General

All the reactions were performed under an atmosphere of argon. $CDCl_3$ or CD_3OD was used as the solvents. Coupling constants (*J*) values are given in Hz and are correct to within 0.5 Hz. Signal patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. All reagents were purchased from Sigma-Aldrich, TCI (Tokyo Chemical Industry Co., Ltd.) or Wako (Wako Pure Chemical Industries, Ltd.). Thin layer chromatography was performed using TLC Silica gel 60 F_{254} (Merck). High-resolution mass spectra (HRMS) were recorded by electrospray ionization (ESI) on a Thermo Scientific Q Exactive instrument. ^{1}H-NMR and ^{13}C NMR (100 MHz) spectra were recorded on a BRUKER (AV-400M) magnetic resonance spectrometer. Electronic absorption and fluorescence spectra were taken on a Jasco V-730BIO UV-Vis spectrophotometer and a Jasco FP-8200 spectrofluorometer respectively. Purification of products was performed on a middle pressure liquid chromatography (MPLC) systems (EPCLC-AI-580S, Yamazen Corporation) equipped with silica gel column (Hi-Flash Column, Yamazen Corporation) and recycling preparative HPLC system (LC-9201, Japan Analytical Industry) with JAIGEL-1H and 2H columns with $CHCl_3$ as an eluent running at 3.0 mL/min. The labeled cells were visualized with a BZ-9000 fluorescence microscopy (KEYENCE) and TCS SP8 confocal laser scanning microscopy (CLSM, Leicamicrosystems). The BODOPY-tetrazine and exo-5-Norbornene-2-carboxylic acid N-hydroxysuccinimide ester were prepared as described previously.[1,2]

Synthesis and characterization of NIR-BODIPY-tetrazine 2

The BODOPY-tetrazine **1** was prepared as described previously.[1] BODIPY-tetrazine **1** (0.072 mmol, 30 mg), Fisher aldehyde (0.072 mmol, 14.4 mg), glacial acetic acid (0.08 mL) and piperidine (0.33 mL) were dissolved in a solution of benzene (5 mL). The reaction mixture was stirred and refluxed for 4 h. Any water formed during the reaction was removed azeotropically in a dean-stark apparatus. The solvent was concentrated *in vacuo* and the crude product was purified using medium pressure liquid chromatography (MPLC) (dichloromethane) and recycling preparative HPLC to afford a blue solid product NIR-BODIPY-tetrazine **2** (7 mg, 16%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ= 8.69 (dd, J = 7.9 Hz, J = 1.2 Hz, 1H, *ArH*), 8.61 (d, J = 1.4 Hz, 1H, *ArH*), 7.72 (t, J = 7.8 Hz, 1H, *ArH*), 7.63-7.57 (m, 2H, *ArH*), 7.12-7.16 (m, 2H, *ArH*), 6.91 (m, 2H, *C=CH*), 6.72 (d, J = 7.9 Hz, 1H, *ArH*), 6.52 (s, 1H, $H_{pyrrole}$), 5.93 (s, 1H, $H_{pyrrole}$), 5.70 (d, J = 12.3 Hz, 1H, *C=CH*), 3.21 (s, 3H, CH_3), 3.11 (s, 3H, CH_3),

2.57 (s, 3H, *CH*$_3$), 1.61 (s, 6H, *2*×*CH*$_3$), 1.49 (s, 3H, *CH*$_3$), 1.42 ppm (s, 3H, *CH*$_3$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ= 167.7, 163.9, 161.7, 156.5, 150.4, 144.6, 142.7, 139.3, 137.9, 137.2, 136.6, 134.8, 133.6, 133.1, 132.7, 130.5, 130.0, 128.5, 128.1, 128.0, 121.7, 120.8, 119.8, 118.4, 114.0, 107.0, 98.6, 46.4, 41.1, 29.4, 28.9, 28.8, 21.3, 15.3, 14.6 ppm. **HRMS** (ESI) for $C_{35}H_{34}BF_2N_7$ $[M+H]^+$: Calcd. 601.2931, Found 601.3217

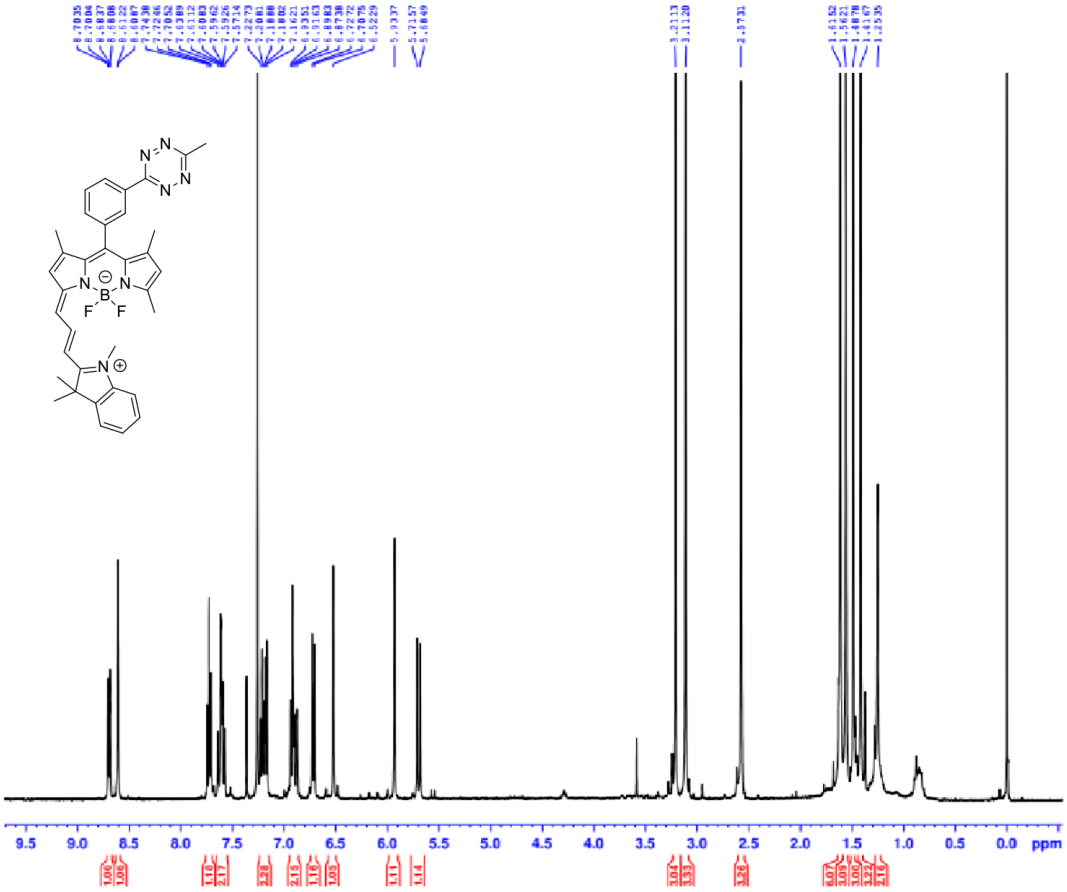

Figure S1. ^{1}H NMR spectrum of NIR-BODIPY-tetrazine **2** in $CDCl_3$

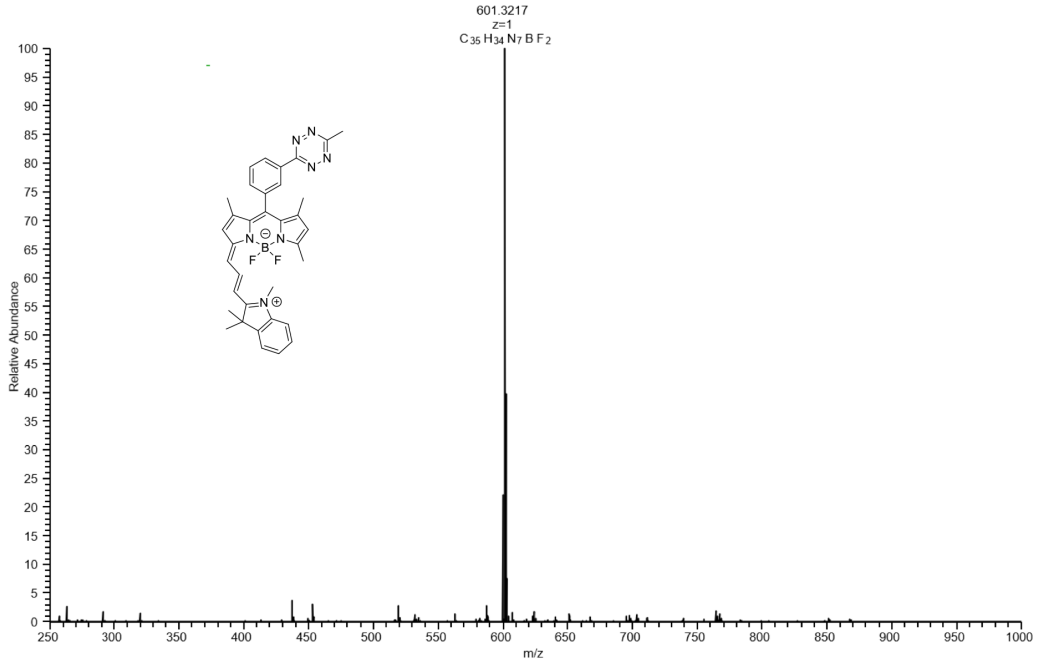

Figure S2. Positive-ion mode High-resolution mass spectrum (HRMS) of NIR-BODIPY-tetrazine **2.**

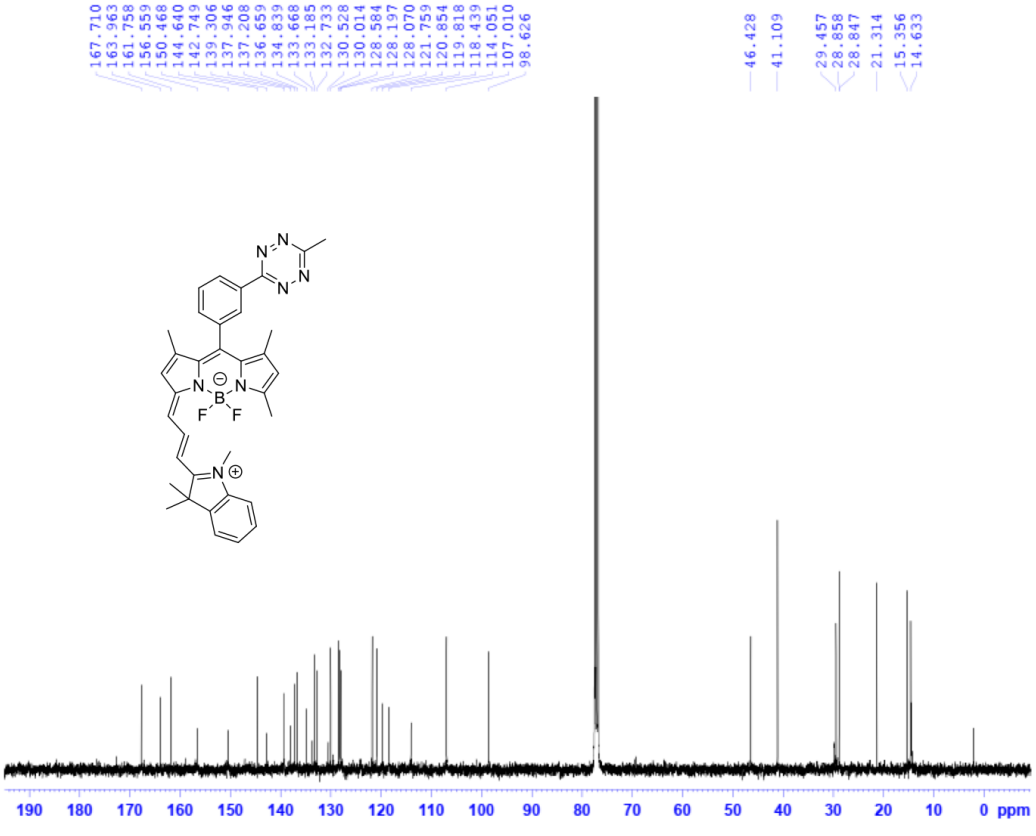

Figure S3. ^{13}C NMR spectrum of NIR-BODIPY-tetrazine **2** in $CDCl_3$.

Synthesis and characterization of Norbornene-modified glucosamine derivative (Nor-GluNOH 4)

The exo-5-Norbornene-2-carboxylic acid N-hydroxysuccinimide ester was prepared referring to literature.[2] Glucosamine hydrochloride **3** (1.92 mmol, 413 mg) was dissolved in anhydrous dimethylformamide (7.0 mL), *N, N*-Diisopropylethylamine (3.81 mmol, 0.664 mL) and exo-5-Norbornene-2-carboxylic acid *N*-hydroxysuccinimide ester (2.09 mmol, 493 mg) were added. The mixture was stirred for overnight at room temperature. The solvent was removed under reduced pressure and the residue was purified by MPLC with the mixture of methanol in dichloromethane (25%, v/v) to afford a white solid product Nor-GluNOH **4** (380 mg, 66%). **^{1}H NMR** (400 MHz, Methanol-d_4): δ= 6.16–6.15 (m, 2H, *C=CH*), 5.00 (d, J=10.0 Hz, 1H, *NH*), 4.29–4.28 (m, 1H), 4.02–3.99 (m, 1H), 3.84-3.82 (m, 1H), 3.77–3.72 (m, 2H), 3.64–3.59 (m, 1H), 3.34 (s, 1H), 3.23–3.20 (m, 1H), 2.87–2.85 (m, 1H), 2.27–2.23 (m, 1H), 1.95–1.93 (m, 1H), 1.73 (t, J=8.4 Hz,1H), 1.38–1.33 (m, 4H), 1.32–1.27 ppm (m, 2H). **^{13}C NMR** (100 MHz, Methanol-d_4) δ= 178.9, 178.8, 139.0, 138.9, 137.4, 137.3, 97.2, 92.6, 78.0, 76.1, 73.1, 73.0, 72.7, 72.6, 72.6,72.6, 62.8, 58.8, 55.9, 47.1, 46.9, 45.5, 45.2, 45.1, 42.8, 42.8, 31.3, 31.0 ppm. **HRMS** (ESI) for $C_{14}H_{21}NO_6Cl$ $[M+Cl]^-$: Calcd. 334.1052, Found 334.1077.

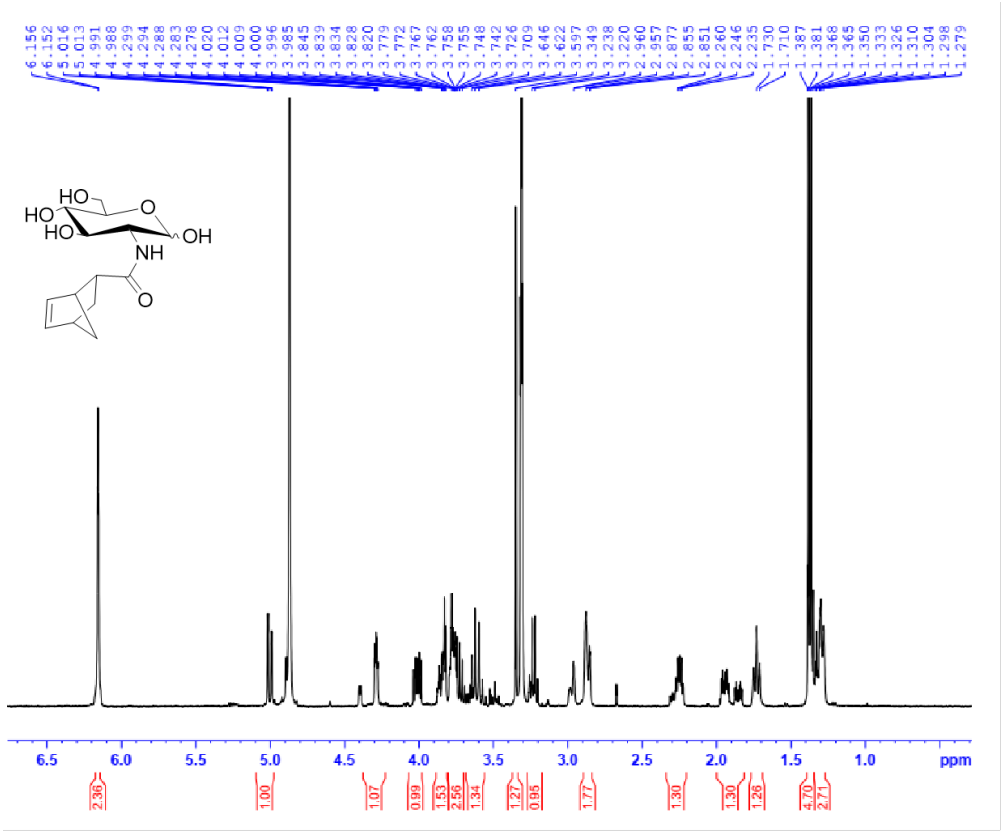

Figure S4 ^{1}H NMR spectrum of Nor-GluNOH **4** in CD_3OD

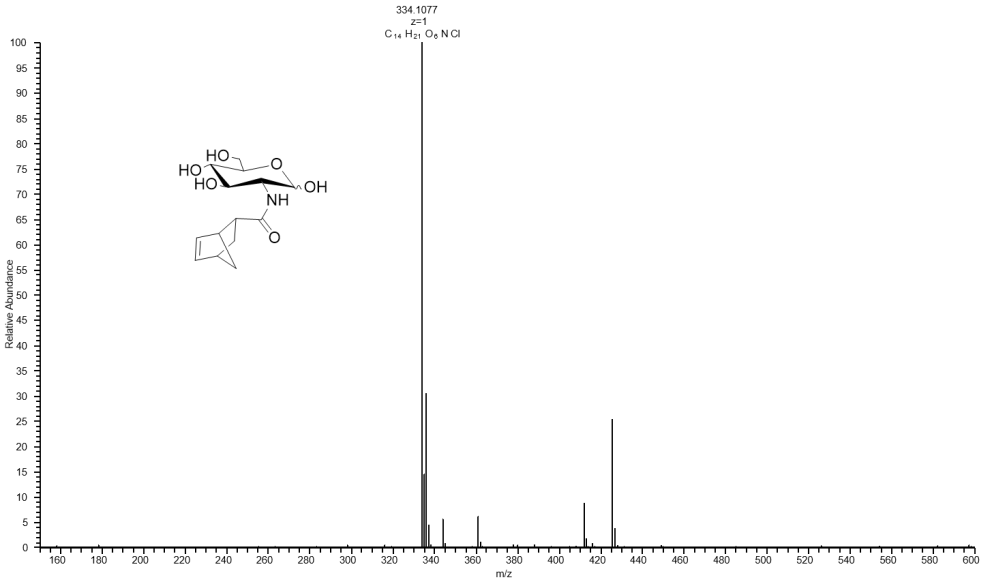

Figure S5. Negative-ion mode High-resolution mass spectrum (HRMS) of Nor-GluNOH **4**.

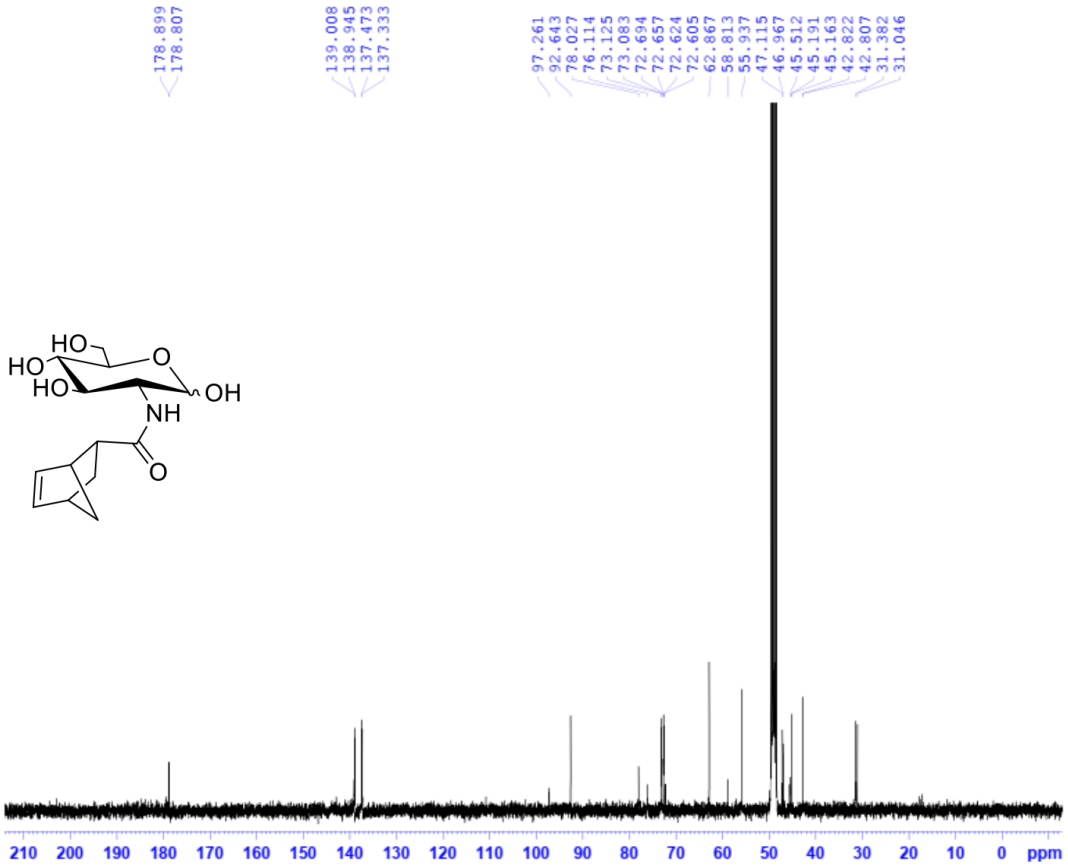

Figure S6. ^{13}C NMR spectrum of Nor-GluNOH **4** in CD_3OD.

UV/vis and emission spectrum measurements

UV-Visible and emission spectroscopy studies were measured using an ultraviolet spectrophotometer (V-730BIO, Jasco, Japan) and a fluorescence spectrophotometer (FP-8200, Jasco, Japan) at room temperature. The slit width was 0.5 nm for both excitation and emission. Quartz cuvettes (1 cm path length) were used for each study. To analyze the data, integrated absorbance against the corresponding solution concentrations were plotted to conclude the extinction coefficients (ε) at the maximum absorption. For each measurement, solvent blank values were subtracted. At least two spectrum scans were collected for each sample.

Inverse-electron-demand Diels-Alder reaction (iEDDA) between NIR-BODIPY-tetrazine 2 and Nor-GluNOH 4

This iEDDA click reaction was carried out in a 3 mm × 3 mm quartz cuvette at 37℃. A 1 mM stock solution of NIR-BODIPY-tetrazine **2** was prepared in DMF. Measurements were immediately started upon the addition of excess Nor-GluNOH **4** (final concentration of 1.2 mM) to the reaction solution. Compound NIR-BODIPY-tetrazine **2** at 1 μM final concentration in DMF/PBS (V/V, 1:1). The fluorescence change of the solution was monitored along with reaction time. The excitation wavelength of NIR-BODIPY-tetrazine **2** was 666 nm and the spectrum was collected from 700 to 900 nm.

Kinetic measurements

Rate constant *k* were measured under pseudo first-order conditions using a constant concentration of tetrazine derivative and 10- to 200- fold excess of the norbornene derivative (Nor-GluNOH **4**). The measurements were performed at 37℃in a mixture of water / dimethyl sulfoxide (90/10) following the decay in the absorption of tetrazine derivative at 280 nm. A typical experimental procedure: an equal volume of tetrazine-COOH in H_2O/DMSO (9:1) and a solution of norbornene in H_2O were mixed in an UV-cuvette and immediately inserted into UV spectrophotometer. Final concentration of tetrazine was 0.05 mM and final concentration of norbornene 0.5- to 10 mM corresponding to 10- to 200- fold excess. The decay in the absorption of the tetrazine was followed 50 min in 2 seconds internals. The data were fit to a single exponential equation. The observed rate constant *k'* was plotted against the concentration of

norbornene. The second-order constant *k* was obtained from the slope of the plot.

Cytotoxicity assay

For all experiment, HeLa and HT-29 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (FBS, 10%), 100 units/mL penicillin, 100 μg/mL streptomycin. The cells were grown at 37 °C in a humidified 5% CO_2 atmosphere.

The day before assaying cytotoxicity of our compounds, 100 μL of HeLa cell suspension (1.5×10^3 cell/μL) in DMEM were seeded per well into clear flat bottom polystyrene 96-well cell culture microplates (Thermo Fisher Scientific, American). The plates were incubated at 37°C in a fully humidified atmosphere of 5 % CO_2 for overnight. The next day, after being washed twice with PBS and 100 μL different concentrations (final concentration 0.0, 1.0, 10.0, 100.0 μM and 1.0, 5.0 mM) of the studied compounds, were added to the cells and mixed properly. After incubation 48 h, the culture media were removed and washed with PBS twice, 100 μL of alarmarBlue reagent (dilute 1:9 in PBS) solution were added to each well and mixed appropriated. After addition in all studied wells, the cells were further incubated 3 h. The fluorescence was next measured using the Immuno Mini microplate reader (NJ-2300, Japan) with the following settings: excitation wavelength: 562 nm, emission wavelength: 595 nm. The OD value of each individual well was calculated and percentage of cell survival in the untreated control was assumed as 100%. Relative cell viability = (experimental OD - background OD)/(OD of untreated control - background OD) × 100 %. All values were obtained at least from three independent measurements. After performing the cell viability assays, IC_{50} values were determined using GraphPad Prism version 5 (GraphPad Software, USA).

Fluorescence imaging in cancer cells

Approximately 5×10^4 HeLa and HT-29 cells in DMEM (1 mL) were seeded on a 35 mm glass bottom dish and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. The medium was then removed. The cells, after being rinsed with PBS, were pre-incubated in the medium for 48 h with or without Nor-GluNOH **4** (100 μM). After sugar moiety labeling, the medium was then removed and washed with PBS, and fixed by 4% formaldehyde for 20 min at room temperature. After that, removed solution, washed PBS again, the cells were incubated with probe in medium for 2 h, and then rinsed with PBS twice, the cells were treated with Hoechst 33342 solution (10 $\mu g \cdot mL^{-1}$) for 15 min. For

control group, the cells were also incubated directly with probe in medium for 2 h. For using PlasMem Bright Green, HeLa cells were grown with Nor-GluNOH **4** (100 μM) for 48 h. Subsequently, cells were incubated with NIR-BODIPY-tetrazine **2** (100 nM, 2 h, 37 ℃), and PlasMem Bright Green (1:100) for 5 min, then the nuclei were stained with Hoechst 33342. The cells were protected from light during the whole process. After that, the cells were washed with PBS once before being examined with a BZ-9000 fluorescence microscopy and a Leica TCS SP8 confocal microscope. The green channel for BODIPY-tetrazine **1** and PlasMem Bright Green were using excitation wavelength of 499 nm, the red channel for NIR-BODIPY-tetrazine was using excitation wavelength of 650 nm, the blue channel for Hoechst 33342 was using excitation wavelength of 350 nm. The images were analyzed using a Leica LAS X software.

***In vivo* fluorescence imaging of tumour-bearing nude mice**

All animal operations were carried out in compliance with the relevant laws and approved by the Institutional Animal Care and Use Committee of University of Miyazaki (Miyazaki, Japan). BALB/c nude mice (aged 6 weeks) were purchased from Charles River Laboratories Japan (Yokohama, Japan). Mice were treated by subcutaneous injection 4T1 cells (1×10^7). The mice were kept under pathogen-free conditions with free access to food and water. Nor-GluNOH (25 mM, 30 μL, in mixed solvent (DMSO: H_2O, v/v, 1:1)) was injected intratumorally (i.t.) to the tumor (upper site) bearded nude mice once daily for three consecutive days (days 1-3), the bottom site tumor was injected i.t. with PBS as control. At day 4, a solution of NIR-BODIPY-tetrazine **4** in mixed solvent (5 mg/kg, DMSO: H_2O, v/v, 1:1) was injected intratumorally. The fluorescence images of the mice were captured before and after the injection of NIR-BODIPY-tetrazine **2** at different time points with an in *vivo* imaging system (Lumazone CMS, Shoshin EM, Japan). Tumors and organs were excised from mouse and imaged ex *vivo*. In vivo labeling of mice in SI, Nor-GluNOH **4** was injected into nude mice bearing a tumor, while PBS was also injected into another mouse as a control. Other experiments are the same as above. The excitation/emission filter of 650-695 nm/720-780 nm was used. Fluorescence intensity was quantified using the SlideBook 6 imaging software (Intelligent Imaging Innovations, Denver, CO, USA). All images were normalized by dividing the fluorescence image by a reference illumination image.

Histological analysis

The tumor tissue was frozen in optimal cutting temperature (OCT) compound and sectioned with 5 μm in thickness. Tumor sections were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) and stained with hematoxylin and eosin (HE). For fluorescence analysis, sections were counterstained with DAPI. Microphotographs were obtained using Olympus BX53 light microscope and Keyence BZ-9000 Fluorescence Microscope. Olympus cellSens Imaging software was used for HE staining.

Table S1 Photophysical properties of NIR-BODIPY-tetrazine **2**

Compound	Solvent	$\lambda_{abs,\ max}$, nm	$\lambda_{em\ max}$, nm	ε, M^{-1} cm^{-1}	Stokes shift (Δλ, nm)
2	MeCN	646	731	71638	85
2	MeCN:DMSO (1:1)	662	733	67235	71
2	DMSO	672	740	29118	68
2	DMF	662	726	26239	64
2	PBS:DMSO (96:4)	676	741	24268	65
2	PBS:DMF (96:4)	674	733	49415	59

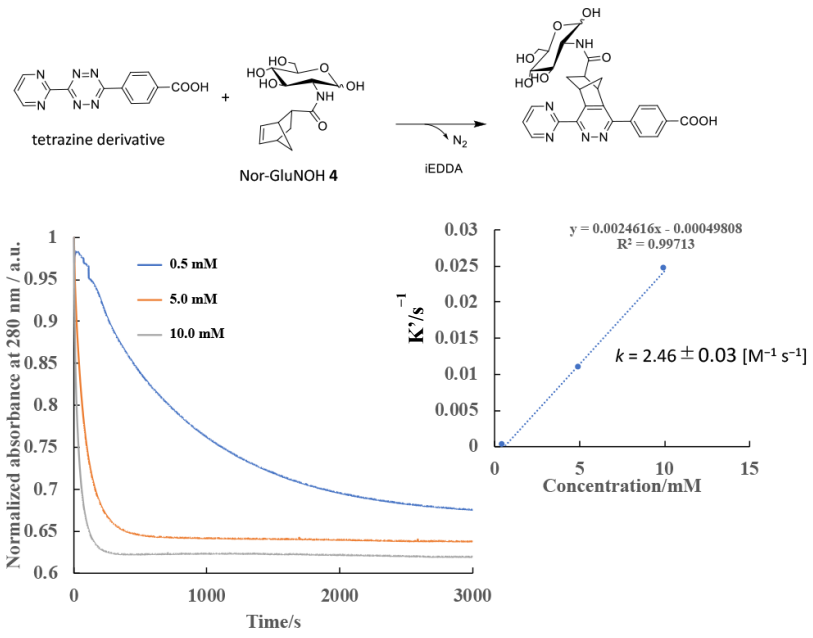

Figure S7. Reaction kinetic measurement based on UV absorption of the reaction product of tetrazine derivative (0.05 mM) and Nor-GluNOH **4** (three separate concentrations in DMSO:H_2O, v/v, 1:9 at 37 ℃). To compare the reaction kinetic with terminal alkenes, we used the tetrazine derivative that has used in previous rate constant study of terminal alkenes.

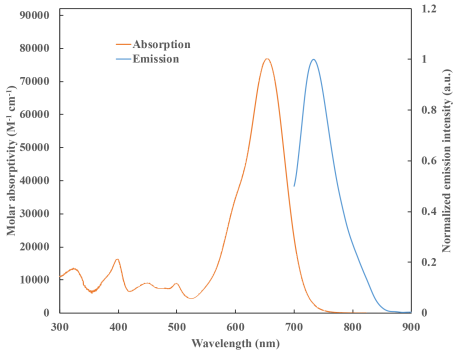

Figure S8. Blue line: emission spectrum of NIR-BODIPY-tetrazine **2** in the presence of Nor-GluNOH **4** (2 mM) in binary solvents (DMSO:MeCN, v/v, 1:1 at 25℃, λ_{em} = 733 nm). Yellow line: absorbance spectrum (10 μM) in acetonitrile at 25℃ (λ_{abs} = 646 nm).

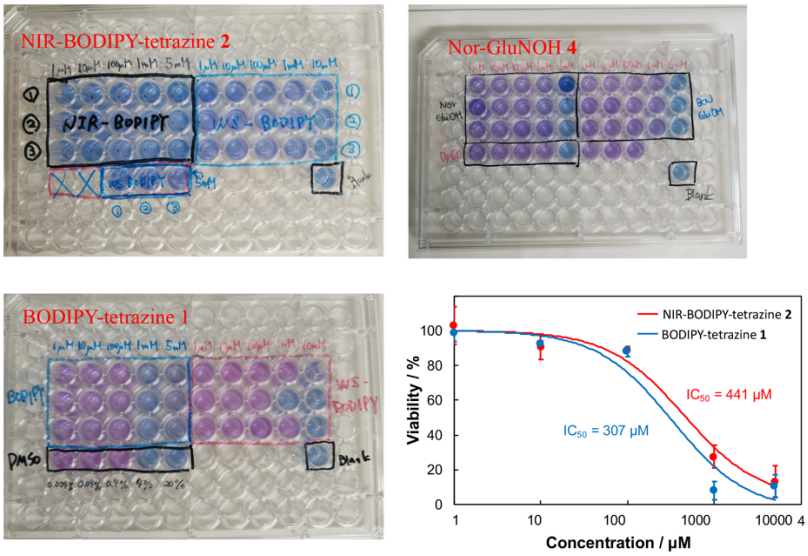

Figure S9. Cell culture microplate for alarmarBlue reagent stain of NIR-BODIPY-tetrazine **2**, BODIPY-tetrazine **1** and Nor-GluNOH **4**. Cytotoxicity against HeLa cells at various concentrations. Data are expressed as the mean ± SD of three independent experiments. IC_{50} values determination are performed using GraphPad Prism software.

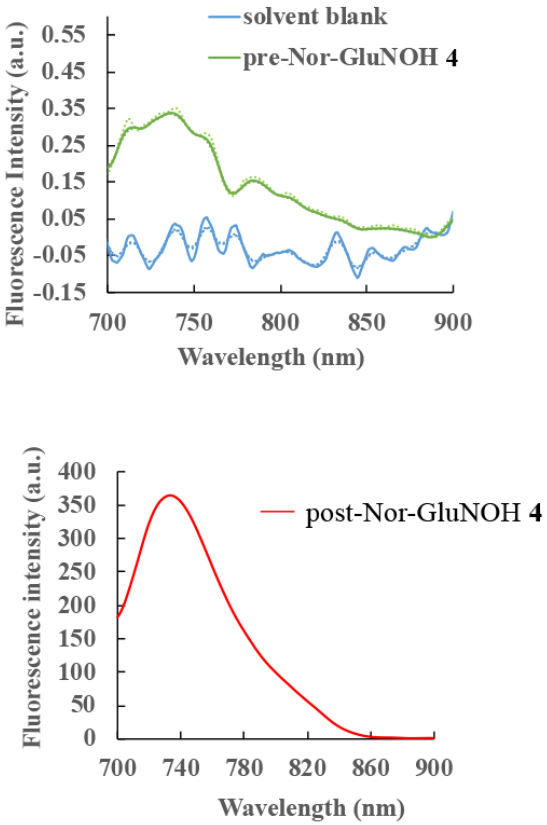

Figure S10. Emission spectra (excitation 666 nm) of NIR-BODIPY-tetrazine **2** in DMF/PBS (V/V, 1:1), before and after addition of Nor-GluNOH **4**. For baseline and pre-Nor-GluNOH spectra, data plotted are means of 2 scans.

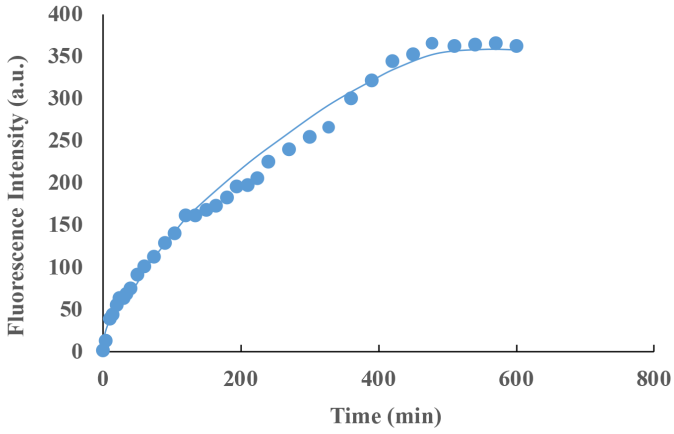

Figure S11. Time courses fluorescence turn-on after addition of Nor-GluNOH **4** to a solution of NIR-BODIPY-tetrazine **2** (DMF/PBS=1:1, v/v, PBS pH=7.4) at 37℃, excitation at 666 nm, emission at 733 nm.

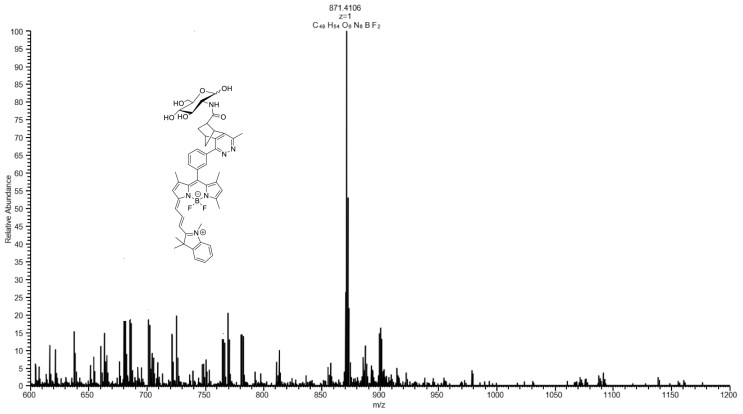

Figure S12. High Resolution Mass Spectrometer (HRMS) result for characterization of iEDDA reaction product. $C_{49}H_{54}N_6O_6BF_2$ $[M+H]^+$: Calcd. 871.4160, Found 871.4106.

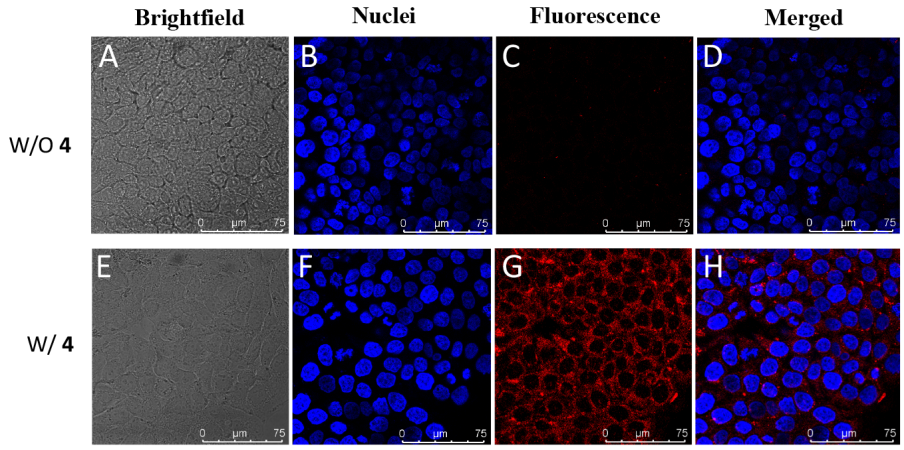

Figure S13. Images in HT-29 cells. Bright field (A/E), nuclei (B/F), fluorescence (C/G) and merged (D/H) images. Cells were treated in the same way as HeLa cells.

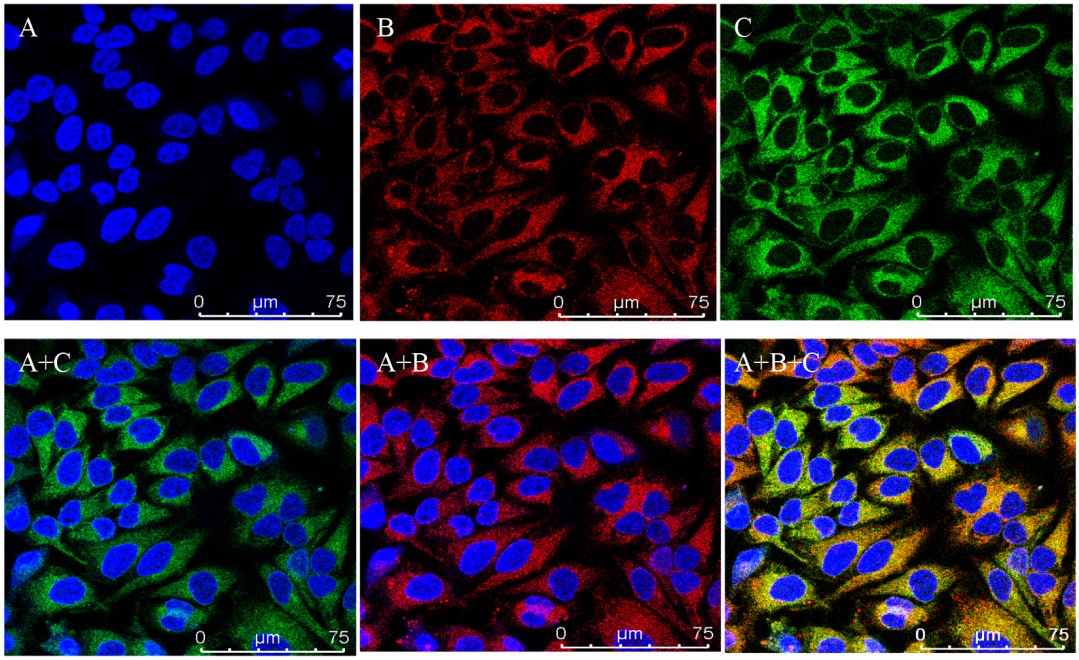

Figure S14. Co-locolization of confocal laser scanning microscopy images in HeLa cells. Blue channel (A) was excited with a 350 nm laser (Hoechst 33342), red channel (B) was excited with a 650 nm laser (NIR-BODIPY-tetrazine **2**), green channel (C) was excited with a 499 nm laser (BODIPY-tetrazine **1**), A+C represents an overlay of blue channel and green channel, A+B represents an overlay of blue channel and red channel, A+B+C represents an overlay of all the channels.

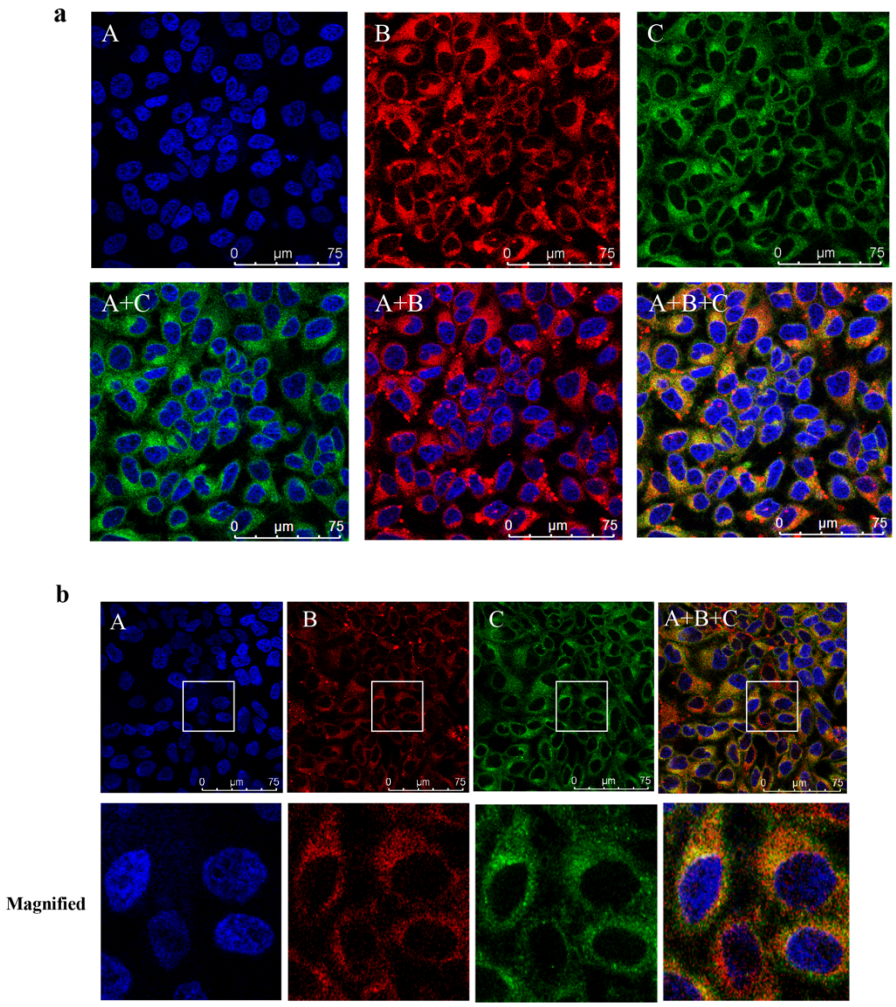

Figure S15. (a) Co-locolization of confocal laser scanning microscopy images in HeLa cells. Blue channel (A) was excited with a 350 nm laser (Hoechst 33342), red channel (B) was excited with a 650 nm laser (NIR-BODIPY-tetrazine **2**), green channel (C) was excited with a 499 nm laser (PlasMem Bright Green), A+C represents an overlay of blue channel and green channel, A+B represents an overlay of blue channel and red channel, A+B+C represents an overlay of all the channels. (b) A, B, C and A+B+C represents the same event with (a). Magnified (150×) image is from the white square region.

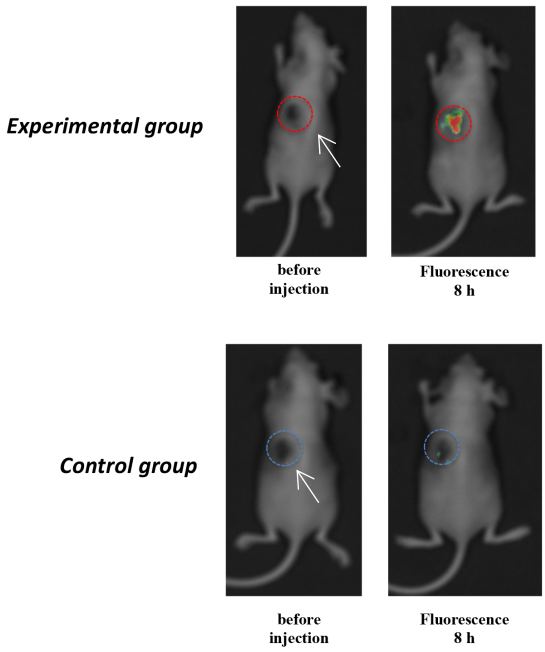

Figure S16. *In vivo* labeling of mice bearing tumor with Nor-GluNOH **4** and subsequent cancer targeting via a light-up strategy using NIR-BODIPY-tetrazine **2** in an iEDDA. *In vivo* fluorescence imaging of mice from Nor-GluNOH **4** and buffer (control) groups. Arrows indicated the sites of injection.

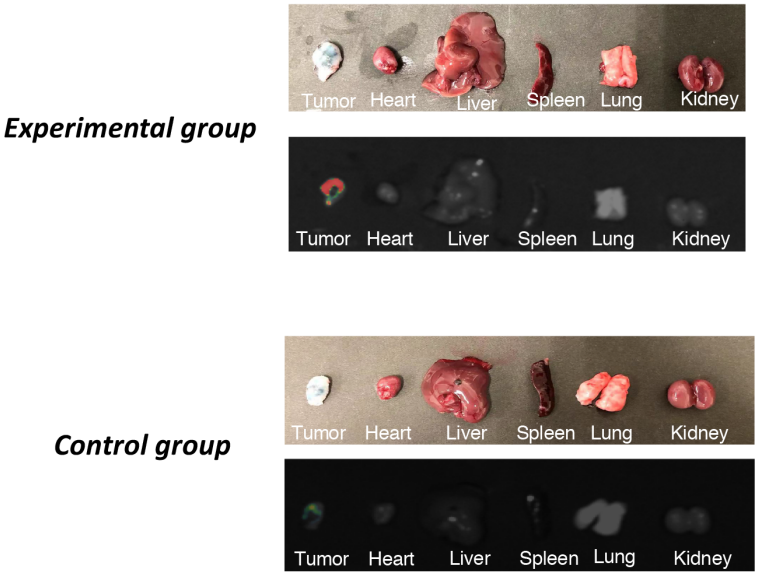

Figure S17. *Ex vivo* fluorescence imaging of tissues excised from mice treated with Nor-GluNOH **4** and buffer (control) groups.

References

[1] Linden G, Zhang L, Pieck F, Linne U, Kosenkov D, Tonner R, Vázquez O. *Angew Chem Int Ed Eng*l., 2019, **58**, 12868-12873.

[2] Melamed Yerushalmi S, Buck ME, Lynn DM, Lemcoff NG, Meijler MM. *Chem Commun.*, 2013, **49**, 5177-5179.