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Whole gene amplification and protein separation from a few cells

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ABSTRACT

Despite the growing interest to explore untapped microbial gene and protein diversity, no single platform has been able to acquire both gene and protein information from just a few cells. We present a microfluidic system that simultaneously performs on-chip capillary electrophoresis for protein analysis and whole genome amplification (WGA), and we demonstrate this by doing both for the same cohort of cyanobacterial cells. This technology opens avenues for studying protein profiles of precious environmental microbial samples and simultaneously accessing genomic information based on WGA.

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Microfluidics technology is being widely adopted in molecular biology and in studies of environmental ecology because it can handle and use small amounts of precious sample. The technology also consumes significantly less quantities of expensive reagents than do conventional approaches. Some reactions may even work more efficiently under microfluidic conditions [1]. Recently, several microfluidic devices have been developed to retrieve either genetic information or protein components from bacteria [2,3]. For example, DNA amplification from individual cells was achieved by performing whole genome amplification (WGA)² reactions in microfluidic environments [3,4]. Besides taking advantage of on-chip cell manipulation, capillary electrophoresis (CE), and a highly efficient detection system based on cylindrical optics, quantification of protein contents from a single cell has been demonstrated in micrometer-sized channels [2].

Although these microdevices are capable of extracting either gene or protein information from individual cells, we have developed a single platform in which both gene and protein information could be acquired from the same cohort of cells. The collection of both gene and protein information from a single sample is likely to be useful in the study of environmental samples of limited

quantity. There is growing interest in finding ways to cultivate and explore untapped microbial gene and protein diversity that has been dubbed the “uncultured microbial majority” [5]. So far, the method of choice has been “metagenomics” that enables the large-scale, high-throughput genomic sequencing of uncultured microorganisms. It provides an “organism-independent” view of gene and protein diversity because there is no requirement for the isolation or cultivation of any particular organism [6,7]. However, metagenomic data are aggregative, and the entire genomic or proteomic information of any one organism cannot be culled from the data. This is critical for in-depth analysis of environmentally important organisms. Combining single cell capture technology with metagenomics has the potential to lead to significant new insights because it can provide detailed information about genomes and partial proteomes from individual cells [8]. Our long-term goal is to develop a low-cost, integrated genomic and proteomic microfluidic platform that can be combined with new methods that promote cell growth under different conditions and maintain actively dividing cells [5,9].

In this article, we describe a microfluidic system that simultaneously extracts both gene and protein information from just a few (~10) cells. We focused on the abundant light-harvesting protein complex, known as phycobilisomes (PBSs), in the photosynthetic cyanobacterium *Synechococcus elongatus* PCC 7942 (sometimes referred to as *S. elongatus* PCC 6301 or *Anacystis nidulans* R2). PBSs are large, supramolecular protein complexes made up of peripheral rods and a core substructure that efficiently collect light energy and transfer it to the chlorophyll-containing photosynthetic reaction centers [10]. A PBS is primarily composed of several phycobiliproteins, which are a family of brilliantly

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E-mail address: zare@stanford.edu (R.N. Zare).¹ Present address: School of Pharmacy, Second Military Medicine University, Shanghai 200433, China.² Abbreviations used: WGA, whole genome amplification; CE, capillary electrophoresis; PBS, phycobilisome; APC, allophycocyanin; PC, phycocyanin; PDMS, poly(dimethylsiloxane); PCR, polymerase chain reaction; DDM, *n*-dodecyl- β -D-maltoside; SDS, sodium dodecyl sulfate; EMCCD, electron-multiplying charge-coupled device; Chl, chlorophylls; EDTA, ethylenediaminetetraacetic acid.

colored, water-soluble proteins that are covalently linked to linear chains of tetrapyrroles known as phycobilins. Each PBS in *S. elongatus* PCC 7942 has two types of pigmented phycobiliproteins: allophycocyanin (APC, $\lambda_{\text{max}} = 650\text{--}655\text{ nm}$) and phycocyanin (PC, $\lambda_{\text{max}} = 615\text{--}640\text{ nm}$). The APC forms the core structure, whereas the PC exists in the peripheral rod structures. In addition, there are various nonpigmented linker peptides that tune the absorption and emission wavelengths of the chromophores and function to hold the APC and PC together.

The composition of PBS in cyanobacteria can change in response to environmental shifts in light intensity, light quality, and nutrient availability [11]. Under conditions where light is limiting, PBSs increase in number; conversely, in strong light or nutrient-depleted conditions, the number of PBSs is reduced. In this study, we investigated the PBS protein components and their corresponding genes by performing CE and WGA reaction on the same sample of cyanobacterial cells in an integrated microfluidic device. To our knowledge, it is the first microfluidic system that integrates protein analysis and WGA on a single chip. It is a step toward a true lab-on-a-chip system with the potential automation of multiple biological experiments.

Materials and methods

Microfluidic chip fabrication

A detailed layout of the integrated microfluidic chip is illustrated in Fig. 1. Photomasks (Fineline Imaging, Colorado Springs, CO, USA) were generated by using computer-aided design software (AutoCAD, Autodesk) and printed on transparency films at a 49,600-dpi resolution. All positive relief masters were fabricated

in the Stanford Nanofabrication Facility under standard photolithographic procedures. The master for the channel layer (red in Fig. 1) was fabricated by spin coating a 10- μm -thick positive photoresist (SPR 220-7) on a silicon wafer. The photoresist was then reflowed on a hot plate at 115 °C for 20 min to generate round-shaped channels. The master for the control layer (blue in Fig. 1) was prepared by spin coating a 35- μm -thick negative photoresist (SU-8, Microchem, Sunnyvale, CA, USA) followed by another layer (10 μm) of positive SPR 220-7 photoresist that served as hydration lines and attached to valve 11. Positive and negative photoresists were exposed by a contact aligner (Electronic Vision 620, EV Group) and developed by MF-26A and SU-8 developers, respectively. The masters were treated with methyltrichlorosilane (Sigma-Aldrich, St. Louis, MO, USA) in a vacuum desiccator to prevent adhesion of poly(dimethylsiloxane) (PDMS) on silicon wafer.

Different layers of the microfluidic chip were prepared by replica molding of PDMS (RTV 615, GE Silicones, Waterford, NY, USA) from the positive relief masters. The channel layer (top layer) was prepared by a 5:1 mixture of PDMS prepolymer (RTV 615A and 615B) followed by curing at 80 °C for 1 h, and holes were punched on this layer for cell/reagent access. The control layer (middle layer) was prepared by spin coating a 20:1 mixture of PDMS prepolymer at 2200 rpm for 45 s followed by curing at 80 °C for 40 min. The channel layer was then aligned onto the control layer, and the assembled layers were cured at 80 °C for an additional 2 h. Holes connecting to a pressure controller were punched on the assembled layer. A bottom layer was prepared by spin coating a mixture of 20:1 PDMS prepolymer with cyclohexane (1:2 mass ratio, spin coating at 2000 rpm for 45 s) on a coverglass and cured at 80 °C for 30 min. Finally, the assembled layer was bonded onto the coverglass and cured at 80 °C overnight.

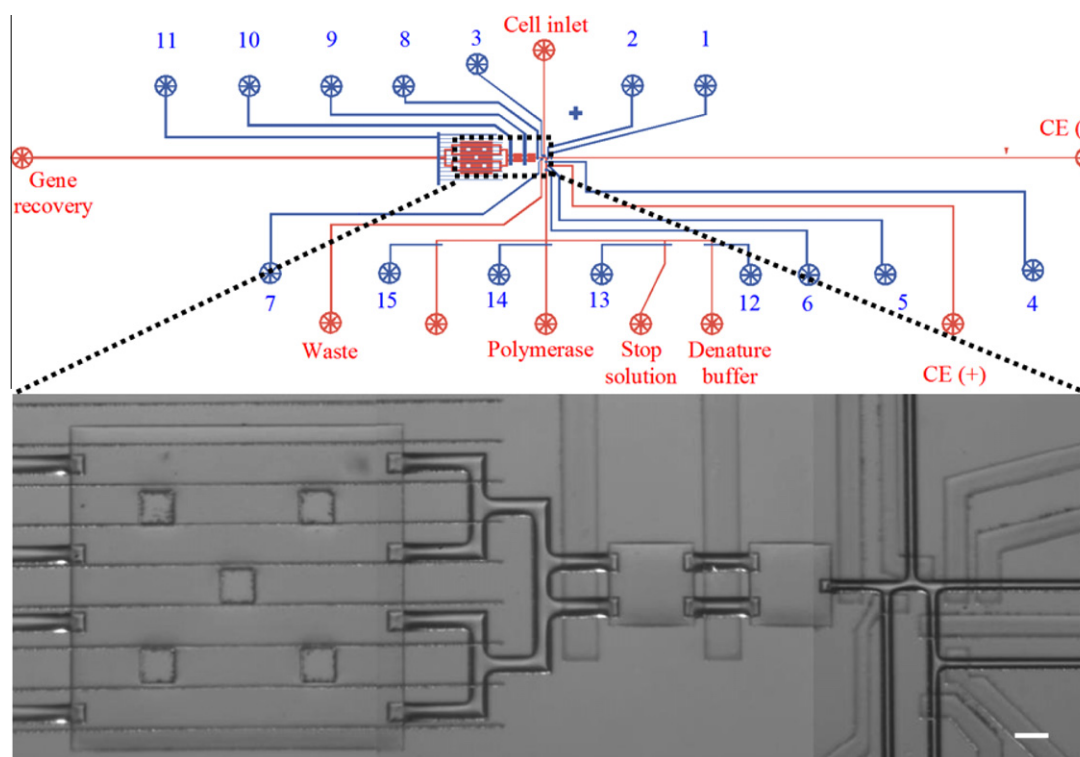


Fig. 1. Mask design of the integrated microfluidic device. The channel and control layers are depicted in red and blue, respectively. Reservoirs of the channel layer were opened for reagent/cell access while reservoirs of the control layer (1–15) were connected to a pressure controller for valve actuation. For laser-induced fluorescence detection, a 532-nm laser beam was focused in the separation channel with position indicated by a red arrow near the CE(–) reservoir. A zoom-in confocal micrograph shows the details of the core microstructures in this microdevice. (For interpretation of the references to color in this figure legend and the description in the text, the reader is referred to the Web version of this article.)

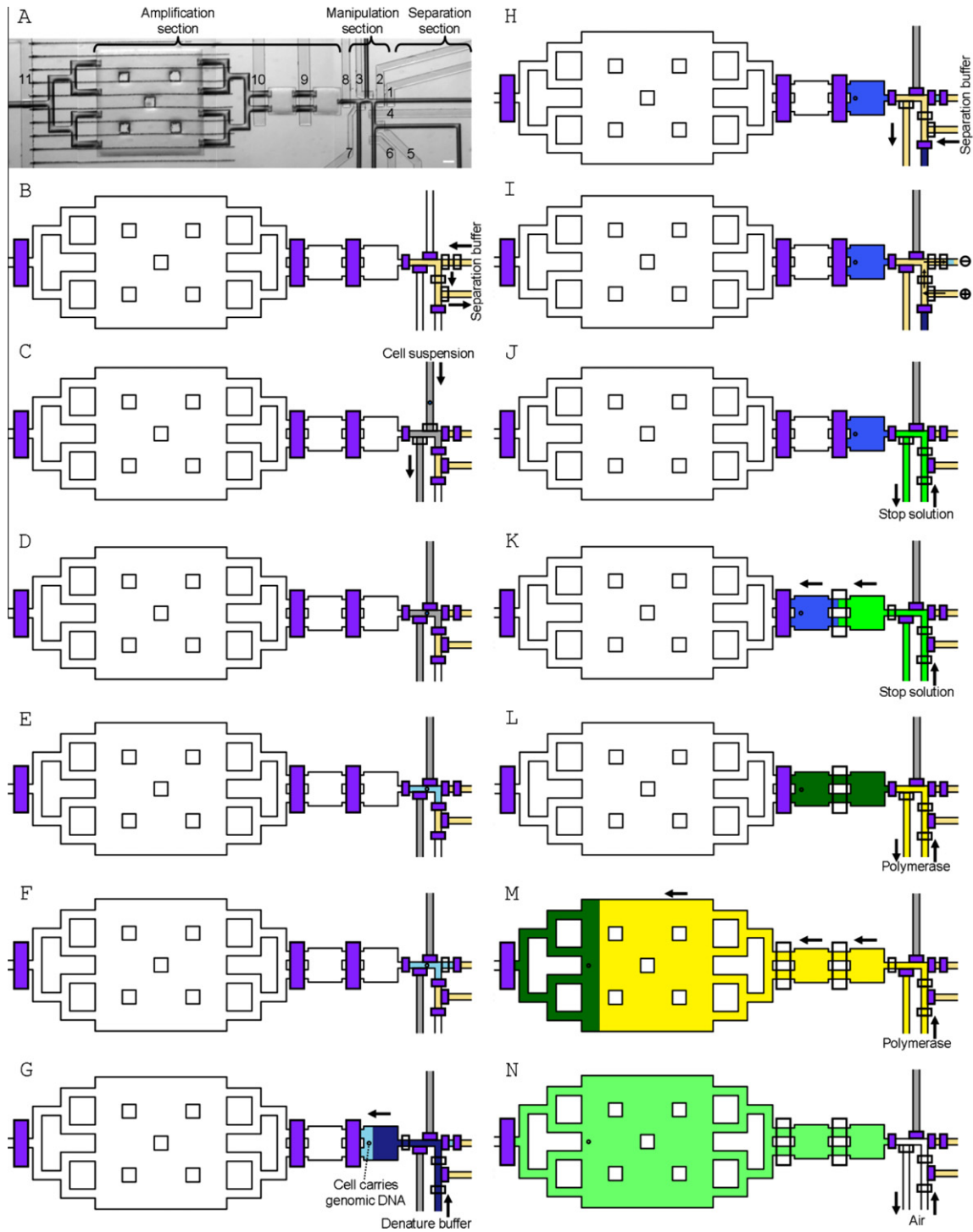


Fig. 2. Architecture of the integrated microfluidic chip showing the separation process: (A) DNA amplification and protein separation controlled by 11 microvalves (scale bar = 150 μm); (B) CE buffer loading; (C) cell injection; (D) cell capture; (E) release of diffusible protein content; (F) protein sample plug formation; (G) denaturing buffer injection; (H) wash with CE buffer; (I) CE separation; (J) wash with stop solution; (K) stop solution injection into manipulation chamber; (L) wash with polymerase solution; (M) polymerase solution injection into manipulation chamber; (N) WGA reaction incubation.

Buffer pretreatment of cyanobacteria

Unlike mammalian cells or enteric bacteria such as *Escherichia coli*, the cyanobacterium *S. elongatus* PCC 7942 has a cell wall that is difficult to lyse. Conventional cell lysing methods that use strong mechanical force or glass bead grinding are incompatible with a microscale device, especially in picoliter-sized microfluidic

chambers. Thus, we first treated cells with lysozyme to weaken and compromise the cyanobacterial cell wall. Cells from a liquid culture were first pelleted by centrifugation, the supernatant was removed, and the cells were resuspended in 10 mg/ml lysozyme solution followed by incubation at 37 $^{\circ}\text{C}$ for 30 min. The suspension was pelleted again to remove the lysozyme and resuspended in protein extraction buffer, B-PER, which weakens the cell wall.

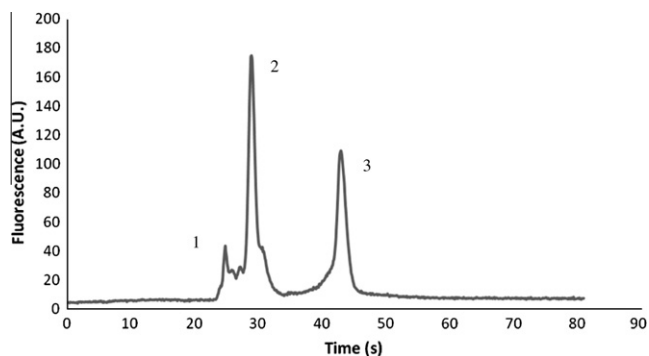


Fig.3. Protein separation from the integrated microfluidic chip showing the electropherogram of protein separation of PBS from 10 cells. The first peak was the APC-containing species, the second peak consisted of the PC species, and the last peak that appeared after 40 s was chlorophylls from photosystem II. A.U., arbitrary units.

PCR-gel electrophoresis

For the “off-chip” WGA, we used the GenomiPhi V2 kit. This particular kit has been shown to yield robust results, and temperature constraints due to the polymer composition of the microfluidic chip are not a constraint in off-chip amplification. DNA (2 μ l) isolated from on-chip amplification was mixed with 8 μ l of sample buffer and heated to 95 $^{\circ}$ C for 3 min in a thermocycler. Samples were cooled to 4 $^{\circ}$ C, and then 9 μ l of reaction buffer and 1 μ l of Phi29 polymerase were added. The WGA reaction was allowed to progress for 16 h at 30 $^{\circ}$ C followed by an inactivation of the enzyme at 65 $^{\circ}$ C for 10 min. The resulting off-chip WGA was then diluted 1:10 and used as the template for subsequent rounds of polymerase chain reaction (PCR). Genes were amplified with the following PCR protocol: (i) denaturing (95 $^{\circ}$ C for 1 min), (ii) annealing (55 $^{\circ}$ C for 30 s), (iii) extension (72 $^{\circ}$ C for 1 min), (iv) repeat (28 times), (v) fill-in (72 $^{\circ}$ C for 6 min), and (vi) hold (at 15 $^{\circ}$ C). PCR products were visualized via electrophoresis on 0.8% agarose in TAE buffer. A 7- μ l aliquot of each sample was loaded, along with 5 μ l of O’GeneRuler Ladder (Fermentas). Gels were run at 100 mV for approximately 20 min.

Results and discussion

The PDMS microfluidic device was fabricated by soft lithography [12] and had three sections for cell manipulation,

Table 1

Primers used and their properties for gene coverage test.

	Primer name	Sequence	Gene annotation	Distance from ORI (Kbp)	Size (bp)
a	Synpcc7942_0001F Synpcc7942_0001R	ATGCTTTGGCAAGATTGCCA CTAGCTGCGCAACTGAATCG	DNA polymerase III	1	1170
b	Synpcc7942_0300F Synpcc7942_0300R	GTGAGTATTCTTCGGCGCTT CTAGGCGCGGAGCTGGCAC	MreB	300	1055
c	Synpcc7942_0611F Synpcc7942_0611R	GTGGCTCCTTTGCTCCCCGT CAGTTTGCCAGTGCCATGGC	MutS2	600	2388
d	Synpcc7942_0892F Synpcc7942_0892R	GTGAAGCACTTCTCAGATCA CTAGCTTGACCTGGACTGCG	MIAB/nifB	900	1008
e	Synpcc7942_1168F Synpcc7942_1168R	ATGATTGATTCACAGGCACT TCATTGGTCGTCGCTTCTTC	CpmA	1200	780
f	Synpcc7942_1449F Synpcc7942_1449R	ATGCGCTGGAGTGAGTTTCT CTAGGAAGTGGGCGGATCCT	UDP3 transferase	1500	1065
g	Synpcc7942_1735F Synpcc7942_1735R	ATGAGCGCCACGGTGCAAGC TTAGCCGACGGAGCCTTCCA	sufB	1800	1437
h	Synpcc7942_2038F Synpcc7942_2038R	ATGGAGTCGCGGACGCTGA TTATTCCACATCTTCTGAGC	Transcription regulator	2100	690
i	Synpcc7942_2333F Synpcc7942_2333R	ATGACGACCCTACTCCCCT TTAGCGTGGGTGCGTTGCC	opcA	2400	1335
j	Synpcc7942_2535F Synpcc7942_2535R	TTGGCATTGTAAAAAGCC TCAGCTGCGGAGCTGACG	GGDEF PAS/Pac	2600	3297

ORI: Origin of Replication.

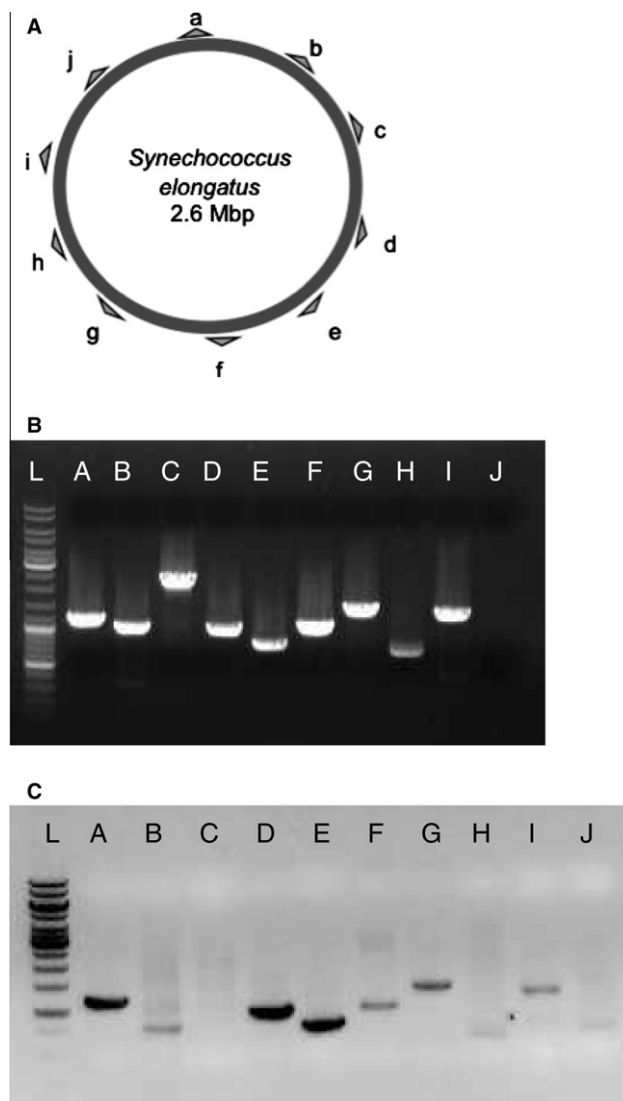


Fig.4. Primers for genome coverage test: (A) positions of genes used for WGA tiled across the circular *S. elongatus* 7942 genome at approximately 300-kbp intervals; (B) test amplification of selected genes from genomic DNA using specific primers presented in Table 1; (C) genome coverage test of specific genes, using primer sequences in Table 1, from 25 cells of *S. elongatus* 7942. L, O’GeneRuler (Fermentas).

electrophoretic separation, and gene amplification (Fig. 2A). To perform both protein separation and gene amplification from the same cells, it was critical to minimize undesirable dilution of the cellular contents during the first manipulation step of cell lysis. Thus, the whole volume of the cell manipulation section, confined by valves 2, 3, 4, 7, and 8, was restricted to approximately 500 pl. Areas confined by valves 1 and 2 were used for injection plug formation instead of using a complicated three-state valve.

We separated proteins from genomic DNA based on their large differences in diffusion coefficient; the molecular weight of PBS (~6 MDa) is much smaller than that of the whole genome (~2.7 Mbp, roughly equal to 1800 MDa). Because phycobiliproteins are strongly autofluorescent, the process of injection plug formation could be monitored before conducting protein separation. In our microdevice, the manipulation section geometrically divided the cell lysate into two fractions, where 50 and 450 pl of cell lysate were employed for protein separation and genome amplification, respectively.

For preconditioning, the separation section was first loaded with CE buffer (Fig. 2B). Then approximately 5 μ l of cell suspension (as mentioned in Section 2) was immediately loaded into the microfluidic device and cell capture was promptly performed (Fig. 2C). We took advantage of the autofluorescence of PBS within individual cells to monitor the injection plug formation in real time and count the total number of cyanobacteria being captured inside the chamber confined by valves 2, 3, 4, 7, and 8. Moreover, by opening valves 3 and 7, it was possible to repeat the capture process until we had captured the designated amount of cells inside the chamber. By manipulating the valves, we were able to selectively capture 10 or 25 cells, depending on the number needed for the experiment (Fig. 2D).

Captured cells were lysed within the chamber while valve 2 was opened. The PBS proteins were allowed to diffuse for 1 h into the area between valves 1 and 2 (Fig. 2E and F), which formed the injection plug when both valves were closed. For the analysis of genomic DNA, the remaining cell lysate (~450 pl) was pushed by compressed air at approximately 5 psi into a 3.5-nl denaturing chamber (confined by valves 8 and 9) that contained a denaturation and lysis buffer (DLB from a REPLI-g kit, Qiagen) (Fig. 2G). Once the chamber was completely filled with buffer, entrance valve 8 was closed (Fig. 2H). Denaturation is a critical step in the WGA reaction so that primers can be annealed to single-stranded DNA templates in a basic solution for subsequent amplification, and the process took 1.5 h to complete at room temperature.

During the denaturing process required, protein analysis was performed concurrently by CE. In the separation section of Fig. 2H, channels involved in electrophoretic separation were then preconditioned by the separation buffer (20 mM Hepes [pH 7.4], 0.1 wt% *n*-dodecyl- β -D-maltoside [DDM], and 0.045 wt% sodium dodecyl sulfate [SDS]) to support electroosmotic flow. The separation used electrical field strengths of approximately 250 V/cm in our experiments (Fig. 2I). At the end of the separation channel, an excitation laser beam (532 nm diode-pumped-frequency-doubled Nd:YAG laser, Compass 215M) was focused using a cylindrical lens at the detection point (2 cm away from valve 1) to form a line at the back focal plane of the 40 \times objective [2]. The fluorescence from molecules that passed through the detection region was recorded by an electron-multiplying charge-coupled device (EMCCD) camera (iXon^{EM} + DU897, Andor Technology) mounted on an inverted microscope (Nikon TE2000-U). The phycobiliprotein complexes from the cell lysate were separated and formed three major peaks in the electropherogram (Fig. 3). The first peak was the APC-containing species, the second peak consisted of the PC species, and the last peak that appeared after 40 s was chlorophylls (Chl) from photosystem II. These peaks have been identified in previous studies [2,10].

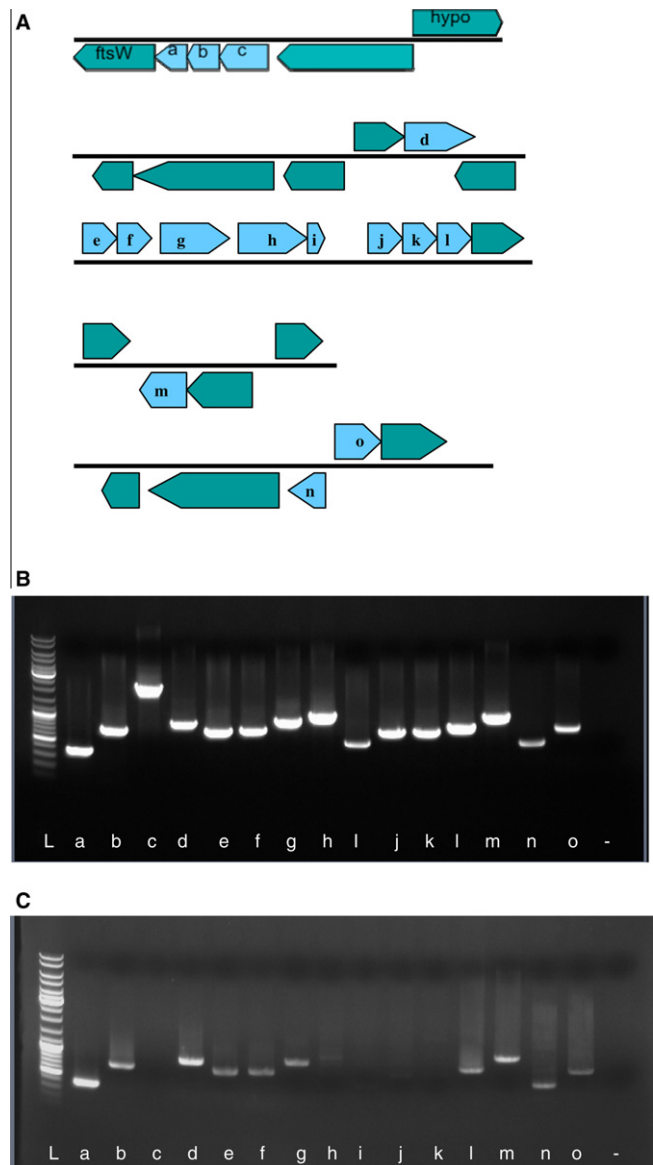


Fig. 5. Primers for amplification of adjacent PC and APC genes: (A) gene neighborhoods of PC and APC genes in *S. elongatus* PCC 7942; (B) test amplification of selected genes from genomic DNA using specific primers presented in Table 2; (C) amplification of adjacent PC and APC genes, using primer sequences in Table 2, from 10 cells of *S. elongatus* PCC 7942. L, O'GeneRuler (Fermentas).

After 1.5 h of incubation for denaturation, 3.5 nl of stop buffer was added in the same way as the DLB was added and the whole device was incubated at room temperature for an additional 1 h (Fig. 2J and K). The stop solution was used to neutralize the strongly basic denaturing buffer. Finally, 50 nl of Master Mix supplemented with 0.2% Tween 20 and 2 \times concentrated Phi29 polymerase (compared with the recommended protocol) was added (Fig. 2L and M) to carry out the WGA reaction [3]. The device was further incubated for 18 h on a hotplate preset at 30 $^{\circ}$ C (Fig. 2N). WGA uses Phi29 polymerases because they are uniquely and highly processive and accurate and they can generate long DNA fragments. Moreover, the strand displacement activity allows the WGA reactions to be implemented isothermally at 30 $^{\circ}$ C. Amplified DNA was pushed with 5 μ l of TE buffer (10 mM Tris [pH 7.5] and 1 mM ethylenediaminetetraacetic acid [EDTA]) toward the device outlet and collected by a gel-loading pipet tip.

To evaluate the genome coverage performance of the WGA reaction on-chip, we collected amplified DNA from the integrated

Table 2
Primers used and their properties for amplification of adjacent PC and APC genes.

	Primer name	Sequence	Annotation	Size (bp)
a	Synpcc7942_0325F Synpcc7942_0325R	CAGCTACTGAGTCTAGGGAATGG GGAGGGCAAGACTCATAGGG	APC beta subunit	390
b	Synpcc7942_0327F Synpcc7942_0327R	GTGAACGGTTTGCTGACTT CGCCACTTTGGAAGTAGCTC	APC alpha chain	737
c	Synpcc7942_0328F Synpcc7942_0328R	GGGAATCTCCATCGCTCTT AATGCCCAAAAAGCTGTGAG	Phycobilisome core-membrane linker polypeptide	2264
d	Synpcc7942_0802F Synpcc7942_0802R	CATCAGCACCTTCGTTGAGA TTGCCTTTCTCACGACCTTT	APC alpha chain-like	922
e	Synpcc7942_1047F Synpcc7942_1047R	CCCTGCTAGAGAATGCGAAA GGAAACGACCAAATGCAACT	PC beta subunit	752
f	Synpcc7942_1048F Synpcc7942_1048R	TCACCAAGGTGACTGTTC TATAGCCGAGAATCCGAGA	PC alpha subunit	771
g	Synpcc7942_1049F Synpcc7942_1049R	AATCATGATCAACCCGAAA TGCAACAGTAATTGCCATCG	Phycobilisome rod linker polypeptide	981
h	Synpcc7942_1050F Synpcc7942_1050R	AGCTGAATGCAACTCTGCAA ACTAGCGACGGCACCCTAC	Phycobilisome rod linker polypeptide	1127
i	Synpcc7942_1051F Synpcc7942_1051R	CAACACTTGGAAACAGGTCA TAGCGGGCTATGGTCTCAGT	PC linker protein 9K	558
j	Synpcc7942_1052F Synpcc7942_1052R	ATCAAACTCAGCCGCTGTT GGAAACGACCAAATGCAACT	PC beta subunit	752
k	Synpcc7942_1053F Synpcc7942_1053R	TCACCAAGGTGACTGTTC CGATCGCTTGTTCGTAGGTC	PC alpha subunit	762
l	Synpcc7942_1055F Synpcc7942_1055R	CTCAAAGGGCTGCTCGATAC GACCGCAGGAGATCTAAAC	PC alpha subunit phycocyanobilin lyase	860
m	Synpcc7942_2030F Synpcc7942_2030R	CCGACAGTGAACCCCTAAAA TCAGTCTGATTGCGAGTTGG	Phycobilisome rod-core linker polypeptide	1119
n	Synpcc7942_2127F Synpcc7942_2127R	GTCACGCCAATTCTGTAGCA GGTCAAGTCCAAGACGGTGT	Phycobilisome degradation protein NblA	559
o	Synpcc7942_2158F Synpcc7942_2158R	AAGCCTGAGTGTGCCATCTT ACGTACTTGGGGAGTTGTCG	Thioredoxin	804

microfluidic chip and followed it up with an off-chip WGA step. To assess the efficiency of the WGA step, we selected 10 genes that were distributed at equal intervals (~300 Kbp apart) around the 3-Mbp circular genome (Fig. 4A and Table 1). These primers were first tested on genomic DNA, and we got a single band from each one (Fig. 4B). Typically we found that seven or eight of the genes were amplified from any sample in replicate experiments, although which genes were amplified did not follow any predictable pattern (Fig. 4C).

We tried the WGA with either 10 or 25 cells and found that we consistently obtained more complete coverage of the genome with the 25-cell sample than with 10 cells. This finding is also consistent with other reports in which the WGA reaction is more reproducible when a greater number of cells (i.e., more template DNA) is present in the chamber [13]. We conclude that using a small number of clonal cells for WGA, as opposed to just one cell, can promote more complete WGA.

It is known that WGA is prone to certain artifacts [14] and amplification is less efficient in certain genomic regions [15]. To assess whether several adjacent genes in a region of interest could be targeted for PCR analysis following WGA, we investigated several genomic regions that contain the genes encoding APC and PC. These genes include the alpha and beta subunits of APC and PC as well as the linker peptides to anchor these subunits. There are five clusters that include these genes (Fig. 5A and Table 2). In two cases, there are several genes in clusters or operons; in other cases, there are single genes or 2 genes in close proximity. Most of these genes were checked by PCR following WGA (Fig. 5B). Of the 15 genes, 11 gave a positive result from the integrated microfluidic chip (Fig. 5C).

Our integrated microfluidic device demonstrated the capability of performing both WGA and protein separation from a few cells. We reduced analyte dilution by manipulating a small amount of cell lysate and further separated genomic DNA and protein content based on diffusion in a picoliter-sized microfluidic chamber. Nevertheless, in our current design, the circuitry required for cell

manipulation and content separation inevitably caused a certain degree of analyte dilution that jeopardized the feasibility of gene and protein analysis at a single cell level. Other techniques such as protein precipitation can be incorporated within our device for better separation and minimized dilution of genomic DNA from cell lysate [16], and we believe that the total analysis of single cell content can be realized in the near future.

Conclusion

We have described the first integrated microfluidic device capable of performing both gene amplification and protein separation from the same cohort of cyanobacterial cells. We took advantage of microfluidics to precisely manipulate the cell lysate for on-chip WGA as well as protein separation. The success of on-chip gene amplification was further evaluated by PCR and gel electrophoresis. Moreover, chip-based CE served as a useful tool for rapid protein separation analysis. The current microfluidic device is just the first prototype of its kind. For future applications, it may be possible to integrate protein labeling in the device so that nonfluorescent proteins can also be studied [17]. This technology may open the door to studying the protein profiles of precious environmental microbial samples and simultaneously access some genomic information based on WGA [18]. As methods to cultivate microbial samples improve, one can envisage coupling these methods with an integrated microfluidic device such as that described here.

Acknowledgments

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