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In situ simultaneous profiling of phosphorylation and ubiquitination by single excitation-duplexed luminescence resonance energy transfer

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Experimental

Materials and reagents. Yttrium(III) nitrate hexahydrate ($Y(NO_3)_3•6H_2O$), ytterbium chloride ($YbCl_3$), erbium chloride ($ErCl_3$), ammonium fluoride (NH_4F), sodium chloride (NaCl), N-hydroxy succinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), polyethylenimine (PEI), cyanuric chloride, potassium carbonate (K_2CO_3), 1,4,7-tris-*tert*-butyloxycarbonyl-1,4,7,10-tetraazacyclododecane, zinc(II)-perchlorate and ethylenediamine were purchased from Sigma-Aldrich Inc. (USA). Ubiquitination antibody was purchased from Abcam plc Co., Ltd. (USA). MCF-7, HeLa and T47D cell lines were purchased from KeyGen Biotech. Co. Ltd. (Nanjing, China). Ethanediol, ethanol, acetone, ethyl acetate (EA), petrol ether (PE), dioxane, dichloromethane, methanol and dimethyl formamide (DMF) were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). Cyanine 3 (Cy3) NHS ester was purchased from Beijing Lipuhui technology co., LTD. Phosphate buffer saline (PBS, pH 7.4) contained 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, and 1.41 mM KH_2PO_4. All aqueous solutions were prepared using ultrapure water (≥ 18 MΩ·cm, Milli-Q, Millipore). The DNA sequences (from

left to right: 5' to 3') were purchased from Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China) with the following sequences:

Carboxylic group-modified oligonucleotide (A30-COOH): COOH-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA;

Fluorescein isothiocyanate conjugated A30-COOH (A30-FITC): COOH-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-FITC;

Cy3 labeled DNA (T6-Cy3): Cy3-TTTTTT;

Cyanine 5.5 (Cy5.5) labeled DNA (T6-Cy5.5): Cy5.5-TTTTTT;

T21-conjugated HER2 aptamer (Apt):

TTTTTTTTTTTTTTTTTTTTTAACCGCCCAAATCCCTAAGAGTCTGCACTTGTCATTTTGTATATGTATTTGGTTTTTGGCTCTCACAGACACACTACACACGCACA;

Fluorescein isothiocyanate conjugated Apt (Apt-FITC):

TTTTTTTTTTTTTTTTTTTTTAACCGCCCAAATCCCTAAGAGTCTGCACTTGTCATTTTGTATATGTATTTGGTTTTTGGCTCTCACAGACACACTACACACGCACA-FITC.

Characterizations. Absorption spectra were obtained from an UV-3600 UV-Vis-NIR spectrophotometer (Shimadzu, Japan). The transmission electron microscopic (TEM) images were recorded on a JEM-2100 transmission electron microscope (JEOL Ltd., Japan). Dynamic light scattering (DLS) was performed on a 90 Plus/BI-MAS equipment (Brook Haven, USA). Zeta potential was observed on a Zetasizer instrument (Nano-Z, Malvern, UK). Fluorescence spectra were recorded on an F-7000 spectrometer (HITACHI, Japan). Upconversion luminescence (UCL) spectra were measured on a ZolixScan ZLX-UPL spectrometer with an external continuous-wave laser (980 nm) as the excitation source. Confocal fluorescence imaging of cells was measured on a TCS SP5 confocal laser scanning microscope (Leica, Germany).

Flow cytometric analysis was obtained from a Coulter FC-500 flow cytometer (Beckman-Coulter). Matrix-assisted laser desorption/ionization time-of-fight mass spectrometry (MALDI-TOF-MS) experiments were carried out on a 4800 Plus MALDI TOF/TOF Analyzer (AB Sciex, U.S.A.) with the Nd:YAG laser at 355 nm, a repetition rate of 200 Hz, and an acceleration voltage of 20 kV. Data was analyzed with a Data Explorer Software from AB Sciex.

Synthesis of PEI-wrapped UCNP. According to the previous protocol,[S1] the PEI-wrapped UCNP was synthesized as follows: $Y(NO_3)_3 \cdot 6H_2O$ (919 mg, 2.40 mmol), $YbCl_3 \cdot 6H_2O$ (92.4 mg, 0.24 mmol), $ErCl_3$ (16.4 mg, 0.06 mmol), NaCl (352 mg, 6.00 mmol), NH_4F (463 mg, 12.5 mmol), and 728.16 μL PEI were added to a 100 mL flask containing 50 mL ethanediol and stirred by a magnet until the solution became clear. Then, the solution was transferred to the reaction kettle and heated to 200 °C for 8 h. After cooling down to room temperature, the mixture was precipitated by adding 10 mL of ethanol, and the precipitate was collected by centrifugation at 10000 *r*/min for 10 min. The as-prepared PEI-wrapped UCNP was purified through repeated washing with ethanol for three times. The obtained solid was dried in vacuum drying oven overnight.

Synthesis of A30-COOH conjugated UCNP (A30-UCNP). The A30-UCNP was synthesized *via* an amide reaction between amino-functionalized UCNP and carboxyl-functionalized DNA (A30-COOH).[S2] After 20 μL of A30-COOH (100 μM) was stirred with 1.5 mg EDC and 1 mg NHS for 20 mins, 800 μL of the UCNP and 200 μL of PBS were added into the mixture and kept stirring at constant speed for another 2 hours at the room temperature to construct the A30-UCNP. Excessive A30-COOH was removed by centrifugation and washing for 3 times. The A30-UCNP was re-dispersed in 800 μL water and stored at 4 °C for further use.

Synthesis of aptamer-functionalized UCNP (Apt-UCNP). After adding 20 μL of 100 μM Apt

into 800 μL of A30-UCNP, the mixture was stirred at constant speed overnight at room temperature to allow the hybridization of Apt with A30 on the UCNP. Excessive Apt was removed by centrifugation and washing for 3 times. The Apt-UCNP was re-dispersed in 800 μL water and stored at 4 °C for further use.

Loading density of aptamer on UCNP. For $NaYF_4$, $NaErF_4$ and $NaYbF_4$, the relative molecular density is 4.31 g/cm^3, 6.11 g/cm^{3}and 6.24 g/cm^3. Assuming the coefficient of crystal lattice of the PEI coated UCNP is 80% Y/2% Er/18% Yb in $NaYF_4$, the mass of PEI coated UCNP with the radius of 43 nm, could be calculated as:[S3]

$$m_{UCNP} = \left(0.8 \times 4.31\frac{g}{cm^3} + 0.02 \times 6.11\frac{g}{cm^3} + 0.18 \times 6.24\frac{g}{cm^3}\right) \times \frac{4}{3} \times \pi \times \left(43 \right.$$
$$= 1.56 \times 10^{-15} g$$

Therefore, the concentration of UCNP (2.0 mg/mL) could be calculated to be 2.1 nM. In order to estimate the loading density of Apt on UCNP, Apt-FITC was used to replace Apt to incubate with UCNP, using A30-COOH as the linker. The concentration of Apt-FITC linked to UCNP was estimated to be 190 nM by fluorescence detection. Thus, the amount of Apt linked to each Apt-UCNP was estimated to be about 90.

Synthesis of compound S1: 98.0 mg (0.531 mmol) of cyanuric chloride and 146 mg (0.106 mmol) of K_2CO_3 were suspended in 3.33 ml acetone in a round bottom flask. Then 500 mg (0.106 mmol) of 1,4,7-tris-*tert*-butyloxycarbonyl-1,4,7,10-tetraazacyclododecanel in 2.5 ml of acetone was slowly added via a syringe to this suspension.[S4,S5] The reaction mixture was heated to 56 °C under reflux for 48 h. The solvent was concentrated by distillation under reduced

pressure and the raw product chromatographed on silica gel (EA/PE 50:50) to yield compound **S1** (350 mg, 0.331 mmol, 58 %), as a light yellow solid (R_f = 0.60, EA/PE 50:50). ^{1}H NMR (400MHz, DMSO): δ = 1.43-1.47 (s, 27H), 3.23-3.67 (m, 16 H). MALDI-TOF-MS: *m*/*z* calculated for $C_{49}H_{86}ClN_{11}O_{12} + K^+$, 1094.7; found, 1094.5.

Synthesis of compound S2: 200 mg (0.189 mmol) of **S1** and 10.0 mg (0.0724 mmol) of K_2CO_3 were dissolved in 5 ml of dioxane in a round bottom flask. 30.0 μL of ethylenediamine was slowly added via a syringe to this suspension.[S4,S5] The reaction mixture was heated to 90 °C under reflux for 21 h. The solvent was concentrated by distillation under reduced pressure and the raw product chromatographed on silica gel (dichloromethane/methanol 90:10) to yield compound **S2** (120 mg, 0.111 mmol, 59 %), as a light yellow solid (R_f = 0.30, EA/PE 20:10). ^{1}H NMR (400 MHz, DMSO): δ = 1.21-1.35 (m, 54 H), 3.31-3.49 (m, 38 H), 8.02 (s, 1 H). MALDI-TOF-MS: *m*/*z* calculated for $C_{51}H_{93}N_{13}O_{12} + H^+$, 1080.7; found, 1080.1.

Synthesis of Cy3-pTag： 100 mg (0.0926 mmol) of **S2** and 47.0 mg (0.0646 mmol) of Cy3-NHS ester were dissolved in 5 ml of DMF in a round bottom flask. The mixture was heated and stirred for 2h at 30 °C and concentrated by distillation under reduced pressure. Then the solid was dissolved in 5 ml of CH_2Cl_2 and 1 ml (12.4 mmol) of TFA was added to this solution. The reaction mixture was stirred for 24 h at room temperature and the solvent was removed in vacuum. After dissolved in 5 ml of MeOH, 432 mg (1.16 mmol) of zinc(II)-perchlorate was slowly added to this solution. The reaction mixture was stirred for 16 h at room temperature and then heated to reflux for another 10 h. The raw product chromatographed on silica gel (dichloromethane/methanol 10:20) to yield compound **Cy3-pTag** (65 mg, 0.0522 mmol, 58 %), as a prunosus solid (R_f = 0.30, EA/PE 20:10). ^{1}H NMR (400 MHz, D_2O): δ = 1.07-1.55 (m, 46H),

1.76-2.24 (m, 10H), 2.50-3.84 (m, 11 H), 6.16-8.22 (m, 16H), ^{13}C NMR (400 MHz, D_2O, ppm): 185.99, 177.93, 164.67, 164.53, 154.56, 146.59, 144.00, 141.96, 141.89, 129.28, 122.24, 120.30, 117.40, 113.94, 105.92, 105.74, 51.63, 46.64, 41.69, 40.63, 39.86, 36.62, 35.56, 29.58, 29.43, 29.07, 28.46, 28.08, 27.46, 27.23, 14.11, and 14.09. MALDI-TOF-MS: *m*/*z* calculated for $C_{54}H_{83}N_{15}O_7S_2Zn_2^{4+}$, 311.86; found, 312.2.

Cell culture. T47D and MCF-7 cells were cultured in RPMI-1640 (GIBCO) supplemented with 10% FBS and 0.2 U/mL bovine insulin at 37 °C in a humidified atmosphere containing 5% CO_2. HeLa cells were cultured with Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. Cell number was calculated using Countess® II Automated Cell Counter (Invitrogen, USA).

Flow cytometric analysis. T47D and HeLa cells were separately seeded on six-well confocal dish at a density of 5.0×10^4 per dish and incubated for 12 h at 37 °C. After washed with PBS for three times, the medium was then replaced by fresh culture medium containing 1.25 μM Apt-FITC or Ran-FITC, and cultured for 30 min. Then the cells were washed, collected, and finally dispersed in the binding buffer for flow cytometric analysis over FL1 (FITC).

Confocal laser scanning microscopic (CLSM) analysis. The T47D and HeLa cells were separately seeded on 4-well confocal dishes and cultured at 37 °C in a humidified atmosphere containing 5% CO_2 for 12 h. After washed by PBS for three times, the cells were incubated with 1.25 μM Apt-FITC or Ran-FITC for 30 min. Then the cells were carefully washed and imaged by CLSM immediately. All images were digitized and analyzed with Leica Application Suite Advanced Fluorescence (LAS-AF) software package.

Duplexed LRET imaging. Cells were seeded at a density of 2×10^5 cells/mL on 4-well confocal

dishes and cultured for 12 h. Then the cells were incubated with Apt-UCNP in culture medium for 3 h and washed with PBS for three times. After fixing with 75% ethanol for 10 min, the cells were incubated with **Cy5.5-UbA** (1:400) overnight and with **Cy3-pTag** for another 2 h. After washed for three times, the treated cells were imaged with CLSM. The equipment parameters remained the same for all the duplexed imaging experiments. The UCL signal was collected by CLSM under 980 nm excitation; the EEB1 and EEB2 channels were collected from 520 to 560 nm, and 640 to 670 nm, respectively. The Cy3 signal was obtained by CLSM under 514 nm excitation and collected from 562 to 600 nm. The Cy5.5 signal was obtained by CLSM under 633 nm excitation and collected from 672 to 720 nm. The LRET1 and LRET2 images under 980 nm excitation were collected from 562 to 600 nm, and 672 to 720 nm, respectively.

Supporting figures

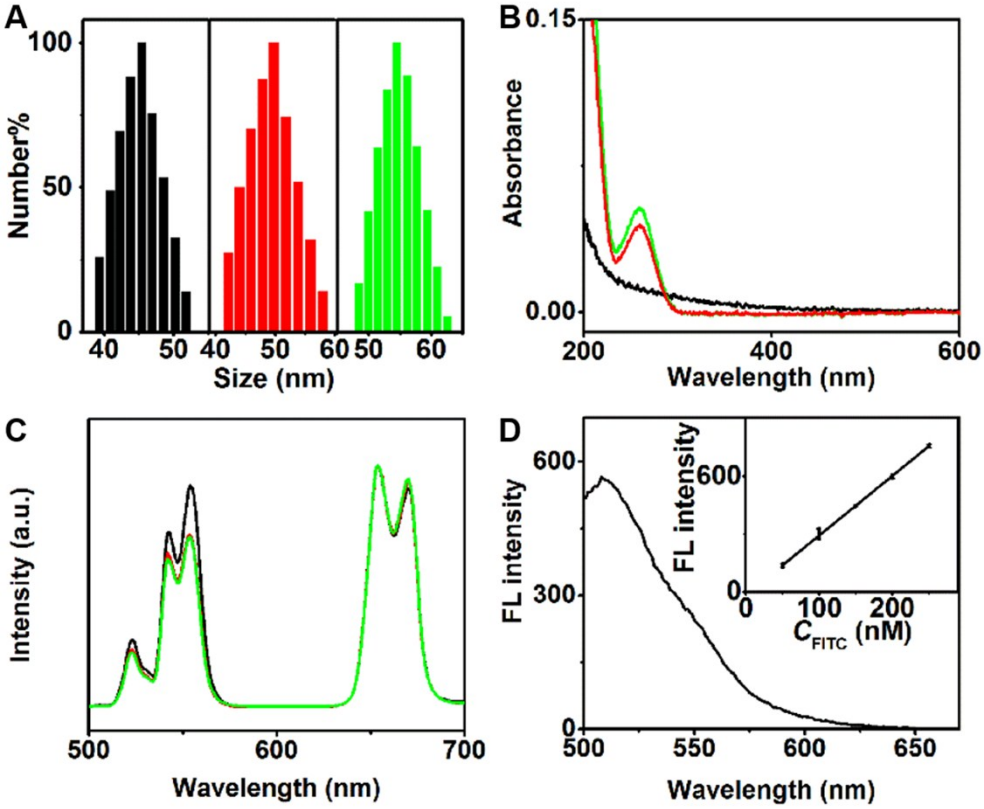

Fig. S1 (A) DLS analysis, (B) UV/vis spectra and (C) upconversion luminescence spectra of UCNP (black), A30-UCNP (red) and Apt-UCNP (green). (D) Fluorescence spectrum of A30-UCNP after hybridization with Apt-FITC. Inset: Fluorescence intensity at 513 nm of different concentrations of Apt-FITC.

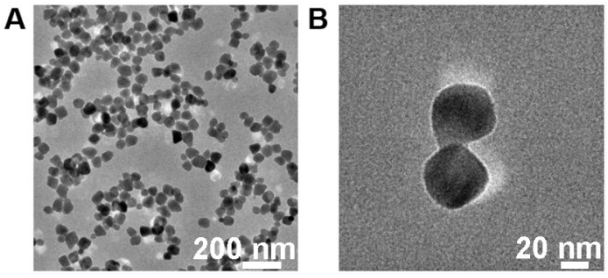

Fig. S2 Transmission electron microscopic images of Apt-UCNP at different scale bars.

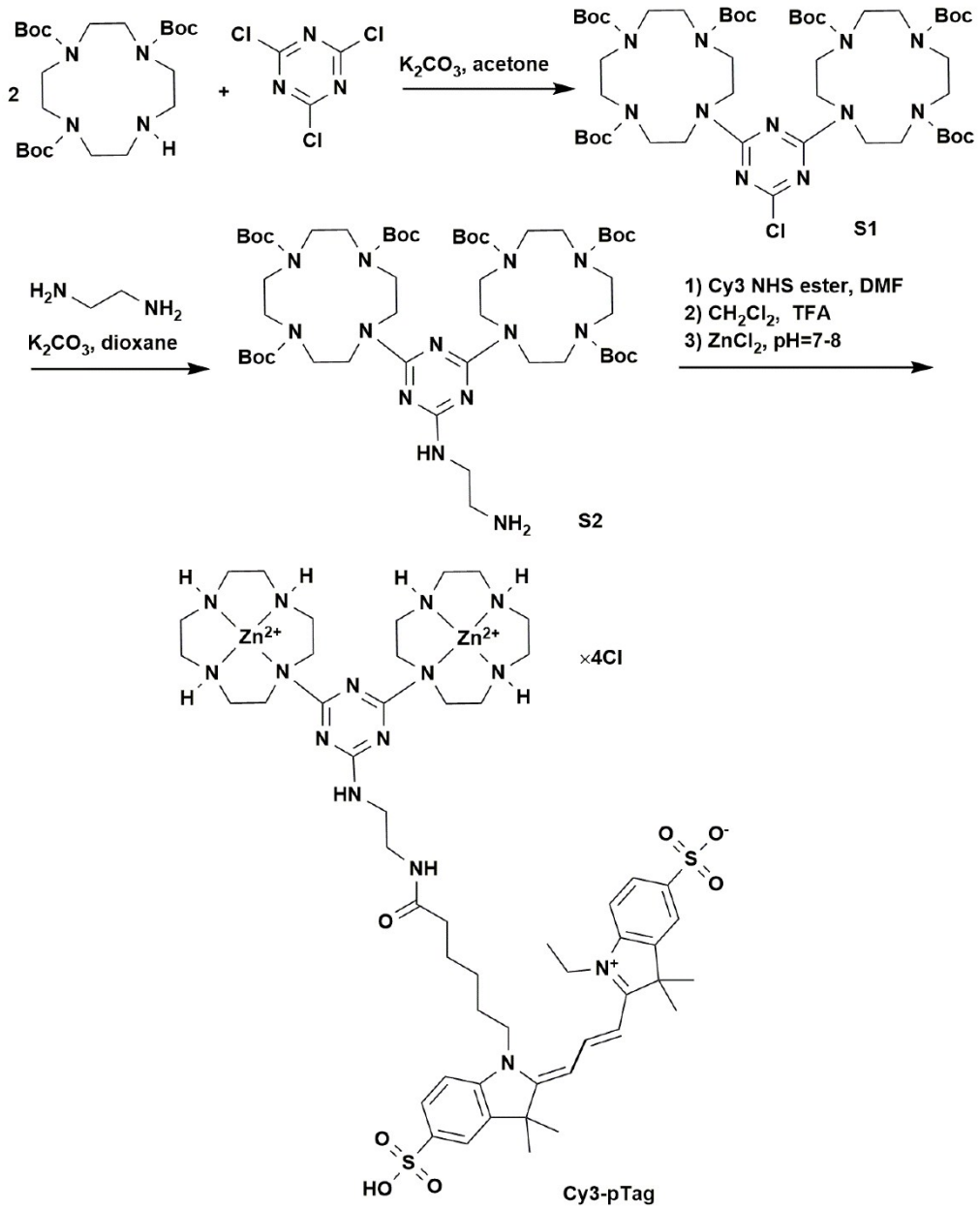

Fig. S3 Synthetic routes of compound **Cy3-pTag**.

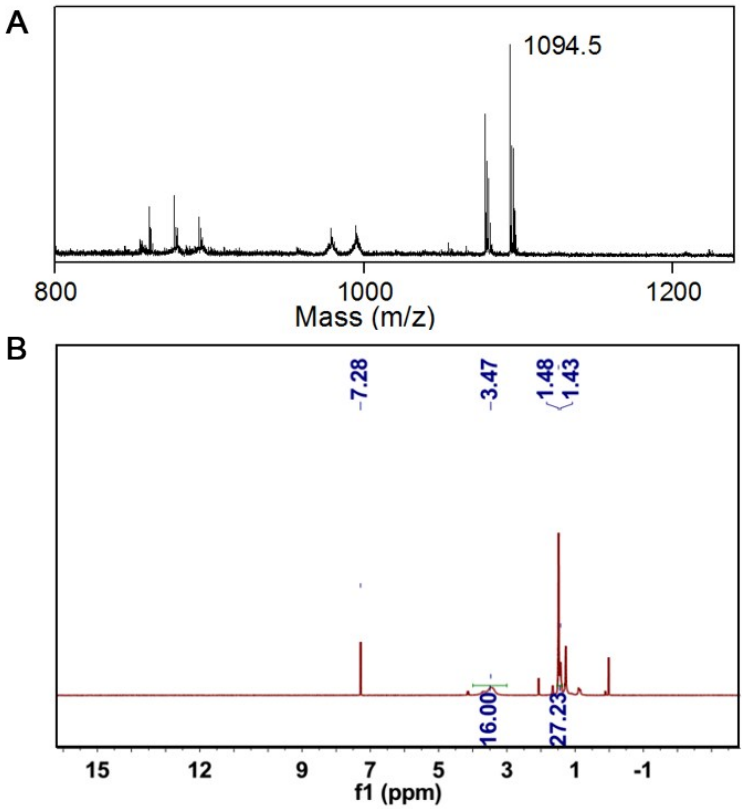

Fig. S4 (A) MALDI-TOF mass and (B) ^{1}H NMR spectra of **S1**.

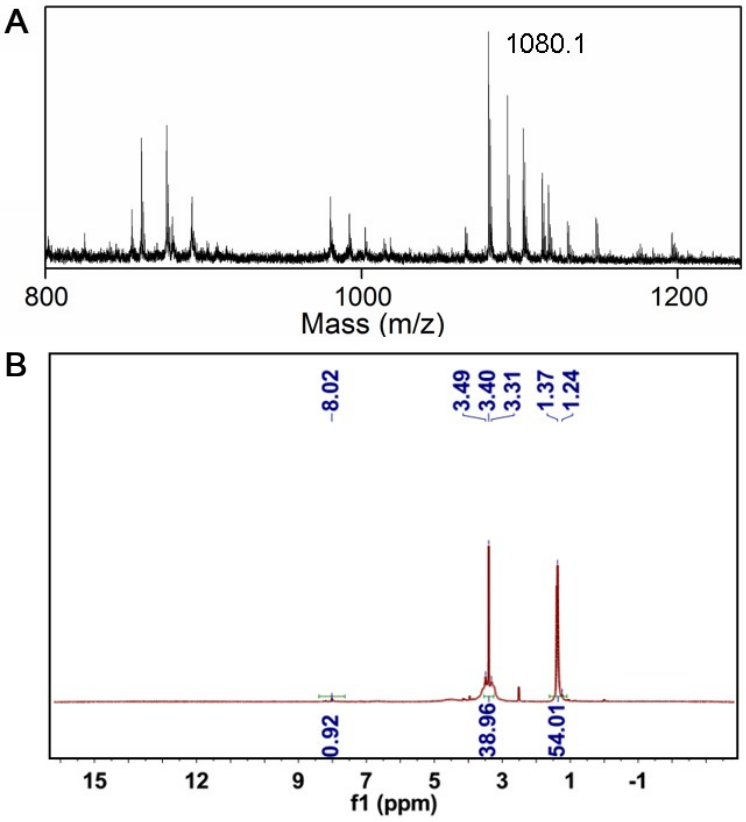

Fig. S5 (A) MALDI-TOF mass and (B) ^{1}H NMR spectra of **S2**.

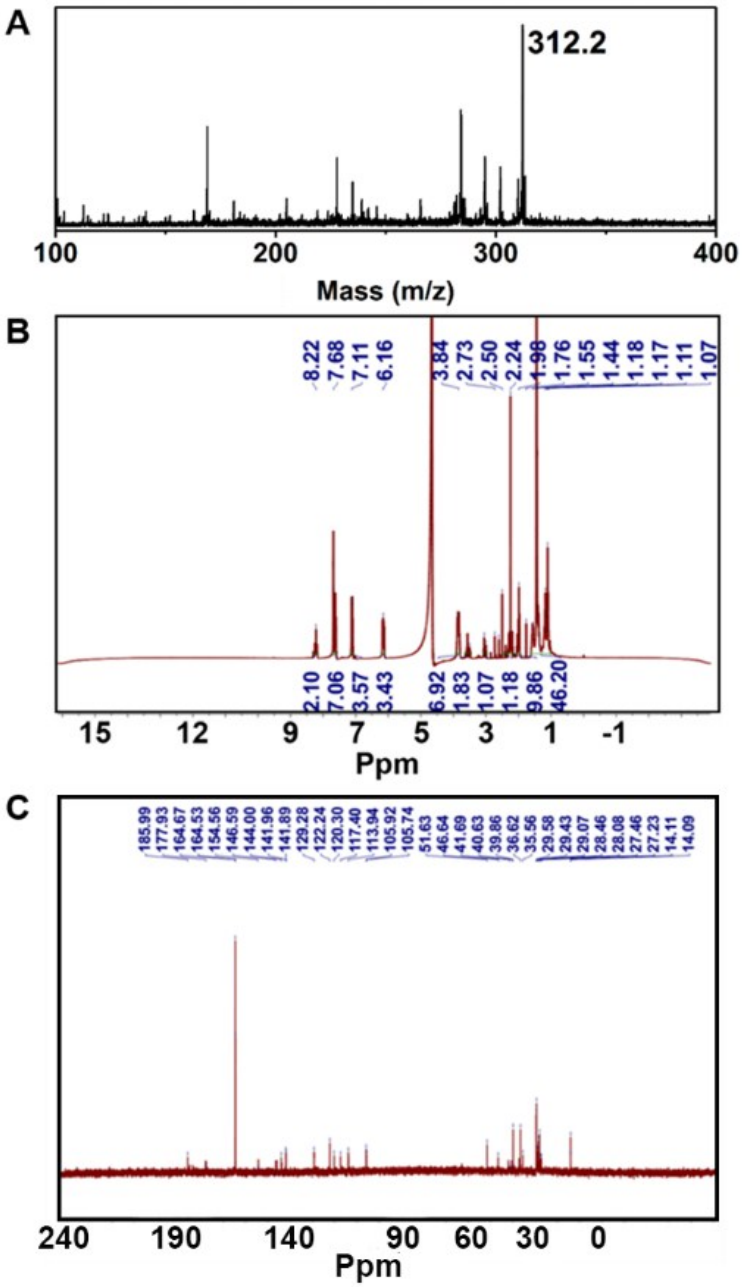

Fig. S6 (A) MALDI-TOF mass, (B) ^{1}H NMR and (C) ^{13}C NMR spectra of **Cy3-pTag**.

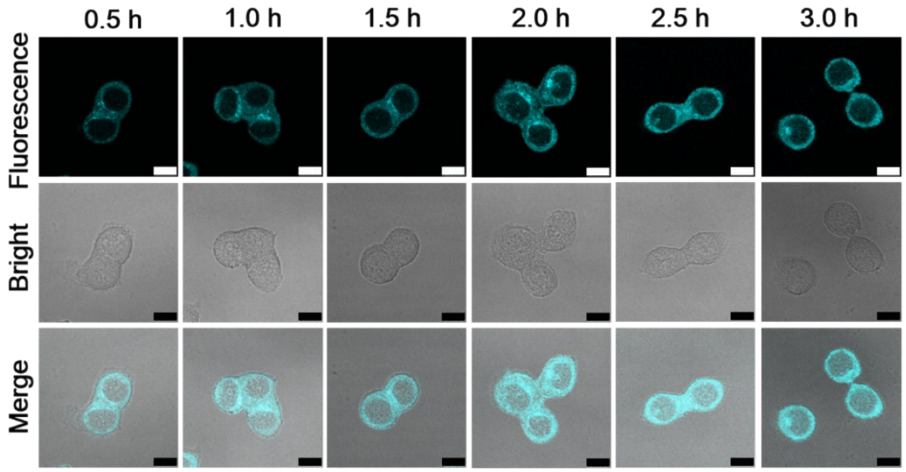

Fig. S7 Confocal fluorescence images of fixed T47D cells after incubated with **Cy3-pTag** (100 μM, 20 μL) for different time at $\lambda_{ex/em}$ of 514/562–600 nm. Scale bars: 10 μm.

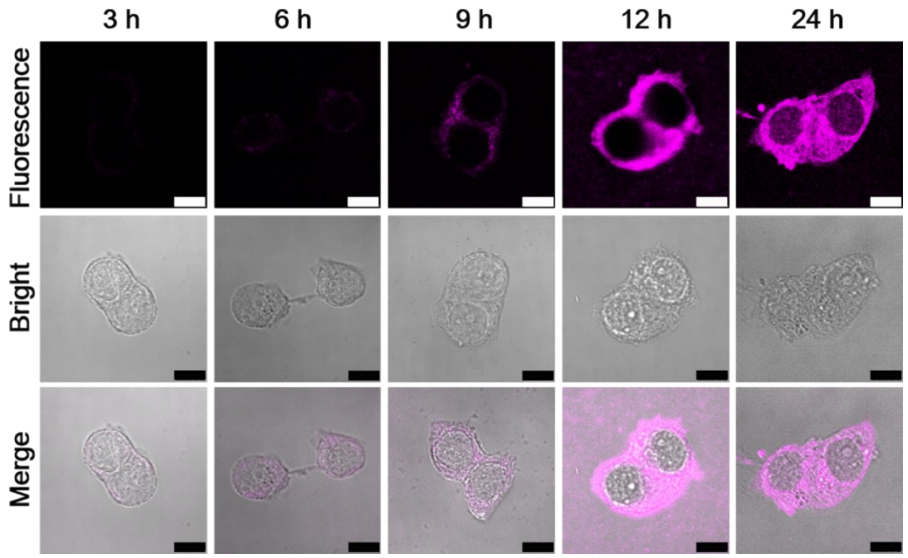

Fig. S8 Confocal fluorescence images of fixed T47D cells incubated with **Cy5.5-UbA** at the dilution ratio of 1:400 for different time at $\lambda_{ex/em}$ of 633/672–720 nm. Scale bars: 10 μm.

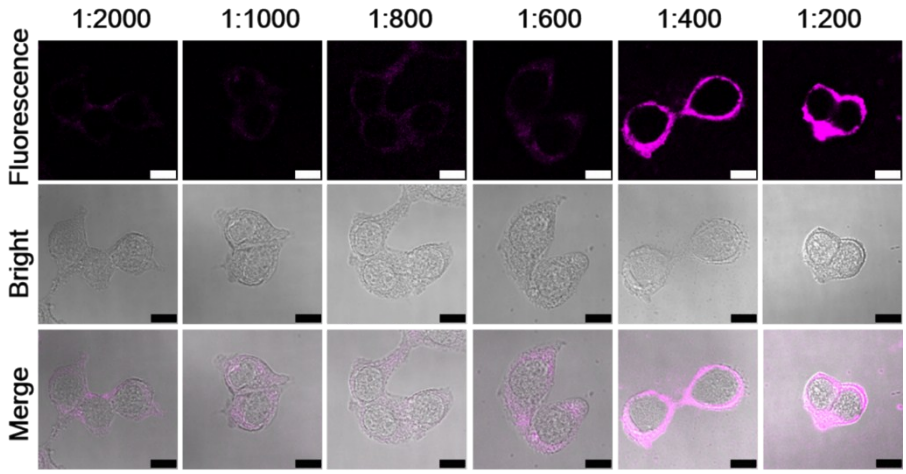

Fig. S9 Confocal fluorescence images of fixed T47D cells after incubated with **Cy5.5-UbA** at different dilution ratios by PBS for 12 h at $\lambda_{ex/em}$ of 633/672–720 nm. Scale bars: 10 μm.

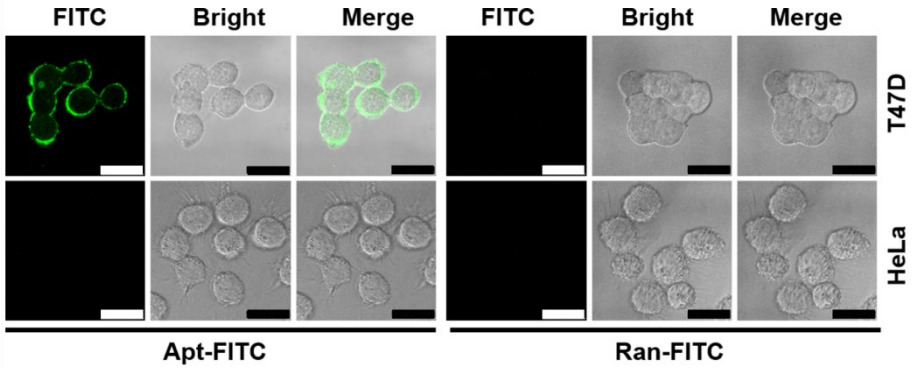

Fig. S10 Confocal images of T47D and HeLa cells after incubation with Apt-FITC or Ran-FITC individually. From left to right: FITC fluorescence excited at 405 nm, bright field and merge. Scale bars: 25 μm.

Supporting references

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