Supporting Information

Improved tumor targeting and longer retention time of NIR fluorescent probes using bioorthogonal chemistry

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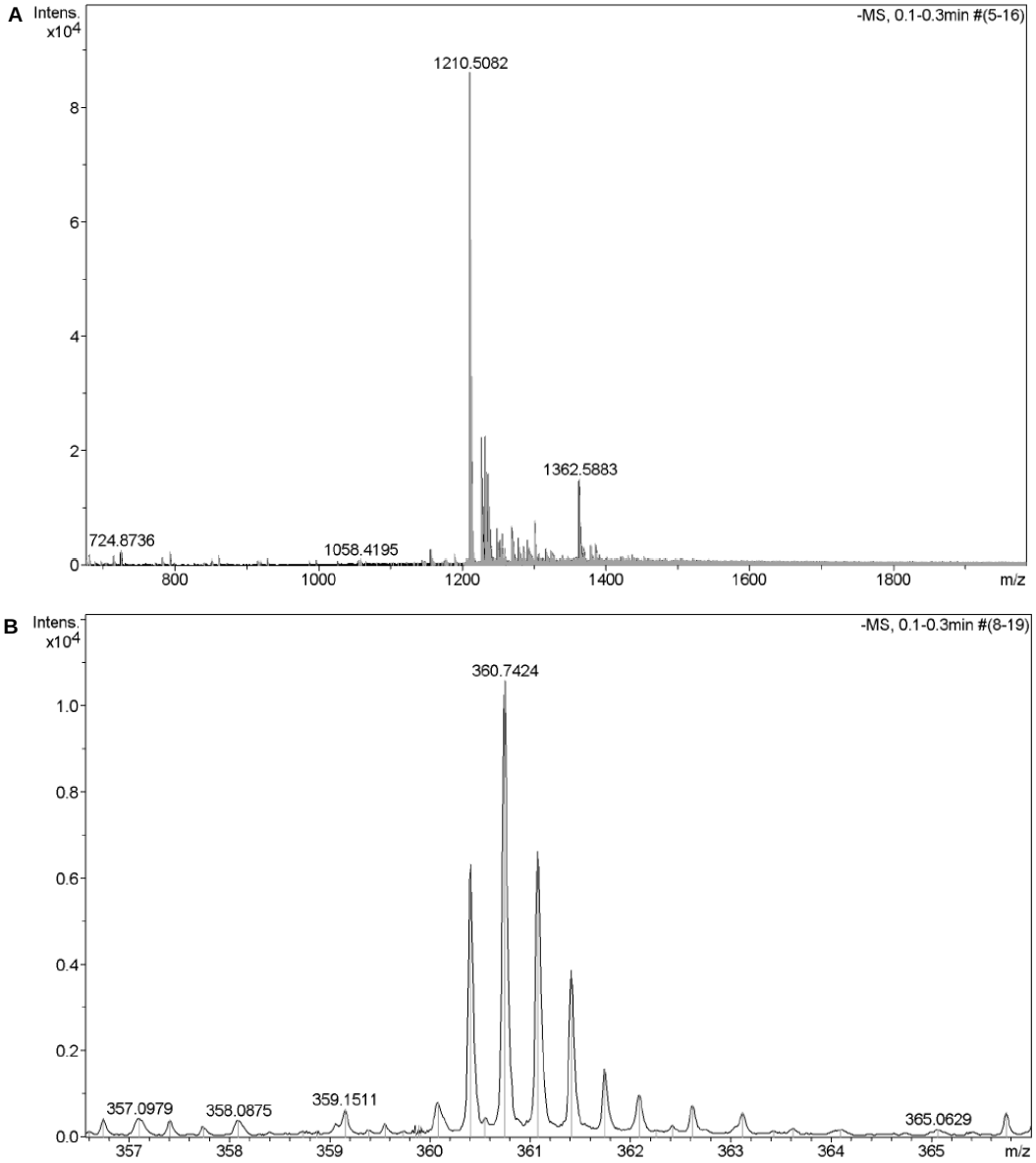

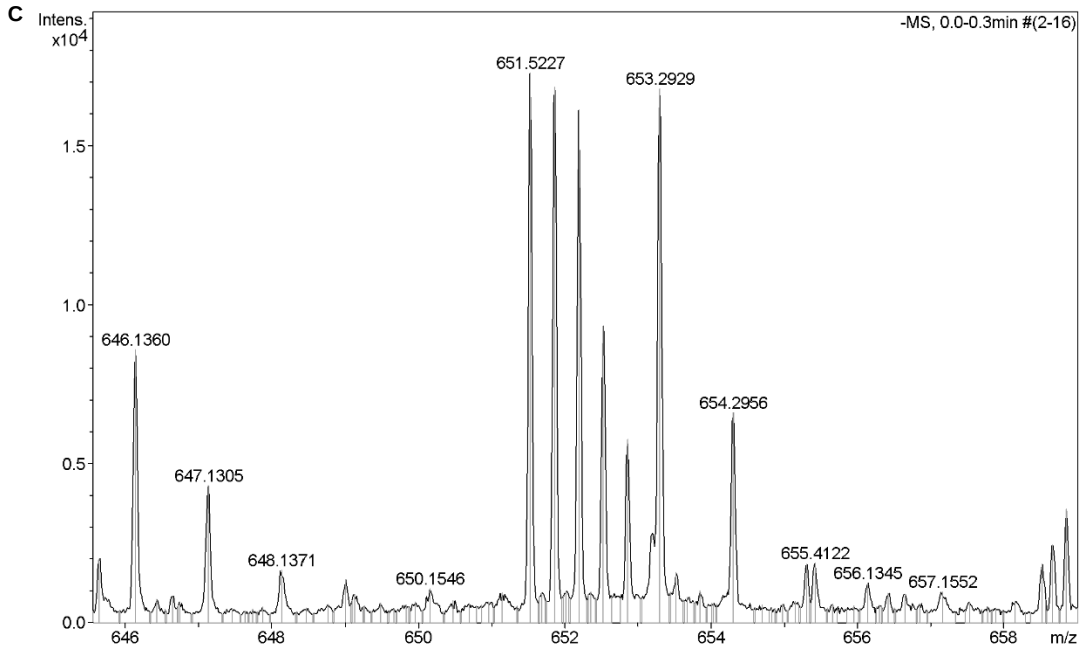

Figure S1. High resolution mass spectra of the probes GEBP11-TCO (A), Cy5.5-Tz (B) and GEBP11-Cy5.5 (C).

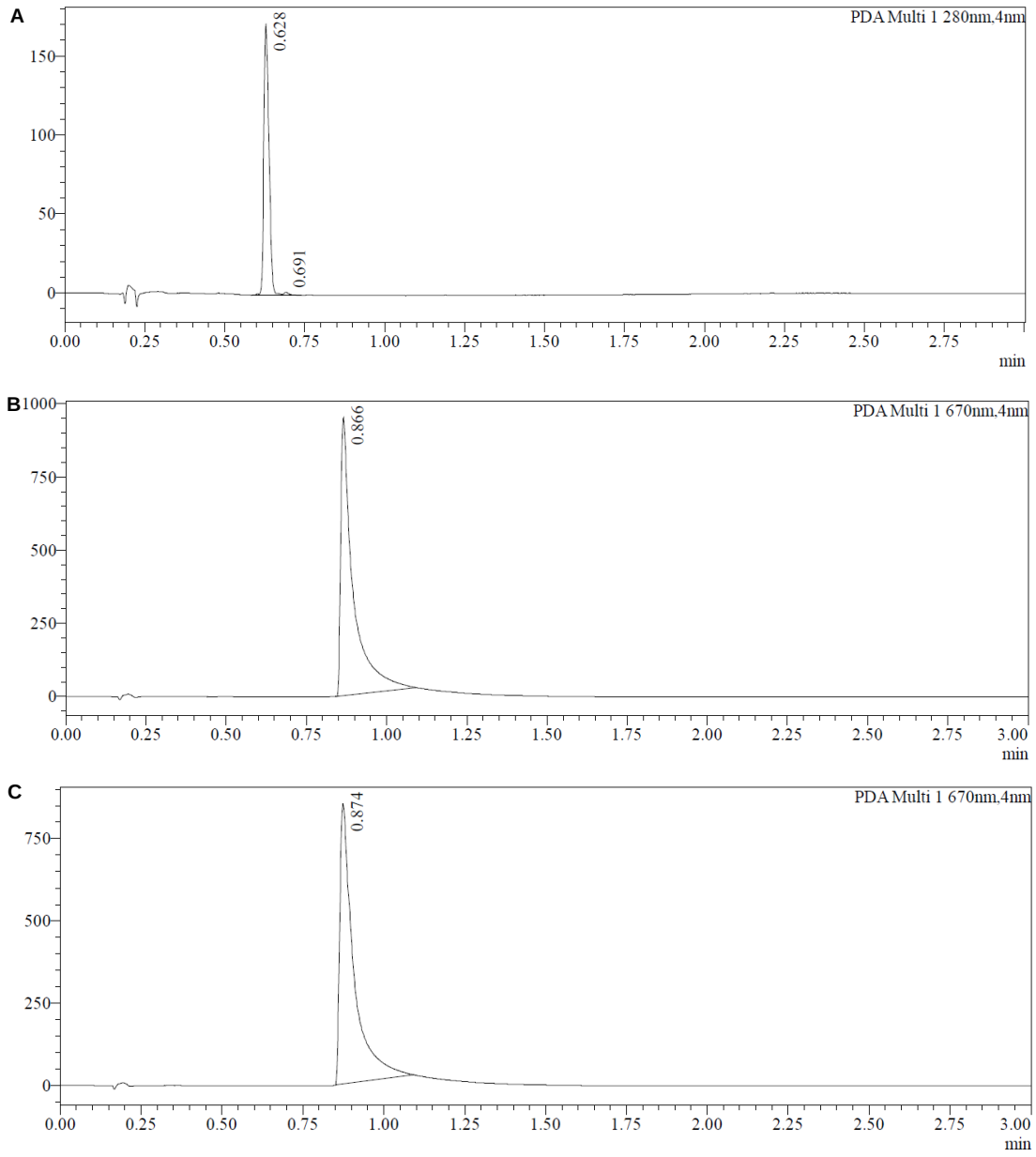

Figure S2. HPLC spectra of the probes GEBP11-TCO (A), Cy5.5-Tz (B) and GEBP11-Cy5.5 (C).

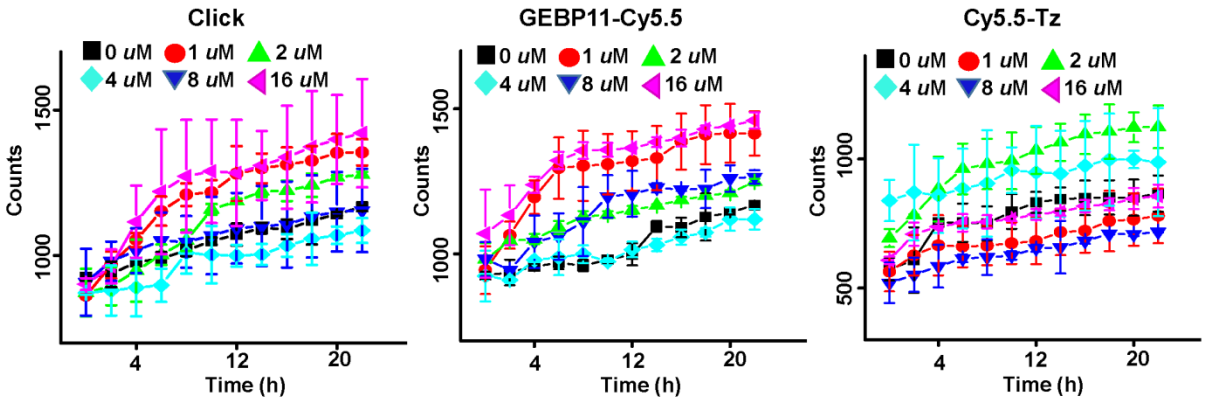

Figure S3. Cytotoxic effect of click, GEBP11-Cy5.5 and Cy5.5-Tz probes for SGC7901-GFP cells measured by high-content analysis (HCA) assay.

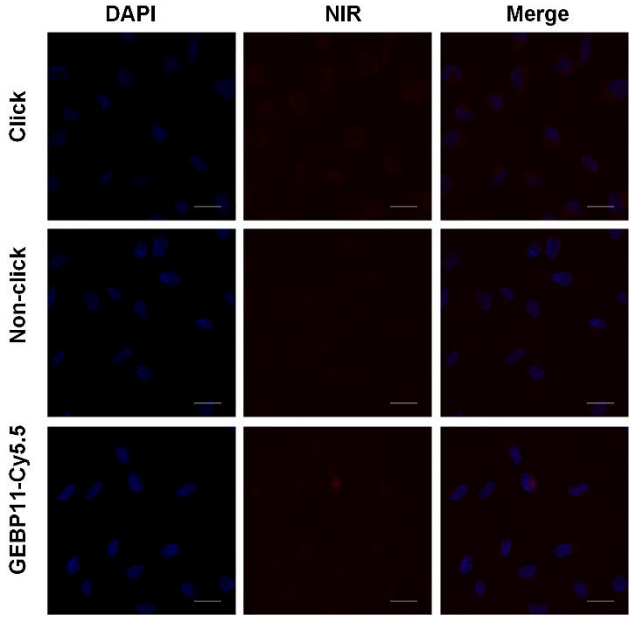

Figure S4. Confocal laser microscopy of GES cells incubated with click probes (pre-labeled 3 h with 5 μM GEBP11-TCO followed by 5 μM Cy5.5-Tzlabeling), non-click probes (pre-labeled 3 h with5 μM GEBP11 followed by 5 μM Cy5.5-Tzlabeling), GEBP11-Cy5.5 (5 μM). Cells were stained with nuclear dye DAPI (blue) and the NIR dye Cy5.5(red).All of the images were acquired at 60$\times$ magnification. The scale bar represents 30 μm.

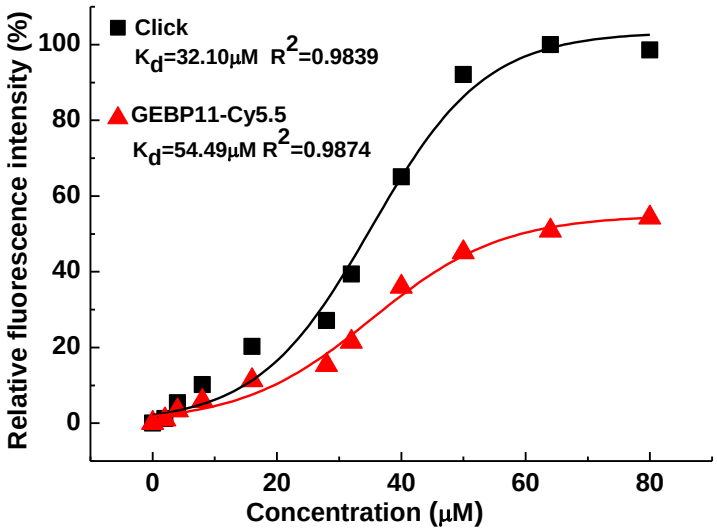

Figure S5. Binding affinity. The affinity for binding of click and GEBP11-Cy5.5 probes to SGC-7901 cells on 4°C is determined from a non-linear fit of the relative fluorescence intensity to concentration of bound probes, and reveals the equilibrium dissociation constant of K_d = 32.10 μM (R^2 = 0.9839) for click probes and K_d = 54.49 μM (R^2 = 0.9874) for GEBP11-Cy5.5 probes.

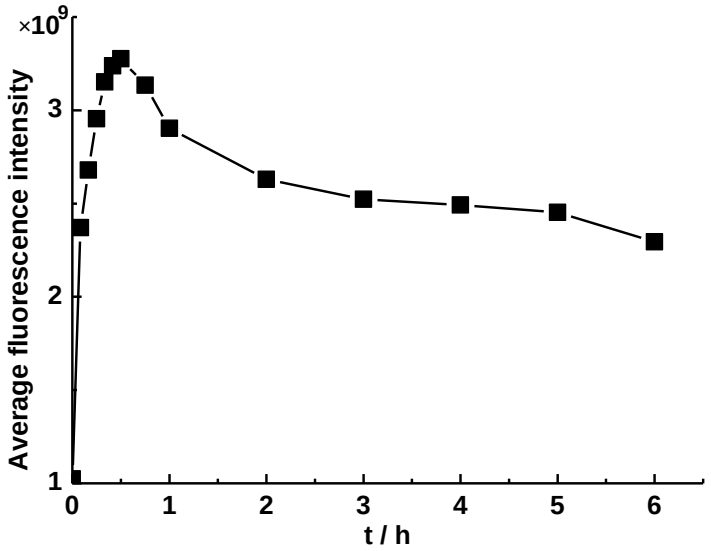

Figure S6. Tumor accumulation of click probes over 6 h.

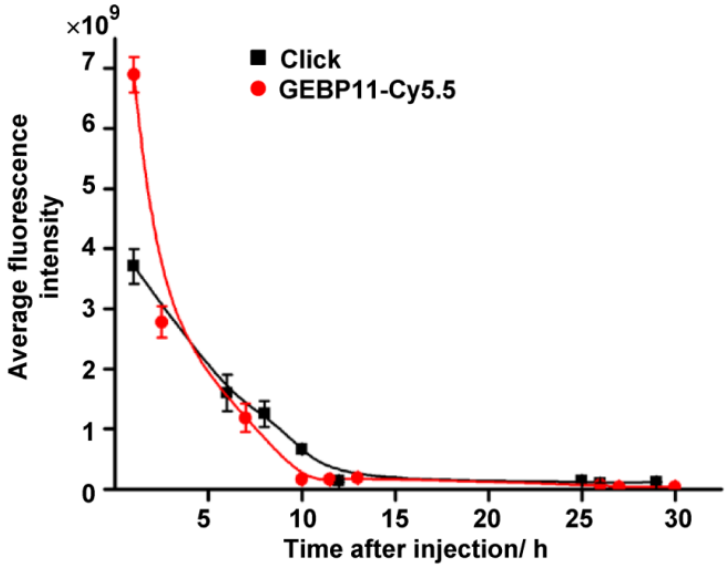

Figure S7. The cumulative urine excretion curve after injection of click and GEBP11-Cy5.5 probes.

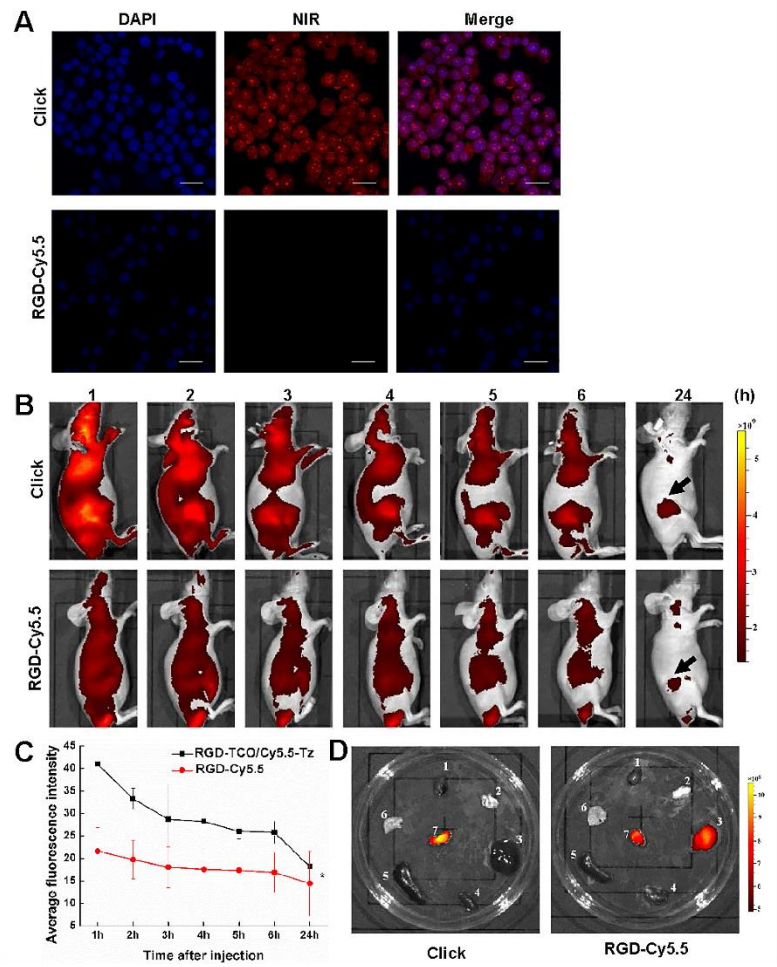

Figure S8. Targeting specificity of click-meditated RGD probes *in vitro* and *in vivo*. (A) Confocal laser microscopy of SGC-7901 cell incubated with click probes (RGD-TCO pre-labeled 3 h with 5 μM, Cy5.5-Tz with 5 μM) and RGD-Cy5.5 (5 μM). All of the images were acquired at 60× magnification. The scale bar represents 30 μm. (B) Fluorescence imaging of subcutaneous tumor-bearing nude mice after intravenous injection with probes. Click group: RGD-TCO pre-injected 3h with 8nmol / Cy5.5-Tz with 4 nmol. RGD-Cy5.5 group: RGD-Cy5.5 with 4 nmol. (C) Average fluorescence signal intensity from tumor tissue over time.* $p< 0.05$. (D) Fluorescence images of tissues harvested from mice 24 HPI (1 heart, 2 lungs, 3 liver, 4 kidneys, 5spleen, 6 stomach, 7 tumor). Net tumor signal in click animals was 1.3 times that in RGD-Cy5.5, liver and kidney signals in RGD-Cy5.5 was 5.4 and 4.2 times that in click groups.

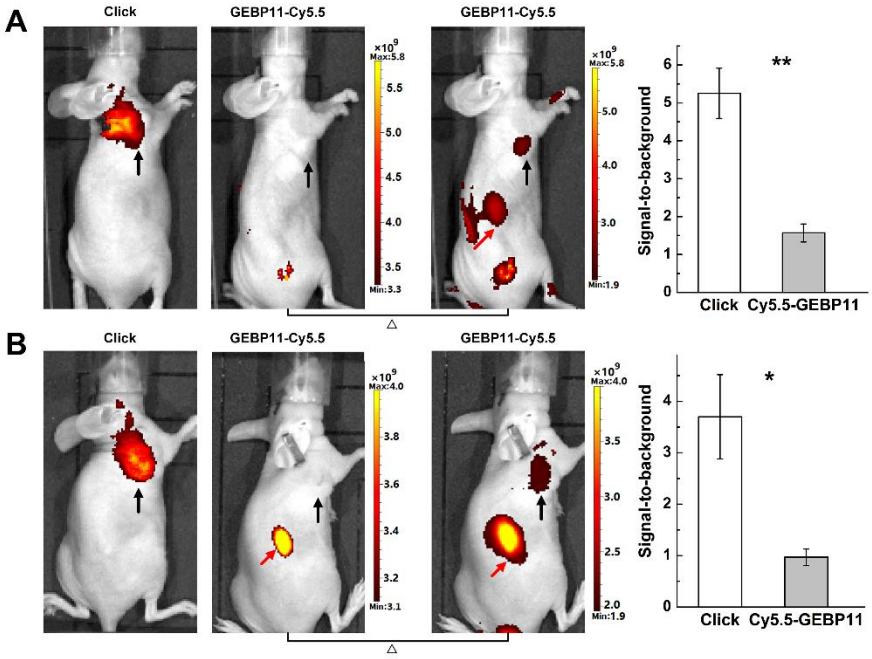

Figure S9. Optical detection sensitivity of twotumour lesions. (A) Fluorescence imaging of subcutaneous tumor-bearing nude mice with 4.3 ± 0.3 mm tumor size at 6 HPI. $^{**}p < 0.01$.ΔThe same mice at different color scale bar.(B) The tumor size was 8.4 ± 0.5 mm at 6 HPI. $^{*}p < 0.05$. Locations of tumors and kidney are indicated by black and red arrows, respectively.

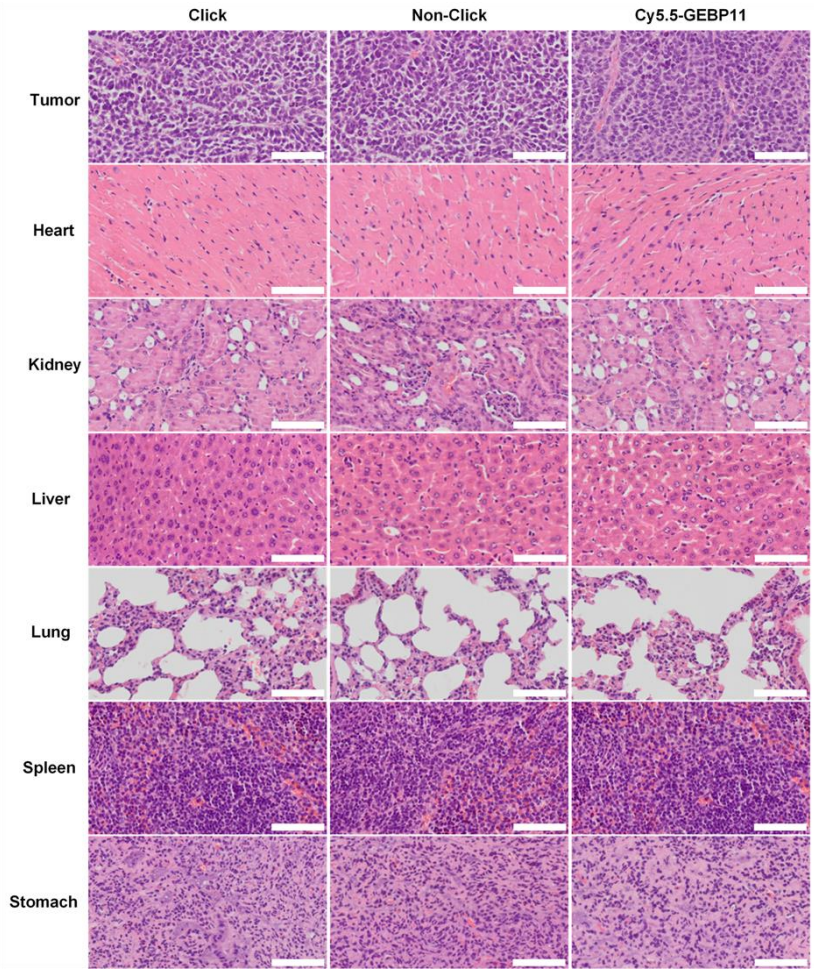

Figure S10. Hematoxylin and eosin (H&E) pathology examinations. The heart, kidney, liver, lung, spleen and stomach of click, non-click, Cy5.5-GEBP11 groups did not show obvious structural changes. The scale bar represents 100 μm.

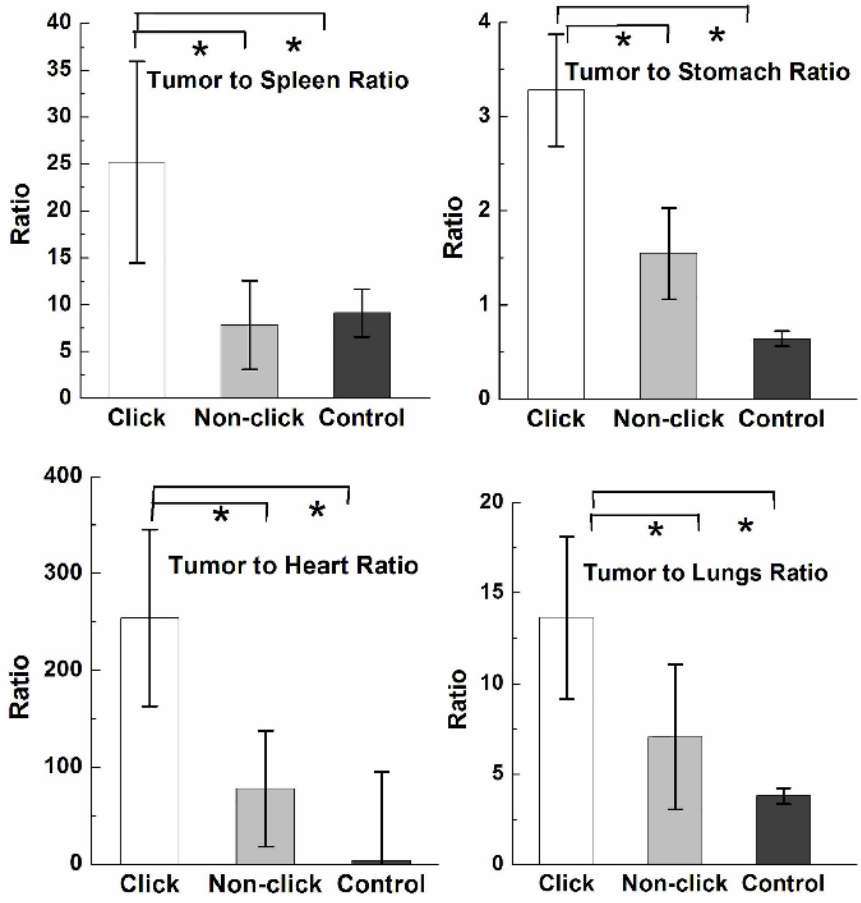

Figure S11. Tumor-to-organ ratios. * $p < 0.05$, $n = 3$.

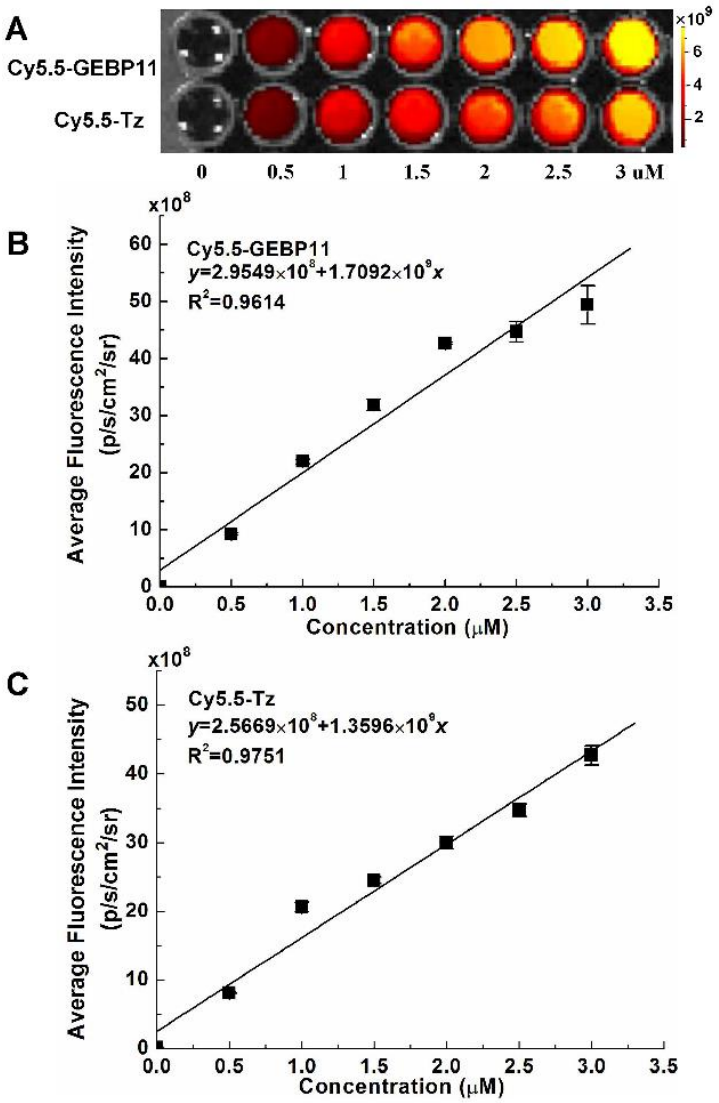

Figure S12. Fluorescence images of of GEBP11-Cy5.5 and Cy5.5-Tz dye. (A) Fluorescence imaging of GEBP11-Cy5.5 and Cy5.5-Tz at different concentrations. (B, C) The linear relationship between fluorescence intensity and dye concentration. Data based on three independent experiments.

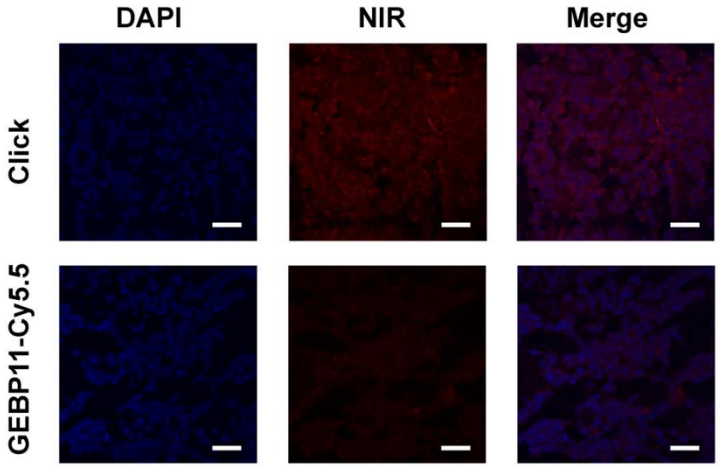

Figure S13. The confocal images were taken from thecryo sectioned tumors.

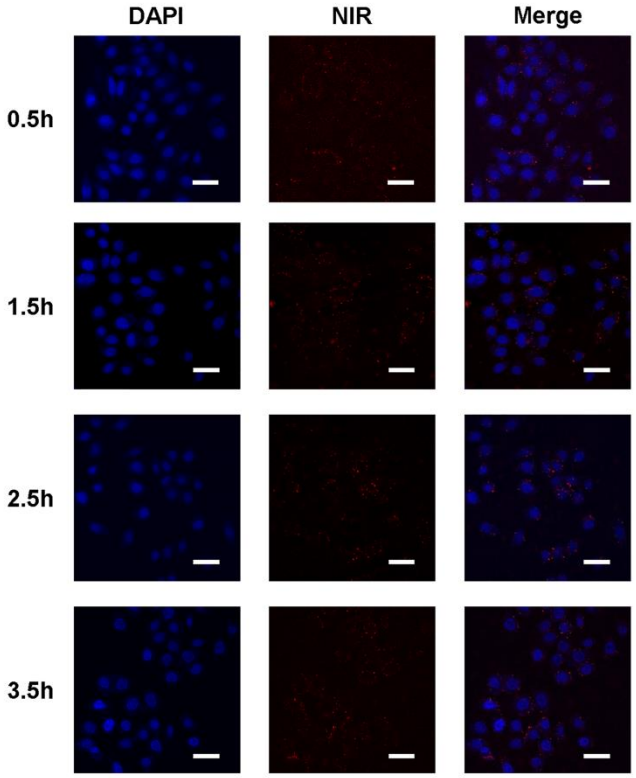

Figure S14. Fluorescence microscopy imaging of SGC-7901 cells labeled with Cy5.5 for 0.5 h, 1.5 h, 2.5 h and 3.5 h, respectively.

In our experiment, we have labeled SGC-7901 cells with directly-labeled probes (GEBP11-Cy5.5) for 0.5 h, 1.5 h, 2.5 h and 3.5 h respectively, and then visualized using confocal laser microscopy. It was expected that the labeling efficiency of SGC-7901cells had no significant differences with time, indicating that half an hour has reached the saturation labeling of the cells. To ensure the same exposure time of fluorescent dye Cy5.5, therefore, the labeling time of both of Cy5.5-Tz or GEBP11-Cy5.5 probes is required for 0.5 h.

Synthesis of Cy5.5-Tz, GEBP11-TCO and GEBP11-Cy5.5.

The Tz was synthesized according to the literature. Cy5.5 (1 mg, 1.0 μmol), *N*,*N'*-diisopropylcarbodiimide (DIC, 7 μL, 0.04 mmol) and *N*-hydroxysuccinimide (NHS, 4.6 mg, 0.04 mmol) were dissolved in 1 mL dry DMSO. The reaction mixture was protected from light and stirred at room temperature for 6 h, and the progress of the reaction monitored by RP-18z TLC. After completion of the reaction ether (1 mL) and ethyl acetate (1 mL) were added to the mixture. A blue solid was obtained which was dissolved in DMSO (300 μL), and added Tz (0.56 mg, 2.8 μmol) and TEA (4 μL). The mixture was stirred for 12 h and then purified on a Prep-HPLC. The Cy5.5-Tz was obtained as a blue solid. HRMS (ESI, negative mode): calculated M_r = 1082.2187 for $C_{50}H_{48}N_7O_{13}S_4$,calculatedM_r/3 = 360.7392, found *m/z* = 360.7424 ([M_r/3]).

TCO-NHS (0.5 mg, 2 μmol) and GEBP1**1** (2 mg, 2 μmol) dissolved in dry DMSO (300 μL) and TEA (4 μL). The mixture was stirred at room temperature for 12 h. After completion of the reaction ethyl acetate (1 mL) were added to the mixture and wash with ether (3×1 mL). The crude white solid was purified on a Pre-HPLC. HRMS (ESI, negative mode) of GEBP11-TCO: calculated M_r= 1210.5179 for $C_{52}H_{82}N_{12}O_{15}S_3$, found *m/z* = 1210.5082 ([M_r]).

Cy5.5-NHS (0.6 mg, 0.5 μmol) and 2 mg GEBP11 (2 mg, 2 μmol) dissolved in dry DMSO (500 μL) and TEA (5 μL). The mixture was stirred at room temperature for 12 h. Ether (1 mL) were added to the mixture and wash with ether (3×1 mL). The crude blue solid was purified on a Pre-HPLC. HRMS (ESI, negative mode) of GEBP11-Cy5.5: = 1954.5711 for $C_{84}H_{109}N_{14}O_{26}S_7$, calculated M_r/3 = 651.5232, found *m/z* = 651.5227 ([M_r/3]).

Cell Lines and Cell Culture.

The human gastric carcinoma cell line SGC7901 and human immortalized fetal gastric epithelial cell line GES were cultured in RPMI1640 (Gibco) culture medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 10 mg/Ml streptomycin. All cells were cultured in a humidified incubator at 37 °C with 5% CO_2. SGC7901 cell line was used to establish the tumor-endothelial cell in vitro co-culture model. Briefly, SGC7901 cells were cultured in M200 complete medium without low serum growth supplement for 24 h. The culture medium was harvested and centrifuged. The supernatant was harvested and the pellets were discarded. The supernatant

was used as the conditioned medium. Then, the conditioned medium and complete medium as described before were mixed well at a ratio of 1:4.