Supporting Information

Enzymatic Labeling of Bacterial Proteins for Super-Resolution Imaging in Live Cells

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Materials and Methods

(A) Synthetic procedures: Unless otherwise stated, all synthetic reactions were performed using oven-dried glassware and PTFE stir bars under an atmosphere of argon. Anhydrous solvents were purchased from Sigma-Aldrich and kept under argon. Other chemicals and reagents for chemical reactions were purchased from Sigma Aldrich and used without further purification. Reactions were monitored with thin layer chromatography (EMD/Merck silica gel 60 F254 pre-coated plates) and UV light for visualization. Flash chromatography purifications were carried out using a Biotage Isolera One purification system with pre-packed SNAP Ultra (silica) or SNAP KP-NH (amine-functionalized silica) columns. The gradient of the eluent is given as a percentage of strong solvent per column volume (CV). ^{1}H and ^{13}C NMR spectra were measured on a Bruker Prodigy 400 spectrometer (at 400 MHz and 101 MHz, respectively) equipped with a cryogenic probe. ^{19}F NMR spectra were recorded on either a Varian 300 (282 MHz) or a Bruker 400 (376 MHz) instrument. ^{1}H, ^{13}C, and ^{19}F chemical shifts are reported as δ in units of parts per million (ppm) relative to tetramethylsilane (TMS, δ = 0) and calibrated using the residual solvent peak in chloroform (δ 7.26, singlet for ^{1}H; δ 77.16 for ^{13}C; and δ 0 for ^{19}F, respectively) or dimethylsulfoxide (δ 2.50, quintet for ^{1}H; δ 39.52 for ^{13}C; and δ 0 for ^{19}F, respectively). Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad, dd= doublet of doublets, td = triplet of doublets, dt = doublet of triplets), coupling constant (Hz), and integration. High-resolution mass spectrometry (HRMS) was performed with either a JEOL JMS-600H High Resolution Mass Spectrometer with fast atom bombardment (FAB) or an LCT Premier XE Electrospray TOF Mass Spectrometer with electrospray ionization (ESI) at the California Institute of Technology Mass Spectrometry Facility in the Division of Chemistry and Chemical Engineering. HMRS data are reported as follows: calculated mass, observed mass, error in ppm. Liquid chromatography (LC) coupled with low-resolution mass spectrometry (LRMS) was performed using an Agilent 1290 UHPLC–MS in positive mode at the Center for Catalysis and Chemical Synthesis in the Caltech Beckman Institute.

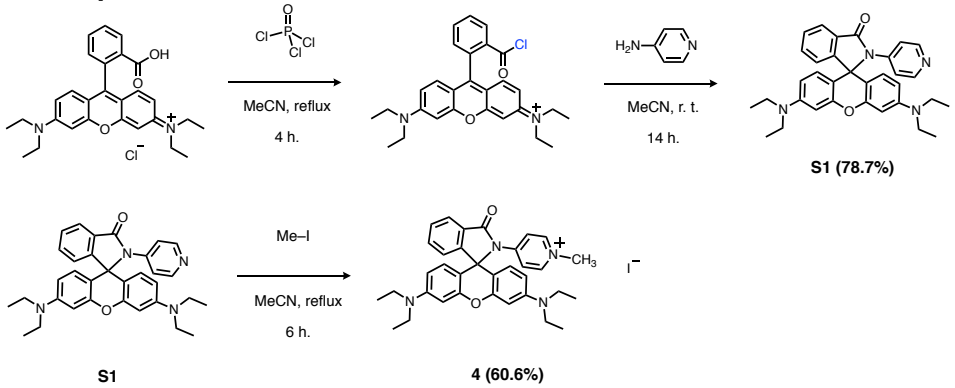
(B) Molecular biology: Lysogeny broth (LB) contained 10 g casein hydrolysate, 5 g yeast extract, and 10 g NaCl per liter of double-distilled water (ddH_2O). Ampicillin sodium salt (BioPioneer, USA) and kanamycin sulfate (BioPioneer, USA) were used at working concentrations of 200 μg/mL and 35 μg/mL, respectively. Phosphate-buffered saline (PBS) was purchased as a 10X stock solution from Thermo Fisher Scientific (USA) and diluted to 1X (pH 7.4) in ddH_2O as needed. Colonies were grown on LB agar plates carrying the appropriate antibiotic supplements. Plates containing colonies were always used within one week or discarded.

(C) Construction of strains without NMT recognition sequence. Primers were used to amplify Tar or FtsA without the NMT recognition sequence, using the modified pBAD24 plasmids bearing sequences for bacterial proteins with the NMT recognition sequence as described in [1]. For Tar, the primers were 5'-ATATATGAATTCACCATGATTAACCGTATCCGCGTAGTCAC-3' and 5'- ATATATAAGCTTCTACAGAT CTTCTTCAGAAATAAGTTTTTGTTC-3'. For construction of FtsA without the recognition sequence, the primers used for amplification were 5'- ATATATGAATTCACCATGATCAAGGCGACGGACAGAAAAC'-3' and 5'-GATCTTCTTCAGAAATAAGTTTTTGTTCGAGCTC'-3'. PCR products were digested with EcoRI/HindIII (for Tar) and EcoRI/SacI (for FtsA) and ligated into the pBAD24 vector using standard restriction enzyme digestion and ligation methods. Plasmids were transformed into E. coli strain BL21 harboring the pHV738-NMT-MetAP plasmids to generate strains SHH013 and SHH014 (**Figure S14**). Colonies were selected against ampicillin (200 μg/mL) and kanamycin (35 μg/mL).

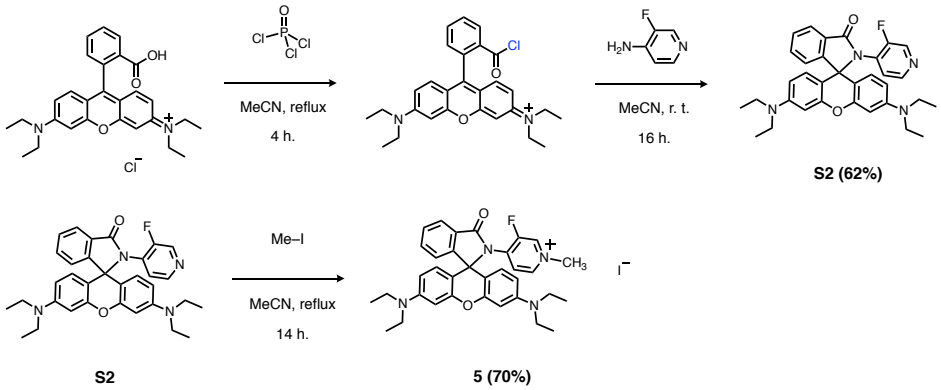
(D) Cell viability with fluorophores 2 and 3. Individual colonies (strain SHH009) were used to inoculate LB medium supplemented with 200 μg/mL ampicillin and 35 μg/mL kanamycin, and cultures were grown overnight at 37 °C with mild agitation (250 rpm). Cultures were diluted to an OD_{600} of 0.01 in LB medium (also supplemented with 200 μg/mL ampicillin and 35 μg/mL kanamycin). To each culture was added 200 nM fluorophore **2** or **3**. Cultures were allowed to continue to grow in the presence of each compound, and OD600 was monitored every 10 min for 14 h using a Varioskan LUX microplate reader (Thermo Fisher, USA). The OD_{600} of cultures grown without the presence of **2** or **3** were also measured as a control.

[1] Ho, S. H.; Tirrell, D. A. *J. Am. Chem. Soc.* **2016**, *138*, 115098–15101.

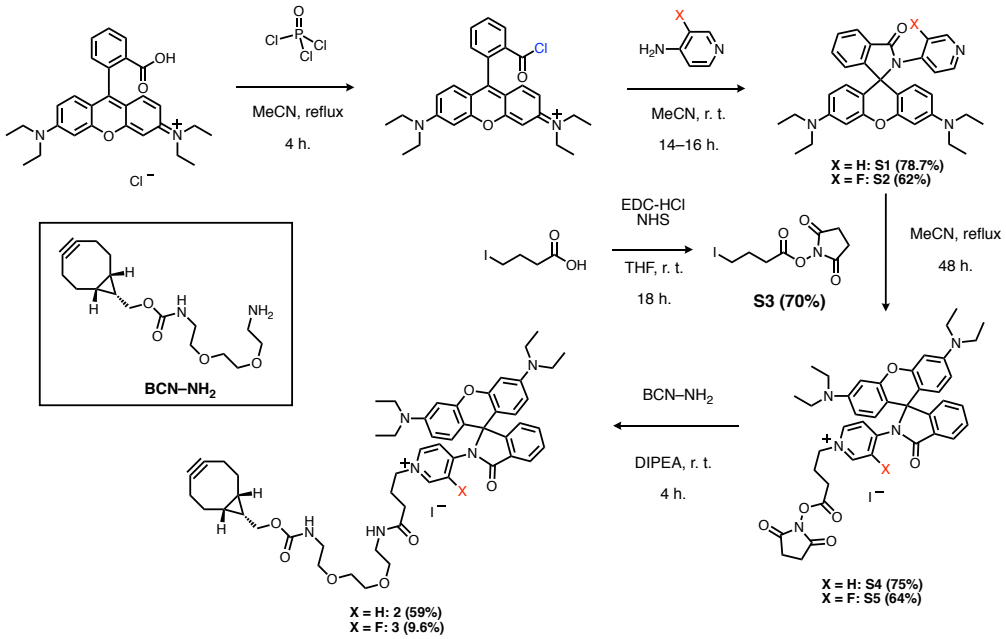
Synthesis of compounds



Scheme S1: Synthesis of rhodamines **S1** and **4**.

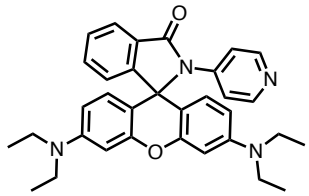


Scheme S2: Synthesis of rhodamines **S2** and **5**.

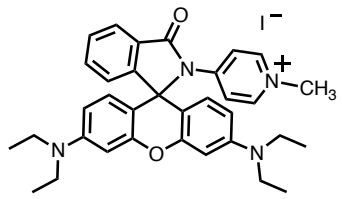


Scheme S3: Synthesis of rhodamine spirolactams **2** and **3**.

Synthesis and characterization of compounds

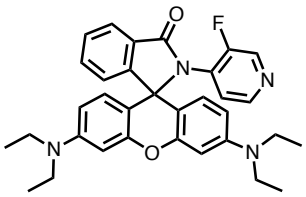


Rhodamine intermediate (**S1**): To a flame-dried 100 mL round bottom flask, was added Rhodamine B (1.5 g, 3.13 mmol, 1.0 eq), PTFE stir bar, and 20 mL acetonitrile at room temperature. Phosphorus(V) oxychloride (0.875 mL, 9.39 mmol, 3.0 eq) was added all at once to the reaction mixture, and the flask was quickly attached to a reflux condenser (14/20 neck size). The solution was heated to 92 °C and allowed to reflux for 4 h, after which the solution was cooled to room temperature and concentrated *in vacuo* to give a purple solid bearing the acid chloride. In a separate 100 mL round bottom flask, 4-aminopyridine (884.1 mg, 9.39 mmol, 3.0 eq) was dissolved in 30 mL acetonitrile. Triethylamine (1.309 mL, 9.39 mmol, 3.0 eq) was added to the solution containing 4-aminopyridine. This pre-mixed solution was then added to the acid chloride via syringe (5 mL per min over the course of 6 min) at room temperature. Gas evolution occurred upon addition of the solution to the acid chloride. The solution was allowed to stir at room temperature for 14 h, after which the solution was concentrated *in vacuo*. Ethyl acetate (50 mL) was added to the resultant solid, and the mixture was transferred to a 1 L separatory funnel. Saturated $NaHCO_3$ (30 mL) was added to wash the organic layer. The organic layer was collected and washed two more times with saturated $NaHCO_3$ (2 x 30 mL). The organic layer was washed three times with saturated NaCl (3 x 50 mL), dried over $MgSO_4$, and concentrated *in vacuo*, resulting in a purple and white solid. Flash chromatography (Biotage SNAP KP-NH, 6–60% ethyl acetate/hexanes over 15 CVs) afforded 1.28 g (78.7%) of **S1** as white solid. ^{1}H NMR (400 MHz, chloroform-d) δ 8.35 – 8.30 (m, 2H), 8.00 – 7.95 (m, 1H), 7.51 – 7.42 (m, 2H), 7.39 – 7.36 (m, 2H), 7.09 – 7.06 (m, 1H), 6.55 (d, J = 8.9 Hz, 2H), 6.37 (d, J = 2.5 Hz, 2H), 6.23 (dd, J = 8.9, 2.6 Hz, 2H), 3.31 (q, J = 7.1 Hz, 8H), 1.15 (t, J = 7.0 Hz, 12H). ^{13}C NMR (101 MHz, chloroform-d) δ 168.96, 154.37, 152.50, 150.05, 149.05, 145.18, 134.00, 128.56, 128.37, 128.05, 123.85, 123.68, 116.92, 108.42, 106.06, 98.01, 66.95, 44.45, 12.73. HRMS (FAB) calculated for $C_{33}H_{35}O_2N_4$ ([M+H]$^+$) 519.2760, found 519.2761 (Δ = 0.2 ppm).

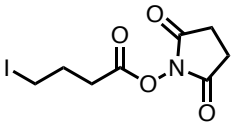


Rhodamine spirolactam (**4**): To a flame-dried 2-neck (14/20) 25 mL round bottom flask, was added rhodamine **S1** (200 mg, 0.386 mmol, 1.0 eq), PTFE stir bar, and 10 mL acetonitrile. Methyl iodide (48 μL, 0.771 mmol, 2.0 eq) was quickly added to the solution all at once and the flask was attached to a reflux condenser. The solution was allowed to reflux at 92 °C for 4 h. TLC was performed to monitor the reaction progress using a solvent system of 2% v/v methanol in methylene chloride. TLC showed that the starting material had not all been consumed. Additional methyl iodide (24 μL, 0.386 mmol, 1.0 eq) was added to the refluxing solution, and the reaction continued to reflux for an additional 2 h. TLC (2% v/v methanol in methylene chloride) indicated the presence of starting material, so additional methyl iodide (12 μL, 0.193 mmol, 0.5 eq) was added to the reaction mixture. The reaction mixture was allowed to reflux for an additional 1 h, after which TLC indicated complete consumption of starting material (total 6 h of reflux with total 3.5 eq of electrophile). Solvent from the reaction mixture was removed *in vacuo* and the resultant brown oil was dissolved in a minimal amount of methylene chloride (2 mL). A mixture of hexanes was added to triturate **4** as a solid. The solid was collected by filtration, washed with hexanes (3 x 5 mL), and dried under vacuum to afford 154.5 mg (60.6%) of rhodamine spirolactam **4** (iodide salt) as a tan solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.68 – 8.64 (m, 2H), 8.08 – 8.04 (m, 2H), 8.01 (dt, *J* = 7.5, 1.0 Hz, 1H), 7.67 (td, *J* = 7.5, 1.2 Hz, 1H), 7.59 (td, *J* = 7.5, 1.0 Hz, 1H), 7.06 (dt, *J* = 7.7, 0.8 Hz, 1H), 6.57 (d, *J* = 8.9 Hz, 2H), 6.47 (d, *J* = 2.7 Hz, 2H), 6.30 (dd, *J* = 9.0, 2.6 Hz, 2H), 4.04 (s, 3H), 3.30 (m, 8H),

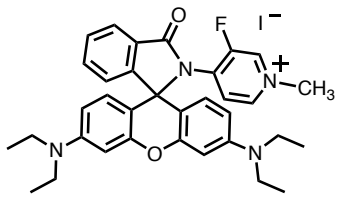
1.07 (t, J = 6.9 Hz, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.11, 154.01, 151.58, 149.34, 148.89, 145.63, 136.00, 129.23, 127.28, 125.53, 124.05, 123.67, 114.70, 108.58, 104.03, 97.43, 66.74, 46.44, 43.63, 12.39. HRMS (FAB) calculated for $C_{34}H_{37}O_2N_4$ ([M]$^+$) 533.2916, found 533.2929 (Δ = 2.4 ppm).



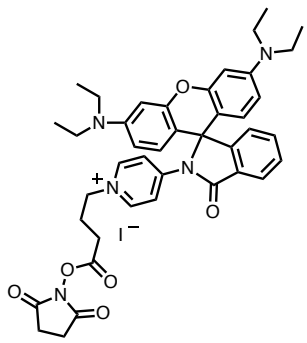
Rhodamine intermediate (**S2**): To a flame-dried 100 mL round bottom flask was added Rhodamine B, (1.5 g, 3.13 mmol, 1.0 eq), PTFE stir bar, and 10 mL acetonitrile. Phosphorus(V) oxychloride (0.875 mL, 9.39 mmol, 3.0 eq) was added all at once to the reaction mixture and the flask was quickly attached to a reflux condenser (14/20 neck size). The solution was heated to 92 °C and allowed to reflux for 4 h, after which the solution was cooled to room temperature and concentrated *in vacuo* to give a purple solid bearing the acid chloride. In a separate 100 mL round bottom flask, 4-amino-3-fluoropyridine (1.053 g, 9.39 mmol, 3.0 eq) was dissolved in 30 mL acetonitrile and triethylamine (1.309 mL, 9.39 mmol, 3.0 eq) was added. The resulting solution was added to the acid chloride (5 mL per min over the course of 6 min) at room temperature. Gas evolution occurred upon addition of the solution containing 4-amino-3-fluoropyridine to the acid chloride. The solution was allowed to stir at room temperature for 16 h, after which the solution was concentrated *in vacuo*. Ethyl acetate (50 mL) was added to the resultant solid, and the mixture was transferred to a 1 L separatory funnel. Saturated $NaHCO_3$ (30 mL) was added to wash the organic layer. The organic layer was collected and washed two more times with saturated $NaHCO_3$ (2 x 30 mL). The organic layer was washed three times with saturated NaCl (3 x 30 mL), dried over $MgSO_4$, and concentrated *in vacuo*, resulting in a purple and white solid. Flash chromatography (Biotage SNAP KP-NH, 6–60% ethyl acetate/hexanes over 15 CVs) afforded 1.04 g (62%) of **S2** as white solid. ^{1}H NMR (400 MHz, chloroform-*d*) δ 8.35 (d, J = 2.2 Hz, 1H), 8.10 (d, J = 5.2 Hz, 1H), 8.05 – 7.99 (m, 1H), 7.56 – 7.47 (m, 2H), 7.17 – 7.11 (m, 1H), 6.67 (d, J = 8.8 Hz, 2H), 6.39 – 6.35 (m, 1H), 6.31 (dd, J = 8.9, 2.7 Hz, 2H), 6.27 (d, J = 2.6 Hz, 2H), 3.32 (q, J = 7.1 Hz, 8H), 1.15 (t, J = 7.1 Hz, 12H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 166.77, 156.59, 154.10, 153.98, 153.04, 149.06, 146.03, 145.98, 139.57, 139.34, 133.54, 132.47, 132.37, 129.69, 128.99, 128.49, 124.24, 123.69, 121.79, 108.30, 105.24, 97.65, 67.87, 44.42, 12.66. ^{19}F NMR (282 MHz, chloroform-*d*) δ -128.72. HRMS (ESI) calculated for $C_{33}H_{34}N_4O_2F$ ([M+H]$^+$) 537.2666, found 537.2642 (Δ = -4.5 ppm).



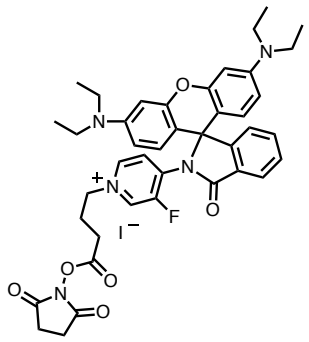
2,5-dioxopyrrolidin-1-yl-4-iodobutanoate (**S3**): To a flame-dried 100 mL round bottom flask was added 4-iodobutyric acid (100 mg, 0.467 mmol, 1.0 eq), N'-ethylcarbodiimide hydrochloride (EDC-HCl) (450 mg, 2.34 mmol, 5.0 eq) N-hydroxysuccinimide (270 mg, 2.34 mmol, 5.0 eq), and PTFE stir bar. The flask was cooled to 0 °C using an ice bath and 15 mL tetrahydrofuran was added. The reaction mixture was allowed to stir and warm to room temperature over the course of 0.5 h. The mixture was stirred for an additional 18 h, after which the solution was diluted with ethyl acetate (20 mL) and extracted with 10% w/v citric acid (20 mL). The organic layer was collected and washed with 10% w/v citric acid (2 x 30 mL) and saturated NaCl (2 x 20 mL), dried over $MgSO_4$ and concentrated *in vacuo* to yield a brown oil. The oil was dissolved in a minimal amount of methylene chloride and loaded onto a Biotage SNAP Ultra cartridge for purification. Purification (6–50% ethyl acetate/hexanes over 15 CVs) afforded 101 mg (70%) of **S3** as a pale yellow solid. This procedure was repeated as needed to generate more **S3**. ^{1}H NMR (400 MHz, chloroform-*d*) δ 3.28 (t, J = 6.7 Hz, 2H), 2.84 (s, 4H), 2.77 (t, J = 7.2 Hz, 2H), 2.24 (p, J = 6.9 Hz, 2H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 169.13, 167.69, 31.93, 28.25, 25.71, 4.01. HRMS (ESI) calculated for $C_8H_{10}NO_4INa$ ([M+Na]$^+$) 333.9552, found 333.9546 (Δ = -1.8 ppm).



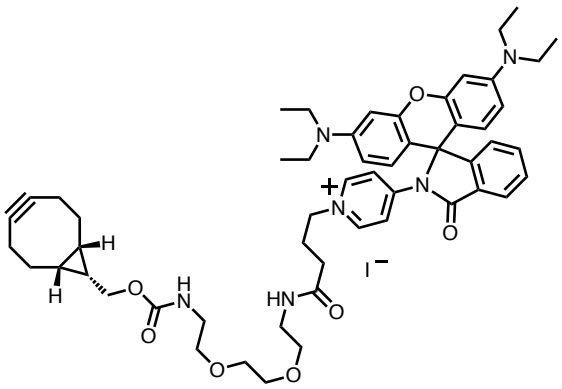
Rhodamine spirolactam (**5**): A flame-dried 2-neck (14/20) 25 mL round bottom flask was charged with rhodamine **S2** (100 mg, 0.186 mmol, 1.0 eq) and PTFE stir bar. Acetonitrile (10 mL) was added to the flask and methyl iodide (23.2 μL, 0.373 mmol, 2.0 eq) was subsequently added. The flask was quickly attached to a reflux condenser and refluxed at 92 °C for 3 h. TLC (2% v/v methanol in methylene chloride) was used to monitor the reaction and indicated the presence of starting material. Methyl iodide (23.2 μL, 0.373 mmol, 2.0 eq) was added to the refluxing solution after 2 h. After an additional 1 h, TLC indicated the starting material had not been completely consumed. Another 2.0 eq of methyl iodide (23.2 μL, 0.373 mmol) was added to the solution. Over the next 6 h, methyl iodide (23.2 μL, 0.373 mmol, 2.0 eq) was added every 2 h. After a total of 14 h, the amount of starting material did not decrease further, as indicated by TLC. The reaction mixture was cooled to room temperature and concentrated *in vacuo*. Flash chromatography (Biotage SNAP Ultra, 1–2% v/v methanol/methylene chloride over 15 CVs) afforded 88.4 mg (70%) of rhodamine spirolactam **5** as a brilliant red powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.36 (d, J = 5.8 Hz, 1H), 8.72 (dd, J = 6.8, 1.5 Hz, 1H), 8.04 – 7.97 (m, 1H), 7.69 (td, J = 7.4, 1.4 Hz, 1H), 7.63 (td, J = 7.4, 1.2 Hz, 1H), 7.13 (d, J = 7.5 Hz, 1H), 6.93 (t, J = 6.9 Hz, 1H), 6.60 (d, J = 8.9 Hz, 2H), 6.44 (dd, J = 9.0, 2.6 Hz, 2H), 6.36 (d, J = 2.6 Hz, 2H), 4.16 (s, 3H), 3.34 – 3.22 (m, 8H), 1.08 (t, J = 6.9 Hz, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.89, 154.86, 154.24, 152.29, 152.08, 151.95, 148.91, 143.86, 143.82, 139.93, 139.83, 137.38, 137.00, 135.25, 129.32, 128.02, 126.70, 124.18, 123.71, 121.27, 108.79, 103.67, 97.13, 68.29, 48.03, 43.66, 12.38. ^{19}F NMR (282 MHz, DMSO-d_6) δ -118.19. HRMS (ESI) calculated for $C_{34}H_{36}N_4O_2F$ ([M]$^+$) 551.2822, found 551.2831 (Δ = 1.6 ppm).



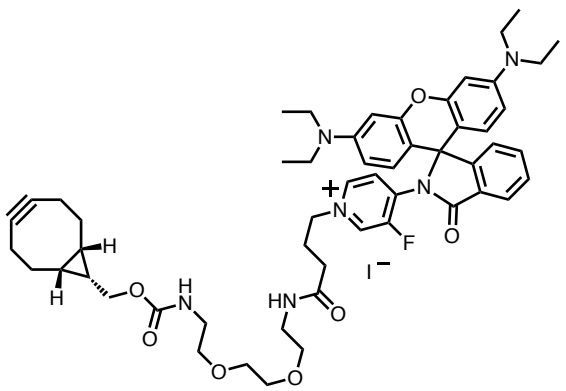
Rhodamine spirolactam NHS ester (**S4**): A flame-dried 2-neck (14/20) 25 mL round bottom flask was charged with rhodamine **S1** (250 mg, 0.482 mmol, 1.0 eq), NHS ester **S3** (300 mg, 0.964 mmol, 2.0 eq), and PTFE stir bar. Acetonitrile (12 mL) was added and the flask was quickly attached to a reflux condenser. The solution was heated to 90 °C and allowed to stir under refluxing conditions for 48 h, after which the solution was cooled to room temperature and solvent was removed *in vacuo*, resulting in a tan precipitate. Ethyl acetate (10 mL) was added to the flask, and the solution was allowed to gently stir for 12 h, after which the solid was collected by filtration. The solid was washed with ethyl acetate (3 x 10 mL) and dried under vacuum to afford 300 mg (75%) rhodamine spirolactam NHS ester **S4** as a tan powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.74 (d, J = 7.6 Hz, 2H), 8.08 (d, J = 7.6 Hz, 2H), 8.03 – 8.01 (m, 1H), 7.67 (td, J = 7.5, 1.2 Hz, 1H), 7.59 (td, J = 7.4, 1.0 Hz, 1H), 7.05 (d, J = 7.8 Hz, 1H), 6.57 (d, J = 8.9 Hz, 2H), 6.48 (d, J = 2.6 Hz, 2H), 6.31 (dd, J = 9.0, 2.6 Hz, 2H), 4.36 (t, J = 7.2 Hz, 2H), 3.33 – 3.22 (m, 8H), 2.80 (s, 4H), 2.79 – 2.74 (m, 2H), 2.17 (q, J = 7.5 Hz, 2H), 1.07 (t, J = 7.0 Hz, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.15, 169.13, 168.17, 154.07, 151.61, 149.87, 148.91, 144.85, 136.05, 129.23, 127.38, 125.47, 124.07, 123.69, 115.09, 108.60, 103.98, 97.41, 66.85, 58.04, 43.63, 27.12, 25.45, 24.89, 12.39. HRMS (ESI) calculated for $C_{41}H_{44}N_5O_6$ ([M]$^+$) 702.3292, found 702.3267 (Δ = -3.6 ppm).



Rhodamine spirolactam NHS ester **S5**: To a flame-dried 2-neck (14/20) 50 mL round bottom flask was added rhodamine **S2** (125 mg, 0.233 mmol, 1.0 eq), NHS ester **S3** (181.2 mg, 0.582 mmol, 2.5 eq), and PTFE stir bar. Acetonitrile (15 mL) was added to the flask and the flask was quickly attached to a reflux condenser. The solution was heated to 95 °C and allowed to reflux for 48 h, after which the solvent was removed *in vacuo*, resulting in a red oil with a purple hue. Ethyl acetate (5 mL) was added to the flask, and the solution was allowed to stir for 24 h at room temperature, resulting in a purple solid. The solid was collected by filtration, washed with ethyl acetate (3 x 5 mL), and dried under vacuum to afford 126 mg (64%) of rhodamine spirolactam NHS ester **S5** as a purple powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.41 (d, J = 5.8 Hz, 1H), 8.79 (d, J = 6.9 Hz, 1H), 8.04 – 8.00 (m, 1H), 7.69 (td, J = 7.5, 1.3 Hz, 1H), 7.63 (td, J = 7.4, 1.1 Hz, 1H), 7.13 (d, J = 7.7 Hz, 1H), 6.99 (t, J = 6.9 Hz, 1H), 6.60 (d, J = 8.9 Hz, 2H), 6.44 (dd, J = 9.0, 2.6 Hz, 2H), 6.37 (d, J = 2.6 Hz, 2H), 4.48 (t, J = 7.1 Hz, 2H), 3.33 – 3.25 (m, 8H), 2.81 (s, 4H), 2.80 – 2.77 (m, 2H), 2.23 (p, J = 7.4 Hz, 2H), 1.08 (t, J = 6.9 Hz, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.14, 168.15, 165.90, 155.02, 154.27, 152.45, 152.05, 148.91, 142.96, 140.41, 140.31, 136.67, 136.29, 135.32, 129.31, 127.96, 126.54, 124.16, 123.75, 121.12, 108.81, 103.78, 97.16, 68.38, 43.65, 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89, 27.07, 25.45, 24.65, 12.37. HRMS (ESI) calculated for $C_{41}H_{43}N_5O_6F$ ($[M]^+$) 720.3197, found 720.3178 (Δ = -2.6 ppm).



Bicyclononyne rhodamine conjugate (**2**): To a 20 mL scintillation vial was added ester **S4** (15 mg, 18.1 μmol, 1.0 eq), 0.5 mL dimethylformamide, and a PTFE stir bar. BCN-NH_2 (see **Scheme S3**) (7.62 mg, 23.5 μmol, 1.3 eq, dissolved in 1.0 mL dimethylformamide) was added to the solution; N,N-diisopropylethylamine (5.6 μL, 32.01 μmol, 1.8 eq) was added subsequently. The reaction mixture was allowed to stir for 2 h at room temperature, after which the crude mixture was directly dry loaded onto Celite. Flash chromatography (Biotage SNAP KP-NH, 0–20% methanol/ethyl acetate over 15 CVs) afforded 11.1 mg (59%) of **2** as a pale yellow film. ^{1}H NMR (400 MHz, chloroform-*d*) δ 8.70 (d, J = 7.0 Hz, 2H), 8.25 (d, J = 7.3 Hz, 2H), 8.01 – 7.98 (m, 1H), 7.57 (td, J = 7.5, 1.2 Hz, 1H), 7.49 (td, J = 7.5, 1.0 Hz, 1H), 7.08 – 7.06 (m, 1H), 6.46 (d, J = 2.6 Hz, 2H), 6.43 (d, J = 8.9 Hz, 2H), 6.22 (dd, J = 9.0, 2.6 Hz, 2H), 5.47 (br s, 1H), 4.75 (t, J = 7.8 Hz, 2H), 4.17 – 4.07 (m, 3H), 3.62 – 3.59 (m, 4H), 3.58 – 3.53 (m, 3H), 3.54 – 3.48 (m, 2H), 3.39 – 3.29 (m, 12H), 2.51 (t, J = 6.8 Hz, 2H), 2.26 – 2.20 (m, 7H), 1.64 – 1.52 (m, 3H), 1.38 – 1.31 (m, 1H), 1.17 (t, J = 7.1 Hz, 12H), 0.92 (t, J = 10.0 Hz, 2H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 171.85, 171.74, 170.12, 157.06, 154.46, 152.49, 152.15, 151.11, 149.66, 143.70, 136.07, 129.08, 127.02, 125.71, 124.42, 124.06, 115.93, 108.77, 108.07, 103.77, 99.00, 98.38, 98.00, 70.41, 70.33, 70.26, 69.59, 68.05, 62.75, 60.55, 58.90, 44.53, 40.85, 40.73, 39.29, 39.16, 32.35, 29.21, 28.10, 21.59, 21.21, 20.23, 17.94, 14.34, 12.74. HRMS (ESI) calculated for $C_{54}H_{67}N_6O_7$ ($[M]^+$) 911.5071, found 911.5060 (Δ = -1.2 ppm).



Bicyclononyne rhodamine conjugate (**3**): To a 20 mL scintillation vial was added ester **S5** (12 mg, 14.2 μmol, 1.0 eq), 0.5 mL dimethylformamide, and a PTFE stir bar. BCN-NH_2 (see **Scheme S3**) (9.19 mg, 28.3 μmol, 2.0 eq, dissolved in 1.0 mL dimethylformamide) was added to the solution; N,N-diisopropylethylamine (3.71 μL, 21.3 μmol, 1.5 eq) was added subsequently. The reaction mixture was allowed to stir for 2 h at room temperature, after which the crude mixture was directly dry loaded onto Celite. Flash chromatography (Biotage SNAP KP-NH, 0–20% methanol/ethyl acetate over 15 CVs) afforded 1.4 mg (9.6%) of **3** as a purple film. ^{1}H NMR (400 MHz, chloroform-*d*) δ 9.02 – 8.97 (m, 1H), 8.02 (d, *J* = 7.4 Hz, 1H), 7.65 – 7.46 (m, 3H), 7.14 – 7.08 (m, 1H), 6.58 (d, *J* = 8.9 Hz, 2H), 6.36 (d, *J* = 2.5 Hz, 2H), 6.31 (dd, *J* = 9.1, 2.7 Hz, 2H), 6.23 – 6.20 (m, 1H), 5.45 (s, 1H), 4.96 (s, 1H), 4.21 – 4.07 (m, 3H), 3.69 – 3.53 (m, 9H), 3.43 – 3.29 (m, 12H), 2.55 – 2.41 (m, 2H), 2.34 – 2.15 (m, 7H), 1.41 – 1.30 (m, 4H), 1.18 (t, *J* = 7.1 Hz, 12H), 1.00 – 0.90 (m, 2H). ^{13}C NMR (101 MHz, chloroform-*d*) δ 173.03, 168.44, 166.70, 157.12, 156.37, 154.54, 153.14, 152.71, 149.67, 144.47, 136.75, 135.20, 134.68, 128.00, 124.41, 124.35, 108.99, 108.27, 108.10, 103.89, 101.60, 99.00, 97.94, 70.41, 70.36, 70.24, 69.62, 68.10, 62.82, 60.56, 57.62, 44.55, 40.90, 40.84, 39.37, 39.10, 31.98, 29.20, 27.95, 21.59, 21.22, 20.25, 17.93, 14.35, 12.75. ^{19}F NMR (376 MHz, chloroform-*d*) δ -111.36. HRMS (ESI) calculated for $C_{54}H_{66}N_6O_7F$ ($[M]^+$) 929.4977, found 929.4978 (Δ = 0.1 ppm).

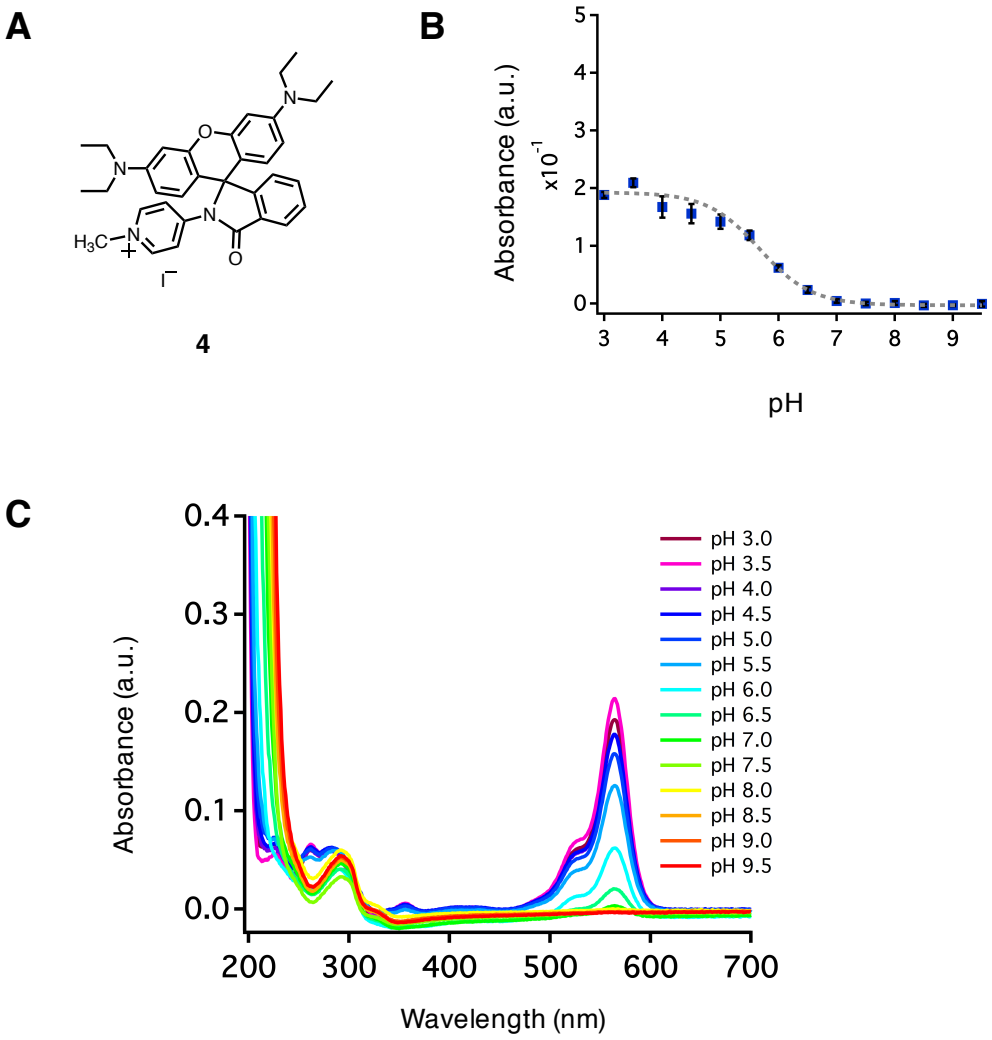


Figure S1: Absorption spectra of rhodamine **4** in bis-tris propane at different pH values. (**A**) Structure of **4**. (**B**) Absorbance at 561 nm as a function of pH. (**C**) Absorption spectra of **4** across different pH ranges. Error bars denote the standard deviation from three independent experiments.

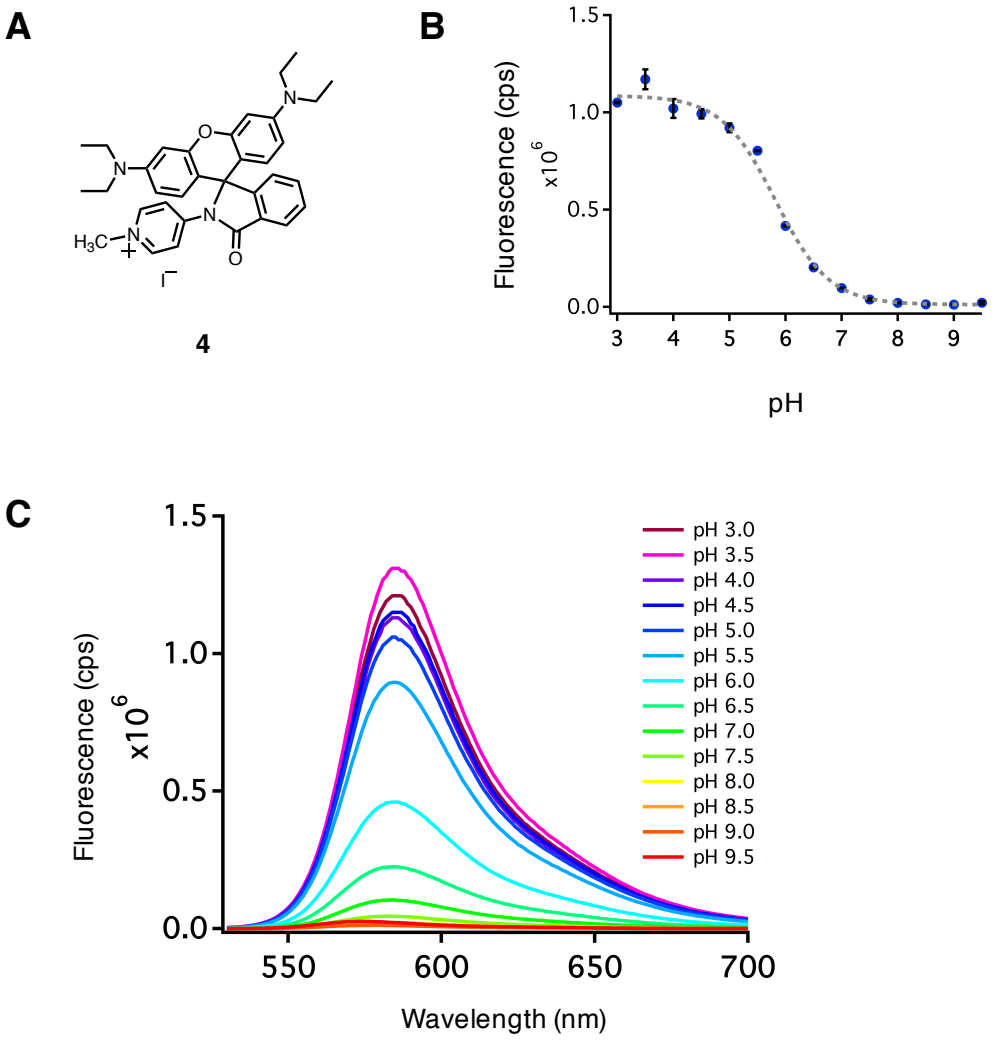


Figure S2: Fluorescence emission from rhodamine **4** in bis-tris propane at different pH values. (**A**) Structure of **4**. (**B**) Fluorescence emission at 577 nm as a function of pH. (**C**) Fluorescence emission spectra of **4** across different pH ranges. Error bars denote the standard deviation from three independent experiments.

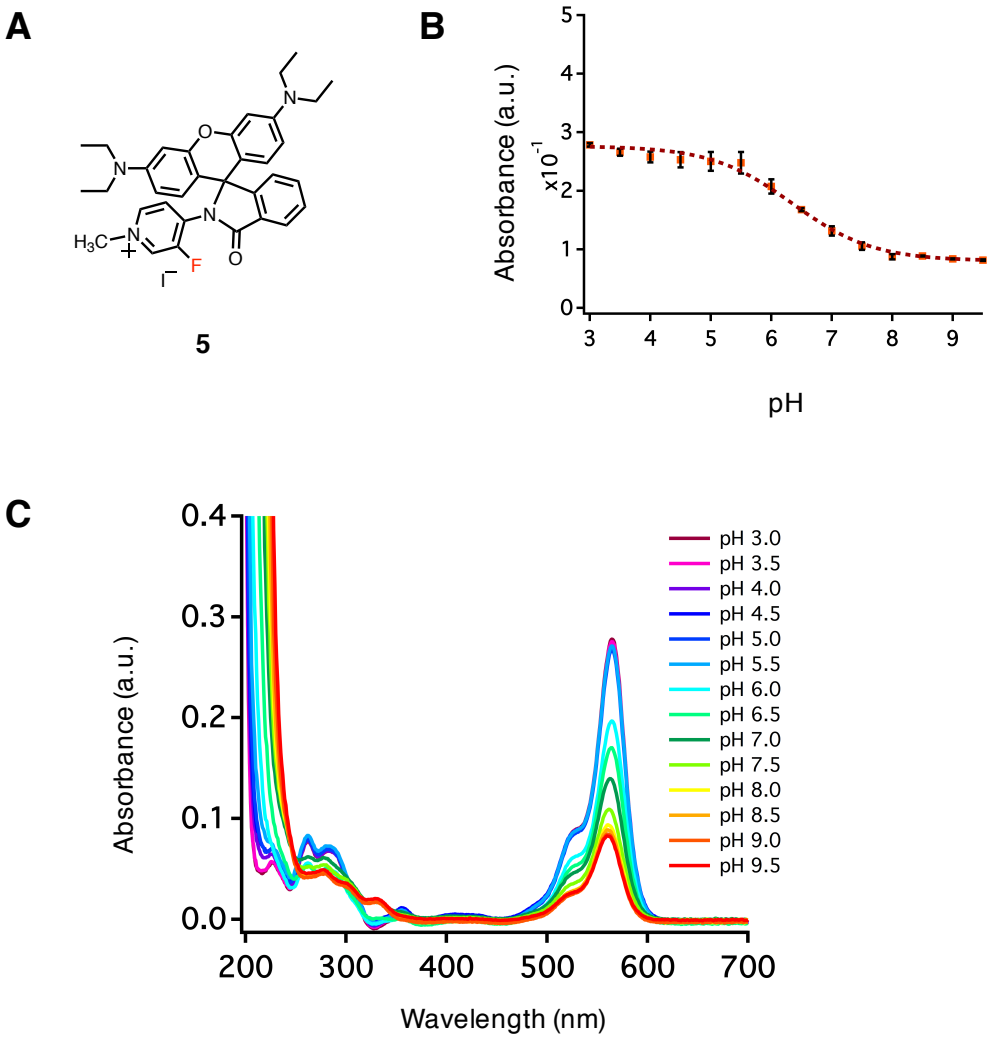


Figure S3: Absorption spectra of rhodamine **5** in bis-tris propane at different pH values. (**A**) Structure of **5**. (**B**) Absorption spectra at 563 nm as a function of pH. (**C**) Absorption spectra of **5** across different pH ranges. Error bars denote the standard deviation from three independent experiments.

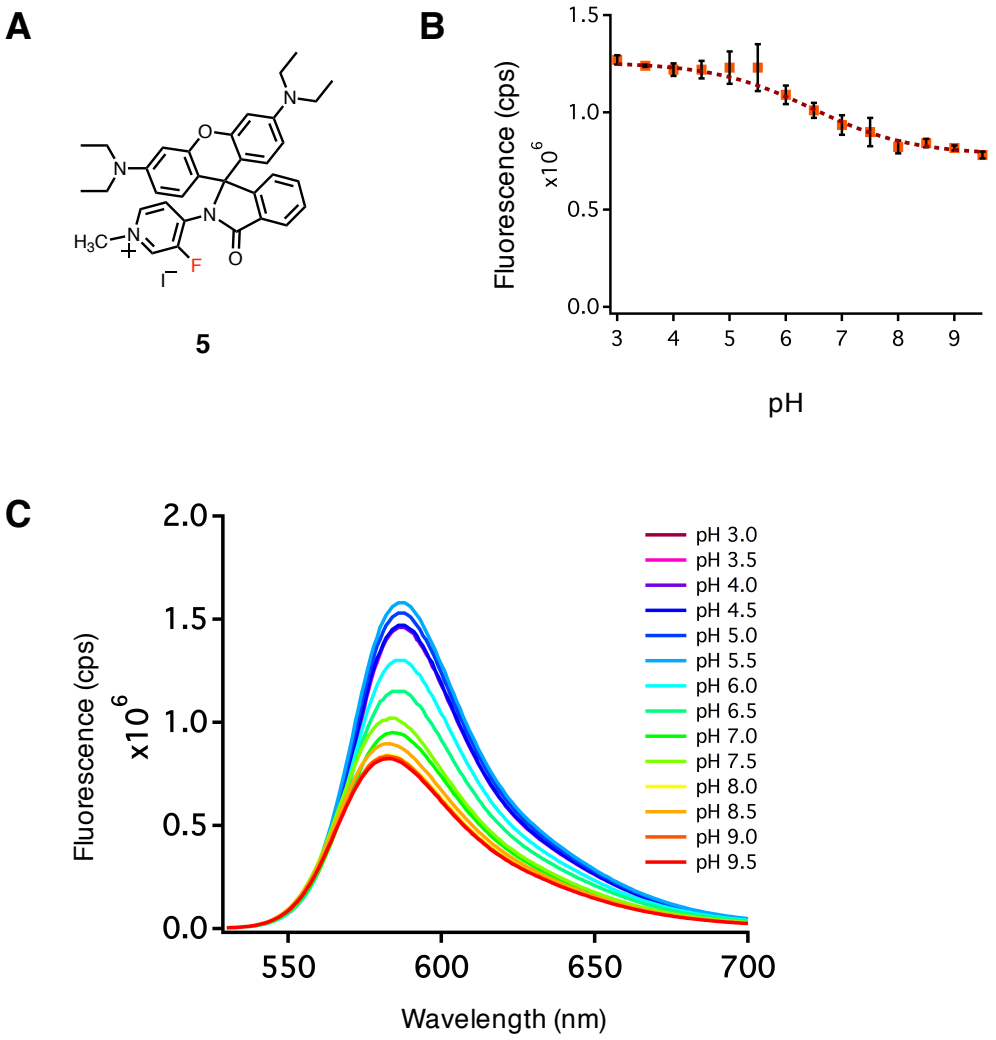


Figure S4: Fluorescence emission from rhodamine **5** in bis-tris propane at different pH values. (**A**) Structure of **5**. (**B**) Fluorescence emission at 578 nm as a function of pH. (**C**) Fluorescence emission spectra of **5** across different pH ranges. Error bars denote the standard deviation from three independent experiments.

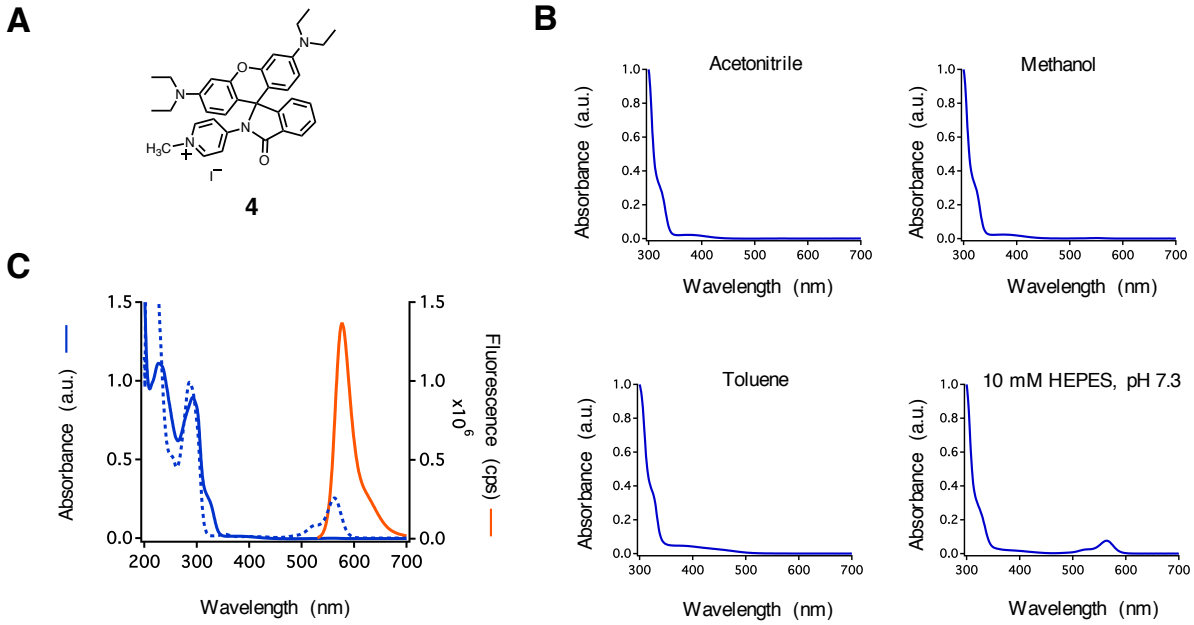


Figure S5: Spectroscopic characterization of rhodamine **4** in different solvents. (**A**) Structure of **4**. (**B**) Absorption spectra of **4** in different solvents. (**C**) Absorption and fluorescence emission spectra of **4**. Absorption spectrum was taken in 1:1 water: acetonitrile (solid blue line) or 1:1 water: acetonitrile with addition of 1 M HCl (dashed blue line). Emission spectrum was taken in acidic ethanol (orange line).

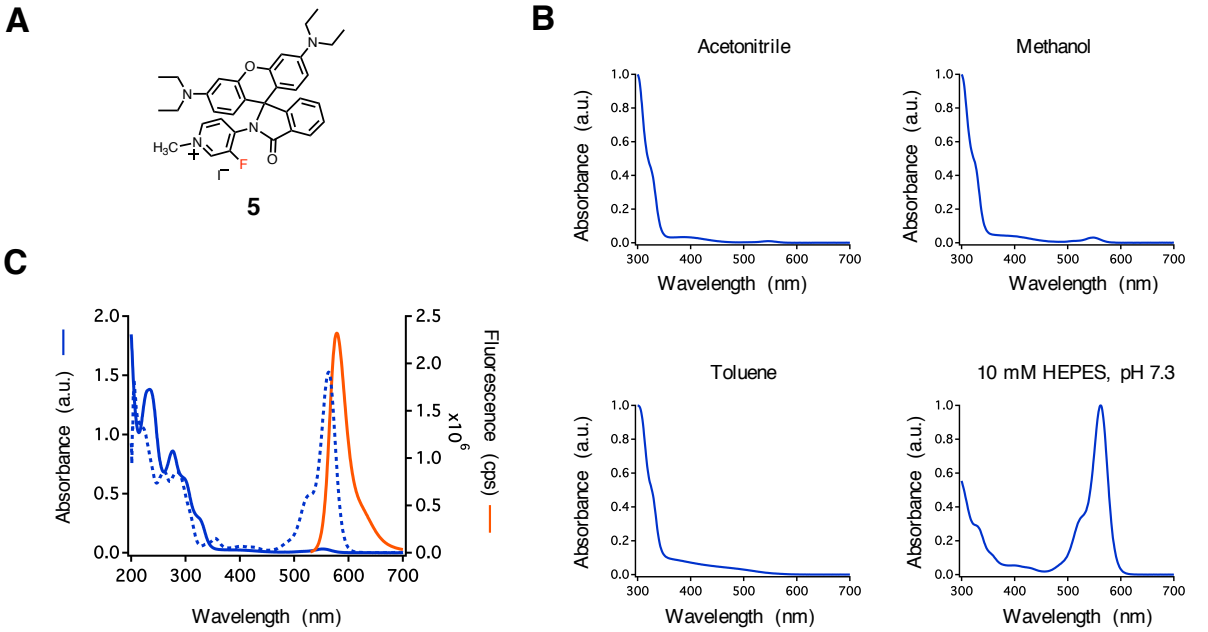


Figure S6: Spectroscopic characterization of rhodamine **5** in different solvents. (**A**) Structure of **5**. (**B**) Absorption spectra of **5** in different solvents. (**C**) Absorption and fluorescence emission spectra of **5**. Absorption spectrum was taken in 1:1 water: acetonitrile (solid blue line) or 1:1 water: acetonitrile with addition of 1 M HCl (dashed blue line). Emission spectrum was taken in acidic ethanol (orange line).

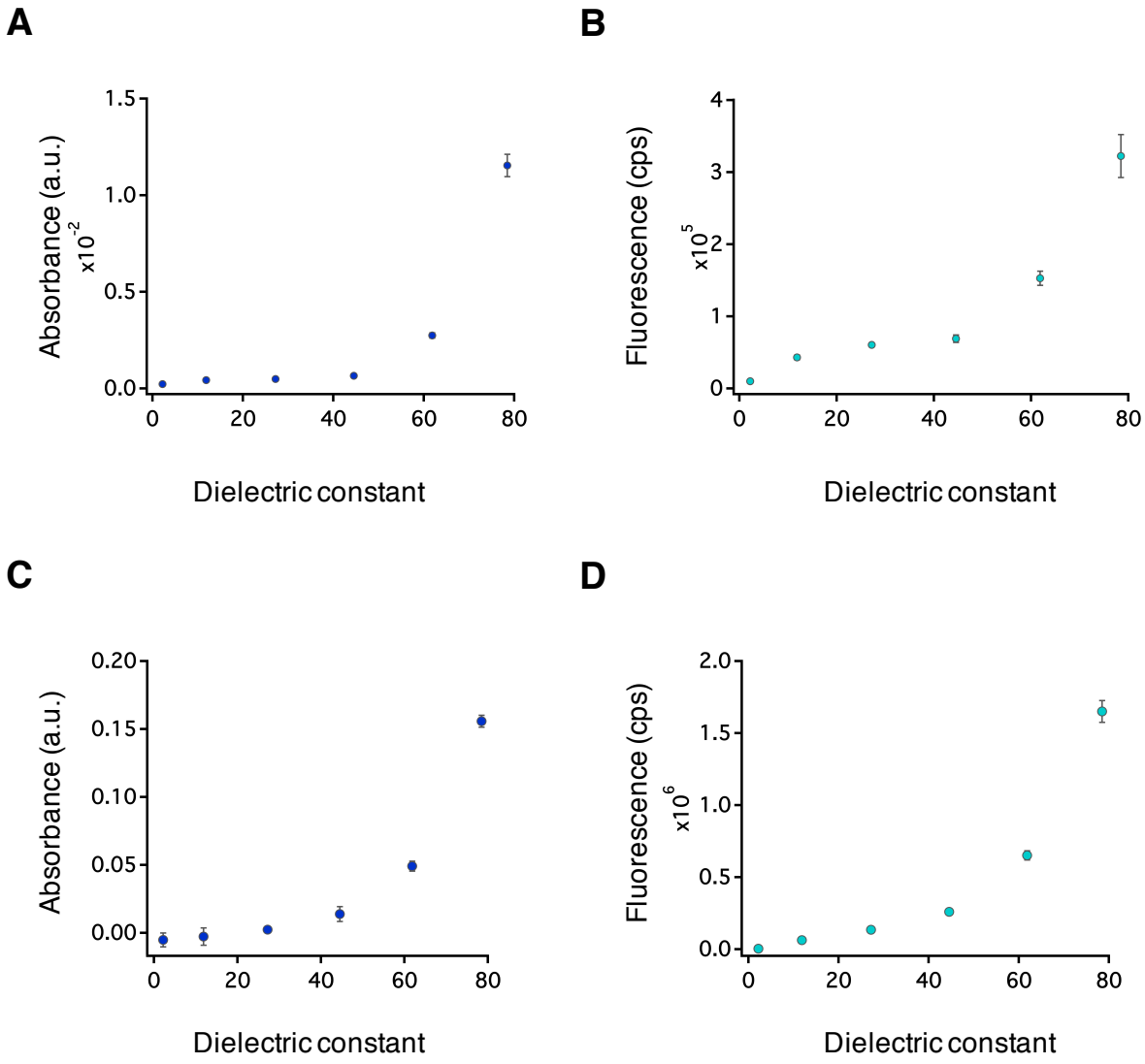


Figure S7: Spectroscopic characterization of rhodamines **4** and **5** in solvents of different dielectric constants. Rhodamines were dissolved in mixtures of dioxane and water and absorbance and fluorescence emission were measured as a function of dielectric constant. (**A**) Absorbance at 561 nm for **4.** (**B**) Fluorescence emission at 577 nm for **4**. (**C**) Absorbance at 563 nm for **5**. (**D**) Fluorescence emission at 578 nm for **5**. Error bars represent the standard deviation from three independent experiments. a.u. = arbitrary units; cps = counts per second.

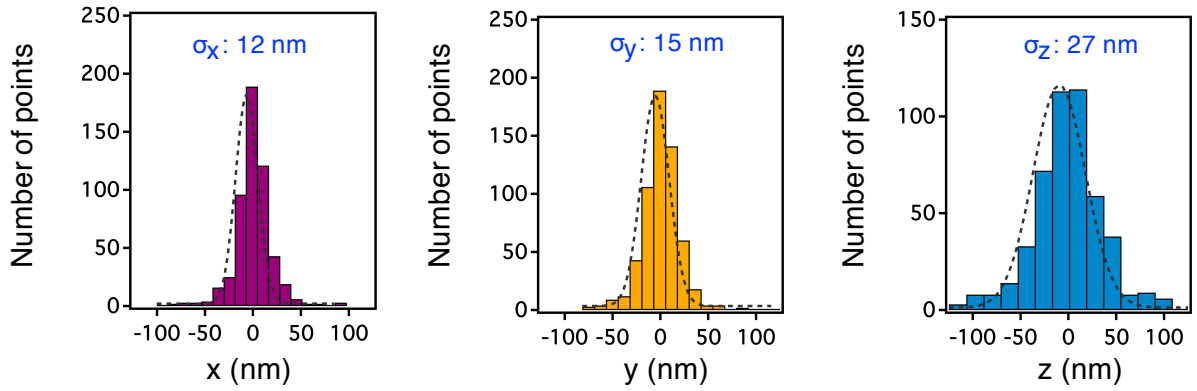


Figure S8: Radial and axial resolutions for *E. coli* cells expressing Tar and labeled with **2**. Precision was calculated with 50 clusters that had ≥5 localizations. Histogram for the X-resolution gave a deviation of 12 nm in the *x* direction (gray dashed line represents Gaussian fit) and a FWHM of 28 nm. Histogram for the Y-resolution gave a deviation of 15 nm in the *y* direction (gray dashed line represents Gaussian fit) and a FWHM of 35 nm. Histogram for the Z-resolution gave a deviation of 27 nm in the *z* direction (gray dashed line represents Gaussian fit) and a FWHM of 64 nm.

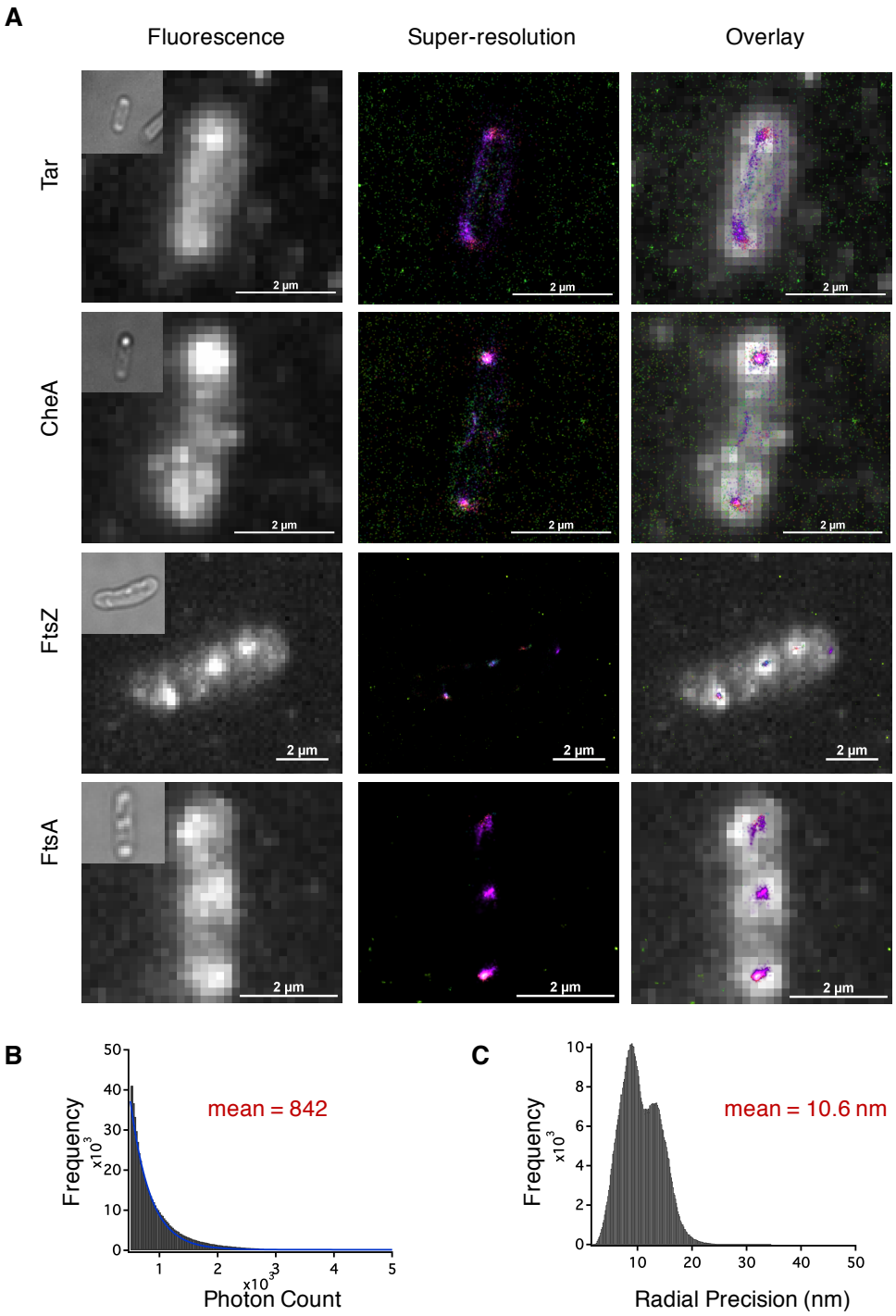


Figure S9: Super-resolution imaging of bacterial proteins in live cells labeled with **3**. Cells expressing one of four bacterial proteins were labeled with **1** and **3**. (**A**) STORM images of bacterial proteins with polar localization (Tar and CheA) or septal localization (FtsZ and FtsA) expressed in *E. coli*. Brightfield images of cells are shown in the top left corner of fluorescence images. (**B**) Histogram indicating number of detected photons during image acquisition with fit to single exponential. The mean number of photons was calculated from the exponential fit (blue curve). (**C**) Mean radial precision for imaging in live bacterial cells.

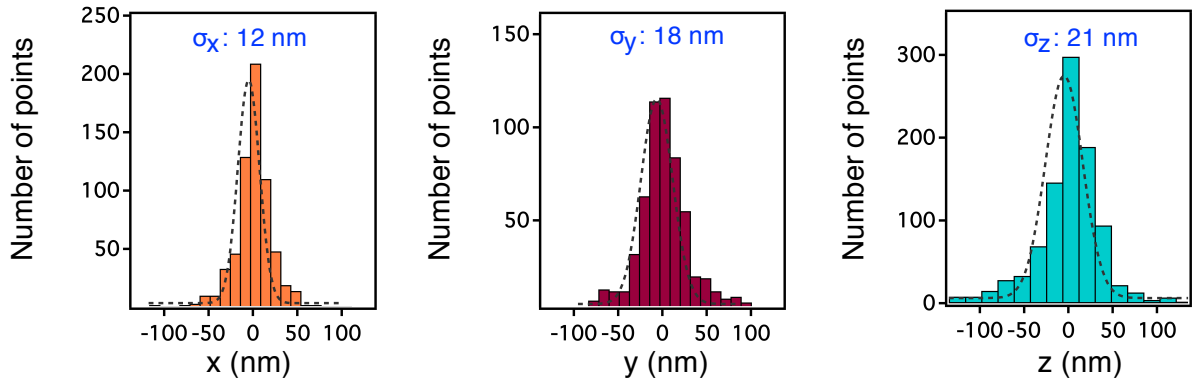


Figure S10: Radial and axial resolutions for *E. coli* cells expressing Tar and labeled with **3**. Precision was calculated with 50 clusters that had ≥5 localizations. Histogram for the X-resolution gave a deviation of 12 nm in the *x* direction (gray dashed line represents Gaussian fit) and a FWHM of 28 nm. Histogram for the Y-resolution gave a deviation of 18 nm in the *y* direction (gray dashed line represents Gaussian fit) and a FWHM of 42 nm. Histogram for the Z-resolution gave a deviation of 21 nm in the *z* direction (gray dashed line represents Gaussian fit) and a FWHM of 49 nm.

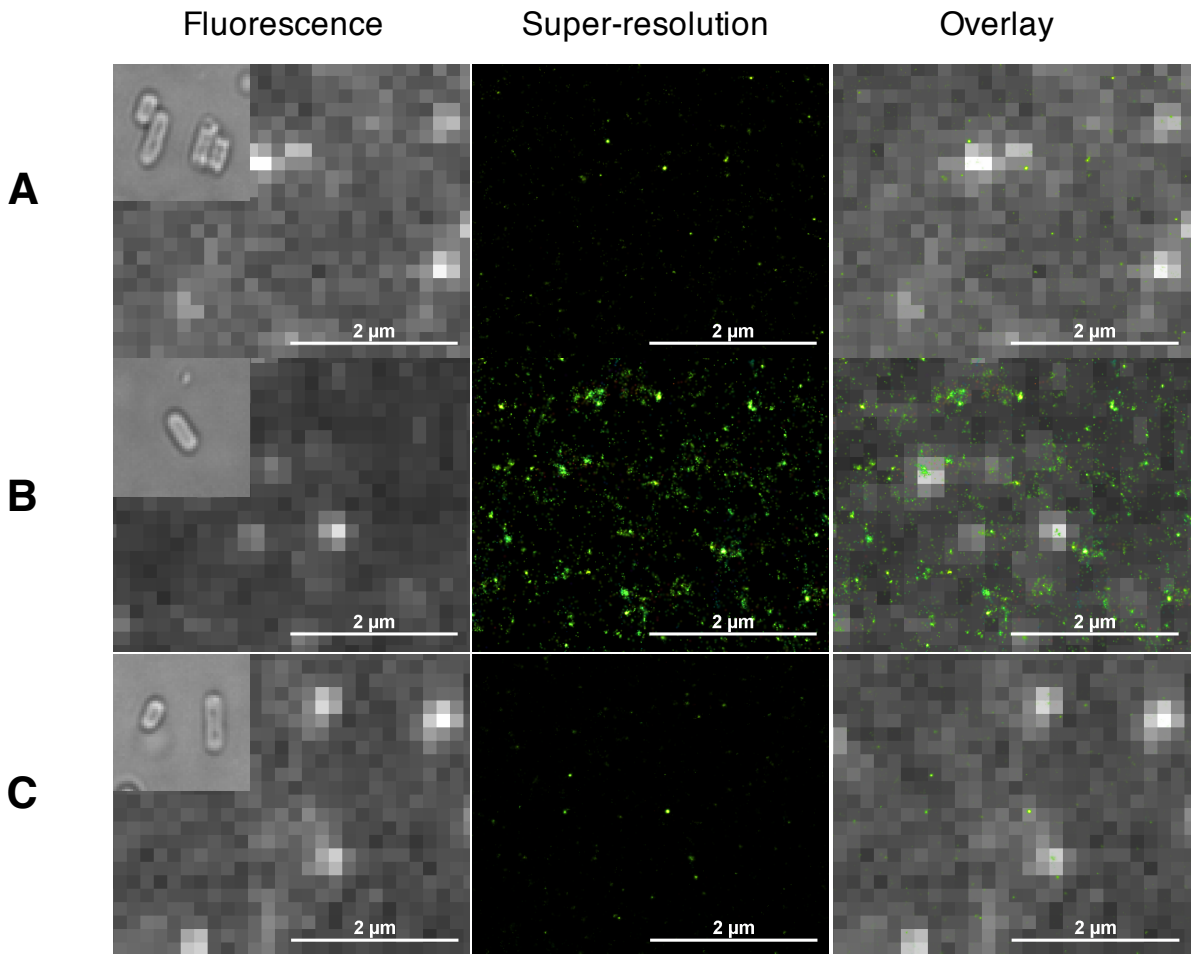


Figure S11: Control imaging experiments with live cells labeled with **2**. (**A**) Cells that did not express the protein of interest (Tar), labeled only with **2** (no added **1**). (**B**) Cells that did not express the protein of interest (Tar), treated with **1** and **2**. (**C**) Cells that expressed Tar labeled only with **2** (no added **1**). None of these controls shows any distinct localization pattern. For all images, brightfield images of cells are shown in the top left corner of the fluorescence image.

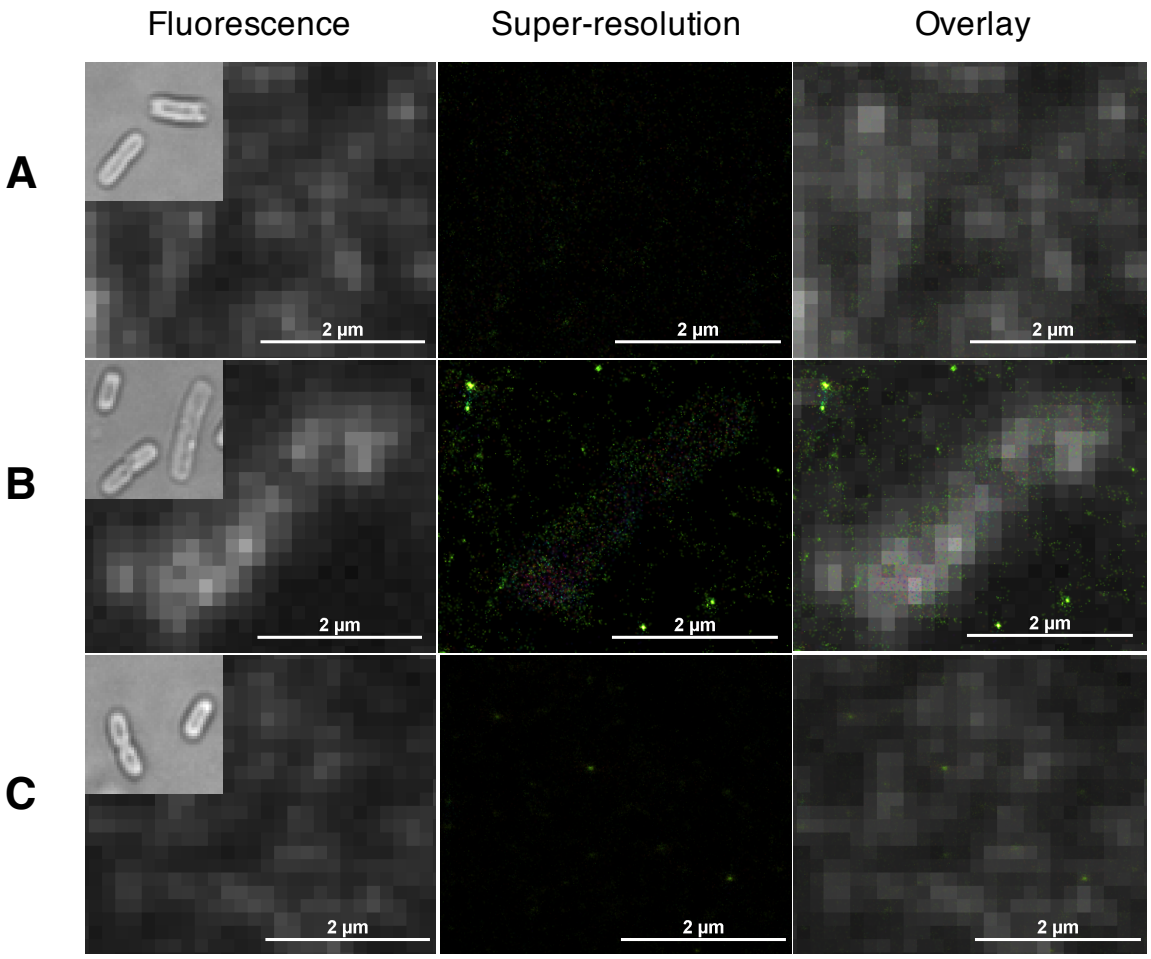


Figure S12: Control imaging experiments with live cells labeled with **3**. (**A**) Cells that do not express the protein of interest (Tar) and not been treated with **1** but labeled with **3** show no distinct localization patterns. (**B**) Cells that do not express the protein of interest (Tar) but have been treated with **1** and **3** show no distinct localization patterns. (**C**) Cells that express Tar but are not treated with **1** and are labeled with **3** show no distinct localization patterns. For all images, brightfield images of cells are shown in the top left corner of the fluorescence image.

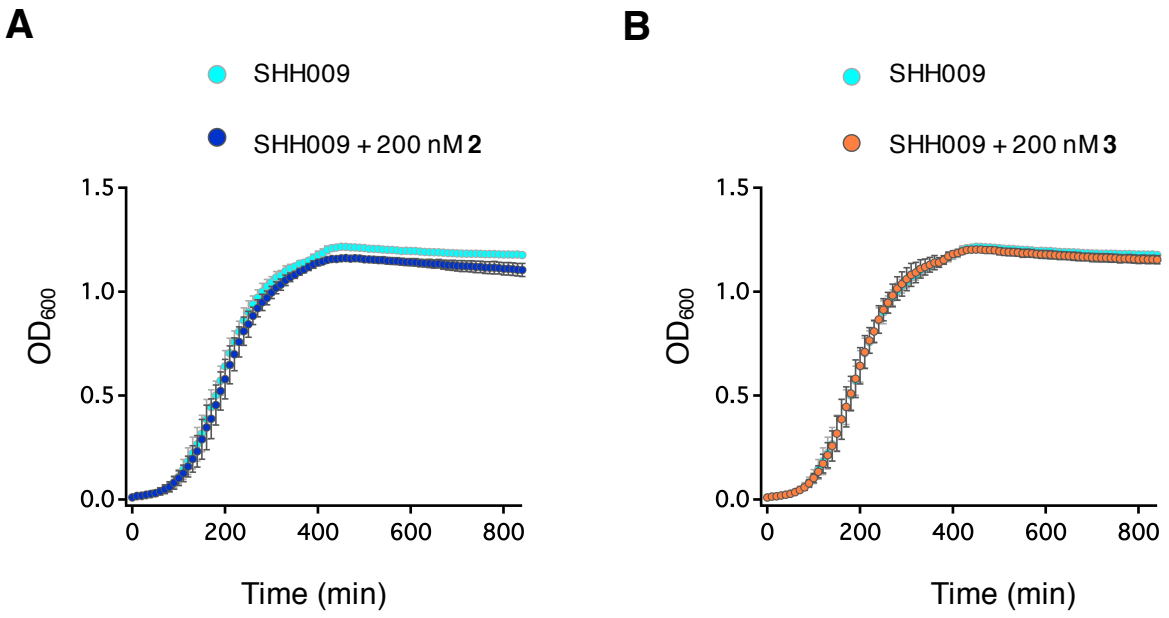
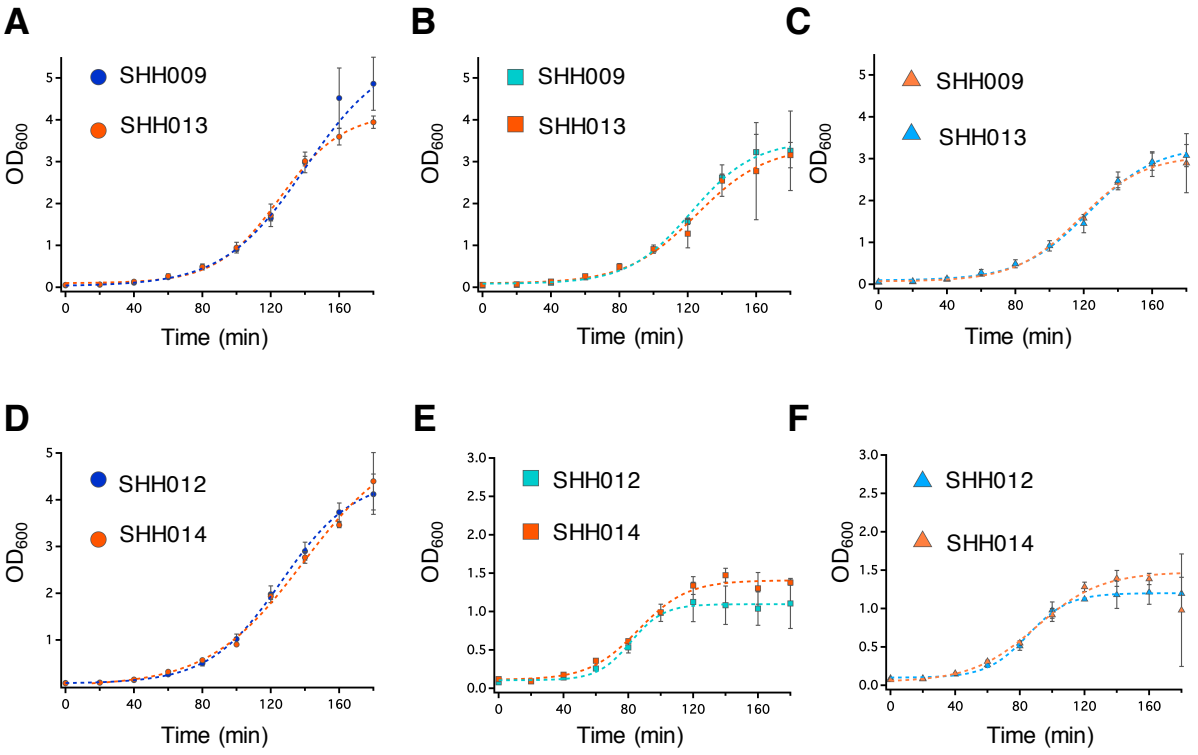


Figure S13: Addition of dyes **2** or **3** does not affect cell growth. Cells were diluted from an overnight culture to an OD_{600} = 0.01 in LB. Cells were then treated with either (**A**) **2** or (**B**) **3** and continued to grow through the course of 14 h. OD_{600} was monitored every 10 min for each culture. *E. coli* strain SHH009 (strain bearing plasmids pBAD24-hCaNB-Tar-cmyc and pHV738-NMT-MetAP) was used in this experiment. Error bars represent the standard deviation from three independent experiments.



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Name	Strain	Genotype	Description	Reference
SHH009	BL21	Amp^R, Kan^R pBAD24-hCaNB-Tar-myc pHV738-NMT-MetAP	Strain expressing Tar with NMT recognition sequence under control of pBAD promoter.	[1]
SHH013	BL21	Amp^R, Kan^R pBAD24-Tar-myc pHV738-NMT-MetAP	Strain expressing Tar without NMT recognition sequence under control of pBAD promoter.	This study.
SHH012	BL21	Amp^R, Kan^R pBAD24-hCaNB-FtsA-myc pHV738-NMT-MetAP	Strain expressing FtsA with NMT recognition sequence under control of pBAD promoter.	[1]
SHH014	BL21	Amp^R, Kan^R pBAD24-FtsA-myc pHV738-NMT-MetAP	Strain expressing FtsA without NMT recognition sequence under control of pBAD promoter.	This study.

Figure S14: Growth effects of protein expression, the N-terminal NMT recognition sequence, and modification with fatty acid. Overnight cultures of cells bearing pBAD24 plasmids encoding inducible expression of Tar (**A–C**) or FtsA (**D–F**) and constitutive expression of NMT were diluted to OD_{600} = 0.1 in LB medium (20 mL culture in 125 mL flasks) and grown until OD_{600} = 0.5. (**B, E**): At OD_{600} = 0.5, induction was performed by addition of 0.2% w/v arabinose; no **1** was added. (**C, F**): Induction and N-terminal labeling (by addition of 0.2% w/v arabinose, 250 μM **1**) was initiated at OD_{600} = 0.5. (**A, D**): Uninduced cells. For all cultures, OD_{600} was measured every 20 min. Error bars denote the standard deviation from three independent experiments. (**G**): Summary of strains generated.

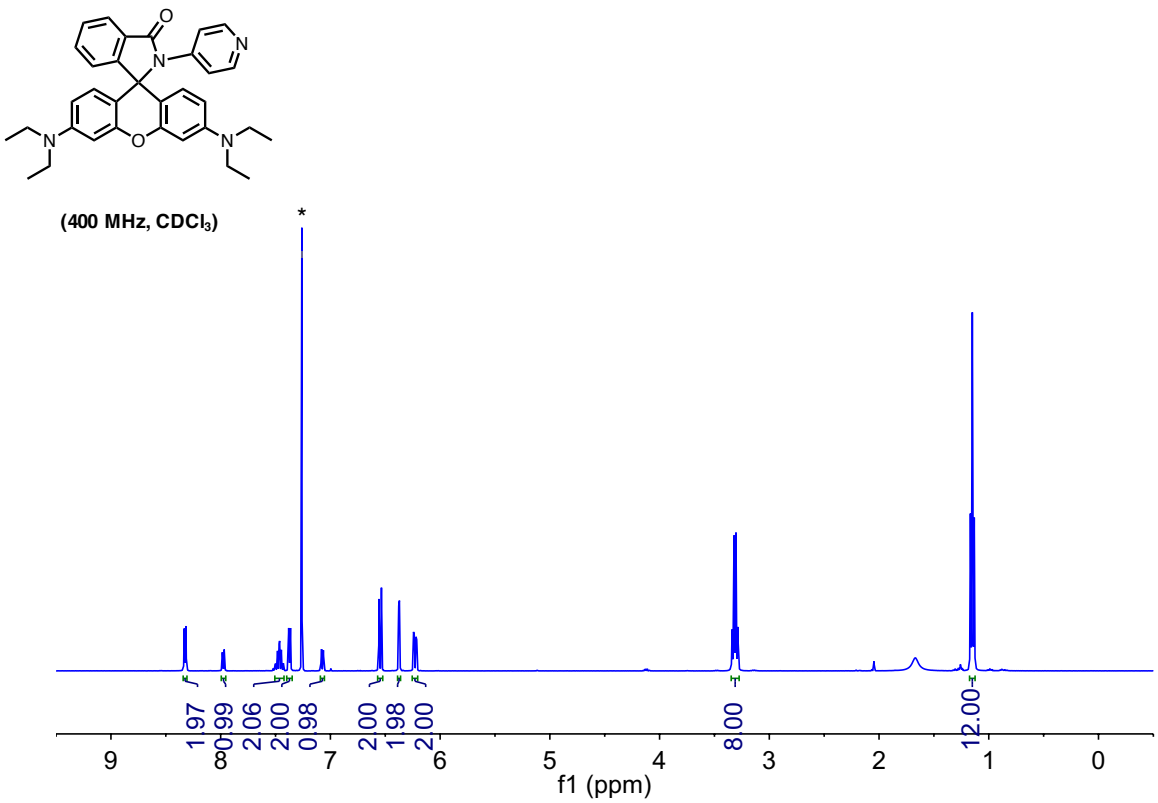


Figure S15: ^{1}H NMR spectrum of compound **S1**. * = chloroform.

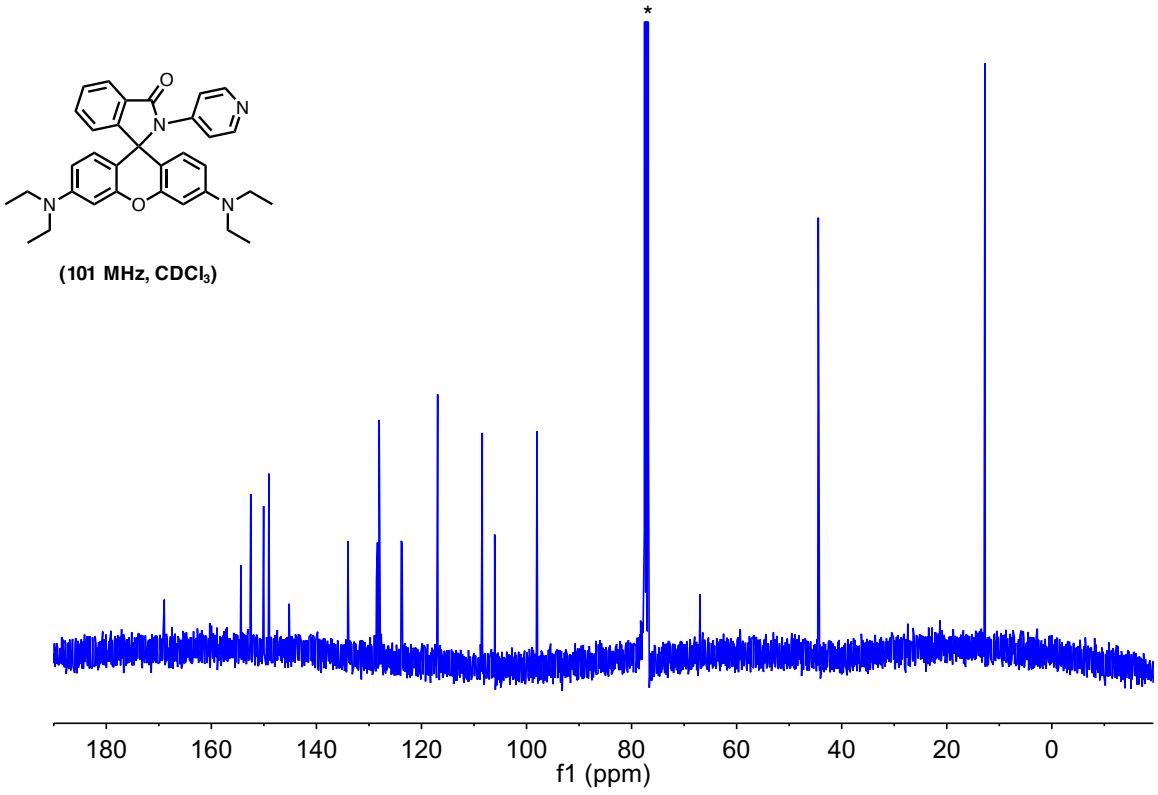


Figure S16: ^{13}C NMR spectrum of compound **S1**. * = chloroform.

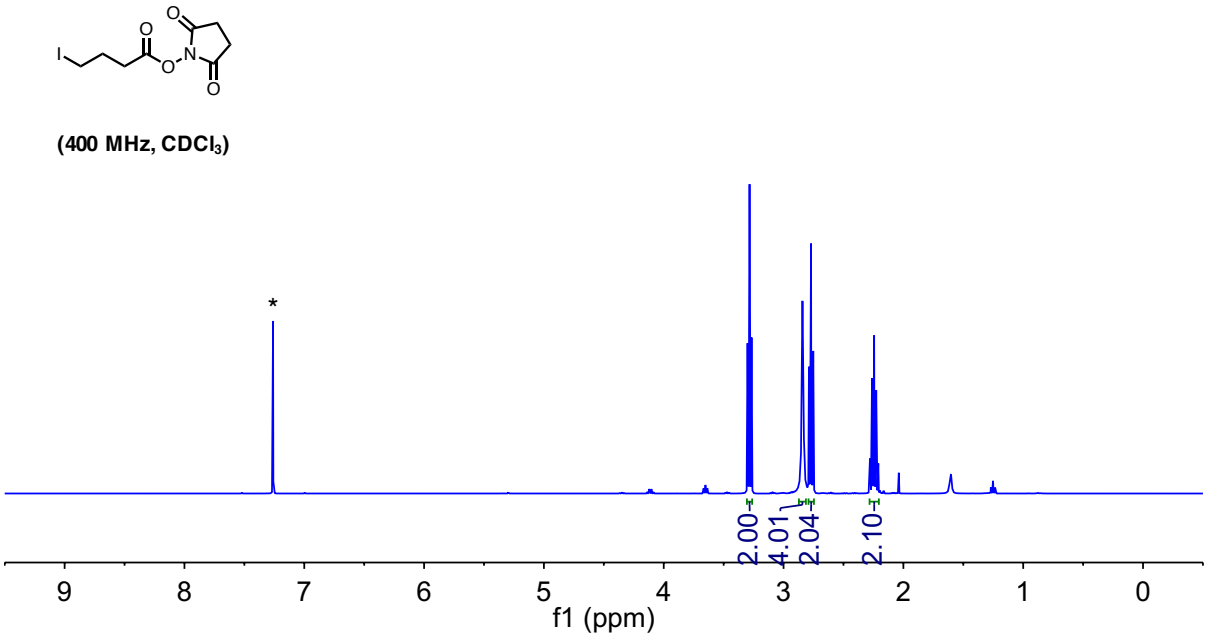


Figure S17: ^{1}H NMR spectrum of compound **S3**. * = chloroform.

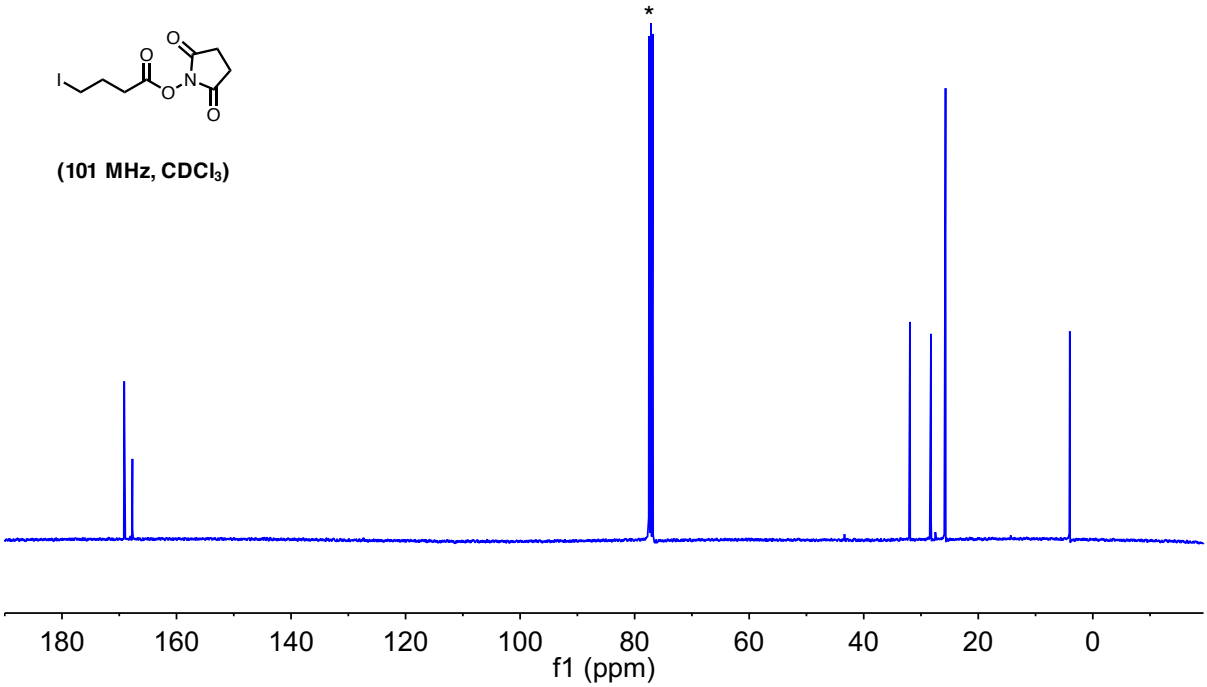


Figure S18: ^{13}C NMR spectrum of compound **S3**. * = chloroform.

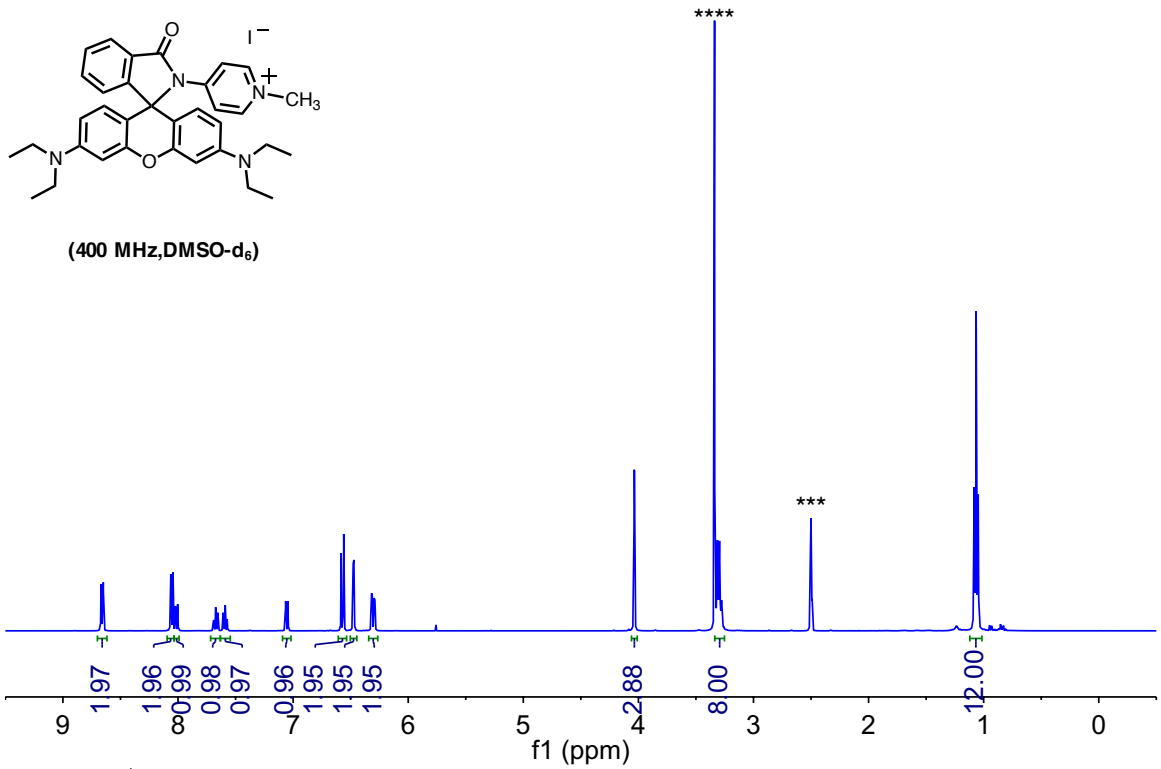


Figure S19: ^{1}H NMR spectrum of compound **4**. *** = dimethyl sulfoxide; **** = water.

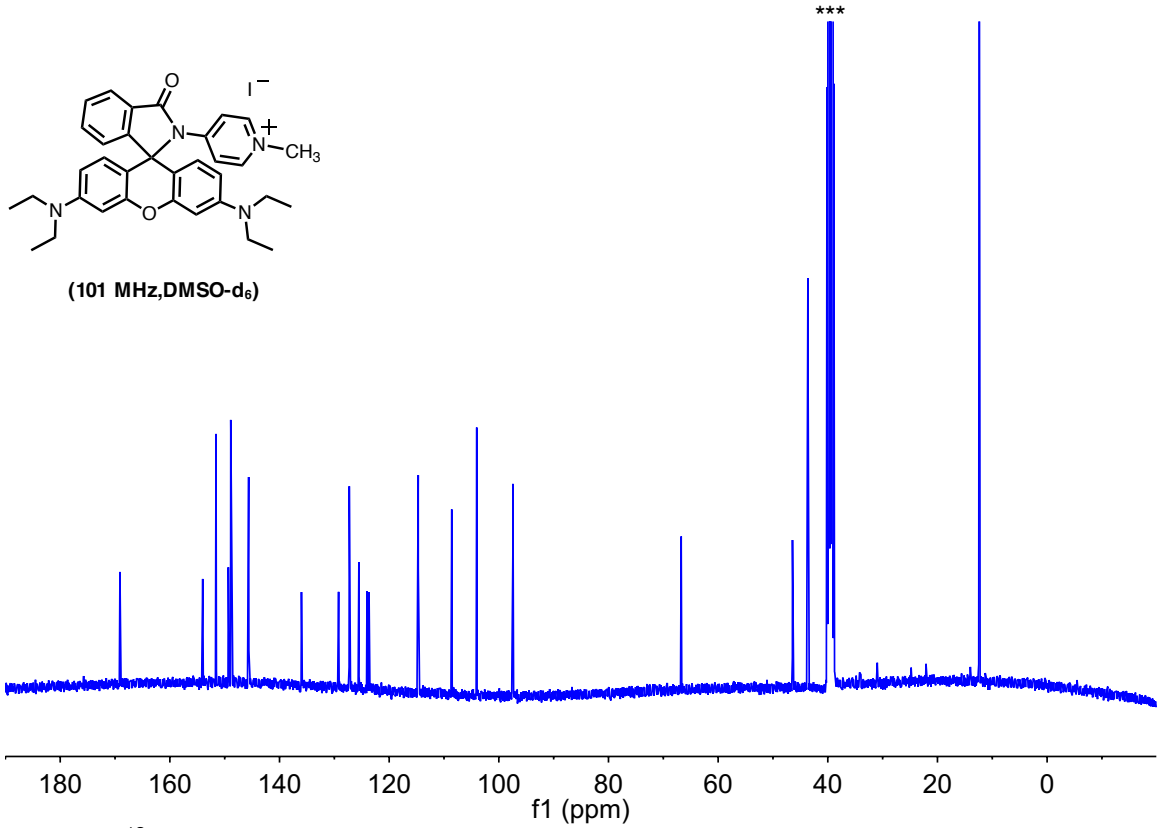


Figure S20: ^{13}C NMR spectrum of compound **4**. *** = dimethyl sulfoxide.

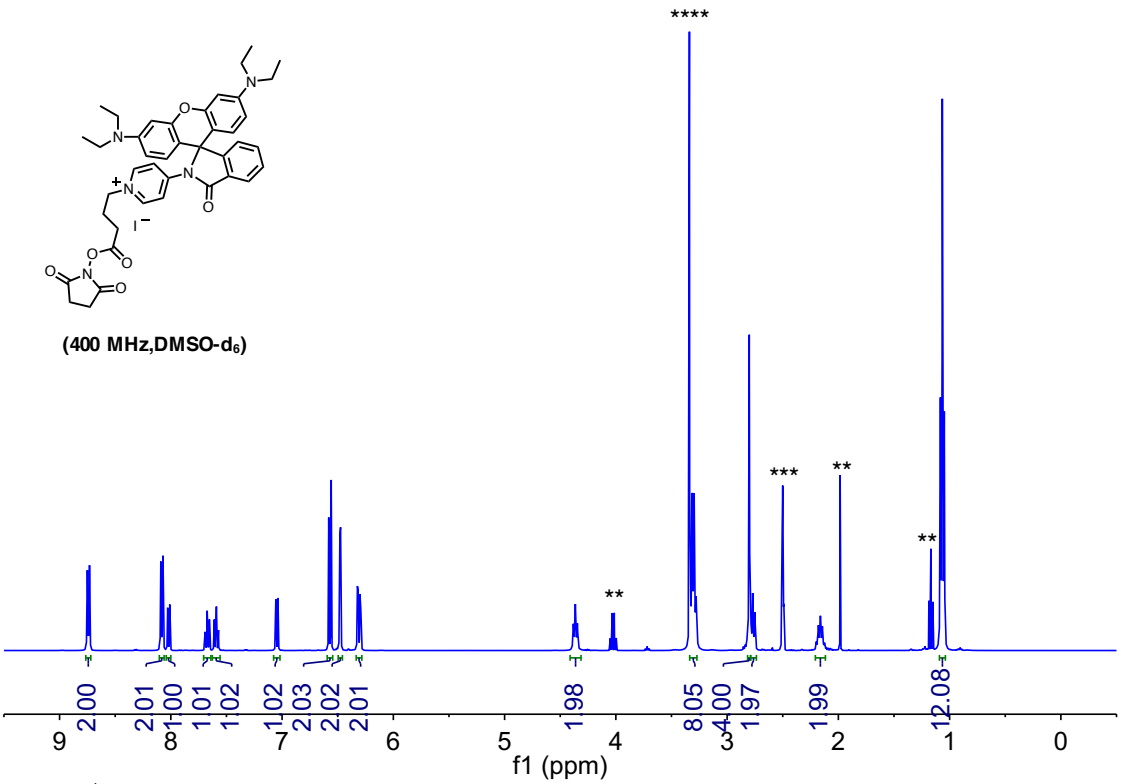


Figure S21: ^{1}H NMR spectrum of compound **S4.** ** = ethyl acetate; *** = dimethyl sulfoxide; **** = water.

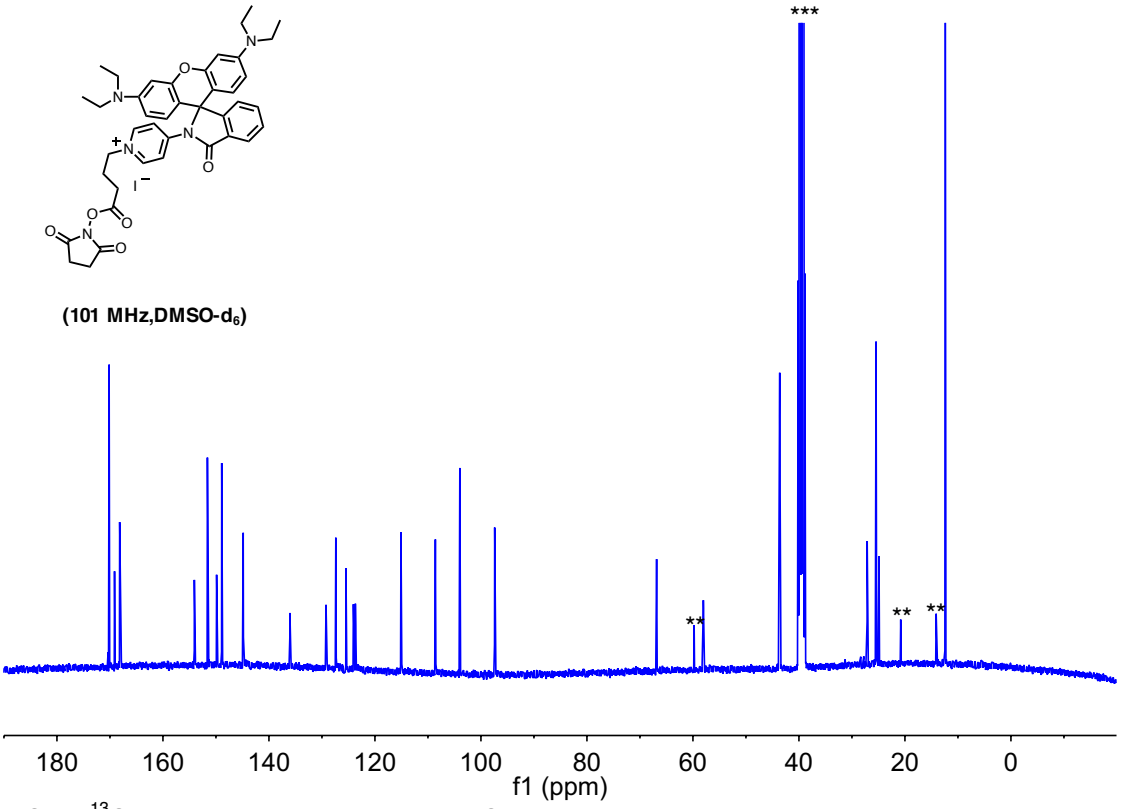


Figure S22: ^{13}C NMR spectrum of compound **S4**. ** = ethyl acetate; *** = dimethyl sulfoxide.

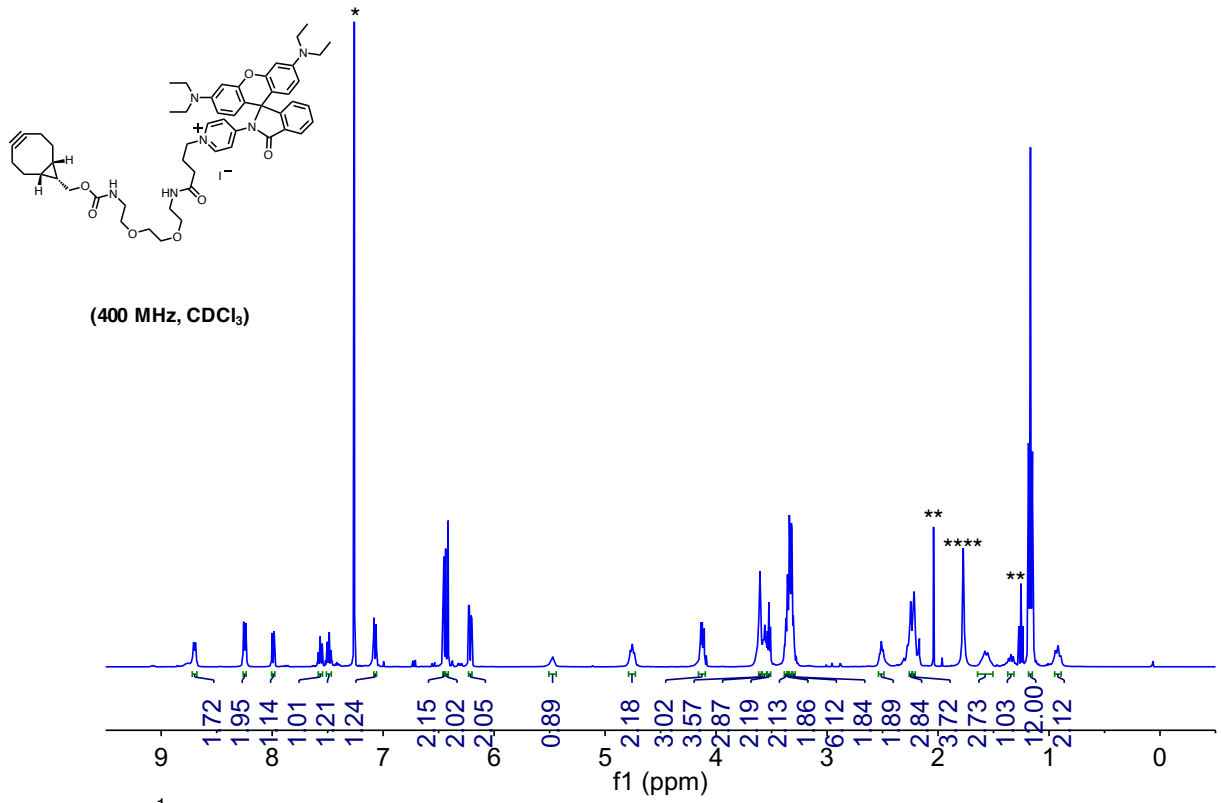


Figure S23: ^{1}H NMR spectrum of compound **2**. * = chloroform; ** = ethyl acetate; **** = water.

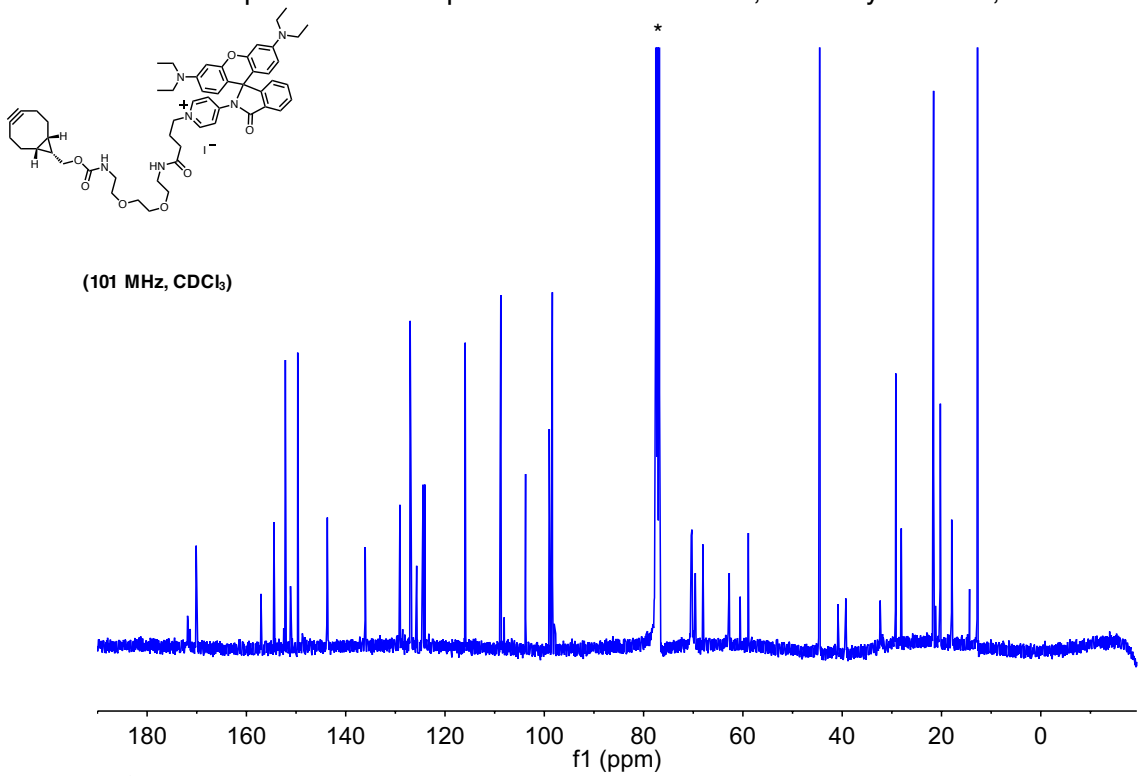


Figure S24: ^{13}C NMR spectrum of compound **2**. * = chloroform.

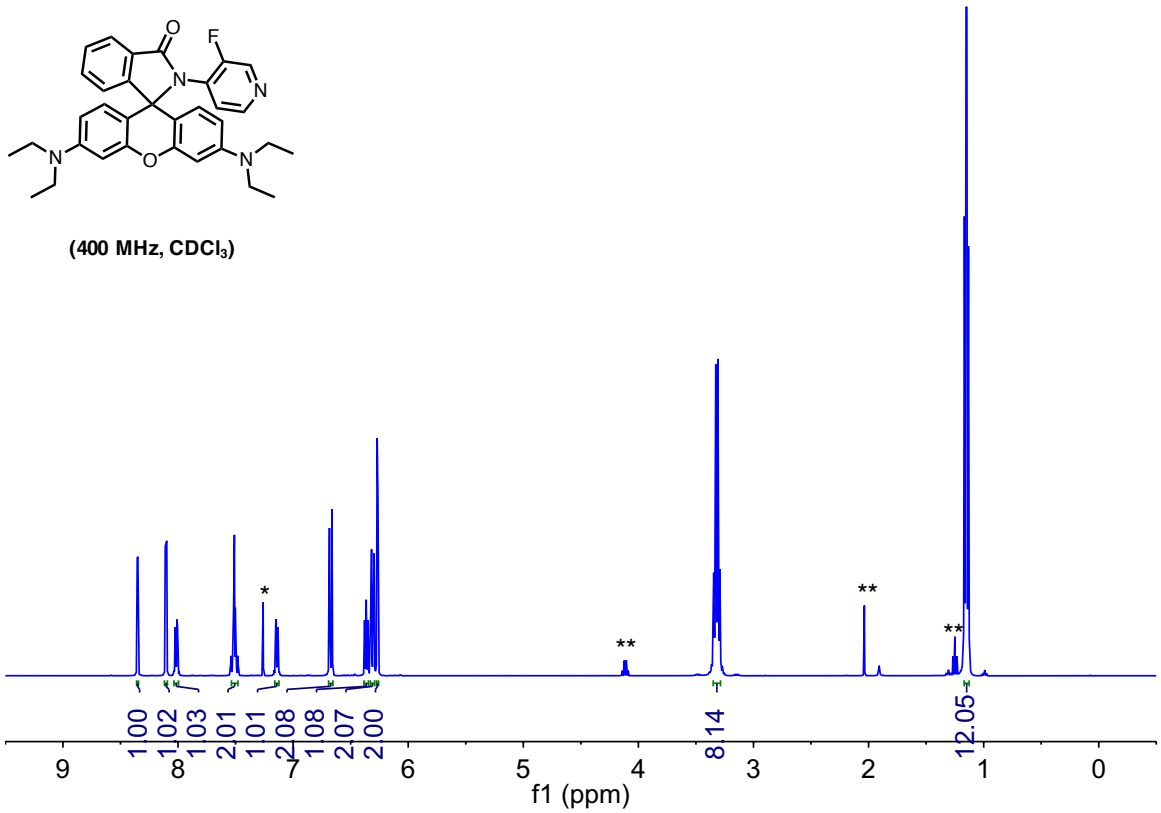


Figure S25: ^{1}H NMR spectrum of compound **S2**. * = chloroform; ** = ethyl acetate.

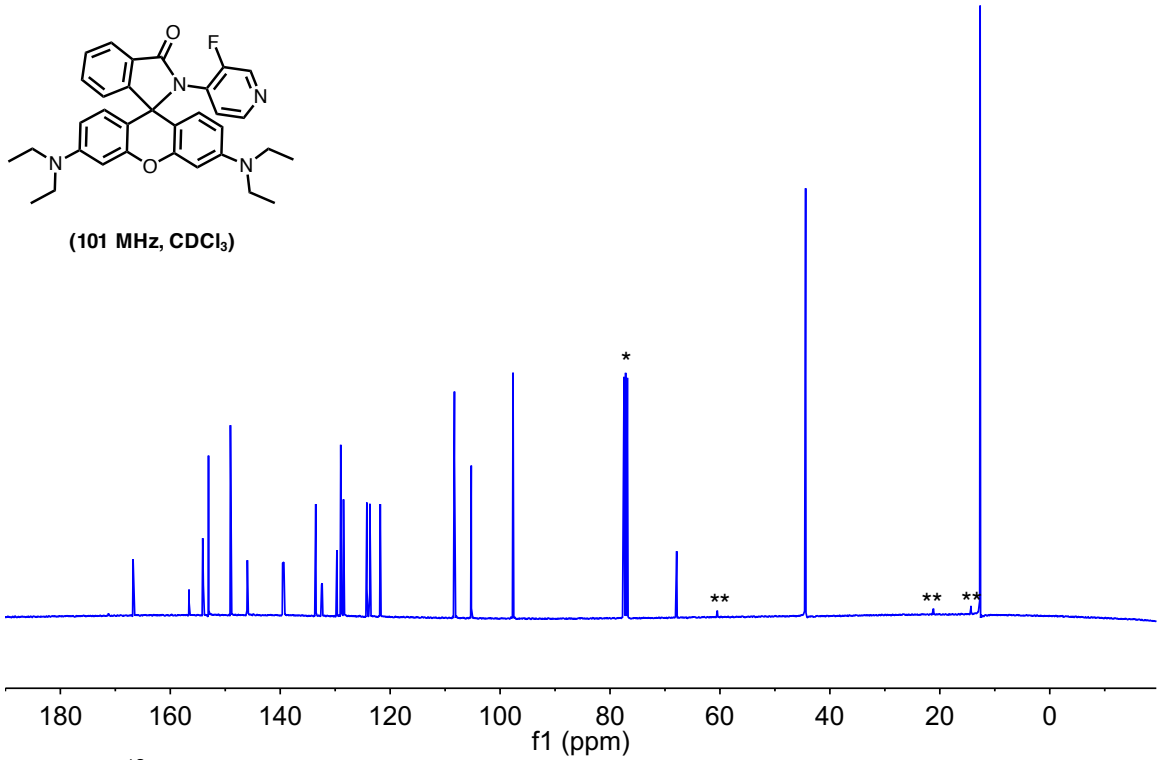


Figure S26: ^{13}C NMR spectrum of compound **S2**. * = chloroform; ** = ethyl acetate.

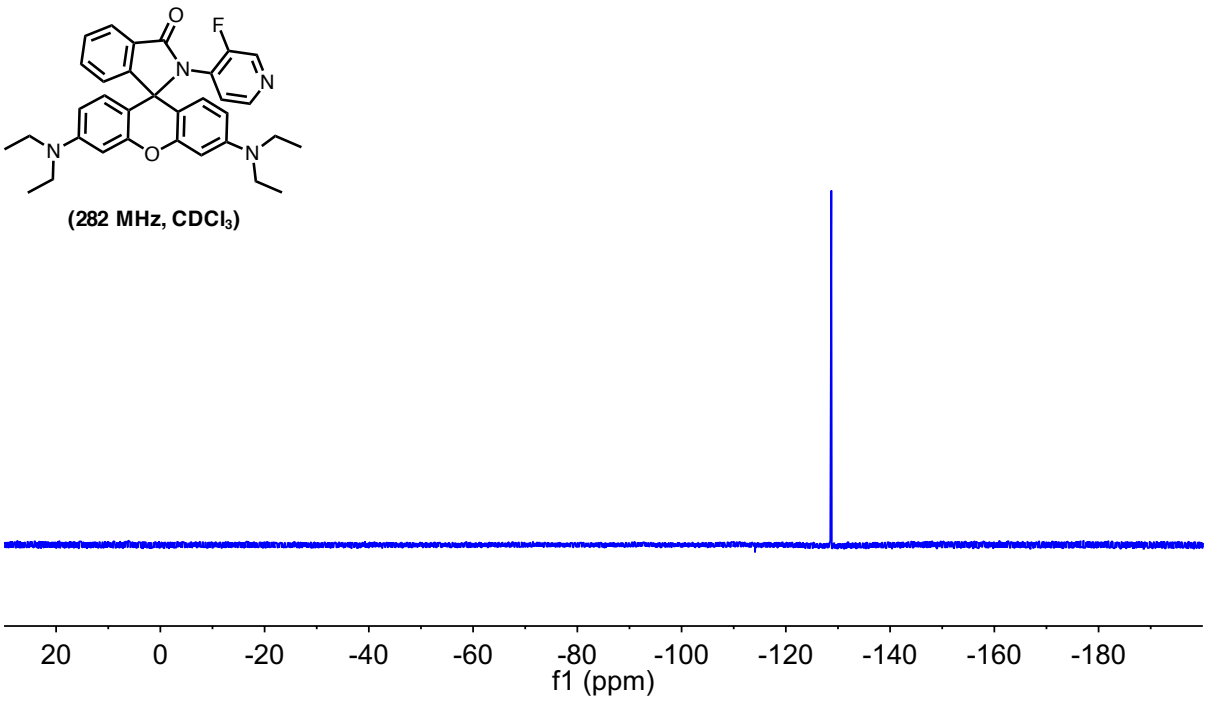


Figure S27: ^{19}F NMR spectrum of compound **S2**.

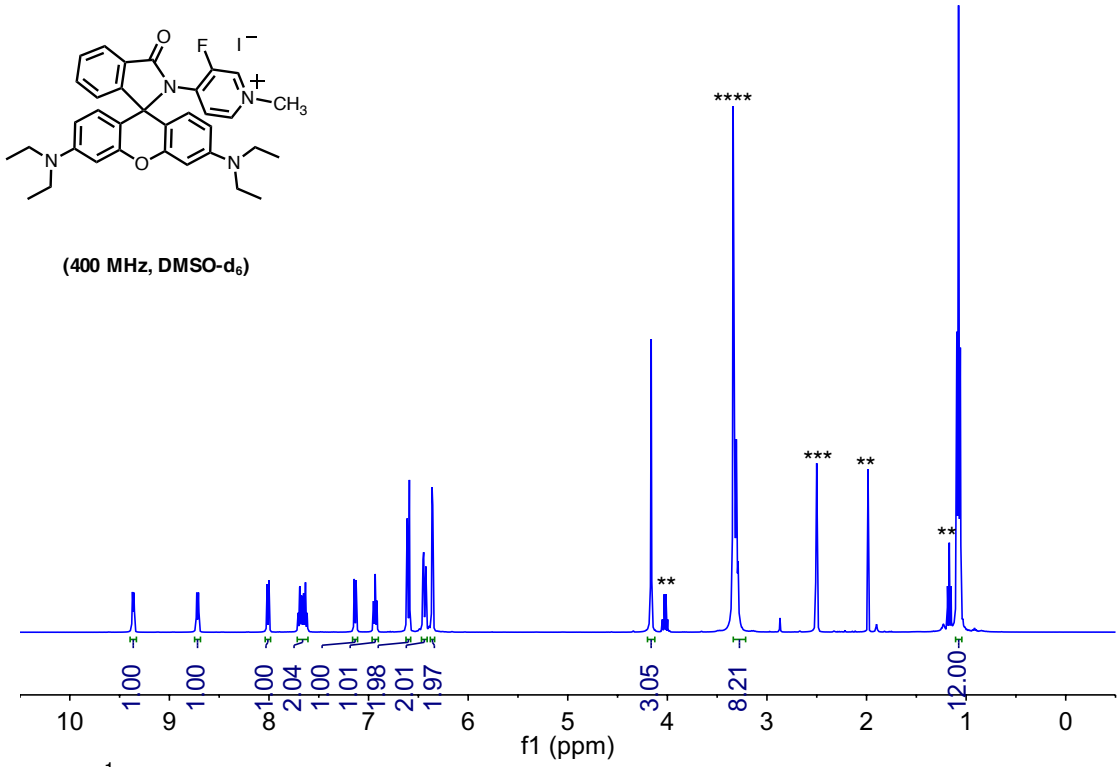


Figure S28: ^{1}H NMR spectrum of compound **5**. ** = ethyl acetate; *** = dimethyl sulfoxide; **** = water.

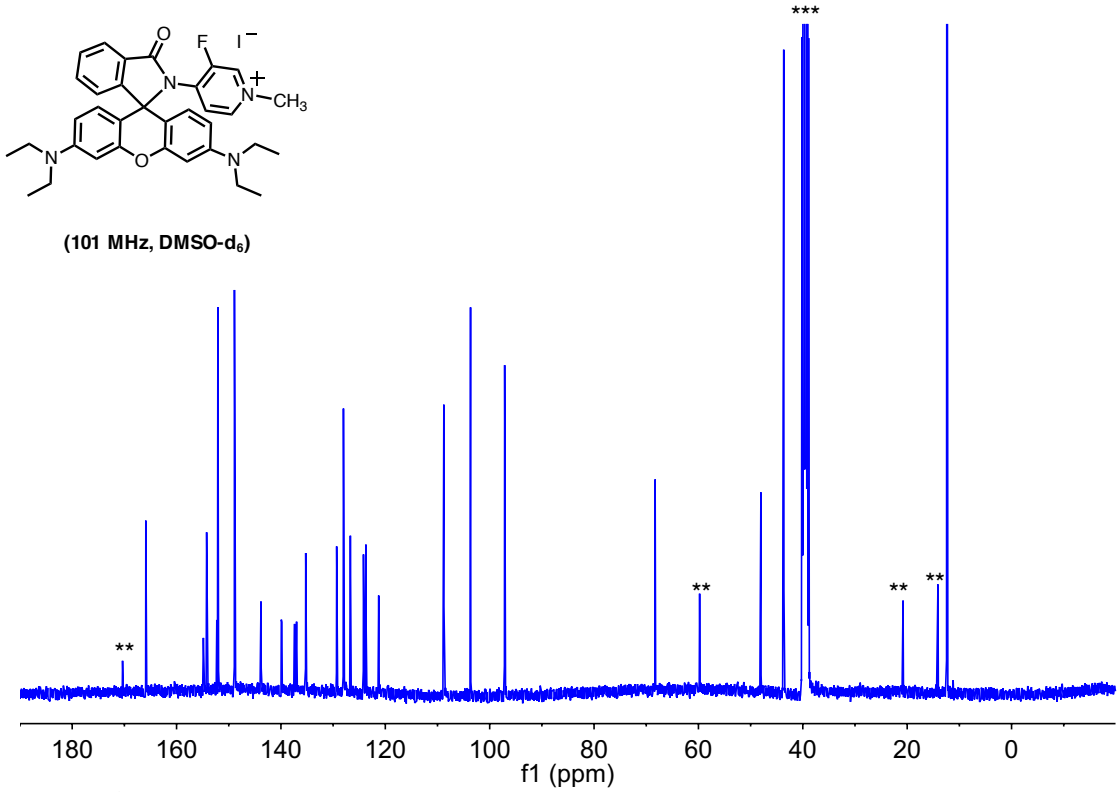


Figure S29: ^{13}C NMR spectrum of compound **5**. ** = ethyl acetate; *** = dimethyl sulfoxide.

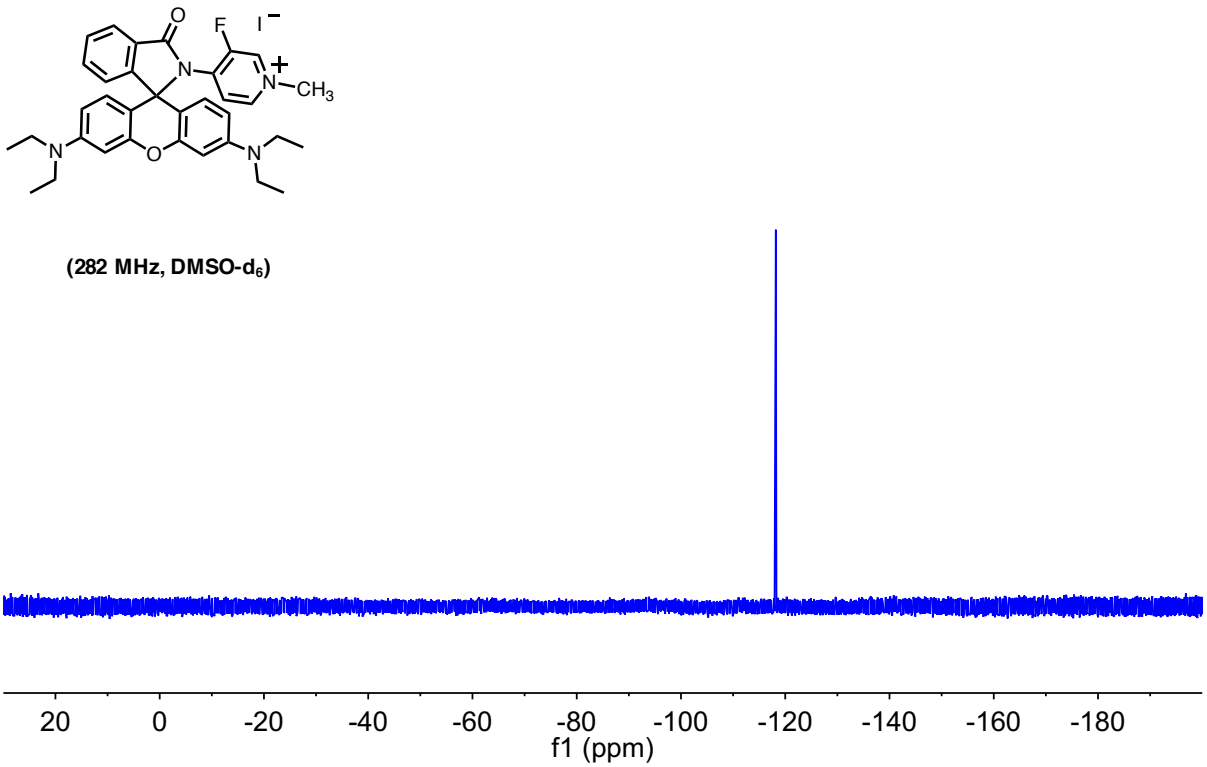


Figure S30: ^{19}F NMR spectrum of compound **5**.

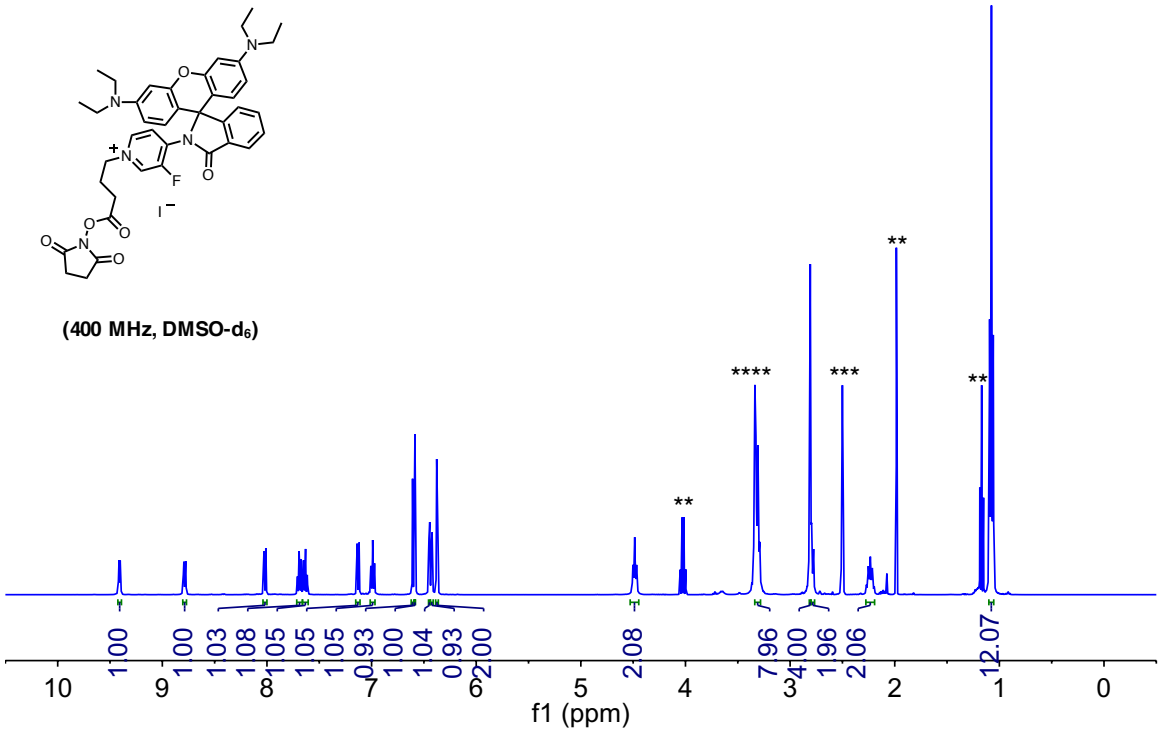


Figure S31: ^{1}H NMR spectrum of compound **S5**. ** = ethyl acetate; *** = dimethyl sulfoxide; **** = water.

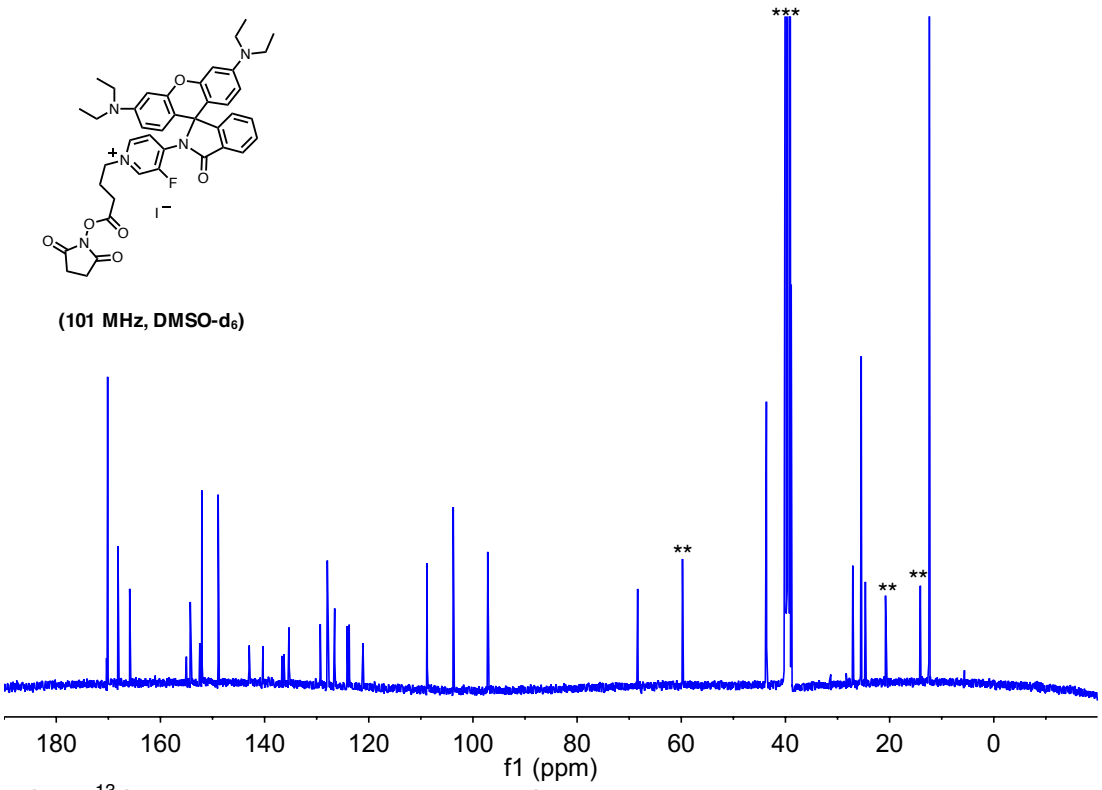


Figure S32: ^{13}C NMR spectrum of compound **S5**. ** = ethyl acetate; *** = dimethyl sulfoxide.

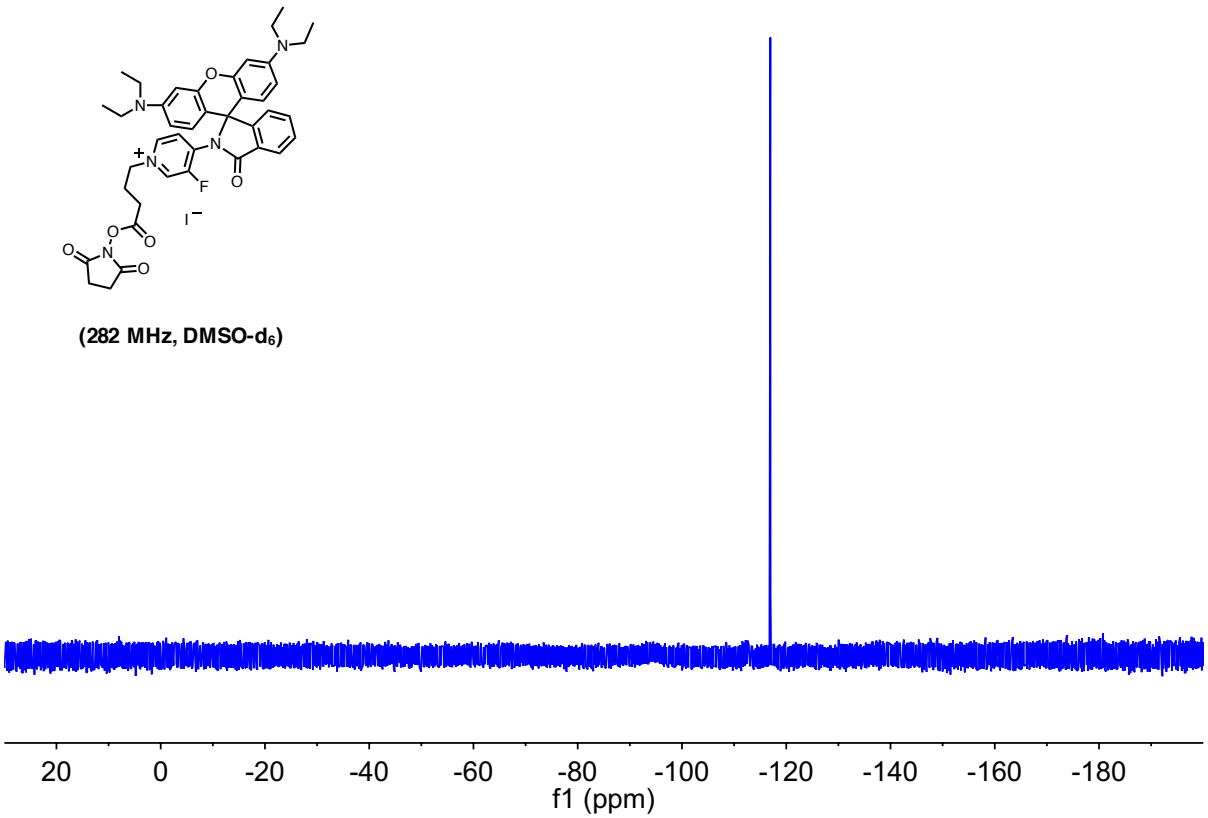


Figure S33: ^{19}F NMR spectrum of compound **S5**.

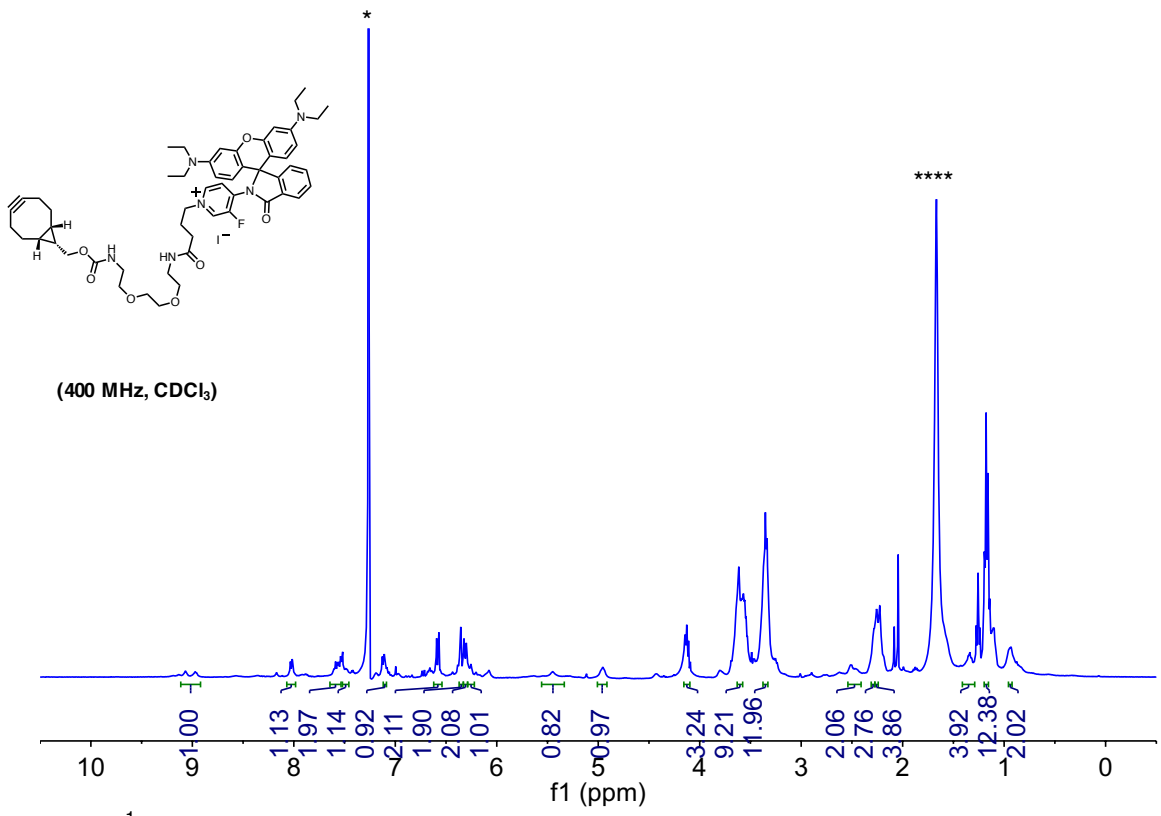


Figure S34: ^{1}H NMR spectrum of compound **3**. * = chloroform; **** = water.

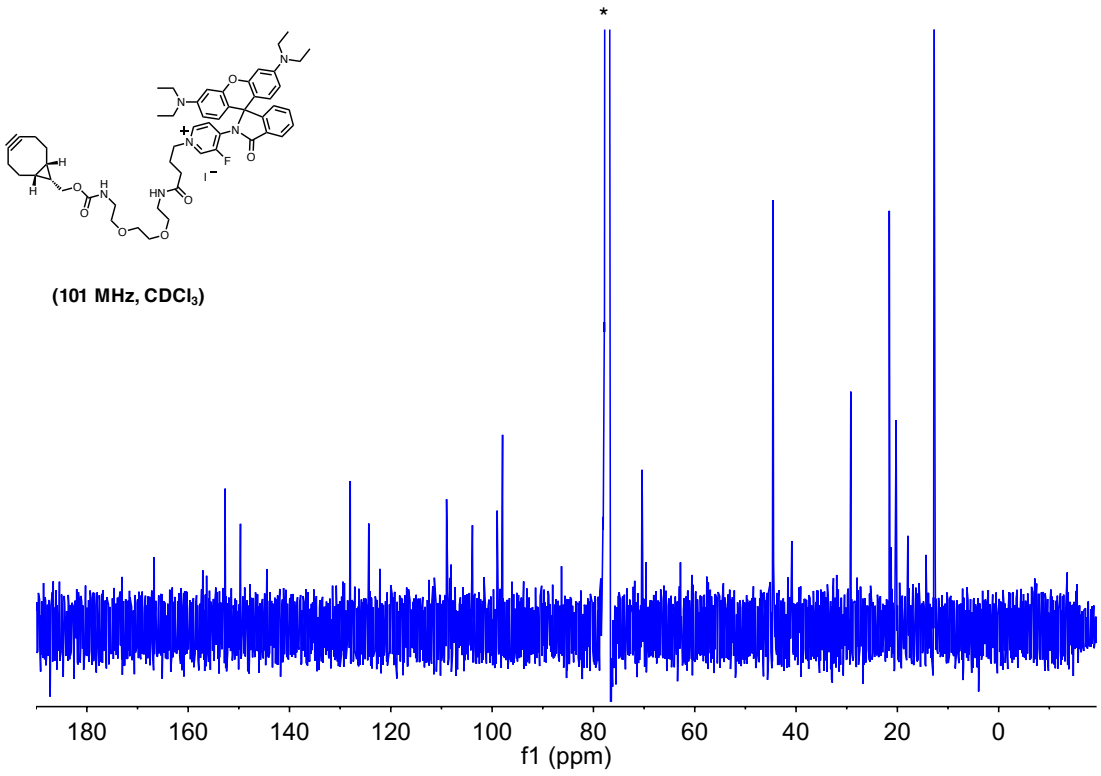
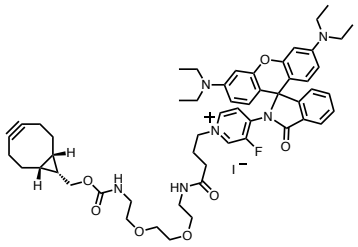


Figure S35: ^{13}C NMR spectrum of compound **3**. * = chloroform.



(376 MHz, $CDCl_3$)

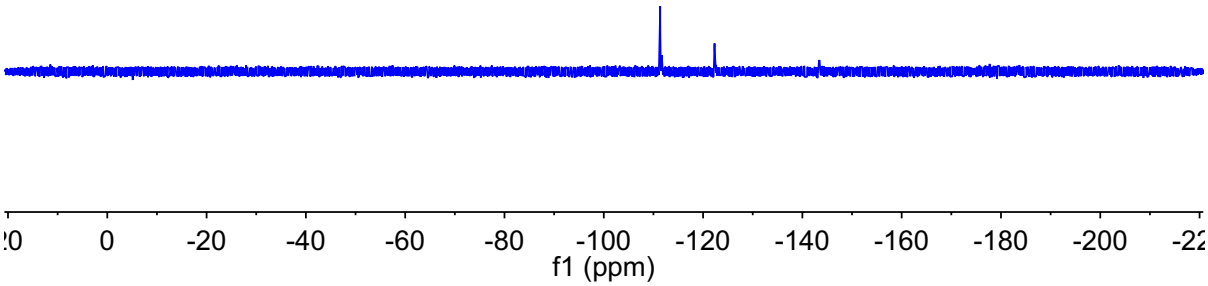


Figure S36: ^{19}F NMR spectrum of compound **3**.

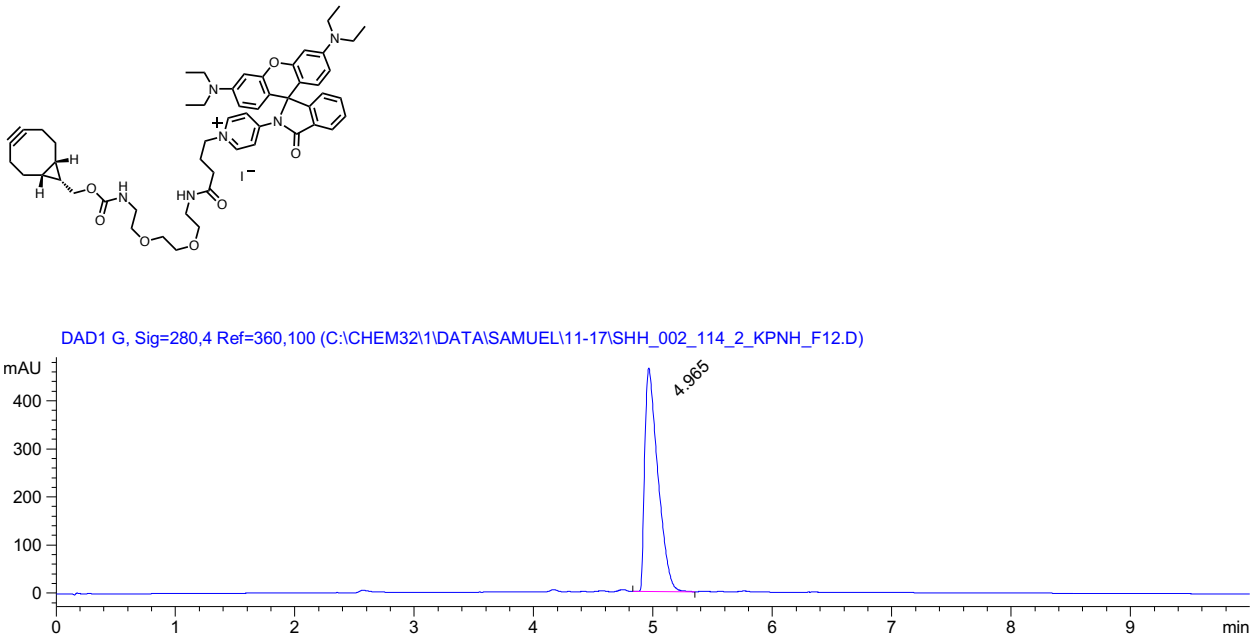
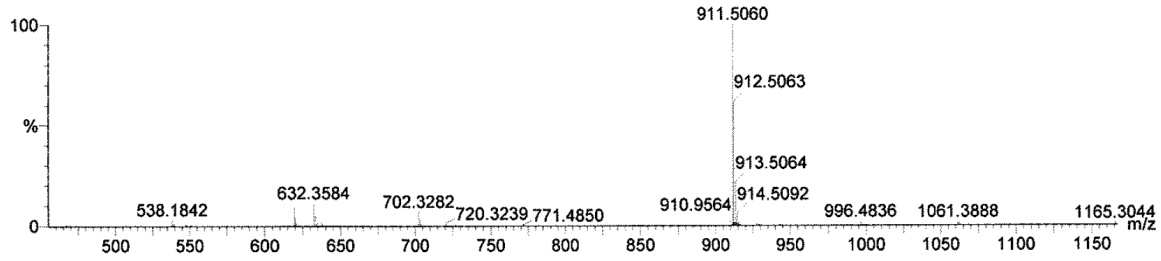


Figure S37: LC-MS trace of **2** (0–95% MeCN in water buffered with 0.1% v/v acetic acid).

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 3.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
256 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-500 H: 0-1000 N: 4-8 O: 4-8
Sample 002-118-2 C54H67N6O7 m/z 912
SamuelHo_181116_03 145 (2.479) AM (Cen,15, 50.00, Ht,4000.0,0.00,0.70); Sm (SG, 2x3.00); Cm (111:166) TOF MS ES+ 1.46e+004



Minimum:				-1.5		
Maximum:		3.0	5.0	100.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
911.5060	911.5071	-1.1	-1.2	24.5	2.9	C54 H67 N6 O7

Figure S38: High-resolution mass spectrum of **2**.

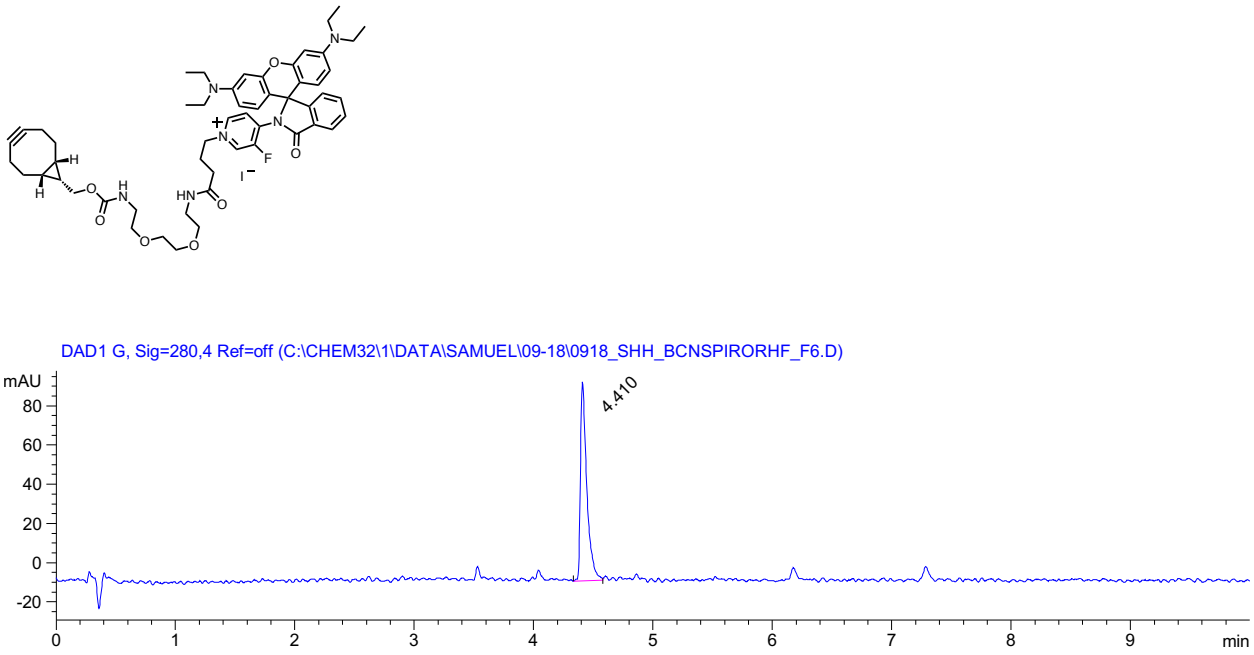
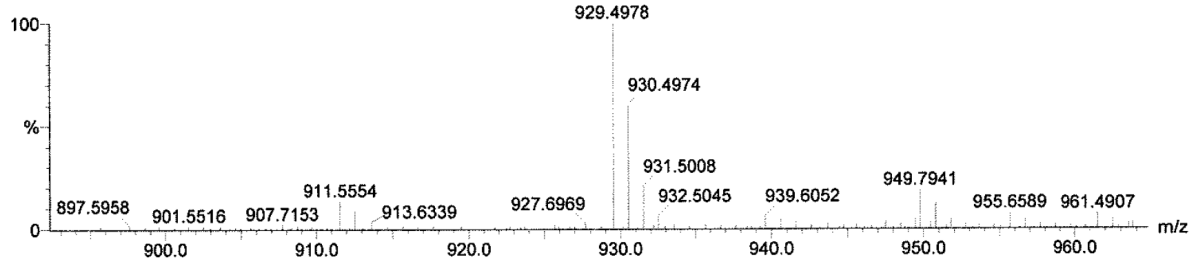


Figure S39: LC-MS trace of **3** (0–95% MeCN in water buffered with 0.1% v/v acetic acid).

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 3.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
123 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-500 H: 0-1000 N: 5-7 O: 5-8 F: 1-1
BCNSpiroRhF1 C54H66FN6O7 (930)
SamuelHo_181119_01 27 (0.462) AM (Cen,15, 70.00, Ht,4000.0,0.00,0.70); Sm (SG, 2x3.00); Cm (10:55) TOF MS ES+ 1.14e+004



Minimum:				-1.5		
Maximum:		3.0	5.0	100.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
929.4978	929.4977	0.1	0.1	24.5	6.6	C54 H66 N6 O7 F

Figure S40: High-resolution mass spectrum of **3**.