

Electronic Supplementary Material (ESI)

BRET-based dual-color (visible/near-infrared) molecular imaging using a quantum dot/EGFP-luciferase conjugate

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1. Materials

Selenium (Se, powder, 99.999%) and Tellurium (Te, shot, 1-2 mm, 99.99%) were purchased from Sigma-Aldrich. Cadmium 2,4-pentanedionate was purchased from Alfa Aesar. n-Octadecylphosphonic acid was purchased from PCI Synthesis. Trioctylphosphine oxide (TOPO), trioctylphosphine (TOP), tributylphosphine (TBP), and hexadecylamine (HDA, 90%) were purchased from Tokyo Chemical Industry (Japan). Sulfur (S, crystalline, 99.9999%), glutathione (GSH, reduced form) and potassium t-butoxide were purchased from Wako Chemicals (Japan). Coelenterazine (CTZ) was purchased from Wako Chemicals (Japan). Herceptin (anti-HER2 monoclonal antibody) and Erbitux (anti-EGFR antibody) were purchased from Chugai Pharmaceutical Co., Ltd, and Merck Serono Co., Ltd, respectively. Other organic solvents were of analytical reagent grades.

2. Synthesis of CdSeTe/CdS QDs

The synthesis of CdSeTe/CdS QDs is reported elsewhere.^{1,2} A typical procedure is as follows: a Se-Te stock solution was prepared by dissolving Se (24 mg, 0.3 mmol) and Te (13 mg, 0.1 mmol) in TBP (1 mL) at room temperature. A Cd-S stock solution was prepared by adding sulfur (40 mg, 1.25 mmol) to TBP (10 mL). After sulfur was completely dissolved, the solution was cooled to room temperature. Then, cadmium 2,4-pentanedionate (388 mg, 1.25 mmol) was added to the sulfur-TBP solution, and the solution was warmed at 100 °C to dissolve cadmium 2,4-pentanedionate. The Cd-S stock solution was stored under an argon atmosphere at room temperature.

A mixture of cadmium 2,4-pentanedionate (150 mg, 0.48 mmol), ODPA (300 mg, 0.90 mmol), TOPO (1 g), HDA (3 g), and TOP (0.5 mL) were loaded into a 25 mL three-necked flask and heated to 330 °C under an argon atmosphere. At this temperature, 0.5 mL of a Se-Te stock solution was quickly injected by using a syringe,

which caused an immediate color change in solution from colorless to brown. By monitoring the QD fluorescence spectra, the formation of QDs (ca. 800 nm emission) was checked. When the desired QDs were formed, the solution was cooled to 60 °C and chloroform (10 mL) was added to the solution. The QDs were precipitated by addition of methanol, and the QD precipitates were separated by centrifugation. The resulting QD precipitates and HDA (3 g) were loaded into a 25 mL three-necked flask and heated to 250 °C. At this temperature, the formation of the CdS shell was performed. The addition of a Cd-S stock solution (0.25 mL) resulted in the formation of CdSeTe/CdS QDs that emit at ca. 830 nm. Then the QD solution was cooled to 80 °C, and chloroform (10 mL) was added. The QDs were precipitated by addition of methanol and separated by centrifugation. To remove excess TOPO and HDA, the QDs were dissolved in chloroform again and precipitated by the addition of methanol. This procedure was repeated three times. The resulting QDs were dissolved in chloroform (20 mL) and stored in the dark.

GSH coating: an aqueous solution (1 mL) of GSH (100 mg/mL,) was slowly added to a tetrahydrofuran solution (1 μM, 2 mL) of CdSeTe/CdS QDs at room temperature under sonication. The precipitates of QDs were separated by centrifugation. To the QD precipitates, an aqueous solution of potassium *t*-butoxide (20 mg/mL, 2 mL) was added under stirring. The solution was sonicated for 5 min and filtered through a 0.45 μm membrane filter. Excess GSH and potassium *t*-butoxide were removed by dialysis using a 10 mM Na₂CO₃ aqueous solution. The resulting GSH-QDs was preserved at 4 °C.

3. Protein Synthesis

His-EGFP-GB1 and His-RLuc-GB1 protein

The syntheses of His-EGFP-GB1 and His-RLuc-GB1 protein were reported elsewhere.^{2,3} For the His-EGFP-GB1 protein, the EGFP sequence and the Protein G B1 sequence were amplified by PCR from pEGFP-C1 plasmid (Clontech) and pET His6 protein G TEV LIC cloning vector (2P-T), Addgene plasmid 29713, which was a gift from Scott Gradia. The PCR fragments were fused with pRSET plasmid (ThermoFisher) by using the InFusion HD cloning kit (Clontech). The constructed pRSET-EGFP-GB1 plasmid was transformed into *E.coli* KRX competent cells (Promega), cultured in large-scale culture, and purified by Ni Sepharose 6 Fast Flow (GE Healthcare) using the method described below. For the His-RLuc-GB1 protein, the RLuc sequence was amplified by PCR from pUC19-RLuc plasmid (NanoLight Technologies). The 6xHistidine sequence was included in the oligonucleotides used

as primers. The PCR fragments were fused with pGEX-6P-1 plasmid (GE Healthcare) by using InFusion HD cloning kit (Clontech). The constructed pGEX-His-RLuc-GB1 plasmid was transformed into *E.coli* KRX competent cells (Promega), cultured in large-scale culture, and attached to Glutathione Sepharose 4B (GE Healthcare). The GST tag was cut and purified by Turbo 3C Protease (Accelagen) using the method described below.

His-RLuc-EGFP-GB1 and His-EGFP-RLuc-GB1 protein

For the His-RLuc-EGFP-GB1 protein, the RLuc sequence was inserted at the N-terminus of the EGFP sequence of the pRSET-EGFP-GB1 plasmid.² For the His-EGFP-RLuc-GB1 protein, the RLuc sequence was inserted at the C-terminus of the EGFP sequence of the pRSET-EGFP-GB1 plasmid. The RLuc sequence was amplified by PCR from pUC19-RLuc plasmid (NanoLight Technologies) and inserted into the pRSET-EGFP-GB1 plasmid by using InFusion HD cloning kit (Clontech). pRSET-RLuc-EGFP-GB1 plasmid and pRSET-EGFP-RLuc-GB1 plasmid were transformed respectively into *E.coli* KRX competent cells (Promega). The transformed cells were grown in 200 mL of LB media with ampicillin (100 µg/mL) at 37°C on shaking table, until they approached to 0.6 of O.D. 600 (absorbance). To induce production of the targeted protein, isopropyl β-D-1-thiogalactopyranoside (0.2 mM) and L-Rhamnose (0.1 %) were added to the LB media, and then incubated with shaking gently for 16 h at 18°C. The cells were harvested by centrifugation at 5,000 ×g for 10 min. The cells were resuspended with 5 mL of binding buffer (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH=8.0). Before cell lysis, EDTA-free protease inhibitor cocktail tablets (Complete, Roche) were added. The solution was sonicated on ice using 10 s bursts at middle-intensity with a 10 s cooling period between each burst. The lysate was clarified by centrifugation at 20,000 ×g for 30 min to eliminate cell debris. The next step was the purification by Ni Sepharose 6 Fast Flow (GE Healthcare). 1mL of Sepharose media equilibrated with binding buffer was added to each 5mL of lysed sample and incubated with gentle agitation at 4°C for 30min. After the solution was transferred to an empty column, the column was washed with five column volumes of binding buffer. Lastly, His-RLuc-EGFP-GB1 and His-EGFP-RLuc-GB1 proteins were drained from the column by the addition of elution buffer (50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH = 8.0). Lastly the eluted fractions were buffer-exchanged using a gel filtration column (PD-10, GE Healthcare).

RLuc-His-EGFP-GB1 and RLuc-GB1 proteins

The pGEX-RLuc-His-EGFP-GB1 plasmid and the pGEX-RLuc-GB1 plasmid were each constructed and the GST tag was cleaved after protein expression. The PCR fragments of each sequences were inserted into the pGEX-6P-1 plasmid (GE Healthcare) by using InFusion HD cloning kit (Clontech). The pGEX-RLuc-His-EGFP-GB1 plasmid and pGEX-RLuc-GB1 plasmid were transformed respectively into *E.coli* KRX competent cells (Promega). The transformants were cultured and extracted as described above to obtain a lysate in which the intended protein was expressed. In this case PBS buffer (phosphate buffered saline, pH = 7.4) was used as binding buffer. The next step was the purification by Glutathione Sepharose 4B (GE Healthcare). 1.5 mL of Sepharose media equilibrated with binding buffer was added to each 5mL of lysed sample and incubated with gentle agitation at 4°C for 1hr. The Sepharose media was then washed with Turbo 3C Protease buffer, followed by digestion of the GST-tag by overnight incubation at 4°C with Turbo 3C Protease (Accelagen) in Turbo 3C Protease buffer. Lastly the RLuc-His-EGFP-GB1 and RLuc-GB1 proteins were buffer-exchanged using a gel filtration column (PD-10, GE Healthcare).

4. Characterization of QDs and proteins

The morphologies of QDs were observed by TEM using a Hitachi H-800 microscope operating at an acceleration voltage of 200 kV. TEM sample (1 μ M QDs in PBS) was prepared by dropping the sample solution onto a copper grid.

The diffusion times of QDs, QD-His-EGFP-RLuc-GB1, and its antibody conjugate were determined by fluorescence correlation spectroscopy. Fluorescence autocorrelation curves were measured on a compact FCS system (C9413-01MOD, Hamamatsu Photonics, Japan) at excitation of 473 nm using a LD pumped solid-state laser. The size of pinhole was 25 μ m and the spectral range of detection wavelengths was 500-900 nm. For the determination of the concentration of GSH-QDs, the number of QD particles in a 10 μ L solution was measured by using FCS, and the QD concentration was estimated by using a 20 nM solution of Rhodamine 6G as a reference.

The purity of recombinant proteins was checked by SDS polyacrylamide gel electrophoresis. The proteins were run on a 10 % polyacrylamide gel in Tris-glycine-SDS buffer, 200 V for 20 min and stained with Coomassie Brilliant Blue (CBB Stain One Super, Nacalai Tesque). The sizes of His-RLuc-EGFP-GB1, RLuc-His-EGFP-GB1, His-EGFP-RLuc-GB1, RLuc-GB1 and His-RLuc-GB1 calculated from the

amino acid sequence were 73.6 kDa, 72.6 kDa, 73.6kDa, 44.7kDa, and 45.5 kDa, respectively. Precision Plus Protein Standard (BIO-RAD) was used as a size marker.

QDs and protein conjugates were run on 1% agarose gel in Tris-Acetate buffer (pH 8.0), 100 V for 15min. Fluorescence emissions of bands were monitored at 830 nm.

5. Flow cytometric analysis

The cells (KPL-4, A431) were collected by trypsinization and resuspended in PBS. The cell suspensions were then incubated without or with 1 μ M of Herceptin, Erbitux or normal human IgG for 10 minutes at 37 °C. Next, the cell suspensions were washed once with PBS and incubated with probe (50 nM QDs-His-EGFP-RLuc-GB1) for 10 minutes at 37 °C. The cell suspensions were washed 5 times with PBS and resuspended in PBS. The cell suspensions were then passed through a 40 μ m cell strainer and analyzed with a flow cytometer (MACS Quant Analyzer, Miltenyi Biotec Inc.). Fluorescence of EGFP was collected through a FL2 (FITC) filter (Ex = 488 nm, Em = 525 \pm 25 nm). Fluorescence of the QDs was collected through a FL7 (APC-Cy7) filter (Ex= 635 nm, Em= 750 nm LP).

6. Western blotting analysis

The cells were lysed with RIPA buffer (Nacalai Tesque) for 15 min on ice and centrifuged at 10,000 x g for 10 min at 4°C to remove insoluble material. Protein concentration was determined by the Quick Start Bradford 1x Dye Reagent (BIORAD). The cell lysates (10 μ g / Lane) were separated by electrophoresis on 7.5 % Extra PAGE One Precast Gel (Nacalai Tesque) at 200 V for 45 min. The protein was transferred by Trans-Blot Turbo Transfer System (BIO-RAD). The membranes to which the protein had been transferred were cut up and down at 75kDa of the molecular weight marker. The membranes were then incubated in 5 % skim milk for 1 hour at room temperature to prevent nonspecific binding. Then, the membranes were incubated with a primary antibody in 5 % skim milk for overnight at 4 °C. primary antibodies used for the membranes more than 75 kDa were anti EGFR A-10 (Santa Cruz Biotechnology, sc-373746, 1:100) or anti HER2 3B5 (Santa Cruz Biotechnology, sc-33684, 1:5000). Primary antibody used for the membranes of 75 kDa or less were anti beta-actin C4 (Santa Cruz Biotechnology, sc-47778, 1:200). After washing with TBST, the membranes were incubated with a secondary antibody

in 5 % skim milk for 1 hour at room temperature. secondary antibody used were horseradish peroxidase conjugated (Millipore, # 12-349, 1: 2000). After washing with TBST, bands were visualized by treating the membranes with Luminata Forte Western HRP Substrate (Millipore) according to manufacturer's instructions and detecting the chemluminescence with MS FX II (Bruker).

7. Bioluminescence and fluorescence measurements

Fluorescence and bioluminescence spectra were measured with a photonic-multichannel analyser (C10027, Hamamatsu Photonics, Japan). For the fluorescence measurement, a 150 W-Xenon lamp was used as an excitation light source at 488 nm. For the bioluminescence measurement, 10 μ L of CTZ (1 mg/mL) was added to the aqueous solution (1 mL) of RLuc recombinant proteins (20 nM). Fluorescence decay curves for EGFP were measured by excitation at 480 nm using a time-correlated single-photon counting (Horiba Fluoro Cube).

8. BRET imaging

Cell pellets: KPL4 and A431 cells (0.5×10^6 cells / well) were incubated with 1 μ M of antibody (none, Herceptin, Erbitux or normal human IgG) for 10 min at 37 °C. After the cells were washed with PBS, incubated with 50nM of probe (QD-His-EGFP-RLuc-GB1) for 10 min at 37 °C. The cells were finally washed five times with PBS. Immediately before the detection of BRET emission, CTZ was added to the cells at a final concentration of 50 μ M. BRET emission images (at 830 ± 20 nm and 530 ± 20 nm) were taken by using an *in vivo* imaging system (Bruker, MS FX PRO). Exposure time was set to 10 min.

Cultured cells: Cellular imaging was performed using a bioluminescence microscope, LV200 (Olympus, Japan). KPL-4 cells were seeded to glass-bottom, collagen-coated dishes (D11134H, Matsunami Glass, Japan) and incubated in Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque) with 10% fetal bovine serum (FBS, Sigma) overnight at 37 °C. Anti HER2 antibody (1 μ M) was added to the cell and incubated for 10 min at 37 °C. After the cells were washed twice with PBS, the probe (50 nM QDs-His-EGFP-RLuc-GB1) was added and incubated for 10 min at 37 °C. Then, the cells were washed with PBS fourth times and filled

with PBS. The cells were observed with optical filters (495-540 nm BP for EGFP emission and a 715 nm LP for QD emission). Exposure time was 3min.

9. Cell viability

HeLa cells were incubated with QDs (0-100 nM, PBS), and QDs-His-EGFP-RLuc-GB1 (0-100 nM, PBS) for 6, 24 and 48 h. MTT assay was performed according to the procedure of a MTT Cell Count Kit (Nacalai Tesque). The MTT reagent was added to each well and the cells were incubated for 2 h at 37 °C. Then, the STOP solution was added to stop the reaction. According to the instruction of the Kit, the absorbance at 570 nm and 650 nm of solubilized MTT formazan products were measured with a Microplate Spectrophotometer (Multiskan GO; ThermoFisher).

References

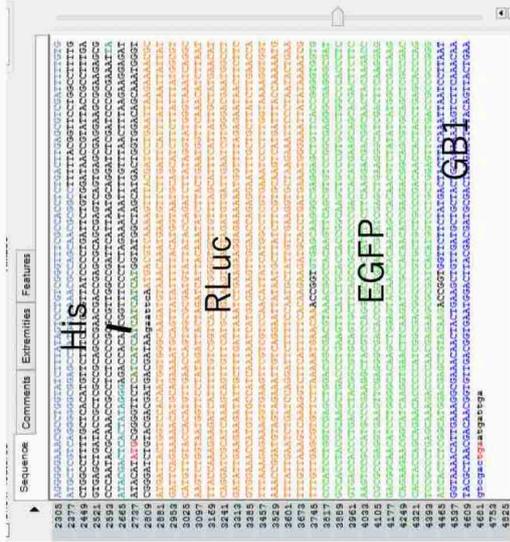
1. S. Tsuboi and T. Jin, *ChemBioChem*, 2017, **18**, 2231-2235.
2. S. Tsuboi and T. Jin, *ChemBioChem*, 2019, **20**, 568-575.
3. S. Tsuboi and T. Jin, *Bioconjugate Chem.* 2018, **29**, 1466-1474.

Supporting Figures



Fig. S1 SDS-PAGE of RLuc recombinant proteins. 1): RLuc-GB1 (44.7 kDa), 2) His-RLuc-GB1 (45.5 kDa), 3) His-RLuc-EGFP-GB1 (73.6 kDa), 4) RLuc-His-EGFP-GB1 (72.6 kDa), and 5) His-EGFP-RLuc-GB1 (73.6 kDa).

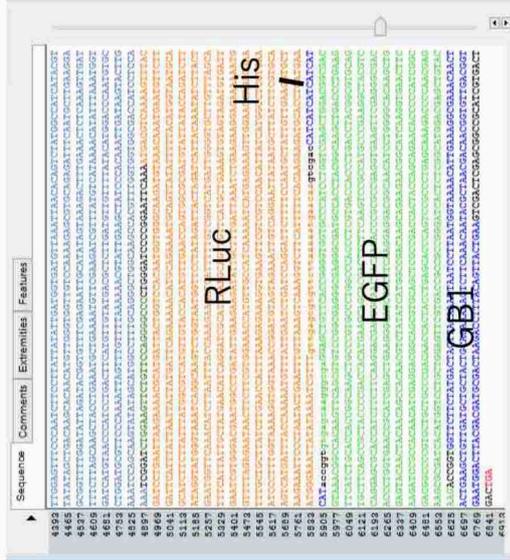
① His-RLuc-EGFP-GB1



His ↔ RLuc: 23aa (2.47kDa).

RLuc ↔ EGFP: 2aa (0.16kDa)

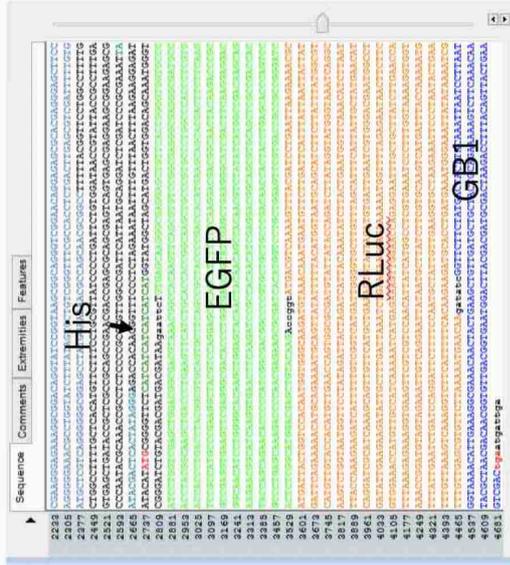
② RLuc-His-EGFP-GB1



RLuc ↔ EGFP: 9aa (1.19kDa)

His ↔ RLuc: 23aa (2.47kDa)

③ His-EGFP-RLuc-GB1



His ↔ EGFP: 23 aa (2.47 kDa)

RLuc ↔ EGFP : 2aa (0.16kDa)

Fig. S2. DNA sequence for 1) His-RLuc-EGFP-GB1, 2) RLuc-His-EGFP-GB1, and 3) His-EGFP-RLuc-GB1.

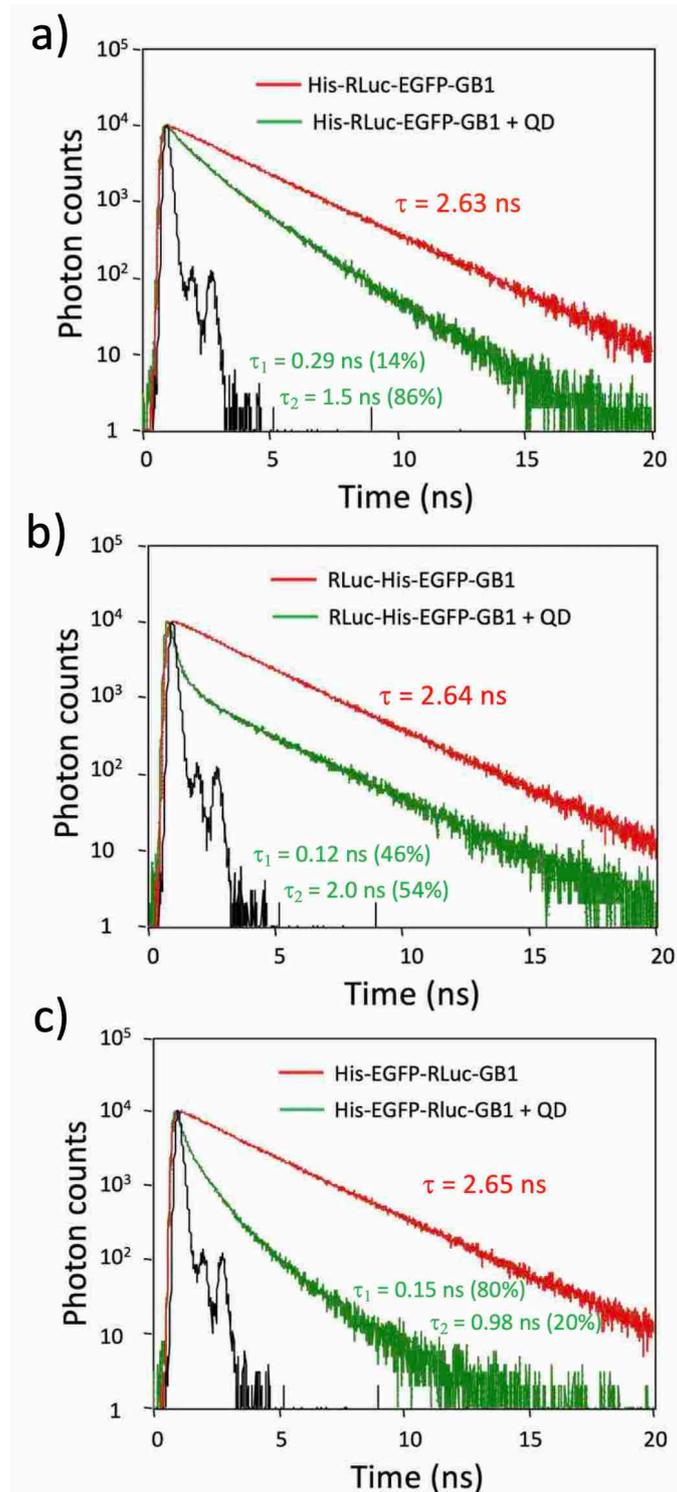


Fig. S3 Fluorescence decay curves of His-RLuc-EGFP-GB1, RLuc-His-EGFP-GB1, and His-EGFP-RLuc-GB1 in the absence and presence of QDs. The molar ratio of QD/protein was 10. Excitation: 484 nm. Emission: 515 nm.

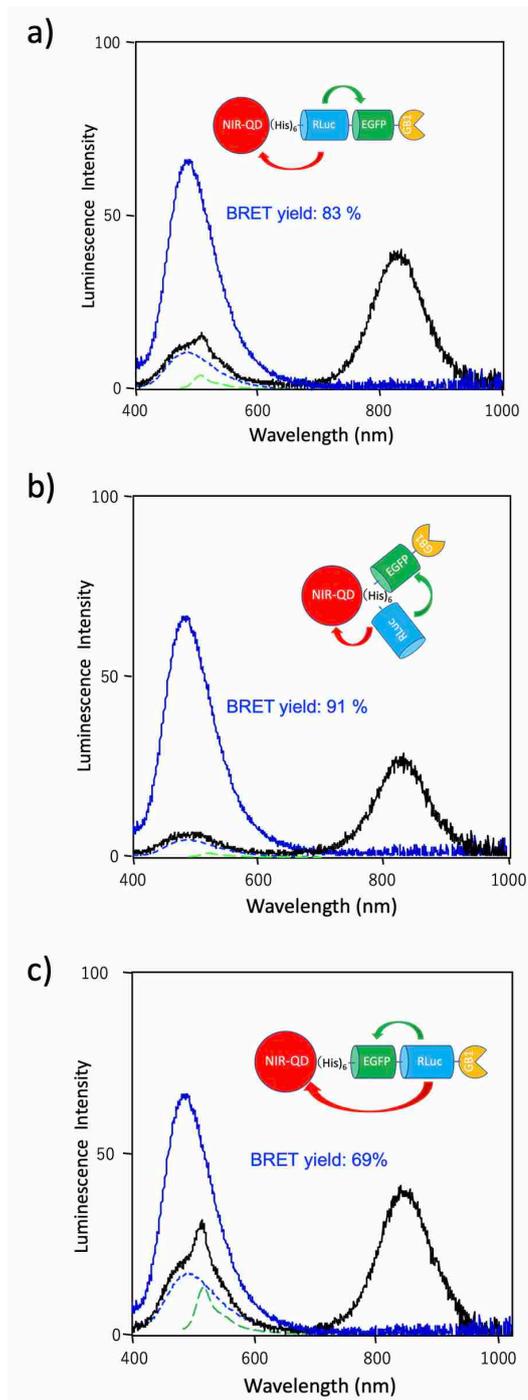


Fig. S4 Bioluminescence spectra (black lines) for (a) His-RLuc-EGFP-GB1, (b) RLuc-His-EGFP-GB1, and (c) His-EGFP-RLuc-GB1. Bioluminescence spectrum for a donor (CTZ/His-RLuc-GB1) is shown as blue lines. Blue dotted line and green dotted lines are show the spectral contribution from CTZ and EGFP. BRET yields were estimated for the following equation: $E = 1 - (I_{DA}/I_D) \times 100\%$, where I_{DA} and I_D represent the emission intensities (blue solid and dotted lines) of CTZ in the presence and absence of acceptor, respectively.

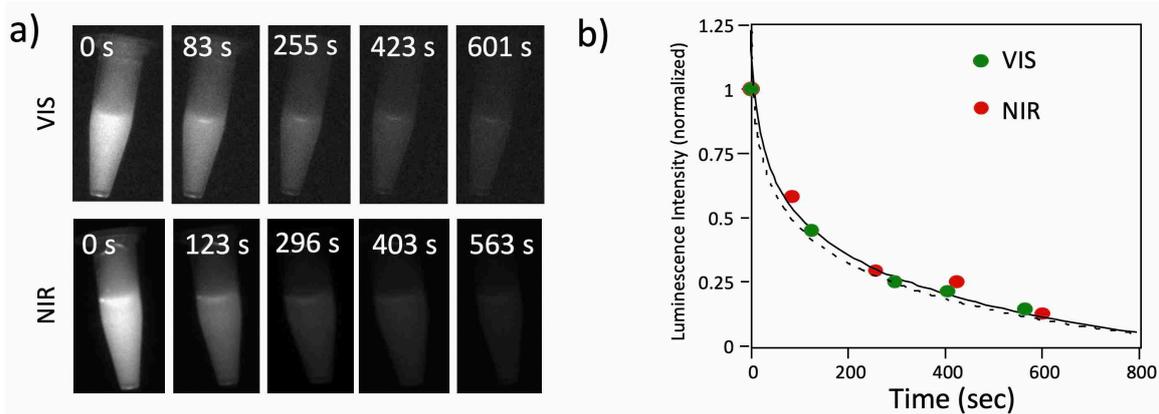


Fig. S5 (a) BRET images of an aqueous solution of QD-His-EGFP-RLuc-GB1 ([QD] = 20nM, [His-EGFP-RLuc-GB1] = 200 nM), where 20 μ L of CTZ solution (20% EtOH, 80% water) was added. (b) Time-course of the intensities of the BRET images at visible (830 ± 20 nm) and NIR (>750nm) region.

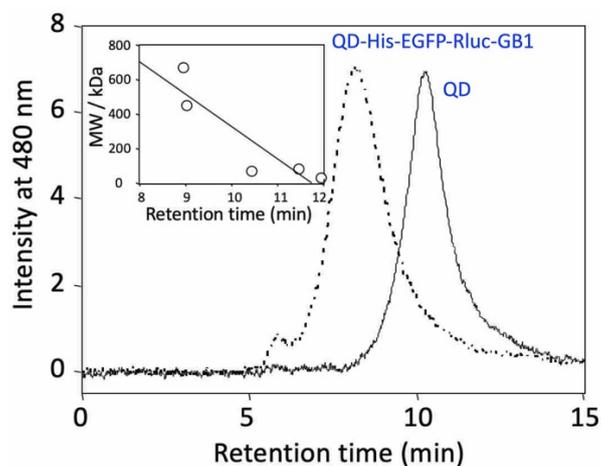


Fig. S6 Size-exclusion HPLC for QDs and His-EGFP-RLuc-GB1 conjugated QDs (QD-His-EGFP-RLuc-GB1). The inset shows a plot of the retention times of standard proteins. Molecular weight: 670 kDa for thyroglobulin, 450 kDa for ferritin, 80 kDa transferrin, 66 kDa for bovine serum albumin, and 30.8 kDa for EGFP.

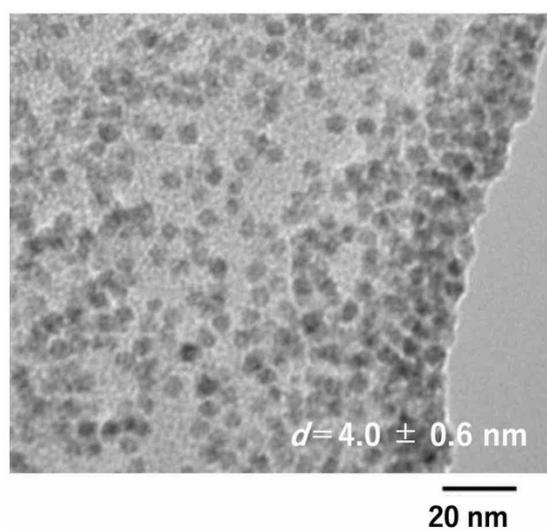


Fig. S7 Transmission electron microscope image of His-EGFP-RLuc-GB1 conjugated QDs (QD-His-EGFP-RLuc-GB1). The mean diameter of the QDs was 4.0 ± 0.6 nm.

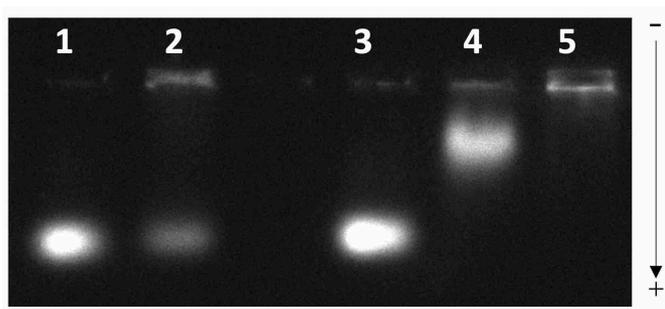
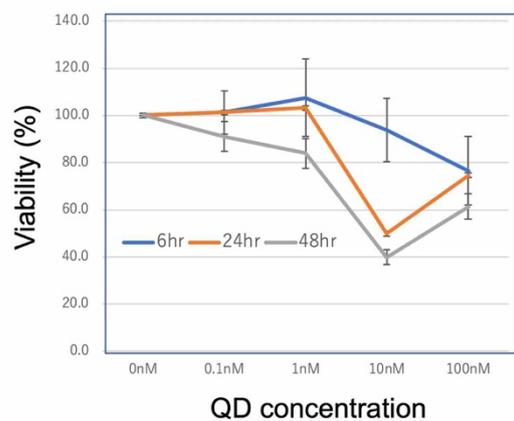


Fig. S8 Agarose gel electrophoresis of 1) & 3) QDs, 2) QDs + antibody (Herceptin), 4) QDs + His-EGFP-RLuc-GB1, and 5) QDs + His-EGFP-RLuc-GB1 + antibody (Herceptin). The molar ratios of antibody/QDs, and His-EGFP-RLuc-GB1/QDs were 5 and 10, respectively.

a) QD



b) QD-His-EGFP-RLuc-GB1

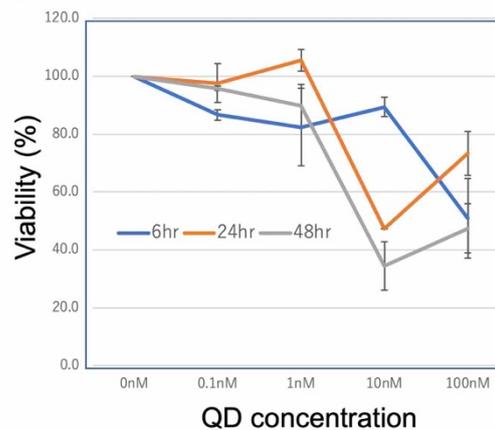


Fig. S9 Viability of HeLa cells in 6, 24, 48 h after treating with 0-100 nM QDs and QD-His-EGFP-RLuc-GB1.