Creating self-illuminating quantum dot conjugates

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Semiconductor quantum dots are inorganic fluorescent nanocrystals that, because of their unique optical properties compared with those of organic fluorophores, have become popular as fluorescent imaging probes. Although external light excitation is typically required for imaging with quantum dots, a new type of quantum dot conjugate has been reported that can luminesce with no need for external excitation. These self-illuminating quantum dot conjugates can be prepared by coupling of commercially available carboxylate-presenting quantum dots to the light-emitting protein Renilla luciferase. When the conjugates are exposed to the luciferase's substrate coelenterazine, the energy released by substrate catabolism is transferred to the quantum dots through bioluminescence resonance energy transfer, leading to quantum dot light emission. This protocol describes step-by-step procedures for the preparation and characterization of these self-illuminating quantum dot conjugates. The preparation process is relatively simple and can be done in less than 2 hours. The availability of self-illuminating quantum dot conjugates will provide many new possibilities for *in vivo* imaging and detection, such as monitoring of *in vivo* cell trafficking, multiplex bioluminescence imaging and new quantum dot-based biosensors.

INTRODUCTION

As fluorescent imaging probes, semiconductor quantum dots overcome certain limitations of organic fluorophores, such as low photostability and broad emission spectra[1–3]. However, they all need excitation from an external illumination source to fluoresce. Although that need is not an issue with transparent samples, it presents an inherent challenge for the imaging of opaque biological subjects because of the substantial background autofluorescence arising from ubiquitous endogenous chromophores as well as the light-absorption and light-scattering properties of tissue. To overcome this challenge a new type of quantum dot conjugate has been reported that can luminesce with no need for external excitation; instead, it luminesces through bioluminescence resonance energy transfer (BRET)[4].

BRET is a naturally occurring phenomenon in which a light-emitting protein (the donor) transfers energy in a non-radiative way to a fluorescent protein (the acceptor) in close proximity[5,6]. For example, in the sea pansy *Renilla reniformis*, the bioluminescent protein Renilla luciferase catalyzes the oxidation of coelenterazine and transfers the released energy through long-range dipole-dipole interactions to a green fluorescent protein, leading to the emission of a green-wavelength photon. BRET is analogous to fluorescence resonance energy transfer (FRET), sharing the characteristics of being highly dependent on the extent of spectral overlap, the relative orientation of the transition dipoles and the distance between the donor and acceptor[7]. However, it differs from FRET in that the excitation energy arises from the catalysis of a substrate rather than a photon derived from an external excitation source[7]. There are many examples of quantum dots serving as the donor in FRET[8]. Quantum dots have also been used as the acceptor in BRET, in which the bioluminescence energy of a luciferase-catalyzed reaction is transferred to the quantum dots to produce quantum dot light emission.

The protocol presented here provides a step-by-step procedure for the preparation of self-illuminating quantum dot conjugates for use in *in vivo* imaging. The chemical principle of creating these self-illuminating quantum dot conjugates is rather straightforward, involving amide couplings between the quantum dot and a stabilized variant of Renilla luciferase (Luc8)[9] (**Fig. 1**). Commercially available quantum dots are capped with negatively charged carboxylate groups. The coupling reagent EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride) is exploited to mediate the amide-bond formation between amino groups present on the surface of the protein and the carboxylates on the quantum dots.

Although the procedure presented here uses a mutant Renilla luciferase, it could potentially be adapted for the use of other light-emitting proteins containing amino groups at the protein surface, such as native Renilla luciferase and horseradish peroxidase[10].

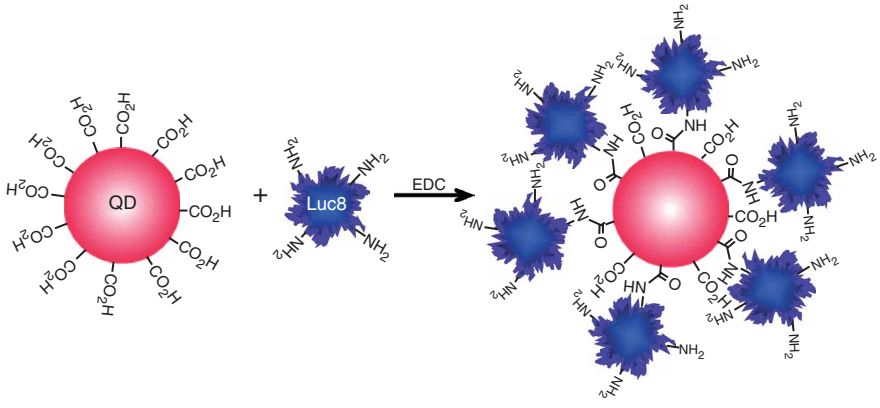

Figure 1 | EDC-mediated conjugation of the bioluminescence protein Luc8 to quantum dots (QD).

In addition, fusion proteins containing Renilla luciferase could be applied as well[11].

A limitation of this protocol is that the simple amide-coupling chemistry is not site specific; for Luc8 there are many amino groups (about 21) present at the surface accessible for conjugation with the quantum dots. Although that has not been an issue with Luc8, it may present problems when other proteins are used. That limitation can be overcome with site-specific conjugation methods such as a HaloTag protein-mediated conjugation[11].

A chief application of these self-illuminating quantum dot conjugates is *in vivo* imaging, particularly with small living animals in which these conjugates show greater sensitivity than standard quantum dots[4]. Other potential applications include the use of the BRET between quantum dots and bioluminescent proteins to detect and image protein-protein interactions[7,12]. The availability of these bioluminescent quantum dot probes should open many new avenues for quantum dot–based imaging, including highly sensitive *in vitro* bioluminescence assays, monitoring of *in vivo* cell trafficking, multiplex bioluminescence imaging and quantum dot–based biosensors in which the BRET emission is modulated by specific biological interactions.

MATERIALS

REAGENTS

- Quantum dots (Qdot ITK™ Carboxyl Quantum Dots; Invitrogen)
- Luc8 (see **Box 1**)
- EDC (Fluka)
- Tris buffer: 10 mM Tris HCl, pH 7.4 (Aldrich)
- NTA buffer: 1 mM NTA (*N*,*N*-bis-(carboxymethyl)-L-lysine hydrate; Fluka)
- Borate buffer: 10 mM sodium borate, pH 7.4 (sodium tetraborate and boric acid, Aldrich)

BOX 1 | PREPARATION OF LUC8

To make self-illuminating quantum dot conjugates, we have used the stabilized Luc8 variant of Renilla luciferase instead of the native enzyme[9]. Luc8 is an engineered mutant with high serum stability and improved catalytic efficiency containing the following eight substitutions: A55T, C124A, S130A, K136R, A143M, M185V, M253L and S287L. Although purified Renilla luciferase is available commercially (NanoLight), Luc8 is not and must be produced in the laboratory. A variety of protein expression systems can potentially be used for the production of Luc8, and the bacterial cytoplasmic method we have used is described here. Native Renilla luciferase can be prepared using this procedure as well.

Reagents:

pBAD-RLuc8 plasmid[9] (available on request)

L-(+)-arabinose (Sigma)

Lysozyme, DNase I and RNase A (Sigma)

Escherichia coli strain deficient in arabinose metabolism (e.g., LMG194; Invitrogen)

Nickel affinity resin (Ni-NTA; Superflow; Qiagen)

Wash buffer: 300 mM NaCl, 20 mM imidazole and 20 mM HEPES, pH 8.0

Elution buffer: 300 mM NaCl, 250 mM imidazole and 20 mM HEPES, pH 8.0

Anion-exchange buffer A: 10 mM NaCl and 10 mM Tris, pH 8.0

Anion-exchange buffer B: 1 M NaCl and 10 mM Tris, pH 8.0

Anion-exchange column (e.g., column packed with Source 15Q resin; GE Healthcare)

Procedure:

1. Transform the plasmid pBAD-RLuc8 into an *E. coli* strain deficient in arabinose metabolism (e.g., LMG194). The pBAD-RLuc8 plasmid contains a gene encoding Luc8 with a C-terminal six-histidine tag[9].

2. Grow an 0.5-liter culture in Terrific Broth to an absorbance at 600 nm of 0.7, induce with 0.2% arabinose, and grow for an additional 12 h at 32 °C. Collect the cell pellets by centrifugation and freeze at −20 °C.

■ **PAUSE POINT** The cell pellet can be left frozen at this point. For periods longer than a few days, it is advisable to store the pellet at −80 °C.

3. Lyse the cells by thawing in 25 ml wash buffer containing 1 mg ml^{-1} lysozyme, 5 μg ml^{-1} DNAse I and 10 μg ml^{-1} RNAse A, slowly mixing for 1 h at room temperature. Follow this by sonication. Clarify the lysates by centrifugation (10,000*g* for 30 min at 4 °C).

4. Mix the lysate supernatant with 5 ml nickel affinity resin and allow the protein to bind for 1 h at 4 °C with gentle mixing.

5. Extensively wash the resin with wash buffer and elute the resin-bound protein with 10 ml elution buffer. The washes and elution can be done either with a disposable column or by successive rounds of centrifugation (1 min at 300*g*) and resuspension. The expected yield of Luc8 at this point is about 200 mg.

6. Dilute the protein 1:5 in anion-exchange buffer A and further purify by anion-exchange chromatography using an appropriate resin (e.g., Source 15Q) and a gradient of 0–30% anion-exchange buffer B. The protein should elute at about 100 mM NaCl.

7. Exchange the protein into Tris buffer using gel-filtration chromatography or another suitable method.

8. Assess the activity of the Luc8 using a calibrated luminometer (20/20n Luminometer; Turner Biosysems). Mix 1 μl protein (diluted appropriately in a buffer containing a carrier protein) with 100 μl of 100 mM sodium phosphate buffer, pH 7.0. Add 0.5 μg coelenterazine (1 μl of a 0.5 mg ml^{-1} stock in propylene glycol), vortex, and measure the activity immediately. Over 10 s, an average flux of 1.4×10^{23} photons per second per mole of enzyme should be observed.

▲ **CRITICAL STEP** Because of the tiny amounts of protein being assayed, a carrier protein must be present in the buffer used to dilute Luc8. We generally use 10 mg ml^{-1} albumin as the carrier. Lack of a carrier will lead to adsorptive losses of Luc8, resulting in underestimation of the protein's activity.

- DNA loading dye (Blue/Orange Loading Dye, 6×; Promega)
- 0.5% agarose gel
- TAE buffer (TAE buffer, 40×; Promega)
- Coelenterazine (Prolume; see REAGENT SETUP)
- Nine-arginine peptide (R9 peptide)
- C6 cells (see REAGENT SETUP)
- Hanks' balanced-salt solution (HBSS, 1×; Gibco)

EQUIPMENT

- Spin filter, 100-kilodalton cutoff (Nanosep; Pall)
- Refrigerated microcentrifuge (centrifuge 5417R; Eppendorf)
- Fluorometer (Fluoro Max-3; Jobin Yvon)
- Glass-bottomed microwell dish (30-mm Petri dish, 14-mm Microwell; MatTek)
- Inverted fluorescence microscope (Axiovert 200M; Zeiss)
- Filter set (420/40 excitation, D660/40 emission and 475DCXR dichroic; Chroma Technology)
- IVIS bioluminescence imaging system (Xenogen)

REAGENT SETUP

Coelenterazine solution Dissolve coelenterazine at a concentration of 1 mg ml^{-1} in methanol and store in small aliquots at –80 °C. Alternatively, propylene glycol can be used as the solvent in place of methanol.

C6 rat glioma cells Culture C6 cells in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic mixture (Gibco). Before incubating cells with quantum dot conjugates, replace culture media with HBSS.

PROCEDURE

Bioconjugation of quantum dots

1| Mix the following reagents in the following order:
Quantum dots: 10 pmol (1.2 µl)
Luc8: 400 pmol (2.6 µl)
EDC: 40,000 pmol (0.6 µl)
Bring the final volume of the conjugation reaction to 20 µl using Tris buffer, pH 7.4.
▲ CRITICAL STEP EDC is hydroscopic and should be dissolved in water immediately before use. Its amount will affect the number of Luc8 molecules immobilized to the quantum dot. Buffers other than Tris, pH 7.4, can be used, such as 10 mM phosphate, pH 7.4; 10 mM borate, pH 7.4; or Tris, pH 8.0 (see ANTICIPATED RESULTS).

2| Vortex to mix and incubate the conjugation reaction solution for 1 h at 22 °C (room temperature).

3| Prewash the spin filter with 100 µl NTA buffer and remove the NTA buffer by centrifugation for 2 min at 4 °C and 5,200*g*. Flush the spin filter with 500 µl Tris buffer to remove any remaining NTA. We have found that using NTA buffer for the prewash reduces loss of quantum dots on the spin filter, especially when small amounts of quantum dots (a few picomoles) are used.

4| Transfer the conjugation reaction solution from Step 2 to the spin filter, add 100 µl Tris buffer and centrifuge for 1 min at 4 °C and 2,700*g*. Repeat the centrifugation for 1 min each time until all the liquid has entered the spin filter. Wash by adding 200 µl Tris buffer and centrifuging as above. Repeat for a total of four wash steps.
▲ CRITICAL STEP In Step 4, it is important to prevent the spin filter from drying out. Use short centrifuge times and check the filter between each spin. Also, high centrifuge speeds (>10,500*g*) can lead to aggregation of the quantum dots on the spin filter and should be avoided.
? TROUBLESHOOTING

5| Recover the conjugate by flushing the spin filter with 20 µl Tris buffer and collecting the conjugate. Store the conjugate on ice until use. This step concludes the preparation of the self-illuminating quantum dot conjugates. These can be characterized by agarose gel electrophoresis (A) and/or spectroscopic measurement of BRET emission (B). To attach cell-penetrating peptides to self-illuminating quantum dot conjugates to label live cells, continue at Step 6.
■ PAUSE POINT The collected conjugate can be stored at 4 °C in borate buffer before use and remains stable for 4 days. Longer storage periods have not been tested.

(A) Agarose gel electrophoresis

(i) Mix the conjugates with standard DNA loading dye (6×). Also prepare unconjugated quantum dots for a control lane.
(ii) Separate samples by 0.5% agarose gel electrophoresis for 20 min at 100 V in 0.5× TAE buffer.
(iii) Visualize the gel using a standard ultraviolet gel box.

BOX 2 | DETERMINATION OF THE BRET RATIO

The FRET efficiency (E) is typically measured using the relative fluorescence intensity of the donor in the absence (F_D) and presence (F_{DA}) of the acceptor: $E = 1 - F_{DA} / F_D$. In BRET, the energy transfer (the BRET signal) is often presented as the BRET ratio, defined by the acceptor emission relative to the donor emission[7]: the larger the BRET ratio, the more efficient the energy transfer. **Figure 2** shows a typical emission spectrum of the self-illuminating quantum dot conjugates containing two peaks, with one peak, at 480 nm, from Luc8, and the other peak, at 655 nm, from the quantum dots. As the two peaks are well separated, no overlap correction is needed. Area A is the integrated total emission (from 600 nm to 710 nm) from the quantum dots and area B is the integrated total emission from Luc8 (390–600 nm). The BRET efficiency is defined by the ratio of A to B. Depending on the coupling conditions, different BRET ratios may be obtained (see ANTICIPATED RESULTS), but they are generally greater than 1.

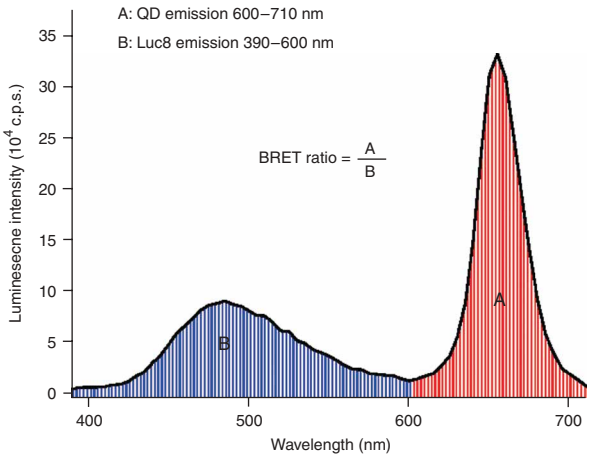

Figure 2 | Luminescence emission spectrum of self-illuminating quantum dot conjugates. Area A is the integrated total emission (600–710 nm) from the quantum dots; area B is the integrated total emission from Luc8 (390–600 nm); the BRET efficiency is defined by the ratio of A to B. c.p.s., counts per second.

(B) Spectroscopic measurement of BRET emission

(i) Dissolve 5 pmol of the conjugate in 60 µl Tris buffer and measure the fluorescence spectra of the quantum dot (480-nm excitation wavelength).

(ii) Add 1 µg coelenterazine to the solution and mix.

(iii) Measure the emission spectrum with the excitation light source of the fluorometer disabled or blocked by a piece of black paper. The wavelength range of the spectrum should cover both the Luc8 and quantum dot emissions. This typically means starting at 400 nm and ending at a wavelength 50 nm longer than the maximum emission wavelength of the quantum dots.

? TROUBLESHOOTING

(iv) Calculate the BRET emission and BRET ratio (**Box 2**).

Attaching cell-penetrating peptides to self-illuminating quantum dots

6| To 10 pmol of the quantum dot–Luc8 conjugate (from Step 5), add 10,000 pmol EDC, vortex and incubate for 5 min at room temperature.

7| Add 2 nmol R9 peptide, vortex and incubate for a further 30 min.

8| Apply the reaction mixture to a spin filter and proceed as in Step 4 of the bioconjugation protocol, with the exception that only one wash is necessary because of the small size of the R9 peptide.

9| Recover the conjugate by flushing the spin filter with 20 µl Tris buffer and collecting the eluate. Store the conjugate on ice until use.

■ **PAUSE POINT** The collected conjugate can be stored at 4 °C in borate buffer for a few days before use.

10| Evaluate the R9 peptide–quantum dot–Luc8 (R9-QD-Luc8) conjugates by agarose gel electrophoresis (Step 5A) and/or spectroscopic measurement (Step 5B).

Labeling live cells with self-illuminating quantum dot conjugates

11| Wash the C6 cells (grown in glass-bottomed dishes) twice with HBSS.

12| Incubate the cells for 1 h at 37 °C in 1 ml HBSS containing R9-QD-Luc8 conjugates (about 2–10 nM).

13| Remove excess R9-QD-Luc8 conjugates by washing the cells five times with HBSS.

14| To visualize the results, image with an inverted fluorescence microscope using the following filter set: 420/40 excitation, D660/40 emission and 475DCXR dichroic.

15| Collect cells using cell scrapers and suspend in 50 µl HBSS.

16| Add 2 µg coelenterazine and image with an IVIS bioluminescence imager both without a filter and with a filter appropriate for the quantum dot emission (acquisition time, 30 s per image).

● **TIMING**

Preparation of self-illuminating quantum dot conjugates takes about 1.5 h.

? TROUBLESHOOTING

In Step 4, occasionally we find the quantum dot conjugates leak into the filtrate. To solve this problem, use a fresh spin

TABLE 1 | BRET ratios of QD655-Luc8 conjugates made with various buffer solutions.

Buffer	BRET ratio	BRET intensity
Borate, pH 7.4	1.29	100%
Borate, pH 8.0	1.70	20%
Phosphate, pH 7.4	1.65	80%
Phosphate, pH 8.0	1.52	30%
Tris HCl, pH 7.4	1.70	110%
Tris HCl, pH 8.0	2.55	15%
PBS, pH 7.4	1.31	10%

The intensity of the BRET emission from conjugates prepared in borate buffer, pH 7.4 (top row), is used as the reference for comparison.

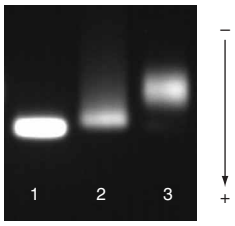

Figure 3 | Typical gel electrophoretogram of components from the conjugation of Luc8 to QD655. Lane 1, unconjugated QD655; lane 2, mixture of QD655 and the coupling reagent EDC; lane 3, purified QD655-Luc8 conjugates.

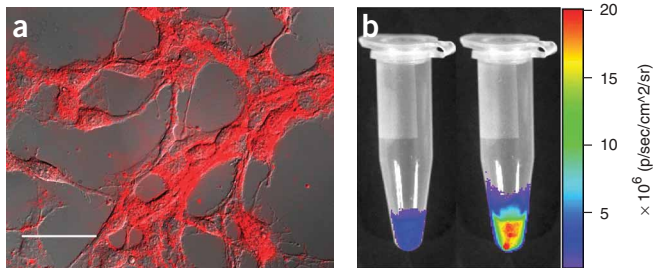

Figure 4 | Imaging of C6 glioma cells labeled with R9-QD655-Luc8. (**a**) Overlay of fluorescence and differential interference contrast images of C6 glioma cells labeled with R9-QD655-Luc8. This fluorescence image was obtained with the following filter set: excitation, 420/40; emission, D660/40; dichroic, 475DCXR. Scale bar, 50 μm. (**b**) Representative bioluminescence images of labeled cells acquired with a filter (575–650 nm; left) and without any filter (right). The intensity scale at right is from 1×10^6 to 2×10^7 photons per second per cm^2 per steradian.

filter and avoid long spin times. The Nanosep spin filters are constructed of polypropylene and contain a low protein-binding membrane for reduced nonspecific adsorption and high recovery. We find that the quality of spin filters varies between batches.

In Step 5B(iii), the BRET emission from the quantum dots may be lower than expected. To solve this problem, ensure that the fluorometer's emission correction file has been applied to the emission spectrum. Additionally, because of the consumption of coelenterazine, the bioluminescence emission gradually decreases to zero in about 15 min (to 50% in 150 s). A fast scan (within 10 s) is recommended to avoid decay of the signal.

ANTICIPATED RESULTS

The prepared quantum dot conjugates should have a typical luminescence emission spectrum like that in **Figure 2** (in **Box 2**), with two peaks: one at 480 nm from Luc8, and the other at the quantum dot emission maximum. Depending on the coupling buffers, the BRET efficiency can vary, as summarized in **Table 1**. During preparation, the typical yield of the conjugation reaction is about 60%, determined from the fluorescence emission.

The mobility of successfully coupled conjugates in the agarose gel will be different from that of the unconjugated quantum dots. **Figure 3** shows a typical gel of the conjugate of QD655 (quantum dots with maximum emission at 655 nm) and Luc8 (QD655-Luc8). **Figure 4** shows typical images of cells labeled with the self-illuminating quantum dot conjugate QD655-Luc8.

This protocol uses the quantum dots that emit at 655 nm as an example. In general, similar results can be obtained with the quantum dots emitting at other wavelengths. However, the quantum dots that emit at a wavelength much longer than 480 nm are preferred to avoid spectral overlap with the donor Luc8 emission.

The quantum dots used here are commercially available from Invitrogen and are coated with a layer of amphiphilic polymers presenting carboxylate functional groups. Other types of quantum dots may be used as well for the preparation of the self-illuminating quantum dot conjugates. However, the BRET ratio may vary. For example, streptavidin-coated quantum dots lead to a smaller value for the BRET ratio[4].

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AUTHOR CONTRIBUTIONS M.-K.S. did the conjugation and imaging experiments and prepared figures; A.M.L. prepared Luc8 and wrote the manuscript; S.S.G. provided discussions; and J.R. designed and coordinated research, analyzed data and wrote the manuscript.

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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