Supplementary Information

Cancer Cell-Selective *In Vivo* Near Infrared Photoimmunotherapy Targeting Specific Membrane Molecules

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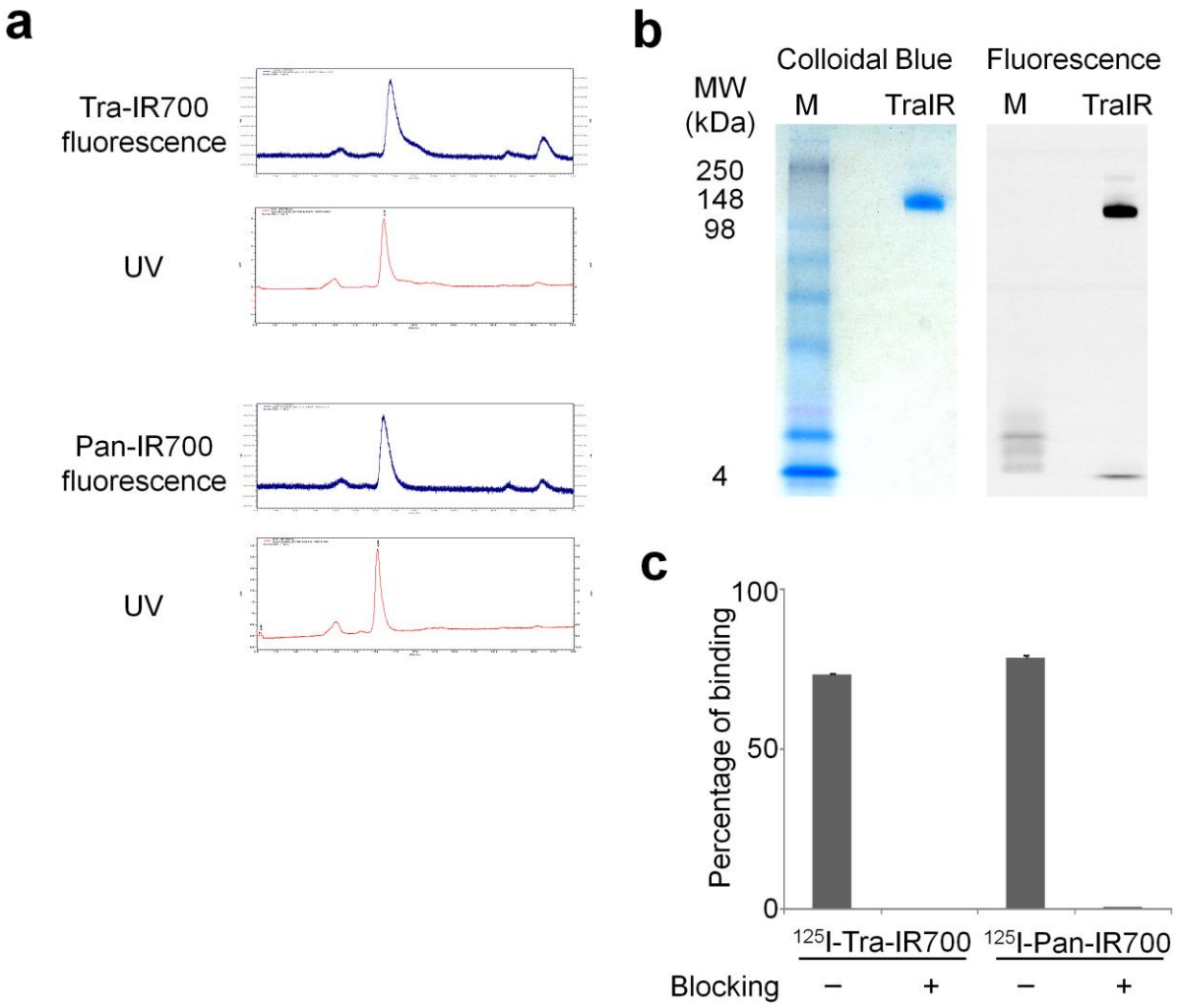
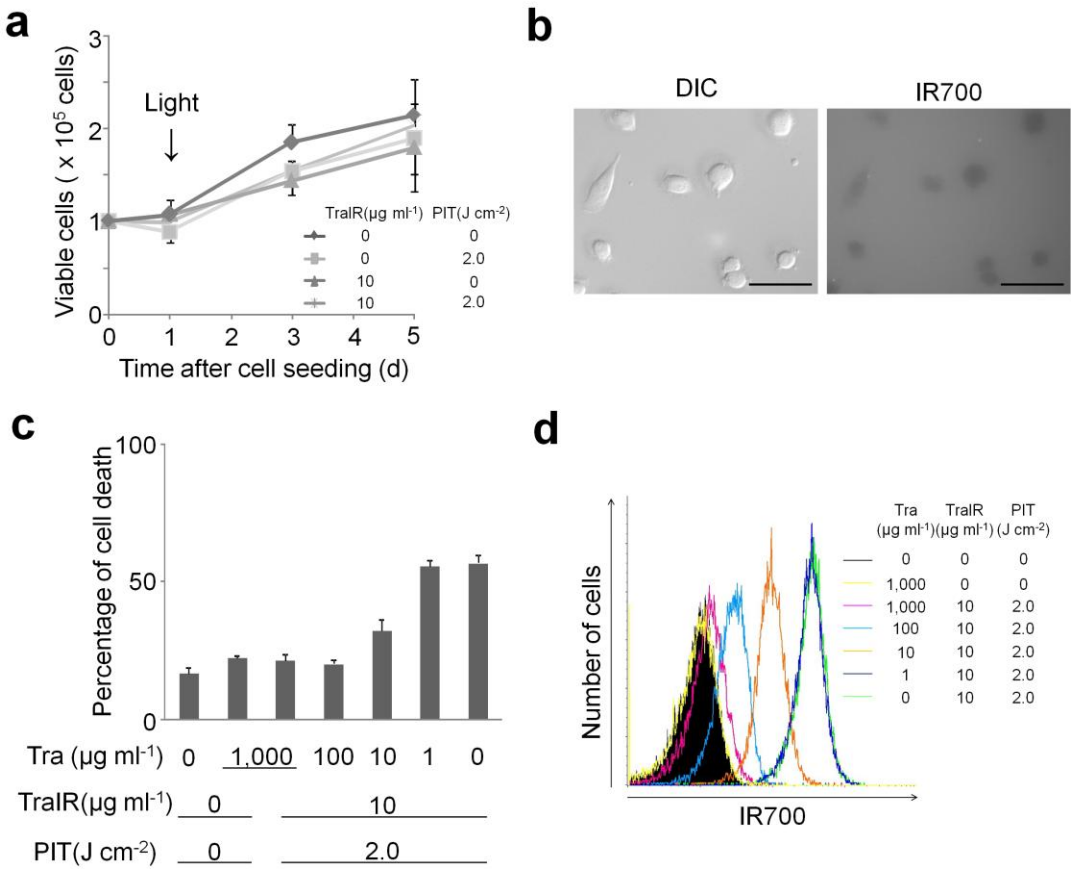


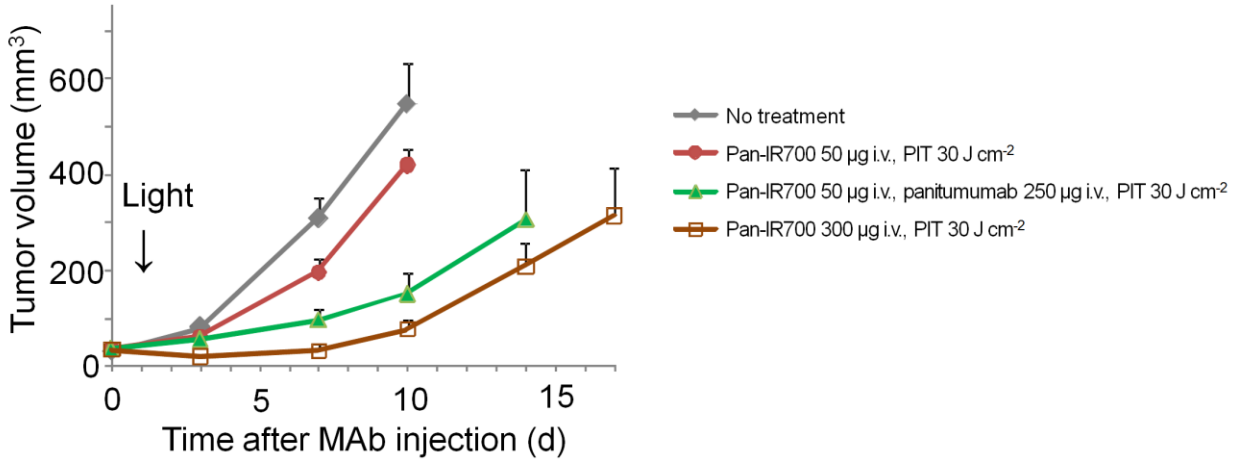
Supplementary Figure 1 Validation of the purity and immunoreactivity of IR700 conjugates.

(**a**) Size-exclusion HPLC and (**b**) SDS-PAGE showed a good association of MAb and IR700. (**c**) Antigen specific binding of ^{125}I labeled MAb-IR700 conjugates confirmed by immunoreactivity assay. Data are means ± s.e.m. (*n* = 3). UV: ultraviolet. TraIR: Tra-IR700.



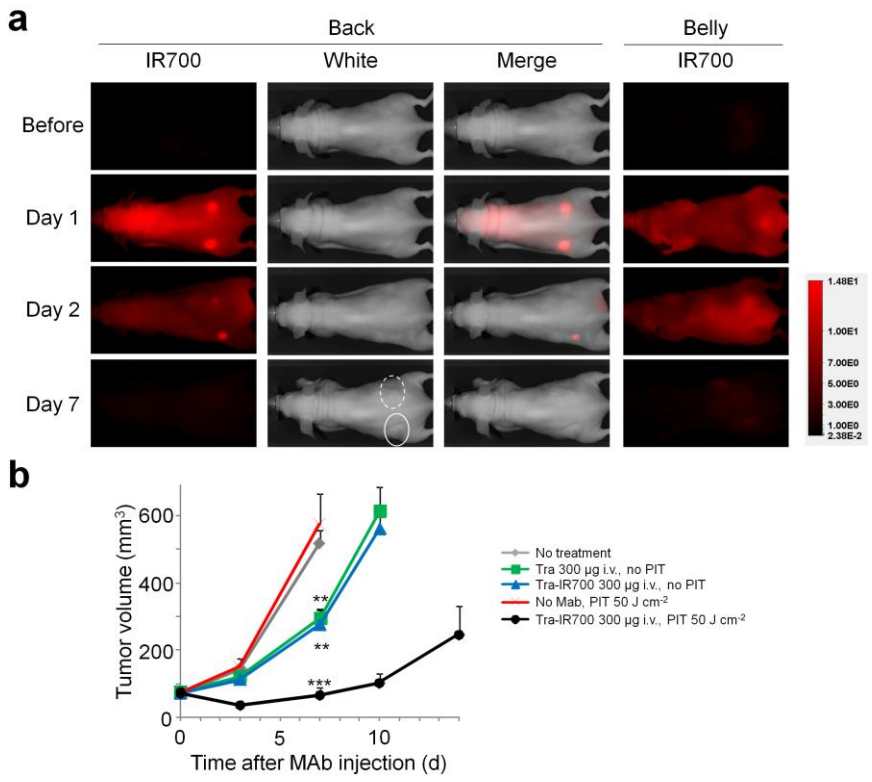
Supplementary Figure 2 Target-specific cell death in response to MAb-IR700 mediated photoimmunotherapy.

(**a**) Long term growth inhibition was not observed in Balb/3T3 (HER2 negative) cells treated with Tra-IR700 (TraIR) and exposed to light. Data are means ± s.e.m. (*n* = 3). (**b**) Free IR700 dye did not incorporate into 3T3/HER2 cells. Fluorescence image was taken without washing the cells. Cells were darker than the media containing free IR700 dye. Scale bar, 50 μm. (**c**) Tra-IR700 mediated phototoxicity was dose-dependently blocked by the excess of unconjugated trastuzumab (Tra). Data are means ± s.e.m. (*n* = 3). (**d**) Tra-IR700 binding for 3T3/HER2 cells was blocked by unconjugated trastuzumab dose-dependent manner (*n* = 3). DIC: differential interference contrast.



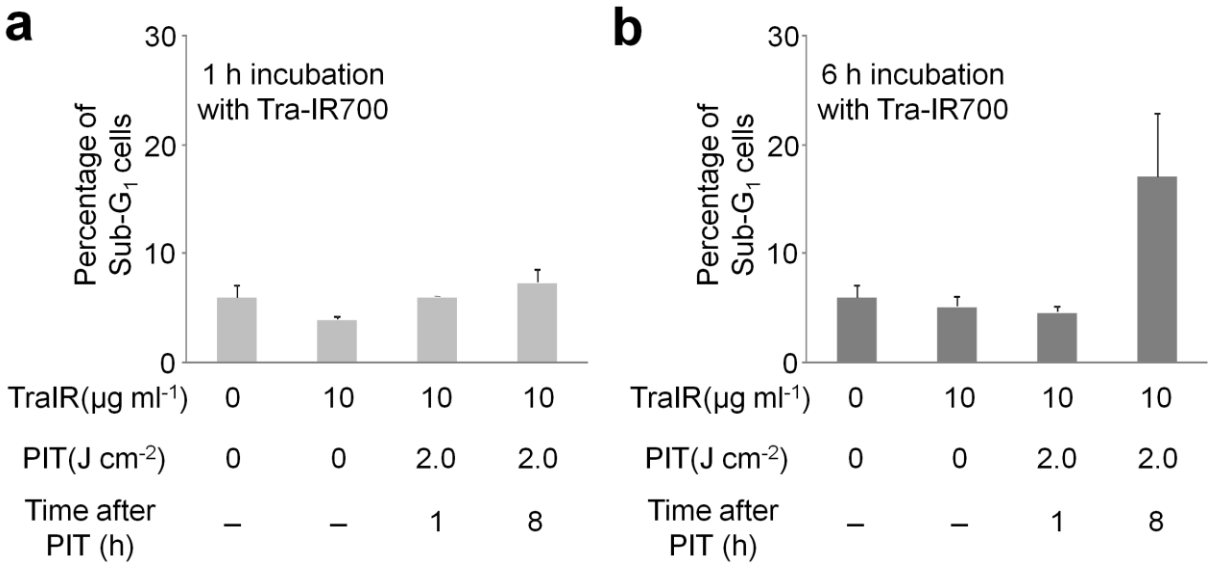
Supplementary Figure 3 High-dose administration of Pan-IR700 lead to higher antitumor efficacy of Pan-IR700 mediated photoimmunotherapy for A431 tumors *in vivo*.

Tumor growth inhibition by Pan-IR700 mediated photoimmunotherapy (PIT) was Pan-IR700-dose-dependently observed. Data are means ± s.e.m. (at least $n = 12$ mice in each group).



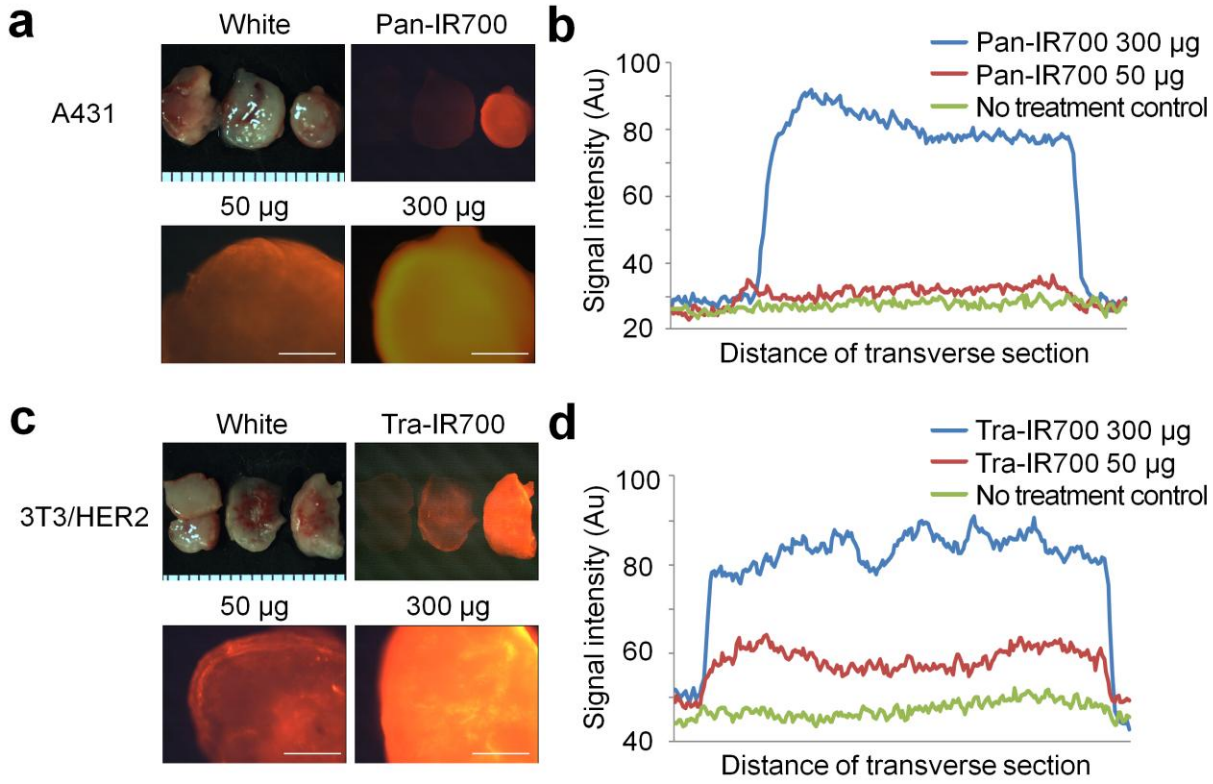
Supplementary Figure 4 Tra-IR700 mediated photoimmunotherapy for 3T3/HER2 tumors *in vivo*.

(**a**) Biodistribution of Tra-IR700. 3T3/HER2 tumors (both sides of dorsum) were visualized with IR700 fluorescence as early as 1 d after Tra-IR700 injection (300 µg). Right side of the tumor was irradiated with near infrared (NIR) light on day 1, while left side of the tumor was covered with black tape. Tumor shrinkage was confirmed on day 7. Dashed line: irradiated tumor, solid line: non-irradiated tumor. No other specific localization of IR700 was found except for the bladder accumulation on day 1 due to the excretion of unbound dye (n = 5 mice). (**b**) Target specific tumor growth inhibition by Tra-IR700 mediated photoimmunotherapy (PIT). Data are means ± s.e.m. (at least n = 12 mice in each group, *** $P < 0.001$, ** $P < 0.01$ vs. non treatment control, Kruskal–Wallis test with post-test). Tra: trastuzumab.



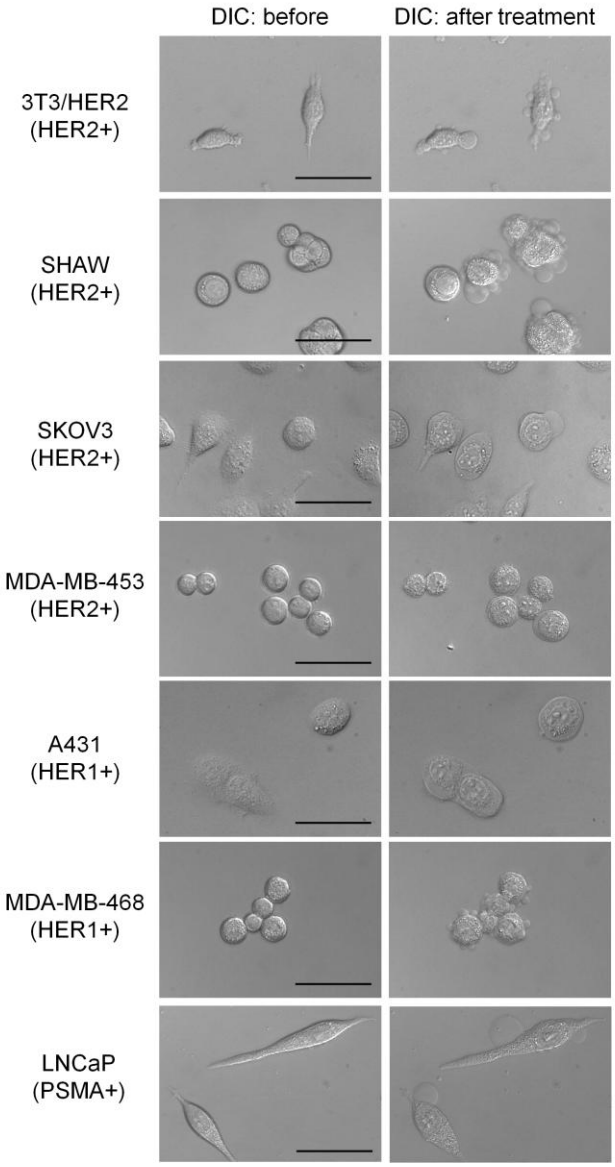
Supplementary Figure 5 Apoptotic cell death does not play a central role in Tra-IR700 mediated photoimmunotherapy *in vitro*.

Flow cytometric analysis was performed after the treatment to identify apoptotic cells. 3T3/HER2 cells were pretreated with Tra-IR700 (TraIR) for (**a**) 1 h or (**b**) 6 h and irradiated. Data showed the percentage of sub-G1 apoptotic population. Data are means ± s.e.m. (n = 3).



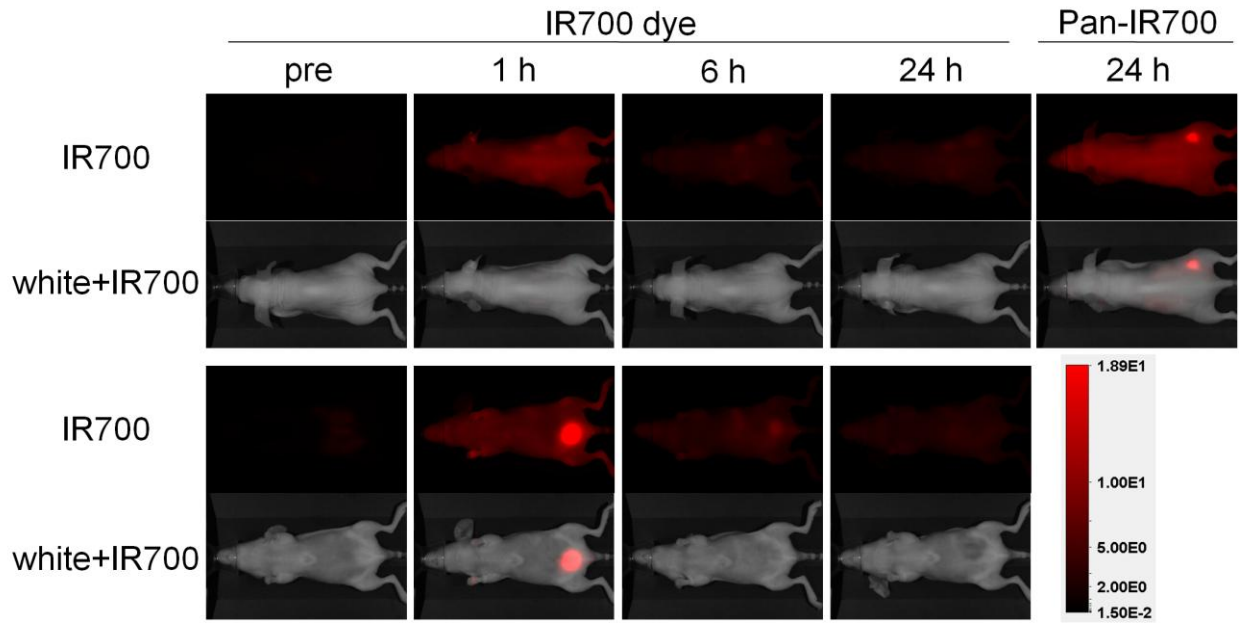
Supplementary Figure 6: Homogeneously high accumulation with high dose (300μg per mouse) of MAb-IR700 in target-specific tumors.

Macro fluorescence microscopic observation of excised tumors 24 h after injecting MAb-IR700. (**a**) White light image of A431 tumors (upper left). From left to right; No treatment control, 50 μg of Pan-IR700, 300 μg of Pan-IR700). The smallest scale indicates 1 mm. IR700 fluorescence image (upper right). Magnified images of intratumor distribution of IR700 (lower left; 50 μg of Pan-IR700, lower right; 300 μg of Pan-IR700). Scale bar, 1 mm (n = 3). (**b**) Quantification of intratumoral distribution of IR700 fluorescence. Graph showed IR700 fluorescence intensity in each tumor. (**c**, **d**) Same experiments were performed with 3T3/HER2 tumor bearing mice injected with Tra-IR700.



Supplementary Figure 7 Photoimmunotherapy for various molecular targets.

Microscopic observation of before and after photoimmunotherapy (PIT) for HER2 expressing 3T3/HER2, SHAW, SKOV3 and MDA-MB-453 cells with Tra-IR700, for HER1 expressing A431 and MDA-MB-468 cells with Pan-IR700, and for prostate specific membrane antigen (PSMA) expressing LNCaP cells with humanized anti-PSMA MAb, huJ591-IR700. Scale bar, 50 μm. DIC: differential interference contrast.



Supplementary Figure 8 Rapid urinary excretion of free IR700 dye without accumulating tumor.

A431 tumor bearing mice at right dorsum were injected with either Pan-IR700 (300 μg) or Pan-IR700 (300 μg) equivalent dose of free IR700 dye. Distribution of IR700 fluorescence was analyzed with fluorescence imager (n = 3).

Supplementary Video 1: Real-time observation of Tra-IR700 mediated phototoxic cell death.

Images were obtained during the irradiation of near infrared (NIR) light after (**a**) 6 h at 37 °C or (**b**) 1 h at 4 °C incubation with Tra-IR700. 3T3/HER2 cells with both preparations showed swelling, bleb formation on the cell membrane upon excitation light. (**a**) showed immediate rupture of intracellular vesicles.

Supplementary Video 2: Target specific phototoxicity in response to Tra-IR700 mediated photoimmunotherapy *in vitro*.

Images were obtained after 6 h incubation with Tra-IR700 during the irradiation of near infrared (NIR) light. Only HER2 expressing 3T3/HER2 cells (fluoresced; 1^{st} and 3^{rd} from the left) showed typical cell shape changes reflecting necrotic cell death, however, co-cultured HER2 negative Balb/3T3 cells (non-fluoresced; 2^{nd} and 4^{th} from the left) did not show any shape changes on the cell membrane after irradiation of NIR excitation light.

Supplementary Methods

Reagents. A water soluble, silicon-phthalocyanine derivative, IRDye 700DX NHS ester (IR700; $C_{74}H_{96}N_{12}Na_4O_{27}S_6Si_3$, molecular weight of 1954.22) was purchased from LI-COR Bioscience. Trastuzumab, a recombinant humanized monoclonal antibody (MAb) directed against the extracellular domain of the human epidermal growth factor receptor (EGFR) 2 (HER2) tyrosine kinase receptor was purchased from Genentech. Panitumumab, a fully humanized IgG_2 MAb directed against the human EGFR, or HER1, was purchased from Amgen. All other chemicals used were of reagent grade.

Synthesis of IR700-conjugated MAbs. Each MAb (1 mg, 6.8 nmol) was incubated with IR700 (66.8 μg, 34.2 nmol, 5 mmol l^{-1} in DMSO) in 0.1 mol l^{-1} Na_2HPO_4 (pH 8.5) at room temperature for 2 h. The mixture was purified with a Sephadex G50 column (PD-10; GE Healthcare). The protein concentration was determined with Coomassie Plus protein assay kit (Pierce Biotechnology) by measuring the absorption at 595 nm with a spectroscopy (8453 Value System; Agilent Technologies). The concentration of IR700 was measured by absorption with the spectroscopy to confirm the number of fluorophore molecules conjugated to each MAb molecule. IR700 was incubated with 50 mM Tris-HCl buffer (pH 7.5) to prepare free IR700 dye.

Determination of the purity of IR700 conjugates. The purity of MAb-IR700 conjugates was confirmed by analytical size-exclusion HPLC (SE-HPLC) and sodium dodecyl sulfate polyacrylamidegel elctrophoresis (SDS-PAGE). SE-HPLC was performed using a Beckman System Gold equipped with model 126 solvent delivery module, a model 168 UV detector, and a

JASCO fluorescence detector (excitation 689 nm and emission at 700 nm) controlled by 32 Karat software. SE chromatography was performed on a TSKgel G2000SWxl (Tosoh Bioscience LLC) eluted for 45 m using phosphate buffered saline (PBS) at 0.5 mL m^{-1}. SDS-PAGE was performed with a 4% to 20% gradient polyacrylamide gel (Invitrogen). Just after separating the proteins, fluorescence intensity was analyzed with a Fujifilm FLA-5100 fluorescence scanner with an internal laser of 670 nm for excitation and 705 nm long pass filter for emission. The fluorescence intensity of each band was analyzed with Multigage software (Fujifilm). The gels were then stained with Colloidal Blue Staining Kit (Invitrogen), and digitally scanned. The protein concentration in each band was analyzed with ImageJ software (http://rsbweb.nih.gov/ij/).

Immunoreactivity of IR700 conjugates. To determine the *in vitro* binding characteristics of IR700 conjugates, we prepared ^{125}I labeling for Tra-IR700 and Pan-IR700 using the Indo-Gen procedure. The specific activities of the radiolabeled antibodies were 8.52 mCi/mg for trastuzumab and 7.84 mCi/mg for panitumumab. Immunoreactivity assay was performed as described previously[1]. Briefly, after trypsinization, 2×10^6 of 3T3/HER2 or A431 cells were resuspended in PBS containing 1% bovine serum albumin (BSA). ^{125}I-Tra-IR700 or ^{125}I-Pan-IR700 (1 mCi, 0.2 μg) was added and incubated for 1 h on ice. Cells were washed, pelleted, the supernatant decanted, and counted in a 2470 Wizard^2 γ-counter (Perkin Elmer). Nonspecific binding to the cells was examined under conditions of antibody excess (200 μg of nonlabeled trastuzumab or panitumumab).

Fluorescence microscopy. To detect the antigen specific localization of IR700, fluorescence microscopy was performed (BX51 or IX81; Olympus America). 3T3/HER2, A431 or 1:1

mixture of 3T3/HER2 and Balb/3T3/DsRed cells were seeded on a cover glass-bottomed dishes and incubated 24 h. trastuzumab-IR700 (Tra-IR700) or panitumumab-IR700 (Pan-IR700) was added to the culture medium at 10 μg ml^{-1} and incubated either for 1 h at 4 °C or 6 h at 37 °C, then cells were washed with PBS. The filter set to detect IR700 consisted of a 590–650 nm excitation filter, a 665–740 nm band pass emission filter. To detect DsRed protein, a filter set consisted of a 480–550 nm excitation filter and a 590 nm long pass emission filter was used. To determine the subcellular localization, the cells were stained with 5 μM of LysoTracker Green (Invitrogen), which detected by a filter set consisted of a 420–480 nm excitation filter and a 520 nm long pass emission filter.

In vivo **photoimmunotherapy for 3T3/HER2 tumors with Tra-IR700.** Four days after 3T3/HER2 cells injection, tumor volumes of approximately 70 mm^3 were selected for the study. Animals were randomized into 5 groups of at least 12 animals per group for the following treatments: (1) no treatment; (2) 300 μg of trastuzumab injected intravenously, no PIT; (3) 300 μg of Tra-IR700 injected intravenously, no PIT; (4) PIT was performed at 50 J cm^{-2} for 3T3/HER2 tumor without Tra-IR700; (5) 300 μg of Tra-IR700 injected intravenously, PIT was performed at 50 J cm^{-2} for 3T3/HER2 tumor.

Apoptosis assay. To determine the role of apoptosis in response to Tra-IR700 mediated PDT, we assessed the accumulation of cells at sub-G1 fraction, representing DNA fragmentation. The treated cells were fixed with 80% ethanol for at least 30 m. After a wash with phosphate-citrate buffer, cells were resuspended with PBS containing propidium iodide (PI) and RNase A. The

samples were then incubated at room temperature for 15 m in the dark and analyzed by flow cytometer.

Intratumoral distribution of IR700 conjugates. To determine intratumoral distribution of MAb-IR700, tumor bearing mice were intravenously injected with 50 or 300 μg of MAb-IR700. Twenty-four hours after injection, mice were euthanized then tumors were excised. Each tumor was sliced with 2 mm thickness and a cross section was observed with a macro zoom fluorescence microscope, MVX10 (Olympus). IR700 fluorescence intensity was quantified by placing an arbitrarily linear region of interest in each cross section of tumor, then analyzed with ImageJ software (n = 3).

Statistical Analysis. Data are expressed as means ± s.e.m. from a minimum of three experiments, unless otherwise indicated. Statistical analyses were carried out using a statistics program (GraphPad Prism; GraphPad Software). Student't t test was used to compare the treatment effects with that of control. For multiple comparisons, a one-way analysis of variance (ANOVA) with post test (Kruskal–Wallis test with post-test) was used. The cumulative probability of survival, determining herein as the tumor volume was failed to reach 500 mm^3, were estimated in each group with the use of the Kaplan–Meier survival curve analysis, and the results were compared with use of the log-rank test with Bonferroni's correction for multiplicity. $P < 0.05$ was considered to indicate a statistically significant difference.

Reference

1. Kobayashi, H., *et al.* L-lysine effectively blocks renal uptake of 125I- or 99mTc-labeled anti-Tac disulfide-stabilized Fv fragment. *Cancer Res* **56**, 3788-3795 (1996).