Chapter 13

Assessing the Affinity Spectrum of the Antigen-Specific B Cell Repertoire via ImmunoSpot®

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Abstract

The affinity distribution of the antigen-specific memory B cell (B_{mem}) repertoire in the body is a critical variable that defines an individual's ability to rapidly generate high-affinity protective antibody specificities. Detailed measurement of antibody affinity so far has largely been confined to studies of monoclonal antibodies (mAbs) and are laborious since each individual mAb needs to be evaluated in isolation. Here, we introduce two variants of the B cell ImmunoSpot® assay that are suitable for simultaneously assessing the affinity distribution of hundreds of individual B cells within a test sample at single-cell resolution using relatively little labor and with high-throughput capacity. First, we experimentally validated that both ImmunoSpot® assay variants are suitable for establishing functional affinity hierarchies using B cell hybridoma lines as model antibody-secreting cells (ASC), each producing mAb with known affinity for a defined antigen. We then leveraged both ImmunoSpot® variants for characterizing the affinity distribution of SARS-CoV-2 Spike-specific ASC in PBMC following COVID-19 mRNA vaccination. Such ImmunoSpot® assays promise to offer tremendous value for future B cell immune monitoring efforts, owing to their ease of implementation, applicability to essentially any antigenic system, economy of PBMC utilization, high-throughput capacity, and suitability for regulated testing.

Key words ELISPOT, FluoroSpot, B lymphocytes, Immune monitoring, Antibodies, Antibody titers, Immune memory, Functional affinity

1 Introduction

1.1 Definition of Affinity, Avidity, and Functional Affinity

Antibodies, also called immunoglobulins (Ig), can specifically bind to nearly any type of molecule (collectively termed an antigen), whereby the antigen-binding domain located in the hypervariable region of the antibody molecule (called the paratope) associates with a defined region of the antigen referred to as an epitope. The specificity and strength of antigen-antibody interactions depends

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on the presence of complementary structures on both surfaces in three-dimensional space ("lock and key principle") with the summation of attractive/repulsive forces contributing to the net binding strength. The binding forces involved in antibody-antigen interactions include (a) pairs of oppositely charged molecular groups, (b) hydrophobic regions capable of attracting each other, (c) coordinated hydrogen bonds, and (d) van der Walls-type interactions. As all four binding forces are reversible, the binding of antibodies to antigens is reversible too. Consequently, there is a constant "on-off" flickering of the paratope-epitope association that follows a second-order biochemical reaction. The association- (K_{on}) and dissociation- (K_{off}) constants of the paratope-epitope interaction jointly determine the equilibrium constant (K_d) that defines "affinity" (*see* **Note 1**).

The affinity of antibodies elicited during the course of an immune response can range from nearly undetectable (e.g., $K_d < 10^{-4}$ M), to extremely high (e.g., $K_d > 10^{-10}$) [1–8]. Consequently, a difference of greater than six orders of magnitude in antibody concentration can be required for low vs. high-affinity antibodies to attain the same level of epitope coverage. Thus, while all such "specific" antibodies can result in "specific" antigen-binding if they are present in sufficiently high concentration, it would take one million times more antibody molecules for a low-affinity antibody compared to a high-affinity antibody to achieve the same density of antigen binding! Therefore, the affinity of an antibody is a critical factor determining its capacity to contribute towards protective immune reactions.

Affinity will suffice for defining the binding strength of an antibody to an antigen when only a single arm (so-called Fab fragment) of the antibody can interact with the antigen. This is the typical scenario when antibodies (immobilized or not) interact with individual antigen molecules in solution. This is because, on most protein antigens, the epitopes are singular unique structures, i.e., there are no other epitopes present on the same antigen molecule for the other arm of the antibody to associate with. Avidity defines the net binding strength when a single antibody molecule (that possesses 2–10 Fab fragments or arms) can attach to multiple epitopes that are structurally connected. This is the case when repetitive epitopes occur on the same antigen molecule and are within the reach of the antibody's arms, or when separate, but physically linked, antigen molecules are sufficiently close in proximity that the antibody can associate with more than one antigen molecule simultaneously (e.g., when two membrane-anchored antigen molecules are in close proximity to each other). The net binding strength attained from such multivalent binding (avidity) is exponentially higher than that resulting from monovalent binding (affinity). This is because, as mentioned above, the interaction of the antibody's paratope with the antigen's epitope is a reversible

"on" and "off" flickering; during the "off" state, in case of a monovalent interaction, the reaction partners can diffuse away. If, however, more than one arm of the same antibody molecule is simultaneously involved in unsynchronized "on/off" flickering, the time window in which two or more arms are dissociated is greatly reduced, and the ligand(s) could separate and diffuse away.

To define affinity, single arms of antibodies (so-called Fab fragments) need to be studied; however, these do not occur naturally. As all naturally occurring antibodies in humans (and other commonly used animal models) have at least two arms (IgG, IgE, and monomeric IgA have two; IgM and dimeric IgA have 10 and 4, respectively), the occurrence of monovalent or multivalent binding (i.e., whether affinity or avidity applies), depends on the special circumstances of the antigen encounter. In terms of immunobiological consequences, however, the overall attachment strength of the antibody molecule with the antigen is fundamental for host defense. The latter is pragmatically called functional affinity. Since, in this chapter, we will also be analyzing the binding of bivalent antibodies to antigens, we adopt the term functional affinity, when appropriate, hereafter.

1.2 Biological Benefits of Antibodies with High Functional Affinity

A critical effector function of antibodies is their ability to bind and neutralize antigens (e.g., toxins or viruses) by preventing their association with cells of the host. Importantly, for this process to occur effectively, these antibodies need to possess a functional affinity for the antigen that surpasses that of the toxin/virus for their endogenous host receptor(s). Thus, the higher the functional affinity of these antibodies (e.g., the lower the K_d), the lower the concentration required for them to exert their neutralizing effector function. Moreover, another effector function of antibodies with high functional affinity is their ability to cross-link soluble antigens and generate immune complexes facilitating the effective elimination of the antigen by phagocytes. Activation of the complement system, another effector function initiated by antibodies, depends on the cross-linking of at least two of the six "arms" of the C1q molecule [9–11]; which is the first component that initiates the classical complement cascade. For this to occur, two antibody molecules need to be bound simultaneously, in close vicinity, so C1q can bind to both at once. As antibodies with high functional affinity bind more stably, even when they occur at low concentrations, they outperform antibodies with lower functional affinity in their capacity to initiate complement activation. The same applies when antibodies bind to antigen and label the latter for FcR-mediated elimination via phagocytosis (opsonization) or destruction (antibody-dependent cellular cytotoxicity, ADCC). For all these effector functions, the concentration of "specific" antibodies in bodily fluids is critical for their efficacy. As antibody

levels tend to decline with time after an immune response, only those antibodies at the high-level end of the functional affinity spectrum will effectively contribute to host defense.

1.3 Affinity Maturation of the Antibody Response

Owing to the fundamental role that antibody functional affinity plays in immune protection, the B cell system has evolved means to maximize the affinity of antibodies deployed in the course of an immune response. This starts with the recombination of V(D)J gene segments within the *IgH/IgL* loci during early B cell ontogeny, a process capable of generating an estimated 10^{14} unique B cell receptors (BCR) [12] (*see* **Note 2**). As B cells are limited to the expression of a single BCR (with rare exceptions), each B cell is endowed with a unique antigen-binding specificity. Importantly, any given specificity occurs at a very low frequency among naive B (and T cells) and such cells continuously recirculate through secondary lymphoid tissues where they may encounter their cognate antigen (*see* **Note 3**). Both naive B and T cells are efficiently retained in secondary lymphoid tissues during the onset of a primary immune response when they first encounter "their" antigen, and, driven by (affinity-based) antigen receptor triggering, these cells are induced to proliferate. In this way, clonal expansions occur resulting in an increase in the frequency of antigen-specific B and T cells among all lymphocytes in the body. The activated T and B cells also acquire specialized effector functions, i.e., T cells become Th1/Th2/Th17/Tfh21 cells, each of which is capable of secreting a distinct cytokine signature, and B cells undergo Ig class switching that is characteristic of antigen-experienced B cells: they switch from IgM-expressing naive B cells to downstream Ig classes such as IgG or IgA, and within the IgG producers, to subclasses IgG1/IgG2/IgG3/IgG4. Of note, the process of Ig class switching is governed by instructive signals provided primarily by T helper cells (*see* **Note 4**).

During the primary immune response, the proliferating antigen-stimulated B cells also undergo an additional fundamental process aimed at improving the efficacy of the ensuing antibody response: the acquisition of somatic hypermutations (SHM) in the *IgH* and *IgL* encoding the antigen-binding variable regions of their BCR. Initially, naive B cells with adequate affinity for the antigen become stimulated; these cells can then acquire SHM as they undergo additional rounds of proliferation within the germinal center reaction. Their progeny, therefore, will consist of subclones that have BCR with a spectrum of affinities for the eliciting antigen: some with higher, and others with lower affinity. Of these daughter cells, only those with the highest affinity for the antigen continue to participate in the germinal center reaction and undergo further rounds of proliferation and acquisition of additional SHM. This process of positive selection for high-affinity subclones (and negligence of daughter cells with lower affinity) continues throughout

the primary immune response and becomes more and more stringent as the antigen becomes gradually eliminated as a consequence of the ensuing successful immune response. This process of affinity maturation through the acquisition of SHM can also be re-initiated at a later time point if the antigen is reencountered, providing the cellular basis for why booster immunizations can progressively raise the affinity of the elicited antibody response. Whether in the context of a primary or secondary (recall) immune response, the B cell progeny endowed with the highest affinity BCR for the eliciting antigen will differentiate into plasma cells (PC) that secrete antibodies with identical specificity of the BCR expressed at the time of their disengagement from the germinal center reaction. If such antibody-secreting cells (ASC) settle into the bone marrow or other suitable niches, they can become long-lived PC (*see* **Note 5**).

Relevant to this chapter, the affinity distribution of B cells for any given antigen is expected to be variable among human subjects, dependent on the dose and duration of antigen persistence during the primary immune response (that dictates the initial affinity-based positive selection process) and on the timing, dose, and duration of secondary, and possibly subsequent, antigen encounters, thus triggering further rounds of affinity-based selection of the antigen-specific B cell repertoire. Based on past efforts generating monoclonal antibody (mAb)-secreting B cell hybridomas, experience supports that the more booster shots that are given, the higher the chance of isolating a clone that secretes antibodies with very high affinity.

1.4 Measuring Antibody Functional Affinity in the Serum vs. the B Cells Themselves

It is important to understand the fundamental differences between immune protection mediated by the B cell system via the first- vs. second walls of adaptive humoral defense. Antibodies already present in serum and other bodily fluids can instantly bind to antigens as soon as they attempt to enter the body. This first wall of defense can prevent reinfections and it can be readily assessed by serum antibody measurements. Serum antibodies, however, are relatively short-lived molecules (*see* **Note 6**) and their continued presence depends on constant replenishment by PC. While PC are potentially long-lived, their lifespans are heterogenous and likely fall on a continuum [13, 14]. During the recent COVID-19 pandemic, we were reminded how rapidly following natural infection, or after vaccination, the induced specific serum antibody levels can decline [15, 16]. We also frequently learned that the detection of memory B cells (B_{mem}) is far more reliable for revealing whether an infection has occurred than measuring serum antibodies [17].

The second wall of B cell-mediated protection is conferred through the reactivation of B_{mem}. If the first wall of adaptive humoral defense fails, and a (re-)infection occurs, antigen-specific B_{mem} (and memory T cells) can rapidly engage in secondary immune responses. These lymphocytes can mount a stronger and

faster counterattack against the offending pathogen because they are present in greatly increased numbers in the body compared to the numbers of naive B (and T) cells in the pre-immune repertoire. Moreover, many of these pre-existing memory cells have already undergone differentiation into effector lineages (Th1/Th2/Th17, etc. for T cells, IgG subclass, or IgA-switched B cells). Additionally, the B cell affinity maturation process re-engages during secondary immune responses, starting, however, from the elevated levels established following the prior antigen encounter(s).

Studying the first wall (existing antigen-specific serum antibodies) and the second wall (the antigen-specific B_{mem} repertoire in the blood, *see* **Note 7**) of adaptive humoral immunity therefore provides fundamentally different information. The former provides a low-resolution and fading image of the past as it still applies to the integrity of the first wall, and the latter permits to gain a high-resolution image of the second wall, thus assessing the immune potential present in case of a subsequent antigen encounter. High-resolution in this context means being able to assess individual B cells within the entire antigen-specific repertoire, the type and quantity of antibody they produce, and, relevant for this chapter, the functional affinity distributions of the individual B cells.

1.5 Measuring Functional Affinity by B Cell ImmunoSpot®

Being able to assess the functional affinity distribution of the antigen-specific B_{mem} compartment in an individual requires defining the functional affinity for hundreds (the more the better) of individual B cells to obtain a representative picture. Doing so by the standard approaches, either through surface plasmon resonance (SPR) or biolayer interferometry (BLI) measurements of mAbs, would be an effort too involved for immune monitoring purposes (*see* **Note 8**). We introduce here modifications of the ImmunoSpot® assay that promise to fill this gap.

The principle of one of the underlying ImmunoSpot® variants suited for this purpose, the inverted assay, is shown in Fig. 1a. In brief, the membrane is coated with an antibody suited for capturing the Ig produced by ASC irrespective of their antigen specificity or affinity: in case of testing human PBMC, the capture reagents typically would be xenogeneic anti-human IgG, A, or E-specific antibodies, or a pan Ig-specific (anti-kappa + anti-lambda light chain) antibody (*see* **Notes 9** and **10**). Onto this lawn of Ig capture reagent, the cells are plated (*see* **Note 11**). During the time in which the ASC resides on the membrane, their secreted antibodies will be captured in close proximity to the respective ASC in the form of a secretory footprint (often also referred to as a "spot forming unit," SFU) (*see* **Note 12**). After a brief period of cell culture during which the secreted Ig analyte is captured, the cells are removed from the plate (*see* **Note 13**), and the antigen probe is added to replicate wells containing the same number of ASC, but decreasing

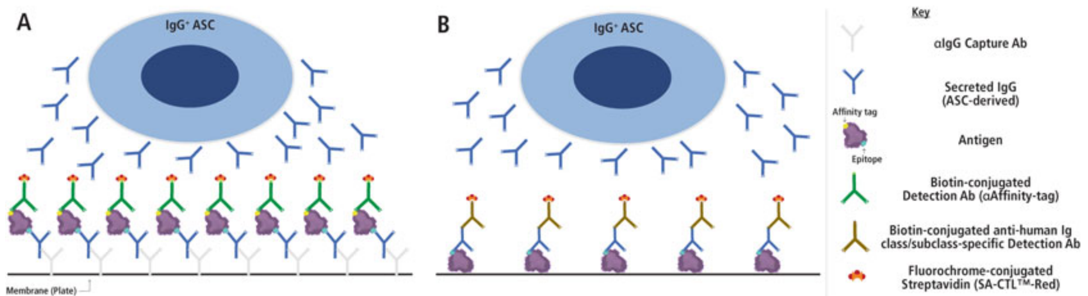

Fig. 1 Schematic representation of antigen-specific inverted or direct ImmunoSpot® assays. The PVDF membrane on the bottom of a 96-well plate is densely coated (**a**) with a pan anti-Ig(G) class-specific (in this example IgG) capture antibody that binds with high-affinity to secreted Ig(G) irrespective of antigen specificity or (**b**) with an antigen. The PBMC are plated on the resulting lawn of (**a**) pan anti-Ig(G) class-specific capture antibody, and individual ASC producing the particular Ig class will generate a secretory footprint or (**b**) antigen and individual ASC-producing antibody with sufficient affinity to the antigen will generate a secretory footprint. After removal of the cells, (A) the affinity-tagged (in this example His) antigen is added at a sufficient concentration to be retained by antigen-specific secretory footprints generated by ASC producing low- or high-affinity antibody or (**b**) antigen-bound antibody is visualized by biotinylated human Ig class/subclass (in this example IgG) specific detection antibodies. (**a**) Antigen-specific secretory footprints are then visualized using a biotinylated anti-affinity tag detection reagent; which is revealed by the addition of a fluorescently-conjugated streptavidin (FluoroSpot, as shown) or via an enzymatic reaction (ELISPOT, not shown). (**b**) Antigen-specific secretory footprints are revealed by the addition of a fluorescently-conjugated streptavidin (FluoroSpot, as shown) or via an enzymatic reaction (ELISPOT, not shown). (**a**, **b**) Counting the spot-forming units (SFU) per well reveals the number of antigen-specific ASC within the PBMC plated

(graded) concentrations of antigen. When the antigen probe is added in excess, all secretory footprints retain the antigen probe: ASC with low- and high-affinity will be revealed alike. As the concentration of antigen probe becomes limiting, however, increasingly only the high-affinity secretory footprints (SFUs) will capture a sufficient quantity of the antigen probe for their detection.

For such affinity measurements, the cells need to be plated at a predetermined "Goldilocks" number that, being assay dependent, is between 50 and 200 SFUs/well (being at the upper end of the linear range between cell numbers plated and SFU counted, so that as many individual ASC can be assessed per well as possible, yet without ASC crowding interfering with the image analysis, see also **Note 14**). Testing a fixed number of replicate wells for each antigen concentration thus permits to assessment of the affinity spectrum within the same number (ideally ≥300) of antigen-specific ASC for each antigen concentration. The percentage of SFU lost with each successive reduction in antigen probe concentration reveals the affinity thresholds for the ASC subpopulations lost, respectively. A detailed protocol for such B cell affinity measurements is provided below.

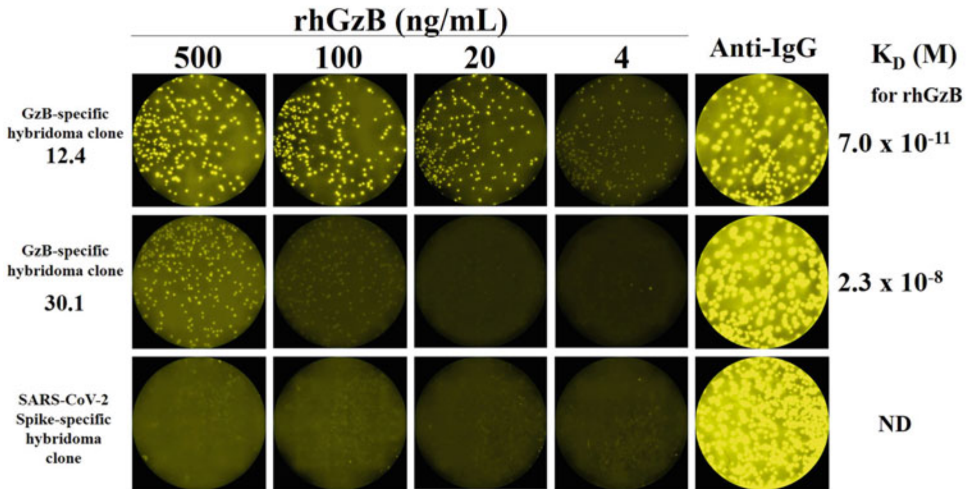

Fig. 2 Assessing the affinity of Granzyme B (GzB)-specific mAb by inverted ImmunoSpot® assay vs. surface plasmon resonance testing. In columns 1–4, the results of an inverted ImmunoSpot® assay are shown. Onto a lawn of anti-murine Igκ capture antibody, B cell hybridoma clones 12.4 and 30.1, each of which secretes GzB-specific mAb, and a third hybridoma clone that secretes a SARS-CoV-2 Spike-specific mAb, were plated at 200 cells per well, in the rows specified. His-tagged recombinant human GzB (rhGzB) was added in decreasing concentrations, as shown, and the captured rhGzB probe was detected via the His-tag. In the column on the right, replicate wells seeded with the B cell hybridomas, respectively, were detected using an anti-murine IgG detection capture antibody to verify the presence of IgG^+ secretory footprints. K_D values of the GzB-specific hybridomas for rhGzB, as established by surface plasmon resonance testing, are indicated on the right. *ND* not determined

1.6 B Cell Hybridoma Studies Validating ImmunoSpot® Affinity Measurements

While performing B cell affinity measurements using the inverted ImmunoSpot® approach outlined above seems simple and intuitive, this strategy has not yet been introduced. To experimentally validate this approach, we tested B cell hybridomas with established affinities for defined antigens. An example is shown in Fig. 2 using a pair of anti-human Granzyme B (GzB) hybridomas we generated during our efforts to raise mAbs against this protein. Among 7 such hybridomas, two clones secreted mAb at opposing ends of the antigen-retention spectrum when tested using an inverted ImmunoSpot® approach: GzB12.4 and GzB30.1. These two clones, along with an additional SARS-CoV-2 Spike-specific control hybridoma (*generously provided by Giuseppe A. Sautto*), were all plated at ~200 cells per well. Visualizing the secretory footprints with an anti-murine IgG detection antibody showed that most cells within each of the three hybridoma clones were capable of generating a secretory footprint and that the per-cell IgG productivity rates were comparable across the three (the SFU sizes and fluorescence intensities were similar between individual ASC within each hybridoma line and between the three B cell hybridoma clones (Fig. 2). When recombinant His-tagged human GzB (rhGzB) was added at a concentration of 500 ng/mL, the secretory footprints of both

GzB12.4 and GzB30.1 bound the antigen revealing ~200 SFU, whereas the footprints of the control hybridoma did not capture rhGzB. When the concentration of rhGzB was decreased in a 1:5 dilution series, footprints of GzB12.4 continued to capture this antigen down to a concentration of 4 ng/mL, whereas the secretory footprints of clone GzB30.1 were no longer discernable when the rhGzB probe was added at concentrations lower than 100 ng/mL. By ImmunoSpot®, clone GzB12.4 was identified as secreting mAb with high affinity for rhGzB, whereas clone GzB30.1 secreted mAb that appeared to possess a substantially lower affinity for this molecule. To verify by an independent method that this was indeed the case, we purified mAb from GzB12.4 and GzB30.1 and established their functional affinity for rhGzB using surface plasmon resonance (Biacore): a K_D value of 7.0×10^{-11} was calculated for mAb GzB12.4, while the K_D value of mAb GzB30.1 was 2.3×10^{-8}. Thus, both techniques concurred that clone GzB12.4 secreted mAb with a substantially higher functional affinity for rhGzB than the mAb produced by clone GzB30.1. Supplementary Figures 1 and 2 (*see* **Note 15**) provide further examples in which inverted ImmunoSpot® assays provided confirmatory results for hybridoma pairs secreting mAb with differences in functional affinity for alternative antigens; influenza hemagglutinin (H1) or SARS-CoV-2 Spike, respectively.

Another semiquantitative approach for assessing the affinity of individual ASC via ImmunoSpot® is based on the direct assay (Fig. 1b), studying the SFU morphologies on antigen-coated wells, in particular when coated with graded antigen densities. In direct B cell ImmunoSpot® assays, the membrane is either coated with the antigen itself, or adapter molecules are used for attaching the antigen to the membrane (*see* **Note 16**). As such, only ASC which produce an antibody with sufficient functional affinity for the membrane-associated antigen will be capable of generating a secretory footprint. Next to the per cell quantity of secreted antibody, the antibodies' functional affinity for the antigen defines the size and density characteristics of the resulting secretory footprint: ASC that produce high-affinity antibodies will create crisp and more intense spots, whereas ASC-producing antibodies with reduced functional affinity yield more diffuse and fainter/sparse footprints [18]. The difference between secretory footprints generated by high- or low-affinity ASC will be further accentuated when the bonus effect of multivalent binding is negated by reducing the antigen-coating density. At low antigen-coating densities, antibodies can only bind using a single "arm," i.e., the binding is defined by affinity alone; if the antigen is coated densely onto the assay membrane, however, the antibody can attach with both arms to neighboring antigen molecules and now avidity can amplify the antibody's functional affinity. Figure 3 illustrates the spot morphologies observed when murine B cell hybridomas secreting mAb with

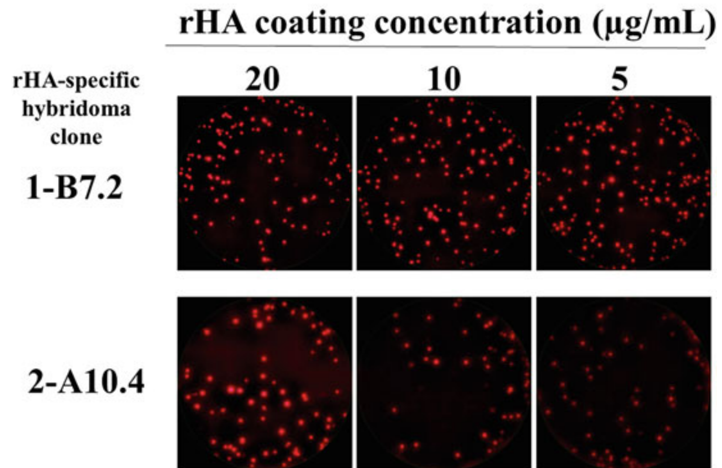

Fig. 3 Direct ImmunoSpot® assay leveraging limiting antigen coating reveals differential mAb functional affinity for influenza hemagglutinin. The specified murine B cell hybridomas, each secreting monoclonal antibody (mAb) reactive with recombinant hemagglutinin (rHA) protein (COBRA P1), however, with previously defined differential affinity [19], were plated at the Goldilocks numbers (100 cells per well) into wells that had been pre-coated with decreasing concentrations of rHA protein, as specified. The captured secretory footprints were visualized using anti-murine IgG1-specific detection antibodies. Secretory footprints from each of the B cell hybridoma lines were readily apparent in wells coated with a high concentration of rHA. However, the number of secretory footprints and their density declined at different rates for the two clones when hybridoma cells were seeded into wells coated with decreasing concentrations of rHA. Based on the results of this experiment, the mAb secreted by the 1-B7.2 B cell hybridoma line has an increased functional affinity for the coated rHA protein compared to the mAb secreted by 2-A10.4, confirming the previously published affinity hierarchy established by Sautto et al. [19]

differential functional affinities for an influenza hemagglutinin (H1) were evaluated for secretory footprint formation on membranes in which the antigen-coating density was progressively limited.

1.7 B Cell Affinity Distribution Measurements in PBMC with Inverted ImmunoSpot® Tests

After having experimentally validated the suitability of both ImmunoSpot® assay variants for B cell affinity measurements using hybridomas as model ASC, we set out to translate these tests for studies of antigen-specific ASC in human PBMC. As described above, B cells endowed with a wide affinity spectrum for the eliciting antigen participate in the primary immune response. However, progressively the engaged BCR repertoire is selected for those with increasingly high affinity, particularly in cases of prolonged or repetitive antigen exposure. Every individual's B_{mem} repertoire at a given time point is therefore a variable defining that person's capacity to engage in an effective anamnestic, adaptive humoral defense

reaction. One critical variable for evaluation is the clonal size of the antigen-specific B_{mem} compartment, as revealed by their frequency in PBMC. A second critical variable for assessment is the antibody class/subclass usage of antigen-specific B_{mem} since this will offer insight into what types of antibodies will be secreted acutely following antigen reencounter. Third, but not least, the frequency of high-affinity B_{mem} capable of producing the desired Ig class/subclass will define the efficacy of the anamnestic antibody response elicited in case of antigen reencounter. Importantly, all three of these parameters can readily be assessed using tailored B cell ImmunoSpot® assays.

As PBMC can be frozen without losing B cell functionality (*see* **Note 11**), we recommend undertaking high-resolution B cell ImmunoSpot® testing using aliquots of the same sample and a tiered approach. In the first experiment, the frequency of antigen-specific B_{mem}-derived ASC is established by performing serial dilutions of the cell input to define the Goldilocks number by linear regression (*see* **Notes 14** and **17**). Of note, this first experiment only requires ~2×10^6 cells per antigen to define the frequency of antigen-specific ASC secreting each Ig class/IgG subclass, respectively (*see* **Note 18** and see the chapter by Yao et al. in this volume [20] that is dedicated to such measurements). In a subsequent experiment, thawing an additional aliquot(s) of the PBMC sample, the affinity distribution of the antigen-specific ASC is established at the Goldilocks number, testing each antigen concentration in the same number of replicate wells. The number of PBMC needed for this second experiment depends on the Goldilocks number, the number of replicate wells needed to obtain a total of minimally 300 SFU, and the concentration range of antigen being evaluated. It can be calculated with high accuracy once the Goldilocks number has been established. In the example provided in Fig. 4, as detailed below, the number of PBMC needed to perform an inverted ImmunoSpot® assay encompassing 4 concentrations of SARS-CoV-2 Spike (receptor-binding domain, RBD) antigen probe, each evaluated in 10 replicate wells with ~50 SFU/well (thus assessing the affinity distribution within ~500 IgG^+ B_{mem} of this specificity) was 4×10^6 PBMC.

In Fig. 4, we present typical results obtained using the inverted ImmunoSpot® assay to assess the affinity distribution of antigen-specific ASC present within a human PBMC sample using limiting quantities of the antigen probe for their detection. In this case, cryopreserved PBMC collected from a healthy human donor 7 days following a second COVID-19 mRNA vaccination were tested. In the first experiment, the Goldilocks number for the SARS-CoV-2 RBD-specific IgG^+ ASC was determined for this PBMC sample (as described above) at 50 SFU/1×10^5 PBMC per well. In the initial test, a saturating concentration of the RBD probe was used and enabled the detection of all antigen-specific IgG^+ ASC;

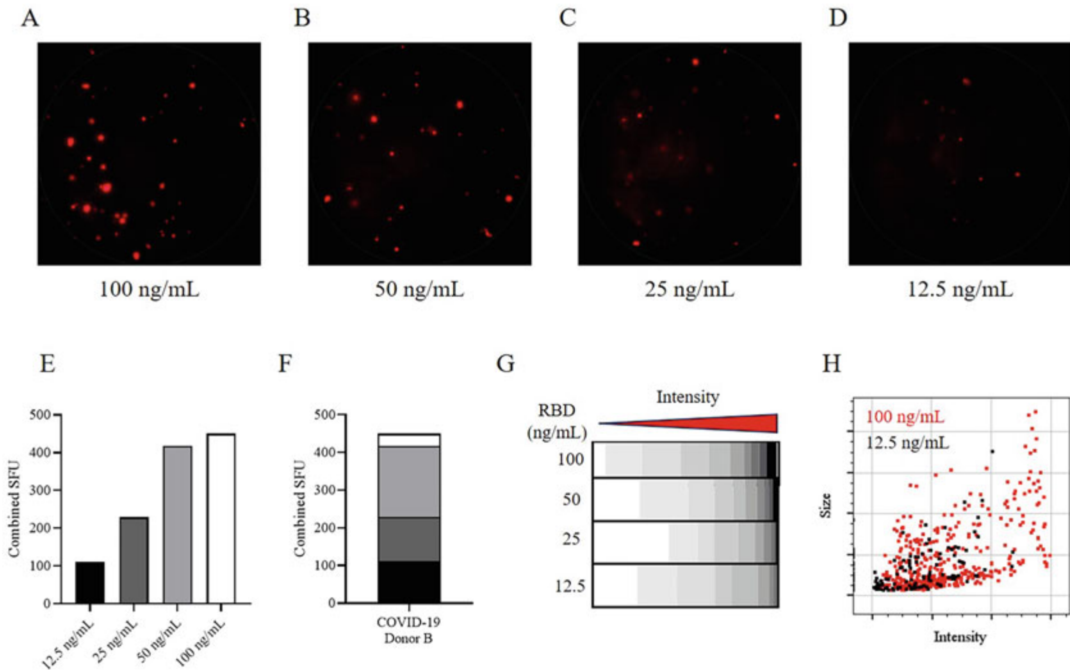

Fig. 4 Measuring B cell affinity distribution in PBMC with inverted ImmunoSpot® assays using limiting quantities of the antigen probe for detection. Cryopreserved PBMC collected from a healthy human donor 7 days following a second COVID-19 mRNA vaccination were tested. The PBMC were seeded into an inverted assay at 1×10^5 PBMC per well, which in a previous experiment was determined to result in ~50 SARS-CoV-2 Spike (RBD)-specific IgG$^+$ spot-forming units (SFU), a number suitable for studying individual secretory footprints. The His-tagged RBD probe was added to 10 replicate wells at the specified concentrations, followed by the detection of the His-tagged, plate-bound antigen. (**a**–**d**) Representative well images are shown for each RBD probe concentration. (**e**) Standard bar graph representation of cumulative SFU counts at each probe concentration. (**f**) Composite bar representation of the same data with color code specifying spot numbers detected at each probe concentration. (**g**) Intensity of spots detected at each RBD probe concentration was stratified into ten intensity groupings (denoted using grayscale) and the percentage of SFU residing in each "bin" is reflected by the width of the corresponding section. (**h**) Flow cytometry standard (FCS)-type intensity by size scatter plot analysis of the cumulative spots detected using 100 ng/mL or 12.5 ng/mL of the RBD probe, respectively

low- and high-affinity alike. Thawing a second aliquot of these cryopreserved cells, the PBMC were plated into 10 replicate wells for each of the four RBD probe concentrations tested at the Goldilocks number. In Fig. 4a–d, representative well images are shown for each RBD probe concentration. A reduction in SFU numbers is readily seen visually as the RBD probe concentration decreases. The corresponding SFU numbers are shown in a conventional bar diagram in Fig. 4e; most of the expected cumulative ~500 SFU were detected at 100 ng RBD/mL. Figure 4f shows these results in a composite bar diagram format that is better suited for comparing affinity distributions between different PBMC samples, e.g., to visualize affinity maturation for a subject's B cell response with repeat immunizations, or for representing such data for cohorts.

Figure 4 also illustrates that, next to the decrease in SFU, the fluorescence intensity of the individual secretory footprints changes as the RBD probe concentration decreases. Footprints of high-affinity ASC continue to stain bright as the RBD probe concentration declines, but ASC whose threshold of binding capability is approximated at the given probe concentration become fainter. Such transitions can be graphically captured. In Fig. 4g, the intensity of spots detected at each RBD probe concentration is stratified into ten intensity groupings, and the percentage of SFU residing in each "bin" is reflected by the width of the corresponding section. Flow cytometry standard (FCS)-type representation of ImmunoSpot® data also permits such further high-content data analysis. Figure 4h shows an FCS-type scatter plot representation (fluorescence intensity vs. size) of spot morphologies, and the difference seen between the highest and lowest RBD probe concentrations.

Inverted ImmunoSpot® assays are particularly well-suited for measuring B cell affinity distributions when the frequency of antigen-specific ASC producing the Ig class/subclass to be captured on the membrane (e.g., IgG) is relatively high within all ASC present in the sample producing that particular Ig class/subclass (*see* **Note 19**). This is often the case when studying plasmablasts that transiently circulate in the blood acutely (5–9 days) after initiation of a B cell response (*see* **Note 20**). If the frequency of antigen-specific ASC vs. total ASC is low in a given sample, direct ImmunoSpot® assays offer an alternative approach for measuring the affinity distribution of B_{mem}-derived ASC.

1.8 B Cell Affinity Distribution Measurements in PBMC with Direct ImmunoSpot® Tests

Direct ImmunoSpot® assays inherently reveal information about ASC affinity. Following basic rules of antigen–antibody binding, ASC that produce high-affinity antibodies will leave dense and sharp secretory footprints on membrane-bound antigens, while ASC-producing antibodies with lower functional affinity for the membrane-associated antigen will form faint and diffuse spots [18]. Performing direct ImmunoSpot® assays under conditions when the membrane is coated with graded antigen densities helps to further enhance such affinity studies. Typical results for these types of tests done on human PBMC are shown in Fig. 5, in this case using PBMC from a COVID-19 mRNA vaccine recipient. Like for the inverted ImmunoSpot® assay, here too the cells were plated into all replicate wells at an assay-specific, Goldilocks number (3×10^4 PBMC/well) needed to achieve 50 Spike-specific SFU/well. A direct ImmunoSpot® assay was performed in which, however, the Spike protein coating density was graded. In Fig. 5a–d, representative well images are shown for each coating concentration of Spike protein. Here too, a reduction in SFU numbers is seen as the coating density was decreased. The corresponding SFU numbers are shown in a conventional bar diagram in Fig. 5e, and a composite bar diagram format in Fig. 5f.

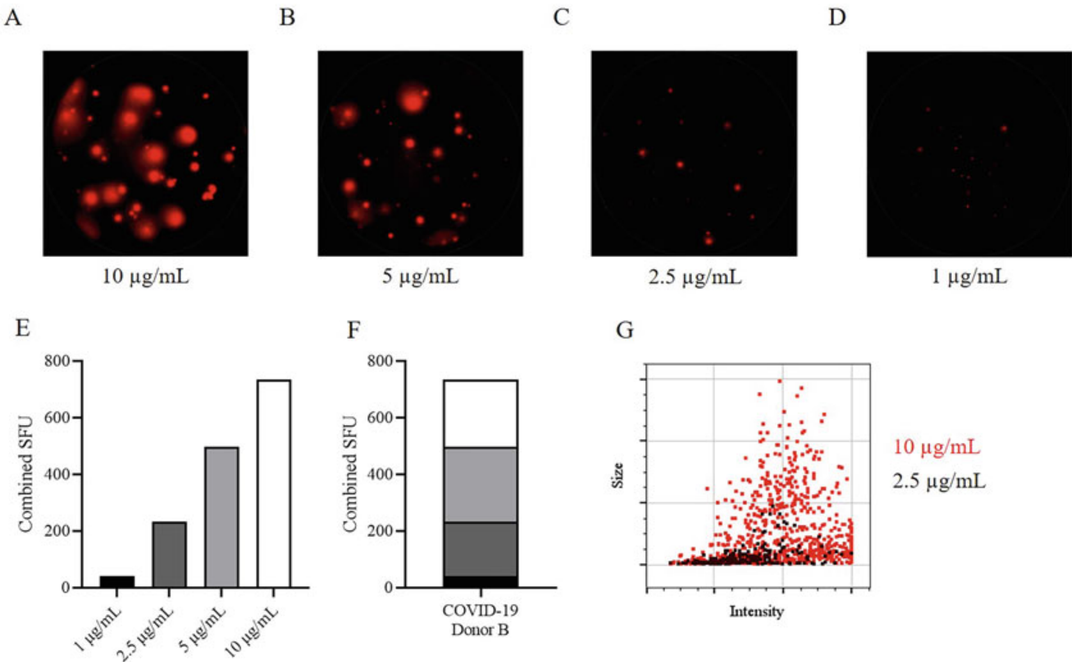

Fig. 5 ImmunoSpot® enables the evaluation of ASC affinity through limiting antigen coating. Cryopreserved PBMC collected from a healthy human following a second COVID-19 mRNA vaccination were tested. The PBMC were seeded into the assay at 3×10^4 PBMC per well, which in a previous experiment was determined to result in ~50 SARS-CoV-2 Spike-specific IgG$^+$ spot-forming units (SFU), a number suitable for studying individual secretory footprints. Assay wells were initially coated with anti-His capture antibody, followed by affinity capture of Spike protein at 10, 5, 2.5, or 1 µg/mL, in 20 replicate wells, respectively. (**a**–**d**) Representative well images are shown for each Spike coating concentration. (**e**) Standard bar graph representation of cumulative SFU counts at each Spike coating concentration. (**f**) Composite bar representation of the same data with color code specifying spot numbers detected at each Spike coating concentration. (**g**) Flow cytometry standard (FCS)-type intensity by size scatter plot analysis of the cumulative spots detected in wells coated with either 10 or 2.5 µg/mL of Spike protein, respectively

In this chapter, we present data demonstrating that B cell ImmunoSpot® assays are capable of distinguishing between model ASC (murine B cell hybridomas) known to secrete mAb with variable functional affinity for defined antigens. Further, through characterizing the B cell response elicited in COVID-19 mRNA vaccine recipients, we highlight the utility of antigen-specific, inverted, and direct ImmunoSpot® assays for characterizing the functional affinity distribution of the antigen-specific B cells present in PBMC. In the following *Materials* and *Methods* section, we provide detailed protocols for performing such inverted and direct ImmunoSpot® assays. Here we also refer to a chapter by Yao et al. [21] in this volume that describes defining of Goldilocks number in detail, and to a chapter by Karulin et al. [29] on high-content image analysis of B cell-derived SFU. The underlying B cell biology is elaborated in greater detail in a chapter by Lehmann et al. [18], also in this volume.

2 Materials

2.1 Thawing of Cryopreserved PBMC

1. Class II biosafety cabinet (BSC)
2. Cryopreserved PBMC sample(s) (*see* **Note 11**).
3. 70% (v/v) ethanol (EtOH)
4. DNase-containing washing medium (pre-warmed to 37 °C) (*see* **Note 21**).
5. Complete B cell medium (BCM) (pre-warmed to 37 °C) (*see* **Note 22**).

2.2 In Vitro Polyclonal Stimulation of B Cells in PBMC

1. B-Poly-S
2. 25 cm^2 sterile culture flask
3. Humidified incubator set at 37 °C, 5% CO_2

2.3 Antigen-Specific, Single-Color Inverted FluoroSpot Assay

1. Commercially available, single-color human IgG Inverted (His) B cell kit (*see* **Note 23**).
2. His-tagged recombinant protein (*see* **Note 24**).
3. 190 proof (95%) EtOH
4. Cell culture-grade water.
5. Reagent reservoir(s) (sterile).
6. 0.05% Tween-PBS wash solution
7. 0.1 μm low-protein binding syringe filter
8. Plate washer.
9. Vacuum manifold.
10. ImmunoSpot® S6 Ultimate 4 LED Analyzer, or a suitable instrument equipped with the appropriate detection channels, running CTL's ImmunoSpot® UV.

2.4 Antigen-Specific FluoroSpot Assay (Affinity Capture Coating)

1. Commercially available, single-color human IgG affinity capture (His) B cell kit (*see* **Note 25**).
2. His-tagged recombinant protein (*see* **Note 26**).

3 Methods

3.1 Thawing of Cryopreserved PBMC (Sterile Conditions)

1. Place cryovial(s) into a 37 °C bead bath, or better, glass bead bath, for 8 min to thaw.
2. Remove cryovial(s) and wipe with 70% EtOH inside the BSC before unscrewing the cap(s).
3. Using a sterile pipette, transfer contents of cryovial(s) into a labeled conical tube (if applicable, up to 5 vials of the same donor's cell material can be pooled in one conical tube).

4. Rinse each of the cryovials with 1 mL of warm anti-aggregate solution. Add the warm rinse solution to the conical tube dropwise while swirling the tube to ensure its adequate mixing with the cells in the thawing medium.
5. Double the volume of the cell suspension by dropwise addition of warm anti-aggregate solution while swirling the tube to ensure adequate mixing of the cells and thawing medium.
6. Continue doubling the volume of the cell suspension by dropwise addition of warm anti-aggregate solution while swirling the tube until the cryopreserved cell material has been diluted ten-fold. If multiple cryovials are pooled, calculate using 1 mL of cryopreserved cell suspension + 9 mL of anti-aggregate solution to determine the necessary final resuspension volume.
7. Centrifuge balanced tubes at 330 × *g* for 10 min with the centrifuge brake on, non-frigerated.
8. Decant supernatant and resuspend the cell pellet(s) using pre-warmed B cell medium (BCM) to achieve a cell density of ~2–5 × 10^6 cells/mL. You may estimate this number assuming a typical recovery of 70–80% of the frozen PBMC.
9. Pipet 15 μL of live/dead cell counting dye onto a piece of parafilm to form a droplet.
10. Remove 15 μL of cell suspension and combine with a droplet of live/dead cell counting dye. Pipet up and down three to five times to mix the sample while avoiding the formation of bubbles.
11. Transfer 15 μL of the cell and dye suspension into each chamber of a hemacytometer.
12. Determine live cell count and viability using CTL's live/dead cell counting suite.
13. Increase volume of cell suspension(s) with additional sterile PBS and centrifuge balanced tubes at 330 × *g* for 10 min with a centrifuge, non-refrigerated, brake on.
14. Decant supernatant and gently resuspend the cell pellet(s) using pre-warmed BCM at a cell density of ~2–4 × 10^6 cells/mL.

3.2 In Vitro Polyclonal Stimulation of B Cells in PBMC (Sterile Conditions)

1. Dilute CTL's B-Poly-S polyclonal stimulation reagent 1:500 into prewarmed BCM to achieve a final concentration of 2X. Into labeled sterile culture vessels, add 50% vol of BCM containing 2X concentration of B-Poly-S.
2. Add the same volume of cell suspension at ~2–4 × 10^6 cells/mL to achieve a final culture at ~1–2 × 10^6 cells/mL with 1X potency of CTL's B-Poly-S polyclonal stimulation reagent (*see* **Note 27**).
3. Transfer culture vessels (flasks or plates) into a humidified incubator set at 37 °C, 5% CO_2 for 4–6 days (96–144 h).

3.3 Antigen-Specific, Single-Color Human IgG Inverted FluoroSpot Assay (Limiting Antigen Probe) (See Notes 19, 28, and 29)

1. One day before plating cells (Day 1), prepare 70% EtOH and anti-human IgG capture antibody solutions.
2. Remove underdrain and pipet 15 μL of 70% EtOH solution into the center of each well (or designated wells) of the assay plate. Immediately after the addition of the 70% EtOH solution to the entire plate (or designated wells), add 180 μL/well of PBS (*see* **Note 30**). Decant and wash wells again with 180 μL/well of PBS.
3. Decant the assay plate, replace underdrain, and immediately add 80 μL/well of the anti-human IgG capture antibody solution into each well (or designated wells) of the low autofluorescence PVDF-membrane plate provided with the kit (*see* **Note 31**).
4. Incubate the plate overnight at 4 °C in a humified chamber.
5. On the day of the assay (Day 0), decant the assay plate and wash wells with 180 μL/well of PBS. Next, decant the plate and add 150 μL/well of pre-warmed BCM to block the plate (≥1 h at RT).
6. If using PBMC following polyclonal activation in vitro, collect the cell suspension(s) and transfer it into labeled conical tube(s). Wash culture vessel(s) interior to recover remaining cells with sterile PBS and transfer to the corresponding conical tube(s). Increase the volume to fill the conical tube with additional PBS and then centrifuge balanced tubes at 330 × *g* for 10 min with the centrifuge brake on. Alternatively, thaw previously cryopreserved PBMC as detailed above if prior in vitro stimulation is not required to elicit antigen-specific ASC activity in the sample(s).
7. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM to achieve a cell density of approximately $2–5 \times 10^6$ cells/mL.
8. Pipet 15 μL of live/dead cell counting dye onto a piece of parafilm to form a droplet.
9. Remove 15 μL of cell suspension and combine with a droplet of live/dead cell counting dye. Pipet up and down three to five times to mix the sample while avoiding the formation of bubbles.
10. Transfer 15 μL of the cell and dye suspension into each chamber of a hemacytometer.
11. Determine live cell count and viability using CTL's live/dead cell counting suite.
12. Increase volume of cell suspension(s) with additional sterile PBS and centrifuge balanced tubes at 330 × *g* for 10 min with centrifuge brake on (*see* **Note 32**).

13. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM at the pre-determined cell density to achieve the so-called "Goldilocks" cell input of 50 SFU/well (*see* **Notes 19, 28,** and **29**).
14. Decant the BCM used for blocking the assay plate and replace it with 100 μL/well of pre-warmed BCM to block the plate.
15. Into a reagent reservoir, add cell suspension adjusted to a density of 500 SFU/mL (*see* **Note 14**). Using a multichannel pipet, transfer 100 μL of the cell suspension(s) into designated wells of the assay plate. Gently tap the edges of the ImmunoSpot® assay plate to ensure equal distribution of the cell input.
16. Incubate cells in the assay plate for 16–18 h at 37 °C, 5% CO_2 (*see* **Note 33**).
17. After completion of the assay incubation period, remove the plate and decant cells. Wash the plate two times with PBS (200 μL/well), followed by two additional washing steps with 0.05% Tween-PBS wash solution (*see* **Note 34**).
18. Prepare His-tagged antigen probe solution at limiting concentrations (*see* **Note 35**) according to kit protocol and pass through a 0.1 μm low protein binding syringe filter to remove any protein aggregates.
19. Decant 0.05% Tween-PBS wash solution, add 80 μL/well of the various His-tagged antigen probe solutions (containing limiting/graded quantities of antigen probe) into designated wells, and incubate for 2 h at RT (protected from light).
20. Wash plate(s) two times with 0.05% Tween-PBS wash solution.
21. Prepare anti-His detection antibody solution according to kit protocol and pass through a 0.1 μm low-protein binding syringe filter to remove any aggregates.
22. Decant 0.05% Tween-PBS wash solution, add 80 μL/well of anti-His detection antibody solution into designated wells, and incubate for 1 h at RT (protected from light).
23. Wash plate(s) two times with 0.05% Tween-PBS wash solution.
24. Prepare tertiary solution according to kit protocol and pass through a 0.1 μm low-protein binding syringe filter to remove any aggregates.
25. Wash plates(s) twice with distilled water.
26. Remove the protective underdrain and place the plate face down on the vacuum manifold. Completely fill the backside of the plate with distilled water and apply a vacuum to draw water through the membrane ("back to front") (*see* **Note 36**).

27. Allow the plate to dry completely, protected from light (*see* **Note 37**).
28. Scan and count plate(s) with a suitable analyzer equipped with the appropriate detection channel (*see* **Note 38**).

3.4 Antigen-Specific (Affinity Capture Coating) FluoroSpot Assay (Limiting Antigen-Coating Concentrations)

1. Two days before plating cells (Day 2), prepare 70% EtOH and anti-His affinity capture antibody solutions.
2. Remove underdrain and pipet 15 μL of 70% EtOH solution into the center of each well (or designated wells) of the assay plate. Immediately after the addition of the 70% EtOH solution to the entire plate (or designated wells), add 180 μL/well of PBS (*see* **Note 30**). Decant and wash wells again with 180 μL/well of PBS.
3. Decant the assay plate, replace underdrain, and immediately add 80 μL/well of the anti-His affinity capture antibody solution into each well (or designated wells) of the low autofluorescence PVDF-membrane plate provided with the kit (*see* **Note 39**).
4. Incubate the plate overnight at 4 °C in a humified chamber.
5. The following day (Day 1) dilute the His-tagged protein (s) into diluent B (provided with the kit) at pre-determined limiting/graded concentrations (*see* **Note 26**).
6. Decant the assay plate and wash wells with 180 μL/well of PBS. Immediately, add 80 μL/well of the corresponding His-tagged protein coating solutions into the designated wells.
7. Incubate the plate overnight at 4 °C in a humidified chamber.
8. On the day of the assay (Day 0), decant the assay plate and wash wells with 180 μL/well of PBS. Next, decant the plate and add 150 μL/well of pre-warmed BCM to block the plate (≥1 h at RT).
9. If using PBMC following polyclonal activation in vitro, collect the cell suspension(s) and transfer it into labeled conical tube (s) (*see* **Note 27**). Wash culture vessel(s) interior with sterile PBMC and also transfer into the corresponding conical tube (s). Increase volu vitrome to fill up tube(s) with additional PBS and then centrifuge balanced tubes at 330× *g* for 10 min with centrifuge brake on. Alternatively, follow the procedures detailed above to obtain freshly isolated PBMC, or to thaw PBMC that were previously cryopreserved, if prior in vitro stimulation is not required to elicit antigen-specific ASC activity in the sample(s).
10. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM to achieve a cell density of approximately 2–5 × 10^6 cells/mL.

11. Pipet 15 μL of live/dead cell counting dye onto a piece of parafilm to form a droplet.
12. Remove 15 μL of cell suspension and combine with a droplet of live/dead cell counting dye. Pipet up and down three to five times to mix the sample while avoiding the formation of bubbles.
13. Transfer 15 μL of the cell and dye suspension into each chamber of a hemacytometer.
14. Determine live cell count and viability using CTL's live/dead cell counting suite.
15. Increase volume of cell suspension(s) with additional sterile PBS and centrifuge balanced tubes at 330× *g* for 10 min with centrifuge brake on (*see* **Note 32**).
16. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM at the pre-determined cell density to achieve the so-called "Goldilocks" cell input of 50 SFU/well.
17. Decant the BCM used for blocking the assay plate and replace it with 100 μL/well of pre-warmed BCM to block the plate.
18. Into a reagent reservoir, add cell suspension adjusted to a density of 500 SFU/mL (*see* **Note 18**). Using a multichannel pipet, transfer 100 μL of the cell suspension(s) into designated wells of the assay plate. Gently tap the edges of the ImmunoSpot® assay plate to ensure equal distribution of the cell input.
19. Incubate cells in the assay plate for 16–18 h at 37 °C, 5% CO_2 (*see* **Note 33**).
20. After completion of the assay incubation period, remove the plate and decant cells. Wash the plate two times with PBS (200 μL/well), followed by two additional washing steps with 0.05% Tween-PBS wash solution (*see* **Note 34**).
21. Prepare detection antibody solution by following kit protocol and pass through a 0.1 μm low protein binding syringe filter to remove any aggregates.
22. Decant 0.05% Tween-PBS wash solution, add 80 μL/well of detection antibody solution into designated wells, and incubate for 2 h at RT (protected from light).
23. Wash plate(s) two times with 0.05% Tween-PBS wash solution.
24. Prepare a tertiary solution by following kit protocol and pass through a 0.1 μm low protein binding syringe filter to remove any aggregates.
25. Decant 0.05% Tween-PBS wash solution, add 80 μL/well of tertiary solution into designated wells, and incubate for 1 h at RT (protected from light).
26. Wash plates(s) twice with distilled water.

27. Remove the protective underdrain and place the plate face down on the vacuum manifold. Completely fill the backside of the plate with distilled water and apply a vacuum to draw water through the membrane ("back to front") (*see* **Note 36**).
28. Allow the plate to dry completely, protected from light (*see* **Note 37**).
29. Scan and count plate(s) with a suitable analyzer equipped with the appropriate detection channels (*see* **Note 38**).

4 Notes

1. The unit in which antibody affinity is measured is molarity (M). It defines the concentration of soluble antibody required to attain 50% of the maximal antigen binding capacity, e.g., by surface plasmon resonance (SPR) or biolayer interferometry (BLI).
2. There are 129 functional V (variable), 27 D (diversity), and 9 J (joining) gene segments available within the human *IgH* locus located on chromosome 14 [21]. In concert with the large number of V and J gene segments present within the Igκ (chromosome 2) and Igλ (chromosome 22) loci, respectively, plus the addition and deletion of nucleotides at the junctional borders of the recombined gene segments, and the pairing of IgH and IgL proteins to generate a functional B cell receptor (BCR), it is estimated that up 10^{14} different BCR specificities can be created through combinatorial diversity. As the adult human body has ~10^{12} lymphocytes, of which 1–10% in the blood are B cells, one can estimate that when an antigen is first encountered, there are very few naive B cells in the entire body that are endowed with a specific BCR.
3. An immune response requires that B and T cells encounter antigens in a secondary lymphoid tissue, such as a draining lymph node (LN) or the spleen. Naive B and T cells continuously recirculate among secondary lymphoid tissues, arriving there via the bloodstream and entering via the "lymph node homing receptors" they express. After searching the secondary lymphoid tissue for the presence of "their" antigen, if such is absent, they return to the blood via the afferent lymph vessels to continue their surveillance of other secondary lymphoid tissues, as well. Antigens are transported to the regional LN (s) that drain these areas of the body. The antigens arrive in that LN(s) either with the efferent lymph fluid or via dendritic cells actively transporting them. In this way, the three essential components of an efficient immune response (antigen, antigen-specific B cells, and antigen-specific T helper cells) are all

brought together in the LN(s). The architecture of the LN is also specialized in that it provides the ideal microenvironment for the ensuing immune response to evolve.

4. Naive T cells undergoing a primary immune response in secondary lymphoid tissues are sensitive to the type of cytokine/chemokine microenvironment created by cells of the innate immune system, as the latter become activated by pattern receptor-perception of the antigen. In this way, through different local microenvironments, Th1, Th2, Th17, and other type of T cell lineages arise. The process of Ig class switch recombination, in turn, is dictated by the type of cytokines these "helper" T cells secrete in response to recognizing cognate peptides presented by antigen-specific B cells. Engaging the "right" class/subclass of T and B cell responses is critical for mounting an effective antigen clearance/control without inciting collateral immunopathology.
5. While still not fully elucidated, plasma cells require specific stimuli provided by stromal and other accessory cells for their longevity and long-term residence in specialized survival niches [13, 22].
6. The half-life of IgG1, IgG2, and IgG4 in humans is 21–28 days, and it is 7 days for IgG3 [23]. The half-life of IgA and IgM is even shorter (3–7 days) [24, 25]. IgE has a half-life in serum of 2–3 days [26].
7. While antibody-secreting plasma cells are tissue-resident in the bone marrow or other specialized survival niches, memory B cells (B_{mem}) recirculates among secondary lymphoid tissues, just as naive B cells do (see also **Note 3**). Returning to the bloodstream via efferent lymph vessels, the B_{mem} are carried with the bloodstream where they egress into remote secondary lymphoid tissues and survey for their cognate antigen. By sampling blood, one can therefore gain insights into the B_{mem} compartment present in the body.
8. Defining the affinity distribution of, e.g., 300 antigen-specific B cells in an individual by SPR (or BLI) would mean having to either establish 300 antigen-specific B cell hybridoma clones, or generate 300 paired *IgH*/*IgL* sequences and suitable expression constructs, to express and purify these 300 monoclonal antibodies (mAb), and then to test the affinity of each of the 300 mAbs, one by one. Compare this effort and cost to the ease with which information on the affinity distribution of antigen-specific B_{mem}/ASC can be obtained via the ImmunoSpot® approach introduced here.
9. To the choice of capture antibody: when plasmablast populations elicited acutely (days 5–9) following induction of a B cell response are being studied, most of the spontaneously

producing ASC in the blood will be antigen-specific. In such a case, the use of a pan Ig-specific (anti-kappa + anti-lambda light chain) capture antibody is justified and enables assessment of all classes and subclasses of antigen-specific antibodies, including IgM. In this case, IgM^+ secretory footprints are inferred to originate from antigen-specific B cells engaging in a primary immune response. Furthermore, such inverted assays performed using pan Ig-specific capture conditions can be multiplexed with detection reagents which enable resolving the Ig class/subclass usage of distinct antigen-specific secretory footprints.

10. When inverted ImmunoSpot® assays are performed with in vitro polyclonally stimulated PBMC, however, third-party IgA and IgM-producing B cells are also induced in high numbers and readily compete for "real estate" on membranes coated with pan Ig capture antibodies. Moreover, the presence of IgM-derived secretory footprints generated by naive B cells possessing broad or highly cross-reactive specificities also hinders the specificity of such inverted assays. To overcome these obstacles, we recommend the usage of Ig class-specific capture antibodies; such as the anti-human IgG capture antibody shown in Fig. 1. In this way, only in vivo class-switched B memory cells are being interrogated in the ImmunoSpot® test.
11. Peripheral blood mononuclear cells (PBMC) can be cryopreserved without loss of spontaneous, or polyclonal stimulation-induced ASC activity ([27] and Becza et al., manuscript in preparation). This permits "direct ex vivo" batch testing of samples irrespective of the time-point of their actual collection. By freezing the cells in aliquots, assay results can be reproduced with high accuracy [17], and/or follow-up experiments can be performed building on the previous test result that establishes the "Goldilocks number" of antigen-specific ASC in a given PBMC sample, as described in **Note 14**. It is important for planning cryopreservation of PBMC to know that any number of PBMC can be frozen per vial between 1 and 10 million PBMC, in each case recovering at thawing about 90% viable and fully functional B cells, and that after 5 days of in vitro polyclonal B cell stimulation about 50% of these cells will be recovered as viable cells for testing (Becza et al., manuscript in preparation).
12. In the inverted assay, as shown in Fig. 1a, the capture of antibodies produced by ASCs occurs at a fixed high affinity, that of the anti-IgG capture antibody for the ASC-derived IgG. Only the net amount of IgG produced per ASC defines the size and density of the resulting secretory footprint. The affinity of the antibody captured in the footprint will be revealed, however, when the antigen is added at various concentrations.

13. The B cells survive the ImmunoSpot® assay unharmed and can potentially be re-used in subsequent functional assays, for cloning, or analysis via means of molecular biology.
14. In the initial test, it is suggested to plate PBMC (or other single-cell suspensions) in serial dilution over a wide range of cell numbers per well because the frequencies of antigen-specific ASC can span orders of magnitudes between individuals [17] and ASC-producing different classes and subclasses of antigen-specific (and total) Ig also spans a wide range within an individual, and in the human population. In this way, not only the frequency of ASC can be reliably established by linear regression analysis [28] within the entire cell material tested but the Goldilocks number for secretory footprints can be defined as the maximal number of cells that can be plated while still being able to discern clearly individual secretory footprints. This number for an optimized, antigen-specific (SARS-CoV-2 Spike RBD) inverted assay is 50 (maximally 100) SFU/well using anti-IgG specific capture antibodies and B_{mem}-derived ASC following in vitro polyclonal stimulation of PBMC [17]. Once the Goldilocks number has been established, in a second experiment, the cell number(s) can be chosen accordingly for creating replicates containing ≥300 individual ASC-derived footprints per antigen concentration for studying the functional affinity distribution.
15. Due to spacing limitations precluding their inclusion, two additional supplementary figures for this chapter can be found at https://immunospot.com/supplfigs-affinity
16. Absorption of biomolecules to the hydrophobic PVDF membrane can occur with variable efficiency and the coating concentration required for reliable detection of antigen-specific ASC may be prohibitive; and even when raising the coating antigen concentration "indefinitely," no, or no satisfactory antigen coating may be achieved. Affinity coating readily circumvents this problem: it is illustrated in Fig. 4 of the chapter by Lehmann et al. in this volume [29].
17. A similar linear regression approach can also be used for defining the Goldilocks number of in vivo-differentiated ASC such as circulating plasmablasts or bone marrow/tissue-resident plasma cells.
18. To establish the frequency, and Ig class or IgG subclass usage, of B_{mem}-derived ASC for one antigen using a direct ImmunoSpot® assay approach, a typical serial dilution experiment requires (with a safety margin) 2×10^6 PBMC that can be obtained from 2 mL of blood, as follows: one starts the PBMC dilution series with 2×10^5 PBMC per well to permit the detection of relatively low frequency B_{mem}-derived ASC as well, and progresses with a 1 + 1 (two-fold) serial dilution of

the cells. Importantly, frequency assessments done with single wells per cell dilution provide frequency assessments with essentially the same accuracy as serial dilutions done with 4 replicate wells (see Yao et al. in this volume [21]). Inclusion of negative control wells seeded with 2×10^5 and 5×10^4 PBMC is also recommended but not required; cell material permitting. Thus, ~6.5×10^5 PBMC will be needed on day 5 from in vitro polyclonal stimulation cultures, for which 1.3 million (for safety margin 2×10^6) freshly isolated PBMC need to be cryopreserved accommodating the 50% cell recovery after in vitro stimulation. The cell numbers recovered on day 5 from such in vitro cultures even permit to establish the frequency of all IgA-, IgG- and IgM-producing B cells in that sample; for such measurements, the serial dilution typically starts with an input of 2×10^4 cells per well. Note the efficiency of PBMC utilization for such B cell ImmunoSpot® assays.

19. Owing to polyclonal stimulation of B_{mem} to trigger their terminal differentiation, a large majority of IgG^+ ASC will not be antigen-specific yet will compete for "real estate" on the lawn of anti-IgG capture reagent used for coating. Consequently, inverted assays aimed at studying lower frequency ASC specificities are directly limited by the maximal number of total IgG^+ ASC that can be input into a single well while still maintaining the ability to resolve individual antigen-specific secretory footprints.
20. The plasmablasts that can be detected circulating in the blood 5–9 days after onset of a B cell response are trafficking from the draining lymph nodes to the bone marrow where they may become resident as plasma cells.
21. Thawing of cryopreserved cells causes a fraction of the cells (up to 20%) to die, and the DNA released from such cells can cause clumping of the thawed cell material. This cell clumping can be reduced, if not completely eliminated, by including an immunologically neutral endonuclease, Benzonase. Ready-to-use Benzonase-containing, serum-free wash solutions are available: CTL Anti-Aggregate Wash™ 20X Solution.
22. A suitable assay medium for use in B cell ImmunoSpot® is RPMI 1640 with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 8 mM HEPES, and 50 μM 2-mercaptoethanol.
23. Kit is suited for detecting either antigen-specific ASC that differentiated in vivo, or antigen-specific B_{mem} that have been polyclonally stimulated in vitro to promote their transition to ASC. Each kit contains an anti-human IgG-specific capture antibody, anti-His detection reagents, diluent buffers, low autofluorescence PVDF-membrane plates, and polyclonal B cell activator (B-Poly-S).

24. The optimal concentration of affinity (His)-tagged antigen probe used for detection of all antigen-specific secretory footprints (e.g., SFU), low- or high-affinity alike, should be determined empirically. Likewise, prior optimization of limiting antigen probe concentrations used to resolve ASC with differential functional affinity is highly recommended.
25. Kit is suited for detecting either antigen-specific antibody-secreting cells (ASC) that differentiated in vivo, or antigen-specific B_{mem} that have been polyclonally stimulated in vitro to promote their transition to ASC. Each kit contains anti-His capture antibodies, detection reagents, diluent buffers, low autofluorescence PVDF-membrane plates, and polyclonal B cell activator (B-Poly-S).
26. For most His-tagged proteins, high-density coating of the assay membrane can be achieved via affinity capture of a 10 μg/mL solution. Lower protein coating concentrations reduce the density of membrane-associated antigens and concomitantly increase the minimal functional affinity of an antigen-specific ASC to generate a detectable secretory footprint.
27. The volume of in vitro stimulation cultures can be scaled up or down but we recommend keeping the cell density of PBMC at ~$1–2 \times 10^6$ cells/mL. If larger numbers of in vitro stimulated PBMC are required for downstream ImmunoSpot® assays, two or more 25 cm^2 flasks can be set up per donor or larger 75 cm^2 culture flasks can be used with a final volume of 20–25 mL. Likewise, smaller in vitro stimulation cultures can be initiated in 24-well plates with a final volume of 2 mL. Be sure to fill empty wells in tissue culture plates with sterile PBS to avoid dehydration of cell cultures.
28. Prior to performing an inverted ImmunoSpot® assay using limiting quantities of antigen detection probe, it is recommended to first determine the Goldilocks cell input to achieve ~50 SFU/well using an aliquot of cryopreserved cell material (see also **Note 11**).
29. In such instances when the population of antigen-specific ASC is low among all ASC, we recommend increasing the number of replicate wells and seeding at lower cell inputs. Moreover, to conserve on cell material required, increasing the fold dilution of the antigen probe and/or testing only at pre-determined concentrations are both valid options.
30. Activation of the PVDF membrane with 70% EtOH is instantaneous and can be seen visually as a graying of the membrane. It is important to be sure that the EtOH solution has spread across the entire membrane before adding the first wash of PBS. If needed, tapping the plate can promote contact of the

EtOH solution with the PVDF membrane. We recommend only pre-wetting one plate at a time with 70% EtOH to ensure that the contact time is ≤1 min; longer contact times may promote leaking of the membrane and result in suboptimal assay performance.

31. If the entire plate will not be coated with the anti-human IgG capture antibody solution, the remainder of the EtOH pre-wet wells should receive 80 μL/well of PBS.
32. Contaminating antibody in the cell suspension(s) is efficiently captured by the anti-Ig capture antibody and may also result in elevated membrane staining that interferes with accurate enumeration of individual antigen-specific ASC in inverted ImmunoSpot® assays.
33. Shorter B cell ImmunoSpot® assay incubation times are suggested if using an enzymatic-based detection approach to avoid merging of spots and/or elevated membrane background staining.
34. Plate washes may also be performed manually. For automated washing, the pin height and flow rate should be customized to avoid damaging the assay membranes, which is the case for the CTL 405LSR plate washer.
35. Antigen probe concentrations can be generated through serial dilution (two-fold, three-fold, or five-fold) or by directly adding fixed concentrations of antigen probe into the diluent provided with the ImmunoSpot® assay kit.
36. Optimal removal of fibers and other debris, along with the reduction of "hot spots" in the center of the assay wells, is achieved through performing the "back to front" water filtration technique.
37. To completely dry plates, blot assay plate(s) on paper towels to remove residual water before either placing them in a running laminar flow hood at a 45° angle for >20 min or placing them face down on paper towels for >2 h. in a dark drawer/cabinet. Do not dry assay plates at temperatures exceeding 37 °C as this may cause the membrane to warp or crack. Fluorescent spots may not be readily visible while the membrane is still wet and the background fluorescence may be elevated. Scan and count plates only after membranes have dried completely.
38. The chapter by Karulin et al. in this volume [28] introduces machine learning-based spot-forming unit (SFU) analysis that can partially compensate for ELISA effects and SFU crowding, thus extending the linear range of accurate quantification for cell numbers plated per well and SFU detected.

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Conflicts of Interest P.V.L. is Founder, President, and CEO of CTL, a company that specializes in immune monitoring by ImmunoSpot®. N.B., Z.L., J.C., and G.A.K. are employees of CTL. The data X.G. contributed to this chapter (Fig. 2) were generated during his employment at CTL.

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