nature portfolio

https://doi.org/10.1038/s41586-026-10789-w

Supplementary information

Modular in vivo antibody–ADC click to reverse drug resistance in tumours

In the format provided by the authors and unedited

SUPPLEMENTARY INFORMATION FOR

MODULAR IN VIVO ANTIBODY–ADC CLICK TO REVERSE DRUG RESISTANCE IN TUMORS

Authors: Cristina Simó[1], Alexander C Vanover[1], Ricardo D'Oliveira Albanus[2], Sandeep Surendra Panikar[1], Shayla Shmuel[1,3], Alex Benton[1,3], Jader Giraldo-Guzman[1], José M. Luna[1,4], Yifei Xu[5], Na-Keysha Berry[1], Nai Keltee[1], Jingxia Liu[5,6], Farrokh Dehdashti[1,7], Patrícia M. R. Pereira[1,4,*]

Affiliations:

[1]Department of Radiology, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO 63110, USA

[2]Department of Neurology, Washington University School of Medicine, St. Louis, Missouri, USA, 63110

[3]Cancer Biology Graduate Program, Washington University School of Medicine, St. Louis, Missouri, USA, 63110

[4]Alvin J. Siteman Cancer Center, Washington University School of Medicine and Barnes-Jewish Hospital, St. Louis, Missouri, USA 63110

[5]Division of Public Health Sciences, Department of Surgery, Washington University School of Medicine, St Louis, Missouri, USA 63110

[6]Division of Biostatistics, Washington University School of Medicine, St Louis, Missouri, USA 63110

*Corresponding author: Patrícia M. R. Pereira (ribeiropereirap@wustl.edu), Phone number: +1 314-273-4898

Table of Contents

Supplementary Figure 1

Figure 1b

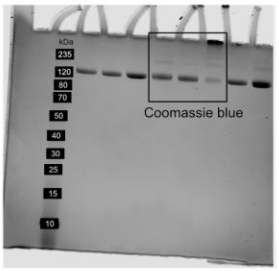
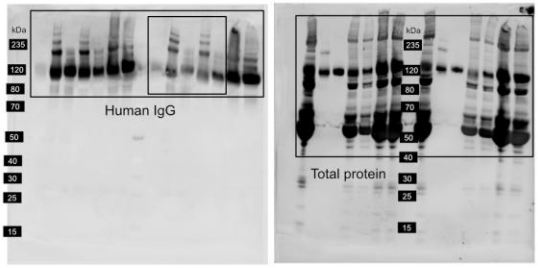


Figure 1c and Supplementary Figure 5d



Extended Figure 1c

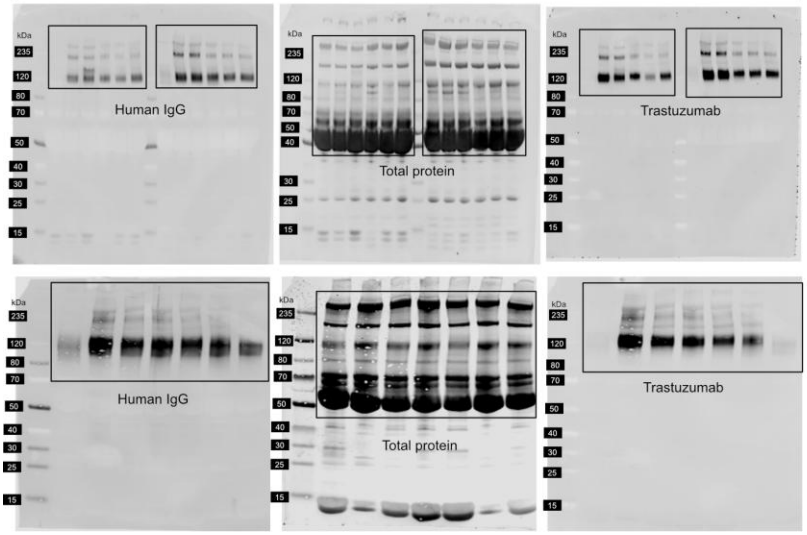


Figure 1e, Figure 2b and Supplementary Figure 7

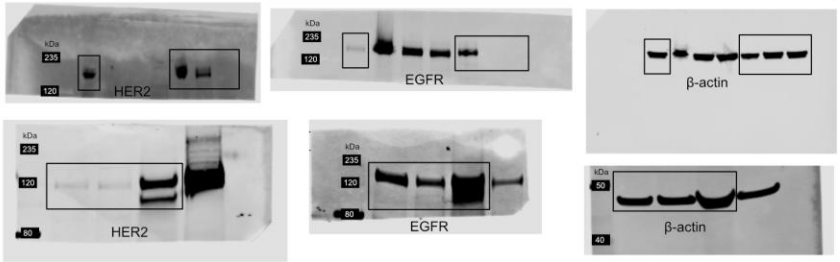
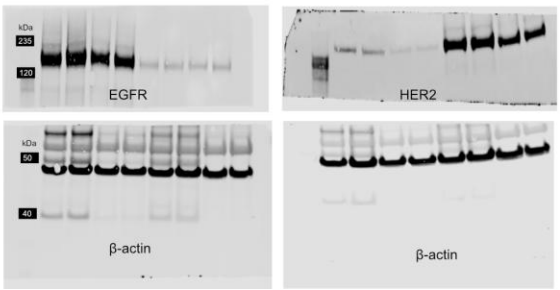
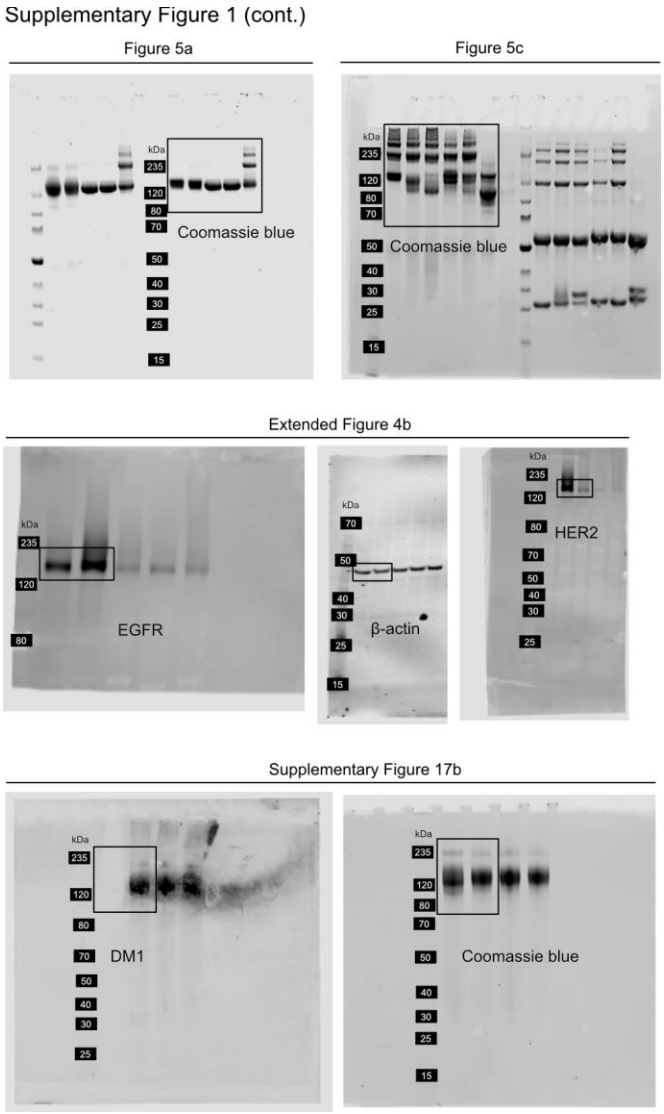
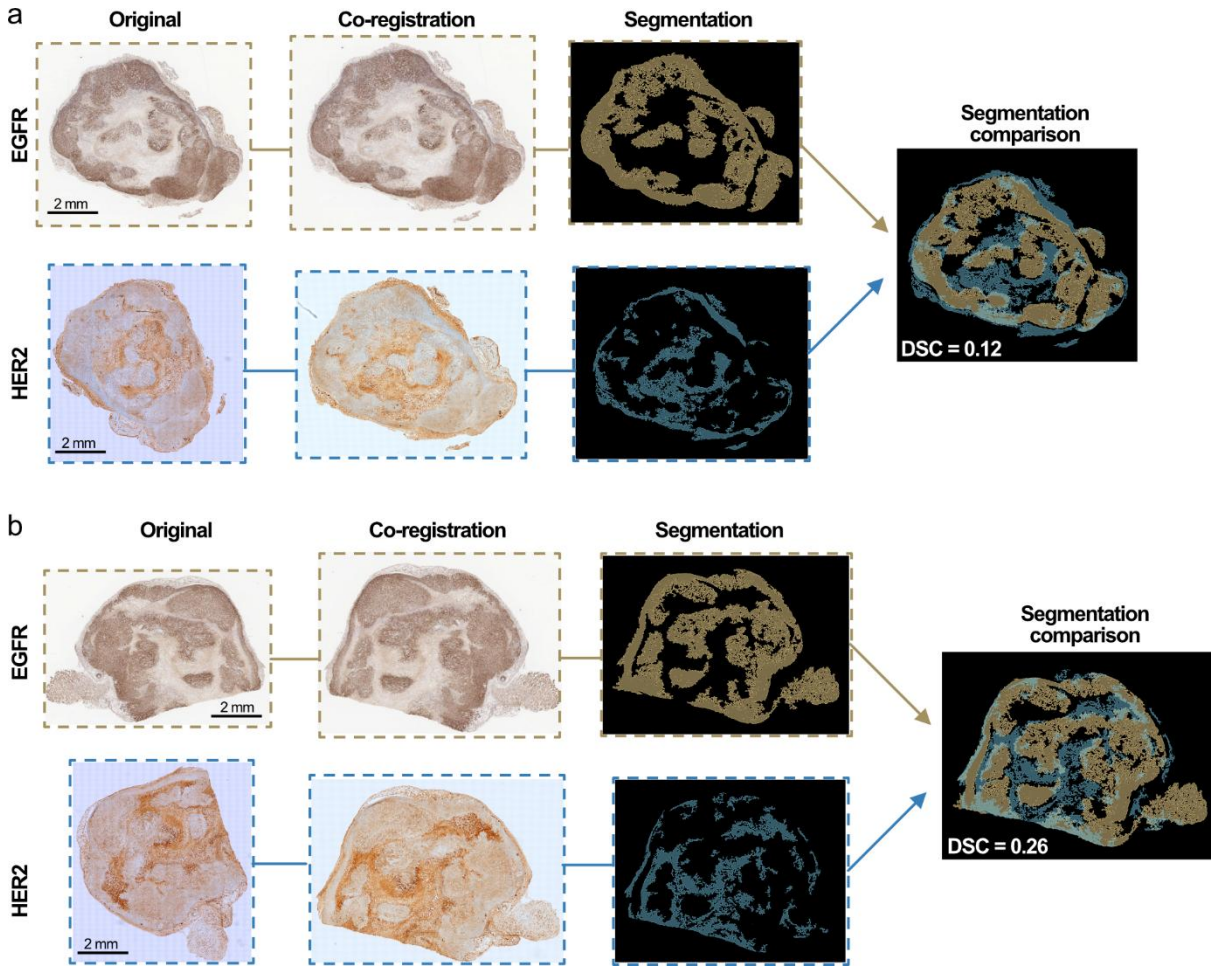


Figure 3c





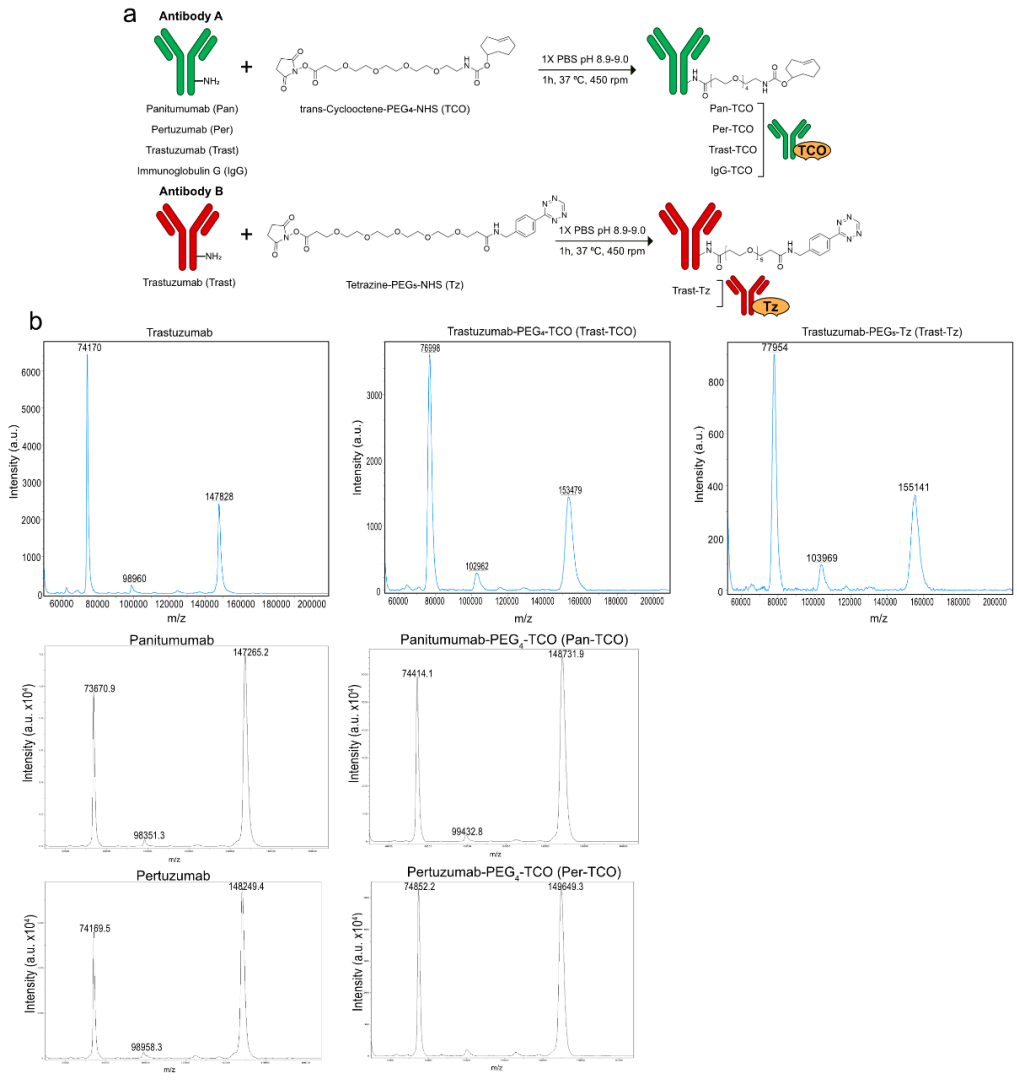
Supplementary Figure 1.
Full membranes of Western blot. Boxes show cropping for representative images.



Supplementary Figure 2.

a) Sequence of processing steps for the first admixed tumor tissue. Original EGFR and HER2 IHC images (left) were co-registered (middle left) and segmented into binary masks (middle right). The final panel (right) shows the overlap between the segmentations, revealing minimal spatial concordance. The dice similarity coefficient (DSC) was 0.12.

b) Same as in **a)**, shown for a second tumor tissue. The DSC was 0.26. The DSC ranges from 0 to 1, with 1 indicating complete spatial overlap; thus, these low values confirm that EGFR and HER2 tend to localize in distinct, largely non-overlapping regions within each tumor section.



c

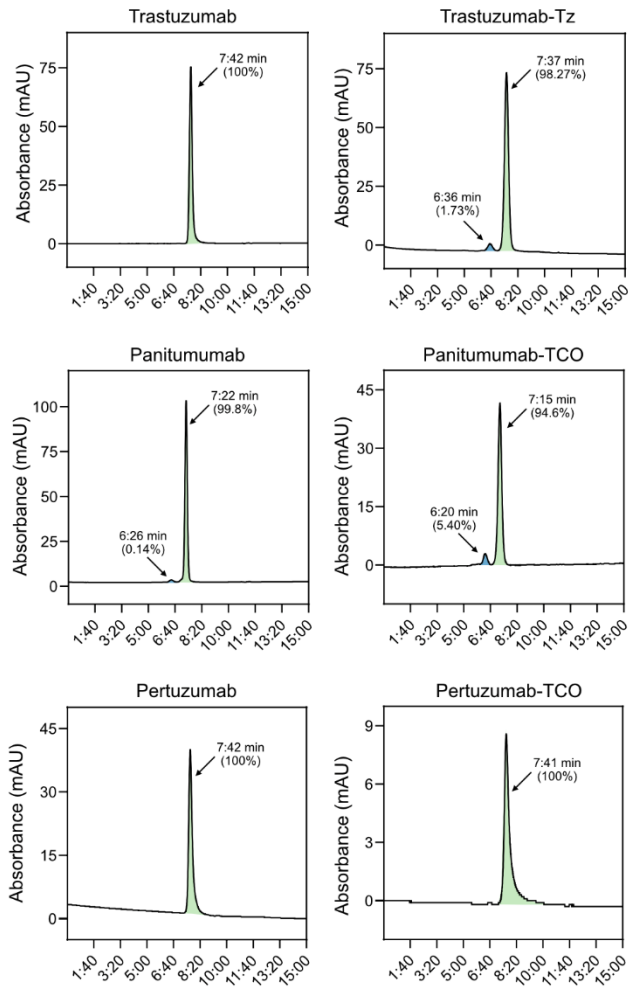
Sample		Mass (Da)	Average mass (Da) ± S.D.	Degree of conjugation (15:1, TCO/Tz:mAbs)
Trastuzumab	Sample #1	148338	148374 ± 50.91	
	Sample #2	148410		
Trastuzumab-PEG4-TCO	Sample #1	153994	153892 ± 144.24	10.72 ± 0.28
	Sample #2	153790		
Trastuzumab-PEG5-Tz	Sample #1	155906	155910 ± 5.65	12.46 ± 0.009
	Sample #2	155914		
Panitumumab	Sample #1	147265	147325.5 ± 85.56	
	Sample #2	147386		
Panitumumab-PEG4-TCO	Sample #1	148731	148752 ± 29.70	2.77 ± 0.06
	Sample #2	148773		
Pertuzumab	Sample #1	148179	148214 ± 49.50	
	Sample #2	148249		
Pertuzumab-PEG4-TCO	Sample #1	149649	149680 ± 43.8	2.85 ± 0.0854
	Sample #2	149711		

Supplementary Figure 3.

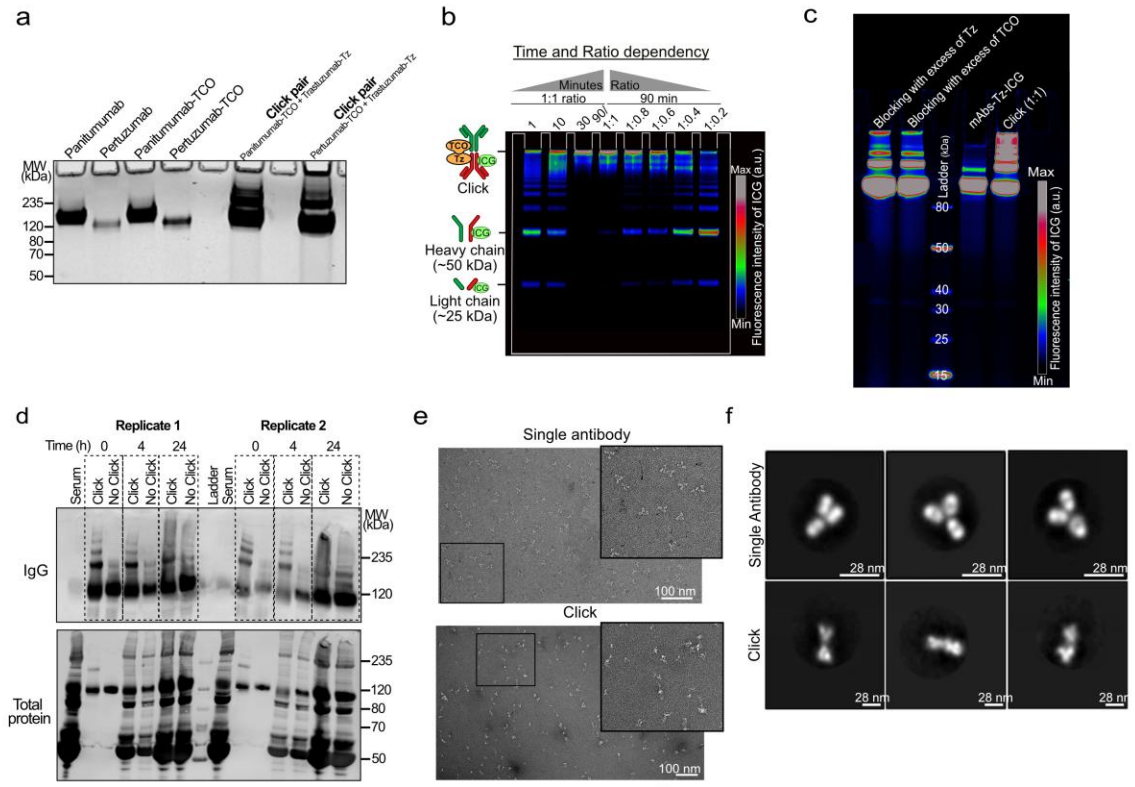
a) Schematic representation of the antibodies conjugated with trans-cyclooctene-PEG$_4$-NHS (TCO) or tetrazine-PEG$_5$-NHS (Tz) with a molar ratio of 15 TCO or Tz per antibody.

b) MALDI spectrum of trastuzumab, trastuzumab-TCO, trastuzumab-Tz, panitumumab, panitumumab-TCO, pertuzumab, and pertuzumab-TCO. *N*=2 conjugates per antibody.

c) Table showing the quantification of TCO or Tz click moieties per antibody obtained by MALDI.



Supplementary Figure 4.
Representative SEC-HPLC chromatograms of 20 µg of trastuzumab, trastuzumab-Tz, panitumumab, and panitumumab-TCO. The green area indicates the antibody, and the blue area indicates the presence of aggregates. SEC-HPLC was performed routinely for every conjugated obtained.



Supplementary Figure 5.

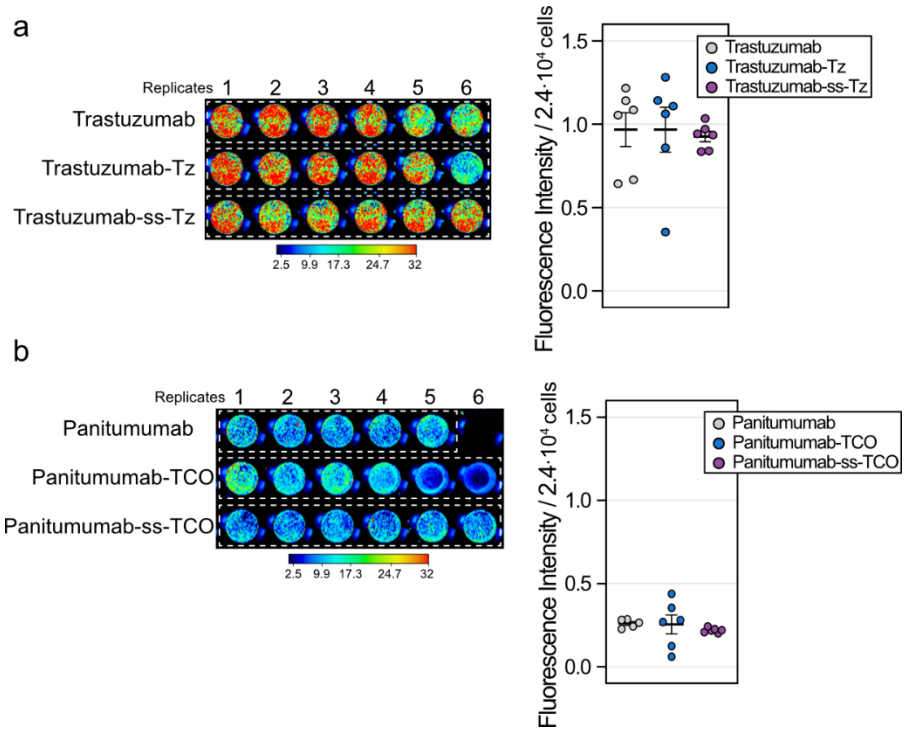
a) SDS-PAGE of antibody click at a 1:1 ratio (panitumumab-TCO plus trastuzumab-Tz or pertuzumab-TCO plus trastuzumab-Tz) for 90 min at 37°C. The gel was stained with Coomassie blue.

b) SDS-PAGE of antibody click at a 1:1 ratio (trastuzumab-TCO plus trastuzumab-Tz) for 0, 10, 30, and 90 min, and different ratios (1:0.2 – 1:0.8) reacting for 90 min at 37°C. Trastuzumab-Tz was conjugated with ICG and clicked with trastuzumab-TCO. The color scale shows the fluorescent signal for indocyanine green (ICG).

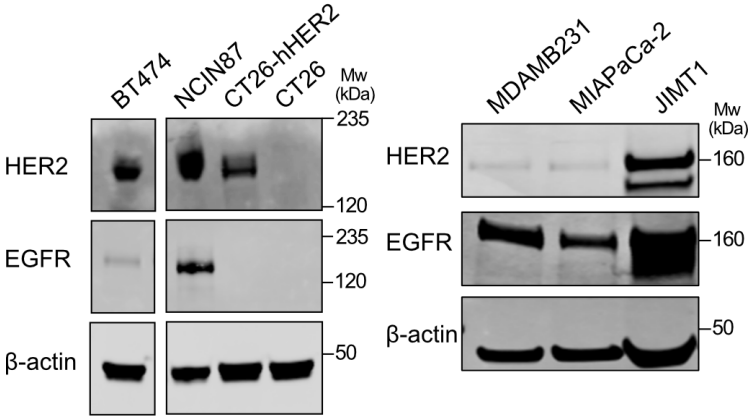
c) SDS-PAGE analysis of the antibody click in the presence of an excess of the unconjugated TCO or Tz. Trastuzumab-TCO (15 µg) or trastuzumab-Tz (15 µg) was first reacted with Tz (0.5 µg) or TCO (0.5 µg), respectively, for 90 min. Next, antibody click was performed at a 1:1 ratio (trastuzumab-TCO plus trastuzumab-Tz) for 90 min at 37°C.

d) SDS-PAGE of antibody click (panitumumab-TCO plus trastuzumab-Tz) or no click (panitumumab plus trastuzumab-Tz) at a 1:1 ratio for 90 min at 37°C. Prior to click reaction incubation, individual antibodies were incubated in mouse serum at 37°C for 0, 4, and 24 h.

e) Transmission Electron Microscopy (TEM) images and **f)** 2D-classes reconstruction of trastuzumab (single antibody) versus trastuzumab-TCO plus trastuzumab-Tz (click). Antibody click was performed by incubating trastuzumab-TCO with trastuzumab-Tz in a 1:1 ratio for 90 min at 37°C.

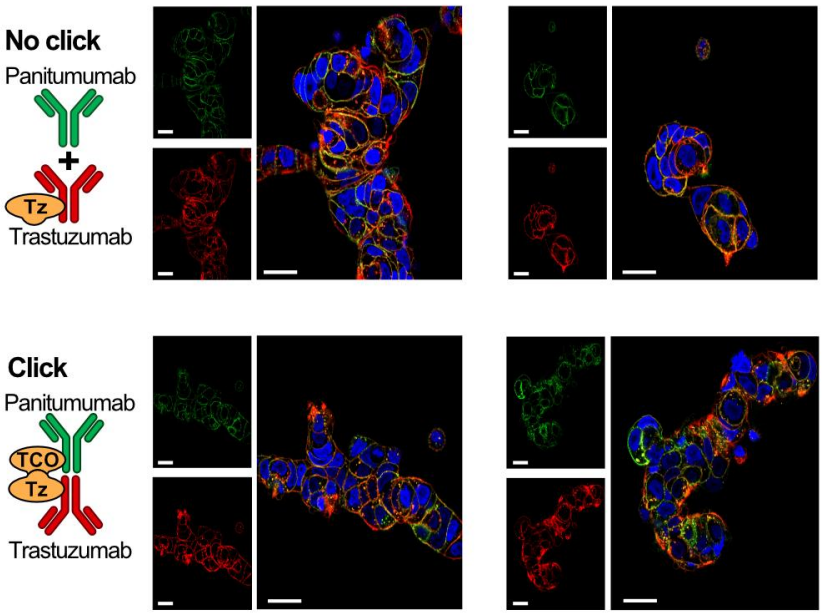


Supplementary Figure 6.
In-cell Western of antibody binding to NCIN87 cancer cells. Cells were treated with 100 nM of trastuzumab, trastuzumab-Tz, trastuzumab-ss-Tz, panitumumab, panitumumab-TCO, or panitumumab-ss-TCO. Quantitative analysis was performed after incubation with an anti-human IgG680 antibody, normalized to the number of cells. Data are shown as mean±S.E.M. (*n*=5-6 wells per group).

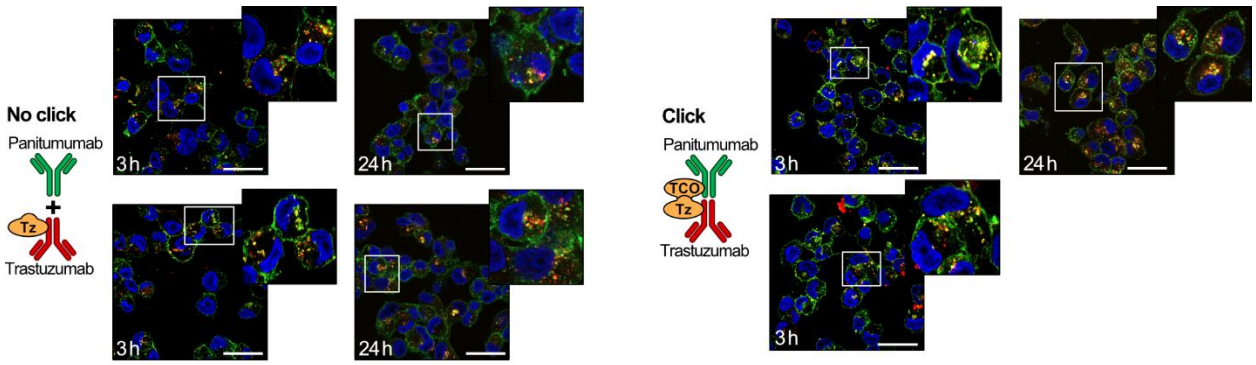


Supplementary Figure 7.

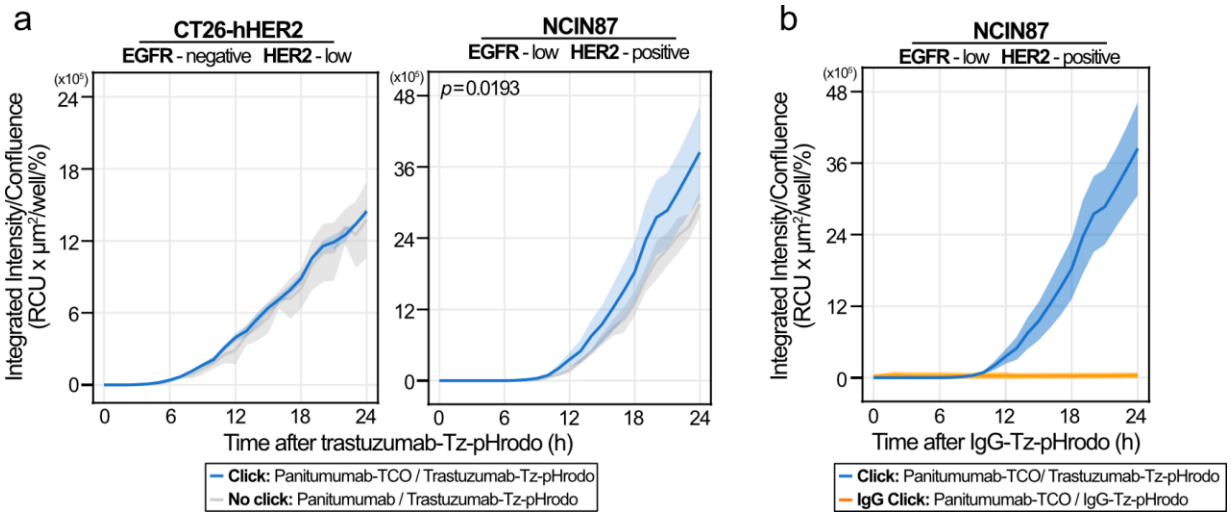
Western blot of HER2 and EGFR in total lysates of cancer cell lines used in this study, and performed routinely for the different experiments.



Supplementary Figure 8.
Fluorescence microscopy images of NCIN87 cells 3 hours post-incubation with either no-click or click panitumumab (green) and trastuzumab (red) antibody pairs. Scale bars=50 μm.



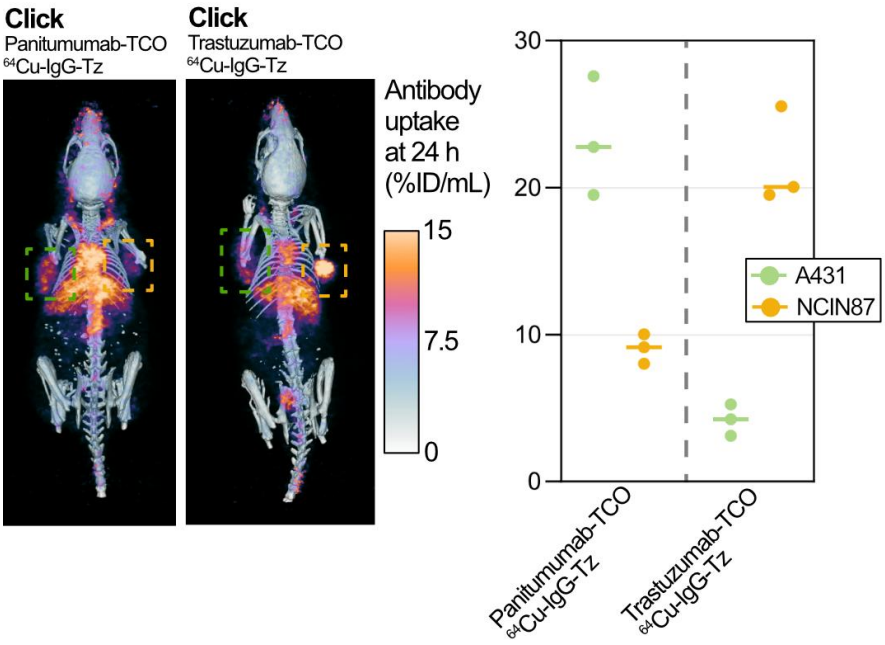
Supplementary Figure 9.
Fluorescence microscopy images of MIAPaCa-2 cells at 3 and 24 hours after incubation with either no-click or click panitumumab (green) and trastuzumab (red) antibody pairs. Scale bars=50 μm.



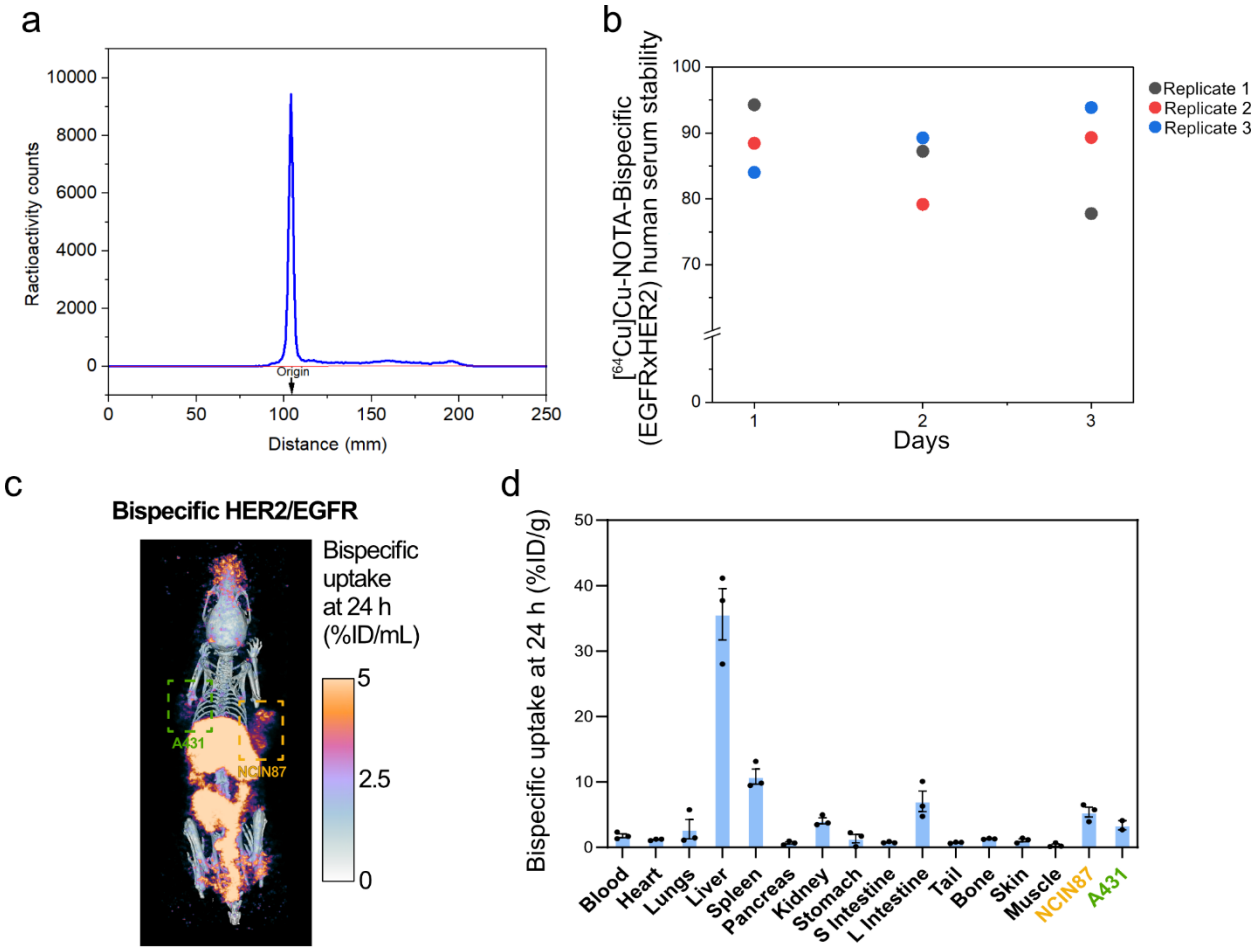
Supplementary Figure 10.

a) Trastuzumab-Tz internalization in CT26-hHER2 and NCIN87 cancer cells (n=3 wells per group) in click and no click conditions using trastuzumab-Tz conjugated to the pHrodo (5 µg/mL). Data are expressed as mean±S.D.; statistical significance between no click and click was determined using Two-way ANOVA. No comparison was performed across cell lines.

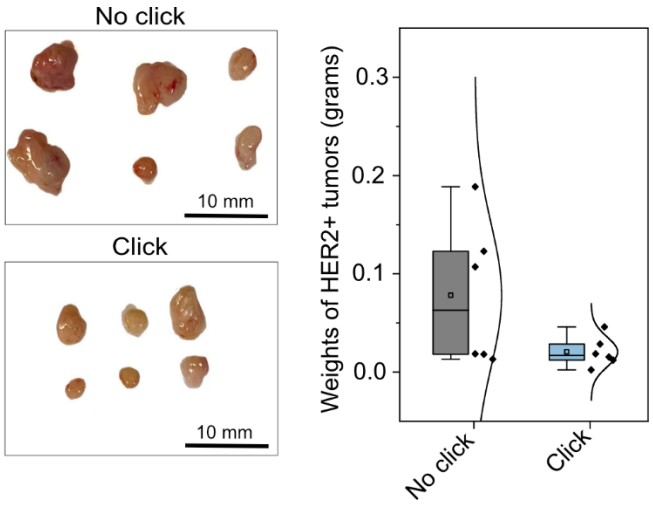
b) Trastuzumab-Tz or IgG-Tz internalization in NCIN87 cancer cell line in click conditions using the respective antibodies conjugated with pHrodo dye (5 µg/mL, *n*=3 wells per group). Data are expressed as mean±S.D.; statistical significance between no click and click was determined using two-way ANOVA. No comparison was performed across cell lines.



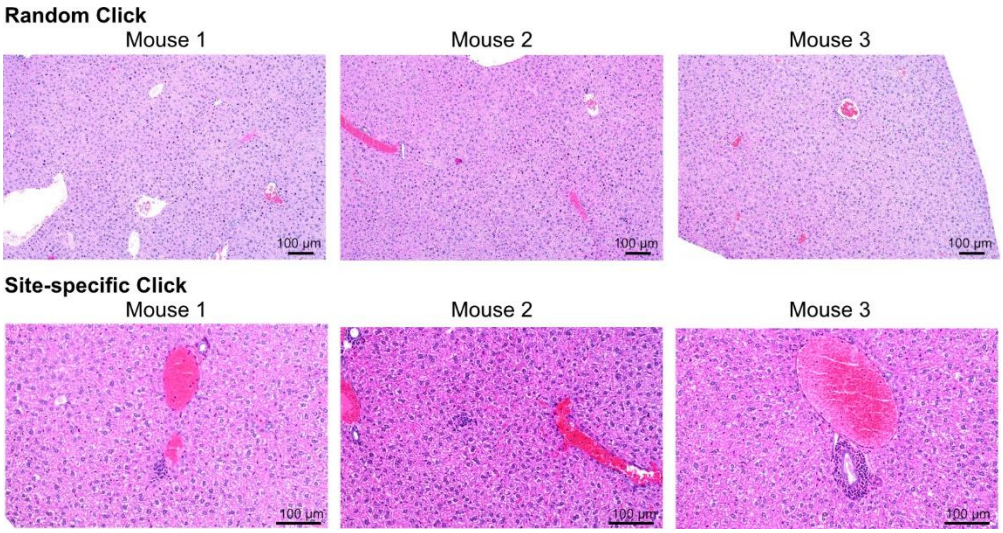
Supplementary Figure 11. PET imaging and tumor uptake of ^{64}Cu-labeled IgG in the bilateral A431/NCIN87 tumor model. ^{64}Cu-labeled IgG was administered 24 h after injection of panitumumab-TCO or trastuzumab-TCO (*n*=3 mice per group).



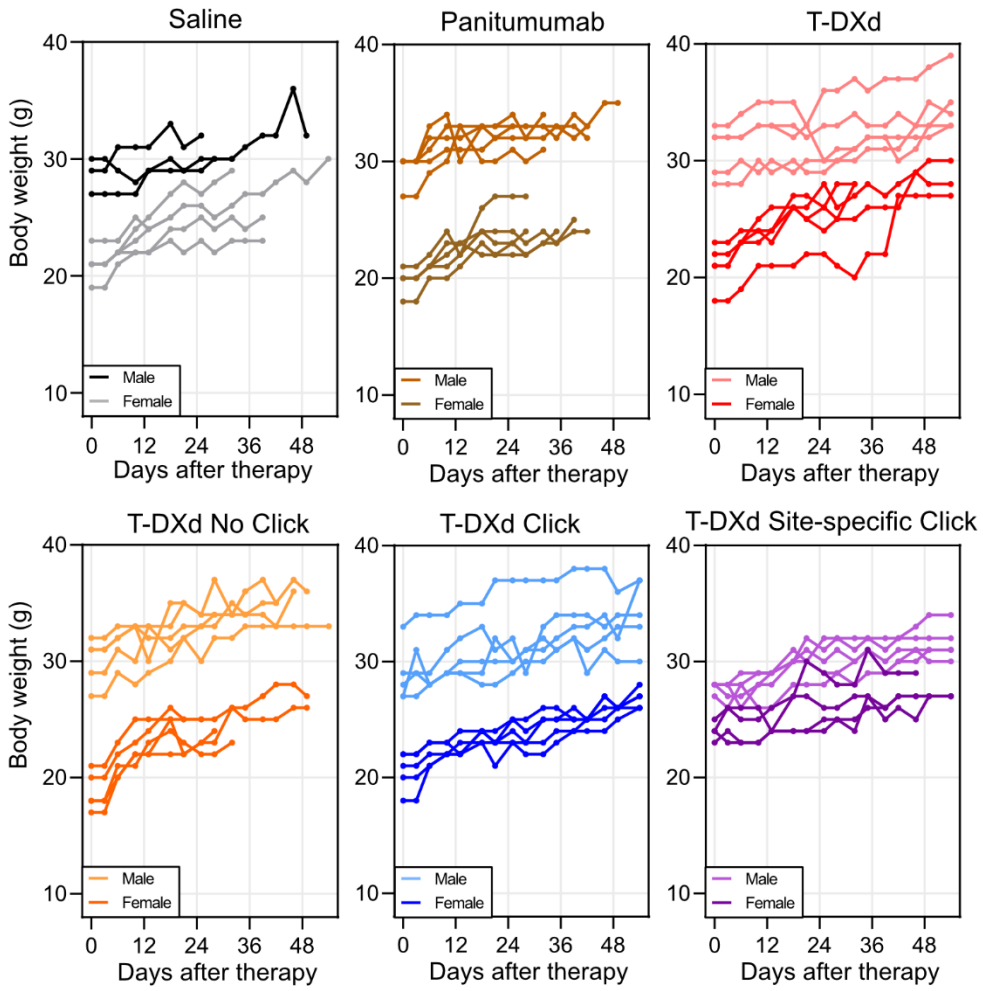
Supplementary Figure 12.
a) iTLC and **b)** serum stability of ^{64}Cu-labeled bispecific antibody (*n*=3 radiolabeled products). **c)** PET imaging and **d)** biodistribution of ^{64}Cu-labeled bispecific antibody at 24 h post-injection in the bilateral A431/NCIN87 tumor model (*n*=3 mice). Recombinant anti-EGFR x anti-HER2 bispecific antibody (scFv-Fc, Creative Biolabs, #BSCFV-155). Data are shown as %ID/g (mean±S.E.M).



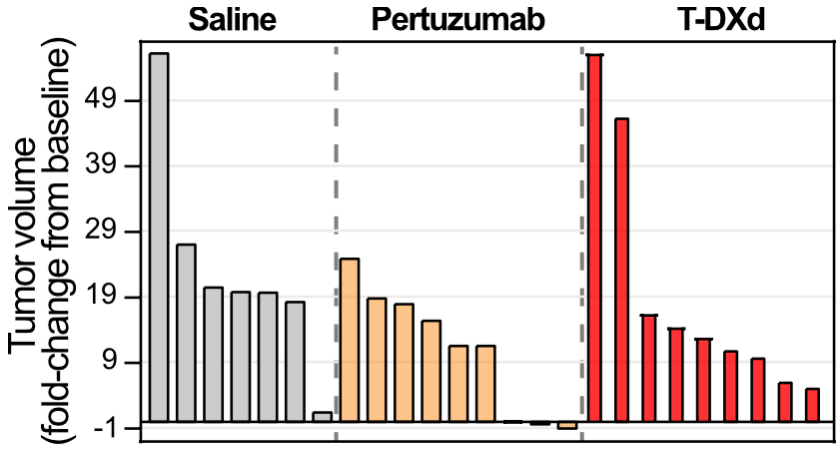
Supplementary Figure 13.
Nu/nu mice bearing NCIN87 tumors were treated with no click (panitumumab plus T-DXd, *n*=6 mice per group), or antibody–ADC click (panitumumab-TCO plus T-DXd-Tz, *n*=6 mice per group). Panitumumab or panitumumab-TCO (10 mg/kg) was injected via the tail vein 24 h prior to the injection of T-DXd or T-DXd-Tz (10 mg/kg). Photographic images and weights of the excised tumors were collected at 26 days post-therapy. Data are shown as weight (mean±S.E.M; *n*=6 mice per group).



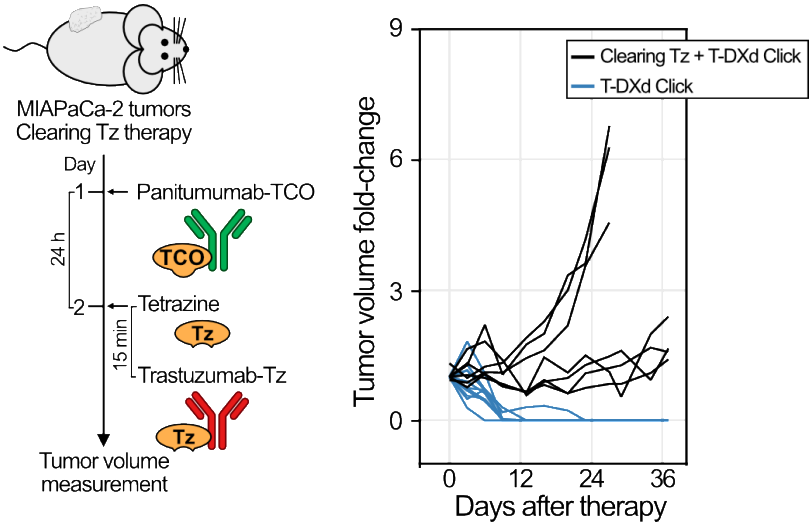
Supplementary Figure 14.
Representative H&E-stained liver sections from female BALB/c mice treated with random (panitumumab-TCO plus T-DXd-Tz) or site-specific (panitumumab-ss-TCO plus T-DXd-ss-Tz) antibody click (*n*=3 mice per group). All antibodies were administered at 20 mg/kg. T-DXd-Tz or T-DXd-ss-Tz was administered 24 h after panitumumab-TCO or panitumumab-ss-TCO. Liver pathology assessment was performed at 7 days after therapy by the Division of Comparative Medicine (scale bars=100 µm).



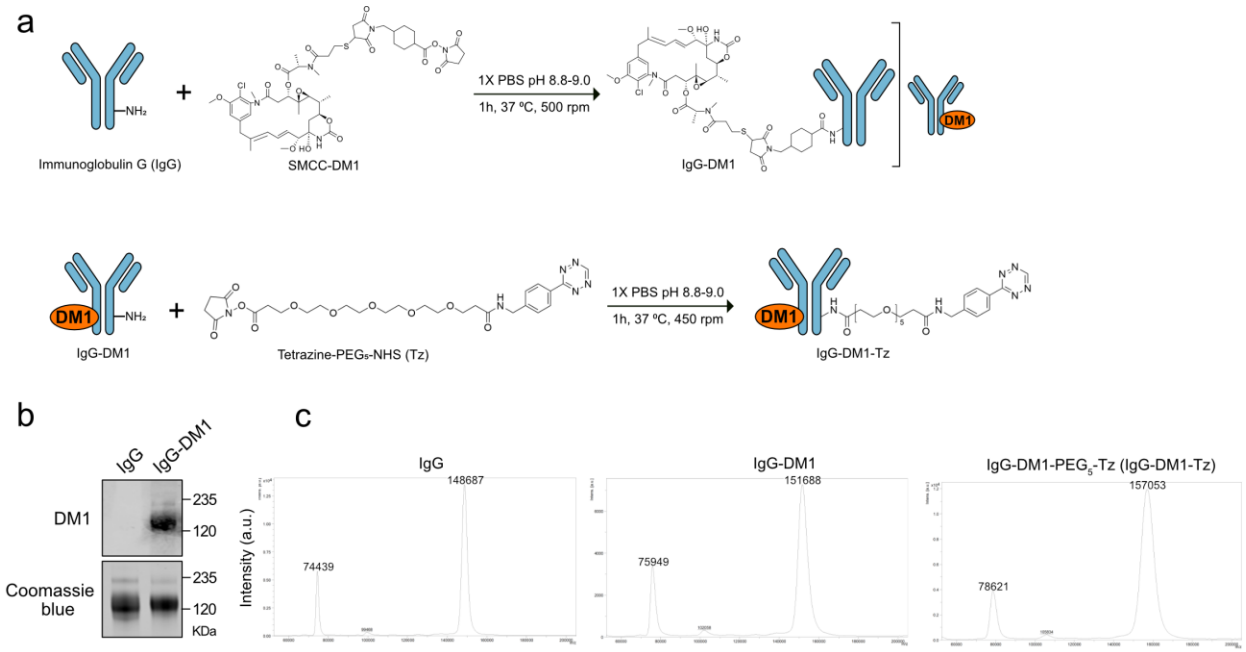
Supplementary Figure 15.
Body weight of female and male *nu/nu* mice implanted with MIAPaCa-2 cancer cells following saline (*n*=8 mice), or 5 mg/kg panitumumab (*n*=10 mice), T-DXd (*n*=10 mice), T-DXd no click (panitumumab plus T-DXd, *n*=10 mice), T-DXd click (panitumumab-TCO plus T-DXd-Tz, *n*=10 mice), or T-DXd site-specific click (panitumumab-ss-TCO plus T-DXd-ss-Tz, *n*=9 mice). Lines represent each individual mouse.



Supplementary Figure 16.
Individual tumor response in the CT26-hHER2 tumor model of saline (*n*=7mice) or following treatment with monotherapies only 5 mg/kg of pertuzumab (*n*=9 mice), or T-DXd (*n*=9 mice). Bars represent fold change in tumor volume relative to baseline at the start of the therapy.



Supplementary Figure 17.
Experimental design and individual tumor growth curves for the MIAPaCa-2 cancer model following a single intravenous dose (5 mg/kg) of panitumumab-TCO plus tetrazine plus T-DXd-Tz (clearing Tz + T-DXd click, *n*=7 mice), or panitumumab-TCO plus T-DXd-Tz (T-DXd click, *n*=10 mice). Lines represent each individual mouse. Illustrations adapted from Servier Medical Art (https://smart.servier.com), under a Creative Commons license CC-BY 4.0 https://creativecommons.org/licenses/by/4.0/.



d

Sample		Mass (Da)	Average mass ± S.D.	Degree of conjugation
IgG	Sample #1	148876	148887 ± 15.56	
	Sample #2	148898		
IgG-DM1	Sample #1	151634	151765 ± 185.26	2.68 ± 0.17
	Sample #2	151896		
IgG-DM1-PEG$_5$-Tz	Sample #1	157240	157316 ± 107.48	9.18 ± 0.18
	Sample #2	157392		

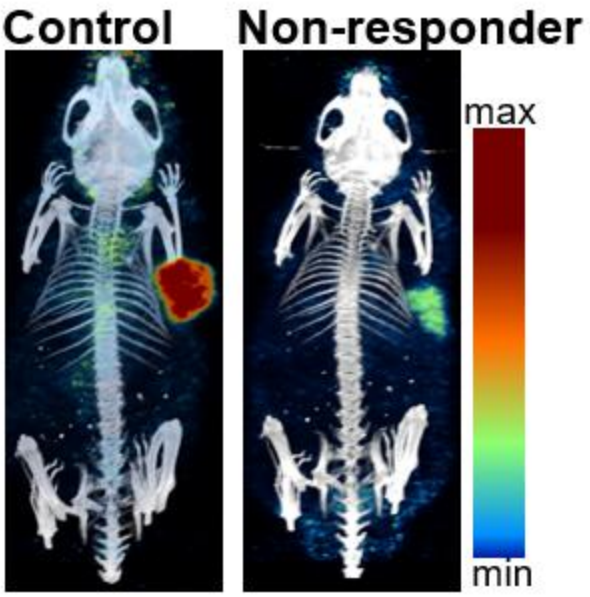
Supplementary Figure 18.

a) Schematic representation of human IgG conjugated with SMCC-DM1 (IgG-DM1; molar ratio of 5 SMCC-DM1 per IgG) and tetrazine-PEG$_5$-NHS (IgG-DM1-Tz; molar ratio of 15 Tz per IgG-DM1).

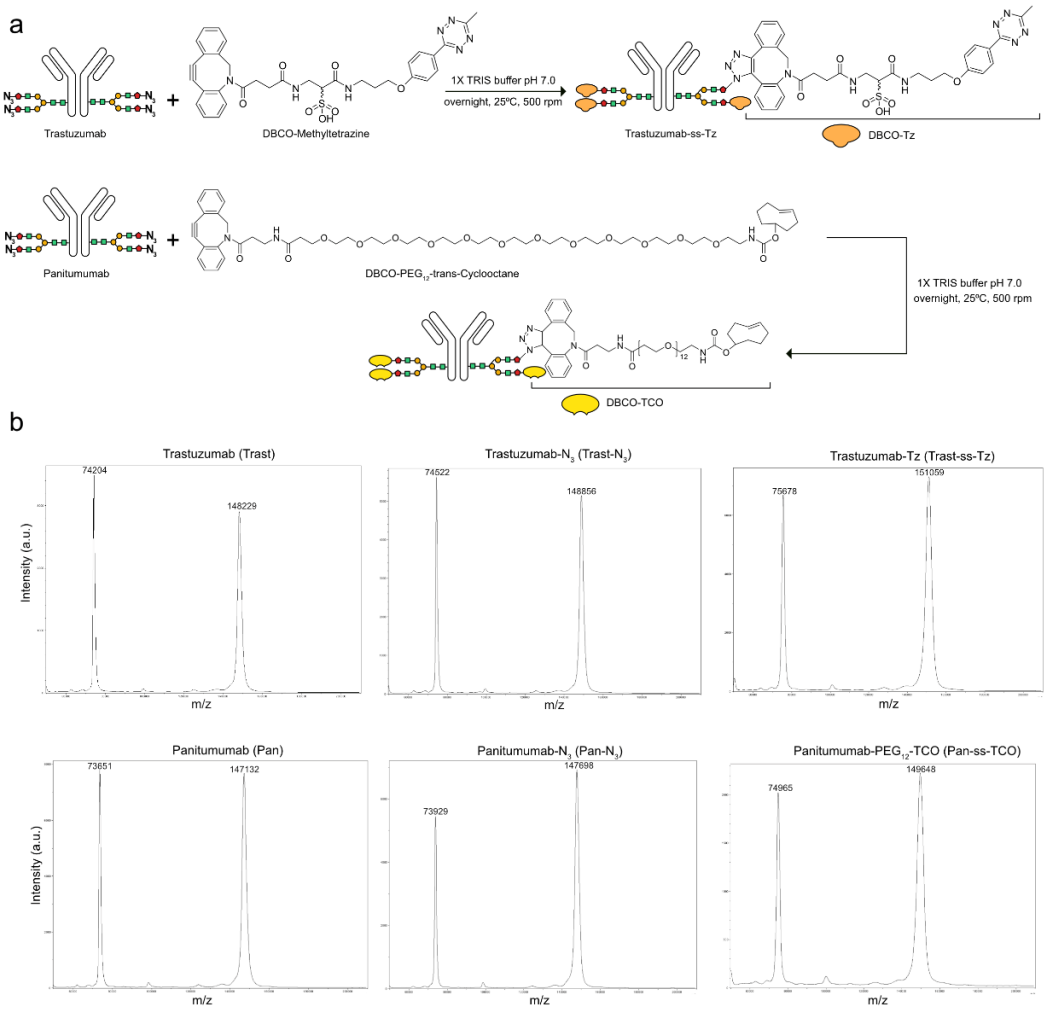
b) Western blots of IgG and IgG-DM1 to determine the presence of DM1 using an anti-DM1 antibody. Coomassie blue staining was used as a control for total protein.

c) MALDI spectrum of IgG, IgG-DM1, and IgG-DM1-Tz. *N*=2 samples per conjugate.

d) Table showing the quantification of DM1 drug and Tz click moiety per IgG obtained by MALDI.



Supplementary Figure 19.
^{64}Cu-labeled trastuzumab PET/CT imaging of NCIN87 tumors and non-responder tumors to T-DXd therapy. PET/CT was performed for a total of 10 T-DXd-treated mice at the moment of dividing them into responders versus non-responders. Color scale, %ID/g.



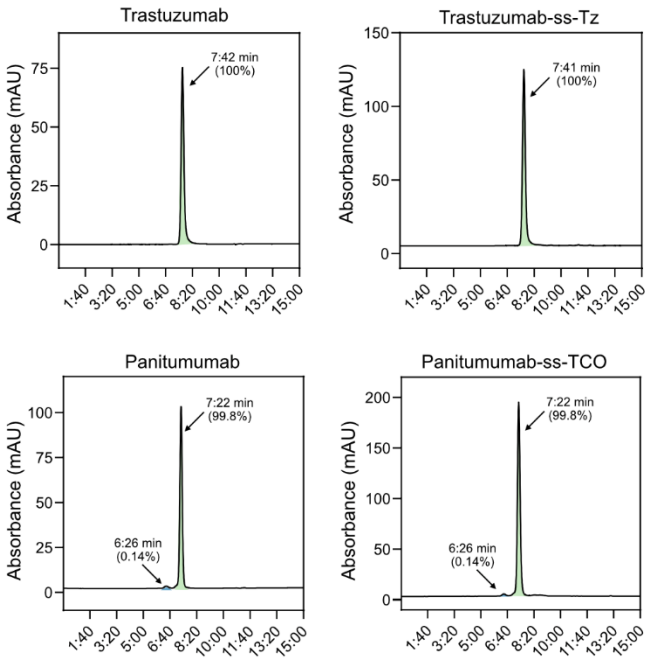
c

Sample		Mass (Da)	Average mass (Da) ± S.D.	Degree of conjugation (15:1; TCO/Tz:mAbs)
Trastuzumab	Sample #1	148428	148417 ± 15.56	
	Sample #2	148406		
Trastuzumab-N_3	Sample #1	149042	149036 ± 8.49	
	Sample #2	149030		
Trastuzumab-Tz	Sample #1	151354	151356 ± 2.83	3.85 ± 0.52
	Sample #2	151358		
Panitumumab	Sample #1	147190	147245 ± 77.78	
	Sample #2	147300		
Panitumumab-N_3	Sample #1	147856	147801 ± 77.78	
	Sample #2	147746		
Panitumumab-PEG_{12}-TCO	Sample #1	149928	149925 ± 4.24	2.20 ± 0.27
	Sample #2	149922		

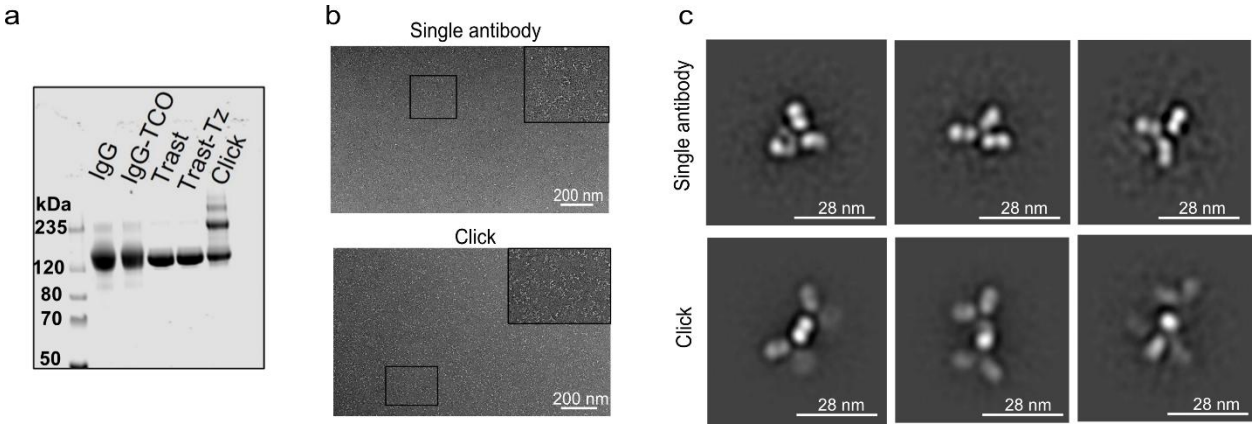
Supplementary Figure 20.

a) Schematic representation of the antibodies conjugated with trans-cyclooctene-PEG_{12}-DBCO (TCO) or methyltetrazine-DBCO (Tz) through site-specific conjugations (molar ratio 15 TCO or Tz per antibody).

b,c) MALDI spectrum of trastuzumab, trastuzumab-N_3, trastuzumab-Tz, panitumumab, panitumumab-N_3, and panitumumab-TCO obtained through site-specific conjugations. *N*=2 conjugates per antibody. Table showing the quantification of Tz and TCO for trastuzumab and panitumumab, respectively.



Supplementary Figure 21.
Representative SEC-HPLC chromatograms of 20 µg of trastuzumab, trastuzumab-ss-Tz, panitumumab, and panitumumab-ss-TCO. The green area indicates the antibody, and the blue area indicates the presence of aggregates. SEC-HPLC performed routinely for each conjugate obtained.

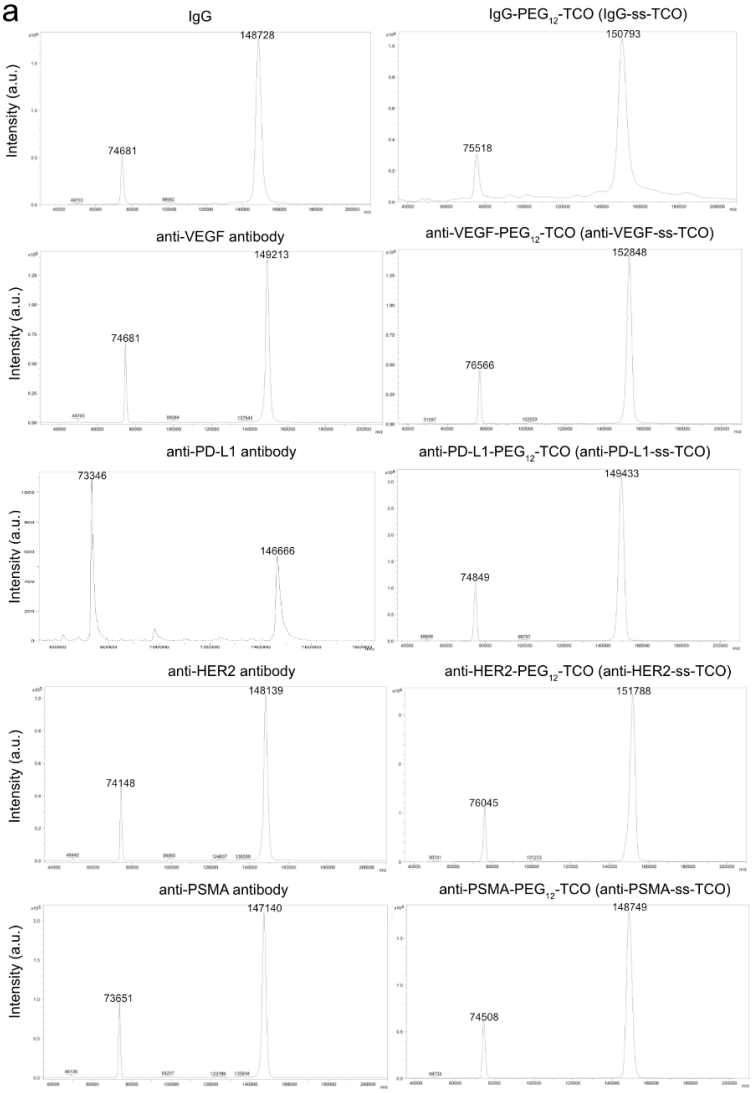


Supplementary Figure 22.

a) SDS-PAGE analyses of antibody click at a 1:1 ratio (IgG-TCO:trastuzumab-Tz) for 90 min. IgG and trastuzumab were conjugated to TCO and Tz through a site-specific approach. Gels were stained with Coomassie blue.

b) Transmission Electron Microscopy (TEM) images and **c)** 2D-classes reconstruction of trastuzumab (single antibody) versus trastuzumab-ss-TCO plus trastuzumab-ss-Tz (click). Antibody click was performed by incubating trastuzumab-ss-TCO with trastuzumab-ss-Tz in a 1:1 ratio for 90 min.

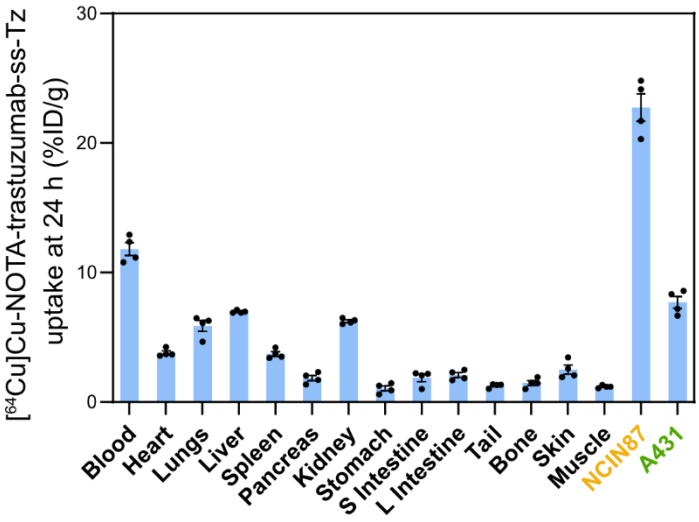
a



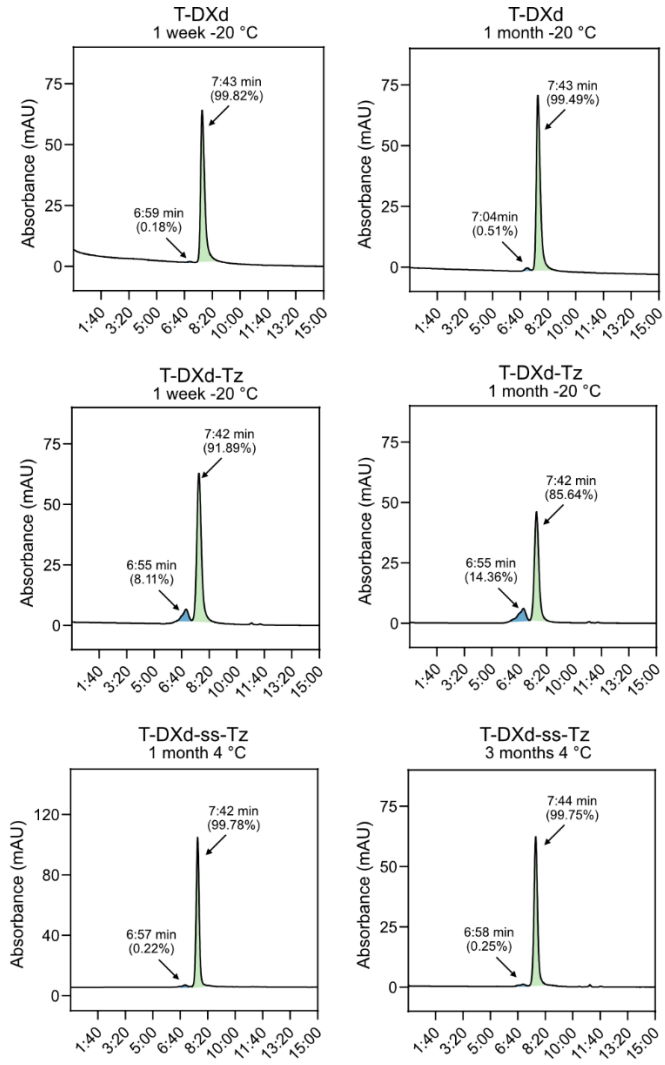
b

Sample	Mass (Da)	Degree of conjugation (15:1; TCO:mAb)
IgG	148930	
IgG-PEG_{12}-TCO	151034	2.05
anti-VEGF antibody	149360	
anti-VEGF-PEG_{12}-TCO	153130	3.67
anti-PD-L1 antibody	146690	
anti-PD-L1-PEG_{12}-TCO	149696	2.92
anti-HER2 antibody	148294	
anti- HER2-PEG_{12}-TCO	152088	3.69
anti-PSMA antibody	147300	
anti-PSMA-PEG_{12}-TCO	149014	1.67

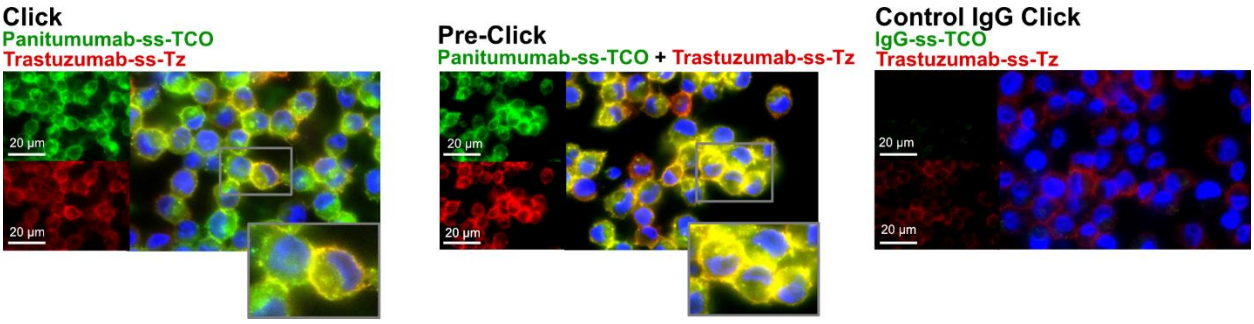
Supplementary Figure 23.
a) MALDI spectrum and **b)** Tz and TCO quantification of the different antibodies used in the study obtained through site-specific conjugations. *N*=2 per conjugate obtained.



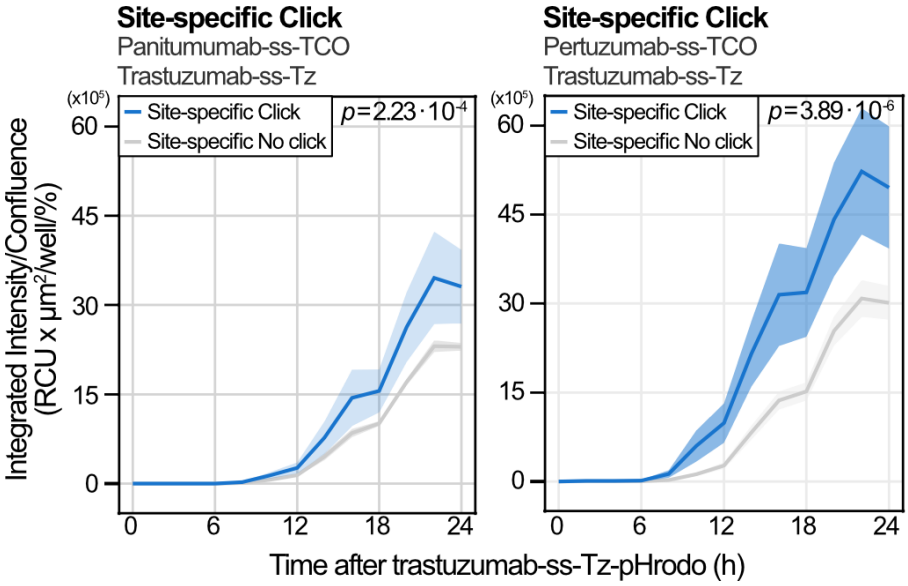
Supplementary Figure 24.
Biodistribution of panitumumab-ss-TCO plus [^{64}Cu]Cu-NOTA-trastuzumab-ss-Tz (click) at 24 h post-injection of [^{64}Cu]Cu-NOTA-trastuzumab-ss-Tz in *nu/nu* female mice bearing a bilateral model of NCIN87 and A431 tumors. Panitumumab-TCO was injected intravenously 24 h before the injection of [^{64}Cu]Cu-NOTA-trastuzumab-ss-Tz. Values are expressed as %ID/g (mean±S.E.M, *n*=4 mice).



Supplementary Figure 25.
Representative SEC-HPLC chromatograms of 20 µg of T-DXd, T-DXd-Tz (random conjugation), and T-DXd-ss-Tz (site-specific conjugation) stored at -20 °C or 4 °C for different periods of time. The green area indicates the antibody, and the blue area indicates the aggregates. SEC-HPLC was performed routinely for each conjugate obtained and monitored over time.



Supplementary Figure 26.
Fluorescence microscopy images of MIAPaCa-2 cells treated with panitumumab-ss-TCO-Alexa488 or IgG-ss-TCO (green) for 30 min at 4°C, followed by trastuzumab-ss-Tz-Alexa594 (red) for 1 h at 37°C. For the pre-click group, both antibodies were reacted at a 1:1 ratio for 90 min at 37°C prior to incubation.



Supplementary Figure 27.
Trastuzumab-ss-Tz internalization in NCIN87 cancer cells in click and no click conditions using the trastuzumab-ss-Tz conjugated to the pHrodo dye (10 µg/mL, *n*=3 wells). Panitumumab, pertuzumab, and trastuzumab were modified with TCO and Tz through site-specific conjugations. Data are expressed as mean±S.D.; statistical significance between no click and click was determined using two-way ANOVA. No comparison was performed across cell lines.

SUPPLEMENTARY TABLES

Supplementary Table 1. FDA-approved antibodies and ADCs used in this work.

Antibody/abbreviation	**Antibody-drug conjugate**	**Target**
Panitumumab		EGFR
Pertuzumab		HER2, domain II
Trastuzumab		HER2, domain IV
	T-DM1	HER2, domain IV
	T-DXd	HER2, domain IV

Supplementary Table 2. Summary of pathology findings of female BALB/c treated with random (panitumumab-TCO plus T-DXd-Tz) or site-specific (panitumumab-ss-TCO plus T-DXd-ss-Tz) antibody click (*n*=3 mice). All antibodies were administered at 20 mg/kg. Analyses were performed in the lungs and liver at 7 days after therapy by the Division of Comparative Medicine.

Random click (panitumumab-TCO plus T-DXd-Tz)			
Mouse	**Lung pathology**	**Liver**	**Interpretation**
1	Mild-moderate pulmonary hemorrhage; alveolar histiocytosis; perivascular lymphocytic infiltration	Microvesicular hepatocellular degeneration; extramedullary hematopoiesis; crystalline intranuclear material	Incidental/background changes; no signs of treatment-induced toxicity
2	Moderate pulmonary hemorrhage	Hepatocellular necrosis and hypertrophy; crystalline intranuclear material	Changes consistent with agonal effects or background findings
3	Mild-moderate pulmonary hemorrhage	Similar to mouse 2, with milder intranuclear inclusions	Comparable to control-level changes
Site-specific click (panitumumab-ss-TCO plus T-DXd-ss-Tz)			
Mouse	**Lung pathology**	**Liver**	**Interpretation**
1	Mild pulmonary hemorrhage	Mild microvesicular hepatocellular degeneration; extramedullary hematopoiesis; Minimal scattered hepatocellular necrosis with mixed inflammation	Changes consistent with agonal effects or background findings
2	Mild pulmonary hemorrhage	Similar to mouse 1	Changes consistent with agonal effects or background findings
3	Mild pulmonary hemorrhage	Similar to mouse 1 and 2	Changes consistent with agonal effects or background findings

Supplementary Table 3. Cancer cell lines and media composition used in this work.

Cell line/Tumor type	Media Composition
NCIN87	RPMI-1640 growth medium + 10% fetal bovine serum (FBS) + 2 mM L-glutamine + 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) + 1 mM sodium pyruvate + 4.5 g/L glucose + 1.5 g/L sodium bicarbonate + 1% of penicillin (10000U) and 10 mg/mL streptomycin
CT26 or CT26-hHER2	RPMI-1640 growth medium + 10% FBS+ 2 mM L-glutamine + 2 g/L sodium bicarbonate + 1% of penicillin (10000U) and 10 mg/mL streptomycin
A431 MDAMB231 MIAPaCa-2	DMEM with high glucose + 10% FBS + 4 mM L-Glutamine + 1 mM Sodium Pyruvate + 4.5 g/L Glucose + 1.5 g/L Sodium Bicarbonate + 1% of penicillin (10000U) and 10 mg/mL streptomycin
BT474-trastuzumab resistant	Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12) + 10% FBS + 5 mL of non-essential amino acid (100X) + 45% glucose + 1% of penicillin (10000U) and 10 mg/mL streptomycin. Cells are maintained in media with 15 μg/ml trastuzumab.
JIMT1	Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12) + 10% FBS + 2.5 mM L-glutamine + 15 mM HEPES + 0.5 mM sodium pyruvate + 3.15 g/L glucose + 1.2 g/L sodium bicarbonate + 1% penicillin (10000U) and 10 mg/mL streptomycin.

Supplementary Table 4. Experimental details on the generation of the animal models used in this work.

Cell line	Cancer type	HER 2 levels (IHC)	Trastuzumab uptake (%ID/mL)	Number of cells (million)	Time after cell implantation	PET, therapy or both	Mouse strain
NCIN87	gastric	3+	19.48±0.98	5	28 days	both	*nu/nu*
CT26-hHER2	colorectal	2+	10.6±2.8	0.25	7-9 days	therapy	BALB/c
A431	epidermoid	1+/0	N/A	0.25	7 days	both	*nu/nu*
MDAMB231:4 JIMT1 (Admix model)	breast	2+	9.90±0.65	1.25 (MDAMB231) : 5 (JIMT1)	9-12 days	therapy	*nu/nu*
MIAPaCa-2	pancreatic	0	9.27±2.42	5	13-14 days	both	*nu/nu*
BT474-trastuzumab resistant	breast	3+	N/A	5	2-3 months	therapy	*nu/nu*

SUPPLEMENTARY ALGORITHM

Algorithm 1. Automated IHC Image Co-registration and ROI Overlap Quantification

Inputs

- I_R: Reference immunohistochemistry (IHC) RGB image
- I_M: Moving IHC RGB image
- c_M: Biomarker-specific channel extracted from the moving image
- r: Rolling-ball radius for background correction
- σ: Gaussian smoothing parameter
- τ: Thresholding rule, e.g., Otsu threshold
- T: Spatial transformation model, e.g., bilinear StackReg
- K: Number of largest connected components retained during ROI refinement

Outputs

- $\tilde{I}_M$: Moving image registered to the reference coordinate system
- M_R: Reference tissue mask
- M_M: Moving tissue mask
- S_R: Reference biomarker-positive ROI mask
- $\hat{S}_M$: Registered moving biomarker-positive ROI mask
- H: Estimated spatial transformation matrix
- D: Dice coefficient

Algorithm

1. Read reference image I_R and moving image I_M.
2. Convert the reference image to grayscale using luminance weighting:
   $G_R \leftarrow 0.299\, I_{R,R} + 0.587\, I_{R,G} + 0.114\, I_{R,B}$
3. Extract the biomarker-specific channel from the moving image:
   $G_M \leftarrow I_{M,c_M}$
4. Normalize G_R and G_M to the intensity range [0,1].
5. Enhance local contrast using contrast-limited adaptive histogram equalization (CLAHE).
6. Convert the enhanced images to 8-bit intensity format.
7. Generate the reference tissue mask M_R:
   a. Correct non-uniform background intensities using rolling-ball filtering with radius r.
   b. Apply Gaussian smoothing with parameter σ.
   c. Compute a binary segmentation using thresholding rule τ.
   d. Retain the largest connected component to isolate the principal tissue region.
8. Generate the moving tissue mask M_M:
   a. Apply thresholding rule τ to the enhanced moving image. Rolling-ball correction may be omitted for the moving channel when not required by image characteristics.
   b. Retain the largest connected component.

9. Resize M_M and the corresponding moving image to match the spatial dimensions of M_R when necessary.
10. Estimate corresponding registration control points between M_M and M_R using bilinear StackReg alignment:
    a. Register M_M to M_R using transformation model T.
    b. Extract corresponding registration control points.
    c. Estimate homography matrix H from the matched control points.
11. Warp the original moving image into the reference coordinate system:
    $\tilde{I}\_M \leftarrow \text{warp}(G\_M, H)$
12. Generate marker-specific high-confidence biomarker-positive ROI masks:
    a. For the reference image, invert intensities when required, apply adaptive histogram equalization, threshold the processed image, and retain the largest connected components to suppress noise.
    b. For the registered moving image $\tilde{I}\_M$, apply intensity inversion, adaptive histogram equalization, Gaussian smoothing, restrictive Otsu thresholding, and morphological refinement.
    c. Refine the moving ROI mask using opening, erosion, and size-based object filtering to suppress isolated noise regions and preserve high-confidence biomarker-positive structures.
13. Segment the registered moving image to obtain the moving ROI mask in the reference coordinate system:
    $\hat{S}\_M \leftarrow \text{SegmentROI}(\tilde{I}\_M)$
14. Compute Dice overlap coefficient:
    $D = 2|S\_R \cap \hat{S}\_M| / (|S\_R| + |\hat{S}\_M|)$
15. Return $\tilde{I}\_M$, M_R, M_M, S_R, $\hat{S}\_M$, H, and D.

Notes

- CLAHE denotes contrast-limited adaptive histogram equalization.
- Tissue masks were generated to constrain the registration to biologically relevant tissue regions and reduce background-induced registration artifacts.
- Marker-specific ROI segmentation was used to account for differences in EGFR and HER2 staining characteristics.
- Registration quality was quantitatively assessed using the Dice overlap coefficient.
- Parameters such as Gaussian smoothing strength, rolling-ball radius, and connected-component filtering thresholds may be empirically selected according to image resolution and staining characteristics.
- All preprocessing and registration procedures were applied consistently across specimens.