Supporting Information

A Novel Strategy for Preparing Dual-modality Optical/PET Imaging Probes via the Photo-click Chemistry

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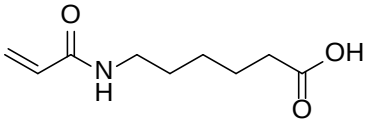
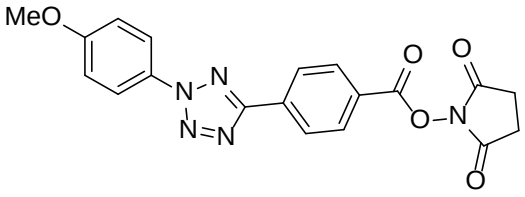
1. Materials and equipments

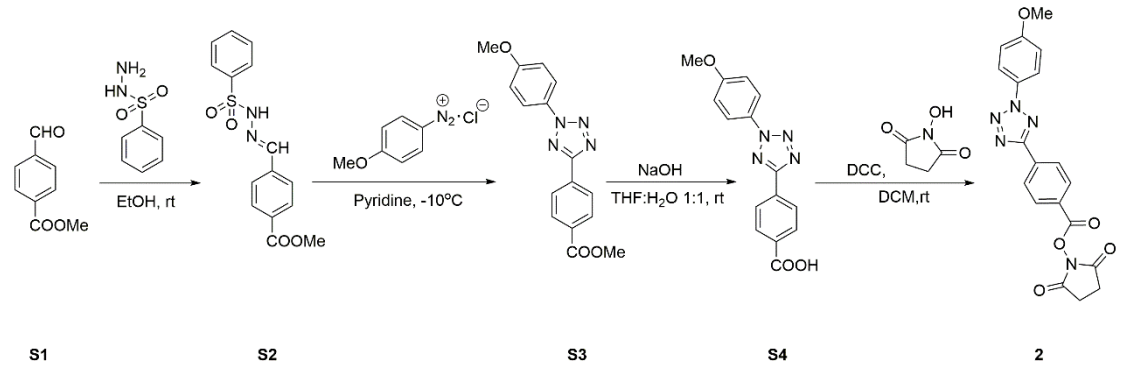
Aqueous solutions were prepared using ultrapure water (resistivity, 18 MΩ*cm). ^{68}Ga (Eckert & Ziegler Isotope Products, Berlin, Germany) was eluted directly to a Modular-Lab (Eckert & Ziegler Isotope Products), concentrated on a Strata-X-C column from Phenomenex (Torrance, CA), and the ^{68}Ga-eluate was collected by desorbing it with 0.8 mL of 0.01 M HCl/98% acetone solution. All other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise specified. ^{1}H NMR spectra were recorded on Bruker DRX 400 MHz or 500 MHz spectrometers (Billerica, MA), and ESI-MS were measured on a Waters LCT-Premier XE LC-MS station (Milford, MA). Luna C-18 HPLC columns were from Phenomenex (Torrance, CA, USA). HPLC were performed on a Waters 1525 Binary HPLC pump (Milford, MA) with a Waters 2489 UV/visible detector and a model 106 Bioscan radioactivity detector for the purification of peptide conjugates and analysis of their ^{68}Ga labeled conjugates using two elution buffers (0.1 v% TFA in de-ionized water as elution buffer A and 0.1 v% TFA in acetonitrile as elution buffer B). PET/CT data were acquired using an Inveon Preclinical Imaging Station (Siemens Medical Solutions).

2. Preparation of functionalization compounds

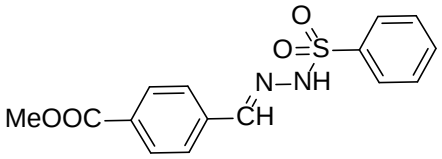
Synthesis of  (**1**)

To 1.3g 6-aminohexanoic acid (10mmol) dissolved in 30ml saturated $NaHCO_3$ was added 450mg acryloyl chloride (5mmol) in 5ml DMF. The reaction mixture was allowed to stir at room temperature for 2h followed by acidified to pH = 1 with 2N HCl. The reaction mixture was then extracted with DCM 20ml × 3. The DCM extraction was washed with brine, dried over Na_2SO_4, and concentrated to give 1.2g product. Yield 65%. ^{1}H NMR (400 MHz, DMSO-d6) δ 1.20-1.32 (2H, m, CH_2), 1.38-1.53 (4H, m, CH_2), 2.19 (2H, t, J = 7.2Hz, CH_2), 3.08-3.13 (2H, m, CH_2), 5.55 (1H, dd, J_1 = 10.4Hz, J_2 = 2.4Hz, CH), 6.05 (1H, dd, J_1 = 16.8Hz, J_2 = 2.4Hz, CH), 6.19 (1H, dd, J_1 = 16.8Hz, J_2 = 10.4Hz, CH).

Synthesis of  (**2**)

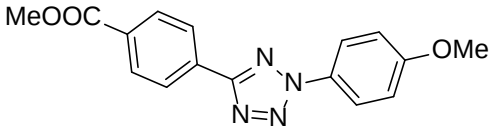


Scheme S1. Synthesis of functionalized compound 2.



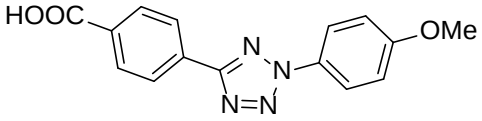
Methyl (E)-4-((2-(phenylsulfonyl)hydrazono)methyl)benzoate (**S2**)

To a solution of 0.98g methyl 4-formylbenzoate (6mmol) in 50ml EtOH was added 1.03g benzenesulfonohydrazide (6mmol). The reaction mixture was stirred at room temperature for 5 hours. Precipitate was filtered to give 1.6g product. Yield 84%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.92 (3H, s, OCH_3), 7.52-7.66 (5H, m, CH), 7.77 (1H, s, NH), 7.99-8.03 (5H, m, CH).



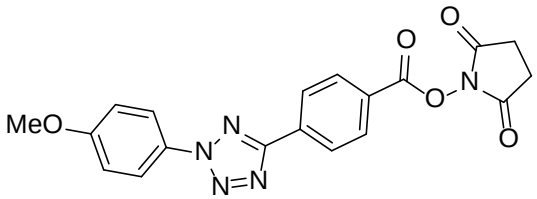
Methyl 4-(2-(4-methoxyphenyl)-2H-tetrazol-5-yl)benzoate (**S3**)

To 0.62g 4-methoxyaniline (5mmol) and 1.3ml concentrated HCl in 8ml 50% ethanol was added 0.35g $NaNO_2$ in 2ml water dropwise at 0^{O}C. The obtained 4-methoxybenzenediazonium chloride solution was then slowly added to a solution of 1.59g methyl (E)-4-((2-(phenylsulfonyl)hydrazono)methyl)benzoate (5mmol) in 30ml pyridine over a period of 30minutes at -10^{o}C. The reaction was subsequently quenched upon the addition of 50ml DCM and water. The DCM layer was separated, washed with 100ml 3M HCl and concentrated. The residue was then purified by column chromotagraphy (HEX:EA = 1:1) to give 450mg product. Yield 29%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.91 (3H, s, OCH_3), 3.97 (4H, m, CH_2), 7.08 (2H, d, J = 9.6Hz, CH), 8.12 (2H, d, J = 9.6Hz, CH), 8.19 (2H, d, J = 8.8Hz, CH), 8.33 (2H, d, J = 8.8Hz, CH).



4-(2-(4-Methoxyphenyl)-2H-tetrazol-5-yl)benzoic acid (**S4**)

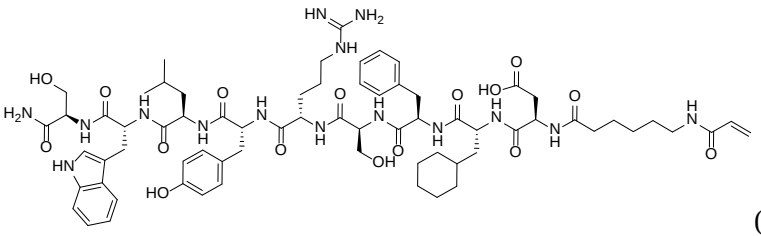
To a solution of 279mg methyl 4-(2-(4-methoxyphenyl)-2H-tetrazol-5-yl)benzoate (0.9mmol) in 12ml THF was added 360mg NaOH (9mmol) in 12ml H_2O. The reaction mixture was stirred vigorously at room temperature for overnight. The reaction was quenched by partitioning the reaction mixture between 10ml EA and 10ml water. The aqueous layer was the separated, washed with 10ml EA, and acidified with 2N HCl to pH =1. Precipitate was collect to give 180mg product. Yield 68%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.88 (3H, s, OCH_3), 7.24 (2H, d, J = 8.8Hz, CH), 8.10 (2H, d, J = 8.8Hz, CH), 8.15 (2H, d, J = 8.0Hz, CH), 8.27 (2H, d, J = 8.0Hz, CH).



2,5-Dioxopyrrolidin-1-yl 4-(2-(4-methoxyphenyl)-2H-tetrazol-5-yl)benzoate (**2**)

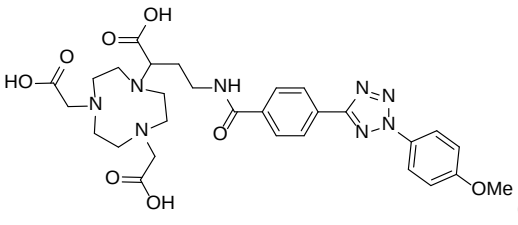
To 85mg 4-(2-(4-methoxyphenyl)-2H-tetrazol-5-yl)benzoic acid (0.29mmol) in 5ml THF was added 60mg DCC (0.29mmol) and 66mg N-hydroxysuccinimide (0.58mmol). The reaction mixture was stirred at room temperature for 24hours. Precipitate was removed by filtration. The filtrate was subsequently diluted with 15ml water, and the precipitate was collected to give 105mg product. Yield 93%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.94 (4H, s, CH_2), 3.89 (3H, s, OCH_3), 7.24 (2H, d, J = 9.2Hz, CH), 8.11 (2H, d, J = 9.2Hz, CH), 8. 33 (2H, d, J = 8.8Hz, CH), 8.43 (2H, d, J = 8.8Hz, CH).

3. Application of functionalization compounds in preparing dual-modality AE105 probe (7)



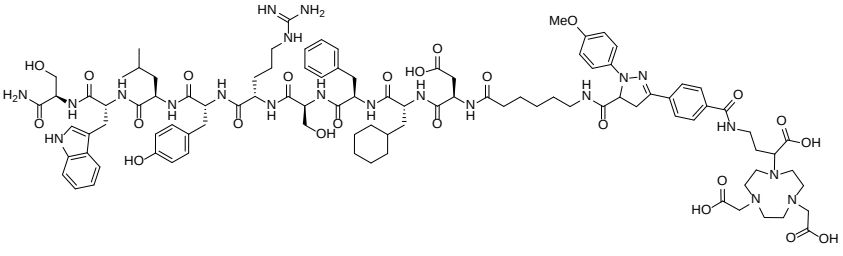
(**4**)

Commercial available AE105 resin 200mg (80 μmol) was swelled in DCM for 10min followed by the treatment of 5equiv HATU, 5equiv HOBt, and 5equiv compound **1** in DMF. The reaction was allowed to stand for overnight, and the excess amount of reagents were removed. The resin was washed with DCM three times and cleaved by 95%TFA to release the modified AE105 peptide. TFA was then removed, and the peptide crude was precipitated from diethyl ether. The crude was subsequently dissolved in acetonitrile and purified by HPLC to get 50mg product. Yield 45%. ESI-MS, M/Z $(M+H)^+$ = 1393.52.



(6)

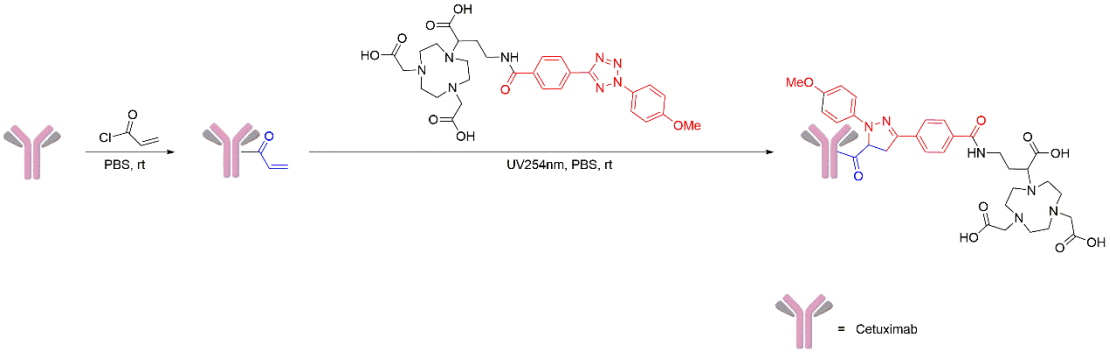
To 0.5mg *tert*-butyl protected NOTA-amine **5** (1μmol) in 0.1ml DMF was added 3equiv compound **2** and 10equiv DIEA, the reaction mixture was allowed to stand at room temperature for 2h. DMF was removed by lyophilization, and the residue was dissolved in acetonitrile and purified by HPLC to isolated the *tert*-butyl protected NOTA tetrazole conjugation which was subsequently treated with 95% TFA to give 0.4mg product. Yield 64%. ESI-MS, M/Z $(M+H)^+$ = 626.25.



(7)

2.8mg **4** (2μmol) and 0.25mg **6** (0.4μmol) were mixed in acetonitrile : water = 1 : 1. The reaction mixture was then irradiated by UV254 for 2min and allowed to stir for another 15min. The reaction mixture was subsequently purified by HPLC to give 0.4mg dual-modality AE105 probe. Yield 50%. ESI-MS, M/Z $(M+2H)^{2+}/2$ = 995.47.

4. Preparation of the cetuximab-based dual-modality optical/PET probe



Scheme S2. Preparation of the cetuximab-based dual-modality optical/PET probe.

To 1mg (6nmol) commercial available cetuximab in 4ml 0.1M Na_2HPO_4 buffer (pH=8.2) was added 54μg (600nmol) acryloyl chloride in 40μl DMF. The reaction mixture was gently rotated for overnight at $4^{O}C$, and the excess amount of acryloyl chloride was removed by centrifuge filters. The alkene functionalized cetuximab 0.8mg in 1ml PBS buffer (pH = 7.0) was subsequently conjugated with compound **6** (200nmol in 20μl DMF) to give 0.6mg dual-modality optical/PET cetuximab.

5. Immunofluorescent Staining of U87MG using prepared dual modality AE105 probe

Cells were seeded in an 8 well chamber slide (100,000 cells per well) 24 h prior to the experiment. Before the experiment, cells were washed twice with PBS, and added with culture medium. Then blocking agent (10 µg AE105) was added to half of the wells to determine in vitro non-specific uptake. After 1h incubation, prepared dual modality AE105 probe (20 pmol per well) was then added to each well, and cells were incubated for another 2 h. Medium was then removed and cells were washed twice with PBS. After fixing the cells using 1% Paraformaldehyde, the slide was sealed and observed under fluorescence microscopy (40 X, oil).

6. Small-animal PET/CT imaging studies for ^{68}Ga labeled dual modality AE105 probe

The ^{68}Ga labeling of dual modality AE105 probe was conducted by incubating the AE105 probe and ^{68}Ga in 0.1 M NaOAc buffer (pH ~ 4. 0) at 90 °C for 10 minutes, with the specific activity (SA) of 1.0 mCi/nmole. All animal studies were conducted under a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. U87MG xenograft tumor–bearing mice were injected intravenously (lateral tail vein) with the prepared ^{68}Ga labeled dual modality AE105 probe. Half of the mice received a dose that was premixed with AE105 (50 µg) for blocking. At 1h and 2h post injection mice were anesthetized with 2% isoflurane, and small-animal PET/CT was performed. Static images were collected for 15 min. PET and CT images were co-registered with Inveon Research Workstation (IRW) software (Siemens Medical Solutions). PET images were reconstructed with the ordered-subsets expectation maximization 3-dimensional/maximum a posteriori probability algorithm, and the analysis of images was done using IRW.

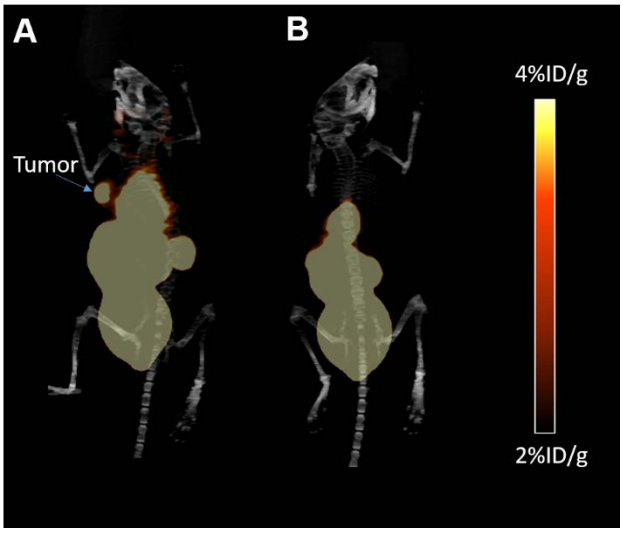


Figure S1. MIP of PET/CT imaging using ^{68}Ga labeled dual-modality AE105 probe: A) 1 h p.i, ; B) with blockade, 1 h p.i.

7. Ex vivo biodistribution studies for dual modality AE105 probe

The 5×10^6 U87MG cells in 150 μl PBS were injected into the right shoulder of NCR nude mice (n = 3). Two weeks after tumor inoculation, 50 μCi of ^{68}Ga labeled dual modality AE105 probe in 150 μl saline were injected intravenously (lateral tail vein). At 1h post tracer injection, all mice were killed, and the major organs as well as tumor were harvested for radioactivity measurement using a gamma counter. Quantification results were expressed as percentage of injected dose per gram (%ID/g) (**Figure S2**).

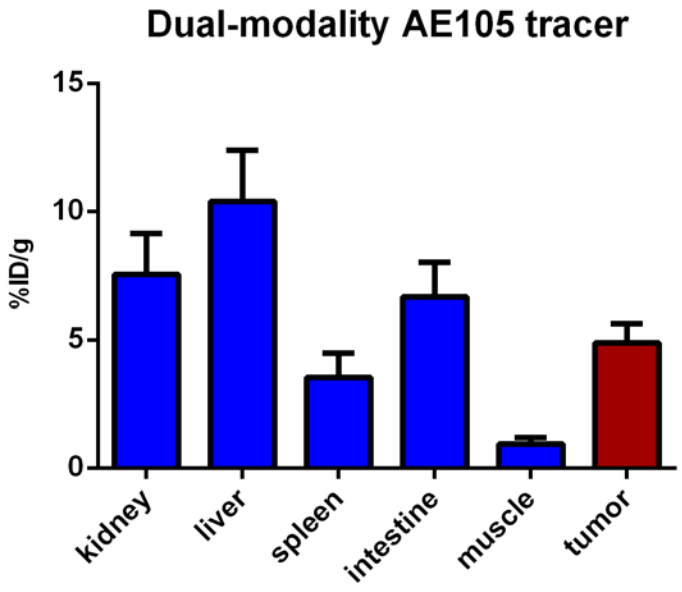


Figure S2. Quantitative uptake of dual-modality AE105 probe in major organs.

8. ^{18}F labeling of synthesized dual-modality AE105 probe

^{18}F- was obtained from UPMC Presbyterian Hospital using a Siemens Eclipse HP medical cyclotron. ^{18}F- was first loaded onto a QMA Sep-Pak Light cartridge (Waters, Milford, MA, USA) and washed with 5 ml of metal-free water. ^{18}F was then eluted from the cartridge with 400 μl of saline, from which the hottest fraction (~ 100 μl) was taken. The pH of the solution was adjusted to 4 with metal-free glacial acetic acid. To the obtained ^{18}F solution (~ 30 mCi) was added $AlCl_3$ (2 mM, 3 μl) in 0.1 M sodium acetate buffer (pH 4), and the resulting mixture was incubated at room temperature for 10 min. 200nmole dual modality AE105 probe[40 nmol/μl in dimethyl sulfoxide (DMSO)] was then added to the Al^{18}F solution. The reaction mixture was incubated at 100°C for another 15 min, and radio-HPLC results showed ~ 60% radiolabeling yield could be obtained.

9. Small-animal PET/CT imaging studies for ^{64}Cu labeled dual modality cetuximab probe

The ^{64}Cu labeling of dual modality cetuximab probe was conducted by incubating the cetuximab probe and ^{64}Cu in 0.1 M NH_4OAc buffer (pH ~ 6. 8) at 37 °C for 30 minutes, with the specific activity (SA) of 10 mCi/1mg. Mice bearing U87MG and 4T1 xenografts (**Figure S3**) were injected intravenously (lateral tail vein) with the prepared ^{64}Cu labeled dual modality cetuximab probe. At 48h post injection mice were anesthetized with 2% isoflurane, and small-animal PET/CT was performed in the same way as for the AE105 probe.

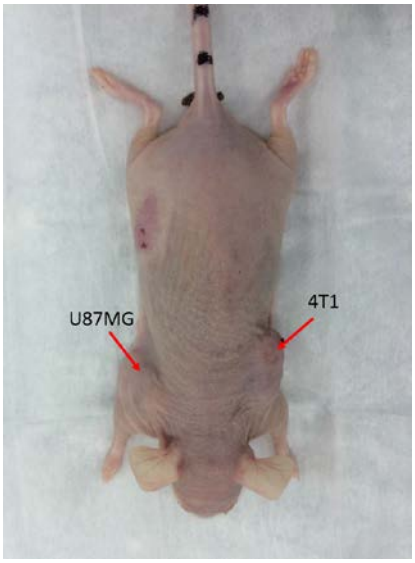


Figure S3. Mouse xenografted with U87MG and 4T1.

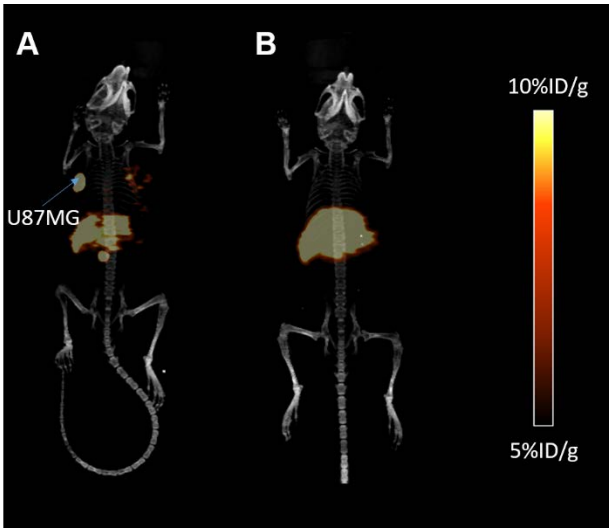


Figure S4. MIP of PET/CT imaging using ^{64}Cu labeled dual modality cetuximab probe: A) 48 h p.i, ; B) with blockade, 48 h p.i

10. Ex vivo biodistribution studies for ^{64}Cu labeled dual modality cetuximab probe

The 5×10^6 U87MG cells in 150 μl PBS were injected into the right shoulder of NCR nude mice respectively (n = 3). Two weeks after tumor inoculation, 100 μCi of ^{64}Cu labeled dual modality cetuximab probe in 150 μl saline were injected intravenously (lateral tail vein). At 48h post tracer injection, all mice were killed, and the major organs as well as tumor were harvested for radioactivity measurement using a gamma counter. Quantification results were expressed as percentage of injected dose per gram (%ID/g) (**Figure S5**).

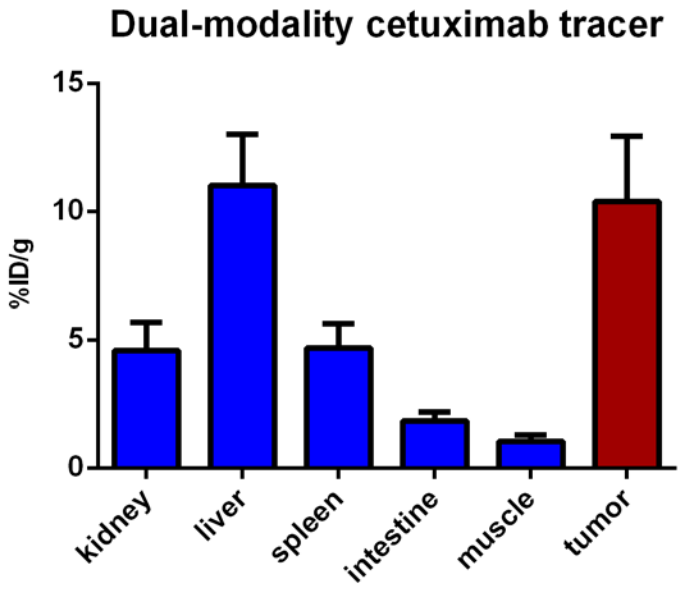


Figure S5. Quantitative uptake of dual-modality cetuximab probe in major organs.