

1 ***Supporting Information***

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

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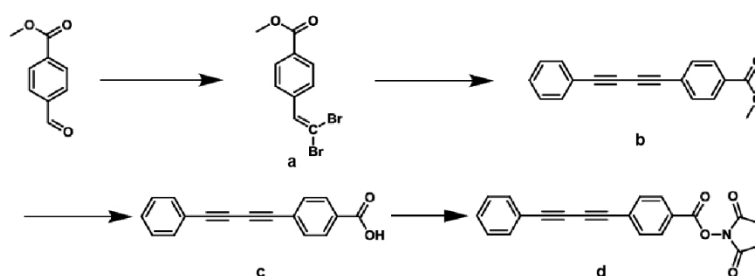
22 *hezy@whu.edu.cn*

## 23 Methods

24 **Reagents:** BCNK and 2'-aTCOK were purchased from Sirius Fine Chemicals SiChem GmbH. N-  
25 (4-(1,2,4,5-tetrazin-3-yl)benzyl)-4-(phenylbuta-1,3-diyne-1-yl)benzamide (**1**) shown in **Fig. 1a** was  
26 home made. Phosphate Buffered Saline (PBS, pH 7.4; Gibco, 8117069), dulbecco's modified  
27 eagle's medium (DMEM; Gibco, 8117156) and fetal bovine serum (FBS; Gibco, 10270) were  
28 purchased from Thermo Fisher Scientific, Inc.

29

### 30 (1) Synthesis of Lyso-BADY



31

32 **Scheme S1.** Synthesis of Lyso-BADY. Reagents and conditions: (a) CBr<sub>4</sub>, Ph<sub>3</sub>P, DCM. (b)  
33 Ethynylbenzene, Ph<sub>3</sub>P, Tris(dibenzylideneacetone)dipalladium, Et<sub>3</sub>N, DMF. (c) NaOH, H<sub>2</sub>O, THF,  
34 MeOH. (d) DSC, DMAP, CH<sub>3</sub>CN.

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

36 To a cooled solution (0 °C) of carbon tetrabromide (9.83 g, 30 mmol) in DCM (50 mL),  
37 triphenylphosphine (15.73 g, 60 mmol) was added slowly. After stirring for 20 minutes at 0 °C,  
38 methyl 4-formylbenzoate (4.92 g, 30 mmol) in DCM (50 mL) was added in slowly. The mixture  
39 was stirred at room temperature for 90 minutes. Then the mixture was poured into ethyl acetate (600  
40 mL), and washed with brine (3 × 300 mL). The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>.  
41 After concentration in vacuo, the residue was purified by silica gel column chromatography, eluting  
42 with PE: EA (20:1) to give **a** as a white solid (8 g, 25.2 mmol, yield 84 %). <sup>1</sup>H NMR (400 MHz,  
43 CDCl<sub>3</sub>) 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); <sup>13</sup>C NMR  
44 (101 MHz, CDCl<sub>3</sub>) δ 166.55, 139.57, 136.04, 129.84, 129.68, 128.35, 91.99, 52.29. ESI-MS Calcd.  
45 for C<sub>10</sub>H<sub>9</sub>Br<sub>2</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 318.9, found: 318.9.

### 46 Synthesis of methyl 4-(phenylbuta-1,3-diyne-1-yl) benzoate (b)

47 To a solution of methyl 4-(2,2-dibromovinyl)benzoate (**a**) (6.4 g, 20.19 mmol), phenylacetylene  
48 (3.1 g, 30.29 mmol), triphenylphosphine (212 mg, 0.81 mmol) and TEA (8.3 mL, 60.57 mmol) in  
49 dry DMF (30 mL), tris(dibenzylideneacetone)dipalladium (180 mg, 0.2 mmol) in dry DMF (4 mL)  
50 was added. The whole mixture was stirred at 85 °C for 4 hours under argon atmosphere. The reaction  
51 mixture was then cooled to r.t. and diluted with Ethyl Acetate: Petroleum Ether (1:1) (600 mL). The  
52 organic layer was washed with 1M hydrochloric acid (2 × 100 mL), 1 M sodium hydroxide (2 × 100  
53 mL), water (2 × 100 mL) and brine (2 × 100 mL), subsequently. After drying over anhydrous

54 Na<sub>2</sub>SO<sub>4</sub>, the organic layer was concentrated in vacuo, and the residue was purified by silica gel  
55 column chromatography, eluting with PE:EA(25:1) to give **b** as a light yellow solid (3.65 g, 14  
56 mmol, yield 69.5 %). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.01 (d, *J* = 8.4 Hz, 2H), 7.58 (d, *J* = 8.4 Hz,  
57 2H), 7.54 (dt, *J* = 3.6, 2.1 Hz, 2H), 7.42 – 7.32 (m, 3H), 3.92 (s, 3H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  
58 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,  
59 73.58, 52.38. ESI-MS Calcd. for C<sub>18</sub>H<sub>13</sub>O<sub>2</sub> [M+H]<sup>+</sup>: 261.1, found: 261.1.

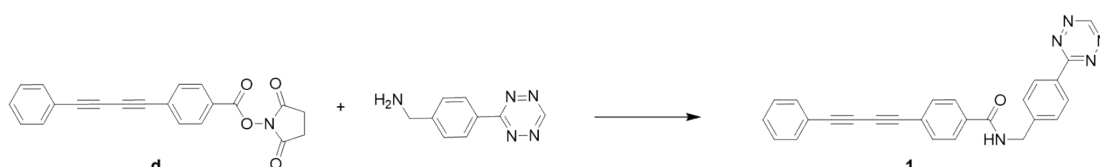
#### 60 Synthesis of 4-(phenylbuta-1, 3-diyn-1-yl) benzoic acid (**c**)

61 To a solution of methyl 4-(phenylbuta-1, 3-diyn-1-yl) benzoate (**c**) (782 mg, 3.0 mmol) in THF (10  
62 mL) and MeOH (10 mL), sodium hydroxide (240 mg, 6.0 mmol) in H<sub>2</sub>O (10 mL) was added. The  
63 reaction mixture was stirred for 1 hour at room temperature. The mixture was then poured into water  
64 (100 mL) and pH of the solution was adjusted below **c** with HCl aqueous solution. The acidic  
65 solution was extracted with ethyl acetate (3 × 60 mL). The combined organic layer was dried over  
66 Na<sub>2</sub>SO<sub>4</sub> and concentrated in vacuo to give **c** as a light yellow solid (676 mg, 2.75 mmol, yield 92  
67 %). <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>) δ 13.31 (br, 1H), 7.97 (d, *J* = 8.4 Hz, 2H), 7.73 (d, *J* = 8.4 Hz,  
68 2H), 7.66 – 7.62 (m, 2H), 7.54 – 7.43 (m, 3H); <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 166.99, 133.08,  
69 132.99, 132.12, 130.75, 130.05, 129.45, 125.13, 120.59, 83.55, 81.35, 76.26, 73.66. HR-ESI-MS  
70 Calcd. for C<sub>17</sub>H<sub>9</sub>O<sub>2</sub> [M-H]<sup>-</sup>: 245.0608, found: 245.0608.

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

72 To a solution of 4-(phenylbuta-1, 3-diyn-1-yl) benzoic acid (**c**) (1 g, 4.05 mmol) in MeCN (30 mL),  
73 DSC (1.56 g, 6.09 mmol) and DMAP (200 mg, 1.63 mmol) was added. The reaction mixture was  
74 stirred for 10 hours at room temperature and diluted with 200 mL EA after the reaction was over.  
75 The mixture was then washed with 1% Sodium bisulfite solution (100 mL ×3) and brine (100 mL  
76 ×3) subsequently. After drying over anhydrous Na<sub>2</sub>SO<sub>4</sub>, the organic layer was concentrated in  
77 vacuo, and the residue was purified by silica gel column chromatography, eluting with DCM:MeOH  
78 (500:1) to give **d** as a light yellow solid (1.3 g, 3.8 mmol, yield 95 %). <sup>1</sup>H NMR (400 MHz, DMSO-  
79 d<sub>6</sub>) 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),  
80 2.91 (s, 4H); <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 170.71, 161.61, 133.80, 133.06, 130.92, 130.75,  
81 129.47, 127.87, 125.39, 120.39, 84.49, 80.68, 77.89, 73.46, 26.03. ESI-MS Calcd. for C<sub>21</sub>H<sub>14</sub>NO<sub>4</sub>  
82 [M+H]<sup>+</sup>: 344.1, found: 344.1.

#### 83 (2) Synthesis of compound 1: N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-4-(phenylbuta-1,3-diyn-1-yl) 84 benzamide



85

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

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

90 (1.01 ml, 5.83 mmol) was added. The reaction mixture was stirred for 2 hours at room temperature.  
91 After removing solvent *in vacuo*, the residue was purified by silica gel column chromatography,  
92 eluting with DCM:Methanol (10 :1) to give **1** as a light pink solid (551 mg, 1.33 mmol, yield 91%).  
93 <sup>1</sup>H NMR (600 MHz, DMF-*d*<sub>7</sub>) δ 9.54 (t, *J* = 6.9 Hz, 1H), 8.75 (dd, *J* = 7.9, 2.6 Hz, 2H), 8.31 (dt, *J*  
94 = 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,  
95 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,  
96 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  
97 – 1.50 (m, 1H), 1.48 (s, 3H), 1.41 (td, *J* = 7.2, 2.7 Hz, 2H), 1.11 – 1.05 (m, 1H). <sup>13</sup>C NMR (151  
98 MHz, DMF-*d*<sub>7</sub>) δ 165.99, 162.13, 158.61, 145.35, 135.66, 132.85, 131.20, 130.39, 129.23, 128.75,  
99 128.20, 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-  
100 ESI-MS Calcd. for C<sub>26</sub>H<sub>18</sub>N<sub>5</sub>O [M+H]<sup>+</sup> : 416.1506, found: 416.1506

101 [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.  
102 Zhou, *Chinese Chem Lett* **2019**, *30*, 1393-1396.

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104 **Plasmids construction:** MmPylRS-AF (Y306A, Y384F double mutant of wild-type PylRS) was  
105 synthesized by Wuhan Tianyi Huiyuan Biotechnology Co., Ltd. and further cloned into the pCMV-  
106 FLAG-IRES2-mCherry plasmid with XbaI and BamHI. 4×U6-PlyT/Htt74Q-EGFP-N150TAG  
107 plasmid was constructed in our laboratory previously. EGFP-N150TAG-Histone 3.3 plasmid was  
108 cloned into the pCMV-Htt74Q-EGFP-N150TAG-6×His plasmid with BsrGI and BamHI in place  
109 of Htt74Q-EGFP-N150TAG. Vimentin fragment was also cloned with XbaI and MfeI to replace the  
110 Htt74Q fragment. TAG codons were introduced into the Htt74Q plasmid at N-terminal and  
111 Vimentin plasmid at N116 position by site-directed mutagenesis. 4×U6-PlyT/TAG-Htt74Q, 4×U6-  
112 PlyT/Histone 3.3-K64TAG and 4×U6-PlyT/Vimentin-N116TAG were generated by introducing the  
113 TAG-Htt74Q, Histone 3.3-K64TAG and Vimentin-N116TAG plasmids into the pCMV-EGFP-  
114 3×HA plasmid, respectively. Site-directed mutagenesis was performed with QuikChange II Site-  
115 Directed Mutagenesis Kit with primers designed on the website <http://bioinformatics.org/primerx/>.

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

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

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

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

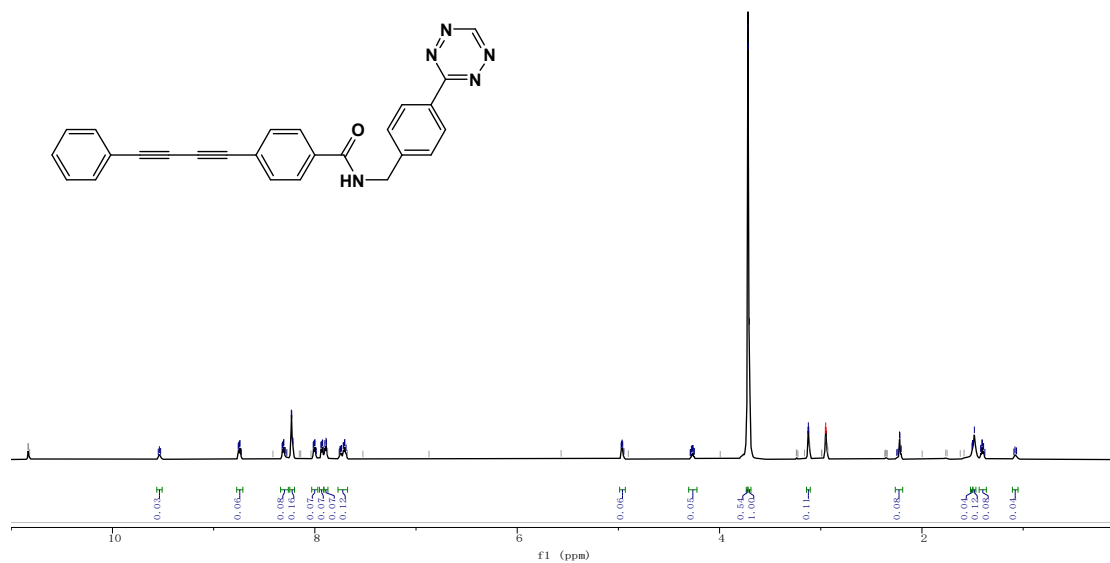
151 ***Multiplex stimulated Raman scattering (SRS) microscopy:*** In SRS imaging of cells, the  
152 pixel settings were 300×300 pixels at 2215 cm<sup>-1</sup> and 2930 cm<sup>-1</sup> with a dwell time of 20 μs (**Fig. 2-**  
153 **4**). Images were averaged 20 times to present better contrast. Multiplex SRS images were acquired  
154 at 300×300 pixels with a dwell time of 20 μs (**Fig. 2d, Supplementary Fig. 4c**). For TPEF imaging,  
155 the pixel settings were 300×300 pixels with a dwell time of 20 μs (**Fig. 2b, Fig. 3b, d, Fig. 4b, d,**  
156 **g, Supplementary Fig. 3a, Supplementary Fig. 4a**).

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

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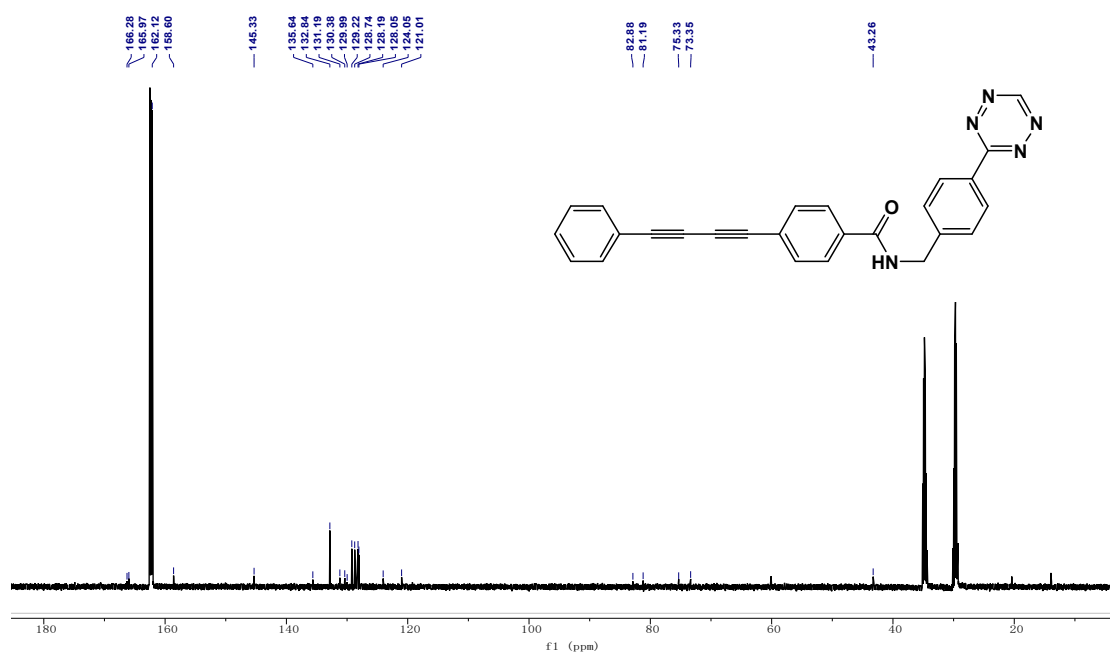
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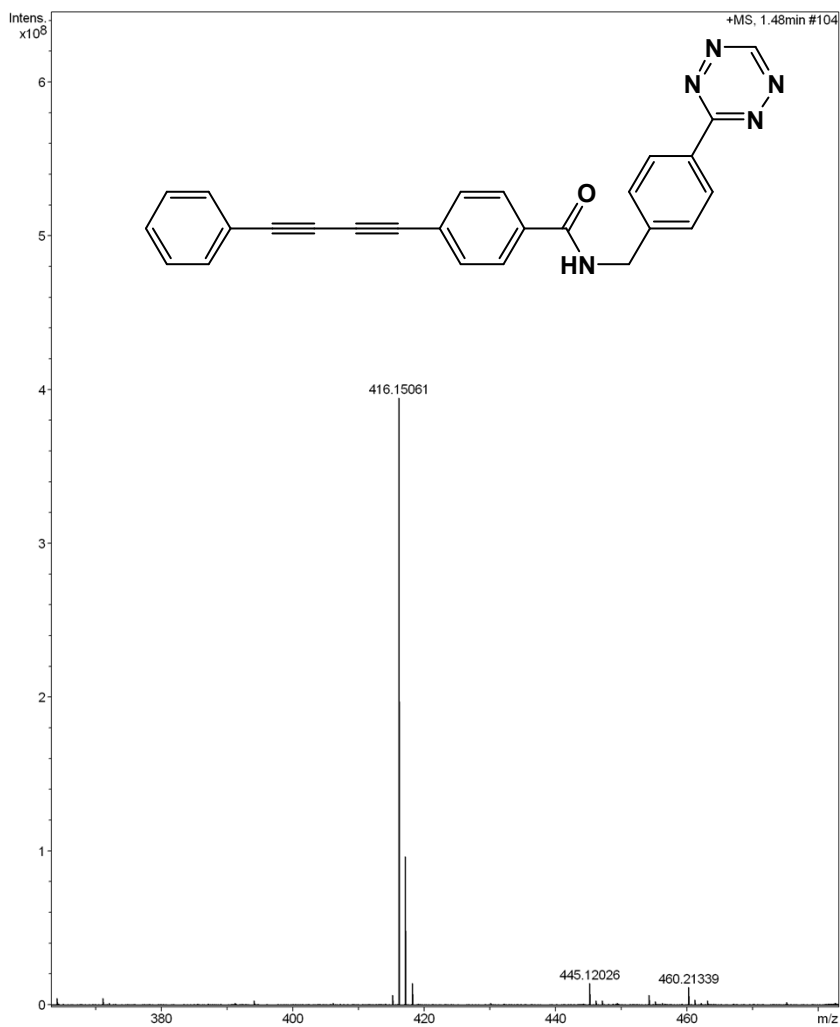
165 **Supplementary Fig. 1.**  $^1\text{H}$  NMR spectrum of **1**.

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168 **Supplementary Fig. 2.**  $^{13}\text{C}$  NMR spectrum of **1**.



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

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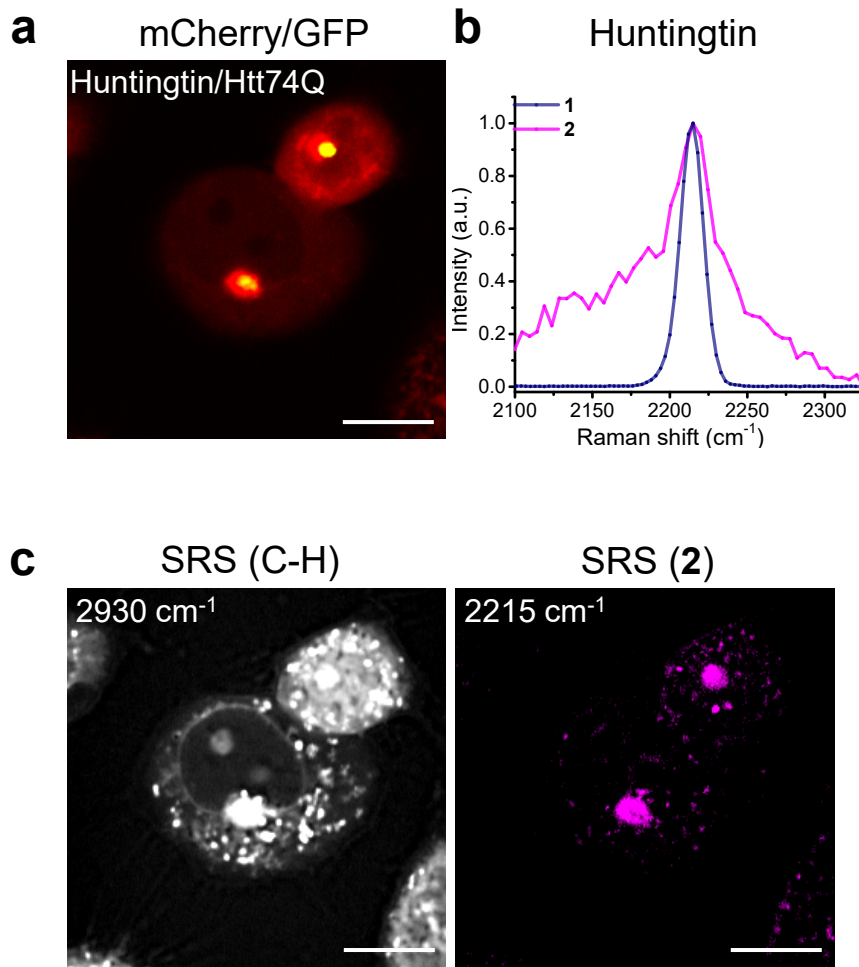
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176 **Supplementary Fig. 4.** A schematic illustration of the instrumental setup of the SRS imaging  
177 system. DM: dichroic mirror; GM: galvanometer; EOM: electro-optic modulator; PD: photodiode;  
178 F: optical filters.

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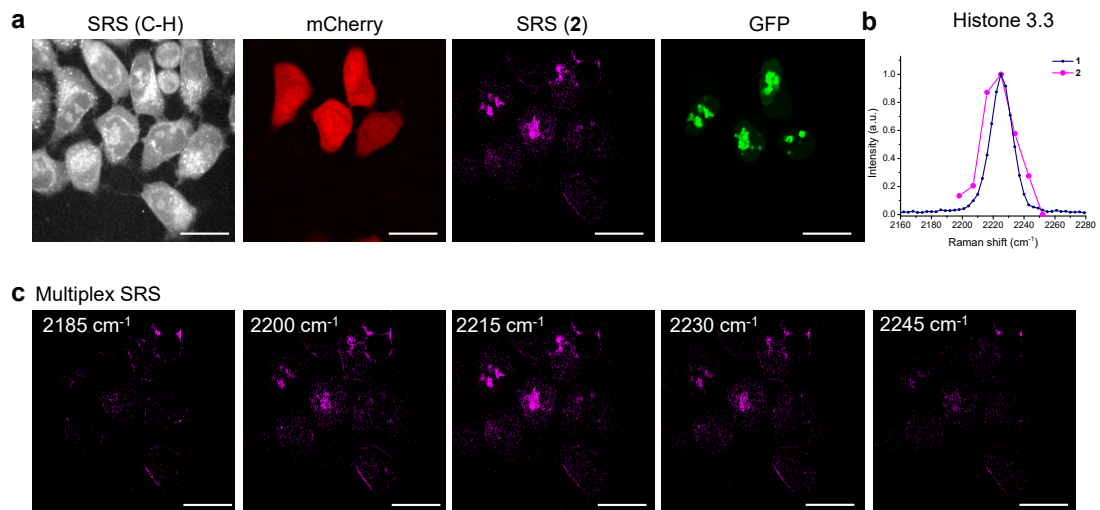


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182 **Supplementary Fig. 5.** Supplementary images of targeted Huntingtin proteins. **a.** The merged  
 183 TPEF image of mCherry and EGFP (yellow). **b.** SRS spectra of Raman tag **2** targeted to  
 184 Huntingtin proteins. Pure compound of **1**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

187





188

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  $\mu\text{m}$ .

193

194 Sequences of all plasmids used in our experiments:

195

196 **4×PlyT/MmBCNRS(Flag)-IRES2-mCherry**

197 ctagaggtagaccgttacataacttacggtaaatggcccgctggctgaccgccaacgacccccgccattgacgtcaatagtaacgcaata  
198 gggactttcattgacgtcaatgggtggagatttacggtaaacgcccacttggcagtagatcaagtgtatcatatgccaagtacgccccatt  
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353

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425

426 **pCMV-Vimentin-eGFP(N150tag)**

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567 **8xHis-Histone3.3(K64tag)**

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629

630 **pCMV-Vimentin(N116tag)**

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