Supporting Information

In vivo monitoring of the ubiquitination of newly synthesized proteins in living cells by combining a click reaction with fluorescence cross-correlation spectroscopy (FCCS)

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Western Blot analysis

Cells were collected after differential adherence selection and washed by PBS, then lysed by 1× glo-lysis buffer (American Type Culture Collection, USA) with protease inhibitor cocktail (Roche, Switzerland). The concentration of sample was measured by BCA Protein Assay Kit (Pierce, USA). The 25ug proteins were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis system (SDS-PAGE) and transferred to PVDF membranes (Millipore, USA). Membranes were blocked in 5% BSA/TBST for 2 h at room temperature. Subsequently, membranes were incubated in the primary antibodies overnight at 4 °C and HRP-conjugated secondary antibodies 2 h at room temperature with gently shake. The information of antibodies was listed in Supplementary Table 2. The results were visualized using ECL start Western Blotting Substrate (GE Healthcare Life Sciences, USA) and analyzed by Image J (National Institutes of Health, USA).

FCS and FCCS System

FCS system is a home-made system which is based on an inverted microscope (IX71, Olympus Optical Co., Japan) using two semiconductor lasers (488 nm and 561 nm, Coherent, USA) as excitation sources. In the FCS/FCCS measurements, the power of laser was about 10 μW for living cell detection to avoid any damage of cell. Two laser beams were coupled with an optical fiber, then expanded and reflected into the objective (UplanApo, 60 × NA 1.2, Olympus, Japan) by a dichroic mirror (ZT405/488/561/640, Chroma, USA). After excitation of fluorescent proteins, the emission fluorescence was split into two detection by a dichroic mirror (540DRLP, Omega Optical, USA). Then fluorescent signals pass through two different emission filters (530DF30/625QM50) and detected by two single photon modules (SPCM-AQR16, PerkinElmer EG&G, Canada). Then collected fluorescence signals were correlated with a digital correlator (Correlator. com, USA). The confocal fluorescence scanning images of living cells were obtained by using the piezoelectric nano-position system (P-733.2CL/P-721.CLQ, Physik Instrumente, Germany) and controller (E-712.3CD, Physik Instrumente, Germany). The detection volume of FCS system is measured using RB (Rhodamine B, its diffusion coefficient: 4.2×10^{-10} m^2/s in water) and RG (Rhodamine Green, its diffusion coefficient: 2.8×10^{-10} m^2/s in water) as standard substance. The detection volume is calibrated before measurement[1,2].

Synthesis of Bodipy-TR-Tz and Puro-TCO

Bodipy-TR-Tz (compound **3**) synthetic route was shown in Fig. S7.

Compound **2**: To a mixture of 4-(aminomethyl)benzonitrile hydrochloride (2.1 g, 12.5 mmol), formamidine acetate (5.2 g, 50 mmol), and elemental sulfur (powder, 400 mg, 12.5 mmol) in a round-bottom flask, anhydrous hydrazine (85%, 7.5 mL) were carefully added, and the mixture was stirred for 20 h at room temperature. Then, 1% aqueous HCl (125 mL) was slowly added to the mixture, stirred for 10 min, and the precipitate was removed by filtration. The resulting red-orange solution was cooled to 0 ˚C, then $NaNO_2$ (4.3 g, 60 mmol in 37.5 mL water) carefully added to the solution, followed by the addition of 1% aqueous HCl (adjust pH ≈ 3.0). After the mixture was stirred for 3 h at room temperature, the solution was concentrated in vacuo, the residue was purified by silica gel column chromatography to give compound **2**. Pink solid, 467.5 mg, 20% yield; ^1H NMR (400 MHz; MeOD) δ = 10.38 (s, 1H), 8.67 (d, J = 8.4 Hz, 2H), 7.74 (d, J = 8.4 Hz, 2H), 4.23 (s, 2H); MS (ESI) *m/z*: Calcd. for $C_9H_{10}N_5$ $[M+H]^+$ 188.09; found: 188.13.

Compound **3**: compound **2** (0.4 mg, 2.2 μmol) was suspended in 40 μL DMF under N_2, 1 μL

TEA and Bodipy-TR-NHS (1mg, 2.0 μmol) in 15 μL DMF were added. After 1 h stirring at room temperature, the reaction was quenched with 6.7 μL NH_2OH (1 mol/L) and the product was purified by column chromatography. Purple solid, 0.3 mg, 30% yield; MS (ESI) *m/z*: Calcd. for $C_{30}H_{22}BFN_7O_2S$ $[M-F]^+$ 574.1633; found: 574.1628.

Puro-TCO (compound **10**) was synthesized according previous literature[3](Fig. S8). White solid, 0.5 mg, 1.5% overall yield from compound **4**; MS (ESI) *m/z*: Calcd. for $C_{31}H_{43}N_8O_6$ $[M+H]^+$ 623.3306; found: 623.3313.

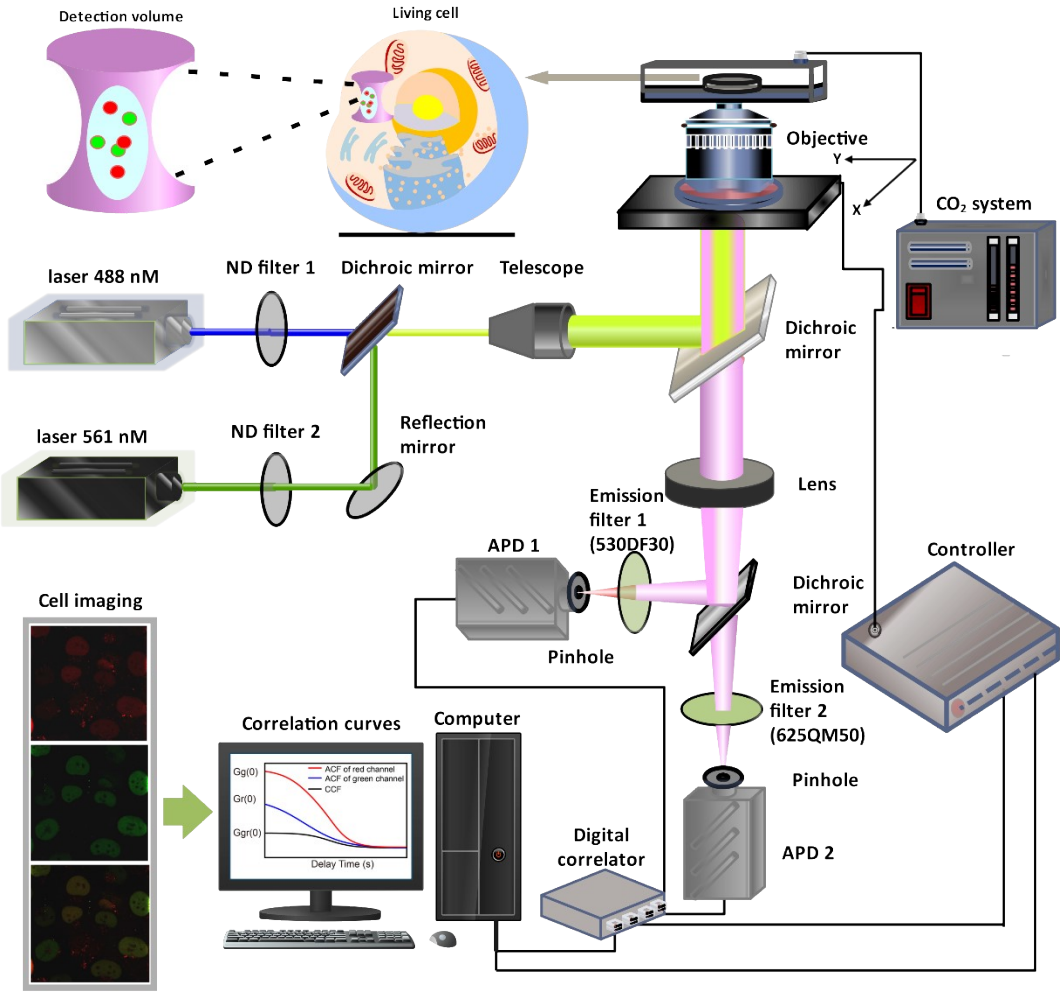

Figure S1. The schematic diagram of FCCS setup.

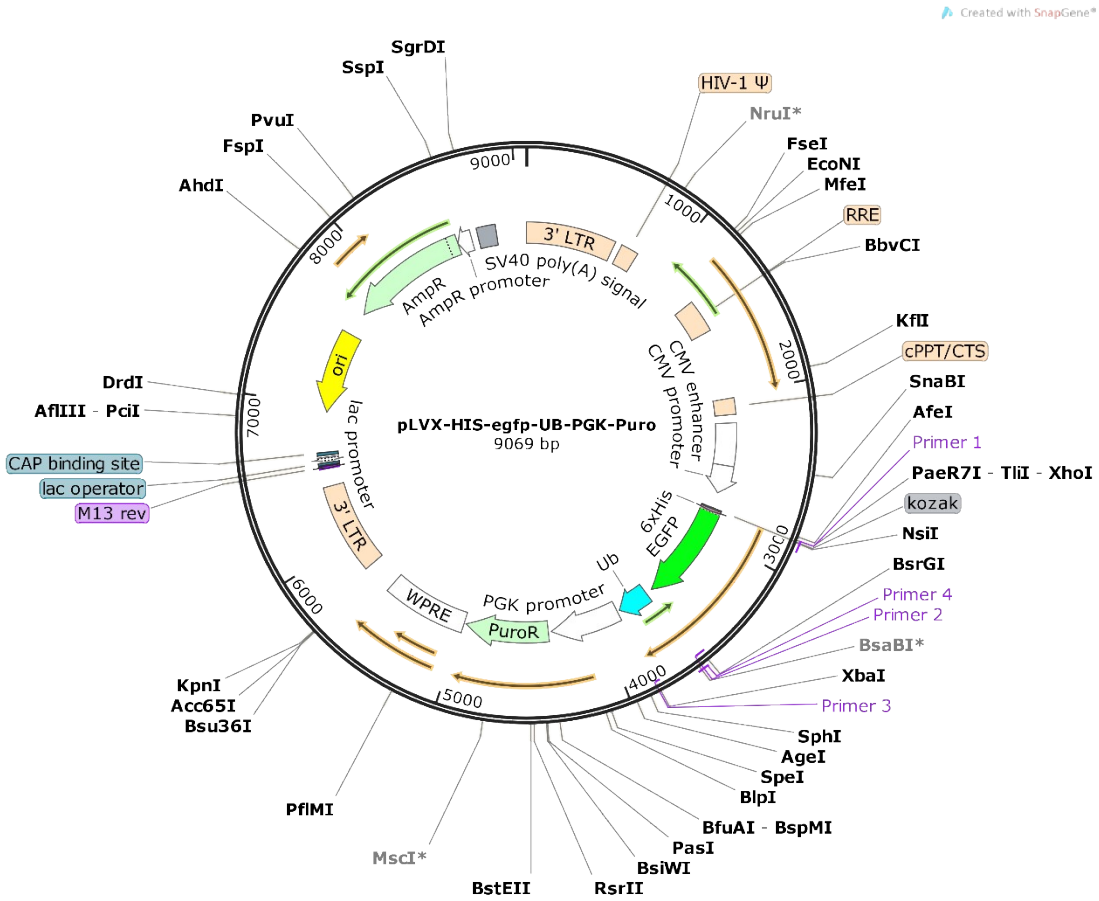

Figure S2. Plasmid structure of EGFP-Ub.

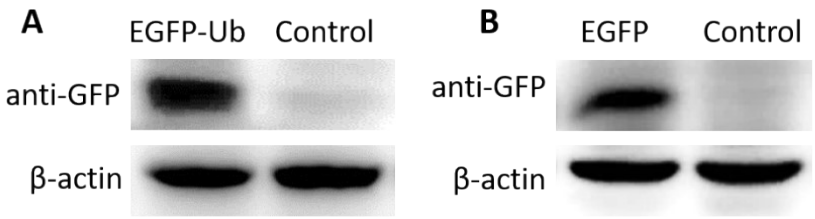

Figure S3. Western blotting images of EGFP-Ub and EGFP. H1299 cell stably expressing EGFP-Ub and EGFP (tetracycline-inducible vector was incubated by Doxorubicin)[4] is lysed. EGFP-Ub (A) and EGFP (B) are detected by anti-GFP. Wild type H1299 cell is used as a negative control.

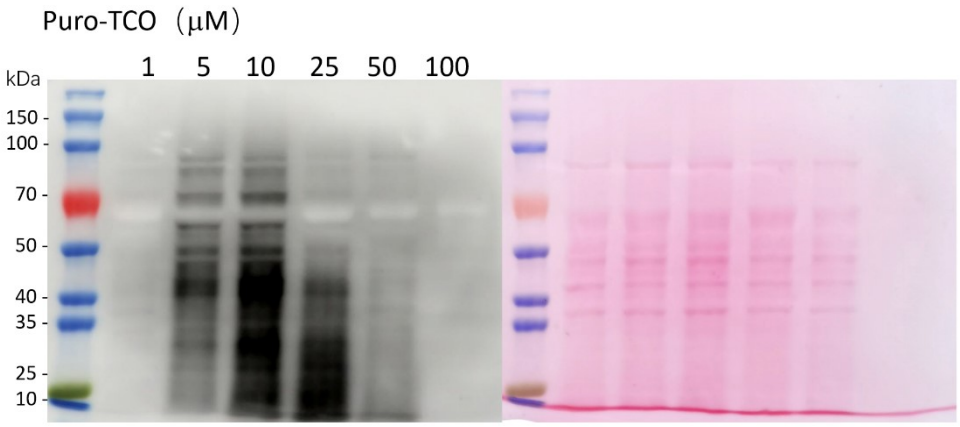

Figure S4. Western blotting images under different concentration of Puro-TCO. Newly synthesized proteins were detected by anti-puro. The same PVDF membrane was also stained with ponceau staining solution to demonstrate equal protein loading.

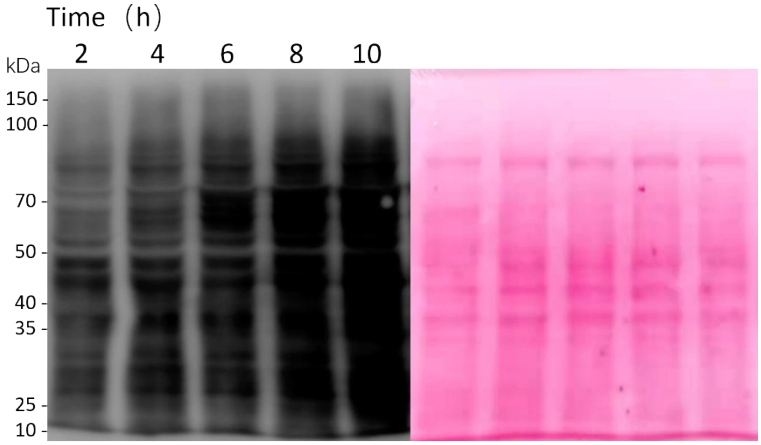

Figure S5. Western blotting images under different incubation time of Puro-TCO. Newly synthesized proteins were detected by anti-puro. The same PVDF membrane was also stained with ponceau staining solution to demonstrate equal protein loading.

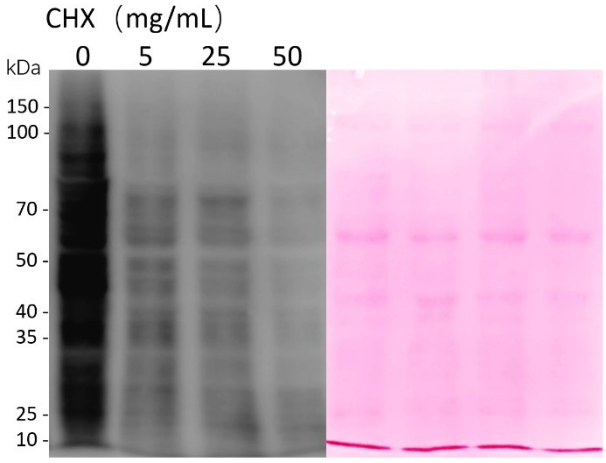

Figure S6. Western blotting images under different concentration of CHX. Newly synthesized

proteins were detected by anti-puro. The same PVDF membrane was also stained with ponceau staining solution to demonstrate equal protein loading.

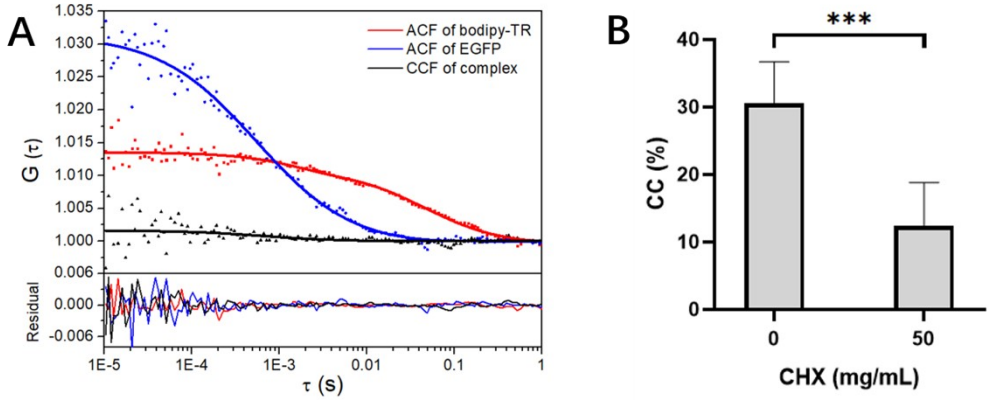

Figure S7. Study the effect of ribosome translocation inhibitor CHX by FCCS. (A) Typical autocorrelation and cross-correlation curves in single living cells and curves of fitted residuals of the experimental group in the presence of 50 mg/mL CHX. (B) Comparison of the CC value of experimental group with CHX and the control experiment without CHX. Data represent means ± the standard deviation. The cell number is about 20. Student's t test was used to represent the differences of statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001.

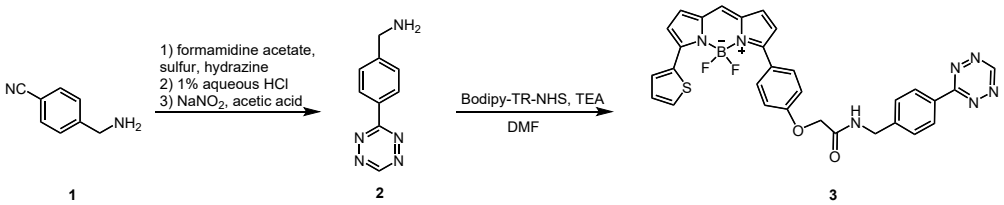

Figure S8. Synthetic route of Bodipy-TR-Tz.

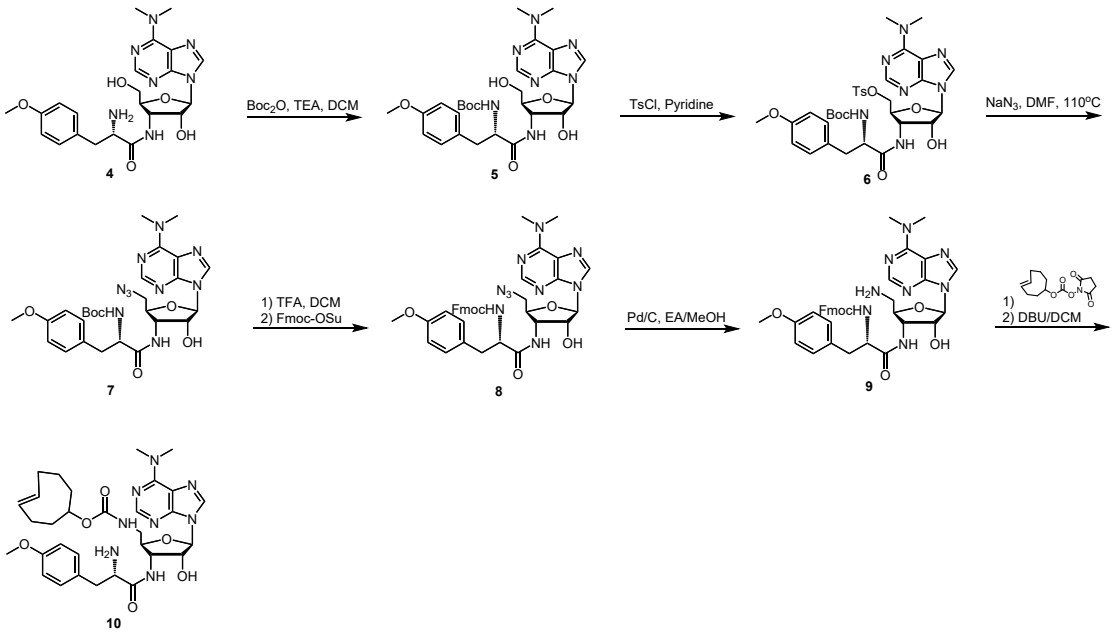

Figure S9. Synthetic route of Puro-TCO.

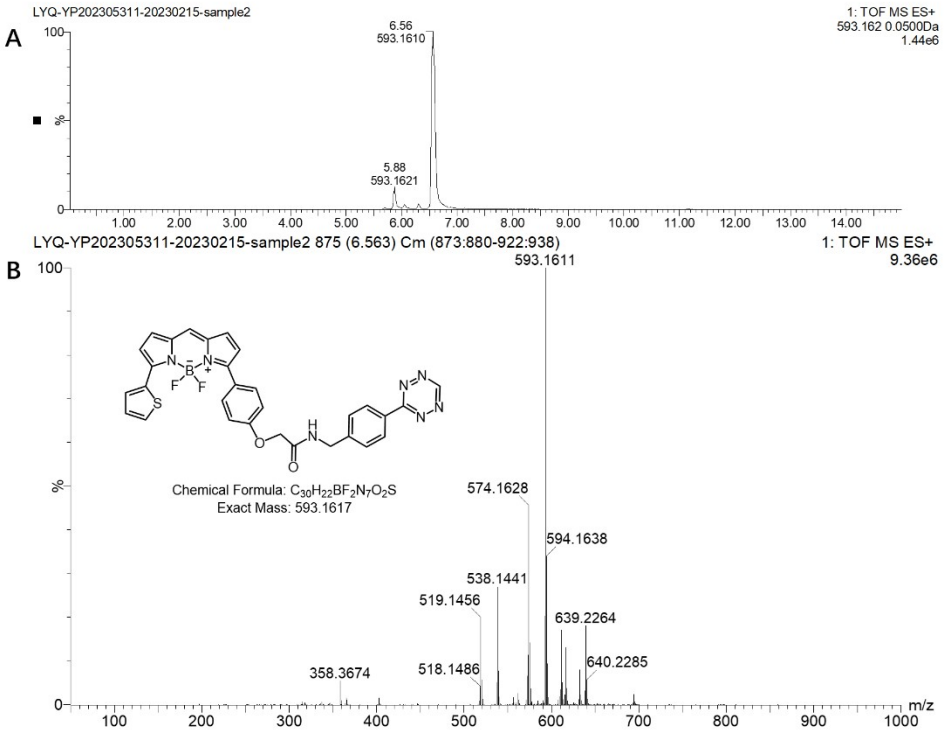

Figure S10. UPLC-MS of Bodipy-TR-Tz. (A) UPLC of Bodipy-TR-Tz with C18 column. (B) High resolution mass spectrum of Bodipy-TR-Tz.

Reference

(1) Su, D.; Hu, X.; Dong, C.; Ren, J. *Anal. Chem.* **2017**, *89*, 9788-9796.

(2) Yu, S.; Li, F.; Huang, X.; Dong, C.; Ren, J. *Anal. Chem.* **2020**, *92*, 2988-2996.

(3) Ge, J.; Zhang, C. W.; Ng, X. W.; Peng, B.; Pan, S.; Du, S.; Wang, D.; Li, L.; Lim, K. L.; Wohland, T.; Yao, S. Q. *Angew Chem Int Ed Engl* **2016**, *55*, 4933-4937.

(4) Du, Z.; Dong, C.; Ren, J. *Methods Appl Fluoresc* **2017**, *5*, 024008.