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Supporting Information

Quantum Dot – NanoLuc Bioluminescence Resonance Energy Transfer Enables Tumor Imaging and Lymph Node Mapping In Vivo

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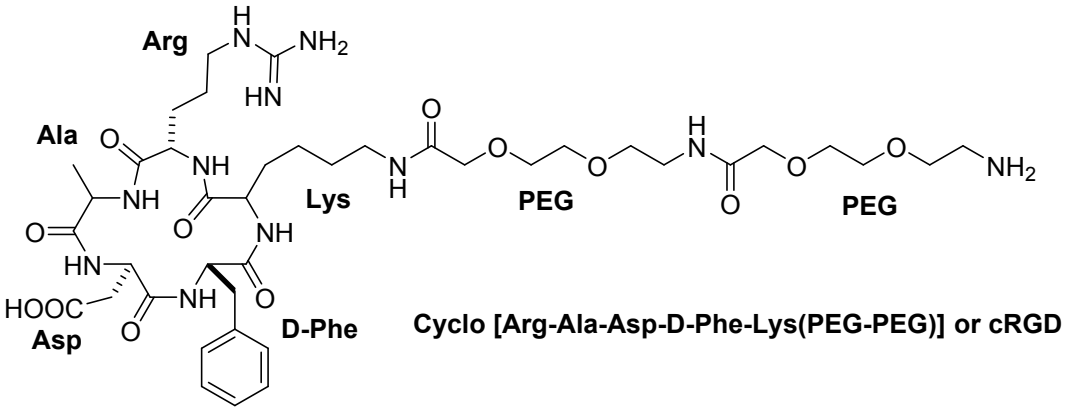
Materials. Quantum dot 705 was obtained from Invitrogen and has typical CdSe/ZnS core-shell structures. The hydrodynamic diameters of QD705 and the conjugates were measured by Malvern Instruments Ltd. with a Zetasizer Nano ZS. The coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was from Fisher Thermoscientific. Nanoluc luciferase (Nluc) and its substrate (Furimazine or NanoGlo) were from Promega. Cyclo [Arg-Ala-Asp-D-Phe-Lys(PEG-PEG)] (cRGD is a short term in this manuscript) was from Peptides international. All other chemicals and solvents were from Sigma-Aldrich and were used without further purification. UV spectrum was collected from a Cary 60 UV-Vis (Agilent Technologies). Fluorescence and bioluminescence emission spectra were collected with a FluoLog-3 (Jobin Yvon Inc.); in case of bioluminescence, the excitation light was blocked, and the emission spectra were corrected with a correction file provided by the company. Animal use protocols were reviewed and approved by the Institutional Animal Care Use Committee of University of Wisconsin-Madison.

Preparation of QD-Nluc and QD-Nluc-cRGD conjugates. QD705 (50 pmol) and Nluc (2 nmol, 40 eq.) were dissolved in Tris-HCl buffer (100 μL, pH 7.4). EDC (200 nmol, 4,000 eq.) was added to the mixture and the reaction was gently shaken at 25^{o}C for 1 h. In case of QD-Nluc-cRGD, cRGD peptides (50 nmol, 1000 eq.) was added to the reaction mixture and continue shaking for another hour. The uncoupled Nluc and excess of EDC and cRGD were removed by 3-4 washes using a 100K Amicon Ultra filter (Merck Millipore Ltd.). The centrifugation was at 5000 rpm for 2 min at 4^{o}C for each washing, and the final conjugates was kept in PBS at 4 ^{o}C.



Gel electrophoresis. After sample purification, unconjugated QD, the QD-Nluc and QD-Nluc-cRGD conjugates were mixed with 6× loading dye. All samples were run on 1 % agarose gel at 100 V in TAE buffer (0.5×) for 40 min. The gel was imaged with Gel Doc XR+ imaging system (Bio-Rad).

Cell culture, cell viability assay and cell imaging. U87MG (human glioblastoma) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (Gibco) supplemented with 10 % fetal bovine serum (FBS, Gibco). Cultures were maintained at 37°C under a humidified atmosphere containing 5% CO_2. Cell viability was measured using MTT assay. U87MG cells were seeded into a 96-well plate at 1×10^4 cells per well and incubated at 37°C under 5% CO_2 for 24 h. QD-Nluc and QD-Nluc-cRGD were added to the wells at varying concentration (0-100 nm). After 72 h incubation with the compounds, 15 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (5 mg/mL) was added into each well of 96-well plate and the plate was incubated for additional 3 h at 37°C under 5% CO_2. Afterwards, the media were removed from each well and then dimethyl sulfoxide (DMSO, 100 μL) was added. The plate was shaken for 15 min at room temperature before measuring the OD

at 570 nm using SpetraMax Plus 384 microplate reader. Cell viability (%) = (mean of OD of treatment group/mean of OD of control group) × 100.

For cell imaging, cells were seeded on 18 mm glass cover slips (1×10^5 cells per well) and cultured for 48 h. QD-Nluc and QD-Nluc-cRGD were added to a separated well and incubated for 3 h. Cells were then gently washed three times with PBS and fixed with cold acetone for 10-15 min. The cells were washed again three times with PBS and mounted with DAPI mounting medium (Vectashield) before imaging with Nikon A1RS confocal microscope and imaging analysis was performed using the NIS-Elements Ar with Deconvolution package. Filter set: excitation 420/40 nm, emission 705/40 nm.

For bioluminescence cell imaging, 1×10^6 U87MG cells were added into 1.5 mL Eppendorf tubes. The cells were washed twice with PBS before incubated with QD-Nluc or QD-Nluc-cRGD (2 nM in PBS/BSA, 2%) for 30 min on ice. After that, the cells were washed three times with PBS and finally suspended in 100 μL PBS/BSA. Before imaging, 20 μL of furimazine substrate (Nano-Glo, 2% v/v in PBS) was added and the bioluminescence images were collected from in vivo imaging system (IVIS).

Lymph node imaging. QD-Nluc (20 μL, ~5 pmol QD) was intradermally injected into a footpad of nude mice. Within 5 min of injection, mice received an intravenous injection of furimazine (50X dilution of Nano-Glo substrate, Promega) in PBS and imaged on an IVIS Spectrum with open filter and optical filter (690-710 nm).

Tumor implantation and in vivo imaging. All animal studies were conducted under an IACUC protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. U87MG cells were harvested using 0.05% trypsin-EDTA when they were 80-90%

confluence. Cells were pelleted by centrifugation at 3000 rpm for 4 min and resuspended in a mixture of 1:1 PBS and Matrigel (BD Biosciences, San Jose, CA). The cells (5×10^6 cells per tumor site) were implanted subcutaneously into the hind leg of 4-5 week-old female athymic nude mice (Envigo, Cambridgeshire, UK). When the tumor reached 4-6 mm in diameter (2-3 weeks after implantation) and, the mice were utilized for *in vivo* studies. The body weights of mice used in experiments ranged from 20-23 g, and the average weight was ~21 g.

In vivo and *ex vivo* fluorescence imaging were performed with an IVIS spectrum imaging system (excitation: 465 ± 15 nm filter; emission collected with 700 ± 10 nm filter). For bioluminescence imaging, the mice were images after injection of furimazine (50X dilution of Nano-Glo substrate in PBS). Images were acquired with 700 ± 10 nm emission filter and without filter and excitation was blocked.

Histology. Tumor-bearing mice were euthanized 2 h after injection with QD-Nluc and QD-Nluc-cRGD. The tumor tissues were frozen in optimal cutting temperature (OCT) medium, cryosectioned at -20°C into 5 μm slices, and the tissue were mounted with DAPI mounting medium to visualize cell nucleus. All images were taken with a Nikon A1RS Confocal Microscope and imaging analysis was performed using the NIS-Elements Ar with Deconvolution package.

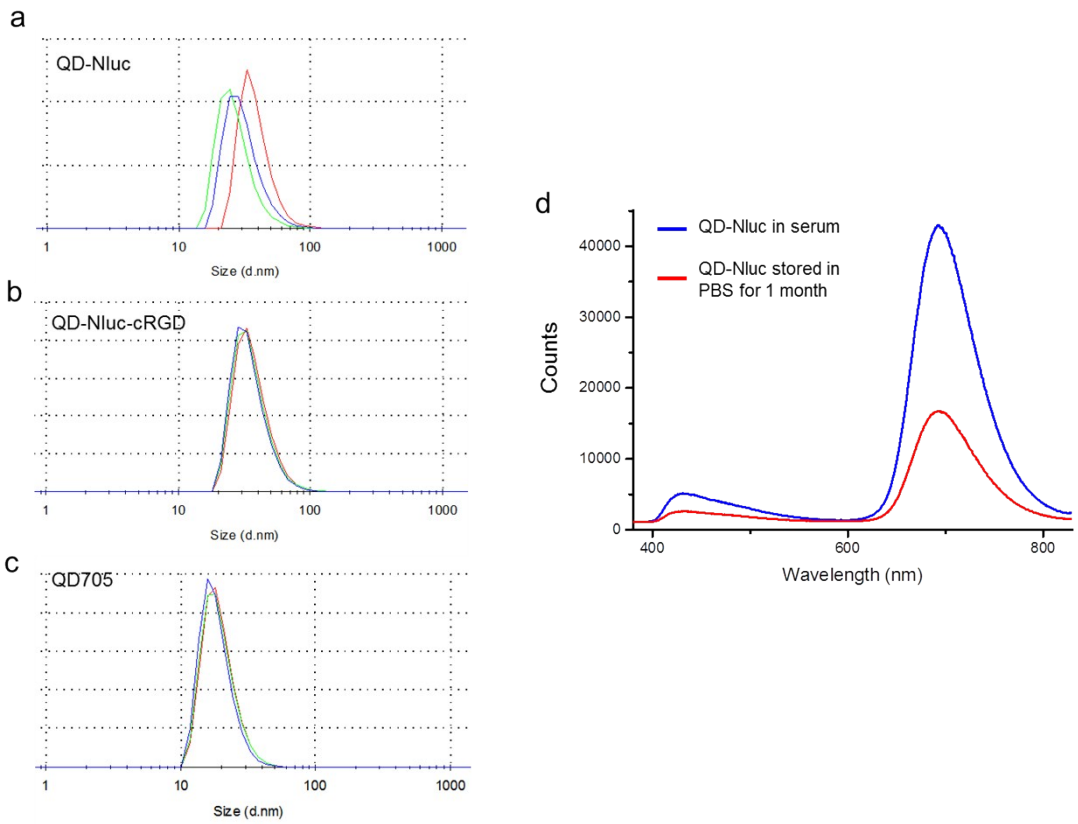


Fig. S1 Characterization of QD-Nluc and QD-Nluc-cRGD conjugates. **a-c**) Dynamic light scattering (DLS) measurement of (**a**) QD-Nluc, (**b**) QD-Nluc-cRGD, and (**c**) unconjugated QD in water by lognalmal size distribution (three times measurements). **d**) Bioluminescence spectra of QD-Nluc in serum (blue) and the sample stored in PBS at 4°C for 1 month (red). Note, the samples in (**d**) were measured in different concentrations.

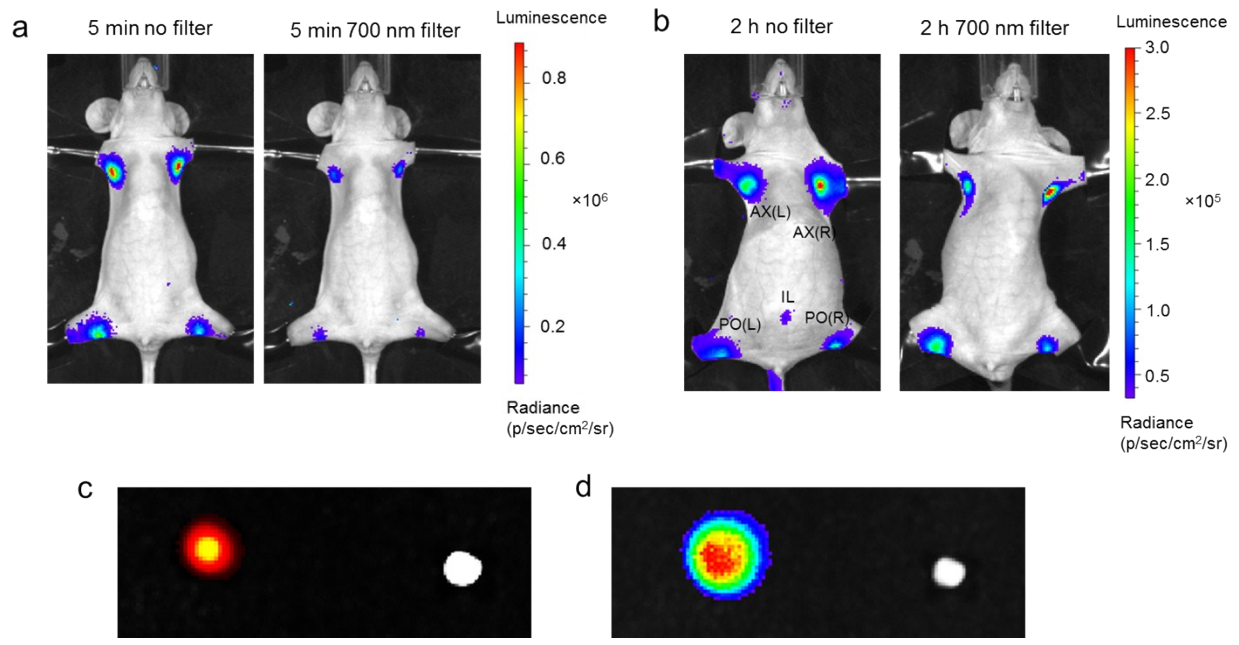


Fig. S2 Bioluminescence imaging of lymph node in a nude mouse. **a,b**) Bioluminescence imaging of lymphatic basins in a mouse after 5 min (**a**) and 2 h (**b**) intradermal injection to four paws. Images were acquired without filter and with 700 ± 10 nm emission filters. **c**) *Ex vivo* fluorescence and **d**) luminescence imaging of PO lymph node compared to a PO lymph node from a mouse without any injection. L, left; PO, popliteal lymph node; LU, lumbar lymph node; IL, iliac; AX, axillary lymph node; R, right.

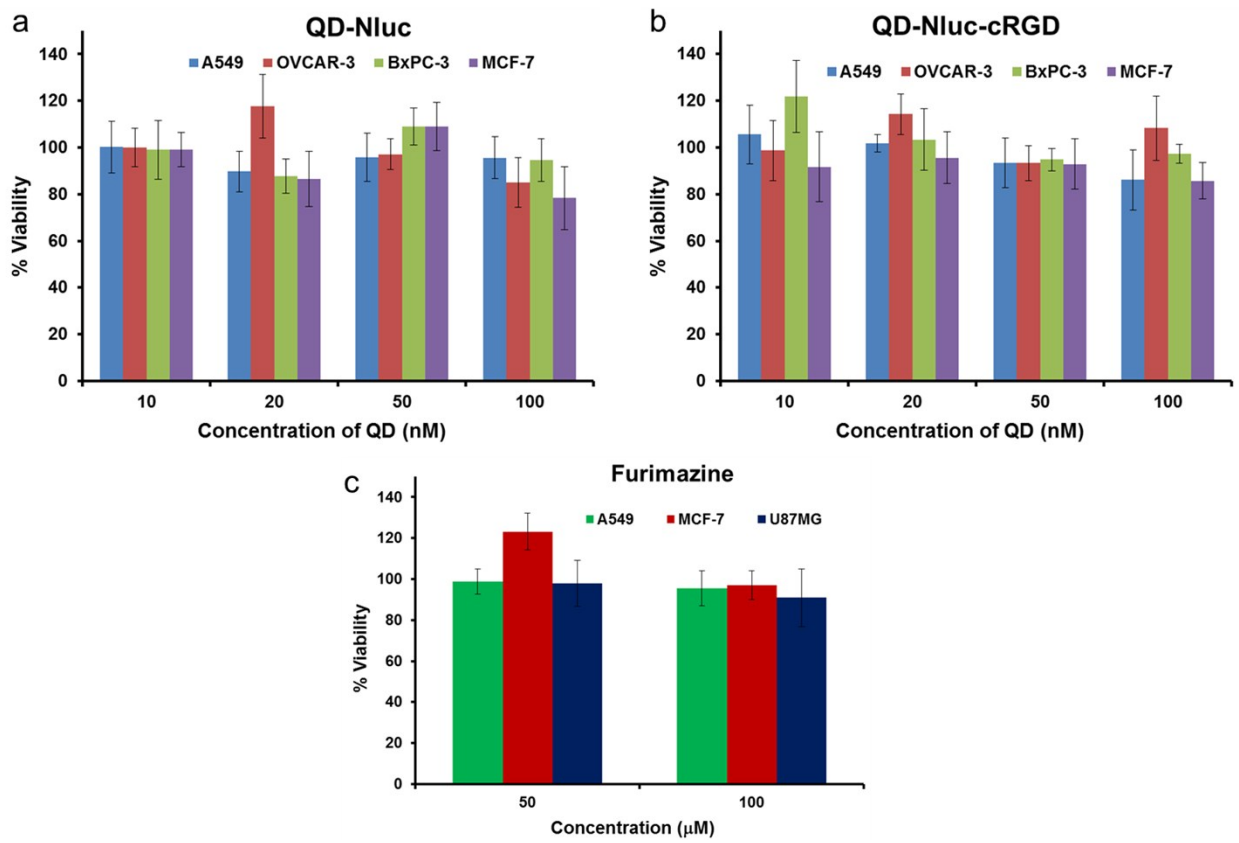


Fig. S3 Cell viability evaluated by MTT assay in a dose-dependent manner of (**a**) QD-Nluc, (**b**) QD-Nluc-cRGD and (**c**) furimazine in different cell lines. Data represent mean ± SD (*n = 4*).

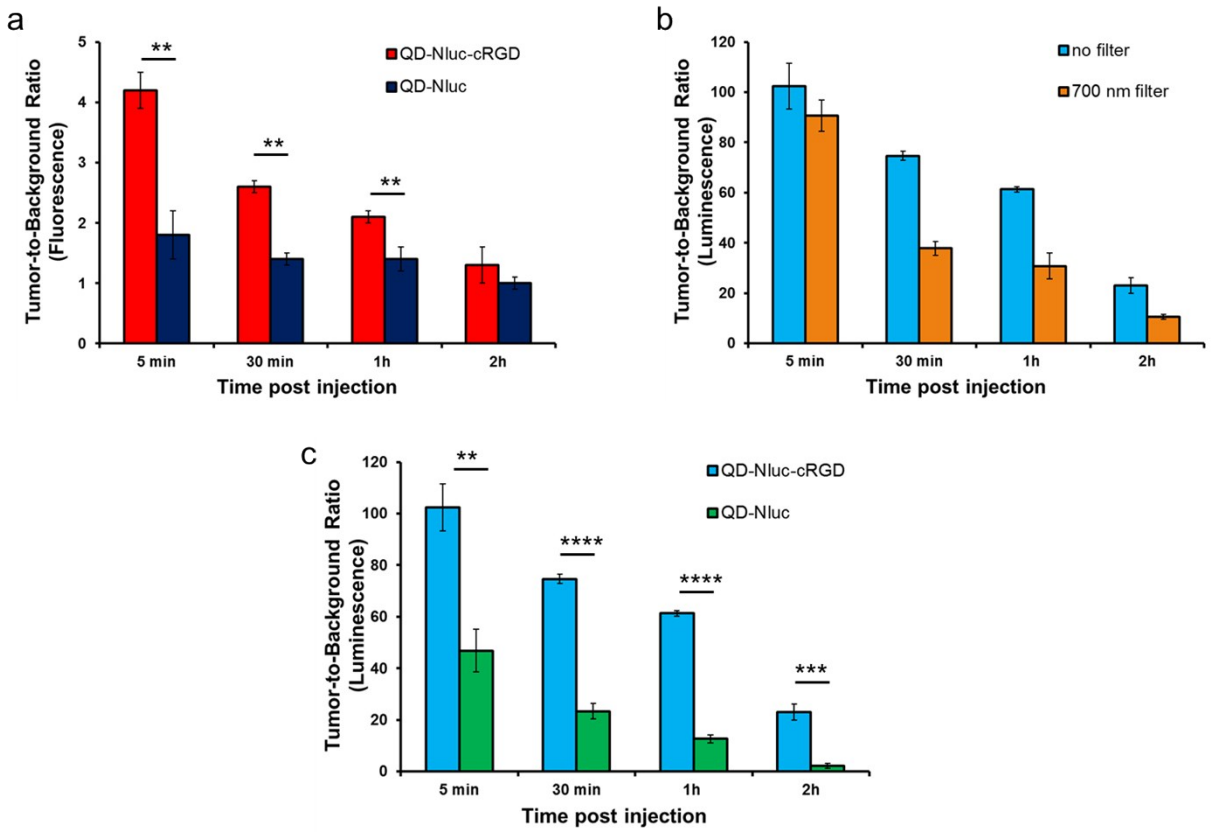


Fig. S4 Region of interest (ROI) analysis of tumor over background of mice injected with QD conjugates. **a**) ROI analysis of fluorescence intensity of tumor over background (region surrounded tumor) of mice injected with QD-Nluc-cRGD (red bars) and QD-Nluc (dark blue bars). **b**) ROI analysis of bioluminescence intensity of tumor over background (region surrounded tumor) of mice injected with QD-Nluc-cRGD, images acquired without emission filter (light blue bars) and with 700 ± 10 nm emission filters (orange bars). **c**) ROI analysis of bioluminescence intensity of tumor over background of mice injected with QD-Nluc-cRGD (blue bars) and QD-Nluc (green bars), images acquired without emission filter. Quantitative data were expressed as mean ± SD. Means were compared using the Student t test with p-values ****< 0.00005, ***< 0.0005, **< 0.005 considered statistically significant ($n = 3$).