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A Fluorescence-Activated Single-Droplet Dispenser for High Accuracy Single-Droplet and Single-Cell Sorting and Dispensing

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Abstract

The ability to sort and dispense droplets accurately is essential to droplet-based single-cell analysis. Here we describe a fluorescence-activated single-droplet dispenser (FASD) that is analogous to a conventional fluorescence-activated cell sorter, but sorts droplets containing single cells within an oil emulsion. The FASD system uses cytometric detection and electrohydrodynamic actuation-based single-droplet manipulation, allowing droplet isolation and dispensing with high efficiency and accuracy. The system is compatible with multi-well plates and can be integrated with existing microfluidic devices and large-scale screening systems. By enabling sorting based on single-cell reactions such as PCR, this platform will help expand the basis of cell sorting from mainly protein biomarkers to nucleic acid sequences and secreted biomolecules.

Rare subtypes within a cell population can be biologically and medically important but are often overlooked due to measurement of average cell properties. Intratumor cell heterogeneity, for example, plays a role in chemoresistance and tumor recurrence.¹⁻² Tumor cell subpopulations exhibit different behavior in response to treatment, confounding cancer therapy.³⁻⁴ To better understand tumor cell heterogeneity, measurements at the level of individual cells are necessary.⁵⁻⁶ Early developments in single-cell analysis began primarily with fluorescence-activated cell sorting (FACS), in which fluorescently-labeled cells in a fluid stream are screened and sorted for downstream applications.⁷ Although conventional FACS is automated and allows cell sorting with high throughput and accuracy, it typically relies on staining protein biomarkers; its inability to isolate single cells based on genetic sequence limits its flexibility. Isolation and recovery of individual cells based on their nucleic acid sequence content is difficult to achieve using existing techniques. In addition, conventional FACS often requires samples containing $>10^5$ cells, which limits applications in which the input cell number is low, such as in the study of rare cells.

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Supporting Information

Optical setup, voltage-controlled droplet generation process, and other supplemental data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Droplet microfluidics-based technologies overcome this limitation by encapsulating single cells in confined nanoliter-to-picoliter aqueous droplets within an oil emulsion, creating microreactors in which independent single-cell reactions can be performed. Droplets of interest are sorted based on a fluorescence signal generated by a reaction such as PCR and are collected for further analysis. This technology is a valuable alternative to flow cytometry, expanding the basis of cell sorting from mainly protein biomarkers to nucleic acid sequences and secreted biomolecules.⁸⁻⁹ Droplet microfluidics has been applied to directed evolution of enzymes,¹⁰⁻¹¹ single-cell sequencing,¹²⁻¹³ screening for secretory phenotypes,¹⁴ rare mutation detection,¹⁵ digital PCR,¹⁶⁻¹⁷ and antibody screening.¹⁸ Nucleic acid cytometry is an emerging field derived from droplet microfluidics which allows identification, sorting, and analysis of cells based on nucleic acid sequences.¹⁹

Wider application of droplet microfluidics-based single-cell analysis would be facilitated by a simple and economical droplet sorting and dispensing method compatible with multi-well plates. Commercial FACS instruments are incompatible with oil emulsions and require re-dispensing water-in-oil emulsion droplets into water carriers to form double emulsion droplets.²⁰⁻²¹ However, these double emulsions are highly polydisperse, limiting quantitative analysis and the types of assays possible. Fluorescence-activated droplet sorting (FADS) is analogous to FACS but applied to droplets in oil rather than cells in water.²² In FADS, droplet microfluidics is integrated with sensitive detection and high throughput microdroplet manipulation to isolate droplets of interest. Approaches used to sort droplets include charging and steering using an electric field,²³ dielectrophoresis,²⁴ laser-induced localized heating,²⁵ hydrodynamic pressure,²⁶ magnetic fields,²⁷ acoustic waves,²⁸ and valves.²⁹ Although some of these methods are promising, they lack a single-droplet collection step to interface with multi-well plates. An automated method for dispensing droplets for single-cell analysis is needed.³⁰⁻³² Here we describe a microfluidic fluorescence-activated single-droplet dispenser (FASD) that isolates individual droplets within an oil emulsion based on a fluorescence signal and dispenses droplets with nanoliter precision into multi-well plates for downstream analysis.

EXPERIMENTAL SECTION

Materials.

QX200 Droplet Generation Oil was purchased from Bio-Rad (Hercules, CA). High voltage power supplies were purchased from Bertan High Voltage Corp. (Hicksville, NY). A 488 nm laser with a CDRH LP 1041441 AG power supply was purchased from Coherent Inc. (Santa Clara, CA). Avalanche photodiodes (SPCM-AQRH-11-FC) were purchased from Excelitas Technologies (Waltham, MA). A Pump 33 DDS was purchased from Harvard Apparatus (Holliston, MA). Trihalo(1H,1H,2H,2H-perfluorooctyl)silane (97%), dimethyl sulfoxide (DMSO), Calbiochem Tween 20, sucrose, and dextrose were purchased from Sigma-Aldrich (St. Louis, MO). FM 1-43 dye was purchased from Thermo Fisher Scientific; 100 µg of FM 1-43 was dissolved in 46 µl of DMSO. Buffer solution was composed of 8.0% sucrose, 0.3% dextrose, and 0.1% BSA in 1 mM Tris pH 8.3. Paraformaldehyde solution (4%) was purchased from Electron Microscopy Sciences (Hatfield, PA). Costar 3595 96-well (~6.9 mm inlet DI) TC-Treated micro-plates was purchased from Thermo Fisher Scientific.

Chip fabrication.

Soft lithography was used for polydimethylsiloxane (PDMS) chip fabrication. SU-8 2050 Epoxy Negative Photoresist (MicroChem, Westborough, MA) was spin-coated onto a 3-inch silicon wafer to form a film of ~50 μm thickness, measured using an interferometer. PDMS was degassed by vacuum, mixed with 10% (w/w) catalyst agent (Dow Corning, Midland, MI), poured onto the master, and cured at 70 $^{\circ}\text{C}$ for 4 h. The PDMS chip was peeled off the master, inlet and outlet holes were punched, and the chip was exposed to oxygen plasma along with a glass slide substrate. The PDMS channel was then sealed irreversibly against the glass substrate. Finally, the chips were stored in a 115 $^{\circ}\text{C}$ oven for 24 h to change the inner surface of the channels from hydrophilic to hydrophobic.

Cell culture.

K562 chronic myelogenous leukemia cells (American Type Culture Collection, Manassas, VA) were cultured at 37 $^{\circ}\text{C}$ at 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Exponential phase cells were centrifuged at 250 rpm for 5 min and resuspended in buffer twice before experiments.

PCR experiments.

On-chip single cell encapsulation was achieved using a flow-focusing method in a T-junction micro-channel by injecting cell solution (4×10^5 cells/ml) and PCR reagents in two separate channels. A mini stir bar was placed in the syringe to prevent cell aggregation. PCR reagents were prepared by mixing 100 μL of SsoAdvanced Universal Probes Supermix (Bio-Rad), 10 μL of primers (Integrated DNA Technologies, Coralville, IA), 20 μL of 20x EvaGreen dye (Biotium, Fremont, CA), and 10 μL of BSA. Droplets were collected off-chip and single-cell PCR amplification was performed in a 96-well plate using 10 μL of PCR reagent mixture per well, with a CFX96 Real-Time PCR Detection System (Bio-Rad). Amplification was performed as follows: hot start at 95 $^{\circ}\text{C}$ for 4 min; 60 cycles of denaturation at 95 $^{\circ}\text{C}$ for 5 s and annealing/extension at 60 $^{\circ}\text{C}$ for 45 s.

Imaging.

An AZ100 Microscope (Nikon, Tokyo, Japan) with a GX1920 CCD camera (Allied Vision Technologies, Burnaby, BC, Canada) was used to acquire brightfield and fluorescence images of cells and droplets. A high-speed GC640 CCD camera (Allied Vision Technologies) was used to record the formation of double emulsion droplets and aqueous droplets. Multi-well plate screening experiments were performed using a Typhoon FLA 9000 imaging system (GE Healthcare, Chicago, IL). Images were analyzed using ImageJ (<http://rsbweb.nih.gov>). Goldwave software (<http://www.goldwave.com>) was used to analyze sound data collected during stage movement.

RESULTS AND DISCUSSION

The automated fluorescence-activated single-droplet dispenser (Figure 1A) employs a programmable combination of capillary-based fluorescence monitoring, high-voltage-triggered single-droplet generation, and a mechanical stage with two-dimensional motion. A fluorescence signal from a droplet is sensed by an avalanche photodiode detector (APD),

triggering movement of the mechanical stage to collect the droplet in a multi-well plate. Precise control of the formation of an oil droplet containing one aqueous droplet of interest at the open end of the capillary is the key step in accurately dispensing single droplets and cells for downstream analysis. To achieve this, we used electrohydrodynamic actuation and applied high voltage to the capillary holder³³⁻³⁵ to exert a controllable force on the emulsion droplets, thereby forming a double emulsion (aqueous-in-oil-in-air droplet) for dispensing into the multi-well plate. Although the mechanism for generating double emulsions using electrohydrodynamic actuation is somewhat different than electrospray³⁵, we note electrospray-based droplet formation has been demonstrated as an efficient technique for the sampling and ejection of single cells or single-cell contents, such as the preparation and analysis of single cells by probe electrospray ionization mass spectrometry (PESI-MS) and single-cell analysis using MALDI-MS analysis³⁶⁻³⁷.

The optical setup of the FASD system (Figures 1B and S1) is similar to flow cytometry but much simplified. We used a 488 nm laser to excite EvaGreen dye (a DNA intercalating dye for real-time qPCR fluorescence detection) in experiments to test the system, and two fiber-coupled APDs, one to collect fluorescence and one to collect a light scattering signal. Nanoliter single-droplet precision was achieved using a capillary-based electrohydrodynamic actuation method (Figure 1C and D). The FASD nozzle consisted of a capillary, a capillary holder made of a modified needle tube, a detection window for laser irradiation, and a copper plate as the ground electrode. The capillary holder was manufactured using commercially available needle tubes of two gauges fixed together using conductive silver paint, which served as the electrode to apply high voltage. The outer tube (N_1) was 18 gauge with an inner diameter (ID) of 0.84 mm and an outer diameter (OD) of 1.27 mm; the inner tube (N_2) was 22 gauge with an ID of 0.41 mm and an OD of 0.71 mm. The purpose of the outer tube in this double layer design was to enlarge the gap between the walls of the capillary and needle tube to minimize light scatter by the needle tube, and to fix the capillary firmly at the center of the holder. The length of the capillary tip outside the capillary holder (L3 in Figure 1C) was optimized to obtain the smallest droplets possible without deformation of droplets by the capillary holder. The size of the droplets was determined by the inner and outer capillary diameters, the applied voltage, and the length between the open end of the capillary tip and the electrode (L3). The capillary used for fluorescence-activated single-cell or single-droplet sorting had a circular cross-section with an ID of 0.1 mm and an OD of 0.15 mm. Since a larger L3 caused a larger droplet size, the detection window was positioned on the electrode instead of at the end of the capillary tip to minimize L3.

Application of an electric field to the capillary polarized the oil, resulting in an electric force between the droplet and grounded electrode (copper plate). When the electric field strength was increased to a certain level, an oil droplet with a specific size was formed and ejected toward the grounded electrode (Movie S1 and Figure S2). Figure 2 shows a series of images captured using a high-speed camera illustrating the relationship between droplet size and voltage. Droplet volume was attenuated by increasing the electric field strength, demonstrating the use of an electric field for enhanced control of droplet formation, which may be useful in other flow-focusing devices.

To test the capability of the FASD system in single-cell analysis based on nucleic acid sequence content, we used the system to sort and dispense droplets containing single K562 (myelogenous leukemia) cells based on the fluorescence signal from a PCR reaction amplifying the FLT3 (FMS-like tyrosine kinase 3) gene. Nucleic acid cytometry is an emerging field built on droplet microfluidics that allows robust identification, sorting and further downstream analysis of cells based on specific nucleic acid biomarkers, which expanded the fluorescent biomarkers from surface to inside of the cell.¹⁹ Since nucleic acids encode the information of life, programming cellular functions and dictating lots of biological information, identifying cells based on their nucleic acids content is a powerful way to unravel the underlying biology of cells, such as the genetic information of many phenotypes. Existing flow cytometry techniques, however, are unable to reliably recover specific cells based on nucleic acid content. Given the important roles of nucleic acids played in all living things, a tool for dispensing single cell based on nucleic acids is essential. Recent developments in nucleic acid cytometry have used barcoding to achieve single-cell resolution,^{12, 38} allowing a library of $\sim 10^9$ droplets to be handled in a single microtube. In contrast, our system takes advantage of the spatial screening possible using two-dimensional microwell arrays by connecting the voltage-controlled microdrop dispensing nozzle to a multi-well plate.

To generate the appropriate water-in-oil droplets, the as-prepared PDMS chip was thermally treated (heated in an oven overnight) to revert its hydrophilic surface back to its hydrophobic form. Figure S3 shows the shape and angle of the water-oil interface at the surface of the PDMS channel, which changed from 28° to 155° after the thermal treatment. Surfactant-stabilized droplets containing single cells and PCR reagents were produced using a flow-focusing T-junction microfluidic device (Figures S4 and S5A), using Droplet Generation Oil as the continuous phase. The flow rates of the oil and aqueous phases were optimized to achieve a small and precisely confined droplet volume sufficient to encapsulate a single cell (~ 2 nL). Emulsion droplets showed a uniform size distribution (Figure S5B), facilitating subsequent quantitative analysis. Droplets were collected off-chip for PCR amplification (Figure S6) and were reinjected into a microfluidic device connected to the FASD system (Figure 3A). The microfluidic device introduced additional oil into the emulsion to further separate droplets for subsequent detection and sorting. Two distinct fluorescence intensity clusters for sample droplets were observed, corresponding to PCR-positive and PCR-negative reactions (Figure 3A and B). A threshold value was set to trigger movement of the mechanical stage for collection of PCR-positive and PCR-negative droplets in two separate wells (Figure 3C and D). A voltage of 1.4 kV was applied to generate an oil droplet with a size sufficient to encapsulate one aqueous droplet. A negative voltage was applied to an electrode positioned beneath the collection container to guide falling droplets straight toward the center of the container. Cells containing the target sequence amplified by the PCR reaction were enriched by the sorting step. Such a cell enrichment step may be useful in genomics applications to eliminate unwanted reads from abundant and uninteresting populations, allowing deeper coverage of populations of interest.

Isolated PCR-positive droplets were reinjected into the T-junction microfluidic chip and were separated by the injected oil (Figure 4A). Movement of the 96-well plate on the mechanical stage was triggered by a fluorescence signal from the droplets, allowing

collection of a single droplet in each well (Figure 4B and C). The plate was covered by a grounded metallic board and placed on an electrode connected to negative voltage to shield the electrostatic interaction between plate and droplets and to achieve successful collection of droplets at the center of each well (Figure S7). Results are assessed using convenient multi-well plate imaging platforms such as Typhoon.

The frequency of single-droplet dispensing was determined by the time required to generate and collect a droplet and the response time of the mechanical stage. The response time of the stage, calculated by analyzing the sound of the movement, was 160 ms for a step in the X direction, and 300 ms for a step in the Y direction (Figure S8 and Movie S2). The time for droplet manipulation was adjusted to match the response time of the stage. The total time to dispense a droplet was ~850 ms: ~67 ms for droplet generation (Figure S9), ~75 ms for collection (Figure S10), and ~700 ms for transit time (traveling from the detection window to the end of the capillary tip). Based on these measurements, the interval time between the detection and trigger events was set to 850 ms to ensure successful collection of the target droplet in a specific well. Single-droplet resolution was achieved by adjusting the flow rate of the droplets while keeping constant the flow rate in the oil channel.

CONCLUSIONS

We developed an automated fluorescence-activated single-droplet dispenser that can sort and dispense single droplets or cells with high efficiency and accuracy. This system provides a general approach for analyzing and dispensing microfluidic droplets in either water or oil phases and allows analysis of samples containing small numbers of cells. This platform achieves high-resolution cytometric detection and dispenses uniform nanoliter droplets, allowing accurate quantitative single-cell analysis. Because most steps in our home-built instrument were automated and controlled by software, our workflow and experiments showed good repeatability and reliability. The system can be integrated with existing microfluidic devices to allow more sophisticated pretreatment and single-cell analysis, and can be embedded into commercially available large-scale screening systems. Compared to previous single-droplet manipulation methods, this system is more automated and provides more accurate droplet dispensing. By facilitating droplet-based single-cell analysis using reactions such as PCR, the FASD platform will enable cell sorting based on nucleic acid sequences and cell secretions. Further developments such as controlled breakage of single droplets could lead to a variety of new single-cell proteomic and genomic analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Schematic of FASD system. (B) Optical setup. An avalanche photodiode (APD1) was used to detect the forward scatter of single cells in water and trigger single-cell dispensing. A second avalanche photodiode (APD2) was used for fluorescence detection, which triggered fluorescence-activated sorting and dispensing events. The main contribution of this system is the electrohydrodynamic actuation-based single-droplet manipulation method described in (C) and (D). (C) Schematic of the capillary-based nozzle. The distance between the capillary tip and copper plate (L_1) is ~6 mm. The diameter of the hole in the copper plate (d) is 3 mm. The thickness of the copper plate (H) is 0.5 mm. The height of the detection window at the end of capillary holder (L_2) is 8 mm. (D) Image showing details of the capillary holder.

Figure 2. Series of micrographs showing the voltage-controlled formation of oil droplets of different sizes. The scale bar is 200 μm .

Figure 3.

(A) Schematic of single-droplet sorting. Emulsion droplet sorting events were activated by the fluorescence signal generated from PCR amplification. Droplets were separated by the injected oil (inset micrograph). The flow rates of the oil and droplets were 1.5 and 0.05 $\mu\text{L}/\text{min}$, respectively. The positive voltage applied to the capillary was 1.4 kV. (B) Segment of an APD trace from emulsion droplets showing results from PCR amplification of the FLT3 gene in single K562 cells. A fluorescence intensity threshold was set to trigger sorting events. Micrographs of sorted (C) PCR-positive and (D) PCR-negative emulsion droplets.

Figure 4.

(A) Schematic of single-droplet dispensing. PCR-positive emulsion droplets were dispensed into 96-well plates for downstream analysis. Droplets were separated by the injected oil (inset micrograph). The flow rates of oil and droplets were 1.5 and 0.05 $\mu\text{L}/\text{min}$, respectively. The positive voltage applied to the capillary was 1.4 kV. (B) Segment of an APD trace from PCR-positive emulsion droplets showing results from PCR amplification of the FLT3 gene in single K562 cells. A fluorescence intensity threshold was set to trigger dispensing events. (C) Fluorescence image of 96-well plate showing successful dispensing results.