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Ultrasensitive Detection of Cellular Protein Interactions Using Bioluminescence Resonance Energy Transfer Quantum Dot–Based Nanoprobes

Gabriel A. Quiñones,[1] Steven C. Miller,[2] Sukanta Bhattacharyya,[2] Daniel Sobek,[2*] and Jean-Philippe Stephan[1**]

[1] *Protein Chemistry Department, Genentech, Inc., MS#98, 1 DNA Way, South San Francisco, California 94080*

[2] *Zymera, 5941 Optical Court, San Jose, California 95138*

ABSTRACT

Sensitive detection of protein interactions is a critical step toward understanding complex cellular processes. As an alternative to fluorescence-based detection, *Renilla reniformis* luciferase conjugated to quantum dots results in self-illuminating bioluminescence resonance energy transfer quantum dot (BRET-Qdot) nanoprobes that emit red to near-infrared bioluminescence light. Here, we report the development of an ultrasensitive technology based on BRET-Qdot conjugates modified with streptavidin ([BRET-Qdot]-SA) to detect cell-surface protein interactions. Transfected COS7 cells expressing human cell-surface proteins were interrogated with a human Fc tagged protein of interest. Specific protein interactions were detected using a biotinylated anti-human Fc region specific antibody followed by incubation with [BRET-Qdot]-SA. The luciferase substrate coelenterazine activated bioluminescence light emission was detected with an ultra-fast and -sensitive imager. Protein interactions barely detectable by the fluorescence-based approach were readily quantified using this technology. The results demonstrate the successful application and the flexibility of the BRET-Qdot-based imaging technology to the ultrasensitive investigation of cell-surface proteins and protein–protein interactions. J. Cell. Biochem. 113: 2397–2405, 2012.

KEY WORDS: CELL-SURFACE PROTEIN; ULTRASENSITIVE DETECTION; IMAGING TECHNOLOGY

All aspects of normal or pathological cell behavior are profoundly influenced by protein–protein interactions. Protein interaction networks, ranging from cell-surface interactions to intracellular signaling cascades, are highly complex and therefore extremely challenging to decipher. Despite tremendous progress in characterizing the functional relationships between proteins, our current understanding of the cellular protein network is at best fragmentary. To gain further insights into many protein–protein interactions, including weak or transient events, the development of new technologies enabling greater sensitivity than current options is necessary. Fluorescence-based technologies remain the cornerstone of most protein interaction detection methods [Yan and Marriott, 2003]. These approaches allow ultra high-throughput evaluation of molecular interactions and use well-established and robust protocols easy to implement. However, fluorescence-based detection suffers from a clear lack of sensitivity when it comes to detecting low affinity interactions [dissociation constant (K_d) higher than 1 μM] or when proteins involved have low level of expression (fewer than 1,000 molecules per cells). Fluorescent semiconductor quantum dots developed in recent years have unique optical properties providing solutions to overcome auto-fluorescence or intrinsic protein fluorescence generated by the aromatic nucleus of amino acid residues, such as tryptophan, tyrosine, or phenylalanine [Lakowicz, 1983]. Quantum dot properties enabled significant

Conflict of interest: Steven C. Miller, Sukanta Bhattacharyya, and Daniel Sobek are Zymera employees or affiliates. Gabriel Quinones and Jean-Philippe Stephan are employees of Genentech.

Gabriel A. Quinones and Steven C. Miller contributed equally to this work.

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*Correspondence to: Daniel Sobek, Zymera, 5941 Optical Court, San Jose, CA 95138. E-mail: daniel_sobek@zymera.com

**Correspondence to: Dr. Jean-Philippe Stephan, PhD, Senior Research Scientist, Department of Protein Chemistry, gRED–Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080. E-mail: stephanj@gene.com

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improvement of the sensitivity of detection in some cases [Michalet et al., 2005]. However, despite these technological advances, application of fluorescent-based detection methods to protein–protein interaction investigations is still limiting.

Recently, a very attractive alternative to fluorescence was introduced with the development of self-illuminating bioluminescence resonance energy transfer (BRET) quantum dot nanoparticles (BRET-Qdot) [So et al., 2006]. The bioluminescent conjugates were assembled through the covalent coupling of a polymer-coated CdSe/ZnS core shell quantum dot, having fluorescence emission at 655 nm, to an improved 8-mutation variant of *Renilla reniformis* luciferase (Luc8), which has an improved catalytic efficiency compared to the wild-type protein resulting in a fourfold improvement in light output over the native enzyme [So et al., 2006]. Upon addition of the luciferase substrate, coelenterazine (CTZ), bioluminescence emission of these nanoparticles ranges from red to near infrared depending on the choice of quantum dot composition. BRET-Qdots offer numerous advantages over traditional fluorescent probes, including multiplexing via quantum dots with different emission wavelengths, and no need for an external light source to induce light emission. In addition, in contrast to conventional quantum dots, BRET-Qdots can be imaged in the presence of intrinsic protein fluorescence that naturally occurs in tissue, thus offering greatly enhanced sensitivity for imaging applications in small animals [Kosaka et al., 2011].

To establish the potential of the BRET-Qdot system for highly sensitive molecular imaging in vitro, we investigated an ultrasensitive imaging approach using [BRET-Qdot] nanoprobes modified with streptavidin ([BRET-Qdot]-SA) to detect cell-surface protein–protein interactions. Using a customized imager we investigated the different experimental parameters critical to achieving the highest level of sensitivity for the detection of cell-surface proteins. Subsequently, we compared the performance of the self-illuminating bioluminescent [BRET-Qdot]-SA and fluorescence-based technologies in detecting cell-surface protein–protein interactions. The technological superiority of the [BRET-Qdot]-SA over the fluorescence-based approach was illustrated through the detection of multiple protein interactions including several members of the IgLON adhesion molecule family. Finally, we demonstrated the flexibility of the [BRET-Qdot]-based detection method comparing three different experimental modalities for the detection of EpCAM on MCF7 breast tumor cells stably expressing HER2. Our investigations clearly demonstrate the significant value the self-illuminating BRET-Qdot could bring to in vitro assays that require high sensitivity, such as the detection of cellular protein interactions.

RESULTS

DESIGN OF [BRET-Qdot]-SA NANOPROBES AND OPTIMIZATION OF THEIR APPLICATION TO DETECTING CELL SURFACE EXPRESS BIOMARKERS

The [BRET-Qdot]-SA nanoprobes consist of quantum dots that emit fluorescence at 655 nm (Qdot® 655, Life Technologies) covalently coupled with a Luc8 and conjugated to SA (Fig. 1a). The produced nanoprobes was estimated to contain, on average, six copies of Luc8 and seven SA molecules per Qdot nanocrystals (see details in Materials and Methods Section). The SA moiety provides a linking chemistry to detect various biological signals through tight binding to specific biotinylated reagents, such as antibodies. Upon addition of the Luc8 substrate CTZ, a bioluminescent signal consisting of two distinct emission peaks from Luc8 and Qdot® 655 at 480 and 655 nm, respectively, was detected. This signal was acquired using a prototype imager (commercial version now available from Zymera) consisting of a light tight imaging chamber and a −20°C cooled charge couple device (CCD) camera with an intensifier tube control through a Zymera proprietary image acquisition/analysis software (Fig. 1b). This imager enables the ultra-sensitive detection of [BRET-Qdot]-SA nanoprobes as low as 0.63 attomole/well at 150 frames or 10 s acquisition time (Fig. 1c).

The [BRET-Qdot]-SA nanoprobes described here allow great flexibility in assay design in both bioluminescence (Luc8 and Qdot signals) and fluorescence (Qdot signal only with the appropriate emission filter) imaging applications and are compatible with high-throughput screening approaches.

To optimize experimental conditions, we first applied the [BRET-Qdot]-SA to the detection of the human epidermal growth factor receptor 2 (HER2/neu) on the cell surface of mouse embryonic fibroblast 3T3 cells stably transfected with the human HER2/neu gene [Di Fiore et al., 1987]. HER2-expressing 3T3 (3T3/HER2) cells were seeded in 96-well plates and cultured overnight before fixation and incubation with increasing concentrations of a biotinylated anti-human HER2 antibody. After washing the cells, 1 nM [BRET-Qdot]-SA was added for a 15-min incubation, and following a wash step the CTZ-activated BRET emission was subsequently imaged for 10 s at 15 fps in a customized imaging platform developed for ultra-fast whole plate imaging. A region of interest (ROI) overlapping each well on the 96-well plate was then overlaid onto the acquisition image (Fig. 2a) and the anti-HER2 antibody-binding signal intensity was measured in each individual well (Fig. 2b). Under these conditions, the limit of detection (LOD) defined as the concentration of biotinylated anti-HER2 antibody generating a signal mean intensity equal to the mean intensity detected in absence of biotinylated antibody (blank) + 3 standard deviation of the blank was 0.07 ng/ml (Fig. 2b). The optimal number of frames required for image acquisition of the CTZ-activated signal using the [BRET-Qdot]-SA nanoprobes without saturating the grayscale image was determined to be 150 frames or 10 s acquisition time (Fig. 2c,d). In addition, we determined that the use of grayscale imaging, or photon counting imaging (using proprietary image analysis software not limited by grayscale imaging) did not affect the intensity results generated (Fig. S1a), nor did the size of the ROI used to acquire those intensity values (Fig. S1b,c). Finally, using SKOV3 cells in the context of EpCAM detection we also assessed blocking of the interaction between the biotinylated detection antibody (anti-EpCAM) and the antigen by increasing amounts of non-biotinylated antibody to the reaction (Fig. S2). This blocking experiment demonstrated the specificity of the SA-conjugated nanoprobe for a biotinylated detection antibody and quantified the background signal due to the nanoprobe alone.

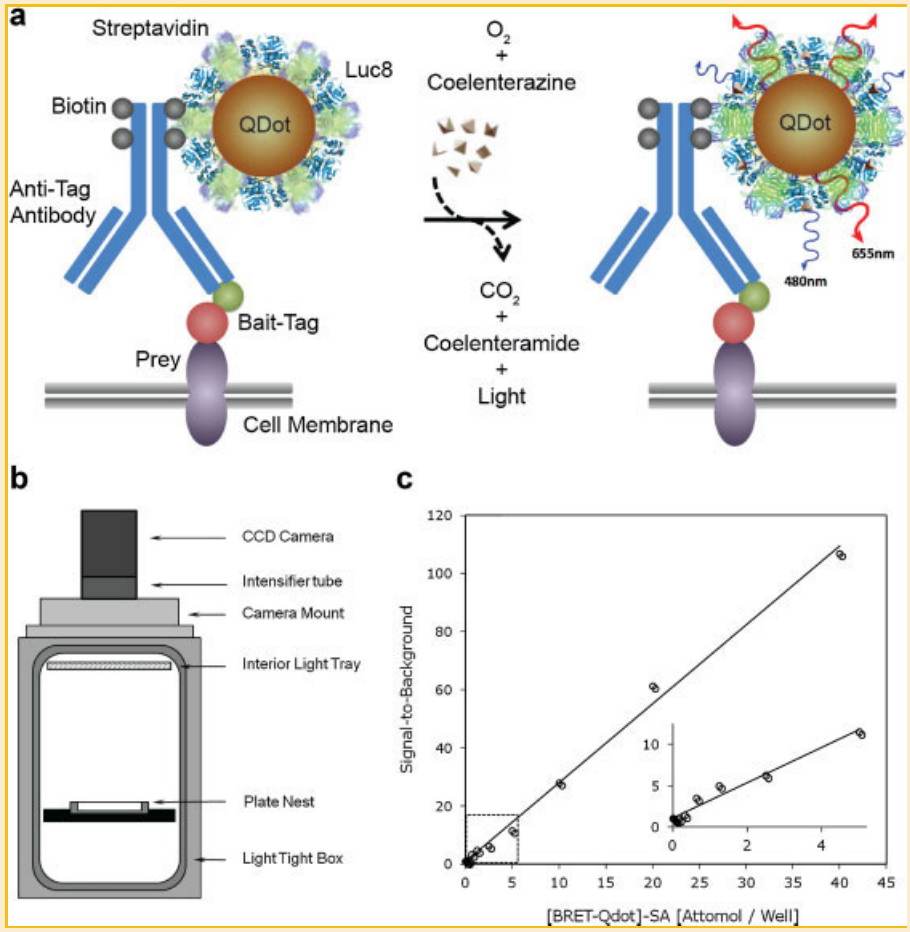

Fig. 1. [BRET-Qdot]-SA nanoprobe technology. a: The [BRET-Qdot]-SA nanoprobe detects cell surface expressed biomarkers by binding to a biotinylated anti-biomarker antibody. Exposure of the cell-bound [BRET-Qdot]-SA nanoprobe to the luciferase substrate, coelenterazine, causes the emission of light of peak wavelength 480 nm from the luciferase protein (Luc8). The energy from the reaction couples to the Qdot acceptor. In this example, the Qdot emits light in the red to near infrared regions (655 nm). b: The Qdot emits light is acquired using a prototype imager consisting of a light tight imaging chamber surrounding a plate nest and surmounted by a −20°C cooled charge couple device (CCD) camera with an intensifier tube. The device is controlled through a Zymera proprietary image acquisition/analysis software. c: Limit of detection using the [BRET-Qdot]-SA nanoprobes using the Zymera imager with a10 s acquisition time (150 frames).

EVALUATION OF THE [BRET-Qdot]-SA NANOPROBES TO DETECT CELL-SURFACE PROTEIN–PROTEIN INTERACTIONS

In addition to detecting cell-surface biomarkers, the [BRET-Qdot]-SA nanoprobes were evaluated for the detection of cell-surface protein–protein interactions using 384-well plates and compared directly to a traditional fluorescence-based detection method carefully optimized in our lab to achieve the best detection sensitivity possible. These studies probed the interaction between the programmed death type 1 (PD1) type 1 membrane receptor and one of its ligands, PD-L1. This ligand-receptor pair is relatively weak with a K_d equal to 770 nM (BIAcore data) [Butte et al., 2008]. COS7 cells transiently expressing PD1 were fixed and then incubated with increasing concentrations of human Fc-tagged PD-L1 bait protein (PD-L1–hFc; Fig. 3b,d,f). Alternatively, the PD-L1 bait protein amount was kept constant while increasing concentrations of biotinylated anti-human Fc detection antibody were added (Fig. 3a,c,e). With a bait protein concentration remaining constant at 5 μg/ml, at least 100 ng/ml of biotinylated anti-hFc antibody is required to detect the PD1/PD-L1–Fc interaction above background using SA-AlexaFluor 488 (Fig. 3a,c). In contrast, with the [BRET-Qdot]-SA bioluminescent technology only 10 ng/ml of detection antibody is sufficient to detect the specific interaction above background (Fig. 3a,e). Notably, despite the 10-fold reduction in detection antibody, 10 ng/ml of biotinylated anti-hFc antibody using the [BRET-Qdot]-SA nanoprobes provide a signal-to-background ratio equal to 5 comparable to 100 ng/ml of antibody with the fluorescence-based detection method that result in a ratio of 4. The [BRET-Qdot]-SA nanoprobe-based detection still performed better than the fluorescence-based approach when the biotinylated anti-hFc detection antibody concentration was kept constant at 5 μg/ml and the PD-L1–hFc bait protein concentration was varied. Under these conditions, the [BRET-Qdot]-SA allowed the detection of PD-L1–hFc, at 1 ng/ml, bound to PD1 with a signal-to-background ratio of 1.5 (Fig. 3f), while the fluorescence-based detection required at least 100 ng/ml with a signal-to-background ratio equal to 1.3 (Fig. 3d). Different biotinylated anti-hFc detection

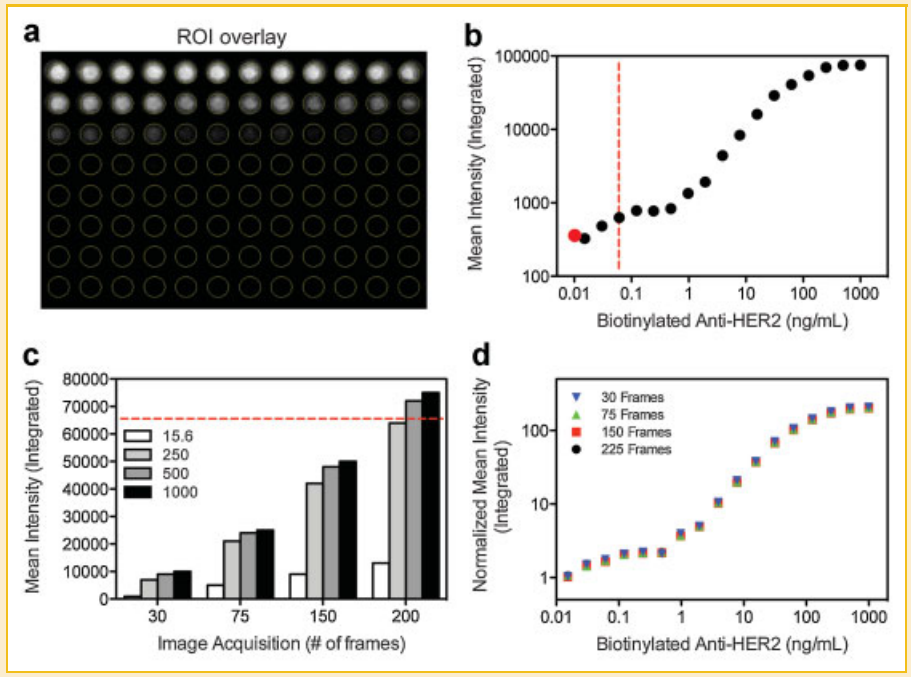

Fig. 2. Detection of coelenterazine-activated BRET signal emission is dependent on the concentration of biotinylated anti-HER2 antibody and image acquisition time. a: Coelenterazine-activated bioluminescence image from a 10 s signal acquisition is shown with the region of interest (ROI) overlay displayed in yellow for NIH 3T3 cells stably expressing HER2 incubated with serial dilutions [in quadruplicate (white box), left to right] of biotinylated anti-HER2 antibody and then with 1 nM [BRET-Qdot]-SA. Wells F9, 10, 11, 12 have no biotinylated Row G and H were empty. b: Quantitation of coelenterazine-activated BRET emission versus incubation with the indicated concentrations of biotinylated anti-HER2 antibody and 1 nM [BRET-Qdot]-SA results in LOD value of 0.07 ng/ml (red dotted line). The red dot indicates the mean intensity from quadruplicate samples with no biotinylated antibody (wells F9, 10, 11, 12). c: The mean intensity integrated value is dependent on the number of frames acquired during the [BRET-Qdot]-SA emission as shown with four concentrations (15.6, 250, 500, and 1,000 ng/ml) of biotinylated anti-HER2 antibody. The red dotted line indicates the saturation point when using grayscale imaging. d: Mean intensity data normalized to the signal in the absence of biotinylated antibody demonstrates that the number of frames collected (30, 75, 150, and 225 frames) does not impact the overall trend of the data.

antibodies from various species were investigated (Fig. S3). Although these anti-hFc antibodies have different non-specific binding to COS7 cells in the absence of bait protein (Fig. S3a), the mean intensity values observed in the presence of 0.1 and 5 μg/ml were similar (Fig. S3b,c).

These initial experiments comparing the [BRET-Qdot]-SA nanoprobe and immunofluorescence-based detection modalities in the context of the relatively weak PD1/PD-L1 interaction demonstrate the significant increase in detection sensitivity when the [BRET-Qdot]-SA nanoprobes are implemented. These results strongly suggest that the [BRET-Qdot]-SA technology is better suited than the fluorescence-based approaches to interrogate cell-surface protein–protein interactions, especially with low affinity interactions or possibly when proteins with low expression levels are involved.

APPLICATION OF [BRET-Qdot]-SA NANOPROBES TO DETECT CELL-SURFACE PROTEIN–PROTEIN INTERACTIONS OF DIFFERENT AFFINITIES

To further evaluate the application of the self-illuminating bioluminescent [BRET-Qdot] technology to the highly sensitive detection of protein interactions on cells we evaluated various interaction among the IgLON family of glycorylphosphatidylinositol anchored neural cell adhesion molecules, comprising OPCML (opioid binding protein/cell adhesion molecule-like) [Schofield et al., 1989], HNT (neurotrimin) [Struyk et al., 1995], LSAMP (limbic system-associated membrane protein) [Levitt, 1984], and NEGR1 (neuronal growth regulator 1/neurotractin) [Funatsu et al., 1999]. These proteins have been shown to be involved in cell-to-cell adhesion and to interact homophilically and heterophilically within the family with various binding affinities [Marg et al., 1999; Lodge et al., 2000; Reed et al., 2004; McNamee et al., 2011]. Here, COS7 cells were transiently transfected either with OPCML, HNT, or LSAMP constructs. No change in cell number or density was observed 48 h after transfection with either of these constructs compared to non-transfected or mock-transfected controls (data not shown). Cell expressing OPCML, HNT, or LSAMP were then incubated with NEGR1–hFc bait protein. Subsequently, NEGR1–hFc binding was detected using a biotinylated goat anti-hFc antibody combined with either SA-AlexaFluor 488 (standard immunofluorescence staining) or the [BRET-Qdot]-SA nanoprobes to detect the cell-surface protein–protein interactions (Fig. 4). As expected, the NEGR1–hFc interactions with HNT, LSAMP, and OPCML were detected over background regardless of the detection method implemented. Both methods showed a more intense binding signal from the interaction between NEGR1 and HNT compared to the NEGFR1/LSAMP interaction. These results correlate with previous data from Marg et al. [1999] generated using NEGR1–

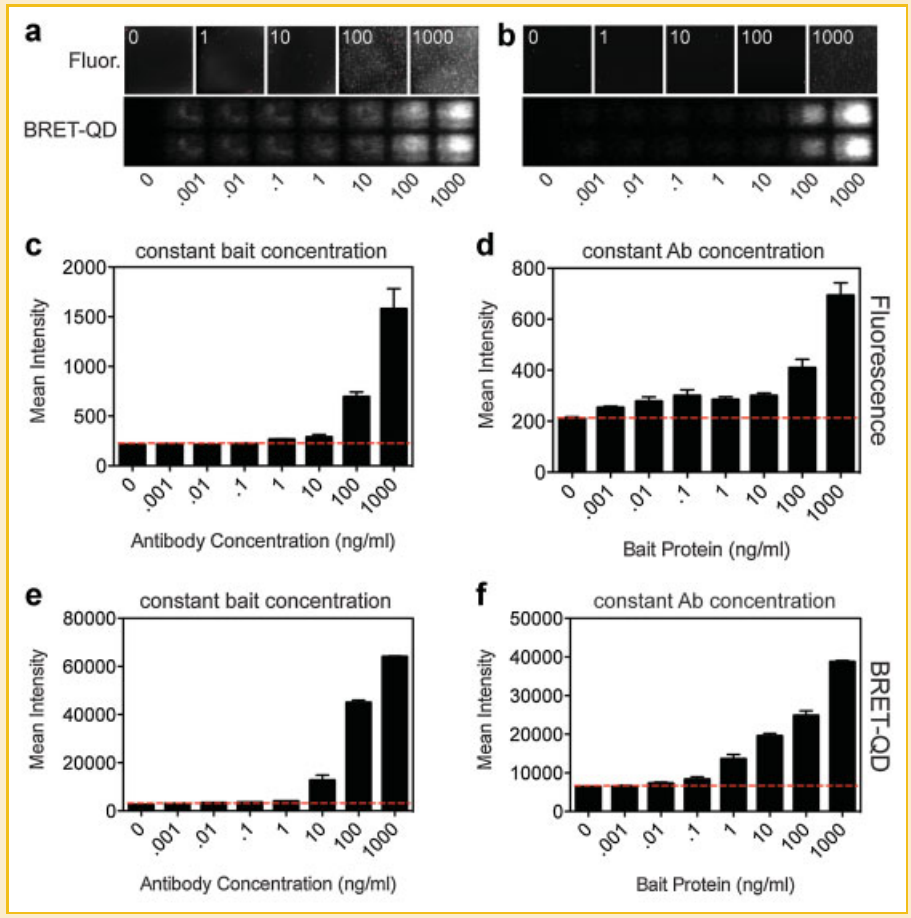

Fig. 3. Comparison between traditional immunofluorescence and [BRET-Qdot]-SA-based detection of cell-surface protein–protein interactions. a: Binding interaction between the receptor PD1 and its ligand PD-L1 on the surface of PD1 transfected COS7 cells. The bait protein, PD-L1–hFc, was kept at a constant concentration of 5 μg/ml while a range of biotinylated goat anti-hFc antibody concentrations (0.001–1,000 ng/ml) was used for detection. Images for both the immunofluorescence staining and the [BRET-Qdot]-SA acquisition are shown. b: The biotinylated goat anti-hFc antibody was kept at a constant concentration of 5 μg/ml while a range of PD-L1–hFc bait protein was tested (0.001–1,000 ng/ml). c,e: Mean signal intensity with increasing amount of biotinylated antibody, (c) fluorescence measurements, (e) [BRET-Qdot]-SA measurements. d,f: Mean intensity with increasing amount of PD-L1–hFc bait protein, (d) fluorescence measurements, (f) [BRET-Qdot]-SA measurements. The red dotted line indicates the background mean intensity detected in absence of biotinylated antibody (c,e) or bait protein (d,f). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

hFc and Cy3-conjugated secondary antibody directed to human IgG demonstrating the NEGR1/LSAMP interaction to be significantly weaker than the NEGR1/HNT interaction. Most importantly, the [BRET-Qdot]-SA nanoprobes were significantly better at detecting the specific interaction between NEGR1–hFc and OPCML, signal-to-background ratio of 24, than the fluorescence-based method that only provides a signal-to-background ratio of 4 (Fig. 4). Both methods detected the interaction between NEGR1–hFc and LSAMP with similar intensity values while the interaction between NEGR1–hFc and HNT had a higher mean intensity value with the [BRET-Qdot]-SA nanoprobe-based method. Interestingly, although NEGR1 interaction with OPCML has been suggested in the literature, in fact it has never been formally characterized [Reed et al., 2004]. One possible explanation for the lack of experimental evidence for the NEGR1/OPCML interaction might be the limited sensitivity of the fluorescence based-detection approach, possibly due to a low expression of OPCML in transfected cells, including COS7 cells. Here, the high sensitivity of the BRET-Qdot nanoprobe technology might enable the true characterization of the relative affinity of the IgLON family members for NEGR1 regardless of their level of expression with NEGR1-Fc/HNT > NEGR1-Fc/OPCML > NEGR1-Fc/LSAMP.

FLEXIBILITY IN ASSAY FORMAT AND STAINING PROCEDURE

Because our data clearly demonstrate the ability of the [BRET-Qdot]-SA nanoprobes to significantly improve the sensitivity of detection of protein interactions on cells compared to fluorescence-based detection methods, we chose to investigate a more direct staining procedure. We therefore tested the use of BRET-Qdots in three different assay formats (Fig. 5) to detect the epithelial cell adhesion molecule (EpCAM) on the surface of MCF7 cells stably expressing HER2 [Keller et al., 2010]. The first method tested was a two-step procedure like the one previously used and similar to the standard immunofluorescence protocols, where the biotinylated antibody was first added to the cells, washed, and then the [BRET-Qdot]-SA nanoprobes were added to the cells (Fig. 5a). This procedure was

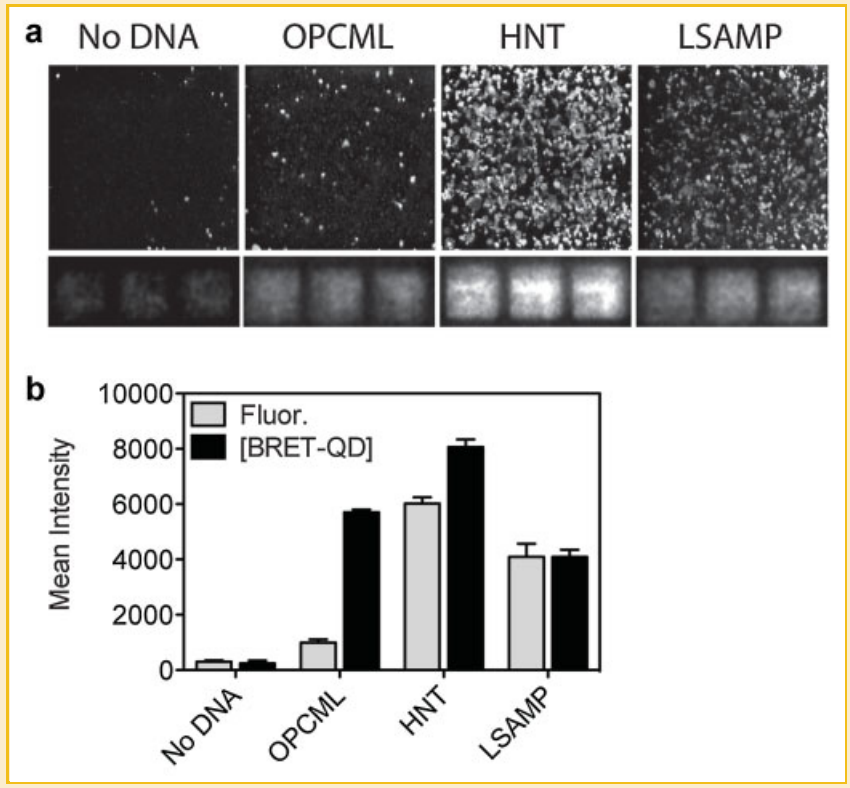

Fig. 4. Comparison between immunofluorescence and [BRET-Qdot]-SA detection of protein–protein interactions of varying affinity. a: Binding interaction between NEGR1 and the IgLON family members OPCML, HNT, and LSAMP. Images from the immunofluorescence assay are shown above the [BRET-Qdot]-SA wells using the same bait protein concentration and staining conditions. In short, COS7 cells were transfected with the indicated constructs, fixed, and the bait protein NEGR1–hFc was incubated with the fixed cells. The cells were washed and treated with an anti-human biotinylated antibody and then either SA-AlexaFluor 488 or the [BRET-Qdot]-SA nanoprobes. Wells were imaged after the addition of coelenterazine. b: Quantitation of the mean intensity values comparing the immunofluorescence and bioluminescence signals. No change in cell number or density was observed after transfection either with OPCML, HNT, or LSAMP.

compared to a one-step detection method relying on preformed, non-covalent complexes between the biotinylated anti-EpCAM antibody and [BRET-Qdot]-SA nanoprobes (Fig. 5b). In that case the complex was formed through biotin–SA interaction and was subsequently purified to remove excess free antibody. Overall this approach resulted in 3- to 4-fold lower mean intensity signal than the two-step procedure at similar detection antibody concentrations (Fig. 5e,h vs. Fig. 5d,g). Finally, a true one-step detection method relying on the direct covalent conjugation of the BRET-Qdot nanoparticles to the anti-EpCAM detection antibody was investigated (Fig. 5c). This procedure resulted in a mean intensity signal comparable to the one observed with the direct conjugation method (Fig. 5d,g), but with significantly higher detection antibody concentration (1.25–20 nM; Fig. 5f,i). This detection method has the advantage of being a one-step procedure that could easily be implemented for the detection of biomarkers or tagged proteins binding to cells. Moreover, the direct conjugation of the antibody to the BRET-Qdot nanoprobe allows for multiplexing applications by using Qdots with different emission wavelengths conjugated to different specific antibodies.

Thus two methods provided the best sensitivity for detecting EpCAM on MCF7/HER2 cells. Covalent conjugation of the [BRET-Qdot]-antibody nanoparticle to the antibody was the preferred one-step procedure. The two-step procedure used incubation with the biotinylated anti-EpCAM antibody, followed by incubation with the [BRET-Qdot]-SA nanoprobe. These results demonstrate the flexibility with which the [BRET-Qdot] technology can be deployed for the ultrasensitive detection of cell-surface proteins as well as protein–protein interactions.

DISCUSSION

The BRET-Qdot nanoprobes offer numerous advantages for ultrasensitive and specific protein detection, including no need for an external light source for light emission, circumventing the problems of tissue auto-fluorescence, and low background emission [So et al., 2006]. The results presented here clearly demonstrate that these features are particularly useful in the context of specific cell-based investigations that could be challenging to carry on using conventional fluorescence-based detection approaches. The BRET-Qdot-based detection method described here deemed to be successful to (i) specifically measure interactions that occurred at the surface of the cell membrane; (ii) detect distinct protein–protein interactions over a wide range of affinities yielding an informative interaction profile for a specific tagged bait proteins; and (iii) detect

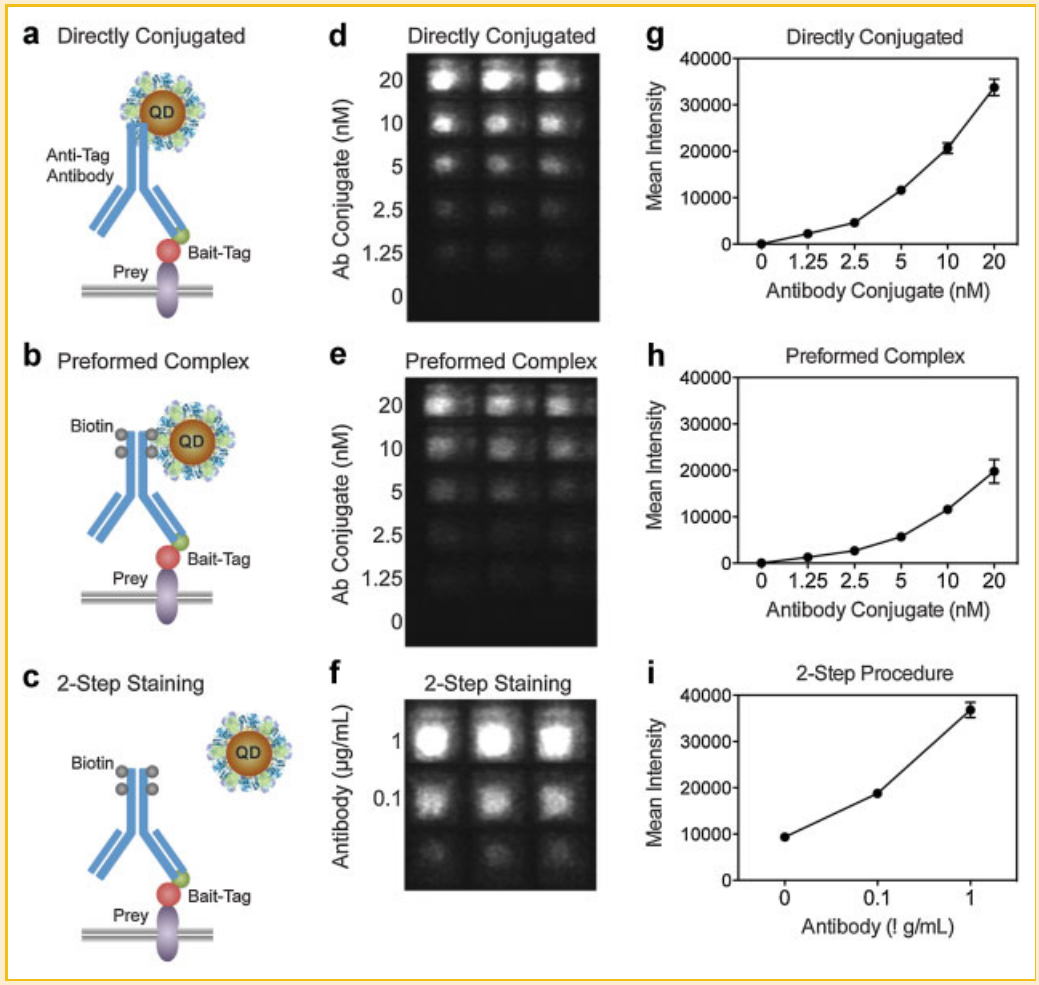

Fig. 5. HER2 positive MCF7 cells were fixed, and then incubated with increasing concentrations of anti-EpCAM antibody conjugates. a–c: Depictions of the three different [BRET-Qdot] staining procedures. a: The biotinylated anti-EpCAM antibody was added to the cells, washed, and then incubated with [BRET-Qdot]-SA. b: The anti-EpCAM antibody was biotinylated, combined with [BRET-Qdot]-SA to make a preformed complex prior to addition to the cells. c: The anti-EpCAM antibody was directly conjugated to the [BRET-Qdot] and added to the fixed cells. d–f: Ten-second image acquisition of the bioluminescence signal from all three staining conditions. Images displayed show three replicate wells for each antibody conjugate concentration. g–i: Quantitation of the mean intensity values (background substracted) over increasing concentrations of the primary antibody (g) or the antibody conjugates (h,i). The direct conjugation of the [BRET-Qdot] nanoprobe to a detection antibody resulted in a higher mean intensity than the preformed complex and the two-step staining procedure.

specific biomarkers using various assay formats that enable greater flexibility in assay design while accommodating possible high-throughput screening approaches. Based on the significant improvement in the LOD, the BRET-Qdot-based method appears amenable to investigating protein interactions, including low affinity interactions or possibly interactions involving binding partners that are either difficult to express or expressed at low levels. Importantly, the BRET-Qdot-based detection method presented here does not require any specific modifications of the conventional immunostaining procedures and offers flexible implementation via the one- or two-step detection methods. Any anti-biomarker or anti-tag specific antibody can be directly conjugated to the BRET-Qdot nanoprobe for a simpler staining reaction when compared to using the [BRET-Qdot]-SA procedure. In addition, the sensitivity of the assay with direct [BRET-Qdot]-antibody conjugates combined with multiple Qdot emission wavelengths of 605, 625, 655, and 705 nm make multiplex imaging applications possible. Although the two-step detection method relying on a biotinylated specific antibody and the [BRET-Qdot]-SA takes slightly longer to perform that the one-step approach and does not allow multiplexing applications, it has the advantage of being implemented rapidly using commercially available and well characterized [BRET-Qdot]-SA. Directly conjugated BRET-Qdot-antibodies are not commercially available yet and would require conjugation method optimization as well as quality control before use as reliable detection reagents.

The customized imaging system developed and implemented to optimally acquire the BRET-Qdot CTZ-activated signal might also be advantageous for automated high throughput screening of whole plate to whole well cellular imaging applications. Therefore, this method is totally compatible with high-throughput research efforts and has great potential for ultrasensitive protein–protein interaction high-throughput screenings. The simple whole plate imaging design

thus yields a versatile system adaptable for multiple imaging applications.

The combination of the BRET-Qdot nanoparticle reagents and the customized imaging platform expands the possibilities for advancing critical cell-based imaging applications requiring sensitivity currently not achieved by the existing fluorescence-based detection methods. Thus, we anticipate that this technology will be broadly applicable in multiple research areas requiring the direct or indirect detection of cell-surface biomarkers, such as, for example, the detection of rare cellular events, and the characterization of new and complex protein–protein interaction networks.

MATERIALS AND METHODS

BRET-Qdot NANOPROBES

BRET-Qdot 655 is commercially available from Zymera Inc (San Jose, CA). The preparation of BRET-Qdot655 probes using carboxylated Qdots (QD655; Invitrogen Corporation, Carlsbad, CA) covalently linked to the Luc8 protein has been previously described [So et al., 2006]. This BRET-Qdot 655 is prepared by an optimized proprietary conjugation protocol using EDC-NHS chemistry. Briefly, the conjugation is done in borate buffer at pH 7.4 and excess Luc8 (MW: 37 kDa), ethyl(dimethylaminopropyl) carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) present in the reaction mixture were separated from the BRET-Qdot655 using a 100 K Amicon spin filter (Millipore Corporation, Billerica, MA). The purified BRET-Qdot 655 conjugates (500 nM) are in 10 mM Tris buffer (pH 7.4) and are stored at 4°C. This BRET-Qdot 655 material has approximately six Luc8 molecules per quantum dot based on its fluorescence and bioluminescence spectral analyses, measured using a Fluoro Max-3 Fluorometer (HORIBA Jobin Yvon Inc., Edison, NJ). The hydrodynamic size of BRET-Qdot 655 particles measured by dynamic light scattering (DLS; Malvern Zeta Sizer Nano Instrument, Malven Instruments Ltd, Malvern, UK) was 22 nm in diameter.

[BRET-Qdot]-SA NANOPROBES

[BRET-Qdot]-SA is commercially available from Zymera Inc. BRET-Qdot 655-SA is prepared by an optimized proprietary conjugation protocol using EDC-NHS chemistry. The conjugation is done in borate buffer at pH 7.4 and excess Luc8 (MW: 37 kDa), SA, NHS, and EDC present in the reaction mixture were separated from the BRET-Qdot655-SA using a 100 K Amicon spin filter (Millipore Corporation, Billerica, MA). The purified BRET-Qdot655-SA conjugates (500 nM) are in 10 mM Tris buffer (pH 7.4) and are stored at 4°C. This BRET-Qdot 655-SA material has approximately six Luc8 molecules per quantum dot based on its fluorescence and bioluminescence, measured using a Fluoro Max-3 Fluorometer (HORIBA Jobin Yvon Inc.). The BRET-Qdot 655-SA material contains approximately seven SA molecules based on a biotin-binding assay using biotin-4-fluorescein, a biotin tethered fluorescein. The fluorescence of biotin-4-fluorescein gets quenched upon binding to 1 of the 4 biotin-binding sites of SA. Thus, the number of biotin-binding sites can be estimated when a known concentration of biothin-4-fluorecein is added to a known amount of SA [Kada et al., 1999].

[BRET-Qdot] CONJUGATED ANTIBODY

The preparation of the direct conjugate formed between the anti-EpCAM antibody and BRET-Qdot was performed according to proprietary conjugation and purification protocols developed by Zymera. The direct conjugates or the preformed complex formed by combining biotinylated anti-EpCAM antibody with [BRET-Qdot]-SA are both supplied as 500 nM in Zymera formulation buffer and stored at 4°C.

CELL LINES AND TRANSFECTIONS

COS7 cells (ATCC CRL-1651) and NIH 3T3/HER2 cells stably expressing HER2 (provided by H. Kobayashi; NCI/NIH) were grown in high glucose DMEM (Invitrogen, 12491-015) containing 10% FBS (Sigma 2442). MCF7 cells stably expressing HER2 (MCF7/HER2) and SKOV3 cells were grown in RPMI-1640 (Invitrogen, 12633-012) containing 10% FBS. For transfections, COS7 cells were grown in 96-well plates, and transfected with cDNA by Fugene 6 (Roche, 11814443001) according to the manufacturer's protocol at a ratio of 6 μl of Fugene 6 per 1 μg of DNA. Cells were typically grown for 48 h before staining.

ANTIBODIES

Primary antibodies used were: anti-EpCAM (R&D systems, AF960). Secondary antibodies used were: biotinylated anti-EpCAM (R&D Systems, BAF960), biotin-SP-conjugated AffiniPure rabbit anti-human IgG (H + L; Jackson ImmunoResearch, 309-065-003), biotin-SP-conjugated AffiniPure donkey anti-human IgG (H + L; Jackson ImmunoResearch, 709-065-149), biotin-SP-conjugated AffiniPure mouse anti-human IgG (H + L; Jackson ImmunoResearch, 209-065-082), biotin-SP-conjugated AffiniPure goat anti-human IgG (H + L; Jackson ImmunoResearch, 109-065-003), Alexa Fluor 488 goat anti-human IgG (H + L; Invitrogen, A-11013), and biotinylated anti-HER2 (trastuzumab, Genentech, Inc.) provided by H. Kobayashi (NCI/NIH).

IMMUNOFLUORESCENCE STAINING

Cells were fixed with 4% paraformaldehyde for 20 min, washed three times with 1× PBS, then blocked with 1% BSA for 30 min. Human Fc-tagged bait proteins or specific biotinylated antibodies (anti-HER2 or anti-EpCAM) were incubated with the cells for 1 h, washed three times in PBS, and incubated with a biotinylated anti-human Fc antibody for 45 min followed by three PBS washes and incubation with SA-AlexaFluor 488 for 15 min or directly incubated with SA-AlexaFluor 488, respectively. After three final PBS washes, assay plates were scanned using an IsoCyte laser imager (ImageXpress Velos, Molecular Devices). All steps were performed at room temperature.

[BRET-Qdot]-SA STAINING

Cells were fixed with 4% paraformaldehyde for 20 min, washed three times with 1× PBS, then blocked with 1% BSA for 30 min. Human Fc-tagged bait proteins or specific biotinylated antibodies (anti-HER2 or anti-EpCAM) were incubated with the cells for 1 h, washed three times in PBS, and incubated with a biotinylated anti-human Fc antibody for 45 min followed by three PBS washes and incubation with [BRET-Qdot]-SA for 15 min or directly incubated with [BRET-

Qdot]-SA, respectively. Cells were washed three times and CTZ was added to each well. Plates were imaged 5 min after CTZ addition on the Zymera Imaging Platform. All steps were performed at room temperature.

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