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High-throughput screening of microchip-synthesized genes in programmable double-emulsion droplets

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Abstract

The rapid advances of synthetic biology and biotechnology increasingly demand high-throughput screening technology, such as screening of the functionalities of synthetic genes for optimization of protein expression. Compartmentalization of single cells in water-in-oil (W/O) emulsion droplets allows screening of a vast number of individualized assays, and recent advances in automated microfluidic devices further help realize the potential of droplet technology for high-throughput screening. However these single-emulsion droplets are incompatible with aqueous phase analysis and the inner droplet environment cannot easily communicate with the external phase. We present a high-throughput, miniaturized screening platform for microchip-synthesized genes using microfluidics-generated water-in-oil-in-water (W/O/W) double emulsion (DE) droplets that overcome these limitations. Synthetic gene variants of fluorescent proteins are synthesized with a custom-built microarray inkjet synthesizer, which are then screened for expression in *Escherichia coli* (*E. coli*) cells. Bacteria bearing individual fluorescent gene variants are encapsulated as single cells into DE droplets where fluorescent signals are enhanced by 100 times within 24 h of proliferation. Enrichment of functionally-correct genes by employing error correction method is demonstrated by screening DE droplets containing fluorescent clones of bacteria with the red fluorescent protein (*rfp*) gene. Permeation of isopropyl β -D-1-thiogalactopyranoside (IPTG) through the thin oil layer from the external solution initiates target gene expression. The induced expression of the synthetic fluorescent proteins from at least ~100 bacteria per droplet generates detectable fluorescent signals to enable fluorescence-activated cell sorting (FACS) of the intact droplets. This technology obviates time- and labor-intensive cell culture typically required in conventional bulk experiment.

TOC image

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Introduction

For over sixty years, the tools to synthesize, manipulate and analyze DNA have grown to encompass new extremes in both scale and precision. Driven by miniaturization technologies, our ability to read and write DNA has improved dramatically over the last decade. High-throughput sequencing technologies such as next-generation sequencing (NGS) have enabled the analysis of many genetic and biochemical processes at unprecedented scale and low cost.^{1–2} Emerging technologies on parallelized and miniaturized synthetic techniques to construct DNA sequences have led to significant improvement in our ability to understand and engineer biology. Following the early demonstrations of gene assembly using microarray-derived oligo pools,^{3–5} exciting developments have been made to improve the quality and efficiency of microarray-based oligo synthesis and gene assembly.^{6–10} We have previously developed a microarray inkjet synthesizer to synthesize pools of thousands of codon-usage variants for protein expression optimization at low cost and high throughput.⁶

Despite tremendous improvement in both DNA synthesis and sequencing, throughput and scale of current experimental workflow in real practice remain limiting. This is due to a bottleneck existing in the screening step where the downstream cost of testing individual biological constructs for function is often far more expensive than the cost of synthesis. In addition, since the engineering information is encoded in the genotype while the selection depends on the phenotype, this requires the genotype and phenotype to be linked in any screening strategy.

Cells are the most commonly used as vehicles to bridge this genotype-phenotype linkage. Synthetic constructs are usually introduced in recombinant forms and individual cells are picked from culture plates and analyzed either by hand or by robotic-pickers.^{6,11} The process is labor-intensive and time-consuming. While eukaryotic cells screened by FACS can potentially access libraries larger than 10^8 ,¹² flow cytometric analysis of bacteria is still infrequent due to their small size and the range of screening is limited to survival or cell-bound products.^{6, 13–14} Automation through the use of colony pickers increases the throughput to 10^4 clones/day but cannot handle larger libraries unless multiple machines are used in parallel.

In vitro compartmentalization (IVC) of individual element of the library in discrete, miniaturized W/O emulsions offers an attractive alternative for coupling genotype and phenotype and simultaneously improves cost-effectiveness and screening sensitivity by

reducing sample consumption and enhancing signal response.^{15–17} Bulk emulsions suffer from polydispersity and the lack of control of reaction volume, timing and generality. These problems can be overcome by using droplet-based microfluidic system which allows the production of homogeneous and uniform droplets.^{18–24} However the W/O emulsions are incompatible with sustained cell culture or any aqueous phase-based analysis (e.g. flow cytometry), as the immiscible oil phase is prone to evaporation and insoluble for polar nutrients typically.²⁵

The problem can be circumvented by entrapping W/O droplets in another aqueous phase, forming W/O/W DE. The external aqueous phase minimizes desiccation and enables droplet sorting via FACS. The middle oil shell functions as a selective barrier to regulate molecule transport, allowing supply of nutrients or input of small inducer molecules, such as anhydrotetracycline (aTC).^{24, 26} Consequently, the droplets are considered “programmable” in the sense that the inner microenvironment can be modified by diffusion of molecules into the core to elicit a change in cell behavior. In this study, we present the development of a high-throughput screening platform for synthetic genes using single bacterium encapsulated in microfluidics-generated DE droplets (Scheme 1). The inducer molecule IPTG will be introduced in the external phase to trigger gene expression upon its diffusion into the droplet and the effect of its diffusion into the droplet on single bacterium proliferation/signal amplification will be studied for the first time.

Fluorescent protein genes were synthesized with a custom-built inkjet synthesizer, starting with oligos synthesis before undergoing isothermal oligonucleotide amplification and parallel gene assembly, and inserted into *E. coli* cells for screening. We then encapsulated individual bacterium bearing individual gene variants into DE droplets. The intrinsic limitation of chemical gene synthesis by stepwise addition of nucleotide monomer results in errors such as deletion and substitution.²⁷ This is especially true for gene synthesis using microarray-produced oligos, where error rates tend to be higher.³ The DE platform allows us to identify bacterial clones with functionally-correct *rfp* sequences. In addition, the diffusion of IPTG into the droplet can trigger the pET vector, under the control of a T7 promoter and a lac operator, regulating the expression of the synthetic *gfp* (green fluorescent protein) gene inserted into the bacteria. Since the pET expression system is one of the most widely used systems for the cloning and expression of synthetic and recombinant proteins in *E. coli*, the induction of pET expression by an external triggering mechanism offers an effective protocol to induce various target protein expression for screening within the droplets. The capacity of DE system (bacterial proliferation and activation of gene by chemical diffusion) combined with high-throughput sorting by FACS provide the basis for screening complex gene libraries for a broad range of functionality and activity.

Experimental

Microfluidics Device Fabrication

Microfluidic devices were fabricated by conventional soft lithography techniques. Patterned silicon mold of 50 μm in height and channel width was prepared from SU-8 2150 (MicroChem, Newton, MA) according to the published protocols.^{26, 28} PDMS prepolymer and curing agent (Sylgard 184 Silicon Elastomer Kit, Dow Corning, Midland, MI) were

mixed at 10:1.05 mass ratio before poured on top of the silicon mold to cure at 80°C for 1 hour. This ratio was chosen based on our experience that the PDMS produced would have optimal stiffness for easy handling. A cover slide was bonded with the device after holes at inlets and outlets were punched and oxygen plasma treatment for 40 s at 20 W (Plasma Asher, Quorum Technologies, West Sussex, and RH). To create a hydrophilic surface along the channels, the devices were coated following a two-step sol-gel coating procedure.²⁶

DE Droplets Generation and Characterization

DE droplets were generated as described in the main text. Bacterial culture medium was used as the inner aqueous phase. The oil phase used was HFE-7500 (Miller-Stephenson Chemical Co. Inc., Danbury, CT) supplemented with Pico-Surf TM 1 surfactant (1%) (Dolomite Microfluidics, Charlestown, MA). The outer aqueous phase comprised culture medium supplemented with Pluronic F-127 (2.5 wt. %). The flow rates of inner aqueous phase (1 µL/min), middle oil phase of HFE7500 (3 M, St. Paul, MN) (2–7 µL/min) and outer aqueous phase (30–70 µL/min) were controlled by a Harvard Apparatus PHD 2000 Syringe Pump.

Gene Synthesis and Error Correction

The construction and error correction of synthetic fluorescent protein genes was detailed in our previous publication.²⁹ Briefly, after polymerase chain reaction (PCR) amplification of the on-chip assembled gene was completed, the gene products were purified by agarose gel electrophoresis and extracted. They were melted by heating at 95°C for 10 min, cooled to 85°C at 2°C/s and held for 1 min. They were then cooled down to 25°C at a rate of 0.3°C, holding for 1 min at every 10°C interval. For ECR using a 20 min Surveyor cleavage incubation, 4 µl (200 ng) of the re-annealed gene product was mixed with 0.5 µl of Surveyor nuclease and 0.5 µl enhancer and incubated at 42°C for 20 min. Two µl of the reaction mixture was used for subsequent overlap extension-PCR (OE-PCR) using the same reaction conditions as the PCR described above. The products were melted again for second iteration following the same procedure.

Cloning of Synthetic Genes

Synthetic gene products (*gfp*, cyan fluorescent protein (*cfp*), and *rfp*) were cloned into pAcGFP1 vector using circular polymerase extension method (CPEC).^{30–31}

To prepare the pET expression plasmid, synthetic *gfp* gene was inserted into pET-28a(+) (Novagen Inc., Madison, WI, USA) vector containing a *lacI* gene, a T7 promoter, a lac operator, an ampicillin resistance gene. Cloning product was transformed into BL21(DE3) chemically competent *E. coli* cells (Invitrogen) according to the manufacturer's instruction. Cells were grown on agar plate with 50 µg/ml kanamycin for approximately 16 h.

Encapsulation of Bacteria Cells

Single colonies containing *rfp*, *gfp* and *cfp* genes were selected from the previously prepared LB agar plate and transferred into 200 mL M9 broth or diluted LB broth (1:1 LB medium: PBS). The inoculated culture was then thoroughly mixed and diluted to reach the desired cell density (e.g. $\sim 10^6$ cells/mL to obtain ~ 0.06 cell per 60 pL droplet) before encapsulated

into DE droplets. The flow rates of three phases (inner aqueous: middle oil: outer aqueous) were set at 1:2:30 $\mu\text{L}/\text{min}$ respectively. The droplets were collected and transferred to 96-well plates containing M9 or diluted LB media for subsequent culture and analysis. >200 droplets were imaged for determining the number of cell-containing droplets.

IPTG Induction of Gene Expression

Single colonies containing pET-gfp plasmid were picked from the LB agar plate and transferred into 200 mL M9 broth or diluted LB broth (1:1 LB medium: PBS) containing 50 $\mu\text{g}/\text{mL}$ kanamycin. Mixture of LB and PBS was used to reduce the effect of autofluorescence of LB. The inoculated culture was then thoroughly mixed and diluted to reach the desired cell density, then encapsulated into DE droplets. The droplets were collected and transferred to 96-well plates containing 200 mL M9 or diluted LB media. IPTG was added immediately to the outer aqueous media to obtain a series of concentrations: 0 mM, 0.5 mM, 2 mM, 5 mM, 10 mM, 20 mM and 40 mM. Droplet fluorescent intensity was then analyzed at various time points with fluorescence microscopy.

Fluorescence Microscopy

Droplets containing fluorescence-bearing bacteria were suspended in a 96-well plate and examined by Nikon Eclipse TE2000-U fluorescence inverted microscope at various time points following encapsulation. Fluorescence intensity was analyzed by Image J. To compare fluorescence signals emitted when different concentrations of IPTG were applied, all data points were normalized against the fluorescence signal measured at the first time point after 2 mM was applied externally at $t=0$.

Flow Cytometry Analysis

E. coli expressing GFP constitutively or under the control of pET expression vector were diluted in PBS buffer and encapsulated in the DE droplets. Equal number of droplets was then suspended in PBS solution or M9 growth medium for comparison of cell growth over time by flow cytometry (FACSCanto II, BD Biosciences, Franklin Lakes, NJ). The FSC/SSC was gated with empty droplets and free bacteria (negative control) to specifically determine the population of droplets with bacteria encapsulated. More than 10,000 droplets were measured each time to ensure reliable statistics. FlowJo (v.7.6, Tree Star, Ashland) was used to analyze the data.

Results and discussion

***In Situ* Gene Synthesis from a Custom Array-Based Oligo Synthesizer**

We synthesized DNA microarrays using a custom-built inkjet DNA synthesizer for on-chip oligo amplification and gene assembly. A microchip made of thermoplastic cyclic olefin copolymer (COC) was functionalized with hydrophilic SiO_2 thin film arrays (Figure S1), creating physically isolated picoliter-sized reactors that constrained liquid via differential wettability (Figure 1A). These functionalized SiO_2 -COC microchips exhibited enhanced droplet confinement and reduced edge-effect during *in situ* DNA synthesis, producing high-quality oligonucleotide arrays (Figure 1B & Figure S2). Oligos were synthesized according to standard phosphoramidite chemistry before they were cleaved and assembled within

isolated hybridization chambers using combined nicking strand displacement and polymerase cycle assembly (nSDA-PCA) reaction (Figure S3). The formation of gene products was confirmed by gel electrophoresis of the PCR reaction products (Figure 1C). We chose *gfp*, *rfp*, *cfp* as test genes for convenient screening of functionally distinguishable genes, which served as an over-simplified model-library to assess the capability of the screening platform. The *gfp*, *rfp* and *cfp* constructs were inserted into a modified pAcGFP1 expression vector (by introducing a stop codon within the multiple cloning site region so that the preinserted GFP sequence would not be expressed) using the CPEC cloning method.³¹ The recombinant products were then transformed into bacteria, and fluorescent colonies were selected and sequence-verified for each gene construct (Table S1).

Single Cell Encapsulation and Amplification in DE Droplets

The high-throughput generation (>200 Hz) of picoliter-sized DE droplets (~60 pL & ~50µm in diameter) was carried out in two polydimethylsiloxane (PDMS) flow-focusing devices connected serially (Figure 2A): the first device produced W/O emulsions, followed by a second device to supplement an outer aqueous phase to form W/O/W emulsions.³² The distribution of cells in a droplet follows Poisson distribution where the probability of finding a droplet with k cells is defined by the equation eq 1:

$$f(k) = \frac{\lambda^k e^{-\lambda}}{k!} \quad (1)$$

Where λ is the average cell number per droplet and k is the specific cell number in the droplet.

To demonstrate single cell encapsulation and the subsequent population enrichment of bacteria in DE droplets, *E.coli* cells expressing synthetic *gfp*, *rfp* and *cfp* fluorescent proteins were loaded into 50 µm-diameter DE droplets (~60 pL) at a density of $\sim 1 \times 10^6$ bacteria/mL, which produced an average of <0.06 bacteria per droplet.

After 24 h of incubation, the proliferation of bacteria generated distinctively red, green and cyan fluorescent signals in individual droplet (Figure 2B). No co-localization of different types of bacteria was observed, indicative of the successful separation of bacteria at single cell level. Analysis over a large pool of droplets indicated a bacterial distribution matching the Poisson distribution of 0.01 bacteria per droplet on average (Figure S4). In this case, the probability of having two or more bacteria per droplet was negligible; suggesting that such a loading cell density could effectively separate all bacteria into single cell per droplet.

When DE droplet was used to encapsulate single bacterium in growth medium (1:1 LB/PBS), fluorescent intensity from the entire droplet was enriched by approximately 100 times over a period of 24 h due to cell proliferation (Figure 2C & D). This observation confirms that culturing bacterium inside the droplets allows both bacteria separation as well as signal amplification from single bacterium.

Screening of Functional Correctness for Microarray-Synthesized and Error-Corrected Genes

To determine how the screening system compared with the conventional culture plate method in terms of productivity and reliability, we applied the platform to estimate the error frequency of microarray-synthesized genes without prior selection and sequence verification. We chose red fluorescent protein (*rfp*) as a test gene for convenient screening of functionally-correct genes, which served as a good approximation of sequence-correct genes. In our previous publication, we reported that Surveyor nuclease, a commercialized form of the CEL endonuclease, was effective in removing errors during chip-based gene synthesis.²⁹ Freshly synthesized *rfp* gene constructs before and after error-correction following our previously reported protocol were transformed into *E. coli* cells which were then encapsulated into DE droplet as single cells. The resultant droplets were incubated in growth medium overnight to allow cell proliferation to saturate the inner droplet. Using droplet counting, it was found that 52.8% of bacteria-positive droplets formed from uncorrected product contained cells that fluoresced brightly. The percentage of fluorescent cells approximated from droplet system was consistent with that calculated using colony counting on agar plates (Figure 3A & B). Employing error correction increased the percentage of brightly fluorescent RFP droplets to 90.6%, which is also consistent with previous results conducted in conventional agar plate condition.²⁹

Tunable Induction of Synthetic Gene Expression through IPTG Diffusion into Droplet

To investigate the potential application of microfluidics-generated DE droplets as a perturbable microenvironment to screen and characterize synthetic gene expression, we first studied the feasibility of inducible gene expression in DE droplet through the diffusion of IPTG from the external aqueous phase. In bulk environment, the expression of GFP in these cells could be activated within a few hours by the application of IPTG. We encapsulated ~30 BL21(DE3) *E. coli* cells carrying a microarray-synthesized *gfp* controlled by both the T7 promoter and lac operator in a pET vector in each droplet. Upon addition of 5 mM IPTG in the external aqueous phase, GFP expression became detectable after 4 h, but not in control droplets without IPTG (Figure 4A). Relative GFP intensity observed per droplet increased over time, which was both contributed by increased GFP expression per cell (indicated by brighter bacteria) and bacterial growth. The appearance of GFP signal in the bacteria suggested effective transport of IPTG molecule across the oil shell.

To further study the induction of GFP expression by IPTG diffusion, we compared the relative fluorescent intensity (normalized against the intensity of bulk culture at 12 h) change over time in droplets with that in bulk culture environment. Gene expression was delayed by about 4 h in droplets compared with the conventional culture environment (Figure 4B). To understand the impact of IPTG concentration on gene expression induction, bacteria-containing droplets were suspended in medium with a gradient of IPTG concentration. A concentration-dependent activation of GFP expression was observed (Figure 4C). However, even at a low IPTG concentration of 0.5 mM, the system was still able to achieve ~80 % of the maximum gene expression level obtained in higher concentration conditions within 8 h of induction. This observation confirms the relatively robust and efficient transport of IPTG across the droplet shells.

We next investigated the effect of IPTG diffusion on gene expression in droplets encapsulated with single cell, and how this process would interfere with bacterial proliferation. Bacteria encoding synthetic GFP in pET vector were suspended in minimal medium (M9) and encapsulated into DE droplets to yield no more than 1 bacterium cell per droplet. The M9 medium was chosen to optimize imaging conditions due to its low autofluorescence property. The resultant droplets were cultured in medium containing a broad range of IPTG concentration from 0.5 to 40 mM.

As shown in Figure 4D & E, IPTG concentration above 5 mM was inhibitive to the growth of bacteria. At 5mM, GFP intensity inside droplets fluctuated over time, suggesting inconsistent effect of inhibition of cell growth and gene activation across the pool of droplets. Lower IPTG concentrations, between 0.5 to 2 mM, were able to activate gene expression under the control of T7 promoter and lac operator in pET vectors without interference with bacterial amplification. Noticeably upon onset, the collective GFP intensity was positively correlated with the supplied IPTG concentration (0.5 to 2 mM) with 2 mM IPTG providing the highest induced expression level. Cell populations at this point were relatively low and uniform across different conditions. The collective GFP intensity was contributed mainly by gene expression in individual cells. Yet this correlation gradually inverted itself as cells proliferated over time. At 12 h post-induction, droplets supplemented with 1 mM IPTG started to exhibit higher GFP intensity than that induced by 2 mM IPTG, whereas droplets activated by 0.5 mM IPTG generated the highest collective GFP intensity among all concentrations tested at the end point. This was partly due to the relatively faster growth of bacteria post-induction than that observed when 2 mM to 40 mM IPTG were applied, generating a large cell population encoding green fluorescent protein.

We next characterized and compared the appearance and morphology of bacterial clusters for droplets in each IPTG concentration (Figure 4D). Besides generating the largest cell density and in turn exhibiting the highest collective GFP intensity, single bacterium induced by 0.5 mM IPTG grew into stable and uniform cell clusters that saturated the entire inner phase of the droplet. Small deviation of intensity was thus observed across individual droplets at the same condition. Nevertheless, as IPTG concentration increased, average cell density dropped significantly while variation across droplets escalated within each condition, as indicated by the error bar on Figure 4E.

To further investigate the optimal induction condition, we switched to a more nutritious medium i.e. LB/PBS (1:1) in an attempt to boost bacterial metabolism and reduce lag time by facilitating the initial growth of bacteria using LB/PBS. We encapsulated single bacterium into DE droplets with either M9 or LB/PBS (1:1) media and suspended the droplets in 0.5 or 2 mM IPTG culture. Fluorescent signal from bacteria cultured in LB/PBS broth increased exponentially after 4 h upon encapsulation, and was much higher at both 12 h and 24 h time points than when M9 media was used (Figure 4F & G). This enhanced signal was partly due to a larger cell population in each droplet. Particularly for 0.5 mM IPTG added to the outer core, intensity achieved at 12 h in LB/PBS broth outweighed that obtained from M9 broth at 24 h time point. Consistent with previous observation, lower IPTG concentration allowed single cell to proliferate into saturated cell density, resulting in uniform and consistent signal in each droplet. Cells induced by higher IPTG concentration,

albeit being brighter themselves, generated enhanced signal variation due to the presence of lower cell number and dispersed cell pattern in each droplet. These observations suggest that IPTG diffusion rate and bacterial growth curve could be synchronized to achieve the best signal amplification effect.

To further understand how IPTG would affect the growth of single bacterium in droplet, we introduced a moderate amount of IPTG to bacterial culture prior to encapsulation. Single cell that came into direct contact with IPTG, even at ultralow concentration of 0.2 mM, proliferated at a much lower rate than one that was activated by the IPTG slowly diffused into the core from the external environment (Figure 4H). This further confirms our previous note that IPTG was inhibitive to bacterial growth when they came into contact with the cell before they reached the lag phase. We also noticed that the effect was proportional to the concentration of IPTG in the environment.

Analysis of Gene Expression by Fluorescence-Activated High-Throughput Droplet Sorting

The high capacity microfluidics-based droplet technology requires automated, high-throughput screening system to process and sort large spectrum of activities. DE droplets are compatible with most flow cytometric analysis platforms. In addition to throughput, the ability to precisely discriminate among DE droplets based on their fluorescence and their uniform size are crucial for accurate screening.

To demonstrate stringent fluorescent-activated sorting of synthetic genes in DE droplets, we encapsulated a mixture of *E.coli* cells carrying synthetic GFP and RFP genes in equivalent amount into DE droplets as single cell. After overnight incubation, we suspended the droplets in PBS and analyzed them with a flow cytometry sorter. As shown in the intensity histograms, both GFP-positive and RFP-positive droplets revealed confined and distinctive peaks, representing strong and uniform signal intensities (Figure 5A). With proper channel compensation, droplets containing GFP and RFP expressing cells were sorted into different reservoirs.

Interesting to note is that unlike conventional mammalian cells, where the Forward Scatter (FSC) vs Side Scatter (SSC) value reveals the size and morphology information of the sample, DE droplets are transparent and thus do not generate similar scatter pattern when excited by the lasers. In contrast, the forward and side scatter information observed here was most likely generated from the spherical structure of the cell cluster confined by the inner droplet. The robust yet confined signal intensity measured during FACS indicated sufficient cell population in each droplet. This is consistent with our previous observation that efficient signal amplification through cell proliferation could be readily achieved from a single copy of bacteria/genotype with this system. The concentrated localization of data points from positive droplets on FSC vs SSC plot further confirms that the emulsion droplets were highly uniform in terms of size and internal composition.

To demonstrate the importance of signal amplification prior to FACS, we conducted a parallel analysis of multiple DE species present in the same sample. DE droplets encapsulated with four different densities of GFP-positive cells at 1, 10, 100, 1000 bacteria/droplet were created and analyzed in FACS. We observed that both signal intensity and

spatial resolution were enhanced with increased average number of cells present per droplet, as indicated by the decreased peak width yet increased peak height. Upon overlaying the intensity histogram for all four emulsions conditions, peaks corresponding to droplets containing 100 and 1000 bacteria/droplet were distinguishable from adjacent peaks (Figure 5B). To further validate the hypothesis, we mixed these four bacteria-loaded droplet samples at equal volumes and analyzed the joint sample in a flow cytometer. As expected, only droplets loaded with higher cell density could be accurately discriminated, while droplets with lower cell population generated faint and broad signals that could not be resolved from each other or from empty droplets.

These observations demonstrated the possibility of coupling FACS with microfluidic DE system to achieve the desired capacity, throughput and automation of synthetic gene screening. More importantly, these findings also highlight the significance of signal enrichment in stringent sorting during FACS, as sufficient cell population is essential to generate robust and uniform signals to guarantee detection sensitivity and resolution.

Discussion

In this study, we demonstrated the application of DE droplets to encapsulate and culture single bacterium carrying synthetic gene generated from high-throughput microarray gene synthesizer. Coupling high-throughput gene synthesis with DE droplet screening is especially advantageous when a known gene library such as a library of codon-usage variants needs to be screened for optimal protein expression.⁶ Efficient proliferation of single bacterium in DE droplets is critical in phenotype screening as sufficient number of target molecules per unit volume of sample is essential to all biological detections. Single bacterium is difficult to be visualized under optical microscope as it measures around one micron in diameter and moves rapidly at erratic trajectory. The signal intensity often falls below the detection threshold of many screening technologies. Furthermore, the rapid and stochastic motion of individual cells in aqueous environment causes them to periodically gather at the center or diffuse to the periphery of the droplet, creating signals that are temporally and spatially unstable. The lack of uniformity and stability of signals from a particular genotype would greatly hinder the detection resolution and sensitivity when phenotyping with high-throughput platforms like FACS. Therefore, efficient enrichment of bacterial population inside the droplet through self-replication significantly amplifies the selective signals and enables stringent screening of enhanced protein expression of synthetic gene. A saturated cell population confines the collective motion of the cells, allowing cell distribution to be spatially uniform and temporally static, which in turn increases both the resolution and sensitivity of the screening process.

The proposed DE system is a tunable microenvironment suitable for single cell analysis as it allows transport of inducer chemicals to activate gene expression and enables efficient signal amplification through robust cell proliferation. The yield of single cell encapsulation is currently low (~1% of droplets contained single cell while ~99% were empty). To increase the yield, the use of a high aspect-ratio channel or a curved continuous microchannel has been proposed to overcome the limitation of stochastic cell loading which may be adopted in future work.^{33–34} The delayed-onset of IPTG-triggered gene activation through molecular

diffusion can be fine-tuned to synchronize with the cell growth curve, facilitating the effective activation and amplification of synthetic genes from single bacterium. The fine-tuning process depends on the optimization of both the bacterial culture medium and IPTG concentration which resulted in the saturation of bacteria in droplets and consequently the creation of a stable, spherical and fluorescent signal originated from the cell cluster. At high IPTG concentration, cells were most likely overburdened with plasmid expression, which diverts cellular resources from making necessary proteins for proliferation and leads to a reduction in the growth rate. This observation is consistent with that reported in the literature.^{27, 35} The inhibition of cell proliferation due to early exposure of bacteria to IPTG again highlighted the importance of diffusion of IPTG from the external aqueous phase into the droplet core to induce gene expression. Analysis of droplets containing different number of bacteria in FACS verified our hypothesis that signal enhancement through bacterial proliferation increases the resolution and sensitivity of the screening process. Compared with conventional agar plate culture, our proposed technology holds potential to replace manual counting and picking bacterial colony with automation, thereby advancing the field of high-throughput synthetic gene screening.

Conclusions

In conclusion, this study demonstrated the development of a microfluidics-based platform that generated well-controlled monodispersed DE droplets for high-throughput synthetic gene screening. We demonstrated single bacterium encapsulation in DE droplets, enabling the screening of functionally-correct genes (which gave an approximation of error-free genes) generated from microarray gene synthesizer before and after the error correction process. The diffusion of IPTG into droplet core induced gene expression, in which 0.5 μ M IPTG applied in the external phase resulted in the creation of a stable, spherical fluorescent signal after 24 h. Fluorescent signals generated from at least 100 bacteria/droplet were distinguishable in FACS, indicative of the importance of signal amplification from single bacterium in DE droplets. The coupling of high-throughput gene synthesis and DE droplet screening system should open up opportunities to produce and screen large amounts of synthetic genes efficiently. This study on single-cell encapsulation and IPTG-triggered gene expression should pave the way for future research of screening complex gene libraries for a broad range of functionalities and activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Scanning electron microscopic image of microchip (scale bar = 500 μm). B) Fluorescein isothiocyanate solution constrained within microwell via differential wettability. C) Gel image showing microchip-synthesized green fluorescent protein (*gfp*), red fluorescent protein (*rfp*) and cyan fluorescent protein (*cfp*) genes.

Figure 2.

A) Schematic diagram of DE droplet generation and bright field images showing W/O and W/O/W droplets generated in 1st and 2nd chip respectively. B) Single cell encapsulation of bacteria carrying *gfp*, *rfp* & *cfp* genes showing no colocalization at 24 h post-encapsulation. C) Relative RFP intensity measured inside the droplets over time. (n = 10) D) Fluorescent microscope images showing the proliferation from a single RFP-expressing *E. coli* cell encapsulated in droplet.

Figure 3.

Characterization of fluorescent cell population transfected with synthetic RFP gene before or after error correction. A) Fluorescent microscope images showing increased percentage of fluorescent droplets after error correction. Circled droplets contain bacteria that are either fluorescent (pink) or non-fluorescent (yellow). B) Percentage of fluorescent clones was measured before and after error correction for RFP gene construct.

Figure 4.

A) Fluorescent microscope images of droplets containing bacteria in absence of IPTG (top panel) and in presence of 5 mM IPTG (bottom panel). (Scale bar: 100 μ m) B) Relative fluorescent intensities of bacteria with pET vector controlling a *gfp* gene in bulk and in droplets after addition of 5 mM IPTG over time. (n=9) Data points were normalized against the intensity of bulk culture at 12 h. C) Relative fluorescent intensities of bacteria with pET vector controlling a *gfp* gene 8 h after introduction of various concentration of IPTG. Data points were normalized against the intensity after 5 mM was applied. D) Fluorescent images

showing GFP expressing cell clusters per droplet at 8 h, 12 h and 24 h time point post-IPTG induction. Images were taken at 20× magnification. E) Average GFP intensity per droplet as a function of time with the external concentration of IPTG ranging from 0.5 mM to 40 mM. (n=9). F) Fluorescent images showing GFP expressing cell clusters per droplet at 12 h and 24 h time point after IPTG induction in minimal M9 vs growth LB/PBS media. Images were taken at 20× magnification. G) GFP intensity per droplet as a function of time upon IPTG induction in minimal M9 vs growth LB/PBS media. H) GFP intensity per droplet as a function of time with IPTG introduced prior to encapsulation at various concentrations. Fluorescent intensity curves are compared to the condition with IPTG introduced from external aqueous environment (red line). All data points for Figure 4E, G & H were normalized against the fluorescence signal measured at the first time point after 2 mM was applied externally at t=0.

Figure 5.

A) Separation of double emulsion droplets containing GFP and RFP expressing cells. Top: Intensity histogram of GFP and RFP channels. Bottom: Overlay of green and red channels showing RFP+-GFP+ populations. B) Flow cytometry analysis of double emulsion droplets loaded with 4 different numbers of GFP-positive cells per droplet. Top: Overlay of signal intensities obtained for each conditions respectively. Bottom: Analysis of droplet mixture containing all four species with 1, 10, 100 and 1000 bacteria/droplet.

Scheme 1.

Workflow of high-throughput screening of microchip-synthesized genes using double emulsion (DE) droplets. A) *In situ* gene synthesis is performed on microchip using inkjet gene synthesizer. Oligos are first printed in microwells, after which enzymatic reactions take place to first amplify and displace overlapping oligos from the original synthesized strand, and then assemble them via polymerase chain assembly to generate full length DNA. B) Synthetic genes are then cloned and transformed into *E. coli*. C) Single bacterium is encapsulated into DE droplets before proliferation for signal enhancement. D) High-throughput screening is achieved with FACS.