Fluid Patterning in a Cavity Array for High-Throughput Screening and Biotechnological Applications

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Abstract

Over the past few decades, fundamental biological understanding has advanced significantly with the help of experimental biotechnologies. Among them, synthetic biology as a rising research field has shown its capability for building new microorganisms from the scratch. In conjunction with synthetic biology, directed evolution techniques seem to be highly useful for industrial purposes such as the over-production of chemical products such as biofuels, which are expected to resolve global energy problems. However, it still requires a high-throughput screening technique and/or compartmentalized environments for cell sorting. In this thesis, two microfluidic technologies are described. First, a novel microdroplet trapping technology is developed that utilizes the difference of specific gravity between two immiscible fluids to offer simple and easy manipulation of microdroplets for time-traceable single microorganism analysis. Second, a high-throughput screening technology is developed by patterning fluid, in which individual *Escherichia coli* cells can be immobilized and cultured in a cavity array format. It is noted that the cavities were coated with parylene and bonded with another parylene layer to secure chemical compatibility. In addition, it was successfully demonstrated that the two technologies hold a high potential to enable not only high-throughput screening but also many biological experiments such as detection of cell-excreted products, long-term cell incubation, cell to cell communication, and detection of target molecules via a whole cell biosensor.

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Chapter I. Introduction

1.1 High-throughput screening for directed evolution

Recently, our understanding of complex genomes, proteomes, bio-molecules, and even many metabolic pathways has been developed significantly as growing a fundamental knowledge of the biochemistry of life. This knowledge enlightened us for new studies of multifaceted biological systems and their evolutionary point of view. Also, these new findings have incredibly influenced in recent bio-technologies, such as synthetic biology having possibly massive potential to solve the global energy problem which is an urgent need for environmentally friendly biofuel energy.

In order to fulfill such needs for the production of biofuel, directed evolution approaches have been introduced to the microbial field [1] to improve the productivity of target chemicals or bio-molecules. Over the last decades, the directed evolution method has proven to be highly useful for both protein engineering [2] and industrial purposes such as the over-production of various chemicals [3-5]. Also, the complex system of metabolic pathway to produce such desired products requires accurate and precise biological experiments. However, most of these methodological approaches are still crucially limited by their need for high-throughput measurements of either target molecule reactions or a desired phenotype among a massive randomly mutated microorganism library.

1.2 Conventional high-throughput screening technologies

When the artificial and random mutagenic reaction is treated on a target microbe, the population of the random mutagenic library usually exceeds more than $10^{5\sim9}$ variants [6]. This massive number of samples is almost impossible to control with conventional experimental instruments such as test tubes, petri dishes, hand pipettes and microplate readers [7]. As discussion of microplate reader which is most widely used in the real laboratory environment, the instrument considerably shows fair sensitivity and good screening performance than hand-based equipment. However, the plate reader shows low-throughput performance because the time-limiting step for assay is ultimately restricted by the microplate reading speed of the instrument. Therefore, some studies have attempted to handle the large population of mutated cells at the genetic level as an alternative. For example, systematic evolution of ligands by exponential enrichment (SELEX) uses the selection force of high-affinity binding and purification of 10^6 nucleic acids variants with its conjugated ligands on a chip basis [8].

Fluorescence-activated cell sorting (FACS) technology was recently introduced [9] for high-throughput screening in the working range of $10^{4\sim6}$ variants [10]. This flow cytometry technique is based on lasers, so the scanning rate can be significantly increased, compared with other conventional experimental methods. Unfortunately, this FACS technology also has some disadvantages. It is very hard to observe individual cell behavior and measure the cell productivity, since only an aggregate level of results can be obtained from FACS analysis. Also, as the flow cytometry technique requires a fluorescence signal inside the cellular membrane, it restricts the analysis to only cells showing higher fluorescence intensity, and is not applicable to the measurement of products from individual bacteria.

Table 1. Comparison of various conventional screening methods.

	Method	Screening performance (Throughput)	Noted	
			Advantages	**Disadvantages**
Conventional hand-based equipment	Test tubes, flasks	$10^{1\sim3}$	Massive absolute sample number (good for mutation)	Require for many labor
	Petri dishes	$10^{2\sim4}$	Detectable with single colony-based growth and expression	Require for many labor
Conventional instrumental –based equipment	Microplate reader	$10^{3\sim4}$	Most widely used	Limited by a plate reading instrument
	GC / LC	$10^{2\sim3}$	Available for post product assay	Low-throughput / limited by analysis instrument
	FACS	$10^{4\sim7}$	High-throughput	Only detectable with fluorescence intensity

1.3 Microfluidic-based fluid patterning technologies

For the past decade, microfluidics has achieved great improvements with the potential to revolutionize high throughput biological assays [11]. Since this microfluidic technique is a suitable platform for patterning liquid with a fully compartmentalized environment and high-throughput performance, many applications were developed to be an appropriate candidate for a biological assay, for example droplet-based microfluidics [12, 13], small bioreactors using complex pneumatic valves [14] and liquid patterning of aqueous phase by immiscible oil separation [15, 16]. These microfluidic platforms provide the enormous benefit of compatibility with fully automated experimental systems, minimized scaling of dimensions, reduced reagent consumption and shortened reaction time.

Because of the advantages listed above, some researchers developed novel microfluidic devices for liquid patterning, in other words, digitization of sample [15], self-priming [16], and droplet patterning [12,13]. Since these liquid patterning technologies fulfill many requirements of the directed evolution by random mutation method, the device can provide a high-throughput screening technique as well as a fully compartmentalized environment for the detection of microbial secreted chemical products.
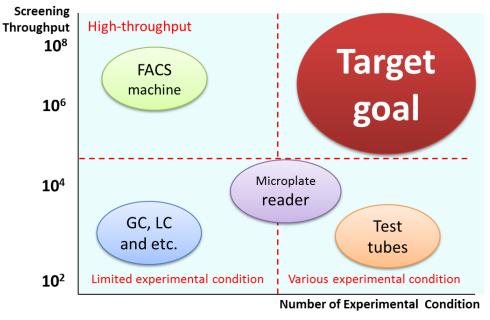
Figure 1 *A hierarchical schematic plot of the comparison with various methods for screening technique in biological research field. Those are categorized in four conditions indicated by x-axis showing availability various experimental condition on the method whereas y-axis indicates screening throughput performance.*

Chapter II. Patterning Microdroplet

2.1 Patterning a microdroplet in microfluidics

Microdroplets were widely used as a great and latent tool for many biological fields including microorganism symbiosis [17], MIC (Minimal Inhibition Concentration) research [18], heterogeneous enzymatic assays [19] as well as molecular works such as high-throughput PCR reactions [20]. However, one of the important issues on the microdroplet in the practical field is a possibility to monitor and identify each microdroplet during long-term and complex experimental process. Owing to the micro-scale small size, random movement of the droplets and complex liquidity, it was considered as a challenge to trap the droplets with incubation or chemical reaction to detect and monitor the experimental processes.

2.2 Trapping of microdroplet on a chip

In order to handle the issue, many research groups have tried to develop an effective method to immobilize the microdroplet with various trapping device. Edd *et al.* trapped microdroplets in a way of sequential manner using orifice structures which are exactly fitted with the target droplet diameter [21]. Huebner *et al.* have reported a two dimensional trapping structure successfully captured droplets, however, caused a serious shrinkage of droplet [22]. Although the trapping devices developed from both Huebner *et al.* [22] and Tan *et al.* [23] showed an excellent droplet trapping efficiency, those devices had disadvantages in device design dependent and not suitable for high-throughput assay. Here, an integrated, versatile microfluidic system is introduced that produces picoliter droplets, traps them on a microcavity array located in a downstream area and then agitates them in a programmable format.

2.3 Experimental setup and methods

2.2.1 Reagents and instruments

Most of oils and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) such as mineral oil, hydrocarbon oil hexadecane, perfluorinated oil FC-40, SPAN 80™ surfactant and trichloro (1H,1H,2H,2H-perfluorooctyl) silane and used without further purification. Polydimethylsiloxane (PDMS, Sylgard 184 silicone elastomer kit) was purchased from Dow Corning (Midland, MI). A FITC fluorescence labeling kit, for visualization of microscopic level, was purchased from Invitrogen (Carlsbad, CA). For microfabrication, oxygen plasma generator CUTE was used from microTECH (Suwon, Korea). Two syringe pumps were prepared from HAVARD APPARATUS (Boston, MA) to provide precise flow rate into the channels. Each device was tightly connected with Tygon® flexible tube purchased from U.S. Plastic Corp (Lima, OH). In order to measure cell density accurately, spectrophotometer Libra S22 was used from Biochrom Inc. (Cambridge, U.K.). All microscopic images were taken with Olympus IX-70 inverted microscope purchased from Olympus America (Melville, NY) installed with X-Cite fluorescence light source.

2.2.2. Fabrication of the microfluidic device

The device fabrication in this work consisted of three steps: device designation, photo-resist fabrication, combination process of two layer of devices into the connected device. Firstly, each of two microchannels, indicated as "top" and "bottom", were specifically designed in order to provide a microfluidic droplet generator at the upstream region of the top device and thousands of microcavities at the downstream region of the bottom device. At the entrance to the microcavities, flow focusing-shaped microfluidic structures were designed with 50~60 μm of widths in order to precisely control the droplet production rate as well as the target droplet volume. Second, photomask that were provided from microTECH (Ansan, Korea) were used in the photo fabrication process. Briefly, a SU-8 2050 master approximately 80 μm thick was fabricated by following the standard photolithographic procedure. Next, a collimated UV light exposed on the silicon wafer using a mask aligner (MA6, SUSS MicroTec, Germany). Trichloro(3,3,3-trifluoropropyl)silane was used for the silanization process of the channel surface within a vacuum jar for an hour in order to minimise the adhesion between the PDMS pre-polymer against the substrate. Poly(dimethylsiloxane) (PDMS) was then cast at room temperature, cured in the 70 ℃ drying oven, and gently peeled off. Both top and bottom PDMS devices were directly bonded each other with the oxygen plasma treatment under 50 $sccm$ of O_2 and 70 W for 90 s (Cute-MP, Femto Science, Korea). Then the combined device were stored in an oven at the temperature of 80 °. This high-temperature treatment was done to make the surfaces of the PDMS channel hydrophobic so that any oil phase flowed along the channel easily.

2.2.3 Experimental procedure and data analysis

The microchannels were filled with hexadecane solution with 2 % Span 80™. Each inlets, for both discontinuous aqueous phase and continuous oil phase, were tightly connected with Tygon® flexible tubes. Using a pair of syringe pumps, it was possible to fully control the production rate and the volume of droplets by varying flow rates to two inlets. Once the droplet generation was done on the top layer while the immobilization of droplets was done at the bottom layer where the microcavity array was fabricated, droplets were sequentially trapped into microcavities achieved by the difference of specific gravity between immiscible substrates. The IX71 microscope from Olympus (Japan) was used for whole imaging process which equipped with a CCD camera from Andor Tech (Clara, CA). A Metamorph 7.7 from MDS Analytical Technologies (Sunnyvale, CA) was used to take the images of microdroplets. All images processing were performed using Image J from NIH (USA) and the quantification of microorganism results were plotted using Origin 8.0 from OriginLab (Northampton, MA).
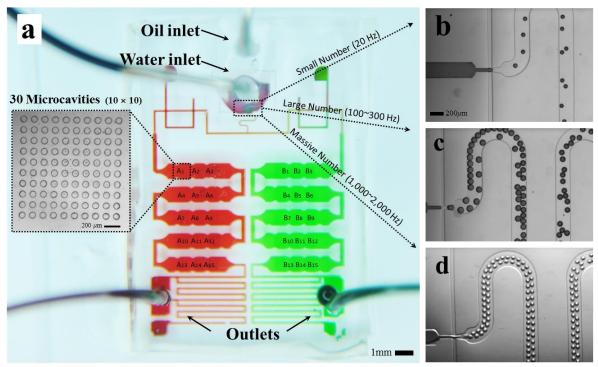

Figure 2.1 *A microfluidic device for high-throughput array. (a) A real figure of the two layered microfluidic device and 30 sets of 100 microcavites. (b-d) Various droplet production mode by varying the flow rates of oil and water inlets.*

2.3 Results

2.3.1 Calibration of microdroplet production rate

The droplet production rate characterised by various combination of flow rates. When the condition for target droplet volume is fixed in approximately 100 μm diameter, which is sufficient for the cell incubation experiment, the droplet production rate was depend on the flow velocity at the flow focusing structure [24]. Flow velocity in the experiment is composed of continuous oil phase flow rate and discontinuous aqueous phase flow rate. *Figure 2.2* shows a linear correlation between the flow velocity and the droplet production rate. The higher flow velocity at the droplet generator structure showed the higher droplet production rate (*Table 2*). The flow rate ratios of discontinuous and continuous phase were basically set as from 1/10 to 1/5.
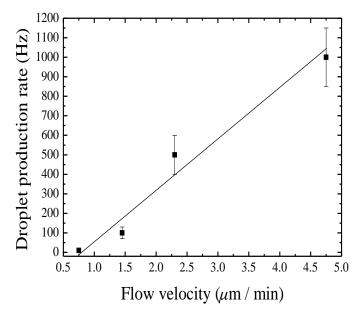
Figure 2.2 *A plot for droplet production rate by precisely controlling flow velocity at the flow-focusing structure.*

Table 2 *A droplet production rate with many values regarding flow velocities from discontinuous and continuous flow rate.*

Production rate(Hz)	Continuous flow rate(μL/min)	Discontinuous flow rate (μL/min)	Flow velocity (μm/min)
10 ($\pm$5)	3	0.3	0.75×10^6
100 ($\pm$30)	5	0.6	1.45×10^6
500 ($\pm$100)	8	1.2	2.3×10^6
1000 ($\pm$200)	12	3	4.75×10^6

2.3.2 Droplet trapping in a cavity array

As briefly described in *Chapter 2.2.3*, droplets can be sinking down to the microcavities because the specific gravity of hexadecane (0.77) and water (1.00) are substantially different. In the hexadecane, the formed water-based droplets should be sequentially trapped into microcavities. In order to obtain actual three dimensional images for the droplet immobilization, Olympus MVX-100 confocal microscope was used in *Figure 2.3b*.

The concept of an immobilization process of droplets in the downstream were shown in *Figure 2.3* in a chronological manner. Since the diameter of microdroplet was approximately 100 μm which is marginally smaller than microchannel, droplet were slightly squeezed and moved along with the channel. When the squeezed droplet reached microcavities on the bottom device, the droplet tended to be a spherical shape which is an original form of droplet. Spherical shape of droplet were stuck the edge of the microcavity, then, drown toward bottom by the difference of specific gravity mentioned earlier.
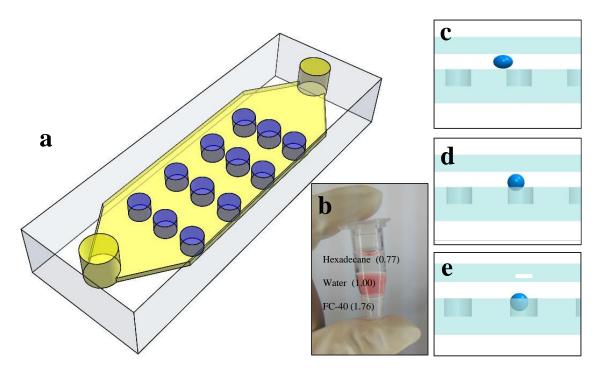
Figure 2.3 *(a) A device schematic concept using a simple fabrication of two layer PDMS devices. (b) The difference of specific gravity between hydrocarbon oil and water. (c-e) Sequential schematic images for droplet trapping in a microcavity.*

Interestingly the immobilized droplets were found to be agitated by oil flow over the microcavities. *Figure 2.4c-e* shows that the droplet containing 2 *μ*m diameter of fluorescence particles shows a trajectory indicating the agitation of liquid inside the droplet. It is a positive proof that the droplet rolling is not only happening on the surface, but also liquid inside. This agitation phenomena within a microcavity has highly co-relation with flow rate over the trap. It is very obvious that faster flow velocity of continuous oil phase drove faster agitation of the droplet inside the trap.
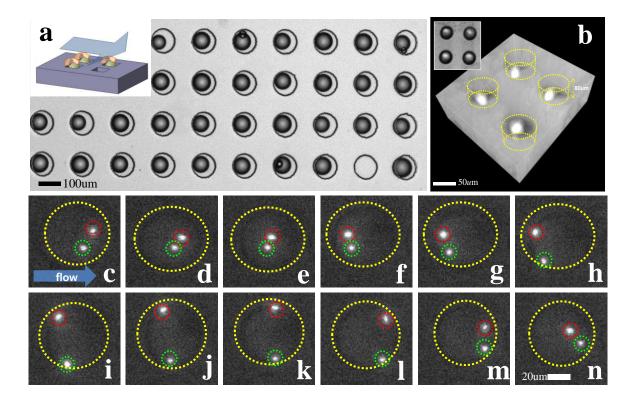
Figure 2.4 *(a) Microdroplet produced in the upstream region were sequentially trapped in microcavites. (b) A confocal image of immobilized droplet within a cavity(c-n) Time-lapse images show the agitation of water-based droplet by oil (hexadecane) flow at approximately 10~30 μL/min.*

2.3 Numerical analysis for agitation of microdroplet

A numerical simulation was performed regarding how the agitation of droplet works in a 2D model by using COMSOL software (version 4.3b). The coupled equations of Navier-Stokes [25] and Cahn-Hilliard [26] (Phase field method) are applied for the simulation experiment. In addition, level set method could provide similar results. *Figure 2.5* shows the flow velocity in the channel at a steady state, and the normalized arrows indicate the direction of the flow. The red circle represents the microdroplet which is rotating in the cavity. Based on the angular velocity of droplet rolling, the RPM value at different flow rates was calculated as shown in *Figure 2.6*.
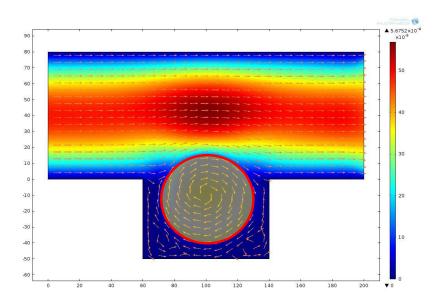
Figure 2.5 *A visualized COMSOL image of a trapped droplet rolling by a continuous flow over a trap.*
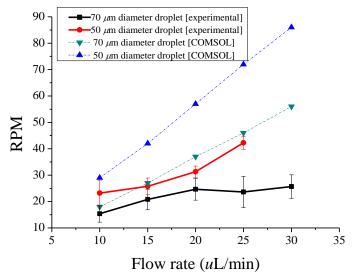
Figure 2.6 *Both experimental and computational simulation results show reasonable trend. Dotted lines indicates computational simulation results, whereas solid lines indicates experimental droplet agitation rate per minutes.*

2.3.4 Single cell level of cell encapsulation in microdroplet

In order to encapsulate a single number of cell inside the target droplet, a cell concentration was precisely changed by using the concept of optical density at 600 nm. In a microbiological field, it is very common that optical density is equivalent to the measurement of cell concentration [27-29]. Both *Figure 2.4* and *Table 3* directly indicate the correlation between the optical density and the cell encapsulation rate. Since encapsulation process using the optical density depends on statistics, it is nearly impossible to avoid small standard deviation among the numerous sample droplets.
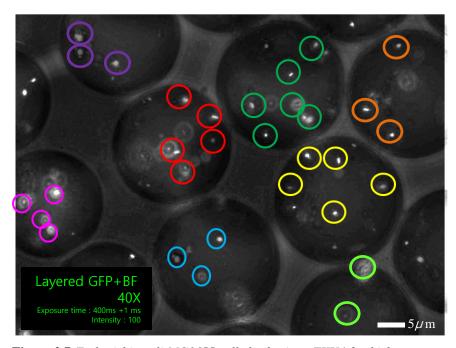
Figure 2.7 *Escherichia coli MG1655 cells harboring pTKU4-2 which expresses green fluorescence protein were encapsulated in each of droplets.*

Table 3 *Varying optical density of the target cell affects to the initial seeding number of pre-designed diameter of microdroplet.*

Initial seeding number	50μm diameter droplet (65 pL)	80μm diameter droplet (270 pL)	120μm diameter droplet (900 pL)
5 (±4)	7.0×10^{-2}	2.0×10^{-2}	5.0×10^{-3}
10 (±6)	0.15~0.2	4.0×10^{-2}	1.0×10^{-2}
100 (±30)	1.5~1.6	0.4	0.1~0.15

2.3.5 Biological applications in microdroplet platform

After successful cell encapsulation step above, it was experimentally tested cell induction by different concentration of inducer chemical. With having three different concentration of inducer, which are 0.2, 12.6 and 50 mM of propionate, the microdroplets were prepared with initial cell seeding number approximately 5 cells per droplet. In the droplet, it was encapsulated that *E. coli* harbouring 'pPro7(s)-GFP' plasmid which has a novel synthetic promoter induced by a certain concentration of propionic acid with a basal expression [30]. As can be seen in *Figure 2.5a-c*, different number of individual cell were gradually induced by different concentration of inducer chemical.
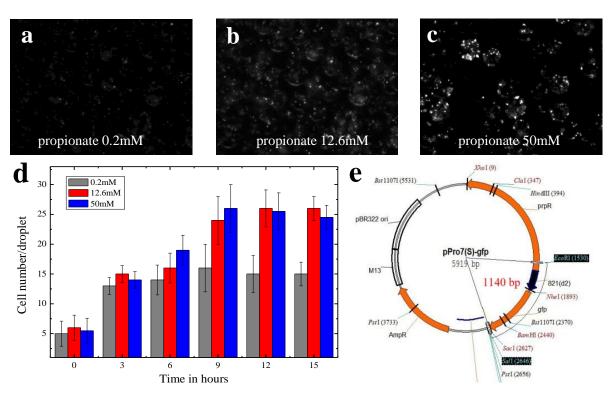
Figure 2.7 *(a-c) Actual microscopic figures by different concentration of inducer within droplet platform. (d) A plot for various concentration of propionate induced cell with sequential intensity. (e) E. coli harboring pPro7s-GFP expressing gradually inducible promoter driven by propionate concentration.*

2.4 Discussion

Microfluidic-based microdroplet patterning technology has attracted especially in biological fields, since it shows great potential to overcome conventional experimental limitations. In this study, the novel trapping technology have been described by using difference of specific gravity between two immiscible liquids. Compared to other droplet devices, this system provides droplet production, transportation and immobilization in the cavity to be integrated into a single set of combined device without any further complex valve system. The system developed in this study offers a simple and easy manipulation for the time-traceable single-organism analysis. Also, it provides the possibility to offer multi-purpose assays for other biological entities.

Chapter III. Fluid Patterning Array

3.1 Fluid patterning for high-throughput array

As discussed previously, synthetic biology can have benefits from the recent significant improvement in the development of microfluidic devices [31]. The novel perspective to study cells effectively was introduced by the microfluidics field. This technology provides the field to enable experiments on both a single- and multi-cellular level as well as the compartmentalization application of individual cell which was not available with conventional experimental instruments. An idea of microfluidic high-throughput compartmentalization device applied in various proto type of experimental forms [32-35]. Particularly, isolation and patterning liquid on a chip was intensively focused to research. Using two immiscible liquids, the concept of patterning liquid was told in many terms such as digitization, and self-priming.

Due to chemical compatibility of oil phase, there have been limited usages in the oil on the PDMS substrate. Representatively, hexadecane is well-known and most widely used oil in the fluid patterning field. However, the porous nature of PDMS, which points out a compatibility issue with some solvents, especially with hexadecane in this study, drives a drawback as the material of choice [36]. Such compatibility leads to swelling on PDMS substrate and causes the buckling and collapse of microchannels [37]. Even though, there have been many devices in the liquid patterning field for the actual biological purpose [15, 38, 39], none of those devices achieved a long-term cell biology experiment on a chip, but only sample preparation, PCR reaction in short time and the chromatography applications [16].

3.2 Parylene conformal coating for chemical durability

Due to having compatibility to small hydrophobic molecules, there have been studies introduced to solve this absorption problem by various modification of PDMS channel such as plasma treatment [40], polymer grafting [41], detergent coating, protein precoating [42], and silanization [43]. It was proposed that poly-*p*-xylylene (parylene) coating applies on microchannel to prevent such hexadecane absorption, because parylene can create non-porous transparent thin layer which prevent any chemical reaction including even gas transportation and solvent diffusion. However, parylene coated surface towards same parylene coated surface is known as difficult to make bonding each other, since parylene is not-reactable with any chemicals. Here, the novel bonding method is introduced between both parylene to parylene coated surface on patterned PDMS substrate in experimental session following.
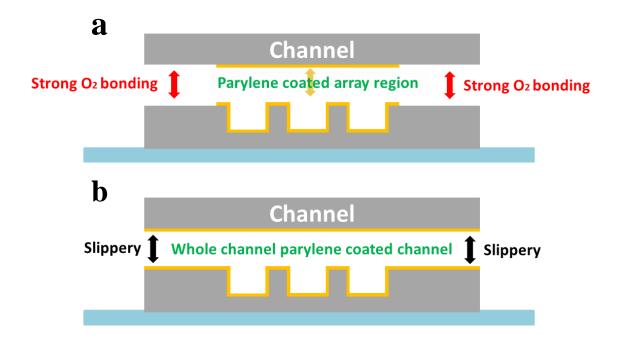

Figure 3.1 *Schematics of different parylene bonding methods. (a) Specific region parylene coated for liquid patterning allows rest of coated region to be strongly bonded by conventional oxygen plasma bonding method. (b) Previously, whole channel parylene coated channel has a difficulty in bonding due to slippery characterness compared to the new bonding method.*

3.3 Experimental setup and methods

3.3.1 Reagents

Most of oils and chemicals were purchased and used as described previously in *Session 2.2* without further purification. Additionally, in this part, Bovine serum albumin (BSA) and 1 X phosphate buffered saline were purchased from Sigma-Aldrich (St. Louis, MO). The BSA concentration in this study was 150 μM (10 mg/mL) unless mentioned specifically. FITC fluorescence labeling kit, which was purchased from Invitrogen (Carlsbad, CA), was dissolved into 10 mg/mL of BSA buffer to reach from 10 μM to 150 μM of its concentration.

For microfabrication, oxygen plasma generator CUTE was used from microTECH (Suwon, Korea). Two syringe pumps were prepared from HAVARD APPARATUS (Boston, MA) to provide precise flow rate into the channels. Each device was tightly connected with Tygon® flexible tube purchased from U.S. Plastic Corp (Lima, OH). In order to measure cell density accurately, spectrophotometer Libra S22 was used from Biochrom Inc. (Cambridge, U.K.). All microscopic images were taken with Olympus IX-70 inverted microscope purchased from Olympus America (Melville, NY) installed with X-Cite 120q LED fluorescence light source from Lumen dynamics (Livermore, CA).

3.3.2 Materials and preparation of cells

In this experiment, *E. coli* strain MG1655 harboring pTKU4-2 (constitutive expression of green fluorescence protein), *tet*R-fatty acid biosensor plasmid was used, which is originally derived from K-12 (a wild type strain). *E.coli* strain DH10B was randomly mutagenized by Tn5 transposon mutagenic kit purchased from Epicentre Biotechnologies (Madison, WI) with following commercial instruction. Also, DH10B without any engineering on genomic DNA was prepared for control experiment. A small colony of *E. coli* grown on a Luria-Bertani broth (LB) medium with 1% of agar plate was inoculated into 5 mL of LB liquid medium in a rotary shacking incubator (36 °C and 150 rpm) for overnight cultivation, then diluted for the OD_{600} (optical density at 600 nm) reading to be approximately 0.2.

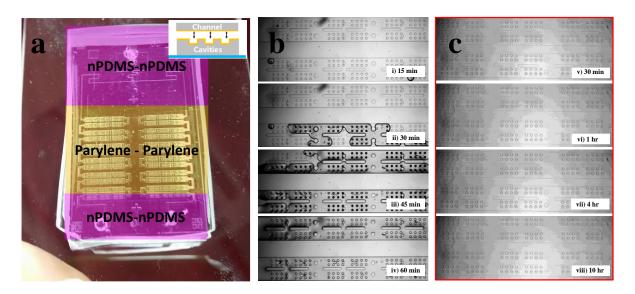*3.3.3 A novel parylene to parylene bonding method*

Figure 3.2 *(a) An actual figure of the parylene to parylene bonding device. Sequential images for parylene to parylene oil durability test. Two layer of Normal PDMS devices were filled with hydrocarbon oil, hexadecane. (b) normal PDMS shows significant adsorption to the hexadecane in 60 minutes. (c) whole parylene conformal coated microchannels were stood for more than 10 hours.*

The parylene conformal coating method was proved to be the successful material of choice to prevent chemical compatibility with many previous studies [44]. However, it is not an easy task to bond two parylene coated layers each other because the coated surface has the slippery character. Therefore, we put on parylene thin layer only on array region in the downstream, so that other part of device has not covered with parylene at all. As we prepared the device only coated on a certain region, oxygen plasma treatment on both top and bottom device were proceeded with 50 *sccm* of O_2 and 70 W for 30 *s*. Among normal PDMS substrates, the bonding process by oxygen plasma treatment works completely well [40]. After bonding two devices, we put the combined device into 165° degree of a furnace for 30 min to reach T_g for bonding with each other [44]. During the incubation in high temperature, an appropriate pressure were given to the device. After 30 min, the device was cooling down very slowly for 4 hours till the temperature reached back at 60 °degree. The combined parylene to parylene coated device were tested hexadecane durability experiments shown in *Figure 3.1*.

3.4 Results and discussion

3.4.1 Fluid patterning for a high-throughput screening assay

The combined parylene coated device offers a very simple method for patterning liquid samples into a three dimensional array with discrete volumes. A sample experiment were proceeded to confirm the performance on fluid patterning technology. Working mechanism can be briefly described that an aqueous sample was completely divided into an array of microcavities by flowing oil phase over the array itself. A time-lapse chorological images of liquid patterning process were shown in *Figure 3.3* below. 100 μM of FITC fluorescence chemical dye was prepared to be aqueous phase which is set as individually isolated arrays and hexadecane with 2 % Span 80^{TM} surfactant was used for the oil phase. The 3,000 chambers in the area of 500 μm × 500 μm were preliminarily designed for high-throughput purpose which can be extended for target experimental purposes. The 3,000 of microcavities were done to be liquid patterned array within only 10 sec with almost 100% of performance. (Except miss microfabricated arrays)
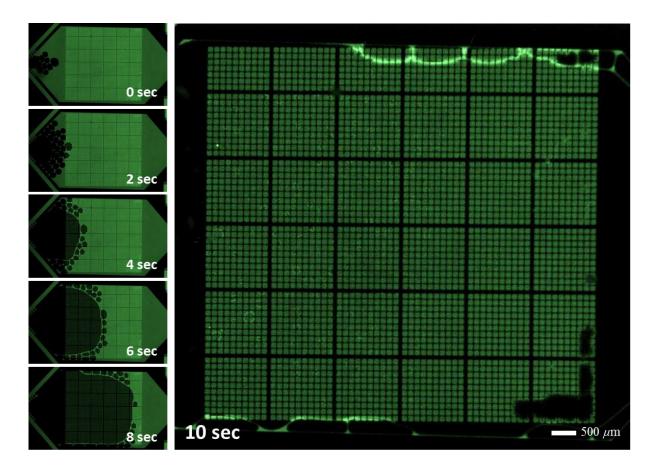

Figure 3.3 *Sequential images for 3,000 arrays of liquid patterning on a chip within only 10 second. For visualization, Fluorescence chemical (100 μM of FITC) used in the experiment. Hexadecane containing 2% of Span 80 surfactant was used to produce arrays as a separation oil phase.*

3.4.2 Characterization of array using food dye with various oil

Not only experimental tests on usage of hexadecane, other form of oils were also tested to confirm its performance for the purposes of both long-term and high-throughput screening array. As shown in *Figure 3.4*, fluorinated oil FC-40 and mineral oil were used in the liquid patterning experiments. Although those oils are known as immiscible to the aqueous phase in general, very small amount of oil can be dissolved into the water. Each of array consisted with food dye for visualization were dissolved out after four and five hours with mineral oil and FC-40.
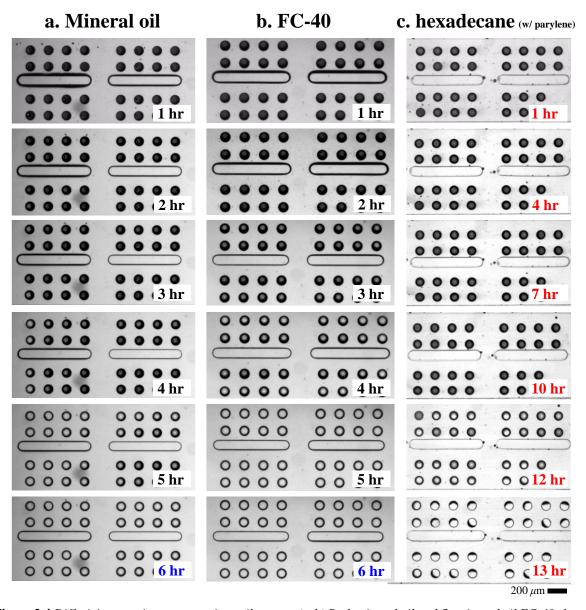

Figure 3.4 *Diffusivity experiments on various oil usages. (a-b) Both mineral oil and fluorinated oil FC-40 showed rapid diffusion to the aqueous phase. (c) Whereas hydrocarbon oil hexadecane showed slower diffusivity to the aqueous phase more than 10 hours.*

3.4.3 Effect of protein concentration on patterned fluid

It is recently reported that dissolved protein tends to relocate outward and near the surface when the phase consists of immiscible liquids.[45] Based on the report, we hypothesized that the protein concentration of aqueous phase may possibly prevent the diffusion of the phase in a patterned array. In order to prove the hypothesis, 100 μM of FITC fluorescence chemical dye were dissolved into 15, 10, 1, and 0 mg/mL of Bovine Serum Albumin (BSA) buffer. This BSA protein was selected because the protein is typically used as standard protein which is consists of 607 amino acids in its length. Then, liquid patterning procedures were proceeded by hexadecane with 2 μL/min of flow rate to prevent oil adsorption toward PDMS substrate. In the results, as protein concentration increased in array, the stability of array also increased together. It concludes that the higher protein concentration minimize an unwanted diffusion of aqueous phase within patterned array.
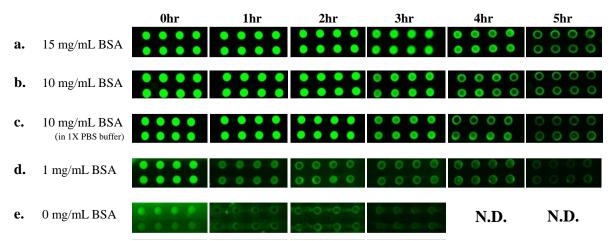

Figure 3.5 *An effect of protein concentration on stability of liquid patterned array. The higher concentration of BSA protein concentration showed the more stable status of liquid patterned array. 1X Phosphate buffered saline was added to confirm whether it can reduce diffusion problem. It seemed that 1X PBS does not affect on diffusion phenomena.*

3.4.4 An array for resveratrol over-production cell

In order to see the real feasibility of this high-throughput microfluidic platform for the multi-purpose as an array itself, engineered microorganism *E.coli* strain MG1655 having 'pET-opTLS' plasmid was incubated in a conventional test tube and injected into the liquid patterning device for the detection of resveratrol molecules. The plasmid 'pET-opTLS' is synthetic biologically engineered and synthesized for over-production of resveratrol (3,5,4'-trihydroxy-trans-stilbene) which is one of the stilbenoid phenol compounds known for increasing lifespan of model organisms [46]. There was a report that resveratrol compound can be detected using specific wavelength of the fluorescence filter. (Approximately excitation at 330 nm and emission at 385 nm) [47]

Although the results showed low-resolution of resveratrol intensity, after 36 hours of resveratrol production, the difference of the cell phenotype among four altered samples can be detectable compared to control without any engineering on genomic DNA. These experiments shows a strong potential of this integrated device to be the multi-purpose microfluidic platform.

a. MG1655 control   **b**. MG1655(pET-opTLS)   **c**. MG1655(pET-opTLS111)   **d**. MG1655(pET-TAL)
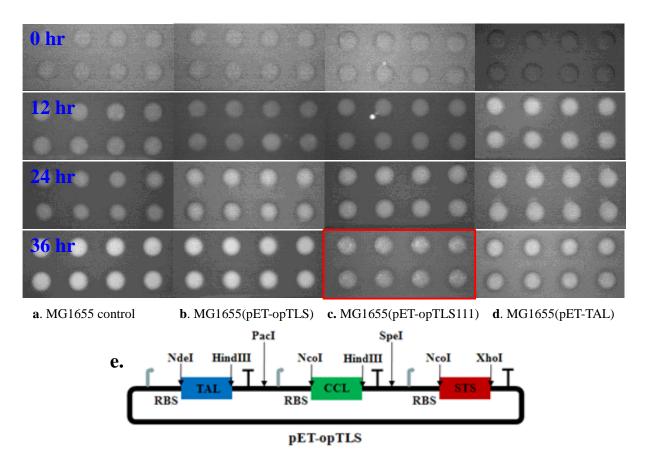
Figure 3.6 *(a) A control MG1655 strain. (b) MG1655 with pET-opTLS, this plasmid contains TAL, CCL, STS that are not E. coli originated gene. (c) pET-opTLS111, the most productive cell in producing cellular resveratrol chemical. (d) pET-TAL, engineered cell for over-production of precursor of resveratrol. (e) schematic plasmid map for pET-opTLS.*

3.4.5 Long-term cell cultivation in a patterned array

A long-term incubation is very important condition of experimental purpose for especially in the microbiology field. However, previously developed liquid patterning devices could not provide long-term experiments and only showed the experiment requires a short-term period such as PCR reactions due to chemical compatibility. Since the novel parylene to parylene bonding method was developed in this study as described in *Session 3.2.3*, we achieved more than 12 hours of the cell incubation experiment on a single chip. The target *E. coli* MG1655 cells harboring pTKU4-2 plasmid, which constitutively expresses green fluorescence protein, were patterned in arrays with nearly single number of initial seeding number. With more than 12 hours of time-lapse images, we speculated fluorescence intensity which directly indicates the cell growth in the liquid patterned arrays. Even it was possible to see the heterogeneity of a single colony seed cell as growing for long-term shown in *Figure 3.7d*.
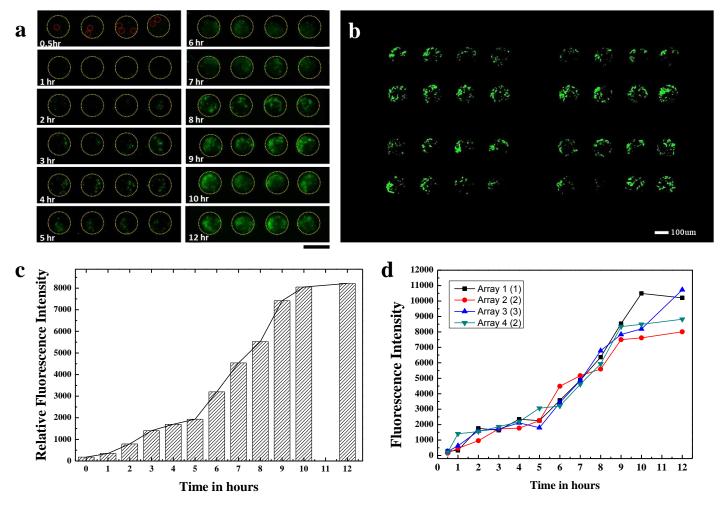

Figure 3.7 *(a) shows time-lapse images of E.coli growing in the patterned array. (b) A lower resolution image at 8 hours showed 32 arrays within an image. (c) Growth curve of target microorganism based on fluorescence intensity from constitutively expressed green fluorescence protein. (d) A plot for the heterogeneity of singular number of cell.*

3.4.6 An array for whole-cell biosensor for fatty acid

Fatty acid production from bacteria has attracted many interests from industrial field. Because fatty acid is famous for the precursor of many forms of biofuel. The fatty acid over-produced microorganism has been one of the goals in the industrial biology field for long time. In order to detect the production of fatty acid, many methods were introduced such as melanin-based [48] and Nile red based colorimetric [49] and post analysis using GC and LC [50]. Besides from previous methods, the whole-cell biosensor were introduced to the fields few years before with its higher resolution. Its working mechanism was based on *fad*R gene specificity to the cellular fatty acid production and the *fad*R relates with expression of red fluorescence protein [51].

In previous session 3.4.5, this device was proved to be the platform for both the long-term incubation and the detection of the signal from cell with high-resolution. Combining the fatty acid biosensor with liquid patterning device makes good synergetic effect in terms of experiment. We prepared two different *E.coli*, the one with control strain without any engineering and another strain has *fad*E gene knock-out region on genomic DNA which is well-known understanding that the knock-out region on *fad*E blocks *β*-oxidation pathway of fatty acid and induces over-production of fatty acid by the accumulation.

Two samples were injected into the device and speculated for 10 hours of measurement for the red fluorescence protein intensity. The measured fluorescence intensities at 10 hours of time point showed different results as expected with conventional experimental data shown in *Figure 3.7d*. Due to accumulation of fatty acid inside cellular membrane, biosensor was induced and clearly expressed more red fluorescence protein than control strain. This experiment proves that the combined parylene coated device can have great advantages in cell to cell communication experiments as well as fully compartmentalization of the individual array.

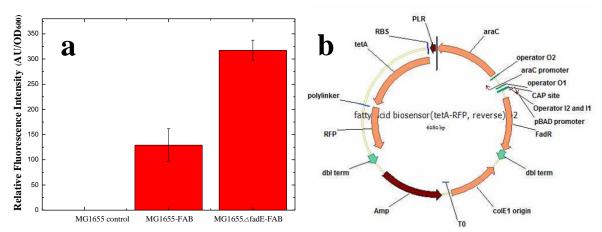**Figure 3.8** *(a) Fluorescence intensity measurement of synthetic biologically engineered fatty acid biosensor plasmid by the TECAN microplate reader. (b) A schematic plasmid map indicating genes and the promoter system of fatty acid biosensor.*

a. MG1655 control [Fatty acid Biosensor]   **b**. MG1655 ΔfadE [Fatty acid Biosensor]
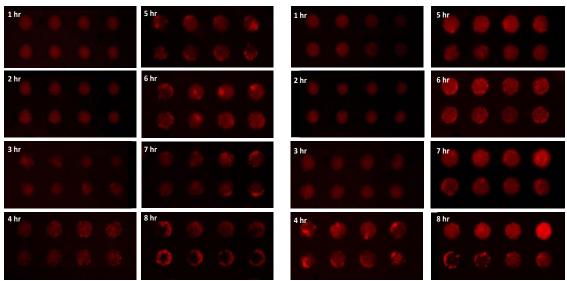

Figure 3.9 *Comparison with (a) MG1655 control and (b) engineered strain MG1655 ΔfadE. Both containing fatty acid biosensor plasmid which expresses red fluorescence protein by accumulation of cellular fatty acid. A noticeable difference between two samples was speculated based on microscopic images.*
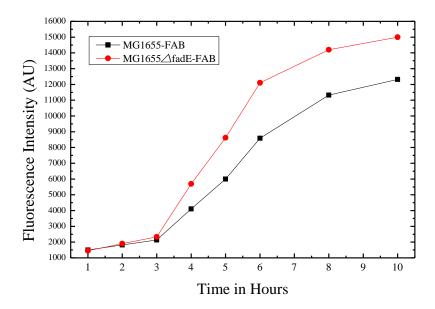

Figure 3.10 *A plot for growth curves based on fluorescence intensity from RFP-fatty acid biosensor for 10 hours of long-term incubation. The red line indicates the growth curve of engineered cell for over-production of fatty acid by gene deletion of fadE.*

3.4.7 Single cell array for Tn5 transposon random mutagenic library

The final purpose of this study is the screening one extraordinary sample out of numerous directed mutagenic library. In order to see the feasibility for screening of random mutagenic library, we prepared Tn5 transposon mutagenic *E. coli* strain DH10B. This transposon mutagenic method provides approximately 3 ~ 4,000 of random library by Tn5 transposon inserting into random locus of genomic DNA [52]. Whether the possibility to screen 4,000 of library works, we patterned arrays containing a single cell of DH10B Tn5 mutagenic with a single cell of DH10B harboring fatty acid biosensor plasmid. Based on statistics, around half of array do not have neither mutagenic cell nor biosensor cell. Among rest of array showing red fluorescence intensity, we could possibly screen the targeted cell which over-produces fatty acid than other mutagenic cell. Below figures only shows the feasibility of screening performance. Unfortunately, we cannot sure that there is an extraordinary mutagenic cell among nearly 4,000 DH10B Tn5 mutagenic library.
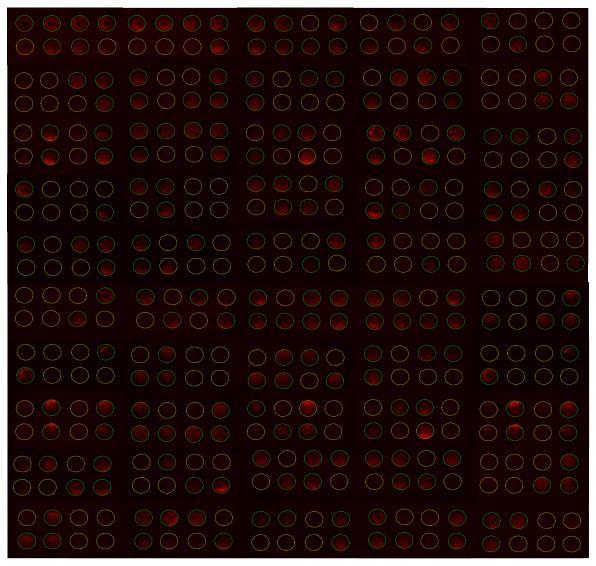
Figure 3.11 *The high-resolution stitched images for 400 individual liquid patterned cell arrays. 203/400(50.7 %) were filled with DH10B Tn5 transposon mutagenic cell and DH10B fatty acid sensor.*

Figure 3.12 *More than 200 of samples were analyzed to confirm the feasibility of high-throughput screening. Each of samples are randomly distributed within arrays. The red line indicates the background noise signal which does not have any cells in the patterned arrays.*

3.5 Discussion and future works

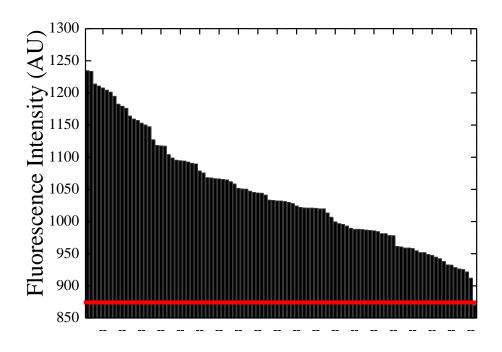In this study, it was demonstrated that the high-throughput screening array of *E. coli* individual cell can be achieved by using a very simple microfluidic device. Since the device consisted in double-layer of PDMS, chemical compatibility problems were increased as well. However, developing the novel parylene to parylene coating method conveyed many advantages in using hydrocarbon oil hexadecane which minimizes diffusion of aqueous phase. Using the device, we successfully tested many possibility such as detection the target chemical product within a chip, long-term cell cultivation of bacteria, whole-cell biosensor detection of random mutagenic library by cell to cell communication. And we were dealing with the actual screening technique by multi-aspect of microfluidic technologies to extract an extraordinary sample out of microchamber.

Chapter IV. Conclusion

Microfluidic-based patterning technology has attracted a lot of interest, especially in the synthetic biological fields, since it shows great potential to overcome conventional experimental limitations of throughput and compartmentalization. In this study, the two layered combined PDMS devices was developed that have multiple uses. Firstly, the novel droplet trapping technology was described by using the difference of specific gravity between two immiscible liquids. Compared to other droplet systems, the device provides droplet production, transportation, and immobilization in the cavity to be integrated into a single combined device without any further complex valve system. The system developed in this study offers a simple and easy manipulation for time-traceable single-organism analysis and only requires a small number of samples. In this work, the device demonstrated the possibility to offer multi-purpose assays for other biological resolves.

Secondly, liquid patterning device for the high-throughput screening array of individual E. coli cells could be achieved by using a very simple microfluidic device. It only requires 10 seconds to make 3,000 liquid patterned arrays which can be extended as required for the experimental purpose. Chemical compatibility problems were the bottleneck in the usage of the hydrocarbon oil hexadecane. However, we solved the compatibility problem by developing a novel parylene to parylene coating method conveying many advantages that minimize the diffusion of the aqueous phase. Using the device, we successfully proceeded many feasibilities such as detecting the target chemical product within a chip, long-term cell cultivation of bacteria, and whole-cell biosensor detection of random mutagenic library by cell to cell communication.

It is strongly believed that this liquid patterning technology can give us a candidates for the breakthrough in the biology field. The simple and multi-purpose microfluidic device was developed which can be used immediately in many actual biological experiments.

Chapter V. REFERENCES

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Chapter VI. Acknowledgements