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An array-based platform to select, release and capture EBV-infected cells based on intercellular adhesion

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Abstract

Micraft arrays were developed to select and separate cells based on a complex phenotype, weak intercellular adhesion, without knowledge of cell-surface markers or intracellular proteins. Since the cells were also not competent to bind to a culture surface, a method to encapsulate non-adherent cells within a gelatin plug on the concave micraft surface was developed, enabling release and collection of the cells without the need for cell attachment to the micraft surface. After micraft collection, the gelatin was liquified to release the cell(s) for culture or analysis. A semi-automated release and collection device for the micrafts demonstrated 100±0% collection efficiency of the micraft while increasing throughput 5 fold relative to that of manual release and collection. Using the micraft array platform along with the gelatin encapsulation method, single cells that were not surface-attached were isolated with a 100±0% efficiency and a 96±4% post-sort single-cell cloning efficiency. As a demonstration, Epstein-Barr virus-infected lymphoblastoid cell lines (EBV-LCL) were isolated based on their intercellular adhesive properties. The identified cell colonies were collected with a 100±0% sorting efficiency and a post-sort viability of 87±3%. When gene expression analysis of the EBV latency-associated gene, EBNA-2, was performed, there was no difference in expression between blasting or weakly adhesive cells and non-blasting or nonadhesive cells. Micraft arrays are a versatile method enabling separation of cells based on complicated and as yet poorly understood cell phenotypes.

Graphical Abstract

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ASSOCIATED CONTENT

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Notes

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By virtue of the ubiquitous requirement to isolate living cells for cloning, genetic analysis, and other assays, there is a need for improved techniques to collect cells displaying properties not amenable to selection criteria using common cell sorting techniques, for example, growth characteristics or cell-cell interactions. Retrieval of cells infected by the Epstein-Barr virus (EBV) is one such example. EBV causes infectious mononucleosis and is associated with a variety of tumors including nasopharyngeal carcinoma, Burkitt's lymphoma, T cell lymphomas and Hodgkin's lymphoma.¹⁻⁴ The virus is highly prevalent among the human population, with 90% of adults having been infected at some point in their life. After initial infection and the establishment of an immune response, the virus enters a latent state and persists within the host's cells without causing obvious host damage. There are at least 4 different modes of EBV latency, each associated with expression of a different pattern of viral genes and linked to various lymphomas and lymphoproliferative disorders.^{3,4} Latent EBV can be reactivated periodically to a state of lytic replication, which is quickly controlled in immunocompetent adults; however, reactivation is life-threatening in immunocompromised populations, such as post-transplant patients and patients with acquired immunodeficiency syndrome (AIDS). Much remains unknown about EBV, its various latency states and progression to reactivation with fulminant infection.

EBV infects both epithelial cells and B lymphocytes. B lymphocytes infected with EBV *in vitro* become immortalized and are often used as a model system to study EBV infection and virus-driven tumorigenesis.¹ These EBV-immortalized lymphoblastoid cell lines (EBV-LCL) recapitulate some of the behaviors of EBV-infected immune cells, including demonstration of features suggestive of early stage progression to "lymphoma-like" cell clusters.⁴ These clusters or groupings of cells with weak intercellular adhesion, more commonly known as clumps in the biology literature, are thought to arise from a single EBV-LCL, display rapid proliferation and maintain cell-cell contact as opposed to other types of EBV-infected cells which lack cell-cell adhesion and clumping behavior.^{4,5} Clumping behavior characterizes these blast-like cells which display increased surface expression of CD23, CD39, CD40 and class II MHC, but a decrease in CD10 expression. The clumped cells also exhibit increased villous projections and vimentin expression.⁴ These characteristics suggest that EBV latency programs can modulate cell cycle as well as cellular adhesion. It is also thought that EBV-LCLs spontaneously undergo latency program switching, altering the proliferation, surface marker expression and intercellular adhesion of the cells in culture. However, the relationship between EBV gene expression, morphology and the clumping phenotype is not fully understood. Technologies to separate EBV-infected cells based on their clustering

behavior would enable a pure sample of cells for analytical study to provide a better molecular understanding of the signaling pathways contributing to these events.

A wide variety of cell separation technologies are available to isolate single, non-adherent cells from a population. Fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) are successful commercialized methods to sort cells. In these techniques, cells are incubated with a fluorescently or magnetically conjugated antibody and sorted based on the presence or absence of an antigen on or within the cell.⁶ FACS and MACS are high-throughput sorting techniques; however, both require prior knowledge of the antigens present to create fluorescently labelled antibodies against the desired cell subpopulation. Furthermore, FACS suffers from low post-sort viability while MACS suffers from low purity.⁷ Microfabricated traps relying on flow for isolation, including mechanical⁸, optical⁹, dielectrophoretic (DEP)^{10,11} and acoustic traps¹² are able to separate individual cells based on size, deformability or membrane capacitance. These methods forgo the reliance on prior knowledge of antigens and separate cells based on morphological characteristics; however, they are unable to segregate cell clusters from singlets. With all of the technologies discussed above, the pre-processing of the sample, as well as the flow forces inherent to each, will split apart weakly adherent cell clusters before they can be isolated. Furthermore, optical, acoustic and DEP-based manipulation¹¹ require complicated and expensive equipment and the mechanical or electrical forces placed on the cell during manipulation can have a negative effect on post-sort viability.^{11,13}

In recent years, technologies have been specifically designed to pick cell colonies grown in agar or the surface of plastic dishes by suction aspiration, *e.g.* Clonepix (Genetix, Hampshire, UK) and CellCelector (ALS Automated Lab Solutions, Jena, Germany). These colony picking platforms generally rely on microscopy techniques to identify individual colonies exhibiting a particular fluorescence signature, morphology or temporal characteristic before the identified cells are isolated, but are most often used for bacterial and yeast colonies.^{14,15} Another approach is laser-capture microdissection (LCM) which uses a laser to cut out a region of cells grown on a specialized membrane, but the technique is most appropriate for tissue sections of fixed cells for genetic analysis rather than isolation of live cell colonies because of issues with cell viability.¹⁶ Arrays of microfabricated wells to trap non-adherent cells followed by an analysis and pipet aspiration have been implemented by a number of groups to identify and isolate cells of interest.¹⁴ Automation of the aspiration step has been implemented to reduce the manpower needed for the isolation step, but cell damage during vacuum aspiration and the potential for cross-contamination from aspirating non-target cells are concerns. An alternative microwell array-based sorting method is the microraft array platform that utilizes the manipulation of individual superparamagnetic cell carriers by a small needle and a magnet to isolate individual cells or cell clusters.¹⁷⁻¹⁹ This sorting method does not require vacuum aspiration or expensive equipment and exhibits very high post-sort purity and viability; however, to date it has been demonstrated only for the isolation of adherent cell types.

In the current work, a technique utilizing the microraft array platform is developed to address the need to pick single non-adherent cells or clonal clusters of non- or weakly adherent cells. The microraft array was originally developed by the Allbritton group as a

cost effective means to sort cells or colonies that readily adhere to the surface of a carrier during culture on the array.^{17,18} The microraft technology also provides an approach to track cells over time, enabling a broad suite of selection criteria for identifying target cells. A limitation of the technique is the need for the cells to be firmly adherent to the microraft carrier in order to release and collect the cell from the array. Here, a strategy has been developed to screen and then collect single, non-adherent target cells from the array, and the technique is then demonstrated for isolation of clonal colonies displaying only weak cell-cell interactions. To accomplish this isolation procedure, non-adherent cells plated on the array were encapsulated in a hydrogel within microwells of the array. This step served to affix the cells to the cell carrier (microraft) at the base of the microwell to allow collection upon release of the microraft. A temperature-sensitive hydrogel formulation was optimized, and collection efficiency and cell viability were characterized. To efficiently release microrafts, a motorized, computer-controlled microneedle assembly mounted on a microscope objective was developed. A small cylindrical device containing a movable magnet was designed to efficiently capture and deposit rafts into a collection vessel. This system was then used to identify and collect single and clumping EBV-LCLs. The utility of the method was demonstrated by assaying the expression of a latency-associated gene, EBV nuclear antigen 2 (EBNA2), in cells that displayed clumping (blasting) and non-clumping (non-blasting) behavior.

EXPERIMENTAL

Materials

The following materials were obtained from Sigma-Aldrich Corp. (St. Louis, MO): γ -butyrolactone (GBL), propylene glycol monomethyl ether acetate, iron(II) chloride tetrahydrate, iron(III) chloride anhydrous, iron(III) nitrate nonahydrate, ammonium hydroxide solution, oleic acid, toluene, styrene, acrylic acid, benzoyl peroxide and gelatin from bovine skin (type B). SU-8 photoresist was purchased from MicroChem Corp (Newton, MA). Sylgard 184 silicone elastomer kit (PDMS) was acquired from Dow Corning (Midland, MI). The following materials were procured from Life Technologies (Carlsbad, CA): RPMI 1640, fetal bovine serum (FBS), phosphate buffered saline (PBS), 0.05% trypsin with EDTA solution, penicillin/streptomycin, TaqMan Probes, TaqMan Gene Expression Assays, Superscript III First-Strand Synthesis System for RT-PCR, TaqMan Universal PCR Mastermix II, Hoechst 33342, Sytox Green, CellTracker Orange CMRA and Oregon Green 488 conjugated gelatin. Polycarbonate and Delrin were purchased from McMaster-Carr (Elmhurst, Illinois). NdFeB (Grade N52) permanent magnets were obtained from K&J Magnetics, Inc. (Plumsteadville, PA). Ethanol was purchased from Decon Labs (King of Prussia, PA). T4 Gene 32 Protein was obtained from New England Biolabs, Inc. (Ipswich, MA). PCR primers were acquired from Integrated DNA Technologies, Inc. (Coralville, IA). Buffer RLT, RNeasy Micro Kit and Carrier RNA were received from Qiagen (Hilden, Germany). All other reagents were from Thermo Fisher Scientific (Waltham, MA).

Fabrication of microraft arrays

PDMS microwells containing superparamagnetic, releasable polystyrene elements were fabricated using previously described protocols.^{17,18} Briefly, a template comprised of an

array of SU-8 pillars (200×200×200 μm , spaced 100 μm apart) on a glass slide was generated by standard photolithography and used to create a PDMS microwell array. A unique 4-digit identifier was transcribed in every 5th pillar that when transferred to the PDMS microwell enabled addressing of individual micrafts based on their position relative to the identifier. Each array contained a total of 4900 microwells (70×70 array). The PDMS microwell array was covered with a solution of poly(styrene-*co*-acrylic acid) (PS-AA) in GBL co-mixed with $\gamma\text{Fe}_2\text{O}_3$ nanoparticles and degassed under vacuum to remove air bubbles.¹⁸ The array was then lowered into a jar of the same solution and slowly pulled out by a stepper motor at a rate of 1 mm/s. This dip-coating procedure resulted in the solution draining from the array while leaving a droplet of solution within each microwell. The GBL solvent was evaporated at 95°C for 12 h producing a hard, concave polystyrene element doped with superparamagnetic nanoparticles, *i.e.* a micraft, within each microwell. The micraft array was then attached to a polystyrene cassette using PDMS.

Microscopy

An Eclipse TE2000-U microscope (Nikon Instruments Inc., Melville, NY) equipped with Nikon CFI Plan Fluor Series objectives was used for all experiments. A CoolSNAP HQ2 CCD camera (Photometrics, Tucson, AZ) was used for all imaging.

Motorized micraft release system

The release system consisted of two pieces of Delrin machined on a CNC mill (MicroMill DSLS 3000; MicroProto Systems, Chandler, AZ). One piece was designed to fit on to a CFI Plan Fluor 4× objective (Nikon Instruments Inc., Melville, NY) and housed a small stepper motor (20M020D1B; Portescap, West Chester, PA). The second Delrin component contained a clear polycarbonate disc with a 5 mm-long needle (150 μm base, 17.5 μm tip) secured to the center. The stepper motor rotated a threaded rod to translate the Delrin piece containing the needle linearly along the vertical axis of the objective. The clear polycarbonate disc permitted bright field and fluorescence microscopy with minimal distortion when the release system was in place. The tip of the needle was located 8.7 mm above the top surface of the objective.

Motorized micraft release system controller

The stepper motor was controlled using a combination of an Arduino Uno (SparkFun Electronics, Boulder, CO) and a motor shield (Adafruit Industries, New York, NY). The Arduino Uno was programmed using the open-source Arduino Software to rotate the stepper motor and move the needle vertically when a pushbutton was pressed. A potentiometer was used to adjust the travel distance of the needle (1–15 mm).

Fabrication of a magnetic micraft collection device

A hollow polycarbonate cylinder (4.76 mm diameter, 63.5 mm length) with a 3.18 mm blind-end hole was machined through the center of its long axis leaving 1 mm of material at the blind end. The open end was tapped and closed with a nylon screw after a cylindrical NdFeB magnet (3.175 mm diameter, 25.4 mm length) was inserted into the cylinder. The magnet was able to move freely along the long axis of the cylinder as described below. The

collection device was either held by hand or secured to a micromanipulator attached to the microscope stage for micraft retrieval.

Cell lines, cell culture

K562 cells, a myelogenous leukemia cell line, and EBV-infected lymphoblastoid cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Prior to cell culture on the micraft array, the array was oxidized in a plasma cleaner (Harrick Plasma, Ithaca, NY) for 5 min. The array was then rinsed once with 100% ethanol and subsequently rinsed $\times 5$ with PBS. The array was coated in a 0.1 wt% bovine gelatin in PBS and incubated at 37°C for 1 h. Before plating cells, the gelatin solution was aspirated and the array was rinsed $\times 3$ with PBS. A suspension of K562 cells or EBV-LCLs were plated on the micraft array at various densities and the array was centrifuged at 400 $\times g$ for 3 min. The array was then rinsed gently $\times 2$ with medium to remove any cells that did not settle on to a micraft. Cells were cultured in a 37°C incubator with 5% CO₂.

Cell staining

Prior to plating, cells were stained with 1 mg/mL Hoechst 33342 or 2 µM CellTracker Orange CMRA following manufacturer protocol. After plating, cells were incubated on the micraft array with 50 nM Sytox Green following the manufacturer's protocol.

Gelatin encapsulation and fluorescent gelatin

In those experiments isolating cells encapsulated in a hydrogel, the culture medium was aspirated after plating cells on micraft arrays coated with 0.1 wt% bovine gelatin, and bovine gelatin in PBS varying in concentration between 0 wt% and 10 wt% was added. The array was then incubated for 10 min at 37°C. The gelatin solution was then aspirated from the arrays which were placed at 4°C for 5 min to solidify the gelatin remaining within the microwells. Cold (4°C) culture medium was added to the arrays, which were then subjected to the cell isolation procedure (see below). For those experiments using fluorescent gelatin, 0.1% Oregon Green 488 conjugated gelatin (Life Technologies, Carlsbad, CA, 505 µL) was mixed with a 10 wt% gelatin solution (495 µL) to generate a 5 wt% gelatin solution, which was added to the cell array as above.

Release and collection of micrafts

Targeted release and collection of micrafts was performed in a manner similar to that described previously.¹⁸ Briefly, after plating and culturing cells on an array, a microwell containing a cell(s) for collection was identified by microscopy. The micraft within the well was then expelled by piercing the PDMS substrate of the microwell array with the needle release device. The magnetic collection device was then placed in the media at a defined distance from the release site, whereupon the superparamagnetic micraft was attracted to the cylindrical magnet within the collection device. After capture of the micraft, the tip of the collection device was placed into a collection vessel such as a PCR tube or a multi-well plate filled with medium and placed over a block NdFeB magnet (101.6 $\times$ 76.2 $\times$ 6.35 mm) oriented such that the top pole of the block magnet was identical to the bottom pole of the cylindrical magnet within the collection device. This procedure led to

the release of the captured micraft into the collection well as described in the Results. In some experiments, a magnetometer (AlphaLab Inc., Salt Lake City, UT) was used to measure the magnetic field of each magnet and the magnetic field gradient (∇B) was calculated in MATLAB. In gelatin encapsulation experiments, the array was immersed in cold (4°C) media to maintain the hydrogel in the gelled state while the gelatin encapsulated cells on the micrafts were being released and collected on the stage of a microscope at room temperature (23°C). Released micrafts were transferred into warm (37°C) medium in the collection wells to melt the gelatin and release the cells.

Growth rate determination

K562 cells were seeded onto micraft arrays at a density of 0.33 cells/micraft. The array was either left in media or subjected to the gelatin encapsulation protocol as described above. Both sets of arrays were subsequently incubated at 23°C for 1 h to simulate the period where release and collection would take place. The arrays were then placed in a humidified, 5% CO₂ incubator at 37°C for 48 h and imaged periodically. The cell number was counted at each time point and the data were fit to an exponential curve to determine the doubling time of the cells on each array.

Single-cell cloning efficiency

The single-cell cloning efficiency of K562 cells was evaluated after successful collection of single cells. After collection, the single cells were cultured 3 days in culture medium at 37°C in a 5% CO₂ incubator. The cells were imaged after the 3 day culture period to assess how many cells successfully proliferated.

Quantitative RT-PCR

Non-blasting cells displaying weak intercellular adhesion were also very slowly dividing so that they were present as singlet or doublets on rafts whereas blasting cells with weak intercellular adhesion divided more rapidly with 20 cells per micraft at the time of analysis. For this reason micrafts with cells were collected and pooled until approximately 20–25 cells were present for each cell phenotype for each time point for the RT-PCR experiments. RNA was isolated using Qiagen's RNeasy Micro Kit. Micrafts containing encapsulated cells were released and transferred to Eppendorf tubes containing 75 μ L Buffer RLT. Carrier RNA (20 ng) was added, the samples were heated at 37°C for 1 min to melt the gelatin then vortexed for 1 min to homogenize the cells. The Superscript III First-Strand Synthesis System for RT-PCR was used to synthesize cDNA with oligo(dT) as the primer. The manufacturer's protocol was followed, except that 4 μ L of T4 Gene 32 Protein was added to each 20 μ L reaction. cDNA synthesis was performed on a Veriti 96 Well Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified using a predesigned assay reagent containing a 6-FAM/MGB-NFQ labeled probe (Single Tube TaqMan Gene Expression Assay). EBV nuclear antigen 2 (EBNA-2) was quantified by TaqMan PCR using forward (5'-GCTTAGCCAGTAACCCAGCACT-3') and reverse (5'-TGCTTAGAAGGTTGTTGGCATG-3') primers and a TaqMan probe (5'-FAM-CCCAACCACAGGTTTCAGGCAAACTTT-TAMRA-3') designed by Bell *et al.*²⁰ The PCR reactions contained 300 nM of each EBNA-2 primer and 250 nM of the EBNA-2

probe. PCR reactions were prepared in a final volume of 20 μ L using TaqMan Universal PCR Mastermix II, and quantitative PCR was performed on an Applied Biosystems 7300 Real Time PCR System with the following thermocycling conditions: 50°C for 2 minutes to activate uracil-N glycosylase and 95°C for 10 min to activate AmpliTaq Gold, followed by 55 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Experiments were performed in triplicate, and no-template controls were used. Fluorescence was measured during the 60°C incubation in each cycle. Data was analyzed using DataAssist v3.01 Software (Applied Biosystems), with the maximum allowable C_T set at 40 and included in the calculations.

Statistical Analysis

MATLAB was used for all statistical analyses. A one-way ANOVA test was performed to determine statistical significance between collection efficiencies and viabilities of K562 cells isolated with the gelatin encapsulation technique. The Tukey-Kramer method was used to determine which groups were statistically different if the one-way ANOVA indicated a group was different.

RESULTS AND DISCUSSION

Overview of hydrogel-based encapsulation and collection of cells on an array

Initially, a strategy to screen and sort individual cells without the need for cell adhesion to the array elements was identified and each step optimized (Fig. 1). Briefly, K562 cells (a nonadherent leukemic cell line), were cultured on an array containing superparamagnetic, concave micrafts (Fig. S-1) and overlaid with a liquid gelatin (Fig. 1A–B). After aspiration of excess liquid from the array, the residual liquid pockets were solidified (Fig. 1C,E,F).

Micrafts with cell(s) encapsulated in hydrogel were released using a motorized microneedle assembly (Fig. 1D,G–J) and transferred to a multiwell plate using a magnetic transfer device.¹⁸ Dissolution of the gelatin then released the targeted cell(s) from the micraft (Fig. 1I–L).

Impact of gelatin encapsulation on cells

Gelatin was tested as a potential hydrogel since it is biocompatible, optically transparent, commonly used in cell culture, and undergoes a reversible, temperature-dependent, liquid (4°C)-to-gel (37°C) transition. To determine if the encapsulation process displaced the cells during the procedure or had an untoward impact on cell viability, K562 cells were cultured at a density of 0.33 cells/micraft on an array so that 1 cell was present on the majority of micrafts. The arrays with cells were imaged, the positions of micrafts containing cells were recorded, and then the array was overlaid with liquid gelatin (5 wt%, 37°C) and incubated for 10 min. Excess gelatin was aspirated and the arrays were placed at 4°C (5 min) followed by addition of cold (4°C) medium. The micrafts were then re-imaged and the cell number for each micraft counted ($n=405$ micrafts). The majority of micrafts ($97.8 \pm 1.1\%$) possessed the same number of cells before and after gelatin encapsulation, demonstrating that most cells remained in place during the encapsulation and gelation steps. Only $1.6 \pm 1.0\%$ of micrafts had an additional cell after gelatin encapsulation. Since cells

were seeded on the micraft array at a density of 0.33 cells/micraft, there was a $0.5\pm0.3\%$ chance that a micraft containing a cell prior to gelatin encapsulation would be contaminated with another cell during encapsulation. Thus gelatin encapsulation was effective in maintaining the cell's location on the array.

The effect of gelatin encapsulation on the growth rate of cells was assessed to determine if the encapsulation process negatively impacted cell replication, a sensitive measure of cell health. K562 cells were seeded and encapsulated on micraft arrays ($n=3$) along with additional arrays ($n=3$) on which the cells were not encapsulated as controls. Media at 4°C was added to both encapsulated and control arrays followed by incubation for 1 h at 23°C to simulate a release and collection period. The arrays were then placed at 37°C , whereupon the hydrogel on the encapsulated array dissolved. The arrays were imaged at multiple time points over the next 48 h and the number of cells at specific sites were counted to determine the average doubling time of the cells under the two conditions (Fig. S-2). The data was fit to an exponential curve and the doubling time of the cells on each array was determined. The doubling time for K562 cells grown on an array after gelatin encapsulation was 18.34 ± 0.04 h, which was not statistically different ($p=0.28$) from that on arrays not exposed to gelatin (19.15 ± 0.65 h). Based on these data, encapsulation with gelatin for up to 1 h did not alter the subsequent growth rate of K562 cells.

Motorized micraft release system

Micrafts have previously been released by manually puncturing the PDMS microwell array with a needle to dislodge an individual micraft.^{17,18} This method, while effective, is slow and labor-intensive. Additionally, the speed and duration of the manual release is user-dependent. For these reasons, a motorized release system was designed to reduce release parameter variability and increase the reproducibility of the release process when paired with gelatin encapsulation. The system used a miniaturized motor mounted on a microscope objective for convenience in microscope mating. A push button actuated the needle, moving it at a user-determined velocity (Fig. 2). The needle velocity was initially optimized in the absence of gelatin to identify the minimum time needed for micraft release. For all velocities tested (1, 5, 10 mm/s), targeted micrafts were released with $100\pm0\%$ efficiency, and $0\pm0\%$ of released micrafts exhibited physical damage ($n=3$ arrays at each speed, 10 micrafts/array). Additionally, no untargeted micrafts were released and there was no observed media leakage from the PDMS at the needle puncture site. The duration of a single release, including upward and downward travel of the needle, using the manual release was 9.5 ± 0.4 s while the motorized micraft release system set to the maximum travel speed of 10 mm/s was 1.9 ± 0.0 s. The motorized release system was then evaluated on a micraft array with gelatin-filled wells to access the impact of encapsulation on micraft release. At all velocities, micrafts were released with $100\pm0\%$ efficiency and none ($0\pm0\%$) showed physical damage. The motorized micraft release system was significantly faster than manual release ($p<0.01$) and was highly efficient irrespective of the presence of a gelatin pocket above the raft. For all further experiments, the release system was set to a travel speed of 10 mm/s.

Magnetic micraft collection device

Micrafts containing superparamagnetic $\gamma\text{Fe}_2\text{O}_3$ nanoparticles have been collected manually using an external magnet after release from the array.¹⁸ This method has been ideal for collecting multiple micrafts into a single container; however, it has proven to be time consuming and labor intensive to collect individual micrafts in separate containers as would be needed to sort different cell populations such as blasting and non-blasting EBV-LCLs. A new magnetic collection system was designed to capture and precisely deposit released micrafts directly into arrayed collection receptacles (Fig. 3). The magnetic transfer device consisted of a non-toxic polycarbonate tube containing a cylindrical magnet free to move within the tube (Fig. 3A,B). To collect micrafts, the magnet was positioned at the tube bottom and to release a micraft the magnet in the tube was repelled upward towards the tube top. During micraft collection, the tube with magnet at its bottom was positioned above the release site in the medium overlying the micraft array to magnetically capture a released micraft (Fig. 3C,D). The capture efficiency of the device was assessed at distances of 7, 8, 9, and 10 mm between the magnet and released micrafts (Fig. 4A). Collection with and without gelatin encapsulation was assessed (3 trials, $n=10$ micrafts/trial). For distances of ≥ 8 mm between the surface of the array and the magnet ($\nabla B = 2.1$ T/m), 100% capture efficiency of released micrafts was attained irrespective of gelatin encapsulation. Thus, the magnetic collection device did not need to be in extremely close proximity to the released micraft to effect capture. It is expected that a stronger magnet would enable even greater capture distances.

To discharge captured micrafts from the collection device, a block magnet was positioned beneath the collection receptacle such that the cylindrical magnet inside the device was repelled by the block magnet (Fig. 3E–H). As the device was moved over the block magnet, the cylindrical magnet was repelled upward within the cylinder and away from the captured micraft. The micraft remained attached to the tip of the collection device due to surface tension effects of a fluid droplet on the tip. Placing the tip of the collection device into the fluid in the collection vessel released the micraft from the collection device whereupon it was attracted by the block magnet to the bottom of the collection vessel (Fig. S-3). The minimum distance (maximum ∇B) between the repelled cylindrical magnet and the micraft on the tip of the collection device required for successful micraft transfer was measured (Fig. 4B). To accomplish this study, the movement of the cylindrical magnet was physically restricted to specific travel distances when repelled of 1, 5, 10, 15, 20 and 30 mm by shortening the long axis of the hole within the cylinder ($n=3$ trials/distance, each with 10 micrafts). For a release efficiency of 100%, the magnet needed to move ≥ 20 mm away from the captured micraft with a corresponding drop in the magnetic gradient to ≥ 0.2 T/m. Under these conditions, a channel length of ≥ 45.4 mm was needed to accommodate the length of the magnet and its required travel distance. A collection device with a channel length of 56.2 mm was used for all further experiments. This magnetic micraft collection device also reduced the labor and reagents needed to isolate many micrafts into individual containers compared to the previous method of collection into a relatively large Petri dish lid.¹⁸

Optimization of gelatin concentration for combined release, capture and deposition of cells

The efficiency of combined release, capture and deposition of non-adherent cells was assessed using various concentrations of gelatin to encapsulate cells on individual microrrafts. Non-adherent K562 cells were seeded on microrraft arrays and the cells were encapsulated in either 0, 3, 5 or 10 wt% gelatin. The motorized microrraft release and magnetic transfer devices were used to release, capture and deposit microrrafts containing single K562 cells into individual wells of a 96-well plate containing medium at 37°C. The collection efficiency of single K562 cells was measured as the percentage of cells that were successfully released, captured, and deposited into a 96-well plate. The successfully collected single cells were cultured for an additional 5 days and the viability of the collected cells were determined based on single-cell cloning efficiency. Collection efficiencies after encapsulation with 0, 3, 5 or 10 wt% gelatin were $25\pm5\%$, $90\pm7\%$, $100\pm0\%$ and $100\pm0\%$ ($n = 4$ arrays, 8 attempted collections per array), respectively. All three gelatin solutions showed a statistically significant increase in collection efficiency compared to the isolation attempts without gelatin ($p < 0.05$) while there was no statistical difference among the collection efficiencies of each gelatin solution ($p = 0.14$) (Fig. 4C). The post-collection single-cell cloning efficiency after encapsulation and collection with 0, 3, 5 or 10 wt% gelatin solution was $100\pm0\%$, $97\pm3\%$, $96\pm4\%$ and $88\pm8\%$ ($n = 4$ arrays, 8 successfully collected single cells per array), respectively. Additionally, there was no statistically significant difference between the post-collection single-cell cloning efficiency of the K562 cells using any of the solutions ($p = 0.34$) (Fig 4C). For this reason, a 5 wt% gelatin solution was chosen to isolate non-adherent cells in subsequent experiments.

Isolation of clumping EBV-LCL cells

Single and clumping EBV-LCLs are readily distinguished by bright field microscopy without the need for specialized stains due to their distinct morphologies and single-cell vs. clumped morphology. Thus the combination of light microscopy-based screening with a non-traumatic, array-based sorting method was expected to enable separation of non-clumping (non-blasting) from clumping (blasting) lymphocytes for subsequent analyses. EBV-LCL cells were seeded on microrraft arrays at a density of 0.33 cells/microrraft. At this density 24% of microrrafts contained single cells and 4.5% of the microrrafts contained >1 cell. The EBV-LCLs were then cultured for 6 days on the microrraft arrays. After 6 days in culture, $43.0\pm4.4\%$ of microrrafts with cells contained a single cell, while $8.4\pm2.7\%$ contained clumping EBV-LCL colonies with >20 cells. The remainder of the microrrafts contained colonies with <20 cells. The gelatin encapsulation procedure was performed on the microrraft arrays and the clumping EBV-LCL colonies were released and collected individually into collection wells. The number of cells in each collected colony was re-counted post-isolation to confirm that all cells in each isolated colony were successfully collected ($100\pm0\%$, $n=3$ arrays, 10 colonies per array) (Fig. S-4). The collected colonies were cultured for an additional 3 days, and $87\pm3\%$ of the colonies continued to expand. These results demonstrate that proliferating cells forming loosely attached clusters were readily isolated with high efficiency and post-isolation viability.

PCR of EBV-LCL colonies and single cells

The expression of EBNA-2 is thought to be important for the continued survival of the immortalized EBV-LCL cells. EBNA-2 is expressed in only 1 of the 4 EBV latency programs (latency III program) identified to date.³ EBV-LCLs cultured in vitro are generally believed to remain in the latency III program; however, EBV infections in vivo and Burkitt's lymphoma cells infected in vitro have been known to switch between latency programs in a manner that is as yet poorly understood.⁴ We hypothesized that EBV-LCLs that do not proliferate in adhesive clumps may have switched latency programs which would be suggested by the down regulation of EBNA-2 expression.

EBV-LCL cells were seeded and cultured on microraft arrays for 6 days. After the culture period, the EBV-LCLs were incubated with Sytox Green to stain dead cells. Microrafts containing living single cells or clumping cell clusters were identified and collected using the gelatin encapsulation method (Fig. S-5). In total, 3 samples of single-cell microrafts (each sample consisted of pooling 25 microrafts containing a single cell) and 4 samples of microrafts with clumped cells (each sample consisted of a single microraft with 25 clumped cells) were isolated for analysis. EBNA-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified by real-time polymerase chain reaction (RT-PCR). GAPDH was successfully amplified in all samples, indicating that the genetic material was effectively isolated from each of the samples. EBNA-2 was successfully amplified in all samples except 1 of the non-blasting single-cell samples (Table S-1). Expression of EBNA-2 was normalized to GAPDH expression to correct for the amount of genetic material collected. Normalized EBNA-2 expression was compared between single cells and cell clusters. There was no statistically significant difference in the expression of EBNA-2 between the two cell populations ($p=0.22$, Mann-Whitney test, Fig. 5C). Analysis of a larger number of samples and characterization of additional viral genes in clumping *vs.* non-clumping cells will need to be performed to identify how the clumping phenotype relates to gene expression, latency program, and EBV infection; nevertheless, these data demonstrate the feasibility of identifying and successfully isolating cells possessing a complex phenotype, weak intercellular adhesion, for further analysis.

CONCLUSION

We have demonstrated a method to adapt a microwell array cell separation platform to selectively identify and select cells based on a complex phenotype without the need for surface attachment all while maintaining a high post-sort purity and viability. Gelatin was used to temporarily capture non-adherent and loosely clustered cells within removable, concave cell carriers (microrafts) on the arrays. The release, capture and deposition of microrafts were greatly enhanced with improvements in speed and reduction in labor by employing a motorized collection system. The utility of the system was demonstrated by performing a sort of clumping EBV-LCLs that display weak cell-cell adhesion. Clonal colonies were identified and isolated as were non-proliferative EBV-LCL single cells for comparison by genetic analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Gelatin encapsulation. A–D) Schematic of cell release using gelatin encapsulation. A) Cells (round objects) were cultured on a micraft array without adhesion to the micraft surface. B) Liquid gelatin (light green) was overlaid onto the array. C) Excess liquid gelatin was aspirated from the array surface and the gelatin solidified. Solidified gelatin is shown as a darker green. D) Medium (light red) was placed over the solidified gelatin and a micraft was ejected by piercing the PDMS substrate with a needle. E–L) Bright field (E, G, I, K) and fluorescence (F, H, J, L) images of the release of a single K562 cell stained with CellTrace Red-Orange and encapsulated in gelatin conjugated to Oregon Green 488. Panels E and F are images of the array before micraft release, while panels G and H are after micraft release. Panel I and J are images of the collected micraft in cold medium while panels K and L show the cell and micraft incubation in warm medium (37°C) for 10 min. Scale bar = 200 μm

Figure 2. Motorized micraft release. A) Schematic of the motorized needle-release system. B) Photograph of motorized micraft release device mounted on a microscope objective. C,D) Side view schematic of raft release with the motorized needle-release system. E) Micraft array with 10 micrafts targeted (red *) for release. F) Micraft array from panel E after releasing the 10 targeted micrafts.

Figure 3. Magnetic transfer system. A,B) Schematic (A) and photo (B) of the magnetic transfer device. The movable magnet is located at the bottom of the hollow tube. C,D) Schematic (C) and photo (D) of the magnetic transfer device placed over a microrraft array. E,F) Schematic (E) and photo (F) of the magnetic transfer device after raft collection. A block magnet underneath the 96-well plate repels the cylindrical magnet upward in the tube. A liquid droplet is present on the device tip securing the microraft in place. G,H) Schematic (G) and photo (H) of the magnetic transfer device after lowering the device into a well on the multi-well plate.

Figure 4.

Efficiency of the magnetic collection device. A) Microraft capture efficiency by the magnetic device as a function of the magnetic field gradient. B) Microraft deposition efficiency by the magnetic device as a function of the magnetic field gradient. C) Cell collection efficiency and single-cell cloning efficiency using 0, 3, 5 and 10 wt% gelatin for encapsulation (n=4, each consisting of 8 collection attempts)

Figure 5.

Separation of blasting from non-blasting EBV-LCLs. A,B) Bright field (A) and fluorescence (B) image of EBV-LCLs cultured on a micraarray. A blasting EBV-LCL colony and a single, non-blasting EBV-LCL cell is shown on the left and right micraarray, respectively. C) Expression of EBNA2 mRNA normalized to GAPDH mRNA expression (y axis) for blasting and non-blasting EBV-LCL subpopulations.