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Dielectrophoresis (DEP) has proven an invaluable tool for the enrichment of populations of stem and progenitor cells owing to its ability to sort cells in a label-free manner and its biological safety. However, DEP separation devices have suffered from a low throughput preventing researchers from undertaking studies requiring large numbers of cells, such as needed for cell transplantation. We developed a microfluidic device designed for the enrichment of stem and progenitor cell populations that sorts cells at a rate of 150,000 cells/h, corresponding to an improvement in the throughput achieved with our previous device designs by over an order of magnitude. This advancement, coupled with data showing the DEP-sorted cells retain their enrichment and differentiation capacity when expanded in culture for periods of up to 2 weeks, provides sufficient throughput and cell numbers to enable a wider variety of experiments with enriched stem and progenitor cell populations. Furthermore, the sorting devices presented here provide ease of setup and operation, a simple fabrication process, and a low associated cost to use that makes them more amenable for use in common biological research laboratories. To our knowledge, this work represents the first to enrich stem cells and expand them in culture to generate transplantation-scale numbers of differentiation-competent cells using DEP. © *2014 AIP Publishing LLC*.
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INTRODUCTION/BACKGROUND

The development of technologies to improve the separation of stem and progenitor cells to generate populations with greater purity holds the potential to increase the efficacy and safety of these cells in transplants and also benefits the study of the basic biology of these cells. Sorting to remove undifferentiated stem cells prior to transplantation could decrease the incidence of tumor development in transplanted patients.[1] A remnant of these cells poses a risk even when most of the stem cells have been differentiated before transplantation. For example, human embryonic stem cells differentiated into dopaminergic neurons prior to transplantation in a rat model of Parkinson's disease still exhibited pockets of undifferentiated cells that can cause tumors.[2] Strategies to purify cells prior to transplantation to remove undifferentiated tumor forming cells are thus highly desirable. Another motivation for sorting cells is to create enriched populations. In the case of stem cells, these biased populations could be used for

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transplantation studies to examine the therapeutic efficacy or regenerative capability of populations enriched for one cell type versus another.

Multiple modalities currently exist to purify stem and progenitor cells. Fluorescence Activated Cell Sorting (FACS) and Magnetic Activated Cell Sorting (MACS) technologies offer rapid rates for cell sorting, at 5000 and 280,000 cells/s, respectively, but they are only useful in sorting cell populations with robust markers that can be used to label the cell populations of interest.[3] Several recent reviews discuss this and other drawbacks of FACS and MACS, including the expense of the machines, the expertise required for their operation, time required for labeling and preparation of samples, and the significant shear stress cells undergo during FACS sorting.[3,4] This shear stress can damage and kill cells, and the effect of antibody labels on cells has not been fully determined.[3] This is a particular concern for cells that will be transplanted into patients.

One technique requiring no cell labeling and thus minimal manipulation of cells prior to sorting is dielectrophoresis (DEP). DEP forces develop in a non-homogeneous electrical field and positive or negative DEP (pDEP or nDEP) in which particles move up or down the electrical field gradient, respectively, can be used to sort cells. The direction of movement at a given applied frequency is governed by the relative polarizability of the cell (based on the cell's inherent electrical properties) compared to that of the medium in which it is suspended, a quantity known as the Clausius-Mossotti factor (see Ref. 33 for supplementary material, Fig. S1). DEP-based devices have been used extensively for cell sorting, as noted in recent reviews.[3,5,6] Such a label-free technique has been very attractive to biological researchers due to its ability to sort cell populations for which few markers have been identified, which is the case for many stem and progenitor cell populations. Furthermore, minimal manipulation of stem cells for applications such as transplantation is of benefit since sorted cells that have not been labeled or genetically modified to enable sorting will be more easily translated to clinical applications. Thus, DEP provides distinct advantages for sorting stem and progenitor cells.

Several different stem and progenitor cell types have been successfully and safely isolated using DEP.[5] These include stem cells from blood or tissue—CD34-positive hematopoietic stem cells have been enriched from bone marrow or peripheral blood[7,8] and NG2-positive human adipose progenitor cells were enriched 14-fold from tissue.[9] DEP-based separation can isolate undifferentiated from more differentiated cells in the same lineage, as shown by the separation of neural stem and progenitor cells (NSPCs) from differentiated neurons[10] and separation of C2C12 myoblasts and more differentiated myotubes.[11] Progenitor cells within the same lineage are also amenable to separation using DEP, and enrichment of neuron progenitors and astrocyte progenitors from a mixed population of NSPCs by DEP provides significantly better enrichment than FACS sorting with PSA-NCAM—a purported marker for neuron progenitors.[12] Sorting by DEP is not toxic for NSPCs, since exposure of these cells to DEP electric fields for the times needed for sorting does not alter cell survival, proliferation, or differentiation.[13] The fact that several types of stem and progenitor cells have been sorted by DEP without deleterious effects on the cells shows the promise of this technique for stem cell isolation.

Many experiments utilizing stem and progenitor cells, such as transplantation into animal models of injury or disease, require relatively large numbers of cells and thus the throughput of DEP separation devices becomes an issue. For example, the number of cells needed for animal transplantation is on the order of 75,000[14] to 1.5×10^6 cells per animal,[15,16] and there are often rv10 animals per group. Therefore, sorting unique stem and progenitor cells using DEP holds great promise, but there is a need to generate large numbers of sorted cells for many applications such as cell transplantation.

The throughput of label-free, DEP-based cell sorting devices has increased over the years and several boast sorting rates of 3000 – 5000 cells/s rivaling those of FACS, which also sorts at 5000 cells/s.[3] However, the use of these devices by biological laboratories has been limited, as has been the case with many engineered devices.[17] Generation of DEP sorting devices that are simple to fabricate, easy to use, able to be incorporated into biological laboratories, and have been validated for real biological applications may increase the use of this methodology by more biological researchers. Ideally, a DEP sorting device would fulfill these criteria while realizing higher levels of throughput.

We aim to address this problem by demonstrating a method to increase the throughput and numbers of sorted stem and progenitor cells for applications such as transplantation while providing a device that is easy to use and generally affordable to the majority of biological labs. Throughput of sorting is increased by device design modifications that augment the numbers of cells obtained at the initial sorting step by one order of magnitude. Following sorting, the number of enriched cells is further increased by expansion of the sorted cells in culture. We show that the expanded cells maintain their post-sorting levels of enrichment and the combination of higher throughput sorting and further expansion in culture generates sufficient quantities of cells to reach transplantation-scale. This is the first known enrichment of stem cells in numbers great enough for transplantation studies using a label-free technique (DEP).

MATERIALS AND METHODS

Device fabrication

The Large Capacity Electrode Array (LCEA) devices consist of a PDMS slab containing the channel features, bonded to a glass slide with the patterned electrodes made of a thin film of titanium and gold, as described previously.[10,13] Briefly, 25 mm × 75 mm glass slides were coated with 200 Å titanium, followed by 1000 Å gold, and the electrode features were patterned with AZ 4620 photoresist (AZ Electronic Materials, Branchburg, NJ, USA). Following patterning, the gold and titanium were selectively etched, and the sacrificial AZ photoresist layer was stripped to yield the electrode features on the glass slide. Electrodes were 50 lm wide with a 50 lm gap between adjacent electrodes. Note that in these experiments, the use of an electron beam was employed to coat standard glass microscope slides with titanium and gold for processing. However, for researchers without access to such equipment, glass slides and coverslips precoated with titanium and gold are commercially available.

The PDMS mold was made by patterning SU-8 2025 photoresist (MicroChem Corp., Newton, MA, USA) to a height of 30 lm. PDMS was casted onto the mold, cured, and cut to the desired size. Inlet holes were punched with a 23 G needle, and an outlet hole of 3 mm diameter was punched using a biopsy punch. Fluidic channels directly after the inlet and before the outlet were 200 lm wide, while the fluidic reservoir where cell trapping takes place was 1500 lm wide (Figure 1).

Following oxygen plasma treatment of the electrodes and PDMS slab, the two layers were irreversibly bonded to one another. Prior to using the device for DEP sorting, prototyping wires

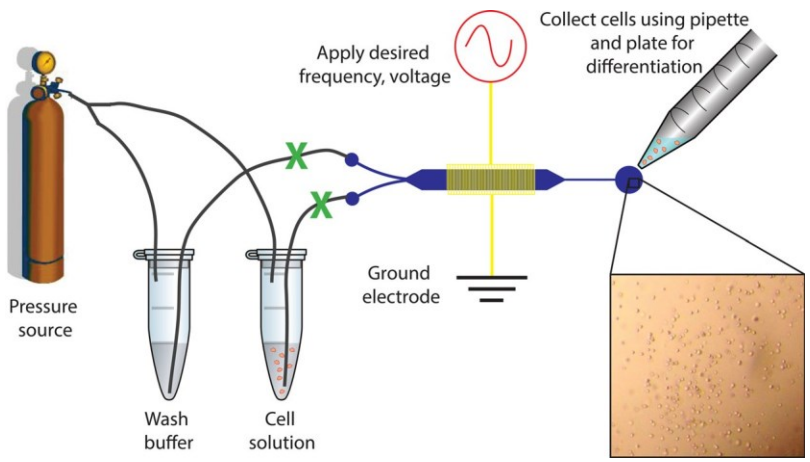

FIG. 1. Schematic of device operation. A common pressure source (compressed gas) is used to induce flow of the wash buffer and cell solution. The valve on each line (green Xs) may be switched off to enable flow of wash buffer or cell solution alternately. Cells flow across the LCEA shown in gold, and untrapped cells can be collected at the outlet using a pipette. During cell collection, flow of cell solution is switched off and wash buffer can flow into the device. Cells are released in fractions by changing the frequency applied to the electrodes, allowing for facile collection of the cell fractions at the outlet. Cells removed from the outlet via pipette are then plated on laminin-coated coverslips for differentiation.

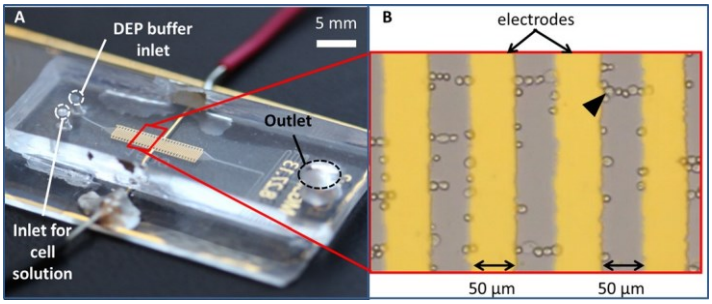

FIG. 2. LCEA device. (a) Photograph of the LCEA device, showing the inlets for DEP wash buffer and cell solution, and the outlet for collecting cells. Electrodes on the chip are connected to a function generator via the red and black wires. (b) Features of the electrode array are shown (electrodes in gold), including the electrode width (50 lm) and spacing (50 lm). Cells trapped by the induced DEP force can be observed at the edges of the electrodes, where the electric field gradient is strongest. Arrowhead denotes cells trapped in a "pearl chain" configuration between electrodes.

were attached to electrode contact pads on the glass slide using a two-part, conductive silver epoxy (MG Chemicals, Toronto, Ontario, Canada) (Figure 2).

Fluid flow was driven from a constant pressure source (a compressed nitrogen tank) and controlled by a digital pressure regulator (ITV series, SMC Corporation, Noblesville, IN, USA). The constant pressure supply was delivered to either the cell solution or buffer solution by switching the pressure source with valves outside of the device (Figure 3). Valves enabled fluid to flow alternately from the cell solution and buffer solution during sorting. To prime the device with fluid and enable rapid washing of the device, both valves were occasionally opened simultaneously. Fluids were driven onto the device by pressurizing the headspace above the fluid in custom 1.5 ml screw cap fluid containers. The pressure source was modulated (typically to 0.6 – 0.8 psi) to obtain a flow velocity of 1 mm/s (3 ll/min), which is similar to the rate of fluid flow in previous work.[10,18]

Device preparation

Prior to beginning a sorting experiment, the microfluidic device and fluid containers for buffer and cell solution were sterilized by flushing with 70% ethanol for at least 15 min. Residual ethanol was then rinsed from the fluid containers and the device by flushing with sterile water for at least 15 min, corresponding to at least 50 times the volume of the channel. To prevent non-specific cell adhesion in the device, a sterile solution of 5% BSA (w/v) in PBS was flushed through the device for at least 15 min. Buffer for the cell sorting experiments (DEP buffer) was prepared as described previously, consisting of 8.5 (w/v) sucrose, 0.3% (w/v) glucose in deionized water, with additions of RPMI-1640 medium in order to bring the final conductivity of the solution to 110 lS/cm.[18] To prepare the device for sorting cells in positive DEP, the BSA solution was flushed from the device by flowing DEP buffer for at least 15 min.

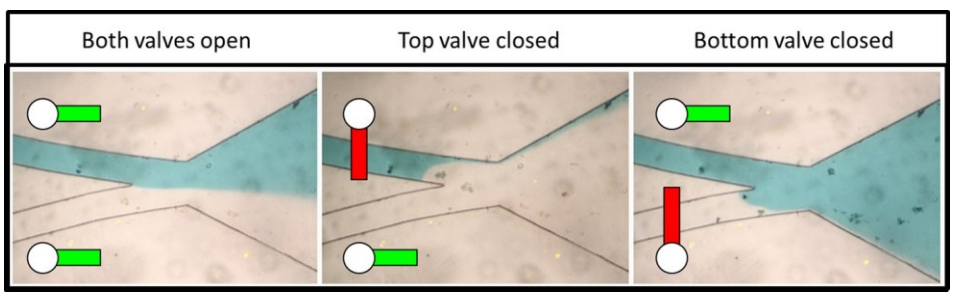

FIG. 3. Operation of valves to control fluid flow in the LCEA device. Solutions of wash buffer (blue) and cells (white) flow into the device alternately when only one valve is open and during normal device operation, using off-chip valves (not pictured but schematized by icons). Green icons denote open valves, and red icons denote closed valves.

Frequency sweeps on interdigitated electrode arrays (IDEs) of various sizes were conducted using an HF2IS impedance spectroscope (Zurich Instruments). These data were used to calculate the electrical impedance of various size IDEs as a function of frequency and are discussed in the supplementary material.[33]

Cell preparation

NSPCs were isolated from the cerebral cortex of the mouse brain at embryonic development day 12.5 (E12 mNSPCs) and grown in NSPC proliferation media (DMEM supplemented with 1x B27, 1x N2, 1 mM sodium pyruvate, 2 mM glutamine, 1 mM N-acetylcysteine, 20 ng/ml EGF, 10 ng/ml FGF, and 2 lg/ml heparin), following prior work.[13,18] In some cases, NSPCs were isolated from E16 cortices to provide cells from a different developmental stage. Prior to sorting, NSPC neurospheres were dissociated using NeuroCult Chemical Dissociation kit (Stem Cell Technologies) and resuspended in DEP buffer, which is low conductivity (110 lS/cm) to enable sorting using positive DEP.

Cell loading and separation

Following rinse of the device with DEP buffer, cells were flowed into the device to initiate the sorting cycle. Electrodes were actuated with a 7 V_{pp} signal at a high frequency (1000 kHz) to trap all viable cells. Non-viable or dead cells do not experience positive DEP and proceed to the outlet of the device (Figure 5(a)). Loading of the large electrode array was optimized by repeated actuation cycles to ensure that the array was saturated with trapped cells. Following array saturation, valves are used to stop flow of the cell solution into the device and flow instead DEP buffer across the array to wash the remaining unbound cells in the device to the outlet. These cells may then be collected manually at the outlet, ensuring high viability in the batch of sorted cells (Figure 5(b)). The frequency applied to the electrodes is then changed in order to elute a fraction of the trapped cells (Figure 5(c)). This fraction of cells can then be collected via pipette at the outlet of the device. This process can be repeated several times (Figures 5(d) and 5(e)) in order to provide several bins of cell populations from the experiment, in a process similar to the techniques of DEP retention and field-flow fractionation[19] (Figure 5(f)).

A trapping curve (Figure 6) was quantified similar to previous work.[18] A high frequency (1000 kHz) was used to trap most of the cells flowing through the device on the electrode array. Then, the frequency was lowered from 1000 kHz to 100 kHz in 100 kHz increments. As the frequency decreases, some of the cells pass their crossover frequency and are released from the array. From videos acquired with a commercial dSLR camera, the percentage of cells that remained trapped on the electrode array at each frequency was determined by manually counting the number of cells. The number of cells trapped initially at 1000 kHz was taken as the maximum number of cells trapped (i.e., 100%), and the "% cells trapped" at other frequencies were normalized to this value. Experiments were conducted using an upright microscope in brightfield, which allowed visualization of cells trapped at the electric field maxima, corresponding to the electrode edges (Figure 2(b)).

Cell recovery and analysis

Cells recovered from the device were cultured in 4 mm diameter PDMS microwells that were contact bonded to glass coverslips as previously described.[12] Glass coverslips were treated prior to cell plating with poly-D-lysine (10 lg/ml in water) followed by laminin (20 lg/ml in EMEM, BD Biosciences). Immediately after removing cells from the sorting device, NSPCs were seeded into wells and cultured for 24 h in NSPC media containing growth factors (NSPC proliferation media). After 24 h, cells were differentiated for 5 days using NSPC media without EGF, FGF, or heparin.

Cells were immunostained, as described previously.[18] Briefly, cells were fixed at 5 days in paraformaldehyde (4% paraformaldehyde, 5 mM $MgCl_2$, 10 mM EGTA, 4% sucrose in PBS)

for 10 min and stored in 0.05% sodium azide in PBS at 4 °C until immunostaining. Cells were permeabilized for 5 min with 0.3% Triton in 1x PBS and blocked for 1 h at room temperature with 5% BSA. Cells differentiated for 5 days were stained with mouse anti-GFAP (Sigma G3893) at 1:200 and Hoechst 33342. Cells were imaged on an inverted Nikon-TE fluorescent microscope. Three randomly selected fields from each coverslip were selected for quantitation. From these fields, the number of nuclei was counted, as well as the number of astrocytes (cells exhibiting a filamentous and cytoskeletal pattern of GFAP expression in the cytoplasm) by counting blinded images manually using ImageJ. Percentages of GFAP-positive cells in each field were determined for each sample. Statistical analysis comparing two samples utilized Student's t-test.

Analysis of cell expansion and enrichment post-sorting

For analysis of cell expansion and enrichment after DEP sorting, cells were sorted into 0 – 100 kHz frequency bins and unsorted 0 – 1000 kHz controls using DEP well devices previously described.[13] E12 or E16 mNSPC neurospheres were dissociated using NeuroCult and resuspended in DEP buffer as described above to a final concentration of rv3 x 10^6 cells/ml. Cells were placed in DEP wells and electrodes were actuated at either 100 kHz (sorted) or 1000 kHz (control) with a 3 V_{pp} signal for 5 min or less to prevent toxicity.[13] Cells not in positive DEP were removed with three washes of DEP buffer. After the washes, the function generator was turned off to release the cells, which were then collected from the well. The number of collected cells was counted on a hemacytometer using trypan blue staining to identify live cells. The enrichment of the collected cells was analyzed by differentiating 7500 cells/well in pDL/laminin-coated microwells as described above in NSPC media without EGF, FGF, or heparin.[12] After differentiation, cells were stained and GFAP-positive cells quantified as described above. In order to assess cell expansion, the remaining collected cells were plated in growth media at 150,000 cells/ml, grown for 3 days, dissociated, and quantified to determine the total number of cells. At each passage 7500 cells/well were plated for differentiation to assess whether enrichment was maintained as the cells were passaged, and the remaining cells were plated for continued expansion. For a schematic representation of the experimental design refer to Figure 8.

RESULTS

Device design

In order to increase sorting throughput, we modified the design of our previous devices shown to successfully enrich neuron and astrocyte progenitors from NSPCs without damaging the cells.[12,13] To overcome previous limitations in terms of cell trapping and sorting capacity, both the number of electrodes as well as the microfluidic channel area available for cell trapping were increased. The dielectrophoretic assisted cell sorting (DACS) device, designed as a proof-of-principle system for enrichment of NSPCs, contained 18 castellated and interdigitated electrode fingers, with a 500 lm wide microfluidic channel for trapping.[10,12] In this device, castellated electrodes were designed to ensure uniform electric field strength in perpendicular flow directions, required for the separation of cells in this configuration. To simplify device operation and fabrication, the LCEA device was designed such that perpendicular flow is not required to separate the cells, and a simple interdigitated electrode design can be used. The use of this simple and more compact electrode design allowed for an increase in the number of interdigitated electrode fingers from 18 (in the DACS device) to 60, a 3.3-fold increase. In addition, due to the simplified channel design, one 1500 lm wide channel is used for cell trapping and sorting, which increases the throughput 3-fold further, for an overall increase in area and potential throughput by over one order of magnitude.

Increasing the electrode array size to increase sorted cell throughput reduced the electrical impedance, which affects the strength of the electrical field and consequently the induced DEP force. A frequency sweep of three devices with different electrode array sizes indicates that increasing the electrode array size decreases the electrical impedance of the array and results in

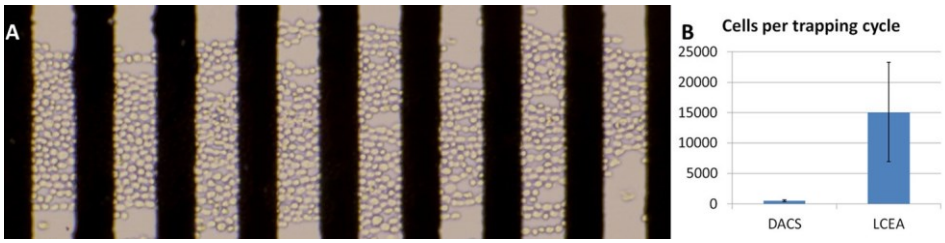

FIG. 4. Optimized loading of electrode arrays to increase throughput. (a) Electrodes load evenly along the array with trapped cells as a result of repeated actuation cycles. (b) By increasing the electrode array area as well as the microfluidic channel area, the area available for cell trapping is increased. The trapping capacity of the previous device (DACS)[10] and the LCEA device is quantified in terms of the number of cells trapped per cycle. The LCEA device provides a 13.2 fold increase in the number of cells trapped per cycle. Error bars represent the standard deviation of 3 measurements.

a lower voltage drop between the electrodes (see Ref. 33 for supplementary material, Fig. S2). We observed in practice that cells would trap on smaller electrode arrays (0.45 – 0.98 mm^2) at 7 V_{pp}, but would not trap on the larger, 9.6 mm^2 array at the same voltage because of the reduction in the induced DEP force. To solve this issue, we decreased the spacing between adjacent electrodes, which increased the electrical field strength and enabled cell trapping and sorting at an applied voltage of 7 V_{pp}. A detailed explanation of these design considerations can be found in the supplementary material (see Ref. 33).

Many microfluidic cell sorting systems employ syringe-pumps, where flow is driven by the application of force to a syringe plunger. The unfortunate side effect of this technique when working with PDMS devices is a pressure buildup due to the compliance of PDMS.[20] This phenomenon prevents rapid switching between fluids without the use of on-chip valves, which introduces complexity to the device fabrication process. However, rapid fluid switching is necessary for loading cells and a separate wash buffer into the sorting chamber of the device. To enable rapid switching between fluids and simplify the device fabrication process, we employ a single constant pressure source (a compressed nitrogen tank with a pressure regulator), which drives flow from the cell solution or wash buffer, depending on the configuration of an off-chip valve (Figures 1 and 3). Thus, the device requires only a single pressure source, two valves, and a single function generator for sorting experiments.

Electrode array loading optimization

Realizing maximum throughput of the LCEA device is dependent upon optimizing the loading of cells along the electrode array. Upon initiation of a trapping cycle, cells begin to trap immediately along the first few (5 – 6) electrode pairs as they first encounter the electrical field gradient. As cell loading continues, these electrode pairs are observed to "saturate" with trapped cells, such that no new cells become trapped. Furthermore, we observed that from the onset of this saturation, cells entering the trapping region would not trap anywhere on the array, even on the unsaturated electrodes. This phenomenon is due to the changing properties of the electrical field as cells trap on the electrodes, eventually forming structures called "pearl chains" that span the region between adjacent electrodes (Figure 2). Although the formation of pearl chains cannot be entirely avoided when achieving high capacity electrode array loading, changes to the electrical field distribution can be mitigated by redistributing the cells trapped on the electrode array.

The simplest way to induce a disruption of the cell trapping patterns that result in pearl chain formation is to cycle the electrical signal delivered to the device on and off intermittently to allow for periods of trapping, followed by re-distribution. When the electrical signal to the device is turned off intermittently, all of the trapped cells on the electrodes are released. Because of the constant hydrodynamic flow through the device, the released cells begin to flow downstream. When the electrical signal is again turned on, the cells immediately re-trap on the closest available electrode (see Ref. 33 for supplementary material, Video 1).

To maximize the number of cells trapped on the array, the optimal loading time, T_{on}, and the optimal re-distribution time, T_{off} are determined for a given set of experimental conditions.

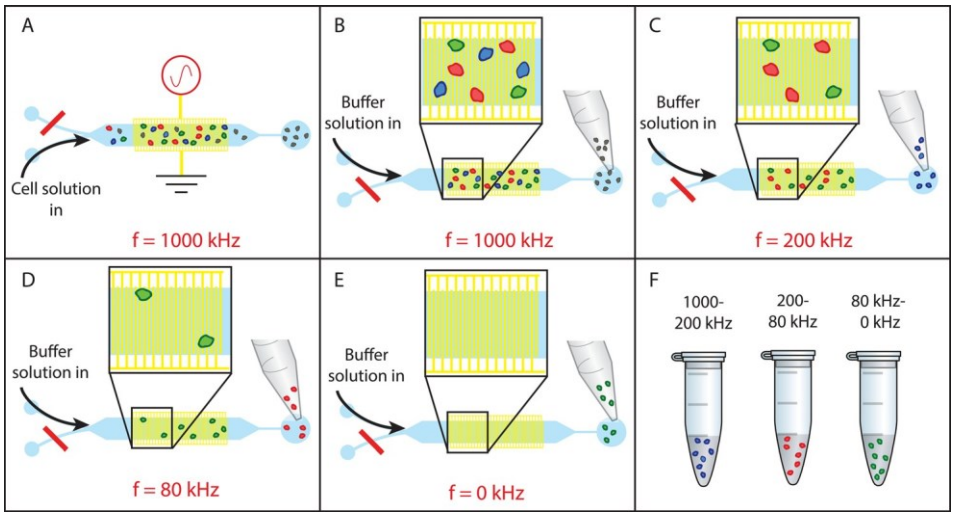

FIG. 5. Schematic showing a cycle of cell sorting in the LCEA device. (a) Cells are loaded in the LCEA device through the cell solution inlet while the wash buffer valve is closed (red line). Viable cells are trapped at a high frequency (e.g., 1000 kHz), while dead cells (gray) do not experience pDEP and flow through to the outlet of the device. (b) Cells that are not trapped on the array are collected via pipette at the outlet as waste. (c) The frequency of actuation is switched to 200 kHz. A portion of the trapped cells release and are collected at the outlet (blue cells). (d) The frequency of actuation is switched to 80 kHz to collect a second portion of the cells (red cells). (e) The electrode actuation is turned off, releasing all remaining cells to be collected (green cells). (f) Three different bins of cells are collected from steps (a) – (e).

The values chosen for T_{on} and T_{off} depend on the density of cells loaded into the device and the flow rate of operation. In previous work where the density of the cell solution was 1×10^6 cells/ml, a period of 30 s was chosen for T_{on}.[10] Although cells continue to trap after this amount of time, the rate at which cells trap begins to decrease. In our experiments, where the density of the cell solution was 5×10^6 cells/ml, a corresponding optimal loading time of T_{on} ¼ 15 s was chosen, due to a diminished cell trapping rate after this amount of time. The ideal T_{off} can be calculated by taking into account the number of electrodes that are saturated with cells, usually between 5 and 8 electrodes. By using the flow rate velocity, and the channel distance occupied by the saturated electrodes, T_{off} can be calculated as the time to completely clear the saturated electrodes as

$$T_{off} \text{ ¼ } \frac{\eth e_w \text{Þ} e_g \text{Þ} x \eth n \text{Þ}}{v}, \tag{1}$$

where e_w and e_g are the electrode width and gap, respectively, v is the flow rate velocity in the device, and n is the number of electrodes to be cleared. For our system, where v ¼ 1000 lm/s, e_w ¼ 50 lm, e_g ¼ 50 lm, and using, for example, n ¼ 5 electrodes, T_{off} ¼ 500 ms.

By intermittent actuation and de-actuation of electrodes, the entire electrode array can be filled with trapped cells (Figure 4). This enables trapping of roughly 15,000 cells on the array in a single trapping cycle, corresponding to a more than one order of magnitude increase in the number of cells trapped per trapping cycle compared to the previously published DACS device[10] (Figure 4), which fits well with the increase in area of the LCEA device over the DACS device. Since the duration of a trapping cycle was 6 min for our experiments, this corresponds to an hourly sorting rate of 150,000 cells/h.

Enrichment of astrocyte progenitors from NSPCs using the LCEA device

The LCEA device can separate cells into discrete bins based on their responses to defined frequencies of the applied electrical field, much like the DACS device.[10] As an example, a heterogeneous population of cells is initially trapped along electrodes at a high frequency (1000 kHz) to trap all viable cells since dead cells do not experience positive DEP (pDEP) and

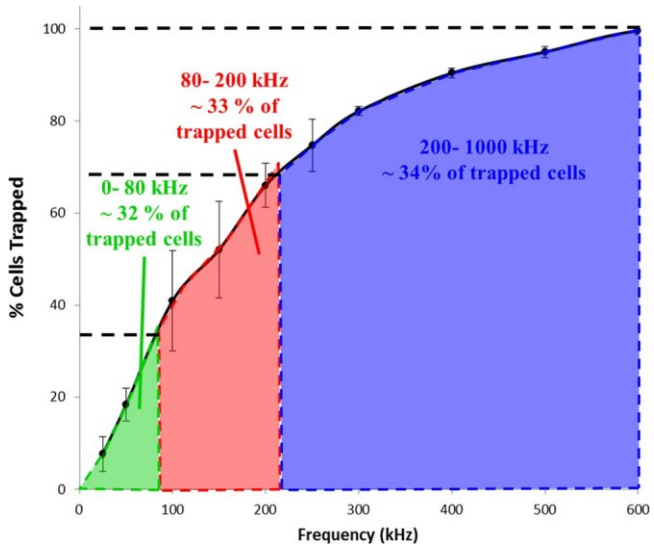

FIG. 6. Trapping curve of mouse NSPCs to determine sorting frequencies in the LCEA device. The DEP response of mouse NSPCs to different frequencies in the LCEA device is shown (n ¼ 3 biological repeats, bars are standard deviation). Cells were trapped at 7 V_{pp} and 1000 kHz, and the frequency was decreased in increments of 100 kHz or less in order to count the number of cells remaining trapped at each frequency. The percentage of cells trapped at each frequency is normalized to the number of cells trapped initially at 1000 kHz which is a reflection of viable cells in the population.

proceed to the outlet of the device (Figures 5(a) and 5(b)). The frequency applied to the electrodes is then lowered (e.g., to 200 kHz) in order to elute a fraction of the trapped cells (Figure 5(c)). This fraction of cells, which in our example is the 200 – 1000 kHz high frequency bin, can then be collected via pipette at the outlet of the device. The process is then repeated with the frequency lowered to 80 kHz to release the 80 – 200 kHz medium frequency bin and again to 0 kHz to generate the 0 – 80 kHz low frequency bin (Figures 5(d) and 5(e)). The entire process provides multiple bins of cell populations from the experiment in a process similar to the techniques of DEP retention and field-flow fractionation (Figure 5(f)).[8,21,22]

To test the sorting fidelity of the LCEA device for stem cell applications, we chose mouse embryonic day 12 (E12) cortical NSPCs as a starting population of cells since we have previously shown specific progenitor populations can be isolated from these cells using DEP and we have defined sorting parameters within which DEP electric fields do not affect the function of these cells.[12,13] Furthermore, we found previously that astrocyte and neuron progenitors in this lineage that can be separated by DEP do not differ in size, helping to clarify that their separation in microfluidic DEP devices is not due to the effects of fluid forces acting on differently sized cells.[12] A trapping curve[18] was generated using these cells in the LCEA device in order to determine appropriate frequencies for subsequent separation. To mimic the operation of the device during sorting, the trapping curve was obtained by starting at high frequency (1000 kHz) then the frequency was lowered by 100 kHz increments to allow cells to release from the electrode array. Since the heterogeneous NSPCs have different dielectric properties, their crossover frequency or the frequency at which the cells switch from pDEP to nDEP differs. In microfluidic devices, cells are also impacted by the Stokes drag force they experience as they change velocity due to the DEP force acting on the cell.[10] Thus, trapping of cells onto electrode arrays in microfluidic channels requires the DEP force to overcome this drag force.[23] Accounting for the combined effects of DEP force and Stokes drag force on a cell, a threshold frequency can be defined, which describes the frequency at which a cell experiences sufficient positive DEP force to overcome the Stokes drag force and is trapped at the electrodes.[10] By graphing the percentage of cells trapped as a function of frequency, we determined the trapping curve of E12 mouse NSPCs in the LCEA device and selected frequencies to roughly divide the populations into thirds during sorting. Specifically, by dividing the cells into a high frequency band from rv200 to 1000 kHz, a medium frequency band from 80 to 200 kHz, and a low frequency band from 0 to 80 kHz we were able to capture approximately equal numbers of cells into the 3 bins (Figure 6).

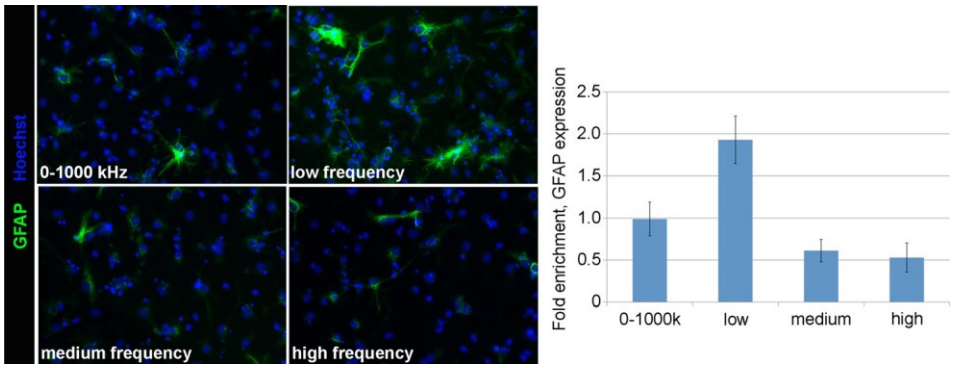

FIG. 7. Astrocyte progenitors are specifically enriched in low frequency fractions recovered from the LCEA device. Undifferentiated mouse NSPCs were sorted in the LCEA device into low, medium, and high frequency fractions and unsorted controls (0 – 1000 kHz). The cells were differentiated post-sorting to generate astrocytes from the astrocyte progenitors and the differentiated astrocytes were detected and quantified using the marker GFAP (shown in green, all cell nuclei detected with Hoechst shown in blue). The data are expressed as fold enrichment relative to control, unsorted cells incubated in DEP buffer, and reflect the results of 3 independent biological repeats with n > 1250 cells counted per condition. Error bars indicate SEM.

We tested the ability of the LCEA device to isolate specific progenitors from mouse NSPCs by sorting the cells into low, medium, and high frequency bins and differentiating the cells to determine which bin generated more astrocytes and thus contained more astrocyte progenitors. We collected separately a population of cells trapping at 1000 kHz as a control, since all viable NSPCs should trap at this frequency (0 – 1000 kHz control). Of note, cells sorted using the LCEA device showed high viability, with 91.4% ± 1.43%, 88.7% ± 2.86%, and 86.4% ± 1.13% viability for cells sorted into the low, medium, and high frequency bins, respectively (n = 7, error represents SEM). NSPC cultures are inherently heterogeneous containing stem cells and progenitors fated to generate one of the differentiated cell types of the central nervous system: neurons, astrocytes, or oligodendrocytes. We found previously that astrocyte progenitors were specifically enriched by low frequency DEP sorting while neuron progenitors were isolated at higher frequencies.[12] After sorting in the LCEA device, astrocyte progenitors were significantly enriched in the low frequency bin compared to control cells incubated in DEP buffer but not exposed to electrical fields (24.6% ± 7.0% GFAP-positive cells in the low frequency bin, and 10.2% ± 1.8% GFAP-positive cells in the DEP buffer control; $p < 0.05$) (Figure 7). In agreement with our previous DEP sorting of these cells using the DACS device,[12] astrocyte progenitors were most enriched in the low frequency bin as compared to cells in the medium or high frequency bins or controls, confirming sorting fidelity of the LCEA device.

DEP-sorted NSPCs expand significantly in culture while retaining post-sorting enrichment

In addition to increasing the throughput of the initial DEP sorting by developing the LCEA device, we investigated the possibility that stem and progenitor cells sorted by DEP could be expanded in culture in order to provide a greater number of enriched cells. We again utilized E12 mouse NSPCs and sorted astrocyte progenitors in a low frequency bin (0 – 100 kHz) and compared them to control, unsorted cells that had also gone through DEP (0 – 1000 kHz bin). For these experiments, we employed another easy to fabricate and simple to use DEP device while we were in the process of developing the LCEA for sorting. This device has open wells, enabling easy loading and retrieval of cells but limiting both throughput, since the electrode trapping arrays are constrained by the size of the well, and sorting fidelity since there are no microfluidic channels for efficient washes to remove non-specifically trapped cells.[13] Furthermore, the well device is best used for isolating a single frequency bin rather than the multiple bins possible with the LCEA. Since we only needed a single, low frequency bin for the analysis of expansion of cells after sorting, the well device was sufficient for these experiments.

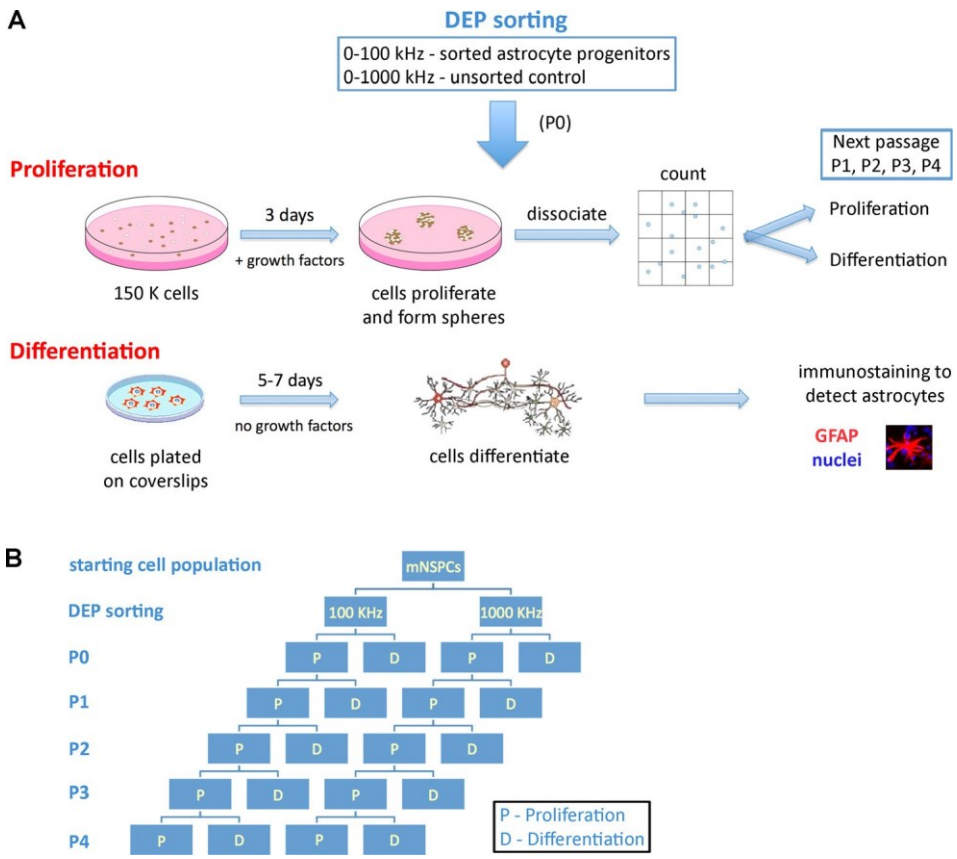

FIG. 8. Experimental strategy to test cell expansion and maintenance of enrichment post-DEP sorting. (a) Schematic depicting proliferation and differentiation assays. Mouse NSPCs are DEP sorted at 0 – 100 kHz to enrich astrocyte progenitors and at 0 – 1000 kHz to serve as unsorted controls. Cells immediately post-sorting are designated P0. Proliferation assays begin with 150,000 cells/ml plated in medium containing mitogenic growth factors (EGF and FGF). The cells expand and form spheres, which are dissociated and counted after growth for 3 days to determine the total number of cells and fold expansion. Dissociated cells are then plated into proliferation media for the next passage or into differentiation media. For differentiation assays, cells are plated on coverslips without mitogenic growth factors and allowed to differentiate for 5 – 7 days to generate astrocytes, which are detected by immunostaining for GFAP. The percentage of GFAP-positive cells is used to determine whether the enrichment of astrocyte progenitors is maintained across serial passaging. (b) Schematic shows the cycles of proliferation and differentiation assays for each passage (P0 - P4) of 0 – 100 kHz sorted and 0 – 1000 kHz unsorted control mouse NSPCs (mNSPCs).

Cells were taken through two parallel assays over 4 passages to determine whether sufficient quantities of enriched cells could be expanded after sorting for applications such as cell transplantation (experimental strategy schematized in Figure 8). In one, the ability of the cells to expand in culture was tested by measuring the total number of cells generated at each passage. The other measured whether the level of post-sorting enrichment was maintained as cells were cultured over time after sorting. For this assay, a portion of the cells at each passage was plated for differentiation in order to determine the percentage of cells forming astrocytes as a means of calculating the enrichment of astrocyte progenitors as cells are expanded post sorting. By taking the cells through multiple passages (P0 – P4) and calculating the degree of cell expansion and the level of enrichment at each passage, we were able to accurately track whether NSPCs sorted by DEP could be further expanded in culture prior to use in additional experiments.

E12 mouse NSPCs sorted at low frequency (0 – 100 kHz) to enrich astrocyte progenitors and controls (0 – 1000 kHz) both expanded well in culture post-DEP (Fig. 9). In general, for these experiments we sorted approximately 1.7×10^6 cells and since about 30% are isolated in the 0 – 100 kHz bin there were on average 5×10^5 cells at P0. We used the same amount of cells at P0 for the control 0 – 1000 kHz bin. The cells were generally passaged every 3 days, so the end of P4 corresponds to less than 2 weeks after sorting. At this point, the total number of E12 sorted cells

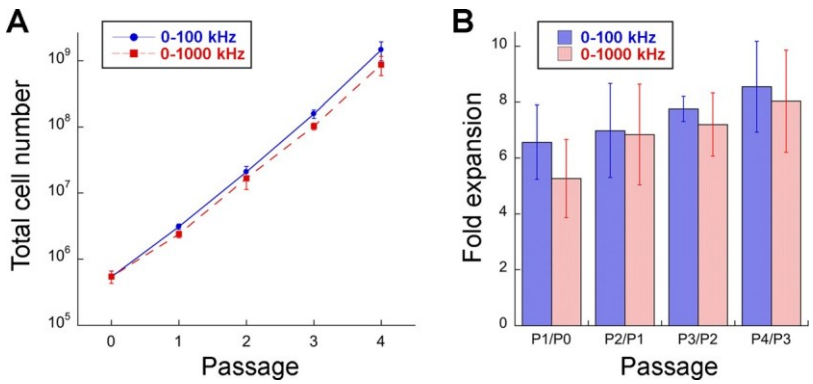

FIG. 9. Sorted cells expand significantly. (a) E12 mouse NSPCs sorted at 0 – 100 kHz or control 0 – 1000 kHz unsorted cells cultured in growth factor containing media continue to proliferate and expand, growing exponentially over 4 passages and rapidly generating large numbers of cells. Cells were passaged approximately every 3 days, so the number of cells generated by passage 4 was obtained within 2 weeks of sorting. (b) Fold expansion of sorted and unsorted control cells does not significantly vary over two weeks in culture post-sorting. Error bars represent SEM. N ¼ 4 independent biological repeats.

is 1.5×10^9 (Fig. 9(a)). We performed analogous experiments with mouse NSPCs isolated from the E16 cortex and found similar results, with the total number of sorted cells at P4 reaching 1.2×10^9 (see Ref. 33 for supplementary material, Fig. S3). Analysis of the fold expansion (fold increase in cell number over the previous passage) revealed that in general there was a 7.5 fold increase in cell number every 3 days and no significant difference in the expansion of 0-100 kHz sorted or 0-1000 kHz control cells (Fig. 9(b)). Again, we found similar results with E16 mouse NSPCs (see Ref. 33 for supplementary material, Fig. S3). The similar rates of expansion of the 0-100 kHz sorted and 0-1000 kHz control cells suggest no toxicity of the low DEP frequencies at the short times needed for sorting, which is consistent with our previous findings.[13] Cells sorted by the LCEA device were also able to proliferate in culture post-sorting. These cells formed neurospheres after being expanded in culture, and there was no difference in the size (see Ref. 33 for supplementary material, Fig. S4) or number of neurospheres (low frequency sorted cells: 39 spheres, 0-1000 kHz control: 32 spheres) between the cells sorted by low frequency or the controls, indicating similar rates of growth. These data demonstrate progenitors isolated from NSPCs by DEP sorting can be expanded in less than 2 weeks to generate approximately 10^9 total cells.

We tested whether the expanded cells maintained their post-sorting enrichment since this will be critical for generating large numbers of sorted cells for experiments. Sorting NSPCs at low frequency enriches for astrocyte progenitors and enrichment at each passage was assessed by differentiating the cells and measuring the formation of astrocytes (Fig. 10(a)). The percentage of GFAP-positive astrocytes at each passage was plotted as a ratio of 0 – 100 kHz sorted cells relative to 0 – 1000 kHz controls. No significant difference in this ratio over the 4 passages was observed, suggesting no loss in enrichment since a downward trend in this ratio over the 2 weeks in culture would indicate a loss of cell purity (Fig. 10(b)). Direct comparison of the percentage of GFAP-positive astrocytes generated over time in culture for the 0 – 100 kHz sorted cells also demonstrates the maintenance of enrichment since the percentages at P0 and P4 did not significantly differ (P0 ¼ 38.4% ± 4.6%, P4 ¼ 37.9% ± 3.2%, p ¼ 0.9298). Sorted E16 mouse NSPCs similarly retain enrichment over continued passaging (see Ref. 33 for supplementary material, Fig. S5). Thus, mouse NSPCs sorted by DEP can be expanded to generate large numbers of cells (10^9) while maintaining enrichment

DISCUSSION

Novel design of DEP sorting device

Throughput

In our group's previous work, we demonstrated separation of NSPC populations at a throughput of ~6000 cells/h using the DACS device.[12] This device was designed to provide

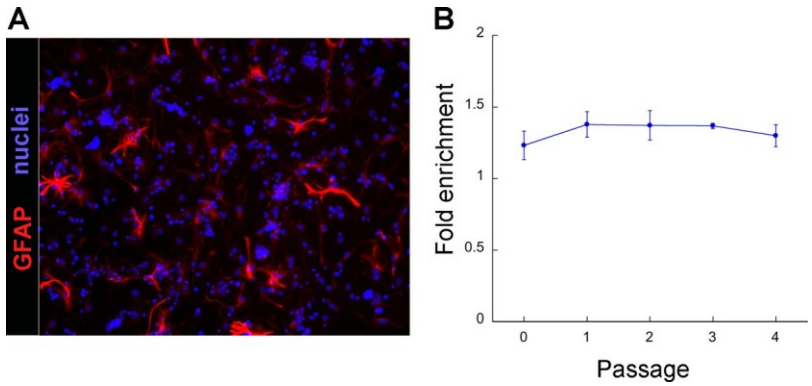

FIG. 10. Sorted cells maintain post-sorting enrichment. (a) E12 mouse NSPCs sorted at 0 – 100 kHz or unsorted 0 – 1000 kHz controls were differentiated in the absence of growth factors to allow formation of astrocytes from astrocyte progenitors. Representative image of 0 – 100 kHz sorted cells with astrocytes immunostained for GFAP in red and all cell nuclei detected by Hoechst in blue. (b) The percentage of GFAP-positive cells in 0 – 100 kHz sorted cells at each passage is expressed relative to that of control 0 – 1000 kHz unsorted cells to determine fold enrichment. Plotting fold enrichment over serial passages indicates no significant shift in level of enrichment with passaging. Error bars represent SEM. N ¼ 5 independent biological repeats.

characterization of the stem cell populations for basic biology research, and the throughput is too low to enable cell transplantation studies. Here, we have shown separation of NSPCs at a throughput of 150,000 cells/h, while maintaining the level of enrichment obtained with our earlier DACS device.[12] Combining this level of throughput with the capability to expand cells in culture after DEP sorting will now provide enough sorted NSPCs to enable transplantation studies.

Several DEP-based cell sorters with sorting rates rivaling those of the gold standard cell sorting method, FACS, have been described in a recent review.[3] Although cell sorting rates of greater than 10^5 cells/s have been demonstrated with DEP-based sorting devices, many of these high throughput devices sorted yeast[24] and bacteria[3] and they have not been validated with mammalian cells. Impressive sorting rates of up to 17,000 cells/s have been demonstrated in mammalian cell separations such as the isolation of breast cancer cells from blood,[25,26] leukemia cells from blood,[22] and circulating colon tumor cells from blood.[27] Many of these separations were aided by labels[28] or a difference in size between the cells of interest and background cells. Here, we have accomplished a biologically relevant enrichment of astrocyte progenitor cells from a population of neural stem/progenitor cells that are homogeneous in size.[12] Therefore, DEP-based sorting need not be limited to applications involving mammalian cell populations that vary significantly in size. Furthermore, we have demonstrated high throughput sorting of biologically viable stem and progenitor cells.

Ease of fabrication and use

The accessibility of many DEP-based sorting platforms for researchers in biology and medicine, who might benefit the most from them, is often impeded by high cost and difficulties in device fabrication, instrumentation setup, and programming required to perform the separations. Here, minimal equipment is required to fabricate and operate the device. If glass slides precoated with metal are purchased, only a photoresist spinner, a UV lamp, and an oxygen plasma source are required to fabricate the chip. To further simplify fabrication of the electrode array, a commercially available flexible circuit could be purchased.[9] Operation of the device is also simple as all that is needed is a compressed gas tank (already available in many biology and medical research labs) with a pressure regulator and a waveform generator. A hemostat performs the function of a valve and enables reliable switching of fluids if pneumatic or digitally controlled fluid valves are not available.

The technology of devices for DEP-based cell sorting and characterization continues to improve. Several DEP systems are now automated, controlled by software that can be adapted

to the particular sorting or characterization needs.[10,29] Other goals have been to design DEP devices for clinical samples (needing eventual approval by the FDA) or to make them amenable to industrial scale up or mass production.[9,24,27] However, many labs are recognizing the need to simplify the operation of DEP sorters to make them more useful for biological separations. To this end, new generations of sorters have addressed issues such as improving cell recovery from DEP devices.[24] As the field of DEP sorting continues to evolve, the challenge will be to develop improved and sophisticated sorting devices while making them easy to use and adopt by biological researchers.

Sorted populations can be expanded to generate large numbers of enriched cells

A main goal of this study was to determine whether cells could be expanded after DEP sorting while maintaining their post-sorting enrichment to generate sufficient numbers of cells for applications such as transplantation of stem and progenitor cells into animal models of disease or injury. In many cases, the number of cells transplanted per animal ranges from 75,000 to 1.5×10^6 and there are often 10 animals per group.[14-16] Thus, 10^6 - 10^7 cells are necessary for an animal transplantation experiment. Since our results demonstrate generation of 10^9 DEP-enriched cells from NSPCs after expansion for less than 2 weeks, there are clearly ample numbers of cells for these types of experiments. Further, the robustness of this finding is demonstrated by the fact that mouse NSPCs from different developmental stages that vary in progenitor ratios (E12 and E16)[18,30] were both expanded after DEP sorting while maintaining enrichment. In addition, cell proliferation was observed after sorting using two different devices in our study (shown by quantification of cell expansion in one case and neurosphere formation in the other), suggesting that this phenomenon is not limited to a particular device design. At least for NSPCs, the proliferative nature of the cells can be exploited to greatly increase the available number of cells for experiments after DEP sorting.

Significant proliferation was observed for both the sorted (0 – 100 kHz) and control (0 – 1000 kHz) cells in our studies, suggesting no significant toxicity or deleterious effects of DEP on the cells at these frequencies and exposure times. These findings are consistent with our previously published studies of human and mouse NSPCs, demonstrating that NSPCs in solution exposed to DEP for short times (5 min or less) had no decrease in survival.[13] Furthermore, our previous studies clarified that exposure for up to 30 min did not affect NSPC proliferation or differentiation.[13] Proper design of experiments to limit exposure times ensures that DEP sorting is a safe technique for NSPCs in solution.

Although other studies of stem and progenitor cells isolated by DEP have not tracked proliferation and continued enrichment over multiple passages as shown here, many have checked viability of the cells post sorting or performed assays demonstrating the continued health and functionality of the sorted cells. CD34-positive hematopoietic stem cells enriched from peripheral blood by DEP were cultured for 2 weeks post-sorting and generated viable cell colonies.[7] Several types of stem and progenitor cells (hematopoietic, adipose-derived progenitors, and myoblasts) have been successfully analyzed by flow cytometry after DEP sorting.[7-9,11] Furthermore, DEP sorted myoblasts have high viability post-sorting.[11] Mouse NSPCs isolated by DEP were analyzed in differentiation assays, demonstrating that the cells retained the ability to generate neurons and astrocytes post-DEP sorting.[12] Taken together, the fact that several studies demonstrate viable stem cells after DEP sorting suggest that other stem cell populations could be expanded post sorting in order to generate sufficient numbers of cells for experiments such as transplantation. Future experiments will be necessary to test this theory with a variety of other stem and progenitor cell types.

Sorting NSPCs by DEP into biased populations of progenitors enables studies to assess the relative ability of each population to provide repair after transplantation. For this purpose, the cells need not be purified to homogeneity and the level of progenitor cell enrichment achieved by DEP sorting is sufficient. In fact, purifying progenitors in this lineage to homogeneity prior to transplant may be deleterious since a mixture of progenitors provides greater support for transplanted cells than a pure population.[31,32] However, too much or undefined heterogeneity

can be problematic for transplants. NSPCs in culture, as generated for transplantation, contain a minority of stem cells and a majority of progenitors linked to glial or neuronal fates. NSPC cultures are thus inherently heterogeneous with varying ratios of astrocyte progenitors and neuron progenitors. Differing ratios of progenitors may underlie the divergent potencies observed across different NSPC transplants and the ideal progenitor ratio for NSPC transplants is not clear. More studies are needed in which the progenitor cell makeup of the transplant is well defined. The techniques developed in this work will allow study of the roles of various progenitor cells in regeneration and tissue repair by enabling transplant of biased populations and assessment of functional improvement in each case.

CONCLUSION AND FUTURE DIRECTIONS

Here, we demonstrate a DEP-based sorting device for NSPCs, capable of sorting cells in preparation for further expansion of the cells in culture. These techniques provide an initial sorting throughput of 150,000 cells/h and the capability to expand cells in culture for at least 4 passages, providing 10^9 cells to perform transplantation studies. In addition to increasing the throughput of sorting, the simplicity of the developed device's fabrication and instrumentation is envisioned to provide enhanced accessibility of DEP-based sorting techniques to biological and medical researchers. Compared to previously developed DEP-based sorting devices, our device design provides a low barrier to entry for non-engineering labs to perform characterization and separation of their cells of interest.

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[33]See supplementary material at http://dx.doi.org/10.1063/1.4902371 for system design considerations, additional figures showing cell culture expansion of cells post-sorting, and a video demonstrating the optimization of electrode loading technique.