Electronic supplementary information

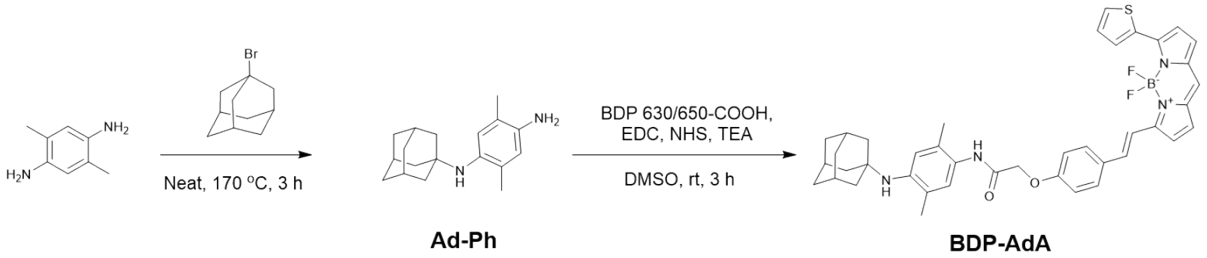
Visualization of lipophagy using a supramolecular FRET pair

Ara Lee, Meng Li, Young Ho Ko, Sanghwang Park, Jongcheol Seo, Kyeng Min Park,* and Kimoon Kim*

Center for Self-assembly and Complexity (CSC), Institute for Basic Science (IBS), Pohang 37673, Republic of Korea. Division of Advanced Materials Science (AMS), Pohang University of Science and Technology (POSTECH), Pohang 37673, Republic of Korea. Department of Chemistry, Pohang University of Science and Technology (POSTECH), Pohang 37673, Republic of Korea, Department of Biochemistry, Daegu Catholic University School of Medicine, Daegu 42471, Republic of Korea.

General information

All the reagents and solvents were commercially available and used as received without further purification. Water purified by a Millipore purification system was used for all experiments. BDP 630/650 carboxylic acid was purchased from Lumiprobe Corporation. Lyso-Tracker and BDP493/503 (LD-tracker) were purchased from Invitrogen. Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone. 1-bromoadamantane was purchased from Sigma Aldrich. 2,5-Dimethyl-1,4-phenylenediamine was purchased from TCI chemicals. HeLa cells were purchased from Korean Cell Line Bank (Seoul, South Korea). GFP-LC3 expressed HeLa cells were kindly supported from Prof. Li Yu research group in Tsinghua University (China). ^{1}H-NMR was recorded using a Bruker Advance III 500 MHz spectrometer and Bruker Ascend 850 MHz spectrometer. High resolution Mass (HR-MS) spectrum was acquired using an Agilent 6560 mass spectrometer (Agilent). Confocal laser scanning microscopy (CLSM) was performed on a FV3000 confocal laser scanning microscope (Olympus). Fluorescence microscopy was performed on an Operetta CLS high-content analysis system (Perkin Elmer). HPLC was performed with a 1260 Infinity II HPLC system (Agilent technologies) equipped with a Luna 10 μm C18 LC column (250 mm × 10mm, 100 Å). The following solvent systems were used: solvent A (0.1% TFA solution in H_2O) and solvent B (0.1% TFA in acetonitrile). Fluorescence images were processed and analyzed by Image J software.



Scheme S1. Synthesis of **Ad-Ph** (adamantylphenylenediamine) and **BDP-AdA** (boron-dipyrromethene 630/650-adamantylammonium)

Ad-Ph: 1-Bromoadamantane (0.71 g, 3.30 mmol) was added to 2,5-Dimethyl-1,4-phenylenediamine (0.5 g, 3.67 mmol). The reaction was conducted neat under pressure in a 170 °C oven for 3 h. The product was purified by silica column chromatography (eluent, DCM:MeOH:NH_3(aq) = 95:4.5:0.5) to afford **Ad-Ph** as a brown solid. **^{1}H NMR** (850 MHz, CD_3OD) δ 6.71 (s, 1H), 6.56 (s, 1H), 2.17 (s, 3H), 2.11 (s, 3H), 2.05 (s, 3H), 1.77 (s, 6H), 1.69 (d, J = 12.1 Hz, 3H), 1.63 (d, J = 11.9 Hz, 3H). **^{13}C NMR** (214 MHz, CD_3OD) δ 140.36, 133.14, 129.14, 127.27, 120.90, 117.78, 53.80, 43.08, 36.13, 29.84, 17.10, 15.91. **HR-ESI-MS** (m/z): $[M+H]^+$ calcd. for $C_{18}H_{28}N_2^+$, 271.2169; found, 271.2176.

BDP-AdA: 630/650 carboxylic acid (6.6 mg, 14.7 μmol) dissolved in anhydrous dimethyl sulfoxide (DMSO, 200 μL) was activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 28.4 mg, 147 μmol) and *N*-hydroxysuccinimide (NHS, 17.0 mg, 147 μmol), and then **Ad-Ph** (12.0 mg, 44.4 μmol) in anhydrous DMSO (200 μL) and trimethylamine (TEA, 40 μL) was added. After stirring the mixture for 3 h, AdA-BDP

was obtained by HPLC purification (6.7 mg, 70%). **^{1}H NMR** (850 MHz, CD_3OD) δ 8.12 (d, *J* = 3.7 Hz, 1H), 7.67 (d, *J* = 8.6 Hz, 2H), 7.62 (d, *J* = 5.0 Hz, 1H), 7.57 (dd, *J* = 16.2 Hz, 2H), 7.37 (s, 1H), 7.22-7.21 (m, 2H), 7.14 (dd, *J* = 3.8, 4H), 7.07 (s, 1H), 6.88 (s, 1H), 6.85 (d, *J* = 4.1 Hz, 1H), 4.76 (s, 2H), 2.16 (s, 3H), 2.14 (s, 3H), 2.09 (s, 3H), 1.91 (d, *J* = 2.0 Hz, 6H), 1.74 (d, *J* = 12.2 Hz, 3H), 1.70 (d, *J* = 11.8 Hz, 3H). **^{13}C NMR** (214 MHz, CD_3OD) δ 169.75, 160.43, 158.22, 150.06, 143.62, 139.01, 138.29, 138.23, 135.64, 132.36, 131.76, 131.50, 130.34, 130.27, 129.93, 129.66, 129.21, 127.97, 126.62, 125.79, 122.89, 120.34, 118.64, 118.37, 116.53. **HR-ESI-MS** (m/z): $[M+H]^+$ calcd. for $C_{41}H_{42}BF_2N_4O_2S^+$, 703.3084; found, 703.3038.

FRET experiments under a fluorometer

Fluorescence emissions of Cy3-CB[7] (donor), BDP-AdA (acceptor) and a mixture of both compounds in methanol were scanned from 540 nm to 750 nm with a same excitation wavelength (530 nm). For both compounds, the final concentration was 250 nM and the same procedure was applied for a control experiment using BDP-NH_2 and Cy3-NH_2. Upon excitation with a 530 nm laser, the percentage fluorescence decrease of the donor and the percentage fluorescence increase of the acceptor were calculated to be P_D = 59.8% (at 564 nm) and P_A = 43.7% (at 640 nm) by following equations,

$$P_D = \frac{I_D - I_{DA} - I_A}{I_D} \times 100(\%)\ (equation\ 1)$$

$$P_A = \frac{I_{AD} - I_A - I_D}{I_{AD}} \times 100(\%)\ (equation\ 2)$$

where, P_D is the percentage fluorescence decrease of the donor, I_D and I_{DA} are the fluorescence intensities of the donor without and with the presence of the acceptor, respectively, I_A is a bleed-through of the acceptor signal in the donor emission range upon excitation with a donor excitation wavelength (530 nm). P_A is the percentage fluorescence increase of the acceptor by FRET, I_{AD} and I_A are the fluorescence intensities of the acceptor with and without the presence of the donor, respectively, I_D is a bleed-through of the donor signal in an acceptor emission range. P_D and P_A were negligible in the control experiment. Additionally, a rate constant for energy transfer was calculated to be 5×10^9 s^{-1} using the following equation 3.[S1]

$$E_{FRET} = \frac{k_{FRET}}{\tau_D^{-1} + k_{FRET}}\ (equation\ 3)$$

Where, τ_D is the lifetime of donor (free Cy3, 0.3 ns),[S2] k_{FRET} is the rate constant for energy transfer, and E_{FRET} is the FRET efficiency (P_D, 59.8%).

Intracellular FRET imaging in HeLa cells

HeLa cells were seeded in a confocal dish with a density of 2×10^5 cells per dish and incubated for 12 h at 37 °C incubator supplemented with 5% CO_2 for adherence. All the samples were

pre-treated with oleic acid (450 μM, complexed with BSA at molar ratio of OA:BSA = 5:1) in culture medium for 2 h and all the compounds were dissolved in culture medium containing OA to induce the formation of lipid droplets.[S3] Each sample was then treated following to the sequence shown in a Table S1. (1) For the Cy3-CB[7] treated sample, cells were incubated for 1 h in 500 μL of cell culture medium with OA, washed three times with PBS and incubated again for 4 h in 500 uL of Cy3-CB[7] (800 nM) in culture medium with OA. (2) For BDP-AdA treated sample, cells were incubated for 1 h in 500 μL of BDP-AdA (150 nM) in medium with OA, washed three times with PBS and incubated for 4 h in 500 μL of medium with OA. (3) For both compound treated sample, cells were incubated for 1 h in 500 μL of BDP-AdA (150 nM) in medium with OA, washed three times with PBS and incubated for 4 h in 500 μL of Cy3-CB[7] (800 nM) in medium with OA. After final incubation, all the cells were washed three times with PBS, and then imaged by CLSM. For image analysis, the mean fluorescence intensity of each cell was used, and the standard deviation was calculated with 40 cells randomly chosen from at least 4 different images. The fluorescence intensity of FRET signals from both BDP-AdA and Cy3-CB[7] treated sample was displayed after subtracting averaged background signals of Cy3-CB[7] in FRET channel from only Cy3-CB[7] treated sample. Fluorescence recovery after photobleaching (FRAP)[S4] experiment was conducted by quenching the acceptor signals with a 640 nm laser (laser transmissivity: 100%) in a selected region.

<table>
<tr><th>Sample</th><th>1st treatment</th><th>Incubation</th><th>2nd treatment</th><th>Incubation</th><th></th></tr>
<tr><td>CB[7]-Cy3 treated</td><td rowspan="2">Culture media</td><td rowspan="3">1 h
@ 37 °C, 5% CO_2</td><td>800 nM Cy3-CB[7]</td><td rowspan="3">4 h
@ 37 °C, 5% CO_2</td><td rowspan="3">CLSM imaging</td></tr>
<tr><td>BDP-AdA treated</td><td>Culture media</td></tr>
<tr><td>Both treated</td><td>150 nM BDP-AdA</td><td>800 nM Cy3-CB[7]</td></tr>
</table>

Table S1. Treatment sequence of FRET components.

Intracellular FRET imaging with an autophagy inhibitor, chloroquine (CQ)

HeLa cells were seeded in a confocal dish with a density of 2×10^5 cells per dish and incubated for 12 h at 37 °C incubator supplemented with 5% CO_2 for adherence. All the samples were pre-treated with CQ in culture media for 2 h followed by the treatment with CQ in culture media with OA for 2 h. All the compounds were dissolved in culture media containing CQ with OA and samples were treated with the same procedure as described in Table S1.

Co-localization assay using organelle trackers

After treating cells with the FRET components as shown in Table S1, the cells were stained with organelle trackers as the following procedure. (1) For staining with lyso-tracker, the cells were incubated for 30 min with lyso-tracker (1 mL, 100 nM) dissolved in culture medium and imaged without further media exchange and washing. (2) While for LD-tracker (BDP 493/503), the cells were incubated for 30 min with BDP 493/503 (1 mL, 0.5 μM) dissolved in HBSS buffer, washed three times with PBS and imaged by CLSM.

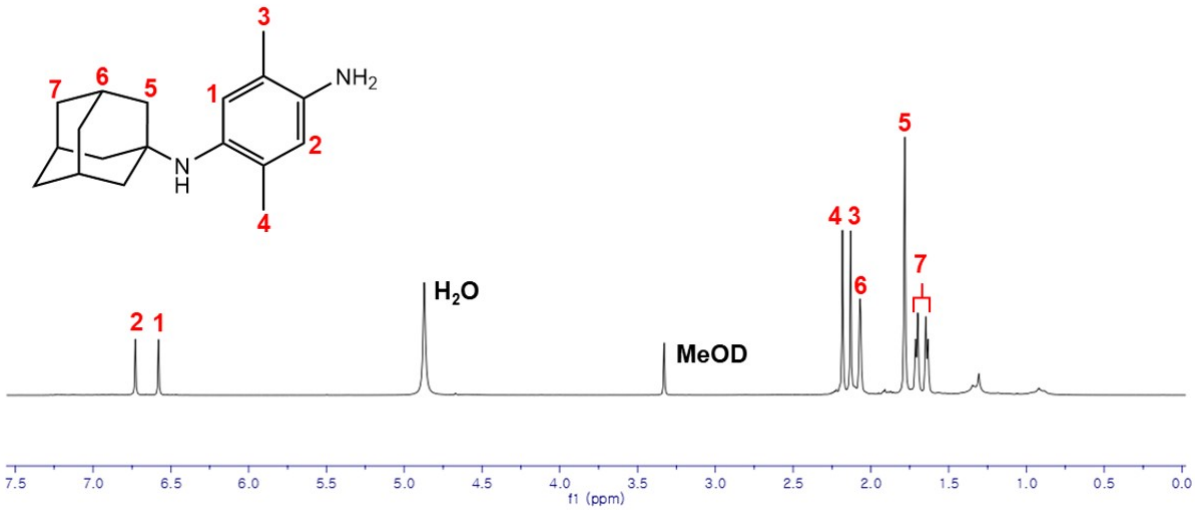


Figure S1. ^{1}H NMR spectrum of Ad-Ph in MeOD.

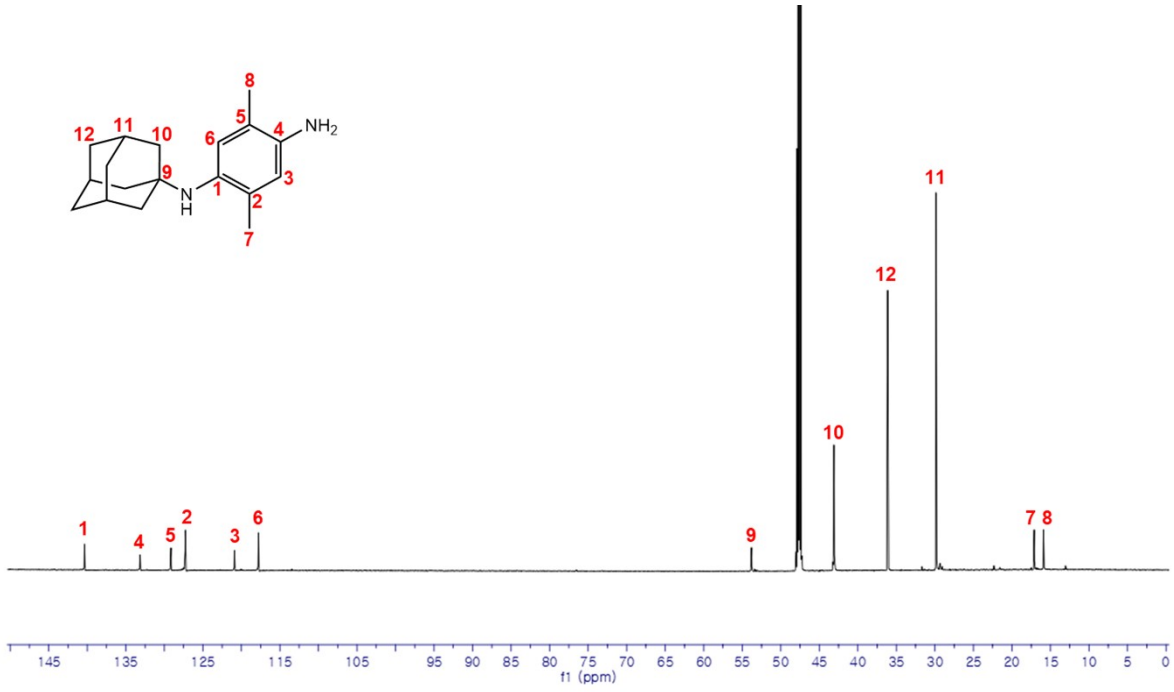


Figure S2. ^{13}C NMR spectrum of Ad-Ph in MeOD.

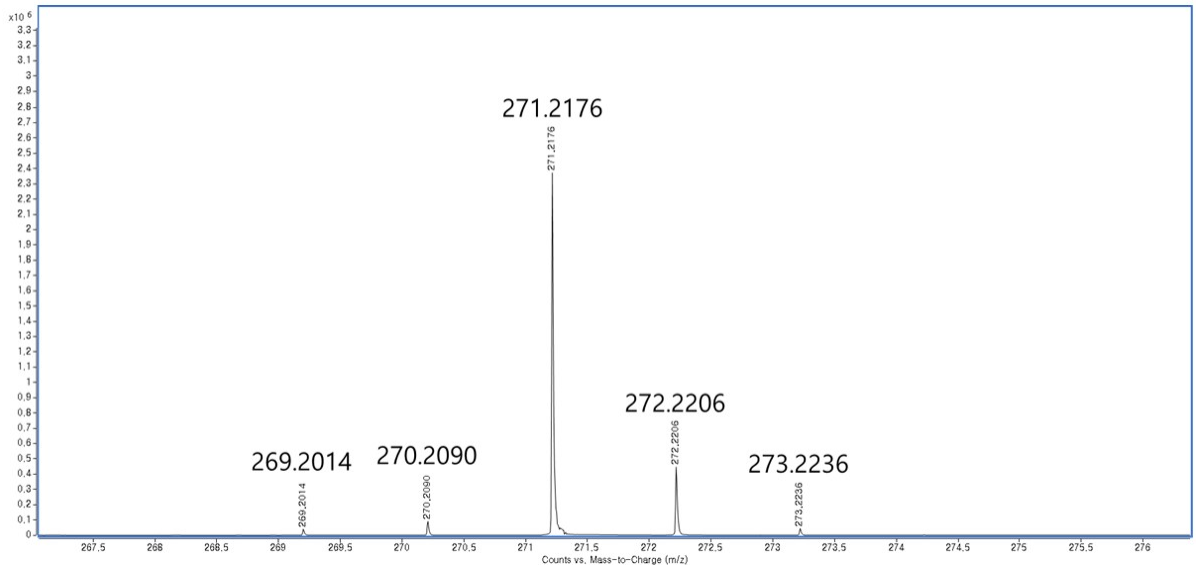


Figure S3. HR-ESI-MS spectrum of Ad-Ph.

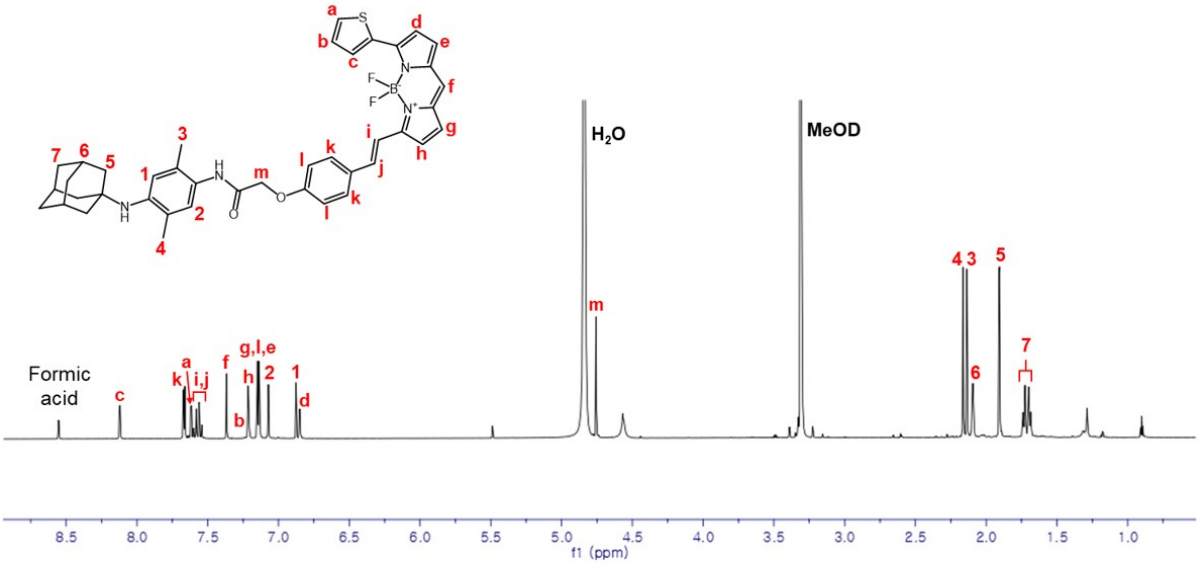


Figure S4. ^{1}H NMR spectrum of BDP-AdA in MeOD.

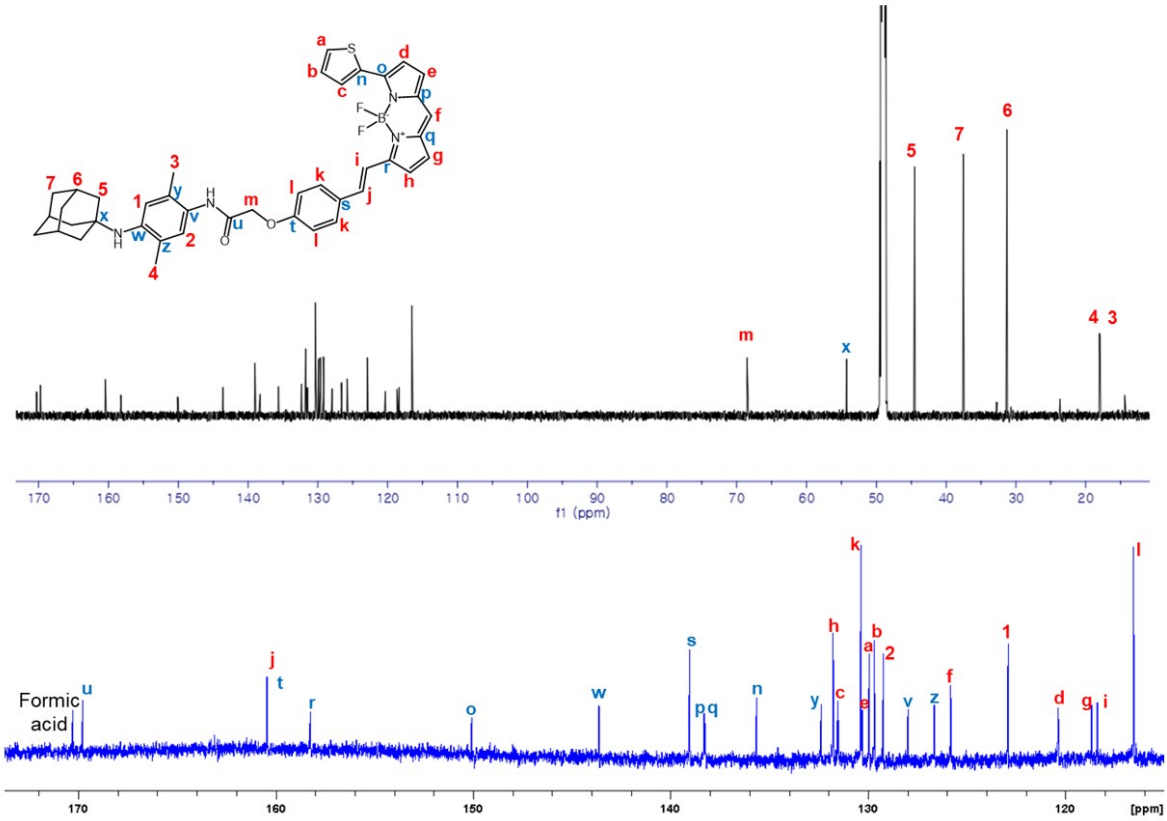


Figure S5. ^{13}C NMR spectrum of BDP-AdA in MeOD.

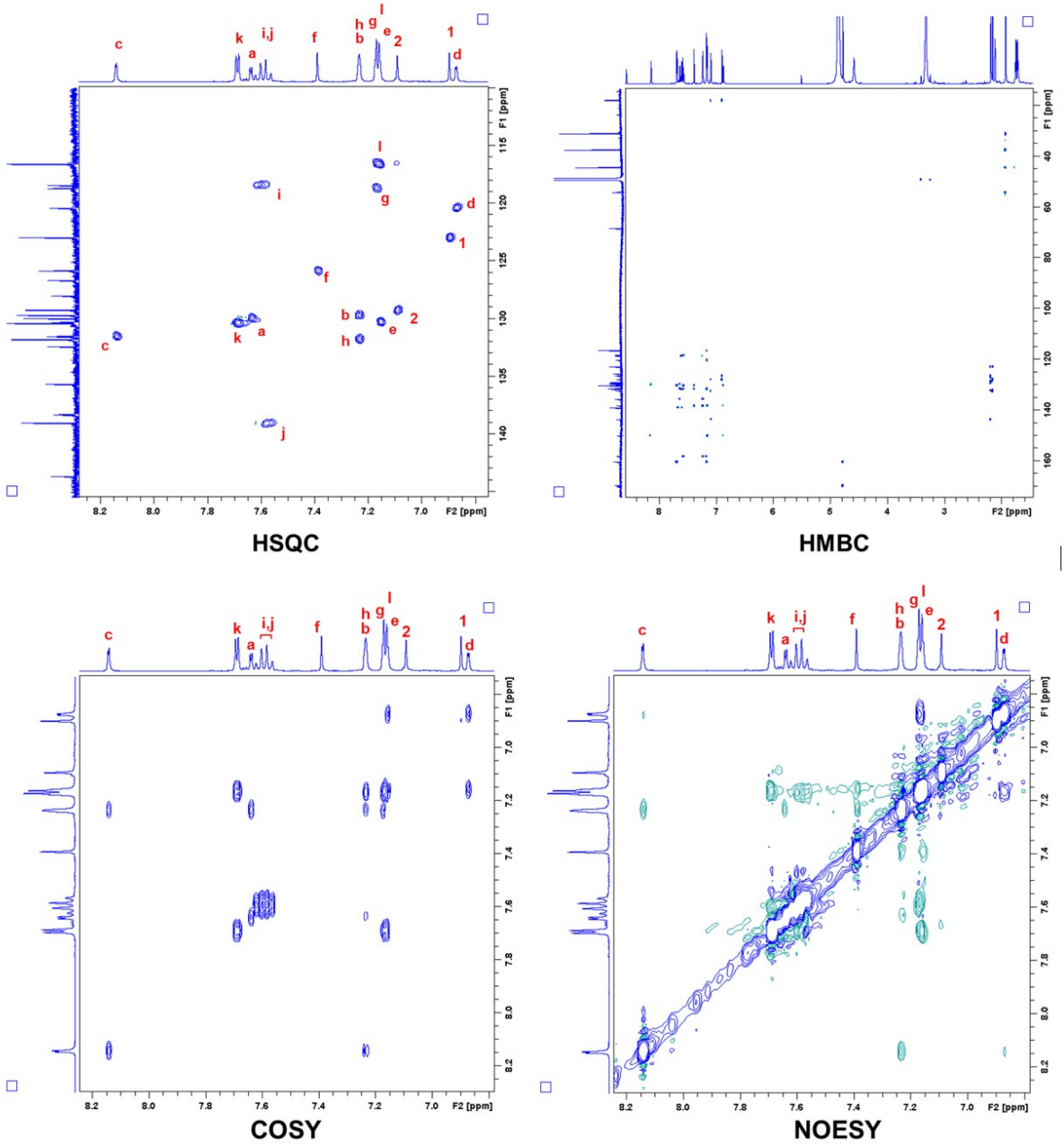


Figure S6. 2D NMR spectra of BDP-AdA in MeOD.

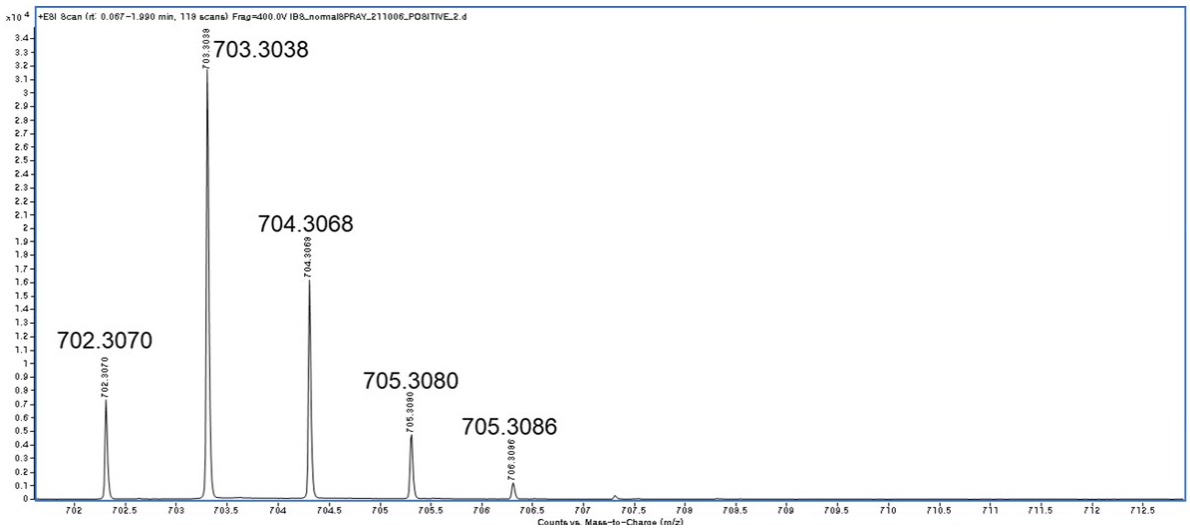


Figure S7. HR-ESI-MS spectrum of BDP-AdA.

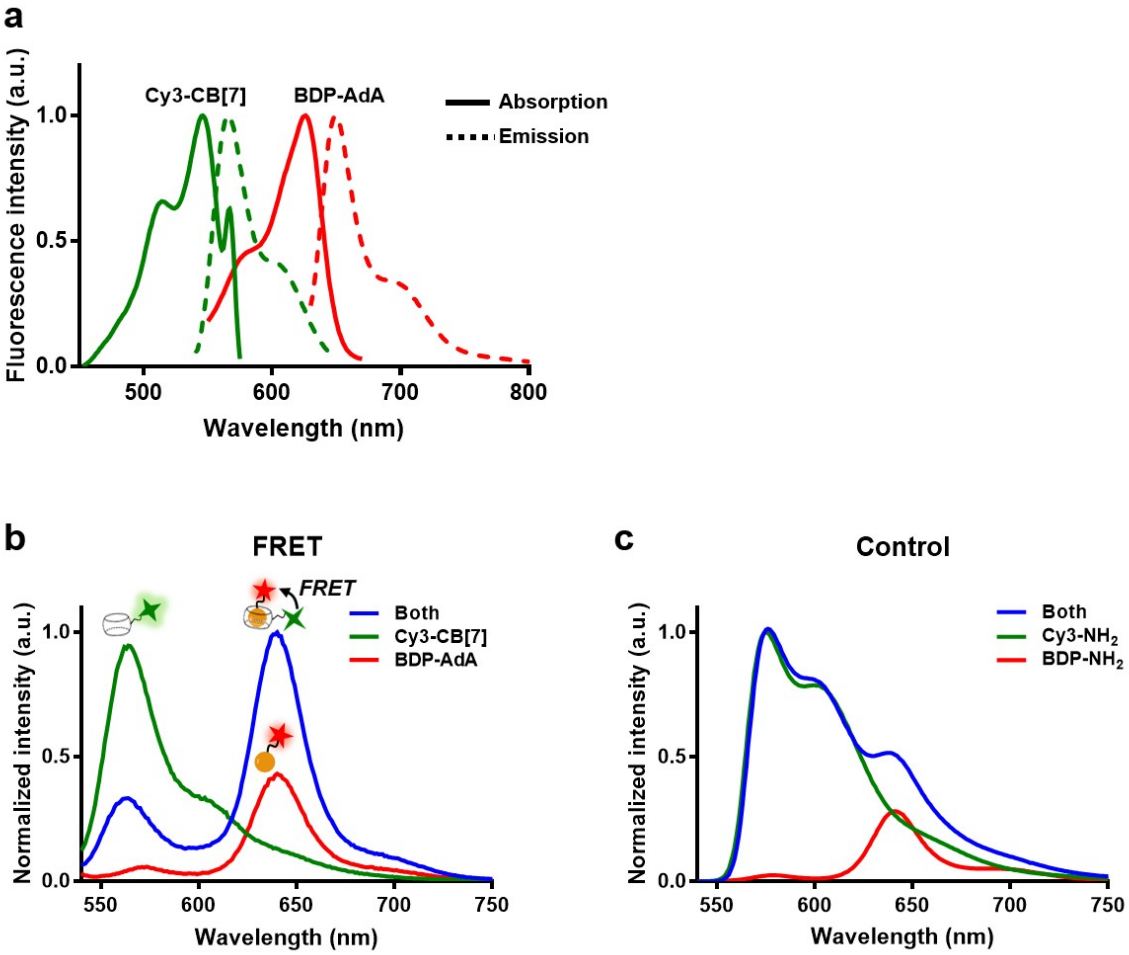


Figure S8. FRET test under a fluorometer. a) Absorption and emission spectra of Cy3-CB[7] and BDP-AdA (extinction coefficients (ε): 5.5×10^4 M^{-1} cm^{-1} and 1.0×10^5 M^{-1} cm^{-1}, respectively). b) Fluorescence emission spectra of Cy3-CB[7] (green), BDP-AdA (red), and Cy3-CB[7] and BDP-AdA complex (blue), and c) Cy3-NH_2 (green), BDP-NH_2 (red), and a mixture (blue) in methanol upon excitation with a 530 nm laser. [Cy3-CB[7]] = [BDP-AdA] = [Cy3-NH_2] = [BDP-NH_2] = 250 nM.

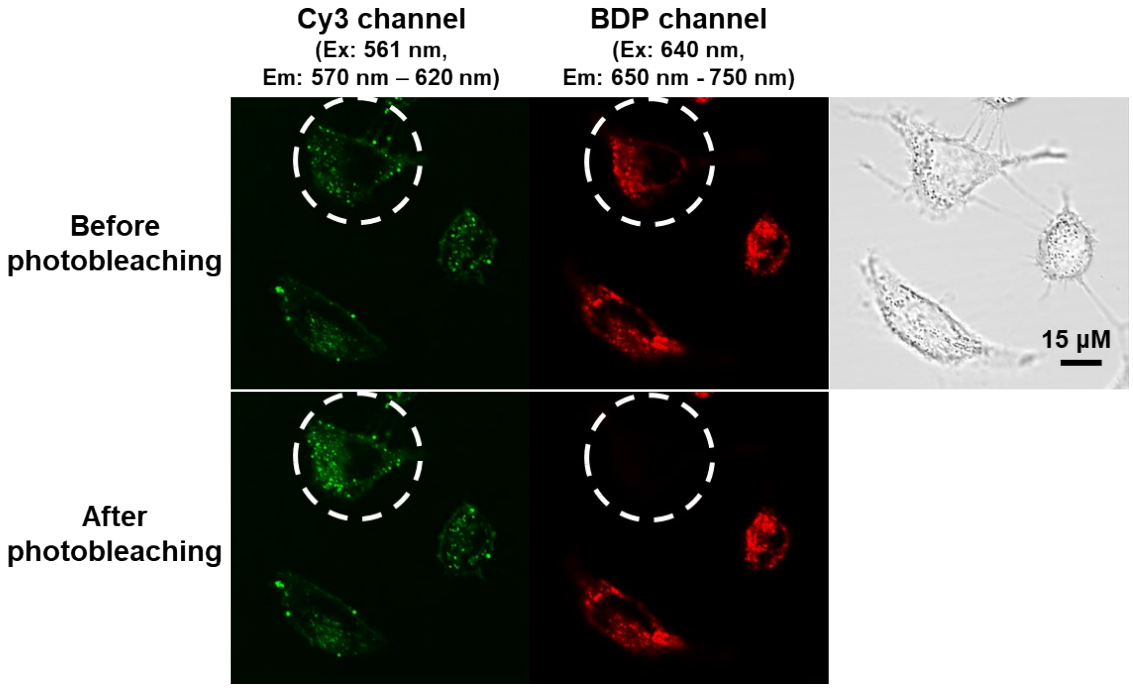


Figure S9. Fluorescence recovery after photobleaching (FRAP). The fluorescence recovery of Cy3-CB[7] (in Cy3 channel) was observed after the quenching of BDP-AdA (in BDP channel) in the selected region.

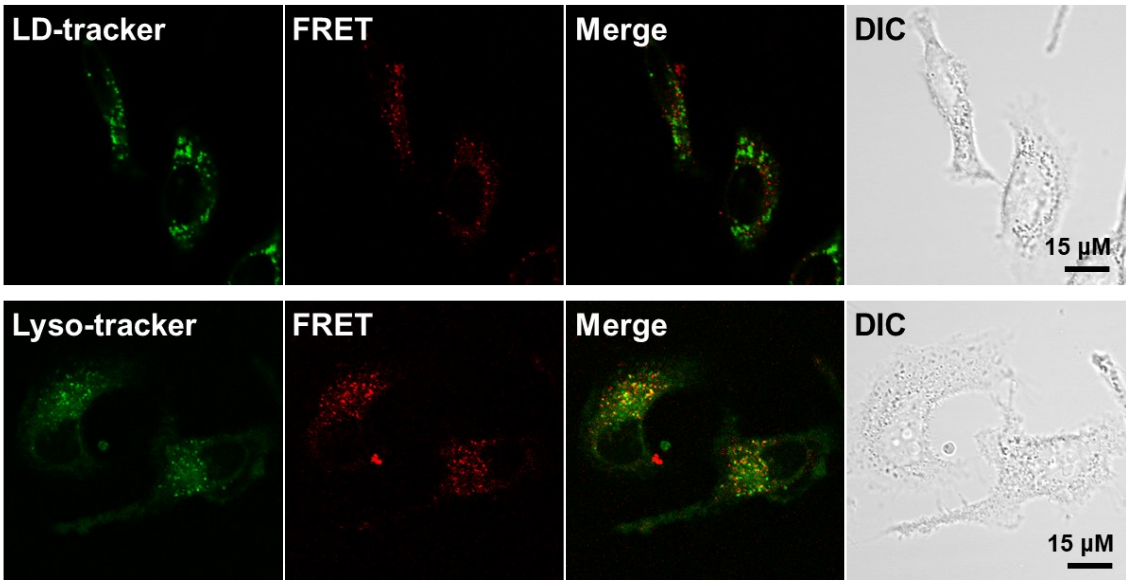


Figure S10. CLSM images of FRET in comparison with organelle trackers inside HeLa cells.

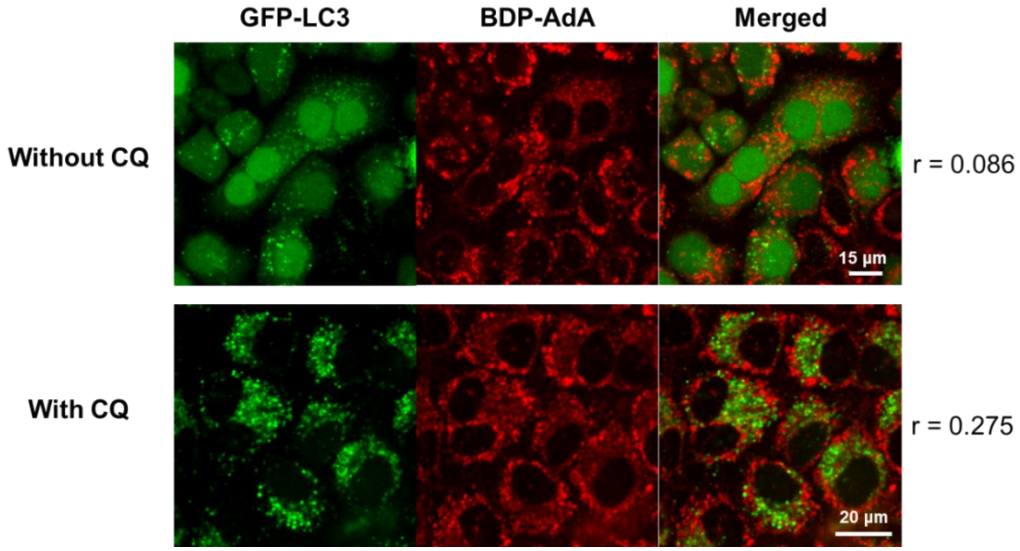


Figure S11. Fluorescence images of GFP-LC3 expressed HeLa cells treated with BDP-AdA for 5 h with or without pre-treatment of CQ. The CQ pre-treated cells showed higher overlap (Pearson's coefficient, r)[S5] between signals from GFP-LC3 and BDP-AdA.

References:

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S3. Y. Fujimoto, H. Itabe, T. Kinoshita, K. J. Homma, J. Onoduka, M. Mori, S. Yamaguchi, M. Makita, Y. Higashi, A. Yamashita and T. Takano, *J. Lipid Res.*, 2007, **48**, 1280-1292.

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