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Development of an open sandwich fluoroimmunoassay based on fluorescence resonance energy transfer

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Abstract

We have developed a sensitive, one-step, homogeneous open sandwich fluoroimmunoassay (OsFIA) based on fluorescence resonance energy transfer (FRET) and luminescent semiconductor quantum dots (QDs). In this FRET assay, estrogen receptor β (ER-β) antigen was incubated with QD-labeled anti-ER-β monoclonal antibody and Alexa Fluor (AF)-labeled anti-ER polyclonal antibody for 30 min, followed by FRET measurement. The dye separation distance was estimated between 80 and 90 Å. The current method is rapid, simple, and highly sensitive, and it did not require the bound/free reagent separation steps and solid-phase carriers. A concentration as low as 0.05 nM (2.65 ng/ml) receptor was detected with linearity. In addition, the assay was performed with commercial antibodies. This assay provides a convenient alternative to conventional, laborious sandwich immunoassays.

Keywords: FRET; Sandwich assay; Quantum dots; Fluorescence labels; Sandwich immunoassays

The quantitative determination of protein is essential in clinical medicine, biochemistry, and laboratory practice. Fluorescence immunoassays have been widely used in this area. Most of these methods are based on the use of organic fluorophores that display fluorescence lifetimes in the range of 1–10 ns. Sensitivity typically is limited by autofluorescence.

Fluorescence resonance energy transfer (FRET)[1] occurs when the electronic excitation energy of a donor chromophore is transferred to a nearby acceptor molecule via dipole–dipole interaction between the donor–acceptor pair [1]. The FRET process is more efficient when there is an appreciable overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor [2]. Compared with conventional chemical analysis, the FRET-based analytical method has higher sensitivity and is simpler in detecting the ligand–receptor binding by observing the quenched fluorescence of the donor and/or the enhanced fluorescence of the acceptor. The distance between the donor and the acceptor usually is less than 100 Å [3–6]. During the past few years, a number of different FRET-based assays have been developed [7–10].

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[1] *Abbreviations used:* FRET, fluorescence resonance energy transfer; OsFIA, open sandwich fluoroimmunoassay; EBFP, enhanced blue fluorescent protein; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; Rluc, *Renilla* luciferase; EYFP, enhanced yellow fluorescent protein; QD, quantum dot; AF, Alexa Fluor; anti-ER-β, anti-estrogen receptor β; McAb, monoclonal antibody; PcAb, polyclonal antibody; IgM, immunoglobulin M; SMCC, 4-(maleimidomethyl)-1-cyclohexanecarboxylic acid *N*-hydroxysuccinimide ester; DTT, dithiothreitol; IgG, immunoglobulin G; CLSM, confocal laser scanning microscope; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; NHS, *N*-hydroxysuccinimide; MBP, maltose-binding protein.

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Conventional immunoassays need solid-phase carriers to immobilize antibody or antigen and several cycles of consecutive binding and washing steps to separate free reagent from bound reagent. To circumvent these limitations, Ueda and coworkers described the open sandwich fluoroimmunoassay (OsFIA) method [11]. This method employs FRET between antibody V_H (variable region of the heavy chain) and V_L (variable region of the light chain) fragments. When a V_H—enhanced blue fluorescent protein (EBFP) chimera and a V_L—enhanced green fluorescent protein (EGFP) chimera are mixed with a sample, antigen concentration could be determined by monitoring the FRET between the two green fluorescent protein (GFP) variants. The measurement is simple and rapid. The detection limit is approximately 1 μg/ml of antigen. A similar method employing FRET between V_H—*Renilla* luciferase (Rluc) and V_L—enhanced yellow fluorescent protein (EYFP) reached a detection limit of 0.1 μg/ml (or 7 nM) [12]. A one-step, noncompetitive FRET immunoassay measuring FRET between Eu-labeled anti-morphine and Cy5-labeled anti-IC (morphine and anti-morphine complex) Fab fragments has been shown to detect 5 ng/ml morphine [13]. All of these methods require the production of special antibody fragments because of the distance limit between two fluorescence dyes. Autofluorescence and the direct excitation of the acceptor fluorophore also limit the usefulness of these methods.

Colloidal semiconductor nanocrystals or quantum dots (QDs) are nanoscale crystals of semiconductor material that glow or fluoresce when excited by a light source such as a laser. QDs offer significant advantages over conventionally used fluorescent markers such as broad absorption spectra, sharp emission spectra, high-emission quantum yield, long lifetimes and tunable spectrum, large Stokes shift, chemical stability, and photostability [14,15]. Advantages of QD technology also include simple excitation (lasers are not required), simple instrumentation, and high sensitivity. These QDs can be detected at concentrations comparable to the best conventional organic dyes by conventional fluorescence spectrometers, and individual bioconjugated QDs are observable by confocal microscopy [16]. Colloidal QDs are approximately spherical nanocrystals with surfaces that can be derived with a variety of functional capping groups (surface ligands), allowing their dispersion in a range of solvents, including aqueous environments [16–19]. Significant advances in the use of QDs as bioanalytical tools for in vitro work have been made in the area of immunoassays [15]. The FRET between QD-labeled antibody and QD-labeled antigen can be used for immunoassay [20]. QD-labeled antibodies have been used in such assays and have been demonstrated to be generally applicable. The benefits of using QD-based labels in immunoassays are that the multiple nanocrystal fluorophores can be excited and several labeled species can be detected simultaneously using a single light source [21]. In addition, proteins conjugated with QD as a donor can reach longer dye separation distances than other fluorescent dyes because of the higher quantum yields of QDs. QD–acceptor distance can reach 91 Å [22].

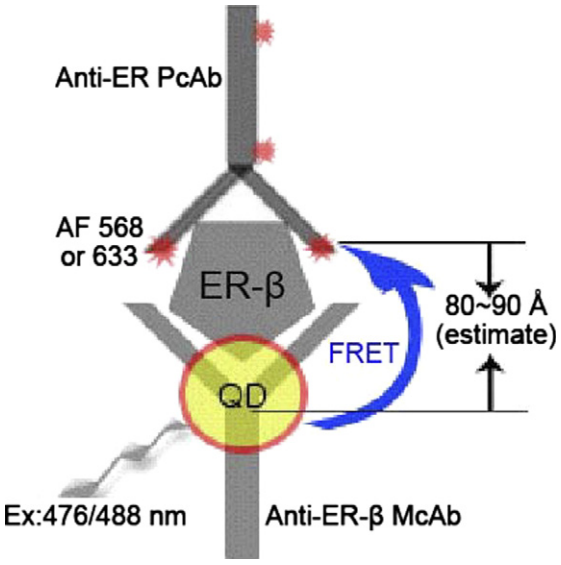

Fig. 1. Schematic illustrations of FRET-based OsFIA. QD 565 is excited by an Ar-visible laser, and the energy transfer occurs from QD to AF that emits red fluorescence. The fluorescence is detected in the FRET channel with the detection window at 590–720 nm (for Alexa 568) or at 610–740 nm (for Alexa 633). The spectra are scanned from 515 to 720 nm (for Alexa 568 acceptor) or from 525 to 730 nm (for Alexa 633 acceptor). ER-β, estrogen receptor β; McAb, monoclonal antibody; PcAb, polyclonal antibody.

Because of the numerous potential applications of immunoassays based on QD–ligand antibodies, we developed the current assay based on FRET between QD- and Alexa Fluor (AF)-labeled antibodies. This OsFIA is illustrated in Fig. 1. The anti-estrogen receptor β (anti-ER-β) monoclonal antibody (McAb) was labeled with the donor QD 565. The anti-ER polyclonal antibody (PcAb) was labeled with the acceptor AF 568 or AF 633. When the two labeled antibodies were incubated with ER-β antigen, the FRET could be measured by a confocal microscope. The objective of this study was to explore the possible application of QD-labeled antibodies in immunoassay on a glass chip model and to develop a fast, simple, and highly sensitive method to detect antigens.

Materials and methods

Labeling of anti-ER-β McAb with QD 565

The anti-ER-β McAb (mouse ascites, immunoglobulin M [IgM] isotype, Sigma, St. Louis, MO, USA) was labeled using a QD 565 antibody conjugation kit (Quantum Dot, Hayward, CA, USA) following the procedures of the product manual. Briefly, the amine groups on the polymer surface of core (CdSe)–shell (ZnS) QD nanocrystals were activated using the hetero-bifunctional crosslinker 4-(maleimidomethyl)-1-cyclohexanecarboxylic acid *N*-hydroxysuccinimide ester (SMCC), yielding a maleimide–

nanocrystal surface. Following a 60-min reaction, the excess crosslinker was removed from the activated QDs by gel filtration chromatography over an NAP-5 column. The McAb was treated with dithiothreitol (DTT), which reduced some of the disulfide bonds of the starting antibody. Removal of the excess reducing reagent was also accomplished by NAP-5 column gel filtration chromatography. The maleimide-activated QDs subsequently were mixed with the thiol-containing antibody. After 1 h of conjugation, the excess maleimide groups were quenched by β-mercaptoethanol. The unconjugated antibody molecules were removed from the QD conjugate by size exclusion chromatography (Superdex 200). The conjugate concentration was calculated by using the extinction coefficient (QD 565 = 300,000 M^{-1} cm^{-1}) and measuring the absorbance of the labeled antibody solution at 556 nm, in which [QD 565] = A_{556}/300,000. The QD 565 concentration was 60 nM with the estimated antibody concentration of 1 μM. The labeled antibody was stored at 4 °C until use.

Labeling of anti-ER PcAb with AF dyes

The rabbit anti-ER PcAb (antiserum, immunoglobulin G [IgG] isotype, Sigma) were labeled with AF 568 and AF 633 protein labeling kits separately (Molecular Probes, Eugene, OR, USA) following the protocol in the kit manual. Briefly, 50 μl of 1 M bicarbonate was added to 0.5 ml of 2 mg/ml antibody solution, mixed with 1 vial reactive dye, and then stirred for 1 h at room temperature. The dye-labeled antibody was purified by BioGel P-30 Fine (Bio-Rad, Hercules, CA, USA) size exclusion chromatography. The labeling ratio of AF dye to antibody (AF 568/antibody or AF 633/antibody) was calculated by the method given in the AF product specification, which called for measuring the absorbance of the labeled antibody solution at 577 nm (AF 568), 632 nm (AF 633), and 280 nm (labeling ratio = [AF 568 or AF 633]/[antibody]). For the AF 568 dye, [AF 568] = A_{577}/91,300, [antibody] = (A_{280} − 0.46A_{577})/203,000. For the AF 633 dye, [AF 633] = A_{632}/100,000, [antibody] = (A_{280} − 0.55A_{577})/203,000. The 91,300 and 100,000 M^{-1} cm^{-1} are the approximate molar extinction coefficients of AF 568 dye at 577 nm and of AF 633 dye at 632 nm, respectively. The 203,000 M^{-1} cm^{-1} is the molar extinction coefficient of a typical IgG. The 0.46 or 0.55 is a correction factor to account for absorption of the dye at 280 nm. The labeling ratio of AF 568 was approximately 7 when the concentration of AF 568 was approximately 6 μM, whereas the concentration of the antibody was 0.85 μM. The labeling ratio of AF 633 was approximately 7.5 when the concentration of AF 633 was approximately 4.5 μM, whereas the concentration of the antibody was 0.6 μM. The labeled antibodies were stored at −20 °C until use.

Open sandwich FRET-based immunoassay

The principle of the FRET immunoassay is illustrated in Fig. 1. QD is attached at the hinge area of McAb as a donor, and AF 568 or AF 633 is chelated at the N terminal of PcAb as an acceptor. The open sandwich immunoassay for detecting ER-β was carried out with either AF 568-labeled or AF 633-labeled anti-ER PcAb and QD 565-labeled anti-ER-β McAb according to the following procedures. For the QD 565 and AF 568 pairing, 2 μl of QD 565-labeled anti-ER-β McAb, 1 μl of AF 568-labeled anti-ER PcAb, and 1 μl of varying concentrations of recombinant human ER-β (Sigma) were mixed. For the QD 565 and AF 633 pairing, 1 μl of QD 565-labeled anti-ER-β McAb, 3 μl of AF 633-labeled anti-ER PcAb, and 1 μl of varying concentrations of recombinant human ER-β were mixed. After incubation for 30 min at room temperature, 2 μl of the reaction solution was added to a cover glass. The fluorescence image was recorded by confocal laser scanning microscope (CLSM). Each experiment was repeated three times. Phosphate buffer (0.01 M, pH 7.4) was used instead of ER-β antigen as a negative control. Three parallel samples were analyzed as negative control. In all assays, phosphate buffer was used for dilution.

Fluorescence microscopy

A TCS-SL confocal system (Leica, Heidelberg, Germany) fitted to a DM IRE 2 inverted microscope (Leica) using a 0.3× numerical aperture objective was employed to record fluorescence images using both *xyz* and *xyλ* modes. The pinhole was set at 1 Airy disc unit, and an appropriate dichroic beam-splitting mirror was used. For the QD 565 and AF 568 pairing, fluorescence was excited by an Ar-visible laser and the laser line was set at 476 nm. The QD 565 fluorescence was observed in PMT-1 (yellow channel) with the detection window at 515–590 nm, and the AF 568 fluorescence was observed in PMT-2 (red channel) with the detection window at 590 to 720 nm simultaneously using the *xyz* mode. Spectral scanning was carried out by recording 40 images between wavelengths 515–520 and 715–720 nm using the *xyλ* mode. For the QD 565 and AF 633 pairing, fluorescence was excited by an Ar-visible laser and the laser line was set at 488 nm. The pictures were taken from PMT-1 at 515–605 nm and from PMT-2 at 610 to 740 nm simultaneously using the *xyz* mode. Spectral scanning was carried out by recording 40 images between wavelengths 525–530 and 725–730 nm using the *xyλ* mode. All images were analyzed using the software supplied by Leica.

For time series experiments, the red/yellow fluorescence ratio as an indicator of FRET was measured in the presence or absence of ER-β antigens. The excitation setting and emission channels were the same as described above. Images from yellow and red channels were recorded every 5 min for up to 70 min. The average intensity of the red and yellow fluorescence was measured at each time point.

Results

Spectral characteristics of QD 565 and AF 568/633

Both the QD 565–AF 568 pair and the QD 565–AF 633 pair were used to detect ER-β antigen. Fig. 2 shows the absorption and emission spectra of QD 565 and AF 568/633-labeled antibodies. Note that the AF 633 dye-labeled antibodies have absorption maxima of approximately 632 nm, with a secondary absorption peak at 580 nm. The absorption spectra of both AF 568 and AF 633 (second absorption peak at 580 nm) have good spectral overlapping on the emission peak of QD 565.

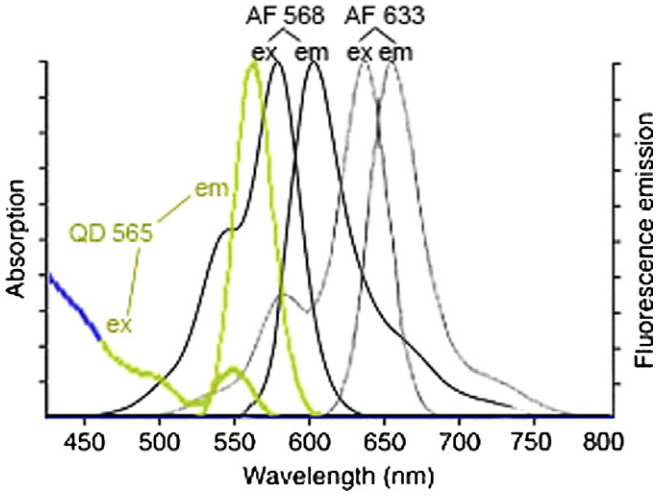

Fig. 2. Spectra of QD 565-labeled antibody and Alexa 568 and Alexa 633 bound to antibody. ex, excitation spectrum; em, emission spectrum.

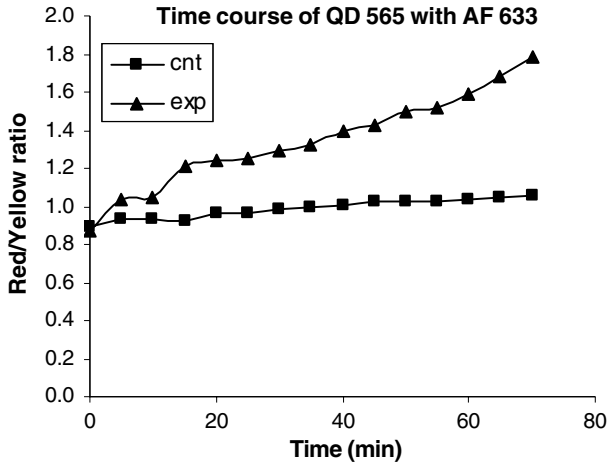

Fig. 3. Time course of FRET-based OsFIA between QD 565 and Alexa 633. Images from the yellow channel (515–605 nm) and the red channel (610–740 nm) were recorded at intervals of 5 min for up to 70 min with the final concentration of ER at 10 nM (control with phosphate-buffered saline). cnt, control; exp, experiment.

Assay conditions

Fluorescence-labeled antibodies were titrated, and the reaction time was optimized for measuring FRET. As shown in Fig. 3, after 15 min of incubation at room temperature, the ratio of acceptor/donor fluorescent density in the experimental group became significantly larger than that in the control group and the difference was not much increased from approximately 30 min. Based on this result, all of the following assays were carried out with 30 min incubation at room temperature. The final concentrations of QD 565-labeled antibody and AF 568-labeled antibody were 0.5 μM (30 nM of QD 565) and 1.75 μM (200 nM of AF 568), respectively, whereas the final concentrations of QD 565-labeled antibody and AF 633-labeled antibody were 0.2 μM (12 nM of QD 565) and 0.6 μM (210 nM of AF 633), respectively.

FRET between QD and AF dyes

As shown in Figs. 4 and 5, FRET occurred when the QD-labeled anti-ER-β and AF 568- or AF 633-labeled anti-ER reacted with ER-β antigen. The fluorescence density of the FRET channel in the experimental sample (Fig. 4D) obviously was higher than that in the control sample (Fig. 4B), whereas the fluorescence density of donor channel in the experimental sample (Fig. 4C) was lower than that in the control sample (Fig. 4A). The spectra scan-

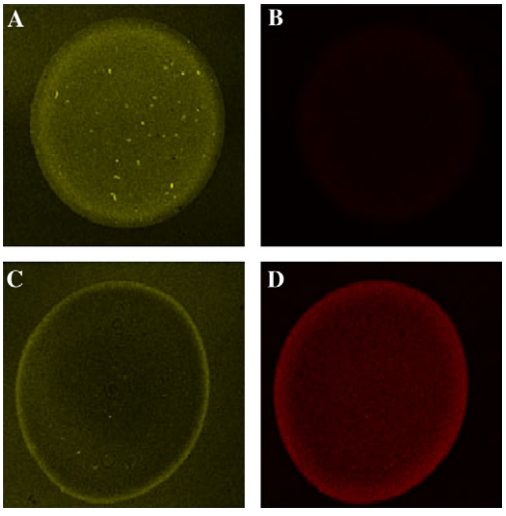

Fig. 4. OsFIA with QD 565 and Alexa 633 dye. (A) Donor fluorescent density of negative control (two fluorophore-labeled antibodies but no antigen). (B) Acceptor fluorescent density of negative control. (C) Donor fluorescent density of experimental sample (two fluorophore-labeled antibodies with antigen). (D) Acceptor fluorescent density of experimental sample. The sample was visualized by illumination using an Ar-visible laser with laser line set at 488 nm. QD 565 fluorescence was detected in the donor channel with the detection window at 515–605 nm. Fluorescence was colored in yellow (panels A and C). Alexa 633 fluorescence was detected in the acceptor channel with the emission window at 610 to 740 nm. Fluorescence was colored in red (panels B and D). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

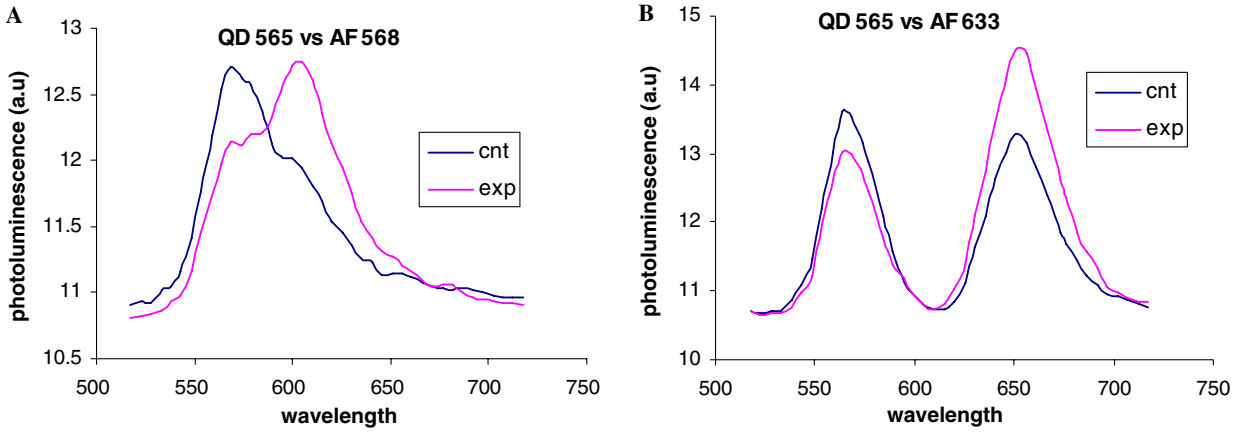

Fig. 5. Spectra scanning of experiment (exp) and negative control (cnt). (A) QD 565–Alexa 568 pair scanned from 515 to 720 nm with 40 images. (B) QD 565–Alexa 633 pair scanned from 525 to 730 nm with 40 images.

ning also showed donor quenching and acceptor enhancement in the experimental samples when compared with the control samples (Fig. 5).

To confirm the complex formation of the McAb (primary antibody), receptor, and PcAb (secondary antibody), we performed enzyme-linked immunosorbent assay (ELISA) and observed that the absorbance (at 405 nm) increased approximately 10-fold after the complex formation. We also tried SDS–PAGE, but because SDS interferes with the binding between the antibody and receptor, we were not able to obtain the evidence. We also used surface plasmon resonance (SPR) with carboxymethyl dextran surface. The binding between the McAb (immobilized) and the receptor was stable, but that between the receptor and the PcAb (free) was very unstable. We believe that the weak secondary binding was due to the fluidic situation that might have influenced it negatively.

Receptor concentration versus FRET

The energy transfer efficiency, FRET efficiency (E), was measured according to the following equation:

$$E = 1 - \frac{F_{DA}}{F_D} \quad (1)$$

where F_{DA} is the integrated fluorescence intensity of the donor in the presence of the acceptor and F_D is the integrated fluorescence intensity of the donor alone. The FRET efficiency between QD 565 and AF 568 was approximately 30%, which was higher than that between QD 565 and AF 633 (~20%). This was because the distance between the emission wavelength of QD and the excitation wavelength of AF 568 was shorter than that between QD and AF 633. Fig. 6 shows the calibration graphs between ER concentration and donor/acceptor density ratio. As shown in Fig. 6A, the density ratio data could be fitted very well with the "two-site binding" model ($r^2 = 0.996$). The K_d value for the first binding was very low (0.007 nM) compared with that of the secondary binding (1.73 nM), indicating that the first binding occurred very rapidly. Fig. 6B shows a linearized plot of the logarithm of the receptor concentration against the density ratio. It also shows that the data points between the lowest concentration (0.05 nM) and

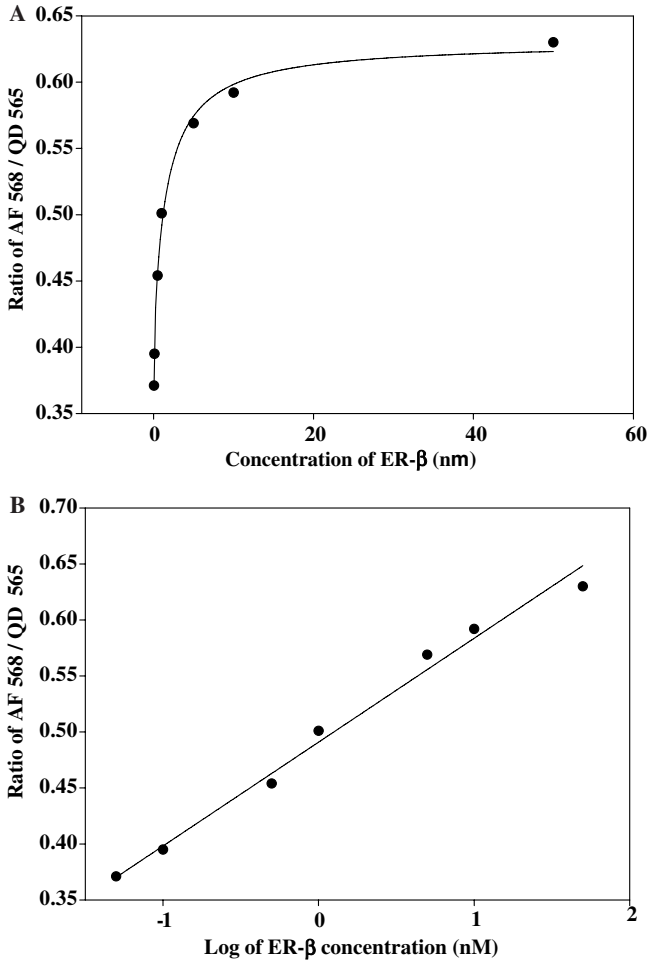

Fig. 6. Calibration graphs between ER concentration and donor/acceptor density ratio. (A) Using two-site binding model. (B) Using semi-log linear model.

Table 1
Lowest concentrations detected in other studies and OsFIA

Method	Description	Detection limit
FRET immunoassay [26]	Sq 635-labeled anti-HSA vs. Sq 660-labeled HSA	100 nM (7 ng/ml)
Open sandwich FIA [11]	Antibody variable region: V_H–EBFP vs. V_L–EGFP	1 μg/ml
ELISA [27]	Enzyme tagged antibody	10 ng/ml
Open sandwich FIA [12]	V_H–Rluc vs. V_L–EYFP	7 nM (0.1 μg/ml)
FRET immunoassay [20]	QD 555–anti-BSA vs. QD 610–BSA	10 nM
Sandwich FIA [13]	Eu-labeled anti-morphine vs. Cy5-labeled anti-IC Fab fragment	5 ng/ml
OsFIA	QD 565–anti-ER-β vs. AF 568–anti-ER	0.05 nM (2.65 ng/ml)

Note. HSA, human serum albumin; BSA, bovine serum albumin; IC, immunocomplex.

the highest concentration (50 nM) were fitted very well ($r^2 = 0.990$). Thus, with 30 min incubation, 0.05 nM was detected with this assay.

Discussion

We have developed a fast and simple FRET-based sandwich immunoassay, OsFIA, for detecting an antigen using McAb labeled with donor QDs and PcAb labeled with AF dyes. OsFIA is a noncompetitive format and can be automated. The method required only a few minutes to mix the antigen and antibodies. After 30 min of incubation, the solutions were added on a cover glass to measure the luminescence. Unlike ELISA, it did not require multiple steps of binding, washing, and blocking. This method can be widely used in other immunoassays and biosensing.

The separation distance of fluorescence dyes is critical in FRET experiment design. A donor–acceptor separation distance within the range of 50–100 Å makes FRET interactions effective. In our OsFIA, QDs were labeled at the hinge area of the antibody by thioether bonds and AF containing *N*-hydroxysuccinimide (NHS) was partly attached at the N-terminal α-amino groups (–NH_2) of the antibodies. The radius of QD 565 is approximately 30 Å. The minimum distance from the hinge area to Fab is approximately 22.5 Å given that the minimum dimension for the antibody is 45 Å (across a single Fab region) [23]. The dimensions of maltose-binding protein (MBP) are $30 \times 40 \times 65$ Å, and the dimensions of avidin are $100 \times 62 \times 25$ Å. The minimum dimensions for 43 kDa of MBP and 68 kDa of avidin were 30 Å [23] and 25 Å [24], respectively, and the estimated minimum dimension of ER-β (MW of 53 kDa) was approximately 25 to 35 Å. The closest distance between the two fluorophores in our method was approximately 80–90 Å (Fig. 1). The distance in our method should be much closer to the distance between the dye-labeled antibody and the antigen reported in other studies [20,23,25]. It is difficult to calculate the real separation distance from the FRET efficiency because the number of the acceptors involved was uncertain. The distance in our method is ideal for FRET assays because the high quantum yield of donor QDs allows the longer distance to acceptor dyes. The small molecules of AF did not affect the activities of antibodies when attached to the N terminal by an amine group. We selected AF dyes because the dyes are brighter and more photostable than any other known organic dyes. Furthermore, our OsFIA method could be used to detect other high-molecular weight antigens in which the distance between two antibodies combining epitopes is not more than 45 Å.

When QD–antibody and AF 568/633–antibody are combined with different antigen epitopes, the antibody–antigen immunocomplex should form. As expected, a significant enhancement of the AF 568/633–antibody's red emission at the FRET window and the corresponding quenching of the yellow emission of QD 565–antibody at the donor window were observed (Figs. 4 and 5). FRET efficiency was particularly high for the QD–AF 568 pairs because of the strong overlap of their emission and absorption spectra. The ratio of fluorescence density at the FRET channel with the density at the donor channel could better represent this change (Fig. 3). Following this approach, we were able to detect 5×10^{-11} M ER-β with QD–AF 568. This OsFIA showed a higher sensitivity than has been seen in other reports (Table 1). Furthermore, the OsFIA employed commercial antibodies, unlike other reported FRET-based immunoassays that require special antibody fragments [11–13]. We expect that optimization of the FRET pair would further improve the signal intensity and sensitivity of the OsFIA.

It is probably useful to mention that the cover glass had a lower background noise than did silicon chips and 96-well fluorescent plates (data not shown). Because QDs have fairly broad excitation spectra (from ultraviolet to red, as shown in Fig. 2), we can choose an excitation spectrum that will not excite the acceptor. Background can be controlled at a lower level, and the sensitivity could be enhanced later.

Conclusions

This QD-based OsFIA is fast and very simple to perform. In addition, it does not need solid-phase carriers or multiple steps of bound/free reagent separation, and it requires only a small volume of sample. The method showed a high sensitivity for detecting antigen and therefore can be easily introduced in routine analysis. A concentration as low as 0.05 nM (2.65 ng/ml) receptor was detected with linearity. Furthermore, it can be performed using commercial antibodies. This method provides a con-

venient alternative to conventional laborious sandwich immunoassays.

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