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Characterization of cell seeding and specific capture of B cells in microbubble well arrays

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Abstract

Development of micro-well array systems for use in high-throughput screening of rare cells requires a detailed understanding of the factors that impact the specific capture of cells in wells and the distribution statistics of the number of cells deposited into wells. In this study we investigate the development of microbubble (MB) well array technology for sorting antigen-specific B-cells. Using Poisson statistics we delineate the important role that the fractional area of MB well opening and the cell seeding density have on determining cell seeding distribution in wells. The unique architecture of the MB well hinders captured cells from escaping the well and provides a unique microenvironmental niche that enables media changes as needed for extended cell culture. Using cell lines and primary B and T cells isolated from human peripheral blood we demonstrate the use of affinity capture agents coated in the MB wells to enrich for the selective capture of B cells. Important differences were noted in the efficacy of bovine serum albumin to block the nonspecific adsorption of primary cells relative to cell lines as well as the efficacy of the capture coatings using mixed primary B and T cells samples. These results emphasize the importance of using primary cells in technology development and suggest the need to utilize B cell capture agents that are insensitive to cell activation.

Keywords

B cell; Cell sorting; Microbubble array; Affinity capture; PDMS; Poisson distribution

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1 Introduction

Microfabricated technologies have become increasingly popular in cell biology and disease state research due to the ability to capture and monitor single cells in physiologically relevant microenvironments (Bhadiraju and Chen 2002; El-Ali et al. 2006; Li et al. 2003; Park and Shuler 2003; Toh et al. 2010; Voldman et al. 1999). Within these *in vitro* microenvironments, heterogeneous cell populations can be sorted and independently interrogated within one device that overcomes many limitations of standard cell culture assay systems (Love et al. 2006; Gong et al. 2010). For example, use of the 96-well plate format imposes the constraint of a high media volume to surface area ratio (Meyvantsson and Beebe 2008) which hinders cell self-conditioning of wells when seeded under limiting dilution conditions (Walker et al. 2004). Relatively large reagent volumes, long processing times, and the necessity to use many plates to assay for minority cell types or secreted soluble factors (e.g. cytokines, antibodies) are additional limitations that can be overcome using microfabricated systems (Love et al. 2006; Giang et al. 2008; Liberskit et al. 2011). The attributes of a low cell culture volume, customizable surface chemistry, and the ability to fabricate high density micro-well arrays, are particularly advantageous for immune system research in which both single cell studies and interactions between B and T cells can be specifically probed (Waldmann 1979; Lanzavecchia 1985; Love et al. 2006; Tangye et al. 2012).

Successful development of a microfabricated technology for high-throughput cell sorting applications requires extensive characterization of the device to predict the appropriate array size and the cell seeding density needed to sustain cell survival, achieve assay detection sensitivity, and relevant statistical analyses in experiments. Systems utilizing micro-well platforms typically claim Poisson-like seeding behaviors (Jin et al. 2009; Love et al. 2006; Nikkah et al. 2011; Rettig and Folch 2005; Zaretsky et al. 2012) but most do not report supporting data or models that describe factors that impact cell seeding or the distribution behavior. Additionally, it is common that cell samples used in technology proof-of-principle studies are sorted prior to use either by the inherent homogeneity of the cell line used or by the expression of cell surface markers using fluorescence activated cell sorting (FACS) (Jin et al. 2009; Kurth et al. 2009; Love et al. 2006; Nikkah et al. 2011; Rettig and Folch 2005). The latter technique is widely used despite the fact that the rigor of sample preparation and analysis can alter cell function and/or viability (Dick 2009). Hence, in developing microfabricated technology platforms for single cell sorting and/or functional studies it is important that the factors impacting micro-well seeding efficiency be determined and controllable and that assays be conducted using minimally manipulated primary cells.

Recently, we introduced microbubble (MB) well array technology and demonstrated its use to sustain single and small cell cultures for extended periods of time (>10 days) (Giang et al. 2008; Chandrasekaran et al. 2011). Microbubbles are spherical cavities formed in polydimethylsiloxane (PDMS) using the gas expansion molding (GEM) process (Giang et al. 2007, 2012). The unique architecture of the MB well provides a low media volume per cell ratio that creates a microenvironmental niche that cells can condition. Factors secreted by cells in MB wells can rise to bioactive levels not attainable in standard culture well

formats thereby facilitating their survival and proliferation (Chandrasekaran et al. 2011). This attribute is highly advantageous and differentiates MB technology from commonly used microfabricated shallow well systems in which cell proliferation and long term culture are hindered by dilution of secreted factor in the bulk media (Rettig and Folch 2005; Jin et al. 2009; Han et al. 2010; Song et al. 2010; Zaretsky et al. 2012; Nikkah et al. 2011). Moreover, fluid stress during media changes could cause cells to dislodge from shallow wells. In previous work we have shown that MB arrays can be used to dissect the heterogeneity of a cell sample at the single cell level (Giang et al. 2012). MB wells have also been used to grow arrays of homogeneously sized microtumors (Giang et al. 2008), to study the epidermal-mesenchymal transition process that is important in cancer metastasis (Chandrasekaran et al. 2011), and to determine the clonogenic potential of samples enriched with cancer stem cells (Chandrasekaran and DeLouise 2011). Furthermore, we have demonstrated a MB well array based perfusion system for culturing multi-cellular tumor spheroids and showed that spheroid cells were more resistant to doxorubicin treatment than adherent cells (Agastin et al. 2011). Here in, we take advantage of the unique properties of MB well architecture and our ability to selectively manipulate the surface chemistry inside the MB wells to enrich and sort B cells. Using B and T cell lines and primary human lymphocyte samples we characterize the cell seeding distribution, cell capture efficiencies, and we elucidate the factors that impact these. We also enumerate differences between primary lymphocyte samples and cell lines in the specific capture of B cells. Our results provide a proof-of-principle that MB well arrays can be developed into a powerful tool for high-throughput sorting of antigen-specific B cells.

2 Materials and methods

2.1 PDMS microbubble fabrication

Polydimethylsiloxane (PDMS) MB arrays were formed according to previously reported methods (Giang et al. 2007, 2012). Briefly, a 10:1 base to curing agent weight ratio from a commercially available silicone elastomer kit (Sylgard®, Dow Corning, USA) was mixed manually. The mix was poured over a modified Bosch coated Si wafer (MEMS and Nanotechnology Exchange LLC, Reston, Virginia) mold and allowed to self-level on the bench top for 30 min at room temperature. PDMS was then cured in a 100 °C oven for 2 h. The PDMS MB cast was then peeled off of the Si wafer after cooling and chips were cut using a razor blade. Chips (0.33 cm×0.5 cm) used for this study contained arrays with 160 MB wells. Each MB had a 60 µm diameter circular opening and a volume of ~0.5 nL. The chips used in these studies had a fractional MB opening area(F_{MB}) of 2.7 % relative to the total chip area.

2.2 Cell culture

A human B cell line expressing surface IgG, ARH-77 (ATCC CRL-1621, BD Drewinko, USA) and a human CD4+ T cell line, CCRF-CEM (ATCC CCL-119, GE Foley, USA) were used to study cell capture statistics and efficiency inside micro-bubble wells. Cell lines were cultured in RPMI1640 (Gibco A10491-01, Invitrogen Corp., USA) supplemented with 5 % heat-inactivated fetal bovine serum (FBS, Gibco 10082-147, Invitrogen Corp., USA), and 1

% Penicillin/Streptomycin (Gibco 15140–122, Invitrogen Corp., USA) at 37 °C and 5 % CO₂.

Primary B and T cells were also used in affinity capture studies. Memory B cells and CD4⁺ T cells were isolated from fresh human blood *via* CPT tubes. Cells were stained with CD19-APC (EBioscience, #17-0198-41), CD4-Alexa488 (BD Pharmingen, #557695), CD14-Pacific Blue (BD Pharmingen, #558121), and 7-Aminoactinomycin D (7AAD, SouthernBiotech, #10042-01). The peripheral blood monocyte cells (PBMCs) were then sorted into B cells (CD4[−], CD19⁺, CD14[−], 7AAD[−]) and T cells (CD4⁺, CD19[−], CD14[−], 7AAD[−]) using FACS (BD Aria LSRII). Both cell types were cultured in stimulatory RPMI 1640 media (Cellgro #10-040-CV, Mediatech, Inc., USA), supplemented with 10 % FBS (PAA #A15-301, GE Healthcare, USA), 1 % P/S (Gibco #15070-063, Invitrogen Corp., USA), 1 % HEPES buffer (Gibco #15630-080, Invitrogen Corp., USA), 1 % NEAA (Gibco #11140-050, Invitrogen Corp., USA), sodium pyruvate (Gibco #11360-070, Invitrogen Corp., USA) with 2.5 µg/ml ODN Cpg2006 (Oligos Etc.), 2.5 µg/ml R848 (InvivoGen tlr-r848-5, USA), 20 ng/ml IL2 (PeproTech, #200-02) and 1/50,000 PWM (kindly provided by Shane Crotty, PhD University of Rochester). All primary cells samples were obtained from healthy adult subjects who provided signed written informed consent. All procedures and methods were approved by the Research Subjects Review Board at the University of Rochester Medical Center.

2.3 Cell seeding distribution analysis

To prepare MB chips for cell culture they were first sterilized using ethanol, then rinsed using deionized water, and blown dry with nitrogen gas. The back and sides of the MB chip were treated with UV/ozone (BioForce Nanosciences, Inc. UV.TC.110, USA) for 30 min. The chip surface, containing the MB openings, was then treated with 2 % bovine serum albumin (BSA, Hyclone SH30574.01, Thermo Scientific, USA) for 10 min to hinder protein and cell adhesion. The BSA solution does not enter the MB wells. MBs were primed with PBS buffer using the vacuum-assisted coating (VAC) technique previously described (Giang et al. 2008). Briefly, ~20 µl of PBS buffer was placed onto the chip surface and then subjected to negative pressure in a bench top vacuum chamber for 45 min. Air is evacuated from inside the MBs and the bulk PDMS chip drawing the liquid into the MB wells. Using the reagent exchange process, excess PBS buffer was pipetted off the chip, being careful not to deprime the MB wells, and replaced with the cell culture media to exchange the PBS inside the MBs. Next, excess media was removed and replaced with a cell stock solution of a specified concentration depending upon the experiment as discussed below. The cell solution was allowed to incubate for 30 min under sterile and humidified conditions. After incubation the number of cells that deposited onto the chip surface and into each MB well in the array were counted by eye under a Nikon phase contrast microscope. Images of the array were captured with a Nikon DS-Fi1 camera.

Eight cell seeding densities (SD) were studied (Table 1) to quantify the effect of SD on cell capture efficiency and the distribution of cells deposited into the MB well arrays. SD is defined as the ratio of the number of cells seeded onto the chip to the number of MBs on the

chip (Eq. 1). Single cell seeding efficiency (SCE, Eq. 2), and total cell seeding efficiency (TCE, Eq. 3) were calculated as defined below.

$$SD = \# \text{cells seeded per chip} / \text{total} \# \text{MB wells per chip} \quad (1)$$

$$SCE = \# \text{MB wells with single cells} / \text{total} \# \text{cells seeded} \quad (2)$$

$$TCE = \text{total cells captured in MB wells} / \text{total} \# \text{cells seeded} \quad (3)$$

The cell distributions in MB well arrays were modeled using the Poisson distribution to identify factors that control the Poisson statistic, λ . A sum of least squares regression analysis was done to fit the Poisson equation to the seeding distribution results to determine an observed λ (λ_{obs}).

2.4 Affinity capture of specific cells

Proof-of-principle studies were conducted to demonstrate affinity capture of B cells by coating MB wells with anti-human IgG antibody (α -IgG). Capture efficiency was tested using both B cell lines and primary human lymphocytes. After preparing sterilized MB array chips as described above, the VAC and reagent exchange processes were used to coat the inside of the MBs with α -IgG. After priming MB arrays with PBS buffer a 15 μ l aliquot of a 0.05 mg/ml streptavidin solution (Rockland Immunochemicals, Inc., S000-01, USA) was applied to the chips and allowed to sit for 2 h. Chips were washed three times by vigorous pipetting with PBS buffer. Next, 15 μ l of 0.2 mg/ml biotinylated α -IgG (Jackson ImmunoResearch Laboratories, Inc. 109-065-088) was pipetted onto chips for 30 min after which the chips were again washed 3 times with PBS buffer. Negative control chips included an uncoated MB well array and an array in which the MB wells were coated with 2 % BSA. An additional negative control included exposing streptavidin coated wells to 0.2 mg/ml biotinylated α -Transferrin (Jackson ImmunoResearch Laboratories, Inc. 015-060-050) as described above. To test for the presence of α -IgG coating we exposed each MB chip to 0.5 mg/ml FITC-labeled human IgG (FITC-IgG, Jackson ImmunoResearch Laboratories, Inc. 009-090-003). Fluorescent images were taken using an inverted microscope (Olympus IX70 with QImaging Retiga EXL camera). Images were integrated for 5.5 s and analyzed using ImageJ software (NIH). Results were analyzed using unpaired student's *t*-test at 95 % confidence.

To validate cell capture specificity we first conducted studies using the ARH-77 (B cells) and CCRF-CEM (T cells) cell lines. Cells were seeded onto chips with α -IgG coated MB wells, uncoated MB wells, and 2 % BSA coated MB wells at a SD=1. Cells were allowed to settle for 30 min, and then chips were gently washed by swirling in cell media. Chips were imaged using a Nikon phase contrast microscope with a Nikon DS-Fi1 camera to quantify the number of cells each MB well (before wash). Chips were washed using three different protocols and remaining cells were counted to determine capture efficiency (cells retained/cells seeded). Wash protocols included (1) pipetting the chip surface with PBS buffer three times (after wash), (2) turning the chip upside down and shaking vigorously (shakeout), (3)

incubating the chip upside down overnight in a reservoir of cell media (overnight). After the wash step, the chips were reimaged to count the remaining cells. Results were analyzed using one-way ANOVA at 95 % confidence.

Affinity capture of B cells was repeated using primary B and T cells freshly isolated from human blood within 5 h of sample collection. A B cell only, a T cell only, and a 1:1 ratio of B:T cells were added to chips at a SD=1. Primary B cells were fluorescently labeled green (PKH67GL-IKT, Sigma Aldrich, USA) and T cells were fluorescently labeled red (PKH26GL-IKT, Sigma Aldrich, USA) to distinguish the each cell type in the mixed population chips. Each cell population was added to α -IgG coated MB chips, uncoated chips, and 2 % BSA coated chips. Chips were subjected to the experimental protocol and data analysis as stated above. Results were statistical analyzed using one-way ANOVA at 95 % confidence.

3 Results

3.1 Cell seeding distributions

Understanding the distribution statistics of cell seeding in MB well arrays and the factors that impact this are essential for developing MB technology as a high throughput tool to capture, identify, and recover rare cells from a heterogeneous sample. For example, antigen-specific B cells in human peripheral blood comprise <1 % of the total B cell population (Kyu et al. 2009) which in turn comprise only 5 to 15 % of circulating lymphocytes (Coila 2010). Hence, a minimum of ~2000 lymphocytes would have to be seeded into the wells of a MB array chip to ensure capture of just one antigen-specific B cell of interest. Therefore, it is important to understand the factors that impact cell capture efficiency (% of cells seeded that deposit into MB wells) and the seeding distribution statistics (number of cells seeded per well) to develop successful assay protocols. The main factors that can affect capture efficiency and the distribution statistics include the cell sample concentration, the sample volume applied to the chip, the number of wells in the array, and the fractional area of MB openings (F_{MB} , area of one MB opening multiplied by the # of MB wells per chip divided by the total chip area). In the following we quantified the effect of cell sample concentration on capture efficiency and cell number distribution in MB wells keeping the sample volume and chip architecture constant (160 MBs per chip, 60 μ mMB diameter opening). Using procedures described in the Section 2, eight cell seeding densities (SD, the # of cells seeded/the number of wells in the array) were explored (Table 1). After seeding, the number of MB wells in the array containing 1, 2, 3, etc. cells/well and the total number of cells deposited onto the planar chip surface were quantified. A representative image of a region of an array depicting a typical cell distribution for SD=0.47 is shown in Fig. 1. A histogram plotting the fraction of MB wells in the array containing a given number of cells divided by the total number of total number of wells per chip is shown in Fig. 2a as a function of SD ($n=3$ chips per SD). Results show that as the SD increases the number of empty MB wells decreases and the number of MB wells containing more than 2 cells increases. The single cell seeding efficiency (SCE, Eq. 2) also decreases with increasing SD (Fig. 2b). These trends are consistent with Poisson statistics given by Eq. 4, where $P(x)$ is the probability of a

specific event x occurring (i.e. the fraction of MB wells containing $x=1, 2, 3$ etc. cells per well).

$$P(x) = e^{-\lambda} \lambda^x / x! \quad (4)$$

The Poisson distribution statistic λ , corresponds to the average number of events that occur in a specified area. Here, we initially hypothesized that λ would equal the SD and that each cell would have an equal chance to deposit into any MB well. Because the volume of the MB is much greater than a cell it is unlikely that the presence of a cell in a MB well would influence the deposition of a second cell into the same well.

To test this hypothesis we plotted the Poisson equation with λ set equal to the experimental seeding densities. Theoretical curves (dotted lines) for SD=5.41, 1.1, and 0.13 are shown in Fig. 3a with the experimental data (solid lines) plotted for comparison ($n=3$ chips). Results show poor agreement for all but the lowest SD. This behavior suggests that additional factors exist that impact the distribution statistic, λ , decreasing its value. To investigate these factors we first fit the experimental data to the Poisson equation to determine the observed λ (λ_{obs}) for each SD (Table 2). An obvious factor that could effectively lower the cell seeding events into wells is the fractional MB opening area (F_{MB}). The F_{MB} of the chips used in these studies was 0.027. With only 2.7 % of the chip area comprised of openings into MB wells it is expected that many cells would deposit onto the chip surface rather than in wells. Multiplying the SD by F_{MB} we calculated a model λ (λ_{model}) reported in Table 2. It can be seen however, that F_{MB} over compensates as λ_{obs} are ~12 times higher than λ_{model} indicating that more cells enter the MB wells than is predicted based on the value of λ_{model} . We observed that during the seeding process cells that deposit onto the chip surface continue to move parallel, presumable do to convective fluid forces in the media. Images of cell motion were recorded every minute over the 30 min incubation time (Fig. S1). These images were used to quantify the magnitudes of the cell displacements from which we calculated an average cell velocity of $42.5 \pm 7.9 \mu\text{m}/\text{min}$. The effect of this parallel motion is to increase the number of MB openings that a cell can encounter. In this study the MB wells were spaced $4\times$ the diameter of the opening ($240 \mu\text{m}$) apart on a square lattice. We estimate that over the 30 min incubation time a cell could encounter ~5.3 MB openings. This effectively increases the fractional area (F_{eff}) to ~0.143. Results plotted in Fig. 3b and summarized in Table 2 show that accounting for parallel motion significantly improves the agreement between λ_{obs} and λ_{eff} . Hence, SD and F_{eff} are key parameters that describe cell seeding statistics in MB well arrays. It is also interesting to note that we observed the total cell seeding efficiency (TCE, Eq. 3) to be independent of SD (Fig. S2). On average of $62.4 \pm 0.08 \%$ of the cells seeded deposit into MB wells. Since we are interested in using MB arrays to capture rare cells and preferable at the single cell level, it will be necessary to use high density MB well arrays and a low cell SD. For example, to capture a rare cell (1:2000) with a 34 % chance to capture it as a single cell in a MB well (Fig. 2b) would require seeding 3205 lymphocytes onto a chip containing 6819 MB wells (SD=0.47). For the low density (1736 MB wells/ cm^2) prototype architecture used in this study, the chip size required would equate to the size of one well of a standard 12 well tissue culture plate (3.8 cm^2).

3.2 Affinity capture surface coating of MB wells

To investigate selective cell capture in MB wells we utilized the VAC and reagent exchange protocols described in the Section 2 to coat the MB wells with an affinity capture agent. Use of selective capture coatings is an approach that can enable seeding MB well arrays with a mixed cell population, such as lymphocytes from human blood, and after washing only the cells of interest would be retained. Here we investigated the selective capture of B cells expressing surface bound IgG by coating the MB wells with anti-IgG antibody. Briefly, the planar chip surface was blocked with BSA and streptavidin (SA) was nonspecifically adsorbed to the MB well surfaces and then exposed to biotinylated anti-human IgG (b- α IgG). To verify the binding of b- α IgG the chip was exposed to FITC conjugated IgG (FITC-IgG) (Fig. 4a). Controls included (1) coating the MB wells with (b- α IgG) alone (no SA pretreatment) followed by exposure to FITC-IgG, (2) uncoated MB wells exposed to FITC-IgG and (3) coating the MB wells with SA followed by biotinylated anti-transferrin (b- α Trans) followed by exposure to FITC-IgG (Figs. 4b–d). The average fluorescence intensity of the 12 MB wells confirms that the (SA) + (b- α IgG) coating enhanced the binding of FITC-IgG over all of the controls with statistical significance ($p < 0.0001$) (Fig. 4e).

3.3 Selective capture of B cells

Our goal is to exploit affinity capture methods to enrich and sort antigen-specific B cells. If a heterogeneous B cell sample is seeding onto an array of MB wells coated with a specific antigen of interest then after washing, ideally only the B cells expressing surface bound antibodies to that antigen should remain. As a proof-of-principle we tested the efficacy of the (SA) + (b- α IgG) coating to selective capture B cells (ARH-77 cell line) expressing surface bound IgG over T cells (CCRF-CEM cell line) that do not express surface bound IgG. After seeding and incubating cells for 30 min the chips were imaged and the number of cells captured in the MB wells were counted (before wash). Three sequential wash steps were tested to remove unbound cells the MB arrays; including (1) pipetting the chip surface with PBS buffer three times (after wash), (2) turning the chip upside down and shaking vigorously (shakeout), (3) incubating the chip upside down overnight (20–24 h) in a reservoir of cell media (overnight). Results from a representative experiment are shown in Fig. 5a. Following the first wash step there is slight (<10 %) increase in the number of cells counted in the MB wells. This arises from cells that were deposited onto the planar chip surface being washed into MB wells. After a brief upside down shakeout about 93 % of the B cells remain in the wells and ~40 % of the T cells wash out. After the overnight upside down incubation ~65 % of the B cells are retained and ~98 % of the T cells are removed validating the affinity capture method. Using the overnight wash protocol the affinity capture coating chemistry was further tested relative to uncoated and BSA coated MB wells ($n=3$). Results (Fig. 5b) confirm that on (SA) + (b- α IgG) coated MB wells an average of ~82 % of the B cells seeded are retained whereas ~92 % of T cells are removed. Interestingly, ~30 % of both B and T cells seeded in uncoated MB wells are retained suggesting strong nonspecific cell adsorption to the hydrophobic PDMS surface. Blocking the MB wells with BSA prior to seeding significantly reduces the nonspecific adsorption to <10 % for both B and T cell types.

To further test the affinity-based capture method we used primary B and T cells freshly isolated from human blood ($n=3$ different donors). Chips were seeded with B and T cells individually and with a 1:1 mix of B and T cells. Cells were seeded onto (SA) + (b- α IgG) coated chips, uncoated chips, and BSA pretreated chips. After seeding, the chips were incubated MB side up for 30 min and then inverted and incubated overnight to remove unbound cells. Results for the individual primary B and T cell chips (Fig. 6) show trends that parallel the cell lines in that (SA) + (b- α IgG) coating enables ~73 % retention of B cells and >90 % depletion of T cells. Coating the MB wells with BSA also helps to block the nonspecific adsorption of B and T cells to uncoated MB wells. Interestingly, unexpected trends emerge when MB wells are seeded with a 1:1 mixed population of primary B and T cells. The (SA) + (b- α IgG) coating does enhance the capture of primary B cells (~63 % captured) relative to primary T cells (~73 % depleted), but the BSA coating was considerably less effective at blocking nonspecific binding, particularly the B cells. This indicates a significant difference between working with primary cells and cell lines which we discuss below.

4 Discussion

The ability to sort and study cells at the single cell level allows for a more accurate elucidation of gene expression and cell function. Studies done on large heterogeneous cell samples yield an average view which can obscure detection and examination of important minority cell types (Holmes and Al-Rubeai 1999; Love et al. 2006; Jin et al. 2009; Gong et al. 2010; Nikkah et al. 2011; Ryan et al. 2011). In developing assays for single cell screening using micro-well arrays it is necessary to identify and fully characterize the factors that determine cell seeding efficiency and distribution statistics. This will allow for a rational design of assay parameters (e.g. array size, cell seeding density, affinity coating chemistry) to enrich and ensure the capture of the rare cells. Many micro-fabricated device designs for single cell analysis have been reported but rarely are the cell seeding statistics modeled or discussed (Love et al. 2006; Jin et al. 2009; Gong et al. 2010; Liberskit et al. 2011; Nikkah et al. 2011; Zaretsky et al. 2012). Studies often claim that cell seeding follows Poisson statistics but evidence supporting this is typically lacking (Love et al. 2006; Jin et al. 2009; Gong et al. 2010; Liberskit et al. 2011; Nikkah et al. 2011). Most use seeding protocols which deposit a significant portion of cells onto the chip surface and many are lost to the external environment. For example, in a study of heterotropic breast cancer cell interactions using shallow micro-wells arrays etched into silicon, Agah and coworkers reported a ~48 % total seeding efficiency by comparing the amount of cells deposited onto the chip surface to the number of cells in the wells (Nikkah et al. 2011). This estimate did not account for cells lost to the external well containing the chip.

In this study we accounted for all cells seeded onto MB well array chips and used the Poisson equation to model the distribution statistics of cells deposited into MB wells. We delineated the important roles that the fractional area of MB openings and the cell seeding density have on determining the λ statistic. Curiously we observed a high cell capture efficiency of ~65 % regardless of seeding density. We attribute this to fluid flow and cell displacement parallel to the chip surface during the seeding step that increases the effective fractional area of MB opening. Furthermore, the unique spherical geometry of the

MB well hinders cells from exiting once they enter. This geometry also assists in the accumulation of secreted factors for conditioning of the microenvironment (Chandrasekaran et al. 2011). Unlike microfabricated shallow cubical or cylindrical wells in which cells can be dislodged by fluid disturbances, such as those occurring during media changes, cells are secured the MB well and they can be sustained in long term culture (>10 days) with media changes as needed (Giang et al. 2008; Chandrasekaran et al. 2011).

In developing methodologies to seed and sort cells for high throughput screening applications it is advantageous to leverage affinity capture coatings to enrich and enhance the collection of the rare cells of interest. The use of antibodies as affinity capture agents has been widely explored for the specific capture of cells found in blood including: CD4 + T cells (Thorslund et al. 2007), CD45.1 positive T cells (Diener et al. 2012), CD20-expressing B lymphoblast (Sherman et al. 2010), circulating tumor cells (King et al. 2009). In this study we demonstrated a proof-of-principle that the high binding affinity streptavidin + biotinylated α -IgG complex (Weber et al. 1989; Chevrier et al. 2004) can be used to enhance the capture of B cells over T cells inside MB wells using both cell lines and primary cell samples obtained from human peripheral blood. The primary B and T cells were tested individually and as a mixed cell sample which revealed added complexities of working with primary cell samples. Our results showed enhanced retention of the B cells (~82 %) over T cells (~8 %) using the ARH-77 and CCRF-CEM cell lines respectively. We observed that BSA pretreatment effectively blocks the nonspecific binding (<10 %) of both cell lines to PDMS. Primary B and T cells behaved similar to the cell lines when seeded individually in MB well chips. However, when primary B and T cells were seeded in a 1:1 mix, we observed enhanced retention of B cells over T cells but the specificity was reduced. It is plausible that T cells may co-deposit with B cells into wells due to formation of cell-cell conjugates. We also observe that the effectiveness of BSA pretreatment to block the nonspecific capture of primary cells was diminished. Although BSA and human serum albumin (HSA) display significant sequence homology (Gelamo and Tabak 2000) it is known that animal albumins can be recognized by the human immune system as allergens (Soichi et al. 2002). Hence, it is plausible that activation of B and T cells through antigen recognition and/or by the addition Toll-Like Receptor Ligands and mitogens in the media may alter cell surface adhesive properties (Cassese et al. 2003; Henn et al. 2012) thereby reducing the effectiveness of the surface coatings, or favor the formation of B-T cell conjugates limiting the selective capture of B cells. Future studies will investigate washing protocols to disrupt cell-cell adhesions. These results exemplify the added complexity of working with primary cells and suggest that further studies are needed to optimize media and/or the surface coating chemistry to enhance selective affinity capture of primary B cells and to minimize nonspecific binding.

5 Conclusions

MB well arrays are being developed for cell sorting applications. In this work we have characterized cell seeding statistics in MB wells and investigated affinity capture coatings using the versatile streptavidin/biotinylated antibody complex to selectively bind B cells from a mixed population of B and T cells. Results confirm that cell seeding distributions can be modeled using the Poisson statistics accounting for key control factors; the cell seeding

density and the fractional area of MB openings on the chip. Single cell capture efficiency depends on the cell seeding density but the total cell seeding efficiency is unexpectedly high (~65 %) and independent of cell seeding density suggesting an effect of convective fluid forces that increases the effective fractional area of MB well opening. The unique architecture of the MB well hinders cells from escaping once they enter and it provides a microenvironmental niche they can condition for sustained long term culture. Cell capture specificity can be enhanced by manipulating the surface chemistry in the MB well, but we observe important differences between cell lines and primary cell samples. On-going studies seek to optimize assay protocols to determine the minimum incubation time needed to remove unbound cells, to explore the effect of additives in the cell culture media to enhance affinity capture efficacy, and to identify alternative capture agents that are independent of B cell activation. Further we are developing methods to identify wells containing cells of interest base on products secreted by the cells that accumulate in the MB well over several days in culture. The proof-of-principle results reported here suggest that scale-up and relevant statistical analyses required for high-throughput sorting of antigen-specific B cells can be achieved using MB well array technology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

An image showing a region within a MB array focused at the bottom of the wells illustrating the distribution of cells in each well ($SD=0.47$). The number of cells per well is indicated by the corresponding number. *White arrows* point to some cells in the wells. Cells deposited onto the planar chip surface are out of focus but some are indicated by *black arrows*

Fig. 2.

a A histogram plot showing the fraction of MB wells within an array (160 MBs/chip, 60 μm diameter opening, $n=3$ chips) containing a given number of cells as a function of cell seeding density (SD=0.13, 0.47, 0.94, 1.11, 1.74, 2.89, 5.41, and 10.41). The resulting trend is consistent with Poisson distribution. **b** A bar graph showing the single cell capture efficiency (SCE, Eq. 2) as a function of SD. Standard error plotted, $n=3$. Results indicate that with increasing SD fewer MB wells are seeded with one cell. Statistical tests were compared to SD=1.11, * $p<0.003$, ** $p<0.0001$

Fig. 3.

Varying the seeding density of cells onto MB chips alters the cell seeding distribution. Cells seeded onto 60 μ m diameter opening chips at SD= (a) 0.13, (b) 0.47, (c) 0.94, (d) 1.11, (e) 1.74, (f) 2.89, (g) 5.41, and (h) 10.41 are shown in the corresponding images. Cells/well were counted on a well basis (160 MBs/chip, $n=3$ chips) and a histogram (i) was plotted as the number of wells with given number of cells over the total number of wells per chip (Eq. 1). The distribution in (i) was modeled using the Poisson equation to find the controlling parameters of in which the effect of σ SD (j) and σ SD $\times$ F σ eff (k) was tested revealing that both SD and F encompassing the cell movement control the distribution

Fig. 4.

Development of PDMS MB well surface coating to enhance capture cells with affinity for α-IgG antibody. FITC-conjugated IgG was added to MB wells coated with **a** streptavidin + biotinylated α-IgG, **b** biotinylated α-IgG only, **c** uncoated MBs and **d** streptavidin + biotinylated α-Transferrin. Quantification of the fluorescent intensity averaged over 12 wells is illustrated in (e). Results indicate that FITC-IgG predominantly binds to the (SA) + b-α(IgG) coating. Bars indicate standard error, $n=12$, $*p<0.0001$

Fig. 5.

ARH-77 B cells expressing surface IgG and CCRF-CEM T cells expressing CD4 were seeding separately onto microbubble chips to test the effect of various surface coatings on cell capture. **a** MB wells were coated with (SA) + (b- α IgG). A representative example of cell count, numerically displayed, before and after pipet washes, shakeout, and overnight incubation with inverted chip. Results show efficient removal of T cells was achieved following overnight incubation. **b** The effect of surface coating chemistry on B and T cell capture following over inverted incubation. Results show selective B cell capture on (SA) + (b- α IgG) coated chips and significant nonspecific adhesion of both B and T cells on uncoated chips which is blocked on BSA coated chips. $n=3$, standard error is plotted, * $p<0.005$

Fig. 6.

The capture efficiency of primary B and T cells in MB wells was tested as a function of surface coating chemistry using chips seeded with B cells only, with T cells only, and with mixed B and T cells. Surface coatings include αIgG affinity capture chemistry, uncoated, and BSA coated MB wells. Chips were seeded with cells and imaged after a 30 min incubation. Chips were then incubated upside overnight and reimaged to count remaining cells. The % cells retained is plotted with standard error ($n=3$ chips, 160 MB per chip) and ANOVA analysis comparing effect of surface chemistry on T and B cell capture, * $p<0.05$ *** $p<0.005$

Table 1

Target and actual cell seeding densities

Target seeding density	0.1	0.5	0.7	1	1.5	3	7	10
Actual seeding density	0.131	0.468	0.944	1.106	1.740	2.888	5.409	10.410
Standard deviation	0.013	0.095	0.213	0.26	0.304	0.495	0.095	0.945
Cell stock (cells/ml)	1.6×10^3	8×10^3	1.1×10^4	1.6×10^4	2.4×10^4	4.8×10^4	8×10^4	1.6×10^5

Table 2Factors affecting the Poisson distribution statistic λ

Target SD	Actual SD	λ_{obs}	$\lambda_{model} = SD \times F_{MB}$	$\lambda_{obs}/\lambda_{model}$	$\lambda_{eff} = SD \times F_{eff}$	$\lambda_{obs}/\lambda_{eff}$
0.1	0.13	0.055	0.004	15.4	0.019	2.9
0.5	0.47	0.227	0.013	17.6	0.068	3.3
0.7	0.94	0.374	0.026	14.5	0.137	2.7
1.0	1.11	0.322	0.030	10.6	0.161	2.0
1.5	1.74	0.485	0.048	10.2	0.253	1.9
3.0	2.89	0.647	0.079	8.2	0.420	1.5
7.0	5.41	1.964	0.148	13.2	0.786	2.5
10.0	10.41	2.016	0.285	7.1	1.512	1.3