Supporting Information

"A Chemoenzymatic Strategy for the Synthesis of Site-Specifically Labeled Immunoconjugates for Multimodal PET and Optical Imaging"

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Reagents and General Procedures: Unless otherwise noted, all chemicals were acquired from Sigma-Aldrich (St. Louis, MO) and were used without further purification. All water employed was ultra-pure (>18.2 $M\Omega cm^{-1}$ at 25 °C), all DMSO was of molecular biology grade (>99.9%), and all other solvents were of the highest grade commercially available. Humanized A33 (huA33) was obtained courtesy of the Ludwig Institute for Cancer Immunotherapy. The GalT(Y289L) enzyme, UDP-GalNAz, and DIBO-DFO with a PEG linker were purchased from Thermo Fisher Scientific (Eugene, OR). All instruments were calibrated and maintained in accordance with standard, manufacturer-recommended quality-control procedures. UV-Vis measurements were taken using a Thermo Scientific NanoDrop 2000 Spectrophotometer.

^{89}Zr was produced at Memorial Sloan Kettering Cancer Center using an EBCO TR19/9 variable-beam energy cyclotron (Ebco Industries Inc., British Columbia, Canada) via the $^{89}Y(p,n)^{89}Zr$ reaction. ^{89}Zr was purified in accordance with previously reported methods to create ^{89}Zr with a specific activity of 5.3 – 13.4 mCi/μg (195 – 497 MBq/μg).[1] A Capintec CRC-15R Dose Calibrator (Capintec, Ramsey, NJ) was used for all activity measurements. To ensure the accurate quantification of activity levels, a calibrated Perkin Elmer (Waltham, MA) Automatic Wizard[2] Gamma Counter was employed, and all experimental samples were counted for at least 1 min. Silica-gel impregnated glass-fiber instant thin-layer chromatography paper (Pall Corp., East Hills, NY) were used for radioTLC experiments, and all radioTLC strips were analyzed on a Bioscan AR-2000 radio-TLC plate reader using Winscan Radio-TLC software (Bioscan Inc., Washington, D. C.). All experiments performed on laboratory animals were performed according to a protocol approved by the Memorial Sloan-Kettering Institutional Animal Care and Use Committee (protocol 08-07-013).

Cell Culture: Human colorectal cancer cell line SW1222 was obtained from Sigma-Aldrich and maintained in Iscove's Modified Dulbecco's Medium, supplemented with 10% heat-inactivated fetal calf serum, 2.0 mM glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin in a 37 °C environment containing 5% CO_2. Cell lines were harvested and passaged weekly using a formulation 0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium.

Xenograft Models: All experiments were performed under an Institutional Animal Care and Use Committee-approved protocol, and the experiments followed institutional guidelines for the proper and humane use of animals in research. Six to eight week-old athymic nude female (Hsd: Athymic Nude-nu) mice were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in ventilated cages, were given food and water *ad libitum*, and were allowed to acclimatize for approximately 1 week prior to inoculation. SW1222 tumors were induced on the right shoulder by a subcutaneous injection of 5.0×10^6 cells in a 150 μL cell suspension of a 1:1 mixture of fresh media: BD Matrigel (BD Biosciences, Bedford, MA). The xenografts reached ideal size for imaging and biodistribution (~100-150 mm^3) in approximately 15-18 d.

Synthesis of *N*-Azidoacetylgalactosamine (UDP-GalNAz): UDP-GalNAz was synthesized according to previously reported methods.[2, 3]

Synthesis of Alexa Fluor® 680-DIBO (DIBO-Dye680): To a solution of Alexa Fluor® 680 (Dye680) carboxylic acid, succinimidyl ester bis(triethylammonium salt) (14 mg, 11 μmol) and N-(2-aminoethyl)-2-(5,6-dihydro-11,12-didehydro-dibenzo[a,e]cycloocten-5-yloxy)acetamide (DIBO amine, 4 mg, 12 μmol) (Thermo Fisher Scientific, Eugene, OR) in 1 mL of anhydrous DMF was added triethylamine (25 μL, 180 μmol) and the mixture was stirred at room temperature for 1 hour. The resulting reaction mixture was added into 10 mL of ethyl acetate slowly over 1 minute period while stirring vigorously at room temperature. The resulting precipitate was collected by centrifugation to give the desired product (DIBO-Dye680, 14 mg, 88 % yield) as a blue solid. TLC (silica gel, 15 % H_2O in CH_3CN): $R_f = 0.50$

Site-specific modification of huA33:

Glycans Modification: huA33 (2.5 mg, 5 mg/mL) underwent a buffer exchange into pre-treatment buffer (50 mM Bis-Tris, 100 mM NaCl, pH 6.0) using a micro-spin column (Amicon Ultra Centrifugal Filter 0.5 mL w/ 50,000 MWCO). The column was first equilibrated in 50 mM Bis-Tris, 100 mM NaCl, pH 6.0, and then spun for 5 minutes at 5000 x g. 500 μl of the huA33 antibody solution was added and then spun down for 5 minutes at 5000 x g. This procedure was repeated 3 times to complete the buffer exchange to 50 mM Bis-Tris, 100 mM NaCl, pH 6.0. After the final spin, the antibody was isolated in ~110-120 μL of buffer. This solution was supplemented with 15 μL of β-

1.4-galactosidase [from S. pneumonia (2 mU/μL), obtained from Prozyme, Inc., Hayward, CA] and placed in an incubator at 37 °C for 6 h.

GalNAz Labeling: After the β-1.4-galactosidase treatment, 75 μL H_2O, 12.5 μL 1 M Tris buffer pH 7.6, 25 μL GalT(Y289L) (from a stock of 2 mg/mL in 50 mM Tris, 5 mM EDTA, pH 8), 2.5 μL 1 M $MnCl_2$ (in 0.1 M HCl), and 10 μL UDP-GalNAz (from a stock of 40 mM in H_2O) were added to the reaction solution to bring the final volume up to 250 μL. This reaction solution contained concentrations of 10 mg/mL huA33, 10 mM $MnCl_2$, 1.6 mM UDP-GalNAz, and 0.2 mg/mL GalT (Y289L) and was incubated for 16 h at 30 °C.

DIBO-DFO and DIBO-Dye680 Ligation: The solution from the GalNAz labeling step was purified via centrifugal filtration using 2 mL Amicon® Ultra centrifugal filters with a 50,000 Dalton molecular weight cut-off (Millipore Corp., Billerica, MA) and TBS buffer pH 7.4. After centrifugation, the modified huA33 antibody (1 mg in 900 μL TBS buffer pH 7.4) was combined with either 100 μL DIBO-DFO (from a 2 mM stock in DMSO), 100 μL DIBO-Dye680 (from a 2 mM stock in DMSO), or 100 μL of a mixture of the two DIBO constructs to yield a final solution containing 1 mg/mL huA33 and 0.2 mM DIBO. This solution was then incubated for 16 h at 25 °C.

Purification: After the click labeling, the completed antibody was purified via size exclusion chromatography (PD10 column, GE Healthcare) and concentrated via centrifugal filtration using 2 mL Amicon® Ultra centrifugal filters with a 50,000 Dalton molecular weight cut-off (Millipore Corp., Billerica, MA).

Determination of Degree of Fluorescent Labeling: To determine the degree of labeling (DOL) of the fluorophore-labeled antibodies, UV-Vis absorbance measurements were taken at 280 nm and 774 nm for three separate antibody concentrations. The degree of labeling was calculated using the following formulas:

$$A_{huA33} = A_{280} - A_{max}(CF)$$

$$DOL = [A_{max} * MW_{huA33}]/[[mAb] * \varepsilon_{Alexa\ Fluor®\ 680}]$$

where the correction factor (CF) for Alexa Fluor® 680 was given as 0.05 by the supplier, MW_{huA33} = 150,000, $\varepsilon_{680,\ Alexa\ Fluor®\ 680}$ = 180,000, and $\varepsilon_{280,\ huA33}$ = 210,000.

SDS-PAGE Analysis of the Antibody Conjugation: 1 μg antibody (2 μL of a 0.5 mg/mL stock) was combined with 18.5 μL H_2O, 3 μL 500 mM dithiothreitol (NuPAGE® 10X Sample Reducing Agent, Life Technologies), and 7.5 μL 4X electrophoresis buffer (NuPAGE® LDS Sample Buffer, Thermo Fisher, Eugene, OR). This mixture was then denatured by heating to 90 °C for 15 min using a heat block. Subsequently, 25 μL of each sample was then loaded alongside an appropriate molecular weight maker (Mark12™ unstained standard, Life Technologies) onto a 1 mm, 10 well 4-12% Bis-Tris protein gel (Life Technologies) and run for ~3 h at 10 V/cm in MOPS buffer . The completed gel was washed 3 times with H_2O, stained using SimplyBlue™ SafeStain (Life Technologies) for 1 h, and destained overnight in H_2O. The gel was then analyzed using an Odyssey Infrared Gel Scanner (Li-Cor Biosciences, Lincoln, NE).

PNGase F Treatment of Antibody: huA33 antibody construct (1 μg) in 10 μL TBS was denatured with 0.5% SDS and 40 mM DTT by adding 17 μl H_2O and 3 μL 10× Glycoprotein Denaturation Buffer (New England Biolabs, Ipswich, MA) and incubated for 10 min at 90 °C on a heat block. Subsequently, 18 μl H_2O, 6 μL 500 mM sodium phosphate, pH 7.5 (G7 reaction buffer from New England Biolabs), and 6 μL 10% NP-40 were added. This solution was then split into two aliquots: one aliquot was supplemented with 1 μl PNGase F (New England Biolabs) and incubated overnight at 37 °C, and the other aliquot was not treated. After incubation, SDS-PAGE electrophoresis was employed to analyze the PNGaseF-treated and untreated samples.

Non-specific Antibody Modification of DFO-^{ns}huA33-Dye680: huA33 (2 mg; 13 nmol) was dissolved in 1 mL of phosphate buffered saline (pH 7.4), and the pH of the solution was adjusted to 8.8-9.0 with $NaHCO_3$ (0.1 M). In parallel, a solution of DFO-NCS in DMSO (5-10 mg/mL) and Alexa Fluor 680-NHS in DMSO (5-10 mg/mL) were prepared. Volumes of these two solutions corresponding to 5 molar equivalents of DFO-NCS (65 nmol) and 4 molar equivalents of Alexa Fluor 680-NHS (52 nmol) were mixed together in a microcentrifuge tube. The pH-adjusted antibody solution was then added to the tube containing DFO-NCS and Alexa Fluor® 680-NHS, and the contents were mixed via vigorous pipetting. The resulting solution was incubated with gentle shaking for 60 min at 37 ºC.

After 60 min, the modified antibody was purified using centrifugal filter units with a 50,000 molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp., Billerica, MA) and phosphate buffered saline (PBS, pH 7.4).[4]

Radiolabeling of Antibody Constructs with ^{89}Zr: For each antibody construct, 1 mg of immunoconjugate was added to 400 μL buffer (PBS, pH 7.4). [^{89}Zr]Zr-oxalate (3000-3250 μCi) in 1.0 M oxalic acid was adjusted to pH 7.0-7.5 with 1.0 M Na_2CO_3. After the evolution of CO_2(g), the ^{89}Zr solution was added to the antibody solution, and the resulting solution was incubated at room temperature for 1 h. After 1 h, the reaction progress was assayed using radio-TLC and an eluent of 50 mM EDTA (pH 5). Subsequently, the reaction was quenched with 50 μL of 50 mM EDTA (pH 5), and the antibody construct was purified using size-exclusion chromatography (Sephadex G-25 M, PD-10 column, GE Healthcare; dead volume = 2.5 mL, eluted with 500 μL fractions of PBS, pH 7.4) and concentrated via centrifugal filtration units with a 50,000 molecular weight cut off (Amicon™ Ultra 4 Centrifugal Filtration Units, Millipore Corp., Billerica, MA). The radiochemical purity of the crude and final radiolabeled bioconjugate was assayed by radio-TLC again using 50 mM EDTA (pH 5.0) as an eluent. In the ITLC experiments, free $^{89}Zr^{4+}$ cations and [^{89}Zr]-EDTA elute with the solvent front, while radiolabeled antibody construct remains at the baseline.

Determination of Chelate Number: Radiometric isotopic dilution assays similar to those described in Anderson, *et al*. and Holland, *et al*.[5, 6] were performed in order to determine the number of accessible DFO chelators appended per antibody. All experiments were performed in triplicate.

Radiolabeled Antibody Stability Assays: The stability of the site-specifically and non-site-specifically labeled radioimmunoconjugates with respect to radiochemical purity and loss of radioactivity from the antibody was investigated via incubation of the antibodies in human serum for 7 days at 37 °C (n = 3). After the prescribed incubation time, the radiochemical purity of the antibodies was determined via radio-TLC with an eluent of 50 mM EDTA pH 5.0.

Immunoreactivity Assays: The immunoreactivity of the ^{89}Zr-labeled radioimmunconjugates was determined using an antigen saturation assay. Briefly, suspensions of 1×10^7 SW1222 colorectal cancer cells in 100 μL media were prepared in microcentrifuge tubes. In parallel, a solution of 0.1-0.4 ng/μL radioimmunoconjugate was prepared in PBS supplemented with 1% bovine serum albumin. 20 μL of the radioimmunoconjugate solution was then added to the cell suspension, and the resulting mixture was agitated via vigorous pipetting and subsequently incubated on ice for 1 h. After 1 h, the cells were then pelleted via centrifugation (600 g for 5 min). After centrifugation, the media was carefully removed and placed in a separate tube, and 1 mL of ice-cold PBS was added to the original tube containing the cells. The cells were then pelleted again via centrifugation (600 g for 5 min). Following centrifugation, the PBS was removed and placed in a separate tube. These PBS washing steps were repeated two more times. After washing, the amount of radioactivity in all five tubes — cell pellet, media, wash 1, wash 2, and wash 3 — was assayed using a gamma counter. The counting data was then background corrected, and the immunoreactive fraction was then calculated by dividing the counts in the cell pellet by the sum of the counts in the cell pellet, media, and three washes. No weighting was applied to the data, and the data were obtained in triplicate. Negative controls for non-specific radioimmunoconjugate binding to the microcentrifuge tube and binding of the radioimmunoconjugates to non-antigen-expressing cells were performed.

PET Imaging: PET imaging experiments were conducted on a microPET Focus rodent scanner (Concorde Microsystems). Mice bearing subcutaneous SW1222 xenografts (right shoulder; 100-150 mm^3) were administered ^{89}Zr-DFO-^{ss}huA33-Dye680 or ^{89}Zr-DFO-^{ns}huA33-Dye680 (180-200 μCi, 72-80 μg, in 200 μL 0.9% sterile saline) via tail vein injection (t = 0). Approximately 5 minutes prior to the PET images, mice were anesthetized by inhalation of 2% isoflurane (Baxter Healthcare, Deerfield, IL)/oxygen gas mixture and placed on the scanner bed. Anesthesia was maintained using 1% isoflurane/gas mixture. PET data for each mouse were recorded via static scans at time points between 24 and 120 h. A minimum of 60 million coincident events were recorded for each scan, which lasted between 5-20 min. An energy window of 350-700 keV and a coincidence timing window of 6 ns were used. Data were sorted into 2-dimensional histograms by Fourier re-binning, and transverse images were reconstructed by filtered back-projection (FBP) into a 128 × 128 × 63 (0.72 × 0.72 × 1.3 mm) matrix. Image data were normalized to correct for dead-time count losses, positron-branching ratio, physical decay to the time of injection, and non-uniformity of response of the PET. No scatter, partial-volume averaging, or scatter correction was applied. A system calibration factor derived from the imaging of a mouse-sized water-equivalent phantom

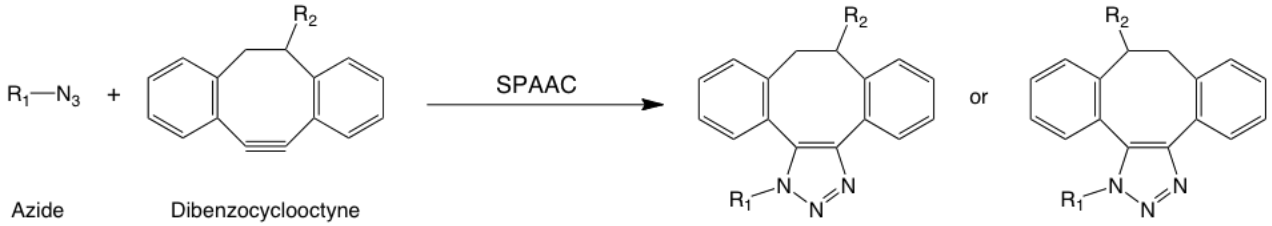
containing ^{89}Zr was used to convert the counting rates in the reconstructed images to activity concentrations (percentage injected dose [%ID] per gram of tissue). Images were analyzed using ASIPro VMTM software (Concorde Microsystems).

Acute Biodistribution: Athymic nude mice bearing subcutaneous SW1222 xenografts (right shoulder; 100-150 mm^3) were randomized before the study and were warmed gently with a heat lamp for 5 min before the administration of ^{89}Zr-DFO-^{ss}huA33-Dye680 (15-20 μCi, 6-8 μg, in 200 μL 0.9% sterile saline) via tail vein injection (t = 0). Subsequently, the mice (n = 4 per group) were euthanized by CO_2(g) asphyxiation at 48 and 96 h post-injection, and 13 tissues (including tumor) were removed, washed, dried, weighed, and counted in a gamma counter. The number of counts in each tissue was background and decayed corrected to the time of injection and converted to activity units (μCi) using a calibration curve generated from known standards. The %ID/g for each tissue sample was then calculated by normalization to the total activity injected and the mass of each tissue. In addition, in order to assay antigen specificity, an additional cohort of animals (n = 4) were administered ^{89}Zr-DFO-^{ss}huA33-Dye680 with significantly lowered specific activity (LSA) ── achieved by co-injection of the standard dose of ^{89}Zr-DFO-^{ss}huA33-Dye680 along with 300 μg of the cold, unlabeled DFO-^{ss}huA33-Dye680 conjugate ── and were euthanized 72 h post-injection, and analyzed in a manner identical to the other cohorts in the study.

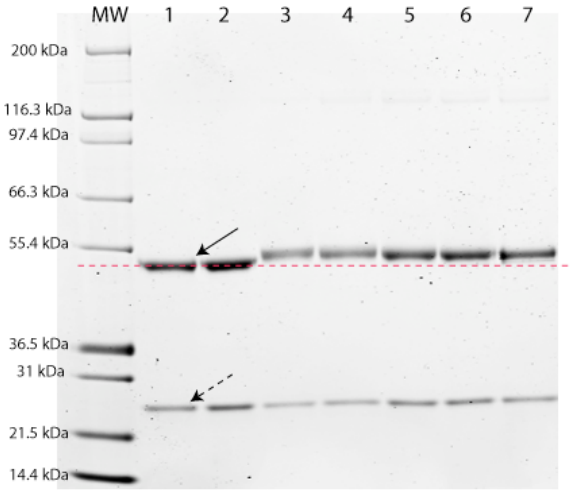
Fluorescence Imaging: Mice bearing subcutaneous SW1222 xenografts (right shoulder; 100-150 mm^3) were administered ^{89}Zr-DFO-^{ss}huA33-Dye680 or ^{89}Zr-DFO-^{ns}huA33-Dye680 (72-80 μg, in 200 μL 0.9% sterile saline) via tail vein injection (t = 0). Approximately 5 minutes prior to the fluorescence images, mice were anesthetized by inhalation of 2% isoflurane (Baxter Healthcare, Deerfield, IL)/oxygen gas mixture and placed on the scanner bed. Anesthesia was maintained using 1% isoflurane/gas mixture. Near-infrared fluorescent images were acquired with an IVIS Spectrum Preclinical Imaging System (Perkin Elmer) using excitation/emission settings of 640nm/704nm. Images were processed using Living Image (v4.4) and values are reported as radiant efficiency to allow direct comparison of images from each experiment. All region-of-interest measurements were generated via automated analysis to avoid user bias.

Ex Vivo **Autoradiography and Fluorescence Microscopy** Following final imaging, tumors were excised, embedded in OCT, and snap-frozen. Series of sequential 1 mm thick cryosections were cut and immediately exposed to a phosphor plate for determination of ^{89}Zr distribution. Digital autoradiographic images at 50 μm pixel size were obtained as previously described.[7] Following autoradiography, sections were fixed in 4% paraformaldehyde. Alexa Fluor 680 images were acquired using an Zeiss Axioplan2 fluorescence microscope connected to a CCD camera, equipped with a motorized stage (Prior Scientific Instruments, Cambridge, UK, USA) and MetaMorph software (Molecular Devices, Sunnyvale, CA) allowing the individual captured image frames to be rendered into a montage of the entire tumor section. Finally, hematoxylin and eosin staining was performed to visualize tumor morphology, and images were acquired in a similar manner. Montage images of each stain, all acquired from the same tumor section, were manually registered using Photoshop CS5 software (Adobe Systems). Image rebinning and pixel-by-pixel correlations were carried out as previously described.[8]

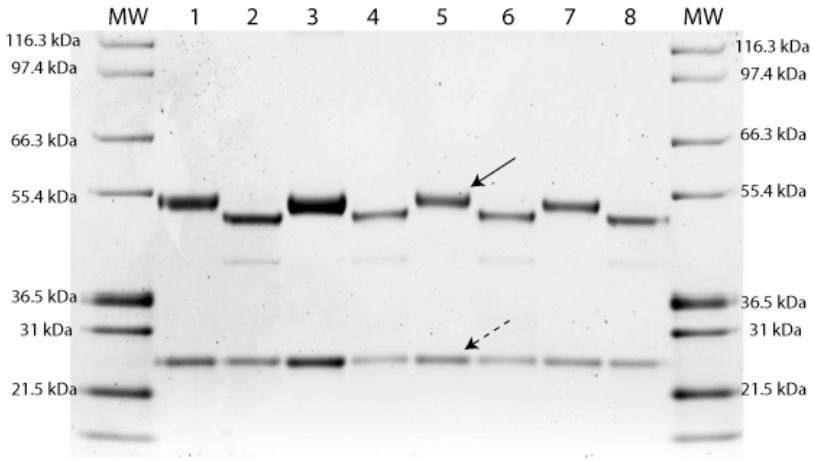
Statistical Analysis: Data were analyzed by the unpaired, two-tailed Student's t-test. Differences at the 95% confidence level ($P < 0.05$) were considered to be statistically significant.



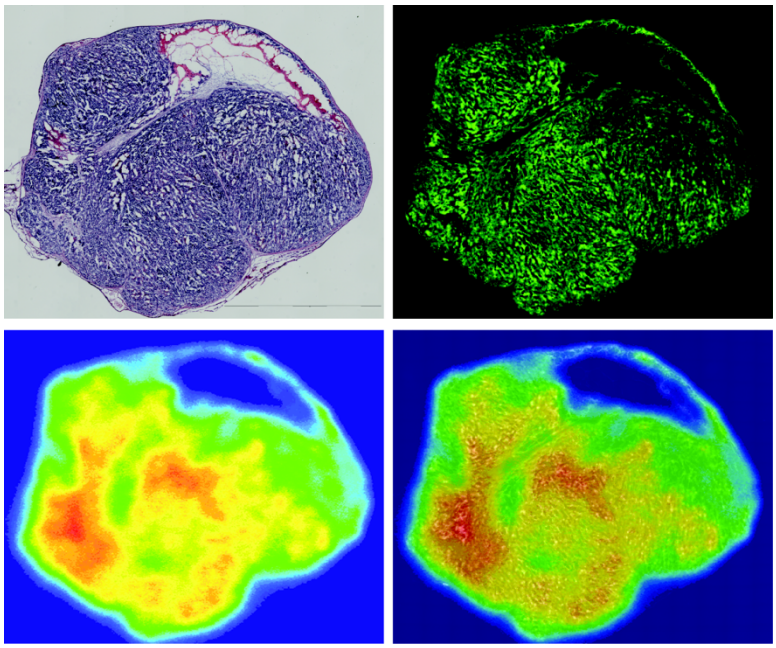
Supplementary Figure S1. Diagram of the strain-promoted azide-alkyne cycloaddition



Supplementary Figure S2. SDS-PAGE analysis of site-specifically labeled immunoconjugates. Lane 1: huA33. Lane 2: ^{ss}huA33-N_3. Lane 3: ^{ss}huA33-DFO. Lanes 4, 5, and 6: DFO-^{ss}huA33-Dye680 synthesized with 3:1, 1:1, and 1:3 mixtures of DIBO-DFO:DIBO-Dye680, respectively. Lane 7: ^{ss}huA33-Dye680. The solid and dotted arrows indicate the antibody heavy chain and light chain, respectively, and the red dotted line indicates the molecular weight of the huA33 heavy chain. MW = molecular weight ladder.



Supplementary Figure S3. SDS-PAGE illustrating the treatment of site-specifically labeled immunoconjugates with PNGase F. Lane 1: untreated huA33. Lane 2: huA33 treated with PNGaseF. Lane 3: untreated ^{ss}huA33-N_3. Lane 4: ^{ss}huA33-N_3 treated with PNGaseF. Lane 5: untreated ^{ss}huA33-DFO. Lane 6: ^{ss}huA33-DFO treated with PNGaseF. Lane 7: untreated ^{ss}huA33-Dye680. Lane 8: ^{ss}huA33-Dye680 treated with PNGaseF. The solid and dotted arrows indicate the antibody heavy chain and light chain, respectively, and the bands at ~40 kDa are the result of excess PNGaseF enzyme. MW = molecular weight ladder.



Supplementary Figure S4. Autoradiography, histology, and fluorescence microscopy of resected SW1222 xenografts from multimodality imaging experiment. Top left: hemotoxyln and eosin staining. Top right: fluorescence microscopy indicating Alexa Fluor® 680 localization (green). Bottom left: autoradiography indicating ^{89}Zr localization. Bottom right: overlay of fluorescence microscopy and autoradiography.

	^{89}Zr-DFO-^{ss}huA33-Dye680		
	48 h	*120 h*	*48 h LSA*
Blood	5.7 ± 1.2	2.4 ± 1.7	9.7 ± 1.4
Tumor	45.9 ± 7.7	45.7 ± 8.1	14.7 ± 1.3
Heart	3.7 ± 1.8	3.2 ± 1.7	4.3 ± 0.6
Lung	3.7 ± 1.6	4.1 ± 2.4	3.7 ± 0.3
Liver	3.9 ± 2.3	2.4 ± 2.1	6.3 ± 1.1
Spleen	2.0 ± 1.1	5.1 ± 1.2	3.1 ± 0.4
Stomach	0.7 ± 0.3	0.9 ± 0.6	0.4 ± 0.4
Large Intestine	0.8 ± 0.2	0.5 ± 0.2	0.7 ± 0.1
Small Intestine	0.9 ± 0.3	1.1 ± 0.4	0.8 ± 0.2
Kidney	3.2 ± 1.8	3.8 ± 1.3	3.5 ± 0.9
Muscle	1.5 ± 0.3	1.4 ± 0.6	1.0 ± 0.3
Bone	2.8 ± 1.2	5.6 ± 1.4	6.9 ± 3.3

Supplementary Table S1. Acute biodistribution uptake values versus time for ^{89}Zr-DFO-^{ss}huA33-Dye680 in mice bearing subcutaneous SW1222 xenografts (n = 4 for each time point). Mice were administered the radioimmunoconjugate (0.55 – 0.75 MBq [15-20 μCi] in 200 μL 0.9% sterile saline) via tail vein injection (t = 0). For the 48 h time point, an additional cohort of animals was given radioimmunoconjugate with dramatically lowered specific activity (LSA), achieved by the co-injection of the standard dose of the ^{89}Zr-labeled construct mixed with 300 μg of cold, unlabeled DFO-^{ss}huA33-Dye680.

	^{89}Zr-DFO-^{ss}huA33-Dye680		
	48 h	*96 h*	*48 h LSA*
Tumor:Blood	8.1 ± 2.2	18.7 ± 13.5	1.5 ± 0.2
Tumor:Heart	12.3 ± 6.3	14.2 ± 7.9	3.4 ± 0.6
Tumor:Lung	12.5 ± 5.9	11.3 ± 6.9	3.9 ± 0.5
Tumor:Liver	11.6 ± 7.1	18.9 ± 16.4	2.3 ± 0.5
Tumor:Spleen	23.3 ± 13.6	8.9 ± 2.7	4.7 ± 0.7
Tumor:Stomach	67.4 ± 27.9	52.5 ± 37.4	35.4 ± 31.8
Tumor:LI	56.1 ± 18.9	88.5 ± 37.9	22.3 ± 3.1
Tumor:SI	53.4 ± 22.5	41.5 ± 17	17.7 ± 5.4
Tumor:Kidney	14.2 ± 8.4	11.9 ± 4.5	4.2 ± 1.2
Tumor:Muscle	30.1 ± 8.2	33.5 ± 15.6	14.7 ± 4.8
Tumor:Bone	16.5 ± 7.8	8.1 ± 2.5	2.1 ± 1

Supplementary Table S2. Tumor-to-tissue activity ratios versus time for ^{89}Zr-DFO-^{ss}huA33-Dye680 in mice bearing subcutaneous SW1222 xenografts (n = 4 for each time point). Mice were administered the radioimmunoconjugates (0.55 – 0.75 MBq [15-20 μCi] in 200 μL 0.9% sterile saline) via tail vein injection (t = 0). For the 48 h time point, an additional cohort of animals was given radioimmunoconjugate with dramatically lowered specific activity (LSA), achieved by the co-injection of the standard dose of the ^{89}Zr-labeled construct mixed with 300 μg of cold, unlabeled DFO-^{ss}huA33-Dye680.

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