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Artificial intelligence-based counting algorithm enables accurate and detailed analysis of the broad spectrum of spot morphologies observed in antigen-specific B cell ELISPOT and FluoroSpot assays

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Abstract

Antigen-specific B cell ELISPOT and multicolor FluoroSpot assays, in which the membrane-bound antigen itself serves as the capture reagent for the antibodies that B cells secrete, inherently result in a broad range of spot sizes and intensities. The diversity of secretory footprint morphologies reflects the polyclonal nature of the antigen-specific B cell repertoire, with individual antibody-secreting B cells in the test sample differing in their affinity for the antigen, fine epitope specificity, and activation/secretion kinetics. To account for these heterogeneous spot morphologies, and to eliminate the need for setting up subjective counting parameters well-by-well, CTL introduces here its cutting-edge deep learning-based IntelliCount™ algorithm within the ImmunoSpot® Studio Software Suite which integrates CTL's proprietary deep neural network. Here, we report detailed analyses of spots with a broad range of morphologies that were challenging to analyze using standard parameter-based counting approaches. IntelliCount™, especially in conjunction with high dynamic range (HDR) imaging, permits the extraction of accurate, high-content information of such spots, as required for assessing the affinity distribution of an antigen-specific memory B cell repertoire *ex vivo*. IntelliCount™ also extends the range in which the number of antibody-secreting B cells plated and spots detected follow a linear function; that is, in which the frequencies of antigen-specific B cells can be accurately established. Introducing high-content analysis of secretory footprints in B cell ELISPOT/FluoroSpot assays therefore fundamentally enhances the depth in which an antigen-specific B cell repertoire can be studied using freshly or cryopreserved primary cell material, such as peripheral blood mononuclear cells.

1. Introduction

Enzyme-Linked Immunosorbent Spot (ELISPOT) assays have emerged as a cornerstone in immunological research, enabling the enumeration and characterization of antigen-specific memory T and B cells at single-cell level. At present, ELISPOT is the only method suitable for detecting rare antigen-specific T and B cells directly *ex vivo*, at frequencies as low as a few per million [1] (Note 1). Alternative flow cytometry-based approaches such as intracellular cytokine staining and/or surface tetramer staining have minimum frequency limitations at least two orders of magnitude higher [2], and are not well-suited for high-throughput studies, such as full virus CD8 T cell epitope mapping or testing a multitude of samples in clinical trials [3]. Over the past decade, the FluoroSpot variant of the assay, based on fluorescence detection, has become increasingly popular due to its multiplexing capabilities [4-7] and potential for high-content analysis (HCA)) [8] (Note 2). Because ELISPOT and FluoroSpot assays only differ in the approach used for detecting the plate-bound analyte (Note 3), which in the case of B cell assays are immunoglobulins produced by antibody-secreting cells (ASC), we collectively refer to both as ImmunoSpot® tests.

The original ELISPOT protocols frequently failed to reveal secretory footprints of individual cells. Only after we introduced the PVDF membrane for ELISPOT analysis [9,10], with its much-enhanced protein retention capacity, did these assays find broad acceptance and become part of standard immune monitoring procedures (Note 4). Moreover, for the reliable performance of B cell ImmunoSpot® assays, our introduction of affinity coating of the antigen was essential [11,12]. More importantly, our key contribution to this field was the introduction of advanced automatic imaging systems and computer image analysis [10,13,14], as is needed for the automated analysis of assay results. Enabled by these advances, ImmunoSpot® tests have become a standard approach for detecting antigen-specific T cells in freshly isolated cell material at single-cell resolution, thus establishing the frequency and cytokine signature of these cells, i.e., defining the magnitude and quality of the antigen-specific T cell repertoire directly *ex vivo* [10,9,15]. Although B cell ELISPOT was introduced prior to T cell assays [16,17], only recently has its real potential been unleashed by making the assay readily adaptable for any antigen [11,12]. In addition, the recent realization that standard serum antibody measurements do not reflect long-term B cell memory, and unlike the latter, frequently provide false negative results regarding the engagement of a B cell immune response [18-21], has increased the utility of this type of assay. The appeal of B cell ImmunoSpot® tests is further enhanced by the realization that they can readily be adapted for establishing, with single-cell resolution, the affinity distribution of an antigen-specific B cell repertoire [8], for studies of its immunoglobulin (Ig) class and subclass usage [22], and for establishing the cross-reactivity profile of memory B cells established following infection/vaccination with the original (homotypic) virus against newly emerging (heterotypic) virus variants [12].

Three common types of B cell ImmunoSpot® assays are the polyclonal total (pan) Ig detection test (Figure 1A) [23,24], which reveals ASC irrespective of their specificity; the inverted assay, (Figure 1B) [7] and the direct assay (Figure 1C) [16,17,11], the latter two detect antigen-specific ASC. Each of these variants individually, or in combination, offer distinct perspectives of ASC at single-cell resolution. A clear understanding of their differences is therefore crucial for selecting the most appropriate approach depending upon the investigator´s research goals and experimental context. Other chapters in this volume describe these assay variants and their utility in detail [12,22,8]. As the pan Ig-detecting assay and the

inverted assay both rely on membrane-bound capture antibodies with a constant and high-affinity for the ASC-derived Ig, the secretory footprints (spot morphologies) revealed by them will be rather uniform and predictable, defined primarily by the quantity and the kinetics with which the individual ASC released their Ig. Thus, image analysis of such spots does not present a major challenge. However, in the most common B cell ImmunoSpot® assay format, the direct assay, the affinity of the ASC-derived Ig will primarily define spot morphology. First *in silico*, using mathematical modeling [25] and then experimentally [8] we showed that the morphology of the ASC-derived secretory footprint in an antigen-specific direct B cell ImmunoSpot® assay reflects upon the individual B cell's functional affinity, next to the quantity of Ig produced and the secretion dynamics. As the affinity spectrum of individual B cells within the antigen-specific repertoire can span a million-fold (ranging from $K_d=10^{-4}$ to $K_d>10^{-10}$) [26], markedly different spot morphologies can be expected to arise. Depending on the extent of affinity maturation that the memory B cell (B_{mem}) repertoire has undergone with respect to an antigen, which increases with repeated and long-lasting exposure [27-29], this affinity distribution (and hence spot morphology in the direct assay) will show fundamental variations from antigen to antigen, between different individuals, and possibly even between sequential bleeds of the same individual. On one hand, this diversity is a challenge to automated image analysis using legacy counting parameters. However, on the other hand, if the individual secretory footprints are accurately assessed for high-content information, such diversity can provide invaluable insights into the affinity distribution of the antigen-specific B_{mem} repertoire in any given individual, at any given time point (for more on this issue, we refer to the chapter by Becza *et al*. in this volume [8].

High-content analysis (HCA) of spot morphologies in antigen-specific direct ImmunoSpot® assays can therefore provide thus far under-exploited information about individual ASC [25]. For practical use, HCA should allow the identification and quantification of subpopulations of B cells within the antigen-specific ASC repertoire, assessing affinity distributions and productivities as shaped by vaccinations, infections, allergens, and auto-antigens.

2.1 More diverse spot morphologies are observed in direct ImmunoSpot® assays than in pan-Ig assays

Due to the diverse affinity of the individual B cells, in assays in which the antigen itself serves as the capture reagent for binding the ASC-derived Ig, antigen-specific ASC produce a wider range of spots with different morphologies than when these ASC-derived Ig are captured using anti-Ig-specific capture antibodies in pan-Ig assays (of which images of representative wells are shown in Figure 2, and see Note 5). Such visually observable heterogeneity of spot sizes and intensities can be quantitatively evaluated using dot plots of spot size vs. average spot intensity (Figure 3). In the pan-Ig assay, spots are bright and compact, and the size vs. intensity dot plots accordingly show a single population of spots with narrow distribution for both parameters. This also applies to inverted B cell, and T cell, assays Note 5). In the antigen-specific direct assay, in contrast, the size/density distributions are much broader and distinct spot (ASC) subpopulations can be seen.

2.2 Size distribution of antigen-specific ASC does not follow log normal distribution and cannot be analyzed using statistics-based size gating

In T cell ELISPOT, the cytokine of interest (e.g., interferon gamma) can also be produced by non-T bystander cells such as monocytes, basophils, dendritic cells, and other cell types [30,31]. However, we have shown that the spot size distribution for antigen-stimulated memory T cells consistently follows a log normal distribution pattern that permits discrimination between T cell- and bystander cell-derived spots in order to count the former while neglecting the latter. This approach is based on an automated gating strategy that subjects spot size distributions to statistical analysis [32,14]. In contrast to T cell cytokines, only B cells can secrete immunoglobulin/antibody and thus all spots exceeding a minimal size (Note 6) must be counted. Establishing the upper limit of the number of spots originating from individual ASC in antigen-specific direct assays is critical: this limit depends upon the morphology of ASC-derived secretory footprints which, in turn, reflects the affinity of secreted antibodies (Note 7). In this same context, ELISA effects (Note 8) and merging of secretory footprints can interfere with accurate quantification. We therefore tested whether ASC-derived spots in antigen-specific direct ImmunoSpot® assays follow a distribution that can be leveraged for automated gating. As shown in Figure 4, spot size distributions for antigen-specific ASC in direct assay do not follow a log normal function. This deviation from normality is related to the polyclonal nature of an antigen-specific B cell repertoire: spot size distributions of individual ASC not only reflect differences in their productivity, as for T cell cytokines, but also in their affinity and fine epitope specificity.

3.4. Artificial intelligence - based IntelliCount™ provides accurate user- and assay-independent counts of antigen-specific ASC-derived spots

Because of the relatively simple spot morphologies involved, traditional thresholding and fixed parameter-based image analysis algorithms are suited for providing accurate and scientifically validated spot-forming unit (SFU) counts for T cell ELISPOT [14], and multicolor T cell FluoroSpot assays [5,13]. Such conventional parametric counting is also suited for providing SFU counts for pan and inverted B cell assays [7,11,21,20,18], but it frequently disappoints when applied to antigen-specific, direct B cell assays. This is the case when frequencies of antigen-specific ASC in test samples (e.g., PBMC) show considerable variability (which is generally observed, see Note 7). In particular, this is the case when the intent of the assay is to extract high-content information on the wide spectrum of secretory footprints, beyond obtaining mere SFU counts present in such assays (e.g., to assess the affinity distribution of an antigen-specific B cell repertoire). Even when counting parameters are fine-tuned manually well-by-well (Note 9), it remains challenging to establish parameters to simultaneously detect fuzzy, spread-out, low-intensity spots along with bright compact spots in the same well, and to accurately establish the boundaries for each SFU. The latter, however, is essential for the precise quantification of the ASC-derived Ig retained within each SFU, *i.e.,* the “spot mass” (Note 10). An artificial-intelligence (AI)-based spot recognition algorithm, however, inherently lends itself better to such a task. IntelliCount™ is built around CTL’s proprietary deep neural network that was trained using thousands of ELISPOT and FluoroSpot images. Thus, it does not require special knowledge for setting counting parameters, and instead, data analysis becomes fully automated and objective.

High-content analysis of secretory footprints may also require high dynamic range (HDR) imaging to accurately quantify fluorescence intensity for spots whose luminosity span a sufficiently wide range and cannot be fully captured using a single fixed exposure image. IntelliCount™ fully supports HDR-based HCA integration to quantitatively assess the characteristics of individual spots. ImmunoSpot® Studio software generates comprehensive HCA data outputs in the form of Flow Cytometry Standard (FCS) files. Such data can be processed and viewed within the ImmunoSpot® software as dot plots and histograms, or can be readily exported into advanced flow cytometry suites for more detailed statistical analysis using multi-dimensional gating and other tools.

3.4.1. IntelliCount™ automatically establishes accurate spot boundaries for high-content analysis

One of the goals of HCA is to quantify the net analyte captured within each secretory footprint i.e. the "mean spot intensity". To obtain this information from antigen-specific B cell assays, spot boundaries have to be defined accurately to include the entire spot area. Images of representative wells that were counted using threshold-based parametric counting vs. IntelliCount™ are presented in Figure 5. In this sample well containing SFU at the "Goldilocks number" (Note 11), both approaches yield similar overall SFU counts, but the spot areas are largely underestimated by intensity threshold-based parametric counting. Importantly, in parametric spot recognition, spot boundaries are defined by an intensity threshold set at a discrete background level above which "mean spot intensity" is calculated (much as the sea level defines the shoreline of an island). Any fluctuations of the background within and between wells (Note 12) will affect spot boundaries and do so dramatically for faint spots. The challenge of properly defining SFU sizes, or even recognizing individual SFU, increases when their frequencies exceed the Goldilocks number since this can result in both local and global ELISA effects (Note 8). In IntelliCount™ mode, in contrast, the morphology of each spot is analyzed in its entirety, and spot boundaries are calculated with precision for each spot's individual modeled shape, irrespective of background fluctuations. These boundaries are used to assess the amount of analyte retained within each secretory footprint via the fluorescent intensity of the captured label.

3.4.2. IntelliCount™ counting mode provides extended linear ranges for accurate calculation of ASC frequencies

Antigen-specific, direct B cell ImmunoSpot® assays are primarily performed to determine the frequency of antigen-specific, memory B cell-derived ASC producing different classes and subclasses of Ig in PBMC (or other primary cell material) [22]. By doing so, one can predict the magnitude and quality of effector functions mediated by B_{mem} upon antigen reencounter, when they engage in secondary-type antibody responses (Notes 13 and 14). A technical challenge in doing so is that frequencies of antigen-specific B_{mem}-derived ASC producing antibodies of a given class/subclass occur at markedly different inter-individual frequencies, even when assessed in individuals at the same time point after infection/vaccination [18] (see also Note 7). Moreover, the frequencies of B_{mem} producing different classes and subclasses of Ig are also typically orders of magnitudes apart in individuals [18]. Furthermore, within an individual, the frequency of B_{mem} recognizing different antigens within individuals is also highly variable, dependent upon the individual's memory status relative to each antigen [12].

True frequencies of antigen-specific B_{mem} can only be established in ImmunoSpot® assays under conditions when secretory footprints of individual ASC are clearly discernable; in such cases, the number of SFU counts per well reveals the number of antigen-specific ASC among all PBMC plated into that well, that is, their frequency. When SFU numbers per well increase, the expected direct linear relationship between numbers of cells plated and spots counted breaks down because of merging of secretory footprints and ELISA effects (Note 8). At low spot counts, however, frequency estimates become imprecise, at least when a limited number of replicate wells are tested, due to the onset of Poisson noise (Note 15 and 16). Therefore, there is a certain range of SFU numbers per well from which reliable data can be extracted for precise ASC frequency calculations (and even more stringently, for high-content spot morphology analysis). Therefore, frequencies of ASC are best estimated when PBMC are seeded in serial dilution into ImmunoSpot® wells, and are calculated by extrapolation from the linear portion of the graph in which SFU counts per well/PBMC seeded per well are plotted [12]. The ImmunoSpot® Studio software implements a Linear Range Finder function for automatic frequency calculations: using statistical analysis, it finds the initial linear part in the cell titration results and calculates frequencies by linear regression from these accurate SFU counts. The chapter by Yao *et al*. in this volume [22] introduces protocols on how to readily measure frequencies of antigen-specific B_{mem}-derived ASC producing distinct Ig classes (or IgG subclasses) with only 4 x 10^5 PBMC per antigen, leveraging four-color ImmunoSpot® analysis; it also established that the frequency of ASC can be established by a single well serial dilution approach as precisely as when done involving 4 replicates in order to maximize utility of precious cell material. With the ImmunoSpot® Software the generation of cell titration graphs and the frequency extrapolations are fully automated, as shown in Figure 6.

Figure 6 shows the results of a serial dilution experiment utilizing PBMC in which the frequencies of pan-IgG^+ memory B cell-derived ASC were determined by the Linear Range Finder regression analysis using legacy parametric counting vs. the IntelliCount™ approach. Note the higher number of data points falling into the linear range with IntelliCount™ (seven with IntelliCount™ vs. four by legacy counting in the example shown) which reduced the regression error and increased the precision of the extrapolated frequency (Note 16). These data also illustrate the robustness of IntelliCount™ to discern individual secretory footprints even as background levels rise with increasing ASC numbers per well.

3.5. Further advantages of AI-based SFU analysis

Unlike any other spot counting algorithm presently available on the market, IntelliCount™ does not require special knowledge for setting counting parameters; thus, data analysis becomes easy, objective, user-independent, and fully automated (Note 17). Moreover, AI-based counting, being more forgiving with fluctuations of image intensity, background staining, and spot contrast over the background, will reduce inter-assay variability of test results when aliquots of the same PBMC are re-tested in a laboratory (Note 18). IntelliCount™, due to the way the deep neural networks are trained, is also rather insensitive to variations in well-image properties resulting from image pixel resolution or varying image acquisition parameters with the same or different reader(s). IntelliCount™ practically eliminates the necessity for harmonization when several instruments are operating in the same laboratory, or in multicenter studies. SFU counts reported by different independent groups should become more comparable using IntelliCount™, representing a major step towards count harmonization [33]. A further advantage of IntelliCount™ is its faster speed of data processing compared to legacy counting, due to the optimized utilization of modern

GPU and TPU accelerator cards. Typically, counting time for a 96-well plate for a single fluorescence channel does not exceed one minute with IntelliCount™ vs. 3-4 minutes with legacy counting. Such time-saving particularly benefits multicolor/channel analysis. With this technology, ImmunoSpot® assays, in particular their multiplexed HCA-inclusive B cell analysis variants, will become truly high-throughput suitable methods that can serve the advanced needs of both immune monitoring efforts in regulated environments and academic research laboratories.

2. Materials

2.1. Single-color ELISPOT assay

1. Commercially available, single-color Human Ig class (IgA, IgE, IgG or IgM) or subclass (IgA1, IgA2, IgG1, IgG2, IgG3 or IgG4) ELISPOT kit
2. 190 proof (95%) EtOH
3. Cell culture-grade water
4. 96-well, round bottom dilution plate
5. 0.05% Tween-PBS wash solution
6. 0.1 µm low protein binding syringe filter
7. Plate washer
8. ImmunoSpot® S6 Ultimate 4 LED Analyzer, or suitable instrument equipped with the appropriate detection channels, running CTL's ImmunoSpot® UV

2.2. Single-, three- or four-color FluoroSpot assays

1. Commercially available, single-color Human Ig class (IgA, IgE, IgG or IgM) or subclass (IgA1, IgA2, IgG1, IgG2, IgG3 or IgG4) FluoroSpot kit
2. Commercially available, three-color Human Ig class (IgA, IgG and IgM) FluoroSpot kit
3. Commercially available, four-color Human Ig class (IgA, IgE, IgG and IgM) FluoroSpot kit
4. Vacuum manifold

2.3. Four-color antigen-specific direct FluoroSpot assay (affinity capture coating)

1. Commercially available, four-color Human Ig class (IgA, IgE, IgG and IgM) affinity capture (His) FluoroSpot kit
2. Commercially available, four-color Human IgG subclass affinity capture (His) FluoroSpot kit
3. His-tagged recombinant protein

2.4. Single-color, antigen-specific inverted ImmunoSpot® assay

1. Commercially available, single-color inverted (His) human B cell ImmunoSpot® kit

3. Methods

3.1. Pan-Ig ImmunoSpot® assay (total ASC, irrespective of specificity)

1. One day before plating cells (Day -1), prepare 70% EtOH and pan anti-Ig 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 addition of the 70% EtOH solution to the entire plate (or designated wells) add 180 µL/well of PBS (Note 19). 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 pan anti-Ig capture antibody solution into each well (or designated wells) of the low autofluorescence PVDF-membrane plate provided with the kit.
4. Incubate the plate overnight at 4°C in a humidified 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 into labeled conical tube(s). Keep the cells warm during processing. Wash culture vessel's interior with sterile warm PBS to collect residual PBMC and transfer into the corresponding conical tube(s). Increase volume to fill the tube with additional warm PBS and then centrifuge balanced tubes at 330 x *g* for 10 min non-refrigerated, centrifuge with brake on (see Notes 20 and 21).
7. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM to achieve a cell density of ~2-5 x 10^6 cells/mL (The cell number recovered at this point can be estimated to be 50% of the number of cells frozen).
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 droplet of Live/Dead cell counting dye. Pipet up and down 3-5 times to mix the sample while avoiding formation of bubbles.
10. Transfer 15 µL of the cell and dye suspension into each chamber of a hemocytometer.
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 warm PBS and centrifuge balanced tubes at 330 x *g* for 10 min with centrifuge brake on, unrefrigerated (Notes 22, and 23).
13. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM at 2 x 10^5 PBMC/mL.
14. Decant the BCM used for blocking the ImmunoSpot® assay plate and replace with 100 µL/well of pre-warmed BCM.
15. Prepare PBMC serial dilution series in a round bottom 96-well polystyrene plate. For this, we recommend the following procedure: Into the round bottom 96-well dilution plate add 120 µL of pre-warmed BCM into all wells, except for row A. Into Row A, add 240 µL of diluted single-cell suspension at 2 x 10^5 PBMC/mL in one or more replicates (Note 24). Using a multichannel pipettor, perform a 2-fold dilution series of the PBMC test sample(s) by transferring 120 µL from each row to the next, diluting the cells by gently aspirating and ejecting twice at each dilution step. Once the cell dilution in the round bottom dilution plate is completed, using a multichannel pipettor and fresh tips, transfer 100 µL of the serially diluted cells from the dilution plate into the actual ImmunoSpot® test plate.
16. Incubate cells in the ImmunoSpot assay plate for 16-18 h at 37°C, 5% CO_2.
17. After completion of the assay incubation period, decant (or reutilize) cells and wash plate two times with warm PBS (200 µL/well), followed by two additional washing steps with 0.05% Tween-PBS wash solution (Note 25).

18. Prepare anti-Ig class/subclass-specific detection antibody solution(s) according to kit protocol and pass through 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 anti-Ig class/subclass-sp26ecific detection antibody solution 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 tertiary solution by following kit protocol and pass through 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 tertiary solution into designated wells, and incubate for 1 h at RT (protected from light).
23. Wash plates(s) twice with distilled water.
24. Remove protective underdrain and place plate face down on vacuum manifold. Completely fill the backside of the plate with distilled water and apply vacuum to draw water through the membrane ("back to front") (Note 26).
25. Allow plate to dry completely, protected from light (Note 27).
26. Scan and count plate(s) with suitable analyzer equipped with the appropriate detection channels.

3.2. Antigen-specific, direct B cell ImmunoSpot®

1. One day before plating cells (Day -1), prepare 70% EtOH and antigen coating solutions (*see* Notes 28 and 29).
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 addition of the 70% EtOH solution to the entire plate (or designated wells) add 180 µL/well of PBS (Note 19). 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 antigen coating solution into each well (or designated wells) of the low autofluorescence PVDF-membrane plate provided with the kit.
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 warm 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 into labeled conical tube(s). Keep the cells warm during processing. Wash culture vessel's interior with sterile warm PBS to collect residual PBMC and transfer into the corresponding conical tube(s). Increase volume to fill the tube with additional warm PBS and then centrifuge balanced tubes at 330 x *g* for 10 min non-refrigerated, centrifuge with brake on (Notes 21, and 22).
7. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM to achieve a cell density of ~2-5 x 10^6 cells/mL. (The cell number recovered at this point can be estimated to be 50% of the number of cells frozen.)
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 droplet of Live/Dead cell counting dye. Pipet up and down 3-5 times to mix the sample while avoiding formation of bubbles.
10. Transfer 15 µL of the cell and dye suspension into each chamber of a hemocytometer.
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 warm PBS and centrifuge balanced tubes at 330 x *g* for 10 min with centrifuge brake on, unrefrigerated.
13. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM at 2-5 x 10^6 PBMC/mL (Note 30).

14. Decant the BCM used for blocking the ImmunoSpot® assay plate and replace with 100 µL/well of pre-warmed BCM.
15. Prepare PBMC serial dilution series in a round bottom 96-well polystyrene plate. For this, we recommend the following procedure. Into the round bottom 96-well dilution plate add 120 µL of pre-warmed BCM into all wells, except for row A. Into Row A, add 240 µL of diluted single-cell suspension at 2-5 x 10^6 PBMC/mL in one or more replicates (Note 24). Using a multichannel pipettor, perform a 2-fold dilution series of the PBMC test sample(s) by transferring 120 µL from each row to the next, diluting the cells by gently aspirating and ejecting twice at each dilution step. Once the cell dilution in the round bottom dilution plate is completed, using a multichannel pipettor and fresh tips, transfer 100 µL of the serially diluted cells from the dilution plate into the actual ImmunoSpot® test plate.
16. Incubate cells in the ImmunoSpot® assay plate for 16-18 h at 37°C, 5% CO_2.
17. After completion of the assay incubation period, decant (or reutilize) cells and wash plate two times with warm PBS (200 µL/well), followed by two additional washing steps with 0.05% Tween-PBS wash solution (Note 25).
18. Prepare anti-Ig class/subclass-specific detection antibody solution(s) according to kit protocol and pass through 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 anti-Ig class/subclass-specific detection antibody solution 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 tertiary solution by following kit protocol and pass through 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 tertiary solution into designated wells, and incubate for 1 h at RT (protected from light).
23. Wash plates(s) twice with distilled water.
24. Remove protective underdrain and place plate face down on vacuum manifold. Completely fill the backside of the plate with distilled water and apply vacuum to draw water through the membrane ("back to front") (Note 26).
25. Allow plate to dry completely, protected from light (Note 27).
26. Scan and count plate(s) with suitable analyzer equipped with the appropriate detection channels.

3.3 Antigen-specific, single-color human IgG inverted ImmunoSpot® assay (see Note 31-33)

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 addition of the 70% EtOH solution to the entire plate (or designated wells) add 180 µL/well of PBS (Note 19). 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.
4. Incubate the plate overnight at 4°C in a humidified chamber.
5. On the day of the assay (Day 0), decant the assay plate and wash wells with 180 µL/well of warm 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 into labeled conical tube(s). Keep the cells warm during processing. Wash culture vessel's interior with

sterile warm PBS to collect residual PBMC and transfer into the corresponding conical tube(s). Increase volume to fill the tube with additional warm PBS and then centrifuge balanced tubes at 330 x *g* for 10 min non-refrigerated, centrifuge with brake on (Notes 21 and 22).

7. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM to achieve a cell density of ~2-5 x 10^6 cells/mL. (The cell number recovered at this point can be estimated to be 50% of the number of cells frozen.)
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 droplet of live/dead cell counting dye. Pipet up and down 3-5 times to mix the sample while avoiding formation of bubbles.
10. Transfer 15 µL of the cell and dye suspension into each chamber of a hemocytometer.
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 warm PBS and centrifuge balanced tubes at 330 x *g* for 10 min with centrifuge brake on, unrefrigerated.
13. Decant supernatant and resuspend the cell pellet(s) using pre-warmed BCM at 1 x 10^6 PBMC/mL (Note 31, 34 and 35).
14. Decant the BCM used for blocking the ImmunoSpot® assay plate and replace with 100 µL/well of pre-warmed BCM.
15. Prepare PBMC serial dilution series in a round bottom 96-well polystyrene plate. For this, we recommend the following procedure. Into the round bottom 96-well dilution plate add 120 µL of pre-warmed BCM into all wells, except for row A. Into Row A, add 240 µL of diluted single-cell suspension at 1 x 10^6 PBMC/mL in one or more replicates (Note 24). Using a multichannel pipettor, perform a 2-fold dilution series of the PBMC test sample(s) by transferring 120 µL from each row to the next, diluting the cells by gently aspirating and ejecting twice at each dilution step. Once the cell dilution in the round bottom dilution plate is completed, using a multichannel pipettor and fresh tips, transfer 100 µL of the serially diluted cells from the dilution plate into the actual ImmunoSpot® test plate.
16. Incubate cells in the assay plate for 16-18 h at 37°C, 5% CO_2 (Note 36).
17. After completion of the assay incubation period, decant (or reutilize) cells and wash plate two times with warm PBS (200 µL/well), followed by two additional washing steps with 0.05% Tween-PBS wash solution.
18. Prepare His-tagged antigen probe solution (*see* Notes 34 and 37) according to kit protocol and pass through 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 His-tagged antigen probe solution 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 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 0.1 µm low protein binding syringe filter to remove any aggregates.
25. Wash plates(s) twice with distilled water.
26. Remove protective underdrain and place plate face down on vacuum manifold. Completely fill the backside of the plate with distilled water and apply vacuum to draw water through the membrane ("back to front") (Note 26).
27. Allow plate to dry completely, protected from light (Note 27).
28. Scan and count plate(s) with suitable analyzer equipped with the appropriate detection channel.

3.4 Automatic scanning and counting of ImmunoSpot® plates

1. ImmunoSpot® plates were scanned on CTL Series 6 Ultimate Analyzer equipped with the appropriate fluorescent detection channels.
2. SFU were counted using ImmunoSpot® Studio Software with integrated IntelliCount™ mode and Linear Range Finder for accurate frequency calculations.

4. Notes

Note 1. In B cell ImmunoSpot® assays there is no inherent lower limit of detection. The PBMC numbers plated per well into a 96-well plate should not exceed 1 x 10^6 cells per well, because with higher numbers the cells no longer form a monolayer on the membrane [34] and the resulting cell layering can interfere with the capture of ASC-derived antibodies. If, e.g., 10 million PBMC are plated at 1 x 10^6 PBMC across 10 replicate wells, 1 in 10 million is the detection limit, etc. Owing to increased Poisson noise occurring at such low frequencies, however, the number of replicate wells needs to be increased accordingly to obtain accurate measurements [35]. As shown in Figure 3 of the chapter by Lehmann *et al.* in this volume [12] antigen-specific memory B cells (B_{mem}) quite frequently occur in low frequencies.

Note 2. In ELISPOT assays, the enzymatic amplification of the signal leads to loss of strict proportionality between the detection antibody bound and the eventual substrate precipitation. Moreover, once the density of the substrate deposition on the membrane reaches a certain point, the spot's optical density/appearance does not increase even if more substrate is deposited (much like applying many layers of paint). With fluorescent detection, however, the number of fluorescent tags bound is proportional to the number of detection antibodies retained on the membrane.

Note 3. As enzyme linked ImmunoSpot (ELISPOT) and FluoroSpot assays differ only in the modality of detecting secretory footprints of cells on membranes, we collectively refer to both as ImmunoSpot® assays. In the former, the detection antibody is tagged to enable the engagement of an enzymatic reaction that results in the local precipitation of a substrate that is visible under white light. In the latter, the plate-bound detection antibodies are visualized via fluorescent tags using appropriate excitation and emission wavelengths. Data provided in a chapter in this volume by Yao *et al.* [22] establish that ELISPOT and FluoroSpot assays have equal sensitivity for detecting numbers of antibody-secreting cell (ASC)-derived secretory footprints. However, they are not equally suited for high-content analysis (HCA) of spot morphologies (see Note 2).

Note 4. Our introduction of the PVDF membrane to ELISPOT assays [9,10], with its by far superior capture antibody adsorption properties [36], was key for improving our ability to detect secretory footprints to the point needed for transforming ImmunoSpot® into the robust immune monitoring platform it has become for detecting rare -- even very rare -- antigen-specific lymphocytes *ex vivo*, in freshly isolated PBMC or other

lymphoid cell material. We refer to Figure 1 in [37] to appreciate the difference in assay performance using the PVDF membrane vs. the previously used mixed cellulose ester membrane.

Note 5. The shape of secretory footprints (spot morphologies) produced by T cells follow defined rules since the capture antibody's (i.e., an anti-cytokine specific mAb) affinity for the analyte to be detected is high and fixed. Consequently, only the quantity of analyte (cytokine) produced by the T cell will define the morphology of the resulting secretory footprint [25]. Predictable (log normal [32]) spot sizes permit objective automated size gating [14,13].

Note 6. Although only B cells can secrete antibody, even in B cell ImmunoSpot® assays, there can be small background spots resulting primarily from aggregated detection reagents. Such artefacts can be reduced/eliminated by filtering or centrifuging the reagents at high speed to eliminate aggregates. To identify such spots, it is important also to include negative controls wells that are subject to the entire test procedure, but do not contain cells. They should and can be readily gated out during ImmunoSpot® analysis.

Note 7. We refer to Figure 3 of the chapter by Lehmann *et al.* in this volume [12] to convey the high degree of variability in frequency of antigen-specific B_{mem} in PBMC.

Note 8. ASC secrete Ig in an undirected fashion into 3D space above the membrane. In ImmunoSpot® assays, the antibody released towards the membrane will be captured as a secretory footprint while the remainder of the secreted antibody will diffuse away from the surface and will be diluted in the bulk of the culture supernatant. As the concentration of such diffused antibody increases in the culture medium, these antibodies are captured on the membrane distantly from the source ASC, increasing the background signal in the assay and undermining the resolution of individual secretory footprints. Such an elevated background in an ImmunoSpot® assay is termed an ELISA effect. If ASC –by chance—settle in clusters on the membrane, local ELISA effects can occur surrounding these cells resulting in regions with increased local background. The ImmunoSpot® software implements powerful local background correction, and therefore such ELISA effects do not interfere with the detection of SFU, however they affect threshold-based detection of spot outlines needed for HCA.

Note 9. Fine-tuning of parameters manually not only requires expert knowledge, but also takes considerable time, and thus it can rarely be done for analyzing an entire assay. Due to global and local ELISA effects in wells, the background level is variable in most assays preventing the accurate detection of outlines of secretory footprints. When using parametric counting for the initial machine reading of the plate, under such conditions well-by-well recounting in Quality Control mode is required for finalizing the results. IntelliCount™ greatly streamlines this process.

Note 10. In FluoroSpot assays, the overall fluorescence intensity of a spot is proportional to the quantity of analyte captured within the secretory footprint, i.e. "mean spot intensity" of a given ASC. In antigen-specific direct assays, spot morphologies can include all possible variations of sizes and intensities (see Fig. 2). A multitude of morphological parameters are readily captured for each SFU, in FCS format, to perform in depth HCA.

Note 11. The so-called “Goldilocks” number is defined as the maximal number of cells that can be plated in a B cell ImmunoSpot® assay well while still being able to discern clearly secretory footprint boundaries derived from individual antigen-specific ASC. For HCA, i.e., for the accurate definition of secretory footprint boundaries, the Goldilocks number is lower than the breaking point for linearity in mere SFU counts. As it is assay-dependent, it needs to be experimentally established by serial dilution of PBMC in the respective assay, but ~50 SFU/well is a safe estimate.

Note 12. Frequently, the background membrane staining of individual wells is not perfectly even in ImmunoSpot® assays and that can interfere with accurate SFU detection, in particular when relying on fixed counting parameters. Lowering non-specific background staining, and reduction of “hot spots” in the center of the assay wells can be achieved through performing the “back to front” water filtration technique. Regarding regional and global ELISA effects, see Note 8.

Note 13. Antibodies occur in four classes (IgM, IgG, IgA and IgE), and in four subclasses (IgG1, IgG2, IgG3 and IgG4). ASC producing all four, can be detected simultaneously in multiplexed ImmunoSpot® assays using only 4 x 10^5 PBMC/antigen (see chapter by Yao *et al.* in this volume, [22]). The different Ig classes and subclasses are endowed with distinct effector functions and each contribute non-redundant roles towards maintaining host defense (reviewed in [38]). Stimulating optimal Ig class usage during an infection or following vaccination is vital to successful host defense and the avoidance of collateral immune-mediated pathology (reviewed in [39]).

Note 14. During the primary immune response, B cells can transition from IgM-expressing naïve B cells into effector cells (antibody-secreting plasma cells) and resting B_{mem} that have undergone class switch recombination (CSR). CSR is an irreversible process that involves the excision of DNA encompassing the exons of the Igμ heavy chain required for expression of IgM and the juxtaposition of upstream variable region genes with downstream exons encoding alternative Ig classes or IgG subclasses [40]. Class switching of the BCR to downstream Ig classes or IgG subclasses is an instructed process and can be influenced by the cytokine milieu and co-stimulation provided by $CD4^+$ T helper cells. Thus far, we have not seen evidence for *in vivo* class-switched B_{mem} to undergo further during short-term polyclonal stimulation *in vitro* using R848 plus IL-2, as needed for their detection in ImmunoSpot® assays. Thus, it has to be assumed that the Ig calls subclass utilization of ASC observed in ImmunoSpot® assays *ex vivo*, reflects on the corresponding B_{mem} commitment for Ig class/subclass utilization upon antigen reencounter *in vivo*.

Note 15. Once activated by polyclonal stimulation, ASC are autonomous. Thus, the well-to-well variation in numbers of ASC in B cell ImmunoSpot® assays is dependent on their concentration in the test sample following the rules of a Poisson distribution: the rarer the cells, the higher the well-to well variation when a set volume is sampled/plated. This knowledge permits to precisely calculate the number of replicate wells needed to establish frequencies with precision when ASC frequencies are low [35].

Note 16. For low frequency antigen-specific B cell assay results, the conventional parametric approach can establish SFU counts (but to a lesser extent HCA-pertaining parameters) with a similar accuracy as

IntelliCount™; however it requires expertise to set up parameters, whereas IntelliCount™ does it automatically.

Note 17. Subjective counting is a considerable challenge for count harmonization among individuals and laboratories [33].

Note 18. Even slight changes in assay conditions (e.g., incubation times and temperature) as well in reagents properties over time (e.g., storage dependent aggregation or decay) can have an effect on the SFU staining intensity seen in repeat ImmunoSpot® assays. By being less sensitive to such qualitative differences, IntelliCount™ helps the assay's robustness in the evaluation phase.

Note 19. 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 of 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 ≤ 1min; longer contact times may promote leaking of the membrane and result in suboptimal assay performance.

Note 20. We refer to the chapter of Yao *et al*. in this volume [22] for detailed procedures covering the isolation of peripheral blood mononuclear cells (PBMC), their cryopreservation and thawing, as well as the polyclonal *in vitro* stimulation culture needed to trigger antibody production by resting memory B cells.

Note 21. PBMC, or other primary cell material, collected acutely following known antigen encounter, which may contain spontaneous (*in vivo* differentiated) ASC can also be evaluated in such assays.

Note 22. If the cells are not washed thoroughly, antibody in the cell suspension(s) can compete with the binding of ASC-derived Ig in the assay, resulting in elevated membrane staining that can interfere with the accurate detection of individual ASC's secretory footprints.

Note 23. Using a serial dilution approach, an ideal starting cell input of 2×10^4 is appropriate for typical pan (total) IgA/IgG/IgM measurements following *in vitro* differentiation of PBMC. However, higher cell inputs may be more appropriate for measurements of spontaneous (in vivo differentiated) ASC.

Note 24. Serial dilutions involving single wells for each cell dilution, progressing in a 1+1 (2-fold) dilution series, is a valid option for establishing accurate SFU frequencies and greatly reduces the cell numbers and reagents required (see the chapter by Yao *et al*. in this volume, [22]. In Figure 5A of that chapter the recommended plate layout for such a serial dilution assay is shown.

Note 25. 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. Plate washes may also be performed manually. See also Note 22.

Note 26. Optimal removal of background staining, fibers and other debris, along with reduction of "hot spots" in the center of the assay wells, is achieved through performing the "back to front" water filtration technique.

Note 27. 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 face down on paper towels for >2hr 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.

Note 28. Direct application of an antigen to the PVDF membrane can result in variable and often low-affinity absorption to the membrane owing to weak, non-specific binding forces (primarily hydrophobicity). Alternatively, our recent introduction of affinity capture coating [11] enables specific and high-affinity binding of antigen to the assay membrane.

Note 29. Optimizing the concentration of His-tagged protein(s) used for affinity capture coating is recommended. A concentration of 10 µg/mL His-tagged protein has yielded well-formed secretory footprints for most antigens, but increased concentrations of the anti-His affinity capture antibody and/or His-tagged protein may be required to achieve optimal assay performance.

Note 30. Using a serial dilution approach, a starting cell input of 2-5 x 10^5 is appropriate for typical antigen-specific ImmunoSpot® tests following *in vitro* differentiation of PBMC. However, higher cell inputs may be more appropriate for measurements of spontaneous (in vivo differentiated) ASC.

Note 31. 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.

Note 32. 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.

Note 33. In instances when the frequency 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.

Note 34. If the Goldilocks cell number input is already known, and the intent of the assay is to assess the affinity spectrum of the antigen-specific ASC compartment, the relevant assay procedures are described in detail in the chapter by Becza *et al*., [8].

Note 35. Using a serial dilution approach, a starting cell input of 1 x 10^5 is appropriate for an antigen-specific, inverted ImmunoSpot® tests following *in vitro* differentiation of PBMC. However, higher cell inputs may be more appropriate for measurements of spontaneous (*in vivo* differentiated) ASC.

Note 36. 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.

Note 37. The optimal concentration of affinity (His)-tagged antigen probe used for detection of all antigen-specific secretory footprints (i.e. SFU), low- or high-affinity alike, should be determined empirically.

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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®. A.Y.K, M.K., Z.M, and G.A.K, are employees of CTL.

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Figures and Legends

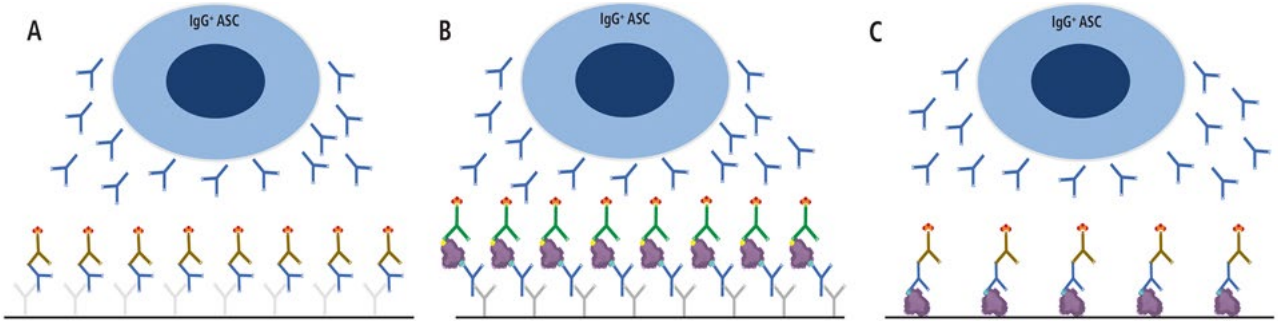

Figure 1. The morphology of secretory footprints captured on the membrane intrinsically differs for (A) pan-Ig detecting ImmunoSpot®, (B) inverted antigen-specific tests, and (C) the direct antigen-specific B cell assays. In both former, the Ig produced by an (in this example human) antibody secreting cell (ASC; the ASC-derived antibodies are depicted in blue) are captured by a high affinity anti-species antibody coated onto the membrane (e.g, a goat-anti human IgG, in grey). This results in ASC footprints of limited diversity, as only the quantity and kinetics of the ASCs' per cell secretory activity define the spot morphology due to the homogenous affinity of the capture antibody for the analyte. In the pan-Ig assay (A), the plate-bound (in this case human IgG) is visualized via an anti-human Ig detection antibody (in brown). In this assay variant, all ASC secreting IgG are detected, irrespective of the IgG^+ ASC's antigen-specificity. In the inverted assay, while IgG is also captured around all IgG^+ ASC with constant affinity (resulting in low-diversity spot morphologies), the added antigen (in purple) will bind only to the footprints generated by antigen-specific ASC. The membrane-bound antigen is detected in a subsequent step via a detection reagent, in the example shown, His-tagged recombinant antigen by an anti-His tag-specific detection antibody (in green). In the direct antigen-specific assay shown in (C) the antigen itself is coated onto the membrane (either directly, or aided by its high affinity capture via a His tag {Koppert, 2021 #1323} as shown here). Only Ig produced by antigen-specific ASC will bind to the lawn of antigen on the membrane, and will be visualized by adding anti-human Ig detection antibody (in brown). In this latter assay variant, the affinity of the "monoclonal" antibody produced by the individual ASC is a major factor for defining the size/shape/and density of the secretory footprint captured, permitting, via high content analysis of the resulting spot morphologies to study the affinity distribution of the antigen-specific B cell repertoire, as detailed in the chapter by Becza *et al.* in this volume {Becza, 2023 #1353}.

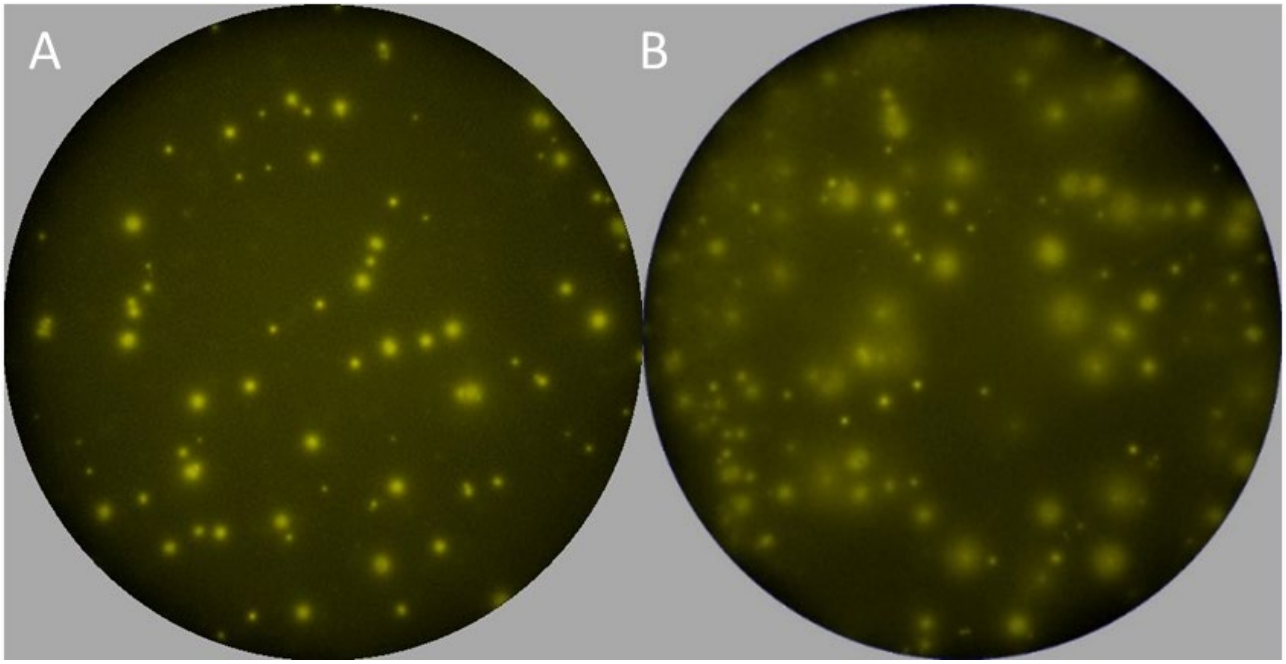

Figure 2. Representative images of (A) pan-Ig and (B) antigen-specific direct B cell ImmunoSpot® assay wells detecting secretory footprints of IgG^+ ASC. For schematic representation of the two assay types, please refer to Figure 1, the protocols are detailed in Materials and Methods. In (A), anti-human Igκ/Igλ-specific antibodies were used to capture the Ig produced, in (B) the membrane was affinity-coated with SARS-CoV-2 Spike protein. Polyclonally pre-activated peripheral blood nuclear cells (PBMC) from a COVID-19 mRNA-vaccinated donor were tested in serial dilution. In both cases, spots were visualized with anti-human IgG-specific detection antibodies. Representative wells containing secretory footprints in the Goldilocks range are shown: (A) 4×10^3 PBMC/well and (B) 2×10^4 PBMC/well. Note the distinct spot morphologies.

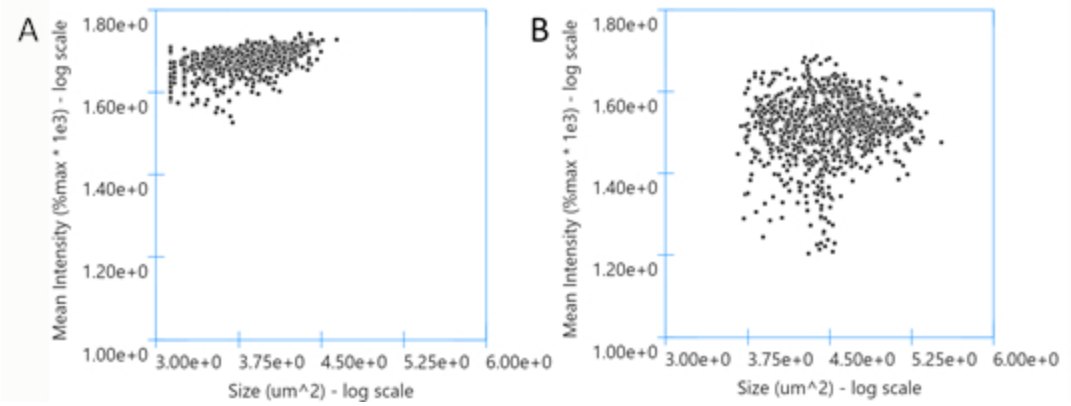

Figure 3. Representative dot-plots of spot sizes vs. mean spot intensities generated by ImmunoSpot® Studio Software for (A) pan-IgG, and (B) SARS-CoV-2 Spike-specific IgG^+ ASC detected in the respective B cell ImmunoSpot® assay. For the raw data, the legend to Figure 2 applies. Replicate wells containing secretory footprints at the respective Goldilocks numbers were subjected to high content image analysis using the IntelliCount® module of the ImmunoSpot® Software by merging data from replicate wells. The individual secretory footprints ("events" in flow cytometry terminology) are represented in flow cytometry standard dot-plot format. Mean spot intensities (Y axis, calculated as total intensity of an individual spot divided by its area) are plotted vs. the respective spot's size (X axis). Such high content spot morphology data are automatically generated as graphs in the ImmunoSpot® software. The raw data containing 12 numeric parameters to detail each SFU's morphology are automatically captured as FCS files and be subjected to more detailed analysis using any FACS software suite.

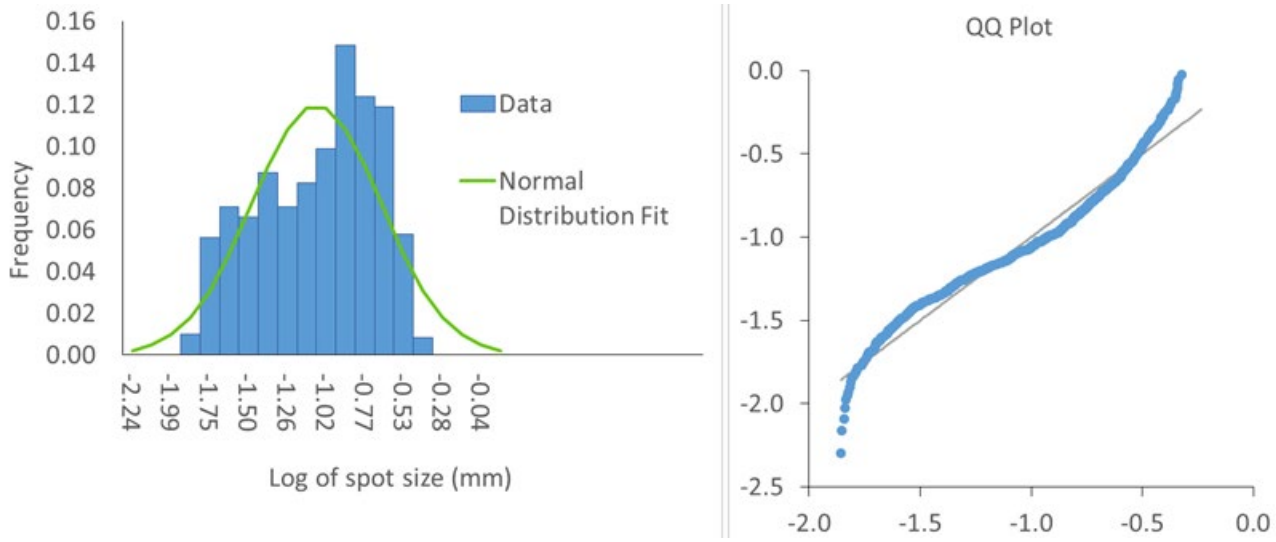

Figure 4. Spot size distributions in antigen-specific B cell ImmunoSpot® assay do not follow a log-normal function. Left panel (A) shows representative spot size (mm^2) histogram calculated for ~800 IgG^+ spots from SARS-CoV-2 Spike-specific B cell ImmunoSpot® assays. For the underlying raw data, legends of Figures 2 and 3 apply. Green line corresponds to the best fit of these data with the log-normal distribution function. Right panel (B) represents QQ plot for experimental vs. theoretical log-normal distributions. Systematic deviation of QQ plot (round dots) from the straight line proves that the experimental spot size distribution does not follow a log-normal function. Shapiro-Wilk (0.96) and Lilliefors (0.09) statistic tests also rejected the log-normal distribution hypothesis with a 5% significance level.

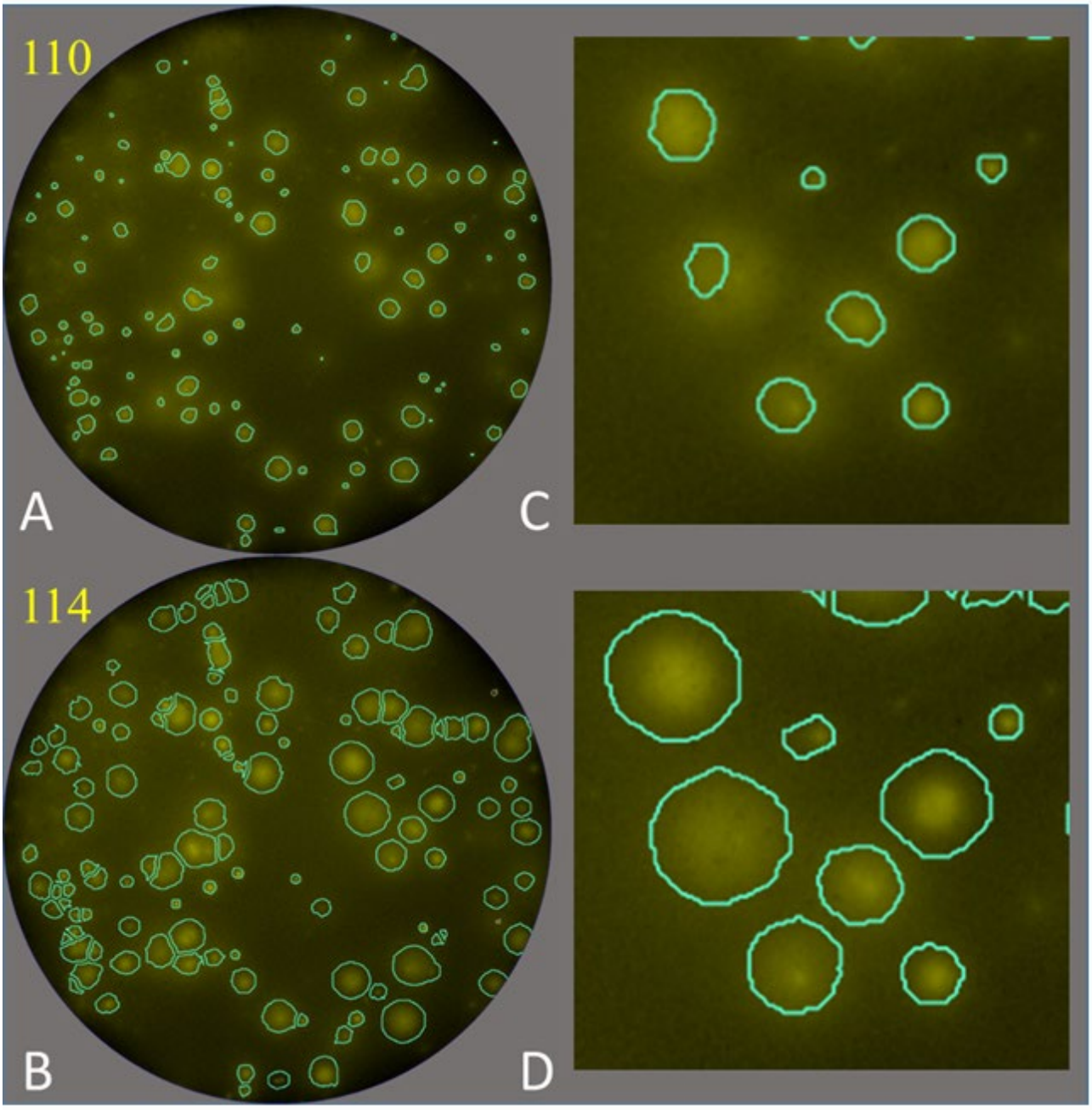

Figure 5. Precision of thresholding-based assessment of secretory footprint outlines (A, showing the entire well, C, an enlarged segment of that well) versus the IntelliCount™-based automated detection of such in the same well (B) and the same well segment (D). The well shown in Figure 1B originates from a direct, SARS-CoV-2 Spike-specific (IgG^+) assay and was analyzed using (A) conventional Parametric or (B) IntelliCount™ counting algorithms in ImmunoSpot® Studio Software. Zoomed-in sections of the same well analyzed with Parametric and IntelliCount™ modes are shown in panels C and D respectively. The spot counts are shown in yellow.

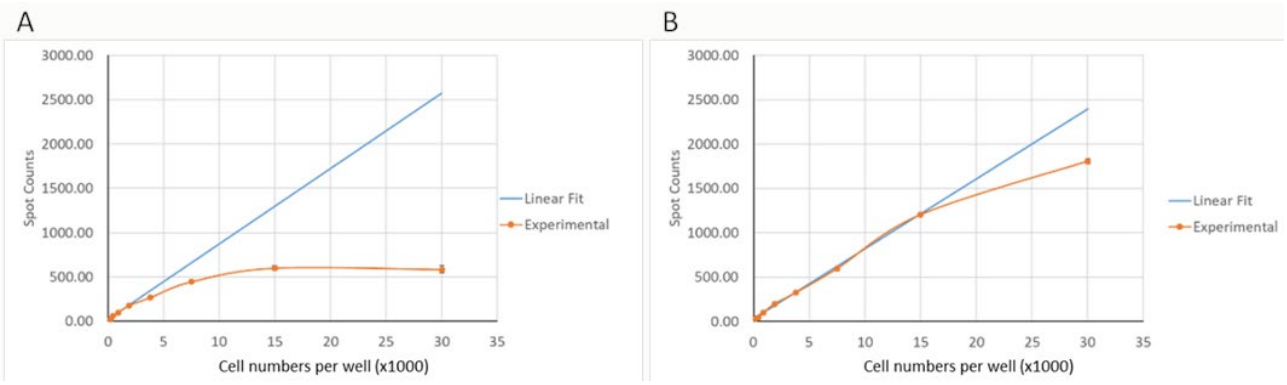

Figure 6. Representative PBMC serial dilution for pan-IgG detecting B cell ImmunoSpot® assay evaluated via (A) parametric counting algorithm *vs.* (B) IntelliCount™. For the raw data analyzed, the legend to Figure 2 applies. SFU counts were established by ImmunoSpot® Studio software automatically using the specified counting module. The linear regression line (in blue) in both cases was automatically calculated by the Linear Range Finder Function integrated into the ImmunoSpot® Studio software. The number of datapoints fitting the linearity range were in this case 4 for parametric counting *vs*.7 for IntelliCount™, respectively. The corresponding frequencies of ASC extrapolated were 8.5 *vs.* 8.0 % of PBMC, respectively. While both numbers are similar (in this case four data points were in the linear range for the parametric count), the precision of frequency calculations is higher for IntelliCount™ with more points in the linear portion of the titration graph. The standard error of regression, calculated as a square root of sum of quadratic errors divided by the number of points in the linear range, were 11.7 and 8.2 for the parametric count and IntelliCount, respectively.