SORT 'N MERGE: A DETERMINISTIC MICROFLUIDIC PLATFORM FOR CO-ENCAPSULATING DISTINCT PARTICLES IN MICRODROPLETS

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ABSTRACT

This paper presents a microfluidic platform for deterministic co-encapsulation of desired combinations of distinct particles in individual droplets. Our microfluidic assay generates droplets co-encapsulating two and three different particles at a yield as high as 87.0% and 80.1%, respectively, by innovative integration of active droplet sorting and downstream merging processes. In conventional approaches, such integration is highly challenging due to the need for precisely handling a small amount of sorted droplets. The developed microfluidic platform is readily implemented for the co-encapsulation of multiple particles of a predetermined number in each droplet at high efficiency and high throughput. Our particle co-encapsulation technique eliminates stochastic pairing processes, for which the Poisson distribution severely limits the yield to < 10%.

INTRODUCTION

The human body is composed of trillions of distinct cells. Understanding cell-to-cell heterogeneity and system-level functions synergistically formed by multiple cells requires biological assays with single-cell resolution. Traditional bio-research protocols using pipettes and microtiter plates fail to efficiently compartmentalize individual cells into microwells for downstream analysis. Recently, droplet-based microfluidics has been applied in various single-cell assays, in which high-throughput analysis is performed with each cell efficiently preserved in a small volume reactor[1]. Many of these assays require pairing distinct objects such as cells or engineered microparticles in a droplet (Table 1). For example, co-encapsulating cells in a droplet with surface modified microparticles that provide solid support to various biomolecular probes enables transcriptomic or proteomic assays[2]–[6]; Co-culturing two or more cells in a confined environment enables the study of how distinct cells interact with one another. Therefore, the ability to precisely control the contents of such microdroplets is expected to open the door to various new applications.

The conventional co-encapsulation method employs co-flowing of cells or beads during the droplet formation process. However, this approach is very inefficient because of its stochastic nature governed by the Poisson statistics[7]. According to the Poisson statistics, the probability of obtaining two distinct particles in a single droplet is 13.4% at best and could be lower if higher accuracy is required. Moreover, the probability of gaining droplets with a desired combination of particles decreases exponentially with the number of particles in each droplet. This prevents the wider use of droplet technology in biological study.

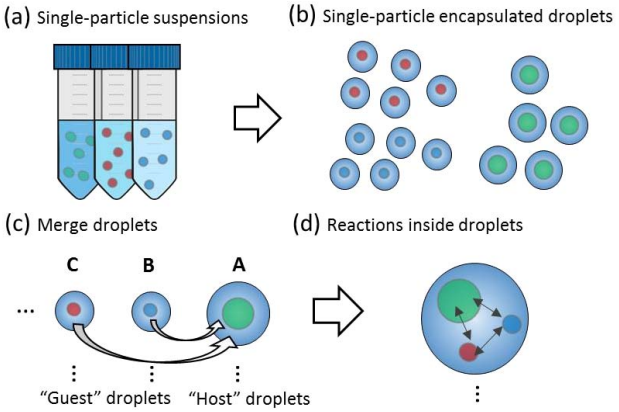

Fig. 1. *Concept of our droplet manipulation process for cells/beads based assay.*

We address this technical challenge through introducing a newly developed microfluidic droplet device that allows the precise merging of multiple droplets with different contents. This device can be seamlessly integrated with a fluorescent-activated droplet sorter so that only droplets containing the desired objects can be captured and merged. Therefore, we can produce micro-droplets that contain desired cells, particles, and buffers from a bulk solution via additive droplet sorting and merging processes (Fig. 1). This guarantees lossless cell or particle capturing, which is no longer limited by the Poisson distribution, and leads to the formation of final merged droplets co-encapsulating desired particles. This paper demonstrates the high-throughput deterministic co-encapsulation of two or more microparticles of arbitrary size in individual

Table 1. Examples of single-cell assays that require pairing two or more particles inside a microreactor.

Particle A	Particle B	Buffer	Applications	Ref.
Cell	Primer coated bead	Lysis and RT buffer	Single-cell RNA-seq	[2][3]
	Antibody coated bead	Fluorescent antibody	Single cell secretion	[4][5]
	Cell(s)	Culture medium	Cell-cell interaction	[6]

droplets for the first time. The high controllability and adaptability of this platform enables versatile microfluidic droplet-based single-cell assay.

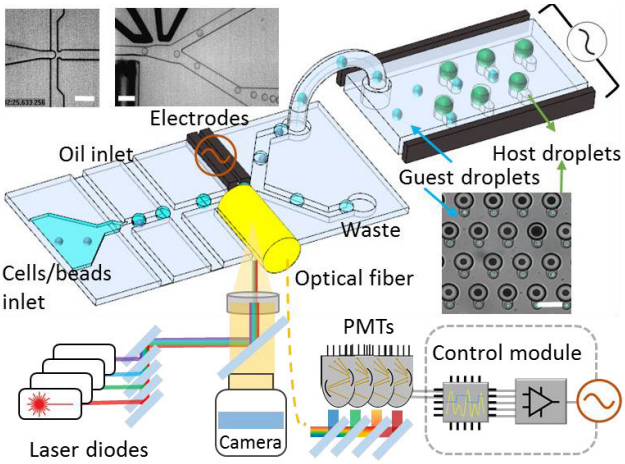

Fig. 2. *Schematics of our microfluidic system composed of droplet generating/sorting and droplet merging devices. Scale bar:100μm.*

SYSTEM DESIGN

Our microfluidic system incorporates two sub-component devices: a fluorescent-activated droplet sorting (FADS) device[8] and a droplet merging device (Fig. 2). The collection outlet of the FADS device is connected to the inlet of the merging device. Water-in-oil droplets containing *Particle A* with an 80 μm diameter are first generated by the flow-focusing structure of the FADS device with a 70×70 (W×H, μm) orifice. Subsequently, the droplets are optically detected and actively sorted into the merging device by a dielectrophoretic force (Fig. 3a, and c). These 80 μm-diameter droplets, called host droplets, are trapped in the larger microwells of the merging device. Next, smaller droplets with a 45 μm diameter that encapsulate *Particle B* are produced by another sorter with a smaller orifice (25×45, μm) and are flowed into the merging device. The 45 μm droplets, called guest droplets, are trapped in smaller microwells adjacent to the larger microwells that hold the host droplets; therefore, they form an array of droplet pairs. Next, the electrodes at the two edges of the merging device generate a uniform electric field across all droplet pairs, triggering the coalescence of all droplet pairs, which leaves the guest droplets absorbed into the host droplets (Fig. 3b, and d). These processes are repeated until all the desired particles or reagents are co-encapsulated within the post-merging host droplets.

DEVICE

All microfluidic devices are made of Polydimethylsiloxane (PDMS) using the standard multi-layer soft lithography methods. The microelectrodes are fabricated by filling the microchannel with a low-melting-point alloy. The excitation light of FADS was composed of 405/473/532/635 nm lasers coupled into an objective lens and focused on the detection zone of the FADS device. An embedded optical fiber near the detection zone transmits a fluorescent or scattering light signal emitted from the droplets to photomultiplier tubes. Feedback control is achieved by a microcontroller (Arduino Due) at a 50 kHz update rate. Once the positive droplet is detected, a finite AC high-voltage pulse (30 kHz, ≤ 2 kV) is triggered to deflect the droplet into the collection channel.

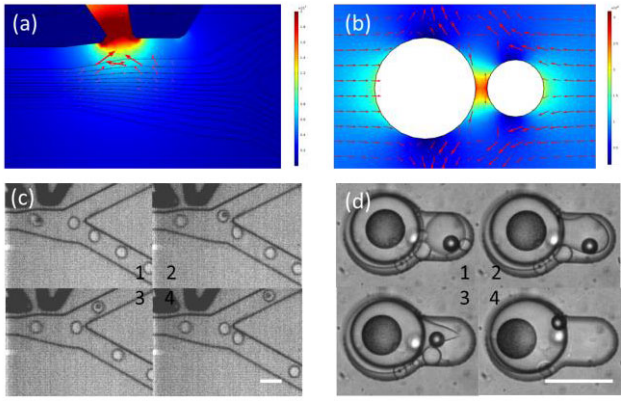

Fig. 3. *Principle of droplet manipulation: (a) positive dielectrophoretic force (pDEP)-based sorting and (b) electrohydrodynamic (EHD) force-based merging. The heat maps indicate the strength of the local electric field. The arrows indicate the relative strength and direction of (a) the force field and (b) the velocity field. (c) Time series images of fluorescence activated droplet sorting. Interval: 0.162 msec. (d) Time series images of droplet merging. Interval: 2.5 msec. Scale bar:50μm.*

RESULTS AND DISCUSSION

Droplet generating and sorting

The single-cell/bead containing droplets are first enriched by the FADS device with the integrated functions of droplet generating and sorting. The encapsulating process of single beads still followed the Poisson distribution. Although there are a few studies demonstrating highly efficient encapsulation that exceeds the Poisson limit, they are all operated under special conditions where deformable hydrogel beads or cells with highly uniform size are required. Thus, it may not be suitable for wider applications.

We characterized the performance of our FADS device using a suspension containing fluorescent beads. The sample was diluted to a low concentration (e.g. one bead per 20 droplets) so that most droplets would be either empty or loaded with a single bead. Since the empty droplets only wasted reagents without affecting the assay readout, our device's sorting accuracy and throughput in excluding empty droplets are critical factors in determining the system performance.

Figure 4a shows a clear contrast in the optical signal between droplets containing either zero or one bead, and only droplets containing beads triggered a sorting pulse. To determine the maximum throughput, we gradually increased the flow rates of both aqueous and oil phases. The ratio of the oil phase to the aqueous phase must be fixed at $Q_{oil}/Q_{aqueous}$ =12 to maintain the same space of two droplets for accurate detection and sorting. The voltage required to deflect the 45 μm droplets into the collection channel was proportional to the flow speed (Figure 4b). We found that at a higher voltage, the droplets could be torn up, which set the upper bound of the sorting throughput to 6,000 droplets per second. Figure 4c shows the accuracy of

the sorting result. More than 99% of the sorted droplets contained at least one particle, and less than 5% of them were doublets. This doublet fraction can be reduced by further diluting the sample concentration if higher accuracy is needed.

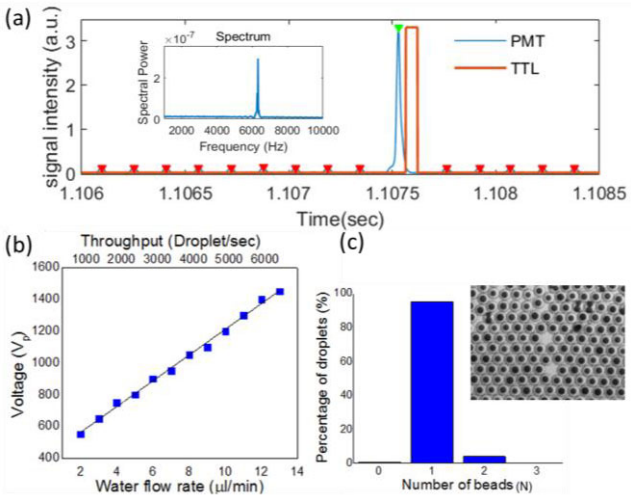

Fig. 4. *(a) Time sequence of fluorescent signals (blue line) detected from empty droplets (red dot) and from droplets containing beads (green dot). The orange line represents the logic signal processed by a microcontroller for triggering the sorting pulse. (b) Relationship of minimum voltage required to deflect droplets into collection channel for given throughput and water (cell culture medium) flow rate. The flow rate of the oil phase is fixed at 11 folds of the water flow rate. (c) The histogram shows the percentage of sorted droplets that contain N beads.*

Droplet paring and merging

The real challenge of the post-sorting droplet merging is not the merging process itself but stems from the need to retain rare sorted droplets and subsequently pair the two types of droplets on a one-to-one basis. Since the sorted droplets are tiny and rare, it is very difficult to handle them once they leave the microfluidic channel. Coupling an additional microfluidic structure downstream of the sorter could increase the back pressure, thus affecting the sorting performance. We addressed the above challenges by employing a buoyancy-based droplet capturing mechanism for the merging device operation. To utilize the buoyancy force for droplet manipulation, the capturing chamber of the device was located at the top of its flow channel. This design did not generate extra fluidic resistance. The shape of each capture chamber only trapped a pair of droplets of distinct sizes. Thus, sequentially loading different types of droplets formed a large array of droplet pairs on top of the channel. Since the buoyancy force-driven droplet capturing process was independent of the fluidic flow, we could connect the FADS and merging devices together without causing any fluidic interference between them.

There are several mechanisms to merge the two captured droplets that destabilize their interface, including electric fields, mechanical squeezing, chemical modifications, and optical heating. In our previous study[9], we found that droplet merging with perfluorobutanol added into the oil phase was convenient and easy-to-implement, but this raised concerns about biocompatibility and the stability of droplets after merging. In this study, we applied a uniform electrical field generated by a pair of parallel electrodes and triggered electrocoalescence across all the droplet pairs. This approach had less impact on the contents inside the droplets because the electrodes did not directly contact the aqueous phase. Our simulation shows the electric field strength in aqueous phase is about one order magnitude lower than that in the oil phase, which is in the tolerance range of living cells.

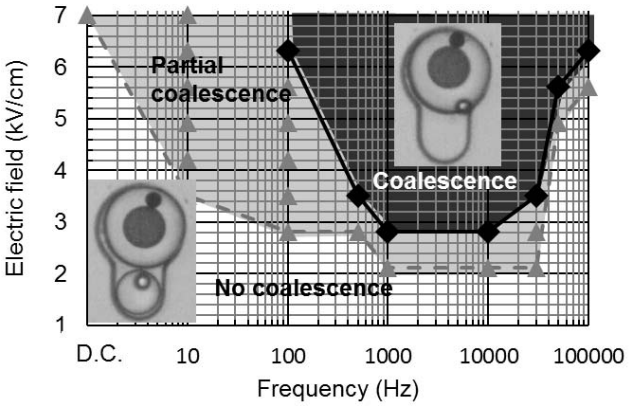

Fig. 5. *Observed coalescence response under different electric field strength and frequency. The dark shaded area indicates all the droplets pairs are merged. Only a fraction of droplets is merged in the light shaded area. No merging event occurred in white area. The electric field is calculated by the applied voltage V across two electrodes with a separation distance of 15mm (E=V/0.15, kV/cm).*

Figure 5 shows the droplet coalescence performance at varying strengths and frequencies of the applied electric field. We found that the merging efficiency was maximized under a 2.9 kV/cm electric field (oil phase) at 1–10 kHz. This frequency dependency can be attributed to both electrostatic and dielectric forces. The electric field near the droplet surface was non-uniform and amplified by dipole–dipole interactions between the two droplets. The host droplets increase their sizes as more guest droplets merge into them. The fixed size of the microwells limits the maximum number of droplets that we could merge; we achieved the merging of up to four guest droplets without observing any problems. The merged droplets could be collected by manually flipping the device chip under gravitation.

Microparticles co-encapsulation

We proved that our platform could process a wide variety of samples by demonstrating the co-encapsulation of microparticles with a size variation of 15–60 μm. The concentrations of all the fluorescent bead suspensions were adjusted to 0.05 bead per droplet volume (λ=0.05). This would result in the formation of doubles for 2.5% of the sorted droplets in theory, and this fraction could be even lower if the bead suspensions are further diluted. Figure 6 shows the co-encapsulation of two or three fluorescent beads with high success rates of 87.0% and 80.1%, respectively. This is a significant improvement over the conventional co-flowing approach that is limited by the Poisson statistics, where only 13.4% and 5.1% of droplets

contained desired particles at most (λ=1). Moreover, most of the single-cell assays require a much lower sample concentration (λ≤0.1) to obtain accurate single-cell readouts. The yield of successful co-encapsulation becomes even worse under these requirements. Thus, it makes the co-encapsulation of 3 or more particles nearly impossible to achieve with the conventional approach. Our method can overcome this limit.

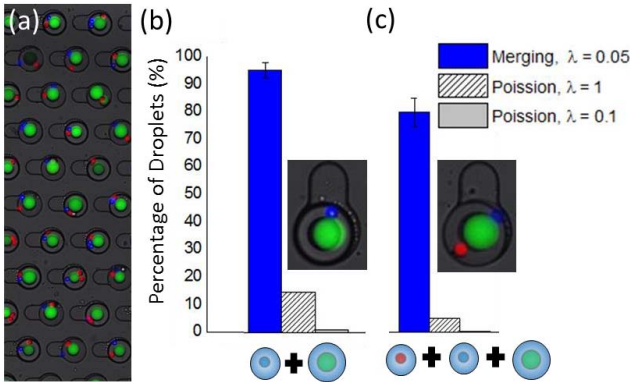

Fig. 6. *(a) Image of merged droplets in merging device. Blue, red, and green florescent microbeads are co-encapsulated in each droplet. (b-c) The histogram shows the success rate of droplets that contain the correct numbers of blue, red, and green fluorescent beads. The data was collected from a total 1176 host droplets in a merging device with 3 repeats. Two cases were demonstrated: (b) Co-encapsulation of blue and green beads and (c) blue, green, and red beads. The estimated co-encapsulation rate resulting from the co-flow method is also plotted based on the Poisson statistics with an event rate λ=1 or 0.1.*

The additive errors in our system during each round of merging can be reduced by changing the microfluidic design or improving the experimental conditions. For example, we observed that some of the doublets were formed in a droplet due to aggregations in the bulk solution but not due to random distributions. The size and shape of the microfluidic chamber design could also affect the droplet capturing efficiency. Furthermore, we could meet more stringent experimental needs by adding other active control mechanisms, such as doublet detection or imaging-based selective droplet fusion.

CONCLUSION

In this paper, we coupled droplet sorting with downstream droplet merging to co-encapsulate the sets of distinct particles inside the droplets. The desired particles were actively sorted by FADS and additively merged into the same droplet without following a stochastic process. This fundamentally overcomes the poor efficiency caused by the conventional Poisson statistics-governed co-flow method. The experiment demonstrated here can be immediately applied for studying single-cell transcriptomics or proteomics using primer-coated or antibody-coated microbeads. In addition, the high-throughput operational procedure using small reactors is similar to those using traditional pipettes and microtiter plates, making it adaptable to many well-developed biological assays. Moreover, the ability to add any desired contents from a bulk sample into droplets without loss makes our method even more suitable for assays analyzing specific cell types that are highly heterogeneous or rare in populations.

ACKNOWLEDGEMENTS

This study was supported by the National Science Foundation (Grant No. ECCS-1708706). We appreciate technical discussions with members of Kurabayashi and Cai labs at the University of Michigan, as well as the assistance of Bo Zhang, a visiting student in Kurabayashi lab from Tsinghua University in China.

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