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Microbubble Array Diffusion Assay for the Detection of Cell Secreting Factors

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Abstract:

The therapeutic potential of monoclonal antibodies (mAbs) makes them an ideal tool in both clinical and research applications due to their ability to recognize and bind specific epitopes with high affinity and selectivity. While mAbs offer significant therapeutic potential, their utility is overshadowed by the cost associated with their production, which often relies on the ability to identify minority antigen specific cells out of a heterogeneous population. To address concerns with suboptimal methods for screening cells, we have developed a cell sorting array comprised of nanoliter spherical cell culture compartments, termed microbubble (MB) wells. We demonstrate a proof-of-concept system for the detection of cell secreted factors from both immortalized cell lines and primary B cell samples. Exploiting the unique ability of the MB well architecture to accumulate cell secreted factors as well as affinity capture coatings, we demonstrate on chip detection and recovery of antibody secreting cells for sequencing of immunoglobin genes. Furthermore, rapid image capture and analysis capabilities were developed for the processing of large MB arrays, thus facilitating the ability to conduct high-throughput screening of heterogeneous cell samples faster and more efficiently than ever before. The proof-of-concept assays presented herein lay the groundwork for the progression of MB well arrays as an advanced on chip cell sorting technology.

Introduction

The ability to sort cells from heterogeneous population and to study them at the single cell level provides unique opportunities for drug discovery and for understanding signaling pathways in disease [1-3]. This capability is particularly advantageous for the production of monoclonal antibodies which requires the sorting of potentially rare (1 in $>10^4$) antibody producing cells from a heterogeneous population. Monoclonal antibodies (mAb) are a rapidly growing class of human therapeutics with a market size of roughly $78 billion in 2012 [4]. Their ability to specifically recognize and bind antigens of interest with high affinity holds vast potential as treatments for diseases ranging from autoimmune disorders to infectious diseases and cancer therapeutics [5-7].

Conventional mAb production involves fusing splenocytes from immunized mice with an immortalized myeloma cell line. The resulting hybridoma cells are cultured under limiting dilution conditions (<1 cell per well) in microtiter plates for 7 to 14 days to allow for clonal expansion. The culture supernatants are then tested for antigen specificity using Enzyme Linked Immunosorbent Assay (ELISA) methods to identify the wells containing cells of interest [8, 9]. While this method is effective, the process is laborious, time consuming and costly. Moreover, relatively few ($\sim10^3$) of the hybridoma cells produced can be tested and therefore potentially high affinity mAbs may be missed.

To expand and simplify hybridoma cell screening, microfabrication technologies have been exploited to develop novel single cell high-throughput methods for screening $>10^5$ hybridoma cells. There are several single cell methods reported for detecting antibody secreting cells (ASC) including antigen arrays [10], droplet based fluidic systems [2], and micro-well techniques including Microengraving [8, 11] and ISAAC [12]. Microengraving utilizes large arrays of shallow cuboidal micron scale pits formed in polydimethylsioxane (PDMS) to seed cells. The array is capped with a glass slide functionalized to bind secreted mAbs. After ~2-4 hours in culture the slide is removed from the array, treated with a secondary reporter and then used as a template to locate positive wells containing the cell(s) producing the mAb of interest [8]. The ISSAC technique similarly uses shallow micro-well arrays formed in PDMS to seed cells, however mAb detection is done through direct binding of cell secretions to an antigen specific surface coating [12]. Direct detection of fluorescence around the exterior of a well greatly simplifies the process of locating positive wells. While the aforementioned techniques make vast improvements over the conventional ELISA cell screening process, they still suffer from various drawbacks. In Microengraving, the array capping process limits the nutrient exchange within the pits and thus limits the time allowed for detecting mAb secretions to only a few hours and therefore only ASC that secrete at a high rate can be detected. While the ISSAC technique does not rely on a cap for signal generation, the open well architecture allows for the loss of cell secretions over time by diffusion and dilution into the bulk media. In shallow well architectures the cells may be easily dislodged by turbulent fluid flow creating uncertainty in being able to recover the specific cell of interest. Neither system allow for clonal expansion of cells which could greatly increase detection sensitivity and thus enable the discovery of potentially high affinity mAbs that are secreted at a low rate.

To overcome these limitations, we have developed a simple micro-well system for culturing cells and sorting them based on what they secrete using Microbubble (MB) well array technology.

MB wells are deep (100-250 μm) spherical compartments with 40-100 μm diameter circular openings fabricated in PDMS using the gas expansion molding process [13, 14]. We have shown that the unique MB well architecture facilitates the accumulation of cell secreted factors while allowing for sufficient nutrient and waste exchange to enable cell proliferation [15]. Although somewhat similar to Microengraving and ISSAC, MBs take the technology two critical steps further by (1) providing an uninhibited niche for cells to proliferate and their secreted factors to concentrate over days without the additional step of capping and (2) simplifying the detection and recovery of cells from wells of interest. Previous studies have shown MB well arrays are an optimum device for culturing various cell lines over time periods ranging from days to weeks with intermittent media changes without concern for dislodging cells from the wells [15-17]. The spherical geometry of the MB well allows for the reduction in shear stresses at the base of the MB both aiding in the growth and propagation of the cells as well as reducing the need for a capping process for cell and secreted factor containment [17]. Cells in MB wells are not easily displaced by fluid flow and in fact it takes several hours for nonadherent cells to exit the wells of inverted chips [16]. We have shown that the interior of the MB wells can be modified to selectively capture cells through antigen specific coatings [16]. Herein, we report on the utilization of this coating process to enhance the detection of ASC using a simple diffusion assay for screening both immortalized hybridoma and primary human B cell samples in a high-throughput manner.

Materials and Methods

Cell Lines

Three cell lines were selected for demonstrating the diffusion assay proof-of-concept that relies on the unique feature of the MB well architecture to accumulate cell secreted factors. All cell lines were cultured at 37 ºC and 5% CO_2 in RPMI 1640 (Gibco A10491-01, Invitrogen Corp., USA) supplemented with 15% heat inactivated fetal bovine serum (Gibco 10082-147, Invitrogen Corp., USA) and 1% penicillin streptomycin (Gibco 15140-122, Invitrogen Corp., USA). The SA-13 cell line (ATCC HB-8501, Cetus Corp.,USA) used secretes IgG with specificity to tetanus toxoid. To validate the growth, proliferation and specific detection of IgG we also used the ARH-77 cell line (ATCC CRL-1621, BD Drewinko) and the CCL-119 T cell line (ATCC CCL-119, GE Foley). ARH-77 cells secrete Ab but with no specificity to tetanus toxoid. The CCL-110 cells do not secrete Ab. A summary of the cell lines with the Ab secretion frequency determined by ELISpot is given in **Table 1**.

Table I: Cell Line Summary – Cells used for proof-of-concept assays with their IgG specific secretion percentages as determined by ELISpot.

Cell Line	Cell Type	Antibody Secretion (Y/N)	Specificity	Secretion %
SA13	Hybridoma	Y	Tetanus Toxoid	25± 6%
ARH77	B lymphoblast	Y	Unknown	7 ± 4%
CCL119	T lymphoblast	N	N/A	N/A
PRIMARY	B cell	Y	Variable	2-4%

Additionally, primary B cells were used for realistic validation of the MB well array diffusion assay. With Institutional Review Board approval and healthy donor informed consent, peripheral blood mononuclear cells (PBMCs) were isolated using BD CPT (BD Biosciences) tubes. B cells were enriched by negative magnetic selection using the B cell Isolation Kit II (Miltenyi Biotec, San Diego, CA) according to manufacturer's instructions. B cells were cultured for three days at $1.5x10^5$ cells per ml in RPMI 1640 media containing 10% FBS and 10 ng/ml IL-2 (Peprotech, Rocky Hill, NJ), 2.5 μg/ml CpG2006 (IDT, Coralville, Iowa), 2.5 μg/ml R848 (Invivogen, San Diego, CA), and 1:100,000 *Staphylococcus aureus* Cowan (SAC) (EMD Millipore, Darmstadt, Germany). After three days, B cells were collected, washed twice, and re-suspended in media above without SAC and seeded into MB well arrays.

Microbubble Array Fabrication

The MB well arrays were formed in PDMS using the gas expansion molding (GEM) process [13, 14]. Briefly, the process utilizes a silicon wafer mold that contains an array of cylindrical pits of 60 μm in diameter and 150 μm in depth (with each feature spaced 4x apart from one another on a square lattice. These features were etched utilizing the Bosch deep reactive ion etch process (Plasma Therm 770, MEMS and Nanotechnology Exchange LLC, Reston, Virginia). To cast the PDMS MB well array, a 10:1 base to elastomer curing agent ratio was used (Sylgard, Dow Corning, USA). The prepolymer was mechanically mixed in a 50 mL conical tube with a plastic pipette for approximately 20-30 seconds or until adequate mixing was achieved, as noticed by the formation of air bubbles within the suspension. The PDMS premix was then poured onto the silicon wafer to achieve a PDMS thickness of ~2 mm. The PDMS is left to self-level at room temperature for ~10 minutes. Following the self-leveling process the residual bubbles left in the PDMS layer are removed by mechanical puncturing with a sterile pipette tip. The silicon wafer with the PDMS premix is then moved to the 100 ºC oven to cure for 1 hour. A spherical MB well forms over each deep pit in the silicon wafer. The size of the MB well produced for this study have circular opening of 60 μm in diameter and overall diameter of ~120 μm (~0.9 nl volume). Following the MB well formation process the wafer and the PDMS cast are removed from the oven and the PDMS cast is peeled away from the silicon wafer mold. The chips are then cut to the desired size using a surgical blade and stored at room temperature in an enclosed Petri dish for future experiments.

Microbubble Array Preparation for Cell Seeding

The MB well array chips were placed on a glass slide with the MB well openings facing down. The slide and the arrays were then treated for 60-90 seconds in oxygen plasma to increase the backside chip wettability (March Instruments Inc., USA). Following the plasma treatment, the chips were moved into separate wells of a 24 well plate and submerged in 1 mL of 1:1 DI water and ethanol. The 24 well plate containing the MB arrays was then placed in a vacuum chamber and degassed for 2-3 minutes or until the MB array became clear, signifying successful priming (infiltration of liquid into the MB wells) of the arrays. This step also serves to sterilize the chip before cell seeding. The MB arrays were then moved to new wells of the 24 well plate and submerged in 1 mL of 1x PBS (BP13351 Fisher BioReagents, USA) under sterile conditions. The 24 well plate with the MBs was then moved to a 37 ºC incubator where they were stored for a minimum of 20 hours. Following incubation in PBS chips were moved to new wells on the 24 well plate and incubated in fresh RPMI 1640 (Gibco A10491-01, Invitrogen Corp., USA) for 10-15 minutes at 37 ºC to allow for priming of the MBs with media. The chips were then moved into new wells and 1 mL of cell suspension was pipette into the wells at a seeding density of 15,000 cells/cm^2 to achieve efficient single cell capture. Chips were incubated for approximately 10 minutes before being rinsed and placed into new wells containing fresh RPMI 1640 (Gibco A10491-01, Invitrogen Corp., USA). Previous studies quantified how the MB well array seeding statistics (% of wells with 0, 1, 2, 3 etc. cells/well) depends on the cell stock concentration, incubation time, and the MB well opening size [15, 16]. The seeding statistics for the conditions used in these studies (n=3 seeding trials) produced arrays with 62%±5% of wells with 0 cells, 28%±2% of wells with 1 cells, 7%±4% of wells with 2 cells, 2%±2% of wells with 3 cells, and 0.3%±0.6% of wells with ≥4 cells/well.

Diffusion Assay for IgG Detection by Immunoprecipitation

SA13 hybridoma cells were used to demonstrate the accumulation of IgG secretions in the MB well using immuno-precipitation detection. Following the seeding of SA13 cells into the MB array, a rabbit α-human IgG -FITC reporter (Rockland antibodies and assays #209-4202, USA) was added to the culture media. The chips were incubated with the reporter over 3-4 days and imaged daily using an inverted fluorescent microscope (Olympus IX70 with QImaging Retiga EXi camera). Images were taken in both bright-field and fluorescence (FITC filter 450-480 nm) for 20 ms and 518 ms respectively. Images were then artificially colored and processed using the enhance contrast function in ImageJ (NIH).

IgG Detection using Affinity Capture Coating

Following the 24 hour incubation in 1X PBS to allow for the removal of residual ethanol, the chips were transferred into new wells of a 24 well plate and 1 mL of a 0.01 mg/mL goat α-human IgG:PBS mixture was added (Rockland antibodies and assays #209-1102, USA) and left to incubate at room temperature for 90 minutes, allowing for the binding of α-IgG to all surfaces. The chips were then transferred to new wells containing a 2% bovine serum albumin (BSA) in 1x PBS for 20 minutes to decrease non-specific interactions. Following the BSA block, the chips

were transferred into wells containing fresh RPMI 1640 (Gibco A10491-01, Invitrogen Corp., USA) for 10-15 minutes at 37 ºC. The cells were then seeded and the rabbit α-human IgG FITC reporter (Rockland antibodies and assays #209-4202, USA) was added to the culture media.

IgG Detection Limit Study

Preliminary studies were conducted in to investigate the IgG detection limit in the MB well array system. MB well chips were fixed in a 48 well plate, sterilized with 50% ethanol and incubated in an oven at 60 °C overnight. The chip surfaces were blocked by placing 600µL of a 2% BSA solution on unprimed chips (air in the MB wells) at room temp for 30 minutes. The chips were rinsed 4 times with 1X PBS. To coat inside of the the MB wells with capture agent 600 µL of a 0.01µg/µL solution of unconjugated anti-Human IgG in PBS was added to the wells of a 48 well plate containing chips. The plate was placed in a desk top vacuum for ~15 minutes until all MB wells were primed with the solution. The plate was then incubated at room temperature on rocker table at low speed for 1.5 hours. After, the chips were rinsed 4 times with 1X PBS and the last rinse was allowed to incubate for ~15 minutes. The chips were then treated with 600µL of 2% BSA for 10 minutes at 37 °C and rinsed 4 times with 1X PBS and again the last rinse was allowed to incubate for ~15 minutes. Next the chips were exposed to 600 µL of either 1µg/mL, 0.1µg/mL, 0.01µg/mL, or 0.001µg/mL Human IgG in 1X PBS and allowed to incubate for 1.5 hrs at room temp on rocker table at low speed, and then at 37 C° for another final 30 minutes. The chips were rinsed 4 times with PBS and the last rinse was allowed to incubate for ~20 minutes. Following this the chips were exposed to 0.002µg/µL TexasRed conjugated anti-Human IgG in 1X PBS and allowed to incubate in at 37°C for ~18hours. The chips were then rinsed 4 times with PBS and fresh PBS was added to each well prior to imaging.

Tetanus Toxoid Antigen Specific Detection using Affinity Capture Coating

The chip preparation protocol was carried out the in same way as described above however the reporter used to detect the tetanus toxoid specific mAb was Clostridium tetani Tetanus Toxoid conjugated to AlexaFluor488. Briefly, 50 µg Tetanus toxoid (#582231, Calbiochem) was dialyzed into PBS and was then adjusted to pH~8.3 with 1M sodium bicarbonate. The conjugation reaction between Tetanus toxoid and AlexaFluor488 dye through tetrafluorophenyl ester was performed at room temperature for 1 hour using the AlexaFluor488 labeling kit (#A20181, Molecular Probes). Then conjugated protein was separated from free dye by spinning through purification resin column. Then the volume was adjusted to 100 µl (0.5 µg/ml) in PBS.

ELISpot

The frequency of Tetanus antigen-specific antibody-secreting cells (ASCs) was measured by Elispot as previously described [18]. Briefly, 96-well Elispot plates (MAIPS4510 96 well,

Millipore, Bedford, MA, USA) were coated overnight at 4 °C in a humidified chamber with either: Tetanus toxoid (1 μg/mL, CAlbiochem, cat#582231), α-human IgG (1μg/mL, Invitrogen, cat#AHI0301), or α-human IgM (1μg/mL, Invitrogen, cat#AHI0601). These antigens and capture antibodies were diluted in sterile PBS to above concentrations. Coated plates were blocked with RPMI and 10% fetal bovine serum for two hours and incubated at 37 °C for 18 to 20 hours with cell line or cultured primary B cells. For antigen plates, 10 to 10^5 cell line or 10^3 to 10^5 cultured primary B cells per well were added in triplicate. For total-IgG and IgM plates, 10 to 10^4 cell line or 50 to 10^4 cultured B cells per well were added in triplicate. After incubation, cells were aspirated and plates were washed with PBS with 0.1% Tween (PBST). Bound antibodies were detected with alkaline phosphatase-conjugated α-human IgG or α-human IgM antibody (1 μg/mL, Jackson Immunoresearch) for two hours and developed with VECTOR Blue, Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA, USA). Spots in each well were counted in a blinded manner using the CTL immunospot reader (Cellular Technologies Ltd, Shaker Heights, OH, USA).

PCR of Recovered Cells

RT-PCR was done on SA13 hybridoma cells retrieved from MB wells to demonstrate amplification of the heavy, kappa and lambda regions of the immunoglobin genes. MB wells were seeded with single SA 13 cells and cultured for 2 to 3 days to proliferate to 5-8 cells. Manual recovery of cells from MB wells was achieved using commercial Eppendorf CellTram and InjectMan® NI 2 Micromanipulator technology. Cells were transferred into a standard 250 μL PCR tube. RT-PCR techniques from Richardson et al. [19] were followed with the following exceptions. Cells were placed in tubes containing 10 μl/well 0.5X PBS containing 10 mM DTT (Invitrogen, Carlsbad, CA), and 8 U RNAsin (Promega, Madison, WI). cDNA was synthesized in a total volume of 20 μl/tube using the qScript cDNA Synthesis Kit (Quanta Biosciences). Primer sequences for the immunoglobin lambda, kappa, and heavy chain genes are given in Supplementary Table S1.

Image Acquisition and Automation

Automated data acquisition and image analysis of large arrays (>10^4 MB wells) is required to minimize operator variability and to enhance research productivity. We have implemented computer control over the x, y, z microscope stage motion, the illumination system and image data acquisition on an Olympus IX70 inverted microscope equipped with QImaging Retiga EXL camera using Objective Imaging's Surveyor Turboscan and Navigator functions. Zero pixel shift filter cubes on the microscope enables concatenation of multiple image channels with enhanced registration quality. This system can image a 1 cm^2 chip (~2K MB wells for current chip layout) in bright-field and one fluorescent channel in ~15 sec at 4x.

Results

Immunoprecipitation

In addition to cell growth within the MBs, cell secreted factors (IgG) were monitored via fluorescence microscopy to demonstrate the utility of MB well arrays for cell sorting using a simple diffusion assay. Uncoated MB well arrays were seeded under limiting dilution conditions with ARH-77, CCL-119, and SA13 cells. The chips were monitored over time by bright-field microscopy for assessing cell proliferation and by fluorescence microscopy to assess the accumulation of secreted IgG. By day 1 the fluorescent images showed that some wells took on a speckled appearance that continued to intensify over time (**Fig. 1**). By day 4 distinct differences could be discerned between the three cell lines. The fluorescence speckle pattern signifies detection of immunoprecipitation (IP) between secreted IgG and FITC labeled α-IgG added to the cell culture media. For multivalent systems, IP occurs through the formation of antibody-antigen agglomerates that precipitate out insoluble fluorescent particles It can be seen that wells with speckled patterns can be detected for the SA13 and ARH-77 cells, albeit with varying frequency, whereas no wells develop a speckled fluorescence signature for the CCL-119 T-cell line as expected. The frequency difference of MB wells containing speckled patterns corresponds with the known Ab secretion variation between the three cell lines determined from ELIspot (**Table I**). Furthermore, it can be seen from the representative images in **Figure 1** that surface bound IgG was also detected in both the ARH-77 and SA13 cell lines. This can be seen by the punctate fluorescent spots within the MB array. This data proves that cell secreted factors accumulate in the MB well and that the MB well array diffusion assay can be used to distinguish heterogeneous states of B cells, suggesting that binding of fluorescently labeled antigen through surface expressed antibody can also be exploited for discovery.

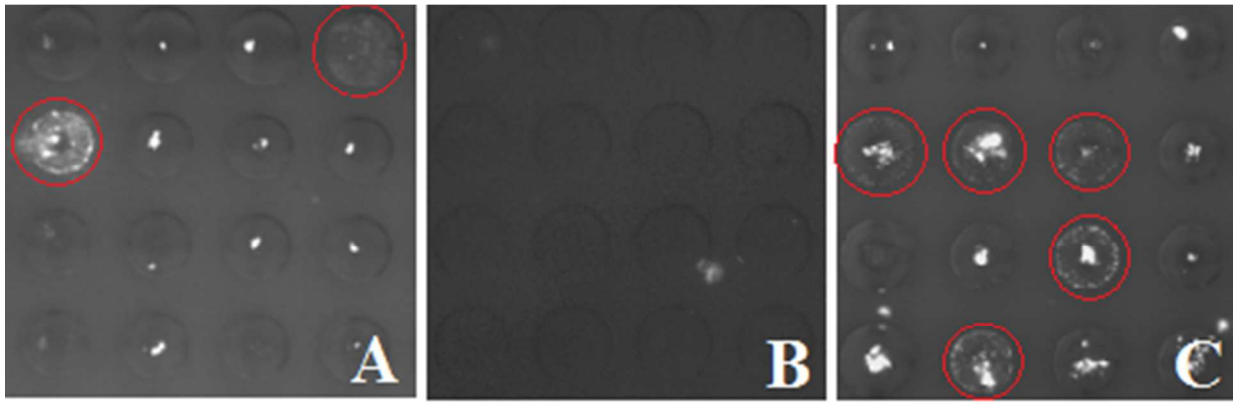

Figure 1: IgG Secretion – A) ARH-77, B) CCL-119, C) SA13. Both ARH-77 and SA13 cell lines display high fluorescence readouts while CCL-119 shows little fluorescence. Red circles correspond to MBs that contained cells and displayed high levels of immunoprecipitation. Light fluorescent spot in (B) is attributed to a reflection off an out of focus bubble on MB surface.

Following the successful detection of IgG through α-IgG mediated IP, a similar protocol was investigated to detect antigen specific Ab detection. Since SA13 cells are a hybridoma line that secrete antibody with affinity toward tetanus toxoid, we added fluorescently conjugated tetanus toxoid to the cell culture media. However, unlike the plentiful speckled fluorescence that was

observed for IgG detection; when the SA13 cells were cultured with tetanus toxoid supplemented media only punctate surface bound detection was observed. No MB wells displaying speckled patterns were observed, even at elevated labeled toxoid concentrations (**Fig. S1**). This result suggest that the avidity of the toxoid was insufficient to initiate IP which we confirmed by combining the SA13 cell culture supernatant with tetanus toxoid and with α-IgG separately and only saw a precipitate form with the latter (**Fig. S2**). Because the IP reaction is limited to detection of multivalent antigens and the potential for difficulties in distinguishing precipitates from background, we did not pursue quantifying the detection threshold for secreted antibody using the IP method. Rather we focused efforts on developing and characterizing a signal detection strategy based on an affinity capture coating method.

Signal Enhancement using Affinity Capture Coatings

For the MB well array diffusion assay to be generally useful for the detection antigen specific B cells a positive signal must be rendered independent of antigen avidity. To circumvent the shortcomings associated with IP detection we investigated an affinity based detection scheme. Here, the MB well array was coated with α-IgG to capture and concentrate IgG secretions from the SA13 cells in and around the MB wells. By utilizing an affinity capture coating we hypothesized that wells containing cells secreting specific antibodies of interest could be detected using the fluorescently labeled antigen as illustrated schematically in **Figure 2.**

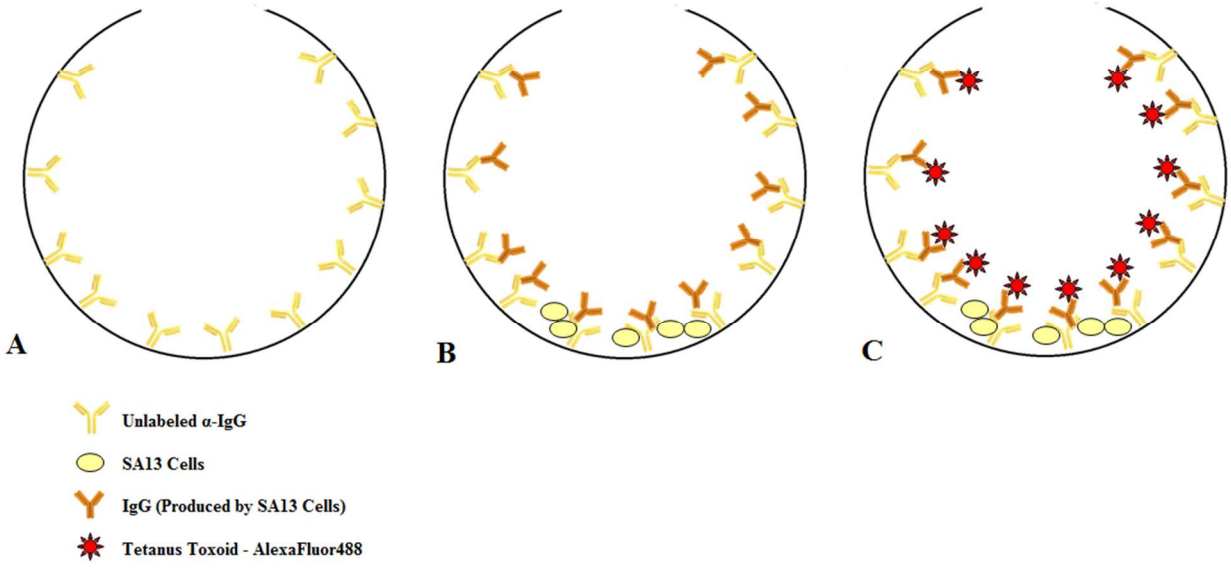

Figure 2: Antigen Specific Coating – Schematic of the coating developed to capture IgG secretions from the SA13 cells onto the surface of the MB. A) Unlabeled α-IgG coated on inner MB. B) Cells seeded, producing IgG that binds coating. C) Fluorescently labeled tetanus toxoid binds IgG layer.

As an initial demonstration, using the protocol outlined in **Figure 2**, SA13 cells were seeded in a MB well array coated with unlabeled human α-IgG and cultured with AlexaFluor488-tetanus toxoid for 4 days. Portions of an array containing (78 MB wells) imaged in fluorescence and bright-field are shown in **Figures 3A and 3B**, respectively. It can be seen that the coating facilitated the capture and concentration of tetanus specific IgG along the surface of the MB well with positive wells indicated by presence of high contrast rings as opposed to a speckle pattern. To confirm this result, affinity coating controls were run with ARH-77 and CCL-119 cells which do not secrete toxoid specific mAbs. Results showed that only the chips containing SA13 cells produced positive fluorescence rings when cultured with AlexaFluor488-tetanus toxoid (**Figure S3)**. The capture coating can also be used to generally detect IgG secretions **(Figure S4)**.

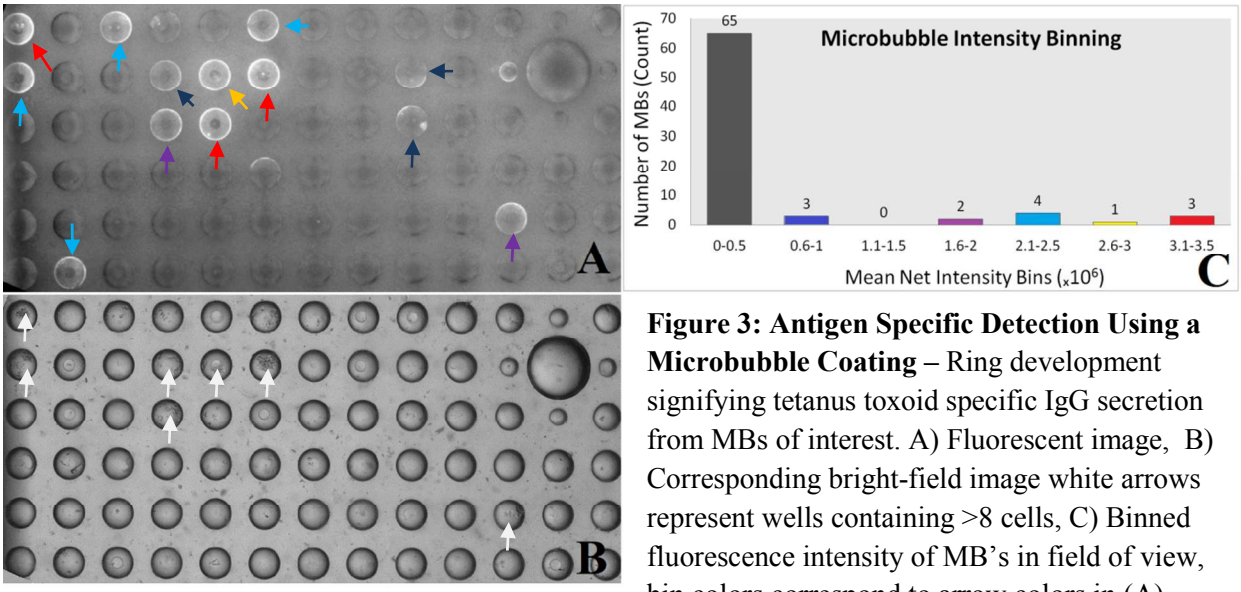

Figure 3: Antigen Specific Detection Using a Microbubble Coating – Ring development signifying tetanus toxoid specific IgG secretion from MBs of interest. A) Fluorescent image, B) Corresponding bright-field image white arrows represent wells containing >8 cells, C) Binned fluorescence intensity of MB's in field of view, bin colors correspond to arrow colors in (A).

It is interesting to note that while some of the MB wells in **Figure 3B** show cell presence more apparently than others (white arrows) the magnitude of the ring intensity does not correlate with the number of cells in the well (**Figure 3**). This is not unexpected as Elispot data indicated that just ~25% of the SA13 cells secrete tetanus toxoid specific IgG (**Table 1, Figure S5**). A further example of this is illustrated in **Figure 4A-F**. MB wells that started with single cells (**Figures 4A, 4D**) and clonally proliferated to a comparable number of cells after 4 days (**Figures 4 B, 4E**) produced markedly dissimilar fluorescence signal (**Figure 4C, 4F).** MB wells which start with single cells that have low secretion rates likely give rise to clonal cell populations that also will have characteristically low secretion rates and thus produce minimal fluorescence regardless of cell number per well. Antibody secretion rates can vary over a wide range; 10 to 10^5 ftg/cell/hr [20]. If antibody is produced by a single cell at ~0.5 pg/hr its concentration in a 0.9 nL MB well could rise to ~13 µg/mL in 24 hr or ~53 µg/mL over 4 days. Preliminary signal detection limit studies **(Figure S6)** indicate that an IgG concentration of 1 ng/ml can easily be detected with the

current imaging system. However, experimental modifications such as reducing the MB well size, increasing the camera integration time, and use of nonphotobleaching fluorescent reporters could easily push the detection limit to the pg/ml level or lower.

Quantification or binning of MB wells based on magnitude of their fluorescence ring intensity is given in **Figure 3C**. Using a custom shape recognition program developed in MATLAB, MB wells were masked and their fluorescence intensity was measured using Equation 1.

$$Net\ Mean\ Intensity = MB_{Area}(MI_{ROI} - MI_B) \quad \text{(Equation 1)}$$

Where MB_{Area} is the area associated with the MB of instance, MI_{ROI} is the mean pixel intensity within the MB of interest and MI_B is the mean pixel intensity of the local background surrounding the MB, net mean intensity was easily calculated. This method for fluorescence quantification enables us to move toward a high throughput screening process in which thousands of MB's can be screened and binned based on their relative fluorescence intensities in seconds. Upon location of highly positive wells (**Figure. 4**), as determined by fluorescence image analysis, cells within the well can be retrieved using a micromanipulator (**Figure S7**). Following retrieval of desired cell population, cells can either be clonally enhanced to produce an original cell line, or the cells can be lysed and their products can be amplified for eventual recombinant mAb expression. In preliminary testing, we have been able to recover cells to conduct RT-PCR, giving us the ability to analyze immunoglobin genes from approximately five SA13 cells retrieved form MB wells which originated from a single cell (**Figure 4G**). The Ig heavy chain variable region (VH) was sequenced from cells recovered from MB1 (**Figure S8**).

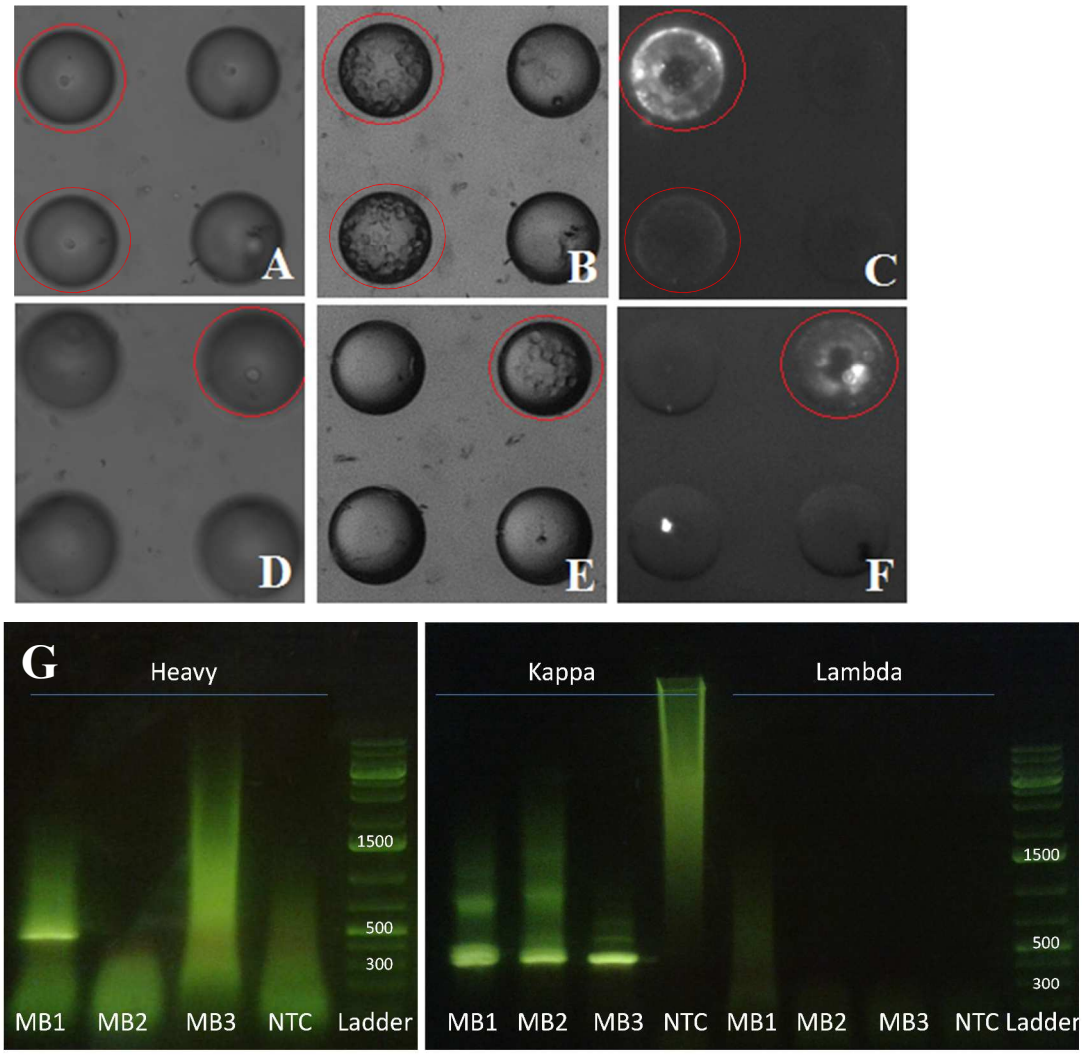

Figure 4: Cell Signal Detection and Retrieval – Two representative MB wells cultured over time that started with single SA13 cells (white arrows). (A&D)– Day 0 bright-field images, (B&E) Day 4 bright-field images and (C&F) – Day 4 fluorescence images signifying tetanus toxoid specific mAb secretions. (G) RT-PCR was done on SA13 cells retrieved from MB wells to amplify the heavy, kappa and lambda regions of the immunoglobin genes (~5-8 cells for each condition). MB1 corresponds to cells showing high fluorescence in Figs. A-C, MB2 corresponds to cells showing high fluorescence in Figs. D-F, No template control (NTC). SA13 cells are a monoclonal hybridoma and only express kappa (not lambda) light chain genes.

Primary B Cell Growth and IgG Detection

While the above results both validate and build on the utility of the MB well array diffusion assay there are major hurdles to translate this technology to clinical applications. For the MB well array technology to excel as a cell sorting platform for screening heterogeneous cell samples

it is imperative to establish a protocol in which primary cells can be sorted and analyzed. While antigen specific detection and isolation of primary B cells from blood samples holds many promising applications in various fields ranging from mAb production to disease detection, the development entails various obstacles [21-23] and complexities associated with the culturing of primary cells [24, 25].

One of the most complex aspects of primary cell culture is the need for unique culture conditions. Unlike the immortalized cell lines discussed above, primary B cells require stimulatory factors to drive memory B cells to generate plasma cells that in turn secrete Abs. Using the protocol outlined in the methods section, Toll Like Receptor (TLR) ligands were used to drive cell differentiation and Ab secretions. Stimulated primary B cell samples were successfully cultured in MB well arrays over a period of 4-5 days (**Figure 5**). It can be clearly seen in the representative image that the MB well that was initially seeded with 2 cells proliferated to >6 cells within the 4 day culture period. Furthermore, a live/dead stain on day 4 confirmed the cells contained within the MB were primarily alive.

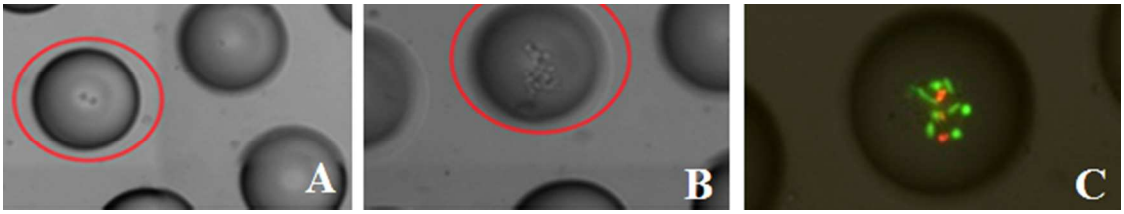

Figure 5: Primary B Cell Growth in MB – A representative MB well was tracked over the course of 4 days to monitor the cell proliferation of primary B cells. A) Bright-field image of the MB on day 0 immediately after cell seeding well contains 2 cells. B) Bright-field image of same MB 4 days later. C) Live/dead stain of MB on day 4. Green represents live cells while red represents dead cells

While primary B cell growth was observed using the MB well platform, the extent of that proliferation was much lower than that seen with the SA13 cells. The clonogenic potential (CP), or the ability for a single cell to proliferate to >5 cells in 4 days within a MB, was determined to be approximately 23±18% for the primary B cells. This is in contrast to the SA13 cells that have a CP of approximately 95±2%.

Having confirmed the survival and proliferation of primary B cells within MB well arrays we moved to the detection of IgG secretion. Using α-IgG affinity capture coating method we observed efficient detection of secreted IgG from primary B cells over the course of 48 hours (**Figure 6**). A magnified view of a well positive **(Figure 7**) shows the IgG secretions in red with the corresponding live cell stain (green). A multiplexed staining approach will for example, enable the ability to screen cells that positive for viability, Ab isotope secretion, as well as antigen specificity at the time of cell recovery.

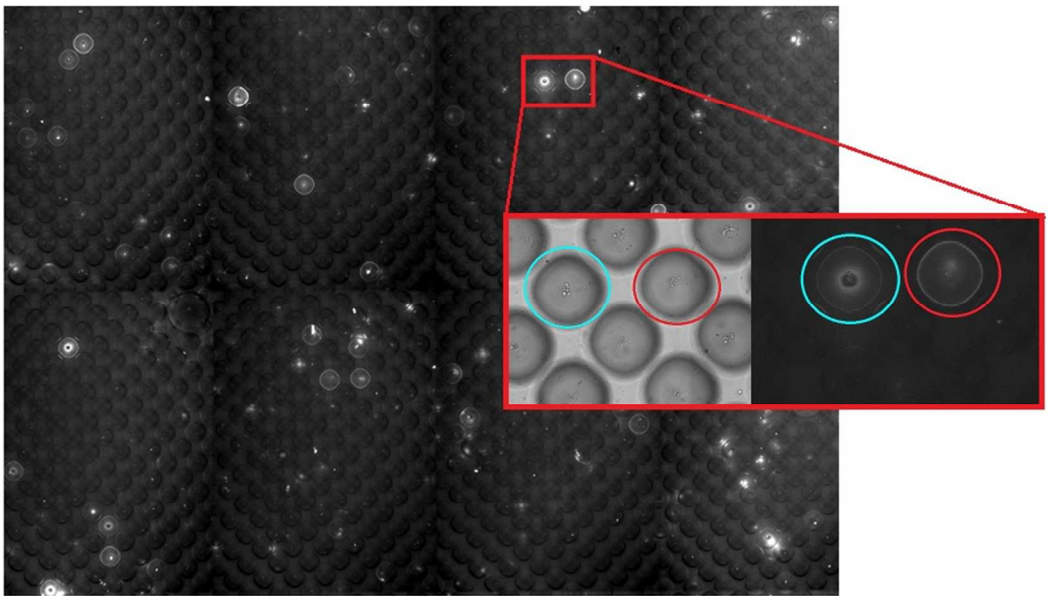

Figure 6: Primary B IgG Capture Coating – Stitched scan of MB array containing approximately 900 MBs. Cells were seeded and the MB array was imaged following 2 days of incubation. MB with fluorescent rings around them are considered positive wells.

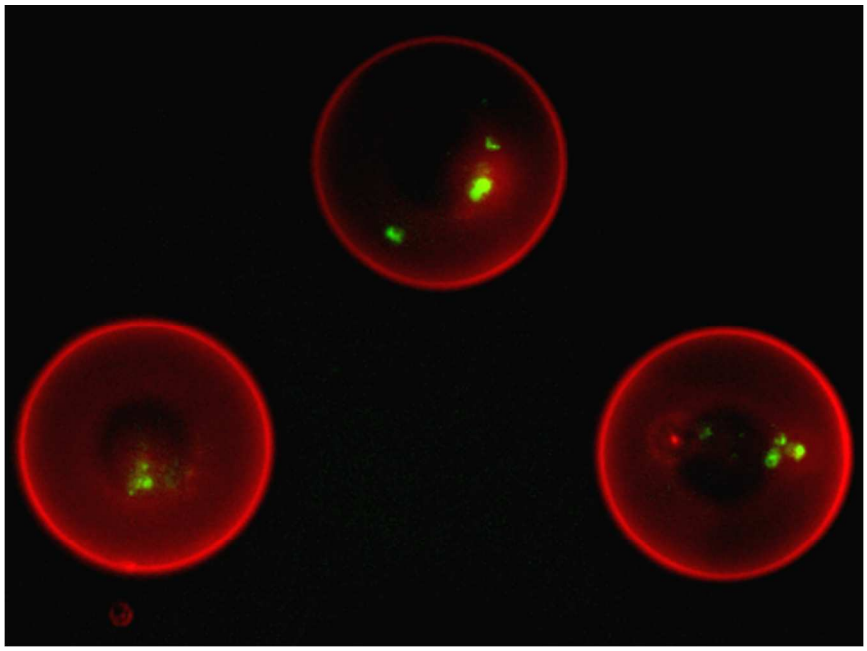

Figure 7: Primary B IgG Capture Coating Live Stain – Representative image from area on a MB array seeded with primary B cells showing MB's positive for live cells (Green) which are additionally secreting IgG (Red).

Discussion

The ability to sort large populations of heterogeneous cells and to assay them on a single cell level in a high-throughput format is critical to the development of both hybridoma screening and mAb production. In this study we present a new method based on MB well array technology to sort cells by what they secrete using a simple diffusion assay. We have shown that in addition to the growth characteristics of SA13 cells (hybridoma that secretes human mAb to tetanus toxoid) within a MB well, their secreted IgG can be monitored over time (~7 days). It is clear from **Figure 1** that secreted IgG accumulates within the MB well to sufficient concentrations to allow detection through immunoprecipitation and fluorescence microscopy. It is also apparent that there are two different types of fluorescence signal present within the MB wells; cell localized spot fluorescence as well as the diffuse punctate speckle fluorescent pattern. For the sake of this assay the spot fluorescence alone is insufficient to be considered a positive readout as this could also result from auto-fluorescent contamination. Imaging at a higher magnification (>4x) would be required to distinguish this from cell surface bound IgG and this would lead to longer imaging times. Conversely, the MB wells that exhibit the speckled fluorescence pattern are considered positive due to the secretion and accumulation of IgG to levels that result polymerization of multiple IgG/α-IgG monomers that precipitate out. Immunoprecipitation is evidence that cell secreted factors accumulate in the MB well.

Following the successful detection of secreted IgG, we attempted antigen specific detection by culturing the SA13 cells in the presence of fluorescently tagged tetanus toxoid. The results were however, in stark contrast from those of the IgG detection as only spot fluorescence signals were observed (**Figure S1**). The lack of speckle or immunoprecipitation for toxoid detection likely reflects the properties of the mAb and toxoid antigen themselves. From the literature, it is known that mAb regardless of affinity rarely form insoluble precipitates [26]. Antigenic avidity (multiple epitopes) is also a required for immunoprecipitation and hence is a contributing factor limiting immunoglobin polymerization as schematically illustrated (**Figure S9**).

Due to monospecificity limitations of mAbs and uncertain antigenic avidity a simple diffusion assay which relies on lattice precipitation between the mAb and the antigen cannot be reliably used for discovery. There are several strategies that can be considered to overcome this shortcoming, including the use of antigen coated polymer nanoparticles as a means to increase antigenic avidity. However, this approach would require extensive effort and cost to generate functionalized nanoparticles that were colloidally stable in cell culture media for several days. Hence, we chose to pursue a much more straightforward approach using affinity capture coatings. The coating procedure acts in a similar way to a sandwich ELISA or the ISSAC [12]. approach whereby a capture reagent binds the cell's secreted factors. Following the secreted factor capture onto the MB surface, a tagged fluorescent reporter is added to the culture media, in this case tetanus toxoid conjugated to Alexa488. Using this approach, we were able to easily identify positive wells, indicated by high contrast fluorescent rings (**Figure 4**) resulting from the concentration of secreted mAb to the MB well surface. This approach not only enabled the detection of antigen specific mAbs for the first time in MB well arrays but it also provides a profound means to easily distinguish positive signals that vary in intensity. It can be noted from **Figure 4** however, that the intensity of the fluorescent signal for a given MB well does not correlate directly with the number of cells within that well. While some MBs have only a few cells and produce high fluorescent signals, other MB wells contain many cells with a very low

signal. This can be attributed to frequency of SA13 cells that secrete antibody as well as to differences in secretion rates amongst SA13 cells. When a cell population is derived from a clonally pure cell that is secreting at a low rate it, is more probable that the resulting daughter cells will mimic the parent cells phenotypic expression [27]. This is something that has been exploited many times throughout the literature for enrichment of cell lines based on surface receptors and phenotypic traits [28, 29].

For MB well array technology to become a successful cell sorting and screening platform technology it is critical to develop and validate protocols to capture, culture and detect signals being secreted from primary cells as well as to automate the data acquisition and image analysis capabilities of very large arrays (>50K MB wells). Herein, we successfully demonstrated the ability to detect IgG secretion from primary B cells derived from human peripheral blood using array size of ~1K MB wells. These initial experiments also examined the ability of the MB well arrays to culture B cells over a period of approximately 7 days. Data from these experiments suggests that the primary B cells in these conditions reach optimum proliferation by day 3-4 and cease proliferation near after. While the immortalized cell lines and primary T cells (data not shown) appear to proliferate within the MBs to confluency (MB well filled and cells proliferate beyond MB well confines), primary B cells never reach confluency. This is not unexpected due to the limited number of divisions that primary B cells undergo before apoptosis [30]. It is important to note as outlined in methods, that following isolation of the cells from the donors they were incubated in stimulatory culture conditions for 24-72 hours before seeding in the MB well array to promote the differentiation of antibody secreting cells.. Therefore, by day 3-4 of MB incubation the cells had been proliferating outside of the donor for nearly 4-6 days, and have likely undergone 1-3 cell divisions within this period [31].

It is evident from **Figure 6,** that similar to the SA13, there is also a range in the level of fluorescence intensity among the positive wells. While primary B cells can vary heavily in their Ab secretion rates, it was expected that primary B cells as a whole would secrete at a lower rate than the hybridoma lines. Recent publications on primary B cell IgG secretion rates over 48 hours show CpG stimulated primary B cells secrete at an average of approximately 145.1 ± 77.5 pg/1000 cells [20]. In contrast, our preliminary data collected on the SA13 hybridoma cell line found them to secrete at approximately 10,000-50,000 pg/1000 cells in 48 hours (not shown); a nearly 70 fold increase at the lower end. Despite this, our ability to detect strong positive signals from MBs wells that typically contain <4 cells is enabled by the use of the affinity capture coating that helps to concentrate the cell secreted product on the surface of the MB well. Future experiments will examine the effect of MB well size on signal detection. Since primary B cells secrete at a lower rate than most immortalized hybridoma lines, decreasing the volume of the MB well may help to increase the concentration of secreted factors and allow for even more efficient detection at earlier time points.

Furthermore, it should be noted that the MB well array system can be used to approximate the frequency of ASC, expressed as a percentage of cells in the heterogeneous sample, which is typically measured using ELISpot. With knowledge of the initial cell seeding distribution (# of MB wells seeded with 1, 2, 3, or 4 cells), the % of ASC can be estimated by simply counting the number of fluorescent rings using Equation 2.

$$\%IgG = \frac{R_*}{\sum_n^4 (C_n P * MB_T)} \quad \text{(Equation 2)}$$

In this equation R_* is the number of fluorescent rings within the field of view while MB_T is the total number of MB wells within that same window of view. The C_nP term is unique to the cell seeding concentration which determines the percentage of MB wells with n= 0, 1, 2, 3 or 4 cells (**Figure S10**). Using this equation and the fluorescent image of the primary B cell array **(Figure 6)** we estimated that the % percentage of IgG secreting was 2.7±1%, versus the actual value obtained from ELISpot which was found to be 6.4 ± 4.2% (**Figure S11**). The accuracy of this prediction relies on the ability to reproducibly seed arrays with a consistent distribution. Studies suggest that the MB well seeding statistics (C_nP) across various cell lines is consistent for a given cell seeding concentration and MB well opening. Hence, MB well array technology has the potential to glean data on the frequency of ASC and the antibody secretion rate in a single assay which enables this technology to compete directly with more conventional techniques such as ELISA and ELISpot, all while doing so more efficiently and with higher throughput.

Future studies will seek to develop protocols to eliminate the 24-48 hour B cell stimulation step prior to MB well array seeding, as well as to expand the array size far beyond 2500 MB wells to increase the number of cells that can be screened in a shorter period of time. Currently, we are developing protocols to image much larger arrays. We estimate that for a rare cell (1 in 5000) we would detect 10 positive wells in a 50K MB well array initially seeded with 37% of the wells containing 0 or 1 cell, 18% with 2 cells, and 6% seeded with 3 cells per well. Such conditions would be more than sufficient for hybridoma screening which for strong immunogens, stimulated B cells are preferentially preserved in the hybridoma formation [32] such that antigen specific cells in the hybridoma screen is typically much higher than 0.1% (1:1000) [33]. The ability to quantitatively screen and bin MB wells based on their fluorescence intensity (**Figure 4C**) will enable future developments of this technology to not only sort cells but return data on the secretion rates of the cell lines as a function of time as well as the secretion percentages. This enables to MB array to glean further data from the sorting process that conventional techniques and other microwell systems can achieve.

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