Supporting Information

Antigen-responsive molecular sensor enables real-time tumor-specific imaging

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Synthesis and characterization of trastuzumab-Alexa 647 conjugate (HER-Alexa647)

Alexa 647-NHS ester was purchased from Thermo Fischer Scientific. Trastuzumab (0.2 mg, 1.36 nmol) and amine-reactive Alexa 647-NHS ester (antibody: dye mole ratio = 1:10) were simultaneously dissolved in 0.3 mL phosphate-buffered saline (PBS, pH 7.4, 10 mM, NaCl, 137 mM) at 25℃ and reacted for 1 h under light-protected conditions with gentle shaking. The unbound fluorophores and byproducts were removed from the mixture using a Sephadex G25 gel filtration column (PD-10; GE Healthcare, Little Chalfont, UK). Next, purified trastuzumab-fluorophore conjugates were concentrated using Amicon Ultra-0.5 mL centrifugal filters (membrane cut off: 50 K, EMD Millipore, Billerica, MA, USA) and stored at 4℃ for use as a stock solution.

The average number of Alexa 647 conjugated to trastuzumab (i.e., degree of labeling, DL) was determined by measuring the absorbance of trastuzumab-Alexa 647 conjugates in PBS using an UV/Vis spectrophotometer. The DL of the synthesized trastuzumab-Alexa 647 conjugates was calculated to be 3.63.

***In vitro* cell studies with HER-Alexa647**

SK-BR-3 cells were plated at a density of 5×10^4/well on a 4-well Lab-Tek II chambered coverglass and incubated overnight for cell attachment. Next, HER-Alexa 647 (10 μg/mL) was added to the medium and incubated for 30 min. Then, confocal fluorescence images (λ_{ex} 633 nm, λ_{em} 647–754 nm) were acquired without washing the cells. The confocal images were analyzed using LSM 5 image browser, and profiling was conducted using Axiovision software (Carl Zeiss).

***In vivo* animal study with HER-Alexa647**

All animal studies were approved by the Institutional Animal Care and Use Committee. Female athymic nude mice (Balb/c-nu, 5- week-old) were used for *in vivo* experiments. Calu-3 (5×10^6 cells/0.1 mL serum-free medium) was implanted subcutaneously into the right hind flank of each mouse. Three mice were used for *in vivo* imaging analysis when tumor sizes reached approximately 190 mm^3. Normal mouse without tumor was used as the untreated control. For *in vivo* NIR fluorescence imaging, three mice with Calu-3 tumors received intravenous injections of HER-Alexa 647 (50 μg conjugate/50 μL PBS/mouse). Normal mouse without tumor received intravenous injections of 50 μL PBS for comparison. NIR fluorescence images were obtained using the IVIS Lumina imaging system (λ_{ex} = 620/10 nm, λ_{em} = 670/20 nm) at 1, 5, and 24 h after injection.

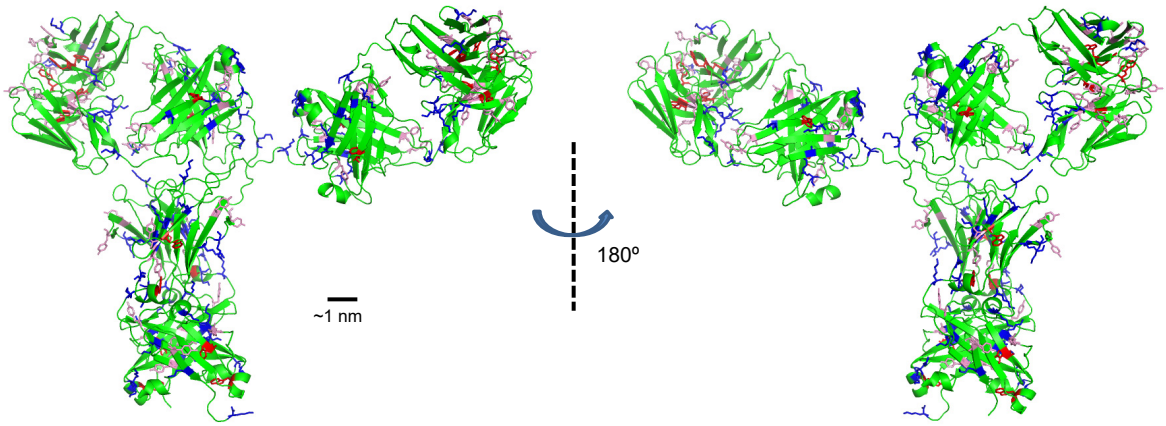

Figure S1. 3D Structure of trastuzumab. Lysine, tryptophan, and tyrosine are marked in blue, red, and pink, respectively. The sequence of trastuzumab was obtained from DrugBank and structures were predicted by modeling. Modeling was performed using SWISS-MODEL [1] and subsequent manual modeling with PDB entry 1HZH as a structural template using program Coot [2]. Trastuzumab contains 22 tryptophan (Trp, shown in red), 62 tyrosine (Tyr, shown in pink), and 90 lysine (Lys, shown in blue) residues.

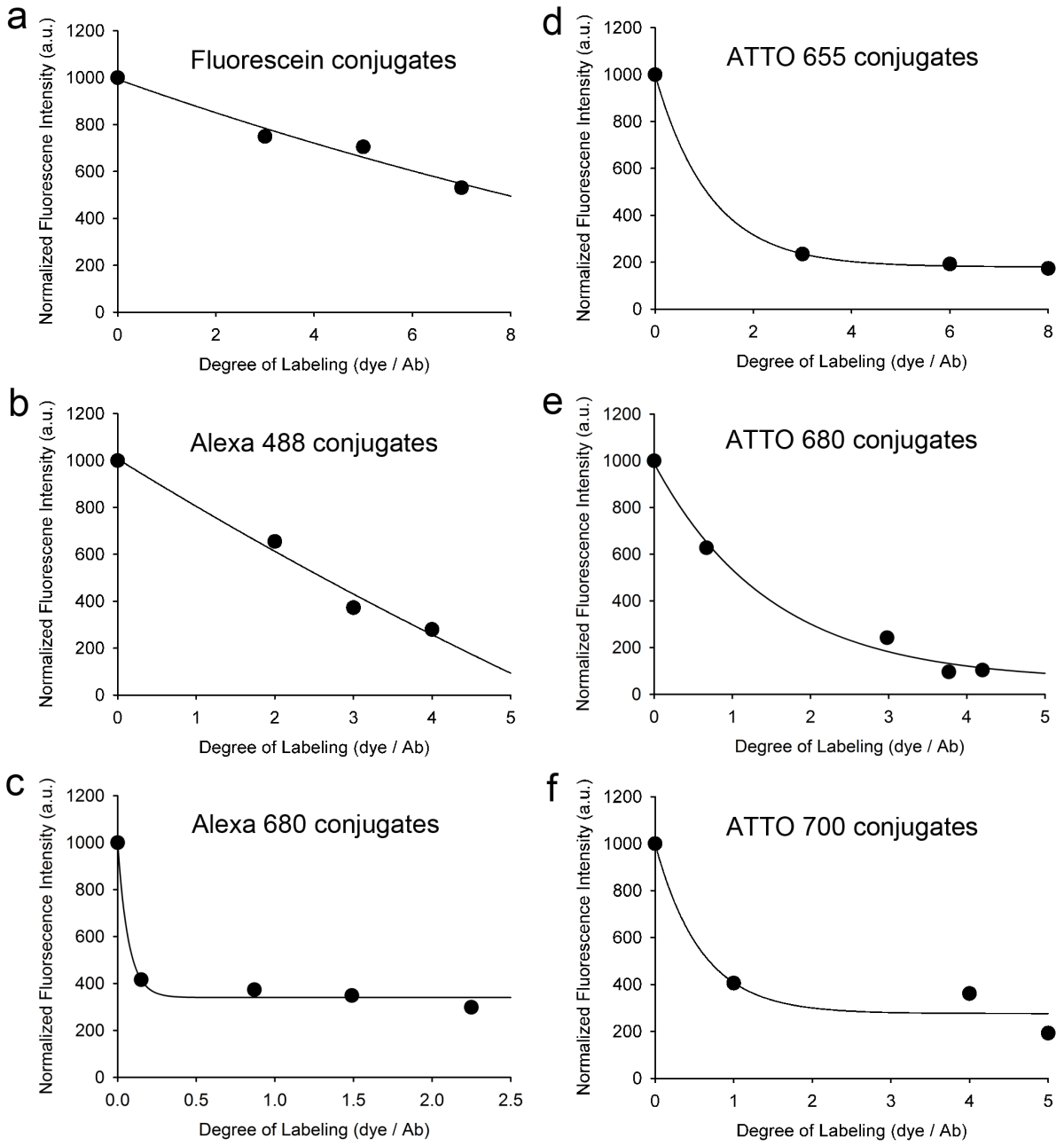

Figure S2. Fluorescence intensities vs. degree of labeling. Fluorescence intensities of trastuzumab-fluorophore conjugates were compared at an equimolar concentration of each dye. a) trastuzumab-fluorescein conjugates, b) trastuzumab-Alexa 488 conjugates, c) trastuzumab-Alexa 680 conjugates, d) trastuzumab-ATTO 655 conjugates, e) trastuzumab-ATTO 680, f) trastuzumab-ATTO 700 conjugates.

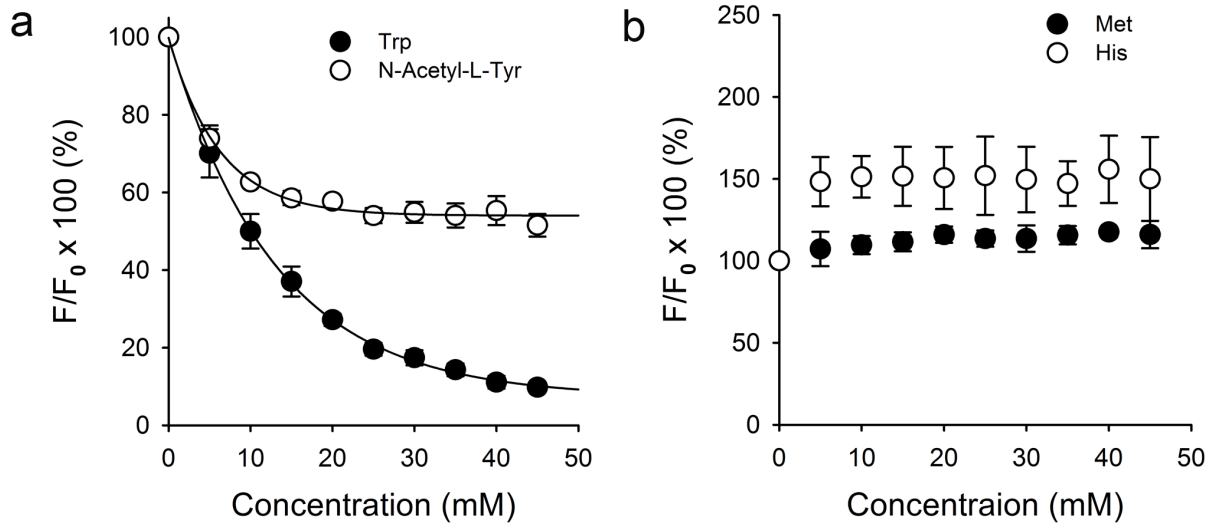

Figure S3. Relevant changes in fluorescence intensity (F) of free ATTO 680 dye in the presence of Trp, N-Acetyl-L-Tyr, Met, and His at various concentrations. Fluorescence intensities of free ATTO 680 in the absence (F_0) of amino acids is set to 100% (n = 3).

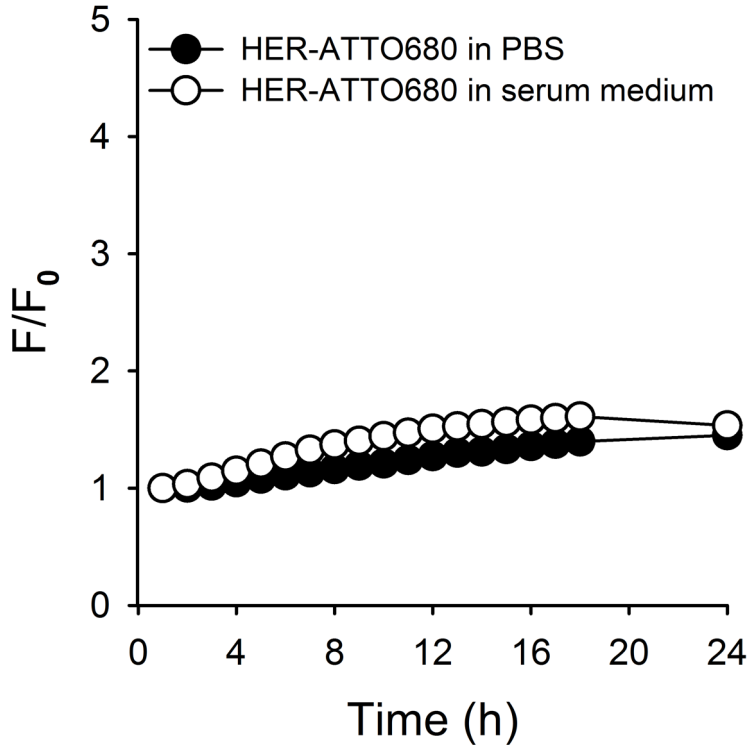

Figure S4. Stability of HER-ATTO680 in the presence of serum proteins. HER-ATTO680 (1 μM dye equivalent) was incubated in either PBS or RPMI media containing 10% fetal bovine serum, and then fluorescence intensity of the solution (λ_{ex} 620 nm, λ_{em} 700 nm) was measured periodically for 24 h. No significant changes in the fluorescence intensities of HER-ATTO680 were observed for 24 h when incubated in PBS buffer (-●-) and serum-containing medium (·○·), indicating that the quenched state of the conjugate was stably maintained in the serum.

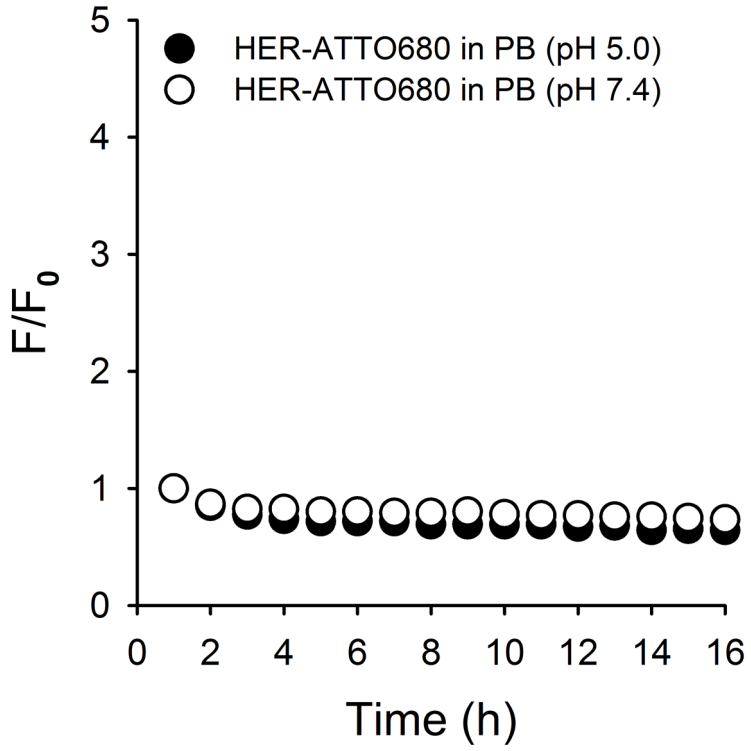

Figure S5. Stability of HER-ATTO680 at different pH conditions. The fluorescence intensities of HER-ATTO680 in phosphate buffer solution was measured periodically for 16 h (λ_{ex} 620 nm, λ_{em} 700 nm).

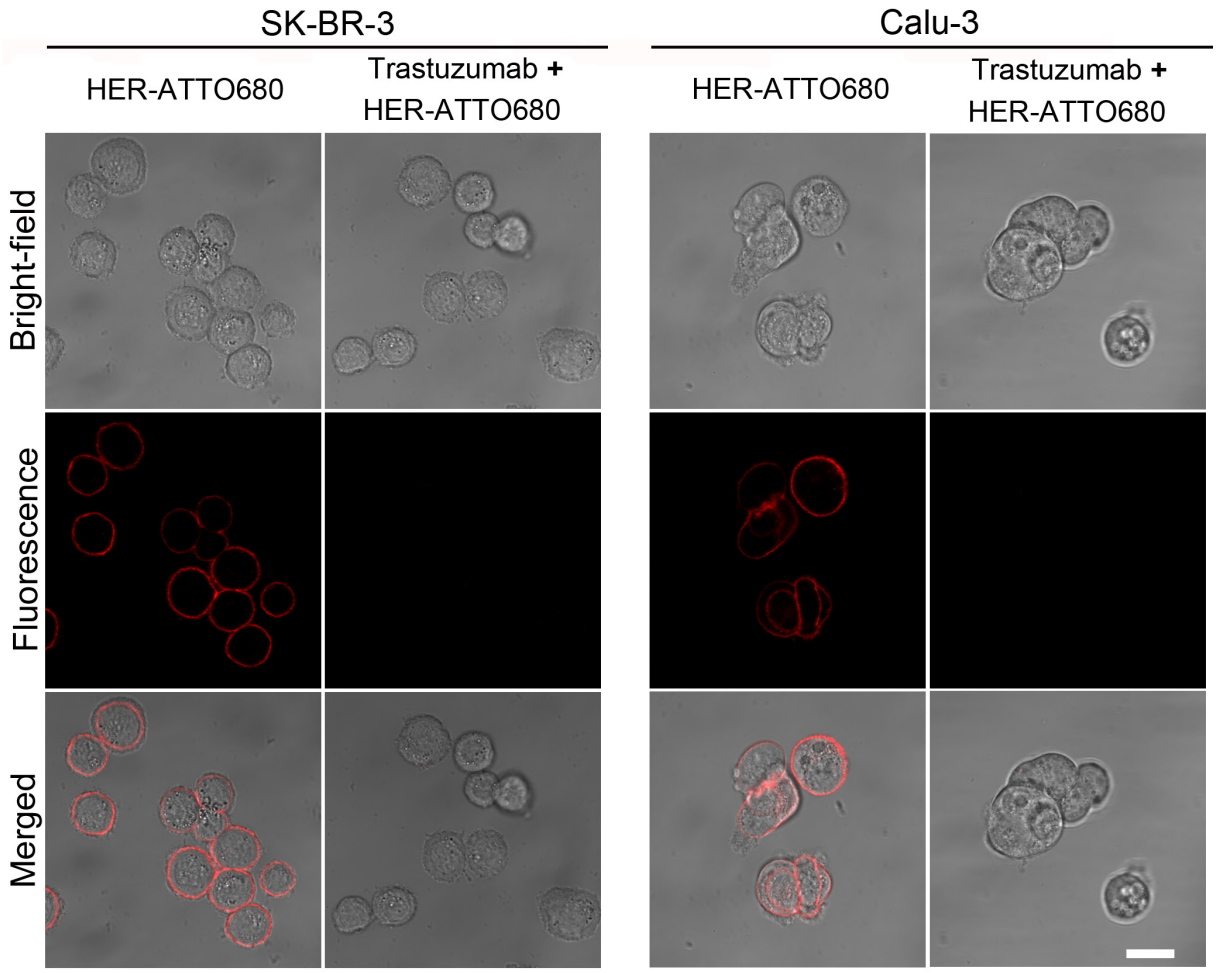

Figure S6. Confocal fluorescence images showing the targeting specificity of HER-ATTO 680. Pretreatment of HER2-positive cell lines with unlabeled trastuzumab inhibited the specific binding of HER-ATTO680 to these cells. Scale bar = 20 μm.

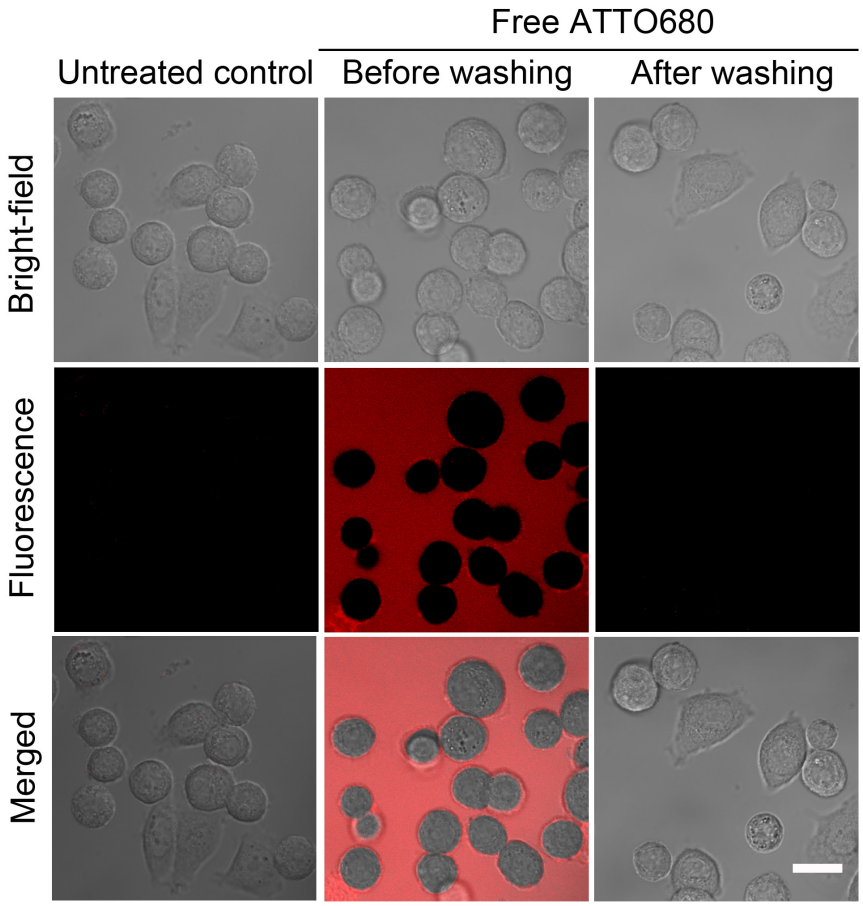

Figure S7. Confocal fluorescence microscopy images of SK-BR-3 cells treated with free ATTO680-COOH. The cells were treated with 1 μM free ATTO680-COOH for 30 min. Next, fluorescence images (λ_{ex} 633 nm, λ_{em} 647–754 nm) were obtained before and after washing the cells. High fluorescence signals were observed in the extracellular region before washing the cells. All fluorescence signals disappeared after washing the cells, indicating no nonspecific adsorption of free dyes on the cell surface under the test conditions (scale bar = 20 μm).

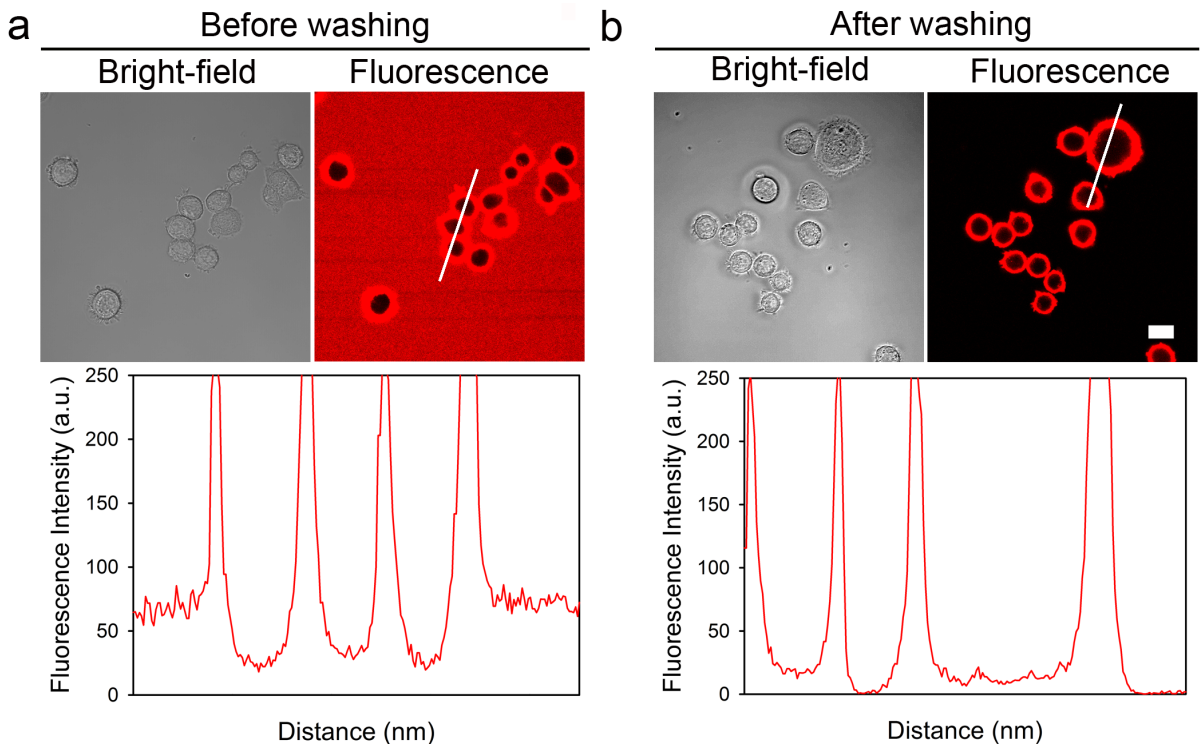

Figure S8. Confocal fluorescence microscopy images of SK-BR-3 cells treated with HER-Alexa647 (λ_{ex} 633 nm, λ_{em} 638–759 nm). HER-Alexa647 (DL of 3.63, 10 μg/mL) was treated for 30 min and confocal fluorescence images were obtained before (a) and after (b) washing the cells. Quantitative analysis of the fluorescence intensities of fluorescence images (i.e., the area indicated by white line in the upper panel) is shown in the lower panel. High fluorescence signals were observed in the extracellular region before washing the cells, which disappeared after washing. Scale bar = 20 μm.

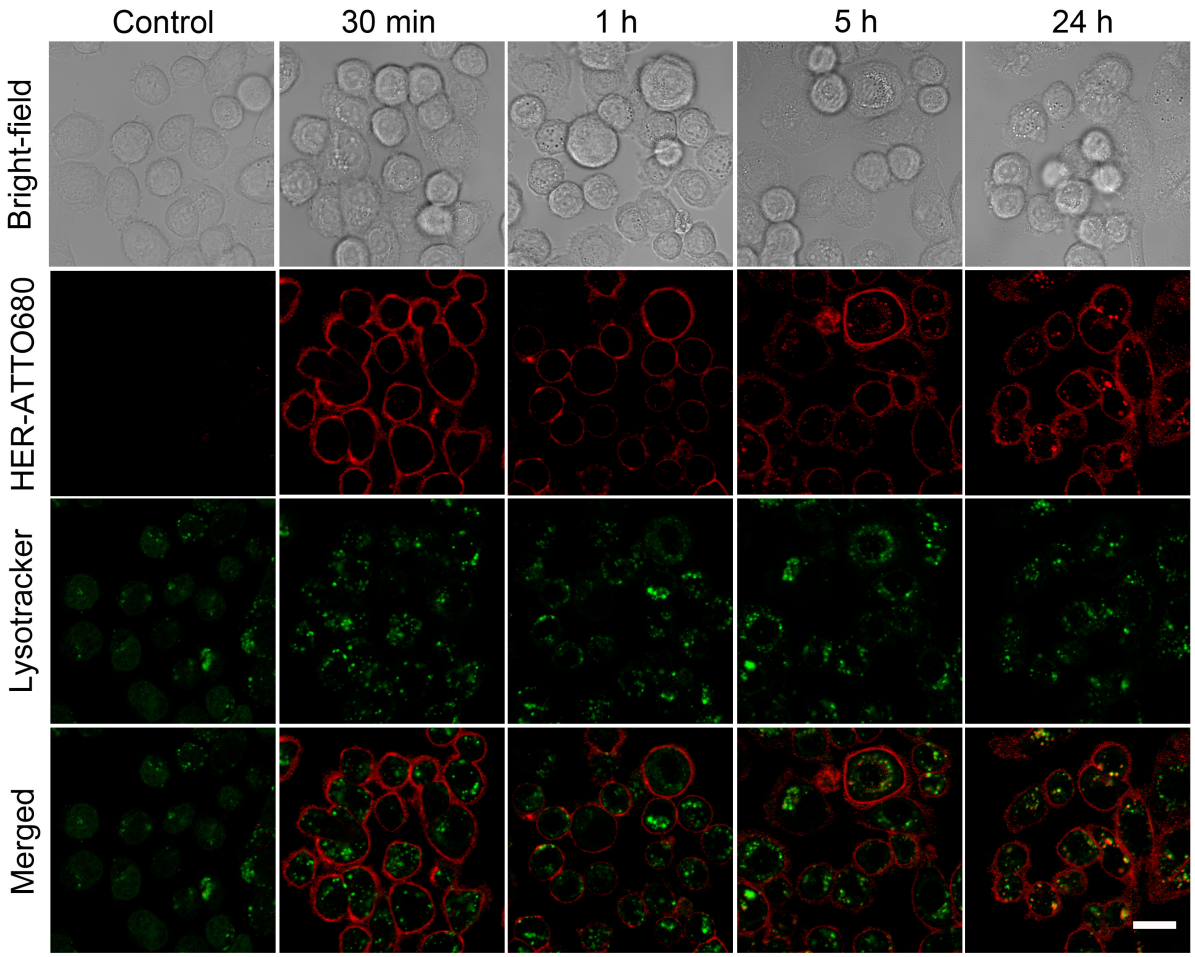

Figure S9. (A) Confocal fluorescence microscopy images showing kinetics of intracellular uptake of HER-ATTO680 into SK-BR-3 cells. The cells were treated with 20 μg/mL HER-ATTO680 for 30 min and then washed three times. After the addition of fresh cell culture medium, fluorescence images were obtained immediately (i.e., 30 min in the above picture) and at 1 h, 5 h, and 24 h, respectively. Lysosomes in the cells were counterstained with 100 nM Lysotracker before CLSM imaging (scale bar = 20 μm).

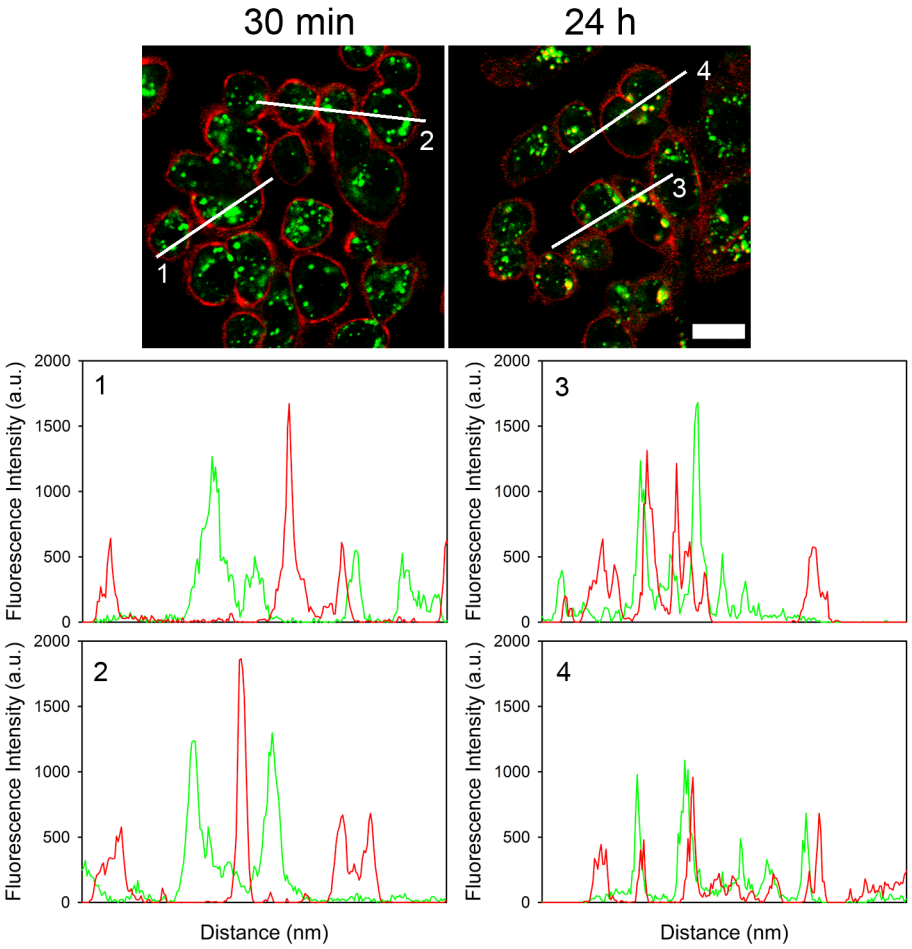

Figure S9. (B) Analysis of fluorescence intensity across the area indicated by the white lines in SK-BR-3 cells after treatment for 30 min and 24 h, respectively. Green colored lines indicate the fluorescence signals of LysoTracker (λ_{ex} 405 nm, λ_{em} 420-480 nm) and red colored lines indicate the fluorescence signals of HER-ATTO680 (λ_{ex} 633 nm, λ_{em} 647-754 nm). Scale bar = 20 μm.

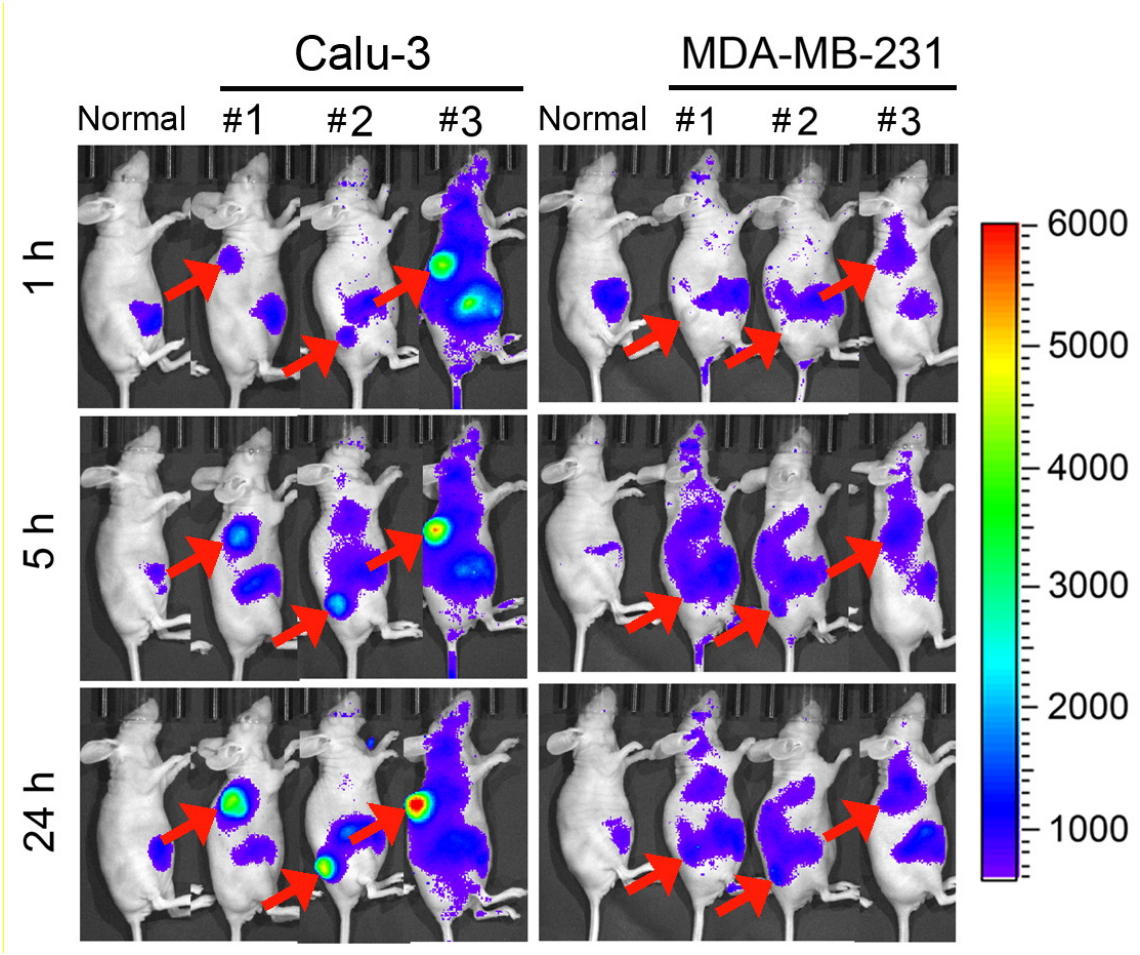

Figure S10. Near-infrared (NIR) fluorescence images of tumor-bearing mice. Mice (n = 3/group) were received intravenous injection of HER-ATTO 680. Normal mice without tumors received intravenous injection of PBS. NIR fluorescence images were obtained at 1, 5, and 24 h post-injection. Arrows indicate tumor sites (λ_{ex} 660/20 nm, λ_{em} 710/40 nm, n = 3).

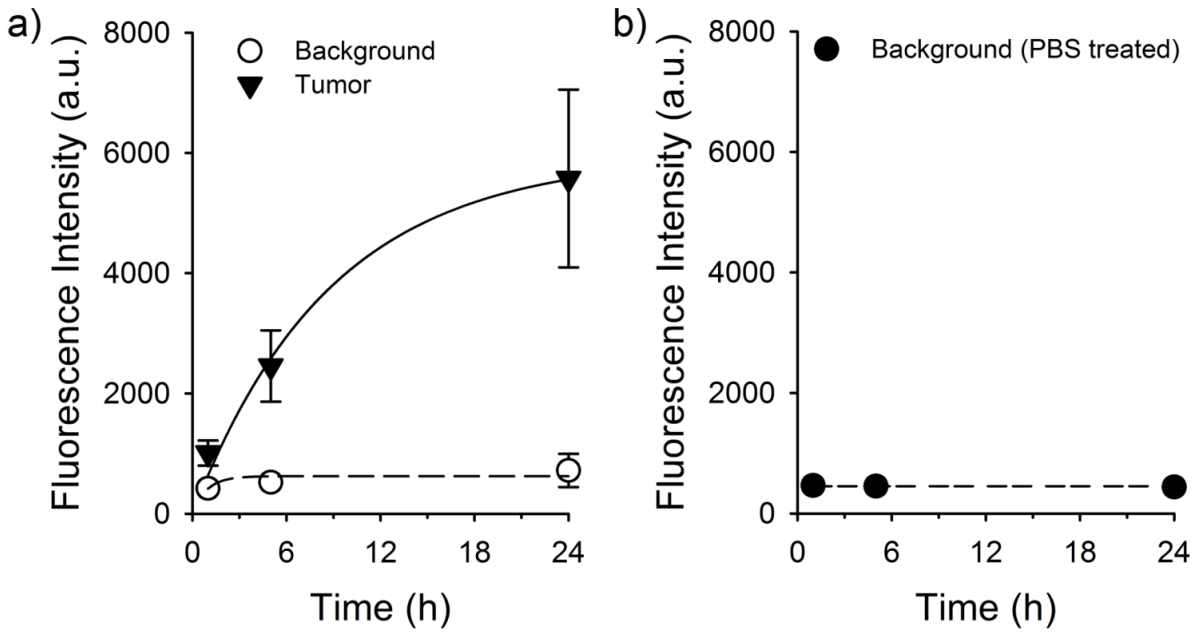

Figure S11. Analysis of NIR fluorescence intensities measured at tumors or surrounding background. a) Fluorescence intensities from the tumors and surrounding normal tissues were analyzed in HER-ATTO680-treated Calu-3-bearing mice. b) Fluorescence intensities observed in normal tissues were analyzed in PBS-treated normal control mice.

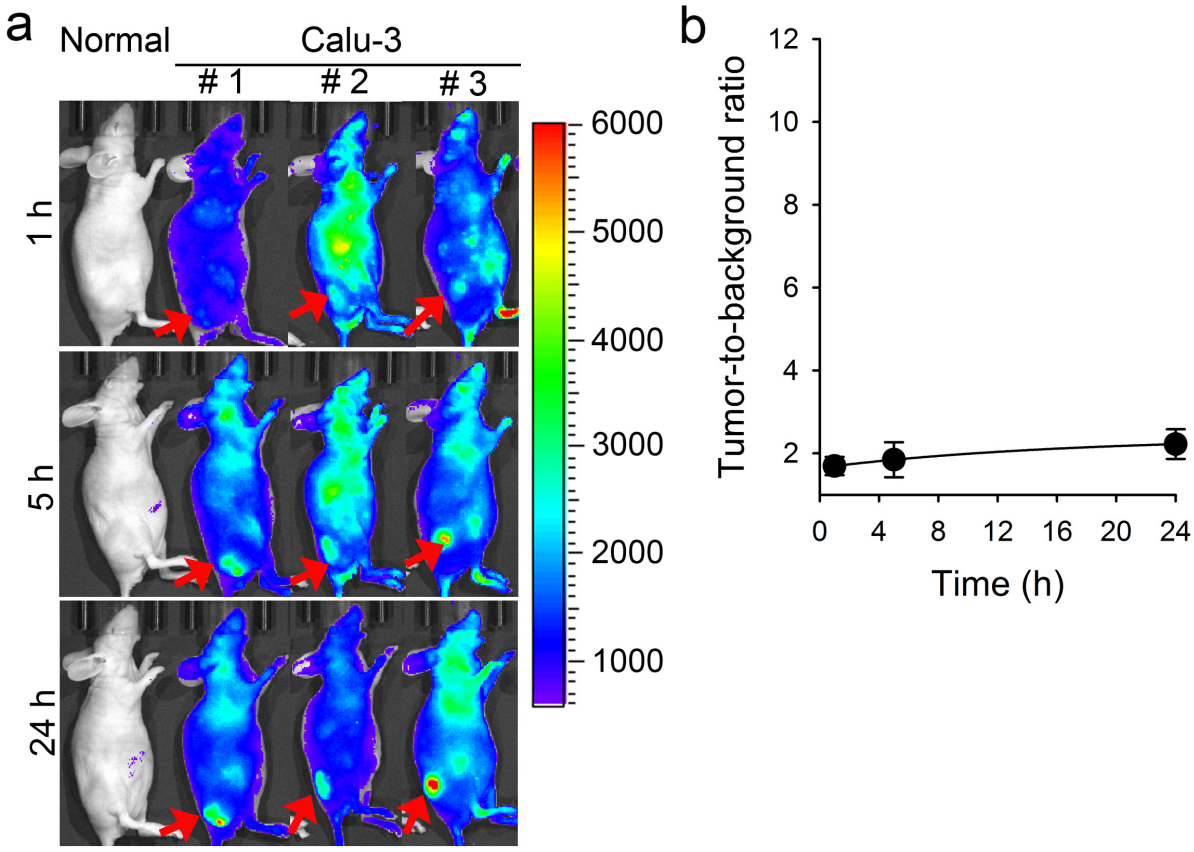

Figure S12. a) *In vivo* NIR florescence images of normal and tumor-bearing mice (λ_{ex} 620/10 nm, λ_{em} 670/20 nm). Mice (n = 3/group) with Calu-3 tumors received intravenous injection of HER-Alexa 647 (50 µg/50 µL). Normal mice without tumors received intravenous injection of PBS for comparison. NIR fluorescence images were obtained at 1, 5, and 24 h post-injection. Arrows indicate the tumor sites. b) Tumor-to-background ratio of fluorescence intensities.

References

1 Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Keifer F, Gallo Cassarino T, Bertoni M, Bordoli L, Schwede T, SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res.* 2014; 42: W252-258.

2 Emsley P, Cowtan K, Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 2004; 60: 2126-2132.