Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Facile and rapid detection of SARS-CoV-2 antibody based on a noncompetitive fluorescence polarization immunoassay in human serum samples

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ABSTRACT

Antibody detection methods for viral infections have received broad attention due to the COVID-19 pandemic. In addition, there remains an ever-increasing need to quantitatively evaluate the immune response to develop vaccines and treatments for COVID-19. Here, we report an analytical method for the rapid and quantitative detection of SARS-CoV-2 antibody in human serum by fluorescence polarization immunoassay (FPIA). A recombinant SARS-CoV-2 receptor binding domain (RBD) protein labeled with HiLyte Fluor 647 (F-RBD) was prepared and used for FPIA. When the anti-RBD antibody in human serum binds to F-RBD, the degree of polarization (*P*) increases by suppressing the rotational diffusion of F-RBD. The measurement procedure required only mixing a reagent containing F-RBD with serum sample and measuring the *P* value with a portable fluorescence polarization analyzer after 15 min incubation. We evaluated analytical performance of the developed FPIA system using 30 samples: 20 COVID-19 positive sera and 10 negative sera. The receiver operating characteristic curve drawn with the obtained results showed that this FPIA system had high accuracy for discriminating COVID-19 positive or negative serum (AUC = 0.965). The total measurement time was about 20 min, and the serum volume required for measurement was 0.25 μL. Therefore, we successfully developed the FPIA system that enables rapid and easy quantification of SARS-CoV-2 antibody. It is believed that our FPIA system will facilitate rapid on-site identification of infected persons and deepen understanding of the immune response to COVID-19.

Introduction

In December 2019, an increase in patients with pneumonia of unknown cause was reported in Wuhan, China (Zhu et al., 2020). The patients' clinical symptoms were very similar to those of SARS-CoV and MERS-CoV, and found to be due to a novel Coronaviridae family (Zhou et al., 2020; Yan et al., 2020a,b). The International Virus Classification Commission (ICTV) classified this virus as "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2) and the World Health Organization (WHO) named the infectious disease caused by SARS-CoV-2 as "COVID-19" (Yang and Wang, 2020). As of April 2021, the total number of COVID-19 cases confirmed worldwide was ~130 million, and the number of deaths was ~2.9 million (World Health Organization. Coronavirus disease (COVID-19) pandemic., https://www.who.int/emergencies/diseases/novel-coronavirus-2019). Patients infected with COVID-19 exhibit a wide range of symptoms, from asymptomatic to

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severe pneumonia. This feature makes it difficult to identify COVID-19 positive patients and it has promoted to the explosive spread of infection (Kronbichler et al., 2020; Lai et al., 2020). Over the past year, vaccines and therapeutic agents effective against COVID-19 have been actively developed (Kaur and Gupta, 2020; Beigel et al., 2020), and several types of vaccines have been approved and inoculations begun (Paltiel et al., 2021; Dal-Ré et al., 2021). However, a considerable amount of time will be needed to spread the vaccines and therapeutic agents world-wide. In addition, there is always a risk of developing highly pathogenic and infectious mutant strains as has happened with influenza viruses (Thomson et al., 2021; Baric, 2020). Therefore, identifying and isolating positive patients including asymptomatic infected patients based on a rapid diagnosis method, and performing contact tracing are still important strategies to prevent the spread of COVID-19 infection (Hellewell et al., 2020; Atalan, 2020).

SARS-CoV-2 is a positive sense, single-stranded RNA virus (Alexandersen et al., 2020). For the diagnosis of RNA virus infection, the reverse transcription polymerase chain reaction (RT-PCR) is used as the gold standard (Hoffmann et al., 2007; Feng et al., 2020). Since RT-PCR is accurate and highly sensitive, it is an essential measurement method for a definitive diagnosis. However, RT-PCR requires well-trained technicians, expensive and large equipment, and several hours of measurement time (Ahrberg et al., 2016; Cheng et al., 2017). These drawbacks limit the applications and environments in which RT-PCR can be used. As a simple and rapid method for diagnosis of COVID-19, the detection of SARS-CoV-2 specific antibody is popular. Lateral flow immunoassay (LFIA) is commonly used to detect SARS-CoV-2 specific antibody for rapid diagnosis and many studies on LFIA for COVID-19 have been reported (Wen et al., 2020; Grant et al., 2020; Liu et al., 2020a,b; Lin et al., 2020; Wang et al., 2020). LFIA is an easy-to-use method; however, typically only qualitative results can be obtained and the measurement sensitivity varies depending on the product (Lisboa Bastos et al., 2020; Torjesen, 2021). In addition, LFIA requires one device per one sample, resulting in low measurement throughput and cost efficiency. Therefore, a rapid and high-throughput quantitative antibody measurement is desirable for reliable and large-scale diagnosis.

Quantitative antibody measurement also plays an important role in confirming the acquisition of immunity by vaccination and the level of herd immunity (Higgins et al., 2021). The objective of vaccination is to induce many SARS-CoV-2 specific neutralizing antibodies, and quantitative measurement of antibody titers is essential for assessing vaccine effect. Obtaining information about the amount of antibody after vaccination and during the recovery period leads to correct evaluation of vaccines and treatment methods for COVID-19. Evaluation of the immune response after vaccination based on antibody titer has already begun (Ewer et al., 2021; Prendecki et al., 2021; Ella et al., 2021). The enzyme-linked immuno-sorbent assay (ELISA) is often used for such evaluation; however, ELISA requires several hours for incubation and many washing procedures. A rapid and high-throughput quantitative antibody measurement greatly contributes to improving the efficiency of vaccine development.

Here, we report a novel method for quantitatively measuring SARS-CoV-2 antibody by fluorescence polarization immunoassay (FPIA). FPIA is a well-known homogeneous immunoassay that uses a dimensionless number of the degree of fluorescence polarization (*P*) as a parameter (Smith and Eremin, 2008). FPIA has the advantages of rapidity and simplicity, and it has been widely used in the food processing and medical fields (Hendrickson et al., 2020). In order to achieve on-site measurement with FPIA for various targets, we have developed a portable fluorescence polarization (FP) analyzer and various applications based on non-competitive FPIA (Wakao et al., 2018, 2019a, 2019b; Nishiyama et al., 2020, 2021a, Nishiyama et al., 2021b; Fukuyama et al., 2020). We thought that if our FPIA system could be applied to the antibody measurement for COVID-19, it would greatly contribute to diagnosis and research on vaccines and treatment drugs. Therefore, we have developed a rapid quantitative on-site measurement system for SARS-CoV-2 antibody based on FPIA and a portable FP analyzer. The fluorescence-labeled SARS-CoV-2 spike protein receptor binding domain (RBD) was used for FPIA, and the total amount of anti-RBD antibody in human serum was measured. RBD is part of an S1 subunit of spike glycoproteins of SARS-CoV-2 and it binds to the peptidase domain of angiotensin-converting enzyme 2 (ACE2) (Yan et al., 2020a,b). Many neutralizing antibodies target RBD, and detection of anti-RBD antibody is an important indicator for the diagnosis of COVID-19 (Indenbaum et al., 2020; Liu et al., 2020a,b; Garcia-Beltran et al., 2021). In addition, some vaccines target S protein including RBD, and measurement of anti-RBD antibody is important from the viewpoint of evaluating the immune response by these vaccines (Chung et al., 2020). We anticipate that our FPIA system will contribute to both diagnosis and fundamental research for COVID-19.

Materials and methods

Materials

Phosphate-buffered saline (PBS; pH 7.4) was purchased from Thermo Fisher Scientific, Inc. (USA). Albumin from bovine serum (BSA) was purchased from FUJIFILM Wako Pure Chemical Corporation (Japan). Recombinant SARS-CoV-2 spike RBD protein (39 kDa) was purchased from SignalChem Biotech, Inc. (Canada). Anti SARS-CoV-2 Spike RBD polyclonal rabbit IgG antibody was purchased from Sino Biological, Inc. (China). Anti-avian influenza A hemagglutinin rabbit IgG antibody was purchased from Abcam, Inc. (USA). Healthy human serum was purchased from Cosmo Bio Co., Ltd., (Japan). COVID-19 positive and negative human serum samples were purchased from Raybiotech, Inc. (USA). Positive serum samples were collected from patients who were positive for COVID-19 by PCR or antigen testing, and have been inactivated. Polydimethylsiloxane (PDMS; SILPOT 184 W/C) was purchased from Dow Corning Toray Co., Ltd. (Japan). A negative photoresist (SU-8 3050) was purchased from Nippon Kayaku Co., Ltd. (Japan). HiLyte Fluor™ 647 Labeling Kit-NH_2 were purchased from Dojindo Molecular Technologies, Inc. (Japan). Two COVID-19 IgM/IgG rapid test LFIA kits, kit A and kit B, were purchased from GenBody Inc. (Korea) and Epigentek (USA), respectively.

Preparation of fluorescence-labeled RBD (F-RBD)

Recombinant SARS-CoV-2 spike RBD was labeled with HiLyte Fluor 647 using the HiLyte Fluor™ 647 Labeling Kit-NH_2. Unreacted fluorescent molecules were separated using a modified polyethersulfone membrane (Nanosep centrifugal filtration device 10K, Pall Corporation, USA). Concentration of F-RBD was determined based on absorbance at 280 nm obtained with a microvolume UV–Vis spectrophotometer (NanoDrop One, Thermo Fisher Scientific, Inc.).

Portable FP analyzer

The portable FP analyzer we previously developed (Wakao et al., 2018, 2019a, 2019b) was used with slight modifications (Fig. S1 (a)). The excitation filter, emission filter and dichroic mirror were changed to correspond to the fluorescence and excitation wavelength of HiLyte Fluor 647. The microdevice (Fig. S1 (b),(c)) which has nine channels was fabricated by the soft lithography technique, in accordance with the literature (Nishiyama et al., 2020).

Assay procedure of FPIA

Analyte samples of three different kinds were used: anti SARS-CoV-2 Spike RBD IgG antibody spiked in human serum, COVID-19 positive serum, and COVID-19 negative serum. Assay conditions were determined by referring to our previous non-competitive FPIA conditions (Nishiyama et al., 2021a). An analyte sample, 1 μg/mL F-RBD in PBS,

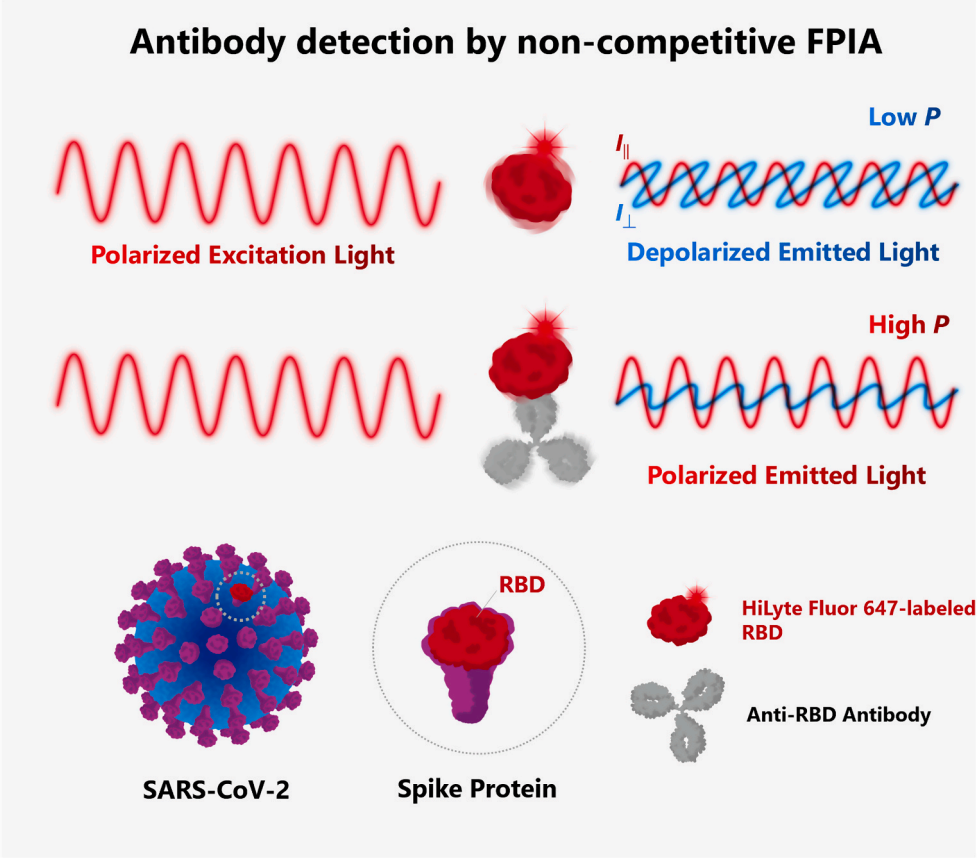

Fig. 1. Principle of FPIA using F-RBD for anti-SARS-CoV-2 RBD antibody.

1% BSA in PBS and neat PBS were mixed in a microtube. The mixing volume ratio was analyte sample:F-RBD:1% BSA-PBS:PBS = 5:20:40:335 (80-fold dilution). When we investigated the dilution rate, the mixing volume ratio was changed. The mixture was incubated at 25 °C for 15 min. Then, the mixture (20 μL) was injected into the microdevice. The microdevice was set on the portable FP analyzer and *P* value was measured. All experiments were performed in a BSL-2 laboratory with biosafety precautions.

Commercial LFIA devices for COVID-19

COVID-19 positive serum sample was tested with the two different COVID-19 IgM/IgG tests kit (kits A and B) to compare the detection sensitivity of FPIA. The sample was diluted by PBS and tested following the procedures described in the kit manufacturer's instructions. For kit A, an aliquot (10 μL) of the original or diluted serum was injected into the lateral flow device. Then, the assay buffer (~100 μL) was added into the device. After the incubation for 10 min, the test results were visually interpreted by reading the test line. For kit B, an aliquot (10 μL) of the original or diluted serum was mixed with the dilution buffer (80 μL) in a microtube. Then, the mixture (90 μL) was added into the device. After the incubation for 15 min, the test results were visually interpreted by reading the test line.

Measurement of fluorescence intensity of the mixture of human serum and F-RBD

Human serum and F-RBD were mixed in a microtube. The mixing volume ratio of the mixture was human serum:F-RBD:1% BSA-PBS:PBS = 5:20:40:335 (80-fold dilution). The mixture (120 μL) was added to a 96-well black microplate (PROTEOSAVE plates, Sumitomo Bakelite, Japan), and the fluorescence intensity was measured with a microplate reader (Infinite 200 PRO, Tecan, Switzerland) at excitation and emission wavelengths of 620 and 670 nm, respectively.

Statistical analyses

All statistical analyses including the receiver operating characteristic (ROC) analysis and the area under the curve (AUC) calculation were performed using BellCurve for Excel (BellCurve Japan).

Results and discussion

FPIA system for the detection of anti-RBD antibody

Fig. 1 shows a schematic illustration of the principle of antibody detection by non-competitive-FPIA. Since there is almost no overlap for the fluorescence wavelength range of HiLyte Fluor 647 with that of the fluorescence emitted by human serum itself, the effect on the measurement of serum components can be greatly reduced (Nishiyama et al., 2021a). When F-RBD in the solution is irradiated with polarized excitation light, the emitted fluorescence is depolarized due to the rotational movement of F-RBD. When the anti-RBD antibody binds to F-RBD, the increase of the hydrodynamic radius of the fluorescent compound suppresses its rotational movement, resulting in emission of polarized fluorescence. By measuring perpendicular polarization ($I_{\perp}$) and parallel polarization ($I_{\parallel}$), the degree of fluorescence polarization (P) is determined as follows (Smith and Eremin, 2008).

$$P = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

When anti-RBD antibodies are added into F-RBD reagent, an antibody-F-RBD complex is formed according to the amount of anti-RBD antibody. Therefore, the P value increases as the amount of antibody

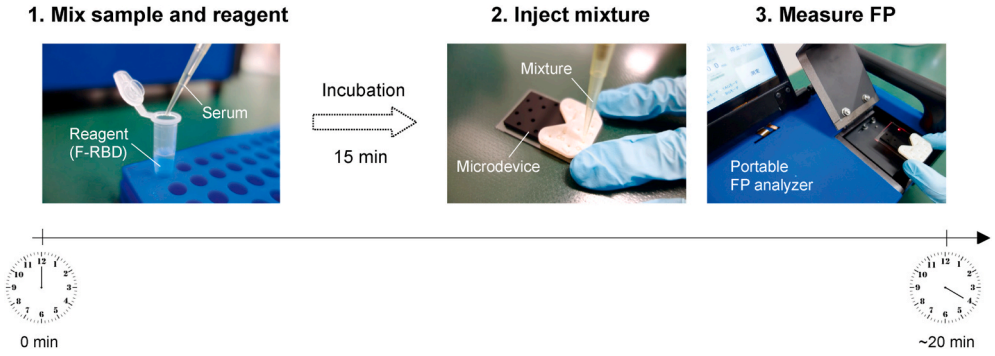

Fig. 2. Measurement procedure of FPIA using the portable FP analyzer and microdevice.

increases.

In this non-competitive FPIA-based assay, the measurement procedure consists simply of adding F-RBD into serum and measuring the *P* value with a portable FP analyzer (Fig. 2). It is possible to perform antibody testing with a sample volume of 20 μL or less per measurement using the microdevice. In our developed method, the total amount of antibody that binds to RBD is determined by one measurement. Almost all LFIA devices for SARS-CoV-2 antibody testing use labeling of secondary antibodies to measure IgG and IgM antibodies separately. However, the importance of detecting the other class of antibody has been reported recently (Cavalera et al., 2021; Infantino et al., 2021; Padoan et al., 2020), and the usefulness of measuring the total amount of antibody independent of the antibody class was also pointed out (Cavalera et al., 2021). It is possible to measure total antibodies regardless of antibody class because FPIA does not use a secondary antibody unlike LFIA.

Optimization and evaluation of the FPIA system

FPIA is a homogeneous immunoassay and bound-free separation is not required. It is easily affected by interferences in the sample, which may cause fluctuation of the *P* value of blank between the samples (Nishiyama et al., 2021a). Therefore, we investigated mitigation of the influence of interferences in serum by diluting serum with F-RBD reagent. In order to determine the appropriate dilution ratio of serum, F-RBD solution was added to healthy donor serum samples ($n = 10$) and the variation of *P* value between samples was evaluated. Fig. 3 shows the mean and standard deviation of the *P* value of the mixture of serum samples and F-RBD solution when diluted by F-RBD solution at each dilution ratio. The dilution ratio means the final dilution ratio of serum in the mixture, where the original serum concentration is 1. It was confirmed that the standard deviation of the *P* value decreased with the dilution ratio and became almost constant when the dilution ratio exceeded 80 times. Also, with 80-fold diluted serum, F-RBD of 1 μg/mL exhibited sufficient fluorescence intensity (Fig. S2). Therefore, we decided to use a dilution factor of 80 times and 1 μg/mL F-RBD in the following experiments. Regarding the decrease in antibody concentration in sample when diluted, we discuss whether the sensitivity is sufficient for antibody detection in COVID-19-positive patients in the next section.

The detection sensitivity of the FPIA system was evaluated with anti-RBD IgG antibody spiked in healthy human serum. F-RBD reagent was added into the anti-RBD antibody in serum and the *P* value was measured after incubation. The calibration curve for antibody in human serum is shown in Fig. 4. The *P* value was confirmed to increase with the increase in antibody concentration and the prepared F-RBD was confirmed to bound with the anti-RBD antibody. The limit of detection (LOD) was calculated to be 2.9 μg/mL (final concentration in the 80 times diluted mixture: 36 ng/mL). Subsequently, we evaluated the selectivity of this FPIA system. The *P* value of anti-RBD antibody was compared with that of an anti-influenza A virus antibody as a negative control (Fig. S3). The *P* value of the anti-RBD antibody was found to be significantly higher than that of the negative control. From the above results, our FPIA system was demonstrated as capable of measuring anti-

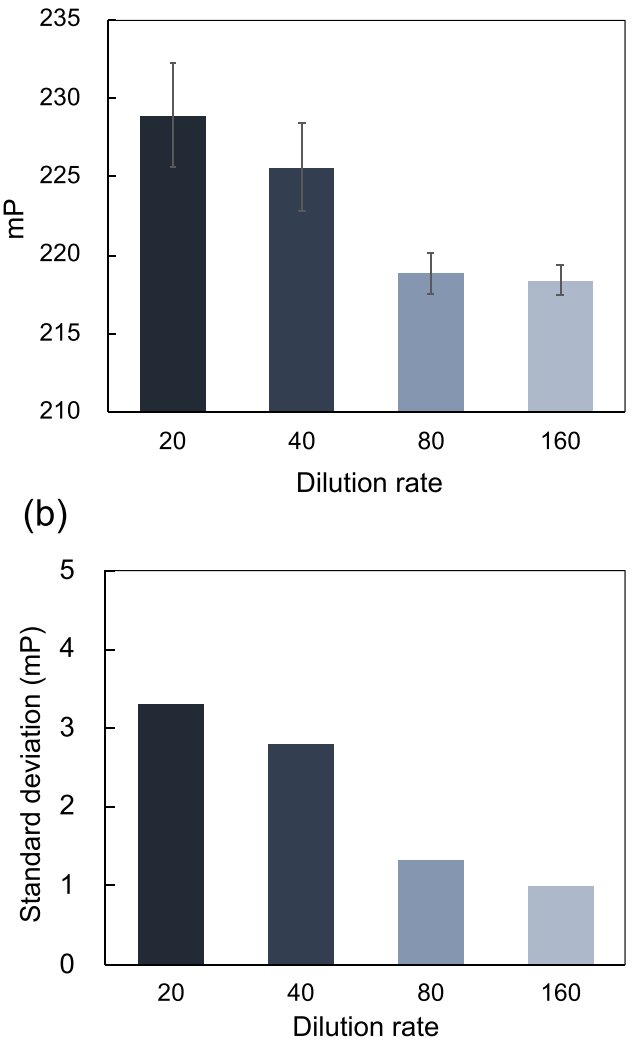

Fig. 3. Optimization of dilution rate of serum. Each serum sample from 10 healthy donors was diluted by PBS and mixed with 1 μg/mL F-RBD and 1% BSA-PBS. (a) Fluorescence polarization of the mixture ($n = 3$). (b) Standard deviation of fluorescence polarization of the mixture. Each data bar means the average value of 10 serum samples from separate donors.

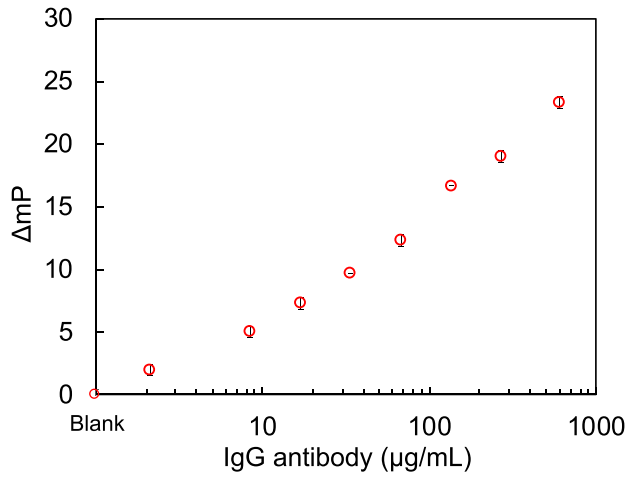

Fig. 4. Calibration curve for anti-SARS-CoV-2 RBD IgG antibody spiked in healthy human serum ($n = 3$). $\Delta mP = 1000 \times (P_{sample} - P_{blank})$.

RBD antibody in human serum selectively with a total measurement time of about 20 min. The required serum sample volume per measurement was only 0.25 μL.

Analytical performance of the FPIA system using positive and negative serum samples

The analytical performance of the developed FPIA system was evaluated with real samples. Commercially available COVID-19 positive and negative serum samples were used for the evaluation. To confirm whether the developed FPIA system has sufficient detection sensitivity to determine the antibody concentration in a real sample, the results obtained by FPIA for positive serum samples at various concentrations (dilution rates) are shown in Fig. 5. Positive serum samples diluted with PBS were prepared, and FPIA was performed under the same conditions as the calibration curve (Fig. 4). The *P* value was confirmed to decrease as the dilution rate increased (the antibody concentration decreased). The difference of the *P* value between the 80-fold dilution and the blank was 14.0 mP, which corresponds to ~70 μg/mL of the antibody in Fig. 4. The antibody concentration in the positive serum can be measured even if serum diluted 80 times is used in FPIA. In order to clarify the analytical performance as a rapid diagnostic method, the LOD was compared using two commercially available LFIA kits (Kits A and B) for detecting

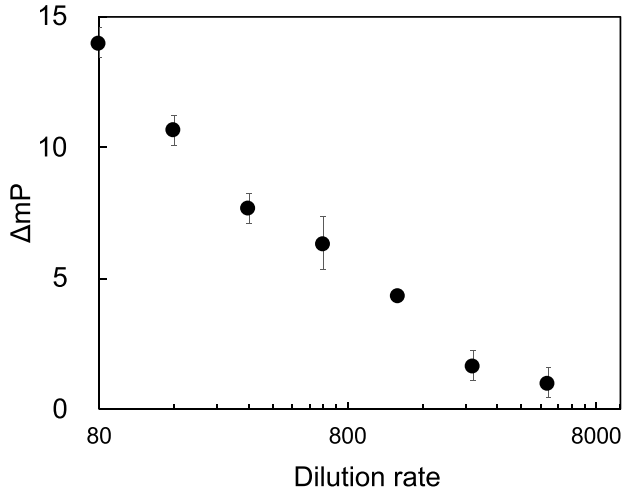

Fig. 5. Fluorescence polarization as a function of dilution rate of COVID-19 positive serum ($n = 3$). $\Delta mP = 1000 \times (P_{sample} - P_{PBS})$.

COVID-19 antibody and the same positive serum sample (Fig. S4 and S5). IgG antibody was detected only in the original serum using Kit A, and up to 16-fold dilution (corresponding to 1280-fold dilution in FPIA) using Kit B. However, in both kits, the IgG line disappeared at the following dilution ratios. Since the types of immobilized antigens in these kits are not disclosed, it is possible that antibodies that bind to epitopes different from FPIA have been detected in these kits. From the viewpoint of simple diagnosis of COVID-19, we judged that the developed FPIA system has a detection sensitivity higher than that of the commercially available LFIA kits for detecting COVID-19 antibody.

We further evaluated analytical performance with 20 positive serum samples from COVID-19 infected donors and 10 negative serum samples from healthy donors. The *P* values obtained from each serum are shown in Fig. 6 (a). The average *P* value of positive sera was higher than that of negative sera. Based on these *P* values, a ROC curve was prepared to evaluate discrimination ability (Fig. 6 (b)). The AUC value, which is an index of discrimination ability obtained from the ROC curve, was 0.965. Generally, an AUC value of 0.900 or higher is considered to indicate a very accurate analysis method (Streiner and Cairney, 2007). Therefore, our FPIA system is highly consistent with the result of PCR or antigen testing. The candidate cut-off value was $\Delta mP = 0.83$ or 2.17, which provided the point with the maximum sensitivity and specificity on the ROC curve. Table S1 summarizes the consistency between the conventional methods (the PCR or antigen testing) and our developed FPIA system at the above cut-off values. However, more data are needed to determine the cut-off value, increasing the measurement data will be implemented in the future.

The above results demonstrated that our FPIA system has the analytical capability for COVID-19 diagnosis that is highly accurate, quick, and easy to do, and that has high throughput, whereas existing quantitative ELISA methods for anti-COVID-19 virus antibody with the comparable AUC value take 1–2 h and require 3–4 washing operations for the assay (Alandijany et al., 2020; Roy et al., 2020). In addition, our developed method capable of multiple simultaneous measurements significantly contributes to high-throughput antibody quantification.

Conclusions

We have developed a FPIA system to detect anti-SARS-CoV-2 antibodies in human serum rapidly. The total amount of anti-RBD antibody in human serum was measured by applying a fluorescence-labeled RBD to FPIA. The total analysis time was ~20 min and the required serum sample volume was 1 μL or less. Since this FPIA can be performed anywhere using a portable FP analyzer, we expect that it will be used as a fast screening method for COVID-19. In addition, based on our previously successful detection of viruses by FPIA (Nishiyama et al., 2021b), we are confident simultaneous detection of viruses and antibodies is possible. By changing the fluorescent label, applicability can be extended to other viral infections. The developed FPIA system can also be used for quantitative evaluation of COVID-19 vaccines and treatments. High-throughput measurement greatly contributes to better research efficiency. In this way, our FPIA system is a highly expandable analysis method, with many purposes in the future.

CRediT authorship contribution statement

Keine Nishiyama: Conceptualization, Investigation, Formal analysis, Methodology, Writing – original draft. **Kazuki Takahashi:** Investigation, Formal analysis, Methodology, Writing – review & editing. **Mao Fukuyama:** Conceptualization, Methodology, Writing – review & editing. **Motohiro Kasuya:** Conceptualization, Methodology, Writing – review & editing. **Ayuko Imai:** Resources, Software, Writing – review & editing. **Takumi Usukura:** Resources, Software, Writing – review & editing. **Nako Maishi:** Resources, Writing – review & editing. **Masatoshi Maeki:** Methodology, Writing – review & editing. **Akihiko Ishida:** Methodology, Writing – review & editing. **Hirofumi Tani:**

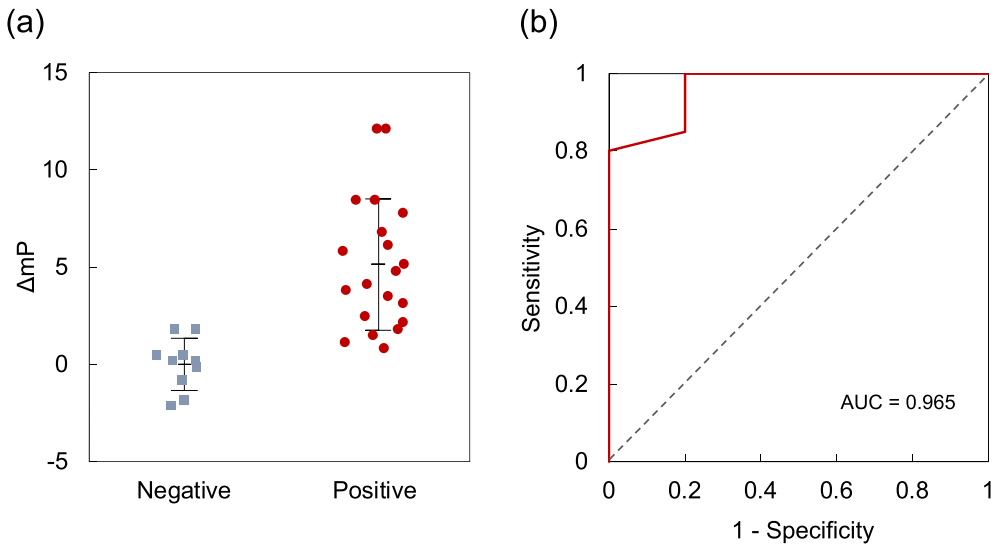

Fig. 6. (a) Fluorescence polarization scatter plot of 30 serum samples: 20 COVID-19 positive serum and 10 negative serum samples. The error bars represent one standard deviation for all data points of positive or negative serum samples. (b) ROC curve analysis of fluorescence polarization of 30 serum samples.

Methodology, Writing – review & editing. **Kyoko Hida:** Resources, Supervision, Writing – review & editing. **Koji Shigemura:** Resources, Supervision, Writing – review & editing. **Akihide Hibara:** Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review & editing. **Manabu Tokeshi:** Conceptualization, Project administration, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2021.113414.

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