP	
DiscoverMP	Refeyn		MP data processing software
Forceps	Electron Microscopy Sciences	78080-CF	soft-tipped forceps for coverslips handling
Human α-thrombin	Haematologic Technologies	HCT-0020	
Immersion oil	Thorlabs	MOIL-30	
Isopropanol	Alfa Aesar	36644	
Microsoft Excel	Microsoft		spreadsheet
OneMP	Refeyn		Mass Photometry instrument
Origin	OriginLab		scientific graphing software
PBS	Corning	46-013-CM	10x stock
Syringe filter	Millipore	SLGSR33SS	buffer and sample filtering

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
National Heart, Lung, and
Blood Institute
Bethesda, Maryland 20892

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
National Heart, Lung, and Blood Institute

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.

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

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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.

SUPPLEMENTARY INFORMATION:

Implementation of the equilibrium constants fitting procedure in Excel

Spreadsheet cells whose values have to be modified before the fitting procedure are highlighted in yellow (rows 1 to 10).

Cells B1 and B2 should be initialized with the estimated dissociation equilibrium constant values K_{d1} and K_{d2} in nanomolar units. After fitting, the optimized K_{d1} and K_{d2} values will be returned here.

Cells B12 and B13 show the association equilibrium constant values calculated from the K_{d1} and K_{d2} according to the formulas: $K_{a1}= (K_{d1})^{-1}$, $K_{a2}= (K_{d2})^{-1}$.

The total concentrations of the antibody and antigen in nanomolar units should be entered into cells D2 and E2, respectively. If several measurements at different concentrations were performed, additional values can be entered in rows 3 to 10 for a global fit. For each set of concentration values entered in columns D and E, three concentration fractions obtained by normalization of the mass distribution peak heights (Protocol step 4.3) should be entered into the adjacent cells. The concentration fraction values of the free antibody, single bound antibody, and double bound antibody are entered in columns F, G, and H, respectively.

Values entered into the yellow-highlighted cells (D2:H10) are copied into cells in columns A to E, rows 21 to 29. Columns G to AB in those rows contain intermediate values used to solve the binding equation as follows:

After substituting K_{a1} and K_{a2} for $f_{Ab\cdot Ag}/f_{Ab}$ and $f_{Ab\cdot Ag2}/f_{Ab}$, respectively, equation (2) can be rearranged to obtain a cubic equation of c_{Ag},:

$$K_{a1}K_{a2}c_{Ag}^3 + \left[K_{a1} + 2K_{a1}K_{a2}(c_{Ab})_{tot} - K_{a1}K_{a2}(c_{Ag})_{tot}\right]c_{Ag}^2 + \left[1 + K_{a1}(c_{Ab})_{tot} - K_{a1}(c_{Ag})_{tot}\right]c_{Ag} - (c_{Ag})_{tot} = 0$$

with coefficients defined as:

$$a = K_{a1}K_{a2}$$

$$b = K_{a1} + 2K_{a1}K_{a2}(c_{Ab})_{tot} - K_{a1}K_{a2}(c_{Ag})_{tot}$$

$$c = 1 + K_{a1}(c_{Ab})_{tot} - K_{a1}(c_{Ag})_{tot}$$

$$d = -(c_{Ag})_{tot}$$

For the given values of K_{d1}, K_{d2}, the total concentration of the Antibody $(c_{Ab})_{tot}$ and the total concentration of the Antigen $(c_{Ag})_{tot}$, the coefficients a, b, c and d are calculated in columns G, H, I and J respectively.

Values of p and q, defined as:

$$p = \frac{3ac - b^2}{3a^2}$$

and:

$$q = \frac{27a^2d - 9abc + 2b^3}{27a^3}$$

are calculated in columns L and M, respectively, and the cubic formula discriminant $\Delta = \left(\frac{q}{2}\right)^2 + \left(\frac{qp}{3}\right)^3$ is calculated in column O.

According to Cardano's formula, when $\Delta > 0$, the real root of the cubic equation is given by:

$$c_{Ag} = \sqrt[3]{-\frac{q}{2} + \sqrt[2]{\left(\frac{q}{2}\right)^2 + \left(\frac{p}{3}\right)^3}} + \sqrt[3]{-\frac{q}{2} - \sqrt[2]{\left(\frac{q}{2}\right)^2 + \left(\frac{p}{3}\right)^3}} - \frac{b}{3a}$$

The value of c_{Ag} is calculated in column T.

When $\Delta < 0$, three real roots for the cubic equation exist:

$$c_{Ag,1} = 2\sqrt[3]{r}\cos\theta - \frac{b}{3a}$$

$$c_{Ag,2} = 2\sqrt[3]{r}\cos\left(\theta + \frac{2\pi}{3}\right) - \frac{b}{3a}$$

$$c_{Ag,3} = 2\sqrt[3]{r}\cos\left(\theta + \frac{4\pi}{3}\right) - \frac{b}{3a}$$

where $r = \sqrt{-\left(\frac{p}{3}\right)^3}$ and $\theta = \frac{1}{3}\cos^{-1}\left(-\frac{q}{2r}\right)$. Values of r and θ are calculated in columns Q and R, respectively, and values of roots $c_{Ag,1}$, $c_{Ag,2}$, and $c_{Ag,3}$ are calculated in columns U, V, and W, respectively. Any root calculations in columns T, U, V and W that result in an invalid value are replaced by 0. If the root values in columns T through W are in the range from 0 to $(c_{Ag})_{tot}$, they are copied to a corresponding cell in column Y to AB. Values outside this range are set to 0. That way, the final value of c_{Ag} can be calculated as the sum of the cells in columns Y to AB. The final c_{Ag} value for the first sample is returned in cell A31. For a global fit, root values for additional data points are displayed in rows 32 to 39 (rows marked in blue). The values of c_{Ag} are used to obtain the concentrations of other reaction components according to the formulas:

$$c_{Ab} = \frac{c_{Ab,tot}}{1 + K_{a1}c_{Ag} + 2K_{a1}K_{a2}c_{Ag}^2}$$

$$c_{Ab\cdot Ag} = K_{a1}c_{Ab}c_{Ag}$$

$$c_{Ab\cdot Ag_2} = K_{a1}K_{a2}c_{Ab}c_{Ag}^2$$

The values of c_{Ab}, $c_{Ab\cdot Ag}$, and $c_{Ab\cdot Ag2}$ are calculated from row 31 to 39 in columns B, C and D, respectively. Column F contains the sum of c_{Ab}, $c_{Ab\cdot Ag}$, and $c_{Ab\cdot Ag2}$, which is the total antibody concentration and, for the first sample, should be identical to the value in cell A21. To avoid Excel calculation errors, very small numbers ($1\text{x}10^{-30}$) are entered into cells in column F for rows not used to fit experimental sample data.

The fractions f_{Ab}, $f_{Ab \cdot Ag}$, and $f_{Ab \cdot Ag2}$—based on the estimated K_d values—are calculated in row 41 in columns C, D, and E, respectively. If data obtained for several samples are utilized in the global fit, additional values will be calculated in rows 42 to 49 (rows marked in gray).

To obtain the sum of squared errors (SSE), the squares of differences between the fit values (rows 41 to 49, marked in gray) and the experimental values (rows 21 to 29, marked in green) are calculated in rows 51 to 59 (rows marked in orange). All of those values are added together, and the final SSE is displayed in cell B15.

The Solver function in Excel is used to minimize the SSE value in cell B15 by adjusting the values of K_{d1} and K_{d2} in cells B1 and B2. Once the minimum value of the SSE is found, the corresponding K_{d1} and K_{d2} values represent the best fit equilibrium constants determined from the MP data.

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Supplemental Coding Files

Kd fit.xlsx

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