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Research Report

An Approach for Multiple Testing of Infectious Diseases in Developing Countries

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Report received on Dec. 12, 2016

ABSTRACT Medical infrastructures in developing countries are ill-equipped for clinical examinations and lack proper instrumentation. We have established an "on-chip enzyme-linked immunosorbent assay (ELISA)" wherein antibodies or antigens are immobilized onto a polymer containing an azobenzene moiety. Immobilized antibodies or antigens capture/recognize biomarkers through an immune reaction on a chip. The advantages of this system are that it uses a small sample volume, is inexpensive, and can simultaneously test multiple components in an examination. In this report, we demonstrated that on-chip ELISA can detect anti-virus antibody in serum using sterilized viruses and developed an inexpensive and portable detecting apparatus with a commercially available digital camera. We anticipate that this system will be particularly useful in developing countries.

KEYWORDS Infection, Disease, Virus, Antibody, Protein, Serum, Assay, Diagnostic, Array, Photo-immobilization, Chemiluminescence

1. Introduction

Various infectious diseases remain a challenge in developing tropical countries, with recent examples including the 2014 Ebola outbreak and 2015-2016 Zika virus epidemic. Medical infrastructures in these countries are ill-equipped for clinical examinations and lack proper instrumentation. Early detection and treatment of infection is important not only to alleviate physical stress, but also to prevent an epidemic or outbreak. Point of care testing shows promise as a quick and efficient treatment in critical events. Furthermore, advancement of PCR and multiplex PCR thermal cyclers has allowed for on-site measurement in an epidemic area, carrier detection, and verification of carrier state.

We have determined that a biological molecule, such as a protein or DNA, can be immobilized in the active state onto a photoresponsive polymer containing an azobenzene (azopolymer).[1-5] The azopolymer "physically" immobilizes the biological molecule with the use of blue light irradiation. Using this immobilization method, we established an "on-chip enzyme-linked immunosorbent assay (ELISA)" wherein antibodies or antigens are immobilized onto an azopolymer-coated glass slide. An overview of this assay system is shown in **Fig. 1**. For a sandwich ELISA, capture antibodies are immobilized onto an antibody array, biomarkers in a sample and enzyme-labeled antibodies are sequentially reacted, and chemiluminescence occurs according to the enzyme concentration in the spots. A charge-coupled device (CCD) detector captures a chemiluminescent image from the antibody array, and the chemiluminescent intensity of each spot is quantified. A representative sample reaction is shown in **Fig. 2**. This antibody array format is designed to test for four diseases in 40 samples per glass slide. Five microliters of a sample can cover four spots of disease-specific antigens, and 40 drops are independently plated onto a glass slide. On-chip ELISA utilizes a small sample volume, is inexpensive, and can simultaneously test multiple components during an examination. Using this system, we have developed a sandwich ELISA that measures adipose tissue hormones in serum and a competitive ELISA that measures representative oxidative biomarkers of low-molecular-weight compounds.[6]

An antibody array system that can be used for multiple testing is an efficacious tool for determining the virus or bacterium causing disease among candidate microorganisms. In this report, we evaluated our on-chip ELISA using viruses immobilized by gamma radiation to determine its ability to detect anti-virus antibodies in serum and demonstrated that this system could be used for simultaneous testing to detect multiple anti-virus antibodies during an examination. Additionally, we

developed an inexpensive and convenient portable detecting apparatus with a commercially available digital camera. Our findings demonstrate that our on-chip ELISA is an inexpensive and effective system that may be particularly useful in developing countries.

2. Experimental Design

2. 1 On-chip ELISA Preparation

2. 1. 1 Azopolymer Synthesis

The azopolymer used is shown in **Fig. 3**. N-ethyl-N-(2-hydroxyethyl)-4-(4-cyanophenylazo) aniline (azobenzene (CN)) was synthesized as previously described.[7] The azomonomer (CN) was synthesized as follows. Methacrylic acid was activated by water-soluble carbodiimide and reacted with azobenzene (CN). After washing with deionized water, the organic layer was dried over magnesium sulfate. The resulting material was then purified by column chromatography and recrystallized. Azomonomer (CN) and methyl methacrylate were polymerized by the addition of azobisisobutyronitrile (copolymerization rate: m = 15, n = 85). The polymer was then precipitated with methanol and obtained by filtration.

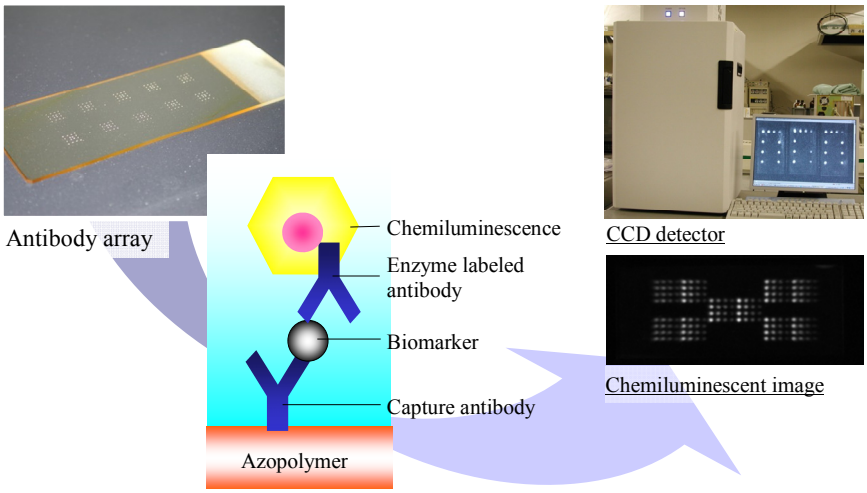


Fig. 1 Overview of the "on-chip ELISA".

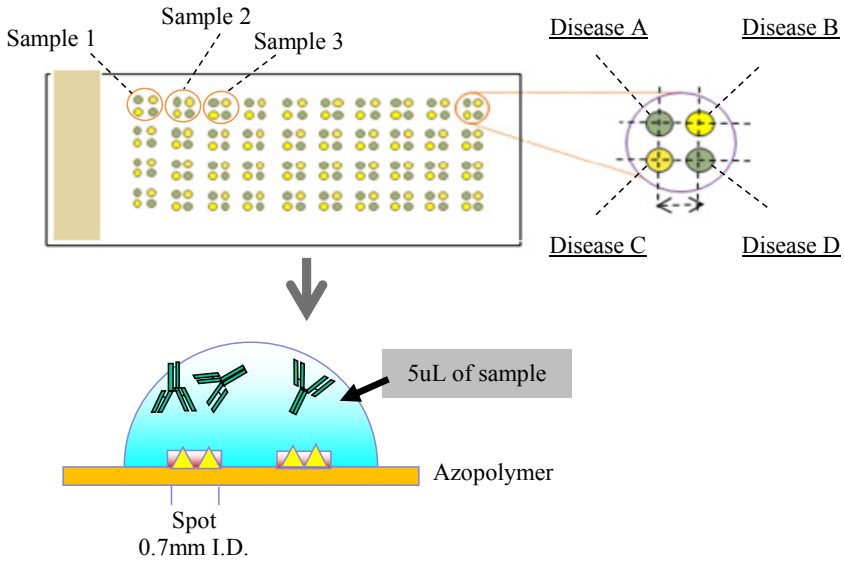


Fig. 2 Representative sample reaction using the on-chip ELISA.

2. 1. 2 Azopolymer Film Preparation

A glass slide with an azopolymer film was prepared as follows. An azopolymer-pyridine solution (12.5 g/L) was spin-coated onto a glass slide. The glass slide was then placed in a vacuum oven at 60°C for 2 h to obtain solvent-free samples, and stocked in a desiccator at room temperature. The resulting film on the glass slide was 40 nm thick.

2. 1. 3 Immobilization of Viruses on the Glass Slide (On-chip ELISA)

Gamma sterilized cytomegalovirus (native cytomegalovirus, PIP005), herpes virus (native herpes simplex virus 2, PIP010), measles virus (native measles virus, PIP013), and rubella virus (native rubella virus, PIP044) were purchased from Cosmo Bio Co., Ltd, Tokyo, Japan. Virus solutions were diluted to 50 μg/mL (protein concentration) with 0.02% Tween 20/PBS (TPBS). Diluted virus solutions were then spotted onto glass slides using a microarray printer (Kubota Corp., Amagasaki, Hyogo, Japan). After spotting, the slide was dried and irradiated for 30 min under blue light (470 nm, 20 mW/cm^2). The slide was then washed twice for 10 min each with TPBS, rinsed for 1 min with PBS, and centrifuged dry. A 1% bovine serum albumin solution was then pipetted onto the dried glass followed by irradiation for 30 min under blue light. Thereafter, the slide was again washed twice for 10 min each with TPBS, rinsed for 1 min with PBS, centrifuged dry, and stocked under refrigeration with dried silica gel.

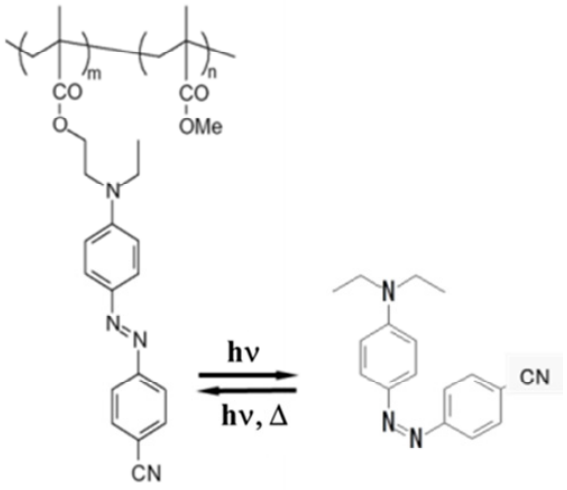


Fig. 3 Chemical structure of a CN azopolymer.

2. 2 Measurement of Anti-virus Antibodies Using On-chip ELISA

Detection kits for cytomegalovirus antibody (viral antibody EIA [Seiken] cytomegalovirus IgG, 322647), herpes antibody (viral antibody EIA [Seiken] herpes IgG, 322654), measles antibody (viral antibody EIA [Seiken] measles IgG, 322685), and rubella antibody (viral antibody EIA [Seiken] rubella IgG, 322966) were purchased from Cosmo Bio Co., Ltd. A chemiluminescence detection solution (SuperSignal ELISA Femto Maximum Sensitivity Substrate, 37075) was purchased from Funakoshi Co., Ltd, Bunkyo-ku, Tokyo, Japan. Droplets (5 μL) of antiserum (0-128 antibody titer) were plated onto a glass slide containing immobilized viruses. The glass slide was then incubated for 16 hours and washed. Droplets (5 μL) of a horseradish peroxidase-labeled anti-human IgG were then plated onto the glass slide. After incubation for an hour, the slide was washed and 300 μL of chemiluminescence detection solution was added to the glass slide with a chamber (Frame-Seal Slide Chambers, SLF-3001, Bio-Rad Laboratories, Inc., CA, USA). Chemiluminescence was measured using a CCD detector (LV-400, Aisin Seiki Co., Ltd., Aichi, Japan) following 15-min exposure.

2. 3 Measurement of Anti-virus Antibodies Using an Enhanced Chemiluminescent System and a Portable Detecting Apparatus with a Digital Camera

A biotin-labeled anti-human IgG (anti IgG + IgM (H + L), human (goat), A119BN) and a horseradish peroxidase-labeled streptavidin (streptavidin poly-HRP80 conjugate, 65R-S119) were purchased from Cosmo Bio Co., Ltd. Droplets (5 μL) of antiserum (0-128 antibody titer) were plated onto a glass slide containing immobilized viruses. The glass slide was then incubated for 20 minutes and washed. Droplets (5 μL) of a biotin-labeled anti-human IgG (100 ng/mL) were then plated onto the glass slide. The slide was then incubated for 30 min and washed. Droplets (5 μL) of streptavidin poly-HRP80 conjugate (200 ng/mL) were then plated onto the glass, then the slide was incubated for

30 min and washed. A chemiluminescence detection solution (300 μL) was plated onto the glass slide with a chamber (SLF-3001). Chemiluminescence was immediately measured using a prototype portable detecting apparatus. A schematic illustration of the portable detecting apparatus for chemiluminescence detection is shown in **Fig. 4**. The apparatus comprised a dark box measuring 150 mm × 150 mm × 300 mm (width × depth × height) containing a digital camera (EOS M, Canon Inc., Tokyo, Japan) and a macro lens (EF-S 60 mm f/2.8 MACRO USM, Canon). The dark box was made using duralumin plates, and the total weight of the apparatus was approximately 2 kg.

3. Results and Discussion

3. 1 Anti-virus Antibody Testing Using On-chip ELISA

We attempted to establish an anti-virus antibody test for serum using our on-chip ELISA system (**Fig. 5**). A sterilized virus is arrayed and immobilized onto an azopolymer-coated glass slide. Anti-virus human antibody in serum and enzyme-labeled antibodies are then sequentially reacted. To measure anti-cytomegalovirus IgG in serum, various concentrations of immunized serum and an anti-human IgG antibody were reacted against immobilized gamma sterilized cytomegalovirus on a glass slide. **Figure 6** shows a chemiluminescent image of the on-chip ELISA and calibration curve. Chemiluminescent intensities of spots were antibody titer-dependent, and the detection limit was lower than an antibody titer of 2 which is past the threshold for indicating a positive infection. **Figures 7**(a) and (b) show calibration curves of conventional ELISA and on-chip ELISA, respectively. On-chip ELISA demonstrated similar sensitivity and dynamic range as conventional ELISA.

3. 2 Multiple Testing of Anti-virus Antibodies in Serum

An antibody array system can simultaneously measure multiple contents. **Figure 8** shows a representative chemiluminescent image and intensity estimation of multiple testing of anti-virus antibodies in serum. One droplet of sample covers four spots of each virus, cytomegalovirus, herpes virus, measles virus, and rubella virus. Various antibody titers of each immunized serum and an immunized serum mix were plated and reacted against each spot on the antibody array. Each spot of virus showed chemiluminescence against specific anti-virus serum, and their intensities were estimated (measles virus: medium, cytomegalovirus: medium, herpes virus: weak, and rubella virus: strong). However, non-specific chemiluminescence was observed for rubella virus with anti-measles serum and anti-cytomegalovirus serum. These non-specific reactions may be caused by the presence of anti-virus antibodies naturally raised in particular anti-virus serum, and were therefore considered acceptable. For

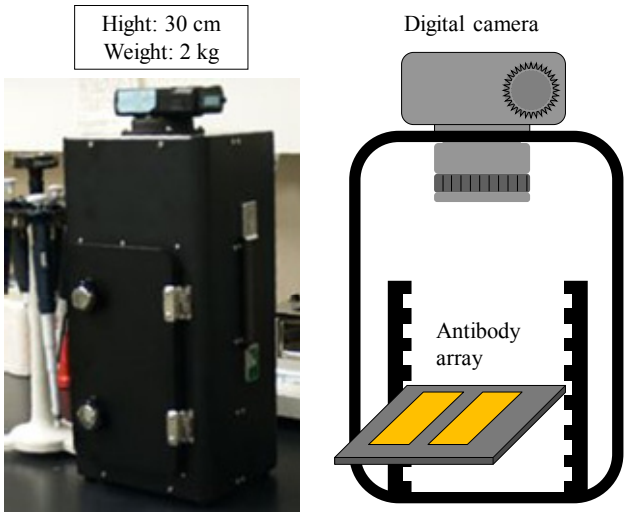


Fig. 4 Overview of a portable detecting apparatus for chemiluminescence detection.

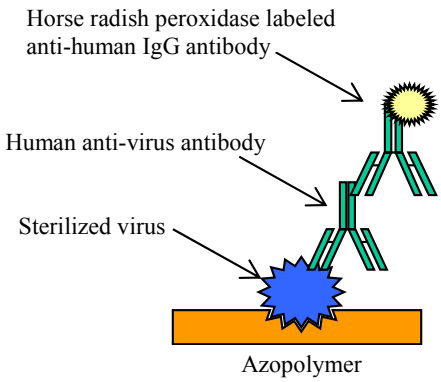


Fig. 5 Measurement of anti-virus antibodies in serum using the on-chip ELISA.

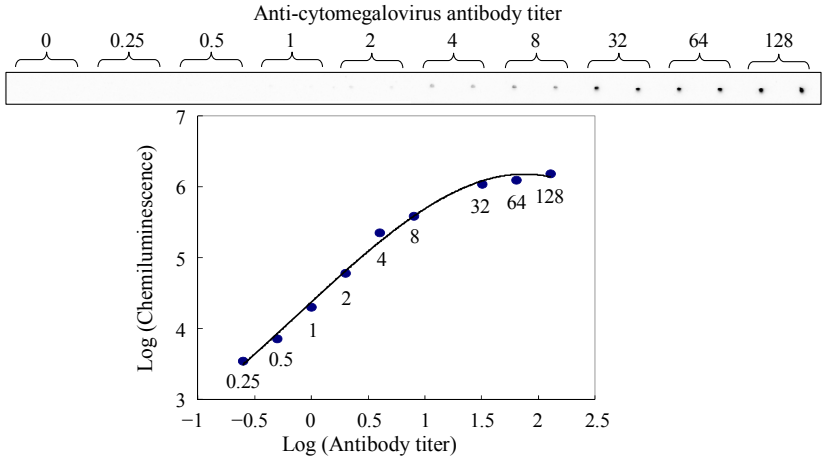


Fig. 6 Chemiluminescent image and calibration curve for measurement of anti-cytomegalovirus IgG in serum.

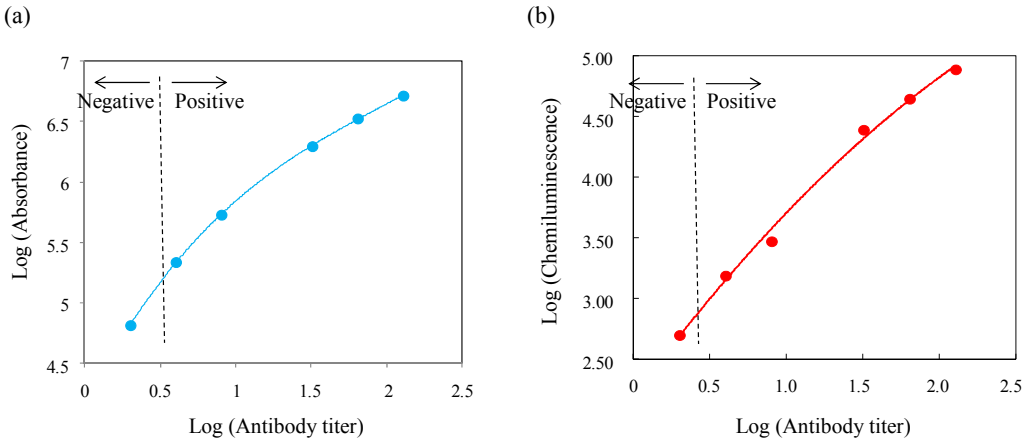
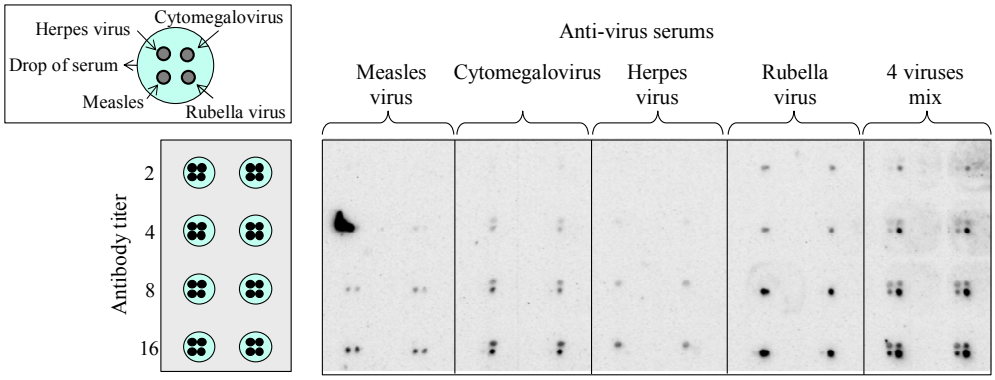


Fig. 7 Comparison of conventional ELISA and on-chip ELISA.
(a) Conventional ELISA. (b) On-chip ELISA.



Measles	++				++
Cytomegalovirus		++			++
Herpes virus			+		+
Rubella virus	++	++		+++	+++

(+++ : Strong response, ++ : Medium response, + : Weak response)

Fig. 8 Multiple testing of anti-virus antibodies in serum.

mixed anti-serum, chemiluminescence of all four spots was observed with intensities reflecting that observed for single anti-virus serum. Thus, multiple testing of anti-virus antibodies in serum was successfully performed. Additionally, we observed that virus inactivation methods influenced immunogenicity. Most formalin- and UV-inactivated viruses demonstrated low immunogenicity.

3. 3 Enhancement of Chemiluminescence Using a Portable Detecting Apparatus

An inexpensive and convenient detecting device is required in developing countries. Using a commercially available digital camera or smartphone instead of a CCD is the best solution for an inexpensive antibody array system. Chemiluminescence demonstrates the highest sensitivity and intensity compared with fluorescence and absorbency; thus, it is suitable for use in an antibody array system with a planar surface. However, general ELISA using horseradish peroxidase does not have sufficient intensity. Therefore, we attempted to enhance the chemiluminescence using a "poly-HRP" system, which increases the amount of horseradish peroxidases available per one molecule of anti-virus antibody. **Figure 9** shows a schematic illustration of measurement of anti-virus antibodies in serum using the enhanced chemiluminescent antibody array. A sterilized virus is arrayed and immobilized onto an azopolymer-coated glass slide. Anti-virus human antibody in serum, biotin-labeled antibody, and poly-HRP are sequentially reacted with the immobilized virus. Chemiluminescent images of

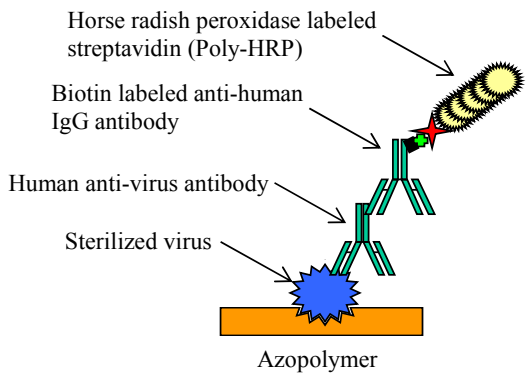


Fig. 9 Measurement of anti-virus antibodies in serum using an enhanced chemiluminescent on-chip ELISA.

on-chip ELISA using a prolonged serum incubation time (16 h) and poly-HRP as the chemiluminescent enzyme are shown in **Fig. 10**. A chemiluminescent image was obtained after 30-s accumulation using a CCD detector (Fig. 10(a)). The chemiluminescent intensity was enhanced over 30-fold compared with the conventional method (Figs. 6 and 8). Chemiluminescent intensities of spots were antibody titer-dependent, and chemiluminescence was observed at antibody titers greater than 2. Using the portable detecting apparatus, a chemiluminescent image could be obtained following 2-min accumulation at ISO 12800, f/2.8 (Fig. 10(b)). Chemiluminescence was observed at antibody titers of 2 or greater. The total running time from serum reaction to chemiluminescent detection was approximately 1 day.

3. 4 Shortening of Assay Run Time

We next attempted to shorten the serum reaction time, which was the longest step in the procedure (16 h). The reaction time for anti-cytomegalovirus serum was fixed at 20 min compared with 16 h, and the concentrations of poly-HRP-labeled streptavidin were then evaluated. Irrespective of poly-HRP concentration, a 20-min reaction time was sufficient to detect chemiluminescence of spots containing antibody titers greater than 2 (**Fig. 11**). Therefore, the total running time was shortened from 1 day to approximately 3 h.

4. Conclusion

We developed an antibody array system (on-chip ELISA) which could measure four contents of anti-virus antibodies in one droplet of serum. Simultaneous testing for all four contents demonstrated similar performance to conventional one-by-one testing. Furthermore, on-chip ELISA had good sensitivity and dynamic range. Enhanced chemiluminescence could be achieved within 3 h using a cheap and portable apparatus. This short total running time enables a rapid diagnosis and proper treatment. In India, for example, patient samples can be collected during the first clinical examination in the morning hours. In the afternoon, patients can then undergo a second examination following the results of the clinical test. Thus, we anticipate that this system will make a significant contribution in developing countries.

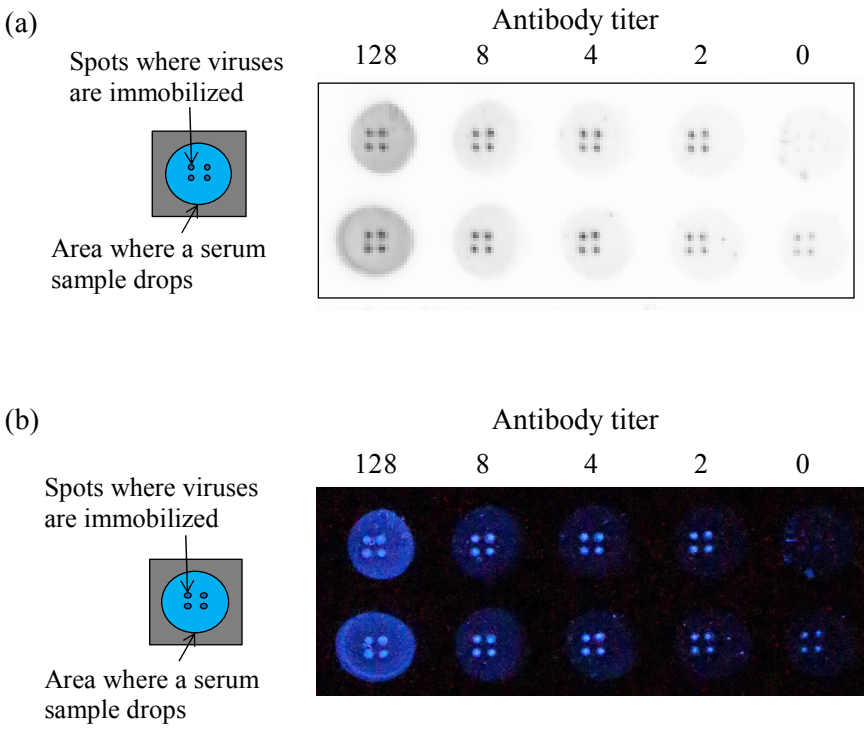


Fig. 10 Chemiluminescent image obtained following enhanced chemiluminescent on-chip ELISA. (a) CCD detector (30-s accumulation). (b) Portable detecting apparatus (2-min accumulation).

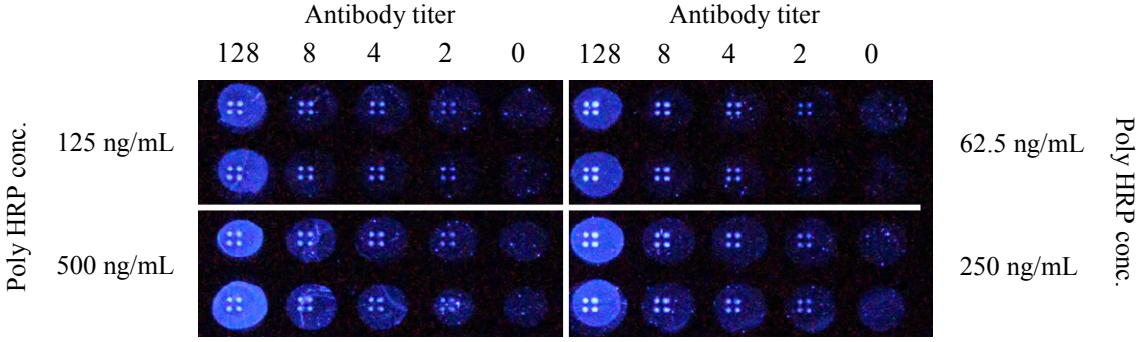


Fig. 11 Chemiluminescent image obtained following shortened serum reaction time.

References

(1) Kawata, Y., Egami, O., Nakamura, O., Sugihara, N., Okamoto, M., Tsuchimori, M. and Watanabe, O., "Non-optically Probing Near-field Microscopy", *Opt. Commun.*, Vol. 161 (1999), pp. 6-12.

(2) Ikawa, T., Mitsuoka, T., Hasegawa, M., Tsuchimori, M., Watanabe, O., Kawata, Y., Egami, C., Sugihara, O. and Okamoto, N., "Optical Near Field Induced Change in Viscoelasticity on an Azobenzene-containing Polymer Surface", *J. Phys. Chem. B*, Vol. 104 (2000), pp. 9055-9058.

(3) Ikawa, T., Mitsuoka, T., Hasegawa, M., Tsuchimori, M., Watanabe, O. and Kawata, Y., "Azobenzene Polymer Surface Deformation Due to the Gradient Force of the Optical Near Field of Monodispersed Polystyrene Spheres", *Phys. Rev. B*, Vol. 64, No. 19 (2001), 195408.

(4) Hasegawa, M., Ikawa, T., Tsuchimori, M., Watanabe, O. and Kawata, Y., "Topographical Nanostructure Patterning on the Surface of a Thin Film of Polyurethane Containing Azobenzene Moiety Using the Optical Near Field Around Polystyrene Spheres", *Macromolecules*, Vol. 34 (2001), pp. 7471-7476.

(5) Ikawa, T., Hoshino, F., Matsuyama, T., Takahashi, H. and Watanabe, O., "Molecular-shape Imprinting and Immobilization of Biomolecules on a Polymer Containing Azo Dye", *Langmuir*, Vol. 22 (2006), pp. 2747-2753.
(6) Hoshino, F., Watanabe, O., Wu, X., Takimoto, Y. and Osawa, T., "Low-cost and Easy-to-use 'On-chip ELISA' for Developing Health-promoting Foods", *Subcellular Biochem.*, Vol. 77 (2014), pp. 151-161.
(7) Ho, M.-S., Natansohn, A., Barrett, C. and Rochon, P., "Azo Polymers for Reversible Optical Storage 8. The Effect of Polarity of the Azobenzene Groups", *Can. J. Chem.*, Vol. 73 (1995), pp. 1773-1778.

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