Supporting Information

A Bioorthogonal Cell Sorting Strategy for Isolation of Desired Cell Phenotypes

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1. Experimental Section

Materials: All chemicals were purchased from J&K Scientific (Shanghai, China), and Bide Pharm (Shanghai, China), and the fluorophore Cy5.5 was conjugated to TCO was purchased from Xi'an Confluore Biological Technology (Xi'an, China), and used without further purification. Immunofluorescent antibodies were purchased from Jackson ImmunoResearch (Pennsylvania, United States) and Abcam (Shanghai, China). Cell counting kit-8 (CCK-8) was obtained from Baoxin Biotechnology Co. Ltd. (Chengdu, China).

Instruments: NMR spectroscopy was performed on a Bruker system operating at 400 MHz (^{1}H) or 101 MHz (^{13}C). High-resolution mass spectrometry with electrospray ionization was performed on a Bruker "micro-TOF-QII" system. Confocal microscopy was carried out using a confocal laser scanning microscope 880 (Zeiss, Germany). Ultraviolet-visible absorption spectroscopy was performed on a scientific Q6000+ microvolume system.

Animals: All animal experiments were approved by the Animal Care and Use Committee and Ethics Committee of West China Hospital, Sichuan University (Approval No. 20230901005).

Preparation of tetrazine plates for cell sorting: An aqueous solution of polylysine (1 mg/mL, 1 mL) was added to the wells of 6-well culture plates and shaken gently at room temperature for 40 min. Excess solution was decanted, the plate was washed three times with deionized water, then tetrazine derivative **4** (100 μM in absolute ethanol, 1 mL) was added to the wells, the plate was shaken gently at room temperature for 120 min, excess solution was decanted, the plate was washed three times with deionized water, then it was allowed to air-dry in a sterile environment. As a negative control, plates were prepared as described above but not treated with **4**.

Confirmation of tetrazine reactivity on plates for cell sorting: The fluorophore Cy5.5 was conjugated to TCO (100 μmol/mL, 200 μL) was added to tetrazine plates and incubated at room temperature for 30 min. The wells were washed three times with deionized water and imaged using a

confocal laser scanning microscope. As a negative control, the experiment was performed in parallel with plates lacking tetrazine.

Cytotoxicity of tetrazine derivative 4 in *vitro*: The toxicity of tetrazine derivative **4** against SKOV3 cells was evaluated using the CCK-8 assay. Cells were seeded into 96-well plates at a density of 1×10^5 cells/well, incubated for 24 h at 37 °C, then treated for up to 72 h with various concentrations of **4** (0, 5, 10, 20, 50 100 μM). After 24, 48, or 72 h of incubation, the culture medium was replaced with 150 μL of fresh medium containing 10% (v/v) CCK-8 solution, and plates were incubated for another 50 min. Absorbance at 450 nm was measured using a microplate reader (Tecan Spark, Maennedorf, Switzerland).

Antibody modification: Cetuximab (100 μL, 40 μM; Merck, Germany), a monoclonal antibody against human EGFR, underwent a 4-hour incubation at room temperature with TCO-NHS ester (5 mM in DMSO, 100 μL) in a total volume of 4 mL of buffer [0.1 M Na_2CO_3/$NaHCO_3$, 10% (v/v) DMF, pH 8.4]. The labeled antibody was separated from unreacted TCO-NHS ester by centrifuging for 10 min at 10000 g using an Amicon Ultra-4 micro spin column with a molecular weight cut-off of 10 kDa (catalog no. UFC8010, Millipore). The separated antibody was then resuspended in PBS, and this concentration and resuspension process were repeated twice more. Following the third centrifugation, the concentrated solution was stored at -20 ºC until use.[1]

A similar procedure was employed for the modification of Ultra-LEAF™ Purified anti-mouse IFN-*γ* Antibody (2.51 mg/mL, BioLegend, United States) with TCO-NHS.

Cell culture: SKOV3 cells, HeLa cells, and MSC cells were cultured with high-glucose DMEM (GIBCO) supplemented with 10% (v/v) fetal bovine serum (GIBCO) and 1% penicillin–streptomycin solution. Cells were maintained in 100-mm culture dishes at 37 °C in an atmosphere containing 5% CO_2.

Cell sorting in three validation experiments: In the first experiment, MSC cells and SKOV3 cells were cultured for 48 h, digested with trypsin, resuspended in a culture medium, and counted using a hematocytometer. The cells were mixed in various ratios of cell number (SKOV3: MSC, 50:50, 40:60, 30:70, 10:90), incubated for 1.5 h at 37 ºC with the conjugate of Cetuximab to TCO (10 μg/mL, 1 mL), centrifuged to remove excess antibody and purified by concentration and resuspension. The cells were applied to tetrazine plates, and the plates were incubated for 30 min at 37 ºC, then the bottom of the plates was blown and washed with cell culture medium several times to promote the shedding of unbound cells. In the end, the residual cells on the plates were incubated in the incubator (37 ºC, 5% CO_2) for further analysis.

In a second experiment, spleens of mice were removed and placed in a 5 mL syringe plunger containing culture medium, then gently pressed syringe plunger through a 70-micron cell strainer and flushed with culture medium into a conical tube under sterile conditions. The single-cell splenocyte suspension was lysed of red blood cells using 1X Red Blood Cell Lysis Buffer for 10 min on ice, and then the solution was neutralized using 1X PBS. The next procedures are similar to the first experiment.

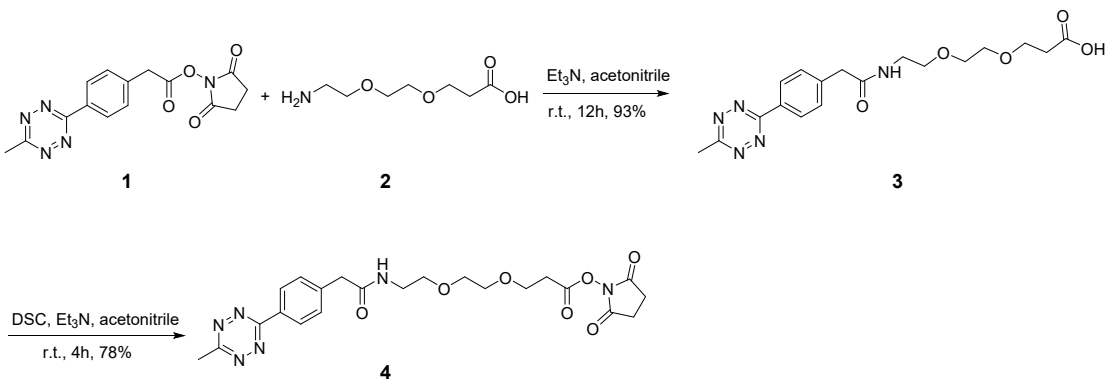
In a third experiment, 1 mL recombinant lentivirus containing the gene for green fluorescent protein (Baoxin Biotechnology Co. Ltd, Chengdu, China) was incubated for 90 min at 37 ºC with 20 μL TCO-NHS ester (5 mM, in DMSO), and unreacted TCO-NHS ester was removed by centrifugation and resuspension in culture medium. HeLa cells were incubated for 30 min at 37 ºC with lentivirus conjugated to TCO, then the cells were centrifuged to remove excess lentivirus conjugated to TCO, purified, and resuspended, by culture medium. The cells were applied to tetrazine plates or no tetrazine plates (control group). The next procedures are similar to the first experiment, except that the bottom of the control group plate was not blown and washed with cell culture medium several times to promote the shedding of unbound cells.

Flow cytometry in three validation experiments: Mixtures of SKOV3 cells and MSC cells were prepared as described above, with sorting on tetrazine plates or without sorting, fixed in 4% paraformaldehyde for 10 min at room temperature, washed once in PBS, blocked for 1 h at room temperature in blocking buffer [0.2% Triton X-100, 5% goat serum (Sigma), 0.01 M PBS], and incubated overnight at 4 ºC with antibody against EGFR (catalog no. ab52894; 1:500; Abcam), followed by 2 h at room temperature with goat anti-rabbit IgG conjugated to Alexa Fluor® 647 (AffiniPure H+L, Jackson ImmunoResearch). Finally, the cells were resuspended in 400 μl PBS and analyzed on a flow cytometer. The same procedure was used in the second validation experiment, except that the primary antibody recognized mouse interferon-*γ* and the secondary antibody was conjugated to Alexa Fluor® 488.In the third validation experiment, the HeLa cells on plates were digested with trypsin, resuspended in 400 μL PBS, and analyzed on a flow cytometer.

Immunocytochemistry: SKOV3 and MSC cells were seeded into 35-mm culture dishes, incubated for 24 h, then fixed in 4% paraformaldehyde, blocked, and treated with primary antibody against EGFR and secondary antibody as described in section 7. The cells were also stained for 5 min at room temperature with Hoechst 33342 dye (4 μM). Fluorescence was imaged with a confocal laser scanning microscope at an excitation wavelength of 633 nm. The same procedure was used for mouse spleen lymphocytes that had been sorted or not (section 6), except that the primary antibody recognized mouse interferon-*γ*, and the secondary antibody was conjugated to Alexa Fluor® 488. Thus, fluorescence was imaged at an excitation wavelength of 488 nm.

Statistical analysis: All data were expressed as mean ± standard deviation (SD). T-test and one-way ANOVA were used to conduct statistical analysis using GraphPad Prism 8.0. $p < 0.001$, and 0.0001 were considered statistically significantly different.

2. Synthesis



3-(2-(2-(2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetamido)ethoxy)ethoxy)propanoic acid **(3):** Compound **1** (9.00 mg, 0.028mmol, 1 equiv) [2] and compound **2** (5.85 mg, 0.033mmol, 1.2 equiv) were dissolved in acetonitrile (1.5 mL). Triethylamine (5.4 µL, 0.041 mmol, 1.5 equiv) was then added, and the mixture was stirred at room temperature for 12 h. After the reaction was completed, the reaction mixture was diluted with water and extracted with DCM. The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo.* The crude product was purified on a silica column (DCM: MeOH, 20:1) to afford compound **3** (10.00 mg, 93%) as a pink solid.

^{1}H NMR: (400 MHz, methanol-d4) δ 8.60 – 8.45 (m, 2H), 7.57 (d, *J* = 8.4 Hz, 2H), 3.73 (t, *J* = 6.2 Hz, 2H), 3.67 (s, 2H), 3.60 (s, 4H), 3.56 (t, *J* = 5.4 Hz, 2H), 3.40 (t, *J* = 5.4 Hz, 2H), 3.04 (s, 3H), 2.54 (t, *J* = 6.2 Hz, 2H).

^{13}C NMR: (101 MHz, methanol-d_4) δ 173.4, 168.7, 165.2, 142.0, 132.1, 131.1, 128.9, 71.4, 71.2, 70.5, 67.9, 43.7, 40.7, 36.0, 21.0.

HRMS: $[M+H]^+$ m/z calcd. for $[C_{18}H_{24}N_5O_5]^+$ 390.1772, found 390.1783.

2,5-dioxopyrrolidin-1-y-l3-(2-(2-(2-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)acetamido)ethoxy)ethoxy)propanoate **(4):** Compound **3** (10.00 mg, 0.026 mmol, 1 equiv) was dissolved in anhydrous acetonitrile (1.5 mL) followed by the addition of Triethylamine (5.4 µL, 0.038 mmol, 1.5 equiv) and *N, N'*-disuccinimidyl carbonate (7.90 mg, 0.031 mmol, 1.2 equiv). This solution was stirred at room temperature for 4 h. After the reaction was completed, the reaction mixture was diluted with water and extracted with DCM. The organic layer was dried over Na_2SO_4, filtered, and concentrated *in vacuo.* The crude product was purified by silica column (DCM: MeOH, 20:1) to afford compound **3** (9.80 mg, 78%) as a pink solid.

^{1}H NMR: (400 MHz, Chloroform-*d*) δ 8.40 (d, *J* = 8.2 Hz, 2H), 8.21 (t, *J* = 5.4 Hz, 1H), 7.54 (d, *J* = 8.2 Hz, 2H), 3.71 (t, *J* = 5.9 Hz, 2H), 3.57 (s, 2H), 3.53 (q, *J* = 7.2 Hz, 4H), 3.43 (t, *J* = 5.7 Hz, 2H), 3.23 (q, *J* = 5.6 Hz, 2H), 2.99 (s, 3H), 2.91 (t, *J* = 5.9 Hz, 2H), 2.80 (s, 4H).

^{13}C NMR: (101 MHz, Chloroform-*d*) δ 170.1, 169.6, 167.4, 167.0, 163.2, 141.3, 130.1, 130.0, 127.3, 69.7, 69.5, 69.1, 65.2, 42.2, 38.8, 31.6, 25.4, 20.8.

HRMS: $[M+H]^+$ m/z calcd. for $[C_{22}H_{27}N_6O_7]^+$ 487.1936, found 487.1943.

3. Supplementary Figures

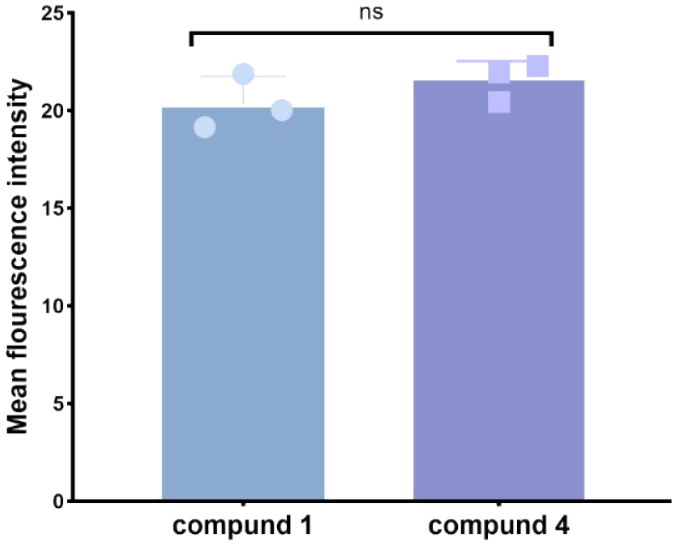


Figure S1. Quantitation of fluorescence from the three tetrazine derivatives after conjugation to the poly-lysine coating on cell culture dishes, followed by incubation with the fluorophore Cy5.5 conjugated to *trans*-cyclooctene. ns, not significant.

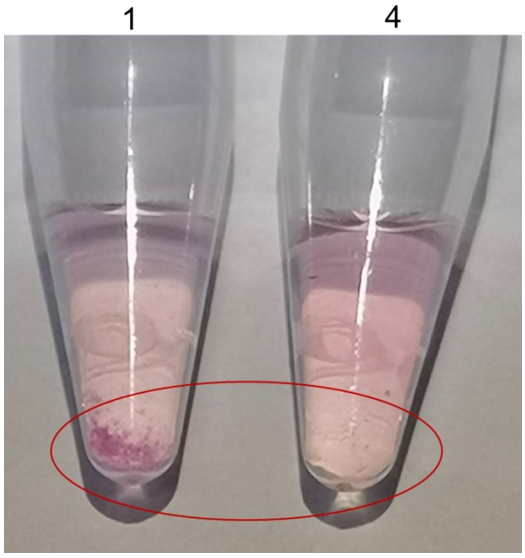


Figure S2. Equimolar solutions of tetrazine derivative **1** and **4** in PBS (10 mM, pH 7.4) were subjected to centrifugation at 15000 rpm for 5 minutes.

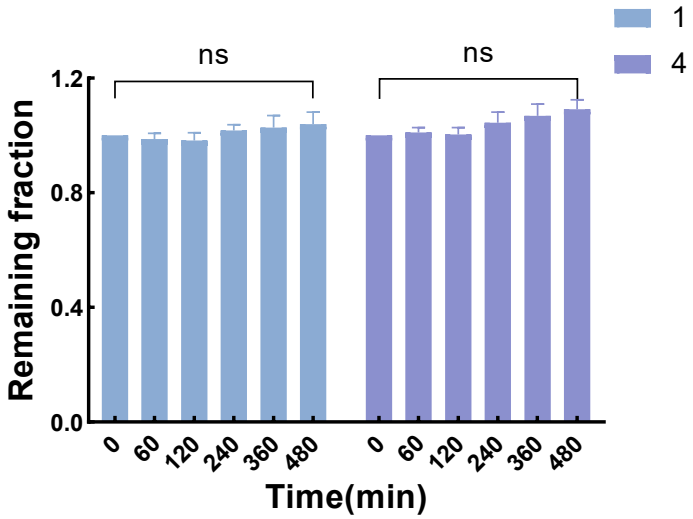


Figure S3. Stability assessment of compound **1** (left) and **4** (right) was conducted using a DNA/Protein analyzer 6000+ (Quawell) equipped with a cuvette. The absorption change of the tetrazine derivative **1** and **4** (100 μM) at 520 nm were measured. The tetrazine derivative **1** and **4** were incubation for 0–8 h at 37 ºC in high-glucose Dulbecco's modified Eagle medium containing 10% (v/v) fetal bovine serum and 10% (v/v) DMSO. The results showed no significant differences (ns).

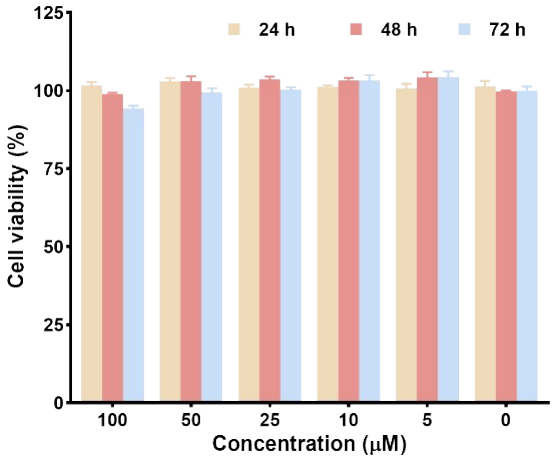


Figure S4. Viability of SKOV3 cells incubated with 0–100 μg/mL **compound 4** lasting 24, 48, or 72 h in high-glucose DMEM, supplemented with 10% (v/v) fetal bovine serum and 1% penicillin–streptomycin solution.

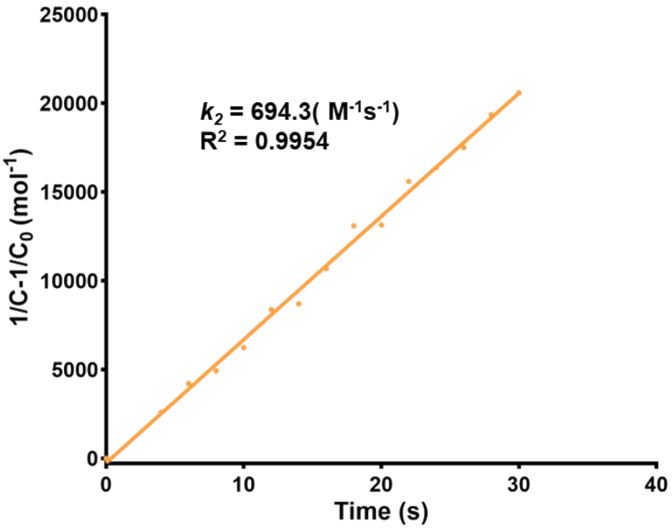


Figure S5. Kinetics of the inverse electron-demand Diels-Alder reaction at 37 ºC between tetrazine derivative **4** and **4e-TCO,** both at 100 µM, in a mixture of fetal bovine serum and PBS (1:9, v/v) in a 1-mL cuvette suitable for ultraviolet spectroscopy. Absorbance at 520 nm was monitored every two seconds.

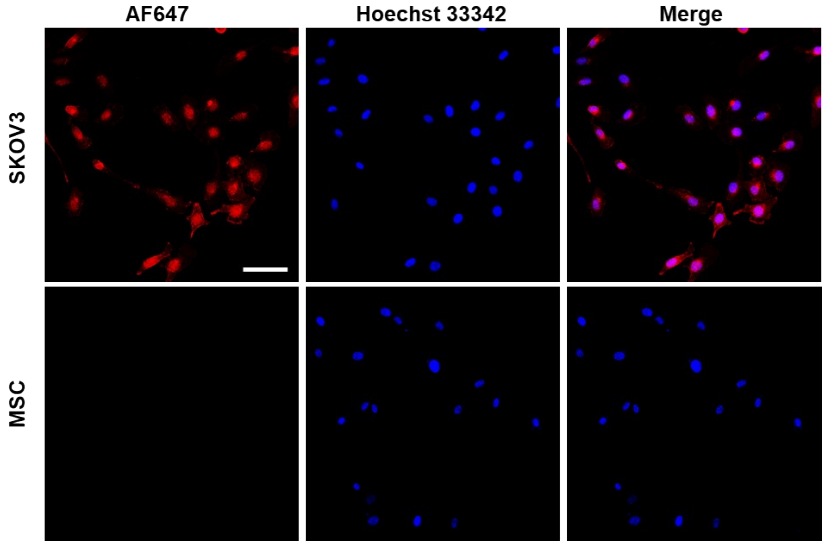


Figure S6. Immunocytochemistry to detect SKOV3 cells and mesenchymal stem cells (MSC). Red fluorescence corresponds to Alexa Fluor® 647 on the secondary antibody, while blue fluorescence corresponds to Hoechst 33342 staining. Scale bar, 50 µm.

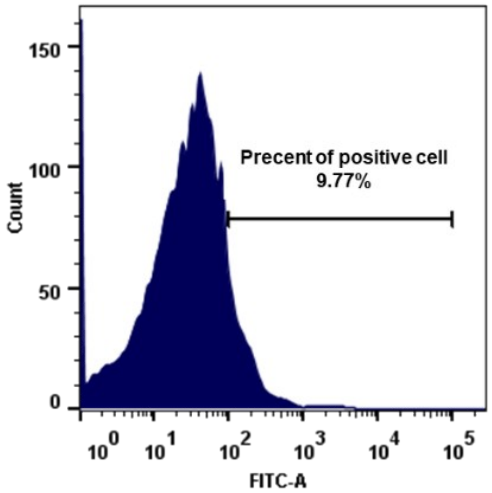


Figure S7. The mouse spleen cells that were not sorting incubated with Alexa Fluor® 488 AffiniPure Goat Anti-Mouse IgG (H+L).

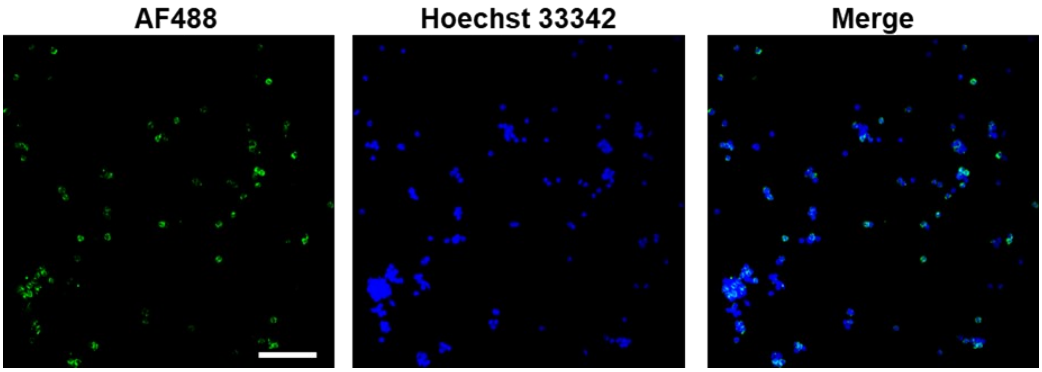


Figure S8. Immunocytochemistry combined with Alexa Fluor® 488 AffiniPure Goat Anti-Rabbit IgG (H+L) (green fluorescence) and Hoechst 33342 staining (blue fluorescence) of the mouse spleen cells which were not sorting. Scale bar 50 μm.

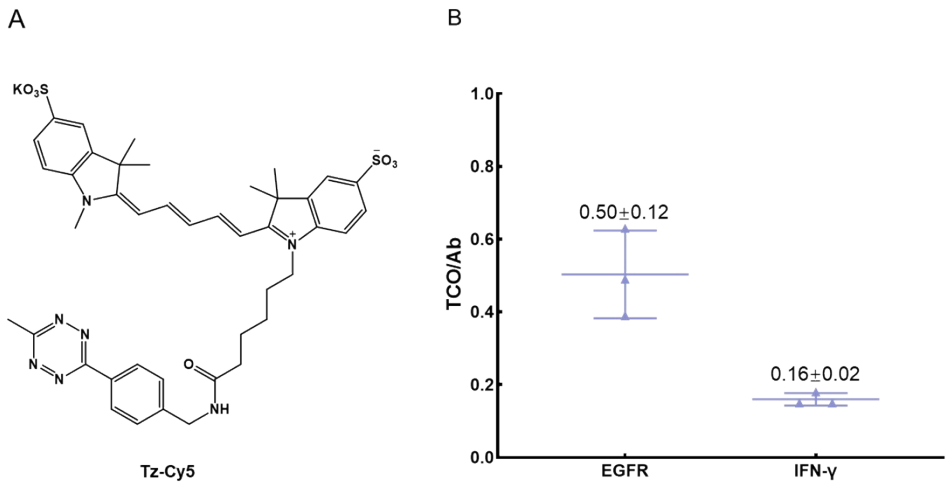


Figure S9. (A) Chemical structure of Tz-Cy5. (B) Measurement of reactive TCO sites per antibody by reaction with a Tz-conjugated fluorophore. Unmodified and TCO-decorated antibodies (0.5 mg/mL) were reacted with an excess (100 μM) of **Tz-Cy5** at room temperature for 6 hours. After the reaction,

the antibody was purified by centrifugation for 10 minutes at 10000 g using an Amicon Ultra-4 micro spin column with a molecular weight cutoff of 10 kDa (catalog no. UFC8010, Millipore) and stored in PBS. The number of fluorochromes per antibody (corresponding to the reactive TCO sites) was determined by ultraviolet spectrophotometric analysis.[3]

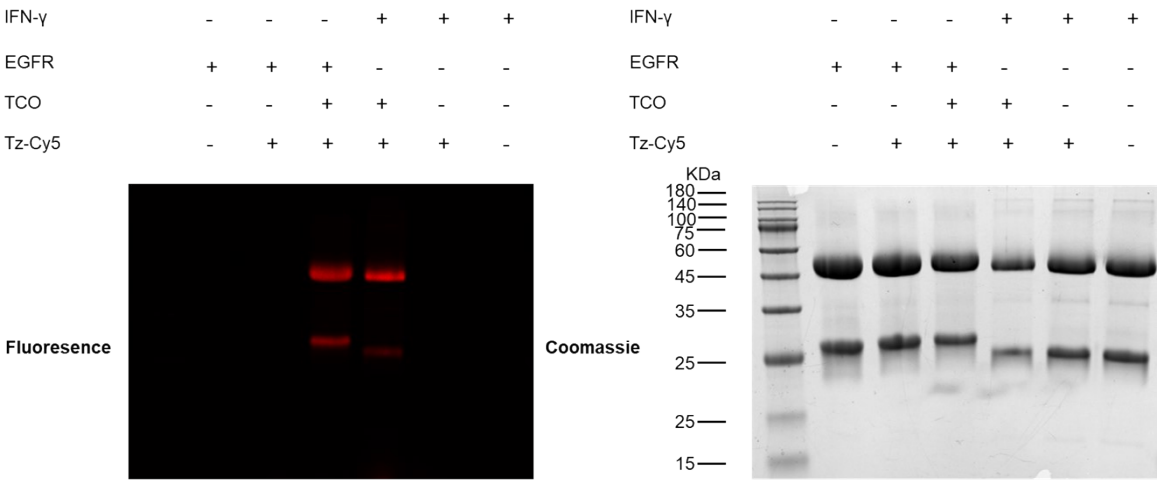
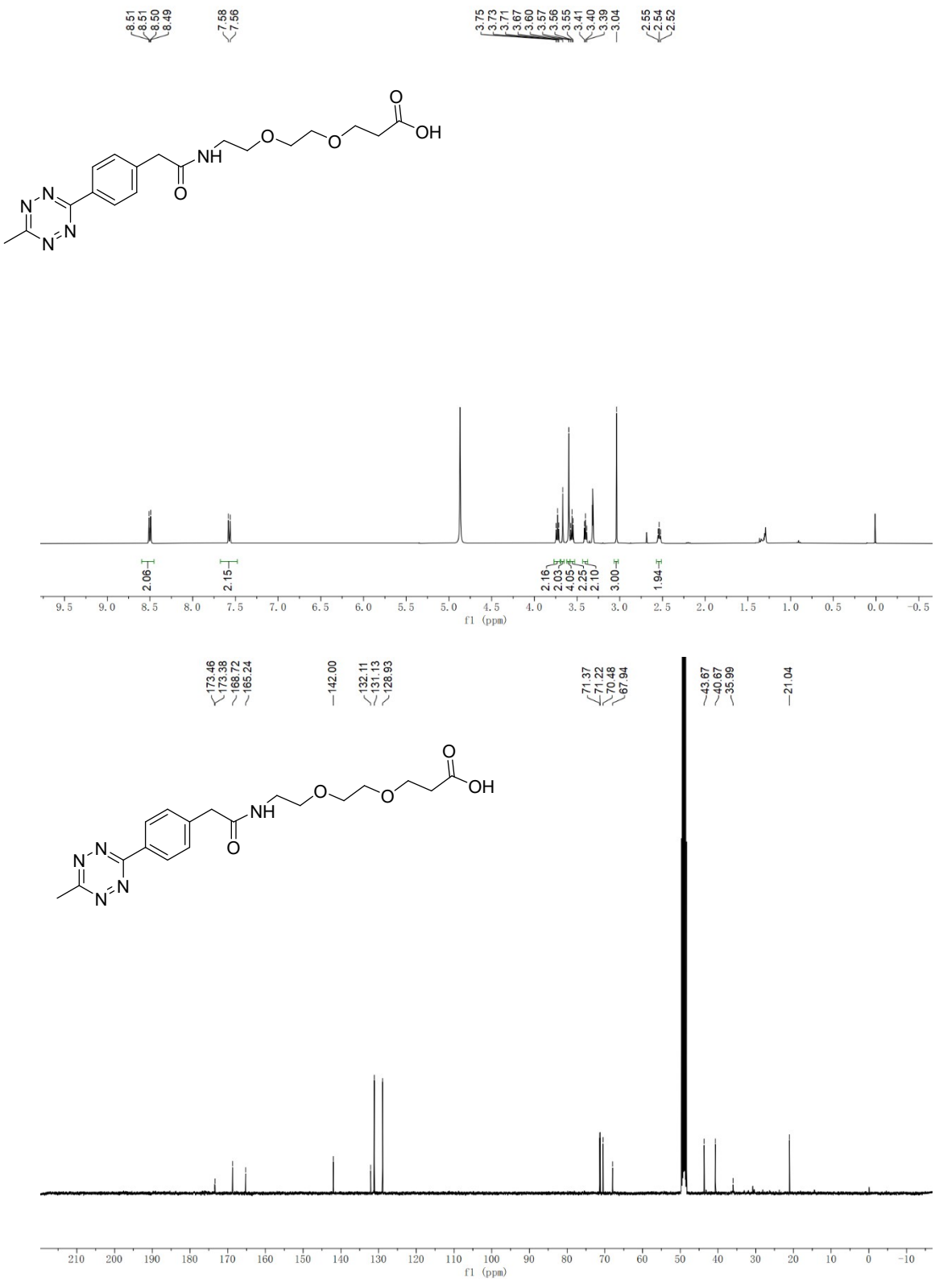


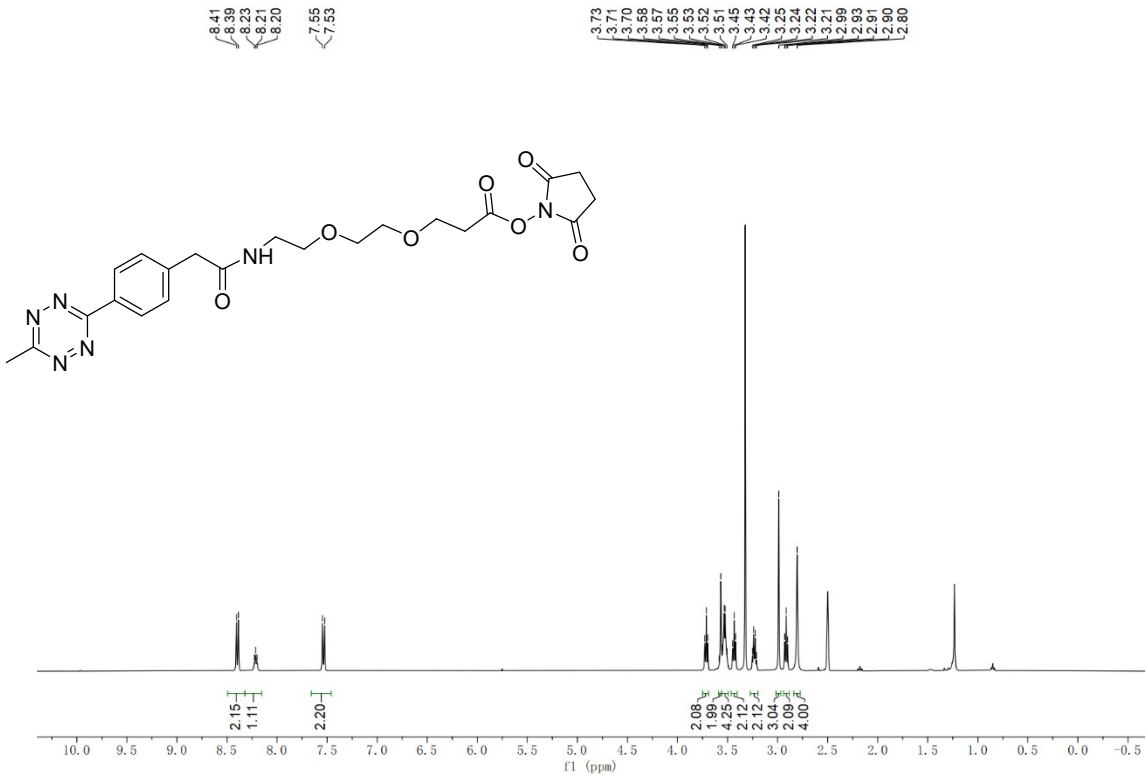
Figure S10. Labeling of **TCO-EGFR** or **TCO-IFN-γ** with **Tz-Cy5**. Unmodified and TCO-decorated antibodies (0.5 mg/mL) were reacted with an excess (100 μM) of **Tz-Cy5** at room temperature for 30 minutes. After the reaction, the antibody was purified by centrifugation for 10 minutes at 10000 g using an Amicon Ultra-4 micro spin column with a molecular weight cutoff of 10 kDa (catalog no. UFC8010, Millipore) and stored in PBS. Proteins were collected and analyzed using SDS-PAGE. In-gel fluorescence labeling was captured using Multiplex Fluorescence (ChemiDoc MP, Bio-rad) with the Cy5 channel (red channel). The image was acquired and processed through the ChemiDoc systems. Subsequently, the gels were stained with Coomassie Blue Fast Staining Solution to assess protein loading.

4. Reference

1. M. K. Rahim, R. Kota and J. B. Haun, *Bioconjugate Chemistry*, 2015, **26**, 352-360.
2. A. La-Venia, R. Dzijak, R. Rampmaier and M. Vrabel, *Chem. Eur. J.*, 2021, **27**, 13632-13641.
3. J. B. Haun, N. K. Devaraj, S. A. Hilderbrand, H. Lee and R. Weissleder, *Nature Nanotechnology*, 2010, **5**, 660-665.

5. NMR Spectra and High-resolution Mass Spectra

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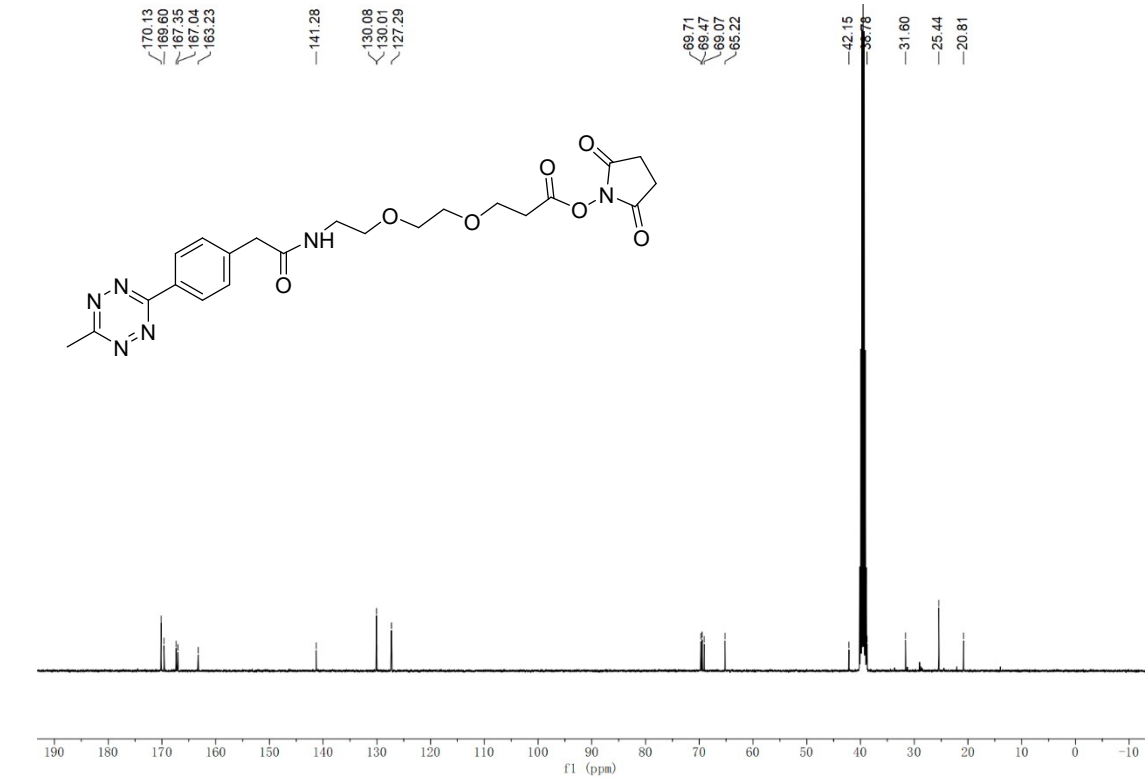
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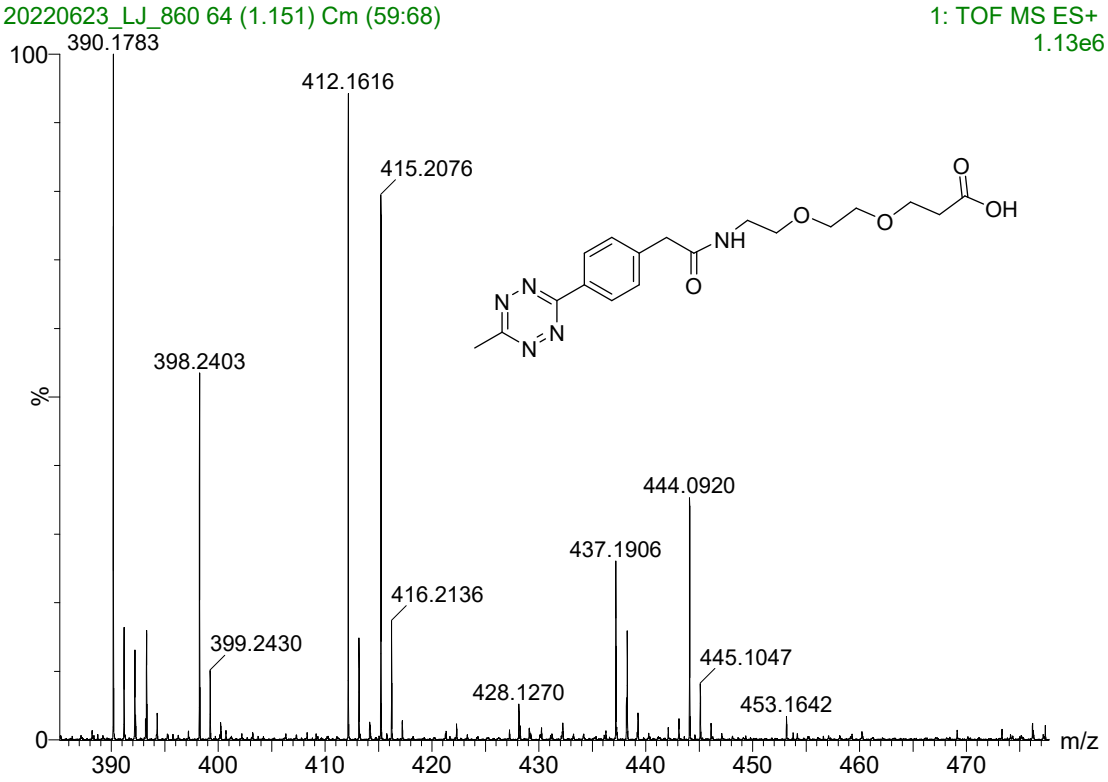
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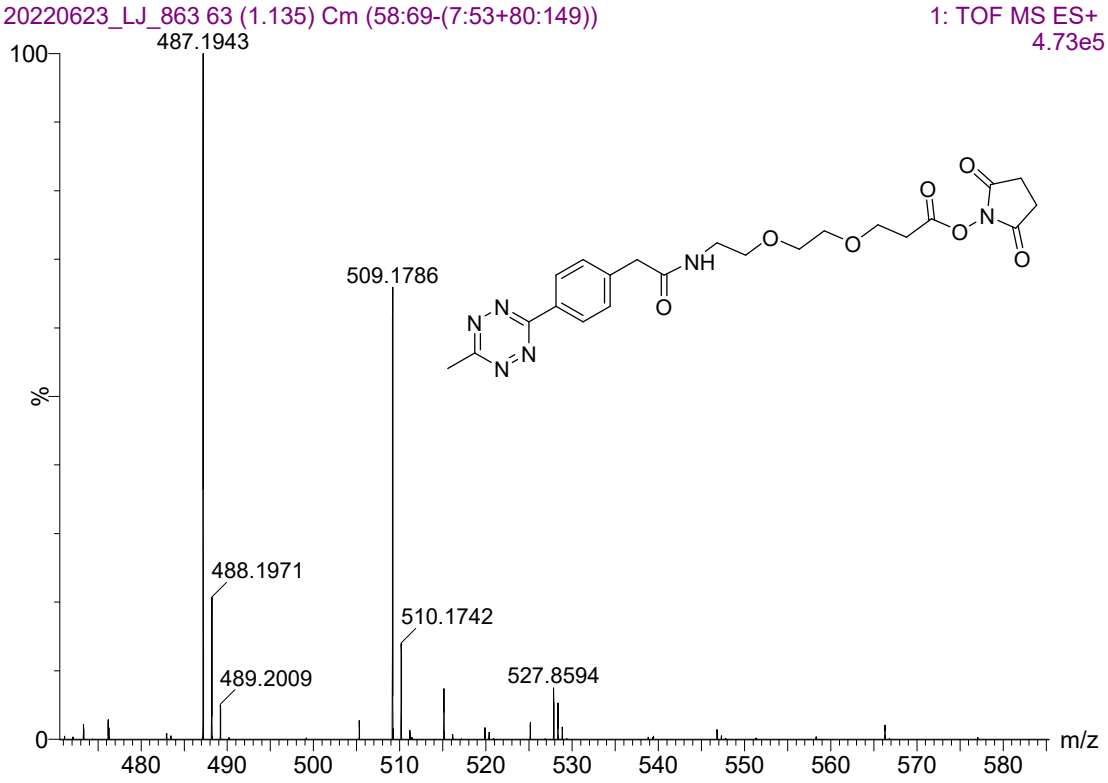
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