

A “Clickable” Photoconvertible Small Fluorescent Molecule as a Minimalist Probe for Tracking Individual Biomolecule Complexes

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1. General Information

1.1 General Information for Synthesis.

Materials. All commercial reagents and solvents were used as received. 1,6-diiodohexane was purchased Alfa Aesar (Haverhill, MA). Sodium azide was purchased from Acros (Waltham, MA). All other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Flash column chromatography was performed using Silicycle silica gel (40-63 μm (230-400 mesh), 60 \AA irregular pore diameter). Thin-layer chromatography was performed on TLC Silica gel 60G F₂₅₄ plate from Millipore Sigma. Reagents were purchased at the highest commercial quality and used without further purification, unless otherwise stated.

Instruments. Accurate mass measurement analyses were conducted on either a Waters GCT Premier, time-of-flight, GCMS with electron ionization (EI), or an LCT Premier XE, time-of-flight, LCMS with electrospray ionization (ESI). The signals were measured against an internal lock mass reference of perfluorotributylamine (PFTBA) for EI-GCMS, and leucine enkephalin for ESI-LCMS. Waters software calibrates and reports by use of neutral atom mass. The mass of an electron is not included. High-resolution mass spectra were obtained by Joomyung Vicky Jun and Dr. Charles Ross III at the University of Pennsylvania's Mass Spectrometry Service Center on a Micromass AutoSpec electrospray/chemical ionization spectrometer. X-ray diffraction data obtained and solved by Dr. Patrick Carroll at the University of Pennsylvania using a Bruker APEX2-DUO CCD X-ray Diffractometer. Photoreactions were carried out in a Rayonet Photochemical Reactor (model RPR-100). UV-Vis absorption spectra were acquired on a Hewlett-Packard 8452A diode array spectrophotometer (currently Agilent Technologies; Santa Clara, CA, USA) using quartz cells with a 1 cm cell path length (Starna Cells, Inc 120ul UV cells) or disposable UV cuvettes. Fluorescence spectra were acquired on a PTI QuantaMaster fluorometer equipped with a Peltier temperature controller. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DMX 500 MHz and AV-II 500 MHz NMR instruments. NMR spectra for **3a-PC** was obtained on a Bruker AVIII 500 MHz (Cryo) NMR instrument. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. High-performance liquid chromatography (HPLC) chromatograms were recorded and compounds were purified on an JASCO SEMI-PREP equipped with a VyDAC HPLC Columns Grace Davison discovery SciencesTM Protein and Peptide Gemini C18 column (cat#218TP510). Labeled proteins were purified using a GE AKTA[®] FPLC system and subsequently purified using a Varian ProStar HPLC equipped with a Vydac C4 218TP C4-semipreparative HPLC column. Analytical HPLC studies were done with a JASCO-FC-2088-30 HPLC or Agilent Technology Infinity II equipped with a Phenomenex Luna Omega 5 μm PS C18(2) 100A; 250 x 4.60 mm column using aqueous (H_2O + 0.1% $\text{CF}_3\text{CO}_2\text{H}$) and organic (CH_3CN + 0.1% $\text{CF}_3\text{CO}_2\text{H}$) phases. The infrared (IR) spectra were obtained with Perkin Elmer Spectrum Two FT-IR by dissolving a small amount of compound in dichloromethane to mount the sample. For certain experiments, anhydrous solvents are obtained from Meyer Solvent Dispensing System (Laguna Beach, CA). MALDI-TOF mass spectra were collected on a Bruker UltraFlex III MALDI-TOF/TOF instrument (Billerica, MA).

1.2 General Information for Cell Imaging

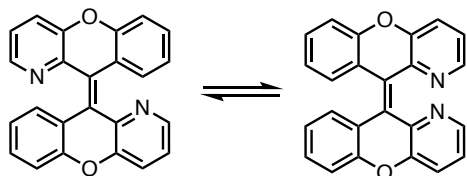
Materials. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Gibco, Life Technologies (Gaithersburg, MD). Penicillin/streptomycin were purchased from Corning Cellgro (Corning, NY). For complete neuronal medium, Neurobasal without phenol red and 5% B27 supplement were purchased from Thermo Fisher. Trypan blue diphosphate was purchased from Sigma-Aldrich.

Primary Neuronal Cell Culture and Photoactivation Experiment. Primary neuronal cultures were prepared from E15-E17 embryos of CD1 mice (Charles River) as previously described (Volpicelli-Daley 2011).¹ All procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Dissociated hippocampal neurons were plated at a density of 100,000 cells in the glass center chamber of a MatTek dish (35 mm dish, 14 mm well, #1.5 coverglass) for live cell imaging, and were allowed to mature for 7-10 d in complete neuronal medium (Neurobasal without phenol red (Thermo Fisher), 5% B27 supplement (Thermo Fisher)). Medium was partially exchanged every 3-4 days. Images were acquired on a Nikon spinning disk confocal microscope equipped with a 100x oil immersion objective lens. Images were processed using Nikon Elements software. *See page S37-S54 for details.*

2. Chemical Synthesis and Characterizations

2.1 Core Diazaxanthilidene Scaffold (1) and Methylated Compound (Me-1)²

Diazaxanthilidene Core (1)



Synthesis of (*E/Z*)-1: Following previously published procedure,² 50 mg (25 % isolated yield) of the title compound.

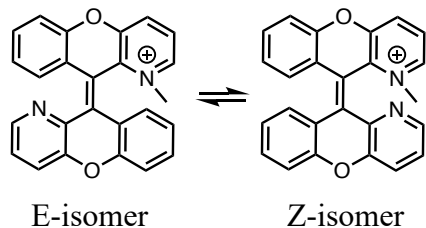
Physical Property: pale yellow solid

TLC: $R_f = 0.26$ (50% EtOAc in Hexanes)

¹H NMR (500 MHz, CD₂Cl₂-*d*₂) δ 8.17 (s, 1H), 8.09 (s, 1H), 7.66 – 7.51 (m, 2H), 7.39 – 7.18 (m, 7H), 6.95 (m, 3H).

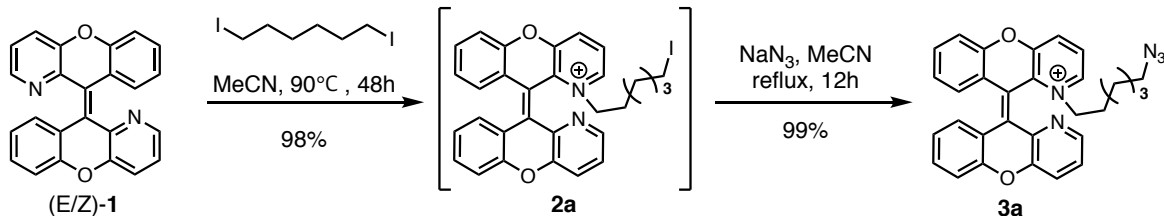
HRMS (ESI-TOF): calc'd for C₂₄H₁₅N₂O₂⁺ [M]⁺ 363.1134; found 363.1128

1N-Methylation of Compound 1 (Me-1)



Full characterizations can be found on previously published paper.²

2.2 6C-linker CPX (3a)



2.2.1 General Linker Synthesis. To a 4.0 mL scintillation vial, 7.5 mg (0.02 mmol, 1.0 equiv) of compound **1** and 66 μ L of excess 1,6-diiodohexane (135 mg, 0.4 mmol, 20 equiv), was added in 2.1 mL of dry acetonitrile (0.01M). The vial was taped with Teflon tape both interior and exterior of the cap, and the mixture was stirred for 48 h at 90 °C. Upon completion of the reaction, the solution turned purple, which is an indication of released iodine compounds. After complete conversion was confirmed by LCMS and analytical HPLC, the reaction vial was cooled down. 1.0 mL of hexane was added to the reaction mixture and mini-extraction was done to remove excess diiodohexane in hexane layer. Once mini-extraction was repeated three times, collected hexane layer was discarded and remaining acetonitrile was removed *in vacuo*. To the same 4.0 mL scintillation vial, excess sodium azide (10.4 mg, 0.16 mmol, 8 equiv) was added in 2.0 mL of dry acetonitrile. The reaction mixture was capped and sealed with Teflon tape, and was stirred for 4 to 8 h at 90 °C. The crude reaction solution was then mixed with 2.0 mL of MiliQ water and filtered prior to HPLC purification. Following the HPLC condition below, 11.6 mg (97 % isolated yield with TFA salt) of the title compound **3a** was obtained.

HPLC-Semi PREP condition: 35 to 70% MeCN (0.1% TFA) in H₂O (0.1% TFA) over 30 min
Product Eluted condition: 55 to 65% MeCN (0.1% TFA) in H₂O (0.1% TFA). 20-26 min

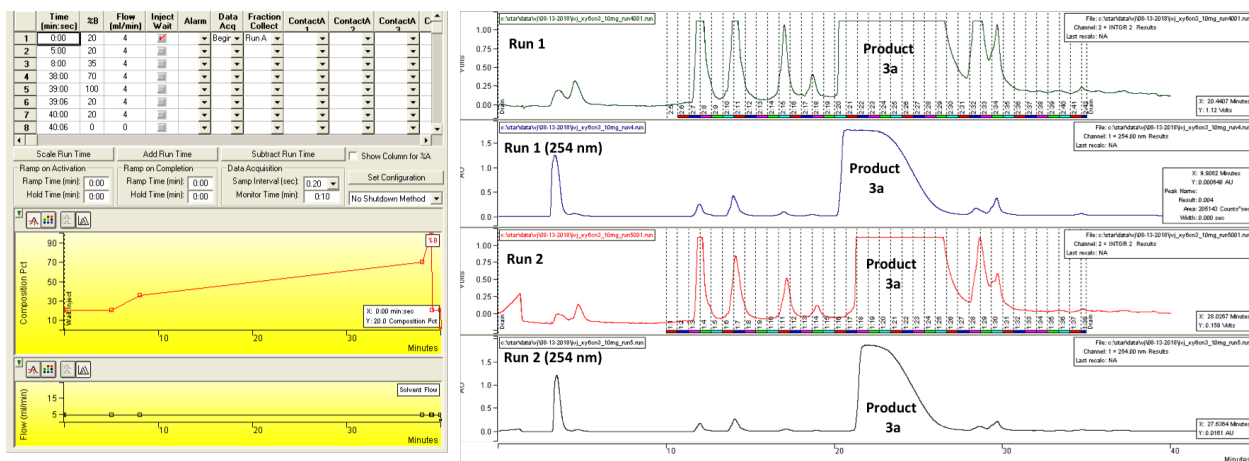
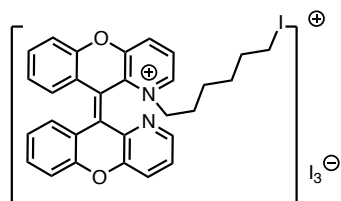


Figure S1. HPLC Semi-Prep gradient and HPLC trace for crude **3a**.

2.2.2 Characterization of iodo-intermediate (2a) and CPX (3a)

Iodo-intermediate (2a)



Physical Property: purple crystal (crystal structure shown in *page S53-S54*)

HRMS (ESI-TOF): calc'd for C₃₀H₂₆IN₂O₂⁺ [M]⁺ 573.1033; found 573.1057

¹H NMR (500 MHz, CD₂Cl₂-d₂) 2a crude (Figure S6)

TLC: R_f = 0.0 (50% EtOAc in Hexanes)

IR (neat): 2981.91, 2929.20, 1715.97, 1452.23, 1388.93, 1367.84, 1272.88, 1251.78, 1167.38, 951.11, 764.00, 743.00 cm⁻¹

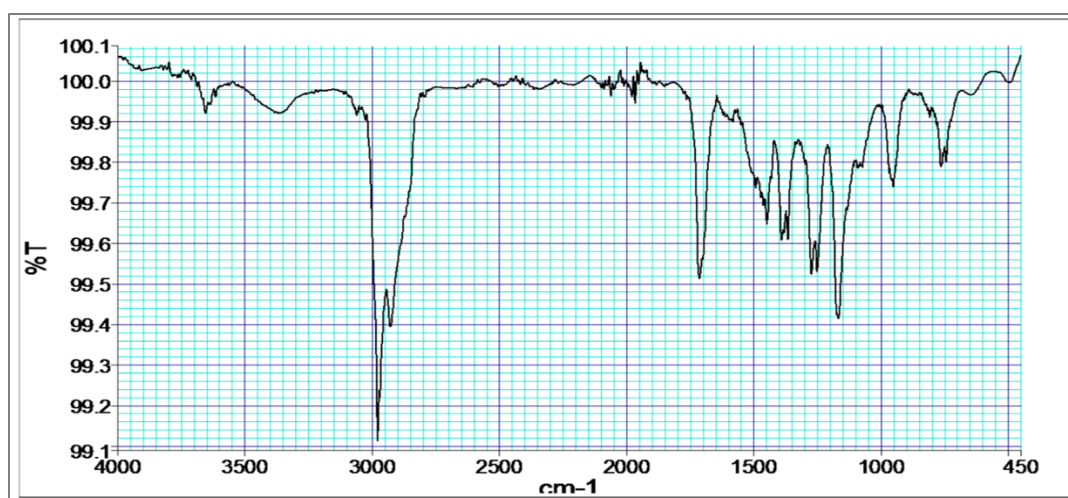


Figure S2. IR spectrum of iodo-intermediate **2a**.

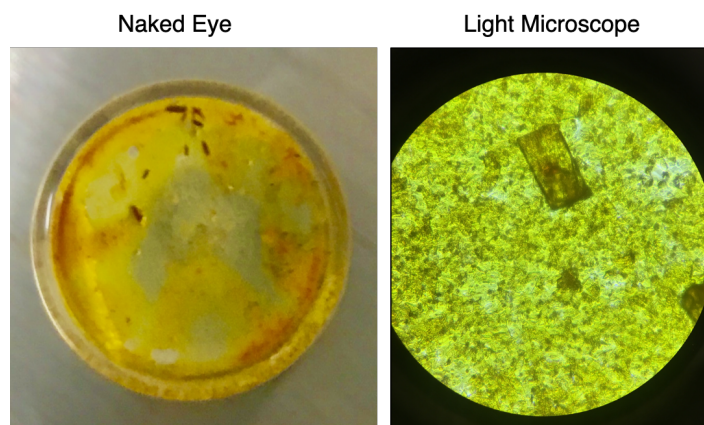
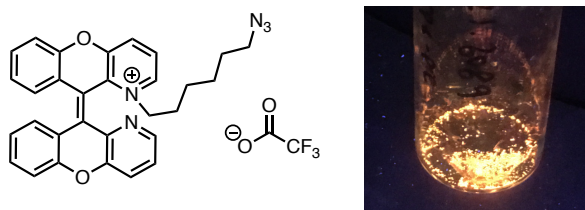


Figure S3. Visualization of **2a** crystals under ambient light (left) and with light microscope (right)

6C-linker CPX (3a)



Physical Property: yellow solid as salt (yellow fluorescence under 365 nm handheld UV lamp)

¹H NMR (500 MHz, CD₃CN-*d*₃) δ 8.39 – 8.33 (m, 2H), 8.13 (dd, *J* = 4.4, 1.4 Hz, 1H), 7.92 (ddd, *J* = 7.7, 6.5, 1.2 Hz, 1H), 7.81 (dt, *J* = 8.3, 1.4 Hz, 1H), 7.58 – 7.44 (m, 5H), 7.46 – 7.40 (m, 1H), 7.32 – 7.25 (m, 1H), 7.13 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.00 (ddd, *J* = 8.2, 7.0, 1.3 Hz, 1H), 4.01 (dt, *J* = 13.5, 7.6 Hz, 1H), 3.51 (dt, *J* = 12.8, 6.2 Hz, 1H), 3.04 (m, 2H), 1.61 – 1.52 (m, 2H), 1.25 (m, 2H), 1.09 – 1.02 (m, 2H), 1.02 – 0.91 (m, 1H), 0.87 – 0.79 (m, 1H).

¹³C NMR (126 MHz, CD₂Cl₂-*d*₂) δ 156.70, 155.73, 154.97, 151.05, 147.08, 144.65, 140.68, 140.44, 133.19, 133.11, 132.22, 130.74, 128.90, 128.73, 128.08, 127.66, 127.64, 127.43, 125.17, 124.15, 120.86, 119.41, 119.29, 113.40, 60.04, 52.36, 32.30, 29.56, 27.09, 27.00.

HRMS (ESI-TOF): calc'd for C₃₀H₂₆N₅O₂⁺ [M]⁺ 488.2081; found 488.2062

IR (neat): 3358.77, 2933.54, 2098.54, 1663.72, 1446.86, 1430.50, 1302.48, 1277.54, 1148.85, 807.75, 762.06, 706.17, 608.34 cm⁻¹

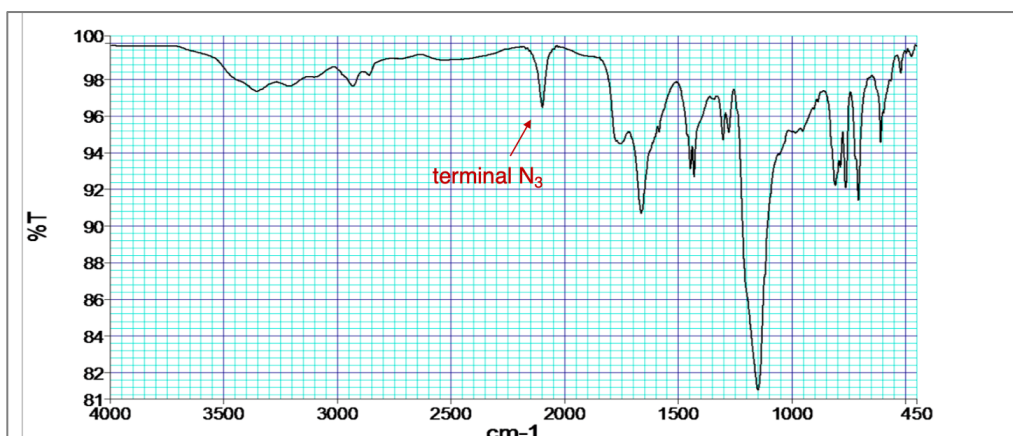


Figure S4. IR spectrum of 6C-linker CPX **3a**. The terminal azide is identified by the N=N asymmetric stretching absorption³ at 2098.54 cm⁻¹.

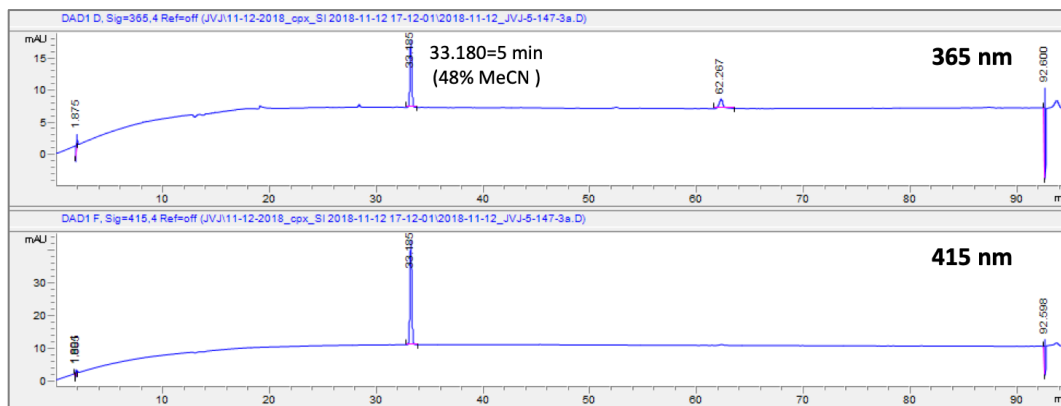


Figure S5. Analytical HPLC (Agilent) of purified **3a** 30-100% MeCN in H₂O over 70 min (15-85 min)

2.2.3 ¹H NMR comparison of core (1), intermediate (2a), and CPX (3a)

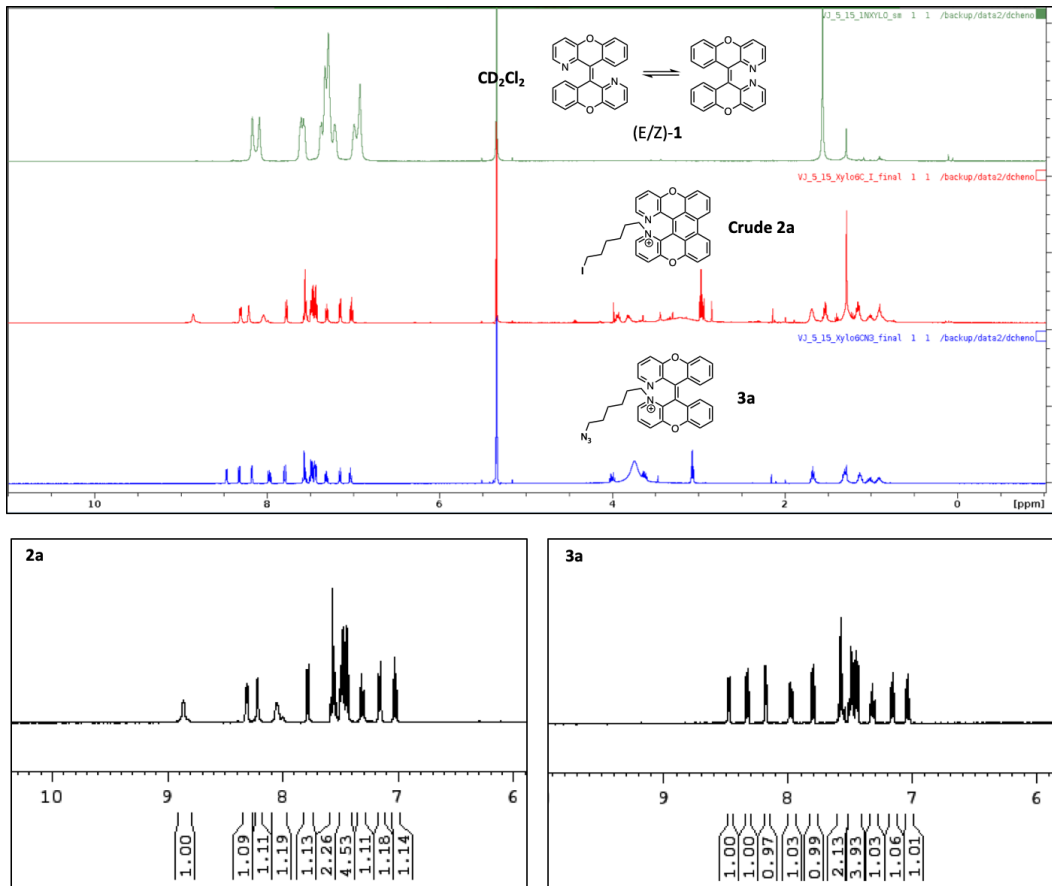


Figure S6. ¹H NMR comparison of aromatic region of **1** vs. **2a** vs. **3a** in CD₂Cl₂.

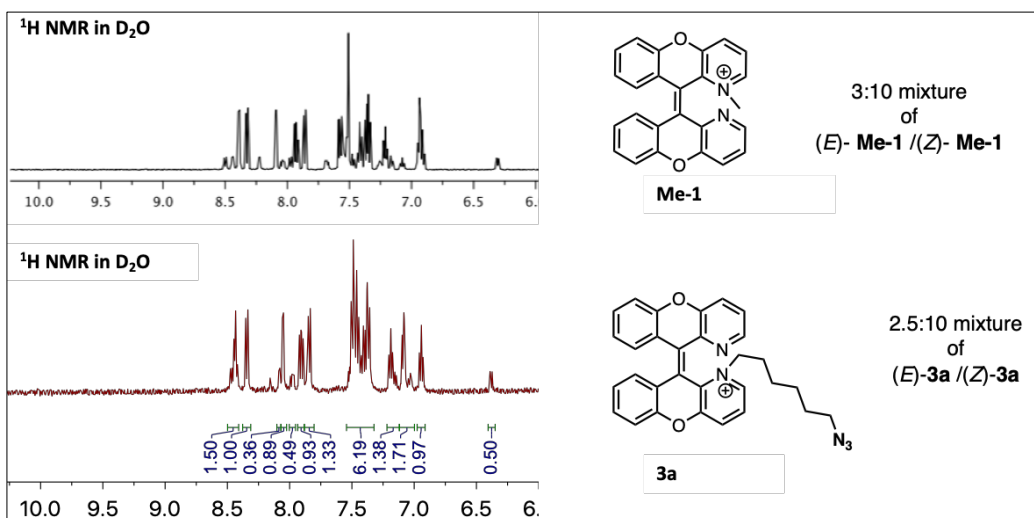
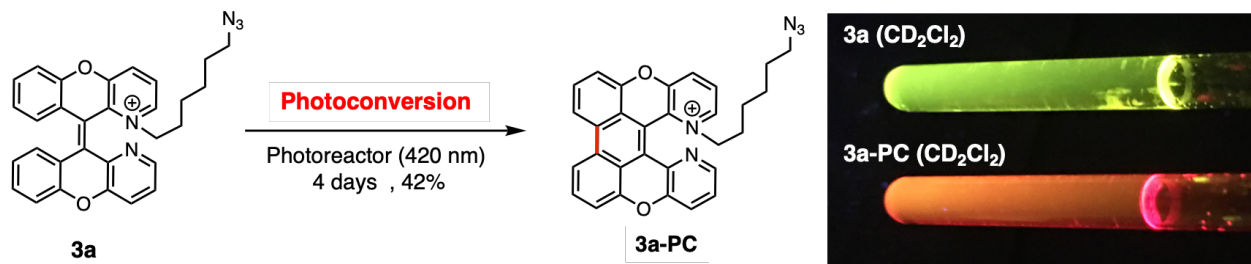


Figure S7. ¹H NMR comparison of Me-1 vs. 3a in aromatic region in D₂O

2.3 Post-activated Product of 3a (3a-PC)



2.3.1 General Photoconversion. To a 20.0 mL scintillation vial, 8.1 mg (13 μmol , 4.5 μM) of compound **3a** in 3.0 mL of 1:2 MeCN: H₂O (0.1M of HCl). The vial was capped with syringe needles on the trifluoroethanol (TFE) septa to allow oxygen flow (see picture below). The vial was stirred and irradiated with four 420 nm (LZC-420) lamps using Rayonet Photoreactor for 4 days. The crude reaction was purified with Semi-Prep Varian HPLC. Following the HPLC condition below, 3.4 mg (5.7 μmol) of the title compound **3a-PC** was obtained in 42 % isolated yield. Masses are calculated with trifluoroacetate as a counter ion.



Rayonet Photoreactor Set Up

Important Notes. The rate of photoconversion will highly dependent on the power of the laser, experimental set up, and irradiation area. With a Rayonet Photoreactor (LZC-420 nm, 8 Watt), complete photoconversion to **3a-PC** takes more than 4 d. With microscope stimulation laser (Spectra X, 440 nm), full photoconversion takes less than 60 s.

HPLC-Semi PREP condition: 45 to 75% MeCN (0.1% TFA) in H₂O (0.1% TFA) over 27 min
Product Eluted condition: 59 to 63% MeCN (0.1% TFA) in H₂O (0.1% TFA). 20-24 min

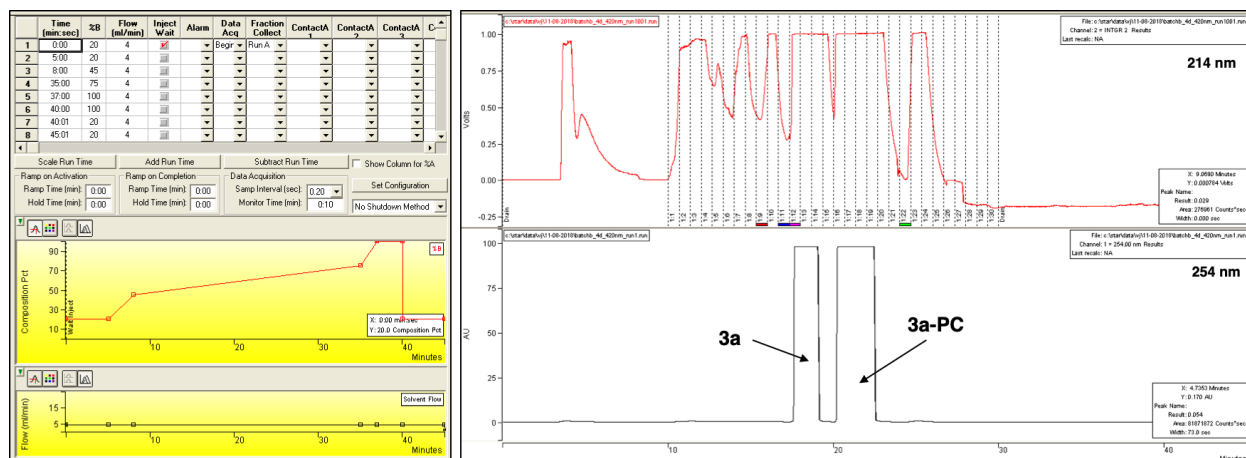
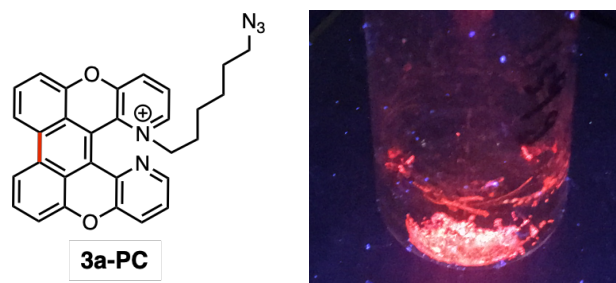


Figure S8. HPLC-Semi Prep gradient and HPLC trace for crude **3a-PC**.

2.3.2 Characterization of photoproduct 3a-PC



Physical Property: red solid as salt (pink fluorescence under 365 nm handheld UV lamp)

¹H NMR (Cryo 500 MHz, CD₃CN-*d*₃) δ 8.42 (d, *J* = 5.8 Hz, 1H), 8.34 (ddd, *J* = 24.7, 8.5, 0.9 Hz, 2H), 8.25 (dd, *J* = 4.4, 1.4 Hz, 1H), 8.19 (dd, *J* = 8.5, 1.2 Hz, 1H), 7.92 – 7.82 (m, 2H), 7.78 – 7.68 (m, 2H), 7.54 (dd, *J* = 8.4, 4.4 Hz, 1H), 7.50 (dd, *J* = 8.1, 0.9 Hz, 1H), 7.40 (dd, *J* = 7.9, 0.8 Hz, 1H), 4.44 (dt, *J* = 13.5, 7.7 Hz, 1H), 3.83 (dt, *J* = 13.0, 6.4 Hz, 1H), 2.89 (t, *J* = 6.9 Hz, 2H), 1.49 (d, *J* = 5.4 Hz, 2H), 1.20 – 1.00 (m, 2H), 0.95 – 0.75 (m, 3H), 0.71 (d, *J* = 8.8 Hz, 1H).

¹³C NMR (Cryo 126 MHz, CD₃CN-*d*₃) δ 154.29, 150.39, 149.07, 148.71, 145.82, 141.22, 140.21, 137.47, 131.46, 131.38, 130.88, 130.03, 129.01, 127.88, 127.64, 126.45, 126.09, 123.26, 120.12, 119.07, 117.88, 113.86, 112.89, 106.57, 59.49, 50.59, 31.37, 27.77, 25.16, 24.57.

HRMS (ESI-TOF): calc'd for C₃₀H₂₄N₅O₂⁺ [M]⁺ 486.1930; found 486.1925

IR (neat): = 3365.54, 2109.20, 1673.60, 1442.00, 1190.2, 1139.9, 802.48, 721.91 cm⁻¹

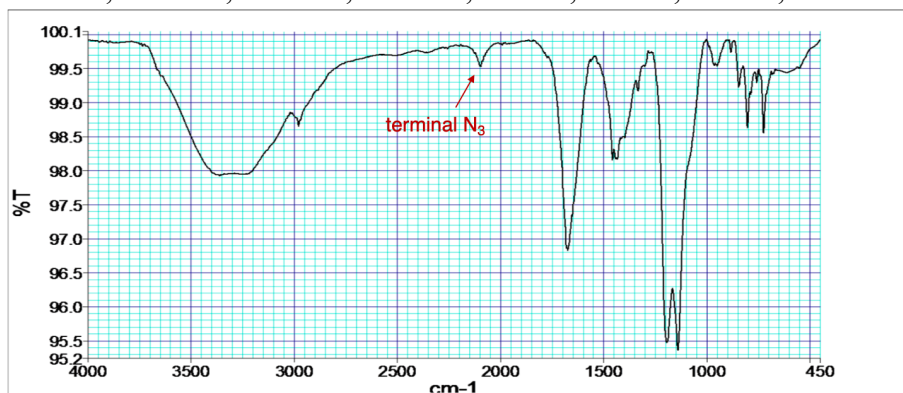


Figure S9. Presence of terminal azide peak (2109.20 cm⁻¹) even after irradiated with photoreactor (420 nm) over 4 days.³

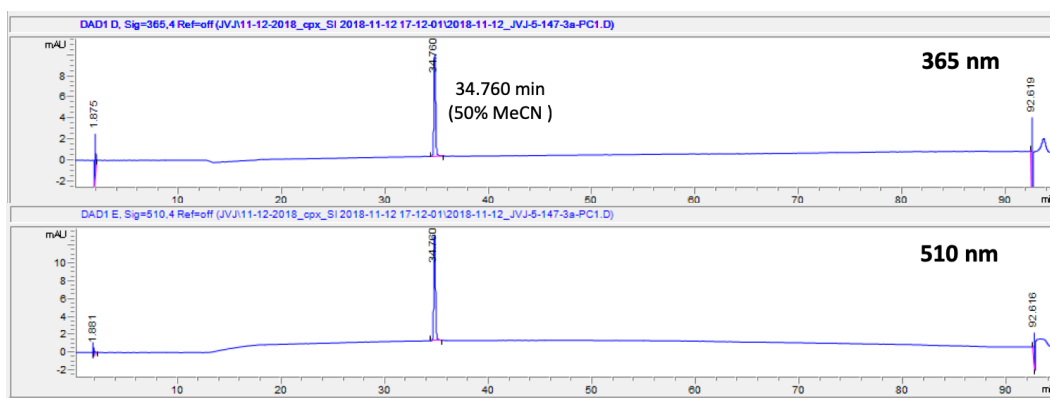
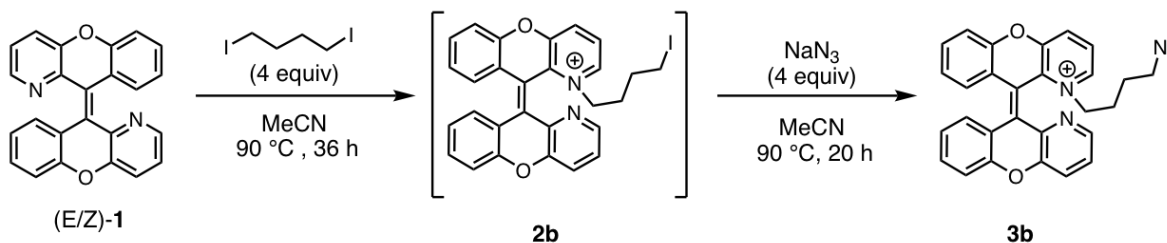


Figure S10. Analytical HPLC of purified 3a-PC 30-100% MeCN in H₂O over 70 min (15-85 min)

2.4 4C-linker CPX (3b)



2.4.1 Synthesis of 3b. Following the general linker synthesis procedure in page S5, 5.0 mg (0.014 mmol) of **1** gave 6.9 mg (87 % isolated overall yield) of the title compound was obtained after HPLC purification. Formation of **2b** (88% analytical HPLC yield) was confirmed by analytical HPLC, HRMS, and crude ^1H NMR. Final product **3b** was purified with Semi-Prep HPLC and eluted around 50-55% MeCN (0.1% TFA) in H_2O (0.1% TFA).

Monitoring Reaction with Analytical (Varian) HPLC

30 to 80% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 10 min (10 min - 20 min retention time)

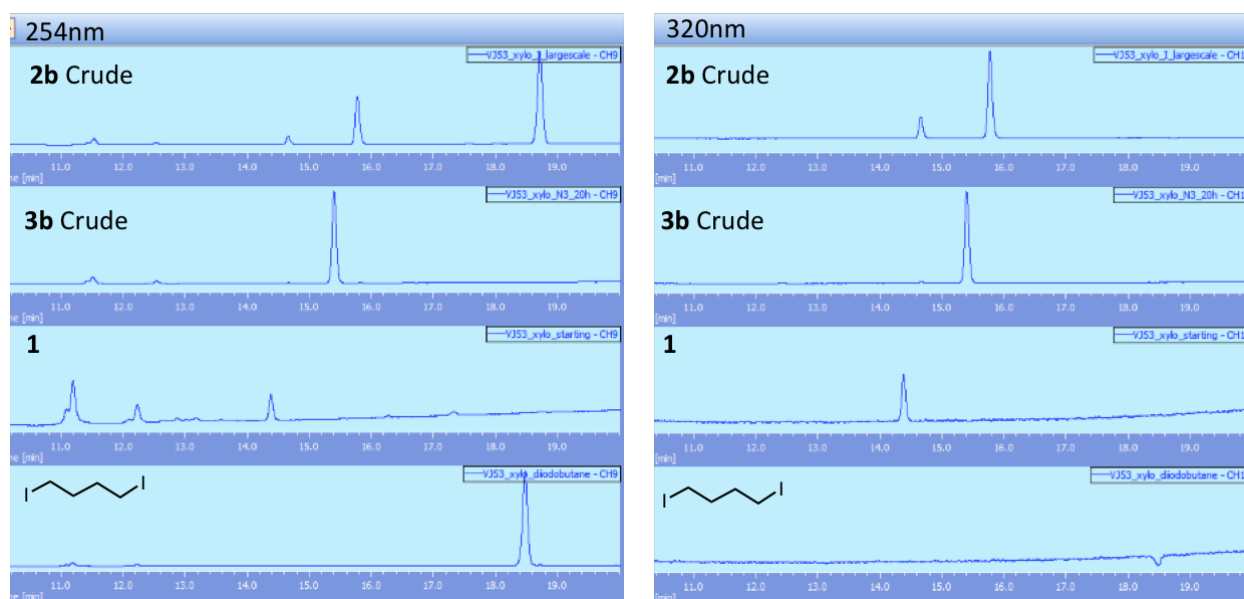
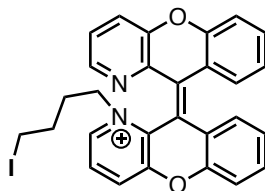


Figure S11. Analytical HPLC of each step with starting material standards in two UV channels (254 nm and 320 nm).

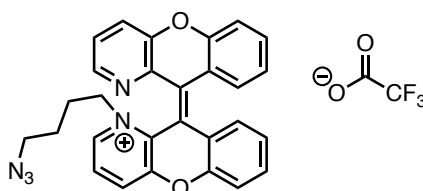
2.4.2 Characterization of Iodo-intermediate (2b) and CPX (3b)

Iodo-intermediate (2b)



HRMS of **2b** (ESI-TOF): calc'd for C₂₈H₂₂IN₂O₂⁺ [M]⁺ 545.0726; found 545.0719

4C-linker CPX (3a)



Physical Property: yellow solid as salt

¹H NMR (500 MHz, CD₃CN-*d*₃) δ 8.38 (s, 2H), 8.13 (dd, *J* = 4.4, 1.4 Hz, 1H), 7.93 (dd, *J* = 8.5, 6.1 Hz, 1H), 7.82 (dd, *J* = 8.4, 1.4 Hz, 1H), 7.56 (s, 2H), 7.49 (s, 3H), 7.44 (s, 1H), 7.30 (d, *J* = 0.7 Hz, 1H), 7.14 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.01 (ddd, *J* = 8.2, 7.0, 1.3 Hz, 1H), 4.05 (s, 1H), 3.53 (s, 1H), 3.07 (t, *J* = 6.6 Hz, 2H), 1.64 (s, 2H), 1.28 (s, 1H), 1.14 (d, *J* = 6.8 Hz, 1H).

¹³C NMR (126 MHz, CD₃CN-*d*₃) δ 155.06, 154.52, 153.54, 149.57, 145.71, 142.64, 139.90, 139.20, 132.00, 131.42, 130.53, 128.99, 127.65, 127.54, 126.43, 126.33, 126.01, 125.72, 123.45, 123.11, 119.92, 112.40, 57.65, 50.25, 28.15, 24.80.

HRMS of **3b** (ESI-TOF): calc'd for C₂₈H₂₂N₅O₂⁺ [M]⁺ 460.1768; found 460.1749

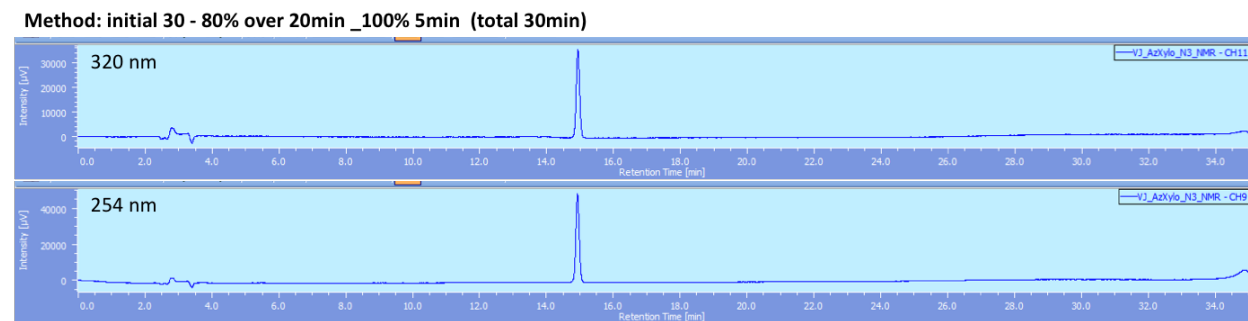


Figure S12. Analytical HPLC of purified compound **3b**

2.4.3 ^1H NMR comparison of intermediate (2b), and CPX (3b)

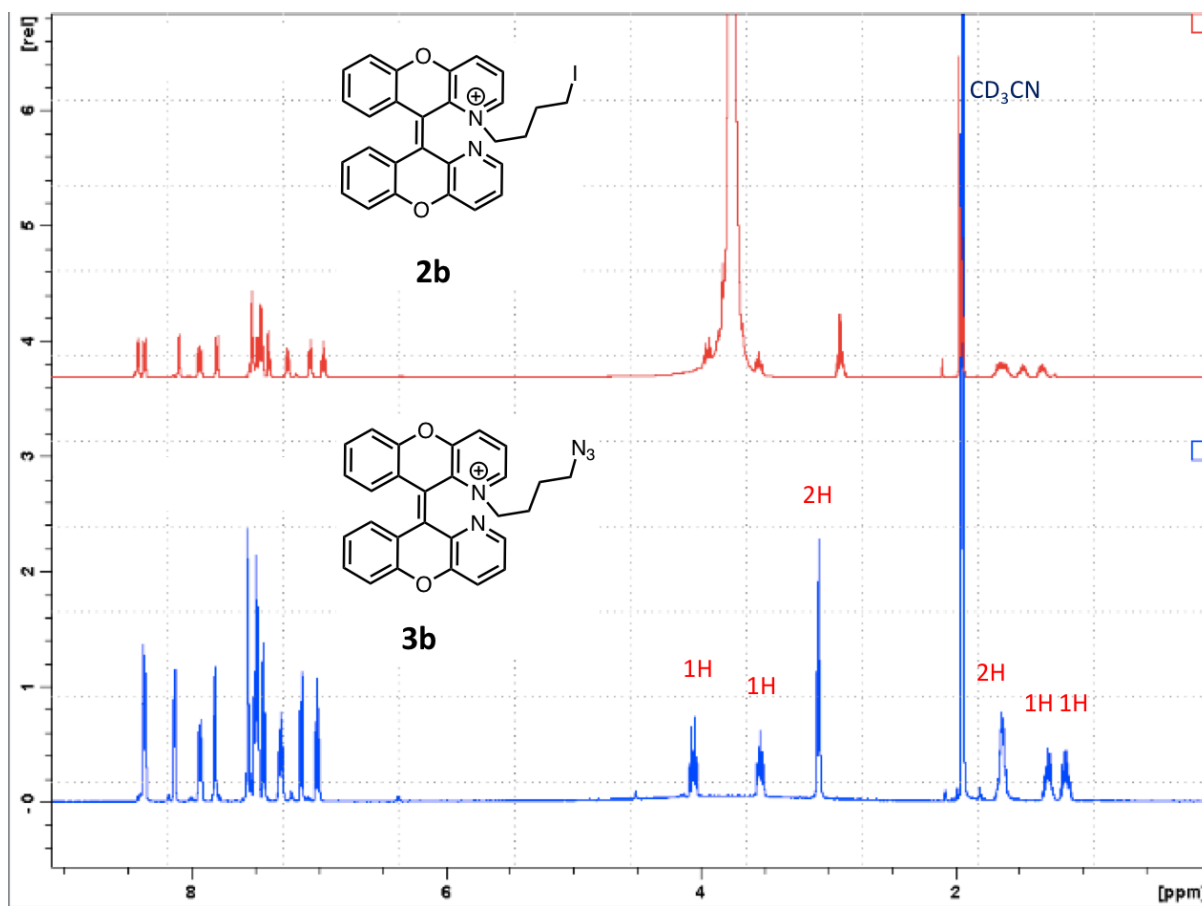


Figure S13. ^1H NMR (500 MHz, $\text{CD}_3\text{CN}-d_3$) analysis of crude **2b** and purified **3b**

3. X-Ray Structure Comparison

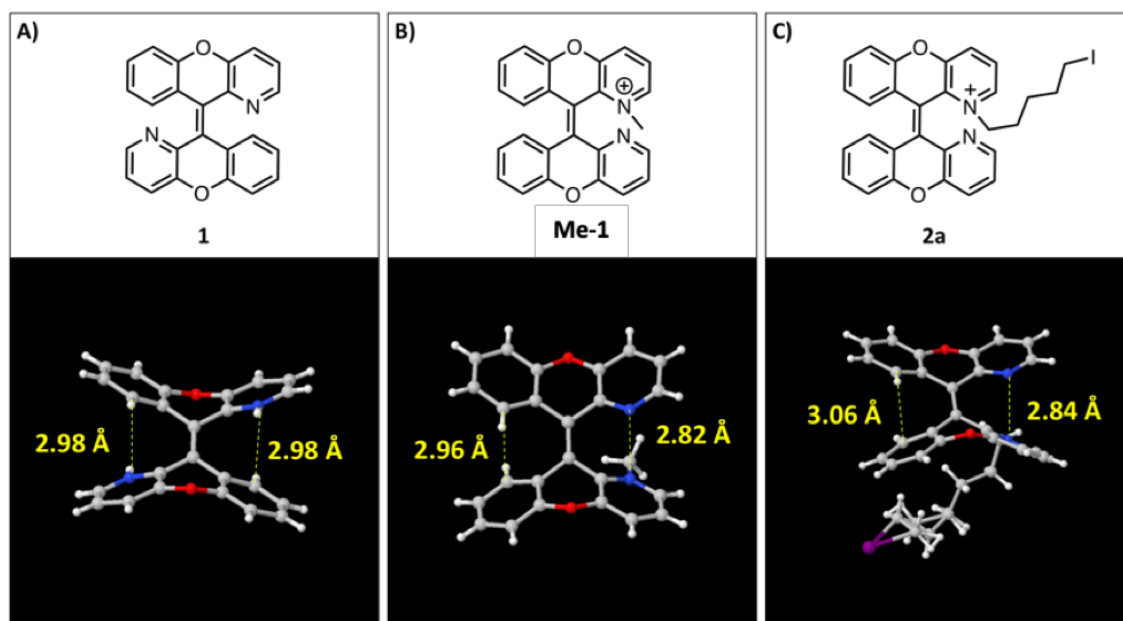


Figure S14. X-Ray Analysis of A) compound **1**², B) N-methylation of **1** (**Me-1**)², and C) **2a**

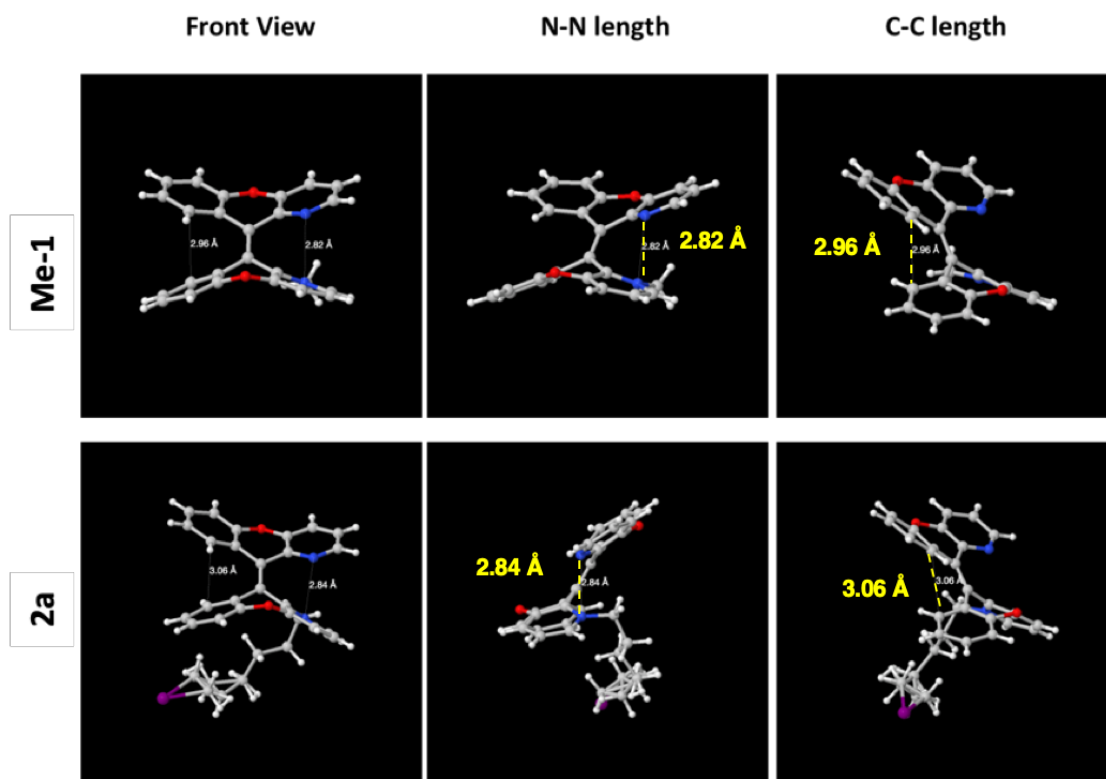


Figure S15. Side views of methylated compound (**Me-1**) and alkylated compounds (**2a**).

4. Photophysical Property of CPX (3a/3a-PC, 3b/3b-PC)

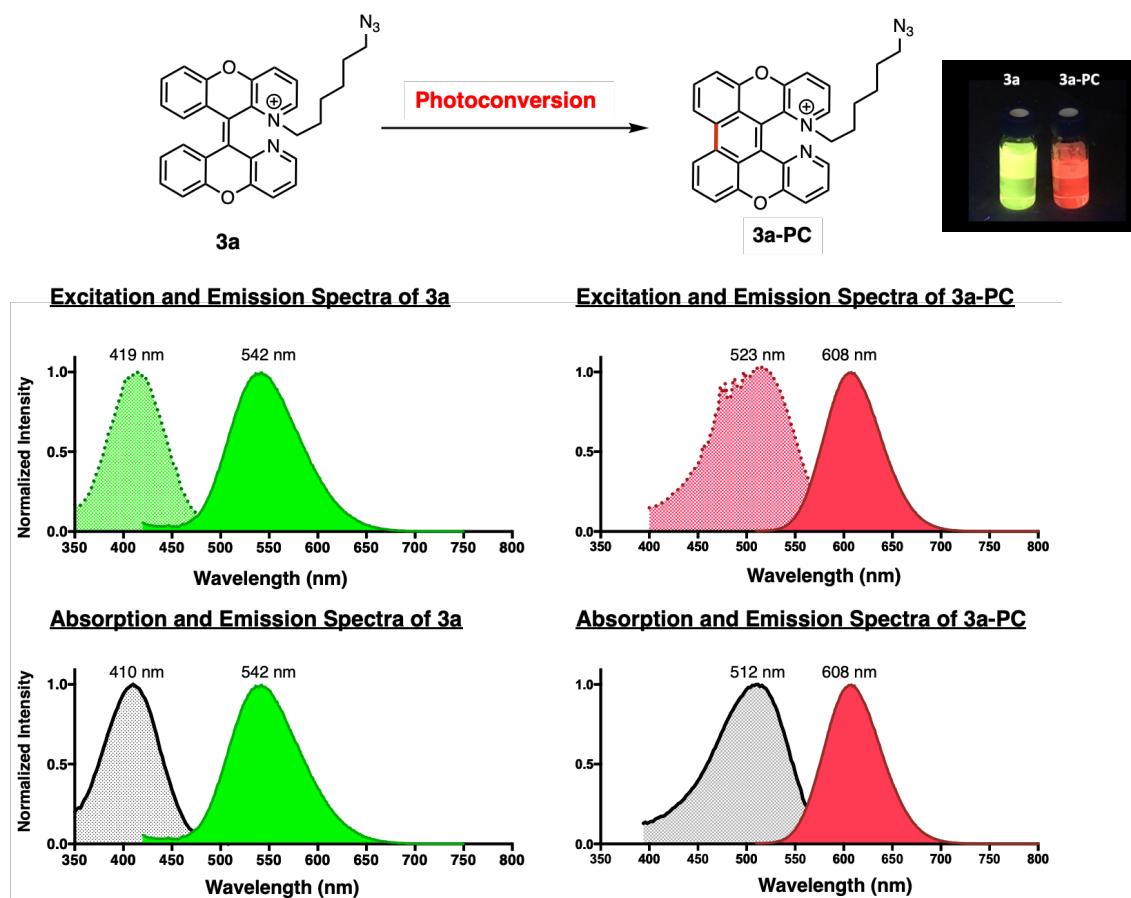


Figure S16. Fluorescence spectra of compound **3a** and **3a-PC** obtained in 1:1 MeCN:H₂O (0.1% TFA)

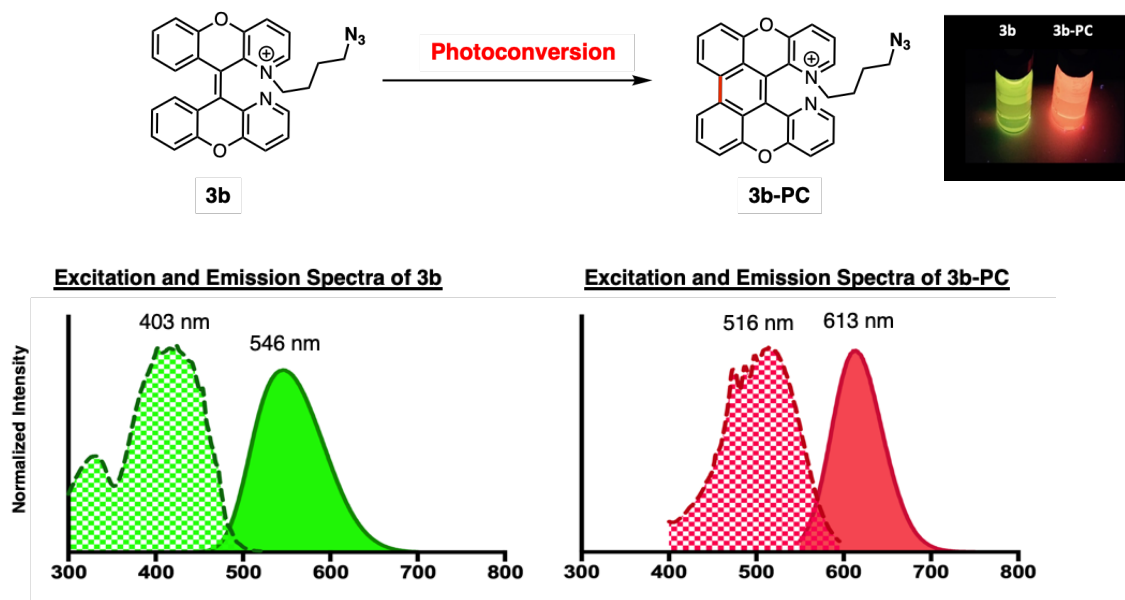


Figure S17. Fluorescence spectra of compound **3b** and **3b-PC** obtained in H₂O

5. General Experiment for Photoactivation

Nikon Microscope Set Up

Microscope: Nikon Eclipse Ti2 Inverted Microscope with ANDOR iXon Ultra 897, CSU-X1 (Yokogawa), Tokai Hit Incubator (STX stage top; temp & CO₂)

Confocal Laser: CSU BA Spinning Disc Emission (Bandpass Filter) Wheel
Nikon LUNV Laser Launch: output power at the fiber end (20mW)

405ex/FITC: Excitation at 405 nm, FITC Em filter (525/36nm)^a
488ex/Cy5: Excitation at 488 nm, Cy5 Em filter (700/72 nm)^a
561ex/Cy5: Excitation at 561 nm, Cy5 Em filter (700/72nm)^a

^a Filter center wavelength / full width at half maximum in nm

Stimulation Laser: 440/20 nm (output power 256 mW) 100% laser power
(Mightex Polygon 400 DMD, Lumencor Spectra X LED Illuminator)
Filter: 442 C-TIRF 97320 (dichroic only)
Note. Exact laser power (Watt) will vary depending on the area (ROI) of stimulation

Objectives: CFI APO 100X Oil TIRF NA 1.49 WD 0.12MM

Image processor: NIS Elements Imaging Software (High Content Package)

Photoactivation Experiment Procedure

Experiment is conducted by looking at two or three channels before (pre-activation) and after (post-activation) irradiation with DMD/ Spectra X laser at the region of interests (ROIs). Green channel (**405ex/FITC**) reveals the initial state of CPX (**3a**), and thus is anticipated to show disappearance of **3a** upon irradiation. Red channels (**488ex/Cy5** or **561ex/Cy5**) will reveal newly formed photoconverted product, **3a-PC**. Merging these channels at pre- and post-activated states will show the successful photoconversion in ROIs. **561ex/Cy5** is used as a second channel for photoactivated species; **561ex/Cy5** shows less background signal (**3a**) but also weaker product signal (**3a-PC**) compare to that of **488ex/Cy5**. **561ex/Cy5** is used as indicator for third dye (e.g., Trypan Blue) in neuronal uptake experiment.

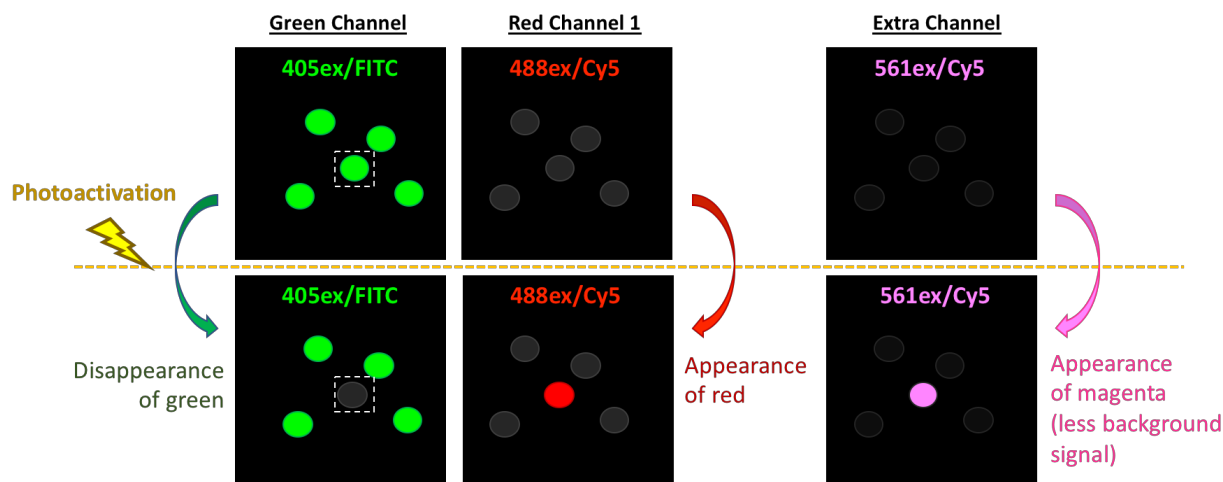


Figure S18. Schematic view of general photoactivation (photoconversion) confocal imaging experiment.

6. Giant Unilamellar Vesicle (GUV) Experiment

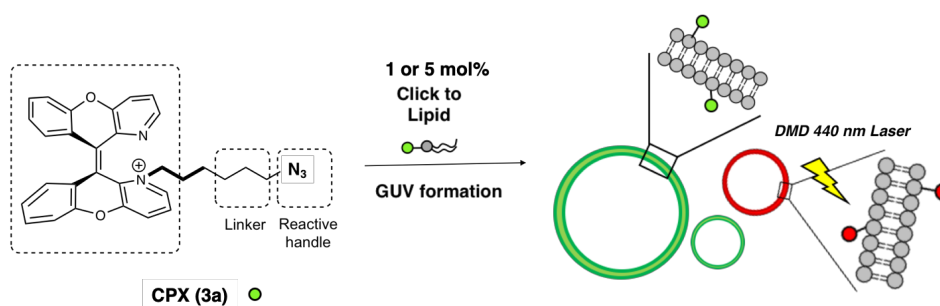


Figure S19. Schematic view of **3a** conjugated GUV and photoactivation

6.1 Materials of GUVs

Materials. Stock material was prepared by commercially available materials. 18:1 (Delta9) Cis PC 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-dibenzocyclooctyl (16:0 DBCO-PE) are purchased from Avanti polar lipids and used without further purification. **3a-PE** was synthesized by mixing DBCO-PE and **3a** in chloroform/methanol (2:1v/v) overnight. ITO glass slides are from Delta Technologies (Stillwater, MN).

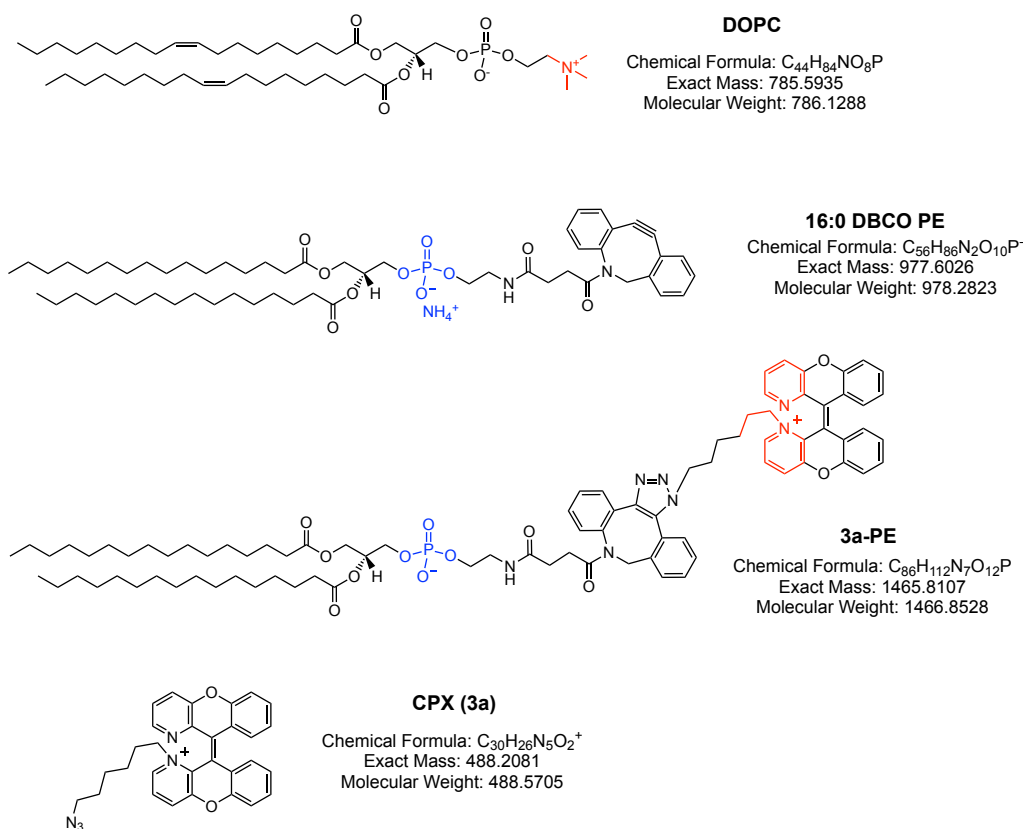


Figure S20. Chemical Structures of GUV materials.

6.2 Synthesis of GUV for Imaging

6.2.1 General Synthesis of GUVs

GUVs were prepared as previously described.⁴⁻⁵ **3a** dye stock (1.0 mM) and phospholipids stock solutions of DOPC, DBCO-PE, and **3a-PE** (1.0 mM) were prepared in chloroform-methanol (2:1 v:v). The following mixtures were used: DOPC/DBCO-PE (95/5, mol ratios); DOPC/**3a-PE** (95/5, mol ratios), DOPC/DBCO-PE (99/1, mol ratios); DOPC/**3a-PE** (99/1, mol ratios). 40 μ L of each lipid mixture in organic solvent was deposited on the microscope cover slide on the conductive face while heating at 60°C. Two slides per sample were prepared. After drying under vacuum overnight to remove the residual solvent, two slides were then combined with a silicone spacer (Grace Bio-Labs, Bend, OR) to enclose 148mM sucrose solution, and incubated at 60°C in the Function generator (AC field 2 V/mm, 5 Hz) for 3 h.⁵ Prepared GUV solutions were diluted in 10-fold and imaged on the MatTek dish (35 mm dish, #1.5 cover glass) at room temperature.

Lipid Mixtures

- GUV 1 : 190 μ L DOPC + 10 μ L DBCO-PE ; then add **3a** dye later (5 mol% in situ labeling)
- GUV 2: 190 μ L DOPC + 10 μ L **3a-PE** (5 mol%)
- GUV 3: 198 μ L DOPC + 2 μ L DBCO-PE ; then add **3a** dye later (1 mol% in situ labeling)
- GUV 4: 198 μ L DOPC + 2 μ L **3a-PE** (1 mol%)

In-situ Click Condition: 2 μ L of **3a** stock solution (1.0 mM) were added to 2.0 mL of sucrose solution in MatTek Dish. (Final 1.0 μ M of **3a**).

6.2.2 Synthesis of **3a-PE**

1.0 mL of DBCO-PE (1.0 mM) and 1.0 mL of **3a** (1.0 mM) in chloroform: methanol (2:1 v/v) was mixed in room temperature and let it sit overnight. The product, **3a-PE**, formation was confirmed by LCMS and HRMS. (Note: Since reaction mixture of **3a-PE** was used without further purification, there might be small amount of unreacted DBCO-PE or **3a** present.)

HRMS of **3a-PE:** calc'd for C₈₆H₁₁₃N₇O₁₂P [M+H]⁺ 1466.8185; found 1466.8190

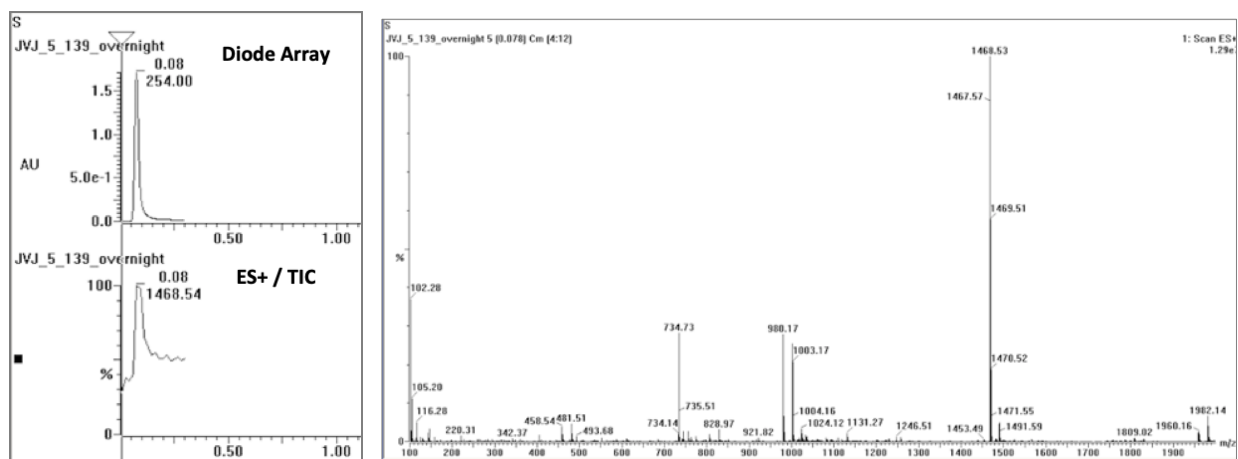


Figure S21. LCMS analysis (loop injection) to see the mass composition of the crude reaction of **3a-PE**. Mass of **3a-PE** is found as the strongest signal and no mass for free **3a** is found.

6.3 Imaging GUV with Nikon Microscope.

Green channel is used for imaging pre-activated **3a**. Upon photo-stimulation, intensity of **3a** will decrease under green channel. Sequentially, red channel is used for imaging post-activated **3a-PC**. Initially, when none of **3a** is activated, **3a** will not be visible or will have low background fluorescence under red channel. After photo-stimulation (photoactivation), fluorescence of post-activated product **3a-PC** will appear under red channel.

Green channel: **405ex/FITC 100%** (excitation laser 405 nm with FITC emission filter)

Red channel: **488ex/Cy5 50%** or **561ex/Cy5 100%**
(excitation laser 488 nm or 561 nm with Cy5 emission)

Merge: Relative intensity between green and red channel indicates photoactivated regions.

Stimulation: 440 nm DMD 100% on ROIs (region of interest)

Note. Imaging under 405ex 100% laser can also activate **3a** probe. Hence capture image under lower laser power (<25%) with 405 nm. Or, find the dark area under 488ex or 561ex laser where no photoactivated **3a-PC** background fluorescence is detected.

Dotted white square: ROIs where Spectra X laser was irradiated

Types of Experiment

- 6.3.1 Tracking experiment:** Find the vesicles under **405ex/FITC** and activate the whole field of view with the 440 nm 100% DMD stimulation laser for 1-2 min. Wait and see if other new (not-photoactivated) vesicle comes. Then capture at **405ex/FITC** (green channel) and **488ex/Cy5** or **561ex/Cy5** (red channel)
- 6.3.2 Selective Turn-On Experiment:** Find the dark region under **488ex/Cy5**, and activate the ROIs with 440 nm 100% Spectra X laser. In order to photoconvert the whole vesicle, make a ROIs (i.e. dotted square) within the vesicle.

6.4 Additional Photoactivation Imaging with 1 mol% 3a-GUV

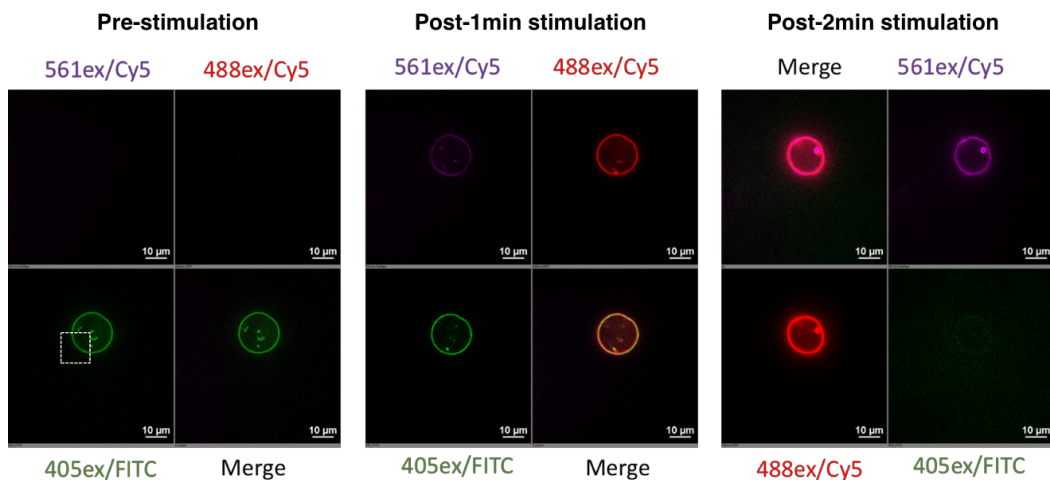


Figure S22. Photo-activation of GUV 3 (1:99 DBCO-PE/ DOPC; then in-situ click with **3a**) in ROI.

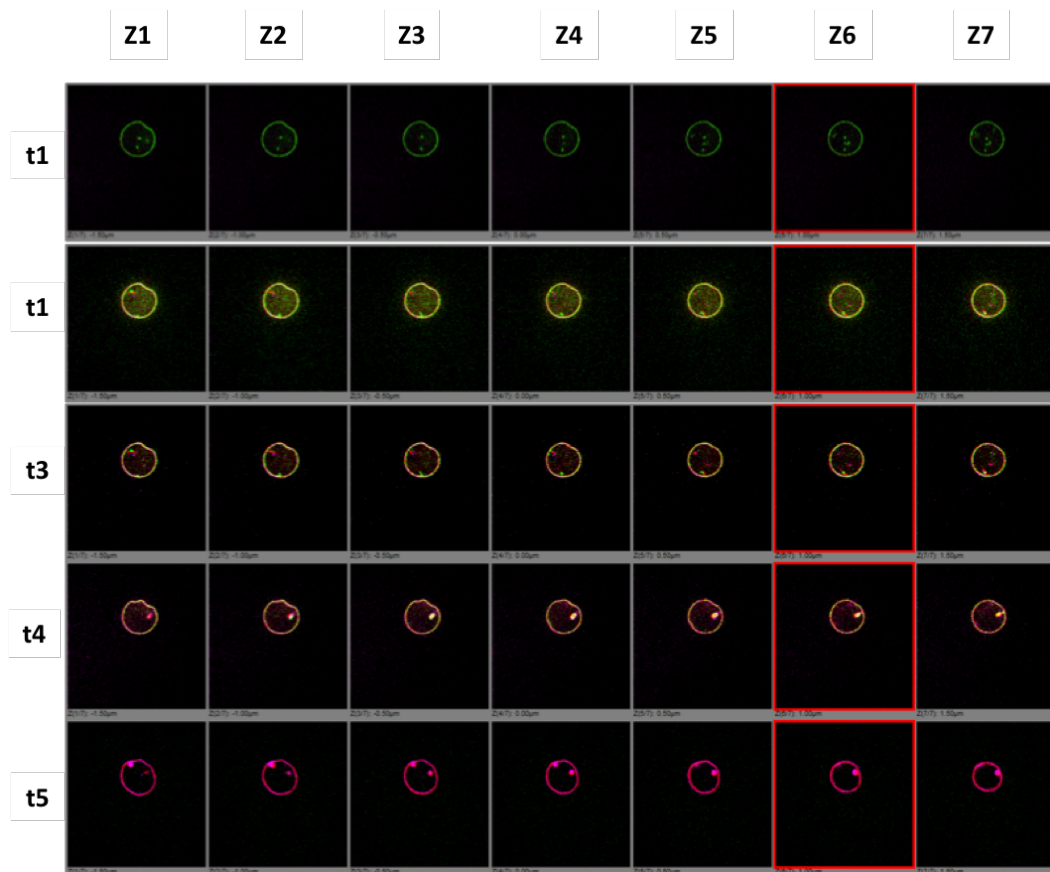


Figure S23. GUV3 sample from Figure S22. Merged images of 405ex/FITC, 488ex/Cy5, and 561ex/Cy5 channels over 7 different Z-positions (Z1-Z7) in increasing stimulation time point over 2 min (t1=0 s, t2=30 s, t3=60 s, t4=90 s, t5=120 s).

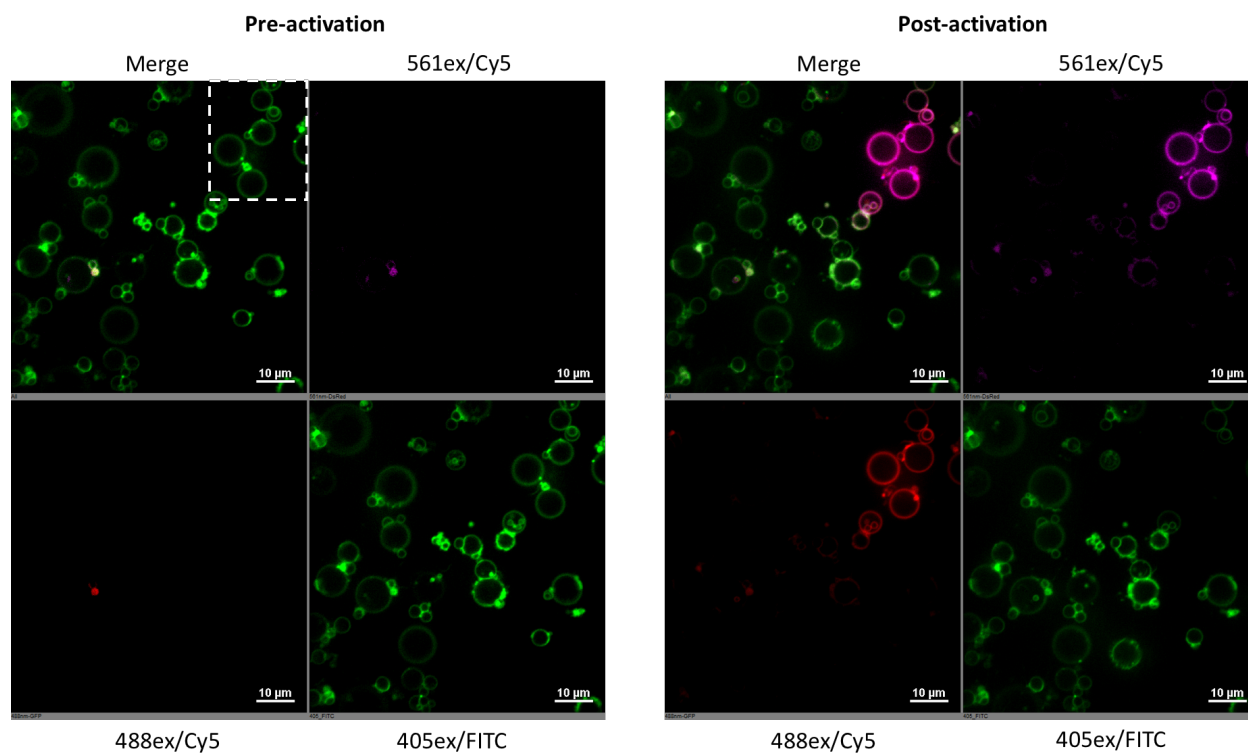


Figure S24. Photoactivation of GUV4 (1:99 **3a-PE**/ DOPC) in ROI.

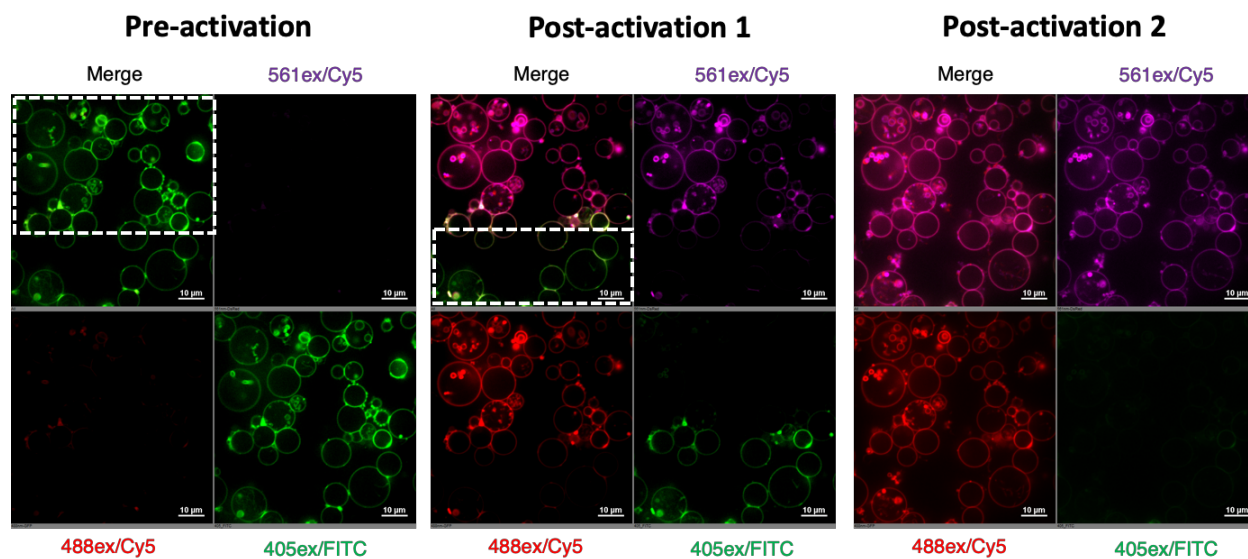


Figure S25. Sequential photoactivation of GUV4 (1:99 **3a-PE**/ DOPC) in ROI.

7. α -Synuclein (α S) Protein Experiment

7.1 General Experimental Workflow

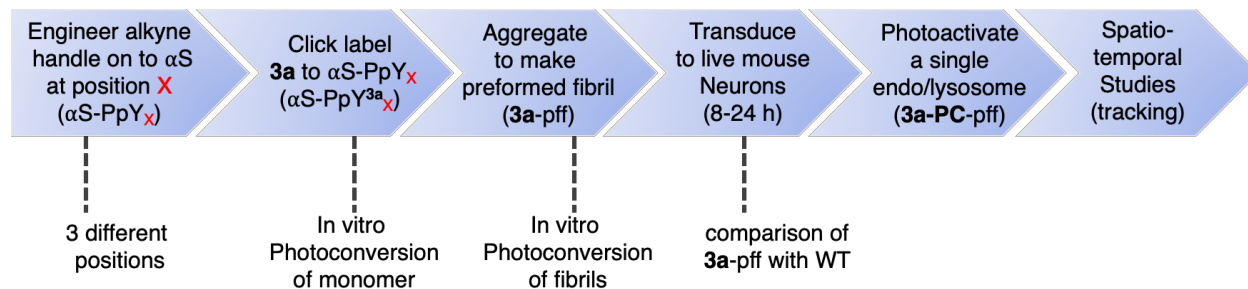


Figure S26. General experimental workflow to study CPX (**3a**) labeled α -Synuclein (α S). In order to study the neuronal uptake of CPX(**3a**)-labeled α S, pre-formed fibrils consisting of **3a**-labeled α S (**3a**-pffs) were systematically synthesized and validated in following order.

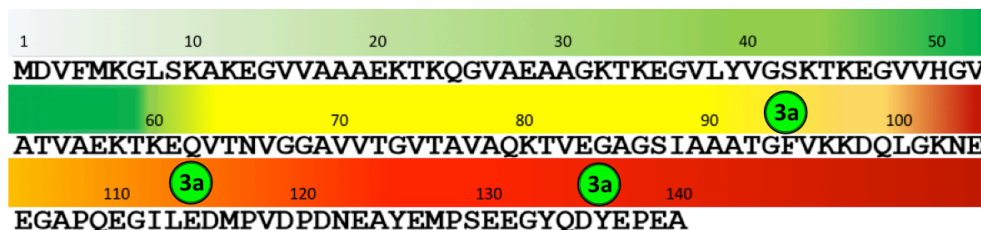
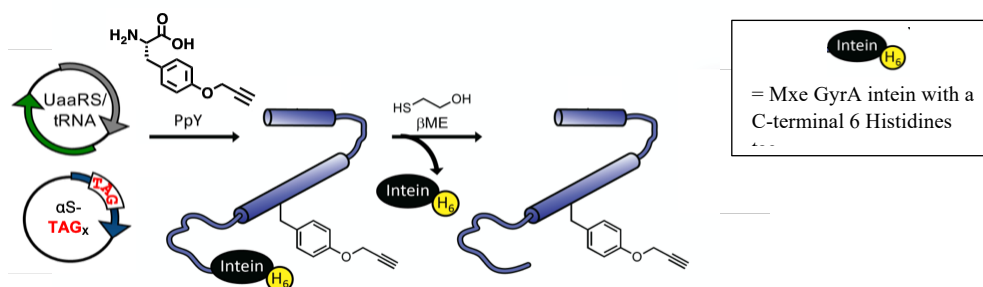


Figure S27. α S sequence with labeling positions highlighted for locations chosen for CPX (**3a**) labeling. **3a** is indicated with green circles at position 94, 114, and 136 of α S. Residues 1-60 (green region) are required for vesicle interactions and contain several key sites of point mutations associated with familial Parkinson's Disease. Residues 61-100 (yellow region) are the non-amyloid component (NAC) region, which is necessary for β -sheet fibril formation. Residues 101-140 (red region) comprise the highly acidic and unstructured C-terminal tail.⁶

7.2 Expression of Engineered α -Synuclein with Alkyne Handle (α S-PpY_x)



Proteins containing propargyl tyrosine (PpY) at residues 94, 114, and 136 have been previously described.⁷⁻⁹ A plasmid encoding for α S-TAG_x-MxeHis₆ was generated via site-directed mutagenesis as previously described using primers with sequences provided below.

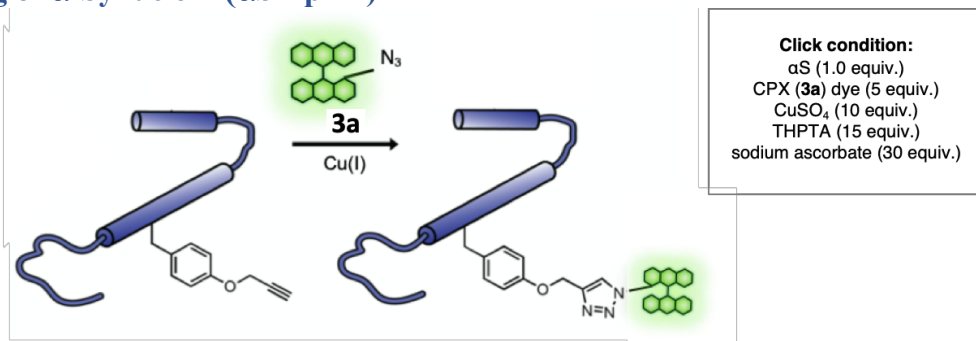
Forward: 5'-CCCACAGGAAGGAATTCTGTAGGATATGCCTGTGGATCCTG-3'

Reverse: 5'-CAGGATCCACAGGCATATCCTACAGAATTCCTTCCTGTGGC-3'

Mutagenesis was confirmed by DNA sequencing. Briefly, plasmids containing DNA sequences encoding for α S-TAG_x-MxeHis₆ were transformed into BL21(DE3) *E. coli* cells bearing the pDULE-pXF expression plasmid encoding for a modified *M. janaschii* tyrosyl synthetase capable of charging a suppressor tRNA^{CUA} with propargyl tyrosine (PpY). Cells (40 μ L) were incubated with α S-encoding plasmid (1 μ L) for 20-30 minutes on ice. Following incubation, cells were heat shocked at 42 °C for 1 minute, then cooled on ice for 2 minutes. After this time, 450 mL of SOC media was added and cells were incubated at 37 °C for 1 hour with 250 rpm shaking. Following this time, cells were plated on LB-agar plates supplemented with ampicillin and streptomycin and incubated at 37 °C overnight. Single colonies were then selected and grown in 5 mL LB media supplemented with 100 μ g/mL ampicillin and 50 μ g/mL streptomycin until visibly cloudy. After this time, cultures were transferred into 1 L of M9 minimal media supplemented with streptomycin (50 mg/L) and ampicillin (100 mg/L) and incubated at 37 °C, 250 rpm shaking for 4-6 hours until OD₆₀₀ = 0.7-1.0. The incubator temperature was then decreased to 18 °C and propargyltyrosine (218 mg/L, 1 mM final concentration) solubilized in water with a few drops of 5 M NaOH was added. After incubation for 10 minutes, isopropyl- β -D-1-thiogalactopyranoside (IPTG) was added to 1 mM final concentration. The culture was then incubated at 18 °C, 250 rpm shaking overnight (15-18 hours). Following incubation, cells were harvested by centrifugation (4000 rpm, 4 °C, 20 minutes) in a Sorvall RC5B centrifuge using a GS3 rotor. The supernatant was then decanted and the resulting cell pellet resuspended in 40 mM Tris pH 8.3 supplemented with one Roche cOmplete Mini protease inhibitor cocktail pill (catalogue #04693159001) and phenylmethanesulfonyl fluoride (PMSF, 1 mM final concentration from a 1 M stock in isopropanol). The resuspended cells were lysed by probe sonication (amplitude 50, 1 second on/off cycles for a total of 5 minutes on), then centrifuged in a Sorvall RC5 centrifuge equipped with an SS34 rotor operated at 14000 rpm for 25 min at 4 °C. Following this time, the supernatant was decanted and incubated with a 3 mL bed volume of Ni-NTA resin (Gold Bio, catalogue #H-350-50) for 1 hour on ice. The liquid was then allowed to flow through, and the resin was washed with 15 mL 50 mM HEPES pH 7.5, then with 20 mL 50 mM HEPES, 5 mM imidazole pH 7.5. Following these wash steps, protein was eluted by three washes consisting of 4 mL each of 50 mM HEPES, 300 mM imidazole pH 7.5. The fractions were combined and treated with β -mercaptoethanol (200 mM final concentration) overnight at room temperature.¹⁰ Following this treatment, the protein was dialyzed into 20 mM Tris pH 8 at 4 °C for 6-8 hours. After dialysis, the protein solution was incubated with a 3 mL bed volume of Ni-NTA resin for 1 hour on ice, then the flow through collected and dialyzed overnight

at 4 °C into 20 mM Tris pH 8. The protein was subsequently labelled as described below. Typical protein yields following this procedure were approximately 5-8 mg of semi-crude protein per culture.

7.3 Click-Labeling of α -Synuclein (α S-PpY^{3a})



Labeling of propargyltyrosine (PpY) containing α S proteins with fluorophore azide has been previously described.^{8-9, 11-12} A similar procedure was followed for labeling with CPX (**3a**) dye as described below. Protein concentrations from stocks purified as described above were determined using a Thermo Fisher Scientific Pierce BCA protein assay kit using bovine serum albumin (BSA) standards at 2, 1, 0.5, 0.25 and 0.125 mg/mL protein. Following incubation and determination of absorbance at 562 nm, a standard calibration curve was generated and used to estimate the concentration of α S-PpY_X containing protein (X indicating PpY at position 94, 114, or 136). Following concentration determination, the following labeling procedure was followed. A catalyst mixture for copper(I) azide-alkyne cycloaddition (CuAAC) was generated by mixing 10 equivalents (relative to protein) CuSO₄ (80 mM stock in water) with 15 equivalents (relative to protein) tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 50 mM stock in water) and briefly vortexing. Sodium ascorbate (30 equivalents relative to protein) was then added from a freshly prepared 100 mM stock in water and the resulting catalyst solution incubated at room temperature for 5-10 minutes. Following this time, CPX (**3a**) dye (5 equiv relative to protein) was added to the protein containing solution from a 25 mM stock in H₂O:MeCN (1:2). Following addition of the dye, the catalyst mixture was added to the protein containing solution and the resulting mixture vortexed briefly, then incubated at 37 °C until complete consumption of the starting (unlabeled) protein was observed by MALDI-TOF MS (typically 3-4 hours). Labeling reactions were then transferred to pre-wetted 3.5 kDa cutoff regenerated cellulose dialysis tubing (Fisher Scientific catalogue # 21-152-10) and dialyzed at 4 °C overnight in 20 mM Tris pH 8. Following dialysis, labelled proteins were purified by FPLC using an AKTA FPLC system and GE HiTrap Q HP column (catalogue #17-1154-01) and gradients between 20 mM Tris pH 8 and 20 mM Tris, 1 M NaCl pH 8. Fractions containing the desired protein were identified by MALDI-TOF MS, pooled, and concentrated using Amicon Ultra 3 kDa cutoff filters (EMD catalogue #UFC900324) to a final volume of ~1 mL. This material was further purified by RP-HPLC on a Varian ProStar system equipped with a Vydac C4 semi-preparative column using gradients between 0.1% TFA in water and 0.1% TFA in acetonitrile. Fractions containing the desired protein were pooled, diluted ~5-fold with α S buffer (20 mM Tris, 100 mM NaCl pH 7.5) and concentrated using Amicon Ultra 3 kDa cutoff filters; following the first concentration, protein was re-diluted and concentrated twice more to remove residual acetonitrile. Following concentration to ~0.5 mL total volume, proteins were stored at -80 °C and thawed only once per assay.

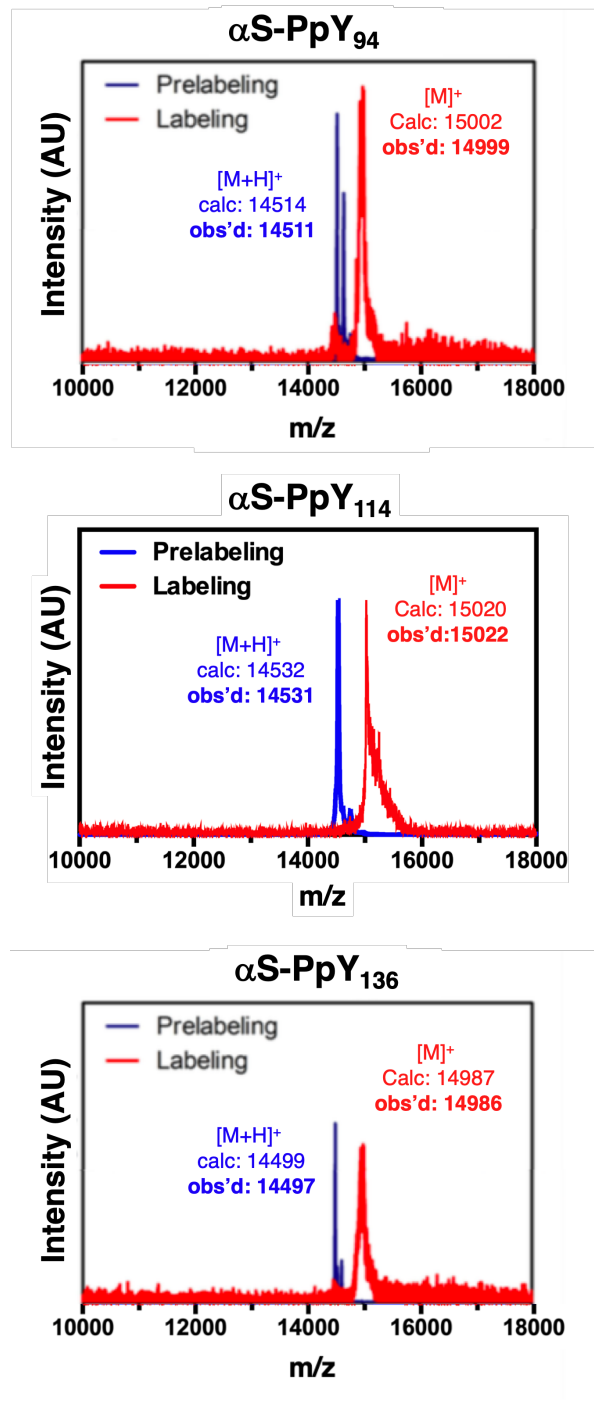
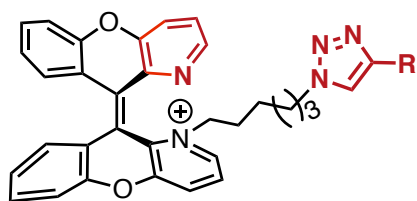


Figure S28. MALDI-MS of Labeling Reactions. Click labeling reactions of aS-PpY₉₄, aS-PpY₁₃₆ and aS-PpY₁₁₄ with **3a** show > 90% labeling after 4 to 6 h. to Click condition: protein (1.0 equiv.), CuSO₄ (10 equiv.), THPTA (15 equiv.) sodium ascorbate (30 equiv.), and CPX (**3a**) dye (5 equiv.).

7.4 Possible Copper binding between triazole and pyridine moiety of CPX probe¹³



Excess of copper catalyst (10 equiv) was used for efficient labeling as we observed possible copper binding to pyridine moiety of CPX probe. Crude labeling reactions through HPLC showed distinct peaks approximating $[M+H]^+$ as well as $[M+H+64]^+$ and $[M+H+128]^+$ Da. These adducts are hypothesized as Cu^+ or Cu^{2+} chelated mass. These adducts were removed following exchange of HPLC fractions into buffer containing 100 μ M EDTA, but MALDI ionization was poor. It should be noted that copper can cause broadening of MALDI-MS.

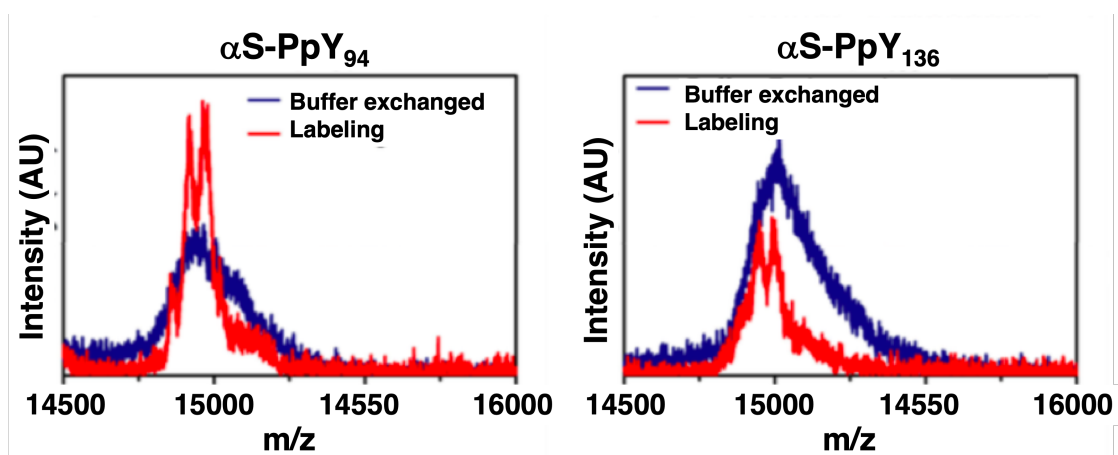
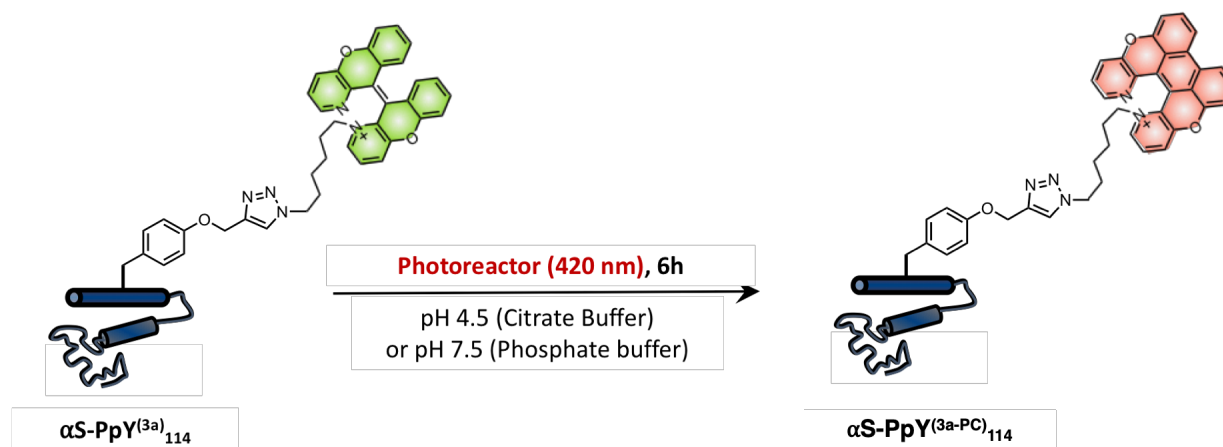


Figure S29. MALDI-MS analysis after and before EDTA buffer exchanged α S-PpY^{3a}₉₄ and α S-PpY^{3a}₁₃₆.

7.5 Photoconversion of Monomer

7.5.1 Photoconversion of α S-PpY^{3a}₁₁₄ with Rayonet Photoreactor



Solutions of α S-PpY^{3a}₁₁₄ at 0.5 μ M protein concentration were transferred to a LCMS vial (Thermo Scientific™ 9mm Wide Opening Clear Vial Convenience kits) and irradiated under Rayonet Photochemical Reactor (model RPR-100). Emission spectra were collected using $\lambda_{\text{ex}} = 410$ nm, $\lambda_{\text{em}} = 420 - 700$ nm or $\lambda_{\text{ex}} = 505$ nm, $\lambda_{\text{em}} = 515 - 700$ nm. Excitation spectra were collected using $\lambda_{\text{em}} = 545$ nm, $\lambda_{\text{ex}} = 350 - 530$ nm; $\lambda_{\text{em}} = 615$ nm, $\lambda_{\text{em}} = 350 - 600$ nm. Following collection of data prior to irradiation, solutions were kept in the vial and irradiated with 420 nm (LZC-420) light bulb using Rayonet Photoreactor for successive periods of 2 hours. After each irradiation time (0, 2, 4 and 6 hours in the vial), spectra were collected using the parameters above. Proteins in pH 7.5 (20mM Tris 100mM NaCl) and pH 4.5 (50 mM citrate buffer) were analyzed and compared. Triplicate samples were measured and irradiation time and averaged in the plots on the next page.

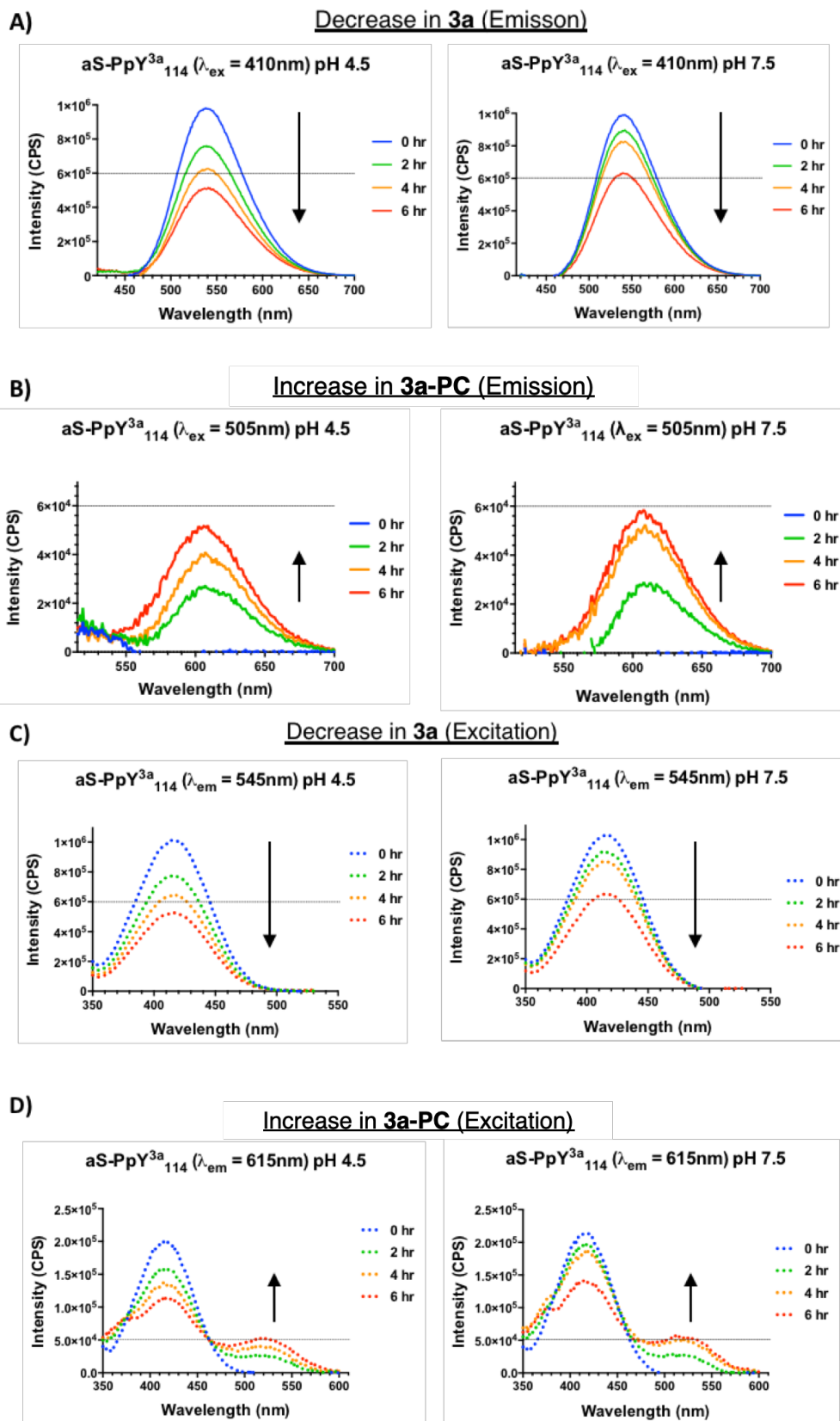
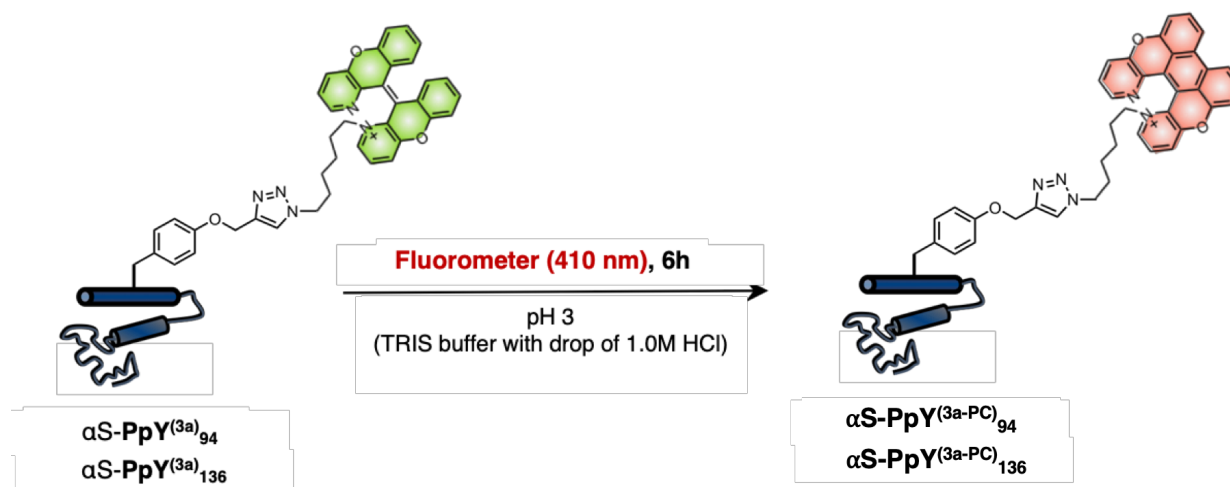


Figure S30. A-B) Emission spectra and C-D) Excitation spectra of α S-PpY^{3a}₁₁₄ monomer.

7.5.2 Photoconversion of monomer α S-PpY^{3a}₉₄ and α S-PpY^{3a}₁₃₆ with Fluorometer



Solutions of α S-PpY^{3a}₉₄ or α S-PpY^{3a}₁₃₆ at 4 μ M protein concentration were transferred to a 1 cm path length fluorometer cuvette. Emission spectra were collected using $\lambda_{ex} = 410$ nm, $\lambda_{em} = 420 - 700$ nm and $\lambda_{ex} = 505$ nm, $\lambda_{em} = 515 - 700$ nm. Excitation spectra were collected using $\lambda_{em} = 543$ nm, $\lambda_{ex} = 350 - 530$ nm; $\lambda_{em} = 612$ nm, $\lambda_{ex} = 350 - 600$ nm. Following collection of data prior to irradiation, solutions were kept in the cuvette and irradiated with 410 nm light using the fluorometer light source (deuterium lamp) for successive periods of 2 hours. After each irradiation time (0, 2, 4 and 6 hours in the cuvette), spectra were collected using the parameters above.

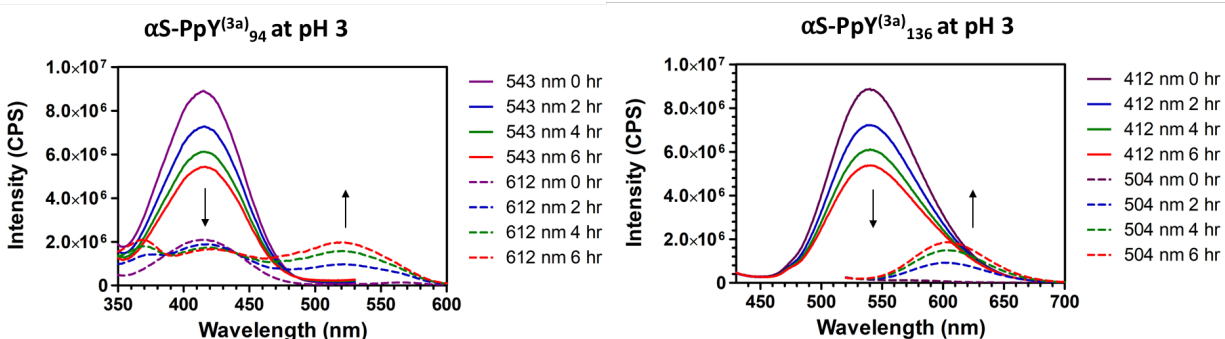


Figure S31. Excitation (solid) and emission (dotted) spectra of α S-PpY^{3a}₉₄ and α S-PpY^{3a}₁₃₆ over 0, 2, 4, 6h of irradiation with fluorometer.

7.6 Photo-degradation Analysis of Monomer α S-PpY^{3a}₁₁₄

Test for photodegradation of α S: 40 μ L of α S-PpY^{3a}₁₁₄ at 70 μ M protein (monomer) were transferred to a LCMS vial as described in page S27 and irradiated up to 11 hours. First 10 μ L of α S-PpY^{3a}₁₁₄ was stopped after 6 h and rest of protein was diluted to 20 μ M for additional 5 h of irradiation (total of 11 h). Emission spectra were collected using $\lambda_{ex} = 410$ nm, $\lambda_{em} = 420 - 700$ nm for disappearance of **3a** (pre-activated) and $\lambda_{ex} = 505$ nm, $\lambda_{em} = 515 - 700$ nm for appearance of **3a-PC** (post-activated) probe on protein (Figure S32 Top). Following the confirmation of photoconversion, MALDI-MS (Figure S32 bottom) and SDS-PAGE (Figure S33) analysis were done to determine presence of severe degradation.

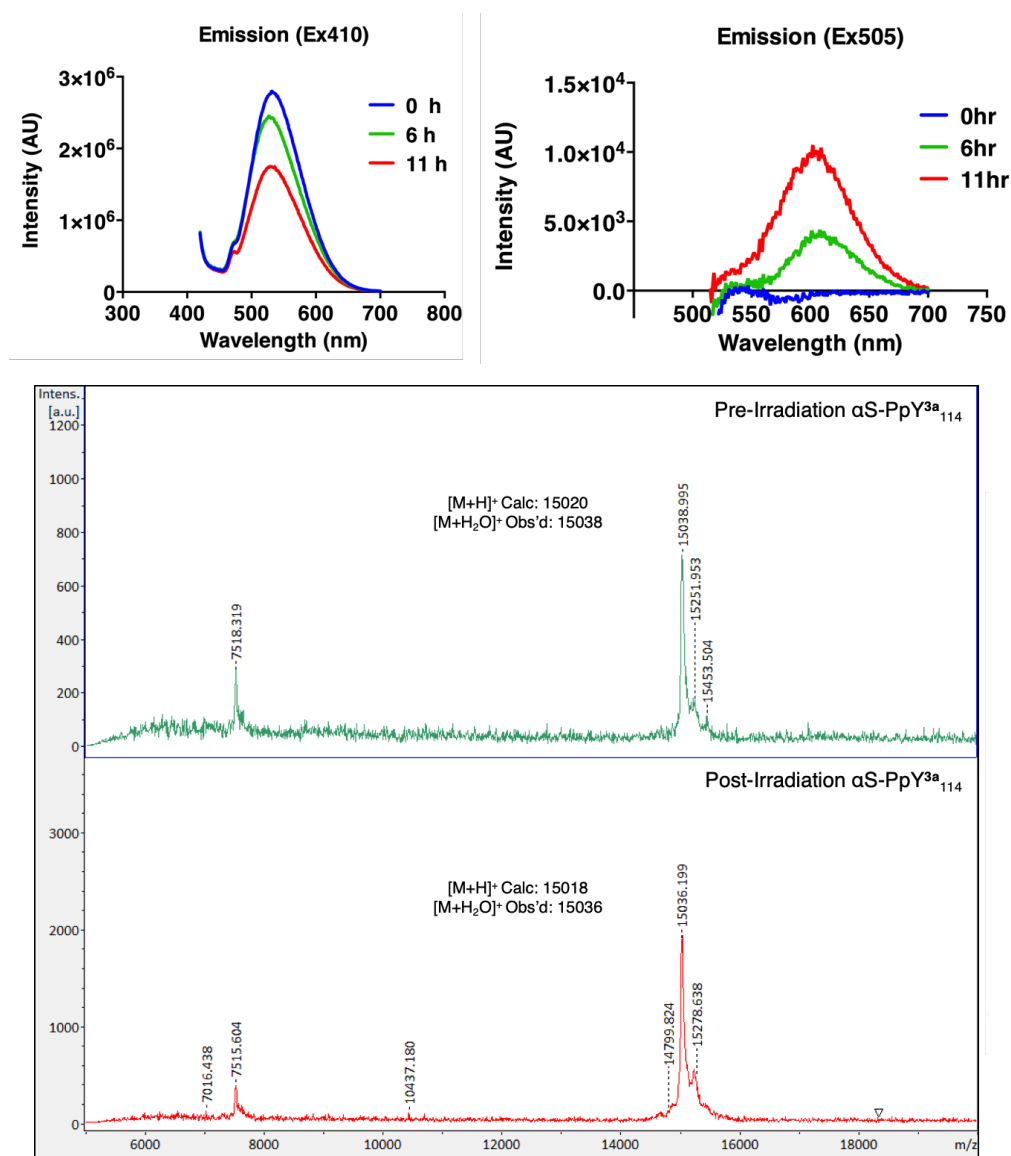


Figure S32. Fluorescence measurement to confirm the photoconversion (top). MALDI-MS analysis for photodegradation of α S-PpY^{3a}₁₁₄. Post-irradiated proteins (bottom) did not show any significant fragmentations. Water adduct masses ($[M+18]^+$) were observed (bottom).

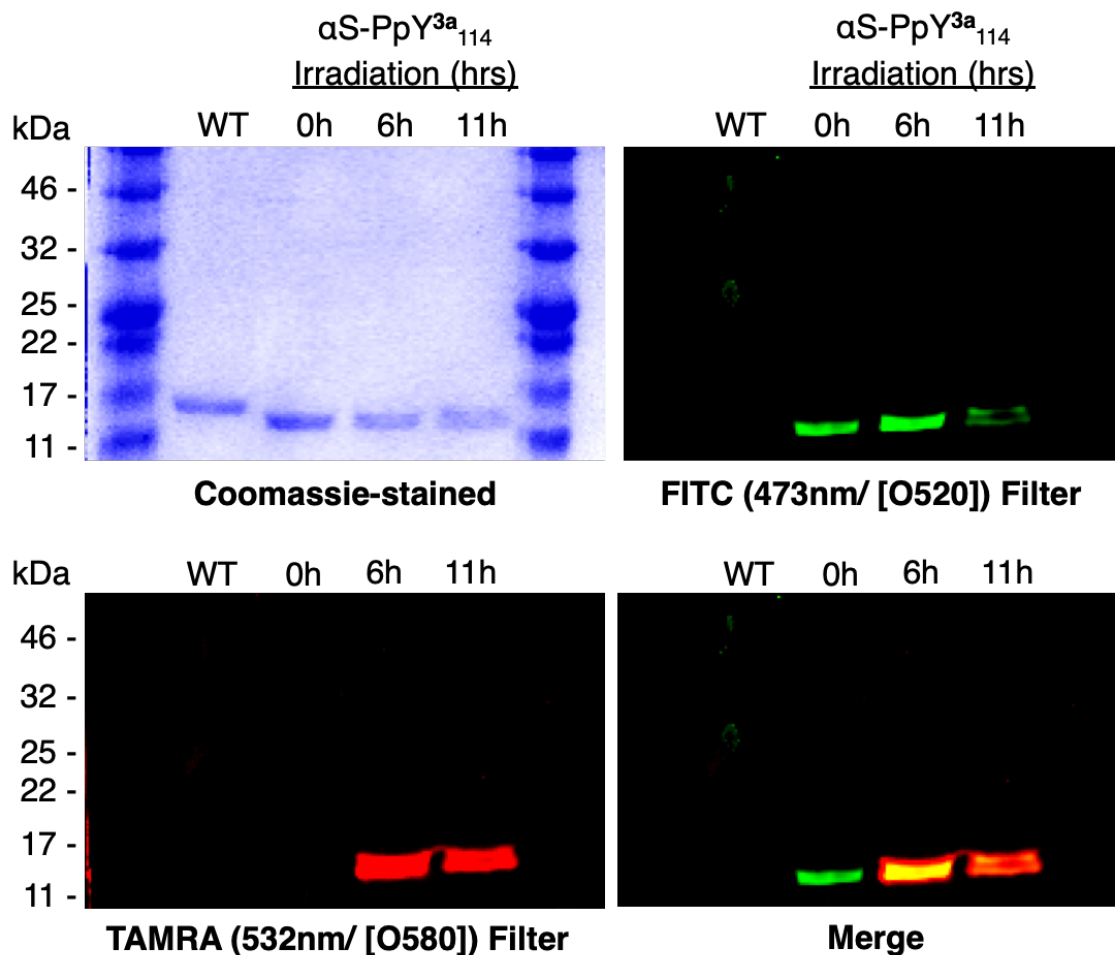
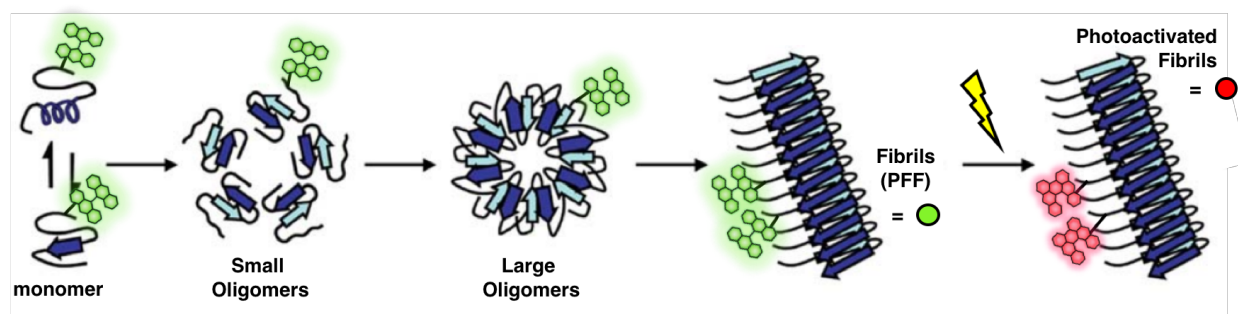


Figure S33. SDS-PAGE of wild-type α S (WT) and α S-PpY^{3a}₁₁₄ after 0, 6, and 11h of irradiation (20 μ M protein). Coomassie stain of gel was done to show any fragmentation or photo-crosslinked high molecular weight protein. Fluorescence gel images were acquired on a Typhoon FLA7000 (FITC filter: λ_{ex} = 473 nm, λ_{em} = 520 nm) for presence of pre-activated **3a** (α S-PpY^{3a}₁₁₄) and TAMRA filter (λ_{ex} = 532 nm, λ_{em} = 580 nm) for presence of post-activated **3a-PC** (α S-PpY^{3a-PC}₁₁₄). No significant aggregation or photo-crosslinked proteins of higher molecular weight observed.

7.7 Photoconversion of fibril in photoreactor



Protein concentrations were determined using UV-Vis spectroscopy on a Hewlett-Packard 8452A diode array spectrophotometer (currently Agilent Technologies). Wild-type α S was quantified using $\epsilon_{280} = 5120 \text{ M}^{-1} \text{ cm}^{-1}$ based on amino acid sequence.¹⁴ α S-PpY^{3a}_X proteins (X = 94, 114, 136) were quantified using $\epsilon_{408} = 11040 \text{ M}^{-1} \text{ cm}^{-1}$ based on previously reported extinction coefficient for dye in water.² Labeled proteins were used to generate fibrils under the following conditions: 100 μM protein in 20 mM Tris, 100 mM NaCl pH 7.5 in a 1.7 mL Eppendorf tube. For α S-PpY^{3a}₉₄ and α S-PpY^{3a}₁₃₆, 5 μM labeled protein and 95 μM mixture is used. For α S-PpY^{3a}₁₁₄, 50 μM labeled protein and 50 μM WT protein mixture is used. Fibrils were formed by agitation at 37 °C, 1500 rpm for 48 hours in an IKA MS3 orbital shaker. Following fibril formation, aggregated protein was pelleted (13,200 rpm, 4 °C, 90 min) in a benchtop fixed-rotor Eppendorf centrifuge. Following centrifugation, the supernatant was carefully decanted and the resulting pellet resuspended by addition of an equivalent volume of the buffer above and repeated vortexing. Resuspended fibrils were then diluted (10x) into 50 mM citrate buffer, pH 4.5 for irradiation & fluorescence acquisition ($\sim 10 \mu\text{M}$ total protein). Irradiation was performed using a Rayonet Photochemical Reactor (model RPR-100) at room temperature 0, 2, 4, 6 hours & $\sim 200 \mu\text{L}$ aliquot removed at given time point, stored shielded from light until fluorescence data acquired. Steady state fluorescence spectra were collected using a PTI QuantaMaster fluorometer equipped with a Peltier temperature controller. All spectra were collected at 20 °C using a 1 nm step size, 0.25 second integration time, and 5 nm excitation and emission slit widths. Triplicate samples were measured for each labeling and irradiation time and averaged in the plots below. Steady-state emission spectra were collected using $\lambda_{\text{ex}} = 410 \text{ nm}$, $\lambda_{\text{em}} = 420 - 700 \text{ nm}$ or $\lambda_{\text{ex}} = 505 \text{ nm}$, $\lambda_{\text{em}} = 515 - 700 \text{ nm}$. Steady state excitation spectra were collected using $\lambda_{\text{em}} = 545 \text{ nm}$, $\lambda_{\text{ex}} = 350 - 530 \text{ nm}$ or $\lambda_{\text{em}} = 615 \text{ nm}$, $\lambda_{\text{ex}} = 350 - 600 \text{ nm}$.

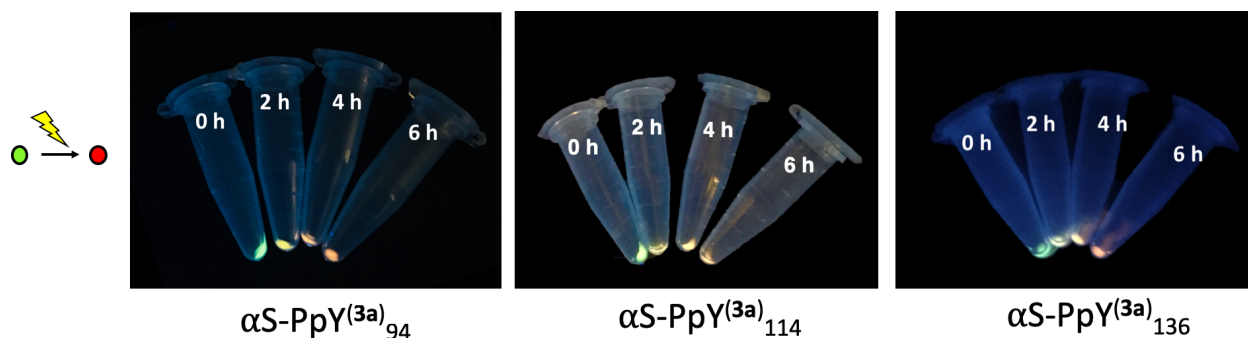


Figure S34. Visualization of change in emissive color of α S-PpY^{3a} Proteins Incorporated into Fibrils. Hand held UV lamp 365 nm to visualize 5% α S-PpY^{3a}₉₄, 5% α S-PpY^{3a}₁₁₄, and 50 % α S-PpY^{3a}₁₃₆ in WT fibrils from left to right. Hours indicate irradiation hours with photoreactor.

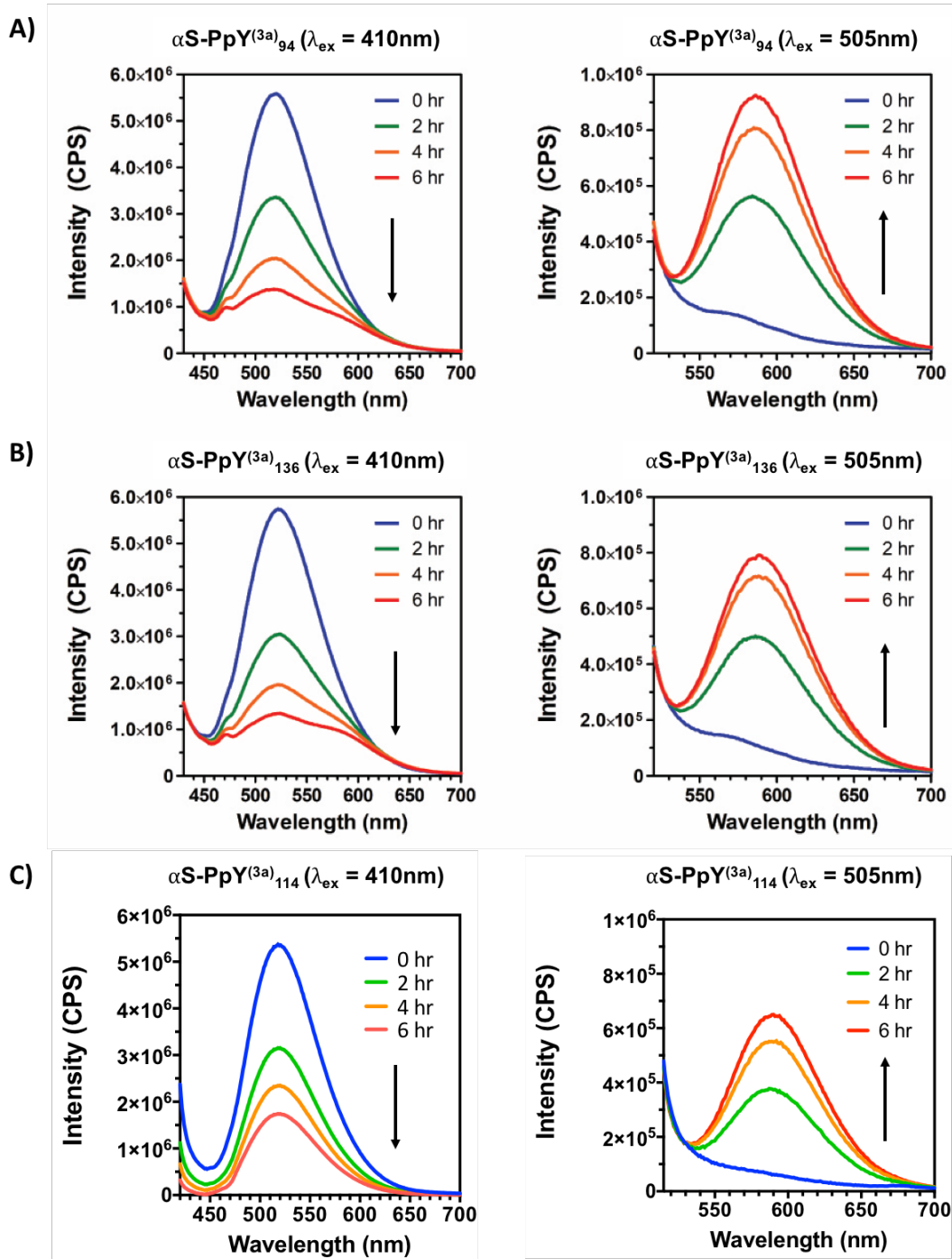


Figure S35. Emission spectra of α S-PpY^{3a} Proteins Incorporated into Fibrils. Fluorescence emission spectra from excitation at 410 nm (left) or 505 nm (right) for A) 5% α S-PpY^{3a}₉₄ fibrils, B) 5% α S-PpY^{3a}₁₃₆ fibrils, and C) 50% α S-PpY^{3a}₁₁₄ fibrils at pH 4.5.

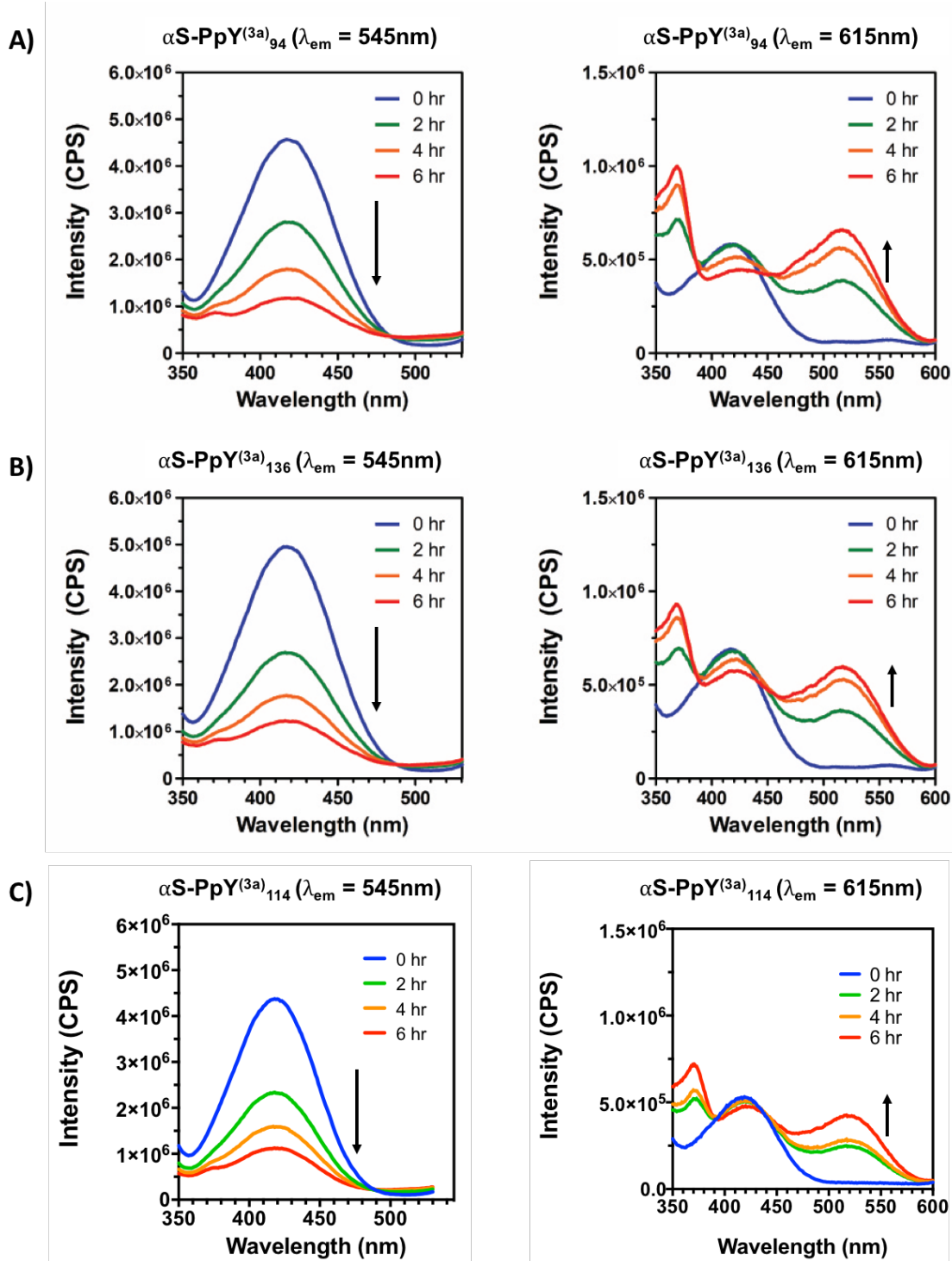


Figure S36. Excitation spectra of $\alpha\text{S-PpY}^{3a}$ Proteins Incorporated into Fibrils. Fluorescence excitation spectra from emission at 545 nm (left) or 615 nm (right) for A) 5% $\alpha\text{S-PpY}^{3a}_{94}$ fibrils, B) 5% $\alpha\text{S-PpY}^{3a}_{136}$ fibrils, and C) 50% $\alpha\text{S-PpY}^{3a}_{114}$ fibrils at pH 4.5.

7.8 SDS-PAGE Analysis of Labeled Protein Incorporation

A 30 μL aliquot was removed and stored at $-20\text{ }^{\circ}\text{C}$ prior to aggregation from the protein mixtures described above to use as a quantification standard. Following fibril formation, insoluble material was pelleted and resuspended as described above. The resuspended pellet (10 μL aliquot) was combined with SDS (2 μL from a 150 mM stock in water; 25 mM final concentration) and boiled for 15-20 minutes. The samples were then cooled on ice for 10-15 minutes, then 3 μL 4X loading dye added. Monomeric samples for calibration were prepared by serial 2-fold dilution of 10 μL with water (standard lanes correspond to 100, 50, 25, and 12.5 μM total protein from left to right with 5% $\alpha\text{S-PpY}^{3a_x}$); 2 μL water was then added to each standard sample, followed by 3 μL loading dye. Standard and pellet samples were then analyzed by SDS-PAGE (15% acrylamide gel run at 150 V for 1.5 hours). Fluorescence images were acquired using a Typhoon FLA7000, and each gel stained with Coomassie Brilliant Blue then destained at room temperature and imaged (Figure S37-38). Gel quantification was performed using ImageJ software. The area of each fluorescent band or total protein by Coomassie staining was determined, and the monomeric standards used to generate a linear calibration; the protein present in the pellet samples was determined relative to the calibration curve, and the fraction relative to the first standard band calculated. The three samples were averaged and plotted as mean and standard deviation.

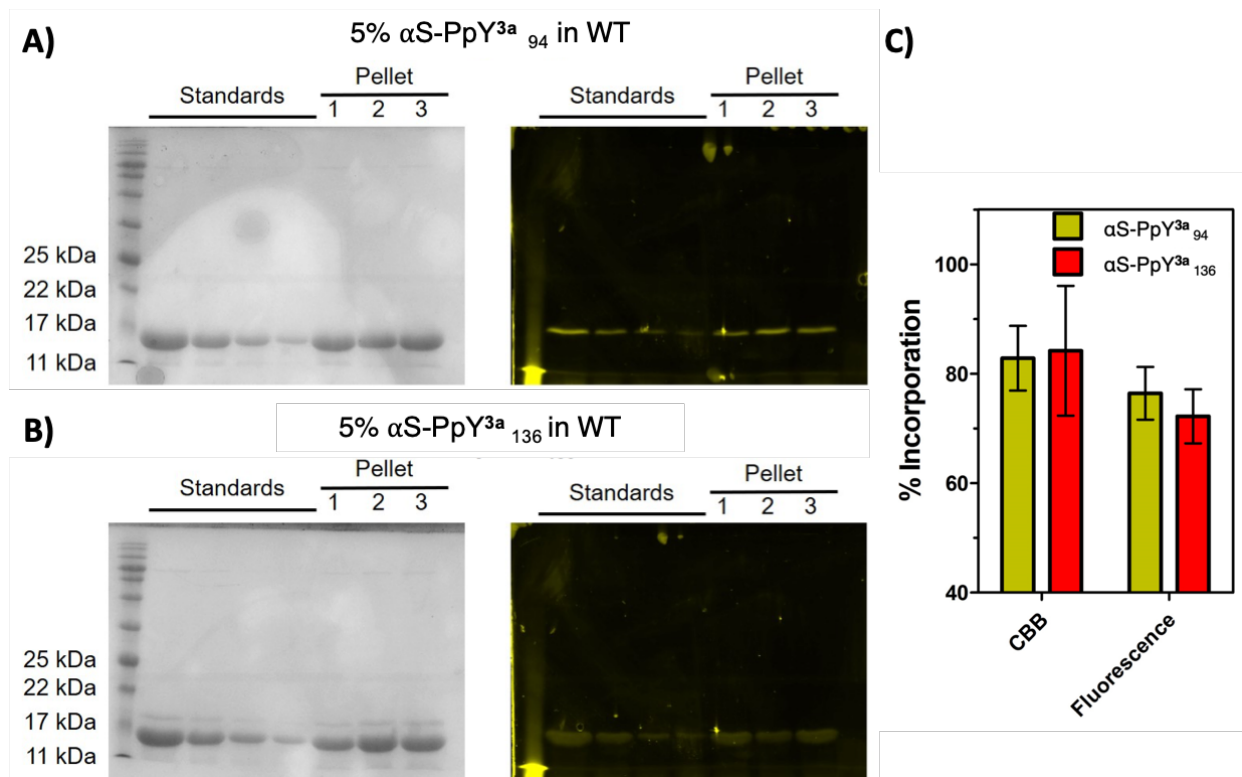


Figure S37. SDS-PAGE Incorporation of 5% $\alpha\text{S-PpY}^{3a}_{94}$ and 5% $\alpha\text{S-PpY}^{3a}_{136}$ Proteins into Fibrils. The identity of each protein is indicated above the plots corresponding to Coomassie-stained gels (left) or fluorescence gel images (middle). Standards refers to monomeric samples, serially diluted. Pellets correspond to triplicate samples of fibrils generated under identical conditions. Fibrils made with A) 5% $\alpha\text{S-PpY}^{3a}_{94}$ B) 5% $\alpha\text{S-PpY}^{3a}_{136}$ in WT. C) Quantification of protein in aggregated samples relative to monomer as determined by gel densitometry. Bars represent mean with error bars representing standard deviation.

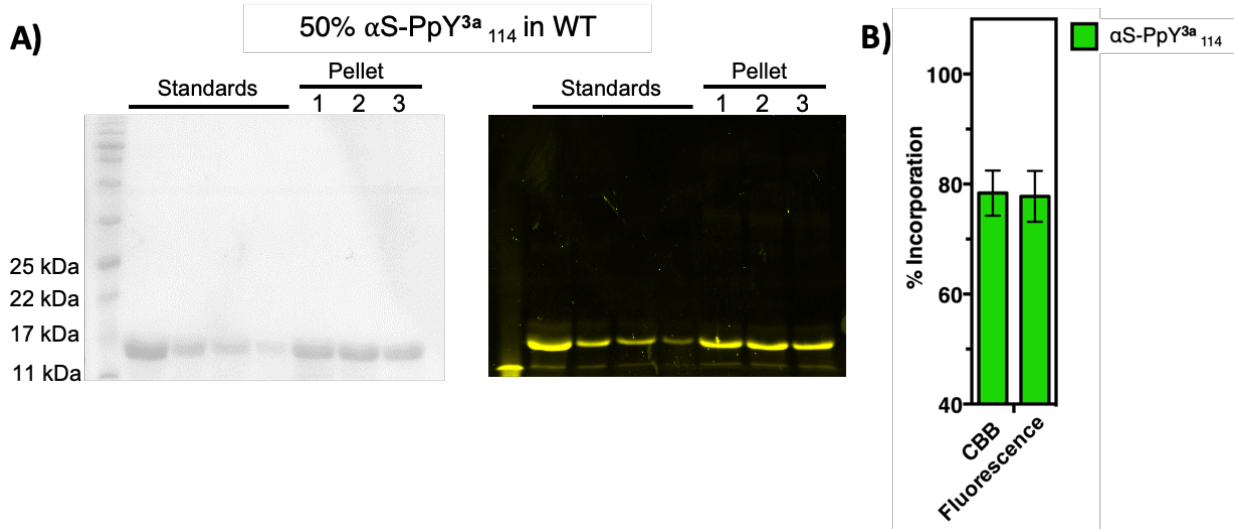


Figure S38. SDS-PAGE Incorporation of 50% α S-PpY^{3a}₁₁₄ Proteins into Fibrils. A) Coomassie-stained gels (left) or fluorescence gel images (middle). Standards refers to monomeric samples, serially diluted. Pellets correspond to triplicate samples of fibrils generated under identical conditions. Fibrils made with 50% α S-PpY^{3a}₁₁₄ in WT. C) Quantification of protein in aggregated samples relative to monomer as determined by gel densitometry. Bars represent mean with error bars representing standard deviation.

8. Live Neuronal Uptake Experiment

8.1 Synthesis and Validation of 3 α -pffs (50% WT and 50% α S-PpY^{3a}₁₁₄)

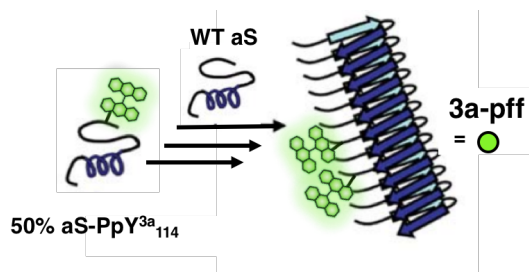


Figure S39. 3 α -pffs were prepared by mixing 50% WT- α S and 50% α S-PpY^{3a}₁₁₄.

Synthesis of 3 α -pffs. 3 α -pffs were prepared by mixing 50% WT- α S with 50% α S-PpY^{3a}₁₁₄ at a total concentration of 2 mg/mL in Dulbecco's Phosphate Buffered Saline (DPBS). The resulting solution was sterile filtered and incubated at 37°C with constant agitation at 1,000 rpm for 7 days.

Circular Dichroism Spectroscopy (Figure S40A). The concentration of purified WT ($\epsilon_{280} = 5120 \text{ M}^{-1} \text{ cm}^{-1}$) and α S-PpY^{3a}₁₁₄ ($\epsilon_{408} = 11040 \text{ M}^{-1} \text{ cm}^{-1}$) were determined by UV-Vis absorbance. CD spectra were acquired on an Aviv model 410 circular dichroism spectrophotometer in 1 mM path length quartz cuvettes at 25 °C. Spectra were acquired as previously described.¹⁵⁻¹⁶ α S-PpY^{3a}₁₁₄ exhibited spectra indicative of an α -helical conformation; the intensity of the helical signal at 208 and 222 nm were similar to wild-type (WT).

In Vitro Aggregation Kinetics (Figure S40B). The aggregation reaction was carried out for 3 α -pffs generated with 50% α S-PpY^{3a}₁₁₄ in WT as previously described.¹⁵⁻¹⁶ Aggregation kinetics of 3 α -pffs were slower than that of WT,¹⁶ but such delayed aggregation has been observed previously, and no deleterious effects were observed for cellular uptake.¹⁵

SDS-PAGE (Figure S40C). Fibrillization was confirmed by sedimentation analysis as previously described for WT α S.¹⁷ Briefly, samples were spun on an ultracentrifuge (Beckman-Coulter) at 100,000x g for 30 min at rt. The supernatant (S) was collected, and the insoluble pellet (P) was resuspended in an equal volume of DPBS. Samples were diluted in 5X Laemmli buffer, boiled, and separated by SDS-PAGE. The gel was stained by Coomassie Brilliant Blue, and bands were quantified using ImageJ (NIH). It was found that $95.7 \pm 1.6 \%$ of the total protein for each sample was found in the pellet fraction (mean \pm SD, n = 2, each measured in duplicate).

Transmission Electron Microscopy or TEM (Figure S40D) For TEM, a 4 μ L drop of sample was applied to a carbon Formvar coated 300-mesh Cu grid. Samples were allowed to rest for 5 min at room temperature. Excess solution was wicked off and the grid was washed 2 x 5 min by inversion over a water droplet. The grids were then stained for 5 min with freshly-prepared and sonicated 0.4-2% w/v uranyl acetate in water. Excess uranyl acetate solution was wicked from the grids with a kimwipe. TEM was carried out on a JEOL-1010 instrument with an accelerating voltage of 80 kV. Images were collected at magnification of 100,000x.

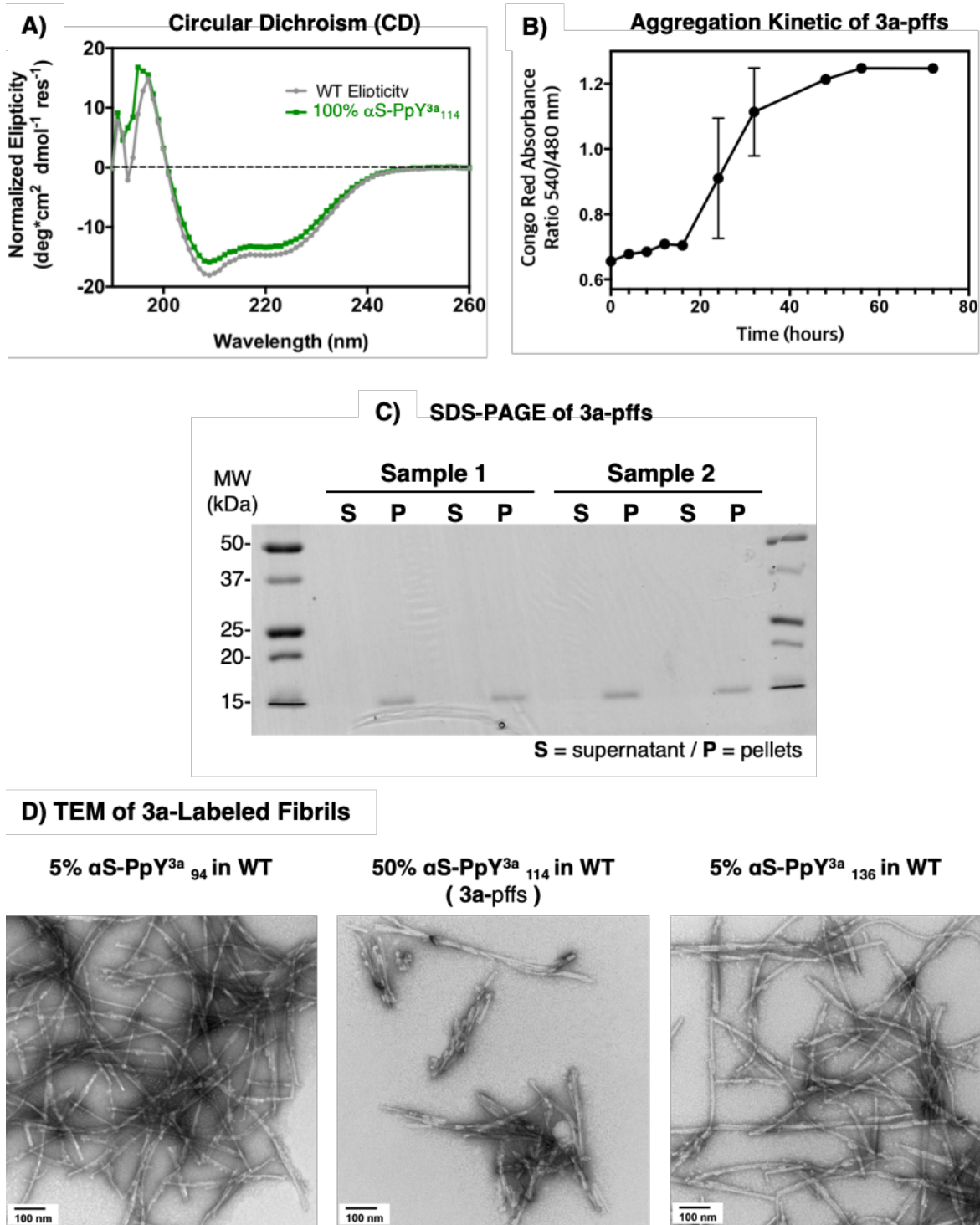


Figure S40. A) Circular dichroism spectra of α S-WT (gray) and 100% α S-PpY^{3a}₁₁₄ (green); B) in vitro aggregation kinetic data of 3a-pffs (50% α S-PpY^{3a}₁₁₄ in WT); C) SDS-PAGE of 3a-pffs, and D) TEM imaging of 5% α S-PpY^{3a}₉₄ in WT, 3a-pffs (50% α S-PpY^{3a}₁₁₄ in WT), and 5% α S-PpY^{3a}₁₃₆ in WT

8.2 Experimental Set Up

8.2.1 Cell Culture.

Primary neuronal cultures were prepared from E15-E17 embryos of CD1 mice (Charles River) as previously described.¹ All procedures were performed according to the NIH Guide for the Care and Use of Experimental Animals and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Dissociated hippocampal neurons were plated at a density of 100,000 cells in the glass center chamber of a MatTek dish (35 mm dish, 14 mm well, #1.5 coverglass) for live cell imaging, and were allowed to mature for 7-10 d in complete neuronal medium (Neurobasal without phenol red (Thermo Fisher), 5% B27 supplement (Thermo Fisher)). Medium was partially exchanged every 3-4 days.

8.2.2 Sample Preparation.

Images were acquired as previously described.¹⁵ Trypan blue diphosphate (Sigma-Aldrich) solutions were freshly prepared on the day of experiment as a 2x solution (1 mM in DPBS without Ca^{2+} , Mg^{2+} , Gibco) and sterilized by filtration. Trypan blue, complete neuronal medium, and neurobasal without phenol red, B27, or antibiotic supplementation were equilibrated at 37°C, 5% CO_2 . **3a**-pffs (50% aS-PpY^{3a}₁₁₄ & 50% WT) were diluted in DPBS to 100 µg/mL for and sonicated (Diagenode Biorupter™ UCD-300 bath sonicator set to high, 30s sonication followed by a delay period of 30s, 10 min total). Sonicated pffs were then diluted in neurobasal to 10 µg/mL. All neuronal medium was collected from the dish and 100 µL of the transduction suspension was directly added to the center chamber. Transduced cultures were incubated for 3-4h before filtered conditioned medium was returned to the dish. Transductions were allowed to proceed for 4 h to 1 d. For trypan blue experiments, immediately before imaging the neuronal culture dish was washed gently with 1 mL equilibrated neurobasal to remove B27. Trypan blue solution (1 mL, 1 mM in DBPS) was added dropwise to the dish and mixed by gentle pipetting for a final concentration of 500 µM trypan blue. For longer-term imaging experiments without trypan blue, neurons were maintained in conditioned neuronal medium. Images were acquired on a Nikon spinning disk confocal microscope.

8.2.3 Imaging Conditions.

Green channel (**405ex/FITC**) was used to image pre-activated **3a**. Upon activation, fluorescence intensity from **3a** decreases under the green channel. Sequentially, red channel (**488ex/Cy5**) was used to image post-activated **3a-PC** upon stimulation. Initially, when none of the probe is activated, **3a** is not detected or displays very low background fluorescence under the red channel. After photo-stimulation (photoactivation) with the Spectra X laser, fluorescence of post-activated **3a-PC** will appear under the red channel. The false-colored blue channel (**561ex/Cy5**) is used to image trypan blue, which fluoresces when bound to extracellular proteins on the cell membrane, enabling the visualization of intact cell membranes. Only fluorescence of internalized **3a**-pffs is detected, as fluorescence of extracellular **3a**-pffs in contact with trypan blue is quenched.

Green channel: **405ex/FITC 100%** (excitation laser 405 nm with FITC emission filter); **3a**
Red channel: **488ex/Cy5 50%** (excitation laser 488 nm with Cy5 emission filter); **3a-PC**
Blue channel: **561ex/Cy5 100%** (excitation laser 561 nm with Cy5 emission); **Trypan blue**
Merge: Green, Red, and Blue channels are merged.
Stimulation: 440 nm Spectra X 100% on ROIs (DMD) for 40 – 60 sec
Incubator: 37°C, 5% CO_2

8.3 Guideline for Imaging Analysis

Red Channel (**488ex/Cy5**) shows fluorescence from both **3a-PC** and **Trypan blue**. Hence, the merged purple region between channels (**488ex/Cy5**) and (**561ex/Cy5**) indicate the cell membrane. Merged images between the green (**405ex/FITC**) and red (**488ex/Cy5**) channels indicate the population of pre- (**3a-pffs**; appearing as green puncta) and post-activated **3a-pffs** (**3a-PC-pffs**; appearing as red puncta).

Note. 405ex 100% laser can also activate **3a** probe. It is best to identify the cell of interest and focus using the blue channel (**561ex/Cy5**). For fine focus to identify specific vesicles, use laser power (<25%) with 405ex for green channel(**405ex/FITC**). The dotted white square below indicates a representative ROI (region of interest) where DMD/Spectra X laser stimulation would be applied.

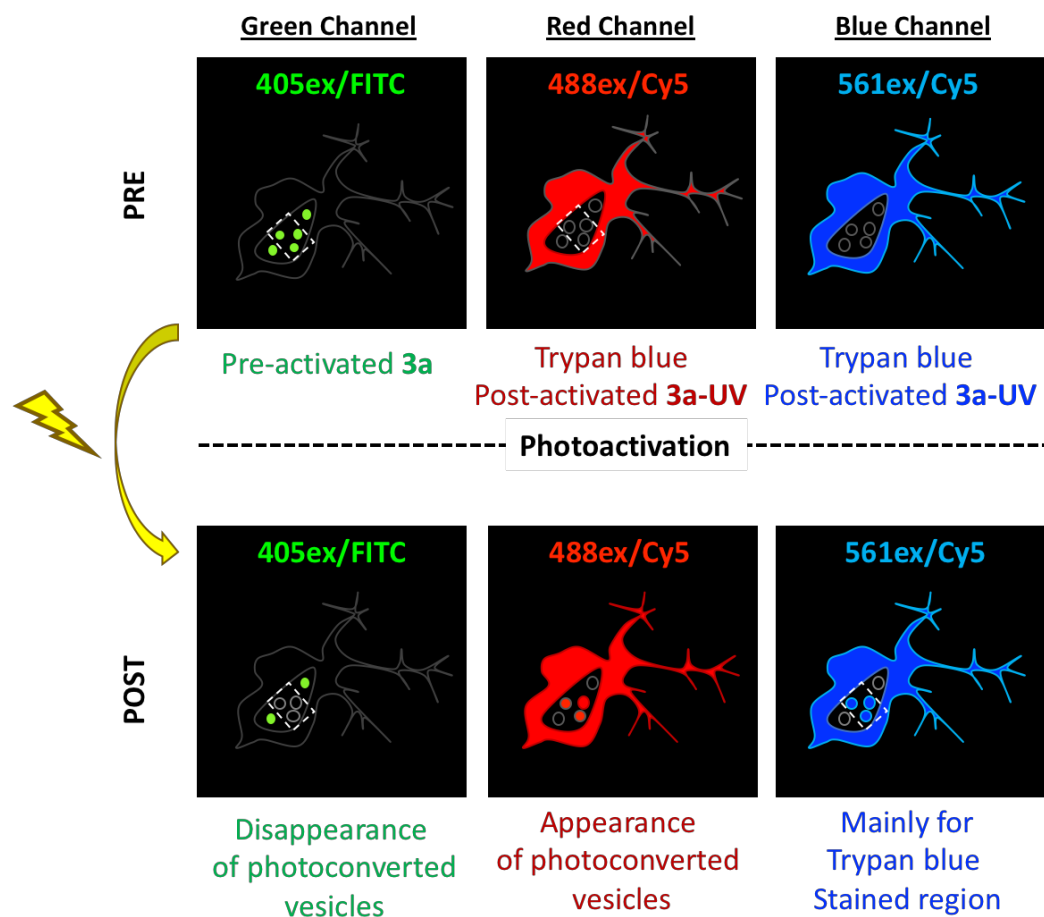


Figure S41. Schematic view of three channels pre- and post-activation of a single neuron. The white dotted square indicates a representative region of irradiation with the stimulation laser (440 nm, DMD).

8.4 Photoactivation and Tracking Protocols

8.4.1 Photoactivation of a single endosome/lysosome experiment (Exp A)

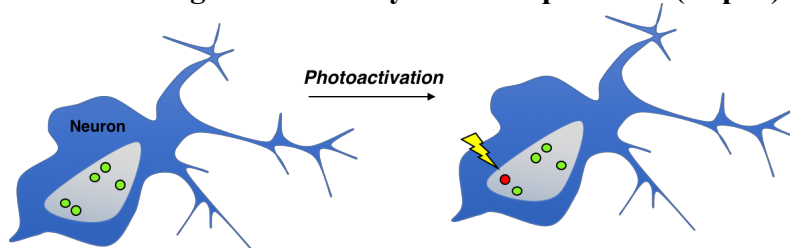


Figure S42. Schematic view of Exp A

The neuronal culture dish (transduced with **3a**-pffs overnight) was carefully washed with 1.0 mL of neurobasal medium. 1.0 mL of neurobasal medium was added to the culture dish, which was placed on the stage-top incubator and allowed to equilibrate for 3-5 min. Neurons were identified under Differential Interference Contrast (DIC) before addition of 1.0 mL of trypan blue solution as described above. The correct focal plane was identified low power 561ex/Cy5. “Pre-activated” images were captured using three channels: green, red, and blue with three z-planes. For stimulation, vesicles of interest in the center of the collected z-stack were identified, and ROIs were drawn to contain these vesicles. Exposure times for imaging were minimized (i.e., images were not collected simultaneously while stimulating the ROI). ROIs were photoactivated with 440 nm Spectra X laser at 100% for 60 s. Images of “post-activated” puncta were captured under three channels: green, red, and blue. Depending on the size of vesicles or focused point of laser, stimulation time required for full conversion may vary.

8.4.2 Tracking photoactivated endosome/lysosome experiment (Exp B)

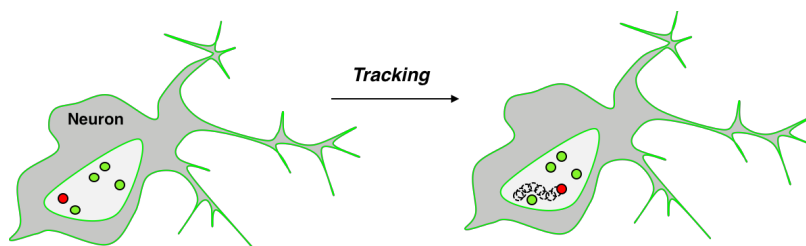


Figure S43. Schematic view of Exp B

The neuronal culture dish (transduced with **3a**-pffs 12 to 48 h) was carefully washed with 1ml of complete neuronal medium. 2.0 mL of complete neuronal medium was then added to the culture dish. Neurons were identified under DIC. Cells with optimal uptake of **3a**-pffs were identified under the green channel at low power (405/FITC, 25%) . “Pre-activated” images were captured under two channels: green (25%) and red (50%) with 3 z-planes. ROIs were photoactivated with the 440 nm Spectra X laser at 100% for 60 s. “Post-activated” images were collected under two channels: green (25%) and red (50%), with 3 z-planes, every 20-30 min over periods of 3 to 8 h as indicated.

8.5 Additional Imaging Data

8.5.1 Data for Exp A: Photoactivation of a single endosome/lysosome

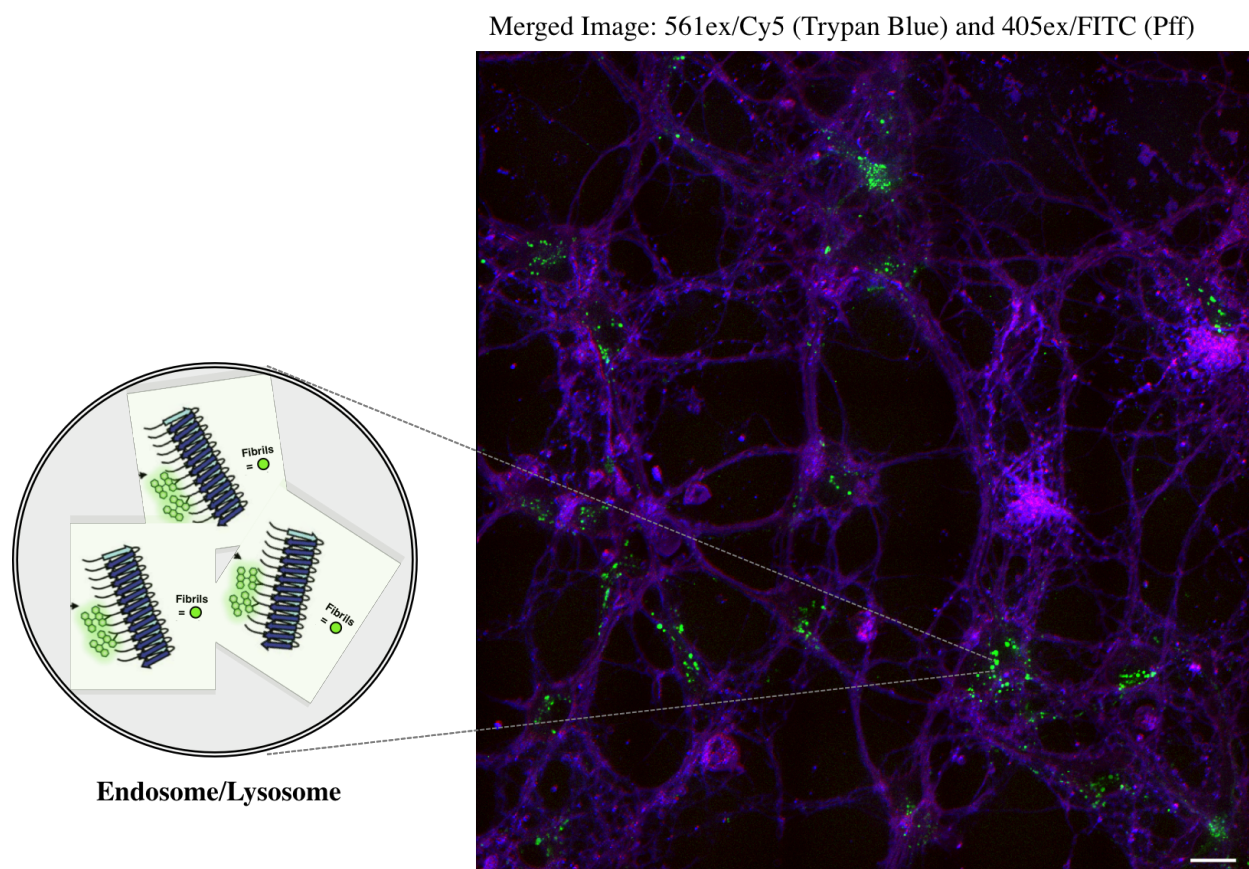


Figure S44. Schematic view of endosomes/lysosomes containing **3a**-pffs (green puncta).

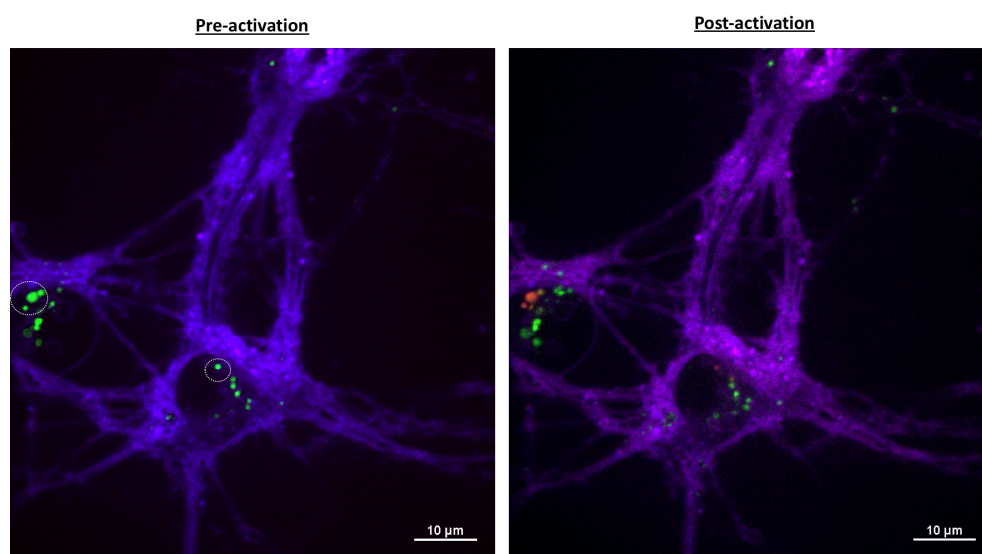
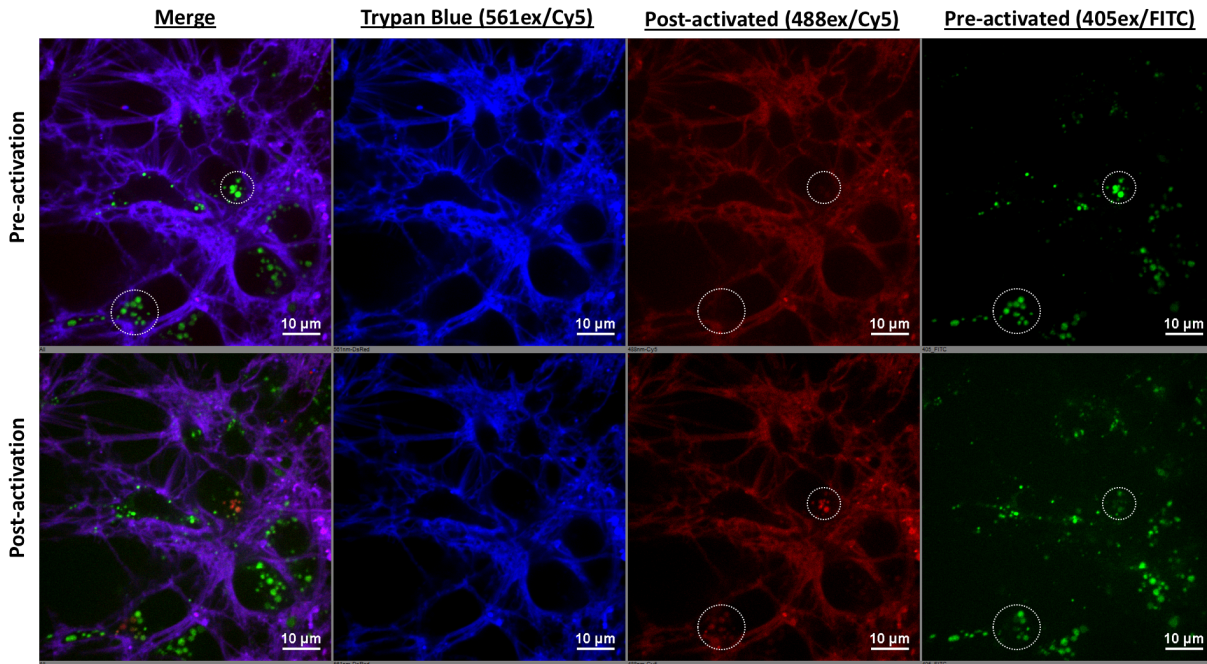


Figure S45. Photoactivation of internalized **3a**-pffs. Merged images of blue, green, and red channels are shown.

Simultaneous Activation of Multiple ROIs

A) Sample 1



B) Sample 2

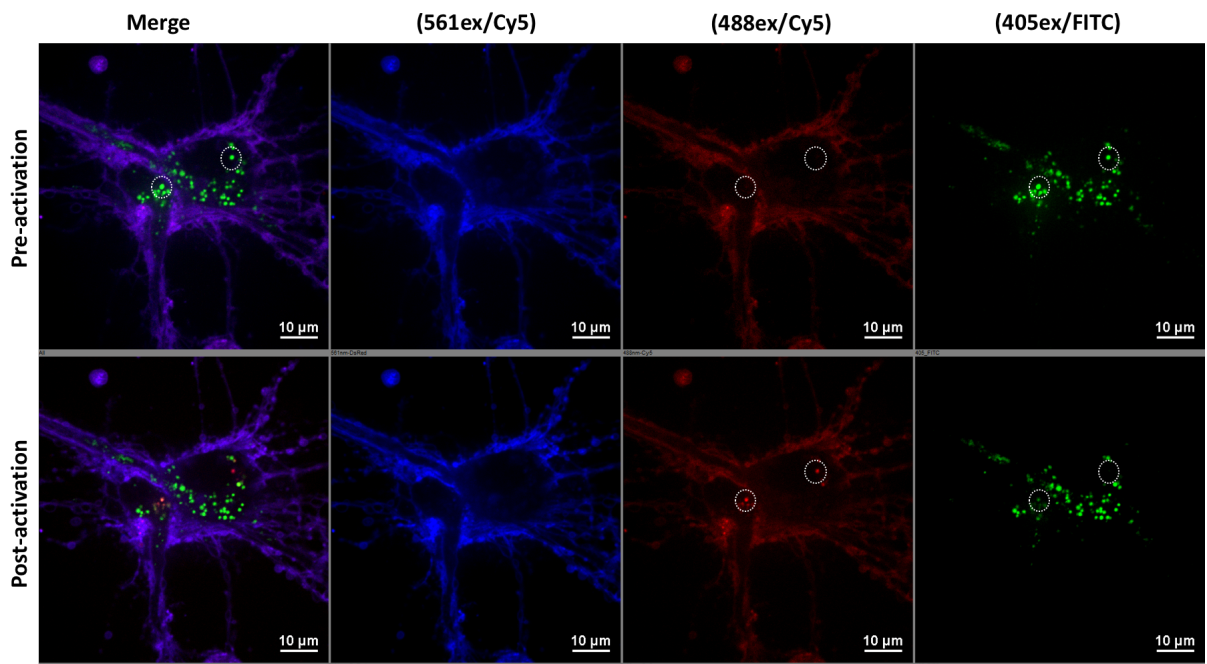


Figure S46. Photoactivation of multiple ROIs with internalized **3a**-pffs in endosomes/lysosomes. Merged, blue, red, and green channels are shown from left to right.

Single Vesicle Activation

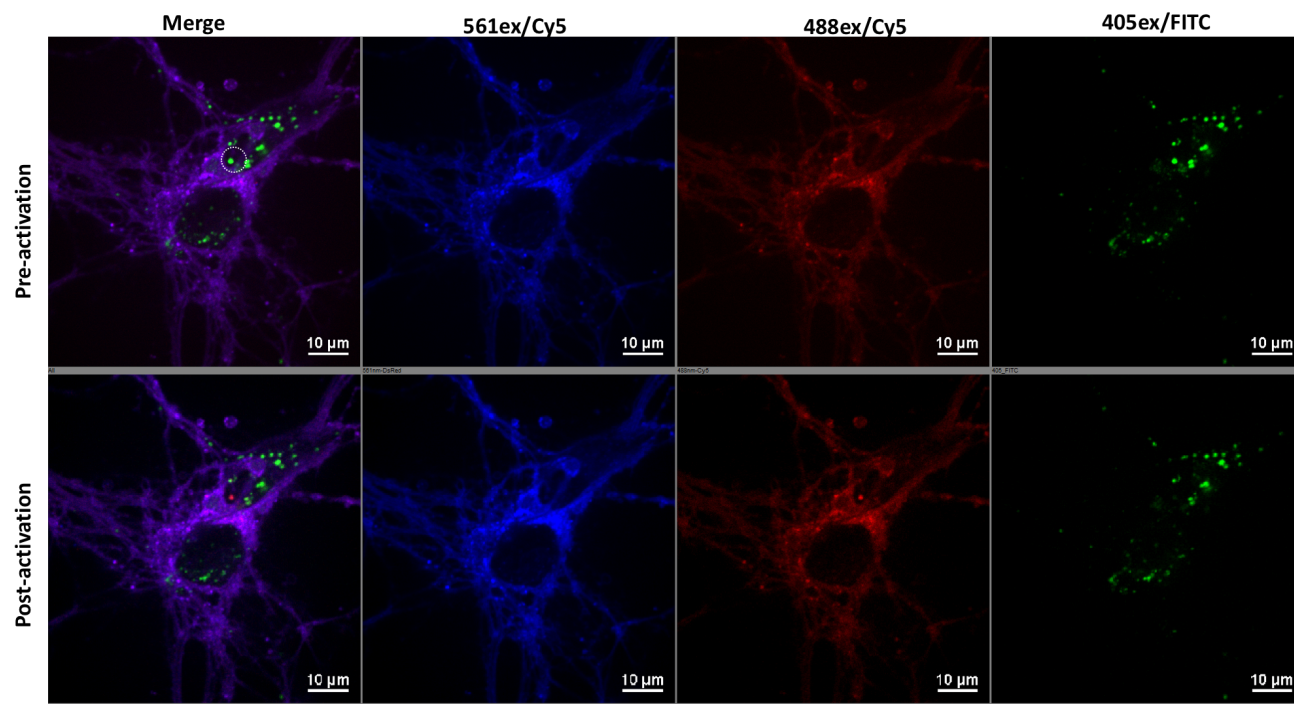
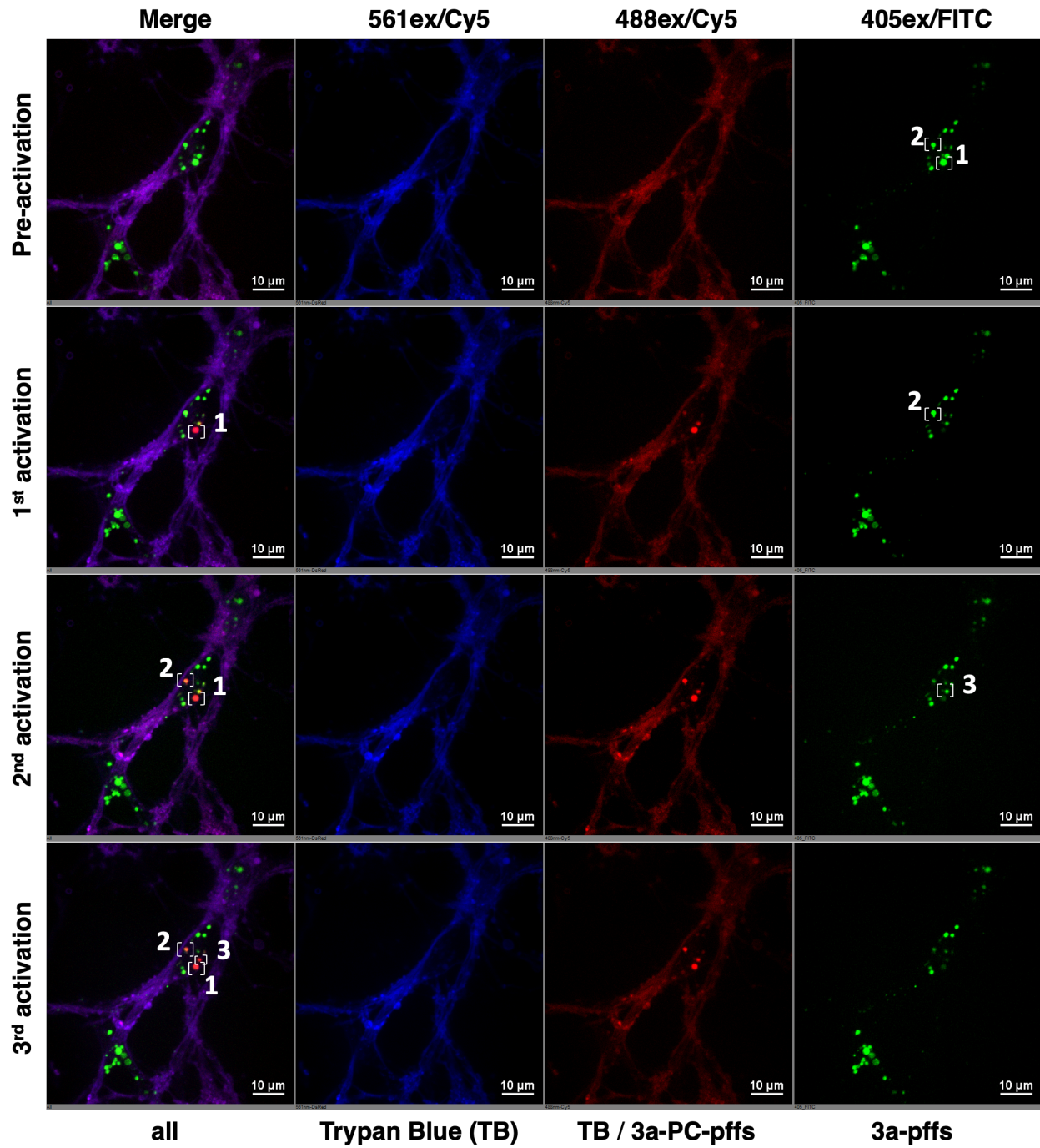


Figure S47. Photoactivation of a single endosome/lysosome containing **3a**-pffs is shown. Merged, blue, red, and green channels are shown from left to right.

Sequential Activation

A) Sample 1 (Main text Figure 5D-O)



B) Sample 2

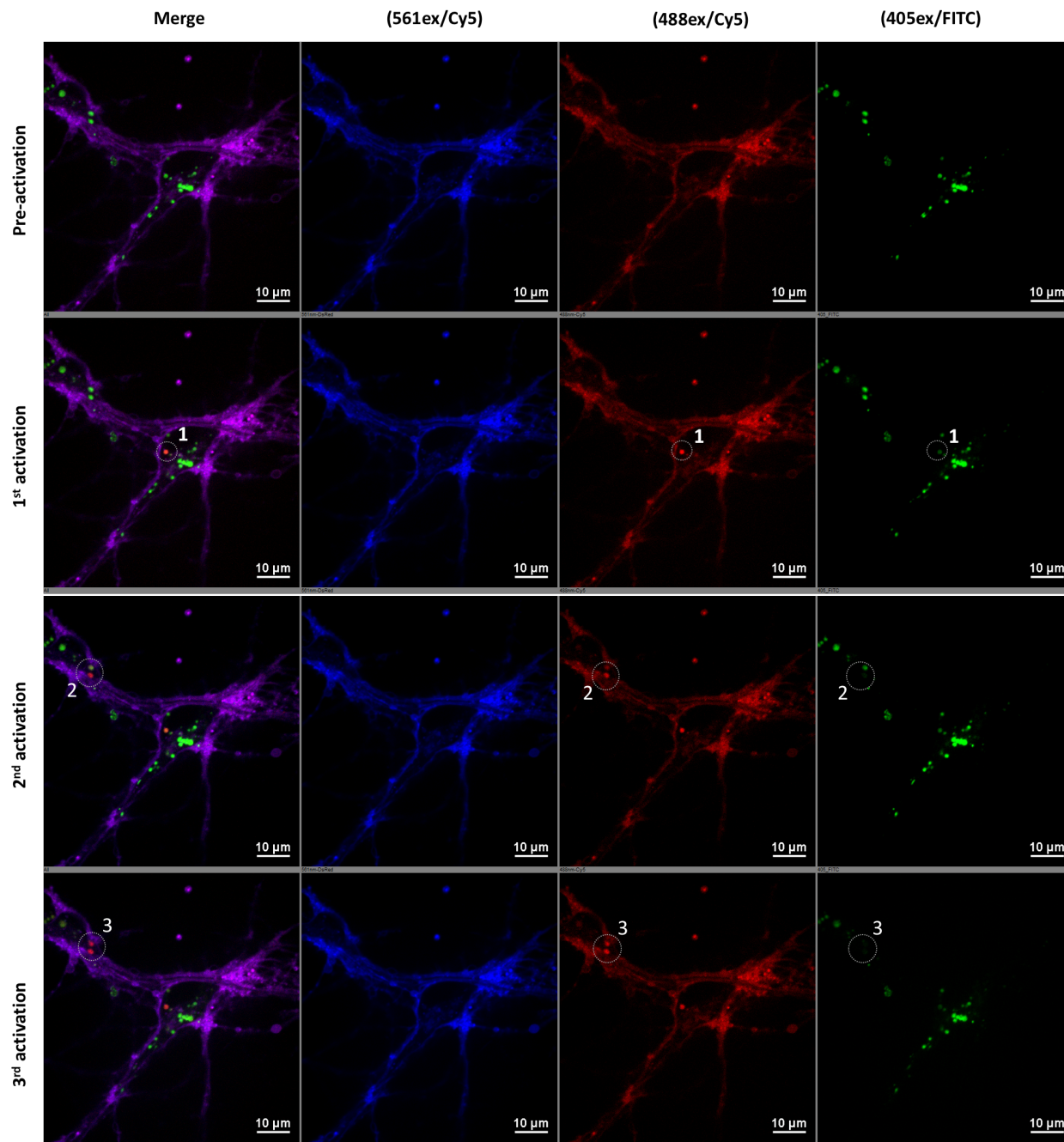


Figure S48. Sequential photoactivation of individual endosomes/lysosomes containing internalized 3a-pffs. Merged, blue, red, and green channels are shown from left to right.

8.5.2 Data for Exp B: Timecourse Tracking Experiment without Trypan Blue

Sample 1. Neurons were transduced with 3a-pffs for 48 h prior to photoactivation

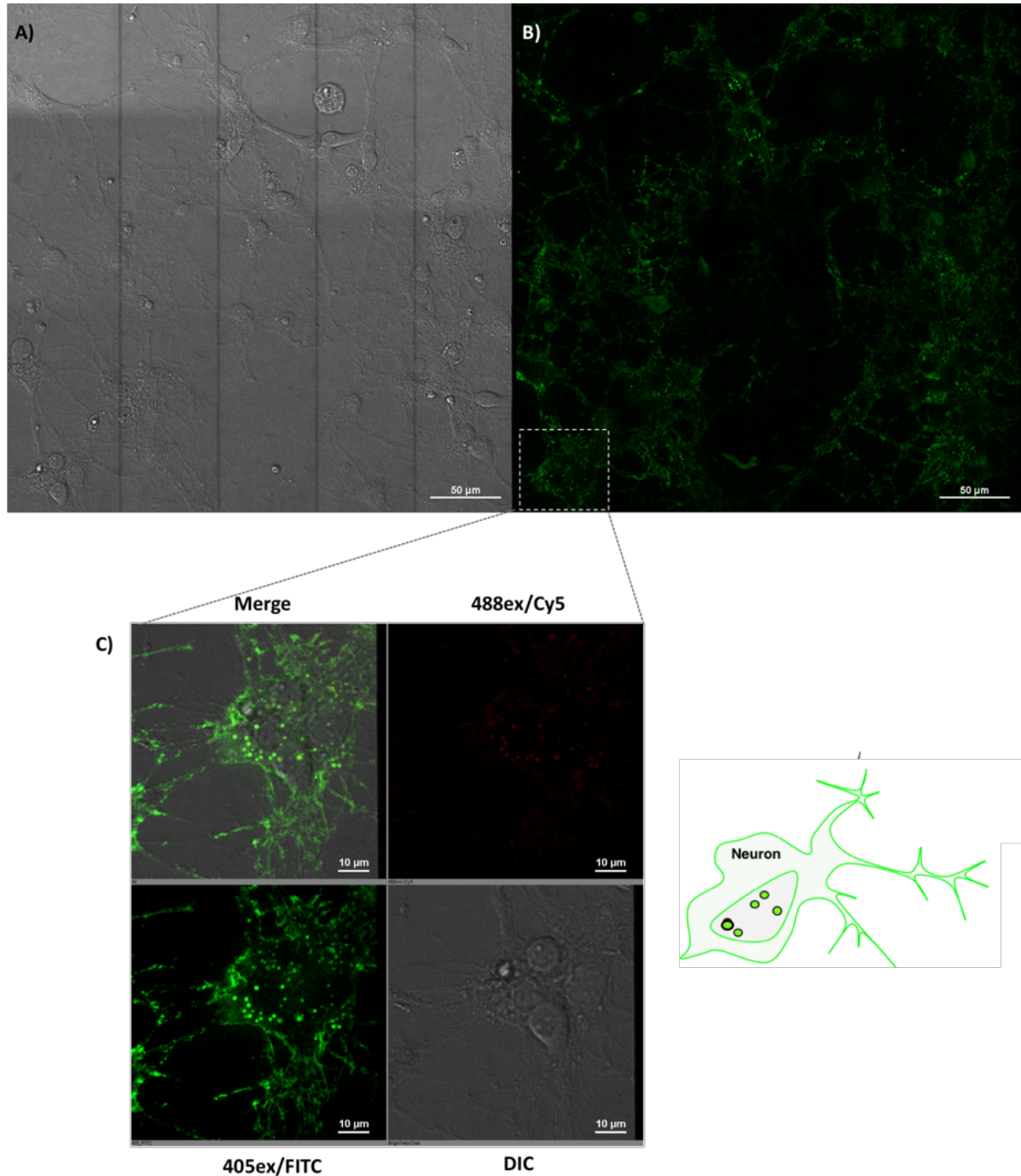


Figure S49. Large population of neurons are shown in A) differential interference contrast (DIC) image, and B) 405ex/FITC channel. C) Pre-activated image before tracking. The majority of signal corresponds to extracellular 3a-pffs, although endocytosed 3a-pffs were identified by spherical, somatic, perinuclear morphology prior to photoactivation.

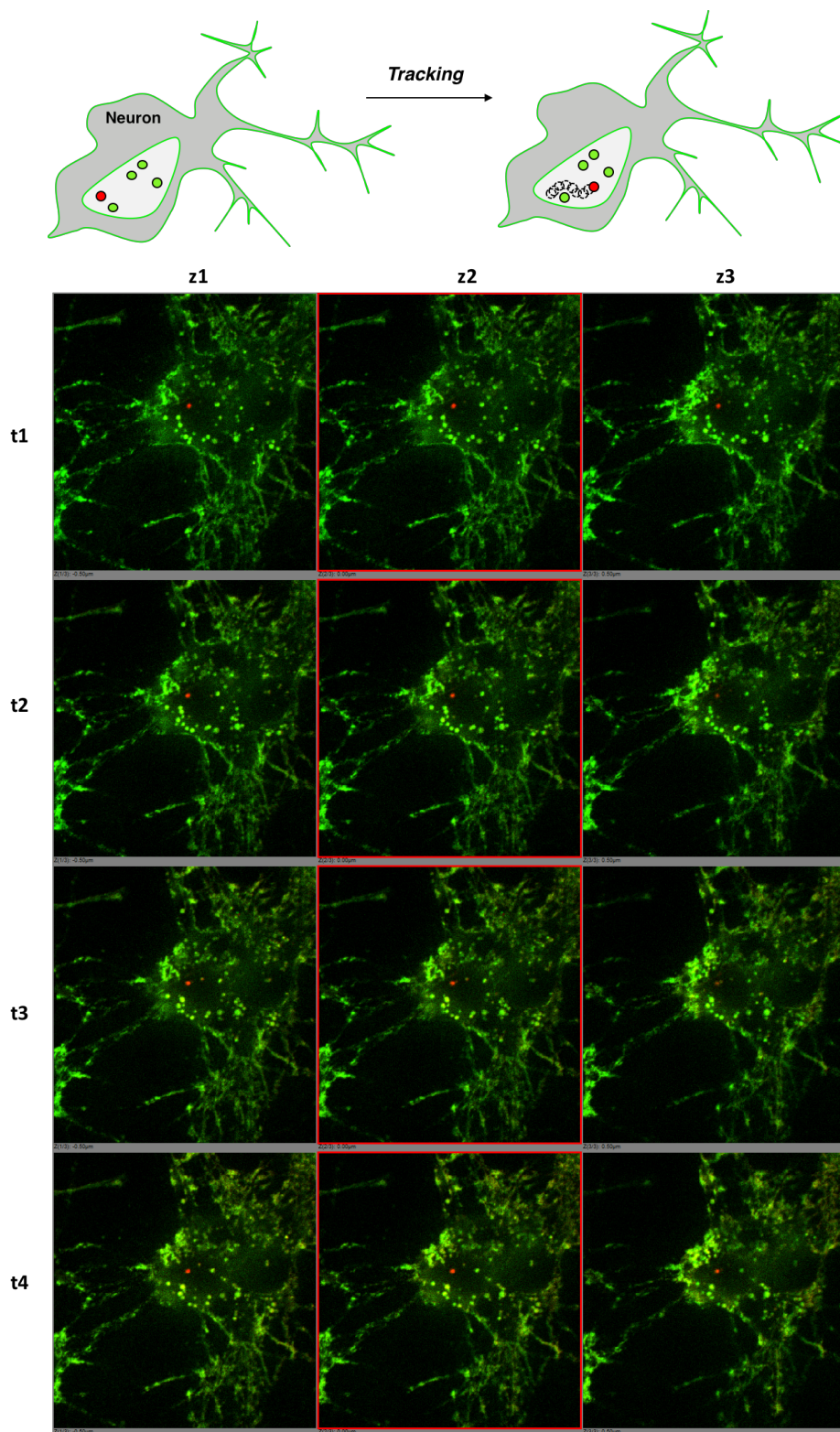


Figure S50. Time point imaging in three z-planes after photoactivation of a single endosome/lysosome containing **3a**-pffs. Internalized **3a**-pffs were quite static, moving only slightly over 8 h. (t1 = 2 h, t2 = 4 h, t3 = 6 h, t4 = 8 h).

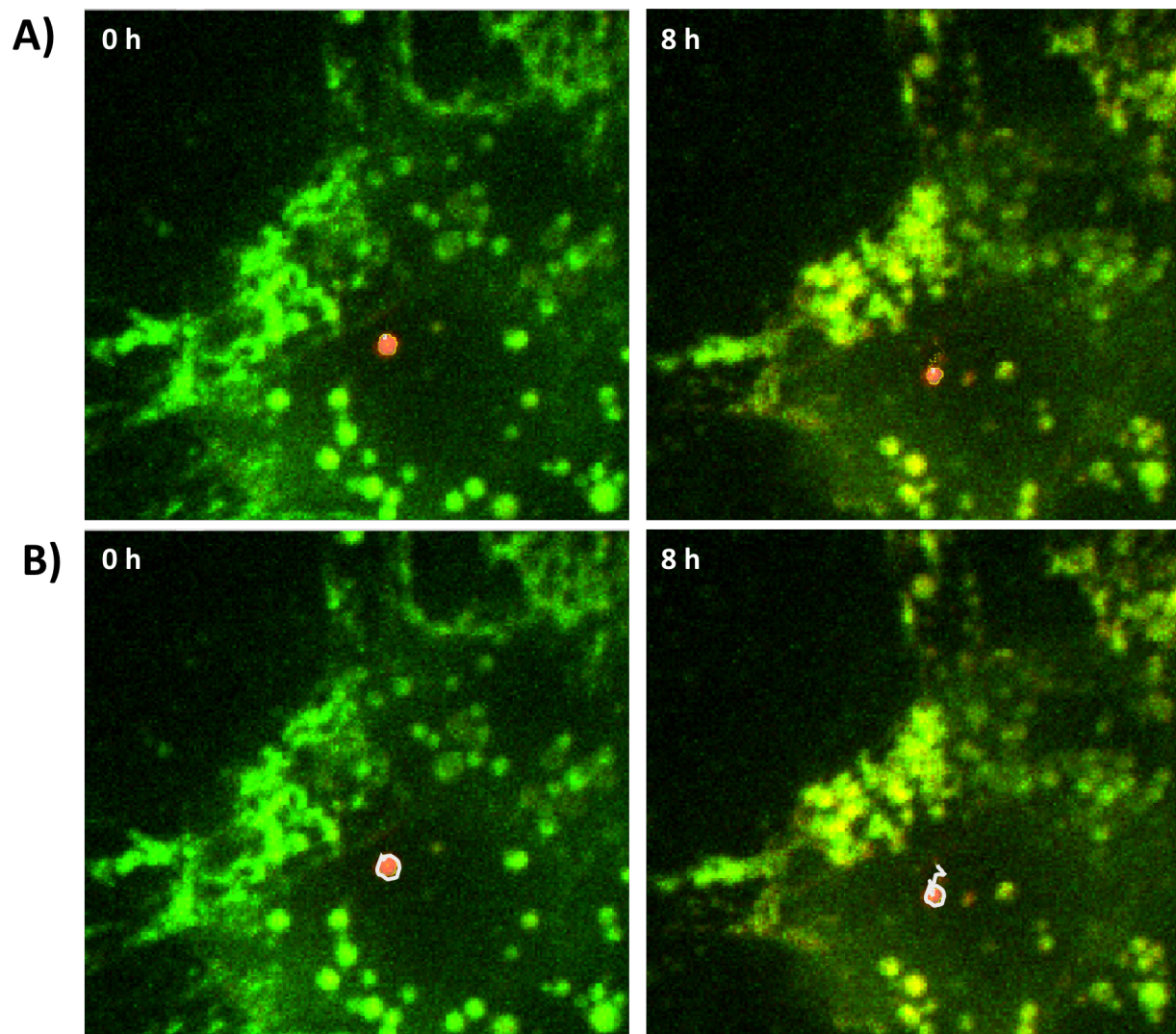
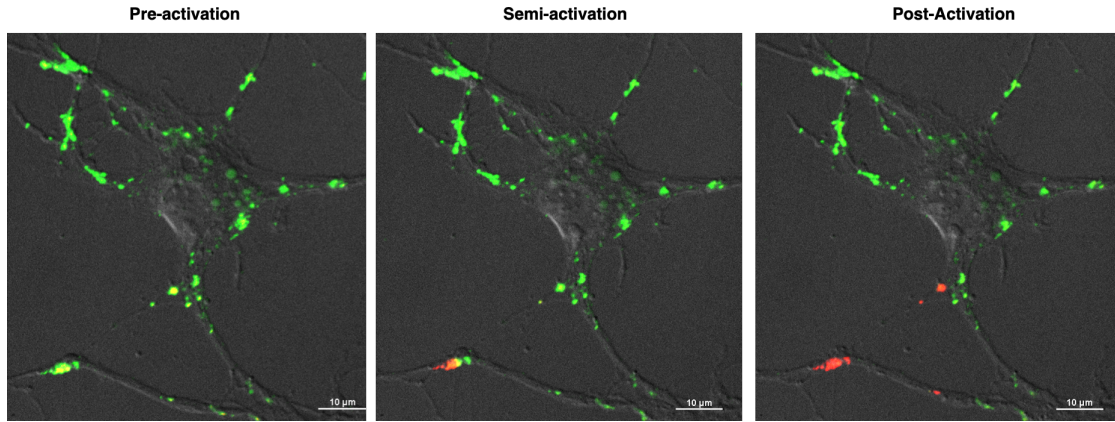


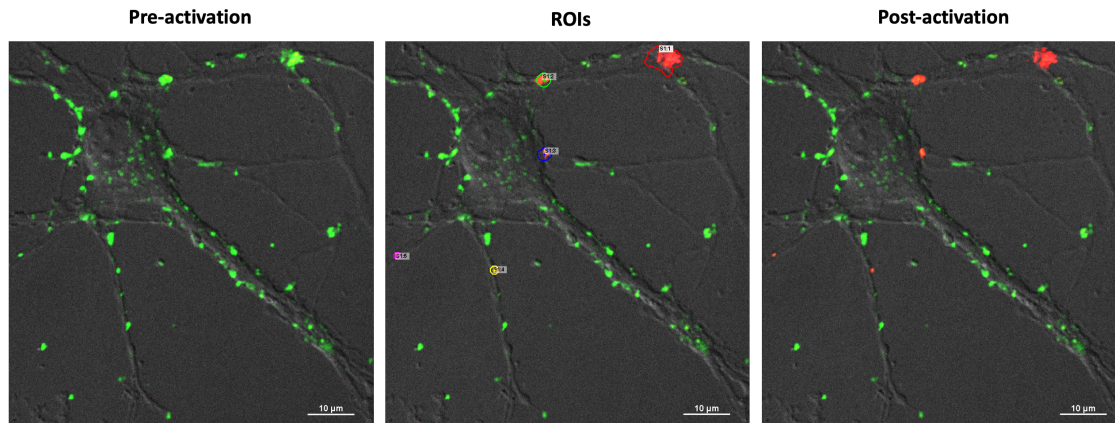
Figure S51. A) Movement of single photoactivated endosome/lysosome containing **3a**-pffs over 8 h. B) White line indicates the recorded distance moved by the punctum.

Sample 2. Neurons were transduced with 3a-pffs for 20 h prior to photoactivation.

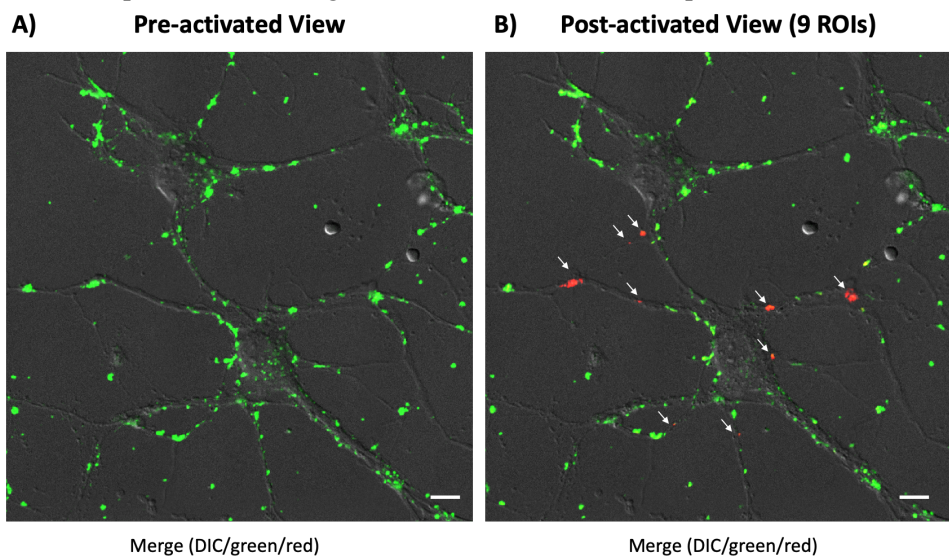
1) Region 1: Photoactivation of multiple regions of interests (ROIs)



2) Region 2: Photoactivation of multiple regions of interests (ROIs)



3) Combined view of photoactivated regions 1 and 2. Scale bar corresponds to 10 μm.



4) Tracking of photoconverted ROIs up to 3 h

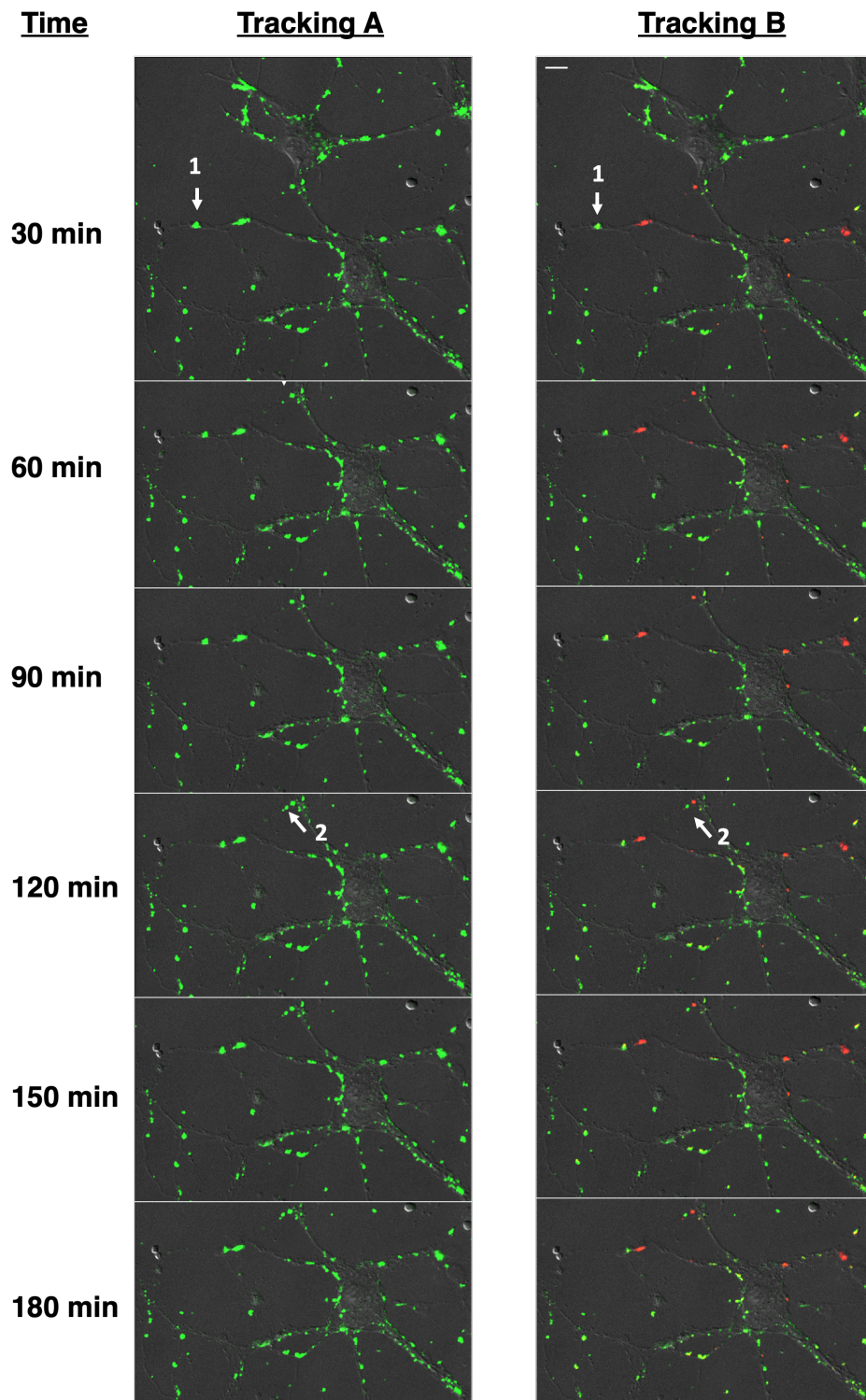
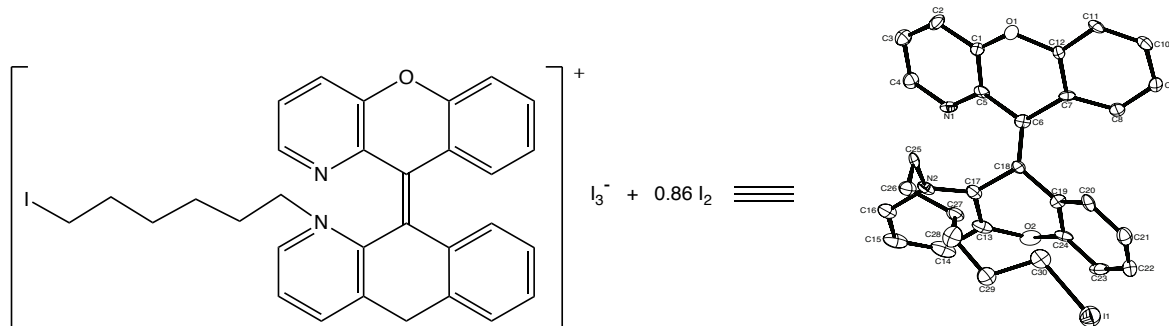
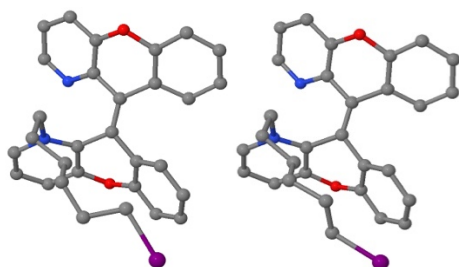


Figure S52. Tracking of individual **3a**-pff puncta **1** and **2**. “Tracking A” images are representative of the appearance of a non-photoconvertible probe. “Tracking B” images represent when a CPX probe is used to mark (via photoconversion) a subset of **3a**-pffs to assist the trafficking of subpopulations of fibrils over 3 h.

9. X-ray Structure Determination of Compound 2a



Compound **2a**, $C_{30}H_{26}I_{4.86}N_2O_2$, crystallizes in the triclinic space group $P\bar{1}$ with $a=10.5582(6)\text{\AA}$, $b=12.7047(8)\text{\AA}$, $c=14.4180(9)\text{\AA}$, $\alpha=64.250(2)^\circ$, $\beta=86.988(2)^\circ$, $\gamma=66.557(2)^\circ$, $V=1581.15(17)\text{\AA}^3$, $Z=2$, and $d_{\text{calc}}=2.233\text{ g/cm}^3$. X-ray intensity data were collected on a Bruker D8QUEST [1] CMOS area detector employing graphite-monochromated Mo-K α radiation ($\lambda=0.71073\text{\AA}$) at a temperature of 100K. Preliminary indexing was performed from a series of twenty-four 0.5° rotation frames with exposures of 10 seconds. A total of 2960 frames were collected with a crystal to detector distance of 33.0 mm, rotation widths of 0.5° and exposures of 5 seconds. Rotation frames were integrated using SAINT [2], producing a listing of unaveraged F^2 and $\sigma(F^2)$ values. A total of 39909 reflections were measured over the ranges $6.05 \leq 2\theta \leq 55.118^\circ$, $-13 \leq h \leq 13$, $-15 \leq k \leq 16$, $-18 \leq l \leq 18$ yielding 7127 unique reflections ($R_{\text{int}} = 0.0425$). The intensity data were corrected for Lorentz and polarization effects and for absorption using SADABS [3] (minimum and maximum transmission 0.5704, 0.7456). The structure was solved by direct methods - ShelXT [4]. There are two tri-iodide ions that lie on crystallographic centers of symmetry (at $\frac{1}{2}, \frac{1}{2}, \frac{1}{2}$ and 1, 1, 0). The tri-iodide ion at 1, 1, 0 is disordered in two different orientations with relative occupancies of 86/14. There is also an I_2 molecule that is not fully occupied, but has an occupancy of 0.86. The hexyl chain is also disordered by a twisting of the 5th and 6th carbons as shown below (the relative occupancies of the two sets of disordered carbons is 50/50):



Refinement was by full-matrix least squares based on F^2 using SHELXL-2017 [5]. All reflections were used during refinement. The weighting scheme used was $w=1/[\sigma^2(F_o^2) + 74.0705P]$ where $P = (F_o^2 + 2F_c^2)/3$. Non-hydrogen atoms were refined anisotropically and hydrogen atoms were refined using a riding model. Refinement converged to $R1=0.0895$ and $wR2=0.1641$ for 5480 observed reflections for which $F > 4\sigma(F)$ and $R1=0.1189$ and $wR2=0.1834$ and $GOF = 1.270$ for all 7127 unique, non-zero reflections and 370 variables. The maximum Δ/σ in the final cycle of least squares was 0.000 and the two most prominent peaks in the final difference Fourier were $+3.44$ and -2.84 e/\AA^3 .

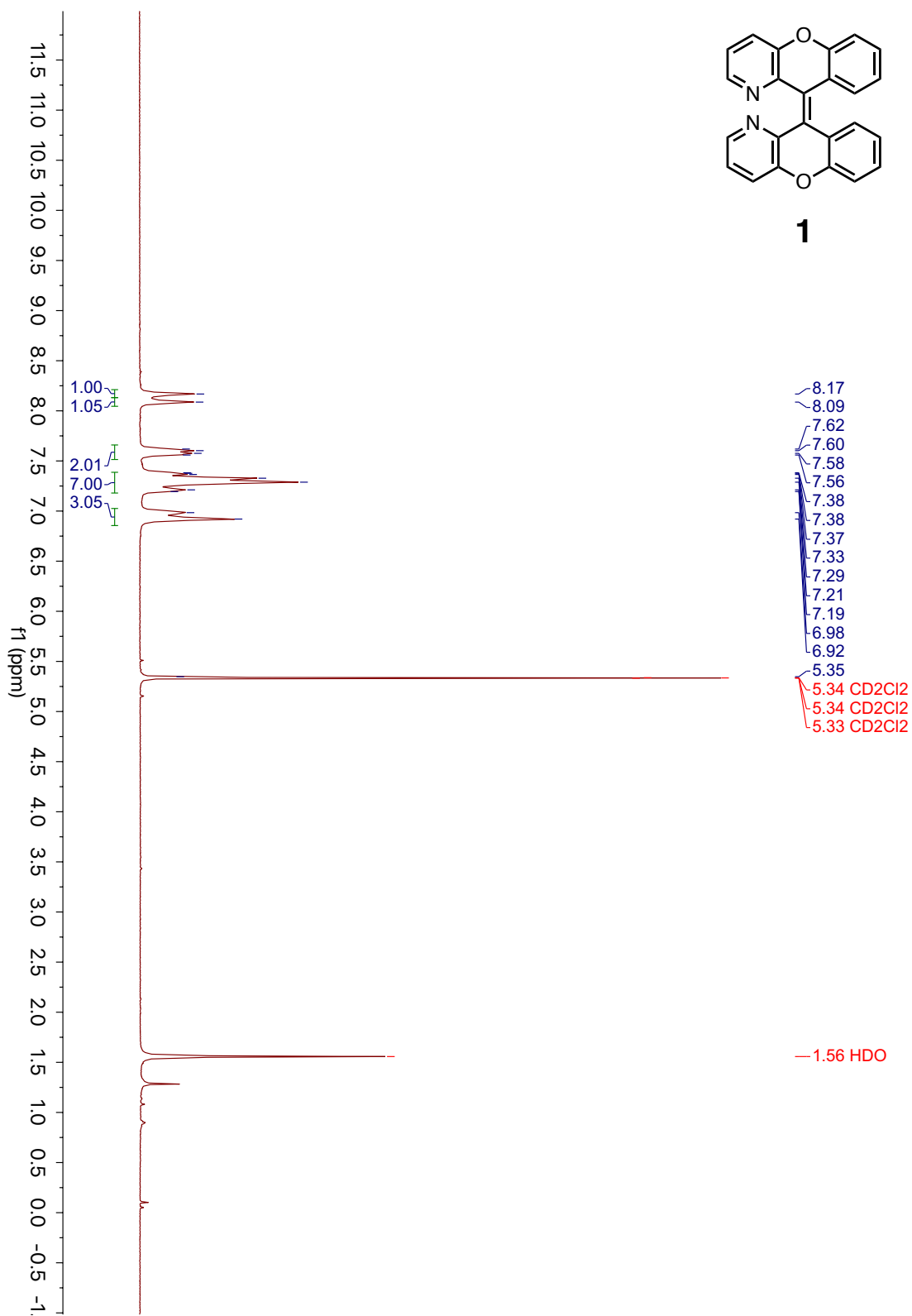
Table 1. Summary of Structure Determination of Compound 9869

Empirical formula	C ₃₀ H ₂₆ I _{4.86} N ₂ O ₂
Formula weight	1063.26
Temperature/K	100
Crystal system	triclinic
Space group	P $\bar{1}$
a	10.5582(6)Å
b	12.7047(8)Å
c	14.4180(9)Å
α	64.250(2)°
β	86.988(2)°
γ	66.557(2)°
Volume	1581.15(17)Å ³
Z	2
d _{calc}	2.233 g/cm ³
μ	4.811 mm ⁻¹
F(000)	987.0
Crystal size, mm	0.22 × 0.2 × 0.08
2 θ range for data collection	6.05 - 55.118°
Index ranges	-13 ≤ h ≤ 13, -15 ≤ k ≤ 16, -18 ≤ l ≤ 18
Reflections collected	39909
Independent reflections	7127[R(int) = 0.0425]
Data/restraints/parameters	7127/343/370
Goodness-of-fit on F ²	1.270
Final R indexes [I ≥ 2 σ (I)]	R ₁ = 0.0895, wR ₂ = 0.1641
Final R indexes [all data]	R ₁ = 0.1189, wR ₂ = 0.1834
Largest diff. peak/hole	3.44/-2.84 eÅ ⁻³

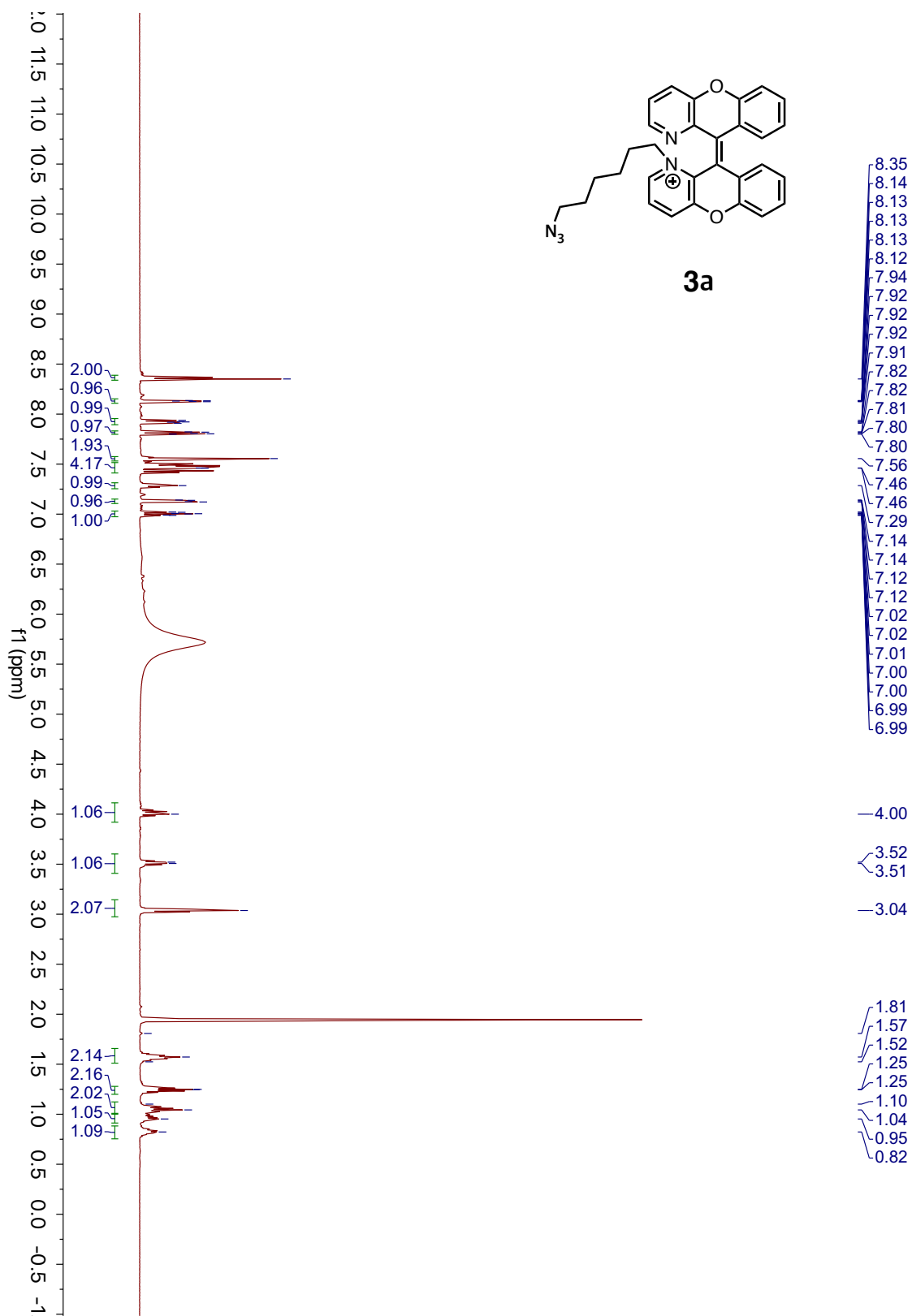
10. Table of Compounds for ^1H and ^{13}C NMR Analysis

^1H NMR Data for Compound 1	S55
^1H NMR Data for Compound 3a	S56
^{13}C NMR Data for Compound 3a	S57
^{19}F NMR Data for Compound 3a	S58
^1H NMR Data for Compound 3a-PC	S59
^{13}C NMR Data for Compound 3a-PC	S60
^1H NMR Data for Compound 3b	S61
^{13}C NMR Data for Compound 3b	S62

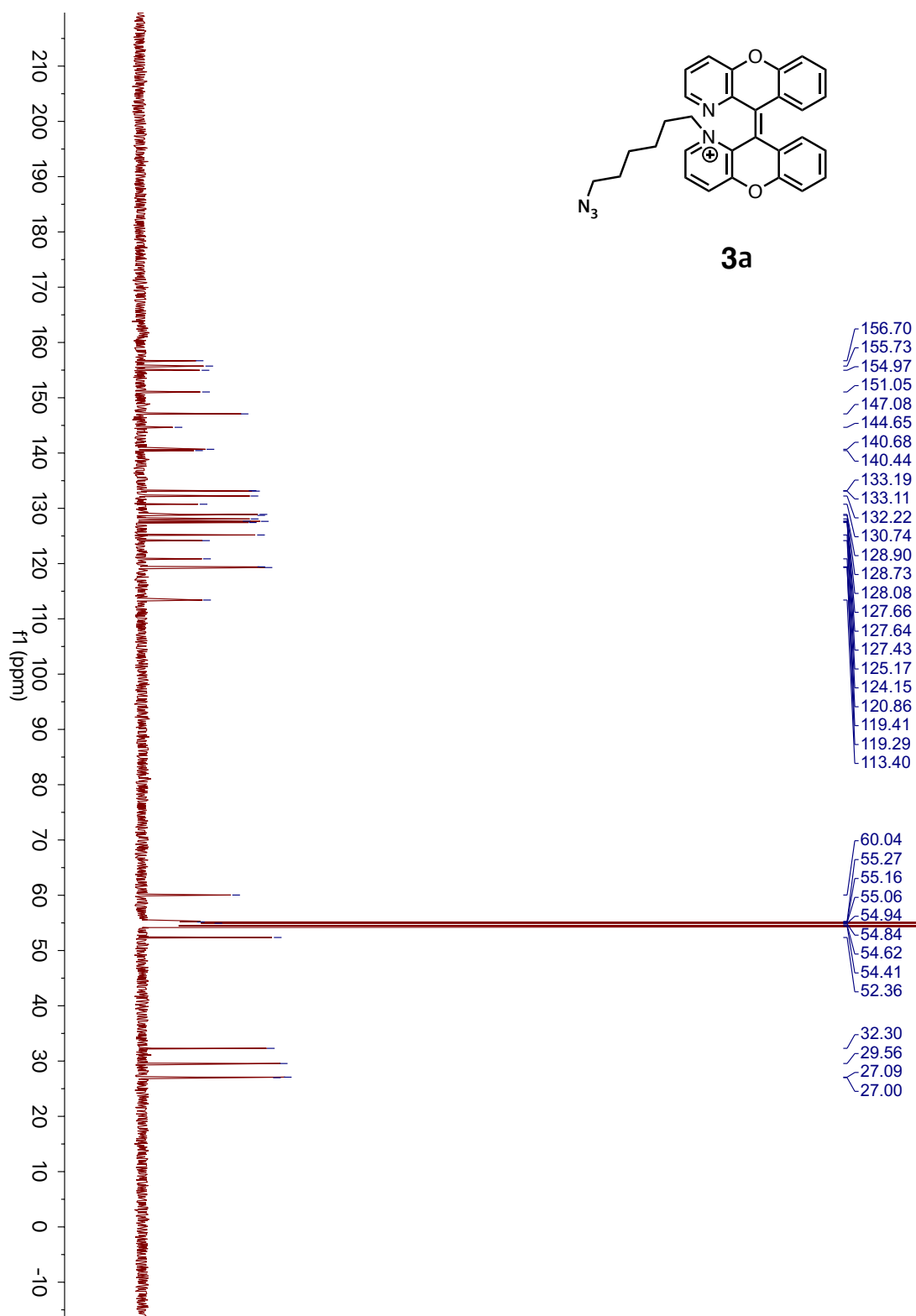
^1H NMR spectrum of **1** in CD_2Cl_2 (500 MHz).



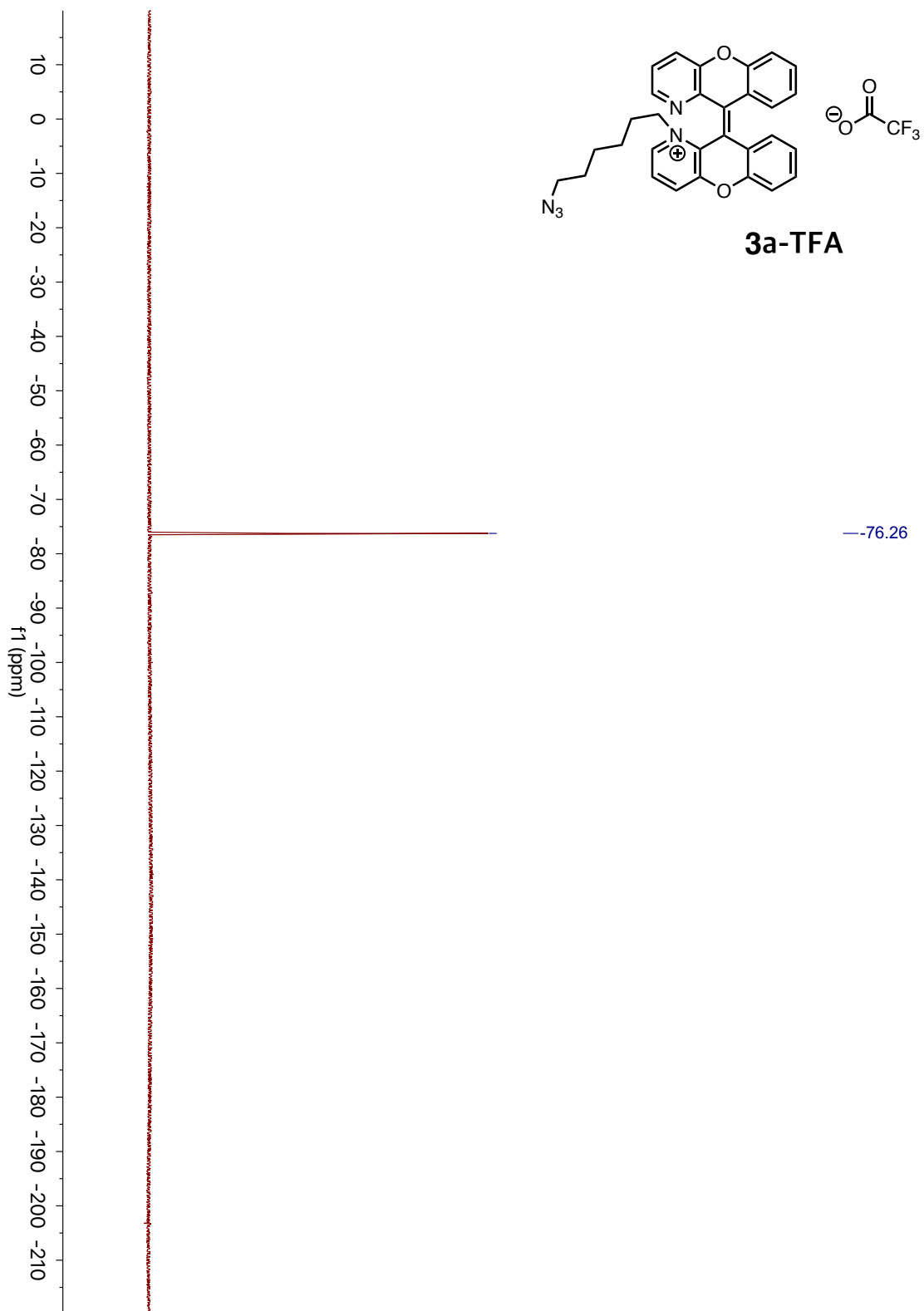
^1H NMR spectrum of **3a** in CD_3CN (500 MHz).



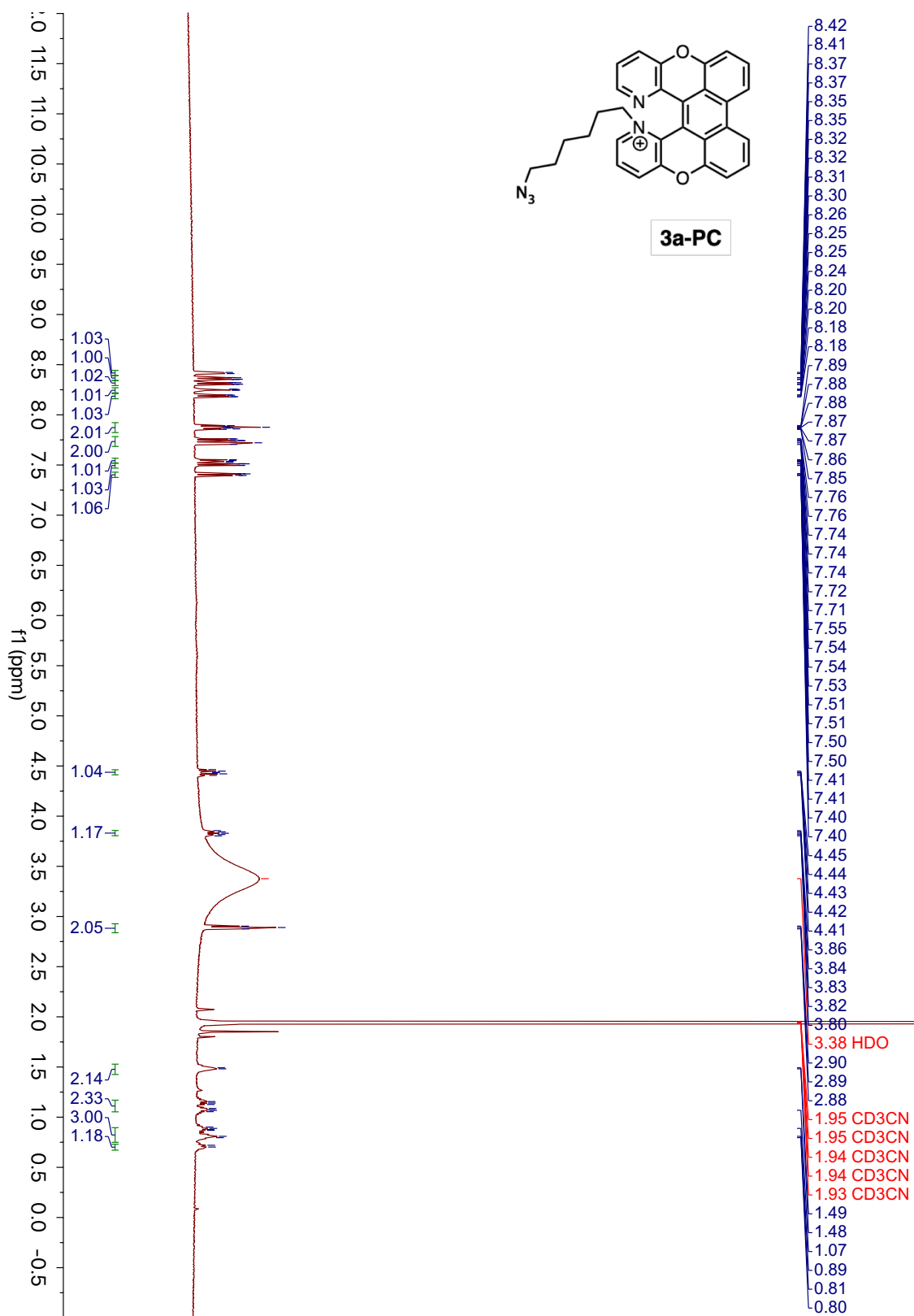
^{13}C NMR spectrum of **3a** in CD_2Cl_2 (126 MHz).



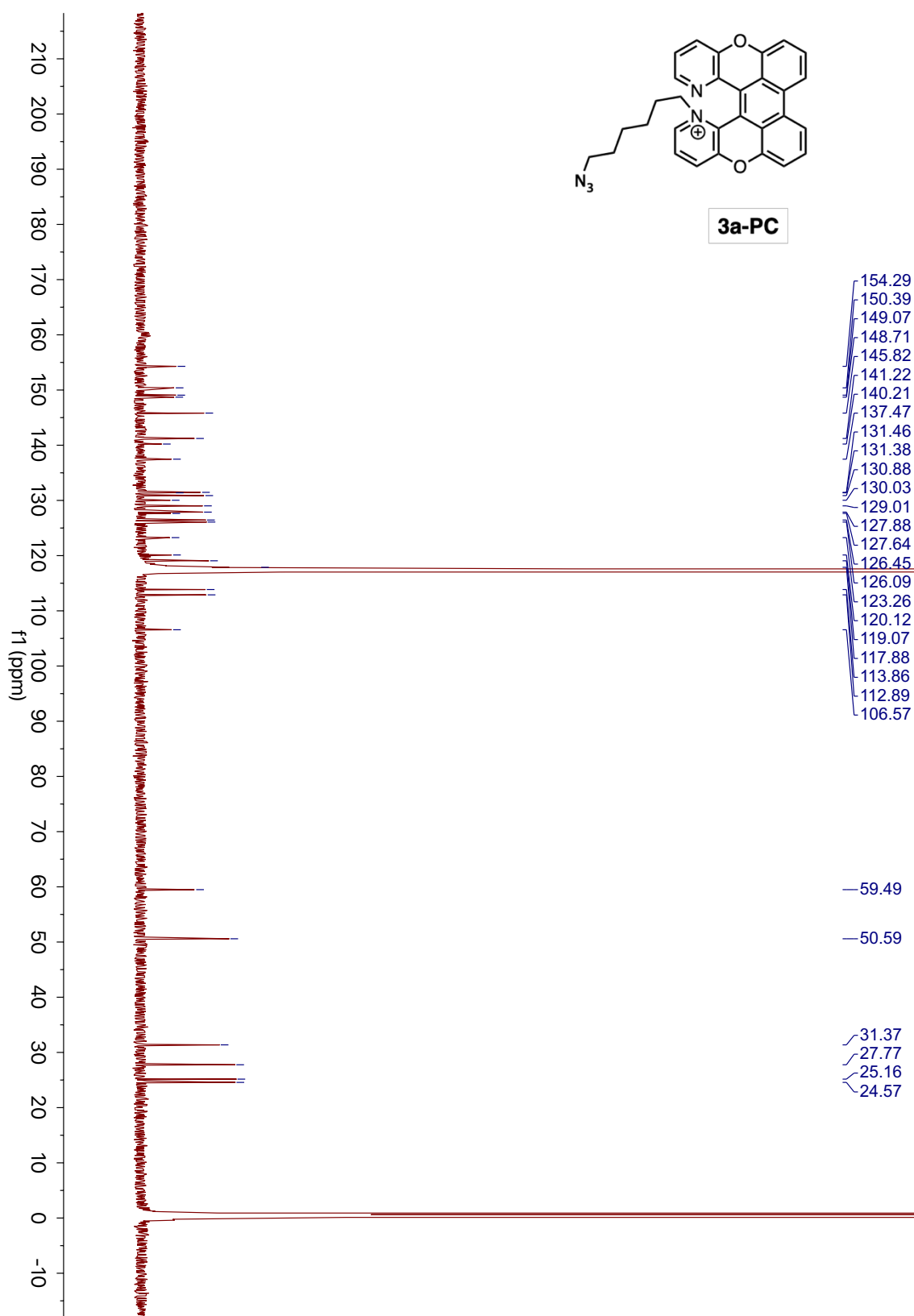
^{19}F NMR spectrum of **3a-Trifluoroacetate (TFA)** in CD_2Cl_2 (500 MHz). 5-15 TFA



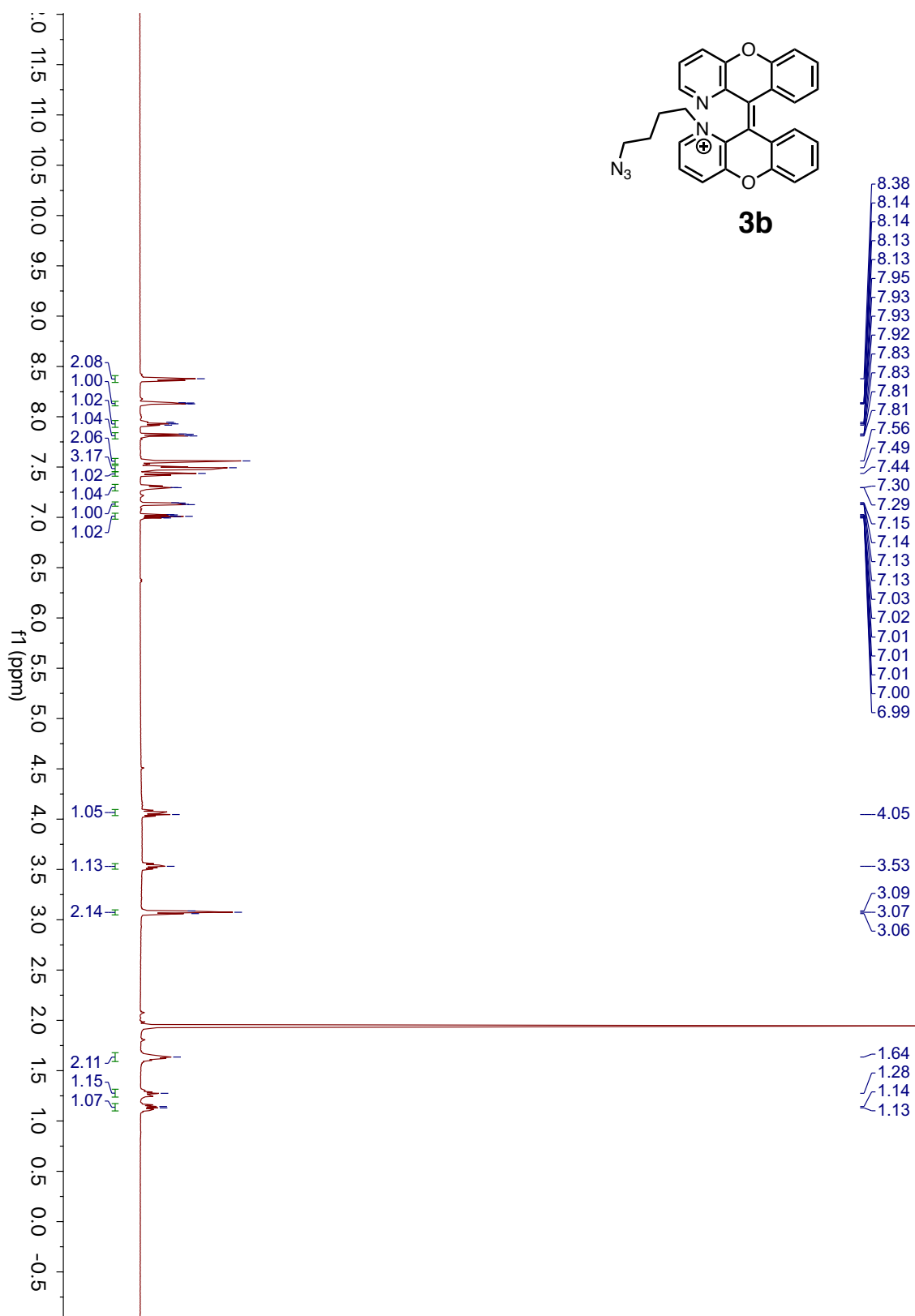
^1H NMR spectrum of **3b** in CD_3CN (Cryo 500 MHz).



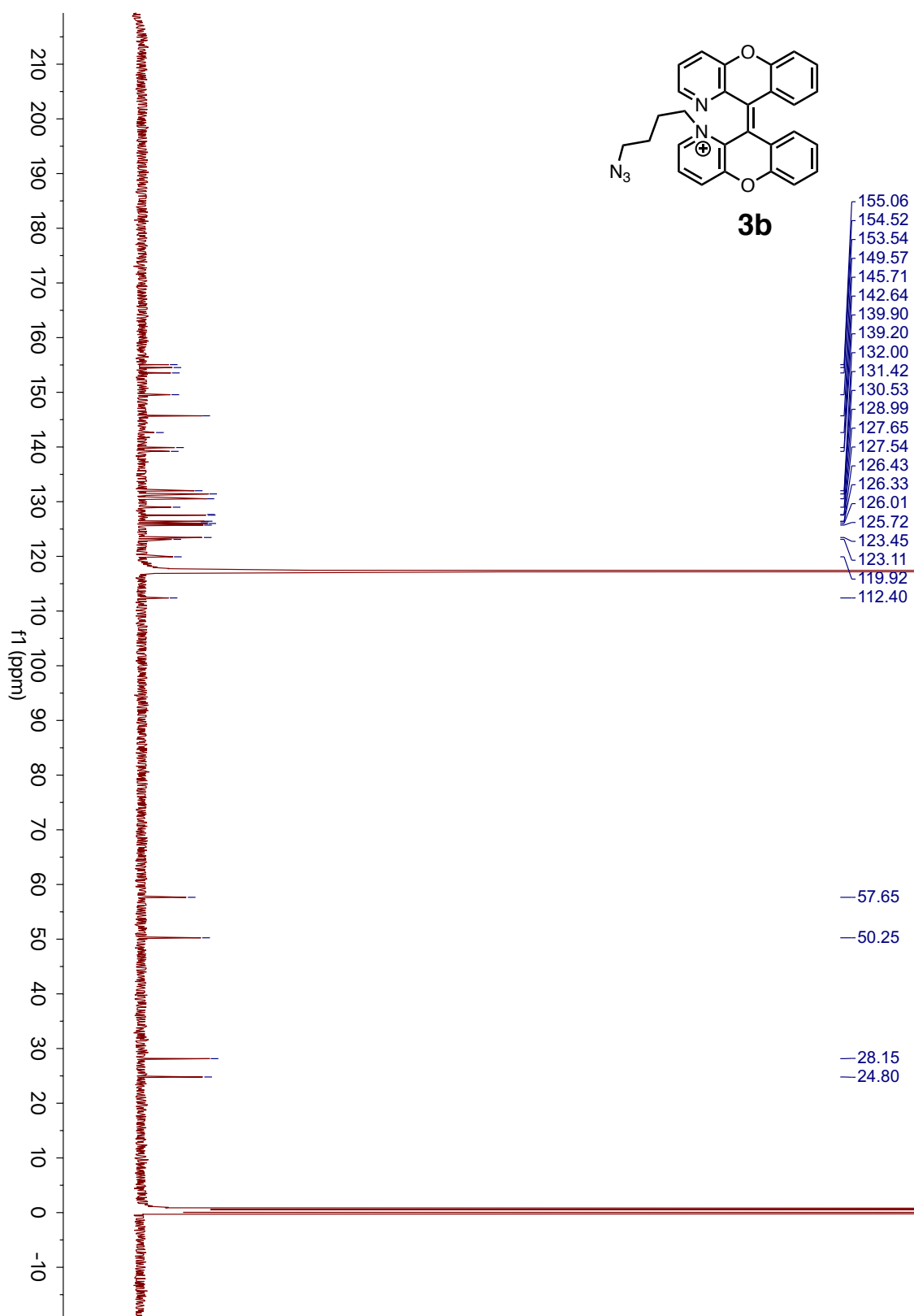
^{13}C NMR spectrum of **3a-PC** in CD_3CN (Cryo 126 MHz).



^1H NMR spectrum of **3b** in CD_3CN (500 MHz).



^{13}C NMR spectrum of **3b** in CD_3CN (126 MHz).



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