Electronic Supplementary Information

Bioluminescence resonance energy transfer coupled near-infrared quantum dots using GST-tagged luciferase for *in vivo* imaging

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Materials and methods

1. Reagents

Cadmium oxide (CdO, 99.99 %), Selenium (Se, powder, 99. 999 %) and Tellurium (Te, shot, 1-2 mm, 99.99 %) were purchased from Sigma-Aldrich. Trioctylphosphine oxide (TOPO), trioctylphosphine (TOP), tributylphosphine (TBP), hexadecylamine (HDA, 90 %) and stearic acid were purchased from Tokyo Chemical Industry (Japan). Sulfur (S, crystalline, 99.9999 %), glutathione (GSH, reduced form) and potassium *t*-butoxide were purchased from Wako (Japan). Other organic solvents were of analytical reagent grades. 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 as follows: sulfur (40 mg, 1.25 mmol) was added to TOP (10 mL) and heated at 100 ºC. After sulfur was completely dissolved, the solution was cooled to room temperature. A mixture of CdO (160 mg, 1.25 mmol) and stearic acid (2 g) was loaded into a 25mL three-necked flask and heated at 300 ºC. After CdO was completely dissolved, the solution was cooled to 80 ºC, and a sulfur-TOP solution was added to the Cd-TOP solution under stirring. The Cd-S stock solution was stored under an argon atmosphere at room temperature. The plamid (pRmLUC) was purchased from NanoLight Technology. Coelenterazine(h) was purchased from Sigma-Aldrich. Hairless mice (HOS: HR-1) were purchased from Japan SLC, Inc.

2. Synthesis of CdSeTe/CdS QDs

NIR-emitting CdSeTe/CdS QDs were synthesized by the previously reported method.[1,2] A typical procedure is as follows: a mixture of CdO (25 mg, 0.2 mmol) and stearic acid (200 mg) was loaded into a 25 mL of three-necked flask and heated at 300 ºC. After CdO was completely dissolved, the mixture cooled to room temperature. Then TOPO (2 g) and HDA (2 g) were added to the flask and heated at 300 ºC. At this temperature, 0.25 mL of a Se-Te stock solution was quickly injected by using a syringe. Immediately, the solution changed from colorless to deeply colored. By monitoring of the growth of QDs with their fluorescence spectra, the formation of the QDs (ca. 780 nm emission) was checked. When the

desired QDs were formed, the solution was cooled to 200 ºC. At this temperature, the formation of CdS shell was performed. Addition of a Cd-S stock solution (0.15-0.25 mL) resulted in the formation of CdSeTe/CdS QDs that emit at 800 nm. Then, the QD solution was cooled to 60 ºC and chloroform (20 mL) was added. The QDs were precipitated by addition of methanol and separated by centrifugation. To remove excess TOPO, HDA and stearic acid, the QDs were dissolved in chloroform again and precipitated by addition of methanol. This procedure was repeated three times. The resulting QDs were dissolved in tetrahydrofuran (20 mL) and stored in dark place.

3. Preparation of GSH-coated CdSeTe/CdS QDs (GSH-QDs)

An aqueous solution of GSH (100 mg/mL, 0.2 mL) was slowly added to the THF solution (0.5 mL) of CdSeTe/CdS QDs (1 μM) at room temperature and the mixture was heated to 60 ºC. The resulting precipitates of QDs were separated by centrifugation. To the QD precipitates, water (1 mL) was added and then potassium *t*-butoxide (5 mg) was slowly added. The mixture was sonicated for 5 min and filtered through a 0.25 μm membrane filter. Excess GSH and potassium *t*-butoxide were removed by dialysis using a 10 mM PBS buffer (pH = 7.4). The quantum yield of the GSH-QDs (excitation at 488 nm) was 0.31 in PBS.

4. Expression and purification of GST-tagged luciferase (GST-RLuc)

Polymerase Chain Reaction (PCR) was used to obtain a gene encoding *Renilla* Luciferase (RLuc) from pRmRLUC based on pUC19 plasmid. The PCR fragments was attached restriction enzyme site of 5'-EcoRI and 3'- SalI at each end. After purification of PCR products, they were digested and ligated with restriction enzyme mentioned above to insert them into pGEX-6P-1 plasmid (GE Healthcare). The pGEX-GST-RLuc plasmid was transformed into *E.coli* KRX competent cells (Promega) to produce efficient cloning and high protein yields.

Next, for large-scale cultures, the transformed cells were grown in 2 L of LB media with ampicillin (1mM) at 37 °C on shaking table, until they approached to 0.5 of O.D. 600 (absorbance). To induce production of the targeted proteins efficiently, isopropyl β-D-1-thiogalactopyranoside (10 mM) and *L*-Rhamnose (1 %) were added to the LB media, and then incubated with shaking gently for 12 hr at 18 °C. The cell pellet of 50 mL of LB cultured media was harvested by centrifugation at 8,000 rpm for 10 min. The cells were lysed and suspended with 2 mL of binding buffer (10 mM Na_2HPO_4, 1.8 mM HH_2PO_4, 140 mM NaCl and 2.7 mM KCl in pH=7.3 PBS, GE Healthcare). Before incubating on ice for 30 min, both lysozyme (2 μg, Wako) and *p*-APMSF (1 mM, Wako) as a protease inhibitor were added. The solution was sonicated on ice using ten 10 s bursts at middle-intensity with a 10 s cooling period

between each burst. The lysate was clarified by high-speed centrifugation at 50,000 rpm for 20 min to eliminate their contaminants.

The next step was the purification of GST-tagged luciferase (GST-RLuc) by Glutathione Sepharose 4B (GE Healthcare) which was designed for purification of recombinant derivatives of glutathione-s-transferase. Two mL of Sepharose media equilibrated with binding buffer was added to each 100 mL of lysed sample, and incubated with gentle agitation at room temperature for 30 min. After the solution was transferred to a disposable column, it was washed with binding buffer three times. Lastly the GST-RLuc was drained from the column by the addition of elution buffer (10 mM glutathione in 50 mM Tris-HCL, pH=8.0). The eluted fractions were further purified by gel filtration column (PD-10 columns, GE Healthcare). Likewise, RLuc alone was prepared with PreScission Protease (GE Healthcare) by means of cleavage of eluted GST-RLuc.

5. Purification of the complex of GST-Rluc and GSH-QDs

An aqueous solution of 0.25 mL of GSH-QDs (1 μM) was mixed with an aqueous solution of 0.25 mL of GST-RLuc (500 μg/mL), and the solution was incubated for 30 min. Then the mixture solution was passed through a Nap™-5 column (GE Healthcare) with PBS as an eluent. The solution of the complex of GST-Rluc and GSH-QDs was centrifuged at 15,000 g for 5 min to remove aggregated QDs. The complex solution was kept in a refrigerator at 5 ºC.

6. Dynamic light scattering (DLS) measurements

The hydrodynamic diameters of all QDs were measured by using dynamic light scattering (DLS) on a Zetasizer Nano-ZS (Malvern Instruments, Inc) with a He/Ne laser at 633 nm. The DLS histograms of GSH-QDs before and after adding GST-RLuc in PBS were obtained at 25 ºC.

7. Fluorescence correlation spectroscopy (FCS) measurements

Fluorescence autocorrelation curves for all QDs 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 μm 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 20 μL solution was measured by using FCS, and the QD concentration was estimated by using a 20 nM solution of Rhodamine 6G as a reference. Assuming the Stokes-Einstein equation ($D = k_BT/6\pi\eta r$) for the diffusion of Rhodamine 6G and QDs in solution, the hydrodynamic diameter of GSH-QDs and GSH-QDs complexed

with GST-RLuc was estimated as 8.5 nm and 17 nm, respectively. For all measurements, pH of the aqueous solution of QDs was set to be 7.4 with PBS.

8. Gel electrophoresis

SDS-PAGE: GST-RLuc and RLuc proteins (100 μg/mL, each) were run on a 12 % polyacrylamide gel (mini-Protean TGX Gels, BIO-RAD) in tris-glycine-SDS buffer, 200 V for 30 min and stained with Coomassie Blue (Quick-CBB PLUS, Wako). A size marker (Precision Plus Protein Standard, BIO-RAD) was used to compare between GST-RLuc and RLuc. The expected size for GST-RLuc and RLuc were 64 kDa and 36 kDa, respectively. Agarose Gel Electrophoresis: GSH-QD and the mixture of GSH-QD and GST-RLuc（50 μg/mL） were run on 1% agarose gel in PBS (pH=8.0), 100 V for 30min and observed under UV light after stained with ethidium bromide.

9. Size-exclusion column chromatography

Size-exclusion column chromatography with a HPLC system (ELITE LaChrom, HITACHI) was performed by using a TSK-gel G4000SW column (7.8 mm × 30 cm, TOSOH). A mobile phase was 20 mM PBS and flow rate was adjusted to 1mL/min. To evaluate the apparent molecular size of GSH-QD and the complex of GSH-QD・GST-RLuc, thyroglobulin (670 kDa), ferritin (450 kDa), bovine serum albumin (66 kDa) and transferrin (80 kDa) were used as standard proteins. HPLC chromatographs were obtained by injection of 20 μL of sample solutions at room temperature.

10. Luminescence and fluorescence spectral measurements

Luminescence and fluorescence spectra for RLuc + coelenterazine, the complex of GSH-QD・GST-RLuc + coelenterazine and GSH-QDs were measured with a photonic-multichannel analyzer (C10027, Hamamatsu Photonics, Japan). In the case of fluorescence measurement, a 150 W Xenon lamp was used as an excitation light source at 488 nm. For luminescence measurement, 0.1 mL of coelenterazine (10 μM) was added to the aqueous solution of RLuc or GSH-QD・GST-RLuc complex (1mL, 0.1 μM).

11. *In vivo* optical imaging

Bright field and fluorescence images were taken with an upright microscope (MVX, Olympus) by using 0.63 × objective lens (N.A. 0.25) and cooled EMCCD camera (iXon3, Andor). Two emission filters (520 ± 7.5 nm and 809 ± 40.5 nm, Semrock) were used for luminescence and BRET NIR-fluorescence.

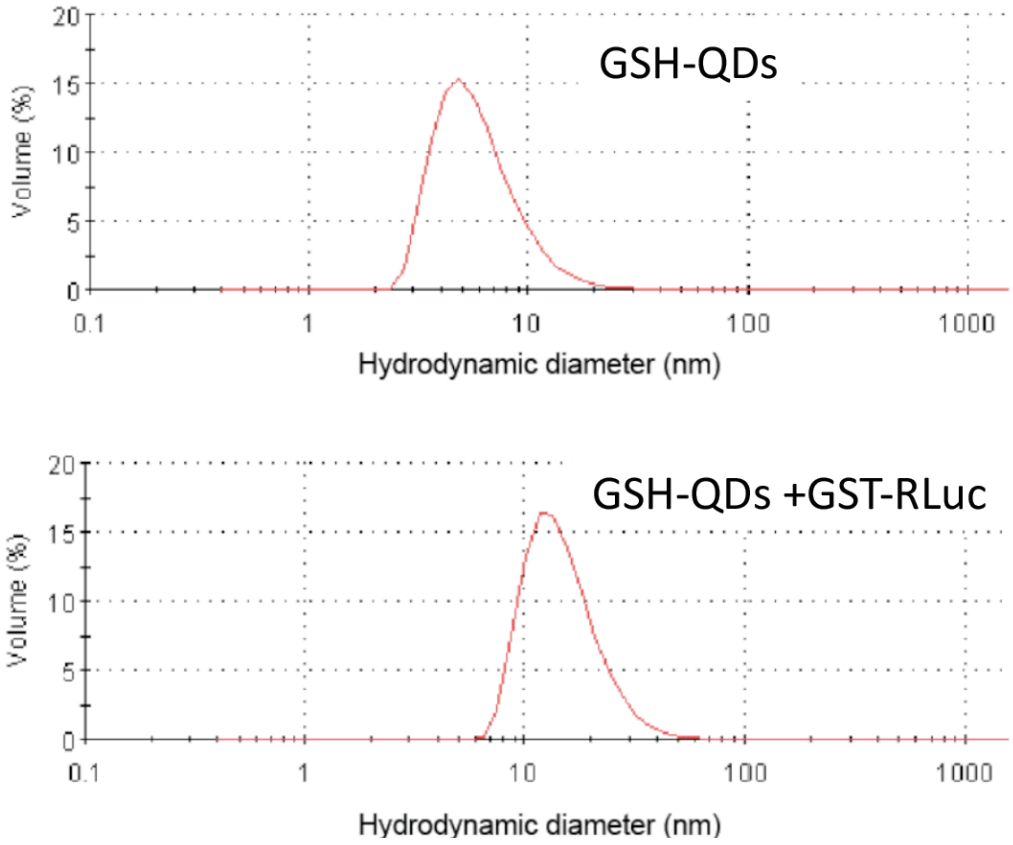

Figure S1 Hydrodynamic diameter of GSH-QDs before and after the addition of GST-RLuc, where [GSH-QDs] = 1 μM and [GST-RLuc] = 10 μM.

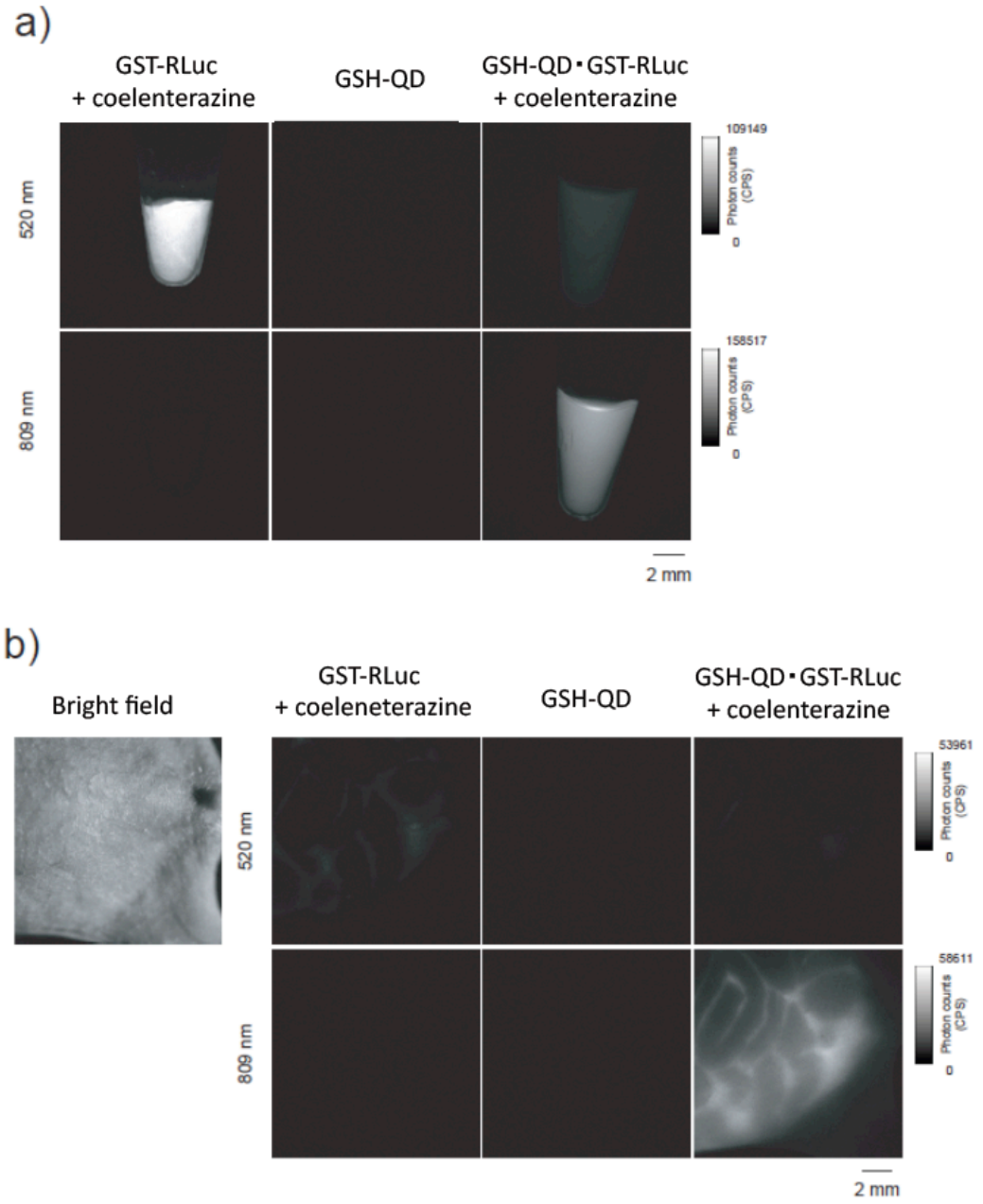

Figure S2 Luminescence images of GST-RLuc + coelenterazine, GSH-QD (with no excitation), GSH-QD・GST-RLuc + coelenterazine *in vitro* (a) and *in vivo* (b). *In vitro* measurements, 50 μL of GST-RLuc (1 μM) + 50 μL of coelenterazine (1 μM), 50 μL of GSH-QD (1 μM), and 50 μL of GSH-QD・GST-RLuc (1 μM) + 50 μL of coelenterazine (1 μM) were used. *In vivo* measurements, 200 μL of GST-RLuc (1 μM) + 200 μL of coelenterazine (1 μM), 200 μL of GSH-QD (1 μM), and 200 μL of GSH-QD・GST-RLuc (1 μM) + 200 μL of coelenterazine (1 μM) were used. Sample solutions were directly injected to a mouse abdomen by using a syringe. Other experimental conditions *in vivo* were the same as those in Fig.6 in the text.

References

1) Jin T.; Fujii F.; Komai. Y.; Seki J.; Seiyama A.; Yoshioka Y. *Int. J. Mol. Sci.*, **2008**, *9*, 2044-2061.

2) Jin T.; Yoshioka Y.; Fujii F.; Komai. Y.; Seki J.; Seiyama A. *Chem Commun*. **2008**, 5764-5766.