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Supporting Information

In situ evaluation of *in vivo* sialylation with a dual-color imaging strategy

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1. Supplementary Methods

Materials and reagents. Bis-MPA-Acetylene dendrimer, trimethylol propane core, generation 5 (Den-Acet) was obtained from Sigma-Aldrich (USA). 11-Azido-3,6,9-trioxaundecan-1-amine (N_3-PEG-NH_2) was obtained from Sigma-Aldrich (USA). Folic acid PEG 1K azide (N_3-PEG-FA) and was obtained from Shanghai Peng Sheng Biotech. Co., Ltd (China). 2-[2-(2-Azidoethoxy) ethoxy] ethyl 2,3,4,6-Tetra-O-acetyl-D-galactopyranoside (N_3-PEG-Gal-4Ac) was obtained from Shanghai Macklin Biochemical Technology Co., Ltd (China). Esterase from porcine liver was obtained from Sigma-Aldrich (USA). (4- {[Bis-(1-tert-butyl-1H- [1,2,3] triazol-4-ylmethyl)-amino]-methyl}- [1,2,3] triazol-1-yl)-acetic acid (BTTAA) was obtained from Aladdin (China). Succinimidyl carboxymethyl ester-Cy5 (NHS-Cy5) was obtained from Meilunbio (China). Cytidine-5'-monophospho-N-acetylneuraminic acid sodium salt (CMP-Sia), α-2,3-sialyltransferase (ST3Gal) expressed in E. coli BL21 from Pasteurella multocid and α-2,6-sialyltransferase (ST6Gal) expressed in E.coli BL21 from Photobacterium damsela were obtained from Sigma-Aldrich Inc. (USA). Fluorescein-5-thiosemicarbazide (FTZ) was obtained from Sigma-Aldrich (USA). Aniline was obtained from Shanghai Macklin biochemical Co., Ltd (China). Sodium periodate ($NaIO_4$) was obtained from Alfa Aesar China Ltd. Glycine was obtained from Sigma-Aldrich (USA). Cy3 and FITC labeld *Sambucus Nigra* lectin (Cy3-SNA and FITC-SNA), and FITC labeld Soybean Agglutinin (FITC-SBA) were obtained from Vector Laboratories (USA). 4',6-Diamidino-2-phenylindole (DAPI) and Triton x-100 were obtained from Wuhan Servicebro Technology Co., Ltd (China). (3-Aminopropyl) trimethoxysilane (APTMS) was obtained from Bide Pharmatech Ltd. (Shanghai, China). RPMI-1640 Medium, Fetal bovine serum (FBS), trypsin, and phosphate buffered saline (PBS) (pH 7.4, containing 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, 1.41 mM KH_2PO_4, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) were purchased from KeyGen Biotech (China). All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water (≥18 MΩ/cm, Milli-Q, Millipore).

Apparatus. Mass Spectrometry (MS) analysis was performed using an LTQ XL ion trap mass spectrometer equipped with an electrospray ion source and an HPLC system (Thermo Scientific, USA). The transmission electron microscopic (TEM) images were obtained on a Low-Voltage electron microscope (Hitachi HT7800, Japan). Dynamic light scattering (DLS) analysis was performed on a 90 Plus/ BI-MAS equipment (Brook haven, USA). Fluorescence spectra were recorded on an F-7000 fluorescence spectrophotometer (Hitachi, Japan). Infrared spectra were acquired on a Nicolet iS50 FTIR spectrophotometer (Nicolet iS50, USA). The fluorescence images of microarray chips were scanned by LuxScan-10k/A (Capital Bio Corp., Beijing, China). Confocal fluorescence imaging of cells was performed on a SP8 STED 3X confocal laser scanning microscope (CLSM) (Leica, Germany). *In vivo* fluorescence imaging of the mice was performed on a PerkinElmer Caliper IVIS Lumina XR III *in vivo* imaging system (Waltham, Massachusetts, USA).

Synthesis of N_3-PEG-Gal. N_3-PEG-Gal were prepared by adding 10 μL N_3- PEG-Gal-4Ac (1 M in DMSO) and 200 μL esterase solution (1 kU in PBS) to 790 μL PBS buffer. The reaction mixture was stirred at 37°C for 12 h. The mixture was cooled down to room temperature and then stored at -20 °C. The reactive components of mixture were detected by the ESI-MS.

Preparation of Den-Gal/Cy5/FA and Den-Cy5/FA. 1 μL Den-Acet (5 mM in DMSO) was mixed with 30 μL N_3-PEG-Gal (10 mM), 10 μL N_3-PEG-NH_2 (10 mM), 10 μL N_3-PEG-FA (10 mM), 10 μL $CuSO_4$ (500 mM), 50 μL BTTAA (200 mM) and 100 μL ascorbic acid (1 M). The mixture was stirred at room temperature overnight to proceed the click reaction. After purified by ultrafiltration at 10000 rpm for 10 min three times, the obtained Den-

Gal/NH_2/FA were added with 10 μL NHS-Cy5 (10 mM) and stirred at room temperature overnight to proceed amide condensation. The obtained Den-Gal/Cy5/FA was purified by ultrafiltration at 10000 rpm for 10 min three times, and dispersed in PBS for further use. The Den-Cy5/FA were prepared with same procedure without addition of N_3-PEG-Gal.

Preparation of lectin chips. Clean glass slides were firstly immersed in 1 M NaOH solution containing 70% ethanol for 2 h, and 1% APTMS in acetone for another 2 min. After ultrasonic cleaning with acetone and water for three times, the slides were immersed in glutaraldehyde solution (5% in PBS) for 2 h at 50 °C. After washing with acetone and water for three times, the obtained aldehyde slides were dried by nitrogen flow at RT and stored at 4 °C. Prior to use, the aldehyde slide was paste with a sticker containing 3 × 3 holes array. The Cy3-SNA solution (0.1mg/ml in PBS containing 3% glycerol) was dropped into each hole on aldehyde slide and incubated at 37 °C for 30 min in a wet box. After washing with PBS for three times, the obtained Cy3-SNA lectin chips were stored at 4 °C for further use.

***In vitro* verification of sialylation.** 100 nM of Den-Gal/Cy5/FA was incubated with 100 μM of CMP-Sia and 5 mU of ST3Gal or ST6Gal in PBS at 37 °C for 2 h. The obtained mixtures were respectively added into different spots on a SNA-Cy3 lectin chip, which was treated with BSA solution (0.5% in PBS) for 30 min to prevent nonspecific adsorption in advance. After further incubation at 37 °C in a wet box for 1 h, the lectin chip was washed with PBS for three times and subjected to scanning with a chip scanner.

Cell Culture. 4T1 and MCF-10A cells were cultured at 37 °C in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 μg/mL) and streptomycin (100 μg/mL) in a humidified atmosphere containing 5% CO_2.

Visualization of intracellular sialylation. 4T1 cells seeded in confocal dishes were respectively incubated with different concentrations of Den-Gal/Cy5/FA or Den-Cy5/FA in culture medium at 37 °C for 1 h. After washing with PBS for three times, the cells in the dishes were sequentially fixed with 4% paraformaldehyde and treated with glycine (22.5 μg/μL) for 30 min to prevent nonspecific adsorption. After washing with PBS, the cell dishes were incubated with 5 mM $NaIO_4$ at RT for 30 min to perform the oxidation of SA groups. After another washing with PBS, the cell dishes were incubated with a mixture containing 10 mM aniline and 100 μM FTZ in PBS at RT for 1h. After carefully washed with PBS for three times, the cells were immediately subjected to CLSM imaging.

As an alternate way to detect SA inside cell, the cells treated with probes and paraformaldehyde fixing were permeated with 0.125% Triton X-100 at RT for 10 min. After washing with PBS, the cells were incubated with 0.01 mg/mL FITC-SNA at RT for 1 h, which were then subjected to CLSM imaging after carefully washing.

Animal experiments. All animal experiments were performed in strict accordance with the Laboratory Animal Management of Jiangsu Province for the care and use of laboratory animals (License No. SYXK 2019-0056) and was approved by the Department of Science and Technology of Jiangsu Province (Nanjing, China). The subcutaneous tumor xenograft models were prepared by inoculating 4T1 mouse breast cancer cells (1×10^6 cells/mouse) into the hind leg of the BALB/c nude mice (female, 6-8 weeks).

Evaluation of sialylation *in vivo*. After the tumor volume risen to 1 cm^3, 100 μL Den-Gal/Cy5/FA (1 μM) or Den-Cy5/FA (1 μM) were firstly subcutaneously injected at the tumor region of the mice. After 1 h, the mice were euthanized to collect the tumor tissue and normal tissue surrounding the tumor. After performing paraformaldehyde fixing operation with 4% paraformaldehyde, the tissue samples were frozen and cut into slices. After performing standard DAPI staining, the tissue slices were incubated with 5 mM $NaIO_4$ at RT for 30 min to perform the oxidation of SA groups. After washing with PBS for three times, the tissue slices were

incubated with a mixture containing 10 mM aniline and 100 μM FTZ in PBS at RT for 1 h. After carefully washed with PBS for three times, the tissue slices were sealed with a cover slide and subjected to CLSM imaging.

As an alternate way to detect SA in tissue, the tissue slices after paraformaldehyde fixing and DAPI staining were permeated with 0.5% Triton X-100 at RT for 20 min. After washing with PBS, the tissue slices were incubated with 0.05 mg/mL FITC-SNA at RT for 1h, which were then subjected to CLSM imaging after carefully washing.

Data processing. The FTZ@Cy5 and FITC@Cy5 signal were processed from the FTZ, FITC and Cy5 fluorescence in CLSM images using MATLAB software. For each pixel, if its red channel (Cy5 fluorescence) value was greater than zero, the green channel (FTZ or FITC fluorescence) value was retained as the original value. If its red channel value was zero, the green channel was processed to be zero, too. After processing all pixels within the image, the FTZ@Cy5 or FITC@Cy5 image was obtained. The statistical intensities of FTZ@Cy5 or FITC@Cy5 were processed from the corresponding images using Photoshop software. For cell and tissue slice images, the statistical intensities were obtained by dividing the sum of the green channel values with the cell numbers. The statistical intensities in the histogram figures were obtained from the images of at least three parallel experiments.

2. Supplementary Figures

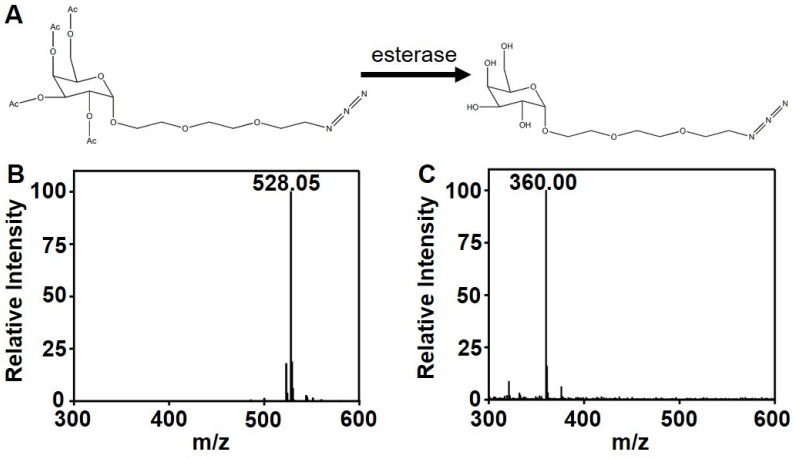

Fig. S1 (A) The synthetic route of N_3-PEG-Gal. (B, C) Mass spectra of N_3- PEG-Gal-4Ac (528.05, + Na^+) and N_3-PEG-Gal (360.00, + Na^+).

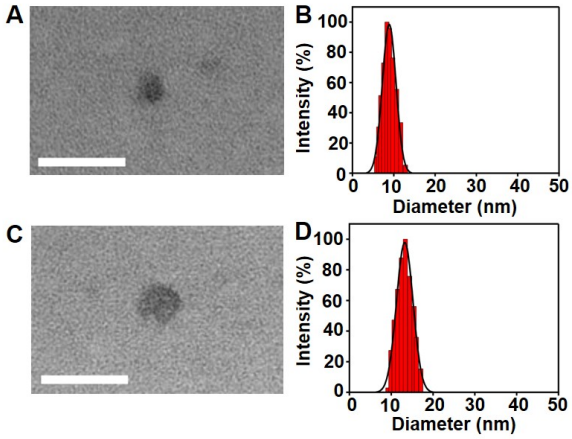

Fig. S2 (A) TEM image and (B) DLS analysis of Den-Acet. (C) TEM image and (D) DLS analysis of Den-Gal/Cy5/FA (C) Scale bar: 50 nm.

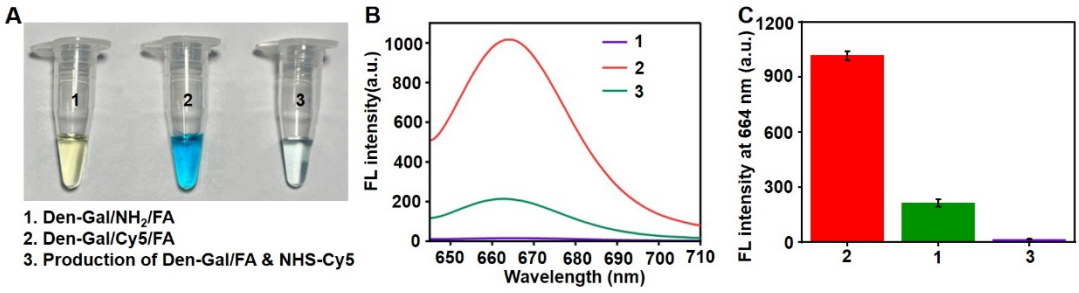

Fig. S3 (A) Photo of Den-Gal/NH_2/FA (1), Den-Gal/Cy5/FA (2), and the production of Den-Gal/FA with NHS-Cy5 (3). (B) Fluorescence spectra of Den-Gal/NH_2/FA (1), Den-Gal/Cy5/FA (2), and the production of Den-Gal/FA with NHS-Cy5 (3). (C) The fluorescence intensities at 664 nm from (B). Error bars represent ± S.D. (n=3).

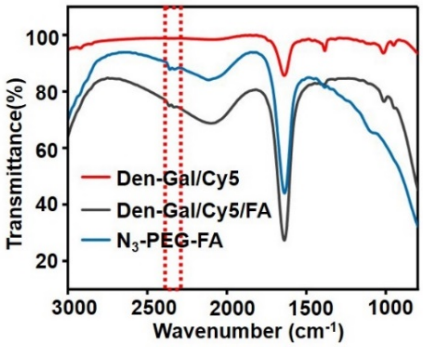

Fig. S4 Infrared spectra of Den-Gal/Cy5, Den-Gal/Cy5/FA, and N_3-PEG-FA.

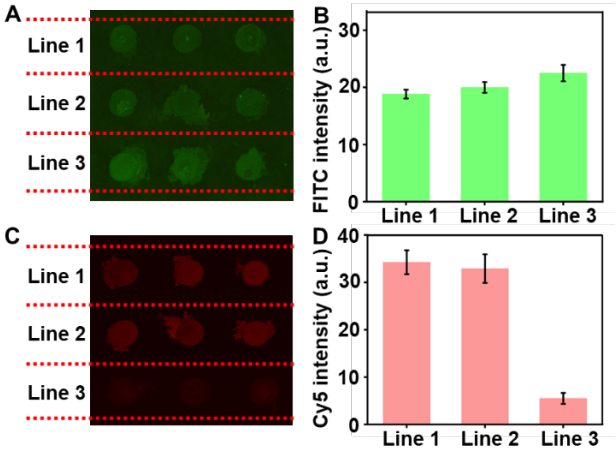

Fig. S5 (A) Fluorescence images of initial SBA lectin chip. Lines 1-3 were same. (B) Average FITC fluorescence intensities from (A). (C) Fluorescence images of SBA lectin chip incubated with Den-Gal/Cy5/FA (line 1), Den-Gal/Cy5 (line 2) and Den-Cy5/FA (line 3). (D) Average Cy5 fluorescence intensities from (C). Error bars represent ± S.D. (n = 3).

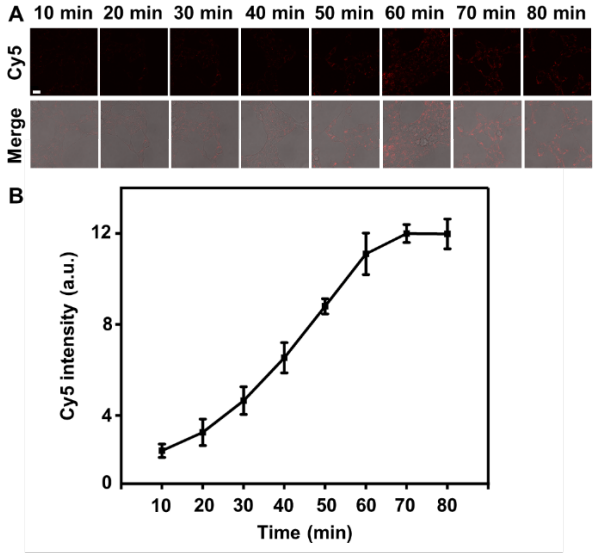

Fig. S6 (A) CLSM images of cells treated with Den-Gal/Cy5/FA for regular time intervals. (B) Average Cy5 fluorescence intensities from (A). Error bars represent ± S.D. (n = 3). Scale bar: 20 μm.

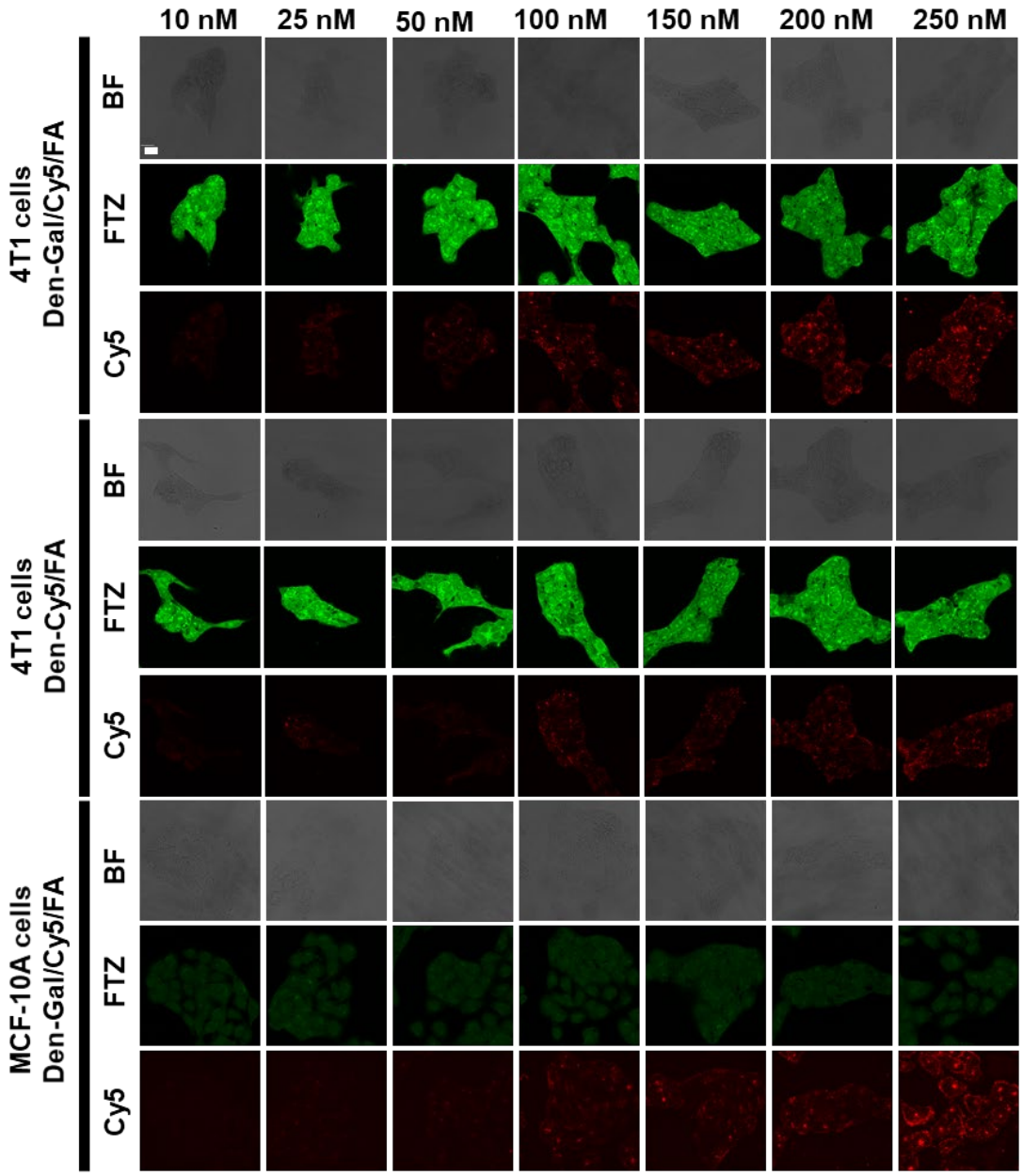

Fig. S7 CLSM images of 4T1 and MCF-10A cells after incubation with 10, 25, 50, 100, 150, 200 and 250 nM Den-Gal/Cy5/FA or Den-Cy5/FA for 1 h, and then treated with paraformaldehyde fixing, $NaIO_4$ oxidation and FTZ labeling. Scale bar: 20 μm.

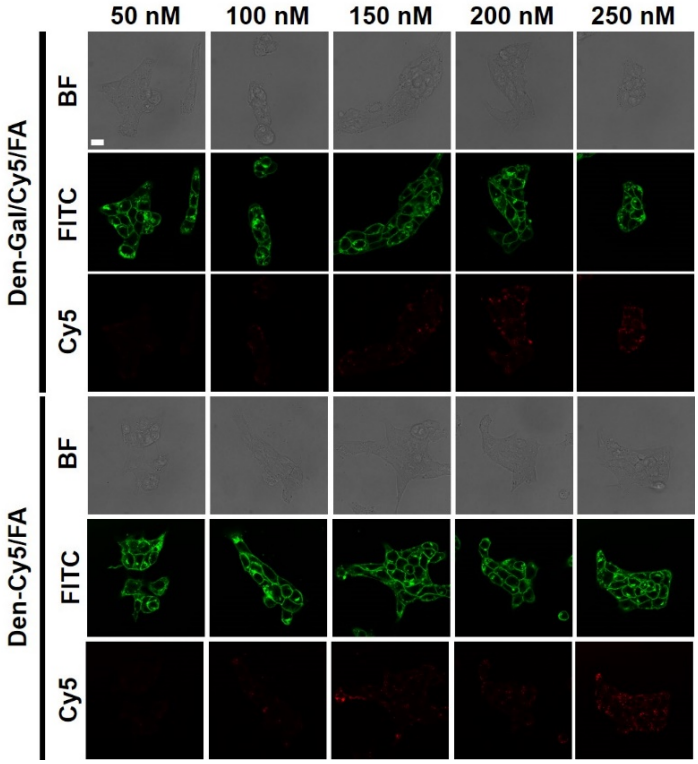

Fig. S8 CLSM images of 4T1 cells after incubation with 50, 100, 150, 200 and 250 nM Den-Gal/Cy5/FA or Den-Cy5/FA for 1 h, and then treated with paraformaldehyde fixing, Triton X-100 permeation and FITC-SNA labeling. Scale bar: 20 μm.

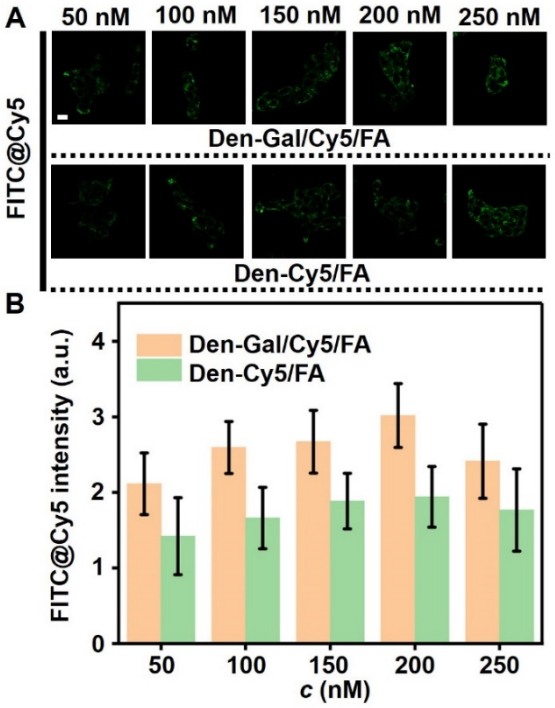

Fig. S9 (A) Colocalization of FITC fluorescence with Cy5 fluorescence obtained by collecting the FITC fluorescence within Cy5 fluorescence area in Figure S8. Scale bar: 20 μm. (B) Statistical FITC@Cy5 intensities from (A). Error bars represent ± S.D. (n = 3).

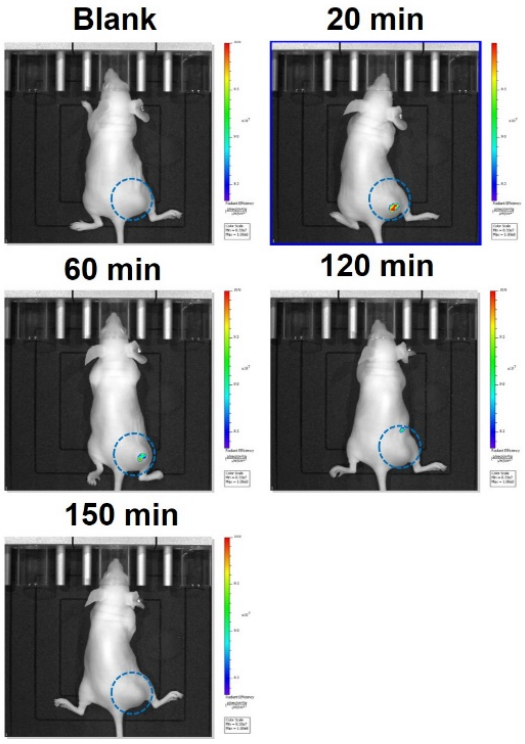

Fig. S10 *In vivo* fluorescence imaging of the 4T1 tumor bearing mouse after subcutaneous injection of 100 μL 1 μM Den-Gal/Cy5/FA for different times.

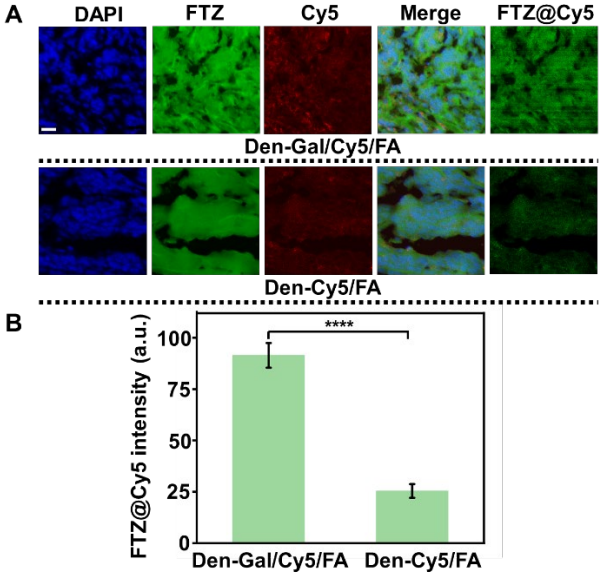

Fig. S11 (A) CLSM images of the tumor tissue slices from Den-Gal/Cy5/FA or Den-Cy5/FA treated mouse after DAPI staining, $NaIO_4$ oxidation and FTZ labeling. Scale bar: 20 μm. (B) Statistical FTZ@Cy5 intensities from (A). Error bars represent ± S.D. (n = 3). Statistical significance is determined by unpaired two-tailed Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

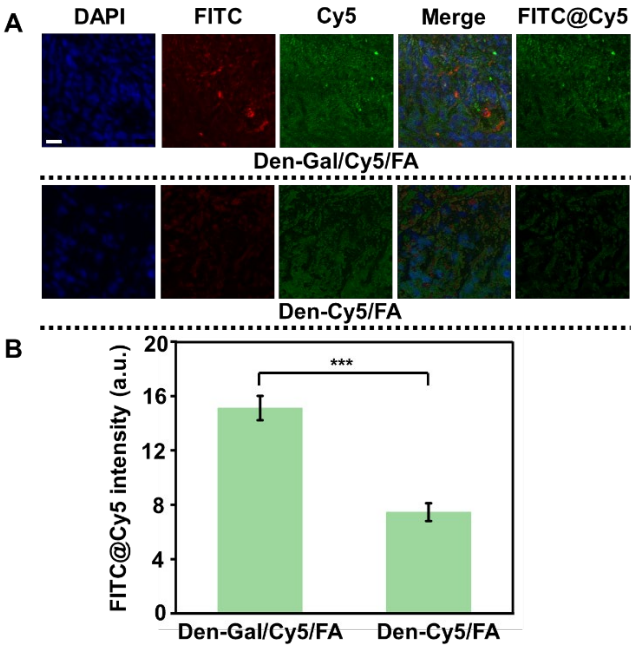

Fig. S12 (A) CLSM images of the tumor tissue slices from Den-Gal/Cy5/FA or Den-Cy5/FA treated mouse after paraformaldehyde fixing, DAPI staining, Triton X-100 permeation and FITC-SNA labeling. Scale bar: 20 μm. (B) Statistical FITC@Cy5 intensities from (A). Error bars represent ± S.D. (n = 3). Statistical significance is determined by unpaired two-tailed Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

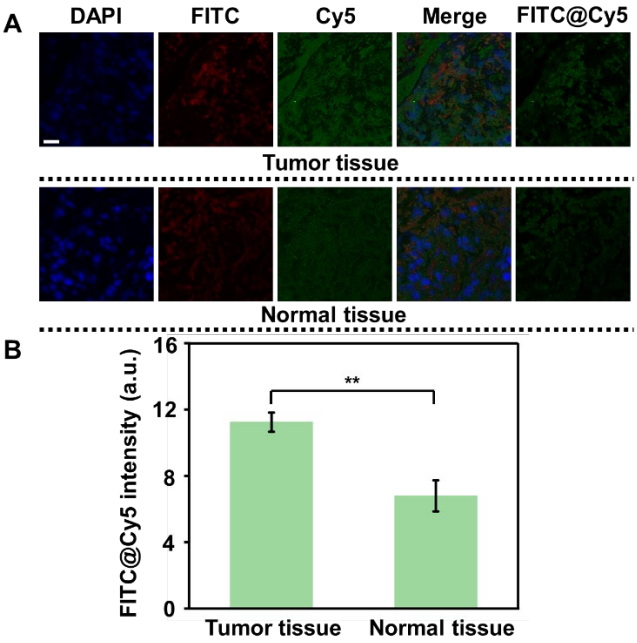

Fig. S13 (A) CLSM images of the tumor and normal tissue slices from Den-Gal/Cy5/FA treated mouse after paraformaldehyde fixing, DAPI staining, Triton X-100 permeation and FITC-SNA labeling. Scale bar: 20 μm. (B) Statistical FITC@Cy5 intensities from (A). Error bars represent ± S.D. (n = 3). Statistical significance is determined by unpaired two-tailed Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).