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Biosensor-enabled droplet microfluidic system for the rapid screening of 3-dehydroshikimic acid produced in *Escherichia coli*

Ran Tu[1,2] · Liangpo Li[1] · Huiling Yuan[1] · Ronglin He[1,3] · Qinhong Wang[1,2]

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Abstract

Genetically encoded biosensors are powerful tools used to screen metabolite-producing microbial strains. Traditionally, biosensor-based screening approaches also use fluorescence-activated cell sorting (FACS). However, these approaches are limited by the measurement of intracellular fluorescence signals in single cells, rather than the signals associated with populations comprising multiple cells. This characteristic reduces the accuracy of screening because of the variability in signal levels among individual cells. To overcome this limitation, we introduced an approach that combined biosensors with droplet microfluidics (i.e., fluorescence-activated droplet sorting, FADS) to detect labeled cells at a multi-copy level and in an independent droplet microenvironment. We used our previously reported genetically encoded biosensor, 3-dehydroshikimic acid (3-DHS), as a model with which to establish the biosensor-based FADS screening method. We then characterized and compared the effects of the sorting method on the biosensor-based screening system by subjecting the same mutant library to FACS and FADS. Notably, our developed biosensor-enabled, droplet microfluidics-based FADS screening system yielded an improved positive mutant enrichment rate and increased productivity by the best mutant, compared with the single-cell FACS system. In conclusion, the combination of a biosensor and droplet microfluidics yielded a more efficient screening method that could be applied to the biosensor-based high-throughput screening of other metabolites.

Keywords Droplet microfluidics · High-throughput screening · Metabolite · Fluorescence signal · Strain engineering

Introduction

In recent years, major advances in metabolic engineering and synthetic biology have led to the construction of increasingly efficient microbial cell factories for the production of high value-added metabolites, such as amino acids, proteins, organic acids and aromatic compounds [22]. However, many of these microbial cell factories could not meet the commercial requirements for the synthesis of these metabolites, thus necessitating strain engineering or evolution to improve the production capacity. Therefore, the development of selection strategies and the ability to screen highly performing variants from massive strain cultures is critical [15].

Fluorescence-activated cell sorting (FACS) has been successfully applied to high-throughput screening (HTS) technology in the context of strain engineering for nearly two decades [9, 17]. A FACS analysis requires the presence of a fluorescent signal either within or on the surface of a cell. Genetically encoded biosensors can be used to convert the production of target metabolites into a measurable intracellular fluorescence signal. These biosensors could therefore be coupled with FACS for screening applications [11, 12, 16, 21]. However, this approach is limited by the measurement of fluorescence signals within single cells, rather than a population of many cells, and the variability among single cells decreases the screening accuracy. Moreover, the measurement is reliant on the detection of an intracellular signal and thus neglects the extracellular concentrations of secreted metabolites that

✉ Ronglin He
he_rl@tib.cas.cn

✉ Qinhong Wang
wang_qh@tib.cas.cn

1 Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China

2 Key Laboratory of Systems Microbial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China

3 Tianjin Key Laboratory of Industrial Biology Systems and Processing Engineering, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China

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are no longer associated with the producing cells [18, 20]. As a result, FACS may be less likely to isolate truly high producers and secretors of metabolites [4, 19]. In contrast, droplet microfluidics (i.e., fluorescence active droplet sorting, FADS) can encapsulate single cells in independent droplet-based micro-reactors, and thus avoids the exchange of metabolites secreted by cells [3, 8, 20]. This approach allows the efficient coupling of the genotype and phenotype and could be applied to enzyme, molecular, and biodiversity screening protocols [1, 2, 5].

In this study, using our previously reported genetically encoded biosensor, 3-dehydroshikimic acid (3-DHS), we established a biosensor-based FADS screening strategy, characterized the resulting model, and compared the effects of FACS and FADS on biosensor-based screening for microbial strains evolution.

Materials and methods

Strains, media, and growth conditions

Escherichia coli ATCC 8739 and its derivative strains TIB02 and M9, which include the cusR-linker-GFP biosensor, were reported in our previous study [10]. All strains for FACS or FDAS analysis were cultivated in LB medium for revival and fermented in NBS, a minimal medium containing KH_2PO_4, 3.5 g/L; $K_2HPO_4{\cdot}3H_2O$, 6.5 g/L; $(NH4)_2HPO_4$, 3.5 g/L; $MgSO_4$, 0.120 g/L; $CaCl_2$, 11 mg/L; thiamine HCl, 5 mg/L; $FeCl_3{\cdot}6H_2O$, 0.160 mg/L; $CoCl_2{\cdot}6H_2O$, 0.200 mg/L; $CuSO_4{\cdot}5H_2O$, 0.015 mg/L; $ZnCl_2$, 0.020 mg/L; $Na_2MoO_4{\cdot}2H_2O$, 0.020 mg/L and H_3BO_3, 0.005 mg/L [6, 13]. The culture flasks were incubated in a rotary shaker at 37 °C and 220 rpm for 24 h.

Strain mutagenesis

The biosensor-expressing strain M9 from our previous study was grown in LB medium containing 25 μg/mL kanamycin until it reached the logarithmic phase [10]. The cells were harvested, washed, and resuspended in 1 mL NaCl, 0.9% (w/v) to an OD_{600} of 1.0. The mutation library was constructed by treating cell suspension using atmospheric and room temperature plasma (ARTP) mutagenesis system for 15 s, in which the positive mutation probability was the best as described previously [10]. The treated cells were revived in LB medium at 37 °C and 220 rpm for 1 h, and then inoculated in NBS fermentation medium for 10-h cultivation prior to FACS screening or encapsulated immediately in droplet and cultured for subsequent FADS screening.

Droplet generation and sorting

Droplets were generated using surfactant-containing fluorinated oil as the oil phase, and a suspension of cells in media at an OD_{600} of 0.1 according to Poisson distribution as the aqueous phase [14]. The flow rates of these two phases were 200 and 80 μL/h, respectively. The generated droplets were stored in a plastic syringe and incubated statically at 37 °C for up to 2 days. The droplets were taken out for analysis at 18–40 h by re-injecting into the sorting chip at a flow rate of 10 μL/h for droplets and 200 μL/h for the separating oil. A total of 300 droplets were sorted using an in-house-developed droplet microfluidics instrument, with the sorting gate set at top 0.1% of the signal droplets [7]. The sorted droplets were then distributed on LB agar plates containing 25 μg/mL kanamycin and incubated at 37 °C for subsequent analysis.

FACS analysis and sorting

The cells were harvested, washed, and re-suspended in phosphate-buffered saline (PBS) at an $OD_{600} < 0.1$. The cell suspension was analyzed immediately using a MoFlo XDP high-speed cell sorter (Beckman Coulter, CA, USA) at an excitation wavelength of 488 nm, emission wavelength of 520 nm, and sample pressure of 70 psi. For sorting, the selection gate was set at 0.05% of the total cells. A total of 300 cells were collected and grown on agar plates containing 25 μg/mL kanamycin at 37 °C overnight.

HPLC analysis

The 3-DHS concentrations produced by the recombinant *E. coli* strains were quantified using high-performance liquid chromatography (HPLC), as described in our previous report [10]. Cell fermentation supernatants were obtained by centrifuging the fermentation cultures at 10,000 rpm for 10 min. Each supernatant was diluted with 10 volumes of ddH_2O, and 20 μL of the diluted sample was subjected to HPLC analysis.

Results and discussion

Establishment of the biosensor-enabled droplet microfluidic screening system

To improve the efficiency of the biosensor-enabled high-throughput screening system for strain engineering, we coupled a biosensor with a droplet microfluidic screening strategy. Using our previously constructed 3-DHS biosensor as a model, we optimized the factors related to biosensor-enabled

droplet microfluidic screening, including the droplet generation parameter, droplet incubation, and sorting time. To reduce the embedding of multiple cells in each droplet, single-cell encapsulation was performed according to the definition of Poisson distribution [14]. When cells at an OD_{600} of 0.1 were used, the most suitable droplet diameter for single-cell encapsulation was ~ 20 μm. The droplet size could be controlled by adjusting the flow rates of the oil and water phase. As shown in Fig. 1a, the droplet diameter decreased gradually from 23.2 to 17.4 μm as the ratio of the water phase flow rate to the oil phase flow rate increased. Next, the stability of the generated droplets was studied. As shown in Fig. 1b, the droplets remained highly stable during a 48-h cultivation period at 37 °C, a characteristic that would be beneficial for subsequent droplet detection and sorting.

We then investigated screening parameters such as the droplet incubation time and sorting time. The proliferation of cells and corresponding fluorescence signals in the droplets were measured at different times to determine the suitable conditions for screening. As shown in Fig. 1c and d, a significant difference in signal was observed between the droplets containing the 3-DHS-producing strain TIB02 and those containing the non-producing strain 8739 after 24-h incubation at 37 °C. These results suggest that a 24-h culture period is optimal for droplet detection and sorting.

Comparison of the sorting efficiencies of droplet FADS and single-cell FACS when used to screen biosensor-enabled DHS-producing cells

The feasibility of the biosensor-enabled FADS screening system was tested by sorting a mutant library generated from M9 cells via ARTP mutation. This mutant library was also sorted by FACS for the purpose of comparison. The mutant library, which contained 5×10^6 mutants, was cultivated in LB medium for cell resuscitation. After overnight incubation, the cells were harvested by centrifugation and washed twice with PBS. The recovered cells were then resuspended and inoculated in fresh NBS fermentation medium for either FADS or FACS sorting. After resuspension, the cells were encapsulated in droplets and incubated for 24 h prior to sorting. The droplets with the highest level of fluorescence (i.e., top 0.1%) were sorted and grown on agar plates. Subsequently, 60 mutants were randomly selected from the plates and inoculated in flasks for further analysis.

Next, a series of protocols, including a fermentation culture in a shaking flask and HPLC, was performed to quantify the 3-DHS-producing capacity of each mutant. Notably, 52% of the selected mutant cells produced greater amounts of 3-DHS than the original strain M9 (Fig. 2a). Compared with M9, which yielded a 3-DHS titer of 2.03 g/L, the highest-producing mutants yielded 2.64 g/L 3-DHS after a 24-h cultivation period, which corresponded to a 30% improvement in 3-DHS productivity relative to strain M9 (Fig. 2b).

The same mutant library was also sorted by FACS for comparison. The sorted cells were incubated on agar

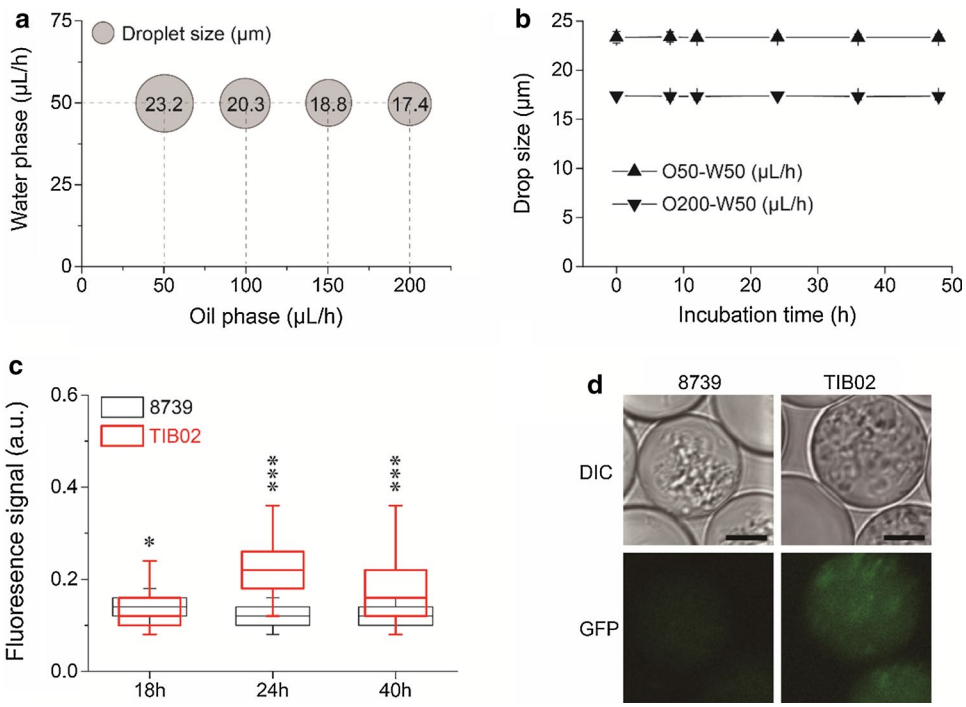

Fig. 1 Characterization of the droplet conditions. **a** Droplet size under different water to oil flow rate. **b** Droplet stability under different incubation time. Statistics were performed by the two-tailed Student's *t* test. $^*P < 0.05$, $^{***}P < 0.001$. **c** Droplet fluorescence signal determined by the microfluidic instrument at different incubation time. **d** Droplet fluorescence signal determined by microscopy

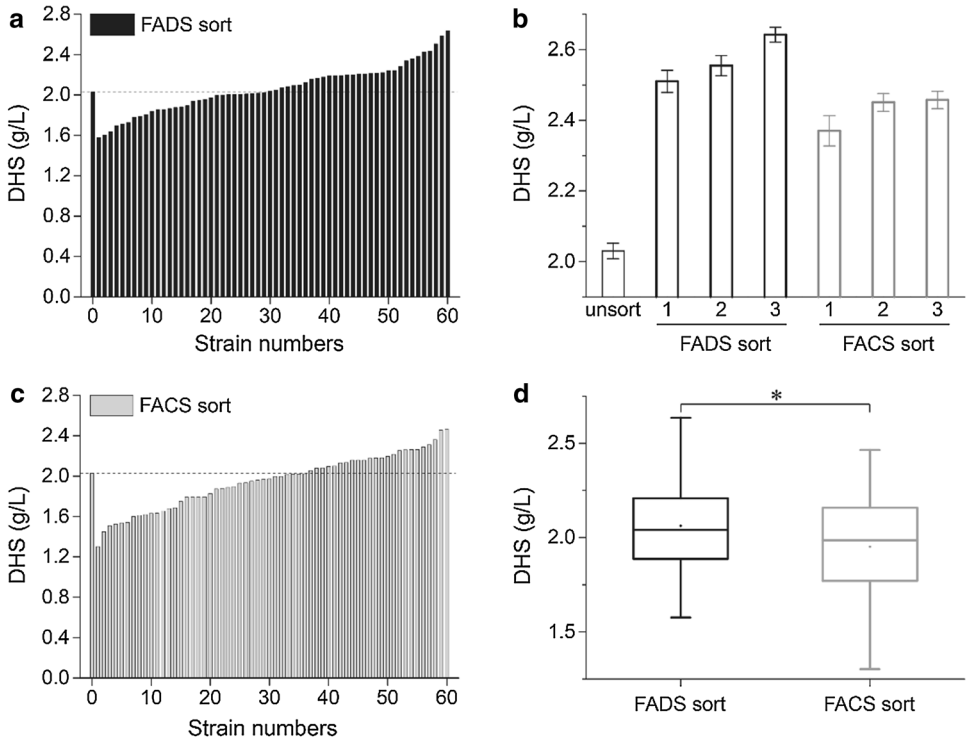

Fig. 2 Comparison of the sorting efficiencies of droplet-based FADS and single-cell FACS. **a** Strains sorted by FADS. **b** Production of 3-DHS by the sorted strains. **c** Strains sorted by FACS. **d** Statistical analysis of the sorted strains by two sorting methods. Statistics were performed by the two-tailed Student's *t* test. $^*P < 0.05$

plates and a total of 60 mutants were randomly selected for analysis. Among these mutants, 24 strains produced larger amounts of 3-DHS than the parent strain M9, resulting in a positive mutant enrichment rate of 40% (Fig. 2c). The best-producing mutant yielded 2.46 g/L 3-DHS after 24-h cultivation, which corresponded to a 21% improvement in 3-DHS productivity relative to the strain M9 (Fig. 2b). Compared with FACS sorting, FADS not only increased the positive enrichment rate of 3-DHS mutants by 12%, but also improved the 3-DHS productivity of the best sorted strain by 9%. These results demonstrated that biosensor-based droplet FADS screening yielded better performances than single-cell FACS in terms of both positive mutant enrichment and the 3-DHS yield (Fig. 2d).

Scheme of the approach that couples a biosensor with droplet FADS

The biosensor was coupled with the droplet-based FADS screening approach as shown in Fig. 3a. Compared with single-cell FACS, biosensors contained in droplets yielded detectably stronger fluorescence signals due to the propagation of multi-cell populations. This proliferation amplified the differences in fluorescent signal intensity among the droplets. Particularly, the target secreted metabolites were limited to the individual microenvironment of the droplet, where they were restricted to communication with the host cells in the droplets, instead of forming a diffused solution and making a material exchange with other cells like FACS [5]. Therefore, a more efficient and accurate method of sorting positive mutants would be beneficial.

To verify the increase in fluorescence signal magnification from the single-cell to the multi-cell level, we incubated single cells in tubes to simulate the growth conditions in droplets. We then demonstrated the effect of the cell density in culture on the fluorescence signal. Two strains, TIB02 and M9, which exhibit differences in 3-DHS productivity, were cultivated under identical conditions. Next, the cultured cells were collected, washed with PBS buffer, and prepared at final concentrations corresponding to an OD_{600} of 0.25, 0.5 or 1, and the fluorescence signal of each was measured. As shown in Fig. 3b, the fluorescence signal produced by M9 was 20 a.u. (arbitrary unit) higher than that produced by TIB02 at an OD_{600} of 1.0, whereas this inter-strain difference decreased to 5 a.u. at an OD_{600} of 0.25. Therefore the growth of multi-cell populations in droplets amplified single cell-level differences in fluorescence. This approach enabled the further enhancement of differences in fluorescence among various mutants and would thus increase the sensitivity of screening for positive mutants.

Besides, droplet compartmentalization also increases the efficiency of coupling the extracellular concentration of a metabolite to the individual output of a biosensor-harboring cell [5, 18, 19]. In the single-cell FACS system, diffusion uncouples the concentration of secreted 3-DHS from its producer cell. In contrast, the droplet FADS system can couple the accumulation of 3-DHS with the respective producer cell and provide feedback to enhance the fluorescence of

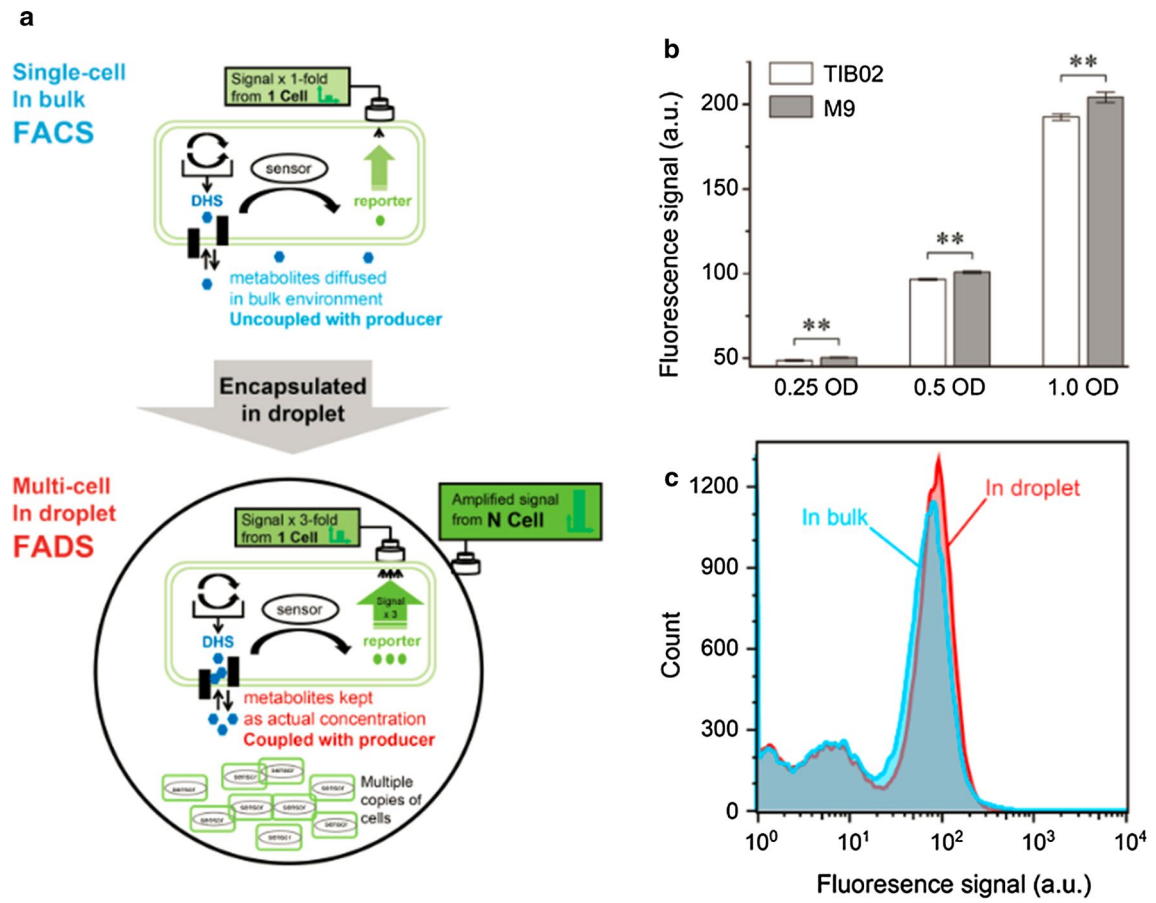

Fig. 3 Analysis of the screening method. **a** Scheme of the method coupling a biosensor with single-cell FACS or droplet FADS. **b** The effect of the cell number on the fluorescent signal divergence. Statistics were performed by the two-tailed Student's *t* test. $^{**}P < 0.01$. **c** The effect of individual droplet compartmentalization on the fluorescent signal divergence

the intracellular biosensor (Fig. 3a and c). Therefore the inclusion of droplet FADS in a biosensor-based screening protocol enhances the sensitivity and accuracy of the sorting of positive mutants and may thus be beneficial for the rapid evolution of metabolite-producing cells.

Conclusions

In this study, we demonstrated that the combined application of a biosensor and droplet microfluidics could increase the sensitivity and precision of screening protocols for microbial producers of metabolites. We used this approach to successfully screen a 3-DHS-producing strain library. When compared with single-cell FACS, droplet screening at a multiple-cell and feedback level could magnify differences in the fluorescent signals produced by various mutant strains and the independent droplet environment, leading to better enrichment and production rates. These results demonstrate that the measurement of cells and their metabolites in an individual droplet microenvironment yields intracellular metabolite concentrations that are closer to the actual situation while excluding interference from other cells. Thus, our described biosensor- and droplet microfluidics-based high-throughput screening method is suitable for the rapid screening of metabolite over-producing microbial strains and will be broadly applicable to strain development campaigns.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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