Biochemical Reactions on a Microfluidic Chip Based on a Precise Fluidic Handling Method at the Nanoliter Scale

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Abstract A passive microfluidic delivery system using hydrophobic valving and pneumatic control was devised for microfluidic handling on a chip. The microfluidic metering, cutting, transport, and merging of two liquids on the chip were correctly performed. The error range of the accuracy of microfluid metering was below 4% on a 20 nL scale, which showed that microfluid was easily manipulated with the desired volume on a chip. For a study of the feasibility of biochemical reactions on the chip, a single enzymatic reaction, such as a β-galactosidase reaction, was performed. The detection limit of the substrate, *i.e.* fluorescein di-β-galactopyranoside (FDG) of the β-galactosidase (6.7 fM), was about 76 pM. Additionally, multiple biochemical reactions such as *in vitro* protein synthesis of enhanced green fluorescence protein (EGFP) were successfully demonstrated at the nanoliter scale, which suggests that our microfluidic chip can be applied not only to miniaturization of various biochemical reactions, but also to development of the microfluidic biochemical reaction system requiring a precise nano-scale control.

Keywords: microfluid, microfluidic handling, microfluidic chip, enzymatic reaction, *in vitro* protein synthesis

INTRODUCTION

Recently, the research area of a micro total analysis system (μ-TAS), named "lab on a chip (LOC)", through the miniaturization of analytical apparatuses and (bio)-chemical reactors, is developing rapidly [1,2]. The advantages of the microsystem include the consumption of a small amount of the sample or reagent, rapid reaction time, applications of hazardous reagents, lower operation cost than conventional instruments, and an increase of throughput compared to macroscopic instruments [1,2].

For the realization of the microsystem, the important hurdle facing the microfluidic system is the manipulation of microfluid on a chip [3]. Several methods have previously been proposed for the manipulation of microfluid; these methods include metering, transport, and mixing of the liquid. Theses techniques are mainly based upon electroosmotic flow [4], electrochemical properties [5], mechanical pumps [6], thermal capillary pumping [7], colloidal valves [8], and surface-directed liquid flow [3,9]. Regardless of the numerous trials, these methods had a weak point in terms of the parallel manipulation of microfluid due to the complicated operation and fabrication processes. Additionally, the real required microfluid volume compared to the volume of used liquid, the so-called "dead volume", was too large. Therefore, a microfluidic chip able to perform repeating and accurate operations without dead volume is needed for the analysis of (bio)chemical reactions. Recently, Quake's group presented the parallel manipulation of microfluid on a single platform, which was based on pneumatic valves and a compute-aided control system. However, this approach was needed to create a number of controllable pumps in consideration of the extremely small microfluidic platform [10,11].

In consideration of miniaturization at the micrometer scale and due to the dramatic increase in the surface-to-volume ratio, the surface tension and viscosity replaced gravity and inertia as the dominant forces in hydrodynamic behavior at the microscale. Therefore, the surface properties, especially surface wetting property, have crucial effects on the hydrodynamic behavior of microfluid in a microchannel. Recent studies [3,7] have curiously implied that hydrophilic and hydrophobic patterned surfaces could be applied to manipulate liquid motions in a microfluidic chip. Additionally, electrowetting methods for droplet dispensing, transport, merging, mixing, and splitting were demonstrated previously. Although the electrowetting method showed flexible droplet handling on a chip, it is necessary to fabricate a delicate, complex elec-

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tric circuit for easy manipulation of liquid drops. It is also quite difficult to measure the desired volume of droplets based on the needs of the user [12-14].

In our previous studies [15-17], we proposed a simple method of passive microfluidic handling, based on patterning surfaces through the lift-off process and pneumatic control, to control microfluid in a microchannel on a microfluidic chip. This method of passive microfluidic handling can offer several strong points, such as the lack of active microfluidic components, the presence of a minimal control system, ease of fabrication, and low power consumption [3,7,15-17]. Because a precise quantitative liquid handling technique for biochemical reaction is one of the essential microfluidic operations performed on a chip, the method of passive microfluidic control can also perform discrete microfluidic operations in microchannel networks. In this case, a hydrophobic surface is used as a valve to control the direction of a microfluid. Several methods using a hydrophobic surface and pneumatic control have been reported to confine liquids in a desired volume for given biochemical reactions [3-9]. However, microfluidic chips have limited metering because their volumes are commonly fixed in advance. If liquid could be manipulated to a desired volume on a chip, the method of passive microfluidic handling will confer advantages, such as the ability to vary the mixing ratios, and will carry out diverse kinetic studies on a chip.

Herein, we presented a microfluidic chip that could perform the rapid microscale analysis of single and multiple biochemical reactions with free manipulation of microfluid on the nanoliter scale. For the application of our microfluidic chip, a qualitative and quantitative single enzyme reaction, *i.e.*, β-galactosidase, and *in vitro* protein synthesis as a model system of multiple biochemical reactions were demonstrated on the nanoliter scale.

MATERIALS AND METHODS

Materials

The β-galactosidase was purchased from Roche Biochemicals (Basel, Switzerland). The fluorescence-di-β-D-galactopyranoside (FDG) was purchased from Sigma Chemicals (St. Louis, MO, USA). Primers were synthesized from Cosmo Corp. (Seoul, Korea). The plasmid pET 23b (+) (Novagen, Germany) was used for the construction of the plasmid-carrying EGFP gene. The structural genes of EGFP were amplified from pEGFP-1 (Clontech, CA, USA) by PCR using synthetic primers. For *in vitro* protein synthesis, an *E. coli* S 30 extract system for circular DNA was purchased from Promega (Madison, USA). Other chemicals of analytical or research grade were used.

The Fabrication Process of the Microfluidic Chip

The microfluidic chip was assembled with polydimethysiloxane (PDMS) microchannels as a lid and hydrophobic patterned glass substrate as the bottom portion.

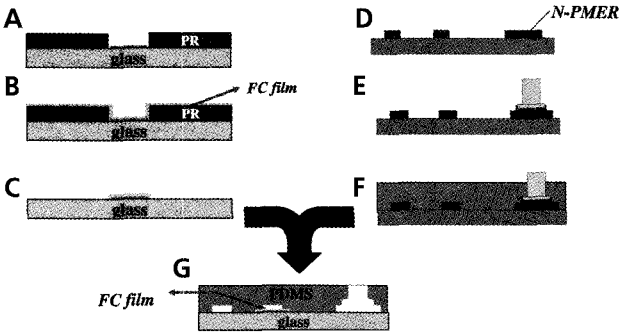

Fig. 1. The fabrication process of the microfluidic chip. A. Photoresist (PR) patterning for the definition of a hydrophobic region. B. Spin-coating of fluorocarbon (FC) and baking in a convection oven. C. FC patterning using the lift-off process. D. Silicon mold fabrication. E. Aligning and adhering metal couplers for connection hole formation. F. PDMS pouring and molding. G. Release of the PDMS replica mold and bonding of mold on the FC film patterned glass substrate.

The patterning method of hydrophobic fluorocarbon (FC) film was developed to control the potential surface wettability on a chip [15-17]. Figs. 1A to C show the hydrophobic thin film patterning on glass substrate using the lift-off method. First, a 2,000 Å aluminum film was patterned using a phosphoric acid-based etchant and a photoresist (PR) mask through the thermal evaporation process (Fig. 1A). Because the used hydrophobic thin film and PDMS lid were both transparent, the patterned aluminum was used as an alignment mark during the assembly process. To create the hydrophobic thin film pattern, positive photoresist (AZ 4330; Clariant Co., USA) was spin-coated onto a glass with a thickness of 3 μm and patterned using the conventional photolithographic method (Fig. 1A). A mixture of Fluorad™ FC 722 and FC 40 (3M, USA) was then spin-coated onto the glass wafer, which was baked in a convection oven at 110°C for 10 min (Fig. 1B). After baking, unnecessary FC film and PR patterns were removed using the lift-off process (Fig. 1C).

To create microfluidic channels, a PDMS lid with microchannel networks was fabricated based on a replica-molding method [18-20] according to the fabrication process flow shown in Figs. 1D to F. A mold was fabricated using a 50 μm thick N-PMER, negative photoresist (TOK Co., Japan) (Fig. 1D). Metal couplers were pasted onto the connection region to form tubing holes (Fig. 1E). For replica micromolding, a mixture of PDMS prepolymer and Sylgard 184 curing agent (Dow Corning, USA) was thoroughly mixed and then degassed in a vacuum chamber. The degassed PDMS mixture was poured onto the silicon master mold and cured for 4 h at 60°C (Fig. 1F). After curing, the PDMS replica was peeled away from the master mold. The PDMS structure was aligned and bonded to the glass wafer (Fig. 1G).

Fig. 2A shows schematic illustration that describes each part of the designed microchip. The microfluidic chip mainly consisted of two regions for microfluidic ma-

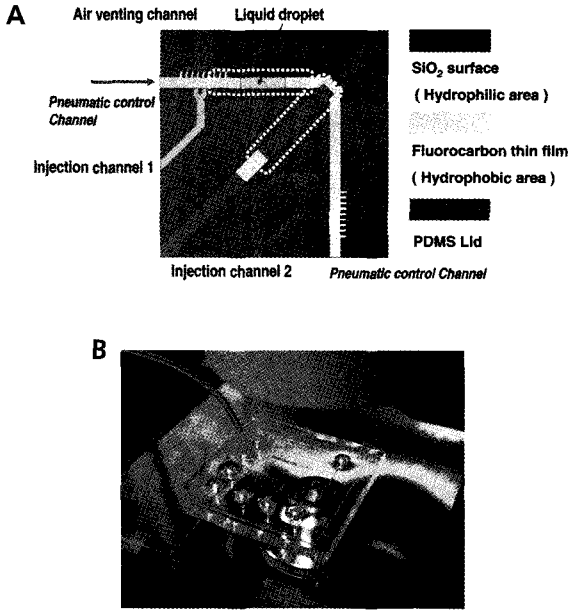

Fig. 2. The design of the microfluidic chip for manipulation of liquid. A. Schematic illustration of the microfluidic chip. B. Photograph of the finally assembled microfluidic chip using confocal fluorescence microscopy.

nipulation and a reaction region for the analysis of the biochemical reaction. Fig. 2B shows the complete, assembled microfluidic chip (28.3 × 28.3 mm^2).

Analytical Instruments

Fluorescence images were observed by confocal fluorescence microscopy with an FITC filter (LSM5 Pascal, Carl Zeiss, Germany). Samples were scanned at a 488 nm wavelength from an Ar laser. To calculate a fluorescent area in the image, we used Kontron Elektronik KS Lite image analysis program 2.0 (München, Germany). The applied pressure was measured by a PT-3300 electronic pressure transmitter (Konics, Seoul, Korea) with an accuracy of ± 0.3% FS (includes non-linearity, hysteresis, and non-repeatability). A profile micrometer (Model VF-7510, Kyence Inc., Japan) was used to examine the prepared chip.

To investigate the properties of patterned hydrophobic film on the glass wafer, we performed an analysis of the contact angle. Prior to the contact angle analysis, the substrates were rinsed with deionized water and dried under a nitrogen stream. A Krüss G10 contact angle analyzer (Krüss GmbH, Hamburg, Germany) was used to investigate the potential surface wettability and surface tension of hydrophobic thin film. All of the measurements were carried out at room temperature and ambient humidity.

Apparatus for Sample Injection and Pneumatic Control at the Nanoliter Scale

The apparatus for executing liquid sample injection and pneumatic control using an external air pressure was homemade consisting of plastic syringes, springs, a micrometer gauge, and acrylic plastic housing. The rotational motion of the micrometer gauge was converted into the linear motion of a piston, and the sample was either injected or withdrawn through Tygon® tubes that were connected to the microfluidic chip [16].

RESULT AND DISCUSSION

The Principle of Microfluidic Handling

The capillary pressure of liquid in a microfluidic channel can be expressed by equation (1), which shows the relationship between the work performed by the pressure (dU_p), the surface free energy of the system (dU_s), and Young's equation [7,9].

$$P = \gamma\cos\theta_{PDMS}\left(\frac{2h+w}{hw}\right)+\gamma\cos\theta_{Glass\ or\ FC}\left(\frac{w}{hw}\right) \quad (1)$$

where, w = width, h = height, θ = the contact angle, and γ = the surface tension of H_2O (0.073 N/m).

A positive pressure indicates capillary filling, and a negative pressure indicates a repellent force at the solid-liquid-gas interface. When external pressure is less than the calculated pressure resulting from equation (1), a microfluid is stably maintained in the microchannel like the state of "valve-close (OFF)". Conversely, in order to switch to the "valve-open (ON)" state against the liquid flow, the external pressure has to be higher than the theoretical pressure needed to push the fluid in a desired direction. The difference in capillary pressure is created by the hydrophobic valving or capillary filling that is used to manipulate the liquid in the microchannel network.

Firstly, the red colloidal ink was introduced through an injection channel, as shown in Fig. 3A. As the liquid droplet grew in the metering channel, we were able to measure the length of the liquid corresponding to a desired volume using the scale bars in the microfluidic chip (Fig. 3A). The liquid pressure was then able to accumulate until liquid flowed into the metering channel. The liquid pressure that had accumulated inside the microfluidic channel compensated for the liquid pressure by diffusing the surrounding air pressure out through the sidewall microchannel array, which had dimensions of 20 (w) × 50 (h) μm^2 in the T-shaped microinjector (Fig. 3A). As shown in Fig. 3A, the sidewall microchannel array provided a helpful additional pathway of airflow, which showed that stable liquid growth was achieved without an abrupt expansion of liquid volume.

Secondly, after the measurement was complete and the liquid injection had stopped, we applied an external pressure of 1.2 kPa to the liquid; the applied external pressure was higher than the minimum pressure of 1.19 kPa calculated using equation (1) on the condition of θ_{PDMS} or θ_{FC} = 110° and a cross-sectional area of 260 (w) × 50 (h) μm^2. Fig. 3B shows the cutting step formed by the intro-

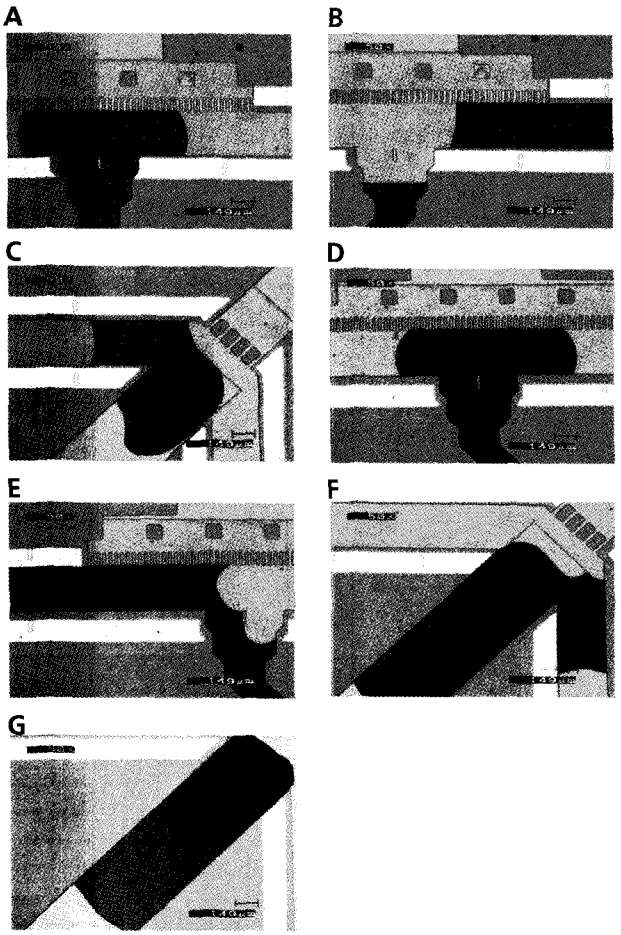

Fig. 3. The principle of microfluidic handling; in this experiment, color colloidal inks were used to obtain clear images. A. The injection of microfluid in a T-shaped microinjector with a sidewall microchannel array. B. Metering of liquid using scale bars and cutting of metered liquid by external air pressure. C. Transport of metered liquid to the reaction region. D. Injection of the second microfluid in the second channel. E. Metering and cutting of the second liquid. F. Merging of two liquids in the reaction region. G. Mixing of two liquids by diffusion.

duction of external air pressure.

Finally, the metered liquid could be transported into the reaction region by the external air pressure. A reaction region enclosed by hydrophobic film is a hydrophilic surface. When the transported liquid met the hydrophilic surface in the reaction region, the liquid rapidly filled the region. In the same manner, the other liquid in the opposite side, blue colloidal ink, could be manipulated (Figs. 3D and E). The metered liquids were merged through the capillary force at the reaction region, which was located at the hydrophilic glass surface (Fig. 3F). In terms of the monitoring of color gradient change at the reaction region, Fig. 3G shows the merged liquids, *i.e.* blue and red colloidal ink, which were mixed by diffusional force.

Consequently, we confirmed that microfluid was freely manipulated, and the desired volume of microfluid was easily metered, cut, transported, and merged on a chip.

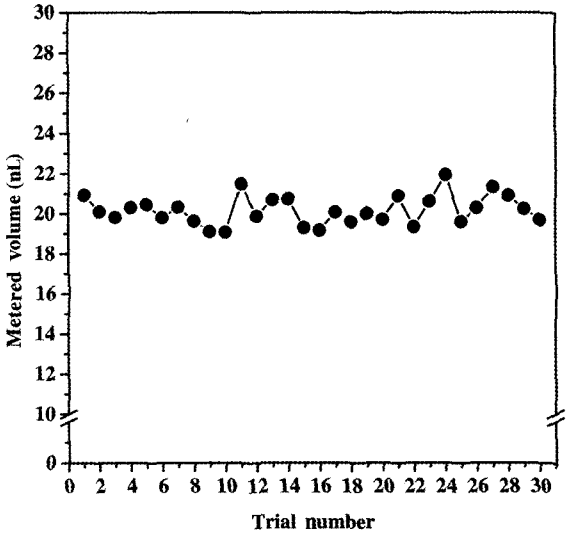

Fig. 4. The quantitative accuracy of metered liquid; the criteria was 20 nL for 30 trials.

The Accuracy of Microfluidic Handling

When the microfluidic chip is applied to the analysis of a quantitative biochemical reaction, the reliability of metered liquid volume must be shown because the variation of metered liquid volume can potentially generate significant error. To investigate the accuracy of the proposed microfluidic handling method on a chip, the microscopic images of the final metered droplet were captured using a CCD camera. The captured images were analyzed using the image analysis software, Kontron KS Lite, in order to determine the droplet area. The measured area was converted to a corresponding volume by multiplying the value of the microchannel depth, 50 μm. In thirty metering runs using a criterion of 20 nL, the accuracy of the metering showed about 4% error (mean = 20.168 nL, standard deviation = 0.730 nL; Fig. 4), which proved that our proposed microfluidic handling method has reliable accuracy at the nanoliter scale.

The Enzymatic Reaction on a Microfluidic Chip

In order to estimate the feasibility of the proposed microfluidic handling method for a biochemical reaction, we examined the β-galactosidase reaction performed on a microfluidic chip. In this experiment, because β-galactosidase hydrolyzes the non-fluorescent FDG to fluorescein via the intermediate compound, fluorescein mono-β-galactopyranoside (FMG), the β-galactosidase reaction was easily detected by fluorescent microscopy. To investigate the non-fluorescence of FDG prior to the enzyme reaction, we carried out a control experiment in a phosphate buffer containing the FDG. As expected, no fluorescence was observed when reacted for 30 min at room temperature, which indicated that the fluorescent signal

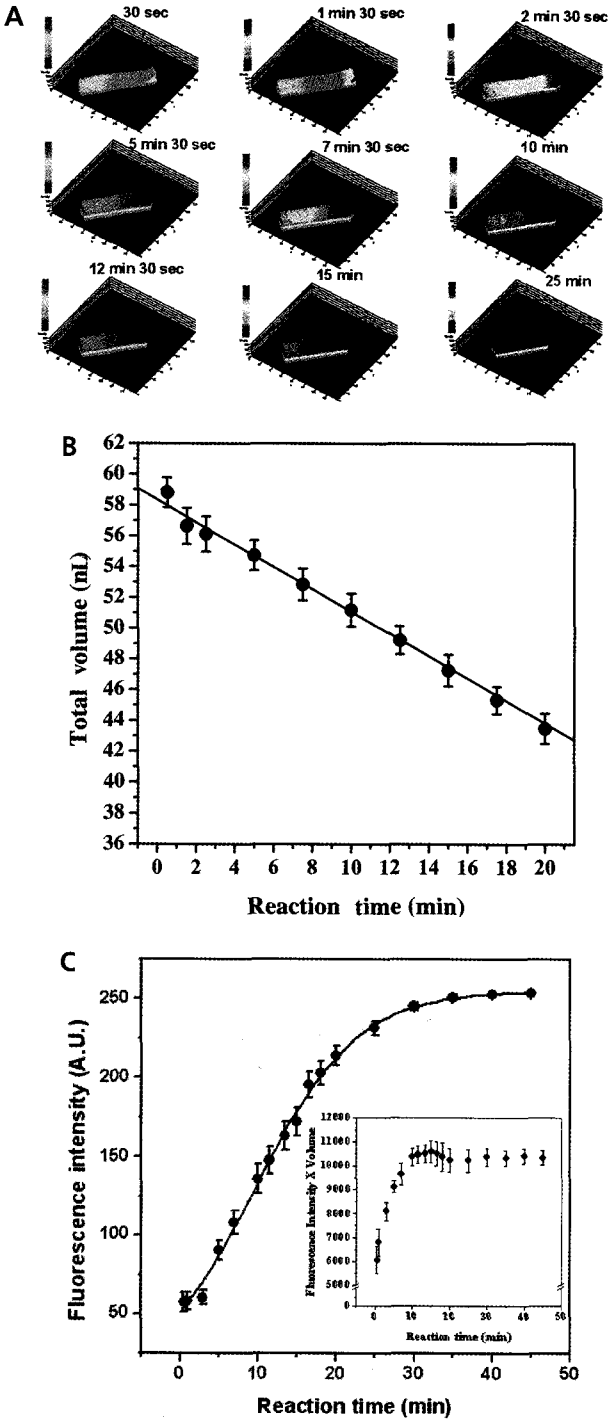

Fig. 5. The reaction profile on the microfluidic chip. A. 3-dimensional fluorescence profile; the vertical color index indicates the fluorescence intensity from blue color (lowest intensity) to red color (highest intensity). B. Evaporation of merged liquids (60 nL) on a microfluidic chip. C. Reaction profile within monitoring time; the plotted points indicate the mean values for three experiments. The error bar indicates the average value from the intra-deviation, attributed to poor diffusion; in the inlet graph, we plotted the corrected fluorescence profile with variation of liquid volume.

was produced only from the β-galactosidase reaction.

The overall sequence of the reaction scheme was operated according the method previously shown in Fig. 3. The substrate of FDG (7.6 pM~760 μM; 30 nL) was sequentially metered, cut, and transported into the reaction region. The β-galactosidase (2 units/mL; 30 nL) was also metered, cut, and transported from the second metering channel. The two liquids, β-galactosidase and FDG, were merged in the reaction region. Induced external air pressure causes the enzyme droplet to be transported into the reaction region, and the FDG droplet and enzyme droplet then merge into a single droplet. Once the β-galactosidase encounters the FDG in the reaction region, the fluorescence signal gradually increased from the top region of the merged liquid.

During the enzymatic reaction on the microfluidic chip, the fluorescence of the merged liquid showed that, at 30 sec, the fluorescence intensity was higher on the upper region compared to the lower region, as indicated by the 3-dimensional profile shown in Fig. 5A, which indicated a poor diffusion reaction between FDG and the enzyme. Since no mixing technique was adopted and only FDG was merged with the enzyme solution to create a single droplet in a reaction region, incomplete mixing of the two different microfluids may be the cause of this heterogeneous reaction. The overall change in fluorescence intensity was presented by a 3-dimensional profile of the merged liquid on the microfluidic chip (Fig. 5A). As the reaction went on, the fluorescence intensity was increased and showed a saturation curve from 30 min by the diffusion and enzymatic reaction in Figs. 5A and C.

However, a decrease in the volume of merged liquid in the microfluidic chip was observed during the monitoring of the reaction. Fig. 5B showed an evaporation of nanoliters of liquid on the microfluidic chip because the total reaction volume (60 nL) was very small compared to that of the high surface area. Although the experiment was performed in a humidified chamber, we could not prevent the problem of evaporation on the microfluidic chip. The total volume diminished linearly, and approximately half of the initial volume was found after a reaction time of 30 min. Therefore, in order to consider the effect of evaporation on the fluorescence intensity, we normalized the fluorescence intensity by multiplying by the volume at each step of data acquisition. In consideration of the evaporation effect, the β-galactosidase reaction was saturated from 10 min in the inset graph of Fig. 5C.

We were also able to change the merging ratio using different volumes of β-galactosidase and FDG. As the ratio of the β-galactosidase increased, the fluorescence intensity was rapidly increased, and reached a saturation point (Fig. 6). As expected, this result indicated that the fluorescence intensity strongly depended not on the concentration of FDG, but on the concentration of β-galactosidase in our experimental condition.

Next, to investigate the limit of detection of the β-galactosidase reaction on the microfluidic chip, various concentrations of FDG (7.6 pM~760 μM; 30 nL) were reacted with a fixed concentration of β-galactosidase (2 units/mL; 30 nL) at 10 min as a criteria. The concentra-

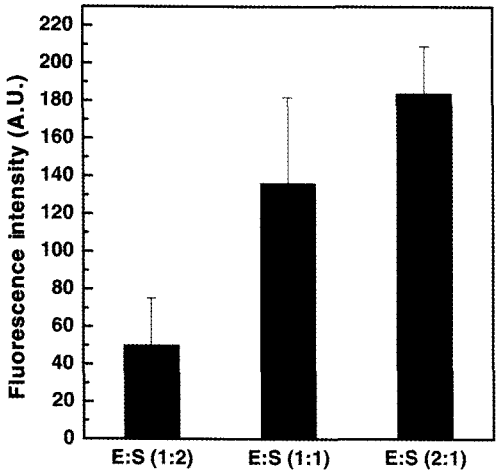

Fig. 6. The effect of the mixing ratio between enzyme (β-galactosidase) and substrate (FDG) for reaction; in order to compare values, these fluorescence intensities were obtained at 10 min.

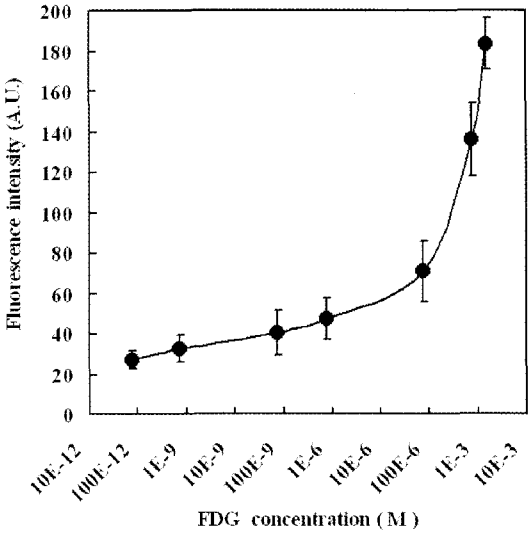

Fig. 7. The limit of detection for the β-galactosidase reaction on a microfluidic chip at a 60 nL scale (β-galactosidase; 30 nL, FDG; 30 nL, respectively); fluorescence intensity was measured at 10 min. The plotted points indicate the mean values for three experiments. The error bar indicates the average value from the intra-deviation, attributed to poor diffusion.

tion of β-galactosidase can be calculated from the activity determined by colorimetric assay, the specific activity of 600 units/mg protein and a molecular mass of 540 kDa. In this experiment, the calculated concentration was 6.7 fM of protein with a specific activity of 2 units/mL. In general, the colorimetric assay using o-nitrophenyl-β-D-galactopyranoside (ONPG) can detect as low as several nM of β-galactosidase [21]. That sensitivity is not sufficient to detect low activity in some instances. The limit of detection of the β-galactosidase (6.7 fM; approximately 120 molecules in 30 nL) reaction within 10 min was 76 pM of FDG on the microfluidic chip under our experimental conditions (Fig. 7). The result showed that the limit of detection could be lowered to five orders of magnitude over a conventional assay, and the amount of consumed reagents could be reduced from microscale to nanoscale. Furthermore, if we adopted a mixing part or mixing method for performing a rapid homogeneous reaction on a chip, the reaction would be faster and the limit of detection would be decreased to a higher degree on a microfluidic chip. We are currently attempting to develop the above mentioned mixing method and the mixer on a chip.

In vitro Protein Synthesis on a Microfluidic Chip

Finally, we examined the possibility of performing multiple biochemical reactions on a microfluidic chip. As evidence for the feasibility of this concept, we performed *in vitro* enhanced green fluorescence protein (EGFP) synthesis on a microfluidic chip because the green fluorescence protein is widely used as an effective indicator in biotechnology fields [22,23]. At first, the mixture (30 nL) of pET 23b-EGFP (plasmid) and complete whole amino acids was introduced from injection channel 1, and then the mixture (30 nL) of the S 30 premix and S 30 extract was introduced from injection channel 2. Because the efficiency of *in vitro* protein synthesis depends upon temperature, *in vitro* protein synthesis on a microfluidic chip was performed in a humidified chamber kept at 37°C (Model 2000, Robbins Scientific, USA) according to the same procedures of the enzymatic reaction. The process of *in vitro* protein synthesis was performed according to the recommended protocols from Promega (WI, USA). The correctly expressed EGFP was excited at 489 nm and emitted at 508 nm, which was easily observed by fluorescence microscopy. Fig. 8 showed that EGFP was correctly expressed on a microfluidic chip, which indicated that multiple complex biochemical reactions, such as *in vitro* coupled transcription and translation, were successfully demonstrated at the nanoliter scale (60 nL). However, even if the reaction was performed in a humidified chamber, the evaporation could not be hindered in this experiment, which seems results from a relatively high temperature such as 37°C or due to the inherent property of the merged liquid, which has a high surface area, considering its small volume of 60 nL, on a microfluidic chip.

Although the fluorescence intensity appeared to increase linearly with reaction time, the corrected fluorescence intensity was saturated from 20 min in consideration of the evaporated volume. This result indicated that the rate of *in vitro* protein synthesis was slower than that of the single enzymatic reaction, which can be attributed to multiple and complex biochemical reactions. The expressed EGFP was routed from transcriptional machinery, translational machinery, and energy regenerating systems, such as the phosphoenol pyruvate and pyruvate kinase, which are very complex and efficient multiple biochemical reactions [24-26]. Many reports have been published

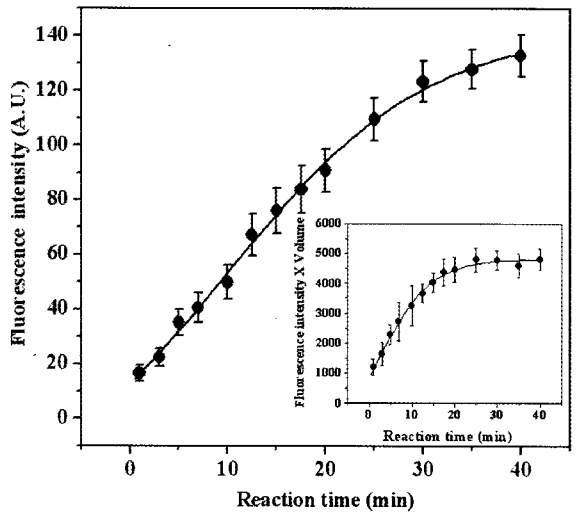

Fig. 8. *In vitro* Enhanced Green Fluorescence Protein (EGFP) synthesis on a microfluidic chip through multiple biochemical reactions (the used purified plasmid of pET 23b-EGFP concentration is about 700 μg/mL); the plotted points indicate the mean values for three experiments. The error bar indicates the average value from the intra-deviation, attributed to poor diffusion.

regarding *in vitro* protein synthesis, but there have been very few studies of *in vitro* protein synthesis on a microfluidic chip. To our knowledge, this is the first time that *in vitro* expression of protein has succeeded through the microfluidic handling method at a nanoliter scale on a microfluidic chip.

CONCLUSION

The tactic of microfluidic handling is an essential for the development of "lab on a chip". In this study, we used the microfluidic handling method to manipulate the motion of microfluid, performing injection, metering, cutting, transport, and merging on a chip. Based on the surface patterning for different wettability and pneumatic pressure balance in chip design, the microfluid is discretely metered to the desired volume depending on the length of the metering channel. The effectiveness of the proposed method was successfully demonstrated to have broad feasibility, from a simple enzymatic reaction with a low limit of detection, to multiple biochemical reactions such as *in vitro* protein synthesis.

Further reactions and diagnostic assays using this methodology are currently in development, and the design of a mixer or mixing tool for efficient biochemical reactions is in progress.

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