Electronic Supplementary Material (ESI) for MedChemComm.

Electronic Supporting Information

Enhancement of aqueous stability and fluorescence brightness of indocyanine green using small calix[4]arene micelles for near-infrared fluorescence imaging

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Experimental details

1. Materials and methods

1-bromohexane and *p*-sulfonatocalix[4]arene were purchased from Tokyo Chemical Industry. Dimethyl sulfoxide and sodium hydroxide were purchased from Wako Pure Chemical Industries. 8-anilino-1-naphthalenesulfonate (ANS) was purchased from Tokyo Chemical Industry. Indocyanine green (ICG) was purchased from Sigma-Aldrich. Anti-HER2 monoclonal antibody (Herceptin) was purchased from Chugai Pharmaceutical. Sulfo-succinimidyl derivative of ICG (ICG-EG4-Sulfo-OSu) was purchased from DOJINDO.

2. Synthesis of hexyl ether of *p*-sulfonatocalix[4]arene (**S4-6**)

The hexyl ether of *p*-sulfonatocalix[4]arene was synthesized according to the literature method.[1, 2] A typical procedure was as follows: *p*-sulfonatocalix[4]arene (1.0 g, 1.3 mmol) was mixed with NaOH (1.0 g, 25 mmol) in 5 mL water and 20 mL dimethyl sulfoxide. Then, 4 mL (29 mmol) of 1-bromohexane was added, and the mixture was heated at 50 °C for 24 h. After cooling, the solution was diluted with methanol to precipitate. The precipitate was dissolved in 5 mL of water and an insoluble material was removed by filtration. The product was precipitated from the filtrate by addition of ethanol. This operation was repeated three times in order to remove NaBr.

3. Preparation of **S4-6** micelles and ICG incorporated **S4-6** micelles

Ten mg of **S4-6** was dissolved to 1 mL of phosphate buffered saline (10 mM PBS), and the solution was sonicated for 1min. The resulting **S4-6** micelle solution was filtered with a 0.45 μm filter. To this solution, an aqueous solution (10 μL) of ICG (1 mM in water) was added under stirring to give ICG incorporated **S4-6** micelles.

4. Preparation of ICG conjugated Herceptin (ICG-Ab) and ICG-Ab incorporated **S4-6** micelles

To a 0.1 M Na_2CO_3 solution of Herceptin (1mg/mL), 20 μL of a dimethyl sulfoxide solution of ICG-EG4-Sulfo-OSu (1 mM) was slowly added. The coupling reaction between ICG-EG4-Sulfo-OSu and Herceptin was performed at 4 ^oC over night. To remove unreacted ICG-EG4-Sulfo-OSu, the solution was passed through a desalting column (PD-10, GE Healthcare) using PBS.

An aqueous solution of **S4-6** micelles (10 mg/mL in 10 mM PBS) was slowly added to an ICG-Ab solution ([Ab] =1mg/mL, PBS) to give ICG-Ab incorporated **S4-6** micelles.

5. Optical measurements

Fluorescence spectra were measured by excitation at 350 nm for ANS and at 760 nm for ICG using a spectrofluorometer (FP8200, JASCO Corporation). Absorption spectra of ICG were measured using a V-670 spectrometer (JASCO Corporation). Absolute quantum yields of ICG and ICG-Ab were measured with a fluorescence spectrometer, C9920-03 (Hamamatsu Photonics, Japan). Fluorescence decay curves for ICG were measured by excitation at 760 nm using a time-correlated single-photon counting (Horiba Fluoro Cube)

6. TEM measurements

The morphologies of **S4-6** micelles were observed by TEM using a Hitachi H-7500 microscope operating at an acceleration voltage of 80 kV. The TEM sample (10 mg/mL **S4-6** in PBS) was prepared by dropping the sample solution onto a copper grid, and then stained with 2% (w/v) uranium acetate.

7. Hydrodynamic size and zeta potential measurements

Hydrodynamic sizes and zeta potentials of **S4-6**, Ab, **S4-6** micelles, ICG incorporated **S4-6** micelles, and ICG-Ab incorporated **S4-6** micelles were determined by using a dynamic light scattering system (Zetasizer Nano-ZS, Malvern).

8. Cell viability

To evaluate the cytotoxicity of **S4-6** micelles, different concentrations of **S4-6** micelles (0-100 μM, PBS) were added to the culturing media and incubated with HeLa cells for 30 min at 37 ^{o}C under 5% CO_2 and a humidified atmosphere. After the incubation, the cells were washed and resuspended with PBS, stained with a trypan blue solution, and their viabilities were determined using a Countess cell counter (Invitrogen).

9. Cellular imaging

Cellular imaging was performed using a fluorescence microscope, BZ-X700 (KEYENCE CORPORATION, Japan). KPL4 cells were transferred to collagen coated glass bottom dishes (D11134H, Matsunami 35 mm), and incubated in Dulbecco's Modified Eagle Media (DMEM) with 20% Fetal Bovine Serum (FBS) overnight to adhere to the dish bottom. The cells were washed with PBS three times and incubated with ICG-Ab or ICG-Ab/**S4-6** ([Ab] = 1mg/mL, [**S4-6]** = 10mg/mL) dissolved in PBS for 15 min. Then, the cells were washed with PBS three times, and filled with Opti-MEM (Life technologies). Microscope setups are as follows: the excitation and emission filters were FF01-769/41 and FF01-832/37 (Semrock) respectively. The objective lens was Nikon PlanApo λ 40x. The exposure time was 30 s.

10. *In vivo* NIR fluorescence imaging

Lymph node imaging: mice (HOS:HR1, 4 weeks aged female) were anesthetized on a microscope stage. Two hundred milliliter of ICG ([ICG] = 5 μM) dissolved in PBS was injected into the footpad. The femoral to lower abdominal area was illuminated by laser light (785 nm, BWF-785-450E/55371, B&W Tek) and fluorescence was detected using an EM-CCD camera (DU-888E, Andor iXon) through an emission filter (> 820 nm).

Liver and tumor imaging: a suspension of KPL-4cells (0.5×10^7 cells per mouse) was

transplanted to the dorsal skins of 5-week old female BALB/c nu/nu mice (Charles River Japan). After several weeks, we selected a mouse bearing a tumor less than 10 mm in diameter for imaging. One hundred μL of ICG-Ab ([ICG] = 5 μM) was injected into a xenografted mouse *via* a tail vein. NIR fluorescence images (ex: 760 nm, em: 830 ± 20 nm) were taken after the injection of ICG-Ab probes using an *in vivo* fluorescence imaging system (Bruker, MS FX PRO). Exposure time of the excitation light was 30 s. All experiments were performed in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by RIKEN Animal Care and Use Committee.

References

1 S. Shinkai, S. Mori, H. Koreishi, T. Tsubaki and O. Manabe, *J. Am. Chem. Soc.,* 1986, **108**, 2409-2416.

2 T. Jin, F. Fujii, H. Sakata, M. Tamura and M. Kinjo, *Chem. Commun.,* 2005, 4300-4302.

Additional figures

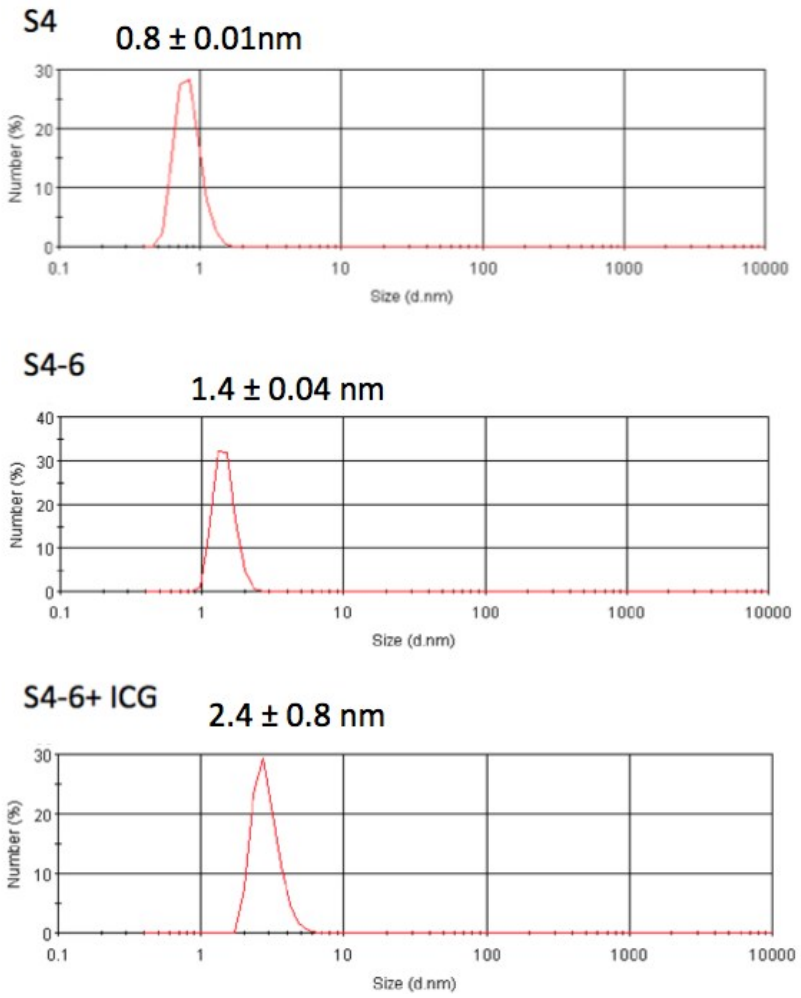
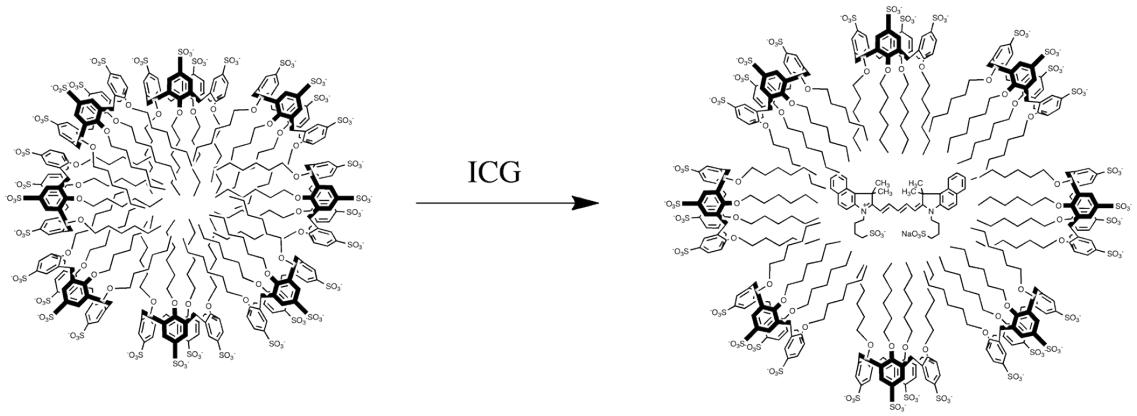


Fig. S1 Hydrodynamic diameter of **S4** (10 mg/mL), **S4-6** (10 mg/mL), and **S4-6** (10 mg/mL) + ICG (1 μM) in PBS (pH = 7.4), determined by DLS.



S4-6 micelle

ICG incorporated **S4-6** micelle

Scheme S1 Schematic representation of **S4-6** micelle and ICG-incorporated **S4-6** micelle.

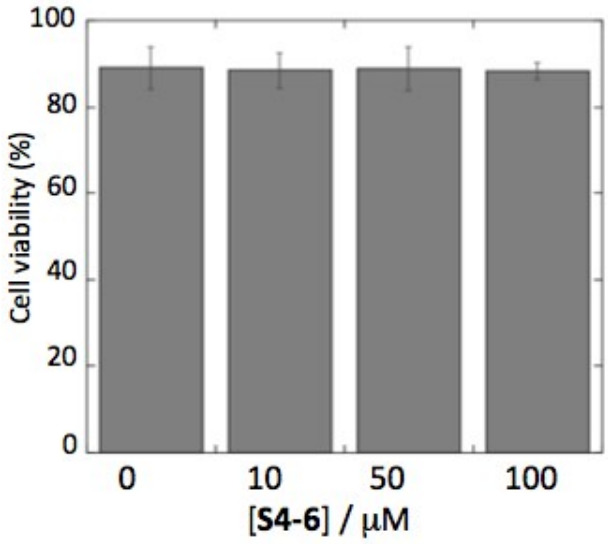


Fig. S2 Viability of HeLa cells in the absence and presence of **S4-6** micelles(0-100 μM). Cell viability was measured after the incubation with **S4-6** micelles for 30 min.

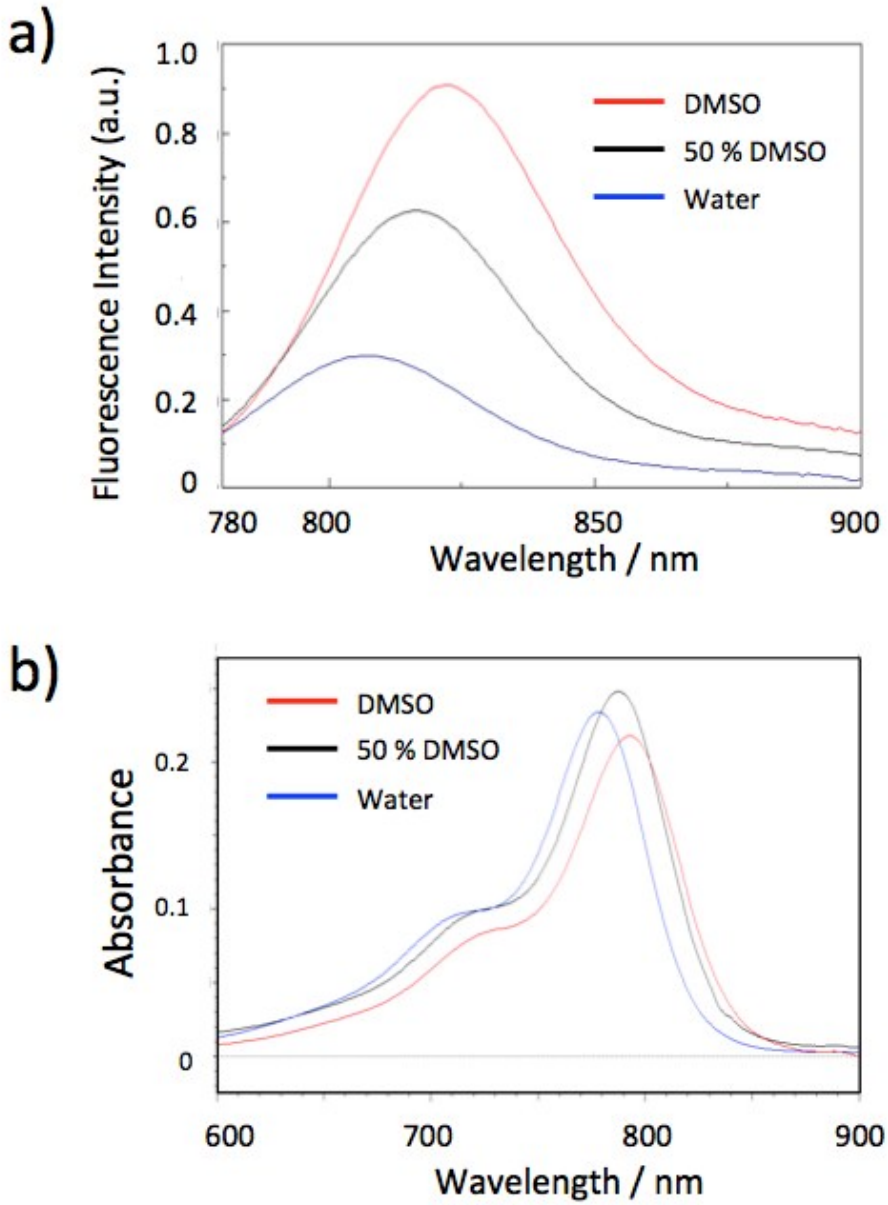


Fig. S3 Fluorescence spectra (a) and absorption spectra (b) of ICG in water, 50 % DMSO (50% water) and 100 % DMSO. [ICG] = 1.5 μM.

Table S1 Zeta potential of **S4-6** micelles, ICG incorporated **S4-6** micelles and ICG-Ab incorporated **S4-6** micelles in PBS (pH = 7.4). [**S4-6**] = 10 mg/mL, [Ab] = 1 mg/mL.

Sample	Zeta Potential
S4-6 micelle	- 24.1 mV
ICG + **S4-6** micelle	- 22.7 mV
ICG-Ab + **S4-6** micelle	-10.6 mV

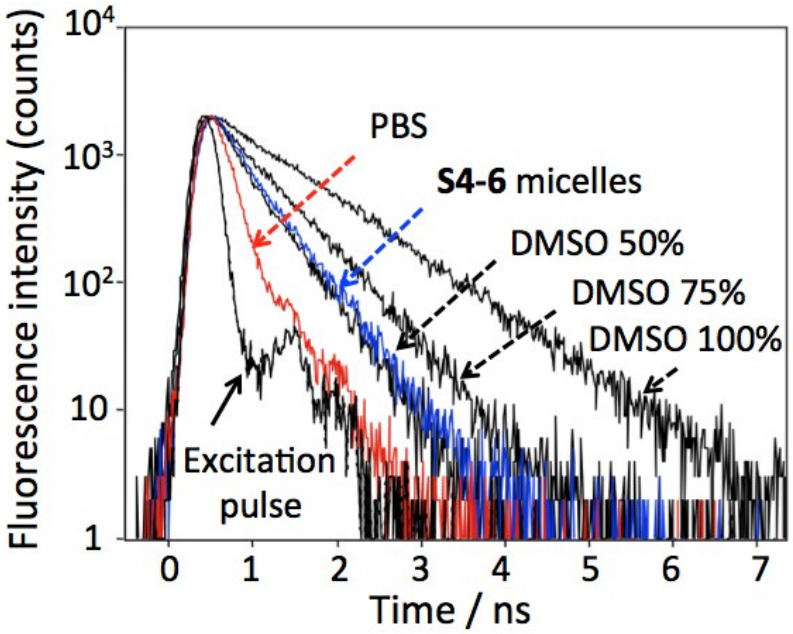


Fig. S4 Fluorescence decay curves of ICG in PBS, **S4-6** micelles (10 mg/mL, PBS), 50 % DMSO (50% PBS), 75 % DMSO (25% PBS), and 100 % DMSO. [ICG] = 1 μM.

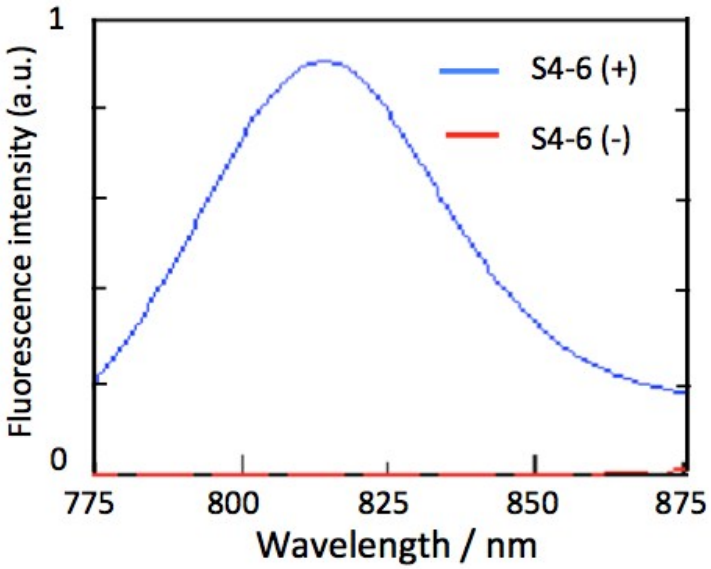


Fig. S5 Fluorescence spectra of ICG (1 μM, PBS) after one month since the preparation of ICG aqueous solution in the absence and presence of **S4-6** (10 mg/mL) micelles.

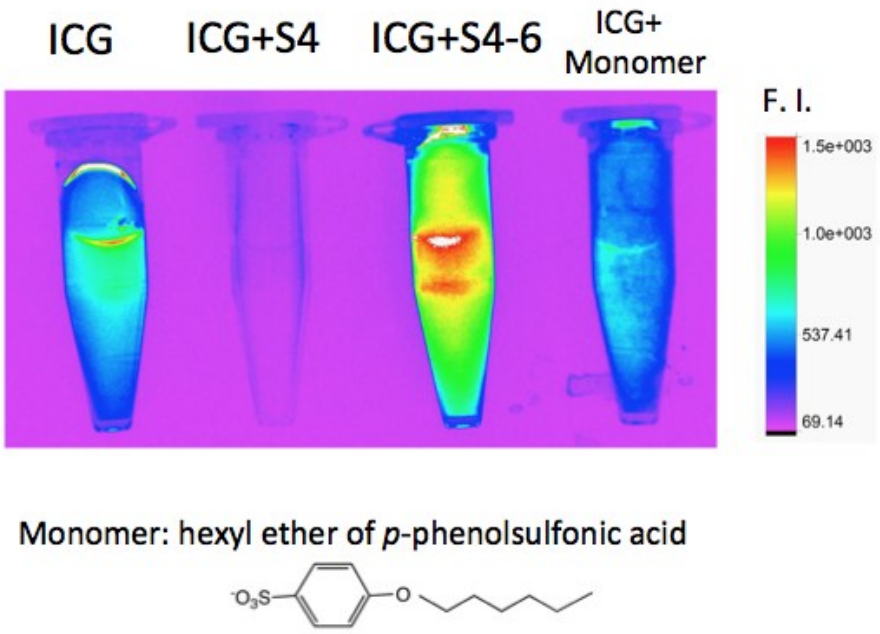


Fig. S6 NIR fluorescence image (830 ± 20 nm) of ICG (1 μM, PBS), ICG (1 μM, PBS)+**S4** (10 mg/mL), ICG (1 μM, PBS)+ **S4-6** (10 mg/mL), and ICG (1 μM, PBS)+ monomer (10 mg/mL). Excitation: 760 nm.

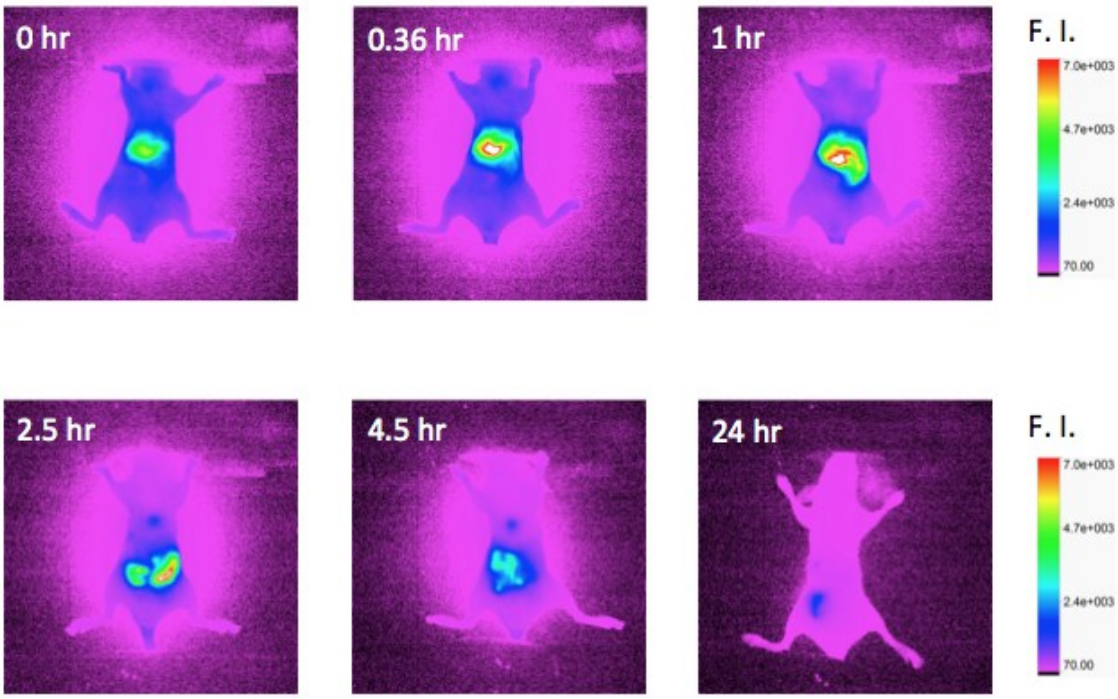


Fig. S7 Time course of NIR fluorescence image of a nude mouse, where ICG (5 μM) incorporated **S4-6** micelles (10 mg/ mL in PBS) was intravascularly injected *via* a tail vein of the mouse. Excitation: 760 nm. Emission: 830 ± 20 nm.

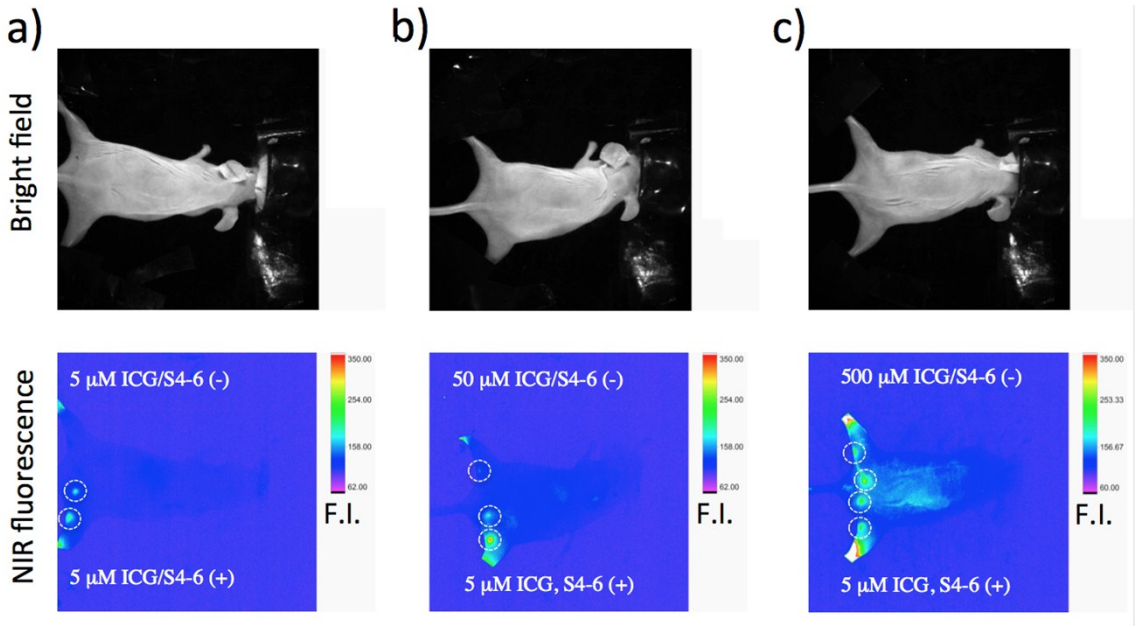


Fig. S8 Comparison of NIR fluorescence visualization of lymph nodes in mice, where aqueous solutions of ICG/**S4-6** (+) ([ICG] =5 μM, [**S4-6**]＝10 mg/mL) and ICG/**S4-6** (-) ([ICG] =5, 50, and 500 μM, [**S4-6**]＝0 mg/mL) were injected to a footpad of the mouse. Upper panel: bright images. Lower panel: NIR fluorescence images. The NIR fluorescence images were taken using an 830 nm band pass filter with excitation at 760 nm. The positions of lymph nodes are shown as white circles.

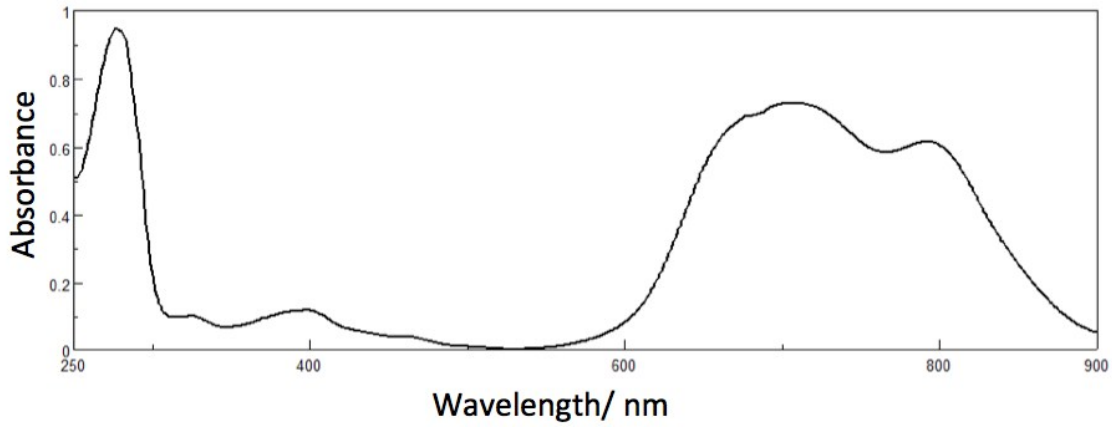


Fig. S9 Fluorescence spectrum of ICG conjugated Herceptin (ICG-Ab) in PBS. Molar extinction coefficients of Herceptin and ICG are is 2.1×10^5 $M^{-1}cm^{-1}$ at 280 nm and 1.28×10^5 $M^{-1}cm^{-1}$ at 795 nm, respectively.

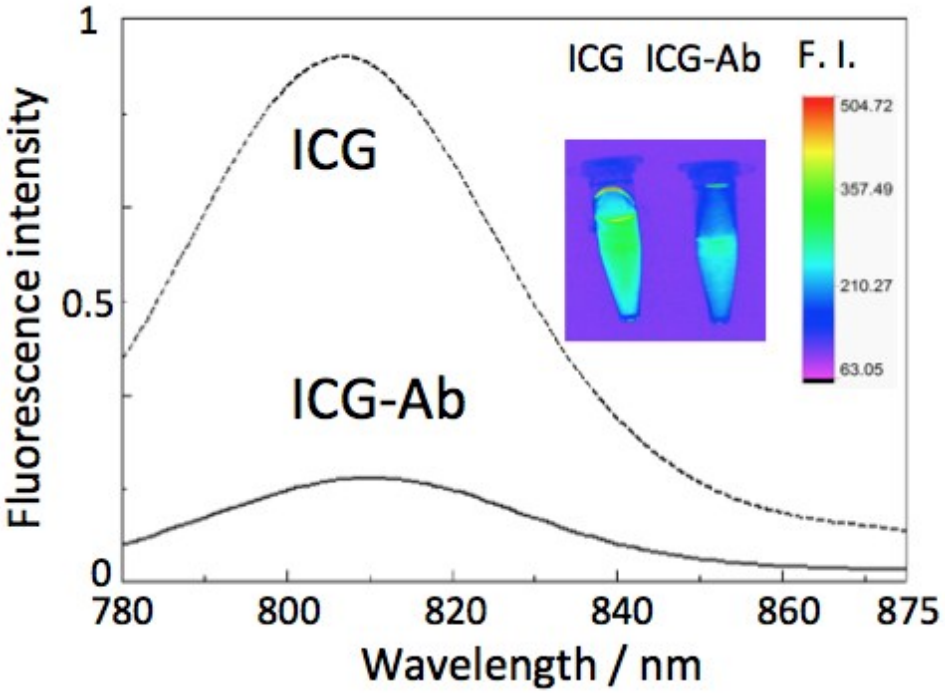


Fig. S10 Comparison of NIR fluorescence spectra of ICG and ICG-Ab in PBS. The absorbance of ICG at 760 nm was adjusted to be 0.2 for both sample solutions. Excitation: 760 nm. Inset is a NIR fluorescence image of the ICG and ICG-Ab solutions, which was taken using an 830 nm band pass filter (± 20 nm) with excitation at 760 nm.

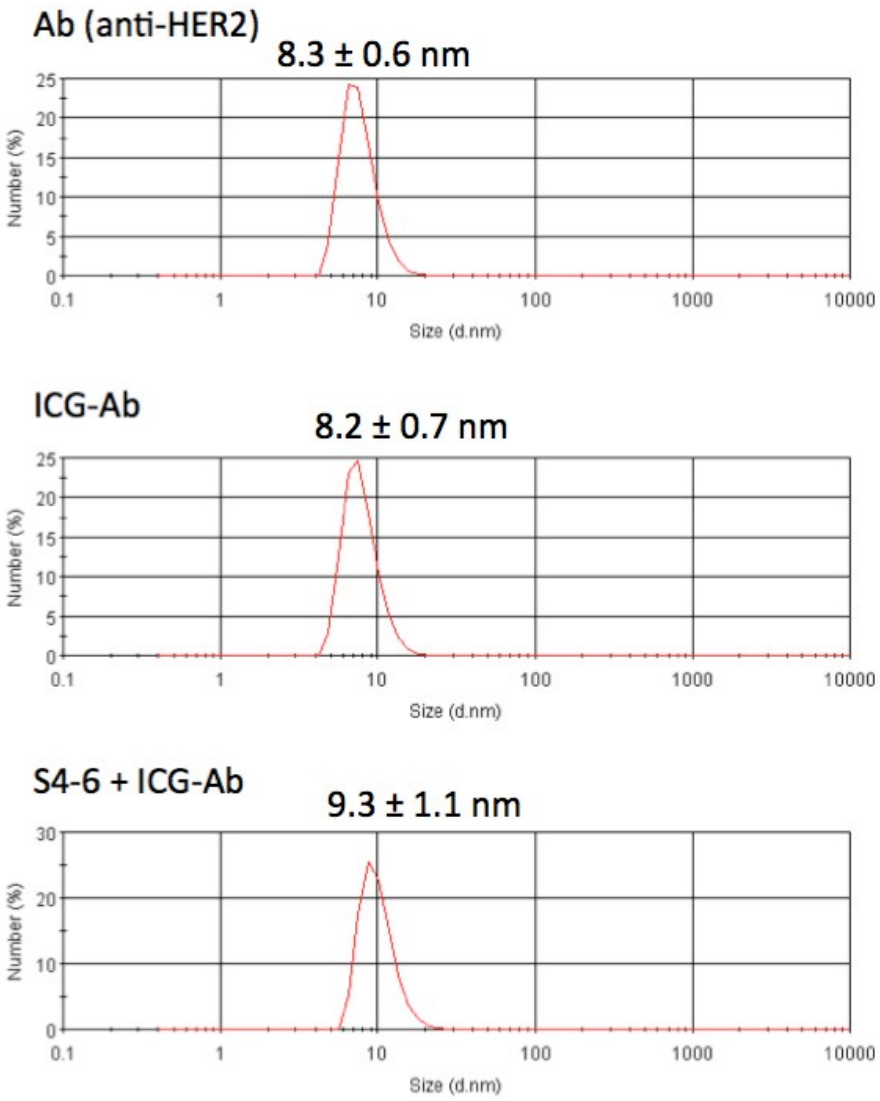


Fig. S11 Hydrodynamic diameter of Ab (Herceptin), ICG-Ab ([Ab] = 1 mg/ mL), and **S4-6** + ICG-Ab ([**S4-6**] = 10 mg/ mL, [Ab] = 1mg/ mL) in PBS (pH = 7.4), determined by DLS. [ICG] = 1μM.

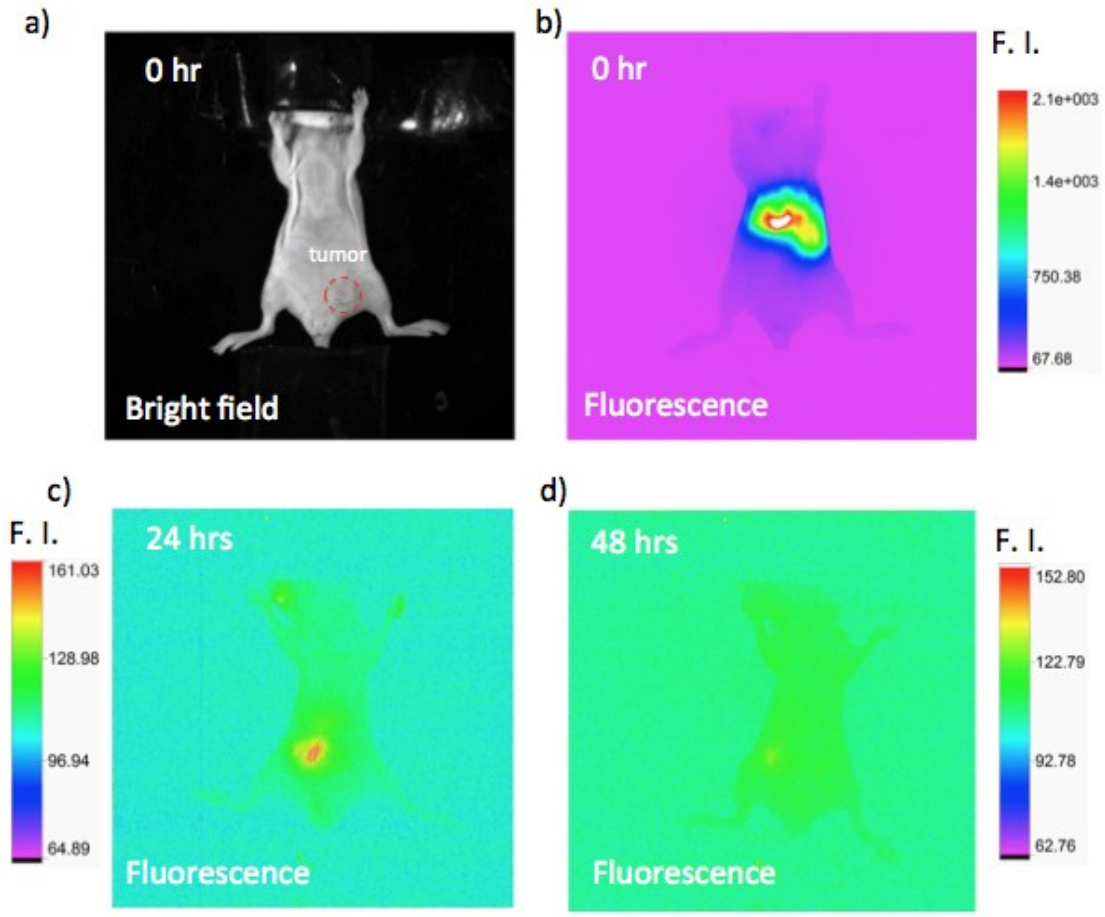


Fig. S12 Time course of the NIR fluorescence image of a nude mouse bearing a breast tumor injected by ICG incorporated **S4-6** micelles ([ICG] = 5 μM, [**S4-6**] = 10 mg/ mL in PBS) was intravascularly injected *via* a tail vein. Excitation: 760 nm. Emission: 830 ± 20 nm.

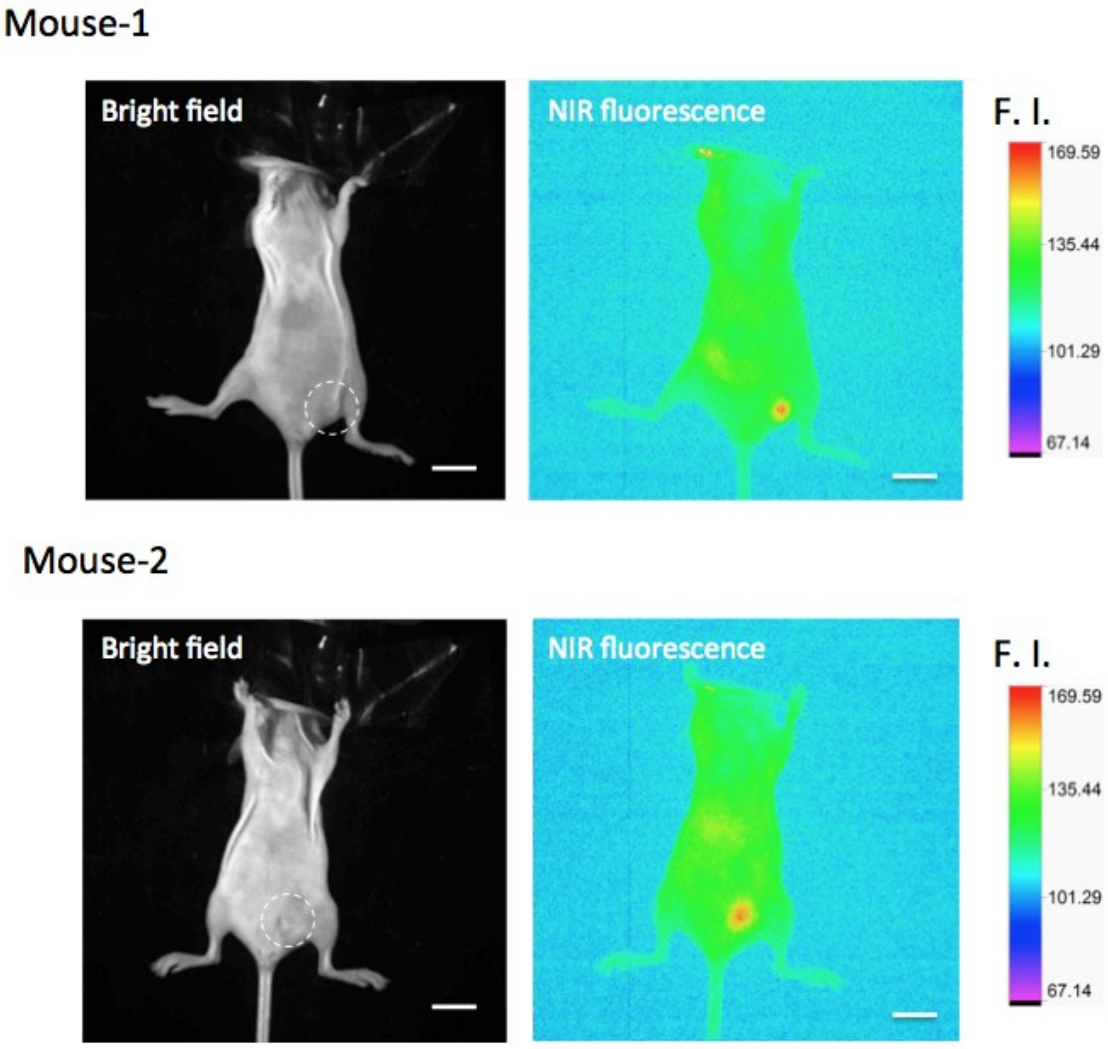


Fig. S13 NIR fluorescence images of nude mice (n = 5) bearing breast tumors with different size from 3-10 mm. The images were taken at 72hrs after the injection of ICG-Ab incorporated **S4-6** micelles ([ICG] = 5 μM, [Ab] = 1 mg/ mL, [**S4-6**] = 10 mg/mL in PBS). Excitation: 760 nm. Emission: 830 ± 20 nm. Scale bar, 10 mm.

Fig. S13 Cont.

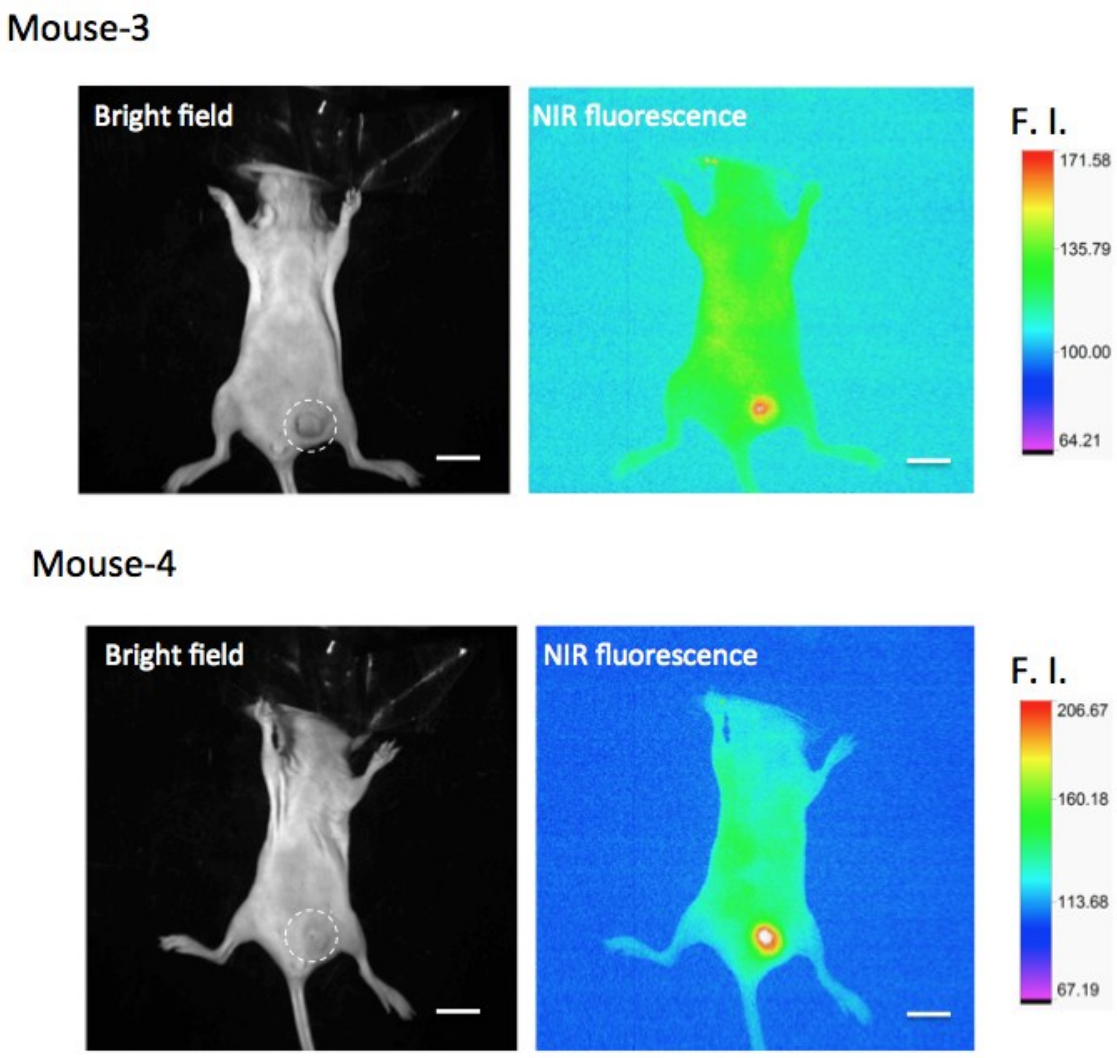
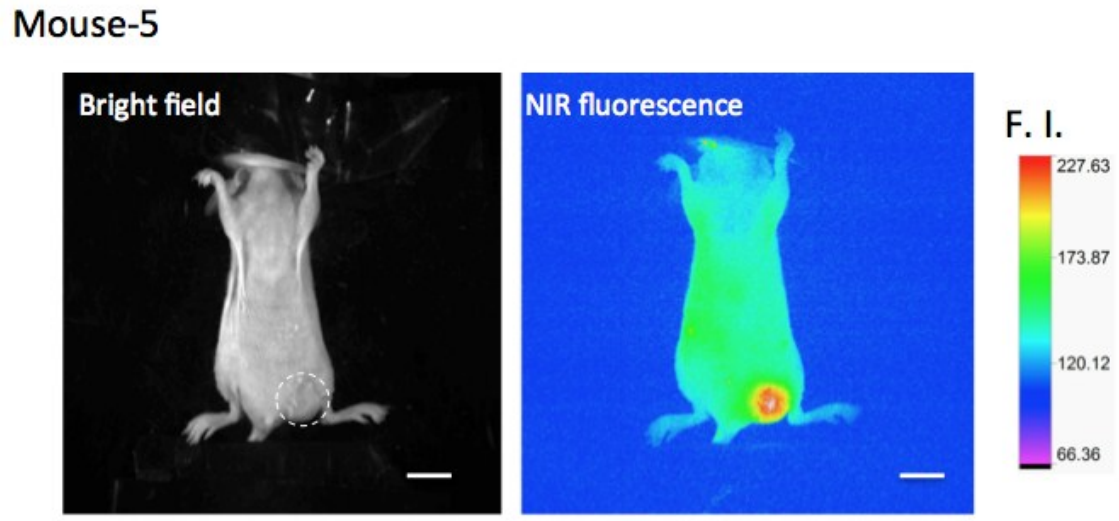

Mouse-3
Bright field
NIR fluorescence
F. I.
171.58
135.79
100.00
64.21
Mouse-4
Bright field
NIR fluorescence
F. I.
206.67
160.18
113.68
67.19

Fig. S13 Cont.


Mouse-5
Bright field
NIR fluorescence
F. I.
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120.12
66.36

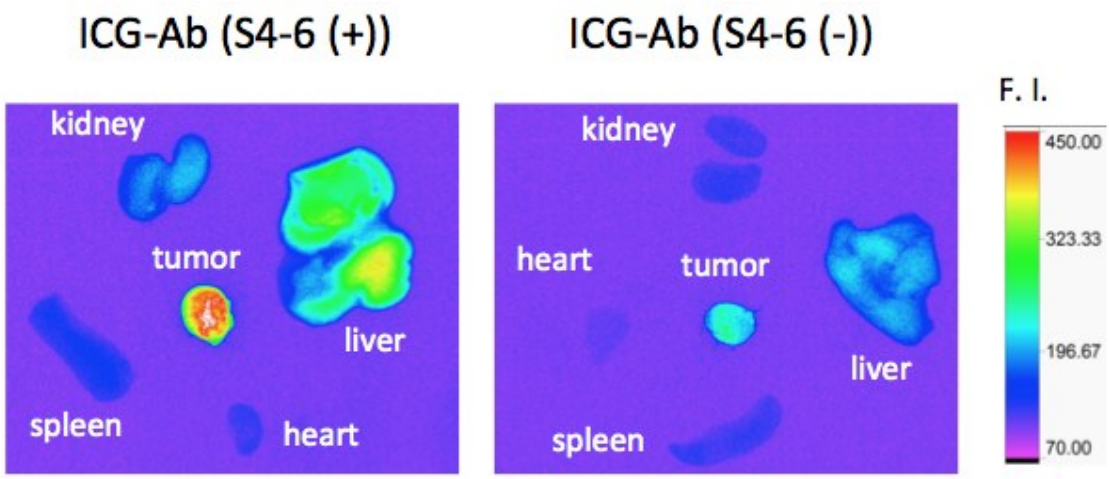

Fig. S14 *Ex vivo* NIR fluorescence images of isolated organs and tumors at 72 hrs after the injection of ICG-Ab incorporated **S4-6** micelles and ICG-Ab, where [Ab] = 1 mg/mL and [**S4-6**] = 10 mg/mL. Excitation: 760 nm. Emission: 830 ± 20 nm.