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Selective Fusion of Anchored Droplets via Changes in Surfactant Concentration

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We present a robust method to fuse in parallel an array of anchored droplets in a microchannel. Pairs of anchored droplets are fused by the removal of surfactant from the droplet interface by reducing the surfactant content in the flowing external oil phase. By controlling the flow of multiple oil inlets, the selective fusion of rows of droplets in a larger array is demonstrated. The technique is compatible with cells as shown with a trypan blue exclusion vitality assay. The method is easy to implement, requires no active components and is applicable to oil/water combinations where the surfactant is soluble in the external phase.

I. Introduction

The applications of droplet microfluidics have rapidly advanced in recent years to a broad range of chemical[1] and biological assays[2]. This progression is driven by the large number of independent measurements made possible by nanoliter droplets. The controlled coalescence of droplets allows the mixture of well-defined ratios of reagents and has been utilized as a basic operation to measure reaction kinetics,[3,4] synthesize chemicals[5] and particles,[6] and add reagents for quantitative biological assays[2,7].

Many different techniques[8,9] have therefore been developed for the controlled fusion of droplets of different content both in the presence and absence of surfactant molecules. These droplet fusion techniques can be broadly separated into two categories. The first uses passive elements to fuse droplets, such as changes in channel geometry[10–13] or regions of different droplet wetting properties to displace surfactant and induce fusion.[14–16] Droplets can also be fused soon after their formation, before the surfactant fully populates the droplet interface as demonstrated by Mazutis *et al.*.[17,18]. Another group of methods use active elements to trigger droplet fusion such as electric fields,[3,19–22] magnetic fields,[23] temperature gradients,[11] and focused lasers.[24] These techniques require extra components and external equipment that can complicate microfabrication and experiments. They can also set constraints on the channel geometries and position of droplet fusion. Droplets are fused in series which often requires synchronization of droplet trains and limits the observation time of droplets after fusion for dynamic measurements. A parallel method of fusing pairs of droplets would simplify and potentially speed-up the addition of reagents or chemicals in droplet platforms.

We present here a general method to easily fuse droplets in parallel in anchored arrays. Two-dimensional arrays simplify the observation, manipulation, and analysis of large sets of separate measurements. Droplet anchors have been previously used to quickly align droplets to form a droplet array.[25] Two different droplets can be placed in the same well.[26] Localized heating by a focused laser was used to displace surfactant and induce droplet fusion to initiate a chemical and enzymatic reaction.[4,26] However, the use of the laser can be difficult to implement, and the heating can often be harmful to cells or proteins encapsulated in the droplets.

Using anchors, pairs of droplets remain in place despite a continued flow of the external oil. Surfactant at the droplet interface inhibits fusion of anchored droplet pairs. As we detail below, pairs of anchored droplets can be fused by removing surfactant from the interface by reducing the surfactant content in the flowing oil phase. This method permits the parallel fusion of all the paired droplets in a droplet array. Also by controlling the relative flow rates of two inlets containing oil with and without surfactant, selective droplet fusion can be achieved among rows of anchored droplets. The application of the technique for cellular assays and the biocompatibility is demonstrated through a trypan blue exclusion test for cellular viability.

II. Materials and Methods

PDMS microfluidic chips with channel depth modulations were fabricated using the dry film photoresist soft lithography technique described by Stephan *et. al.*[27] since it enables rapid prototyping of multi-level structures. To render the internal channel surface hydrophobic, Novec™ 1720 Electronic Grade Coating (3M) was flowed into the microchannel and the chip was heated for 30 minutes at 150 °C. The surface treatment ensured that the aqueous droplets did not wet or make contact with the channel walls.

Droplets were formed using a flow focuser and flowed into a 3mm wide channel containing an array of anchors (supplemental figure S1). For all measurements described the channel depth was 50 µm. The anchors had a diameter of

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150µm and a depth of 15µm. The external phase was fluorinated oil containing surfactant, QX100 (Biorad), and oil without surfactant, Novec 7500 (3M). The aqueous droplets contained 0.0025 – 0.25 % m/v pluronic F-68 (Affymetrix) for all experiments. Pluronic F-68 was found to reduce undesired droplet fusion. Reactions were performed with droplets containing 0.27 M $FeCl_3$ and 0.8 M KSCN. For this combination of channel depth, anchor depth and fluids, droplets would remain in the anchors for external oil flows of less than about 25µL/min. The array could be entirely cleared of droplets at higher external oil flows (about 200µL/min). Fluid flow was controlled using computer-controlled syringe pumps (Nemesys, Cetoni). Cell experiments were performed with the AX2 strain of *dictyostelium discoideum* obtained from the Dictyostelium Stock Center (http://www.dictybase.org/)[28]. Cells were suspended in HL5 media with glucose (Formedium). Trypan blue exclusion assay was performed with 0.4% of trypan blue (MP Biomedicals) dissolved in HL5 media.

III. Results and Discussion

Most droplet experiments use surfactant to avoid droplet fusion due to collisions or contact while flowing in a microchannel. Surfactant inhibits droplet fusion by creating a steric barrier to fusion and by preventing drainage of oil between droplets.[29] Therefore, only directly after the droplet formation (within the first few milliseconds for the combination of fluorinated oil and surfactant[18]) is the interface of a droplet not fully enveloped with surfactant and will readily fuse with another droplet. However, if droplets are pinned in place, the surfactant coverage can be reduced by decreasing the concentration of surfactant in the flowing external oil. The desorption rate coefficients from the oil/water interface have not been characterized for most surfactants used in microfluidics, but in one example an alcohol surfactant in mineral oil it has been determined to be about 70 sec^{-1}.[30]

Flattened droplets can be positioned using a reduction in surface energy due to the expansion into a well in the channel. This method called "Rails and Anchors"[25] allows the quick and passive formation of an array of droplets of arbitrary shape using microfabricated wells. The chip used for experiments uses flow focusers to produce droplets (supplemental figure S1). These droplets flow into a wide channel containing an anchor array, depressions in the channel. Droplets expand into the anchors and are therefore anchored in place despite the external flow of oil. If the size of the anchor is large enough, multiple droplets can be positioned within the same anchor as shown in the first panel of Figure 1.

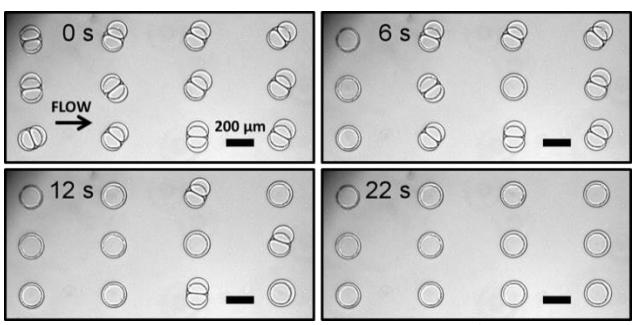

Figure 1. Image sequence showing a subsection of the anchor array containing pairs of identical aqueous droplets. Oil without surfactant is flowed into the array at a flow rate of 15 µL/min from left to right in the chip and triggers fusion of the pairs of droplets.

Once the array is prepared, droplet contact is limited to the droplet pairs in the anchors. To provoke fusion, oil containing no surfactant is flowed into the droplet array (Figure 1, Movie S1). By flowing oil without surfactant, the surfactant at the interface of the droplets is progressively removed. The removal of surfactant at the interface can be noticed by a subtle compression of the droplets towards each other as the interfacial tension of the individual droplets increases. The fusion occurs predominantly from left to right in the direction of the flow of oil. Surfactant can be transferred from upstream droplet pairs, delaying the downstream droplet fusion. All pairs within the 6 by 6 array fuse with a delay of 8 to 35 seconds after the introduction of oil without surfactant for the flow rate of 15 µL/min. This exceeds the expected time for the oil without surfactant to reach the array under these flow conditions (less than 3 seconds). The average delay time for droplet fusion after the introduction of oil without surfactant decreases at lower flow rates (< 7.5 µL/min) but is relatively constant at higher flow rates (supplemental figure S2). Previous work using fluorinated surfactants have shown that moving spherical droplets will fuse when interfacial surfactant coverage is below a critical value of 66%.[18] Variability in fusion time of 10-30 seconds is observed for pairs of droplets within the same column. This is likely an indication that static droplets may be metastable below a critical interfacial surfactant coverage leading to variation in fusion time. The method provides a simple platform to study the chemical and physical components leading to droplet fusion.

The fusion of the droplets can be spatially selective by controlling the flow of two oil inlets, one with and one without surfactant. This is demonstrated in Figure 2. The total oil flow in the channel is kept constant at 20 µL/min while the relative flow of oil without surfactant increases linearly as the flow rate of the oil with surfactant is decreased over a period of 180 seconds. The inlet of oil with surfactant is entering from the top position relative to the image, while the inlet of oil without surfactant is entering from the bottom (Figure 2a). In this co-flow geometry, array surfactant transfer between the streams will be limited to diffusion prior to the array. The rows of anchored droplets fuse sequentially from bottom to top with the increased flow of oil without surfactant (Figure 2b and MOVIE S2). Droplets within the same row fuse closely in time, typically within the same 10 second time window. On average, upstream droplet pairs fuse before downstream droplet pairs. As an alternative to time varying flow, a constant flow of the two oil inlets can be used to fuse only certain rows of droplets (data not shown). The relative flow of the two oils would thus

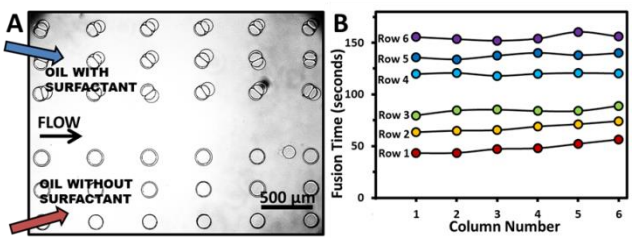

Figure 2. a) Image showing the selective fusion of rows of paired droplets by gradually increasing the flow of oil without surfactants while decreasing the flow of oil with surfactant over 180 seconds. Total flow of oil is kept constant at 20 µL/min. Oil with surfactant enters in the upper portion of the channel while oil without surfactant enters at the bottom portion. Image taken after 90 seconds when both oil flow rates are at 10 µL/min. Rows are numbered from bottom to top and columns from left to right. **b)** Fusion time for individual pairs of droplets in the array.

be calculated from simple geometric considerations to expose only certain rows of paired droplet to oil without surfactant.

This method can be used to initiate a chemical reaction containing two reactant droplets. Droplets of different content containing 0.27 M $FeCl_3$ or 0.8 M KSCN were placed in the same anchor using the method described in Figure 3 of Fradet et al., 2011.[26] Briefly, the droplets containing KSCN were flowed into the anchor array. At this stage, two KSCN droplets occupy each anchor. The external oil flow was then increased, displacing one of the two droplets occupying each anchor. Droplets containing $FeCl_3$ are then flowed into the anchor array to produce the array shown in Figure 3a, containing one droplet of KSCN and one of $FeCl_3$ in each well. At this stage, one of the oil inlets is replaced by oil without surfactant (Oil inlet 2 in supplemental Figure S1). Using two oil inlets and the same flow profile as described above for Figure 2, droplet fusion is triggered sequentially to produce different start times of the reaction. Droplet fusion produces the colored product, $FeSCN^{+2}$ (Figure 3, MOVIE S3). Mixing of the reactants and products is partially aided by the flow of external oil.[31]

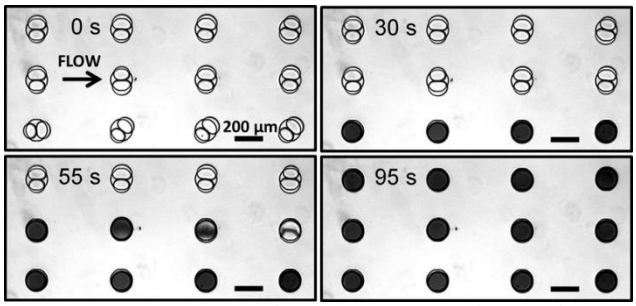

Figure 3. Image sequence of sequential droplet fusion of pairs of droplets containing 0.27 M $FeCl_3$ or 0.8 M KSCN by progressive increase in the flow of oil without surfactant in the channel while decreasing the flow of oil with surfactant. Fusion of droplets initiates the reaction to form the colored product, $FeSCN^{+2}$.

The method is not limited to fusion of pairs of droplets of equal size. Multiple droplets of different sizes can be fused together as shown in Figure 4 and MOVIE S4. Small droplets of KSCN fuse sequentially with the larger drop of $FeCl_3$. The three droplets of KSCN fuse with the larger droplet of $FeCl_3$ within 0.25 seconds of each other. This shows the suitability of the technique for pico-injection where a small volume of reagent can be added to larger droplet with little dilution of the larger droplet. The method also enables complicated operations involving multiple droplet reagents and the dosing of reagents as a function of number of fused droplets.

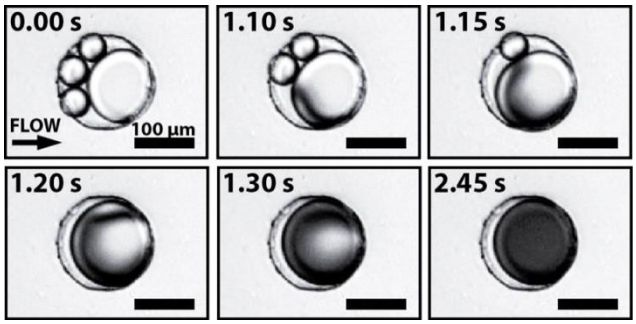

Figure 4. Image sequence of the fusion of large droplet containing 0.27 M $FeCl_3$ with three small droplets containing 0.8M KSCN by the introduction of external oil without surfactant.

We demonstrate the biocompatibility and suitability for cellular assays of the fusion technique by a trypan blue exclusion viability assay. The viability assay is based on the principal that live cells with intact cell membranes can exclude trypan blue while dead cells with damaged membranes are stained blue by the molecule.[32] Cells of the amoeba, *dictyostelium discoideum*, in media were encapsulated into droplets and anchored into the wells. Within the same anchor, a droplet containing media and trypan blue was also placed (Figure 5a). Droplet fusion of the pair of droplets was initiated by the flow of oil without surfactant (Figure 5b). After droplet fusion, oil with surfactant was flowed back into the droplet array to minimize direct cell exposure to the oil/droplet interface. There was no noticeable staining of the cells after droplet fusion either in the greyscale image (5b) or color image (5c). Also, after fusion, the cells maintained their ability to extend and retract pseudopods (inset of Figure 5b and MOVIE S5). Observation of live cells for over 30 minutes after droplet fusion show no cell staining and continued pseudopod extraction and retraction (data not shown).

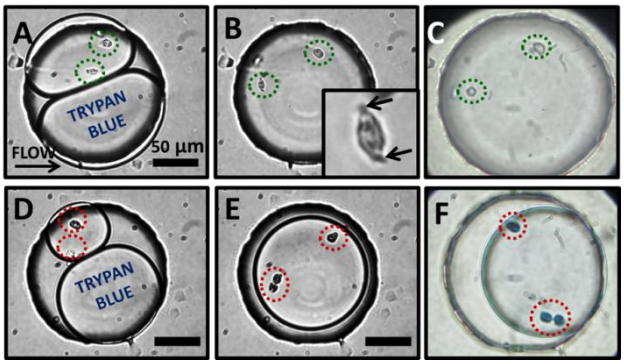

Figure 5. a) The image shows a pair of droplets in an anchor. Top droplet in the image contains live *dictyostelium discoideum* cells circled in green. The bottom droplet in image contains trypan blue. **b)** Droplets containing live cells are coalescence by flowing oil without surfactant. Inset shows close-up of cell with arrows indicating extended pseudopods. **c)** Color image of live cells after droplet fusion. Cells remain alive and show no blue staining of trypan blue. d) Pair of droplets with top droplet in image containing dead cells circled in red. **e)** Droplet containing dead cells after droplet coalescence. Cells darken due to absorption of trypan blue. f) Color image of dead cells after droplet fusion. Dead cells are stained blue by trypan blue.

A control experiment was performed under similar conditions with dead *dictyostelium discoideum* cells. Cells were first killed by exposure to 40% ethanol. The cells were then resuspending in media and encapsulated into droplets. Figure 5d, shows a droplet of the dead cells and trypan blue droplet within the same well. After droplet fusion, a quick darkening of the cells (within 10 seconds of the droplet fusion) is observed from staining with trypan blue (Figure 5e). The blue staining of the cells is more pronounced in the color image (Figure 5f). These experiments show that the technique can be used for a biological assay by fusing a reagent droplet to a droplet encapsulating cells. The punctual removal of surfactant from the droplet interface did not hinder the cells.

V. Conclusions

In summary, we demonstrate a robust method to spatially- and temporally- fuse droplets in an anchored array. The simplicity of implementation and reproducibility makes this method particular attractive. It does not require any active

elements and can be used in virtually any device containing anchored droplets. Unlike other fusion techniques, there are no inherent size limitations to the number or area of droplets that can be fused. These characteristics make the technique suitable for a wide range of applications including combinatorial synthesis, cell viability assays and compound screening.

The technique effectively decouples the formation, flow and positioning of droplets with the fusion of droplets. Since the pairs of droplets are stationary, it is an attractive platform for the study of surfactant absorption/desorption and the physics of droplet fusion. Although demonstrated here for the combination of perfluorinated oil containing aqueous droplets, it would be broadly applicable to two-phase systems where the surfactant can be dissolved in the external phase. We have found the method to be appropriate for all oil/surfactant combinations that we have tested that fit this criteria including FC40/PEG-PFPE,[33] mineral oil/Span80 and hexadecane/Span80.

The fusion of droplets in a planar array format is well suited for biological assays. The position of droplets in the array is predetermined, and therefore the method is adapted to measuring dynamics initiated by the mixing of reagents or reactants in a fused droplet. Since the creation and fusion of mixed droplet arrays is solely dependent on fluid flow, experiments can be automated using programmable syringe pumps. If required, surfactant can be returned to the droplet interface after fusion by flowing oil with surfactant into the array region, an important consideration if the oil/water interface may hinder droplet contents such as protein or cells. The external flow of oil can be used to mix the reagents after fusion, or higher flows can be used to eject droplets from the anchor for collection or to prepare for subsequent measurements.

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