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Development of a rapid immunoassay system: Luminescent detection of antigen-associated antibody-luciferase in the presence of a dye that absorbs light from free antibody-luciferase

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In this report, we developed a rapid immunoassay system, designated the bioluminescent interference gathering optical (BINGO) assay, which required no time-consuming washing steps for removal of unbound antibodies. This system employed a luciferase (Luc)-conjugated antibody (LucAb) and a dye that absorbed light from the LucAb. The antigen-associated LucAb was localized by transfer of an antigen to the detector-side of a chamber where a detector photomultiplier tube (PMT) was installed. In contrast, the free LucAb was distributed throughout the solution, and the light emitted by the free LucAb was absorbed by the dye. Therefore, only light from LucAb associated with antigen could be detected by the PMT. The new system could be used to rapidly detect the amount of antigen-antibody-Luc complex by collecting steps, such as centrifugation or magnetic collection of antibody-coated magnetic beads. Proof-of-principle experiments were performed using a model system with streptavidin beads and biotinylated Luc. The feasibility of the system was demonstrated using magnetic beads coated with anti-*Escherichia coli* O157 antibody, enabling detection of 4×10^3 cells in only 15 min. Thus, this system may have applications in a variety of biomedical fields.

[**Key words:** Rapid immunoassay; Antibody localization; Luciferase; Dye; Luminescence]

Immunoassays have been widely applied in various fields, including food security control, diagnosis, and clinical research (1). Enzyme-linked immunosorbent assays (ELISAs) are the most frequently conducted immunoassays, but require several rounds of time-consuming washing steps. Although immunochromatography strip tests and latex agglutination can be applied to detect antigen rapidly, these methods are easily influenced by coexisting materials and are not easily applicable for quantitative analysis of antigens (2,3). Additionally, various methods have been developed to enable rapid, simple, and quantitative immunoassays (4,5).

For development of immunoassays with less time requirements, shortening or elimination of laborious washing steps is essential. An early study reported the detection of morphine from urine samples, using a competitive assay system developed for detection of the binding of an anti-morphine antibody to morphine and morphine labeled-lysozyme (6). Moreover, Ueda et al. reported the development of an open sandwich system (7), in which fluorescence is emitted only when both V_H and V_L bind to the antigen, effectively utilizing fluorescence resonance energy transfer (FRET). The same research group also reported Quenchbody, an antibody modified with a fluorophore (8). In the presence of antigen, the fluorophore is released from Trp residues of Quenchbody, resulting in the emission of fluorescence. These techniques have been designed to recognize only one epitope of an antigen and are difficult to apply for sandwich assays. Although sandwich immunoassays for bacterial detection have been reported using surface-enhanced Raman scattering (9), antibody-immobilized quantum dots (10), and antibody-immobilized carbon particles (11), these assays require complicated instruments or washing steps to remove unbound antibodies.

In this study, we developed a novel immunoassay system, designated the bioluminescent interference gathering optical (BINGO) assay, which does not require washing steps for removal of unbound antibodies. This system used a luciferase (Luc)-conjugated antibody (LucAb), a dye that absorbed light from LucAb, and a photomultiplier tube (PMT) detector (Fig. 1). Without antigen, LucAb was uniformly dispersed in the solution, resulting in absorbance of most of the luminescence from LucAb by the dye. In contrast, in the presence of antigen, the antigen-LucAb complex was localized to the bottom of the PMT side, using centrifugation or magnetic force. We demonstrated the principle of the system by a biotin-streptavidin-based assay, followed by application to the detection of *Escherichia coli* O157, using LucAb.

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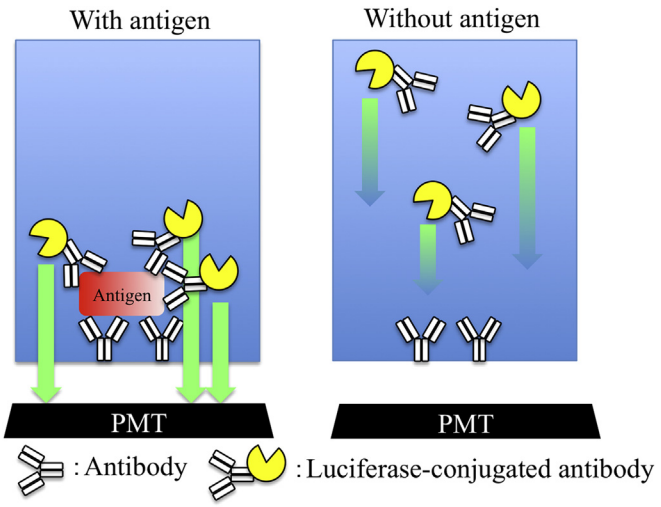

FIG. 1. Schematic illustration of BINGO assays. The luciferase-conjugated antibody and dye are uniformly distributed in the solution. In the presence of antigen, luciferase-conjugated antibodies are localized around the PMT, causing light to reach the PMT. The luminescence of Luc is indicated by an arrow. In the absence of antigen, the luminescence of the luciferase-conjugated antibody is absorbed by the dye, resulting in a low signal from the PMT.

MATERIALS AND METHODS

Expression and purification of Luc For expression of Luc, a pCold I vector (Takara Bio, Otsu, Japan) containing the *Luciola cruciata luc* gene (accession no. E05447.1) was prepared (12). The codon usage was optimized for expression in *E. coli*, using Optimizer (http://genomes.urv.es/OPTIMIZER/), and a mutation (Thr217Ile) was included to enhance thermostability and pH stability (13). The cloned sequence of the *luc* gene is shown in Fig. S1. *E. coli* BL21(DE3) was transformed with the plasmid, cultured, and treated to facilitate protein expression according to the manufacturer's instructions for pCold I. Cells were harvested by centrifugation at 8000 ×*g* for 5 min at 4°C and resuspended in 10 mL of 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 2 mg phenylmethylsulfonyl fluoride. Cells were then disrupted by sonication and centrifuged at 6000 ×*g* for 5 min at 4°C. The supernatant containing Luc was purified with Ni-NTA agarose (Qiagen, Hilden, Germany), using 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl and 500 mM imidazole. Purified Luc was dialyzed with phosphate-buffered saline (PBS) (pH 7.4), and the protein concentration was measured with Quick Start Bradford Protein Assays (Bio-Rad Laboratories, Hercules, CA, USA). For biotinylation of Luc, 1.0 mg/mL Luc was modified using Biotin Labeling Kit − NH_2 (Dojindo, Mashiki, Japan).

Selection of dye for absorbance of Luc luminescence The dye used for absorbance of Luc luminescence was selected as follows. Each of 10 dyes was dissolved in PBS, which had an absorbance of 0.4 at 590 nm. The concentrations of each dye were as follows: 18 μM bromothymol blue (Wako), 60 μM crystal violet (Katayama Chemical Industries, Osaka, Japan), 5.4 μM bromophenol blue (Kanto Chemical, Tokyo, Japan), 0.006% blue dextran 2000 (blue dextran; GE Healthcare UK, Little Chalfont, UK), 10 μM brilliant blue FCF (Wako), 17 μM Coomassie brilliant blue R-250 (Nacalai Tesque, Kyoto, Japan), 56 μM Congo red (Wako, Osaka, Japan), 59 μM Remazol brilliant blue R salt (Nacalai Tesque), 50 μM methyl blue (Tokyo Chemical Industry, Tokyo, Japan), and 0.24 mM neutral red (Wako). For analysis of the appropriate dye, 12.5 μL of 0.1 μg/mL biotinylated Luc, 12.5 μL PBS containing each dye, and 25 μL ONE-Glo Luciferase Assay System substrate (Promega, Madison, WI, USA) were mixed, and luminescence was measured using an Infinite 200 PRO (Tecan, Mannedorf, Switzerland).

Immunodevice for BINGO assays We designed and constructed an original device for BINGO assays, as illustrated in Fig. 2A. A PMT (H7360-01; Hamamatsu Photonics K. K., Hamamatsu, Japan) was attached beneath the lightproof sample holder in a dark box (Fig. 2B). The PMT was connected to a computer through a photon counting unit (C8855-01; Hamamatsu Photonics K. K.). Recording of the photon counts was carried out using software accompanying the photon-counting unit. The average of the photon counts in 30 s was employed for measurement of antigen amount.

Model assay system using biotinylated Luc and streptavidin-coated magnetic beads Ten microliters of 1 μg/mL biotinylated Luc in PBS was mixed with 10 μL of streptavidin-coated magnetic beads (1.5–1.8 × 10^9, 6–7 × 10^8, 6–7 × 10^7, 6–7 × 10^6 beads/mL; Dynabeads M270 streptavidin; SA beads; Veritas, Tokyo, Japan) in PBS, 80 μL of 2.5% blue dextran in PBS, and 100 μL of substrate in 0.2-mL clear polypropylene polymerase chain reaction (PCR) tubes with dome caps, and the mixtures were pipetted for several seconds at room temperature. The mixed reagents were placed on a magnet for 10 s to collect magnetic bead-biotinylated Luc complexes, followed by detection of luminescence using the PMT for 30 s. PBS including no beads or 6.5 × 10^8 beads/mL carboxylic acid-coated magnetic beads (Dynabeads M270 carboxylic acid; CA beads; Veritas) was used as a negative control instead of PBS including SA beads.

Modification of anti-*E. coli* O157 antibodies with streptavidin Ten microliters of 1 mg/mL BacTrace anti-*E. coli* O157:H7 antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) derived from a goat was modified with streptavidin, using a Streptavidin conjugation kit (Abcam, Cambridge, UK).

Detection of *E. coli* O157 in BINGO assays Five microliters of 4 μg/mL biotinylated Luc and 5 μL of 100 fold-diluted SA-conjugated anti-*E. coli* O157 antibody were mixed to prepare LucAb. After addition of 5 μL *E. coli* O157 GTC03904 cell suspension (the National BioResource Project GTC Collection, Gifu University, Gifu, Japan) in PBS (4 × 10^5, 4 × 10^4, or 4 × 10^3 cells), the solution was incubated on a rotator for 10 min at room temperature. Next, 145 μL of 2.5% blue dextran and 40 μL substrate were mixed, and samples were centrifuged to collect the bacteria and LucAb at the bottom of the sample chamber.

Detection of *E. coli* O157 in BINGO sandwich assays Twenty microliters of microbeads with anti-*E. coli* O157 antibody (Dynabeads anti-*E. coli* O157 beads; 1.5 × 10^8 beads/mL; Veritas) was mixed with 50 μL cell suspension (*E. coli* O157 GTC03904: 4 × 10^7, 4 × 10^6, 4 × 10^5, 4 × 10^4, 4 × 10^3, or 4 × 10^2 cells; *E. coli* DH5α: 4 × 10^5 cells) in PBS and incubated for 5 min. Then, 20 μL LucAb solution, which was prepared by mixing equal amounts of 2 μg/mL biotinylated Luc and 200 fold-diluted SA-conjugated anti-*E. coli* O157 antibodies, was added to the solution and incubated for 5 min. Subsequently, 70 μL of 2.5% blue dextran in PBS and 40 μL of substrate were added to the tube, which was then placed on the magnet to collect beads, at the bottom of the sample chamber.

RESULTS

Screening of the dye utilized in BINGO assays The dye used in BINGO assays should efficiently absorb the luminescence of Luc and not inhibit Luc activity. To select the appropriate dye for BINGO assays, Luc activity was measured in the presence of a low concentration of dye (Abs 590 nm = 0.1), which barely absorbed Luc luminescence. When using bromothymol blue, crystal violet, bromophenol blue, and blue dextran, Luc activities were 116%, 80.5%, 80.2%, and 73.5%, respectively, compared with that in the absence of dye. In addition, other dyes significantly reduced Luc activity (Fig. 3). However, bromothymol blue, bromophenol blue, and crystal violet are often used as pH indicators and are pH sensitive. These dyes are considered unsuitable for the BINGO assay, as the test sample may cause pH fluctuations. Finally, blue dextran was chosen as the dye to be used for the BINGO assay, taking into account the low inhibitory effect on Luc activity and the insensitivity to pH.

Examination of the model system based on the SA-biotin interaction To demonstrate the feasibility of BINGO assays, we attempted model experiments using the biotin-SA interaction (Fig. 4). After mixing SA beads and biotinylated Luc, SA beads were collected at the bottom of the sample chamber with a magnet, and Luc luminescence was measured from the bottom. Signal intensity increased with the amount of SA beads and peaked when 6–7 × 10^6 SA beads were used, which was 3.1 times that of the negative control in the absence of beads. In contrast, when 6.5 × 10^6 CA beads that could not bind biotinylated Luc were added to the detection system, the signal was 0.3 of the value in the absence of beads.

Immunodetection of *E. coli* O157 in BINGO assays Next, *E. coli* O157 was detected by BINGO assays using anti-*E. coli*-O157 antibodies labeled with Luc (LucAb; Fig. 5). Importantly, 4 × 10^5 *E. coli*-O157 cells yielded a 1.7-times higher signal than the negative control in the absence of the cells, whereas the presence of 4 × 10^4 or 4 × 10^3 cells did not result in any significant increase in the signal, compared with the negative control.

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