Supporting Information

Imaging Specific Proteins in Living Cells with Small Unnatural Amino Acid Attached Raman Reporters

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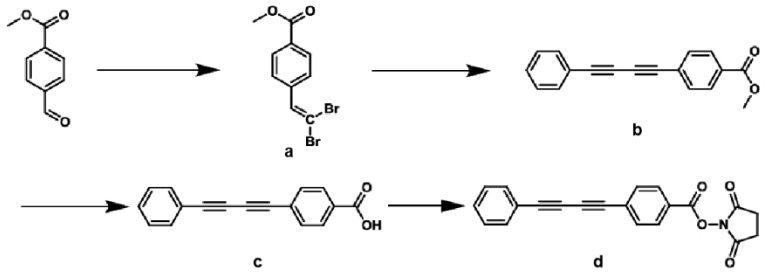
Methods

Reagents: BCNK and 2'-aTCOK were purchased from Sirius Fine Chemicals SiChem GmbH. N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-4-(phenylbuta-1,3-diyn-1-yl)benzamide (**1**) shown in **Fig. 1a** was home made. Phosphate Buffered Saline (PBS, pH 7.4; Gibco, 8117069), dulbecco's modified eagle's medium (DMEM; Gibco, 8117156) and fetal bovine serum (FBS; Gibco, 10270) were purchased from Thermo Fisher Scientific, Inc.

(1) Synthesis of Lyso-BADY



Scheme S1. Synthesis of Lyso-BADY. Reagents and conditions: (**a**) CBr_4, Ph_3P, DCM. (**b**) Ethynylbenzene, Ph_3P, Tris(dibenzylideneacetone)dipalladium, Et_3N, DMF. (**c**) NaOH, H_2O, THF, MeOH. (**d**) DSC, DMAP, CH_3CN.

Synthesis of methyl 4-(2, 2-dibromovinyl) benzoate (a)

To a cooled solution (0 ℃) of carbon tetrabromide (9.83 g, 30 mmol) in DCM (50 mL), triphenylphosphine (15.73 g, 60 mmol) was added slowly. After stirring for 20 minutes at 0 ℃, methyl 4-formylbenzoate (4.92 g, 30 mmol) in DCM (50 mL) was added in slowly. The mixture was stirred at room temperature for 90 minutes. Then the mixture was poured into ethyl acetate (600 mL), and washed with brine (3 × 300 mL). The organic layer was dried over anhydrous Na_2SO_4. After concentration in vacuo, the residue was purified by silica gel column chromatography, eluting with PE: EA (20:1) to give **a** as a white solid (8 g, 25.2 mmol, yield 84 %).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, J = 8.4 Hz, 2H), 7.59 (d, *J* = 8.3 Hz, 2H), 7.51 (s, 1H), 3.92 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.55, 139.57, 136.04, 129.84, 129.68, 128.35, 91.99, 52.29. ESI-MS Calcd. for $C_{10}H_9Br_2O_2$ $[M+H]^+$: 318.9, found: 318.9.

Synthesis of methyl 4-(phenylbuta-1, 3-diyn-1-yl) benzoate (b)

To a solution of methyl 4-(2,2-dibromovinyl)benzoate (**a**) (6.4 g, 20.19 mmol), phenylacetylene (3.1 g, 30.29 mmol), triphenylphosphine (212 mg, 0.81 mmol) and TEA (8.3 mL, 60.57 mmol) in dry DMF (30 mL), tris(dibenzylideneacetone)dipalladium (180 mg, 0.2 mmol) in dry DMF (4 mL) was added. The whole mixture was stirred at 85 ℃ for 4 hours under argon atmosphere. The reaction mixture was then cooled to r.t. and diluted with Ethyl Acetate: Petroleum Ether (1:1) (600 mL). The organic layer was washed with 1M hydrochloric acid (2 × 100 mL), 1 M sodium hydroxide (2 × 100 mL), water (2× 100 mL) and brine (2 × 100 mL), subsequently. After drying over anhydrous

Na_2SO_4, the organic layer was concentrated in vacuo, and the residue was purified by silica gel column chromatography, eluting with PE:EA(25:1) to give **b** as a light yellow solid (3.65 g, 14 mmol, yield 69.5 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, J = 8.4 Hz, 2H), 7.58 (d, J = 8.4 Hz, 2H), 7.54 (dt, J = 3.6, 2.1 Hz, 2H), 7.42 – 7.32 (m, 3H), 3.92 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.36, 132.61, 132.42, 130.27, 129.57, 129.54, 128.53, 126.50, 121.45, 83.05, 80.49, 77.26, 73.58, 52.38. ESI-MS Calcd. for $C_{18}H_{13}O_2$ $[M+H]^+$: 261.1, found: 261.1.

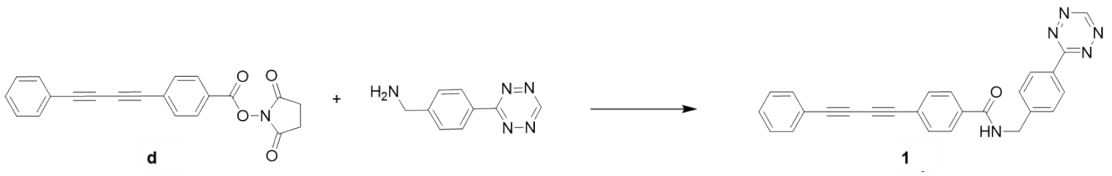
Synthesis of 4-(phenylbuta-1, 3-diyn-1-yl) benzoic acid (c)

To a solution of methyl 4-(phenylbuta-1, 3-diyn-1-yl) benzoate (**c**) (782 mg, 3.0 mmol) in THF (10 mL) and MeOH (10 mL), sodium hydroxide (240 mg, 6.0 mmol) in H_2O (10 mL) was added. The reaction mixture was stirred for 1 hour at room temperature. The mixture was then poured into water (100 mL) and pH of the solution was adjusted below **c** with HCl aqueous solution. The acidic solution was extracted with ethyl acetate (3 × 60 mL). The combined organic layer was dried over Na_2SO_4 and concentrated in vacuo to give **c** as a light yellow solid (676 mg, 2.75 mmol, yield 92 %). ^{1}H NMR (400 MHz, DMSO-d6) δ 13.31 (br, 1H), 7.97 (d, J = 8.4 Hz, 2H), 7.73 (d, J = 8.4 Hz, 2H), 7.66 – 7.62 (m, 2H), 7.54 – 7.43 (m, 3H); ^{13}C NMR (101 MHz, DMSO-d6) δ 166.99, 133.08, 132.99, 132.12, 130.75, 130.05, 129.45, 125.13, 120.59, 83.55, 81.35, 76.26, 73.66. HR-ESI-MS Calcd. for $C_{17}H_9O_2$ $[M-H]^-$: 245.0608, found: 245.0608.

Synthesis of 2, 5-dioxopyrrolidin-1-yl 4-(phenylbuta-1, 3-diyn-1-yl) benzoate (d)

To a solution of 4-(phenylbuta-1, 3-diyn-1-yl) benzoic acid (**c**) (1 g, 4.05 mmol) in MeCN (30 mL), DSC (1.56 g, 6.09 mmol) and DMAP (200 mg, 1.63 mmol) was added. The reaction mixture was stirred for 10 hours at room temperature and diluted with 200 mL EA after the reaction was over. The mixture was then washed with 1% Sodium bisulfite solution (100 mL ×3) and brine (100 mL ×3) subsequently. After drying over anhydrous Na_2SO_4, the organic layer was concentrated in vacuo, and the residue was purified by silica gel column chromatography, eluting with DCM:MeOH (500:1) to give **d** as a light yellow solid (1.3 g, 3.8 mmol, yield 95 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (d, J = 8.1 Hz, 2 H), 7.87 (d, J = 8.1 Hz, 2H), 7.65 (d, J = 7.2 Hz, 2H), 7.55-7.43 (m, 3H), 2.91 (s, 4H); ^{13}C NMR (101 MHz, DMSO-d6) δ 170.71, 161.61, 133.80, 133.06, 130.92, 130.75, 129.47, 127.87, 125.39, 120.39, 84.49, 80.68, 77.89, 73.46, 26.03. ESI-MS Calcd. for $C_{21}H_{14}NO_4$ $[M+H]^+$: 344.1, found: 344.1.

(2) Synthesis of compound 1: N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-4-(phenylbuta-1,3-diyn-1-yl) benzamide



Scheme S2. Synthesis of compound **1**. Reagents and conditions: 2, 5-dioxopyrrolidin-1-yl 4-(phenylbuta-1, 3-diyn-1-yl) benzoate, DMF,tetrazine-amine,N,N-Diisopropylethylamine.

To a solution of 2, 5-dioxopyrrolidin-1-yl 4-(phenylbuta-1, 3-diyn-1-yl) benzoate[1] (500 mg, 1.46 mmol) in DMF (10 ml), tetrazine-amine (300 mg, 1.60 mmol) and N,N-Diisopropylethylamine

(1.01 ml, 5.83 mmol) was added. The reaction mixture was stirred for 2 hours at room temperature. After removing solvent *in vacuo*, the residue was purified by silica gel column chromatography, eluting with DCM:Methanol (10 :1) to give **1** as a light pink solid (551 mg, 1.33 mmol, yield 91%). ^{1}H NMR (600 MHz, DMF-d_7) δ 9.54 (t, J = 6.9 Hz, 1H), 8.75 (dd, J = 7.9, 2.6 Hz, 2H), 8.31 (dt, J = 10.7, 5.4 Hz, 2H), 8.25 – 8.20 (m, 4H), 8.01 (dd, J = 8.0, 2.5 Hz, 2H), 7.93 (dd, J = 8.0, 2.5 Hz, 2H), 7.89 (t, J = 4.7 Hz, 2H), 7.77 – 7.68 (m, 3H), 4.96 (dt, J = 9.6, 4.6 Hz, 2H), 4.27 (qt, J = 9.6, 4.8 Hz, 1H), 3.72 (s, 14H), 3.71 (d, J = 7.2 Hz, 26H), 3.14 – 3.10 (m, 3H), 2.26 – 2.19 (m, 2H), 1.52 – 1.50 (m, 1H), 1.48 (s, 3H), 1.41 (td, J = 7.2, 2.7 Hz, 2H), 1.11 – 1.05 (m, 1H). ^{13}C NMR (151 MHz, DMF-d_7) δ 165.99, 162.13, 158.61, 145.35, 135.66, 132.85, 131.20, 130.39, 129.23, 128.75, 128.20, 128.06, 124.06, 121.02, 82.88, 81.19, 75.33, 73.36, 60.09, 43.27, 34.40, 20.41, 13.85. HR-ESI-MS Calcd. for $C_{26}H_{18}N_5O$ $[M+H]^+$: 416.1506, found: 416.1506

[1] C. Ding, Y. G. Chen, H. Z. Li, B. Y. Wang, Q. Wei, H. J. Tang, S. K. Jia, Z. Y. He, P. Wang, X. Zhou, *Chinese Chem Lett* **2019**, *30*, 1393-1396.

Plasmids construction: MmPylRS-AF (Y306A, Y384F double mutant of wild-type PylRS) was synthesized by Wuhan Tianyi Huiyuan Biotechnology Co., Ltd. and further cloned into the pCMV-FLAG-IRES2-mCherry plasmid with XbaI and BamHI. 4×U6-PlyT/Htt74Q-EGFP-N150TAG plasmid was constructed in our laboratory previously. EGFP-N150TAG-Histone 3.3 plasmid was cloned into the pCMV-Htt74Q-EGFP-N150TAG-6×His plasmid with BsrGI and BamHI in place of Htt74Q-EGFP-N150TAG. Vimentin fragment was also cloned with XbaI and MfeI to replace the Htt74Q fragment. TAG codons were introduced into the Htt74Q plasmid at N-terminal and Vimentin plasmid at N116 position by site-directed mutagenesis. 4×U6-PlyT/TAG-Htt74Q, 4×U6-PlyT/Histone 3.3-K64TAG and 4×U6-PlyT/Vimentin-N116TAG were generated by introducing the TAG-Htt74Q, Histone 3.3-K64TAG and Vimentin-N116TAG plasmids into the pCMV-EGFP-3×HA plasmid, respectively. Site-directed mutagenesis was performed with QuikChange II Site-Directed Mutagenesis Kit with primers designed on the website http://bioinformatics.org/primerx/.

Mammalian cell culture and transfection: HeLa cells were cultured at 37 ℃ in a 5% CO_2 atmosphere in DMEM supplemented with 10% (v/v) FBS, and were subcultured every 3-4 days. Neofect™ DNA transfection reagent (Neofect™ biotech Co., Ltd.) was used by 1:1 (Neofect™: total amount of DNA) for transient transfection, following the manufacturer's protocol. Cells were transfected in the cell culture dishes (glass bottom φ20 mm, Cellvis) at a density of 5×10^4 per well with the Mm-PylRS-AF/Pyl-$tRNA_{CUA}$ plasmid (0.5 μg) and the plasmid of interest (0.5 μg). Transfection of both plasmids was performed using equal amount. The medium was replaced by fresh medium, when necessary, after overnight incubation with BCNK or 2'-aTCOK (1 mM). Cells were used for experiments at least 24 h after transfection. Compound **1** (5 μM) was added for incubation 30 minutes at 37 ℃ before imaging.

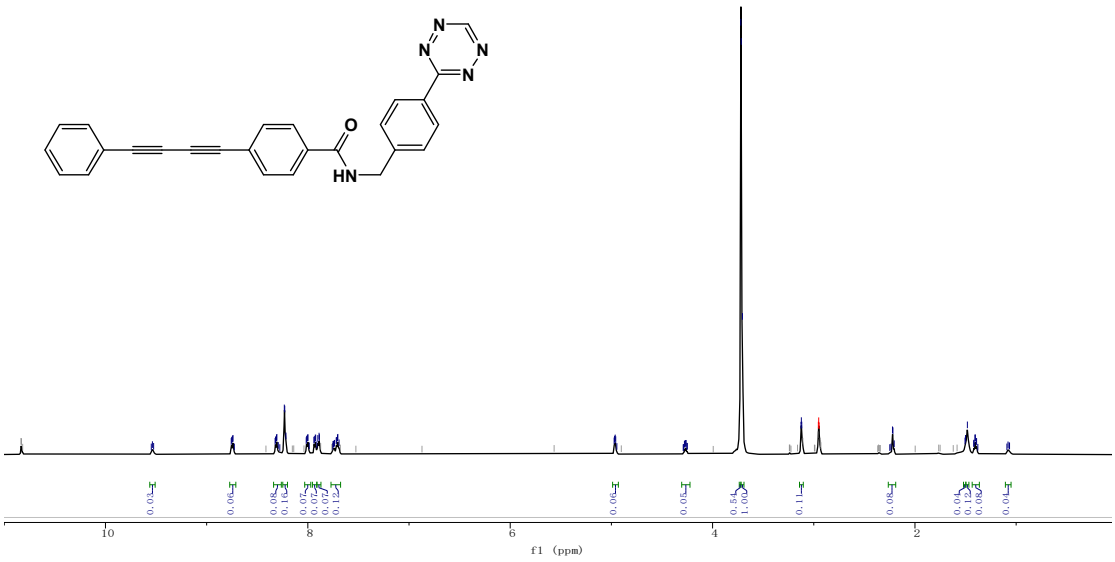
Multiplex stimulated Raman scattering microscope: The dual-output femtosecond laser (InSight DeepSee, Spectra-Physics) provided both pump (680–1300 nm, ~120 fs) and Stokes (1040 nm, ~220 fs) laser beams with a repetition rate of 80 MHz. The Stokes beam was modulated by a resonant electro-optical modulator (EO-AM-RC2, Thorlabs) at 10.55 MHz with about 95% modulation depth. The temporal overlap between pump and Stokes pulse trains was ensured with a

time delay line. The pump beam was spatially overlapped with Stokes beam by a dichroic mirror (DMSP1000L, Thorlabs). Pump and Stokes pulses were linearly chirped to ~3 ps by 64 cm long SF57 glass rod. The multiplex SRS image stacks were obtained by scanning the relative time delay between pump and Stokes pulses. Then the two laser beams were guided into a laser scanning microscope equipped with a two-axis galvanometer (GVS002, Thorlabs). A ×60 water immersion objective (N.A. 1.1, LUMFLN 60XW, Olympus) was used for all cell imaging, and the transmitted pump beam was collected with a 1.4 N.A. oil condenser and detected by a large area Si photodiode (S3994-01, Hamamatsu). Two short-pass filters (ET980SP, Chroma) were installed in front of the photodiode to completely block the Stokes beam. The SRS signal from PD was amplified by a home built 10.5MHz resonant amplifier and demodulated by a digital lock-in amplifier (LIA, HF2LI, Zurich Instrument). Two optical filters were equipped for two photon exited imaging of mCherry (FF02-641/75-25, Semrock) and EGFP (ET525/70m, Chroma), respectively. The integration time for a pixel of 250 nm × 250 nm was 10 μs, the typical acquisition time for each frame with an field of view of 100 μm× 100 μm was around 1.6 s.

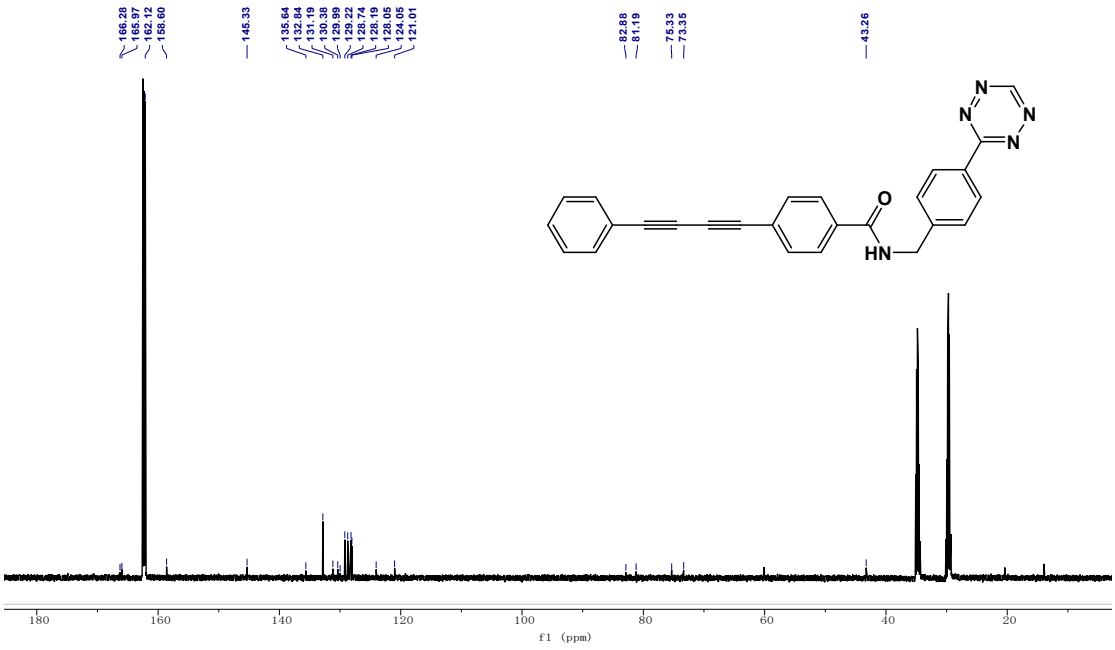
Sample preparation for SRS and TPEF imaging: Cells were transfected in a 24-well plate at a density of 1 × 10^5 per well. After reaching ~80% confluence, cells were added with BCNK/TCOK (5 mM), and then transfected with Neofect™ DNA transfection reagent with 1 μg of two plasmids at a ratio of 1:1. After incubation at 37 °C for 36 h, the transfected cells were washed once with PBS, and cultured with **1** (5 μM) for 30-60 min, and then washed three times over 1h by fresh complete-cell-growth medium to minimize the background signal during SRS imaging.

Multiplex stimulated Raman scattering (SRS) microscopy: In SRS imaging of cells, the pixel settings were 300×300 pixels at 2215 cm^{-1} and 2930 cm^{-1} with a dwell time of 20 μs (**Fig. 2-4**). Images were averaged 20 times to present better contrast. Multiplex SRS images were acquired at 300×300 pixels with a dwell time of 20 μs (**Fig. 2d, Supplementary Fig. 4c**). For TPEF imaging, the pixel settings were 300×300 pixels with a dwell time of 20 μs (**Fig. 2b, Fig. 3b, d, Fig. 4b, d, g, Supplementary Fig. 3a, Supplementary Fig. 4a**).

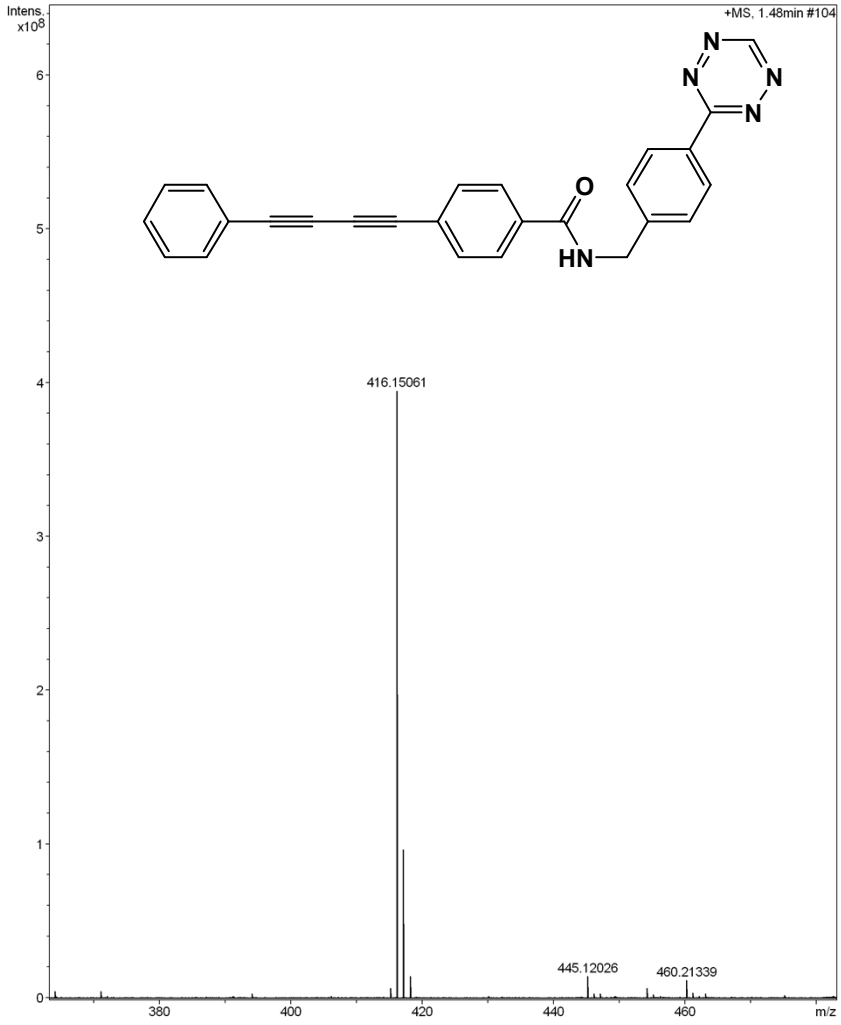
Spontaneous Raman spectroscopy: The spontaneous Raman spectra were acquired by a confocal Raman microscope (LabRAM HR800, Horiba Jobin Yvon). A 785 nm laser was focused on the samples with a 50× objective (N.A. 0.55, Olympus). The integration time for spectra acquisition was about 30 seconds.



Supplementary Fig. 1. ^{1}H NMR spectrum of **1**.

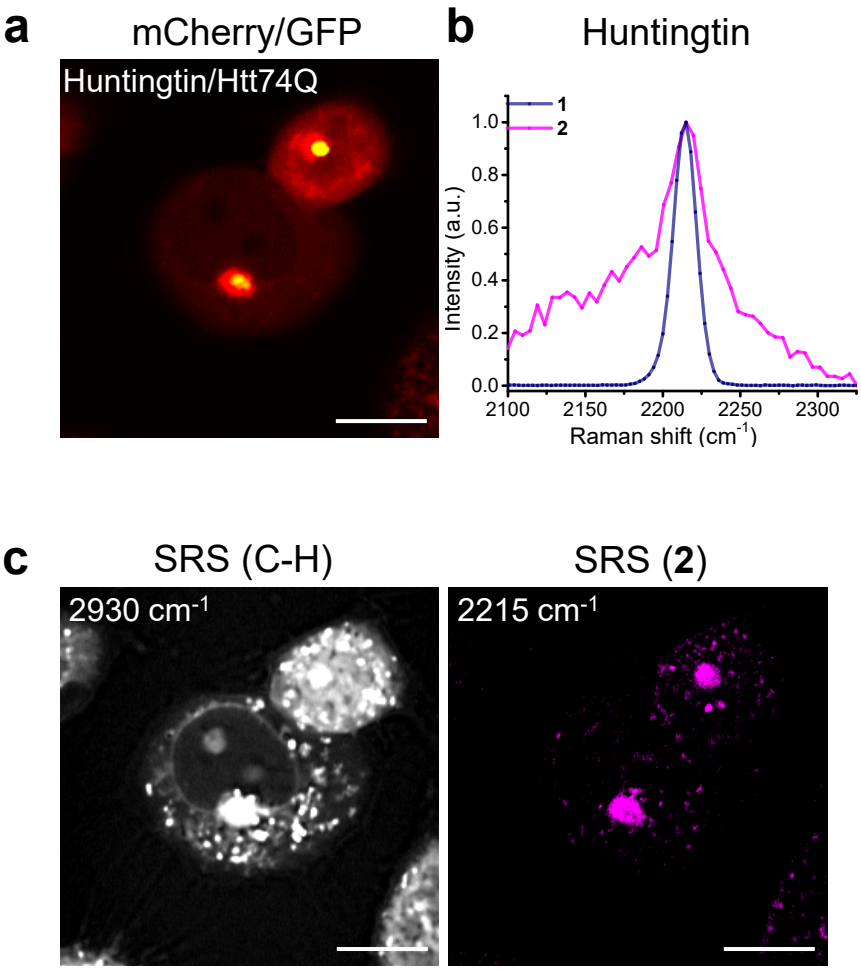


Supplementary Fig. 2. ^{13}C NMR spectrum of **1**.

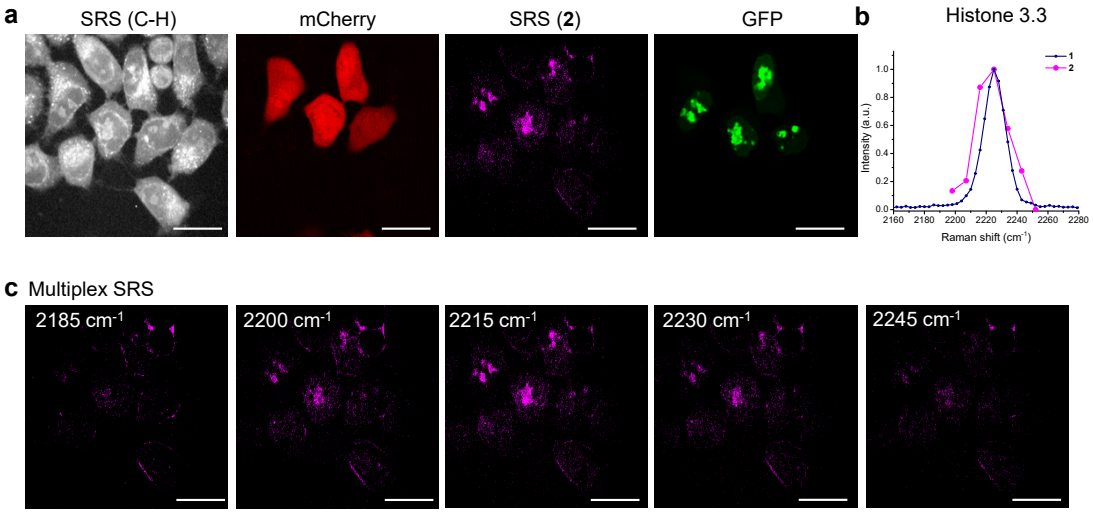


Supplementary Fig. 3. FT-MS spectrum of **1**.

Supplementary Fig. 4. A schematic illustration of the instrumental setup of the SRS imaging system. DM: dichroic mirror; GM: galvanometer; EOM: electro-optic modulator; PD: photodiode; F: optical filters.



Supplementary Fig. 5. Supplementary images of targeted Huntingtin proteins. **a.** The merged TPEF image of mCherry and EGFP (yellow). **b.** SRS spectra of Raman tag **2** targeted to Huntingtin proteins. Pure compound of **1** is shown as reference. **c.** SRS imaging of CH vibrations (2930 cm^{-1}) and Raman reporters (2215 cm^{-1}) targeted Huntingtin proteins in living HeLa cells. Scale bar: 15 μm.



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189 **Supplementary Fig. 6.** Multiplex SRS imaging of Histone 3.3 proteins. **a. SRS** and TPEF
190 imaging of Histone 3.3 proteins. **b.** Multiplex SRS spectra of **2** targeted to Histone 3.3 proteins.
191 **c**. Multiplex SRS image stacks, which show the Raman reporters in EGFP-Histone 3.3 in HeLa
192 cells. Scale bar: 15 μm.

193

Sequences of all plasmids used in our experiments:

4×PlyT/MmBCNRS(Flag)-IRES2-mCherry

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