Supporting Information for

Lipid Expansion Microscopy

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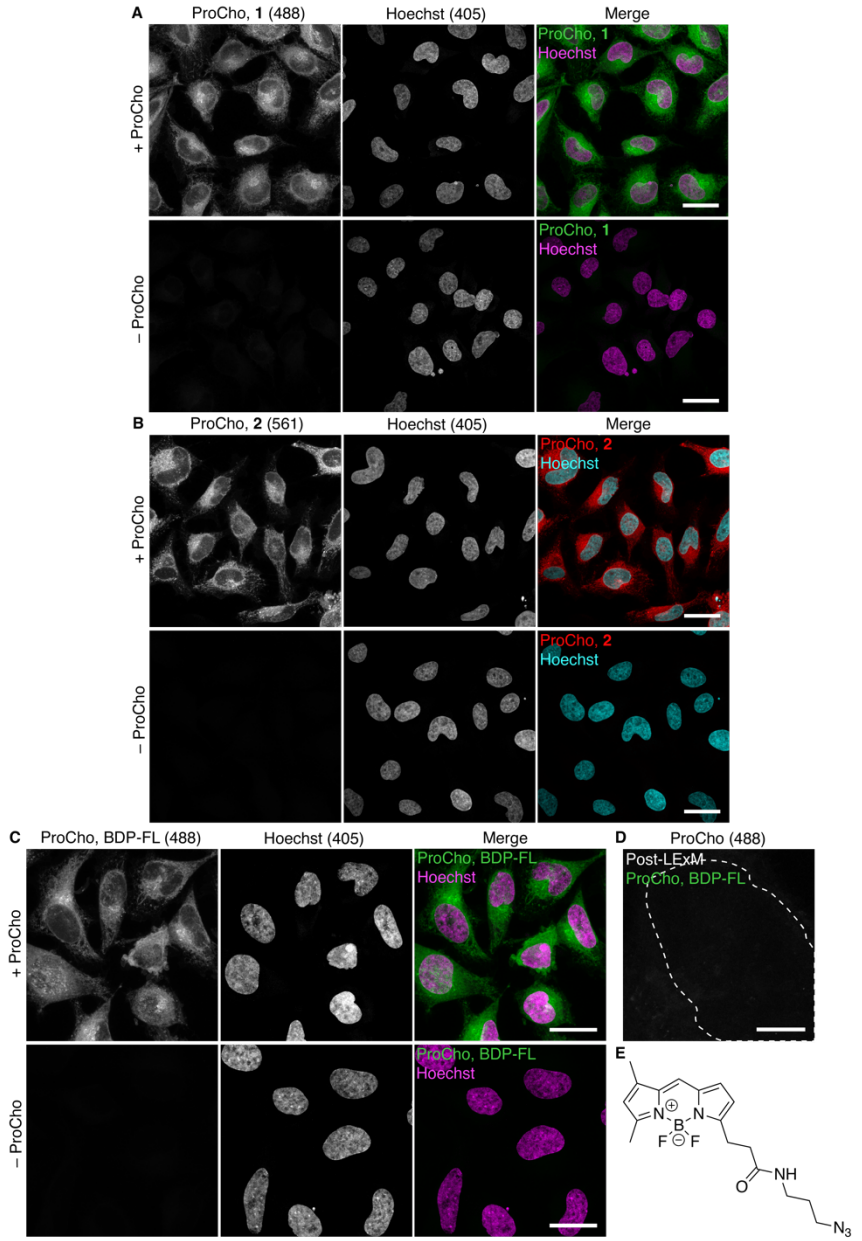


Figure S1. Control experiments of cells labeled with ProCho and tagged with 1, 2, or BDP-FL-azide. Confocal microscopy images of cells metabolically labeled with ProCho (top row) or without ProCho (bottom row) and tagged with **1** (A), **2** (B) or BDP-FL-azide (C) via CuAAC. In the merged images, labeled lipids are shown in green (A and C) or red (B) and Hoechst 33342 is shown in magenta (A and C) or cyan (B). (D) A confocal microscopy image of an expanded cell labeled with BDP-FL-azide. The nucleus of the cell is outlined with the dotted line. (E) The chemical structure of BDP-FL-azide. Scale bars: 30 μm.

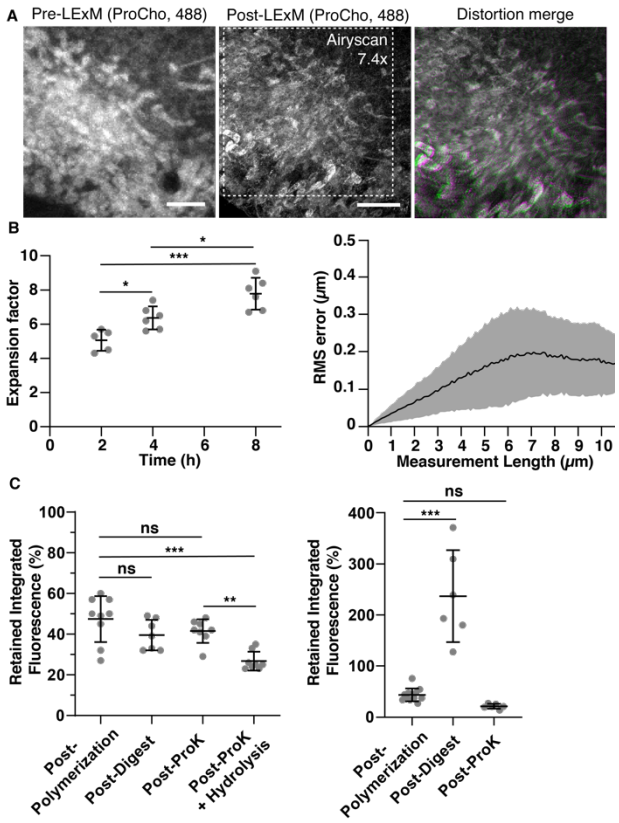


Figure S2. Representative images used for distortion analysis, expansion factor and RMSE graph of cells labeled with ProCho and tagged with 1, and label retention of cells tagged with 1 and 2. (A) Representative image of a pre-LExM and post-LExM cell, labeled with ProCho and tagged with **1**, used for distortion analysis shown in (B). Pre-LExM images (left) were compared to post-LExM images (middle) with non-rigid registration using the area highlighted by the box in the post-LExM image. In the distortion image (right), the result of the non-rigid registration (green) is overlaid with the undistorted post-LExM area (magenta). The post-LExM image was acquired with Airyscan confocal microscopy. The expansion factor is listed in the upper right corner of the post-LExM image. (B) Expansion factors of samples labeled with ProCho and tagged with **1** that were hydrolyzed for 2, 4 and 8 h (left). Expansion factors were determined with rigid registration. Root mean square (RMS) error as a function of measurement length generated by non-rigid registration of pre-LExM and post-LExM images labeled with ProCho and tagged with **1** (right, n=6). The expansion factor is listed in the upper right corner of the post-LExM image. (C) Fluorescence retention of cells metabolically labeled with ProCho then tagged with **1** (left) or **2** (right) via CuAAC for each step in the LExM procedure. Note that the fluorescence of **1** is retained when proteinase K (ProK) is used for homogenization, making LExM compatible with ExM methods that require strong proteolysis. Fluorescence retention was determined by comparing the integrated fluorescence of a sample to the same area post-CuAAC. Images were acquired using confocal microscopy. Statistical significance: ANOVA (one-way, Tukey). *** $p < 0.0001$, ** $p < 0.005$, ns = not significant. Scale bars: 5 μm (A, pre-LExM distance).

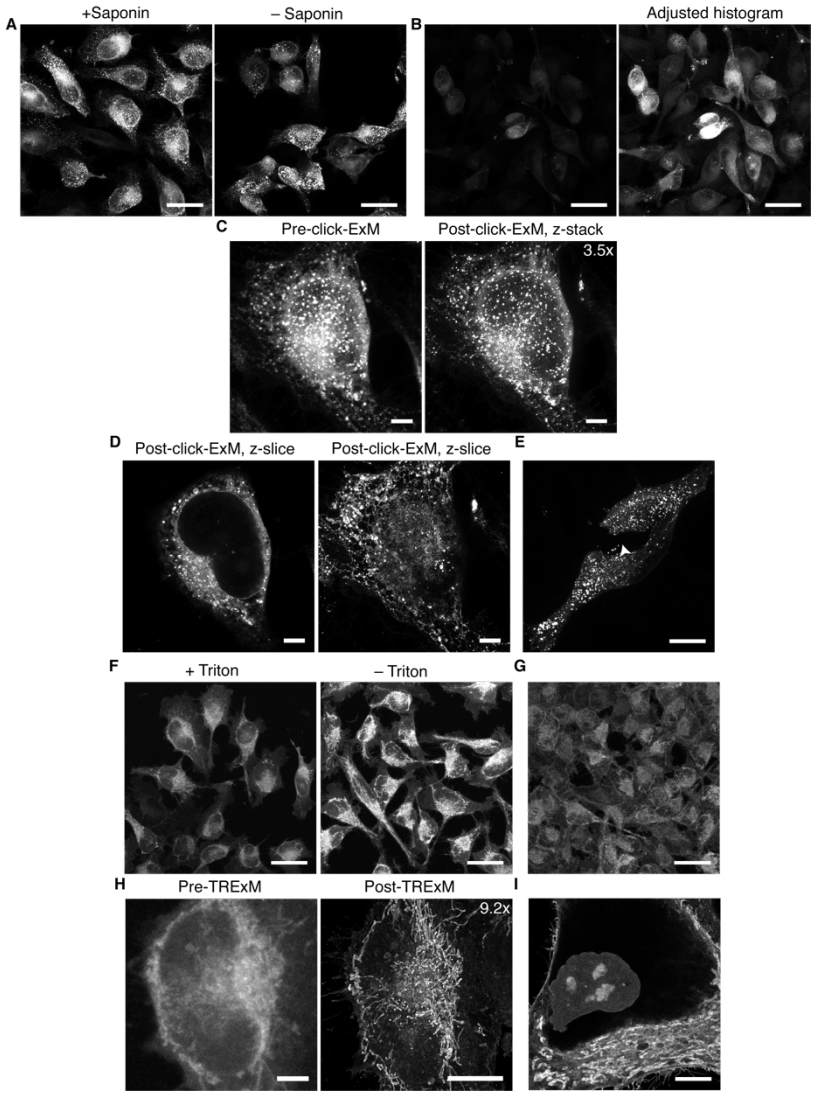
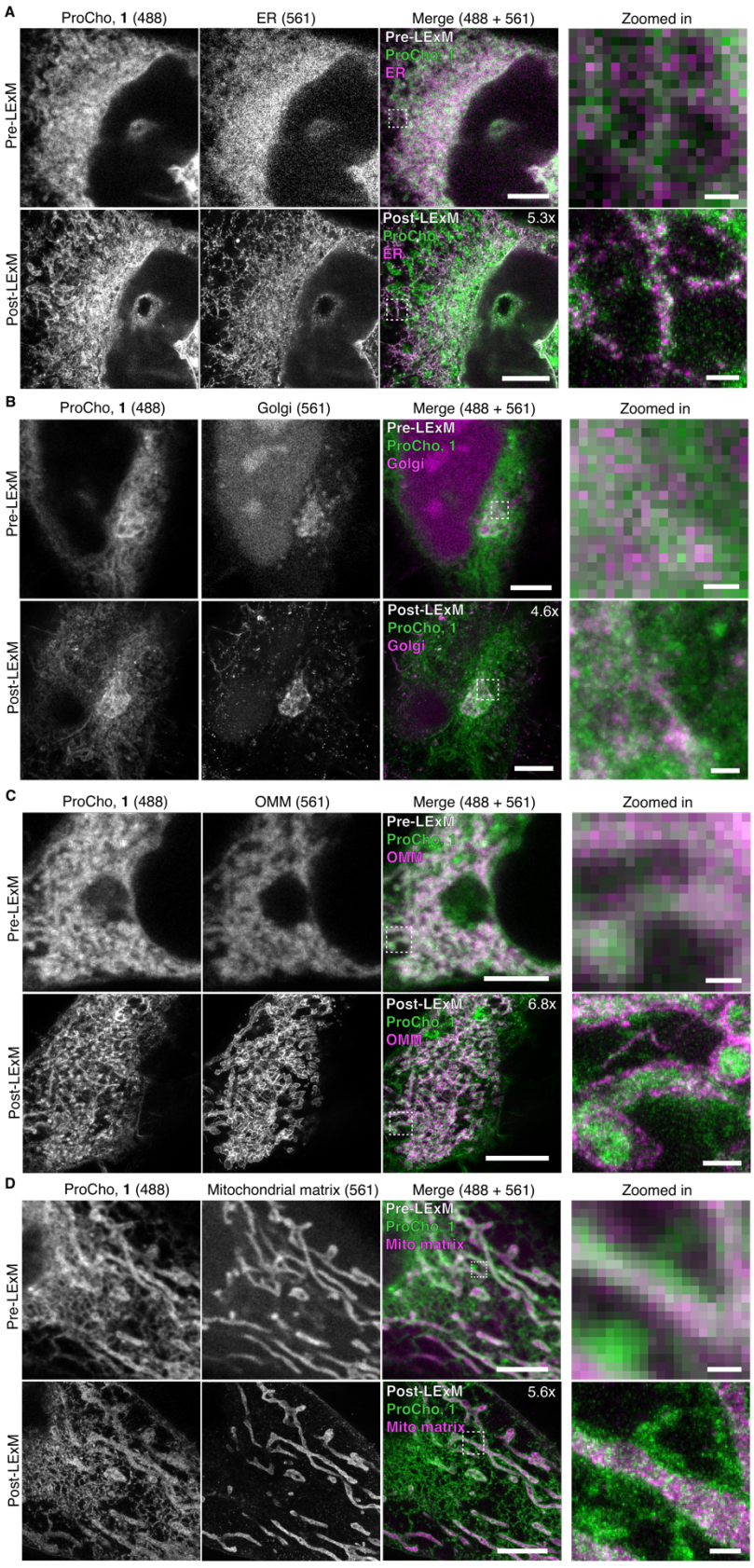


Figure S3. ExM imaging using click-ExM and TRExM. (A–E) Confocal microscopy images of cells metabolically labeled with ProCho, tagged via CuAAC with biotin-PEG_3-azide, and labeled with streptavidin-488 according to the click-ExM procedure.[1] (A) Pre-click-ExM images of cells treated with (left) and without saponin (right) prior to CuAAC labeling with biotin-PEG_3-azide. (B) Images show an area of confluent cells that was acquired under identical imaging conditions as (A) (left) and with an adjusted histogram (right), illustrating diminished fluorescence signal observed in click-ExM in regions of high cell confluence. (C) Pre-click-ExM and post-click-ExM z-stack images of the same cell in a sample treated with saponin during the click-ExM procedure. (D) Two different z-slice images of the post-click-ExM image (C, right) for comparison to images reported in click-ExM. (E) A post-click-ExM image of a sample where the saponin step in click-ExM was omitted. Arrowhead indicates tear in the gel, which was encountered in the majority of cells throughout the gel in samples that were labeled in this manner. (F–I) Confocal microscopy images of cells labeled with mCling-ATTO-488 according to the TRExM procedure.[2] (F) Pre-TRExM images of cells treated with (left) or without (right) Triton X-100 prior to pre-TRExM imaging. (G) An area of confluent cells from samples prepared and imaged as in (F); note variability in subcellular localization of the TRExM probe, with the image of high cell confluence exhibiting a greater fraction of plasma membrane as opposed to intracellular labeling. (H) Pre-

TRExM and post-TRExM images of the same cell in a sample treated with Triton X-100. (I) A slice of a post-TRExM image from a sample treated without Triton X-100; note detachment of nucleus and failure of nucleus to expand. Expansion factors are listed in the upper right corner of the post-ExM images. Scale bars: 30 μm (A, B, E, F, G and I), 5 μm (C, D and H, pre-ExM distance).


A
ProCho, 1 (488)
ER (561)
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Pre-LExM
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ProCho. 1
ER
5.3x
B
ProCho, 1 (488)
Golgi (561)
Merge (488 + 561)
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Pre-LExM
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Golgi
Post-LExM
ProCho. 1
Golgi
4.6x
C
ProCho, 1 (488)
OMM (561)
Merge (488 + 561)
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Pre-LExM
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OMM
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ProCho, 1 (488)
Mitochondrial matrix (561)
Merge (488 + 561)
Zoomed in
Pre-LExM
Post-LExM
Pre-LExM
ProCho. 1
Mito matrix
Post-LExM
ProCho. 1
Mito matrix
5.6x

Figure S4. Colocalization studies of organelle markers and LExM with ProCho and 1. Pre-LExM (top rows) and post-LExM (bottom rows) images of cells that were transfected and labeled with ProCho as described in the Figure 3 legend. In merged images, organelle markers are shown in magenta and lipids metabolically labeled with ProCho are shown in green. Cells were transfected with the ER marker mRFP-Sec61β (A), Golgi complex marker mCherry-PH(OSBP) (B), outer mitochondrial membrane (OMM) marker OMP25TM-mCherry (C), or mitochondrial matrix marker (Mito matrix) mCherry-Mito7 (D). Boxes in merged images indicate area shown in zoomed in images. Post-LExM images were acquired with Airyscan confocal microscopy. Expansion factors are listed in the upper right corner of the post-LExM merged images. Scale bars (pre-LExM distance): 5 μm (merged images), 400 nm (zoomed in images).

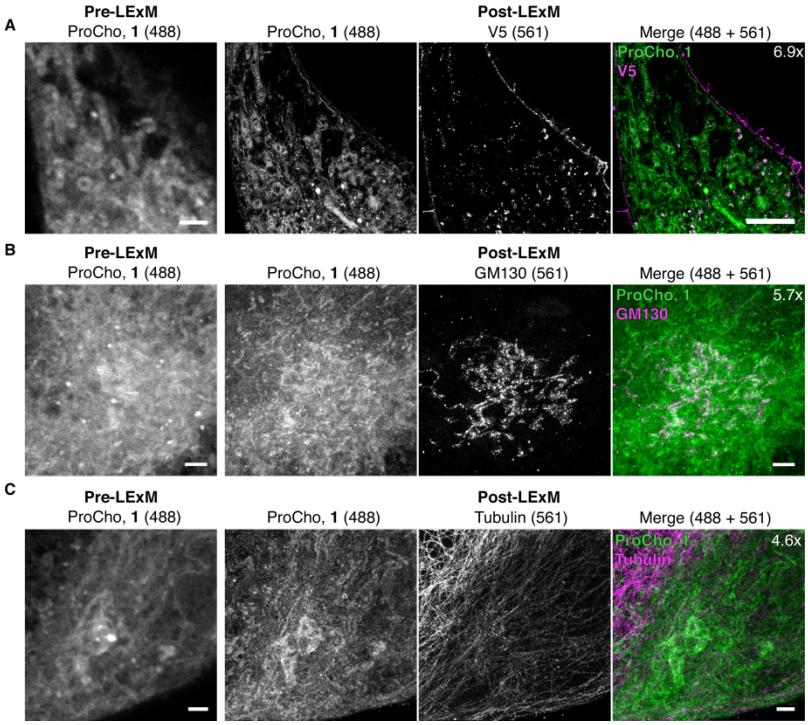


Figure S5. Colocalization studies of LExM with ProCho and 1 with immunofluorescence detection of V5 tagged or endogenous proteins. Pre-LExM (left) and post-LExM (right) images of cells labeled with ProCho then tagged with **1** via CuAAC and treated with an organelle marker. (A) Cells were transfected with the plasma membrane marker Lyn10-miniTurbo-V5 prior to ProCho labeling. The V5 organelle marker was visualized by staining gels post-digest with mouse anti-V5 primary and anti-mouse-Alexa Fluor 594 secondary antibodies. Endogenous proteins were visualized by staining gels post-digest with anti-GM130 (B) or anti-tubulin (C) primary, biotinylated anti-mouse secondary, and streptavidin-Alexa Fluor 568 tertiary antibodies. In the merged images, lipid signal is shown in green and organelle markers are shown in magenta. Post-LExM images were acquired with Airyscan confocal microscopy. Expansion factors are listed in the upper right corner of the post-LExM merged images. Scale bars (pre-LExM distance): 2 μm.

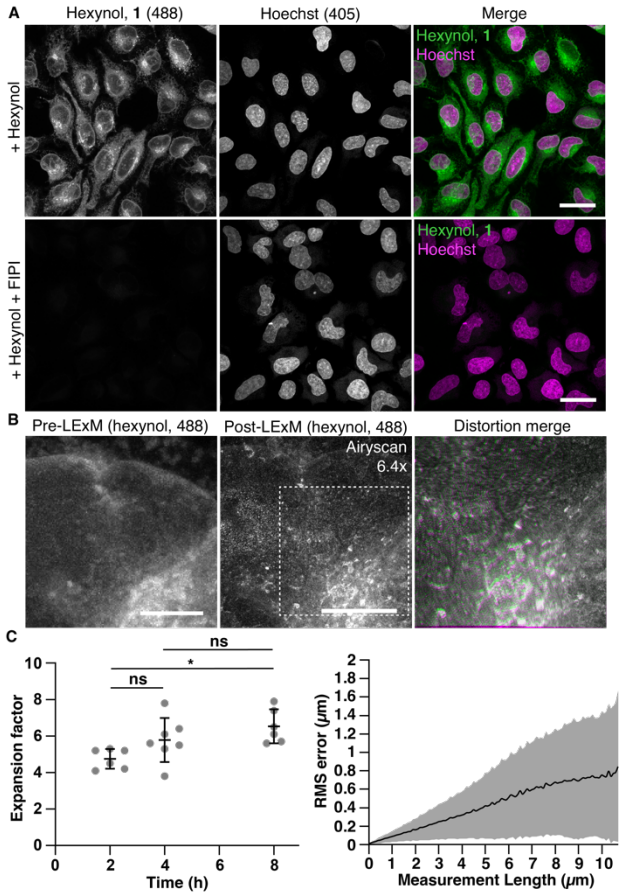


Figure S6. Control experiments, representative image used for distortion analysis, and expansion factor and RMSE graphs of cells labeled with IMPACT and tagged with 1 for LExM. Images of cells labeled via IMPACT with hexynol in the absence (top row) or presence (bottom row) of the pan-PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) that were then tagged with **1** via CuAAC. In the merged image, the IMPACT-labeled lipids are shown in green and Hoechst 33342 is shown in magenta. (B) Representative image of a pre-LExM and post-LExM cell used for distortion analysis shown in (C). Pre-LExM images (left) were compared to post-LExM images (middle) with non-rigid registration using the area highlighted by the box in the post-LExM. In the distortion image (right), the result of the non-rigid registration (green) is overlaid with the undistorted post-LExM area (magenta). The post-LExM image was acquired with Airyscan confocal microscopy. (C) Expansion factors of samples hydrolyzed for 2, 4 and 8 h (left). Expansion factors were determined with rigid registration. Statistical significance was determined using ANOVA (one-way, Tukey). * $p < 0.05$, ns = not significant. Root mean square (RMS) error as a function of measurement length generated by non-rigid registration of pre-LExM and post-LExM images (right, n=7). Expansion factors are listed in the upper right corner of the post-LExM images. Scale bars: 30 μm (A), 5 μm (B, pre-LExM distance).

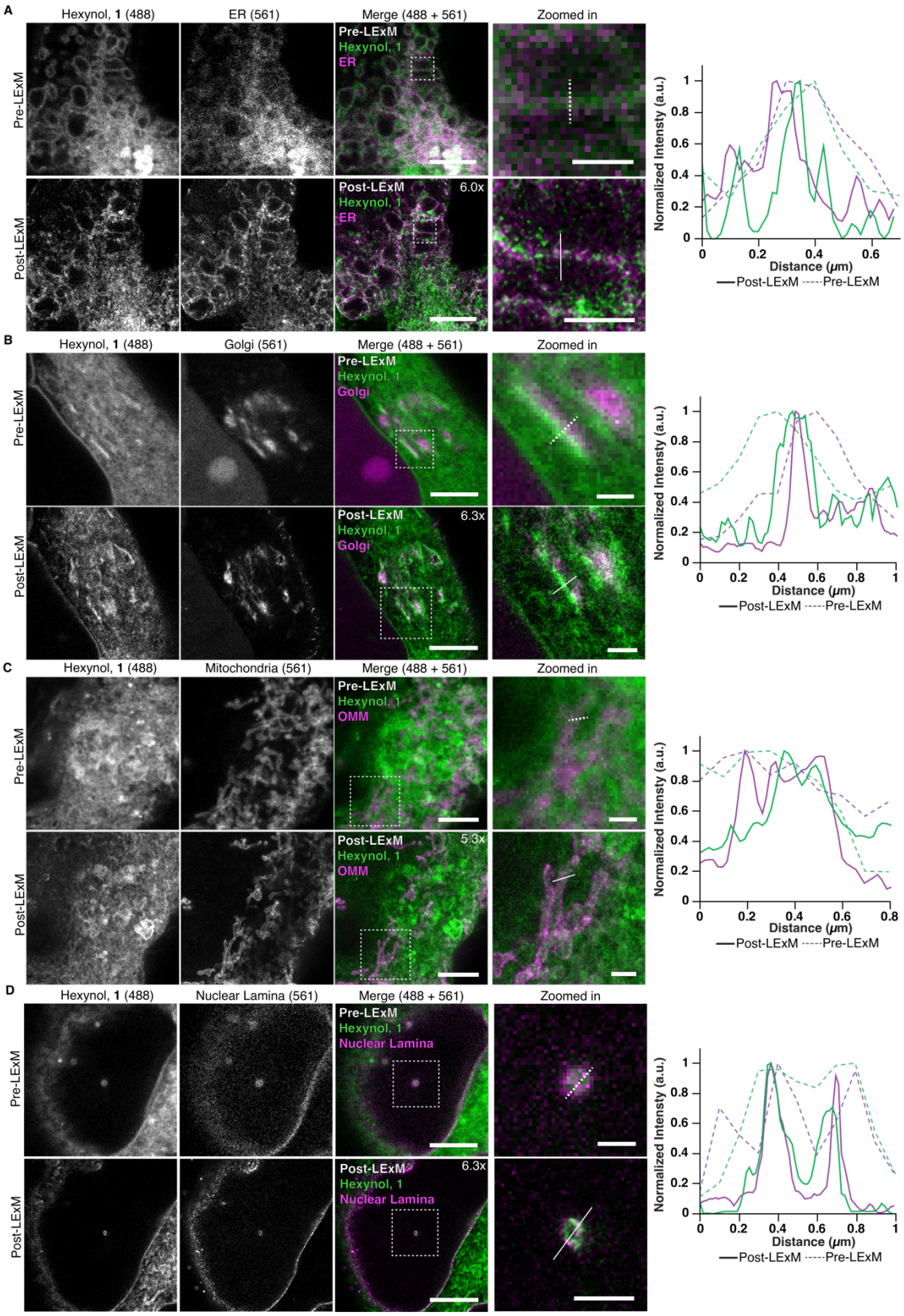


Figure S7. Colocalization studies of organelle markers with IMPACT labeling and tagging with 1 for LExM. Pre-LExM (top row) and post-LExM (bottom row) images of representative

cells transfected with organelle marker (magenta in merge) then labeled via IMPACT with hexynol and **1** (green in merge), with fluorescence intensity profile line plots of the pre-LExM (dotted line) and post-LExM (solid line) images. Cells were transfected with the ER marker mRFP-Sec61β (A), Golgi complex marker mCherry-PH(OSBP) (B), outer mitochondrial membrane (OMM) marker OMP25TM-mCherry (C), or the nuclear lamina marker mRFP-Lamin A (D). Dotted (pre-LExM) and solid (post-LExM) lines on zoomed in images indicate profiles measured for line plots. Boxes in merged images indicate area shown in the zoomed in image. Post-LExM images were acquired with Airyscan confocal microscopy. Expansion factors are listed in the upper right corner of the post-LExM merged images. Scale bars (pre-LExM distance): 5 μm (merged images), and 1 μm (zoomed in images).

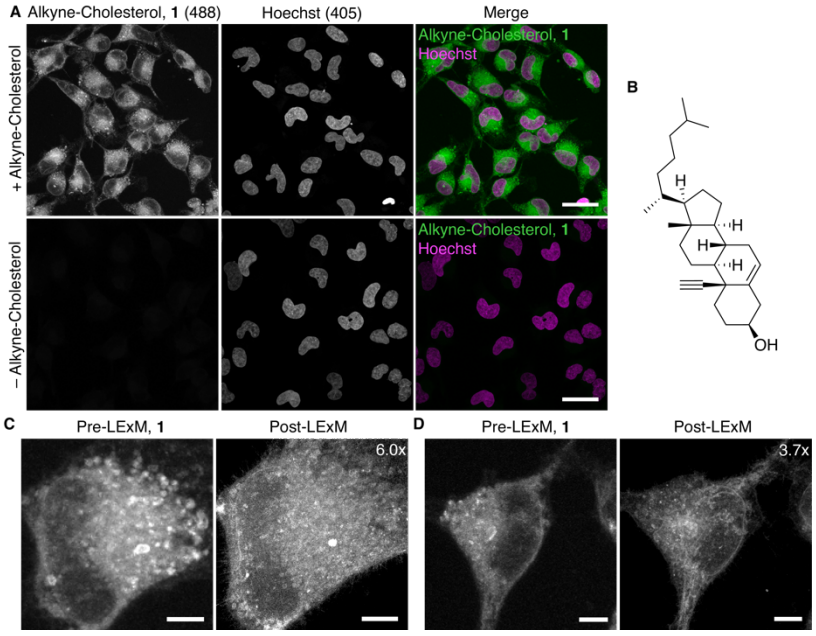


Figure S8. Control experiments and expanded images of cells labeled for LExM with alkyne-cholesterol and 1. (A) Confocal microscopy images of cells labeled with (top row) or without (bottom row) alkyne-cholesterol and tagged with **1** via CuAAC. In the merged images, labeled lipids are shown in green and Hoechst 33342 is shown in magenta. (B) Structure of alkyne-cholesterol. (C) Pre-LExM (left) and post-LExM (right) images of the same cell polymerized under standard LExM conditions. (D) Pre-LExM (left) and post-LExM (right) images of the same cell polymerized using lower concentrations of TEMED and APS (1.7 mg/mL each) to slow polymerization and a higher temperature (37 °C) incubation to increase monomer diffusion into the sample for an overall increased incorporation of **1**. Expansion factors are listed in the upper right corner of the post-LExM images. Scale bars: 30 μm (A), 5 μm (B and C, pre-LExM distance).

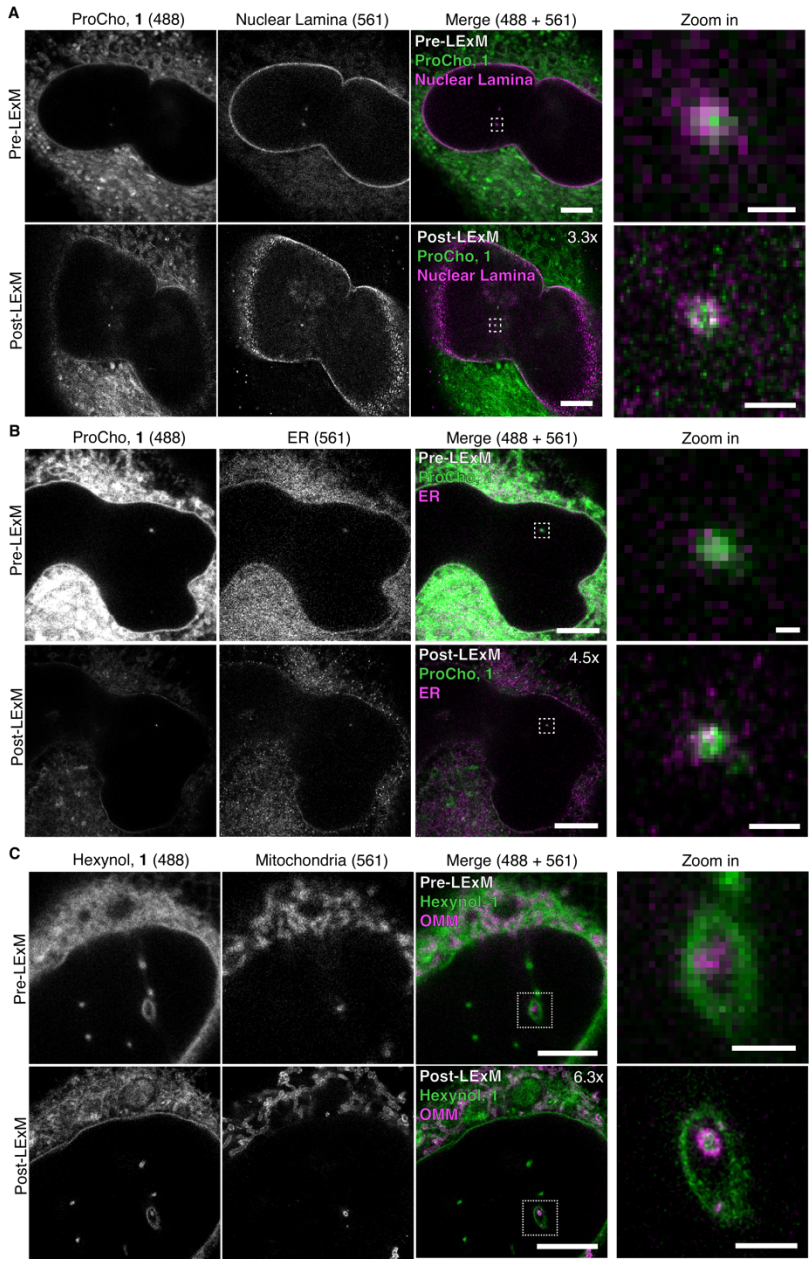


Figure S9. LExM imaging of nuclear invaginations tagged with 1. Pre-LExM (top rows) and post-LExM (bottom rows) images of cells that were transfected and metabolically labeled as described the Figure 5 legend. Organelle markers are magenta in merged images and labeled lipids are green in merged images. Cells were transfected with the nuclear lamina marker mRFP-Lamin A (A), the ER marker mRFP-Sec61β (B), or the outer mitochondrial membrane (OMM) marker OMP25TM-mCherry (C). Boxes in merged images indicate area shown in the zoomed in images. Post-LExM images were acquired with Airyscan confocal microscopy. Expansion factors are listed in the upper right corner of the post-LExM merge images. Scale bars (pre-LExM distance): 5 μm (merged images), 500 nm (A, zoomed in image), 300 nm (B, zoomed in image), and 1 μm (C, zoomed in image).

Video S1. A post-LExM z-stack of a cell labeled with ProCho and tagged with **1**, demonstrating channels traveling through the nucleus. The image was acquired with Airyscan confocal microscopy. Expansion factor: 5.3x. Scale bar (pre-LExM distance): 3 μm. Spacing between each z-slice is 0.5 μm.

Supplementary Note 1. Comparison of LExM with click-ExM and TRExM, alternative ExM methods for imaging membranes.

Click-ExM: Like LExM, click-ExM uses metabolic labeling with ProCho to install bioorthogonal handles onto lipid membranes. Subsequent fixation and additional permeabilization with saponin, followed by tagging with biotin and streptavidin-conjugated fluorophores that are anchored to the polymer network through acryloyl-X-SE treatment, enables expansion of lipid-containing membranes. Whereas click-ExM results in bright images and is capable of further signal enhancement through the biotin-streptavidin conjugation, we noted that z-stack projections of click-ExM samples contained bright puncta that differ from the reported localizations and patterns of ProCho-labeled lipids within cellular membranes (Figure S3C). Conversely, we were able to recapitulate the reported localizations and patterns of ProCho-labeled lipids with LExM. We note that, in our hands, puncta were less apparent in single z-plane slices of the click-ExM images (Figure S3D), and that these images more closely resembled reported click-ExM images with ProCho labeling, with fewer but still some observable puncta. Additionally, areas of high cell confluency resulted in lower overall fluorescence tagging of membranes (Figure S3A–B), potentially due to the failure of impermeable CuAAC reagents to effectively access azido lipids in our hands, a phenomenon that we do not observe in LExM. In the absence of saponin permeabilization, as required by the click-ExM protocol, the fluorescent signal did not appear to diminish, suggesting that click-ExM labeling does not require permeabilization for successful CuAAC tagging. However, samples generated in this manner, when subjected to expansion, resulted in highly distorted and torn samples, indicating the requirement for detergent in this protocol. Finally, in colocalization experiments, we observed signal from ProCho-labeled lipids in the inner mitochondrial membrane (Figure 3D and S4D) in LExM, which is consistent with the reported localization of ProCho-labeled lipids but differs from reported localizations obtained using click-ExM.[3,1] We hypothesize that this difference in localization pattern may be due to inefficient membrane permeabilization that does not allow streptavidin-based macromolecular labels to access the inner mitochondrial membrane, a phenomenon that may be avoided in LExM, where membrane tagging involves only small-molecule probes.

TRExM: Unlike the LExM and click-ExM, which use metabolic labeling of native phospholipids, Ten-fold Robust ExM (TRExM) uses the fluorescently tagged, unnatural lipid probes mCling-ATTO-488 and mCling-ATTO-647 to label membranes. These probes are anchored to the polymer network through lysine residues that are functionalized upon treatment with acryloyl-X-SE. When compared to LExM and click-ExM, TRExM exhibited higher localization of fluorescence signal at the plasma membrane (PM) and mitochondria, with apparent lower levels of labeling at the Golgi and ER (Figure S3F–I). Additionally, samples treated with the nonionic detergent Triton X-100, the permeabilization reagent that is required for this protocol, resulted in lower levels of fluorescent labeling, indicating that this treatment somewhat reduces the overall signal of the sample (Figure S3F). Akin to our observation in samples treated with click-ExM, areas of high confluency resulted in lower levels of fluorescent labeling, and further, we noticed that the label

was more concentrated at the PM as opposed to intracellular membranes (Figure S3F–G). We did not note any cell confluence-dependent changes in probe localization or labeling efficiency with LExM. Finally, samples that were labeled via TRExM but not treated with Triton X-100 also resulted exhibited severe delamination (note in particular the separation of the nucleus from the cytoplasm and failure of the nucleus to expand in Figure S3I), demonstrating the necessity of detergent-based permeabilization in this protocol. The requirement in click-ExM and TRExM for additional permeabilization beyond fixation, which can perturb membrane structure and integrity, stands in contrast to LExM, which uses soluble small molecules for lipid labeling and polymerization to preserve molecular details of membrane environments.

Summary of advantages and limitations of LExM:

Advantages:

1) LExM does not require additional permeabilization of membranes beyond fixation, enabling labeling while preserving the natural structure and molecular detail of membrane environments.
2) Direct chemical anchoring of the lipid to the polymer network reduces the linker length when compared to other membrane expansion methods that use macromolecule-based detection reagents, such as click-ExM.
3) LExM, like click-ExM, uses incorporation of close structural mimics of native lipids into membranes to report on natural membrane environments in contrast to methods such as TRExM and mExM, which use unnatural hydrophobic probes for labeling.

Limitations:

1) LExM has not been demonstrated in tissue samples, in contrast to click-ExM and mExM. LExM may require additional optimization steps to ensure that the fluorophore and CuAAC reagents effectively diffuse into and out of tissues without additional permeabilization steps.
2) LExM is not capable of signal amplification of the CuAAC product, in contrast to click-ExM.
3) LExM results in moderately higher signal loss when compared to click-ExM, which may limit the sensitivity and thus resolution level that can be achieved, especially under conditions where higher expansion factor values are desired.

Materials and Methods

General Experimental Details

Unless otherwise noted, reactions were carried out under an inert atmosphere of nitrogen using standard Schlenk technique. All reagents used were analytical grade and obtained from commercial suppliers without further purification. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (P/S), sodium pyruvate, phosphate-buffered saline (PBS), and 0.05% trypsin-EDTA (trypsin) were purchased from Corning. Lipofectamine 2000, streptavidin-488, streptavidin-568 and 4-well Nunc™ dishes (item #267061) were purchased from Thermo Fisher. Human plasma fibronectin, sodium acrylate, acrylic acid N-hydroxysuccinimide (NAS), 16-well Grace Bio-Labs CultureWell™ plate with removable no. 1.5 DESAG coverglass, and Grace Bio-Labs CultureWell™ removal tool were purchased from

Sigma-Aldrich. Acryoyl-X-SE was purchased from Invitrogen. 5-hexyn-1-ol (heyxnol) was purchased from Alfa Aesar. 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) was purchased from Cayman Chemical. Phorbol 12-myristate 13-acetate (PMA) was purchased from Santa Cruz Biotechnology. BDP-FL-azide was purchased from Lumiprobe. Biotin-PEG_3-azide, BTTAA and alkyne-cholesterol (E-cholesterol) were purchased from Click Chemistry Tools. Methyl-β-cyclodextrin was purchased from Acros Organics. Saponin was purchased from J&K Scientific. mCling-ATTO-488 was purchased from Synaptic Systems. Proteinase K was purchased from New England Biolabs. Acrylamide, bis-acrylamide, ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were purchased from VWR. Ethyl 3-(1H-pyrrol-2-yl)propanoate[4], and propargyl choline (ProCho)[5] were prepared according to previous reports.

For **1** and **3–8**, silica gel purification was performed with 60 Å, 40- 63 μm SiliCycle SiliaFlash irregular silica gel. For the synthesis of **2** and **9–11**, reactions products were purified either by flash chromatography on a Biotage Isolera automated system with pre-packed silica gel columns or with preparative HPLC on an Agilent 1200 Infinity system with a Phenomenex Gemini–NX, 105 mm x 4.6 mm, 5 μm C_{18}, 110 Å column with a 1 mL/minute flowrate, fitted with a mass spectrometer and diode array detector or on a Shimadzu HPLC with identical column conditions and fitted with diode array detector. Thin layer chromatography (TLC) was performed using EMD Millipore Silica gel 60 F_{254} plates for normal phase chromatography. For all intermediates and final compounds, developed TLC plates were visualized by UV light at wavelengths of 254 and 265 nm. For **2** and **9–11**, some TLC plates were visualized by treatment with ceric ammonium molybdate (CAM) or $KMnO_4$ stains. For the synthesis of **2**, reactions were monitored by TLC or with a Shimadzu 2020 LCMS with a Phenomenex Kinetes, 30 mm x 2.1 mm, 2.6 μm C_{18} column with 1–10 μL injections, and a 6 minute run using a 5-98% MeCN/H_2O + 0.1 v/v acetic acid linear gradient and 1mL/minute flow rate in ESI positive ion mode. For **1**, HPLC purification was performed on a Shimadzu LC20AR HPLC equipped with an SPD-20AV UV/Vis detector and a ES Industries Epic Polar C18 reverse phase column (25 cm x 10 mm, 5 μm). For **1** and **3–8,** NMR spectra were recorded on a Bruker 500 MHz spectrometer with a BBO H&F cryoprobe at ambient temperature. For **2** and **9–11**, NMR spectra were recorded on Bruker 400 MHz spectrometer. All ^{1}H NMR spectra were obtained in $CDCl_3$ (referenced to TMS, δ 0.00 ppm), or MeOD-d_4 (referenced to residual MeOD, δ 3.31 ppm). All ^{13}C NMR spectra were taken in $CDCl_3$ (referenced to chloroform, δ 77.16 ppm) or MeOD-d_4 (referenced to methanol, δ 49.00 ppm). For **1** and **3–8,** DART MS was performed on an Exactive Plus Orbitrap Mass Spectrometer with a DART SVP ion source from Ion Sense. For **2** and **9–11**, ESI MS was performed at the High Resolution Mass Spectrometry Facility at the University of Iowa. Confocal imaging was performed on a Zeiss LSM 800 confocal laser scanning microscope equipped with a Plan Apochromat 40X 1.4 NA and Plan-Apochromat 63X 1.4 NA f/ELYRA oil immersion objective oil immersion objectives, 405, 488, 561, and 640 nm solid-state lasers, and two GaAsP PMT detectors. Airyscan confocal imaging was acquired on the same Zeiss LSM 800 microscope equipped with a Plan-Apochromat 63X 1.4 NA f/ELYRA oil immersion objective and an Airyscan module. Images were acquired using Zeiss Zen Blue 2.3 and analyzed using ImageJ/FIJI.[6]

Cell culture

HeLa cells were cultured in a 5% CO_2, water-saturated atmosphere at 37 ºC in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin. Cells were maintained between 10^5 and $1.6x10^6$ cells/mL.

Plasmids

mCherry-PH(OSBP) was prepared through cut and paste of iRFP-$PH_{(OSBP)}$ and mCherry-C1 empty vector. Lyn10-miniTurbo-V5 was prepared by replacement of C1 with the Lyn10 sequence in C1-miniTurbo-V5. mCherry-Mito-7 and C1-miniTurbo-V5 were purchased from Addgene (#55102 and #107174). The remaining plasmids were obtained from the following sources: mCherry-OMP25TM, mRFP-Sec61β, and iRFP-PH(OSBP) were obtained from P. De Camilli (Yale University, New Haven, CT); mRFP-Lamin A from Jan Lammerding (Cornell University, Ithaca, NY).

Antibodies

Mouse anti-mCherry (IC51), Sigma-Aldrich (MAB131873); Alexa Fluor™ 594 donkey anti-mouse, Thermo Fisher Scientific (A21203); Anti-V5 antibody (SV5-PK1), BioRad (MCA1360GA); Anti-GM130 antibody, BD Biosciences (610822); Anti-tubulin, Millipore Sigma (T5168); Biotinylated anti-mouse antibody, Vector Laboratories (BA-9200-1.5).

Preparation of monomer solution and polymerization components

Not more than one day before polymerization, the monomer solution was prepared with acrylamide (300 mg/mL) and bis-acrylamide (0.1 mg/mL, prepared from a 10 mg/mL stock solution) in PBS buffer along with the TEMED (100 mg/mL) and APS (100 mg/mL) solutions in deionized water. The solutions were stored at –20 ºC overnight and thawed immediately before use.

Metabolic labeling of cultured cells with ProCho

A 16-well Grace Bio-Labs CultureWell plate with removable coverglass was treated with human plasma fibronectin (0.5 mg/mL) in PBS for 35 min in a cell incubator. The solution was aspirated and the wells were rinsed once with PBS then seeded with HeLa cells (7,500 cells) with 250 µL of media in each well. The cells were grown overnight (16–24 h) then the media was aspirated and replaced with media containing 500 µM ProCho, prepared from a 1 M solution of ProCho in DMSO, or regular media for no ProCho negative controls. Cells were incubated in the labeling media for 24 h before treatment with the LExM protocol.

For colocalization experiments, one day prior to metabolic labeling, each well of a fibronectin-coated 16-well plate was seeded with HeLa cells (12,500 cells) with 250 µL of media. Cells were grown overnight (16–24 h) then transfected with the appropriate plasmid using Lipofectamine 2000 according to the manufacturer's instructions. After 6 h, the transfection media was exchanged for fresh media then cells were incubated for 18 h before metabolic labeling with ProCho.

Metabolic labeling of cultured cells with hexynol via IMPACT

A 16-well Grace Bio-Labs CultureWell plate with removable coverglass was treated with human plasma fibronectin (0.5 mg/mL) in PBS for 35 min in a cell incubator. The solution was aspirated, and the wells were rinsed once with PBS then seeded with HeLa cells (15,000 cells) with 250 µL of media in each well. The cells were grown overnight (16–24 h) then negative control cells were treated with FIPI to a final concentration of 750 nM from a 750 µM stock in DMSO for 30 min. Each well was aspirated, rinsed with PBS buffer three times, then incubated with Tyrode's-HEPES imaging buffer (135 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $CaCl_2$, and 100 µM $MgCl_2$ adjusted to pH = 7.4, plus 1 mg/mL BSA and 1 mg/mL glucose) containing 100 nM PMA,

15 mM or 10 mM hexynol, and FIPI for negative controls for 30 min before continuing with the LExM protocol. Cells labeled with 15 mM hexynol were used for imaging experiments without transfection and 10 mM hexynol for imaging experiments with transfection.

For colocalization experiments, one day prior to metabolic labeling, each well of a fibronectin-coated 16-well plate was seeded with HeLa cells (12,500 cells) with 250 µL of media. Cells were grown overnight (16–24 h) then transfected with the appropriate plasmid using Lipofectamine 2000 according to the manufacturer's instructions. After 6 h, the transfection media was exchanged for fresh media then cells were incubated for 18 h before IMPACT labeling with hexynol.

Labeling of cultured cells with alkyne-cholesterol

Alkyne-cholesterol treatment was performed following a protocol by Jao *et al*.[7, 8] Briefly, alkyne-cholesterol (0.50 µmol) from a 20 mM stock in DMSO was added to an Eppendorf tube, and the solution was lyophilized to give a white solid. Methyl-β-cyclodextrin (6.55 mg, 5.00 µmol) was added to the Eppendorf tube along with 131 µL of absolute ethanol, and the solution was heated at 10 min at 30 ºC to prepare a 10:1 methyl-β-cyclodextrin:alkyne-cholesterol complex. The solution was lyophilized to give a white solid and stored at 4 ºC until use.

A 16-well Grace Bio-Labs CultureWell plate with removable coverglass was treated with human plasma fibronectin (0.5 mg/mL) in PBS for 35 min in a cell incubator. The solution was aspirated, the wells were rinsed once with PBS and then seeded with HeLa cells (10,000 cells) with 250 µL of media in each well, and the cells were grown overnight (16–24 h). Immediately before use, the methyl-β-cyclodextrin/alkyne-cholesterol complex was dissolved in 500 µL of DMEM without FBS and P/S then heated at 37 ºC for 10 min to prepare a 1 M solution in alkyne-cholesterol. A 1 mM solution in DMEM without FBS and P/S was prepared from the 1 M solution, and the media in each well of the 16-well plate was aspirated and replaced with 250 µL of the 1 mM solution. The cells were incubated for 16 h before treatment with the LExM protocol.

CuAAC-mediated tagging of labeled cells

Cells labeled with ProCho, hexynol, or alkyne-cholesterol were rinsed with PBS two times and then fixed by treatment with a solution of PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde for 20 min at room temperature. The fixative was removed, and the cells were quenched by treatment with 0.1% $NaBH_4$ in PBS for 7 min followed by 100 mM glycine for 20 min. The cells were rinsed with PBS two times for 5 min each then labeled with **1** or **2** using a protocol described by Jao *et al*.[5] Briefly, cells were rinsed with tris-buffered saline (TBS) three times and then labeled with 50 µL of a CuAAC solution. The CuAAC solution was prepared immediately before use and contained the following reagents added in the order listed: 100 mM Tris pH 8.5, 5 µM **1** or **2** for ProCho or hexynol labeling or 20 µM **1** or **2** for alkyne-cholesterol labeling (prepared from a 5 mM stock in DMSO), 1 mM $CuSO_4$ (prepared from a 20 mM stock in deionized water), and 50 mM sodium ascorbate (prepared from a 500 mM stock in deionized water). After 1 h of treatment in the dark at room temperature, the CuAAC solution was aspirated and cells were rinsed with TBS three times, 500 mM NaCl three times, TBS again three times.

Protein conjugation and polymer formation

Samples were polymerized according to a protocol modified from Park *et al*.[9] Immediately after tagging with **1** or **2**, cells were treated with a 2.5 mM solution of NAS in PBS buffer for 3 h at room temperature. The NAS solution was prepared from a freshly prepared 50 mM stock of

NAS in DMSO that was added to PBS pre-chilled to 4 ºC. During the incubation period, a mask, prepared by cutting defined shapes corresponding to each well into a strip of Scotch tape, was placed on the bottom of the plate, and cells were imaged by confocal microscopy in the defined regions to obtain pre-LExM images. Cells were then rinsed three times with PBS for 5 min each, followed by careful removal of the wells using the manufacturer's removal tool. Cells were then treated with the monomer solution described above for one 1 h at room temperature in the dark by adding 35 µL of the solution to each area defined by the gasket that remained on the cover slide. This step was followed by treatment with 35 µL of the polymerization solution, four areas at a time, at 4 ºC in the dark. The polymerization solution was prepared immediately before use in a microcentrifuge tube pre-chilled to 4 ºC with the following reagents that were kept at 4 ºC and added in order as listed: 135 µL of monomer solution, 7.5 µL of 100 mg/mL TEMED to a final concentration of 5 mg/mL, and 7.5 µL of 100 mg/mL APS to a final concentration of 5 mg/mL. The polymerization solution was vortexed and centrifuged briefly before use, and areas were immediately covered with parafilm-coated glass slides with caution to avoid the formation of air pockets in the polymerization solution. The cover slide was then kept at room temperature for 1 h in the dark before the parafilm-coated glass slides were carefully removed, and the resulting gels were cut to include the areas defined by the Scotch tape mask. Each gel was removed from the cover slide using a PBS-saturated paint brush, placed in a 35-mm dish, and then rinsed with PBS three times for 5 min each. The gels were stored submerged in PBS in a humidified chamber at 4 ºC overnight in the dark.

Gel denaturation and hydrolysis

The gels were removed from the humidified chamber and placed into 13 x 100 mm test tubes with 2 mL of denaturation buffer (0.2 M sodium dodecyl sulfate (SDS), 0.05 M boric acid, pH = 9). The tubes were then placed in a heat block at 95 ºC and maintained at this temperature for 30 min in the dark. At this time, the temperature of the heating block was decreased to 80 ºC and the samples were heated for an additional 4 h, except in cases where the hydrolysis time was adjusted to 2 or 8 h as indicated. The gels were removed from the heat block and cooled to room temperature for 10 min, placed into individual wells of 4-well Nunc dishes, and then rinsed with 5 mL of PBS four times for 30 min each at room temperature on a shaker in the dark. After the final rinse, fresh PBS was added to each well and the gels were placed on a shaker at 4 ºC for overnight storage in the dark.

Antibody staining of transfected samples

For colocalization studies with mCherry-transfected organelle markers, intrinsic fluorescent protein fluorescence was too dim for detection by ExM in this protocol, so immunofluorescence staining for the fluorescent protein marker was necessary and was performed as described. Samples were stained with antibodies according to a protocol modified from the reported literature procedure by Park *et al*.[9] After the denature and hydrolysis steps, gels were placed in individual wells of 4-well Nunc dishes for the blocking step with 5% BSA in PBS with 0.1% Triton X-100 (PBST) and placed on a shaker at 4 ºC overnight (12–16 h) in the dark. The gels were then placed in individual wells of a 24-well plate with 300 µL of freshly prepared mouse anti-mCherry in PBST (1:100) and placed on a shaker at 4 ºC in the dark for 24 h. The antibody solution was carefully removed, the gels were rinsed 3 times with PBST, and incubated again for 24 h with 300 µL of freshly prepared mouse anti-mCherry in PBST (1:100) on a shaker at 4 ºC in the dark. The gels were placed in individual wells of 4-well Nunc dishes and rinsed with PBST

four times for 1 h each at room temperature on a shaker in the dark. After the final rinse, fresh PBST was added, and the gels were rinsed overnight (12–16 h) on a shaker at 4 ºC in the dark. The gels were placed in individual wells of a 24-well plate with 300 µL of freshly prepared Alexa Fluor 594 donkey anti-mouse IgG in PBST (1:100) and placed on a shaker at 4 ºC in the dark for 24 h. The antibody solution was carefully removed, the gels were rinsed 3 times with PBST, and the gels were incubated again for 24 h with 300 µL of freshly prepared Alexa Fluor 594 donkey anti-mouse IgG in PBST (1:100) on a shaker at 4 ºC in the dark. The gels were placed in individual wells of 4-well Nunc dishes and rinsed with PBST four times for 1 h each at room temperature on a shaker in the dark. After the final rinse, fresh PBST was added, and the gels were rinsed overnight (12–16 h) on a shaker at 4 ºC in the dark. An identical staining protocol was followed for cells transfected with lyn10-miniTurbo-V5 organelle marker, except an anti-V5 antibody was used in place of the anti-mCherry antibody.

Sample were stained for endogenous proteins by treating gels with 300 µL of freshly prepared mouse anti-GM130 or anti-tubulin in PBST (1:100) and placed on a shaker at 4 ºC in the dark for 24 h after the blocking step. Gels treated with anti-GM130 were stained once more with primary antibody solution for 24 h rinsing 3 times with PBST in between treatments, whereas gels treated with anti-tubulin were stained two additional times with primary antibody for 24 h each, rinsing with PBST 3 times in between each treatment. After primary antibody treatment, the gels were placed in individual wells of 4-well Nunc dishes and rinsed with PBST four times for 1 h each at room temperature on a shaker in the dark. After the final rinse, fresh PBST was added, and the gels were rinsed overnight (12–16 h) on a shaker at 4 ºC in the dark. The gels were then placed in individual wells of a 24-well plate with 300 µL of freshly prepared biotinylated anti-mouse in PBST (1:100) and placed on a shaker at 4 ºC in the dark for 24 h. Gels treated with anti-GM130 were stained once more with secondary antibody solution for 24 h, rinsing 3 times with PBST in between treatments, whereas gels treated with anti-tubulin were stained two additional times with secondary antibody for 24 h each, rinsing with PBST 3 times between each treatment. After secondary antibody treatment, the gels were placed in individual wells of 4-well Nunc dishes and rinsed with PBST four times for 1 h each at room temperature on a shaker in the dark. After the final rinse, fresh PBST was added, and the gels were rinsed overnight (12–16 h) on a shaker at 4 ºC in the dark. The gels were then placed in individual wells of a 24-well plate with 300 µL of freshly prepared streptavidin-568 in PBST (1:100) and placed on a shaker at 4 ºC in the dark for 24 h. Gels treated with anti-GM130 were stained once more with streptavidin solution for 24 h, rinsing 3 times with PBST in between treatments, whereas gels treated with anti-tubulin were stained two additional times with streptavidin for 24 h each, rinsing with PBST 3 times between each treatment. After streptavidin treatment, the gels were placed in individual wells of 4-well Nunc dishes and rinsed with PBST four times for 1 h each at room temperature on a shaker in the dark. After the final rinse, fresh PBST was added, and the gels were rinsed overnight (12–16 h) on a shaker at 4 ºC in the dark.

Gel expansion and post-LExM imaging

Several 35-mm, glass-bottomed imaging dishes were incubated at 37 ºC with solutions of poly-L-lysine (0.1 mg/mL) in PBS overnight (12–16 h). The dishes where then aspirated, rinsed with PBS once and dried under high vacuum for 2–4 h. The dishes were used immediately or stored at 4 ºC for up to a week. Gels were placed in individual wells of a 4-well Nunc dishes and expanded by treatment with 10 mL of deionized water four times for 1 h each. The expanded samples were carefully removed and cut to an area of interest using a razor blade, mounted on the

poly-L-lysine-coated dishes, and then imaged by standard confocal or Airyscan confocal microscopy. All gels transfected with organelle markers were imaged with Airyscan confocal microscopy, whereas non-transfected cells were imaged either with standard confocal or Airyscan confocal microscopy, as indicated.

Expansion factor determination and root mean square error analysis

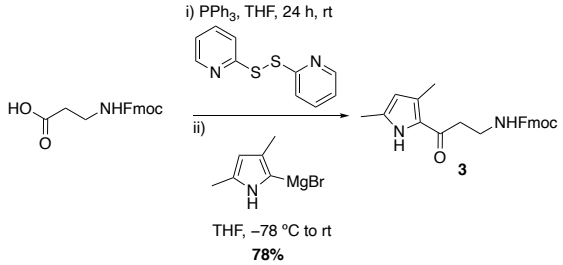
Z-stacks of pre-LExM and post-LExM images acquired with the same frame size and objective were imported into ImageJ/FIJI as .czi files and converted to maximum intensity z-projections. Post-LExM images were further processed with the despeckle function in ImageJ while pre-LExM images were cropped to a square area containing the same features as the post-LExM image. Post-LExM images acquired with Airyscan confocal microscopy were cropped to remove the black 12-pixel border. The initial expansion factor was calculated by dividing the frame size of the post-LExM image by the frame size of the cropped pre-LExM image. This number was used to describe the expansion factor of images shown in Figures 3, 5, S3, S4, and S5. Final expansion factors were determined with rigid registration using the protocol described by Chozinski *et al*.[10] Briefly, the pre-LExM image was scaled to match the frame size of the post-LExM image using the scale function in ImageJ with bicubic interpolation. The pixel width and height of the pre-LExM and post-LExM images were adjusted to 1, and the images were converted to 16-bit images and saved as .raw and .mhd files in the same folder as the similarity function scripts. Using terminal, the images were submitted for rigid analysis in Elastix using the similarity parameters described in the protocol to give a "TransformParameters.0.txt" output file where the first number listed on the transform parameters line represents the scaling factor of the image. This scaling factor was multiplied by the initial expansion factor, calculated by comparing the pre-LExM and post-LExM frame sizes, to give the final expansion factor that was used to describe the expansion factors show in Figures 2, 4, S1B, and S2B. Final expansion factors were also used for the statistical comparison of expansion factors show in Figures 2 and S2.

Continuing the protocol developed by Chozinski *et al*,[10] the adjusted post-LExM image resulting from the similarity transform was compared to the pre-LExM image using nonrigid registration for distortion analysis. Briefly, the image resulting from the similarity transform was converted to a 16-bit image then overlayed with the pre-LExM image. An area of interest was defined using the crop function in ImageJ, the cropped images were converted back to grayscale and split into individual images then saved as pre-LExM and post-LExM .raw and .mhd files in the same folder as the b-spline parameters. The images were submitted for rigid analysis in Elastix using the b-spline parameters described in the protocol to give a "result.0" distorted image which was saved as a 16-bit tiff file in the same folder as the Mathematica notebook, along with the post-LExM image submitted to b-spline analysis. Using a binary mask to define analyzed pixels, the root mean square error (RMSE) was determined using the Mathematica notebook with a binsize of 0.5 to generate more points and exported as an Excel file. To generate the root mean square error plots show in Figures 2 and S2, multiple outputs were combined, sorted by distance then binned into groups of 50 to give an average distance with associated average root mean square and standard deviation values that were used to generate the line and error bands of the RMSE plots respectively.

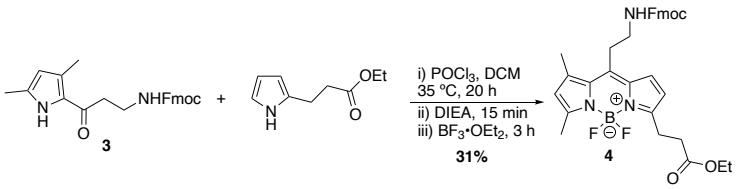
Click-ExM and TRExM

Click-ExM[1] with ProCho labeling and biotin/streptavidin–488 tagging and TRExM[2] with mCling-ATTO-488 labeling were performed according to literature procedures.

Synthetic Methods

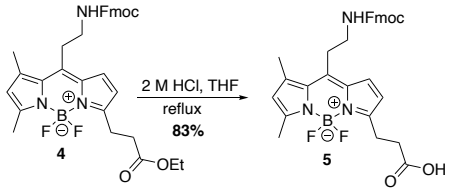


Fmoc-protected pyrrole **3**. This procedure was adapted from a previous report.[11] Fmoc-β-AlaOH (1.65 g, 5.28 mmol, 1.00 equiv), 2,2'-dipyridyl disulfide (1.78 g, 7.92 mmol, 1.50 equiv), and triphenylphosphine (2.77 g, 10.56 mmol, 2.00 equiv) were dissolved with dry tetrahydrofuran (30 mL) in a flame dried flask, and the mixture was stirred for 24 hours at room temperature. In a separate flame dried flask, 2,4-dimethylpyrrole (3.27 mL, 31.7 mmol, 6.00 equiv) in dry tetrahydrofuran (30 mL) was cooled to –78 ºC and methylmagnesium bromide (7.9 mL, 23.8 mmol, 4.50 equiv) was added dropwise to the solution. The mixture was stirred at –78 ºC for 30 minutes then warmed to room temperature and stirred for another 30 minutes. The pyrrole solution was again cooled, the thioester mixture was added dropwise and reaction was stirred at –78 ºC for 30 minutes then warmed to room temperature and stirred for another 30 minutes. The reaction was then quenched by the addition of a saturated solution of ammonium chloride (40 mL) and diluted with diethyl ether (40 mL). The organic layer was extracted then washed with water (3 x 60 mL), brine (1 x 60 mL), dried over magnesium sulfate, and then concentrated to give a yellow tinted oil that solidified upon standing. The product was purified by recrystallization in acetone (100 mL) to give **3** as an off-white crystalline powder after rinsing with hexanes (1.60 g, 78%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.98 (brs, 1 H), 7.75 (d, J = 7.5 Hz, 2 H), 7.58 (d , J = 7.5 Hz, 2 H), 7.38 (dd, J = 8.1, 7.5 Hz, 2 H), 7.29 (dd , J = 8.1, 7.5 Hz, 2 H), 5.83 (d , J = 2.9 Hz, 1 H), 5.47 (t , J = 5.5 Hz, 1 H), 4.36 (d , J = 7.2 Hz, 2 H), 4.20 (t , J = 7.2 Hz, 1 H), 3.61 (dt , J = 5.9, 5.5 Hz, 2 H), 2.93 (t , J = 5.9 Hz, 2 H), 2.23 (s, 3 H), 2.26 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$): 187.72, 156.61, 144.15, 141.43, 134.78, 129.17, 128.40, 127.78, 127.16, 125.26, 120.09, 113.02, 66.83, 47.41, 39.49, 36.22, 14.66, 13.20. HRMS (DART) m/z: [M+H] calcd. for $C_{24}H_{25}N_2O_3^+$, 389.18597; found 389.18756.

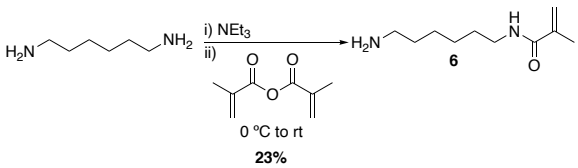


Fmoc-protected BODIPY ester **4**. Compound **3** (184 mg, 0.550 mmol, 1.00 equiv) and ethyl 3-(1H-pyrrol-2-yl)propanoate[4] (100 mg, 0.60 mmol, 1.10 equiv) were added to a flame-dried flask and dissolved in dichloromethane (6 mL) then cooled to 0 ºC. Phosphoryl chloride (0.10 mL, 1.1 mmol, 2.0 equiv) was added to the reaction flask and the mixture was stirred 1 hour at 0 ºC. The reaction was warmed to room temperature then heated to 40 ºC and stirred for 20 hours. After cooling to room temperature, diisopropylethylamine (DIEA, 0.43 mL, 2.47 mmol, 4.50 equiv) was added and the reactions as stirred for 15 minutes. Boron trifluoride diethyl etherate (0.310 mL,

2.47 mmol, 4.50 equiv) was then added and the reaction mixture stirred for 3 hours at room temperature, at which time the reaction mixture was concentrated to give a crude black oil that solidified upon standing. The product was purified via silica gel column chromatography (0-5% ethyl acetate in dichloromethane) to give **4** as a sticky red solid (99 mg, 31%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.77 (d, *J* = 7.6 Hz, 2 H), 7.55 (d, *J* = 7.5 Hz, 2 H), 7.41 (dd, *J* = 7.6, 7.5 Hz, 2 H), 7.31 (m, 2 H), 7.09 (d, *J* = 4.2 Hz, 1 H), 6.25 (d, *J* = 4.2 Hz, 1 H), 6.12 (s, 1 H), 5.04 (brs, 1 H), 4.43 (d, *J* = 6.9 Hz, 1 H), 4.43 (d, *J* = 6.9 Hz, 1 H), 4.20 (t, *J* = 6.9 Hz, 1 H), 4.15 (q, *J* = 7.2 Hz, 2 H), 3.48 (td, *J* = 7.5, 7.1 Hz, 2 H), 3.28 (t, *J* = 7.6 Hz, 2 H), 3.14 (t, *J* = 7.1 Hz, 2 H), 2.73 (t, *J* = 7.6 Hz, 3 H), 2.56 (s, 3 H), 2.43 (s, 3 H), 1.25 (t, *J* = 7.2 Hz, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$): 172.68, 158.85, 156.46, 156.39, 143.91, 143.27, 141.49, 141.14, 134.52, 132.91, 127.89, 127.19, 125.69, 125.16, 123.21, 120.16, 116.59, 66.95, 60.68, 47.37, 42.97, 33.58, 30.03, 23.98, 16.22, 14.98, 14.37. HRMS (DART) m/z: $[M-F]^+$ calcd. for $C_{33}H_{34}BFN_3O_4^+$, 566.26264; found 566.26456.

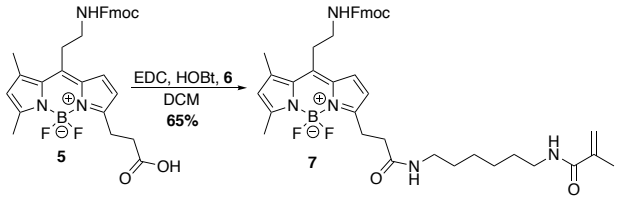


Fmoc-protected BODIPY acid **5**. BODIPY ester **4** (165 mg, 0.284 mmol, 1.00 equiv) was added to a flame-dried flask equipped with a reflux condenser and purged with N_2 for 1 hour. Tetrahydrofuran (14.2 mL) was added to the flask while a solution of 2 M hydrochloric acid in deionized water (2.84 mL) was sparged with N_2 for 30 minutes. The sparged 2 M hydrochloric acid solution was then added to the reaction flask and the mixture refluxed and stirred for 16 hours before cooling to room temperature. The reaction was quenched with the addition of deionized water (10 mL) and extracted with dichloromethane (3 x 10 mL). The combined organic layers were washed with brine (1 x 10 mL), dried over magnesium sulfate, and concentrated to give a brownish red residue. Purification of the desired product via silica gel column chromatography (10-20% ethyl acetate in dichloromethane with 0.1% acetic acid) gave **5** as a sticky red solid (130 mg, 83%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.76 (d , *J* = 7.6 Hz, 2 H), 7.55 (d, *J* = 7.5 Hz, 2 H), 7.40 (dd, *J* = 7.6, 7.4 Hz, 2 H), 7.30 (dd, *J* = 7.5, 7.4 Hz, 2 H), 7.07 (d, *J* = 4.1, 1 H), 6.25 (d, *J* = 4.1, 1 H), 6.12 (s, 1 H), 5.06 (t, *J* = 6.4, 1 H), 4.43 (d, *J* = 6.9, 2 H), 4.19 (t, *J* = 6.9, 1 H), 3.49 (td, *J* = 7.6, 6.4, 2 H), 3.28 (t, *J* = 7.6, 2 H), 3.13 (t, *J* = 7.6, 2 H), 2.79 (t, *J* = 7.6, 2 H), 2.56 (s, 3 H), 2.43 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$): 177.43, 159.13, 156.50, 155.61, 143.89, 143.51, 141.47, 141.26, 134.52, 133.02, 127.89, 127.19, 125.60, 125.16, 123.32, 120.16, 116.47, 66.95, 47.33, 42.93, 33.04, 30.00, 23.68, 16.23, 15.01. HRMS (DART) m/z: [M-H] calcd. for $C_{31}H_{29}BF_2N_3O_4^-$, 556.22247; found 556.22017.

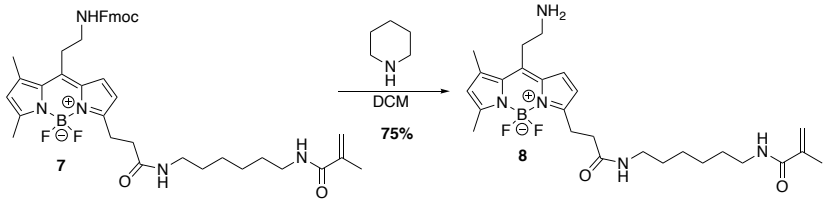


Methacrylamide amine **6**. This procedure was adapted from a previous report.[12] 1,6-Hexanediamine (2.00 g, 17.2 mmol, 1.50 equiv) was dissolved in dichloromethane (6 mL) in a flame dried flask then cooled to 0 ºC. Triethylamine (1.60 mL, 11.5 mmol, 1.00 equiv) was added dropwise to the solution followed by the dropwise addition of methacrylic anhydride (1.70 mL,

11.5 mmol, 1.00 equiv). The reaction was stirred at 0 ºC for 1 hour. The now milky white reaction mixture and was warmed to room temperature and stirred for an additional 16 hours. The reaction was quenched with the addition of deionized water (20 mL) and extracted with dichloromethane (3 x 25 mL). The combined organic layers were washed with brine (1 x 20 mL), dried over magnesium sulfate, and then concentrated to give a white, oily residue. Purification of the crude reaction mixture via silica gel column chromatography (10% methanol in ammonium saturated dichloromethane) afforded **6** as a white gummy solid (495 mg, 23%). Characterization of the compound was consistent with previous reports.[12]

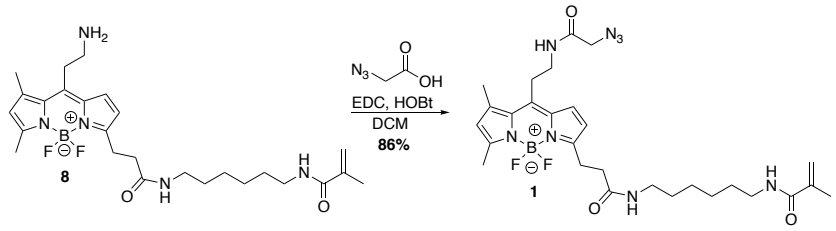


Fmoc-protected BODIPY methacrylamide **7**. Compound **5** (111 mg, 0.199 mmol 1.00 equiv), compound **5** (72 mg, 0.40 mmol, 2.0 equiv), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 54 mg, 0.28 mmol, 1.4 equiv), and hydroxybenzotriazole (HOBt, 33 mg, 0.25 mmol, 1.2 equiv) were added to a flame-dried flask and then suspended in dichloromethane (13 mL). The reaction flask was briefly sonicated to promote dissolution of N-(6-aminohexyl)methacrylamide, and then the mixture was stirred for 23 hours at room temperature. The reaction mixture was cooled to room temperature then extracted with DCM (3 x 10 mL). The combined organic layers were washed with brine (1 x 50 ml), dried with magnesium sulfate and concentrated. The product was purified via silica gel column chromatography (25-75% ethyl acetate in dichloromethane) to give **7** as an orange solid (94 mg, 65%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.76 (d, *J* = 7.5 Hz, 2 H), 7.56 (d, *J* = 7.5 Hz, 2 H), 7.40 (dd, *J* = 7.5, 7.5 Hz, 2 H), 7.30 (ddd, *J* = 7.5, 7.5, 1.1 Hz, 2 H), 7.13 (d, *J* = 4.2 Hz, 1 H), 6.26 (d, *J* = 4.2 Hz, 2 H), 6.14 (s, 1 H), 5.95 (t, *J* = 5.3 Hz, 1 H), 5.73 (t, *J* = 5.2 Hz, 1 H), 5.69 (brs, 1 H), 5.60 (t, *J* = 6.7 Hz, 1 H), 5.31-5.30 (m, 1 H), 4.41 (d, *J* = 7.0 Hz, 2 H), 4.19 (t, *J* = 7.0 Hz, 1 H), 3.47 (td, *J* = 7.0, 6.7 Hz, 2 H), 3.29-3.23 (overlap, 4 H), 3.20-3.15 (overlap, 4 H), 2.60 (t, *J* = 7.3 Hz, 2 H), 2.56 (s, 3 H), 2.48 (s, 3 H), 1.96 (brs, 3 H), 1.49 (tt, *J* = 7.3, 7.3 Hz, 2 H), 1.38 (tt, *J* = 7.1, 7.1 Hz, 2 H), 1.31-1.19 (overlap, 4 H). ^{13}C NMR (125 MHz, $CDCl_3$): 171.88, 168.73, 158.76, 156.58, 156.15, 143.95, 143.70, 141.45, 141.20, 140.29, 134.57, 132.93, 127.86, 127.16, 125.75, 125.22, 123.19, 120.13, 119.50, 117.28, 66.90, 47.35, 43.10, 39.65, 39.19, 36.14, 30.24, 29.67, 29.58, 26.52, 26.22, 24.93, 18.87, 16.18, 14.98. HRMS (DART) m/z: [M-F] calcd. for $C_{41}H_{48}BFN_5O_4^+$, 704.37779; found 704.38107.

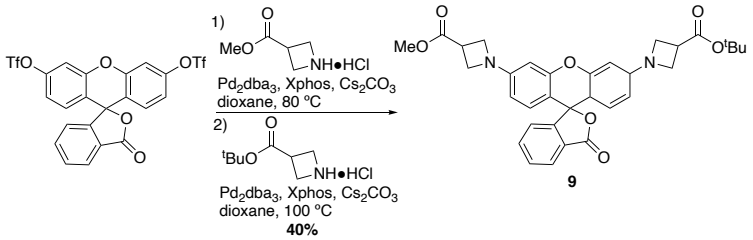


BODIPY methacrylamide **8**. Compound **9** (59 mg, 0.037 mmol, 1.0 equiv) was dissolved in dichloromethane (6 mL) in a flame-dried flask. Piperidine (75 µL, 0.37 mmol, 10 equiv) was added dropwise and the mixture stirred for 18 hours at room temperature. The product was diluted with 50 mL of dichloromethane then washed with 0.5 M aqueous sodium hydroxide (3 x 25 mL) before drying over magnesium sulfate. The product was purified via silica gel column chromatography (15% methanol in dichloromethane) to give **8** as a dark red, sticky solid (30 mg, 75%). ^{1}H NMR

(500 MHz, MeOD-d_4): δ 7.30 (d, J = 4.1 Hz, 1 H), 6.36 (d, J = 4.1 Hz, 1 H), 6.27 (s, 1 H), 5.67 (brs, 1 H), 5.36-5.35 (m, 1 H), 3.25-3.16 (overlap, 8 H), 2.59 (t, J = 7.7 Hz, 2 H), 2.51 (s, 3 H), 2.48 (s, 3 H), 1.93 (brs, 3 H), 1.56-1.46 (overlap, 4 H), 1.36-1.31 (overlap, 6 H). ^{13}C NMR (125 MHz, MeOD-d_4): 174.56, 171.27, 160.32, 157.78, 145.04, 141.84, 141.50, 135.46, 133.94, 126.70, 124.13, 120.17, 117.53, 43.75, 40.56, 40.28, 35.97, 30.77, 30.37, 30.28, 27.68, 27.57, 23.84, 23.11, 18.83, 16.05.

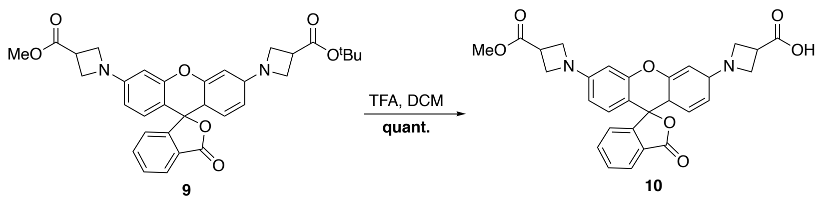


Trifunctional BODIPY probe **1**. Compound **8** (10 mg, 0.020 mmol, 1.0 equiv), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 6 mg, 0.03 mmol, 1.4 equiv), and hydroxybenzotriazole (HOBt, 4 mg, 0.024 mmol, 1.2 equiv) were added to a flame-dried flask and then suspended in dichloromethane (5 mL). The solution was briefly sonicated to promote dissolution of the fluorophore, and then azidoacetic acid (20 μL, 0.27 mmol, 14 equiv) was added dropwise and the mixture was stirred for 19 hours at room temperature. The reaction mixture was concentrated then the product was purified via silica gel column chromatography (0-6% methanol in dichloromethane) to give **1** as a red orange solid (10 mg, 86%). The compound was purified via reverse phase HPLC prior to cells studies (50-100% acetonitrile in water). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.18 (t, J = 6.3 Hz, 1 H), 7.12 (d, J = 4.1 Hz, 1 H), 6.29 (d, J = 4.1 Hz, 1 H), 6.15 (s, 1 H), 6.00 (t, J = 6.4 Hz, 1 H), 5.78 (t, J = 5.1 Hz, 1 H), 5.69 (s, 1 H), 5.33 (brs, 1 H), 3.94 (s, 2 H), 3.57 (td, J = 7.9, 6.4 Hz, 2 H), 3.28-3.24 (overlap, 4 H), 3.20-3.15 (overlap, 4 H), 2.62 (t, J = 7.3 Hz, 2 H), 2.56 (2, 3 H), 2.50 (s, 3 H), 1.97 (brs, 3 H), 1.49 (tt, J = 7.2, 7.2 Hz, 2 H), 1.35 (tt, J = 7.2, 7.1 Hz, 4 H), 1.31-1.19 (overlap, 4 H). ^{13}C NMR (125 MHz, $CDCl_3$): 171.90, 168.81, 167.30, 159.04, 156.22, 143.83, 140.77, 140.26, 134.56, 132.92, 125.62, 123.28, 119.57, 117.31, 52.80, 41.44, 39.71, 39.20, 36.11, 29.70, 29.62, 29.53, 26.58, 26.23, 24.93, 18.85, 16.16, 15.00. HRMS (DART) m/z: [M+H] calcd. for $C_{28}H_{40}BF_2N_8O_3^+$, 585.32790; found 585.32552.

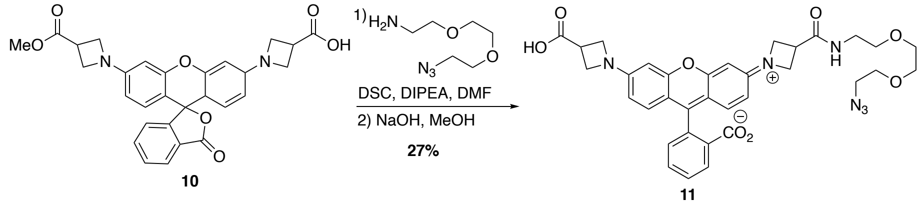


JF_{549} methyl t-butyl ester **9**. An oven dried μ-wave vial containing a magnetic stir bar was charged with fluorescein ditriflate (299 mg, 0.5 mmol, 1.0 equiv), methyl azetidine-3-carboxylate hydrochloride (83.3 mg, 0.55 mmol, 1.1 equiv), Tris(dibenzylideneacetone)dipalladium (Pd_2dba_3, 22.9 mg, 0.025 mmol, 0.05 equiv), dicylcohexyl[2', 4', 6'-tris(propan-2-yl)[1, 1'-biphenyl]-2-yl]phosphane (Xphos, 43.4 mg, 0.075 mmol, 0.15 equiv), and cesium carbonante (Cs_2CO_3, 454.3 mg, 1.4 mmol, 2.8 equiv). After sealing the vial, it was flushed with Argon (3x) and suspended in degassed dioxane (10 mL). The reaction mixture was heated at 80 ºC for 3.5 hours, cooled down to room temperature, filtered through a pad of celite (~5 g) using EtOAc, concentrated under reduced pressure, and purified by flash chromatography over silica gel (0–50% EtOAc/hexanes)

to obtain 149 mg of a faint-green foamy solid. This solid (120 mg, 213 μmol, 1.00 eq) was added to an oven dried μ-wave vial containing a magnetic stir bar and charged with tert-butyl azetidine-3-carboxylate hydrochloride (62.0 mg, 319 μmol, 1.50 eq), Pd_2dba_3 (20.0 mg, 21.0 μmol, 0.100 eq), Xphos (37 mg, 64 μmol, 0.30 eq), and Cs_2CO_3 (194 mg, 596 μmol, 2.80 eq). After sealing the vial, it was flushed with Argon (3x) and suspended in degassed dioxane (10 mL). The reaction mixture was heated at 100 ºC for 6 h, cooled down to room temperature, filtered through a pad of celite (~ 5g) using MeOH, concentrated under reduced pressure, and purified by flash chromatography over silica gel (0–8% ammoniated MeOH/DCM) to obtain a dark red foamy solid (114 mg, 80.4% yield) in 40% yield over two steps. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, J = 7.6 Hz, 1H), 7.64 (td, J = 7.4, 1.3 Hz, 1H), 7.58 (td, J = 7.4, 1.1 Hz, 1H), 7.15 (d, J = 7.6 Hz, 1H), 6.60 (dd, J = 8.6, 1.6 Hz, 2H), 6.24 (t, J = 2.0 Hz, 2H), 6.12 (dd, J = 8.6, 2.3 Hz, 2H), 4.18 – 3.98 (m, 8H), 3.75 (s, 3H), 3.57 (tt, J = 8.6, 6.1 Hz, 1H), 3.47 (tt, J = 8.6, 6.1 Hz, 1H), 1.47 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.06, 171.87, 169.61, 153.21, 153.07, 153.01, 134.65, 129.55, 129.25, 129.22, 127.76, 125.37, 124.44, 109.03, 108.77, 108.20, 98.07, 97.95, 81.64, 54.44, 52.46, 34.39, 33.38, 28.17. HRMS (ESI) calculated for $C_{33}H_{35}N_2O_7$ $[M+H]^+$ = 569.2282, found 569.2281.

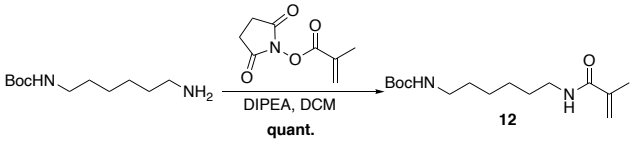


JF$_{549}$ methyl ester acid **10**. Trifluoroacetic acid (TFA, 0.5 mL, 6.5 equiv) was added to a solution of **9** (23.0 mg, 40.3 μmol, 1.00 equiv) in DCM (4 mL). This red-orange solution was stirred at room temperature overnight. Upon completion, excess TFA and DCM were removed under reduced pressure to obtain TFA salt of **10** in quantitative yield (25.2 mg) as a red solid which was used without further purification. ^{1}H NMR (400 MHz, MeOD-d$_4$+0.03%TMS) δ 8.35 (dd, J = 7.7, 1.5 Hz, 1H), 7.88 – 7.78 (m, 2H), 7.40 (dd, J = 7.4, 1.5 Hz, 1H), 7.13 (s, 1H), 7.11 (s, 1H), 6.67 (t, J = 2.4 Hz, 1H), 6.65 (t, J = 2.4 Hz, 1H), 6.57 (dd, J = 2.2, 1.2 Hz, 2H), 4.51 – 4.43 (m, 4H), 4.42 – 4.34 (m, 4H), 3.79 (s, 3H), 3.78 – 3.68 (m, 2H). ^{13}C NMR (101 MHz, MeOD-d$_4$+0.03%TMS) δ 175.17, 174.03, 167.96, 162.58, 158.94, 158.88, 157.95, 157.87, 135.33, 133.92, 132.60, 132.54, 132.51, 132.17, 131.52, 131.43, 115.45, 115.41, 113.81, 113.72, 95.58, 95.53, 55.31, 55.19, 53.01, 33.80, 33.75. ^{19}F NMR (376 MHz, MeOD-d$_4$+0.03%TMS) δ -77.19. HRMS (ESI) calculated for $C_{29}H_{25}N_2O_7$ $[M+H]^+$ = 513.1656, found 513.1661.

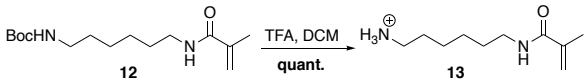


JF$_{549}$ methyl ester azide **11**. To a solution of **10** (25.2 mg, 40.0 μmol, 1.00 equiv) in DMF (2 mL) was added N,N-diisopropylethylamine (DIPEA, 52.0 mg, 70.0 μL, 403 μmol, 10.0 equiv) and N, N'-disuccinimidyl carbonate (DSC, 25.8 mg, 101 μmol, 2.50 equiv) and the reaction mixture was stirred at room temperature for 1hour. N_3-PEG$_2$-NH_2 was then added (21.0 mg, 121 μmol, 3.00 equiv) and the reaction mixture was stirred overnight. The crude reaction was removed under

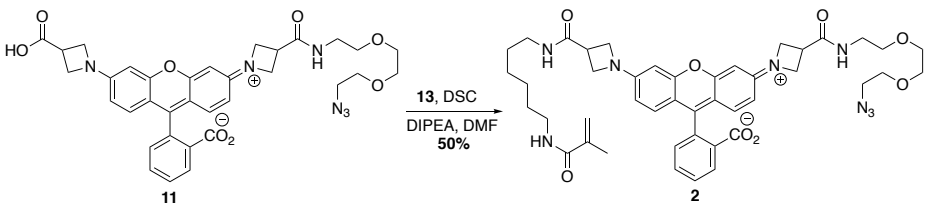
reduced pressure, redissolved in methanol (5 mL) then charged with 2M NaOH (600 μL) and stirred overnight. The reaction mixture was neutralized with 6M HCl, filtered, purified by HPLC (20–40% MeCN/H_2O over 18 mins w/ 0.1% TFA) and lyophilized to obtain TFA salt of **11** as a red solid in 27% yield over two steps. ^{1}H NMR (400 MHz, MeOD-d_4+0.03%TMS) δ 8.33 (dd, *J* = 7.7, 1.5 Hz, 1H), 8.25 (t, *J* = 5.6 Hz, 1H), 7.82 (dtd, *J* = 20.8, 7.6, 1.4 Hz, 2H), 7.39 (dd, *J* = 7.4, 1.5 Hz, 1H), 7.11 (d, *J* = 9.2 Hz, 2H), 6.66 (dd, *J* = 9.2, 2.2 Hz, 2H), 6.61 (dd, *J* = 5.7, 2.2 Hz, 2H), 4.51 – 4.31 (m, 8H), 3.79 – 3.62 (m, 8H), 3.59 (t, *J* = 5.4 Hz, 2H), 3.44 (q, *J* = 5.2 Hz, 2H), 3.38 (t, *J* = 4.9 Hz, 2H). ^{13}C NMR (101 MHz, MeOD-d_4+0.03%TMS) δ 175.68, 174.08, 173.82, 168.65, 158.78, 158.76, 157.83, 157.76, 135.75, 133.63, 133.08, 132.49, 132.24, 131.40, 131.16, 115.23, 115.21, 113.54, 95.61, 95.52, 71.56, 71.34, 71.14, 70.49, 55.40, 55.28, 51.74, 40.69, 34.60, 34.08. ^{19}F NMR (376 MHz, MeOD-d_4+0.03%TMS) δ -76.98. HRMS (ESI) calculated for $C_{34}H_{35}N_6O_8$ $[M+H]^+$ = 655.2511, found 655.2510.



Boc protected methylacrylamide amine **12**. To a solution of tert-butyl (6-aminohexyl)carbamate (510 mg, 2.36 mmol, 1.00 equiv) and N,N-diisopropylethylamine (DIPEA, 742 mg, 5.75 mmol, 2.40 equiv) in DCM (15 mL) was added methacrylic acid NHS ester (455 mg, 2.48 mmol, 1.05 equiv). This solution was stirred overnight, concentrated under reduced pressure, and purified by flash chromatography over silica gel (0–50% EtOAc/hexanes) to obtain a white solid in quantitative yield.^{1}H NMR (400 MHz, $CDCl_3$) δ 6.01 (br s, 1H), 5.66 (t, *J* = 1.1 Hz, 1H), 5.28 (p, *J* = 1.5 Hz, 1H), 4.58 (br s, 1H), 3.27 (td, *J* = 7.1, 5.8 Hz, 2H), 3.08 (q, *J* = 6.0 Hz, 2H), 1.94 (t, *J* = 1.2 Hz, 3H), 1.55 – 1.43 (m, 4H), 1.41 (s, 9H), 1.32 (tt, *J* = 5.5, 2.6 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.56, 156.19, 140.31, 119.27, 79.15, 40.30, 39.43, 30.10, 29.53, 28.51, 26.38, 26.21, 18.82. HRMS (ESI) calculated for $C_{15}H_{28}N_2O_3$ $[M+H]^+$ = 285.2173, found 285.2177.



Methylacrylamide quaternary amine **12**. TFA (3 mL, 18 equiv) was added to a solution of tert-butyl (6-methacrylamidohexyl)carbamate (669 mg, 2.3 mmol, 1.00 equiv) in DCM (10 mL). This clear solution was stirred at room temperature for 1.5 hours and the reaction progress was monitored through TLC. Upon completion, excess TFA and DCM were removed under reduced pressure to obtain TFA salt of *N*-(6-aminohexyl)methacrylamide in quantitative yield as a sticky oil which was used without further purification.^{1}H NMR (400 MHz, MeOD-d_4+0.03%TMS) δ 5.67 (t, *J* = 1.2 Hz, 1H), 5.42 – 5.29 (m, 1H), 3.24 (t, *J* = 7.1 Hz, 2H), 2.92 (t, *J* = 7.7 Hz, 2H), 1.93 (t, *J* = 1.2 Hz, 3H), 1.71 – 1.61 (m, 2H), 1.57 (p, *J* = 7.1 Hz, 2H), 1.47 – 1.34 (m, 4H). ^{13}C NMR (101 MHz, MeOD-d_4+0.03%TMS) δ 171.29, 141.41, 120.24, 40.60, 40.29, 30.17, 28.44, 27.33, 26.98, 18.80. ^{19}F NMR (376 MHz, MeOD-d_4+0.03%TMS) δ -77.04. HRMS (ESI) calculated for $C_{10}H_{21}N_2O$ $[M]^+$ = 185.1648, found 185.1650.



JF_{549} methylacrylamide azide **2**. To a solution of **11** (8.2 mg, 11 µmol, 1.0 equiv) in DMF (2 mL) was added N,N-diisopropylethylamine (DIPEA, 14.0 mg, 18.0 µL, 107 µmol, 10.0 equiv) and N, N'-disuccinimidyl carbonate (DSC, 10.9 mg, 42.6 µmol, 4.00 equiv) and the reaction mixture was stirred at room temperature. After 1 hour, **13** was added (12.7 mg, 42.6 µmol, 4.00 equiv) and the reaction mixture was stirred overnight. DMF was removed under reduced pressure and the mixture was redissolved in acidified methanol (5 mL), filtered, and purified by HPLC (20−45% MeCN/H_2O over 18 mins w/ 0.1% TFA) and lyophilized to obtain the TFA salt of **2** as a red solid (5.0 mg) in 50 % yield. ^{1}H NMR (400 MHz, MeOD-d_4) δ 8.34 (dd, *J* = 7.7, 1.5 Hz, 1H), 8.25 (t, *J* = 5.6 Hz, 1H), 8.21 (t, *J* = 5.6 Hz, 1H), 7.82 (dtd, *J* = 21.3, 7.5, 1.4 Hz, 2H), 7.40 (dd, *J* = 7.4, 1.5 Hz, 1H), 7.11 (dd, *J* = 9.2, 1.0 Hz, 2H), 6.66 (dd, *J* = 9.2, 2.2 Hz, 2H), 6.61 (t, *J* = 2.0 Hz, 2H), 5.66 (t, *J* = 1.1 Hz, 1H), 5.35 (p, *J* = 1.5 Hz, 1H), 4.43 (t, *J* = 9.3 Hz, 4H), 4.37– 4.34 (m, 4H), 3.70 – 3.62 (m, 8H), 3.59 (t, *J* = 5.4 Hz, 2H), 3.46 – 3.41 (m, 2H), 3.38 (t, *J* = 4.9 Hz, 2H), 3.26 – 3.20 (m, 4H), 1.93 (t, *J* = 1.2 Hz, 3H), 1.54 (tt, *J* = 7.2, 3.5 Hz, 4H), 1.38 (p, *J* = 3.5 Hz, 4H). ^{13}C NMR (101 MHz, MeOD-d_4) δ 173.81, 173.58, 171.26, 167.94, 162.36, 158.94, 157.89, 141.50, 135.38, 133.88, 132.57, 132.48, 132.23, 131.47, 131.43, 120.15, 115.37, 113.69, 95.43, 71.56, 71.34, 71.15, 70.49, 55.29, 51.74, 40.70, 40.56, 40.43, 34.62, 34.59, 30.32, 30.23, 27.55, 27.54, 18.83. HRMS (ESI) calculated for $C_{44}H_{53}N_8O_8$ $[M+H]^+$ = 821.3981, found 821.3974.

Supporting Information References

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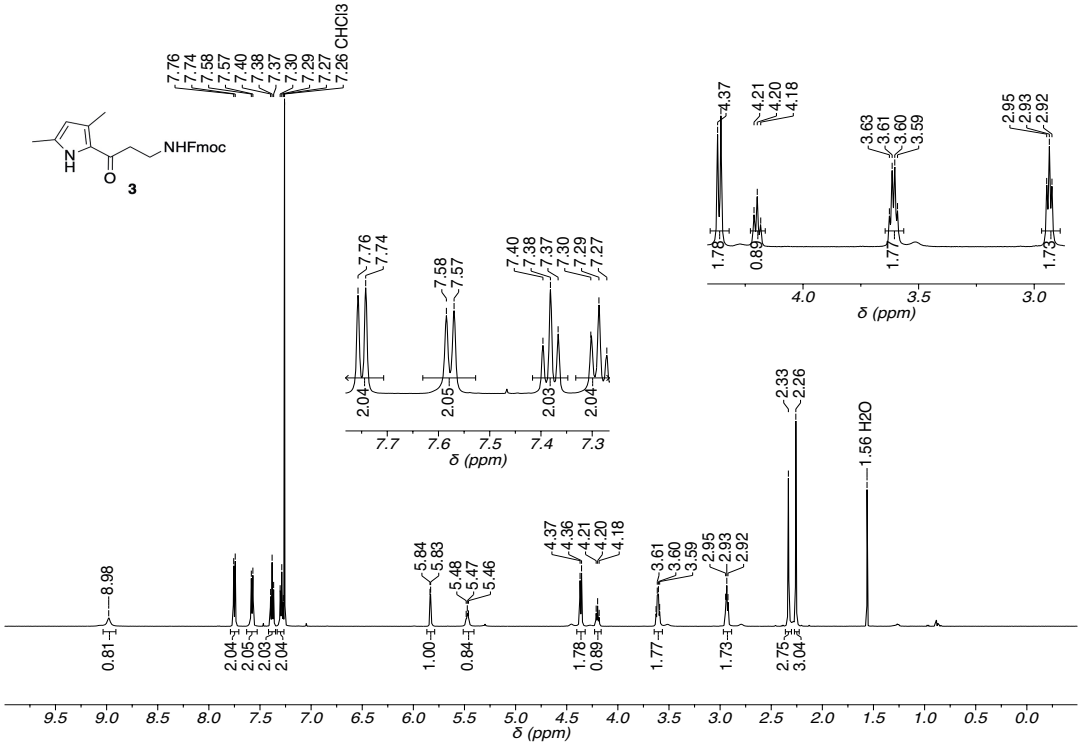
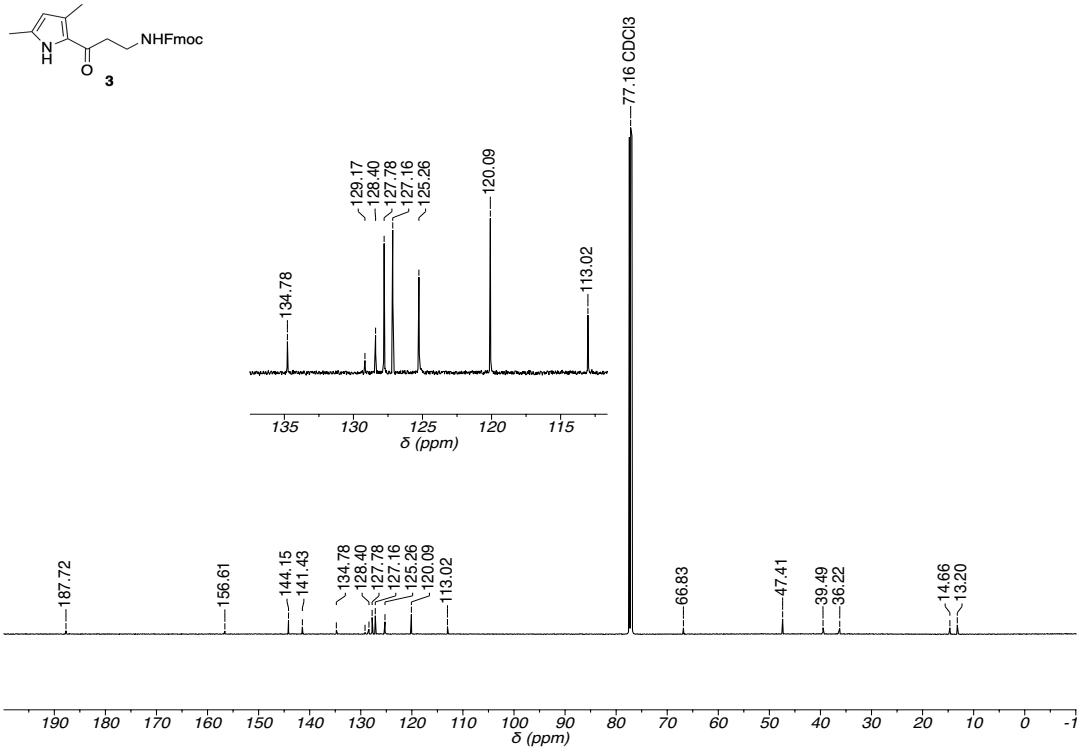
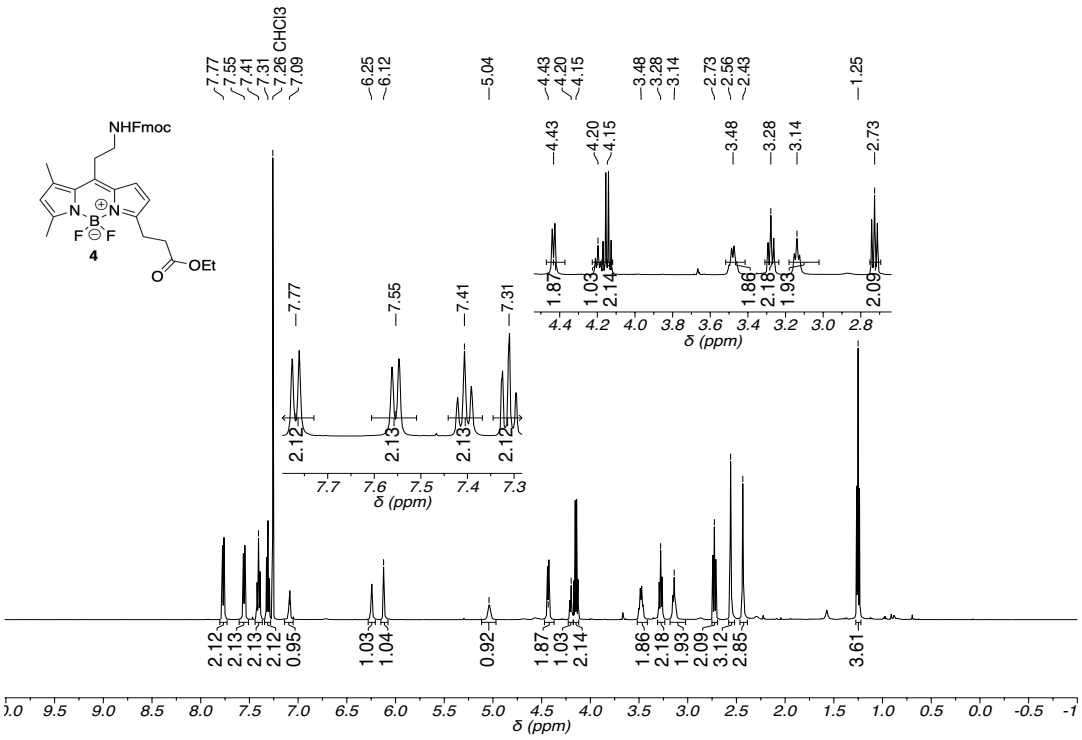
^{1}H and ^{13}C NMR Spectra



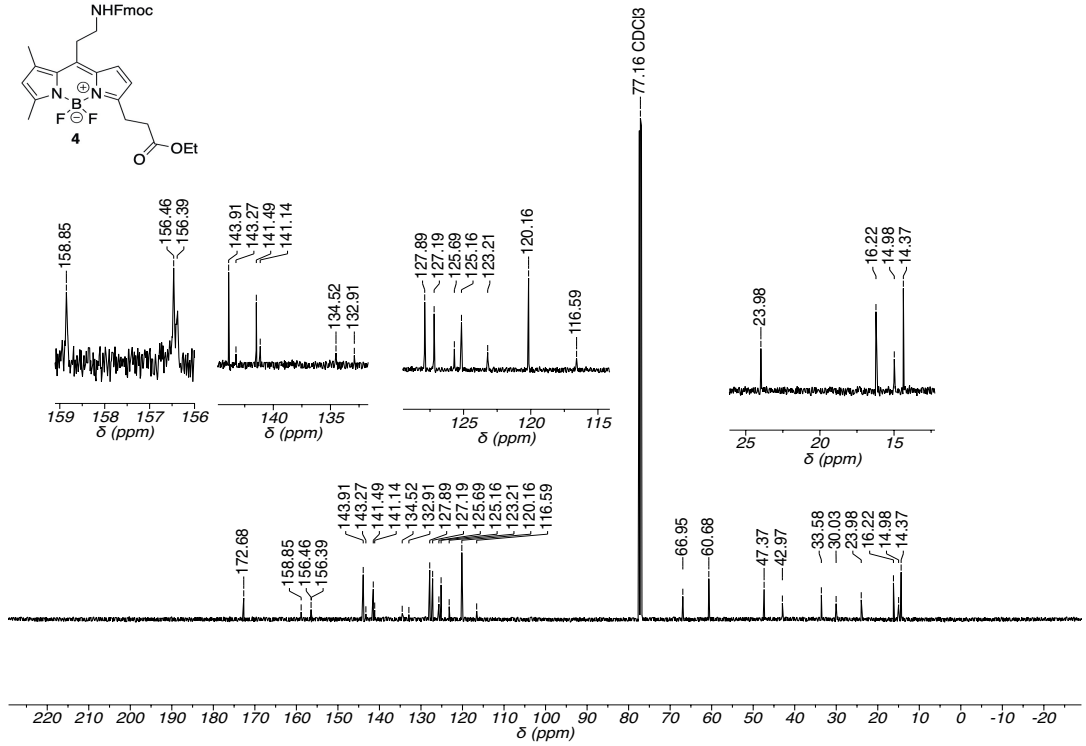
^{1}H NMR of compound **3** (500 MHz, $CDCl_3$).



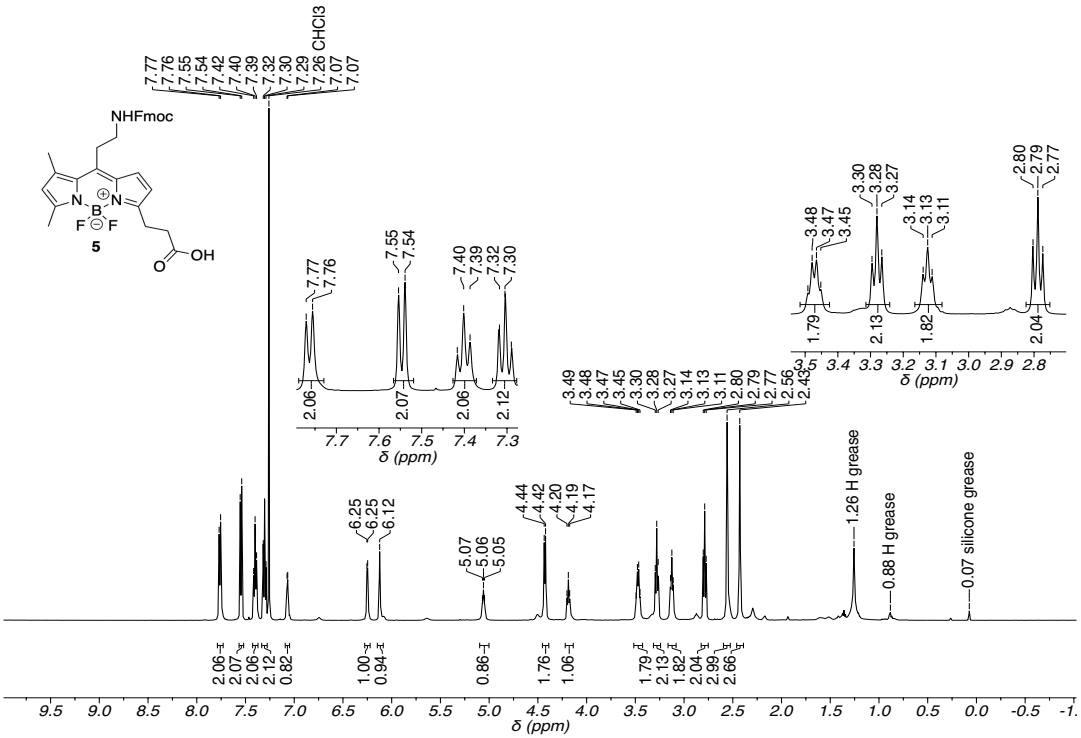
^{13}C NMR of compound **3** (125 MHz, $CDCl_3$).



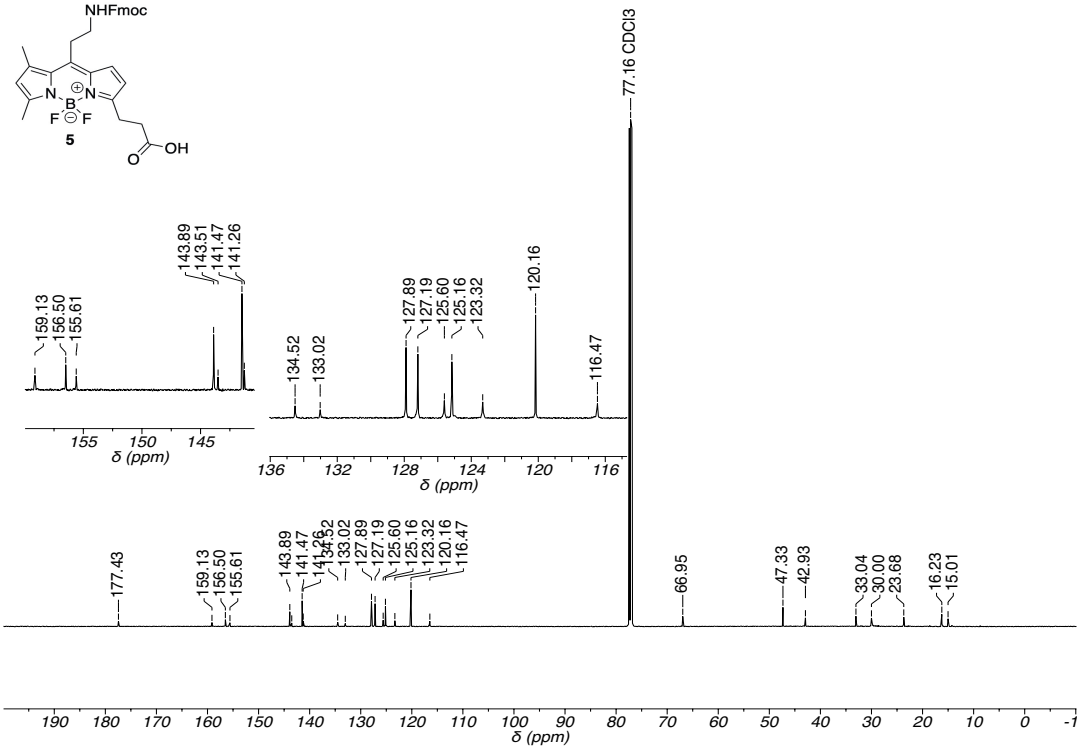
^{1}H NMR of compound **4** (500 MHz, $CDCl_3$).



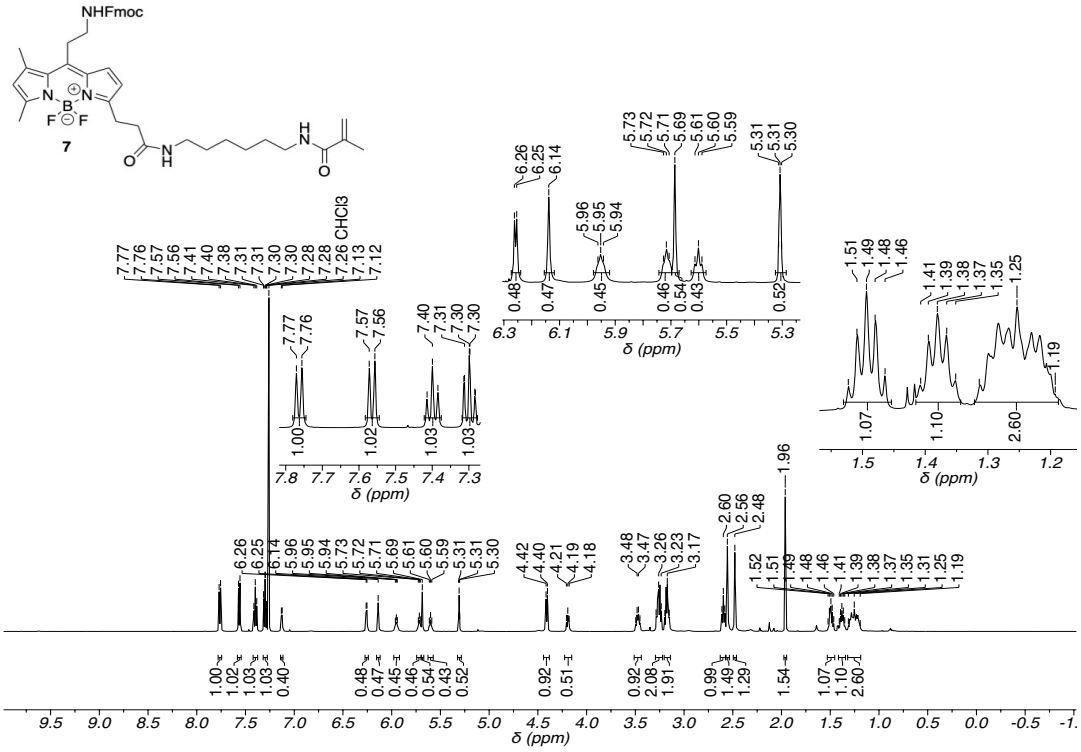
^{13}C NMR of compound **4** (125 MHz, $CDCl_3$).



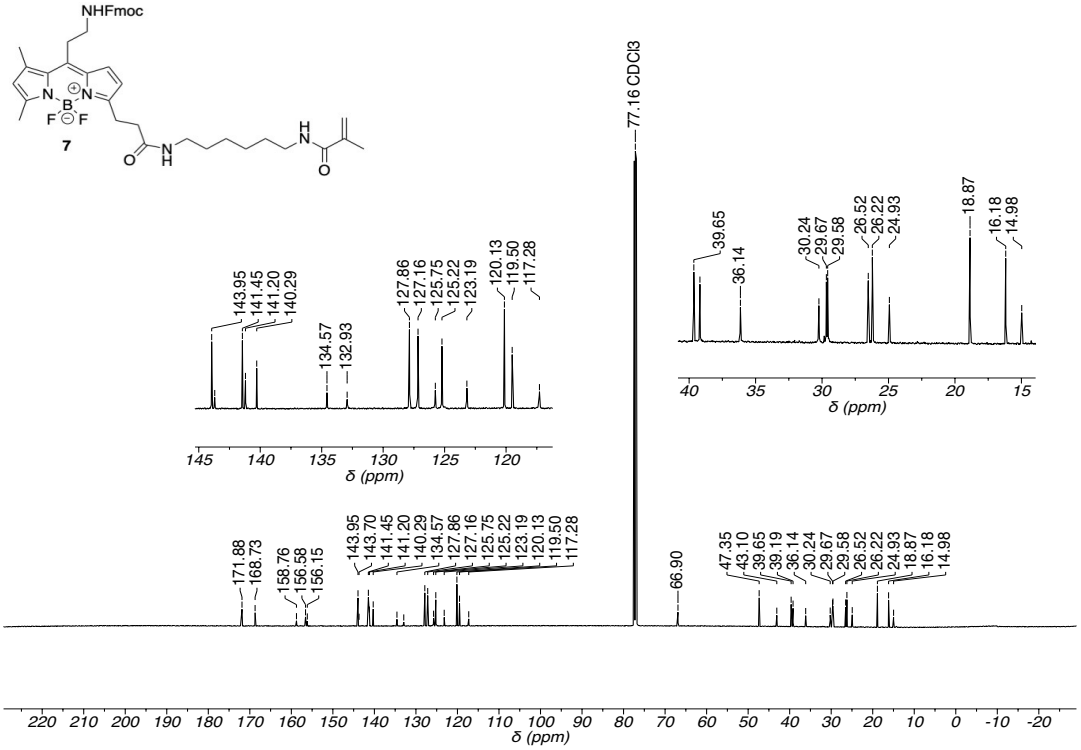
^{1}H NMR of compound **5** (500 MHz, $CDCl_3$).



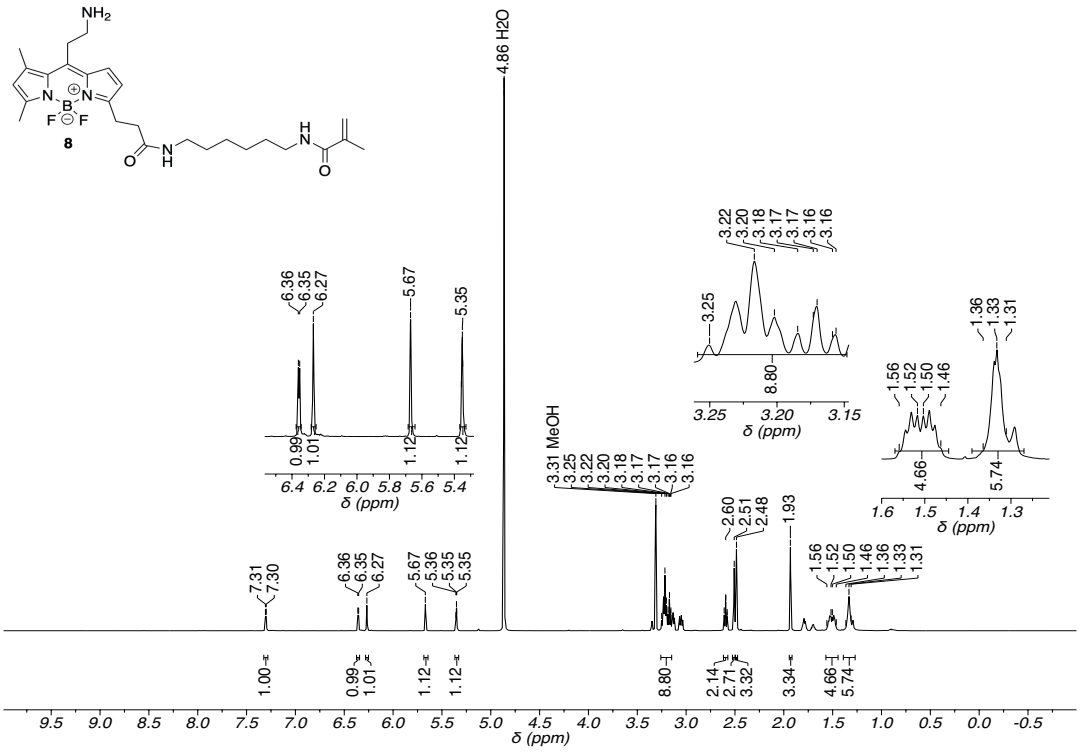
^{13}C NMR of compound **5** (125 MHz, $CDCl_3$).



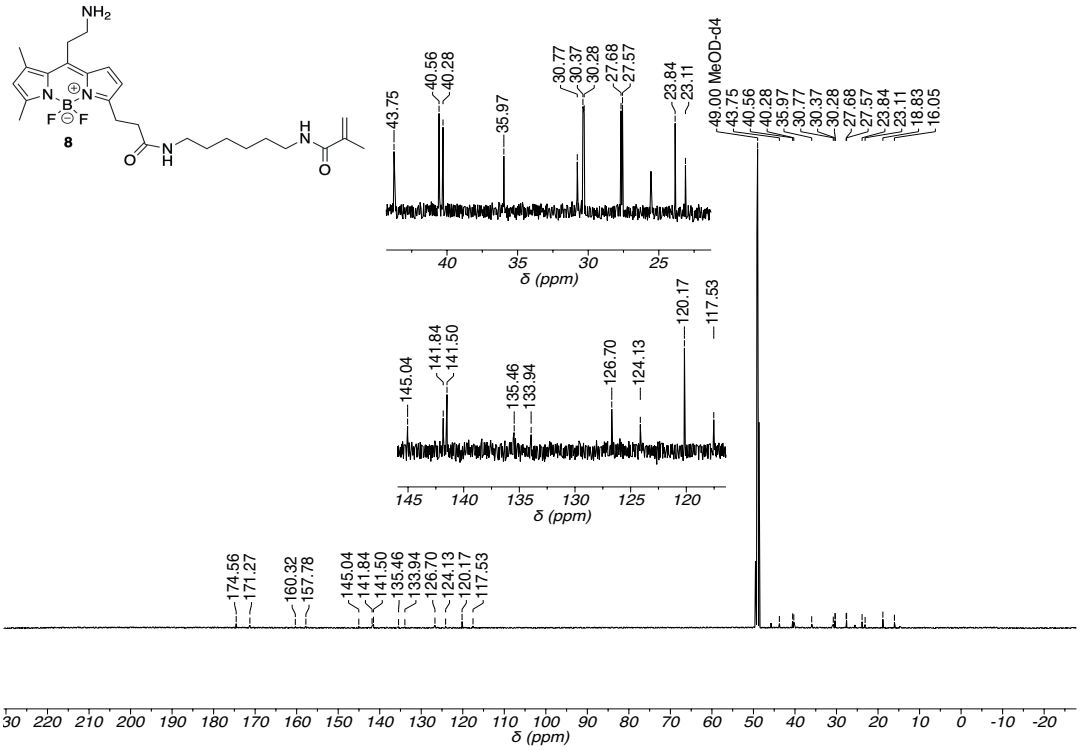
^{1}H NMR of compound **7** (500 MHz, $CDCl_3$).



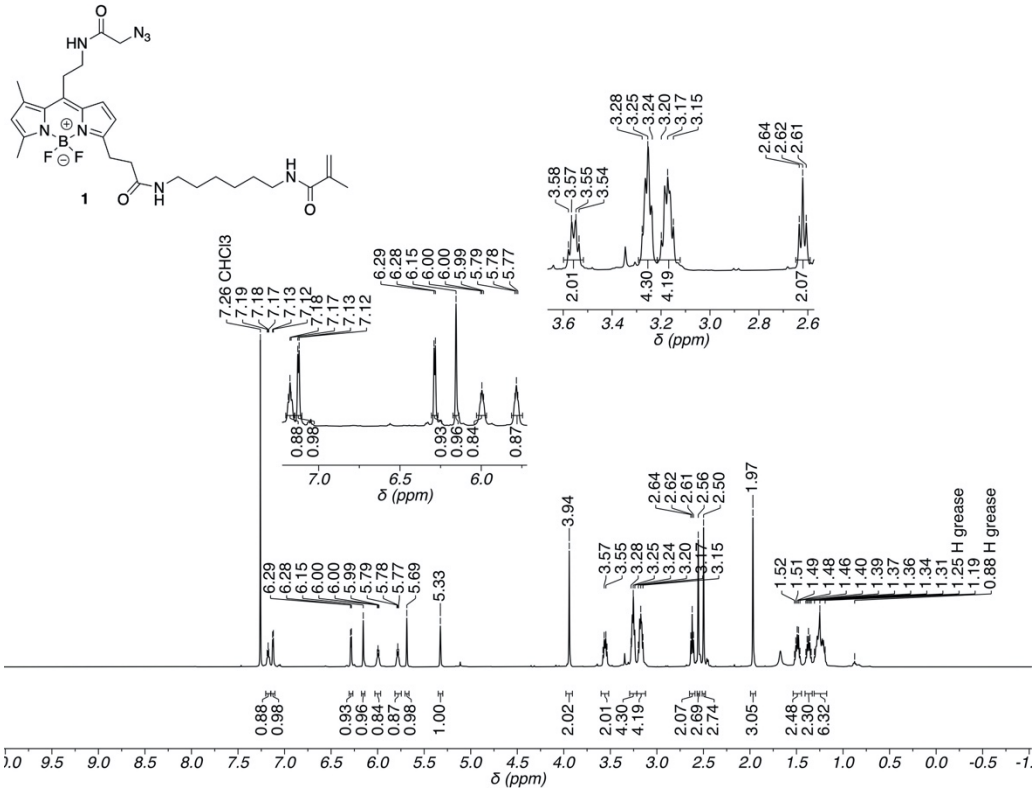
^{13}C NMR of compound **7** (125 MHz, $CDCl_3$).



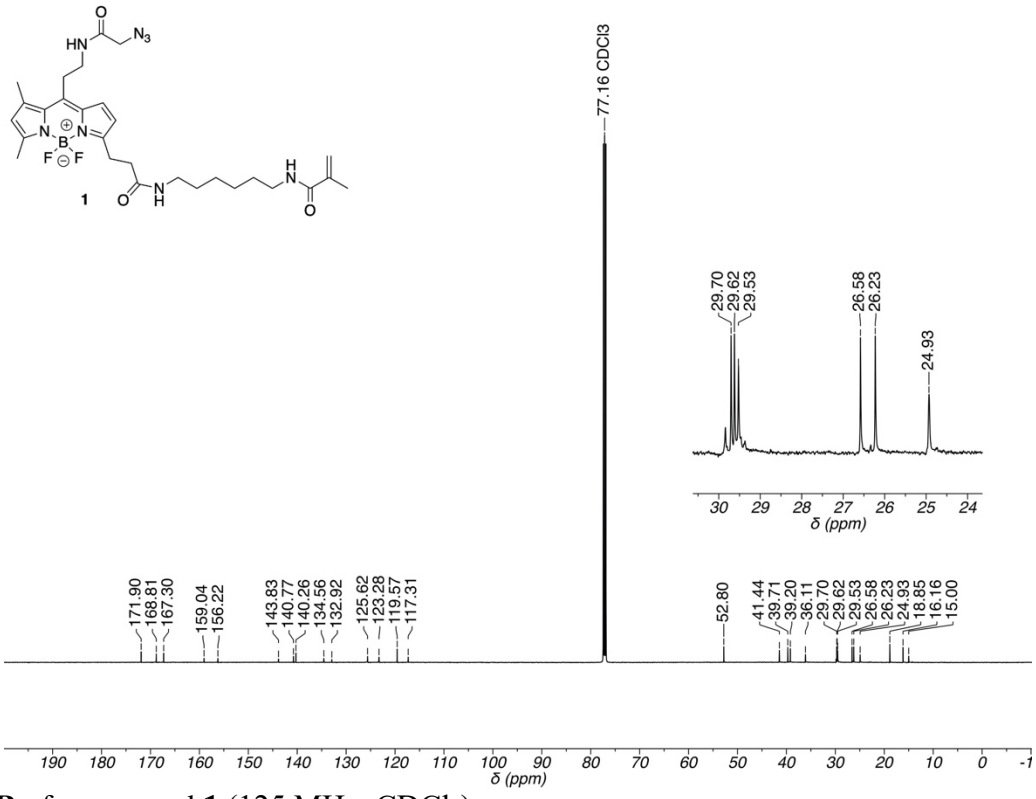
^{1}H NMR of compound **8** (500 MHz, MeOD-d_4).



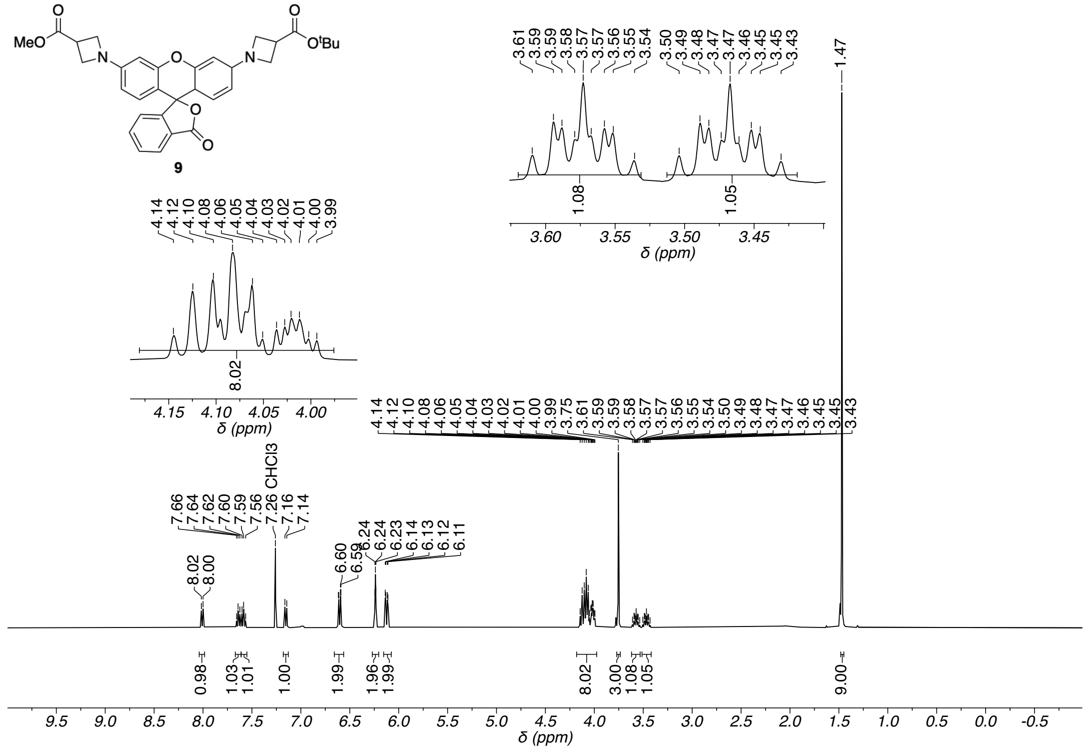
^{13}C NMR of compound **8** (125 MHz, MeOD-d_4).



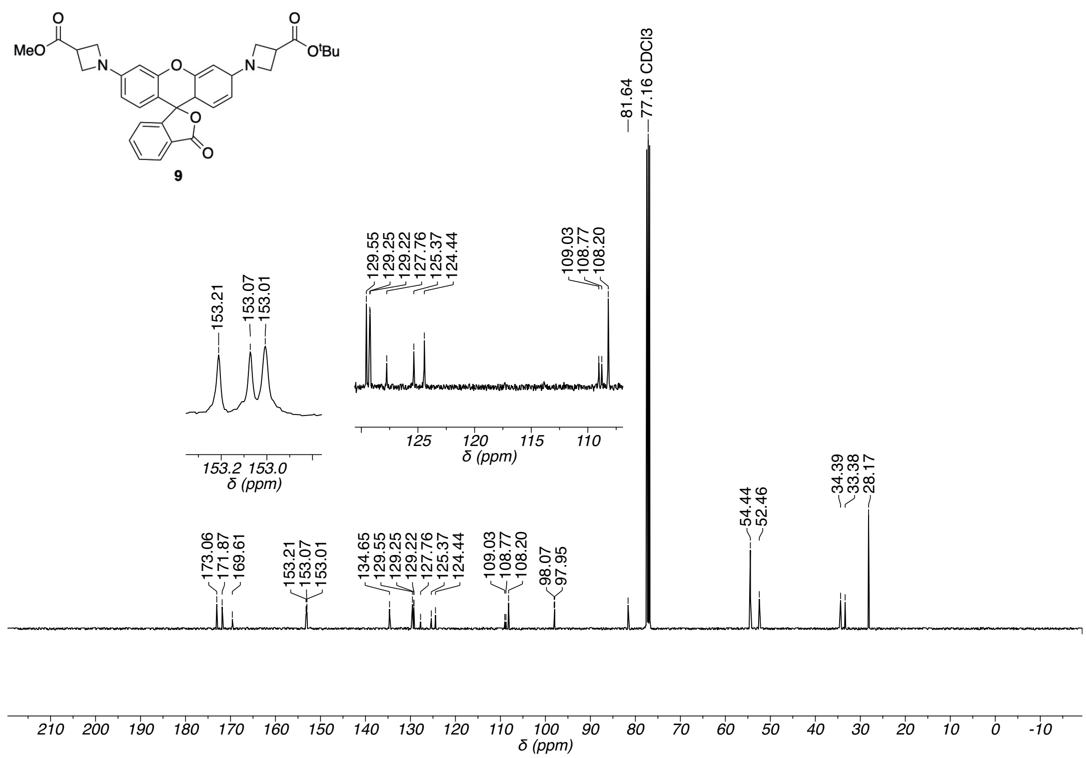
^{1}H NMR of compound **1** (500 MHz, $CDCl_3$).



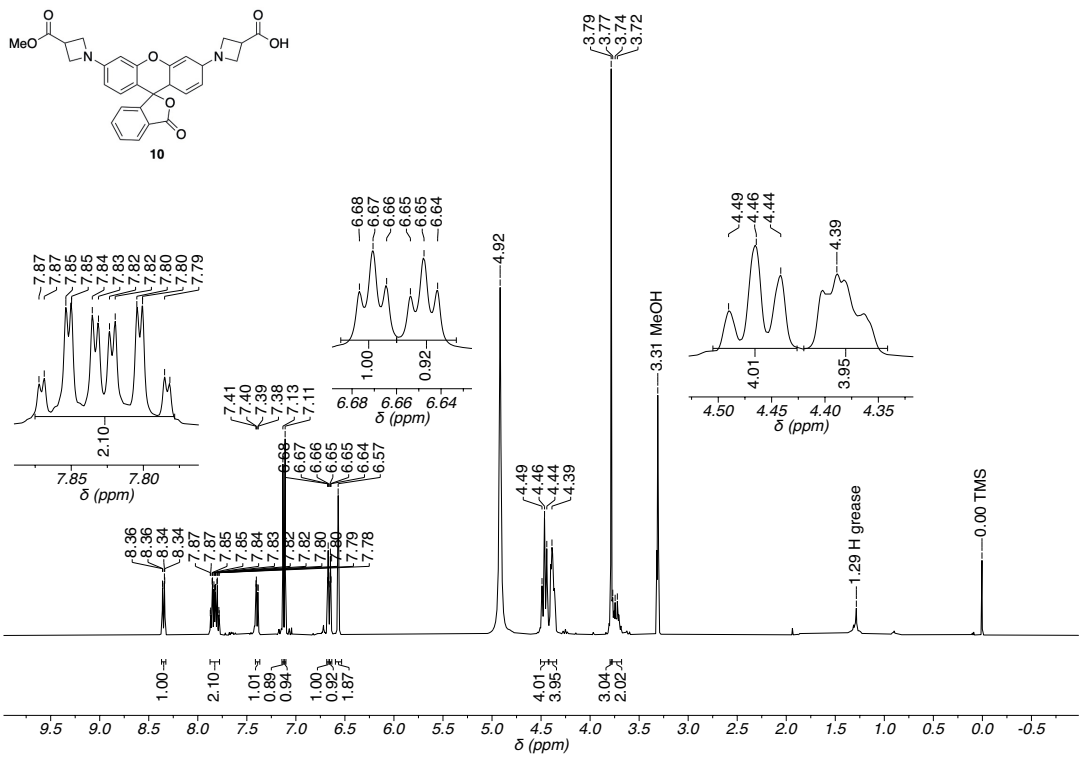
^{13}C NMR of compound **1** (125 MHz, $CDCl_3$).



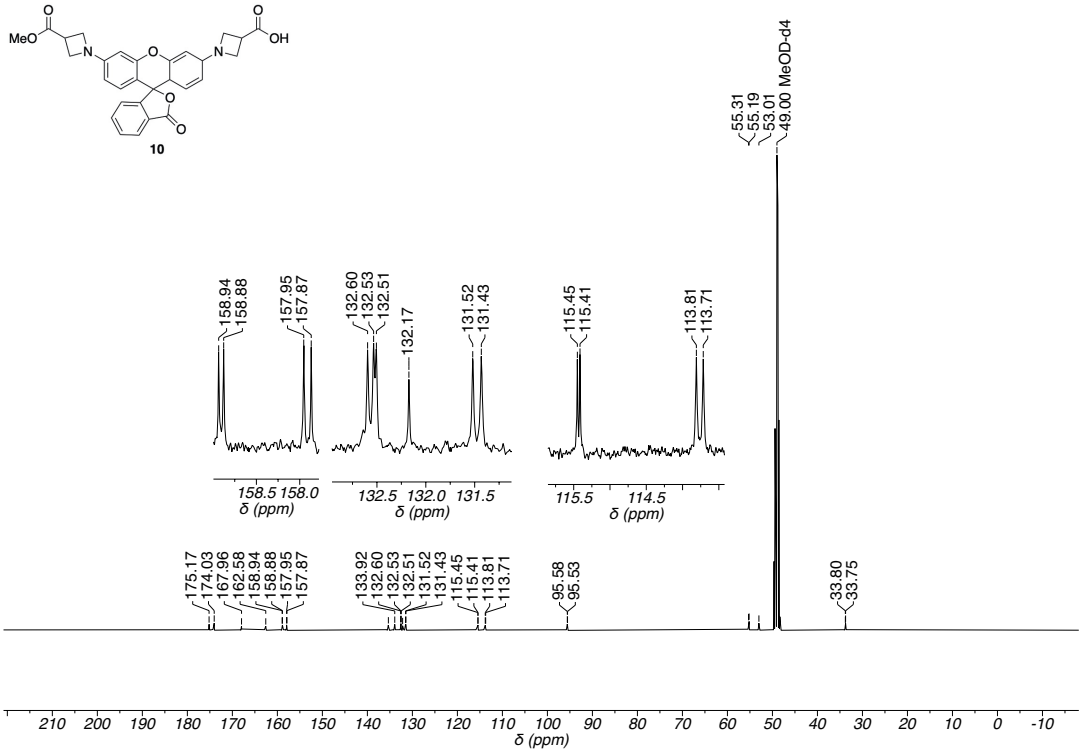
^{1}H NMR of compound **9** (400 MHz, $CDCl_3$).



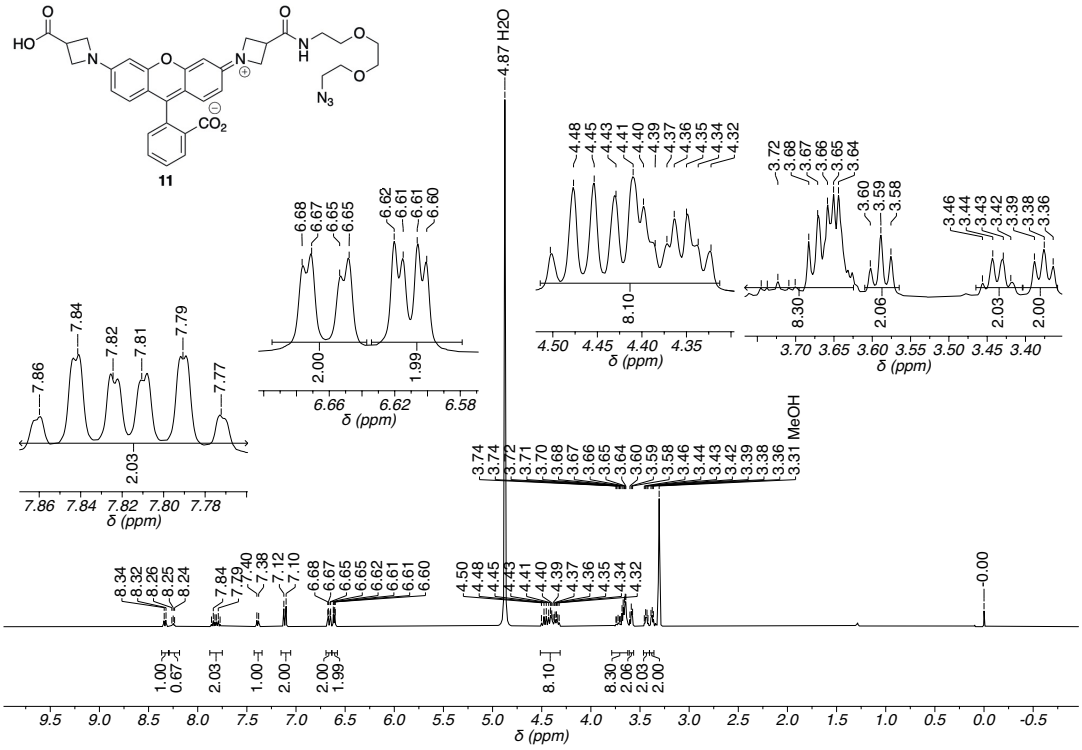
^{13}C NMR of compound **9** (101 MHz, $CDCl_3$).



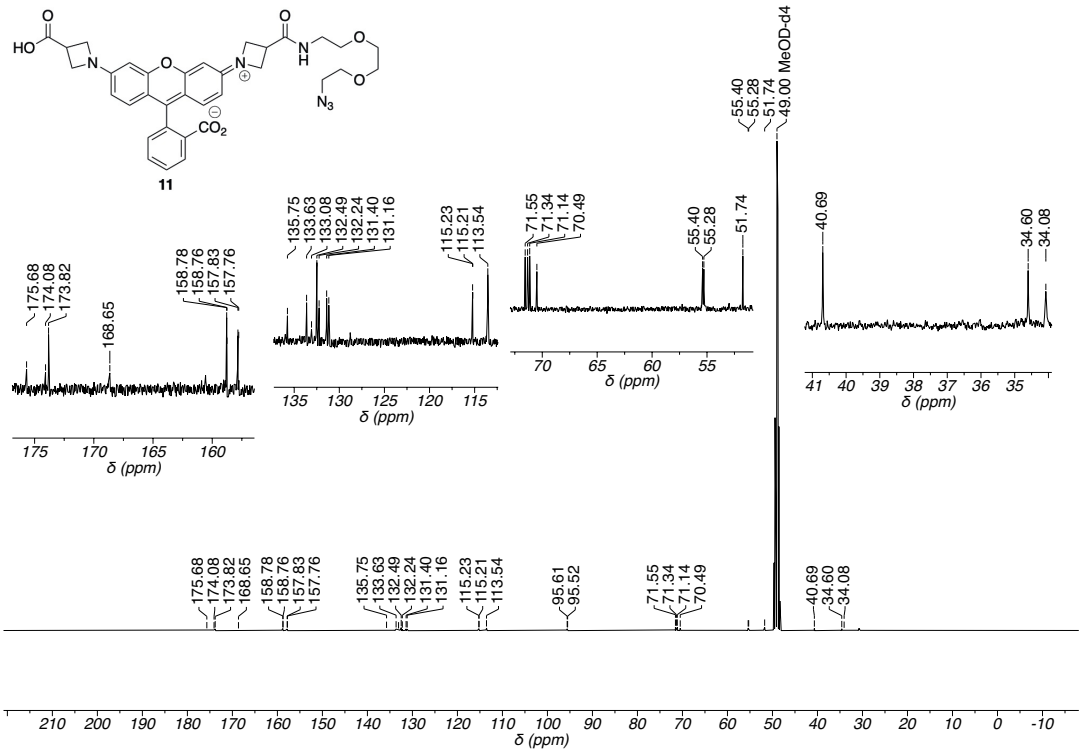
^{1}H NMR of compound **10** (400 MHz, MeOD-d_4+0.03%TMS).



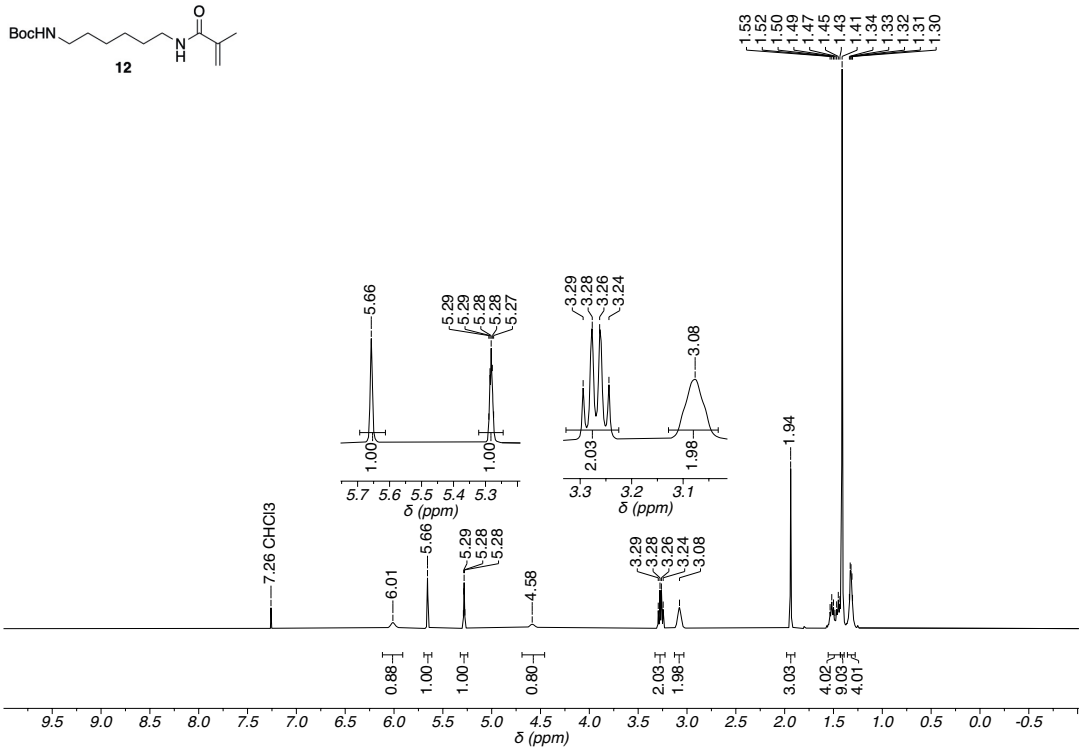
^{13}C NMR of compound **10** (101 MHz, MeOD-d_4+0.03%TMS).



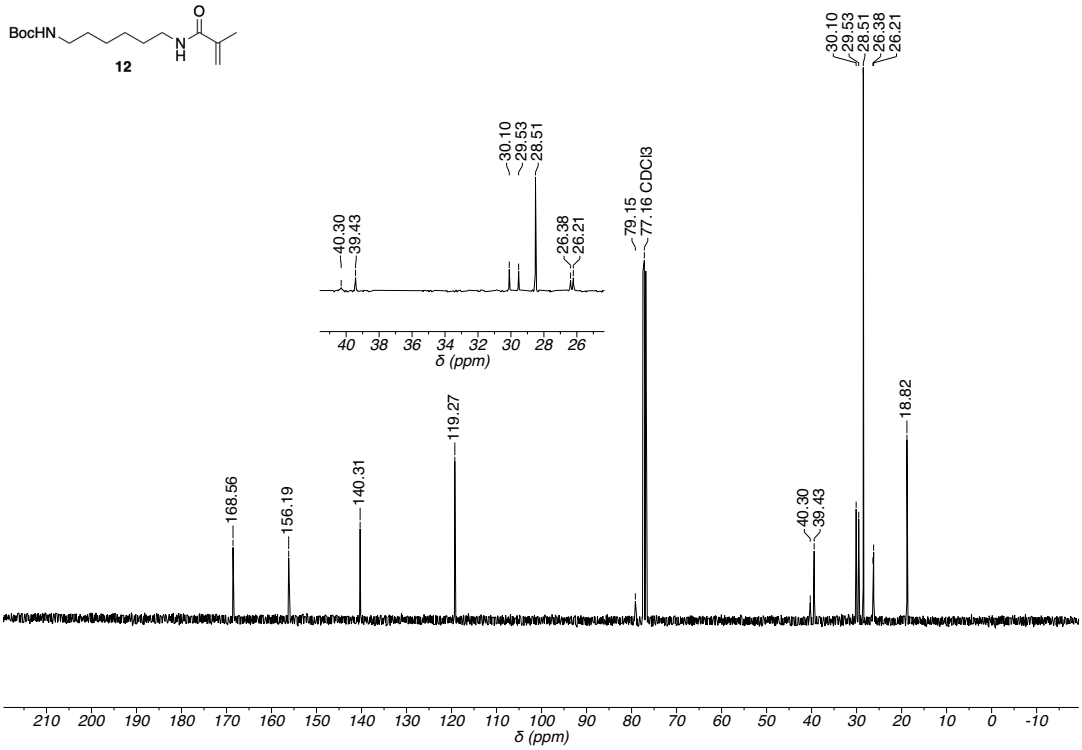
^{1}H NMR of compound **11** (400 MHz, MeOD-d_4+0.03%TMS).



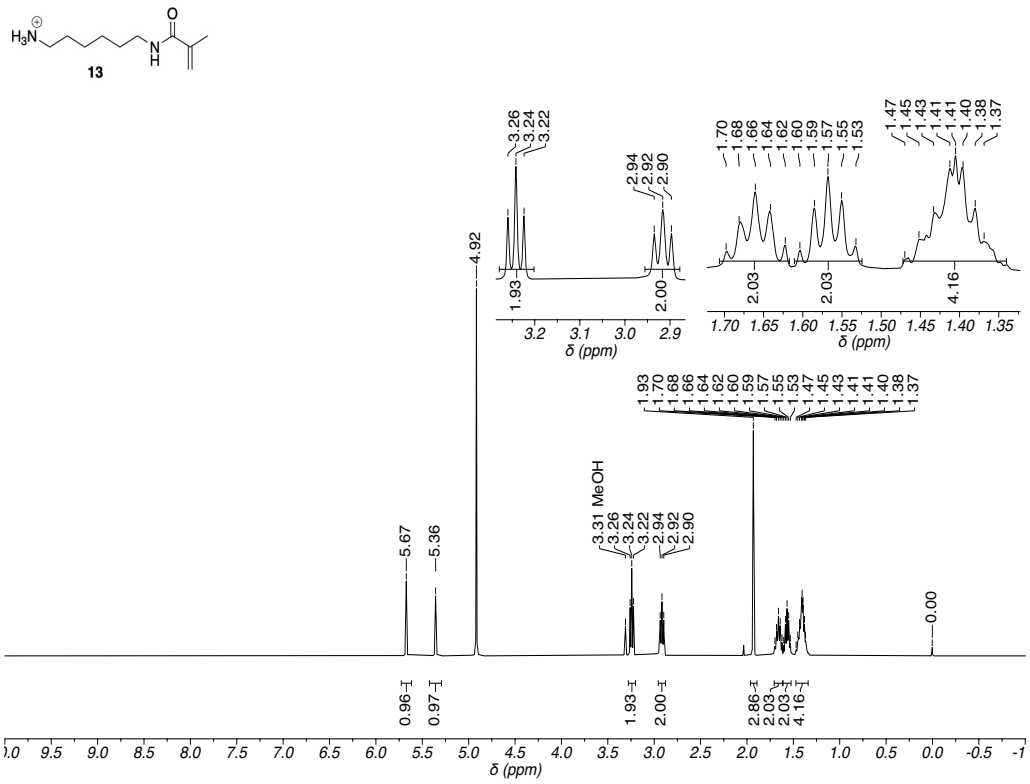
^{13}C NMR of compound **11** (101 MHz, MeOD-d_4+0.03%TMS).



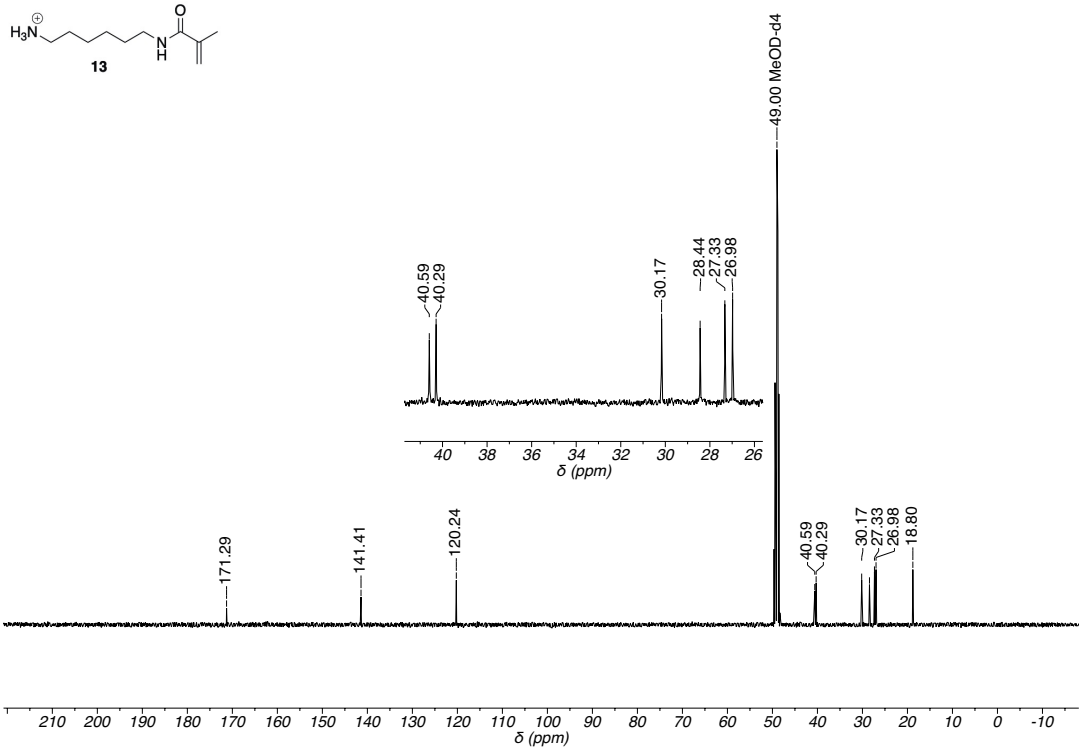
^{1}H NMR of compound **12** (400 MHz, $CDCl_3$).



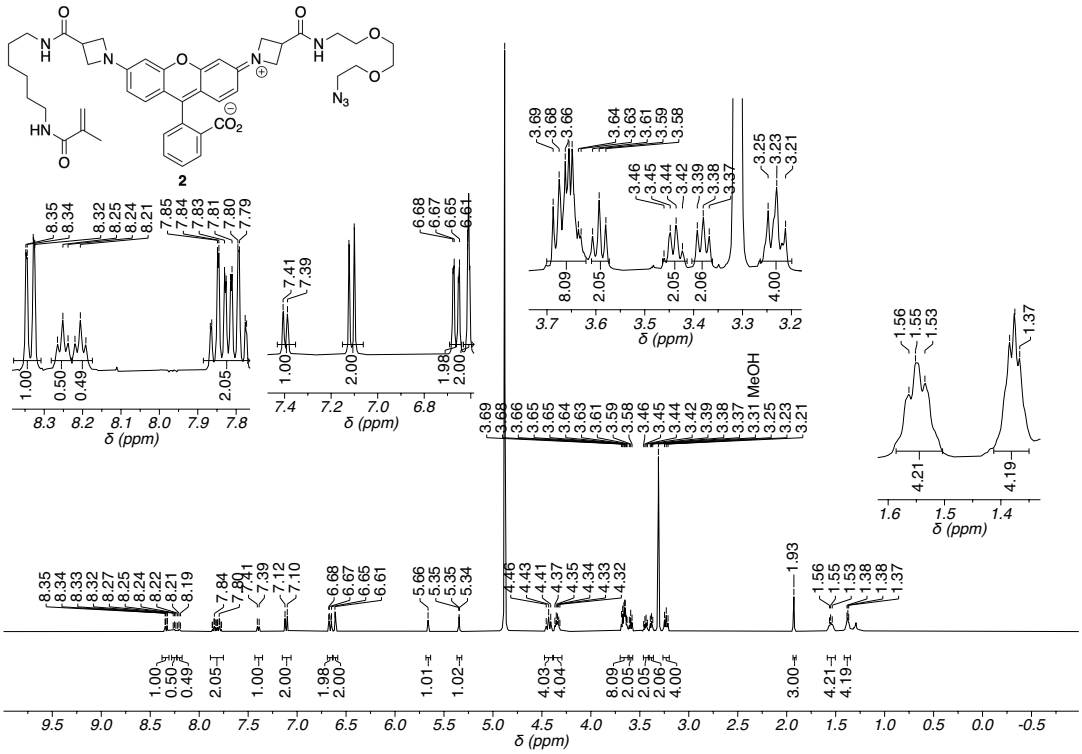
^{13}C NMR of compound **12** (101 MHz, $CDCl_3$).



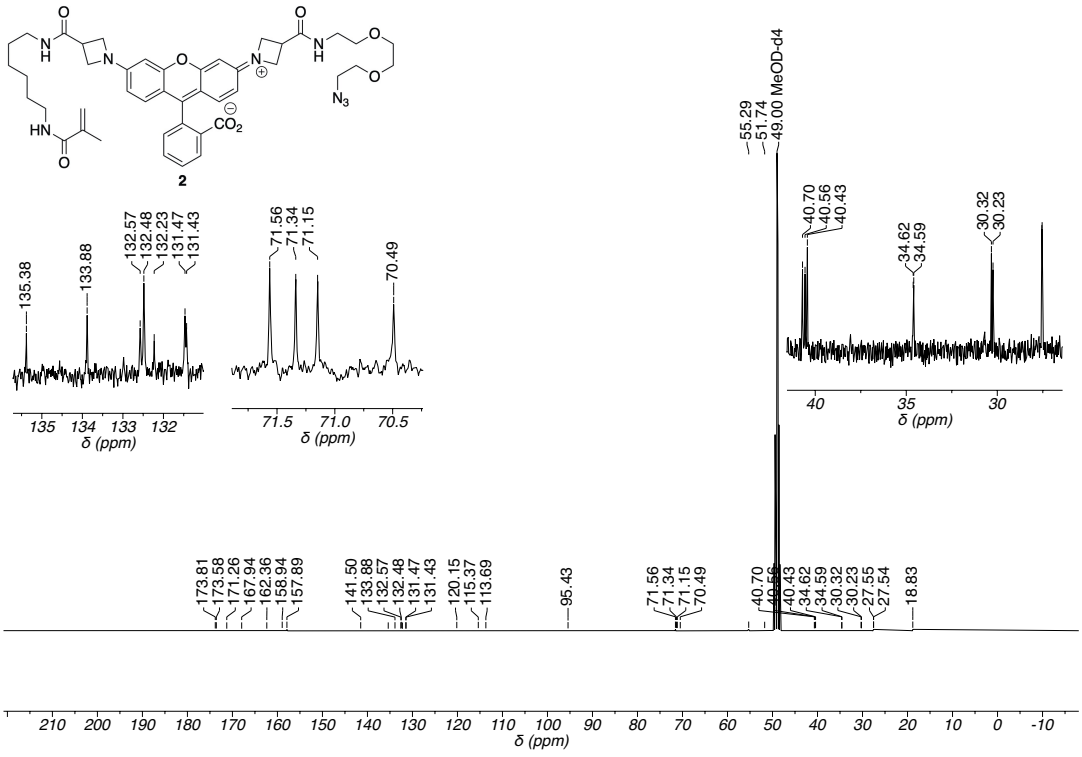
^{1}H NMR of compound **13** (400 MHz, MeOD-d_4+0.03%TMS).



^{13}C NMR of compound **13** (101 MHz, MeOD-d_4+0.03%TMS).



^{1}H NMR of compound **2** (400 MHz, MeOD-d_4).



^{13}C NMR of compound **2** (101 MHz, MeOD-d_4).