The application of microfluidic-based technologies in the cycle of metabolic engineering

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A R T I C L E  I N F O

Article history:
Received 13 July 2016
Received in revised form 15 August 2016
Accepted 27 September 2016

Keywords:
Metabolic engineering
Microfluidics
High-throughput
Single-cell analysis

A B S T R A C T

The process of metabolic engineering consists of multiple cycles of design, build, test and learn, which is typically laborious and time-consuming. To increase the efficiency and the rate of success of strain engineering, novel instrumentation must be applied. Microfluidics, the control of liquid flow in microstructures, has enabled flexible, accurate, automatic, and high-throughput manipulation of cells in liquid at picoliter to nanoliter scale. These attributes hold great promise in advancing metabolic engineering in terms of the phases of design, build, test and learn. To promote the application of microfluidic-based technologies in strain improvement, this review addressed the potentials of microfluidics and the related approaches in DNA assembly, transformation, strain screening, genotyping and phenotyping, and highlighted their adaptations for single-cell analysis. As a result, this facilitates in-depth understanding of the metabolic network, which in turn promote efficient optimization in the following cycles of strain engineering. Taken together, microfluidic-based technologies enable on-chip workflow, and could greatly accelerate the turnaround of metabolic engineering.

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hinders the advancement of sustainable and green industry.

To speed up the process of strain construction and exploit the potential of cell metabolism, a series of techniques have been developed and applied to different levels of metabolic engineering, such as the tuning of the promoter strength at the gene level [2], the elimination of the competing pathways [3], and the identification of additional beneficial enzymes by omics analysis of the whole cell system [4]. However, conventional methods are often limited by the throughput, precision, cost, convenience and the compatibility with other assays. These defects were overcome by microfluidics, which is the technology of manipulating and controlling the flow of liquids inside micrometer-sized channels, reaction wells and reaction chambers. It usually appears in two forms: stream of fluids and droplet. The latter is comprised of two immiscible fluids such as buffer and oil, droplets are formed via shearing one into the other [5]. The microfluidics are characterized by laminar flow, surface effects, short diffusion length and small volume [6,7]. Laminar flow induces a predictable stream behavior and minimal mixing, and surface effects facilitate capillary liquid drawing, interfacial transport and reactions. Meanwhile, the minute length and volume scale enable rapid changes in temperature and chemical composition. These attributes ensure accurate control over the flow at picoliter to nanoliter scale under automated mode, hence higher throughput and precision (Table 1). Along with reduced cost and labor, as well as flexible integration with sensors, actuators, and controllers, the microfluidic methods exhibit promising applications in the systems level of strain optimization, offering unprecedented opportunities for improving yield and strain stability [8].

To encourage the implementation of microfluidics in metabolic engineering, we reviewed the revolutions brought by this approaches in the key processes of strain development including design, build, test and learn. Meanwhile, we emphasized the realization of single-cell analysis for metabolic engineering thanks to the combined utilization of microfluidics and the downstream approaches. We believe that the microfluidic technologies could accelerate the cycle of metabolic engineering toward a rapid turnaround by following its own “Moore’s law”, just like the electronic industry.

2. Microfluidics in the realization and decision-making of metabolic design

The process of metabolic engineering begins with the step of design. It includes the selection of host and the creation of the synthetic pathways capable of overproducing the chemicals of interest. The blueprint of metabolic pathways is usually drafted in silico, with the aid of computer algorithms to design the most feasible and efficient routes using the data from a set of databases.

Although the design phase does not involve with in vivo or in vitro operations, it is linked with microfluidic-based technologies (Fig. 1). Their application in metabolic design was attributed to the high-throughput and high-resolution of microfluidics. Due to their higher throughput than conventional approaches, combining microfluidics with supporting algorithms will reduce labor in the process of metabolic design [9]. For instance, microfluidic trapping devices have recently been used to characterize the biophysical properties of transcription factor binding sites at a throughput up to 4000 [10]. With the aid of computational tools capable of detecting motifs in large dataset [11], the proper binding strength could be easily selected and incorporated into the genetic design. The advantage of high-resolution enables precise design of the metabolic pathway. For instance, metabolic information collected at the scale of single cells by droplet microfluidics considers the variation of individuals rather than the metabolic state of the whole cell population. This increases the accuracy of the estimated coefficients in the flux balance analysis and may also uncover new pathways, enabling more reliable calculation of the theoretical yield and the allocation of metabolites [12], thus guiding the design of the optimal metabolic pathway that achieves the highest yield. Moreover, the resources, rules and tools for metabolic designs are increasing rapidly, which broaden the diversity of chemicals that could be produced by metabolic engineering [13]. This also calls for coupled improvement of the analytical technologies to fulfill the design space in order to exploit the potentials of metabolic engineering in modern industry. Compared with conventional approaches, microfluidics is one of the most cutting edge technologies featuring high sensitivity, accuracy, high-throughput and other advantages, which can expedite the realization of metabolic design. At last, the initial design is mostly always flawed. The errors or bottlenecks must be identified and revised in the subsequent redesigning. Microfluidics enables the integration of the omics platform [14]. Data acquired from the systematic studies of metabolomics, proteomics, genomes and transcriptomes provides quantitative information of the interactions within the complex cell network, offering new strategies for strain improvement. For example, the integration of transcriptomic and proteomic analysis has been used to uncover the competing pathway of phosphoenol pyruvate carboxykinase in the production of threonine, deletion of the related targets increased the productivity of threonine by more than 40% [15].

3. Microfluidics facilitates strain building

The build step introduces exogenous pathways into the host and directs the metabolic fluxes towards the production of desired chemicals in accordance to the prior design. This phase involves the synthesis, assembly and transformation of DNA into the chassis host, for which microfluidic platforms have recently been introduced to expedite this process.

3.1. DNA assembly

To obtain novel pathways, fragments of DNA encoding different functions must be assembled into the vector. The traditional manual procedures were laborious and time-consuming. Although the automatic robotic techniques have reached the level of high-throughput, their application was limited by the high cost in equipments and consumable assays [16]. Fortunately, microfluidics overcome these defects by reducing processing time, reagents consumption (100-fold), DNA loss, and by offering facile control

Table 1

| Approaches          | Sample volume | Throughput   | Integration | Manipulation | Cost | Labor | Solution mixing | Molecule adsorption |
|---------------------|---------------|--------------|-------------|--------------|------|-------|-----------------|-------------------|
| Conventional        | Microliter    | Low (usually <10⁻³) | Complex     | Difficult    | High | High  | Fast            | Difficult          |
| Microfluidics       | Pico to nanoliter | High (can reach 10⁻³) | Flexible    | Easy         | Low  | Low   | Slow            | Easy              |
| Pros or cons        |               |             |             |              |      |       |                 |                   |

* Molecule adsorption is the adsorption of hydrophobic molecules to the surface due to the large surface-to-volume ratios of microfluidic devices, which may affect the concentrations of reagents.
over multiple flows simultaneously. For example, microfluidic chips with different functional components have been designed to integrate DNA digestion and ligation into a single run [17]. Digital microfluidic devices imbedded with electrodes have achieved even higher precision and reproducibility [18]. These platforms have been successfully adopted to generate large libraries of plasmids following protocols such as Gibson assembly, Golden gate assembly, and yeast assembly [19].

3.2. Transformation

After construction, the plasmids must be transferred into the cell to perform functions. The efficiency of transformation ensures the size of the genetic libraries, which correlates positively with the probability of the occurrence of the desired mutant. By incorporating either heat-shock or electroporation procedures, transformation using microfluidic platforms have been proved to achieve at least the comparable efficiencies for *E. coli* and *S. cerevisiae* as the tube-based approaches [19,20]. Moreover, the microfluidic-based approaches are much cheaper and convenient. Further optimization such as the utilization of magnetic beads can improve the transformation efficiency up to 2-fold [21]. These advantages allowed routine construction of large genetic libraries.

4. Increased efficiency and accuracy in the test step

The test step validates the efficacy of the design and build phases in achieving the desired properties. It includes the characterization, screening and analysis of the engineered strain in terms of cell phenotype and genotype.

4.1. High-throughput screening

The build process generates large genetic libraries with high diversity, which have to be screened based on the phenotypic or genotypic characteristics of the cells. Since the occurrence of the target properties can be rather infrequent, usually $10^{-4}$, the size of the mutant libraries is typically $10^5$, with maxima up to $10^{12}$ [22]. Accordingly, high-throughput analysis is essential for detecting the desired mutant from such a large pool. Traditional screening was performed using microtiter well plates, and the contents of the metabolites of interest are measured as indicators for screening. This method is low-throughput, time consuming, laborious and costly, thus is less likely to acquire the optimal strains.

In contrast, microfluidic platforms can achieve high efficiency by using the fluorescent properties of the cells as reporters. For small molecules that emit no spectral signature, fluorescence could be realized by linking the fluorescent gene to the gene of interest. Another strategy is to use biosensors. These are substrates capable of entering cells or attached to the cell membranes, and can be converted to fluorescent reporters by enzymatic reactions to reflect the contents of the desired metabolites [5]. These enabled the application of fluorescence-activated cell sorting (FACS) for the screening of genetic libraries [22]. The FACS is suitable for stream-
based microfluidics and works as microscaled flow cytometry, which increases the throughput to 10^8 variants per day [23]. However, this method only allows the detection of intracellular metabolites, or metabolites attached to the cell membrane without diffusing into the fluid. For secretory metabolites, the latest fluorescence-activated droplet sorting (FADS) serves the purpose [24,25]. The droplet microfluidics isolates single cell into individual compartment and preserves the unique phenotypic characteristics of the cell. By employing electrical, acoustic, magnetic, optical, hydrodynamic and mechanical approaches [26], the FADS droplet microfluidics has enabled a high screening rate of 10^6 cells per hour. Further modification such as device parallel can increase the throughput by another three orders of magnitude [27]. Due to this merit, microfluidic platforms have been successfully applied to strain screening. For instance, yeast strains capable of over-consumption of xylose were acquired using microfluidics from a library of 10^6 clones [27], and at a rate of 300 droplets per second, strains with high α-amylase yield were isolated in a total of 10^6 cells within 3 h [25].

Another major advantage of microfluidics is the facilitation of fine-scale spatial and temporal control of the cells. By using microwells, microchambers, droplets, hydrogels and other contact or contactless trappings, cells could be easily manipulated in zero to three dimensional spaces [5,26]. This enables batch cultivation of single or multiple cells in the upstream or downstream of the microfluidics workflow. For instance, the droplet trapping has been used for short-term culturing of the single cells, allowing secretory metabolites to accumulate and ready for FADS screening [27,28]. Moreover, the high controllability of microfluidics enables facile changes in the microenvironment around the cell. This could be adapted to optimize strain stability, such as the ability to withstand concentrated end product, as well as the ability to sustain high productivity over the course of cell growing phases.

4.2. Single cell analysis

Strain screening is often coupled with phenotype and genotype of the cells to validate the manipulations of the pathway, which enable global analysis of the cellular metabolites. Conventionally, the measurement is based on the average information of a whole cell population. However, cells are highly heterogeneous systems. Due to stochastic gene expression, cell cycle, aging, epigenetic regulation and microenvironments, they can present unique properties that differ significantly from the mean levels in terms of gene translation and expression, even for isogenic populations in identical environments [26,29]. Thus, single cell analysis at the systems level can provide new possibilities to uncover rare mechanisms of the metabolic regulations, which may lead to great improvement in strain performances.

The feasibility of single cell analysis in metabolic engineering depends on two factors: cell sorting and downstream assays. The throughput issue concerning cell sorting is better fulfilled by automated droplet microfluidics using FADS method, in comparison to the conventional approaches such as optical tweezers, serial and microwell dilutions [30]. On the other hand, the sensitivity, fidelity and throughput become the major challenges for downstream measurements. Fortunately, these issues have been addressed by the recent development in analytical technologies.

The analysis of proteomics and metabolomics for single cells has been realized thanks to the increased sensitivity and resolution of the latest mass spectrometry (MS). The MS has been the ideal device to identify and quantify the complex mixtures containing thousands of analytes in biological samples [31]. The detection limit of the latest MS devices has reached attomole level [32], which is suitable for the analysis of proteins and metabolites from single cells. However, the low throughput of MS-based approaches still confines their utilization in large-scale analysis. Recent solutions such as mass spectrometry imaging has increased the number of measured samples per run [33]. Nevertheless, future development of MS-based approaches should incorporate both the sensitivity and throughput. One promising solution is the integration of microfluidics, by reducing sample volume, it improves MS sensitivity and resolution, and facilitate parallel detection for large-scale analysis. This will allow unrestricted access to diverse types of trace molecules, which could be more relevant to the desired metabolic characteristics. The protein expression, enzymatic activity and metabolite levels can also be extracted from the identical cells, enabling the establishment of direct metabolite-protein interactions, and subsequent evaluation of their feedbacks on gene expression and signaling [34].

For the measurements of cell genome and transcriptome, one strategy to increase throughput is double barcoding, which tags both the cell and the genetic molecules. The method is based on droplet microfluidics and is hence referred to drop sequencing (drop-seq) [35]. Take transcriptome for instance, the cell barcode usually consists of 12 bases that has the potential to distinguish 4^12 (16,777,216) individual cells, for each cell barcode, a unique molecular identifier of 8 bases is then attached, enabling it to mark 4^8 (65,536) different mRNAs in a single cell. The barcode and cell are co-encapsulated in a droplet, after the binding of mRNAs and reverse transcription. The transcriptome from a single cell is tagged uniquely, allowing parallel sequencing in a single run [35,36]. The current throughput for drop-seq is 10^8 cells per day, although still an order of magnitude lower than the typical size of a mutant library, it already shortened the processing time by > 100-fold comparing with the existing methods [35], and has realized routine genotyping of single cells. This can rapidly reveal significant differences in the gene transcription level among individual cells, aiding parallel validation of the efforts in modifying targeted genes. The comparative analysis of genome and transcriptome among different cells helps to identify the key genetic factors that endow the desired phenotypes, facilitating directed manipulation of other strains in the process of inverse metabolic engineering [37]. Moreover, the single-cell omic analysis captures a “snap-shot” of the cell status, a series of “snap-shots” will create a dynamic record of the cellular metabolism, offering timing and causal analysis of the metabolic regulation mechanisms.

The fidelity of the amplified genome is crucial for single-cell analysis since the initial copy number is only one. The traditional PCR-based method is highly biased due to its low coverage and high rate of amplification error [30], thus is not suitable for single-cell analysis. A better strategy is to use isothermal approaches such as multiple displacement amplification (MDA) [38]. This method generates greater genome coverage with lower error rate compared to PCR-based methods. However, the lack of uniformity for MDA method may introduce bias in the final data. To compensate these defects, hybrid methods such as multiple annealing and looping-based amplification cycles (MALBAC) has been proposed to strike a balance between the PCR-based and the MDA methods. The MALBAC achieves medium coverage and error rate with high uniformity, and is considered an alternative for MDA method [38]. In terms of single cells, the amplification bias for both MDA and MALBAC methods are rather similar, but with different preferences. The former one has much lower error rate whereas the latter is better at detecting variations in copy numbers. In all, the amplicon-based methods are intrinsically flawed for single cell genotypic analysis.

The third-generation sequencing technologies realized an amplicon-free, single molecule-based direct DNA or RNA sequencing [39], which eliminates the bias resulted from the
amplification step. One of the most well-known methods is the single molecule, real-time (SMRT) sequencing. It adopts a sequencing-by-synthesis approach in which a circular template, usually a DNA molecule, is targeted by a single DNA polymerase. The type of nucleotide is identified when it is incorporated by the DNA polymerase, according to changes in the fluorescence of the nucleotide. Another advantage of SMRT is the long reads length up to 20 kb [40], which facilitates accurate genome assembly. The error in SMRT sequencing derives primarily from insertions, followed by deletions, and a small chance of mismatch, which are all random and will not increase with the increase of read length [41]. It has been claimed that the SMRT was of low accuracy [42], in fact, this only applies to a single read (~90% accuracy) [43]. By increasing sequencing depth and implementing a circular consensus sequence, the SMRT can achieve a final accuracy up to 99.999% [44,45].

An alternative single molecule sequencing method is the nanopore sequencing, in which the nucleotide is identified by detecting the changes in current when strands of DNA pass through the nanopore [46]. Besides advantages of amplification-free and long read length, this method also offered a high portability and the nanopore [46]. Besides advantages of amplification-free and long read length, this method also offered a high portability and the nanopore. Moreover, the combined application of fluorescent-based cell sorting, barcoding and single molecule sequencing opens the gate of single cell analysis for metabolic engineering, which holds great promise in the near future. The era of microfluidic-based metabolic engineering has just begun, and the microfluidic platforms should move toward modularity to speed up its application. This will allow standard components for various steps such as strain build, screening and sequencing to be assembled into an engineering line for different purposes. The seamless and flexible integration of functional units facilitates the refinement of targeted information. The single cell analysis may be the most significant advancement for metabolic engineering. It enables a closer look at the diverse regulatory mechanism of the cell, and reveals the rare but key pathways which are easily masked by the mean data. Together with the facile manipulation of cells in microfluidic platforms, it is able to study the genetic and phenotypic responses of cells to different micro-environments and cell cycles. For instance, microfluidic coupled with optical tweezers was able to manipulate the microenvironment around single cells in less than 2 s, and has been used to study the intracellular responses of single yeast cells to changes in glucose availability [49]. Using microfluidic bioreactor, single cells such as yeast can also be cultivated for generations in constant environment, allowing unbiased investigation of the effects of cell cycle on cell biochemical heterogeneity [50,51]. Findings from these studies can offer new insights for pathway modeling, design and strain construction, and ultimately increase the rate of success.

6. Outlook and conclusions

The microfluidic-based technologies are characterized by high-throughput, small volume, easy control, low cost, and flexible integration with other assays. These qualify microfluidics as the ideal choice for metabolic engineering, which covers the steps of DNA assembly, transformation, strain screening, genotyping, phenotyping, and in-depth systems analysis. Moreover, the combined application of fluorescent-based cell sorting, barcoding and single molecule sequencing opens the gate of single cell analysis for metabolic engineering, which hold great promise in the near future. The era of microfluidic-based metabolic engineering has just begun, and the microfluidic platforms should move toward modularity to speed up its application. This will allow standard components for various steps such as strain build, screening and sequencing to be assembled into an engineering line for different purposes. The seamless and flexible integration of functional units can avoid extra procedures for sample handling and transferring, and enable automation. Taken together, the microfluidic-based technologies hold the potential to miniaturize the process of metabolic engineering to on-chip scale, and greatly reduce the turnaround of the engineering cycle.

Acknowledgements

This work was supported by the Recruitment Program for Young Professionals (1000 Plan), and the Financial Aid Project for
Outstanding Young Teachers of Beijing Institute of Technology (2015YG1607).

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