ON-CHIP BIOLUMINECENCE ASSAY OF ATP AND KINASES USING IMMOBILIZED FIREFLY LUCIFERASE IN THREE-DIMENSIONAL MICROFLUIDIC CHIP

Hirofumi Tani, Atsuki Morisaki, Akihiko Ishida, Manabu Tokeshi
Faculty of Engineering, Hokkaido University, Japan

ABSTRACT

We describe an on-chip firefly bioluminescence (BL) assay in a three-dimensional microfluidic platform. In the assay, firefly luciferase was immobilized in a perforated well chip placed between two poly(dimethylsiloxane) (PDMS) microchannel chips. Then, BL reaction in each well was successively carried out by filling the channels with substrate solutions containing adenosine 5'-triphosphate (ATP) and luciferin. Based on the BL intensity obtained by a charge-coupled device, ATP was detected as low as sub-fmol level in this format. Finally, by using reaction mixtures of kinase instead of ATP, the on-chip format was applied to the simultaneous BL assay of kinases.

KEYWORDS

Bioluminescence, ATP, Kinase, Three-dimensional microfluidics, High-throughput assay

INTRODUCTION

The firefly BL reaction (Scheme 1) has been widely used for the determination of ATP and luciferase due to its high sensitivity and specificity. [1] Since there is no necessity of a light source in the BL-based assay, it is one of the most promising methods for microscale analytical systems. The BL is emitted in the reaction of oxidative decarboxylation of D-luciferin catalyzed by luciferase in the presence of ATP, Mg^{2+}, and molecular oxygen. [2] The BL method can be applied to the estimation of cell number, because all living cells containing ATP as an energy

$$\text{Luciferin} + \text{ATP} + \text{O}_2 \xrightarrow{\text{Luciferase, } Mg^{2+}} \text{oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO}_2 + \textit{light}$$

Scheme 1. Firefly bioluminescence reaction.

source in the same concentration level. Based on this fact, the detection of microbial contamination is carried out by the BL method for hygiene purposes in food and medical fields. [3] The BL-based ATP assay is also applicable to measurement of ATP-dependent enzymes, such as a series of kinases. [4] They play a key role in cellular processes by involving in signal transduction and metabolic pathways. More than 500 different kinases were found in human genome, but only a fraction has been characterized. [5] Thus, there is a great need for measuring each kinase activity with high-throughput methods for understanding of their association with human diseases and for drug discovery.

We have previously reported a microchip-based BL assay format in which a chip assembly consists of a well array chip with five-by-five perforated microwells placed between two channel chips with five microchannels was used. [6, 7] It enables a simultaneous assay of several samples using several analytical reagents introduced into and immobilized in a three-dimensional microfluidic network constructed in the chip assembly as shown in Fig. 1. The on-chip format was successfully applied to SOS-based whole-cell genotoxicity assay using sensing bacteria expressing Luc as a reporter enzyme. [8]

In this study, we applied BL reaction catalyzed by Luc to the on-chip format for the assay of ATP and ATP-dependent enzymes. In the assay, Luc, one of the analytical reagents, was immobilized in the well chip by gelation of mixture of Luc and agarose loaded from the reagent injection port into the wells. To retain Luc in agarose gel without leakage, we first tested three types of Luc with different sizes. Then, ATP assay was carried out by filling the channels with substrate solutions containing various concentrations of ATP and luciferin. Moreover, the proposed method was applied to the measurement of three ATP-dependent kinases, creatine, acetate, and pyruvate kinases (CK, AK, PK).

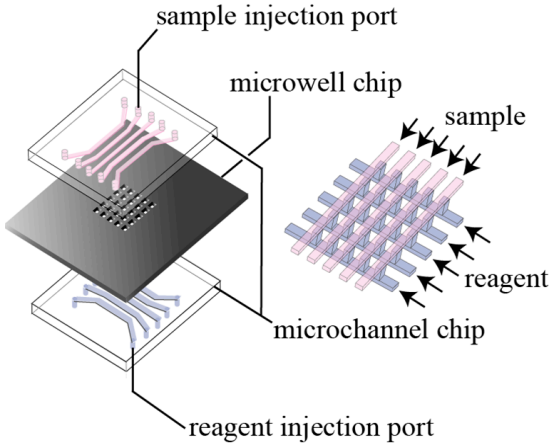

Fig. 1. Schematic of the on-chip assay system.

EXPERIMENTAL

In this study, three types of Luc with different size were used. Normal Luc (Luc_f) was obtained from Promega. Glutathione S-transferase-fused Luc (GST-Luc_f) was expressed in genetically engineered *Escherichia coli* and purified by using glutathione Sepharose 4B (GE Healthcare) column. Bead-bound GST-Luc (GST-Luc_b) was prepared by using glutathione Sepharose 4B beads. A perforated five-by-five well array chip was fabricated by

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photolithography and wet-etching using a silicon substrate with a thickness of 625 μm, and two microfluidic five-channel chips were made by softlithography from PDMS as reported previously. Each well has a size of 700 x 700 μm^2, and each channel of 700-μm width and 200-μm depth. One of the channel chips was first sealed on the well chip. Mixed solution of Luc and agarose was then loaded from each reagent injection port into the channels and the wells, and was allowed to stand at 4°C for gelation. Another channel chip was sealed on the well chip, followed by filling the sample channels with mixed solutions of luciferin and sample (ATP or mixture of kinase and its substrates). BL from the wells was measured with a charge-coupled device for 30 min.

RESULTS AND DISCUSSION

First, we measured BL emission to confirm ATP detectable in the on-chip assay format by using three types of Luc with different sizes, Luc_f, GST-Luc_f, and GST-Luc_b. After gelation of a mixture containing 20 μg/mL each Luc and 1.5% agarose in the wells, BL was observed from the wells for any types of Luc when the channels were filled with luciferin/ATP (10^{-7} M) mixtures. The result indicates ATP is detectable in the proposed format. However, in the case of Luc_f and GST-Luc_f, BL was observed not only on the wells but also on the channels. This is due to the leakage of them from agarose gel in the well into the ATP/luciferin solution filling in the channel. Therefore, bead-bound GST-Luc was used in the on-chip assay.

CCD images of BL emission from the wells are shown in Figure 2 (A and B). As seen in this figure, five samples with different concentrations of ATP can be tested, and for each sample five BL data obtained in single chip assembly. Figure 2 also shows BL emission was dependent on ATP concentration. A linear relationship between ATP concentration and average BL integration was obtained with the detection limit of 1.8×10^{-9} M (2.7×10^{-16} mol) (Fig. 2C), which is comparable to those in the batch methods.

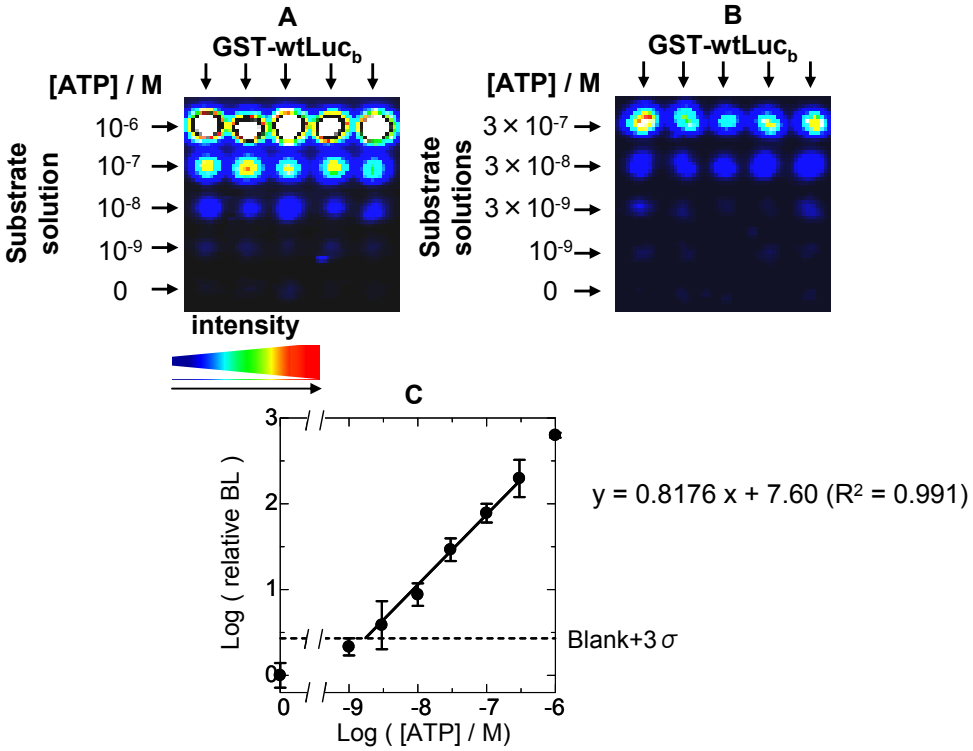

Fig. 2. CCD images of the on-chip BL at various concentrations of ATP (A and B), and calibration curve for ATP (C).

To extend this format to the assay of kinases, ATP produced by kinase reaction and luciferin should be brought into the wells where Luc is immobilized. Additionally, to exploit a characteristic of this format, i.e. ability of a simultaneous assay of five samples using five analytical reagents, each substrate of kinases tested should be immobilized in the wells on each reagent line (Fig. 1). For these purposes, creatine kinase (CK) was used as a model. Creatine phosphate was immobilized together with Luc in the wells on two reagent lines while in the remaining wells on other three reagent lines on the same chip, Luc was immobilized. Then, all of the sample channels were filled with a mixture containing CK, adenosine 5'-diphosphate (ADP), and luciferin. In this method, BL emission was observed in the wells having both Luc and creatine phosphate. However, BL from the wells without creatine phosphate on the same reagent line was also detected, indicating creatine phosphate leakage from agarose gel. The result means possible interference of BL with the neighbor wells in the case of simultaneous assay of several kinases. Thus, for the kinase assay in this format, a reaction mixture containing kinase, kinase substrate, ADP, and luciferin was used instead of ATP solution in the ATP assay.

Various concentrations of CK, acetate kinase (AK), and pyruvate kinase (PK) were tested by using a mixture of each kinase and its substrates after optimization of the experimental condition. As results, linear relationships between kinase concentration and BL emission were obtained, and the detection limits were $3.0\sim130\times10^{-12}$ M ($0.47\sim20\times10^{-18}$ mol) depending on type of kinase. Finally, we examined simultaneous assay of CK, AK, and PK. When a mixture of three kinases as a model sample was loaded into the channels with each substrate and BL reagents, BL was obtained from the wells for any substrate loaded (Fig. 3). Since the BL intensities using kinase mixture were the same as in the use of one kinase and its substrate, kinases used in this work specifically recognized each substrate and produced ATP followed by Luc reaction. The on-chip assay system using immobilized Luc will thus be applicable to a simultaneous assay for kinases.

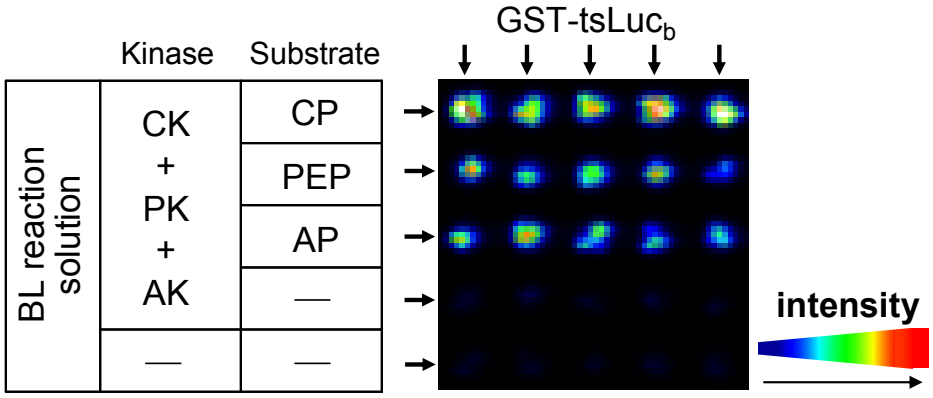

Fig. 3. CCD image of the on-chip BL in simultaneous assay for kinases. CK; creatine kinase, PK; pyruvate kinase, AK; acetate kinase, CP; creatine phosphate, PEP; phosphoenolpyruvate, AP; acetyl phosphate.

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CONTACT

***H. Tani tani@eng.hokudai.ac.jp**