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Title

On-chip Genotoxic Bioassay Based on Bioluminescence Reporter System Using Three-Dimensional Microfluidic Network

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Abstract

Microchip-based genotoxic bioassay using sensing *Escherichia coli* strains has been performed. In this method, the assay was conducted in three-dimensional microfluidic network constructed by a silicon perforated microwell array chip and two poly (dimethylsiloxane) (PDMS) multi-microchannel chips. The sensing strains having firefly luciferase reporter gene under transcriptional control of *umuD* as an SOS promoter were put into the channels on one of the PDMS chips and immobilized in the silicon microwells. Samples containing genotoxic substances and substrates for luciferase were into the channels on the other PDMS chip. The optimum conditions of the assay in the on-chip format have been investigated using mitomycin C (MMC) as a genotoxic substance. As a result, the dose-dependence of bioluminescence intensity was obtained at once on the chip. Additionally, the response ratios of the bioluminescence between mutagen- and non-induced strains were successfully enhanced by improving the on-chip assay methods and conditions. Several well-known genotoxic substances were subjected to the on-chip assay, and were detected with the detection limits comparable to those in the conventional method with reduced time.

1. Introduction

A genotoxic bioassay based on the induction of the bacterial SOS system has been used for genetic risk assessment in various fields [1-3]. In this assay, genotoxicity is estimated by measuring the amount of reporter protein synthesized under transcriptional control of an SOS promoter in response to chemical substances. The SOS genes are responsible for error-prone DNA repair following the DNA-damage under the chemical and/or physical genotoxic stress. Thus, placing reporter gene which codes for an enzyme that catalyzes colorimetric and bioluminescent reaction or a fluorescent protein under the control of the SOS promoter enables the determination of genotoxic potential by measurement of the expressed reporter protein activity [1-9]. However, this assay requires relatively long time (ca. 8 h) and complicated handling under sterile conditions. Therefore, to provide the risk assessment for the substances with unknown genotoxic potential, development of rapid and sensitive genotoxic bioassay with high-throughput system have been expected.

Microchip-based micro total analytical system (μ -TAS) has attracted great attention because of its advantage including high-throughput analysis, low-energy requirement, and on-site monitoring, etc [10, 11]. Recently, various microsystems targeted biomaterials, such as DNA [12-14] and protein [15-19] have been developed. These devices have been widely studied due to expectation as a robust tool in post-genome and/or clinical fields. In addition, a few living cell-based systems have also been studied because the μ -TAS introduced living cell could be utilized for various application, such as biosensor, bioreactor, gene or protein analysis, and medical diagnosis, etc [20-24]. In these cell-based microsystems, cells are brought into the microchannel and/or immobilized using hydrogel [25, 26] or printing method [27]. These systems seem to be good for study of cell function, but not to be suitable for

quantitative bioassay such as genotoxic test because a small amount of cells in the device could bring less reproducible results. Therefore, to conduct quantitative assay using cells in microsystems, selecting a proper cell-based bioassay system and applying appropriate amounts of cells could be important.

We previously developed a chip-based reporter bioassay system using microfluidic device and immobilized sensing *Escherichia coli* strains having bioluminescence reporter gene [28]. This system is based on the micromosaic assay format [16], which provides high-throughput capabilities using a simple linear channel and a flat chips. The channels make a grid of probe and sample lines on the flat chip, and thus every couple of probes and samples interacts at each intersection of the lines. We have expanded the on-chip mosaic assay format for the application to cell-based bioassays. In our on-chip bioassay system, two poly(dimethylsiloxane) (PDMS) multichannel chips were sealed on both sides of a silicon substrate having an array of perforated microwells, in which every pairs of channels on separate PDMS chips were connected *via* each well to make a grid-like three-dimensional microfluidic network. The three-dimensional microfluidic network allows retaining a large number of cells enough for quantitative and reproducible bioassay. It was shown that the system has the potential to conduct a high-throughput reporter assay for genotoxic substances. Bioluminescent emission observed in mosaic format on the chip was highly reproducible because an appropriate amount of sensing strains was immobilized by using agarose gel in the microwells as mentioned above. However, to use this system as a genotoxic test based on the reporter system, assay characteristics in the on-chip format should be clarified.

In this paper, we investigated several conditions and methods for the on-chip genotoxic bioassay, such as bioluminescence detection and induction time, strain type

and concentration, and use of nutrient. In these experiments, we used the sensing *E. coli* strain, which was constructed in our laboratory, having a plasmid with firefly luciferase gene (*luc*) under transcriptional control of *umuD* as an SOS promoter [29]. Furthermore, to evaluate the assay performance in the on-chip format, the detection limits of various genotoxic substances were compared to those in the conventional method using test tubes.

2. Experimental

2.1. Materials

Si wafer (100) with thickness of 625 μm used for the microwell array chip was obtained from Osaka Tokusyu-gokin (Osaka, Japan). Negative photoresist SU-8 used for the template of poly (dimethylsiloxane) (PDMS) microchannel chip was from MicroChem Corp (Newton, USA). Poly (dimethylsiloxane) prepolymer (Sylgard 184) and curing agent were from Dow Corning (Midland, USA). Agarose type VII with low gelling temperature ($<30^{\circ}\text{C}$) and polytetrafluoroethylene (PTFE) membrane filter used for immobilization of cells were from Sigma (St. Louis, USA) and Advantec (Tokyo, Japan), respectively. Beetle luciferin potassium salt and Passive lysis buffer as a cell lysis agent were from Promega (Heidelberg, Germany). Adenosine-5-triphosphoric acid disodium salt (ATP) was from Wako Pure Chemical Industries (Osaka, Japan). *Escherichia coli* KT1008 wt, and KT1008 *tolC*⁻, all harboring the plasmid pRSSL, which has a firefly luciferase gene (*luc*) under the transcriptional control of an *umuD* as an SOS promoter, were prepared in our laboratory [29]. All other chemicals were reagent grade and were used as received.

2.2. Genotoxic substances

Mitomycin C (MMC), hydrogen peroxide (H_2O_2), 4-nitroquinoline-*N*-oxide (4-NQO), and nalidixic acid (NA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Acridine mutagen (ICR191) and nitrofurantoin (NFO) were obtained from Sigma (St. Louis, USA). Mitomycin C, H_2O_2 , and ICR191 were dissolved in sterilized water. 4-Nitroquinoline-*N*-oxide, NA, and NFO were dissolved in 20 % (v/v) DMSO.

2.3. Chip design and fabrication

The microwell array for immobilization of the sensing strains, and the microchannel for introduction of the strains and the reagents were fabricated on Si and PDMS, respectively. The microwell set in 5 x 5 array with each size of 700 x 700 μm^2 was perforated. The channel dimensions are 700- μm width and 200- μm depth with two access holes at both ends of each channel.

2.4. On-chip genotoxic bioassay

The sensing strains were grown in Luria-Bertani (LB) broth (10 g l⁻¹ Bacto Tryptone, 5 g l⁻¹ Bacto Yeast Extract, and 10 g l⁻¹ NaCl) containing 100 $\mu\text{g ml}^{-1}$ ampicillin with shaking at 37°C until OD₆₀₀ was 0.4. An antibiotic, ampicillin, was used for selective growth of the sensing strain containing reporter plasmid pRSSL conferring antibiotic resistance on the strain. The cells were collected by centrifugation, and were suspended 10 mM Tris-HCl containing 150 mM NaCl (pH7.0) (TBS) or LB medium. The cell suspension was mixed with the same volume of agarose at 37°C. The final concentration of agarose was 1.5 % (w/v). The method of on-chip genotoxic bioassay was shown in Figure 1. Firstly, one of the two PDMS channel chips was sealed on the Si microwell chip. The sensing strain mixed with agarose was put into the channel from one of the two access holes at the ends of each channel by using a micropipette after the backside of the well array chip was covered by a membrane filter for preventing leakage of the mixture from the wells. The mixture filled in the channels and wells was allowed to stand at 4°C for gelation. After the filter was peeled off, another PDMS channel chip was sealed on the backside of the microchip immobilizing the cells, the channel lines of the PDMS chip crossing the cell lines in the former channel chip at each well. Then, solutions of genotoxic substance were filled in the channels from the access hole. The immobilized strains were exposed to the solutions,

and were incubated at 37°C. After the genotoxic substances were removed from the access hole of the channel by a pipette, luciferin/ATP solutions (1 mM luciferin, 2 mM ATP, 20 mM MgSO₄, and 40 mM Tris-HCl, pH 8.0) with or without the lysis buffer were put into the same channels similar to doing for the genotoxic substances. Bioluminescence thus obtained was measured by a charge-coupled camera (C4800, Hamamatsu Photonics). The integrated luminescence value for 15 or 30 min subtracted background noise was defined as the bioluminescence emission. The ratio of bioluminescence emission of the induced strain to that of the non-induced one was defined as response ratio. The minimal concentration of the genotoxic substance, which gives the response ratio of two or above in any replicated assay, was defined as the detection limit.

In the conventional method, after genotoxic substances were added to 2 ml of sensing strain cultures, the strains were cultivated with shaking at 37°C for 2 h, and then bioluminescence was measured by using Kikkoman Lumitester C-100 after mixing the cell homogenates with luciferin/ATP solution for 10 s [29].

The concentrations of the genotoxic substance were indicated as initial ones in both methods. All experiments were repeated at least two times from replicate aliquots of a single culture and from those of separate cultures.

3. Results and discussion

For the genotoxic bioassay in this study, we used the same chip set as that developed for reporter assays in our previous work [28], which consists of a silicon chip with 5 x 5 perforated microwells and two PDMS chips each with 5 microchannels. Prior to the application of this chip set for the genotoxic assay, the assay methods and conditions for the on-chip format were investigated.

3.1. Assay methods and conditions

Cell lysis and bioluminescence measurement: In this method, substrates of luciferase such as luciferin and ATP have to enter the cells through the agarose gel and cell membrane for bioluminescence reaction. This could cause a long time for the detection of bioluminescence. Therefore, cell lysis buffer was tested in the on-chip assay. The bioluminescence response curves with and without the lysis buffer were shown in Figure 2. In this experiment, 2×10^{10} cells ml^{-1} KT1008 wt/pRSSL was used, and the cells were induced for 60 min by $2.0 \mu\text{g ml}^{-1}$ MMC. From Fig. 2, the bioluminescence emission was not observed until 15 min after addition of luciferin/ATP mixed solution without the lysis buffer, and was increased over 15 min. On the other hand, in the case of using lysis buffer, the bioluminescence emission was increased until 10 min and then was decreased. The rapid bioluminescence emission in the presence of the lysis buffer is due to enhanced accessibility of luciferase to luciferin and ATP by release of the enzyme from the cells. The cell lysis buffer, thus, can be available for reducing bioluminescence measurement time even for the cells entrapped in agarose gel. The integrated bioluminescence intensity for 30 min without the lysis buffer was almost same as that for 15 min with the lysis buffer. Therefore, the optimum bioluminescence measurement time was determined to be 15 min in the presence of the lysis buffer.

Induction time of luciferase gene in the immobilized cells: The effect of induction time on bioluminescence emission was shown in Figure 3. The 2×10^{10} cells ml^{-1} KT1008 wt/pRSSL were induced by $2.0 \mu\text{g ml}^{-1}$ MMC, and then bioluminescence emission was measured for 30 min in the absence of lysis buffer. The bioluminescence emission was increased with increasing the induction time up to 60 min, and was nearly constant over 60 min. In the conventional method using test tubes under its optimal condition, induction time at which bioluminescence emission became constant was approximately 4 h. Thus, in this assay, induction time can be shortened to one-quarter of that in the conventional method. In the on-chip assay, reduced induction time seems to be attributable mainly to suppression of cell growth because a high concentration of the cells was immobilized in the wells. The optimum induction time was determined to be 60 min.

Concentration of immobilized cell: Effect of immobilized cell concentration on bioluminescence emission and response ratio was shown in Figure 4. The bioluminescence generated from the cell induced by MMC was increased with increasing cell concentration over the range tested. Additionally, the non-induced bioluminescence emission showed only a little increase in the range of 0.2 to 2.0×10^{10} cells ml^{-1} , resulting that the response ratio was also increased at this range. Therefore, 2×10^{10} cells ml^{-1} of the immobilizing cells was used in this method.

Addition of nutrients to agarose gel: In the on-chip assay, immobilized cells are not cultivated, and may not live at high concentration of genotoxic substances. This could cause to lower the response ratio and the detection limit. Thus, we tested the effect of the addition of LB medium as nutrients to the agarose gel on the response ratio. As listed in Table 1, the presence of the Luria-Bertani (LB) resulted in lowering the bioluminescence emissions. The response ratios, however, were approximately 2-fold

higher with LB at 20 ng ml⁻¹ MMC. Low bioluminescence emission is due to the presence of some interfering factors for bioluminescence reaction among LB constituents, because LB showed inhibitory effect even in the reaction using purified luciferase (data not shown). On the other hand, increased response ratio could be explained by the growth and the extinction of the cells. In the absence of LB, densely packed cells in the chip are in poor growth conditions, and thus apt to die with MMC, resulting in low response ratio. By the addition of LB, the growth conditions are improved at all, so that the cells could be viable even in the presence of MMC, and thus the ratio increased.

3.2. On-chip genotoxic assay for MMC

The on-chip genotoxic bioassay for Mitomycin C (MMC) as a well-known genotoxic substance was performed at the assay conditions optimized above. After the 2 x 10¹⁰ cells ml⁻¹ of tester strain KT1008 wt/pRSSL mixed with agarose containing Luria-Bertani (LB) medium was immobilized to all microwells, different concentration of MMC was introduced into the channels crossing the channel lines of the strain. After the induction for 1 h, bioluminescence from each well was measured by a CCD camera for 15 min upon addition of luciferin/ATP solution containing lysis buffer. In Figure 5, the effect of MMC concentration on response ratio was examined in the range of 0.2 ng to 20 µg ml⁻¹ of MMC (Curve A). The response ratio was increased with increasing MMC concentration until 2.0 µg ml⁻¹, and then decreased because of cell inviability at excess concentration of MMC. This result indicates that MMC was permeated into the agarose gel and cell membranes, and then damaged DNA, thereby expressing of *luc* depending on the degree of DNA damaging by MMC. Relative standard deviation for bioluminescence emission in different wells and chips was

smaller than 20 %. The detection limit of MMC using KT1008 wt/pRSSL in this method was 2.0 ng ml^{-1} , which was improved to 10-fold by increasing the response ratio due to the nutrient addition. Although the ratios are still lower than those in the conventional method, the detection limit is at the same level as that obtained by using test tubes.

Escherichia coli gene *tolC* codes for an outer membrane protein which pumps out undesirable substances from the cell. Thus, the mutation of *tolC* causes that the substances permeable into the cell cannot be pumped out, and are thus they concentrated in the cell. The *tolC* mutants have already been used in other bacterial genotoxic tests for an improvement in sensitivity for the substances. In the on-chip format, the *tolC* mutant was tested for the sensitive bioassay of MMC. The effect of MMC concentration on the response ratio using KT1008 *tolC*⁻ harboring pRSSL [29] was shown in Figure 5 (Curve B). As compared to two curves in Figure 5, a dose-response using KT1008 *tolC*⁻/pRSSL shifted to lower MMC concentration than that using KT1008 wt/pRSSL. The result is similar to that in the conventional method, indicating that the *tolC* mutant is exploitable for a sensitive genotoxic test even in the on-chip format. The detection limit of MMC using *tolC* mutant was improved to be below 0.20 ng ml^{-1} , 10-fold lower than that using wild type.

3.3. Detection of other genotoxic substances

The other well-known genotoxic substances, such as ICR191 and H_2O_2 as hydrophilic substances and 4-NQO, NFO and NA as hydrophobic ones, were also tested in the on-chip assay. Their detection limits were listed in Table 2 with those in the conventional method. In both the conventional and the on-chip methods, KT1008 *tolC* harboring pRSSL was used as a sensing strain. Additionally, in the on-chip

method, the Luria-Bertani was added, and bioluminescence measured for 15 min in the presence of cell lysis buffer. As a result, the genotoxicity of all substances except for NFO can be detected with the detection limits comparable to those in the conventional method.

In this experiment, nutrient addition was effective for increasing the response ratios of all substances tested. However, NFO cannot be detected in the on-chip method even under the improved conditions because the response ratio is below 2 at any concentration of NFO. This indicates the method is not applicable to NFO genotoxicity studies. For the detection of NFO in the on-chip method, further improvement in the response ratio is required.

Despite the case of NFO, the results obtained were strongly suggested that the presented assay could be available for the genotoxic test.

4. Conclusions

In this paper, we have presented a new format for a reporter assay system for genotoxic substances based on the SOS response using microchips having three-dimensional microfluidic network. The microfluidic network enabled us to retain a large number of tester strains in the chip for the quantitative and reproducible bioassay. After investigation of the assay conditions and methods, well-known genotoxic substances can be detected with reduced time and comparable detection limits to those in the conventional method. In the on-chip assay, several types of the sensing strain can be immobilized and different samples can be subjected to each of the strains in the single chip. Thus, all of the combination of the strains and the samples can be examined all together on the chip. The results are obtained as a mosaic bioluminescence signal pattern as well as those in a DNA array. If the cells are immobilized on the chip in advance and are alive for a long time by controlling conditions, one can carry out assays by simply introducing his samples into the chip. The on-chip format using three-dimensional microfluidic network presented here could be one of the promising methods for a convenient and a high-throughput bioassay by sensing bacteria.

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Table 2 Detection limit of genotoxic substances. A: Conventional method using 2-ml culture, B: On-chip method.

Substance name	Molecular weight	Detection limit / $\mu\text{g ml}^{-1}$	
		A	B
<i>Hydrophilic</i>			
Mitomycin C (MMC)	334.2	2.0×10^{-4}	2.0×10^{-4}
Hydrogen peroxide (H_2O_2)	34.01	2000	200
Acridine mutagen (ICR191)	451.2	20	2.0
<i>Hydrophobic</i>			
4-Nitroquinoline- <i>N</i> -oxide (4-NQO)	190.2	20	20
Nalidixic acid (NA)	232.2	2.0	2.0
Nitrofurantoin (NFO)	238.2	2.0	n.d.

n.d. not detected

Table 1 BL emission and response ratio under different compositions of the agarose gel (with and without LB). Each value indicates the average of duplicate or triplicate measurements and the error is the standard deviation of those measurements.

MMC / ng ml ⁻¹	BL emission / counts (Response ratio)	
	-LB	+LB
0	216 ± 23 (1.0)	25 ± 4 (1.0)
20	432 ± 40 (2.0)	95 ± 12 (3.8)

Figure captions

Fig. 1 Method of on-chip genotoxic bioassay. (A) A PDMS microchannel chip was sealed on one side of a Si microwell array chip, in which perforated microwells connected to the microchannel. (B) The backside of the Si chip was covered by a membrane filter. The sensing strains mixed with agarose were put into the channels at 37°C. (C) The chips were allowed to stand at 4°C for immobilization of the strains in the channels and the wells by gelation. Then, a set of the chips was upended and the filter was peeled off. (D) Another PDMS channel chip was sealed on the other side of the Si chip immobilizing strains, the channel lines of the PDMS chip crossing the cell lines in the former channel chip at each well. Genotoxic substances were put into the channels, and then the chips were incubated at 37°C. (E) After removing the substances from the channels, luciferin/ATP solutions were into the same channels, followed by bioluminescence measurement.

Fig. 2 Bioluminescence response in the on-chip method. On addition of a luciferin/ATP solution with (○) and without (●) the lysis buffer into the channel, bioluminescence emission from the microwell was measured by CCD, and was integrated every 5 min. Before the measurement, 2×10^{10} cells ml⁻¹ KT1008 wt/pRSSL was induced by 2.0 µg ml⁻¹ MMC for 60 min. Experimental points are the average of duplicate or triplicate measurements and the error is the standard deviation of those measurements.

Fig. 3 Bioluminescence emission as a function of the induction time in the on-chip (●)

and in the conventional (\circ) methods. In both methods, 2×10^{10} cells ml^{-1} KT1008 wt/pRSSL and $2.0 \mu\text{g ml}^{-1}$ MMC were used. Bioluminescence emissions were integrated for 30 min in the absence of the lysis buffer. Experimental points are the average of duplicate or triplicate measurements and the error is the standard deviation of those measurements.

Fig. 4 (A) Bioluminescence imaging of the on-chip bioassay using various concentrations of KT1008 wt/pRSSL. The sensing strains in the wells on odd rows were induced by $2.0 \mu\text{g ml}^{-1}$ MMC, and those on even rows were not. The induction was carried out for 60 min, and then the bioluminescence emissions were measured for 15 min in the presence of the lysis buffer. (B) Bioluminescence emission and response ratio calculated from the imaging as a function of cell concentration. The bars of dark and light grey show Bioluminescence emission with and without induction, respectively, by $2.0 \mu\text{g ml}^{-1}$ MMC. Experimental points are the average of duplicate or triplicate measurements and the error is the standard deviation of those measurements.

Fig. 5 Effect of the response ratio on MMC concentration using KT1008 wild type (A) and *tolC* mutant (B) harboring pRSSL in the on-chip system under the optimum condition. The assay was conducted for 60 min of induction time and 15 min of bioluminescence integration time in the presence of the lysis buffer. Experimental points are the average of duplicate or triplicate measurements and the error is the standard deviation of those measurements.









