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Bai, Yunpeng; Weibull, Emilie; Jönsson, Håkan; Andersson-Svahn, Helene

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Interfacing picoliter droplet microfluidics with addressable microliter compartments using fluorescence activated cell sorting

Yunpeng Bai, Emilie Weibull, Haakan N. Joensson, Helene Andersson-Svahn*

Division of Proteomics and Nanobiotechnology, Science for Life Laboratory, KTH-Royal Institute of Technology, Stockholm, Sweden

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Droplet microfluidic platforms have, while enabling high-throughput manipulations and the assaying of single cell scale compartments, been lacking interfacing to allow macro scale access to the output from droplet microfluidic operations. Here, we present a simple and high-throughput method for individually directing cell containing droplets to an addressable and macro scale accessible microwell slide for downstream analysis. Picoliter aqueous droplets containing low gelling point agarose and eGFP expressing Escherichia coli (E. coli) are created in a microfluidic device, solidified to agarose beads and transferred into an aqueous buffer. A fluorescence activated cell sorter (FACS) is used to sort agarose beads containing cells into microwells in which the growth and expansion of cell colonies is monitored. We demonstrate fast sorting and high accuracy positioning of sorted 15 μm gelled droplet agarose beads into microwells (14 × 48) on a 25 mm × 75 mm microscope slide format using a FACS with a 100 μm nozzle and an xy-stage. The interfacing method presented here enables the products of high-throughput or single cell scale droplet microfluidics assays to be output to a wide range of microtitter plate formats familiar to biological researchers lowering the barriers for utilization of these microfluidic platforms.

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1. Introduction

Recently, droplet microfluidics, a technology which enables the manipulation of biocompatible and monodisperse nano- or picoliter droplets as independent micro-reactors in an immiscible phase, has been drawing attention from molecular and cell biology [1–3]. The volume of the typical droplets is in the range of a few picoliters to several nanoliters, dramatically decreasing the reaction volume by a million times compared to the conventional microtiter plate well. Compartmentalization of biological components, e.g. single copies of DNA, RNA, proteins, or single cells, in picoliter droplets enables the coupling of two components without a physical link such as a genotype and extracellular phenotype, enzyme and dissolved product, or the DNA, RNA and protein from an in vitro transcription-translation process. Thus droplet microfluidics provides a high-throughput platform for directed evolution experiments [4–6], single-cell analysis [7–9], proteomics [10], biological reactions [11-13], digital polymerase chain reactions (digital PCR) [14–16], diagnostics [17–19] and drug screening [20–22]. The applications of droplet microfluidics rely on manipulations of droplets in various ways. Droplets can be generated [23,24], split [25], added new contents by passive diffusion [26,27], picoinjection [11,16,28,29] or electrocoalescence [30,31], and incubated for a short or long term in on-chip delay lines or off-chip reservoirs [32–34]. Molecules secreted by the encapsulated cells largely remain in the droplets during incubation and detected by fluorogenic assays, enabling the high-throughput dielectrophoretic sorting of droplets that contain cells producing molecules of interest. High-throughput droplet sorting is important because it can provide the capacity of selecting improved activity of enzymes or screening antibody-secreting cells with a reduction in reagent and time cost as compared to conventional assays [35–37].

In spite of the growing number of droplet sorting techniques, little attention has been paid to the reliable retrieval of the sorted cells or colonies of interest in a controlled manner for downstream processing. For example, fluorescence activated droplet sorting (FADS) can sort individual droplets at frequencies of up to 2 kHz with a 1000-fold increase in speed and a significant reduction in reagent cost [4]. However, the sorted droplets are usually broken to retrieve cells, yielding a cell population as the output of the sorting experiment [38]. In some cases, it is important to isolate cells of interest after sorting for further processing [39]. Although arrays for droplet trapping have been developed for time scale study of single cells [38,40,41], these do not allow for the retrieval of cells of interest to a macro scale accessible format.

Fluorescence activated cell sorting (FACS) is a method widely used in the biological community for high throughput cell sorting and biomarker detection, which can be used as a chip-to-world
output format [42]. Compared to FADS, state-of-the-art FACS provides at least an order of magnitude higher throughput, but it lacks the compartmentalization of biological components offered by droplet microfluidics [43]. Agarose beads are emerging as new carriers generated in microfluidics for the detection of rare pathogens [44], single cell reverse transcription PCR [45], and diagnostics [46]. The sol–gel transition of agarose depending on temperature allows it to be an ideal soft material for high-throughput FACS [47]. Eun et al. described a microfluidic technique for encapsulating and sorting *Escherichia coli* in agarose microparticles by FACS to measure the minimum inhibitory concentration of an antibiotic [48]. However, using flow cytometry to sort agarose beads containing single cells and isolate them in an addressable micro-compartment is more challenging, which needs careful control over factors influencing the precise patterning of sorted particles of interest.

Previously we have developed a microwell slide for high-throughput, long-term single cell/clone cultivation and analysis [49], and demonstrated its application for PCR amplification and genetic analysis [50]. Here, in this technical innovation, we present a high-throughput method to interface picoliter droplet microfluidics for single cell analysis with the microwell slide. By adding agarose to droplets and patterned positioning of the resulting agarose beads via FACS, we show that cells encapsulated in droplets could be retrieved and cultured in the microwell slide. We optimized the size of agarose beads and the width of the FACS nozzle, which are key factors in determining a controlled, precise sorting of agarose beads into microwells. Cells encapsulated in agarose beads developed into single colonies after 3 h incubation, providing the ability for subsequent clonal analysis. This protocol combines the advantages of droplet microfluidics and flow cytometry, and provides an interface between the emerging droplet microfluidics tools and the macroscale working format of microwell slides more familiar to the molecular biology community.

2. Materials and methods

2.1. Materials

FACS-7500 was obtained from 3M. Polydimethylsiloxane base (PDMS, Sylgard 184) and curing agent was purchased from Dow Corning. EA surfactant and surfactant destabilizer was obtained from Raindance Technologies. Agarose (Type IX) was obtained from Sigma–Aldrich. Phosphate buffered saline (PBS, pH 7.2) and TSB + Y bacterial growth medium were prepared in the lab. *E. coli* transformed with His6-ABP-SOP1 eGFP (abbreviated as eGFP E. coli) was cultured in the lab. Isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin were obtained from Alfa Aesar. Green fluorescent polymer microspheres, 15 μm (Duke Scientific Corp.) were used as a control. Microscope glass slides were obtained from VWR.

2.2. Fabrication of microfluidic chips

Microfluidic chips were manufactured in PDMS and glass according to standard soft lithographic techniques [51]. Briefly, masters were fabricated on 4 in. silicon wafers using SU8 photore sist. PDMS base was mixed with curing agent in a 10:1 ratio and poured onto the master template. After baking for 4 h at 65 °C, the PDMS was peeled off and holes for the inlets and outlets were punched with biopsy punches (Harris Uni-Cure) and cleaned using scotch tape. The PDMS slabs were subsequently cleaned in an ultrasonic bath (15 min) to remove debris, and followed by surface activation in oxygen plasma (Femto Scientific). The PDMS slabs and glass slides were bonded together and incubated at 65 °C for 2 h. Finally, a fluorophilic surface treatment was applied to each circuit by injecting Aquapel (PGP Industries), flushing with pure HFE-7500 oil and immediately purging with the filtered N₂.

2.3. Preparation of agarose beads containing eGFP E. coli

A small amount of eGFP E. coli was inoculated at 37 °C in a 10 ml TSB + Y medium containing 50 μg/ml kanamycin overnight before each experiment. A bacterial sample from the inoculated culture was re-cultured in fresh medium 3 h before the generation of droplets. The fresh culture was induced with 1 mM IPTG for the expression of eGFP from *E. coli*. 20 mg Agarose (Type IX) powder was dissolved in a 0.5 ml medium by incubating and shaking the sample at 65 °C/1000 rpm in an Eppendorf Thermomixer. A 0.5 ml eGFP E. coli culture was diluted to OD₉₀₀ = 1.0 and mixed 1:1 (OD₉₀₀ = 0.5) with the 0.5 ml agarose solution, which was vortexed and transferred in a 1.0 ml syringe (BD Plastipack) as the aqueous phase. HFE-7500 with 1.0% EA surfactant was injected by NEMESIS syringe pump (Cetoni GmbH) into the microfluidic circuit as the oil phase. The aqueous phase was injected in the device after air bubbles have been displaced, and a final flow configuration of 3000 μl/h and Faqueous 100 μl/h was used to generate 15 μm droplets. The microfluidic device was kept over 17 °C. The generated emulsion was collected in an eppendorf tube, and placed in an ice bath to solidify droplets to form agarose beads. A 10 μl surfactant destabilizer was added in the sample to break the emulsion and agarose beads were transferred to a 300 μl PBS buffer before FACS sorting.

2.4. Microwell slide

The single agarose beads were collected on a microwell slide. The slide has the same dimensions as a traditional microscopic slide (25 mm × 75 mm) and contains 672 (14 × 48) wells. It is made out of a 175 μm thin glass slide, which is anodically bonded to a microwell-etched silicon grid. The wells have tilted walls and can hold a volume of 500 nl each. A thin semi-permeable membrane made out of PDMS was used to seal the wells after FACS sorting.

2.5. FACS setup and operation

The FACS (Moflo Astrios, Beckman Coulter) utilizing the software IntelliSort was used to sort single polymer microspheres and agarose beads with and without *E. coli* into individual microwells on the microwell slide. The FACS housed a 100 μm nozzle and a laser/photonmultiplier system with excitation at 488 nm and emission at 513/26 nm. The pressure was set to 25 psi and the temperature to 4 °C (sample holder and microwell slide holder). The software sorting accuracy was set to single events and the event rate was adjusted to around 300 events per second (EPS). The software sorting mode was set to plate sorting and a sorting device with 14 × 48 positions was designed to accommodate the microwell slide format.

2.6. Cell culture in microwells and micrograph processing

Before sorting, the microwell slides were pre-filled with TSB + Y medium. After sorting, the agarose beads were left to settle in the wells for 10 min in the FACS microwell slide holder before a 200 μm PDMS membrane was placed over the wells. The microwell slides containing agarose beads were incubated at 37 °C. The agarose beads were monitored using an inverted fluorescent microscope (Eclipse TS 100, Nikon) with 10 × and 40 × objectives. The micrographs were taken after 0, 2 and 5 h incubation with a Stingray F-145B ASG camera (Allied Vision Technologies).
3. Results and discussion

3.1. Workflow

The workflow of interfacing droplet microfluidics with addressable microcarrier microwells is illustrated in Fig. 1. A two-phase flow-focusing microfluidic device was used to produce uniform monodisperse picoliter agarose droplets containing eGFP expressing E. coli cells in fluorinated oil. The collected droplets were solidified to gel beads by cooling the emulsion below the gelling point of agarose. A surfactant destabilizer (Raindance Technologies) and PBS buffer were added to the emulsion in order to break the droplets. The oil/water mixture was then gently vortexed and allowed to phase separate. The upper aqueous phase containing agarose beads was isolated and beads were sorted for cell content by FACS into the microwell slide. After FACS, beads containing cells were isolated in the microwells and further culture and analysis were performed with the moveable and addressable microwell slide.

3.2. Generation of microbeads containing single cells

The average number of cells per droplet could be adjusted by diluting the cell solution with PBS prior to injection of cell sample. To generate droplets containing on average 1.7 cells per droplet, the cell solution was diluted to achieve an OD$_{600}$ of 0.5. With this method, millions of agarose droplets can be generated at the frequency of 3000 droplets per second. E. coli cells can be encapsulated into droplets at the flow-focusing geometry and remain in the agarose beads following gelation (Fig. 2). It should be noted that viscosity is a key factor that influences the size distribution of droplets during the emulsion generation. The high viscosity of the agarose containing aqueous phase can lead to the formation of satellite droplets (Fig. S1A). The viscosity is influenced by the concentration of agarose and temperature of the liquid agarose. The gelling points of agarose IX at different concentrations (1–5%) were measured showing that the gelling point (°C) increased with the concentration of agarose (Fig. S1B). An acceptable size distribution (15±3 μm) was accomplished when limiting the agarose concentration to 2% (wt%) while keeping the temperature of the microfluidic device above the gelling temperature (17°C). The flow rates of the oil phase and aqueous phase were controlled at a 10:1 ratio, which also helped reducing the influence of flow fluctuation caused by viscosity.

3.3. Sorting and delivery of cell containing microbeads in microwells by FACS

Polymer microspheres as well as agarose beads were sorted onto glass slides in order to optimize parameters for bead sorting. This allows us to calculate the ratio of FACS droplets that contain microspheres or agarose beads in the whole population of sorted droplets. First, the influence of the bead diameter on pattern accuracy was examined by FACS sorting 10, 15 and 25 μm fluorescent microspheres on to glass slides. The sorted microspheres were arrayed in a pattern, which was visible after the solvent had evaporated. The pattern accuracy was considered acceptable for 10 and 15 μm but the stream was clearly affected when sorting 25 μm microspheres using a 100 μm nozzle (Fig. S2). Hence, the 15 μm green fluorescent polymer microspheres met all our requirements and were therefore selected and used as a control, validating the FACS alignment, when sorting agarose beads. It was found that 96.4% of the spots on the glass slide contain polymer microspheres (Fig. 3A). After the setup-validating step ensuring high pattern accuracy of the microspheres, picoliter agarose beads with a diameter of 15 μm were sorted. The statistical result was similar: 95% of the spots held a single agarose bead, 0.5% had two beads and 0.2% had multiple beads and only 4.3% of the spots had no beads at all (Fig. 3B). The deviation of beads in the individual spots can be caused by instrumental errors or alignment fluctuation, which affects the sorting stream. Each spot matched the position of one well in the microwell slide (Fig. 3C). In a zoom-in of the glass slide, a grid, illustrating the well borders (top view), was placed over the wells allowing us to calculate the probability that the beads were hitting the wells (Fig. 3D). A dried in droplet or spot will have a larger area then before glass impact. It was considered to be hitting the well if more than 80%
of the spot was within the grid. The accuracy was calculated to 97.9%. In Fig. 3E a single dried spot containing an agarose bead illustrates that agarose beads were intact after sorting. It is important to keep the agarose beads at 4 °C to maintain good sorting accuracy. Increasing temperature may soften the agarose beads, which could lead to charge and sorting stream instability.

In addition to the size of the agarose beads, the quality and operational parameters of the FACS and the dimension of the microwell influence sorting accuracy. The microwell slide has an array pattern of 14 × 48 microwells with the side length of 1360 μm at the top, 650 μm at the bottom and a 1500 μm center-to-center distance (Fig. S3). The microwell slide was in part designed to fit the mechanical limitations of a particular FACS instrument (FACSVantage, Becton Dickinson). The center-to-center distance can be decreased slightly for newer instruments but is in the end instrument-dependent. The microwell slide used here is in the format of a microscope slide and the FACS stage is that of a 96-well plate, hence a larger array can be used, such as the one used by Lindström et al. holding 3243 wells [49]. The EPS was controlled at 300 EPSs, which can easily distribute beads to all the wells in 2–5 min. Because most commercial flow cytometers can be operated at frequencies of tens of kilohertz, the sorting rate is usually limited by the density of targets in the samples, which in turn depends on the application. When screening for rare samples where it is important to retrieve as many beads as possible, a more dilute sample and a lower EPS is required to ensure the highest efficiency possible.

3.4. Cell culture in microwells after sorting

The agarose beads containing E. coli expressing eGFP were sorted into microwells in 2 min and its growth was monitored over time (Fig. 4). After FACS sorting, a PDMS membrane was placed on top of the slide and the slide was placed in an incubator. At the start of the incubation, single cells were trapped in the agarose beads without free cells in the microwell (Fig. 4A). After 2 h, colony formation was found around the agarose bead in the microwell (Fig. 4B). The single colony continuously expanded and was monitored after 5 h (Fig. 4C). The formation of an E. coli colony outside agarose beads was observed in 13.6% of the microwells. The same experiment was performed in a 96-well plate where 20.8% of the wells had E. coli colonies outside beads. The E. coli growing out of the agarose beads show signs of being under stress, e.g. due to high cell density, by forming filaments [52]. Here, because E. coli is encapsulated in agarose beads and no free cells were found in the microwell at the beginning of the incubation, the filamentation and expansion of colonies can be due to the release of cells from agarose beads. There are agarose beads, which did not release the E. coli cells during the incubation. However, E. coli cells can still grow to form microcolonies within these agarose beads, which is shown in Fig. 4D and has been reported in literature [48]. Our work shows that single E. coli encapsulated in agarose beads can be sorted and isolated precisely in microwells, and its colony expansion can be followed in the microwell. To analyze the contents of the wells, one possibility would be to extract the desired E. coli colonies for further expansion off-chip. Alternatively PCR amplification could be performed directly in the wells to analyze the genetic contents of each of the wells. Moreover, the microwell slide has 672 wells with a relatively small volume (500 nL vs. 100 μL in 96 plate), which significantly improves the image resolution, due to the decreased path length and the thin glass slide (175 μm).

The chip-to-world concept, provided by this technique, can be used for any assay where cells need to be retrieved after running a
droplet microfluidics assay e.g. screening populations of single cells for rare cell types. An application that can greatly benefit from this technique is directed evolution of secreted enzymes [53]. An advantage with agarose beads is that the cells and their secreted enzymes are kept isolated in the beads during sorting, creating a physical link between genotype and extracellular phenotype, which is not feasible using conventional FACS.

4. Conclusions

We have demonstrated the potential of using FACS to interface microfluidic droplets with a microwell slide by retrieving selected cells encapsulated in a large droplet population to the slide. By carefully choosing the size of agarose beads and the nozzle equipped in the flow cytometer, a well-defined 2D spot pattern can be achieved, which can match the microwells in the slide. The sorted cells were found to grow out of the agarose beads and to expand into colonies in the microwells. Cells trapped in agarose beads can also grow to microcolonies. Using FACS to interface between droplet microfluidics and accessible μl-compartment will enable cells to be further analyzed and screened, for e.g. gene expression, in conventional instruments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2013.12.089.

References


Biographies

Yunpeng Bai received his M.Sc. degree in nanotechnology in 2009 at the Faculty of Engineering LTH at Lund University, Sweden. She is currently pursuing her Ph.D. degree, working with miniaturized microwell-based cell assays at the division of Proteomics and Nanobiotechnology at the Royal Institute of Technology in Sweden, focusing on development and applications of droplet microfluidics for high-throughput biological screenings.

Emilie Weibull received her M.Sc. degree in nanotechnology in 2009 at the Faculty of Engineering LTH at Lund University, Sweden. She is currently pursuing her Ph.D. degree, working with miniaturized microwell-based cell assays at the division of Proteomics and Nanobiotechnology at the Royal Institute of Technology in Stockholm, Sweden. Since 2010 she has in parallel been working at the start-up company Picovitro AB as an R&D Scientist (part time).

Haakon N. Joensson studied engineering physics at Lund University, Sweden, and the University of Illinois at Urbana-Champaign. He completed his Ph.D. in biotechnology from KTH, focusing on biological applications of high-throughput droplet microfluidics. In 2006–2007 he was a visiting scientist at RainDance Technologies, Boston, MA, USA. He is currently a scientist in the division of Proteomics and Nanobiotechnology and group leader for Droplet Microfluidics in the Novo Nordisk Foundation Center for Biosustainability, Royal Institute of Technology, Sweden.

Helene Andersson-Svahn is professor in Nanobiotechnology at the Royal Institute of Technology in Sweden. She has a M.Sc. in molecular biotechnology at Uppsala University, and received her Ph.D. degree in electrical engineering at the Royal Institute of Technology in 2001. In 2011 she was elected as chairman for the Young Academy of Sweden and she is also a member of The Royal Swedish Academy of Sciences and the Editorial board of the Lab on a Chip journal. Dr Andersson-Svahn is also scientific director of the new national lab Science for Life Laboratories and member of the board of the Swedish Foundation of Strategic Research. Her main research focus is micro- and nano-fluidic devices for biotech and medical applications.