Design and Development of High Bioluminescent Resonance Energy Transfer Efficiency Hybrid-Imaging Constructs

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ABSTRACT. Here we describe the design and construction of an imaging construct with high bioluminescent resonance energy transfer (BRET) efficiency that is comprised of multiple quantum dots (QDs, λ_{em} 655 nm) self-assembled onto a bioluminescent protein, *Renilla* luciferase (Rluc). This is facilitated by the streptavidin-biotin interaction, allowing the facile formation of a hybrid-imaging-construct (HIC) comprising up to 6 QDs (acceptor) grafted onto a light emitting Rluc (donor) core. The resulting assembly of multiple acceptors surrounding a donor permits this construct to exhibit high resonance energy transfer efficiency (~64.8%). The HIC was characterized using fluorescence excitation anisotropy measurements and high resolution transmission electron microscopy. To demonstrate the application of our construct, a generation-5 (G5) polyamidoamine dendrimer (PAMAM) nanocarrier was loaded with our HIC for *in vitro* and *in vivo* imaging. We envision that this design of multiple acceptors and bioluminescent donor would lead to the development of new BRET-based systems useful in sensing, imaging, and other bioanalytical applications.

KEYWORDS. Bioluminescence, bioluminescence resonance energy transfer, quantum dots, *Renilla* luciferase, imaging construct.

Introduction

Extensive effort has been invested in the design, development, characterization, and application of efficient light emitting constructs utilizing energy transfer [1]. Such optically-active constructs have a strong impact on many scientific fields, such as in the development of sensitive and selective biosensors, [2-6] molecular medicine, [7-10] imaging, and others [1, 6, 7, 11-16]. Part of this motivation for advanced resonance energy transfer (RET) platforms arises from the difficulty in tuning self-illuminating imaging constructs such as bioluminescent proteins for red-shifted emission. While the Gambhir laboratory has made important advances in this regard [17], the limited (66 nm) bandwidth of these *Renilla* luciferase mutants combined with their intrinsically broad emission leaves little opportunity for multiplexing. However, the increased complexity of fabrication for RET systems naturally dictates the need for facile, rapid, and reproducible synthesis to truly revolutionize the application of these constructs. This can be achieved by developing methodologies in which individual reagent-components are modified in such a way as to support self-assembly [18] of the desired light emitting constructs. These light emitting constructs often utilize fluorescent resonance energy transfer (FRET) [3, 19] or bioluminescent resonance energy transfer (BRET) [4, 20-23] mechanisms.

The optimization of a resonance energy transfer system involves proper matching of donor-to-acceptor energy levels in order to exhibit the desired emission characteristics.

For example, in order to obtain high quantum yield and a high signal-to-noise ratio of the emitted energy, a large molar extinction coefficient, narrow emission, and good photostability from the acceptor is desired. Many commercially available organic dyes fulfill some of these requirements, however, they can be either sensitive to photobleaching, unstable under different pHs, exhibit small Stokes shifts, have narrow excitation and broad emission peaks, or have small molar extinction coefficients. Recent developments in the synthesis of colloidal [semiconducting nanoparticles](#) have expanded their use in various optically-demanding applications such as FRET, BRET, and chemiluminescent resonance energy transfer (CRET) [20]. These luminescent “quantum dots” (QDs) have good photostability and high fluorescence quantum yield. Presently, commercially available QDs such as the widely used CdSe/ZnS and CdSe/CdTe, or the biocompatible InP/ZnS dots, can cover the full optical absorption spectrum while maintaining a narrow emission band (~45-50 nm) for application in sensing, imaging, and energy production (dye sensitized solar cells or solar concentrators) [24]. Their broad UV-Vis excitation band allows simultaneous excitation of multiple color QDs, allowing them to be considered as universal resonant energy donors. At the same time, their narrow emission allows for highly-selective energy transfer to the appropriate fluorophore. However, the application of QD-based, UV-Vis FRET systems is limited in biological medium (tissues, organs or cells) due to high biological attenuation of these wavelengths [15, 21, 22, 25], which results in weak and diffuse emission. Additionally, the utilization of high power sources for QD excitation is required in order to penetrate the tissue. [Consequently, high energy excitation results in photobleaching of the acceptor dye and significant background from the biological media \(autofluorescence\).](#) All of

these factors are detrimental to the observed signal-to-noise ratio (S/N). Additionally, the broad emission of most fluorophores restricts the degree of multiplexing available for sensing or imaging constructs [26]. While the broad UV-Vis absorption band of QDs makes them suitable as energy donors, their application as energy acceptors remained relatively limited until recently [4, 27] and is mainly limited to sensing applications [28, 29]. Du *et al.* [20] reported the design of QD-decorated chemiluminescent (CL) nanocapsules employing vinyl-encapsulated horseradish peroxidase conjugated to QDs. CL was generated using hydrogen peroxide and *p*-iodophenol as substrates. However, *in vivo* imaging using this construct was not shown, possibly due to the weak CL signal and complexity of the system. Herein we describe a construct using QDs as the energy acceptor from a bioluminescence [21, 23] (BL)-based excitation energy source placed in close proximity to the QDs.

Renilla luciferase (Rluc) is a bioluminescent protein that generates light as a result of a chemical reaction with its “luciferin” substrate, coelenterazine. This radiative energy can be used for direct imaging or as the excitation source for organic dyes or QDs. The emission spectrum of coelenteramide overlaps with the QD absorption spectra to provide resonance energy transfer that results in near-infrared QD emission for efficient biological matrix penetration (mm-cm) [7] and higher optical imaging sensitivity. A variation of this concept has been previously demonstrated using self-illuminating QDs [21]. In this work, multiple Rluc (donors) were linked to a single QD (acceptor) through a zero-length cross-linker. This multiple donor-single acceptor method utilizing a short Rluc-QD separation distance resulted in 56% BRET efficiency. The optimized construct was utilized for bio-imaging using the In-Vivo-Imaging System (IVIS). This novel

design allowed tissue imaging without the need for an exogenous excitation source. However, this application of multiple donors around a single acceptor required time-consuming conjugation chemistry.

In the present work, a new design is presented for a hybrid-imaging-construct (HIC) based on BRET principle that demonstrates high efficiency, self-assembly, and near-infrared illumination. The design is based on facile streptavidin-biotin chemistry, which enables a multiple acceptor–donor construct for superior BRET-based imaging. The HIC incorporates a network of streptavidin-modified QDs (QDSV) surrounding a biotin-modified Rluc (bRluc). A multiple-acceptor/single-donor configuration has not been demonstrated previously in a BRET study. Our system capitalizes on the fact that an increase in fluorescence resonance energy transfer (FRET) efficiency was observed when multiple energy acceptors surround a single energy donor in several studies [3, 5, 6, 30-32]. Due to the high quantum yield, large molar extinction coefficient, and broad absorption band [33] of the QD acceptors, a well-resolved, red-shifted, and stable emission is obtained. Bioluminescence emission from the donor (bRluc) in combination with the high excitation efficiency of the multiple QD acceptors results in low signal scattering and superior image quality for *in vitro* and *in vivo* imaging applications. The major advancement of this work comprises the ability to assemble the complete HIC using a facile technique that does not require complicated conjugation while exceeding the BRET efficiency of similar platforms that require significantly more synthetic manipulation. Additionally, this is the first report of a BRET-based single-donor/multiple-acceptor construct, and this design was found to exceed the BRET efficiency observed in previously reported work.

Materials and Methods

All purchased chemicals were used without further purification. Streptavidin-modified QDs emitting at 655 nm (QDSV) were purchased from Invitrogen (Carlsbad, CA). Biotin-N-hydroxysuccinimide ester (98% HPLC purified) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), ethylenediamine core generation-5 polyamidoamine dendrimer (G5-PAMAM), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich. Targeted peptide (AKXVAAWTLKAAAZC)-G5 dendrimer conjugate was purchased from 21st Century Biochemicals. 96-well microtiter plates with non-binding surface were purchased from Corning (NY). Microcon YM-100 (100 kDa MWCO) spin-columns were purchased from Millipore (Billerica, MA). Native coelenterazine was obtained from Prolume (Pinetop, AZ). Zeba desalting spin columns (7 kDa MWCO), monobasic sodium phosphate anhydrous, and dibasic sodium phosphate heptahydrate were purchased from Thermo Fischer Scientific (Rockford, IL). Dulbecco's modified Eagle's medium/Ham's F12 50/50 mix, 1% antibiotic-antimycotic solution, and trypsin-EDTA were purchased from Cellgro (Manassas, VA).

***Renilla* Luciferase-Biotin (bRluc) Conjugation.**

Rluc was expressed and purified using methods developed in our laboratory [4]. Biotin-NHS ester was used to conjugate biotin to the free amine groups on Rluc. In a typical reaction, 1.1 nmol of Rluc was reacted with 11 nmol of biotin-NHS ester in 200 μ L of 100 mM phosphate buffer pH 8. The reaction was allowed to continue 15 min prior to buffer-exchange through Zeba desalting spin-columns (7 kD MWCO). The bRluc conjugates were buffer-exchanged to 100 mM phosphate buffer pH 7.0 and kept at

4 °C until further use. The bioluminescence emission of b-Rluc was measured and compared with Rluc showing no significant effect of conjugation on the bioluminescence of Rluc.

Bioluminescence Measurements.

To synthesize self-assembled HIC, 1 pmol of bRluc was added to 100 mM phosphate buffer pH 7 in a 96-well microtiter plate followed by incremental addition of QDSV from a 2 μ M stock solution. This mixture was incubated for 15 min at 25 °C. To the final mixture volume of 150 μ L, 50 μ L of a 2 μ g/mL solution of coelenterazine was injected, and luminescence was recorded using 486 ± 10 nm and 680 ± 10 nm emission filters using bioluminescent measurements on a Victor X Light luminometer (Perkin Elmer). Additionally, the spectral output of the HIC was recorded under similar conditions using a Labsystem Luminoskan Ascent Microplate Reader (LabSystems, Franklin, MA) with a water-cooled CCD.

Given the potential for extrinsic signal losses *in vivo*, we evaluated the possibility of further signal loss due to the inner-filter effect using an Agilent UV-Vis spectrophotometer. Briefly, 200 μ L of the HIC complex was drawn from each well of the 96-well microtiter plates following completion of the BRET characterization, and the transmittance and absorbance of the solution was measured. A solution of 100 mM phosphate buffer pH 7.0 was used as a blank.

Fluorescence Excitation Anisotropy Measurements.

Fluorescence excitation anisotropy measurements were carried out using a Quantum Master 40 fluorescence spectrophotometer (Photon Technology International; Burmingham, NJ). The excitation spectrum of coelenteramide was achieved through an excitation polarizer (385 nm), while emission was collected through a 500 nm emission polarizer using a 4 nm slit opening. Fluorescence excitation anisotropy data was collected for each sample at a rate of 0.1 points/sec for 200 sec. Briefly, 1 pmol of bRluc was added to 100 μ L of 100 mM phosphate buffer pH 7.0, followed by the addition of 0.5 μ g/mL of coelenterazine. Fluorescence excitation anisotropy was recorded after a 5 min incubation to allow for the conversion of coelenterazine to coelenteramide. To this mixture, a stock solution of 0.5 μ M QDSV was added in increments of 1-2 μ L until reaching 8 pmol. After each QDSV addition, fluorescence excitation anisotropy measurements were recorded at 15 min intervals.

HR-TEM Analysis

For TEM analysis, 1 μ L of either a 6 μ M QDSV stock solution or a 6 μ M HIC solution was drop-casted on a copper grid and allowed to dry. Samples were analyzed using a high resolution JEOL (JEM-2100) transmission electron microscope equipped with a LaB6 filament operating at 200 kV.

Synthesis of Hybrid Imaging Construct (HIC) PAMAM Dendrimer Complex.

For imaging analyses, the HIC was encapsulated with G5-PAMAM dendrimer (HIC-D). For this, a solution containing 6 pmol of QDSV was added to 1 pmol of bRluc and incubated for 15 min at room temperature. Subsequently, 10 pmol of a G5-PAMAM solution was added [34]. The control consisted of 1 pmol of bRluc and 10 pmol of G5-

PAMAM in the absence of QDSV. Dendrimer encapsulation of the construct was performed overnight and used for imaging the following day. For *in vivo* imaging, the HIC complex was encapsulated with targeting peptide G5-PAMAM dendrimer (PDD) to create HIC-PDD using a similar procedure. A control conjugate of bRluc-PDD was also prepared.

***In vitro* Imaging.**

ARPE-19 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 50/50 mix supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. Cells were incubated at 37 °C in 98% humidified air containing 5% CO₂. For HIC-D imaging, cells were grown to 90% confluence, washed with phosphate-buffered saline (PBS), and detached with 0.25% Trypsin-EDTA. Cells were then counted and resuspended at a density of 2×10^6 cells/mL in Opti-MEM Reduced-Serum Medium. 500µL aliquots containing 1×10^6 cells were treated with the complexes (9.96 pmoles) and incubated at 37 °C for 30 min. The cell/complex mixture was centrifuged for 5 minutes at 500 x g, washed with 500µL of PBS twice, divided into two aliquots of 250µL, and centrifuged for 5 minutes at 700 x g. Prior to imaging, cells were resuspended by pipetting, followed by the addition of 10 µg of coelenterazine. The images were acquired with an IVIS Spectra (Caliper Life Science, Inc.) in the auto mode using 660 and 500 ± 20 nm emission filters, and the acquired images were analyzed using Living Image[®] 4.2 software available free of cost at <http://www.caliperls.com/support/software-downloads.htm>.

***In vivo* Mice Imaging**

Two C57BL/6 mice were injected subcutaneously with HIC-PDD and bRLuc-PDD as imaging construct and control, respectively. After 4 hours, 100 μ L of 100 ng/ μ L coelenterazine was administered by IP injection, and the mice were imaged for 60 sec. Prior to imaging, the peritoneum of mice was incised dorsally and imaged by IVIS. The emission from HIC-PDD and bRLuc-PDD was collected using a 660 ± 20 nm filter. The obtained images were analyzed using Live Imaging[®] 4.2 software available at the IVIS website.

Figure 1: A simplified schematic representation of the imaging construct synthesis. While enlarged slightly to show detail, the diameter of Rluc measures approximately half the length of the cylindrical quantum dots, with axial crystallographic dimensions of $8.92 \times 5.1 \times 7.4$ nm for Rluc (PDB ID 2PSJ, [35]) versus ~ 20 nm (Invitrogen) for the streptavidin-functionalized nanocrystals. Biotinylated Rluc (1 pmol) mixed with streptavidin-modified QDs (6 pmol) results in formation of the HIC.

Results and Discussion

The general scheme for the HIC (Figure 1) relies on multiple energy acceptors surrounding a central bioluminescent donor core for efficient energy transfer. The initial

optical characterization of BRET in the HIC demonstrated high intensity emission at 655 nm from QD with a concurrent decrease in the emission from Rluc (Figure 2a). The inner filter effect, or a decreasing linearity between the HIC concentration and fluorescent output due to reabsorption of the signal at higher concentrations [32], was evaluated for any effect on the BRET efficiency. Using aliquots drawn from the titration experiment, we were able to show that signal reabsorption had a negligible contribution toward overall BRET signal (data not presented). In Figure 2a, “ I_{nc} ” represents the normalized intensity following correction for the inner filter effect [32]. As shown in Figure 2b, optimization of the QDSV:bRluc ratio led to a BRET efficiency maximum at 6 pmol of QDSV when plotted against a bRluc concentration of 1 pmol. A reproducible BRET efficiency curve was obtained with different batches of HIC construct. BRET efficiency of the system was calculated using Equation 1 [32],

$$E = 1 - (I_{DA} / I_D) \times 100 \% \quad (1)$$

where I_D and I_{DA} represent the emission intensity of the donor in the absence and presence of acceptor, respectively. As shown in Figure 2b, BRET efficiency increased to a maximum of 64.8% at a QDSV:bRluc ratio of 6 and remained unchanged upon further increase in QDSV concentration.

Figure 2: Optical characteristics of the hybrid imaging construct (HIC) showing (a) the normalized intensity of the QD (—) and Rluc (---) emissions for increasing QDSV:bRluc ratios, (b) variation in BRET efficiency with respect to an increase in QDSV:bRluc ratio.

A certain degree of non-specific interaction between Rluc and QD can be expected. However, the strong streptavidin-biotin affinity and the reproducible BRET

signal saturation ratio strongly indicated the self-assembly of multiple QDSV and bRluc. Although it is possible that these HIC constructs could lead to mixtures of ratio from 1:1 to 1:6 or higher, the higher ratio does not affect BRET efficiency. The use of a 1:6 ratio between bRluc:QDSV resulted in reproducible BRET efficiency for different batches, indicating that a majority of complexes follow this configuration. Furthermore, to ensure that the formation of the HIC was complete, steady-state fluorescence excitation anisotropy experiments were performed using the fluorescence excitation of coelenteramide. Coelenteramide is produced from the oxidation of coelenterazine within the Rluc pocket [36, 37] and remains bound to Rluc after oxidation due to its higher binding affinity [36]. The fluorescence properties of coelenteramide (Figure 3a) provided a means to correlate QDSV binding to bRluc and fluorescence excitation anisotropy changes (Figure 3b). Anisotropy (r) can be loosely defined by the Perrin equation as the ratio of the maximum polarization exhibited by a static molecule to the loss of polarization due to rotational diffusion. When comparing anisotropy within the same system, fluorescence lifetime and maximum polarization are constant, so only a change in rotational diffusion will perturb the system and provide an anisotropy change. As QDSV became immobilized on the surface of bRluc, the rotational diffusion was expected to decrease incrementally until all binding sites were occupied or further binding of QDSV was sterically hindered. In either case, fluorescence excitation anisotropy was expected to increase toward a limit defined by the maximum coordination of the HIC. It was observed that the anisotropy value for bRluc without QDSV was on the order of 0.05, which resulted from the high internal flexibility and rapid rotational diffusion of the protein. Upon subsequent addition of QDSV, the anisotropy values increased from 0.05

to ~0.09, likely as a result of decreases in both intrinsic flexibility and rotational diffusion. Anisotropy of the system reached its maximum at a QDSV:bRluc ratio of ~6 and remained invariant to further ratio increases. This corroborated the interaction of multiple QDSV around a single bRluc with a maximum ratio of 1:6. Based on steady-state fluorescence excitation anisotropy, the fraction of QDSV attached to bRluc was calculated using Equation 2 [32],

$$f_b = (r - r_F) / (r_B - r_F) \quad (2)$$

where r is the measured anisotropy, r_F and r_B are the anisotropy of free and bound bRluc, respectively, and f_b represents the fraction of bound bRluc. Based on this equation, the average f_b was calculated to be ~80.6%.

Figure 3: Characterization of multiple QDSV around bRluc using anisotropy experiments. (a) Absorbance (---) and emission (—) of coelenteramide. (b) Anisotropy change upon increasing the ratio of QDSV:bRluc.

We also performed HR-TEM analysis using our HIC complex (Figure 4). The TEM shows the formation of HIC ranging from 1:3 to 1:6 ratio of bRluc:QDSV.

Figure 4: HRTEM image of Rluc-b QDSV (HIC) complex.

The utility of the HIC prepared in this study was demonstrated for *in vivo* imaging applications. First, we verified that the HIC could be successfully transported into an *in vitro* model tissue. For this study, we chose G5-polyamidoamide (PAMAM) dendrimer nanocarrier for transport of the HIC into cells and tissue, as the uptake via endocytosis of G5-PAMAM dendrimer-conjugated cargo has been well established in the literature. The

possibility of non-specific adsorption of the HIC complex on cell surface was reduced by performing several wash steps. G5-PAMAM dendrimer is a tree-like polymeric structure that terminates in primary amines, allowing for efficient complexation of DNA, proteins, and drugs through ionic interactions. Daftarian et al. [34] demonstrated that G5-PAMAM-DNA complexes (larger than 200 nm in size) efficiently crossed cell membranes. Thus, we relied on the same platform for transfection [34] of the HIC complex into human retinal pigment epithelium (ARPE-19) cells [38]. Following complexation with G5-PAMAM, no change in the optical properties of the HIC was observed, likely as a result of external complexation of the HIC given their highly disparate sizes (>50 nm for the HIC versus ~ 5 nm for the dendrimer). The zeta potential measurement of the HIC prior to incorporation with the dendrimer was found to be -9.9 mV, and this value decreased to -6.5 mV after incorporation, indicating that dendrimer complexation occurred as anticipated. In order to generate a calculated BRET efficiency in a biological matrix and compare it to the HIC efficiency in deep-tissue imaging applications, these HIC-dendrimer complexes were used for cellular imaging on an IVIS (Caliper Technologies, CA) instrument. Images were acquired from the HIC-dendrimer complex using 660 ± 20 nm and 500 ± 20 nm filters (for QD and Rluc, respectively) and demonstrated strong emissions (Figure 5a-b) at both wavelengths, whereas the control imaging construct of bRluc-G5-PAMAM (bRluc-D) without QDSV displayed only Rluc emission (Figure 5c-d). Based on the photon flux obtained from the control (bRluc-D) and the imaging construct (HIC-dendrimer) using Living Image[®] 4.2 software (CALIPER), a preliminary BRET efficiency of $\sim 72\%$ was calculated, indicating that the HIC exceeded our expectations in a biological matrix. We believe that this improvement

over the HIC alone could be due to stabilization of the HIC within the G5-PAMAM. Based on the success of our *in vitro* results, *in vivo* imaging using C57BL/6 mice was performed (Figure 6a-b) to evaluate the imaging prospects of the HIC in live animals. For this, the HIC was encapsulated within PDD, a dendrimer complex displaying the targeting peptide (AKXVAAWTLKAAAZC), to generate an *in vivo* imaging complex (HIC-PDD). The dendrimer with targeting peptide was chosen for this study because it allows for specific targeting of drugs to antigen-presenting cells in the spleen. We anticipated that targeting of the HIC to a specific organ may yield better imaging output. Electrostatic encapsulation of the HIC within the PDD was achieved by incubating the two components together in a phosphate buffer (pH 7).

Figure 5: Self-assembled, encapsulated hybrid imaging construct (HIC-D) transfected into ARPE-19 cells was imaged using (a) 660 ± 20 nm and (b) 500 ± 20 nm filters.

Similarly, the encapsulated bRluc control (bRluc-D) was imaged using (c) 660 ± 20 nm and (d) 500 ± 20 nm filters. All images were obtained using an IVIS imaging system.

For *in vivo* imaging, C57BL/6 mice were subcutaneously injected with ~ 0.4 μ g of HIC-PDD or bRluc-PDD, and images were acquired 4 hours post-injection. Mice injected with HIC-PDD showed strong emissions without a filter (Figure 6a) as well as with the 660 ± 20 nm filter (Figure 6b) for QD emission. The bRluc-PDD complex did not show detectable bioluminescence emission (Figure 6a-b, right mice) at similar concentrations – likely a result of biological attenuation of the Rluc signal at these low concentrations.

Figure 6: Imaging the HIC-PDD. The mouse on the left in each frame was injected with the targeted imaging construct HIC-PDD, while the mouse on the right was injected with control bRluc-PDD. Images were acquired post-subQ injection of coelenterazine using (a) no filter or (b) 660 ± 20 nm filter.

Recent reports [39] have demonstrated that constructs of similar size and chemistry (based on streptavidin-biotin interaction) can be used for *in vitro* and *in vivo* studies. Furthermore, in the case of HIC-PDD, energy transfer to the red emitting QDs

can overcome any issues with blue-green light absorption. As shown from the IVIS images in Figure 6a-b, the HIC-PDD complex appears to have localized near the spleen where a high concentration of MHC class II cells is anticipated. However, the main scope of the current work was to demonstrate that the designed HIC complex can be used for *in vivo* and *in vitro* imaging applications and not necessarily targeting; therefore, further histological studies were beyond the scope of the current study.

Conclusion

In this work we demonstrated the facile synthesis, characterization and application of a BRET-based hybrid imaging construct (HIC). Our studies demonstrated the formation of imaging constructs comprised of a central bioluminescent donor core (bRluc) decorated with multiple acceptors (QDSV). Furthermore, the formation of these imaging constructs utilized reproducible self-assembly of QDSV and bRluc, successfully producing high BRET efficiency (64.8%). Finally, we were able to apply these nanostructures directly to *in vitro* imaging applications while demonstrating that a recently-developed encapsulation method provided for *in vivo* imaging.

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Notes: Authors declare no competing financial interest.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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REFERENCES

- [1] Cai W, Chen X. Nanoplatforms for targeted molecular imaging in living subjects. *Small* 2007;3:1840-54.
- [2] Chan WC, Nie S. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. *Science* 1998;281:2016-8.
- [3] Clapp AR, Medintz IL, Mauro JM, Fisher BR, Bawendi MG, Mattoussi H. Fluorescence resonance energy transfer between quantum dot donors and dye-labeled protein acceptors. *Journal of the American Chemical Society* 2004;126:301-10.
- [4] Kumar M, Zhang D, Broyles D, Deo SK. A rapid, sensitive, and selective bioluminescence resonance energy transfer (BRET)-based nucleic acid sensing system. *Biosensors & bioelectronics* 2011;30:133-9.
- [5] Medintz IL, Clapp AR, Mattoussi H, Goldman ER, Fisher B, Mauro JM. Self-assembled nanoscale biosensors based on quantum dot FRET donors. *Nature materials* 2003;2:630-8.
- [6] Medintz IL, Uyeda HT, Goldman ER, Mattoussi H. Quantum dot bioconjugates for imaging, labelling and sensing. *Nature materials* 2005;4:435-46.
- [7] Gao X, Cui Y, Levenson RM, Chung LW, Nie S. In vivo cancer targeting and imaging with semiconductor quantum dots. *Nature biotechnology* 2004;22:969-76.
- [8] Kim S, Lim YT, Soltesz EG, De Grand AM, Lee J, Nakayama A, et al. Near-infrared fluorescent type II quantum dots for sentinel lymph node mapping. *Nature biotechnology* 2004;22:93-7.
- [9] Macfarlane RJ, Lee B, Jones MR, Harris N, Schatz GC, Mirkin CA. Nanoparticle superlattice engineering with DNA. *Science* 2011;334:204-8.
- [10] Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC. Nanoparticles in medicine: therapeutic applications and developments. *Clinical pharmacology and therapeutics* 2008;83:761-9.

- [11] Bruchez M, Jr., Moronne M, Gin P, Weiss S, Alivisatos AP. Semiconductor nanocrystals as fluorescent biological labels. *Science* 1998;281:2013-6.
- [12] Chudakov DM, Lukyanov S, Lukyanov KA. Fluorescent proteins as a toolkit for in vivo imaging. *Trends in biotechnology* 2005;23:605-13.
- [13] He X, Gao J, Gambhir SS, Cheng Z. Near-infrared fluorescent nanoprobe for cancer molecular imaging: status and challenges. *Trends in molecular medicine* 2010;16:574-83.
- [14] Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, et al. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science* 2005;307:538-44.
- [15] Rice BW, Cable MD, Nelson MB. In vivo imaging of light-emitting probes. *Journal of biomedical optics* 2001;6:432-40.
- [16] He Y, Wang RK. Improvement of low-level light imaging performance using optical clearing method. *Biosensors & bioelectronics* 2004;20:460-7.
- [17] Loening AM, Wu AM, Gambhir SS. Red-shifted *Renilla reniformis* luciferase variants for imaging in living subjects. *Nature methods* 2007;4:641-3.
- [18] Mattoussi H, Mauro JM, Goldman ER, Anderson GP, Sundar VC, Mikulec FV, et al. Self-Assembly of CdSe-ZnS Quantum Dot Bioconjugates Using an Engineered Recombinant Protein. *Journal of the American Chemical Society* 2000;122:12142-50.
- [19] Kagan CR, Murray CB, Bawendi MG. Long-range resonance transfer of electronic excitations in close-packed CdSe quantum-dot solids. *Physical review B, Condensed matter* 1996;54:8633-43.
- [20] Du J, Yu C, Pan D, Li J, Chen W, Yan M, et al. Quantum-dot-decorated robust transducible bioluminescent nanocapsules. *Journal of the American Chemical Society* 2010;132:12780-1.
- [21] So MK, Xu C, Loening AM, Gambhir SS, Rao J. Self-illuminating quantum dot conjugates for in vivo imaging. *Nature biotechnology* 2006;24:339-43.
- [22] Xia Z, Rao J. Biosensing and imaging based on bioluminescence resonance energy transfer. *Current opinion in biotechnology* 2009;20:37-44.
- [23] Xu Y, Piston DW, Johnson CH. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. *Proceedings of the National Academy of Sciences of the United States of America* 1999;96:151-6.
- [24] Sholin V, Olson JD, Carter SA. Semiconducting polymers and quantum dots in luminescent solar concentrators for solar energy harvesting. *Journal of applied physics* 2007;101:-.
- [25] Roda A, Guardigli M, Michelini E, Mirasoli M. Bioluminescence in analytical chemistry and in vivo imaging. *TrAC Trends in Analytical Chemistry* 2009;28:307-22.
- [26] Jaiswal JK, Simon SM. Potentials and pitfalls of fluorescent quantum dots for biological imaging. *Trends in cell biology* 2004;14:497-504.
- [27] Cissell KA, Campbell S, Deo SK. Rapid, single-step nucleic acid detection. *Analytical and bioanalytical chemistry* 2008;391:2577-81.
- [28] Algar WR, Ancona MG, Malanoski AP, Susumu K, Medintz IL. Assembly of a concentric Forster resonance energy transfer relay on a quantum dot scaffold: characterization and application to multiplexed protease sensing. *ACS nano* 2012;6:11044-58.
- [29] Algar WR, Wegner D, Huston AL, Blanco-Canosa JB, Stewart MH, Armstrong A, et al. Quantum dots as simultaneous acceptors and donors in time-gated Forster

resonance energy transfer relays: characterization and biosensing. *Journal of the American Chemical Society* 2012;134:1876-91.

[30] Fabian AI, Rente T, Szollosi J, Matyus L, Jenei A. Strength in numbers: effects of acceptor abundance on FRET efficiency. *Chemphyschem : a European journal of chemical physics and physical chemistry* 2010;11:3713-21.

[31] Koushik SV, Blank PS, Vogel SS. Anomalous surplus energy transfer observed with multiple FRET acceptors. *PloS one* 2009;4:e8031.

[32] Lakowicz JR. *Principles of fluorescence spectroscopy*. 3rd ed. New York: Springer; 2006.

[33] Leatherdale CA, Woo WK, Mikulec FV, Bawendi MG. On the Absorption Cross Section of CdSe Nanocrystal Quantum Dots. *The Journal of Physical Chemistry B* 2002;106:7619-22.

[34] Daftarian P, Kaifer AE, Li W, Blomberg BB, Frasca D, Roth F, et al. Peptide-conjugated PAMAM dendrimer as a universal DNA vaccine platform to target antigen-presenting cells. *Cancer research* 2011;71:7452-62.

[35] Loening AM, Fenn TD, Gambhir SS. Crystal structures of the luciferase and green fluorescent protein from *Renilla reniformis*. *Journal of molecular biology* 2007;374:1017-28.

[36] Matthews JC, Hori K, Cormier MJ. Substrate and substrate analogue binding properties of *Renilla luciferase*. *Biochemistry* 1977;16:5217-20.

[37] Matthews JC, Hori K, Cormier MJ. Purification and properties of *Renilla reniformis luciferase*. *Biochemistry* 1977;16:85-91.

[38] Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Experimental eye research* 1996;62:155-69.

[39] Sen D, Deerinck TJ, Ellisman MH, Parker I, Cahalan MD. Quantum dots for tracking dendritic cells and priming an immune response in vitro and in vivo. *PloS one* 2008;3:e3290.