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Title: Rapid Determination of Antibody-Antigen Affinity by Mass Photometry

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: All screen captures provided, do not film

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **40**

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Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Grzegorz Piszczek**: Antibodies are routinely used in laboratory techniques and their therapeutic and diagnostic applications are quickly expanding. Fast and accurate antigen-antibody binding characterization is critically important for these applications **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Grzegorz Piszczek**: MP quickly measures binding affinities using extremely small amounts of material. No labeling or immobilization is required and information on antigen-antibody complex oligomerization and purity can be obtained from the same experiment **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Di Wu**: This protocol can be used not only to study antibodies, but also to measure strong protein-protein binding for proteins with a molecular mass larger than 50 kDa **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

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Protocol

2. Flow Chamber Preparation and Assembly

2.1. Begin by using soft-tipped forceps and wash bottles to sequentially rinse 24 by 50-millimeter coverslips from top to bottom with distilled water, ethanol, distilled water, isopropanol, and distilled water **[1]**.

2.1.1. WIDE: Talent rinsing coverslip(s), with water, ethanol, and isopropanol bottles visible in frame *Videographer: Important step*

2.2. After the last wash, dry the coverslips with a stream of clean nitrogen in the same direction to avoid transferring contamination from the forceps **[1]**.

2.2.1. Talent drying coverslip(s) *Videographer: Important step*

2.3. Rinse 24 by 24-millimeter coverslips with distilled water and ethanol as demonstrated **[1]** followed by drying with a stream of clean nitrogen **[2]**.

2.3.1. Coverslip being rinsed, with water and ethanol bottles visible in frame

2.3.2. Coverslip being dried

2.4. After drying, place a 24 by 24-millimeter coverslip onto a piece of aluminum foil **[1]** and place strips of double-sided tape onto the coverslip **[2]**.

2.4.1. Talent placing coverslip onto foil

2.4.2. Talent placing tape onto coverslip

2.5. Then cut the 24 by 24-millimeter coverslip out of the foil **[1]** and gently press the foil onto the working side of the 24 by 50-millimeter coverslip **[2]**.

2.5.1. Talent cutting the coverslip out of the foil

2.5.2. Talent positioning the coverslips and gently pressing them together

3. Antibody-Antigen Samples Preparation

3.1. To prepare antibody-antigen samples for the affinity measurements, filter at least 2 milliliters of PBS through a 0.22-micron syringe filter to remove dust particles and aggregates **[1]** and centrifuge the antibody and antigen stocks of interest **[2-TXT]**.

3.1.1. WIDE: Talent filtering PBS, with PBS container visible in frame

3.1.2. Talent placing tube(s) into centrifuge **TEXT: 10 min, max speed**

3.2. Measure the 280-nanometer UV absorbance of the antibody and antigen stocks to determine their actual concentrations **[1]** and prepare a titration series of antibody-antigen mixtures at the appropriate range of antigen concentrations in a final volume of 50 microliters per sample **[2-TXT]**.

3.2.1. Talent loading sample onto spectrophotometer *Videographer: Important step*

3.2.2. Talent adding antibody to antigen, with antibody and antigen containers visible in frame *Videographer: Important step* **TEXT: If Ab binding affinity not known and one sample used, mix 30 nM Ag + 20 nM Ab**

3.3. Then incubate the antigen-antibody mixtures for approximately 10 minutes at room temperature to allow the binding reaction to reach chemical equilibrium **[1-TXT]**.

3.3.1. Talent placing tubes at room temperature *Videographer: Important step* **TEXT: Here 6-sample titration shown**

4. Mass Photometry Measurement

4.1. To assess the antibody-antigen affinity by mass photometry, apply a drop of microscope immersion oil to the instrument objective **[1]** and place the flow chamber onto the stage of the microscope **[2]**.

4.1.1. WIDE: Talent adding oil to objective

4.1.2. Talent placing chamber onto stage

4.2. Add 10 microliters of filtered PBS to one end of the flow chamber channel. The buffer will enter the channel by capillary action **[1]**.

4.2.1. PBS being added to end of chamber/PBS entering chamber

4.3. In the **Focus Control** tab of the data collection software **[1]**, use the coarse stage movement **Up** and **Down** buttons to make the initial adjustments and click **Sharpness** to view the sharpness signal readout **[2]**.

4.3.1. Talent at computer, clicking tab in software, with monitor visible in frame

4.3.2. SCREEN: screenshot_1: 00:00-00:15 *Video Editor: please speed up*

4.4. Use the fine **Up** and **Down** adjustment buttons to maximize the **Sharpness** value and click **Set Focus** and **Lock Focus** to activate the focus tracking function. An image of a clean slide should have a signal value equal to or below 0.05% **[1]**.

4.4.1. SCREEN: screenshot_1: 00:16-00:38 *Video Editor: please speed up and please emphasize 0.5% signal value when mentioned*

4.5. Load 20 microliters of the first antibody-antigen sample as demonstrated and blotting the liquid from the other end with a small piece of blotting paper **[1]**.

4.5.1. Sample being added to chamber and the other end blotted. *Videographer: Important step*

4.6. Immediately upon loading, click **Record** to acquire the appropriate number of frames equivalent to 100 seconds of data **[1]**.

4.6.1. SCREEN: screenshot_2: 00:00-00:03

4.7. At the end of the data collection period, enter a file name for the data and click **OK,** then **Sample,** to save the file **[1]**.

4.7.1. SCREEN: screenshot_2: 01:44-02:06 *Video Editor: please speed up*

5. Mass Photometry Data Analysis

5.1. To analyze the mass photometry data, open the file of interest **[1]** and click **Analyze [2]**.

5.1.1. WIDE: Talent opening file, with monitor visible in frame

5.1.2. SCREEN: screenshot_3: 00:00-00:14 *Video Editor: please speed up*

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5.2. Click **Load** to load the calibration function and click **File** and **Save Results As** to save the analyzed data **[1]**.

5.2.1. SCREEN: screenshot_3: 01:14-01:40

5.3. To obtain the relative concentrations of each species in the sample, open the **events Fitted .csv** file, copy the data in column M into the appropriate plotting and analysis software, and use the **Plot-Statistics-Histogram** function to plot the molecular mass distribution **[1]**.

5.3.1. SCREEN: screenshot_4: 00:00-00:35 *Video Editor: please speed up*

5.4. Double click on the histogram to open the **Plot Properties** window, disable the automatic binning, and select a bin size of 2.5 kilodaltons. To create the **Bin Centers** and **Counts** data, click **Apply** and **Go [1]**.

5.4.1. SCREEN: screenshot_4: 00:36-00:55 *Video Editor: please speed up*

5.5. To fit the histogram with Gaussian functions, select the **Bin Centers** and **Counts** columns and click the **Analysis-Peaks and Baseline-Multiple Peak Fit** menu function. Double click to indicate the approximate peak positions on the distribution plot and click **Open NLFit** *(pronounce 'N-L-fit')* **[1]**.

5.5.1. SCREEN: screenshot_4: 00:55-01:34

5.6. Check the **Fixed** checkboxes for the **xc** *(pronounce 'X-C')* peak centers and set their values to the expected molecular masses of the free antibody and the single and double antigen-antibody complexes **[1-TXT]**.

5.6.1. SCREEN: screenshot_4: 01:35-02:16 *Video Editor: please speed up* **TEXT: *e.g.*, 150 kDa for Ab, 187 kDa for 1:1 complex, and 224 kDa for 2:1 complex**

5.7. Check the **Share** option for the width parameters and click **Fit**. The fitted peak height values of the Gaussian components represent the relative concentration of each species in the sample **[1]**.

5.7.1. SCREEN: screenshot_4: 02:17-02:44 *Video Editor: please speed up and please emphasize fitted peak height values when mentioned*

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5.8. Then use the equation to calculate the concentration fraction of each species from the peak height values **[1-TXT]**.

5.8.1. BLACK TEXT WHITE BACKGROUND: $f_i = \frac{h_i}{h_{Ab}+h_{Ab\cdot Ag}+h_{Ab\cdot Ag_2}}$

6. Equilibrium Constant Value Calculation

6.1. To calculate equilibrium constants using a spreadsheet program, open the **dissociation constant calculation.xlsx** worksheet **[1]**. In this worksheet, the yellow-highlighted cell values in rows 1 to 10 can be modified to perform the equilibrium constant calculations **[2]**.

6.1.1. WIDE: Talent opening worksheet, with monitor visible in frame

6.1.2. SCREEN: screenshot_5: 00:00-00:08 *Video Editor: please emphasize highlighted rows*

6.2. Enter the estimated dissociation constant values in nanomolar units into cells B1 and B2. If the estimated dissociation constant values are not known, leave the default values in cells B1 and B2 unchanged **[1]**.

6.2.1. SCREEN: screenshot_5: 00:09-00:14

6.3. Enter the total antibody and total antigen concentrations in nanomolar units into cells D2 and E2 and enter the calculated fraction values for each species into cells F2, G2, and H2. When performing global analysis of the titration, add the appropriate data fraction values into rows 2-10 **[1]**.

6.3.1. SCREEN: screenshot_5: 00:15-00:50

6.4. In the **Solver Parameters** window, select cell B15 for the **Set Objective** box and cells B1 to B2 for the **By Changing Variable Cells** box **[1]**.

6.4.1. SCREEN: screenshot_5: 00:59-01:18

6.5. Select the **Min** button for the **To:** option and check the **Make Unconstrained Variables Non-Negative** checkbox **[1]**.

6.5.1. SCREEN: screensot_5: 01:24-01:32

6.6. Then select **GRG** (G-R-G) **Nonlinear** as the solving method and click **Solve**. The best fit dissociation constant values will be shown in cells B1 and B2 and the final sum of the squared errors will be shown in cell B7 **[1]**.

6.6.1. SCREEN: screenshot_5: 01:32-01:49 *Video Editor: please speed up and please emphasize cells B1, B2, and B7 when mentioned*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.1., 2.2., 3.2., 3.3.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.5. (sample loading)

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Results

7. Results: Representative Antibody-Antigen Affinity

7.1. In this representative experiment, a titration series with the anti-human thrombin antibody at a fixed 25-nanomolar concentration **[1]** and 0-, 7.5-, 15-, 30-, 60-, and 120-nanomolar human alpha-thrombin concentrations were performed **[2]** to obtain the molecular mass distributions of the antigen-antibody mixtures **[3]** and the antibody-only sample **[4]**.

7.1.1. LAB MEDIA: Figure 3
7.1.2. LAB MEDIA: Figure 3 *Video Editor: please sequentially emphasize Figure 3A-3F graphs*
7.1.3. LAB MEDIA: Figure 3 *Video Editor: please emphasize peaks in Figures 3B-3F*
7.1.4. LAB MEDIA: Figure 3 *Video Editor: please emphasize peak in Figure 3A*

7.2. Best-fit peak height parameters of the Gaussian components were normalized to obtain species concentration fractions **[1]**. The concentration fraction values were then fit globally to obtain the antigen-antibody binding affinities **[2]**.

7.2.1. LAB MEDIA: Table 1
7.2.2. LAB MEDIA: Table 1 *Video Editor: please sequentially emphasize f_ab, f_AbAg, and f_AbAg2 columns*

7.3. The experimental concentration fractions and the global fit results **[1]** for the free antibody **[2]**, single antigen-antibody complex **[3]**, and double antigen complex could then be plotted **[4]**.

7.3.1. LAB MEDIA: Figure 4
7.3.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize blue data line*
7.3.3. LAB MEDIA: Figure 4 *Video Editor: please emphasize red data line*
7.3.4. LAB MEDIA: Figure 4 *Video Editor: please emphasize black data line*

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Conclusion

8. Conclusion Interview Statements

8.1. **Di Wu**: To obtain accurate Kd values, protein concentrations have to be carefully selected to populate all of the reaction species. We recommend preparing a titration series using a range of antigen concentrations **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.2.)

8.2. **Grzegroz Piszczek**: The error projection method can be used to obtain the fitting error, but it is better to repeat the experiment and to calculate the standard deviations of the average Kd values **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

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