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Biosensing and Imaging Based on Bioluminescence Resonance Energy Transfer

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Abstract

Bioluminescence resonance energy transfer (BRET) operates with biochemical energy generated by bioluminescent proteins to excite fluorophores and offers additional advantages over fluorescence energy transfer (FRET) for *in vivo* imaging and biosensing. While fluorescent proteins are frequently used as BRET acceptors, both small molecule dyes and nanoparticles can also serve as acceptor fluorophores. Semiconductor fluorescent nanocrystals or quantum dots (QDs) are particularly well-suited for use as BRET acceptors due to their high quantum yields, large Stokes shifts and long wavelength emission. This review examines the potential of QDs for BRET-based bioassays and imaging, and highlights examples of QD BRET for biosensing and imaging applications. Future development of new BRET acceptors should further expand the multiplexing capability of BRET and improve its applicability and sensitivity for *in vivo* imaging applications.

Introduction

Bioluminescence resonance energy transfer (BRET), first discovered in marine creatures such as the jellyfish *Aequorea victoria* and the sea pansy *Renilla reniformis*, is a nonradiative process of transferring energy from a donor (usually a light emitting enzyme during the catalysis of the oxidation of its substrate such as luciferase) to an acceptor (for example a fluorescent protein that absorbs the donor energy and emits light at a longer wavelength) [1–4]. BRET resembles fluorescence resonance energy transfer (FRET) in many aspects except that it does not require external light source for the donor excitation. Efficient resonance energy transfer requires that the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor. The efficiency of BRET is inversely proportional to the sixth power of the distance between the donor and the acceptor, as described in the equation $E = 1/(1+R^6/R_0^6)$ where R_0 is the distance leading to 50% of energy transfer from the donor to the acceptor [5–6].

With the dependence of the BRET efficiency on the distance between the donor and acceptor, BRET is a powerful technique to evaluate and image protein-protein interactions [7]. In the original BRET description, the donor was *Renilla* luciferase, and green fluorescent protein (GFP) was the acceptor. Both can be genetically fused to proteins of interest for use in BRET or other applications [8–9]. Energy transfer occurs when proteins of interest interact bringing the donor and acceptor into close proximity. The acceptor energy emission can then be detected

relative to the donor emission. Since its initial description, BRET has evolved into several new forms by employing novel donor and acceptor pairs.

BRET donors

Renilla luciferase (Rluc) is widely used in the artificial BRET systems as the donor. Rluc catalyzes the oxidation of its substrate coelenterazine with an emission maximum at 480 nm. Coelenterazine is hydrophobic and cell permeable, which allows the BRET imaging in living cells and animals. Synthetic analogs of coelenterazine may also be used to generate emission at a different wavelength with different efficiency [10]. For example, DeepBlueC or coelenterazine 400a, produces an emission peak at ~395 nm, which is 100 nm away from the donor emission of GFP (GFP², a mutant form that can be excited at 395 nm and emit at 510 nm); this large spectral separation of two emission peaks improves the sensitivity of the BRET assay [11]. However, DeepBlueC is a poor substrate generating about 300 times less signal than coelenterazine [12].

Another blue bioluminescent protein aequorin, a complex of 22kD apoaquorin with coelenterazine and molecular oxygen, emits photons at 466 nm in the presence of Ca²⁺, and can similarly act as a BRET donor [13]. Highly efficient resonance energy transfer has been reported between aequorin and GFP in a protease assay [14]. The observation of a nearly complete shift in the spectral peak emission from aequorin to that of GFP suggests that aequorin is a superior BRET donor for GFP.

Firefly luciferase (Fluc) from *Hotaria parvula* have also employed as the BRET donor [15]. Fluc oxidizes its substrate D-luciferin in the presence of ATP, O₂, and Mg²⁺, producing an emission peaked between 560–580 nm [16]. With its long wavelength emission, no suitable fluorescent proteins could serve as its acceptor until the discovery of red fluorescent proteins. An early example uses DsRed fluorescent protein (ex: 558 nm; em: 583 nm) as the acceptor in a protein-protein interaction assay [17]. However, due to the substantial overlap of two emission peaks, only a small shift in the luminescence emission peak (from 565 nm to 583 nm) was observed in the study. The oligomerization of DsRed further complicates its use as a BRET acceptor. With monomeric red and far-red fluorescent proteins now available [18–20], the perspective of Fluc as the BRET donor should be largely improved.

BRET acceptors

Fluorescent proteins are common BRET acceptors due to the ease of genetically fusing them with other proteins of interest (Figure 1a). Many GFP mutants with varying spectral properties have become available [21–22], and the choice of a GFP variant for individual BRET systems is heavily influenced by the donor emission which is determined by both the donor protein and its substrate. For example, for the same donor Rluc, yellow fluorescent protein (YFP) is preferred when coelenterazine h is the substrate; this system is generally referred to BRET¹. The combination of the substrate DeepBlueC, Rluc and GFP² (ex: 395/475 nm; em: 510 nm) makes up BRET², another popular BRET system. The Rluc-based BRET¹ and BRET² have been applied to many studies, e.g. characterizing dimer and/or oligomer formation of G protein-coupled receptor (GPCR) [23–24], investigating subunit dynamics of two major protein kinase A (PKA) isoforms side by side [25], examining oligomerization of transcription factor complexes in the nuclear compartment [26], and detecting protease activity [27–28]. By combining two systems, BRET and FRET, a sophisticated sequential BRET-FRET (SRET) was created for the purpose of identifying heteromers formed by three different proteins [29]. Gambhir et al. even successfully applied the BRET² technology to image small molecule mediated protein-protein interactions in living mice [30].

In addition to fluorescent proteins, other fluorophores can serve as the BRET acceptor as well such as small organic dyes and fluorescent nanoparticles (Figure 1b & c). A number of examples exploited small fluorescent dyes as the BRET acceptor through chemical modification of the donor protein. Two fluorophores—IANBD ester (ex: 478 nm; em: 536 nm) and Lucifer Yellow (ex: 428 nm; em: 531 nm)—have been chemically conjugated to cysteine-containing aequorin mutants, and the position where the fluorophore is conjugated to aequorin strongly influenced the BRET efficiency [31]. In a homogeneous immunoassay [15], Cy3 (ex: 547 nm; em: 563 nm) or Cy3.5 (ex: 591 nm; em: 604 nm) was conjugated to an antibody that could bind to an epitope fused to firefly luciferase such that binding induced a BRET signal. The bioluminescence emission in the constructs can be quenched by a non-emitting energy absorber and become dark [32]. Dyes QSY-7 and dabcy1 have been conjugated to avidin whose binding to biotinylated aequorin led to 50% quenching in the bioluminescence emission. This quenched dark state offers another level of control that may be of use in both homogenous immunoassays and in *in vivo* imaging.

Both fluorescent proteins and small-molecule fluorophores share one common shortcoming as the BRET acceptor, that is, small Stokes shifts, which result in poor spectral separation of the acceptor emission from the donor emission. A new class of fluorophores based on semiconductor materials, quantum dots (QDs), on the other hand, possesses a large Stokes shift and are excellent BRET acceptors.

QDs are fluorescent semiconductor nanocrystals with a typical size of 2–8 nm in diameter, and can be excited at essentially any wavelength ranging from UV to the visible region [33–35]. Their broad excitation spectra allow them to be excited by nearly all the bioluminescent proteins in BRET constructs. Their emission spectra are often narrow and tunable by particle size; these features contribute to a large Stokes shift and excellent separation from the donor emission peak. Demonstration of QDs as the BRET acceptor for a mutant of *Renilla* luciferase (Luc8 with improved chemical stability and light efficiency [36]) has been recently realized both *in vitro* and *in vivo* [37].

With previous reviews on BRET primarily focusing on the fluorescent protein-based BRET and its application in imaging protein-protein interactions [7,38–41], the rest of this short review will emphasize on the development of new BRET systems using non-fluorescent protein acceptors and their applications for biosensing and *in vivo* imaging.

Chemical methods for establishing the QD-BRET system

Unlike the BRET system using fluorescent proteins, QDs cannot be genetically fused to bioluminescent proteins or to other proteins of interest, and have to be chemically introduced into the BRET construct. Several strategies have been developed to configure the QD-BRET systems.

The first approach is to couple the carboxylate groups presented on the QDs to the amino groups on the luciferase proteins, Luc8, and form the amide-linked nanoconjugates (Figure 2a) [37, 42]. The resulting conjugates displayed the fluorescence emission from QDs in addition to the bioluminescent emission from Luc8 upon the addition of coelenterazine. With the QD655 that emits at a maximum of 655 nm, the two emission peaks are clearly separated by 175 nm, which is much larger than that with GFPs as the acceptor. The BRET ratio calculation thus becomes straightforward with no need for correcting the overlap from the donor emission. It has been observed that the coupling condition strongly influenced the BRET efficiency and intensity of the final conjugates [42]; this is probably due to the different lysine amino groups on the bioluminescent protein that were conjugated to the QDs, resulting in different dipole-dipole orientation and modification on the luciferase activity.

Metal-mediated complexation between QDs and the bioluminescent proteins can also induce BRET (Figure 2b) [43]. Carboxylate-presenting QDs can associate with a 6×His tag fused to the C-terminus of Luc8 in the presence of Ni²⁺, resulting in a strong BRET emission from QDs. This Ni²⁺-dependent BRET system may be applied to develop homogeneous assays for sensitive detection of metal ions.

Similar to BRET that is based on fluorescent proteins for imaging protein-protein interactions, the QD-BRET may be also applied to sense protein-protein, or receptor-ligand interactions (Figure 2c) [44]. In one study we genetically fused Luc8 to the HaloTag protein (HTP, an engineered bacteria haloalkane dehalogenase), which binds aliphatic halogenated compounds (HaloTag ligands) irreversibly. HaloTag ligands were immobilized on the QDs, and their binding with the HTP in the fusion protein induced the BRET. With a larger distance between Luc8 and QDs than that in the above two examples, a generally smaller BRET ratio has been observed.

Direct site-specific conjugation of the bioluminescent proteins to the QDs can be achieved with an intein-mediated traceless ligation (Figure 2d) [45]. Intein is a polypeptide sequence inside a protein that is able to excise itself and rejoin the remaining portions with an amide bond [46]. It catalyzes the splicing reaction through formation of an active thioester intermediate, and has been widely applied to protein conjugation and immobilization in literature. In establishing the intein-mediated nanoconjugation strategy, a *Mex* GyrA intein (*Mycobacterium xenopi* gyrase A intein, a 198-aa natural mini intein, which lacks a central intein endonuclease domain) [47] was fused to the C-terminus of Luc8. Carboxylate coated QDs were coupled to adipic dihydrazine (ADH) and generated hydrazide coated QDs. A simple mixing of the two entities led to the site-specific conjugation of the C-terminus of the fusion protein to the hydrazide QDs with the intein excised out. This intein-mediated site-specific thus allows the precise attachment of proteins of interest to QDs much like that of the genetically fused constructs.

Protease sensing based on the QD-BRET system

An important application of the QD-BRET system is to sense the proteolytic activity of proteases. Using the intein-mediated nanoconjugation chemistry, we linked the C-terminus of the bioluminescent protein Luc8 to the QDs via a protease peptide substrate (Figure 3a) [45]. The protease cleavage breaks down the BRET process by releasing the bioluminescent protein from the QDs and this leads to the decrease in the BRET ratio. This QD-BRET sensing system has been successfully demonstrated in the detection of matrix metalloproteinase 2 (MMP-2) activity in mouse sera and tumor cell lysates, and was able to sense a few nanograms of MMP-2 in a milliliter (ng/mL; Figure 3b). By varying the peptide substrate, many other proteases can be similarly detected in biological media such as matrix metalloproteinase 7 (MMP-7) and urokinase-type plasminogen activator (uPA). Furthermore, by coupling the QD emission wavelength with the protease substrate sequence, it is possible to sense multiple proteases in the same sample, e.g. multiplex detection of MMP-2 and uPA with QD655 and QD705 BRET systems in one sample (Figure 3c). The two probes were mixed together for simultaneous detection of MMP-2 and uPA: when MMP-2 was added, only the BRET signals at 655 nm decreased; the presence of uPA led to the decrease in the BRET emission at 705 nm; the decrease in both BRET signals indicated the presence of both MMP-2 and uPA. The multiplex assay provides straightforward detection of multiple analytes, simplifies experiment procedures and eliminates systematic errors.

While this example concerns the protease as the sensing target, the QD-BRET system may serve as a general sensing platform for many other targets (Figure 3d). The forward reaction brings the QD and bioluminescent proteins together through the interaction of X and Y (each

of which is fused or conjugated to a QD, or bioluminescent protein) for BRET to occur, and it may be designed to detect protein-protein interactions, analytes (e.g. Ni^{2+} , DNA, RNA that mediate the interaction of X and Y), or enzymatic activities that act to join X and Y directly (e.g. ligases) or indirectly (e.g. kinases that catalyze the phosphorylation dependent X-Y complexation). The backward reaction breaks BRET and thus may be used to sense analytes (via competing off the binding site in the X-Y complex), and cleaving enzymes (such as proteases, nucleases).

In vivo imaging with the QD-BRET system

BRET based *in vivo* imaging does not require external excitation light source, thus the issue of autofluorescence, which significantly reduces signal to noise ratios (SNR) in fluorescence based *in vivo* imaging, is not of concern in BRET-based strategies. However, light scattering by tissues and absorption by hemoglobin still exist, and can significantly affect short-wavelength (<600 nm) emission [48]. In BRET systems that have been described using fluorescent proteins as the acceptor, both the donor and acceptor peak emissions are less than 600 nm, which makes *in vivo* BRET imaging a significant challenge, especially in deep tissues. BRET configurations with QDs as acceptors, on the other hand, have the potential to overcome this obstacle due to the significantly longer wavelengths of emission.

With QDs that emit at wavelengths longer than 600 nm, it has been shown that the absorbance of whole blood did not significantly affect BRET emission from QDs. In contrast, the emission of donor bioluminescent proteins was nearly eliminated by the absorption of their short wavelength emission due to hemoglobin (Figure 4a) [37]. *In vivo* detection of QD-BRET signals has been demonstrated with conjugates at superficial sites and at deep tissue locations. Using a conjugate prepared by direct coupling of QD655 with Luc8 (Figure 2b), we showed that the BRET emission from QD655 could be detected from conjugates at both subcutaneous and intramuscular sites (Figure 4b). When C6 glioma cells were labeled by the QD-BRET conjugates and injected through the tail vein into a nude mouse, their trafficking into the lungs was readily imaged from using BRET emission from the QDs (Figure 4c). These successful examples highlight the advantage of QDs as the BRET acceptors largely due to the longer wavelengths of emission relative to that of BRET constructs that use fluorescent proteins as acceptors.

The features of broad excitation spectra and size-tunable emission of QDs enable the creation of many possible BRET pairs. For example, the same bioluminescent protein Luc8 can pair with QD605, QD655, QD705, and QD800 for multiplexed BRET imaging (Figure 4d). Each of these has displayed efficient BRET upon conjugation, and all can be imaged *in vivo* upon injection. When two groups of cells were labeled with different QD-BRET conjugates (e.g. QD655 and QD800) and introduced into the same mouse, both can be imaged and differentiated from their QD-BRET emission (Figure 4e). With the capability of tuning the emission of QDs by controlling the size and composition, more BRET pairs may be available, which may allow more interactions and events to be imaged simultaneously in the same animal.

Concluding remarks

Like FRET, BRET is a broadly applicable method that is increasing in the number of applications, and has become a widely used technique for identifying and imaging protein-protein interactions in living systems. By eliminating the need for external light source for the donor excitation, BRET offer additional advantages over FRET: it produces no photodamaging to cells, no photobleaching of the fluorophores, no autofluorescence background, and no direct excitation of the acceptor.

While the present BRET systems primarily use fluorescent proteins as the acceptors, new BRET acceptors with improved features such as large Stokes shifts and fluorescence emission at a wavelength of more than 600 nm are needed to expand the multiplexing capability of BRET and improve its applicability and sensitivity for *in vivo* imaging applications. A new class of fluorophores, semiconductor nanocrystals or QDs, has emerged as an excellent BRET acceptor with bright and well-separated BRET emission, and largely increased the number of BRET pairs for multiplexing. While QDs are incompatible with genetic fusion, versatile biochemical and chemical methods are available for their conjugation to proteins of interest, and for their delivery into cells [49–50]. As research in nanotechnology continues to produce novel fluorescent nano materials, exploitation of these new fluorophores as potential BRET acceptors would further empower BRET and expand its utility as an important biosensing and imaging tool.

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Figure 1.

BRET occurs between a light-emitting protein (donor) and a fluorophore (acceptor) that can be (a) a fluorescent protein, (b) a small organic dye, or (c) an inorganic fluorescent nanoparticle (quantum dot).

Figure 2.

Chemical methods for establishing BRET between a bioluminescent protein and a quantum dot. **(a)** Non-covalent complexation mediated by Ni^{2+} between carboxylated quantum dots and the donor protein fused with a 6xHis tag. **(b)** Non-specific covalent coupling between a quantum dot and the donor protein. **(c)** Specific interaction mediated by the receptor fused to the donor protein and the ligand immobilized on a quantum dot. **(d)** Intein-mediated traceless site-specific ligation of the donor protein to a quantum dot.

Figure 3.

Biosensing applications with the QD-BRET system. **(a)** Schematic of protease sensing by a QD and luciferase protein (Luc8) that are linked together through a peptide substrate. Protease cleavage removes the donor protein from QD and disrupts BRET. **(b)** Detection of MMP-2 activity; representative emission spectra of a QD-BRET conjugate (QD655-MMP-2-Luc8) incubated with indicated amount of active MMP-2 in 20 mM Tris buffer (pH 7.5) for 1 h at room temperature. **(c)** Simultaneous detection of MMP-2 and uPA with a mixture of two QD-BRET conjugates (QD655-MMP-2-Luc and QD705-uPA-Luc8) that are incubated with MMP-2 (1 $\mu\text{g/mL}$; red), uPA (10 $\mu\text{g/mL}$; black), MMP-2 (1 $\mu\text{g/mL}$) + uPA (10 $\mu\text{g/mL}$) (green), or no enzyme (blue) at room temperature for 1 h in 20 mM Tris buffer. **(d)** QD-BRET system as a general platform for biosensing; X and Y are interacting partners that are linked to a QD and luciferase protein, respectively.

Figure 4.

In vivo imaging applications with QD-BRET (a) Bioluminescence emission spectrum of the QD-BRET conjugate (QD655-Luc8) in the mouse serum and whole blood. (b) Bioluminescence imaging of a live mouse injected with QD655-Luc8 subcutaneously (top) and intramuscularly (bottom) with an emission filter (650–660 nm). (c) Imaging C6 glioma cells labeled with the QD-BRET conjugate *in vitro* and injected into a living mouse via tail vein, acquired with a filter (575–650 nm). (d) Bioluminescence emission spectra of four QD-BRET conjugates (QD605-Luc8, QD655-Luc8, QD705-Luc8, and QD800-Luc8). (e) Multiplexed imaging of two groups of C6 glioma cells labeled by QD655-Luc8 conjugate and injected via tail vein, or labeled by QD800-Luc8 conjugate and injected intramuscularly; left image is collected without any filter, middle with 575–650 nm filter, and right with 760–790 nm filter.