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Research paper

Multiplex bead binding assays using off-the-shelf components and common flow cytometers

Takamitsu Hattori^{a,b,1}, Akiko Koide^{a,c,1}, Tatyana Panchenko^a, Larizbeth A. Romero^b, Kai Wen Teng^a, Alexis D. Corrado^a, Shohei Koide^{a,b,*}

^a Perlmutter Cancer Center, New York University Langone Health, New York, NY 10016, USA

^b Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, New York, NY 10016, USA

^c Department of Medicine, New York University School of Medicine, New York, NY 10016, USA



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ABSTRACT

The ability to quantify protein-ligand interactions in an accurate and high-throughput manner is important in diverse areas of biology and medicine. Multiplex bead binding assays (MBBAs) are powerful methods that allow for simultaneous analysis of many protein-ligand interactions. Although there are a number of well-established MBBA platforms, there are few platforms suitable for research and development that offer rapid experimentation at low costs and without the need for specialized reagents or instruments dedicated for MBBA. Here, we describe a MBBA method that uses low-cost reagents and standard cytometers. The key innovation is the use of the essentially irreversible biotin-streptavidin interaction. We prepared a biotin-conjugated fluorescent dye and used it to produce streptavidin-coated magnetic beads that are labeled at distinct levels of fluorescence. We show the utility of our method in characterization of phage-displayed antibodies against multiple antigens of SARS-CoV-2, which substantially improves the throughput and dramatically reduces antigen consumption compared with conventional phage ELISA methods. This approach will make MBBAs more broadly accessible.

1. Introduction

Assays for quantifying antibody or antigen levels as well as for quantifying the strength of antibody-antigen interactions have broad utilities in biomedical science. Multiplex bead binding assays (MBBAs) utilize microbeads that have different levels of fluorescence to which different antigens (or antibodies) are immobilized (Morgan et al., 2004; Chen et al., 1999). The amount of an antibody (or an antigen) captured by each of the beads is quantified by a secondary reagent with a fluorescent label that is orthogonal to that for marking the beads. Thus, MBBA is a powerful technology that enables simultaneous detection of multiple antibody-antigen interactions.

Although MBBA is powerful, the technology often requires a dedicated instrument and specially formulated microbeads, which limits its accessibility. Here, we report the development of an MBBA method that utilizes readily available reagents and a standard flow cytometer.

We previously developed a bead binding assay for quantifying the affinity and specificity of anti-histone antibodies. The assay uses protein

A-coated polystyrene beads to capture an antibody to be characterized. Binding of a biotinylated peptide corresponding to a histone sequence and a post-translational modification of interest is detected using a fluorescently labeled streptavidin (Nishikori et al., 2012). Subsequently, we developed a variant of this assay using Dynabeads M-280 magnetic beads where a biotinylated antigen is immobilized on the beads and a bound protein is detected with a fluorescently labeled secondary reagent (Nady et al., 2015; Hattori et al., 2016). We found that only a small fraction of the biotin-binding capacity of the M-280 beads is necessary to produce robust signals for the detection of the antigen-antibody interaction. We also found that the immobilized biotinylated molecule does not dissociate at a substantial level within the time frame of the assay, as expected from the exceptionally tight interaction between biotin and streptavidin. Thus, we hypothesized that the remaining biotin-binding capacity of the beads could be utilized for labeling with a biotin-fluorescent dye conjugate and that we could produce beads with multiple, distinct levels of fluorescence by adjusting the amount of the biotin-fluorescent dye conjugate loaded on beads. We describe the

Abbreviations: MBBA, multiplex bead binding assay; IMAC, immobilized metal ion affinity chromatography; RBD, receptor binding domain.

* Corresponding author at: 522 First Avenue, New York, NY 10016, USA.

E-mail address: Shohei.Koide@nyulangone.org (S. Koide).

¹ Equal contribution.

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development of a biotin-fluorescent dye conjugate and MBAs using dye labeled magnetic beads using readily commercially available reagents. We demonstrate their utility in characterizing purified antibodies as well as phage-displayed antibodies.

2. Materials and methods

2.1. Reagents

Monoclonal antibody CR3022 was purchased from Absolute Antibody (catalog number Ab01680-10.0). Biotin-PEG4-Amine (Nanocs, catalog number B-P4A-1) was dissolved in distilled water at a concentration of 10 mM (4.36 mg/ml). Fifty micrograms of DyLight650 NHS Ester (Thermo Fisher, catalog number 62266, MW = 1066) was dissolved in 100 μ l phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄), resulting in a 0.47 mM solution. Fifty micrograms of DyLight488 NHS Ester (Thermo Fisher, catalog number 46403, MW = 1011) was dissolved in 100 μ l PBS, resulting in a 0.47 mM solution. Eighty microliters of the DyLight650 NHS Ester or DyLight488 NHS Ester solution were mixed with 2.4 μ l of the biotin-PEG4-Amine stock solution, corresponding to a dye:amine ratio of 1.57:1. After 1 h at room temperature, the reaction was quenched by the addition of 1 μ l of 2 M Tris-HCl pH 8.0. We assumed that the reaction was complete, resulting in a 0.288 mM solution of biotin-PEG4-dye. These solutions were used without further purification, because the unreacted dye is not expected to interfere with the assay, as it is removed in multiple washing steps in the MBBA procedure.

2.2. Preparation of recombinant proteins

The amino acid sequences of the Spike ectodomain (residues 16-1213) of SARS-CoV-2 was obtained from Genbank entry **MN908947.3**. A synthetic gene encoding a stabilized version of Spike described by Amanat et al. (Amanat et al., 2020), including the removal of the furin cleavage site, K986P and V987P mutations, the addition of a T4 foldon trimerization domain, a His₆-tag and a biotinylation tag (Avi-tag) at the C-terminus was synthesized (Integrated DNA Technologies) and cloned into the pBCAG mammalian expression vector. Similarly, the codon-optimized genes encoding the receptor binding domain of SARS-CoV-2 (RBD, residues 328–531; Genbank entry: **MN908947.3**), and the RBD of SARS-CoV (residues 315–517; GenBank entry: **AFR58742.1**) with the His-tag and the Avi-tag at the C-terminus were synthesized (Integrated DNA Technologies) and cloned into the pBCAG vector. The G476S mutation, common in SARS-CoV-2 samples in North America (Li et al., 2020), was introduced in the expression vector for SARS-CoV-2 RBD using PCR.

The Expi293 cells (Thermo Fisher) were transiently transfected with the vectors according to the manufacturer's protocol, and the cells were grown at 37 °C with 8% CO₂ for 7 days. The recombinant proteins were purified from the filtered cell culture supernatants by immobilized metal ion affinity chromatography (IMAC) using a HisTrap excel column (GE Healthcare). The purified proteins were biotinylated using the BirA enzyme produced in-house in the presence of 500 μ M biotin and 10 mM ATP. After biotinylation, the BirA enzyme was removed by IMAC and the recombinant proteins were dialyzed against PBS and stored at –80 °C. High purity of the recombinant proteins was confirmed by SDS-PAGE. Analysis of the recombinant proteins by size exclusion chromatography using a Superdex 200 10/300 Increase or Superdex 75 10/300 Increase column (GE Healthcare) showed a single, monodispersed peak with the predicted molecular mass of each protein.

Synthetic antibodies in the biotinylated Fab format were expressed and purified as described previously (Miller et al., 2012) except that we used a GORE protein A capture device (GORE, catalog number PROA102) for purification in place of protein G Sepharose.

2.3. Preparation of conjugated beads

The capacity of Dynabeads™ M-280 Streptavidin (Thermo Fisher catalog number 11206D) was estimated to be 8 nmol of biotin per ml beads (per 10 mg beads) according to the datasheet supplied by the manufacturer. Typically, 10 μ l of the stock bead suspension for each labeling reaction was mixed with 90 μ l of PBSB (PBS containing 0.1% (w/v) BSA (GeminiBio, catalog number 700-100P)). We avoided using skim milk, a common blocking reagent, because it contains biotin that would interfere with conjugation of biotinylated ligands to the beads. The supernatant was removed using a magnetic stand and the beads were resuspended in 100 μ l of PBSB. To prepare a “1% saturated” beads, we mixed the beads with an equal volume of the buffer containing 8 nM biotinylated ligand, i.e. a biotin-PEG-dye. To prepare beads with different levels of saturation, the ligand concentration was adjusted accordingly. Typically, beads were prepared with biotin-PEG4-DyLight650 with 0, 16, 56, 136 and 400 nM biotin-PEG-dye to achieve 0, 2, 7, 17 and 50% nominal saturation, respectively. To achieve uniform labeling of beads, the bead suspension was vortexed, the biotinylated ligand solution was immediately added, and the solution was mixed by pipetting quickly. After rotating the tube containing the solution for 10 min at room temperature, the supernatant was removed using a magnetic stand and the beads resuspended the beads in PBSB. Dynabeads™ M-270 Streptavidin (Thermo Fisher, catalog number 65305) was used in the same manner.

2.4. Multiplex bead binding assay

A second biotinylated ligand, i.e. a biotinylated antigen was added to the beads in the same manner as described in the above section. A biotinylated antigen was immobilized at 2% nominal saturation to each biotin-PEG-dye conjugated beads, by mixing a 10-fold diluted bead suspension with an equal volume of 16 nM antigen solution. The beads were then blocked with biotin at a final concentration of 1 μ M, and incubated for 5 min at room temperature. The beads were washed once using a magnetic stand and resuspend at a concentration of 0.5 mg/ml, and then beads with different dye levels, typically five levels, were mixed so that the concentration of each bead type is 0.1 mg /ml, i.e. 100 times diluted with respect to the stock suspension.

Five microliters of the bead mixture were aliquoted in each well of a 96-well polypropylene plate (Greiner Bio-One, catalog number 650261). Then 20 μ l of PBSB containing an appropriate concentration of the antibody of interest was added and incubated for 30 min at room temperature with mixing on a shaker. The samples were transferred to the wells of a 96-well filter plate (Millipore MultiScreen HTS HV, 0.45 mm, Thermo Fisher, catalog number MSHVN4550), the liquid was removed using a vacuum chamber and the wells were washed twice with 150 μ l of PBST-BSA (PBS containing 0.5% (w/v) BSA and 0.05% (v/v) Tween 20). The secondary antibody staining was performed in a total volume of 25 μ l for 30 min at room temperature with shaking. The samples were protected from light by covering with aluminum foil during the incubation. The wells were washed again with PBST-BSA and the beads resuspended in 100 μ l buffer and analyzed on an iQue screener (Sartorius) or a Guava BGR (Millipore).

2.5. Phage MBBA and phage ELISA

Phage display library sorting and preparation of Fab-displaying phage clones were performed as described previously (Miller et al., 2012; Hattori et al., 2020). For phage MBBA, one-tenth volume of 10xTBS (500 mM Tris-HCl (pH 7.5), 1.5 M NaCl) and 5% (v/v) BSA were added to the culture supernatants containing phage. In each well of a 96-well polypropylene plate, 5 μ l of the bead mixture and 45 μ l of the phage solution were combined. Binding, washing and detection were performed as described above. Anti-M13 antibody (Sino Biological, catalog number 11973-MM05T) was chemically conjugated with

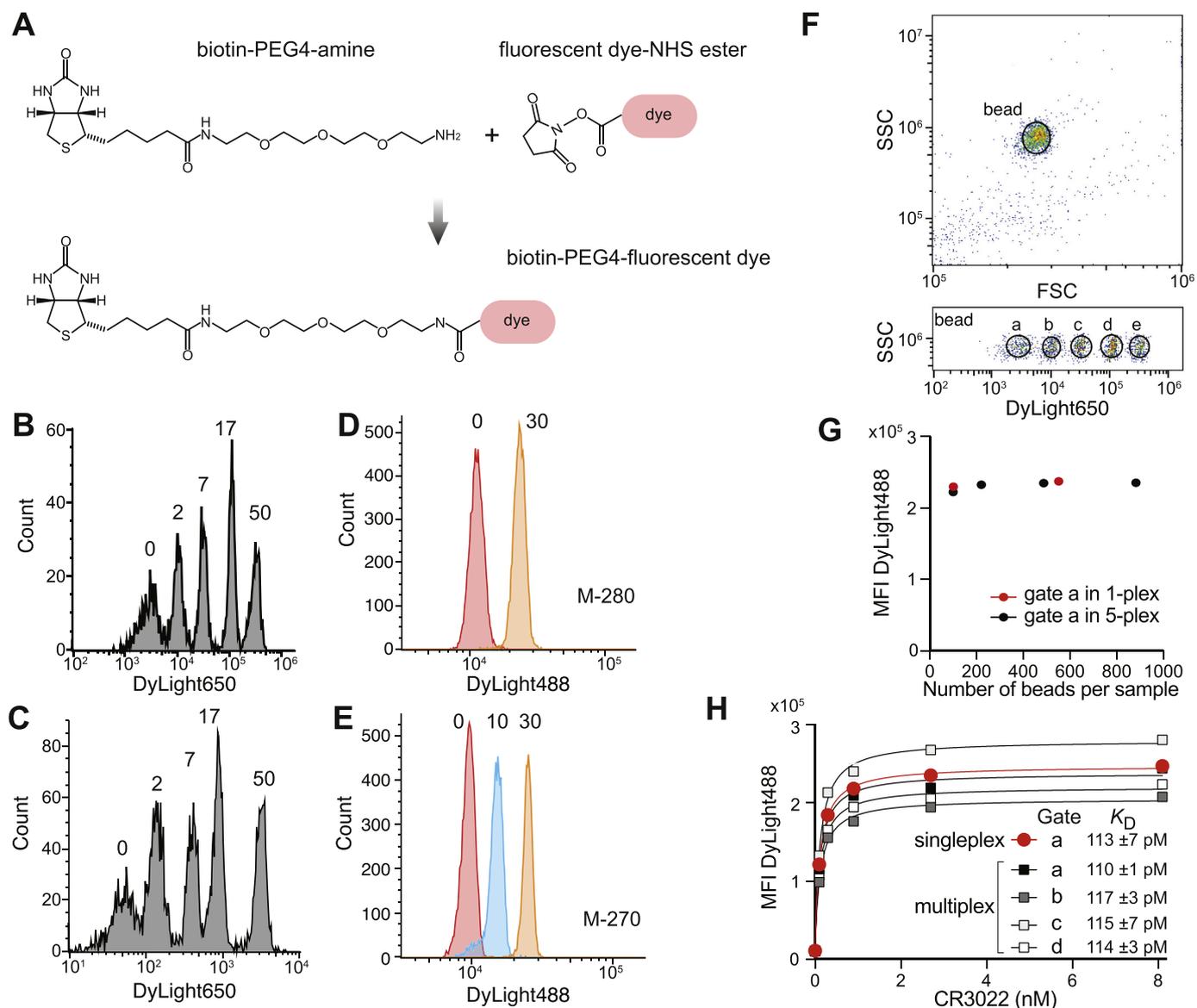


Fig. 1. MBBA technology design. (A) Preparation of biotin-PEG4-dye. (B, C) Histograms of fluorescence of a 5-plex mixture of M-280 beads conjugated with biotin-PEG4-DyLight650 recorded on a Sartorius iQue cytometer (B) and on a Millipore Guava BGR (C). The numbers indicate the nominal saturation levels. (D, E) An overlay of histograms of fluorescence of M-280 beads (D) and M-270 beads (E) conjugated with biotin-PEG4-DyLight488 recorded on an iQue cytometer. The data were recorded separately for each bead preparation and overlaid. The numbers indicate the nominal saturation levels. (F) Gating of flow cytometry data used for 5-plex experiments. (G) The median fluorescence intensity (MFI) of antibody binding of RBD-loaded beads versus the number of beads per sample, showing that MFI is independent of bead number. (H) Antibody binding titration performed in the singleplex format (red circles) and multiplex format (squares). Bead populations (a-d) are according to panel F. Data for gate e, a no-antigen control, are not shown. The K_D values are from curve fitting of a 1:1 binding model. The errors shown are the s.d. from curve fitting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

DyLight 488 NHS Ester (Thermo Fisher, catalog number 46420) in-house, and used at a 1:100 dilution.

Phage ELISA was performed as described previously (Miller et al., 2012). Briefly, the wells of the 96-well ELISA plate (Thermo Fisher, catalog number 655081) were coated with neutravidin (Thermo Fisher catalog number 31000), and blocked with 0.5% (w/v) BSA in PBS. Fifty microliters of 20 nM biotinylated antigens were added to the wells, and the plate was washed with PBST (PBS containing 0.1% (v/v) Tween20) three times using the plate washer (BioTek). The culture supernatants containing phage were diluted 1/5 in PBS with 0.5% (w/v) BSA, then transferred into the wells. After washing the plate with PBST three times, a secondary antibody (anti-M13-HRP: Sino Biological, catalog number 11973-MM05T-H) was added to the wells. One-step ultra TMB-ELISA solution (Thermo Fisher, catalog number 34028) was used as a substrate, then reaction was stopped by adding 2 M sulfuric acid. The

absorbance at 450 nm was measured using a BioTek Epoch plate reader (BioTek).

3. Results

3.1. Preparation of biotinylated fluorophore

To minimize nonspecific interactions due to a fluorescent dye bound to streptavidin, we designed a biotin-dye conjugate that is less hydrophobic and thus water soluble. We chemically conjugated biotin-PEG4-amine with an amine reactive dye, DyLight650 NHS Ester (Fig. 1A). We used the reaction product without purification, because excess dye would be removed during the preparation of dye-labeled beads. We found that this biotin-dye conjugate produced strong fluorescence signals, which enabled us to prepare a total of five distinctly labeled bead

Table 1
Negligible effects of incorrectly gated beads on the median fluorescence intensity (MFI) of the population of interest.

	Gate	Sample 1		Sample 2		Sample 3	
A. Antigen Loading	a	+		-		+	
	b	-		+		+	
	c	+		-		+	
	d	-		+		+	
	e	-		Not included		-	
		DyLight488 high (antigen +)	DyLight488 low (antigen -)	DyLight488 high (antigen +)	DyLight488 low (antigen -)	DyLight488 high (antigen +)	DyLight488 low (antigen -)
B. Number of events (% events)	a	1533 (96.2%)	60 (3.8%)	39 (4.5%)	837 (95.5%)	1512 (97.9%)	32 (2.1%)
	b	33 (2.3%)	1402 (97.7%)	1660 (98.0%)	34 (2.0%)	1630 (98.1%)	32 (1.9%)
	c	1900 (94.7%)	107 (5.3%)	242 (16.8%)	1195 (83.2%)	1843 (95.7%)	83 (4.3%)
	d	25 (2.2%)	1117 (97.8%)	1640 (98.4%)	27 (1.6%)	1654 (96.9%)	53 (3.1%)
	e	13 (0.8%)	1661 (99.2%)		5	20 (1.2%)	1595 (98.8%)
C. MFI of DyLight488-gated events	a	123158	10215	107274	10256	120194	10595
	b	120683	10638	115879	10901	114942	11775
	c	125685	10812	123660	10856	123158	11124
	d	180412	11079	127744	10901	129836	11215
	e	139683	10681			121174	10638
D. MFI of DyLight488-ungated events and % of gated MFI	a	121376	98.6%	10154	99.0%	118858	98.9%
	b	10587	99.5%	113629	98.1%	115961	100.9%
	c	123226	98.0%	10509	96.8%	120633	97.9%
	d	10949	98.8%	128186	100.3%	129742	99.9%
	e	10528	98.6%			10440	98.1%

* MFI's for the fractions containing the correct type of beads, determined after gating on the DyLight488 intensity with a threshold of 27,000, are in bold. In D, the percentages of the ungated MFI values with respect to the corresponding gated MFI values in C are shown.

populations that we were able to detect on two different flow cytometers (Fig. 1B and C). Another useful feature of this conjugate is that it is highly soluble in aqueous solutions in the absence of an organic solvent that could affect the stability of proteins and protein-protein interactions.

We also prepared biotin-PEG4-DyLight488. We found that its

fluorescence emission with respect to the unlabeled beads was not as strong as that of biotin-PEG4-DyLight650 and we were able to produce only two distinct populations (Fig. 1D). Subsequently, we found that we could prepare a total of three distinctly labeled populations with biotin-PEG4-DyLight488 using more hydrophilic Dynabeads M-270 (Fig. 1E).

All subsequent experiments were performed using the M-280 beads

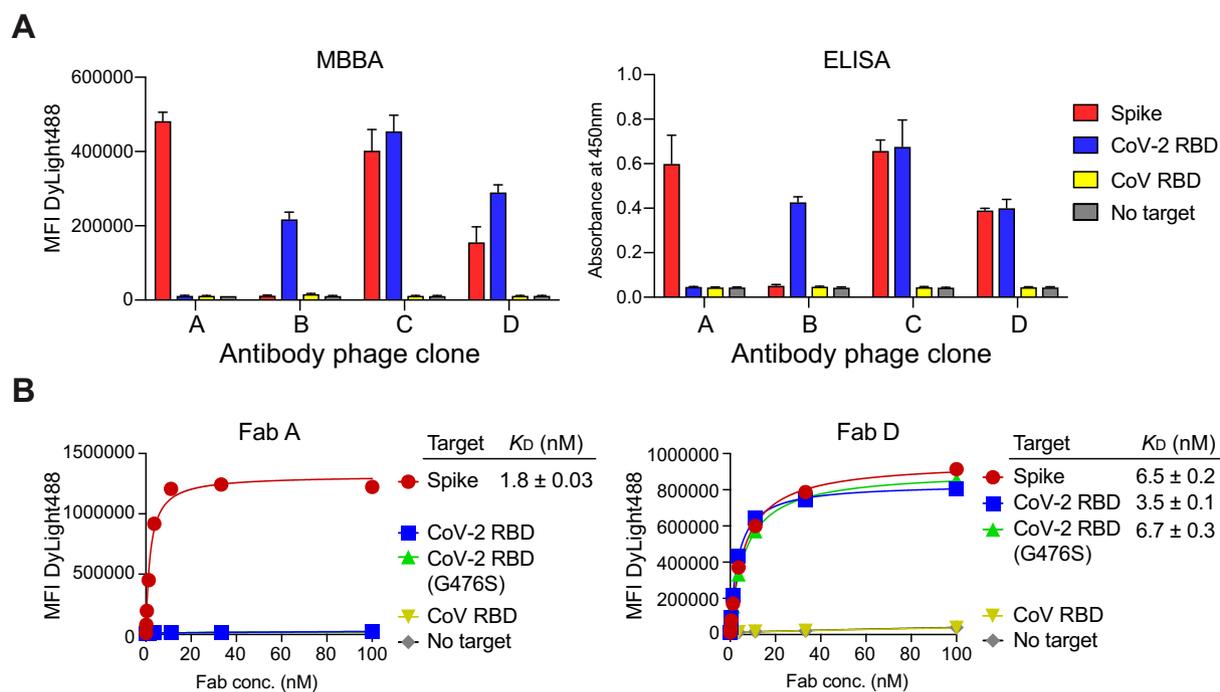


Fig. 2. Application of MBBA to screening phage-displayed antibodies. (A) Detection of antigen binding of phage-displayed antibodies by MBBA (left panel) and ELISA (right panel). The concentration of each phage clone in the binding reaction in MBBA was 1.7×10^{11} particles/mL (clone A), 9.9×10^{10} particles/mL (clone B), 2.0×10^{11} particles/mL (clone C) and 1.2×10^{11} particles/mL (clone D) respectively. The MBBA and ELISA showed essentially the same binding pattern for each clone. (B) Antigen binding titration of purified Fab performed in the 5-multiplex format. CoV-2 RBD (G476S) is a mutant RBD containing a naturally occurring mutation. The K_D values are from curve fitting of a 1:1 binding model. The errors shown are the s.d. from curve fitting. Error bars are within the size of the symbols. All data shown here are from triplicate measurements.

labeled with biotin-PEG4-DyLight650. Antibody binding was detected using a secondary antibody labeled with DyLight488. This fluorophore combination can be detected using a cytometer equipped with two lasers at 488 and 633 nm with no need for applying compensation.

3.2. Assay development and validation

We used a model antigen-antibody pair to establish a 5-plex bead binding assay (Fig. 1F). We used the receptor-binding domain (RBD) of SARS-CoV-2 that is site-specifically biotinylated and a positive control monoclonal antibody, CR3022, that potently binds to the RBDs of both SARS-CoV and SARS-CoV-2. As the assay readout, we used the median fluorescence intensity, rather than the mean fluorescence intensity, in order to minimize contributions of few events with anomalous fluorescence intensities. The median is essentially insensitive to such events but the mean is not. We refer to the median fluorescence intensity as MFI hereafter.

First, we examined the independence of the assay readout on the number of beads in the reaction. The MFI did not change when we changed the number of beads (Fig. 1G), and hence bead concentrations, from 4 to 36 per μl , indicating that deviations in the number of beads in reactions do not lead to errors in assay readout.

Second, we examined effects of overlaps of different bead populations. As can be seen in Fig. 1B, some bead populations are not completely separated, and thus each gated fraction may contain beads originating from the adjacent populations. However, as described above, the use of the MFI should minimize contributions of such erroneously gated beads. To critically test this prediction, we designed an experiment in which adjacent bead populations gave drastically different binding signals in the DyLight488 channel. The observed MFI values of DyLight488-ungated signals from the DyLight650-gated populations (Table 1, section D) were nearly identical to those of signals from the correct population, i.e. either DyLight488-low or -high, even when as much as 17% of beads from an adjacent peak are included in the DyLight650-gated population (sample 2, gate c in Table 1).

Finally, we compared antibody binding titrations in the singleplex and multiplex formats (Fig. 1H) by immobilizing the biotinylated RBD antigen on the beads and measuring antibody binding. All the measurements gave identical apparent K_D values, indicating the feasibility of performing affinity measurements in a multiplex format.

3.3. Screening of phage-displayed antibodies

We tested whether our MBBA is applicable to the detection of antigen binding of phage-displayed antibodies. Conventionally, phage ELISA is widely used for detecting binding of phage-displayed antibodies. However, phage ELISA cannot be multiplexed and it consumes substantial amounts of antigens if one wishes to screen many clones. Schofield et al. pioneered the use of flow cytometry detection for phage binding but they did not report multiplexed detection of multiple antigens (Schofield et al., 2007). We prepared a DyLight488-conjugated anti-M13 antibody and performed MBBA using the RBD and Spike ectodomain of SARS-CoV-2, and the RBD of SARS-CoV as antigens. The assay robustly detected binding of phage-displayed antibodies and enabled us to discriminate antibody clones with different profiles of antigen specificity (Fig. 2A). Importantly, the binding specificity profiles of the antibodies determined with MBBA quantitatively agreed with those determined with phage ELISA. MBBA using a purified antibody confirmed the results from phage MBBA and demonstrated the ability of MBBA to determine the apparent affinity of an antibody against multiple antigens (Fig. 2B). In addition, Fab D was insensitive to a mutation in the RBD frequently found in SARS-CoV-2 samples in North America, G476S (Li et al., 2020).

4. Discussion

We have extended a conventional bead binding assay to include multiplexing capability using simple, off-the-shelf reagents. This method fills a gap in current technologies between singleplex immunoassays such as ELISA and high-end multiplex assays such as Luminex™ (Schwenk et al., 2007). It is particularly effective in testing antibody binding to a handful of antigens, a situation that our laboratory encounters frequently. The preparation of biotin-dye conjugates is simple and each measurement requires as little as 50 nl of stock bead suspension, which keeps the cost low. It can be performed on a small benchtop flow cytometer, as well as expanded into a high-throughput assay by utilizing a flow cytometer equipped with an autosampler, as we demonstrated in Fig. 1B–E.

Compared with ELISA, MBBA dramatically reduces antigen consumption, in addition to reducing the total experimental time. In the experiment shown in Fig. 2, ELISA used 1000 fmol of antigen per well, whereas MBBA used 1.6 fmol of each antigen, i.e. a 625-fold reduction. This substantial reduction in antigen consumption may expand the types of phage-display antibody discovery campaigns, because limited sample quantities often present a bottleneck in screening a large number of individual clones, which is crucial for identifying a diverse set of antibody clones.

We were pleasantly surprised that beads with high levels of DyLight650 did not exhibit elevated levels of nonspecific binding, as fluorescent dyes contain multiple aromatic groups and may form nonspecific interactions. However, one should pay close attention to this potential artifact due to the presence of a dye on the bead surface. By using both unlabeled and highest labeled bead populations as controls with no conjugated antigen, one can readily detect such an artifact.

We envision that the degree of multiplexing of our MBBA can be further increased by adding another dimension in bead labeling, for example using both DyLight650 and DyLight488. However, in such a setting, care must be taken in selecting detection reagents to avoid interference from the dyes used for labeling beads.

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Declaration of Competing Interest

All authors declare no competing interests.

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