Electronic Supplementary Information

Nitrobenzyl-based fluorescent photocages for spatial and temporal control of signaling lipids in cells

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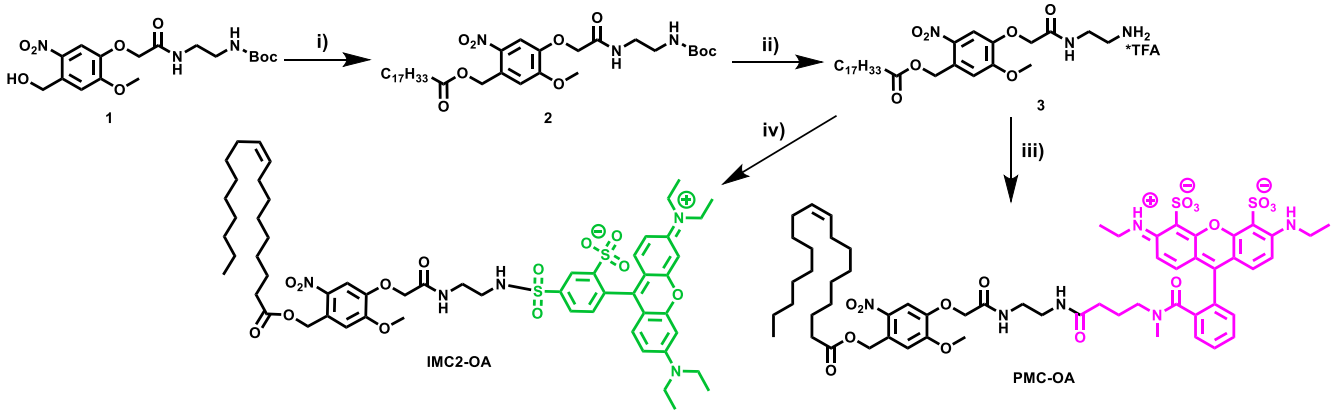
1. Synthesis and characterization of caged lipids

Structural elucidation of synthesized compounds

^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance III HD 400 MHz spectrometer. HRMS-ESI spectra were recorded on LTQ Orbitrap XL spectrometer (Thermo Fisher Scientific).



Scheme S1 i) oleic acid, DMAP, DCC in DCM, 2h (86%); ii) DCM/TFA/H_2O (20/2/0.1 v/v), 5 h (99%); iii) Atto 532 Carboxy, HBTU, DIEA in DCM/CH_3CN, overnight (64%) and iv) sulforhodamine B acid chloride, DIEA in DCM, overnight (27%).

Compound 1

Tert-butyl (2-(2-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy)acetamido)ethyl) carbamate was synthesized as described by Baker. [1] Product is pale-yellow sticky solid. NMR data is in good accordance with literature. **^{1}H NMR** ($CDCl_3$, 400MHz), ppm: 7.76 (s, 1H), 7.28 (s, 1H), 7.21 (br s, 1H, *amide*), 5.00 (s, 2H), 4.88 (br s, 1H, *amide*), 4.58 (s, 2H), 4.03 (s, 3H), 3.53-3.45 (m, 2H), 3.35-3.27 (m, 2H), 1.43 (s, 9H). **^{13}C NMR** ($CDCl_3$, 100MHz), ppm: 167.98, 154.46, 145.57, 139.49, 134.22, 111.86, 111.45, 79.85, 69.00, 62.61, 56.45, 40.37, 28.30. **LCMS** (m/z): ESI, found $[M+Na]^+$ = 422.2 (calcd for $C_{17}H_{25}N_3O_8$+Na = 422.15).

Compound 2

4-(2-((2-((Tert-butoxycarbonyl)amino)ethyl)amino)-2-oxoethoxy)-5-methoxy-2-nitrobenzyl oleate

Compound 1 (140 mg, 1.0 eq), oleic acid (109 mg, 1.1 eq) and DMAP (9 mg, 0.2 eq) were dissolved in DCM (15 mL). The last reagent DCC (94 mg, 1.3 eq) was added to the solution, and reaction mixture was stirred overnight. The formed precipitate was filtered off. The organic solvent was evaporated. The obtained residue was purified by column chromatography (eluent DCM/Et_2O = 5/1, the first fraction) to give the product **2** as colorless oil that solidifies upon standing. Yield is 200 mg

(86%). **^{1}H NMR** ($CDCl_3$, 400 MHz), ppm: 7.76 (s, 1H), 7.18 (br s, 1H, *amide*), 7.05 (s, 1H), 5.52 (s, 2H), 5.40-5.30 (m, 2H), 4.87 (br s, 1H, *amide*), 4.58 (s, 2H), 4.01 (s, 3H), 3.53-3.45 (m, 2H), 3.35-3.27 (m, 2H), 2.43 (t, J = 7.5 Hz, 2H), 2.05-1.97 (m, 4H), 1.74-1.65 (m, 2H), 1.43 (s, 9H), 1.40-1.20 (m, 20H), 0.88 (t, J = 6.8 Hz, 3H). **HRMS** (m/z): ESI, found $[M+1]^+$ = 664.4168 (calcd for $C_{35}H_{57}N_3O_9$+H = 664.4173).

Compound 3

4-(2-((2-Aminoethyl)amino)-2-oxoethoxy)-5-methoxy-2-nitrobenzyl oleate, TFA salt

Compound 2 (100 mg, 1.0 eq) was dissolved in DCM (5 mL). Then some TFA (400 µL) and water (25 µL) were added. The reaction mixture was stirred at RT for 5 h. The organic solvent was evaporated under vacuum. The obtained residue was treated with diethyl ether (3 mL), and the mixture was evaporated. Such treatment was repeated 2 times to remove the excess of TFA. The crude product **3** is white solid. Yield is 99%.

^{1}H NMR ($CDCl_3$, 400 MHz), ppm: 7.77 (s, 1H), 7.73 (br s, 1H, *amide*), 7.0 (s, 1H), 5.44 (s, 2H), 5.39-5.32 (m, 2H), 4.63 (br s, 2H), 3.94 (s, 3H), 3.70 (br s, 2H), 3.25 (br s, 2H), 2.41 (t, J = 7.5 Hz, 2H), 2.07-1.97 (m, 4H), 1.72-1.62 (m, 2H), 1.31-1.27 (m, 20H), 0.88 (t, J = 6.8 Hz, 3H). **HRMS** (m/z): ESI, found $[M+1]^+$ = 564.3644 (calcd for $C_{30}H_{49}N_3O_7$+H = 564.3649).

PMC-OA

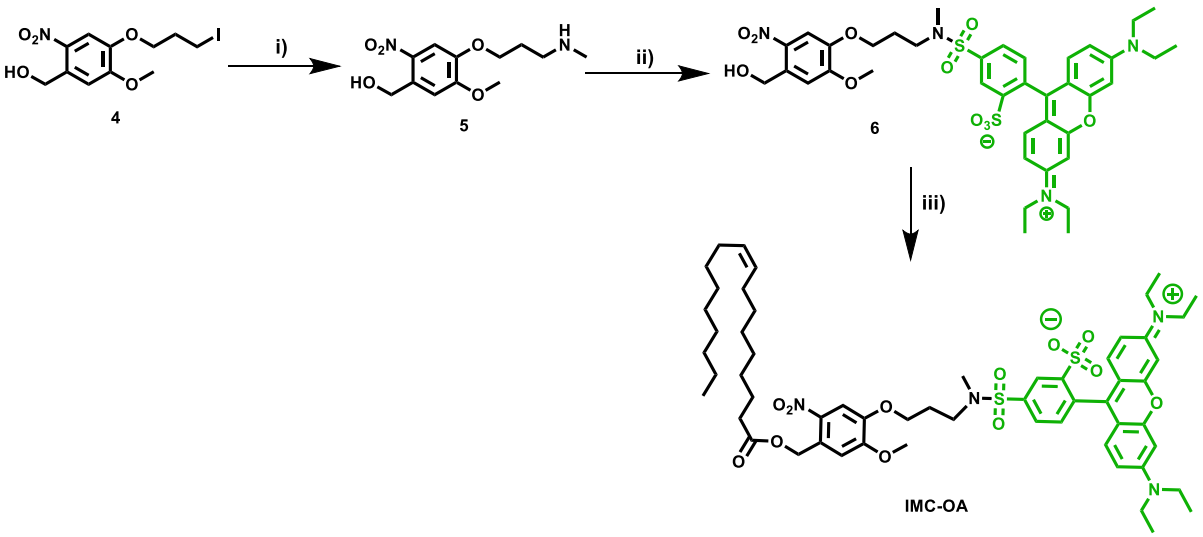
3,6-Bis(ethylamino)-9-(2-((4-((2-(2-(2-methoxy-5-nitro-4-(oleoyloxy)methyl) phenoxy)acetamido)ethyl)amino)-4-oxobutyl)(methyl)carbamoyl)phenyl)-4,5-disulfoxanthylium

Atto 532 carboxy (AD 532-21) was supplied by Atto-tec GmbH. Atto 532 carboxy (1 mg, 1eq) was suspended in DCM (3 mL) and acetonitrile (3 mL), followed by the addition of HBTU (1 mg, 2 eq) and DIEA (0.7 µL, 3 eq). The obtained mixture was added to the mixture of compound **3** (2 mg, 2 eq) and DIEA (0.7 µL, 3 eq) in DCM to get some vine-red solution. The reaction mixture was stirred overnight at RT. In the morning the residue of Atto-dye completely disappeared. The homogeneous reaction mixture was concentrated under vacuum. The obtained residue was washed with diethyl ether (1mL twice), and then purified by column chromatography (eluent MeOH/DCM = 1/9, Rf = 35%) to give the final product as red solid. Yield is 1 mg (64%). Product is soluble in DCM. **HRMS** (m/z): ESI, found $[M]^+$ = 1191.4999 (calcd for $C_{59}H_{79}N_6O_{16}S_2$ = 1191.4994). ESI, found $[M-2]^-$ = 1189.4828 (calcd for $C_{59}H_{79}N_6O_{16}S_2$-2H = 1189.4838).

IMC2-OA

2-(3,6-Bis(diethylamino)xanthylium-9-yl)-5-(N-(2-(2-(2-methoxy-5-nitro-4-((oleoyloxy)methyl)phenoxy)acetamido)ethyl)sulfamoyl) benzenesulfonate

The previously obtained compound **3** (22 mg, 1.0 eq) and DIEA (16 µL, 3.0 eq) were mixed together in DCM (5 mL). Then, sulforhodamine B acid chloride [2] (26 mg, 1.5 eq as solution in DCM) was added, and the reaction mixture was stirred at RT overnight. The red homogeneous solution was formed. Organic solvent was evaporated under vacuum. The obtained residue was purified by column chromatography (eluent DCM/MeOH = 98/2, then 96/4) to give the final product as a dark red solid. Yield is 9 mg (27%). **^{1}H NMR** ($CDCl_3$, 400 MHz), ppm: 8.76 (d, J = 1.7 Hz, 1H), 7.97 (dd, J = 8.0 Hz, J = 1.7 Hz, 1H), 7.73 (t, J = 5.5 Hz, 1H, *amide*), 7.65 (s, 1H), 7.37 (d, J = 9.4 Hz, 2H), 7.23 (d, J = 8.0 Hz, 1H), 6.99 (s, 1H), 6.94 (dd, J = 9.4 Hz, J = 1.0 Hz, 2H), 6.68 (d, J = 1.0 Hz, 2H), 5.47 (s, 2H), 5.38-5.32 (m, 2H), 4.53 (s, 2H), 3.98 (s, 3H), 3.65-3.48 (m, 8H), 3.41 (br s, 2H), 3.21 (br s, 2H), 2.44 (t, J = 7.5 Hz, 2H), 2.05-1.97 (m, 4H), 1.72-1.62 (m, 2H), 1.40-1.20 (m, 32H), 0.88 (t, J = 6.8 Hz, 3H). **HRMS** (m/z): ESI, found $[M+1]^+$ = 1104.5037 (calcd for $C_{57}H_{77}N_5O_{13}S_2$+H = 1104.5038).



Scheme S2 i) NaI in acetone, reflux 2h, then $MeNH_2$ in EtOH, overnight (51%); ii) sulforhodamine B acid chloride, DIEA in DCM, 1h (42%); iii) oleic acid, DMAP, DCC in DCM, 2h (48%).

Compound 4

(4-(3-Iodopropoxy)-5-methoxy-2-nitrophenyl)methanol

(4-(3-Chloropropoxy)-5-methoxy-2-nitrophenyl)methanol[3] (2.24 g, 1.0eq) was dissolved in the saturated solution of NaI in acetone (15 mL). The clear transparent solution was stirred under reflux for 2h. After cooling some precipitate of NaCl was filtered off (approximately 400 mg). Organic solvent was evaporated, and the residue was treated with DCM. White precipitate of NaI was filtered off, and the filtrate was evaporated to give the crude product. It was purified by column chromatography

(eluent DCM, then DCM/MeOH = 98/2, finished with 95/5) to give the compound **4** as white powder. Yield is 2.4g (80%). **^{1}H NMR** ($CDCl_3$, 400MHz), ppm: 7.75 (s, 1H), 7.18 (s, 1H), 4.97 (s, 2H), 4.17 (t, J = 5.9 Hz, 2H), 3.99 (s, 3H), 3.40 (t, J = 6.6 Hz, 2H), 2.63 (br s, 1H, *OH*), 2.36 (p, J = 6.2, 2H). **^{13}C NMR** ($CDCl_3$, 100MHz), ppm: 154.32, 147.08, 139.66, 132.52, 111.31, 109.83, 68.89, 62.87, 56.41, 32.57, 1.90. **LCMS** (m/z): ESI, found $[M+Na]^+$ = 390.0 (calcd for $C_{11}H_{14}INO_5$+Na = 390.0).

Compound 5

(5-Methoxy-4-(3-(methylamino)propoxy)-2-nitrophenyl)methanol

Compound **4** (1.05 g, 1.0 eq) was dissolved in MeOH (5 mL), and then it was poured into the 33% solution of $MeNH_2$ in ethanol (5 mL, 15.0 eq) at 0°C. Reaction mixture was stirred at RT overnight. Flash TLC (eluent DCM/MeOH = 9/1) showed complete transformation of the starting material. Organic solvent was evaporated under vacuum. Obtained residue was treated with 20 mL of saturated aqueous solution of $NaHCO_3$, and then solution was extracted with DCM (10 mL x 3 times). Combined organic layer was dried with $MgSO_4$, filtered and evaporated. Crude product was purified by short column chromatography (eluent DCM/MeOH = 9/1, then 1/1) to give the product **5** as pale-yellow powder. Yield is 400mg (51%). **^{1}H NMR** (CD_3OD, 400MHz), ppm: 7.74 (s, 1H), 7.41 (s, 1H), 4.95 (s, 2H), 4.14 (t, J = 6.0 Hz, 2H), 3.97 (s, 3H), 2.77 (t, J = 6.9 Hz, 2H), 2.40 (s, 3H), 2.03 (p, J = 6.5 Hz, 2H). **^{13}C NMR** (CD_3OD, 100MHz), ppm: 155.78, 148.35, 140.48, 135.09, 110.98, 110.62, 69.34, 62.33, 56.92, 49.36, 36.22, 29.83. **LCMS** (m/z): ESI, found $[M+1]^+$ = 271.1 (calcd for $C_{12}H_{18}N_2O_5$+H = 271.1).

Compound 6

2-(3,6-Bis(diethylamino)xanthylium-9-yl)-5-(N-(3-(4-(hydroxymethyl)-2-methoxy-5-nitrophenoxy)propyl)-N-methylsulfamoyl) benzenesulfonate

Sulforhodamine B acid chloride [2] (170 mg, 1.0eq) was mixed with DCM (15 mL) at 0°C. Then DIEA (76 µL, 1.5 eq) was added, followed by the addition of N-methylamine derivative **9** (88 mg, 1.1 eq) as solution in DCM (5 mL). After 1h of stirring at RT, the homogeneous solution was concentrated under vacuum. The obtained residue was purified by column chromatography (eluent DCM/MeOH = 98/2, the first fraction). The obtained substance was washed with MeOH (1 mL) to give the key intermediate 6 as red crystals. Yield is 100 mg (42%). **^{1}H NMR** (DMSO-d_6, 400 MHz), ppm: 8.32 (d, J = 2.0 Hz, 1H), 7.95 (dd, J = 8.0 Hz, J = 2.0 Hz, 1H), 7.71 (s, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.40 (s, 1H), 7.05-6.90 (m, 6H), 5.58 (t, J = 5.5 Hz, 1H, *OH*), 4.82 (d, J = 5.5 Hz, 2H), 4.14 (t, J = 6.2 Hz, 2H), 3.93 (s, 3H), 3.65 (q, J = 7.0 Hz, 8H), 3.28 (t, J = 6.9 Hz, 2H), 2.84 (s, 3H), 2.04 (p, J = 6.5 Hz, 2H), 1.20 (t, J = 7.0 Hz, 12H). **HRMS** (m/z): ESI, found $[M+1]^+$ = 811.2679 (calcd for $C_{39}H_{46}N_4O_{11}S_2$+H = 811.2683).

IMC-OA

2-(3,6-Bis(diethylamino)xanthylium-9-yl)-5-(N-(3-(2-methoxy-5-nitro-4-((oleoyloxy) methyl)phenoxy)propyl)-N-methylsulfamoyl)benzenesulfonate

Compound **6** (50 mg, 1.0eq), oleic acid (19 mg, 1.1 eq) and DMAP (1.5 mg, 0.2 eq) were dissolved in DCM (15 mL). Then, the last component DCC (14 mg, 1.1 eq) was added, and then reaction mixture was stirred at RT for 1h. Clear red solution was evaporated under vacuum. Residue was purified by column chromatography (eluent DCM MeOH = 98/2 then 95/5, the first fraction) to give the final product as dark red powder. Yield is 32 mg (48%). **^{1}H NMR** ($CDCl_3$, 400 MHz), ppm: 8.77 (d, J = 1.7 Hz, 1H), 7.94 (dd, J = 8.0 Hz, J = 1.7 Hz, 1H), 7.76 (s, 1H), 7.27 (d, J = 9.2 Hz, 2H), 7.23 (d, J = 8.0 Hz, 1H), 7.01 (s, 1H), 6.80 (dd, J = 9.2 Hz, J = 1.8 Hz, 2H), 6.68 (d, J = 1.8 Hz, 2H), 5.51 (s, 2H), 5.38-5.32 (m, 2H), 4.22 (t, J = 6.0 Hz, 2H), 4.00 (s, 3H), 3.56 (q, J = 7.0 Hz, 8H), 3.47 (t, J = 6.6 Hz, 2H), 2.97 (s, 3H), 2.42 (t, J = 7.5 Hz, 2H), 2.20 (p, J = 6.3 Hz, 2H), 2.05-1.97 (m, 4H), 1.71 (m, 2H), 1.40-1.20 (m, 32H), 0.89 (t, J = 7.0 Hz, 3H). **HRMS** (m/z): ESI, found $[M+1]^+$ = 1075.5131 (calcd for $C_{57}H_{78}N_4O_{12}S_2$+H = 1075.5136).

2. Photophysical properties of caged lipids

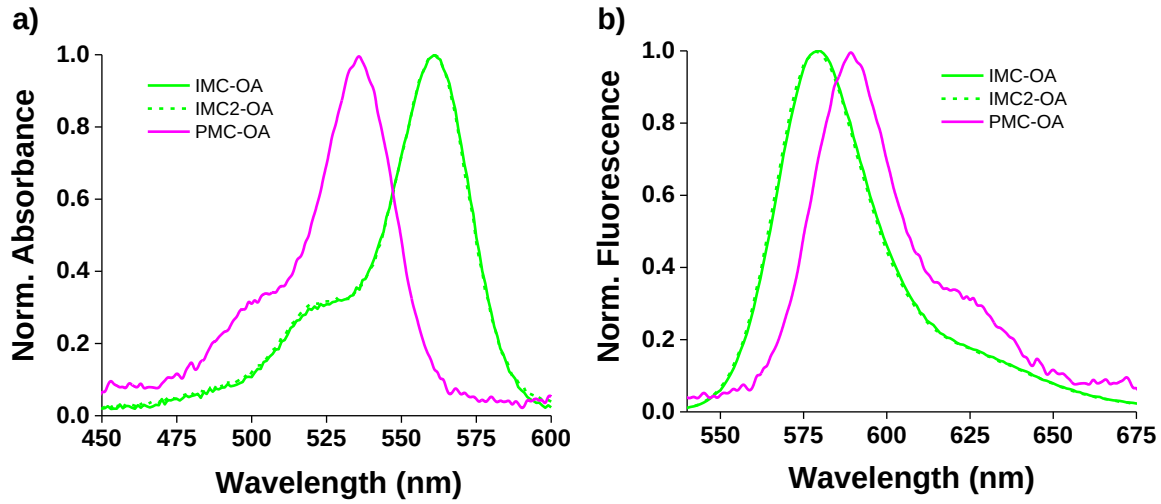


Fig. S1 Normalized absorption (a) and emission (b) spectra of caged oleates IMC-OA, IMC2-OA and PMC-OA. Spectral measurements were made in methanol (concentration = 0.3 µM for IMC-OA/IMC2-OA and 0.2 µM for PMC-OA).

Absorption and emission spectra were recorded on Cary-100 spectrophotometer (Varian) and FluoroMax-4 spectrofluoremeter (Horiba Scientific), respectively. Stock solutions of caged lipids (1 mM concentration) were prepared in DMSO. Measurements were performed in 1 cm quartz cuvette (3.5 mL). The excitation wavelength was 520 nm, slits were 2 nm. Fluorescence quantum yield of the caged lipids was determined using Rhodamine B (ϕ_R = 0.31 in water) as the standard.[4]

$$\phi_S = \phi_R \left(\frac{A_R}{A_S} \right) \left(\frac{D_S}{D_R} \right)$$

Where ϕ_S and ϕ_R are the quantum yields of sample and the reference; A_S and A_R are absorbance of the sample and the reference at the excitation wavelength; and D_S and D_R are the integral fluorescence intensities.

Table S1 Photophysical properties of caged lipids.

Compound	λ_{abs} (nm)	λ_{em} (nm)	Quantum yield, ϕ
PMC-OA	536	589	0.22
IMC-OA	561	579	0.34
IMC2-OA	561	579	0.31

3. Uncaging study in solution

A stock solution 10 mg/mL of IMC-OA was prepared in a mixture of solvents CH_3CN/CH_3OH (1:1). A six set of solutions R_0-R_5 in CH_3CN (1 mg/mL, 70 µL each) were exposed under LED-365B (350 mW) lamp at regular time intervals (1, 2, 3, 5, 7 min) to monitor the dynamics of uncaging. Progress of uncaging reaction was monitored by TLC (ALUGRAM Xtra SIL G/UV_{254}). Control sample and exposed sample were submitted for mass analysis. Further, the solution of IMC-OA in $CDCl_3$ was exposed at regular interval and the uncaging was monitored by measuring ^{1}H NMR.

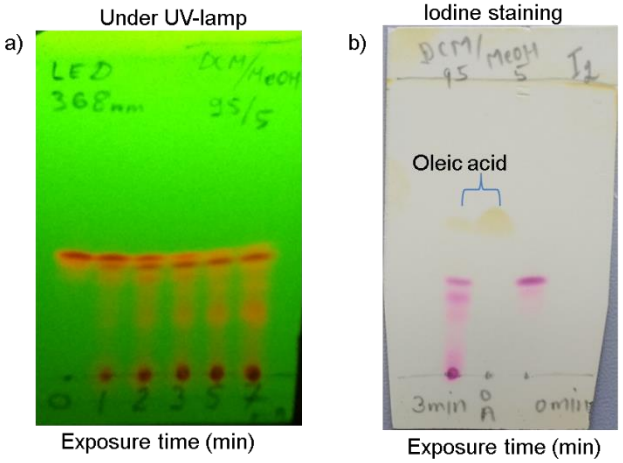


Fig. S2 Uncaging of IMC-OA under 365 nm LED lamp (350 mW) monitored by TLC (ALUGRAM Xtra SIL G/UV254). a) TLC of the irradiated samples visualized under UV-lamp; b) TLC of the irradiated sample and pure oleic acid visualized by iodine staining. Solution of IMC-OA in MeCN/MeOH was illuminated. Small aliquots were spotted on ALUGRAM and TLC was run in 5:95 MeOH/DCM.

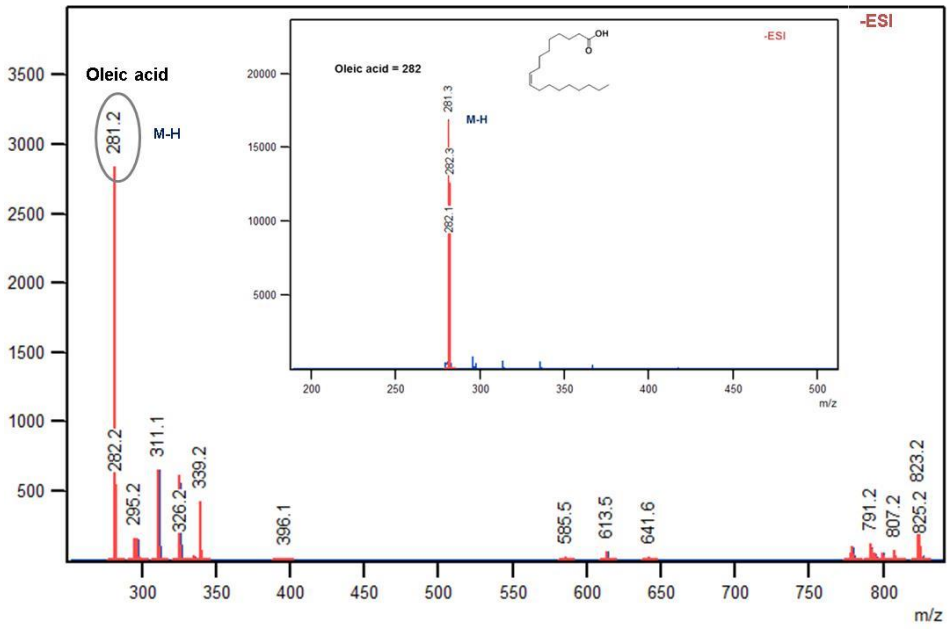


Fig. S3 Mass spectrum of IMC-OA exposed for 2 min upon illumination with 365 nm. Inset presents the mass spectrum of pure oleic acid.

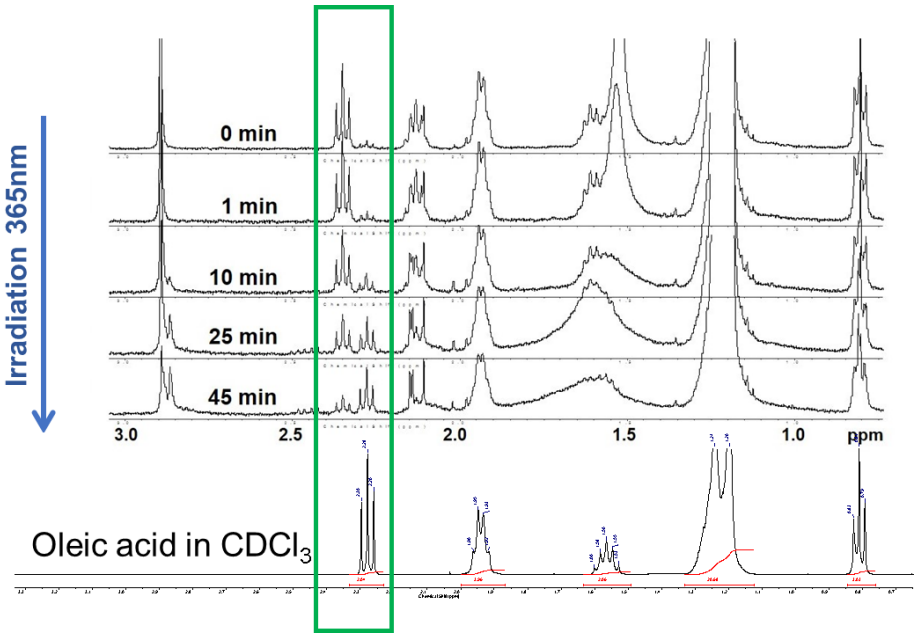


Fig. S4 Monitoring the uncaging of IMC-OA by NMR upon illumination with 365 nm LED. Green rectangle highlights the region of α-protons.

4. Cell studies

Cell growth

HeLa cells were grown in growth media containing complete DMEM (DMEM (Gibco # 11965092), supplemented with 10% FBS (BioWest South America #S1810), 2 mM L-Glutamine (Gibco # 25030081), 20 mM sodium pyruvate (Gibco # 11360070) and antibiotic (Penicillin, Streptomycin, Gibco # 15140122, 100 U/ml). First, the cells were seeded in an 8-well Lab-Tek chambered coverslip (ThermoScientific #155411) 24 h before transfection at 37°C and 5% CO_2, 50 000 cells/ml, 300 µL per well.

Transfection protocol

Transfection was carried out with FugeneHD (Promega, cat # E2311) in OptiMEM free of FBS and antibiotics.

First, the media was aspirated and the cells were washed with PBS. The wells were transferred in OptiMEM media (250 µL per well) with 4 mM L-Glutamine. Transfection mixture was prepared by mixing OptiMEM (50 µL per well) with plasmid DNA (0.5 µg of DNA per well, 1:1 plasmid ratio for cotransfection) with addition of FugeneHD (1.5 µL per well) (protect from additional contact with plastic, sticks to plastics!) was then added dropwise to each well of the 8-well Lab-Tek (use 200 µL tips). After 10-24 h cells were washed with complete DMEM, and 300 µL complete DMEM was added into each well.

The cells were incubated at 37°C and 5% CO_2 for 24 h before the microscopy experiments were performed. Total transfection time before imaging 24-48 h.

Used Plasmids

Name	Characterization
GCaMP6s	Green genetically-encoded fluorescent Ca^{2+} sensor
GPR40	Free fatty acid receptor
BFP-KDEL	Endoplasmic reticulum marker
HyPer-Mem	Plasma membrane marker, Hydrogen peroxide probe

Live-cell imaging

The cells seeded in eight-well Lab-Tek chambered coverslip were washed with PBS and charged with imaging buffer (11 mM glucose) containing (mM): 20 HEPES, 115 NaCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$ and 1.2 K_2HPO_4. Imaging was performed in eight-well Lab-Tek at 37 °C and 5% CO_2 on a dual scanner confocal microscope Olympus Fluoview 1200, with 63× (oil) objective. This microscope

houses two independent, fully synchronized laser scanners for simultaneous laser stimulation and confocal observation and permits capturing of cellular responses that occur during or immediately following laser stimulation. Microscope settings were adjusted to generate images displaying background fluorescence values slightly larger than zero in order to capture the complete signal stemming from the respective fluorescent dyes or proteins.

Image J Fiji was used to analyse the cell imaging data. Fluorescence intensities were measured with respect to time and calculated relative to the maximum detected fluorescence intensity after background substraction (F/F_{max}). The average of three regions of interest were used for statical analysis. Cells with represenative behavior were selected per condition and their responses were averaged.

Cellular localization of PMC-OA, IMC-OA and IMC2-OA

BFP-KDEL (Endoplasmic reticulum marker), HyPerMem (Plasma membrane marker) and Con A-FITC (Concanavalin A conjugated with FITC, plasma membrane tracker) were used to check the localization of caged oleates. BFP-KDEL was excited with 405 nm laser and emitted light was collected between 425 and 475 nm. HyPerMem was excited with 488 nm laser and emitted light was collected at 495–555 nm. PMC-OA, IMC-OA and IMC2-OA were excited with 561 nm lasers and emitted light was collected at 585–645 nm. Con A-FITC was excited with 488 nm laser and emitted light was collected at 495–555 nm. Images were acquired at 37°C and 5%CO_2.

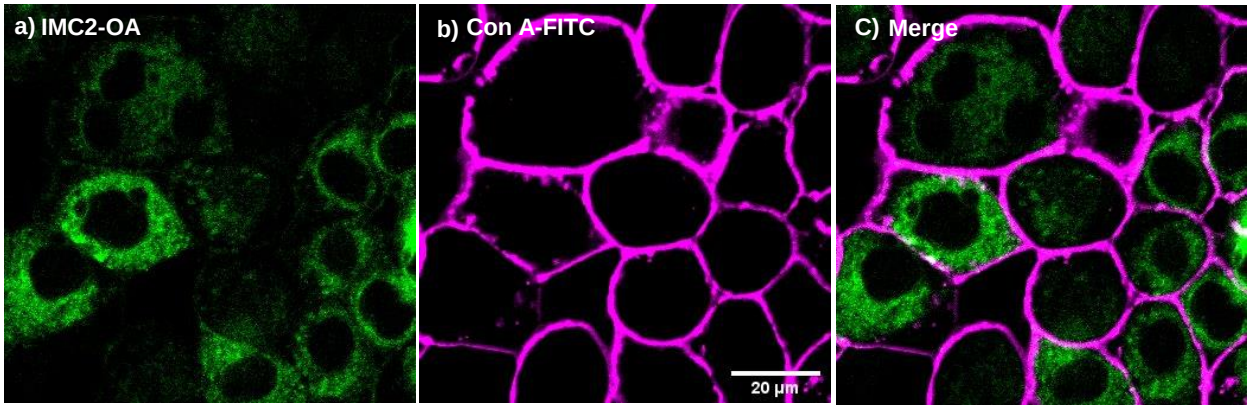


Fig. S5 Cellular localization of IMC2-OA (30µM) with Con A-FITC in live HeLa cells. All images were acquired after incubation of 30 min. a) IMC2-OA, λ_{ex} = 561nm, λ_{em} = 585-650 nm; b) Con A-FITC (50 µg/mL, Concanavalin A conjugated with FITC), λ_{ex} = 488 nm, λ_{em} = 495-555 nm and c) merge image of a), b). Magnification 63x, scale bar is 20 µm.

GPR40 activation upon addition of free OA

To check if GPR40 is activated by free OA, we used live HeLa cells expressing GCaMP6s (Ca^{2+} sensor) only, and cells expressing GCaMP6s and GPR40. Intracellular Ca^{2+} levels in response to exogenous OA (100 µM) was measured using GCaMP6s fluorescence (λ_{ex} = 488 nm, λ_{em} = 495-555 nm).

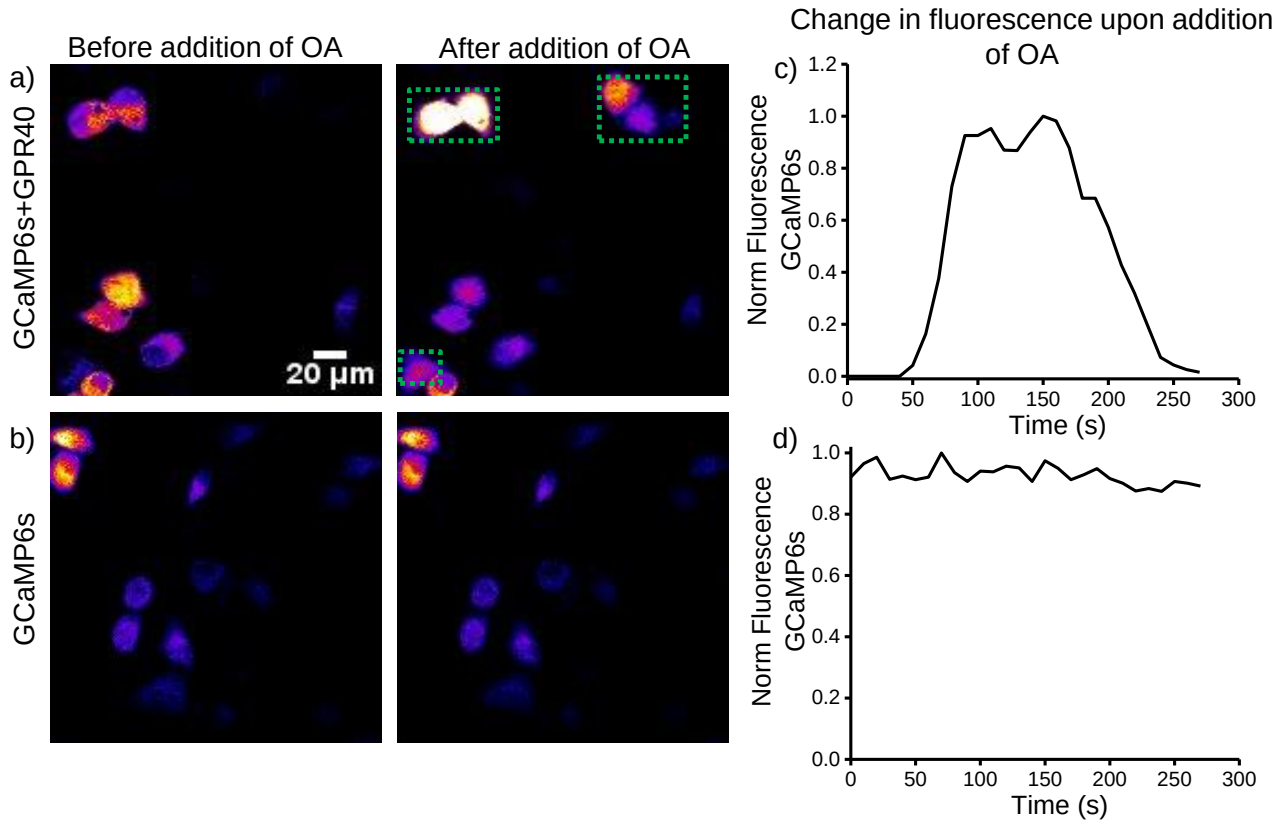


Fig. S6 a, b) Time-lapse imaging of GCaMP6s before and after addition of free OA (100 µM) in live cells expressing GCaMP6s and GPR40, and cell expressing GCaMP6s respectively. Green rectangles highlight the elevation in Ca^{2+} levels in cells. c, d) Normalized fluorescence intensity of GCaMP6s upon addition of free OA to cells transfected with GPR40 plasmid (c) and cells not transfected with GPR40 plasmid (d).

Uncaging in cellular system

Uncaging of PMC-OA was performed using 375 nm laser (5 frames, 6 sec/frame) at 37ºC and 5%CO_2 in live HeLa cells expressing GCaMP6s and GPR40. Fluorescence of GCaMP6s was used as the example for the elevation in Ca^{2+} levels in the response to uncaging of free Oleic acid. λ_{ex} = 488 nm, λ_{em} = 495-555 nm were used for the measuremet of GCaMP6s fluorescence. λ_{ex}= 561nm, λ_{em} = 585-650 nm were used to measure the fluorescene of PMC-OA.

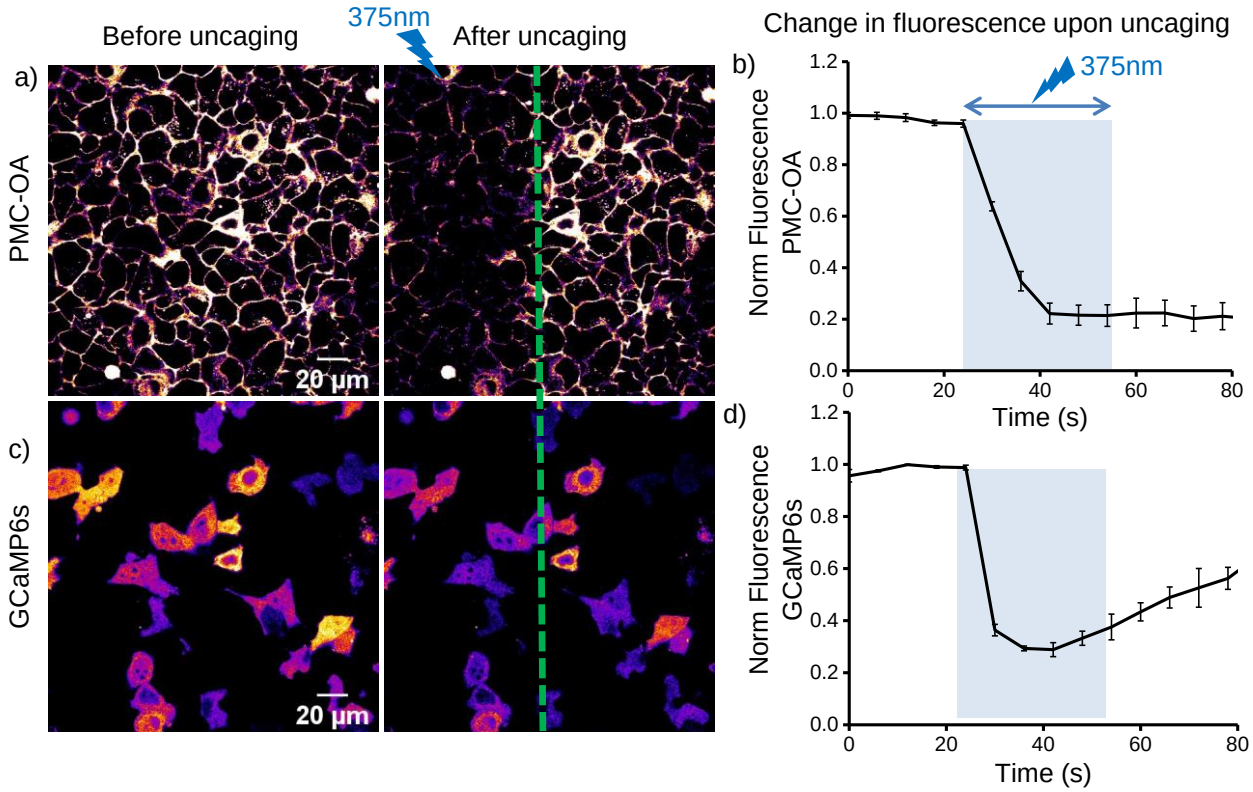


Fig. S7 a) Time-lapse imaging of PMC-OA upon illumination with 375 nm laser; b) normalized fluorescence intensity of PMC-OA upon uncaging. c, d) Time-lapse imaging and normalized fluorescence intensity of GCaMP6s in the response to uncaging of PMC-OA. An exponential drop down in the fluorescence intensity of GCaMP6s due to unrepairable photodamage of the chromophores of GCaMP6s pool was observed upon uncaging of PMC-OA. Gray rectangles showed the time frame of exposure (24-54 sec). Uncaging experiment was peroformed at 37ºC and 5%CO_2 in live HeLa cells expressing GCaMP6s only. Data represent the average of three regions of interests. Error bars represent SD. Magnification 63x, scale bar is 20 µm.

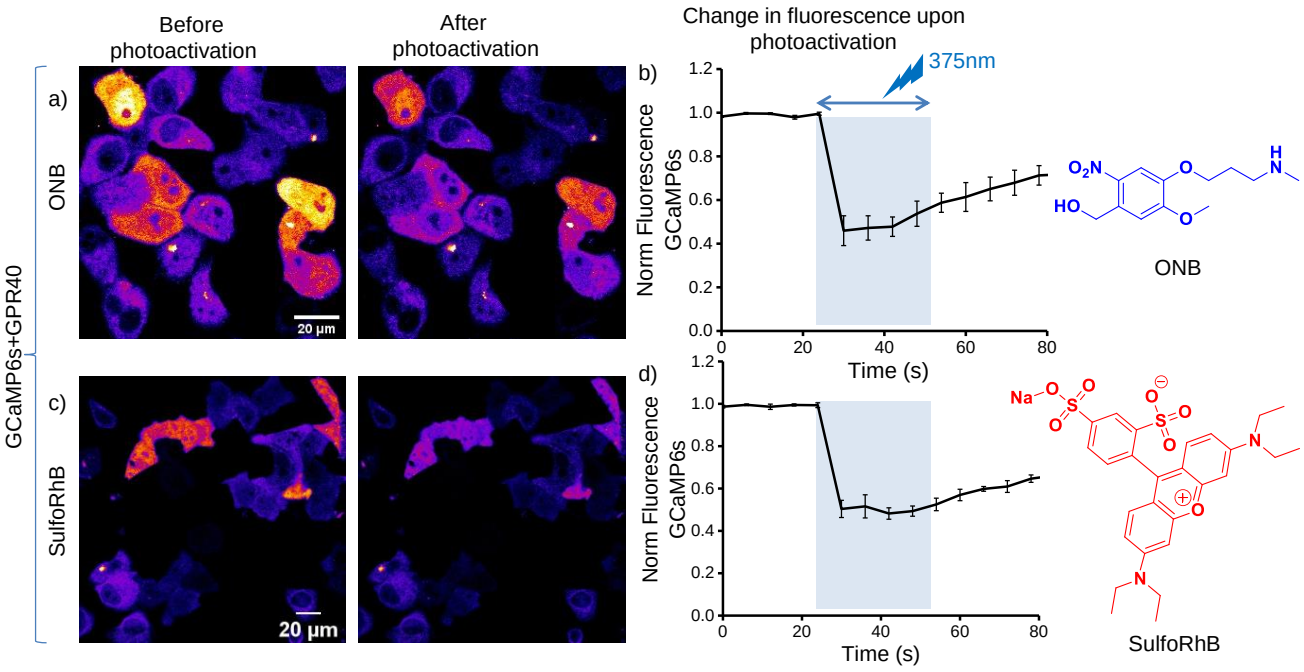


Fig. S8 a, c) Time-lapse imaging of GCaMP6s in live HeLa cells incubated with ONB (30 µM) and SulfoRhB (30 µM); b, d) normalized fluorescence intensity of GCaMP6s in response to photoactivation of ONB and SulfoRhB respectively. Imaging was peroformed in live HeLa cells at 37ºC and 5%CO_2. Data represent the average of three regions of interests. Gray rectangles showed the time frame of exposure. Error bars represent SD. Magnification 63x, scale bar is 20 µm.

Bleaching vs uncaging

HeLa cells were incubated with 30 µM PMC-OA for 10 min at 37°C and 5% CO_2. After 10 min loading solution was removed and cells were washed with PBS. Imaging media 300 µL was added and cells were kept for 90-180 min at 37°C and 5% CO_2 to ensure the efficient uptake of PMC-OA in vesicular structures. After 3 h, uncaging of PMC-OA was performed at 37°C and 5% CO_2 in live HeLa cells using 375 nm laser to dissect uncaging from bleaching.

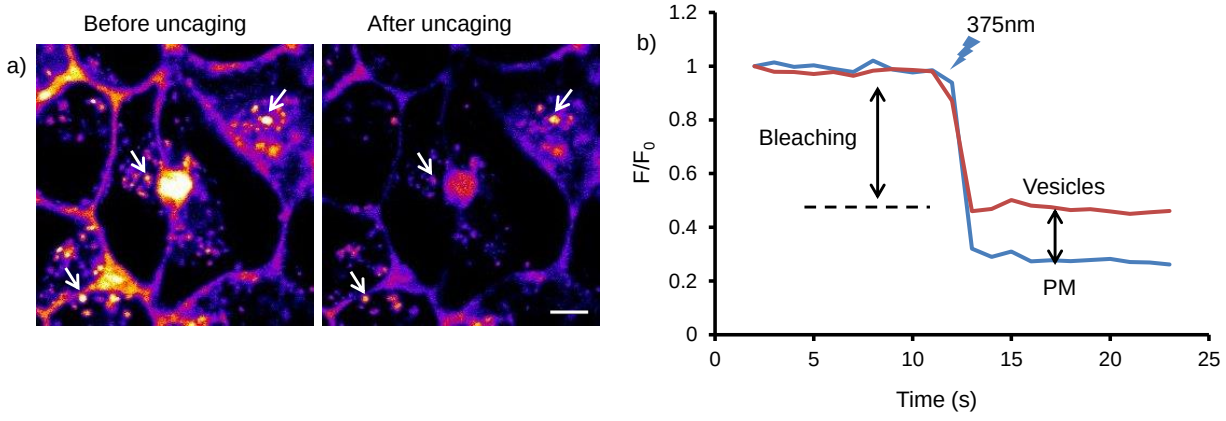


Fig. S9 **Bleaching vs uncaging.** a) Distinctly different decrease in fluorescence intensity of PMC-OA at the plasma membrane and in vesicles upon photoactivation (Arrows mark the vesicles to compare the fluorescence intensity of PMC-OA in the vesicles before and after uncaging); b) normalized fluorescence intensity for plasma membrane and vesicles over time. Magnification 63x, scale bar is 20 µm.

Uncaging during imaging

Time-lapse imaging was performed using PMC-OA and ScC-OA separately in live HeLa cells expressing GCaMP6s and GPR40 to check the possibility of unintended release of oleic acid during imaging. First, the cells loaded with PMC-OA (30 µM) and ScC-OA (90 µM) were exposed using 488 nm laser only and fluorescence of GcaMP6s was measured in both cases. Thereafer, the cells loaded with PMC-OA were illuminated simultaneously with 488 nm, 561 nm to measue the fluorescence of GCaMP6s and PMC-OA. Similarly, the cells loaded with ScC-OA were exposed with 488nm, 405 nm lases to measue the fluorescence of GCaMP6s and ScC-OA. Under both conditions, the difference in the fluorescence of GCaMP6s was measured for PMC-OA and ScC-OA to check the unintended uncaging during imaging.

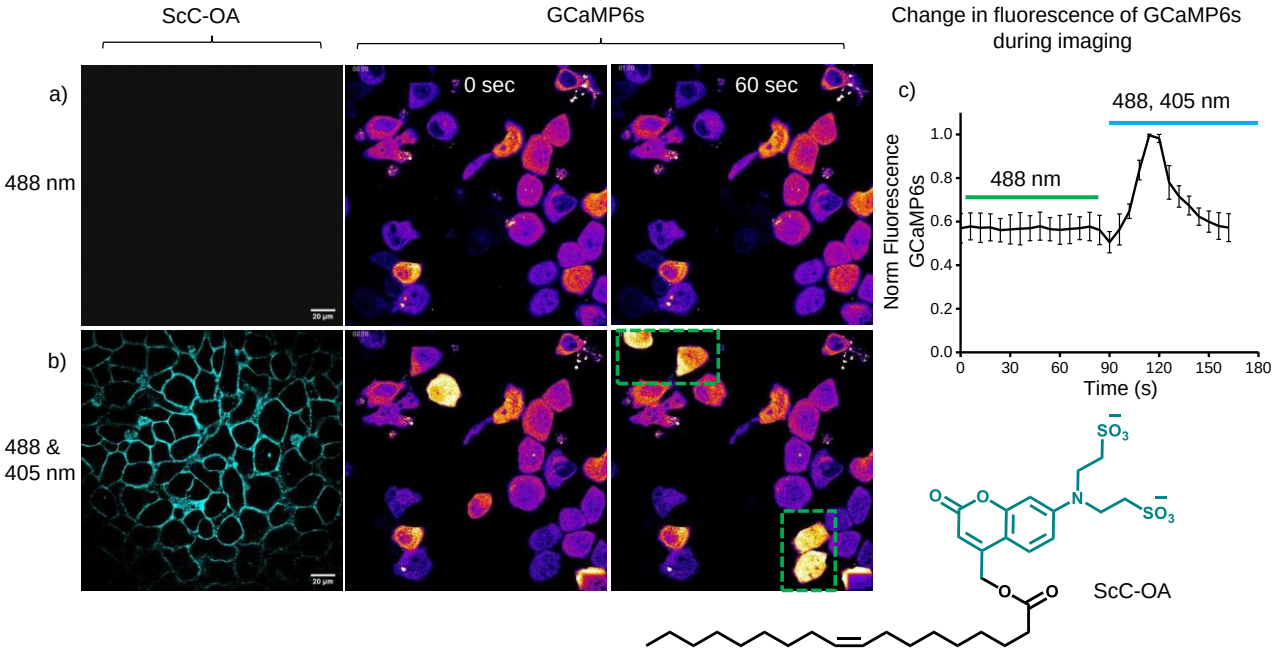


Fig. S10 Time-lapse imaging of HeLa cells expressing GCaMP6s after incubation with ScC-OA: a) λ_{ex} = 488 nm, λ_{em} = 495-555 nm; b) λ_{ex} = 488 nm, λ_{em} = 495-555 nm and λ_{ex} = 405 nm, λ_{em} = 425-485 nm for GCaMP6s and ScC-OA respectively and c) change in Ca^{2+} level during time-lapse imaging. Green rectangles show the elevation in Ca^{2+} levels due to unintended release of oleic acid in the presence of 405 nm laser. Imaging was performed in live HeLa cells at 37°C and 5%CO_2. Data represent the average of three regions of interests. Error bars represent SD. Magnification 63x, scale bar is 20 µm.

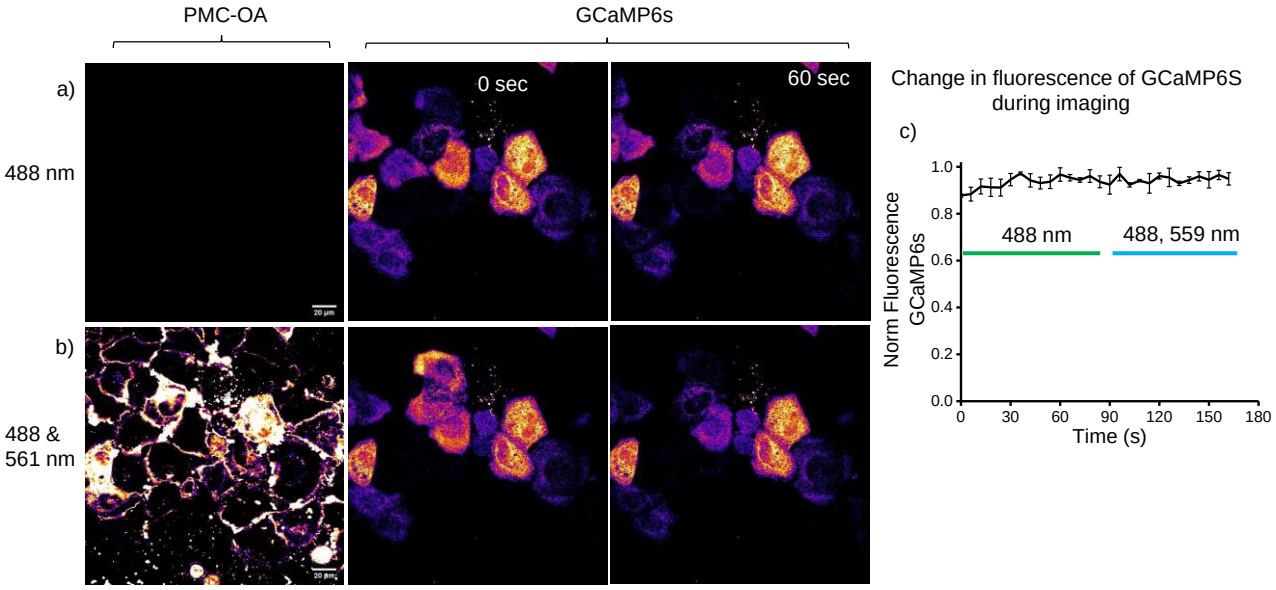


Fig. S11 Time-lapse imaging of HeLa cells expressing GCaMP6s after incubation with PMC-OA: a) λ_{ex} = 488 nm, λ_{em} = 495-555 nm; b) λ_{ex} = 488 nm, λ_{em} = 495-555 nm and λ_{ex} = 561 nm, λ_{em} = 585-650 nm for GCaMP6s and PMC-OA respectively and c) unaffected Ca^{2+} levels due to stability of ONB-Rh scaffold to the uncaging during imaging. Imaging was peroformed in live HeLa cells at 37ºC and 5%CO_2. Data represent the average of three regions of interests. Error bars represent SD. Magnification 63x, scale bar is 20 µm.

Semi-orthogonal uncaging

HeLa cells expressing GCaMP6s and GPR40 were loaded with 90 µM ScC-OA and 30 µM PMC-OA10 min before the experiment. Time-lapse imaging was performed at 37ºC and 5%CO_2 using three lasers 405 nm, 488 nm and 561 nm to measure the fluorescence of ScC-OA, GCaMP6s and PMC-OA. First, the uncaging was performed in the left half of the cells using 405 nm laser (3 frames, 6 sec/frame) and the fluorescence of ScC-OA, GCaMP6s and PMC-OA was measured. Then the uncaging was performed in the right half of the cells using 375 nm laser (5 frames, 6 sec/frame) and the fluorescence of ScC-OA, GCaMP6s and PMC-OA was measured.

5. NMR spectra

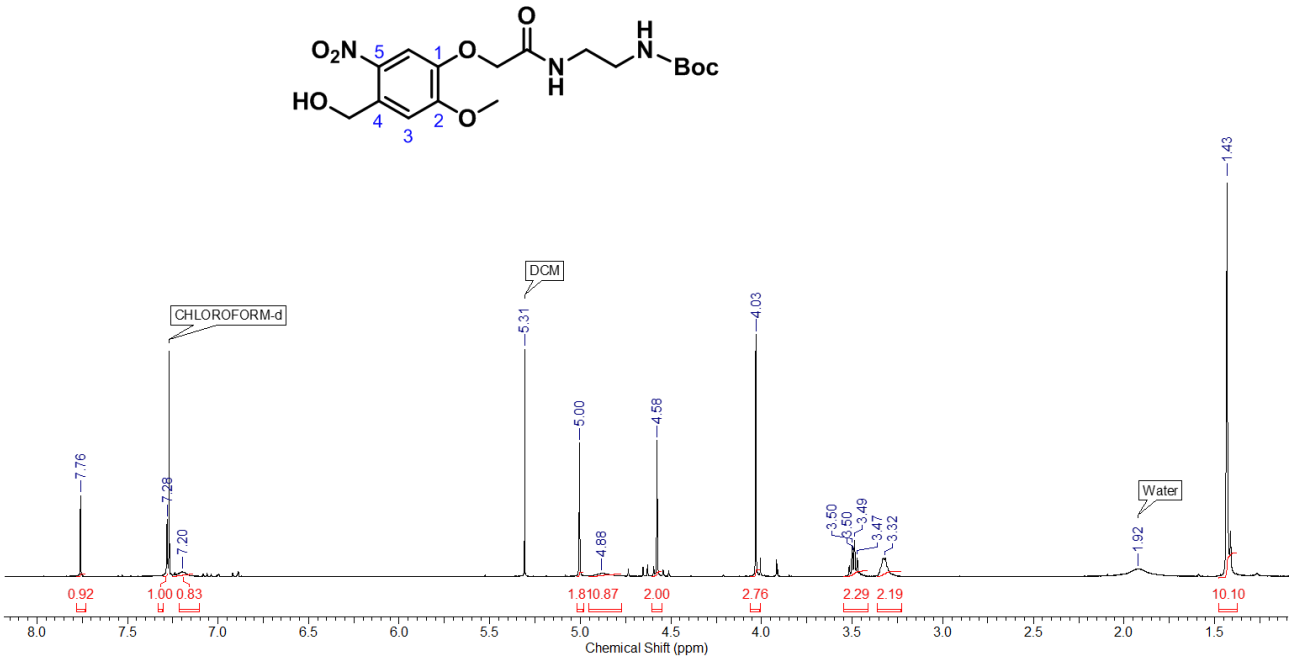


Fig. S12 ^{1}H NMR spectrum of **Compound 1** in $CDCl_3$

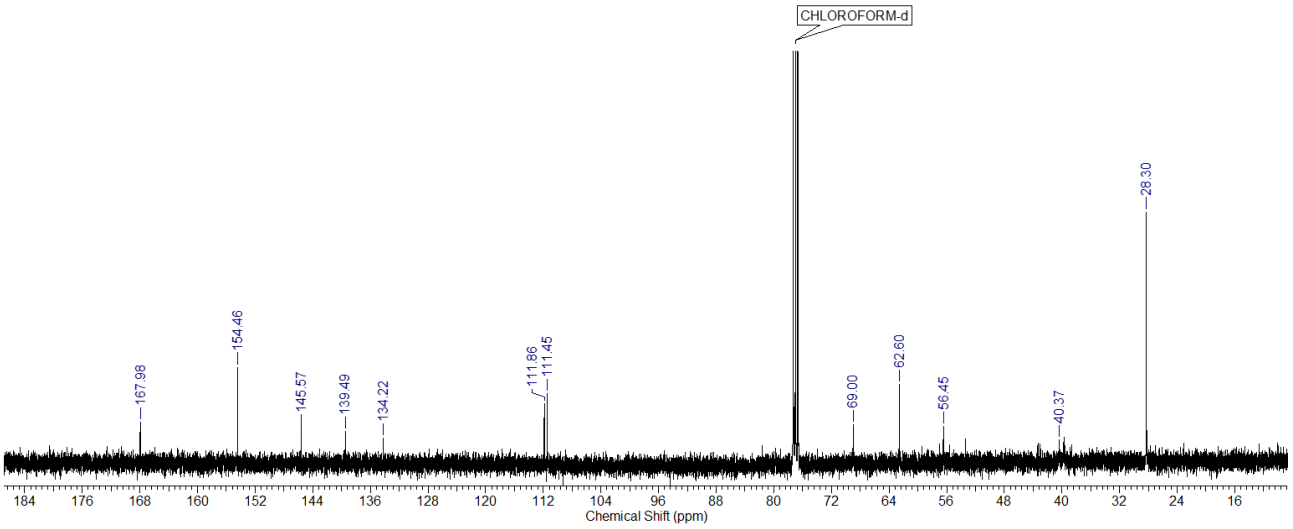


Fig. S13 ^{13}C NMR spectrum of **Compound 1** in $CDCl_3$.

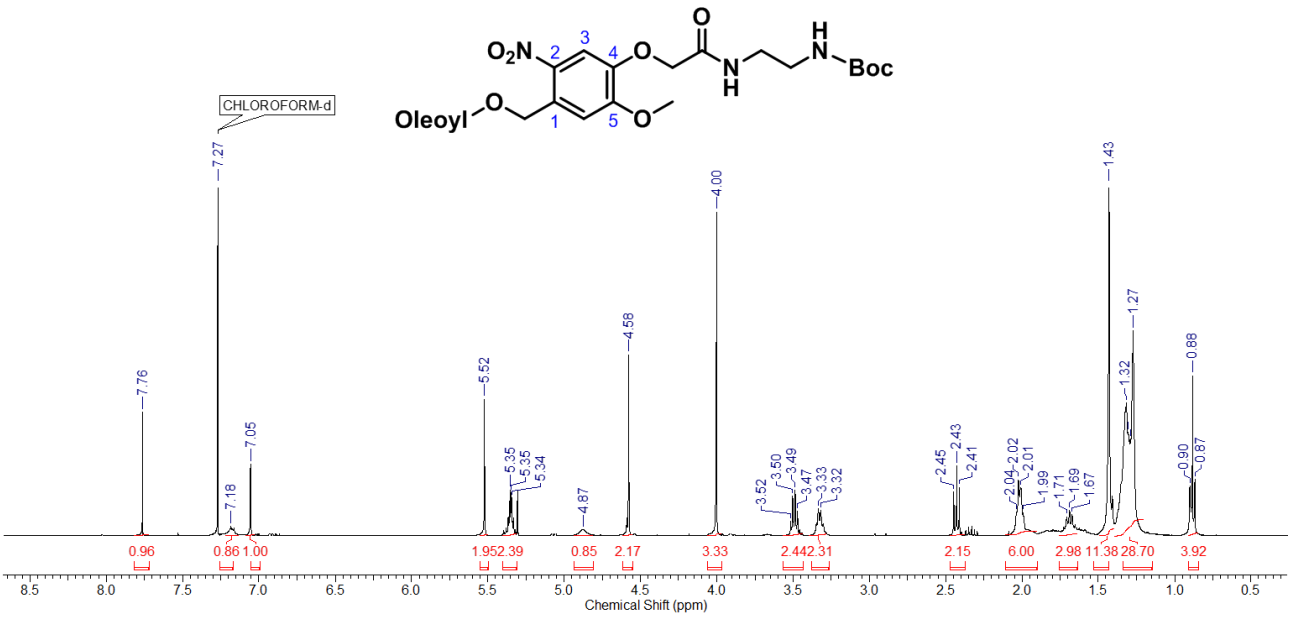


Fig. S14 ^{1}H NMR spectrum of **Compound 2** in $CDCl_3$

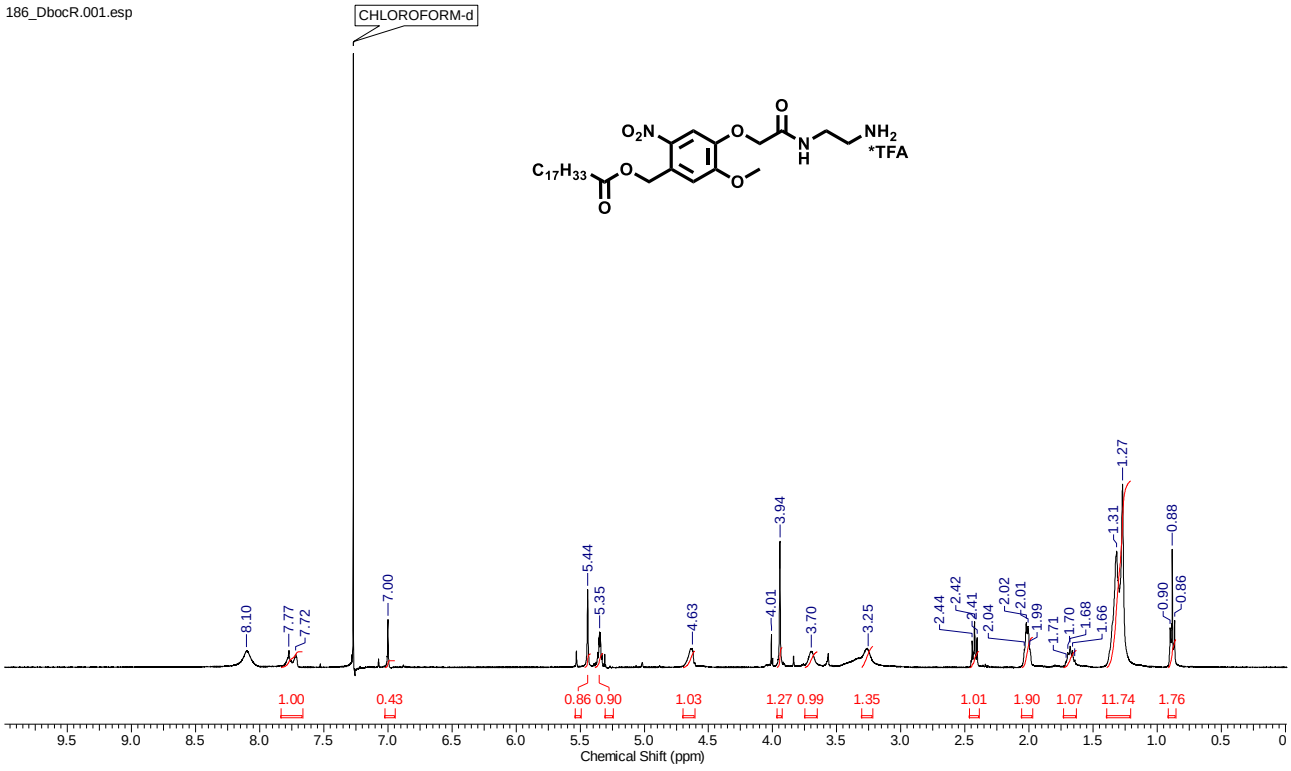


Fig. S15 ^{1}H NMR spectrum of **Compound 3** in $CDCl_3$

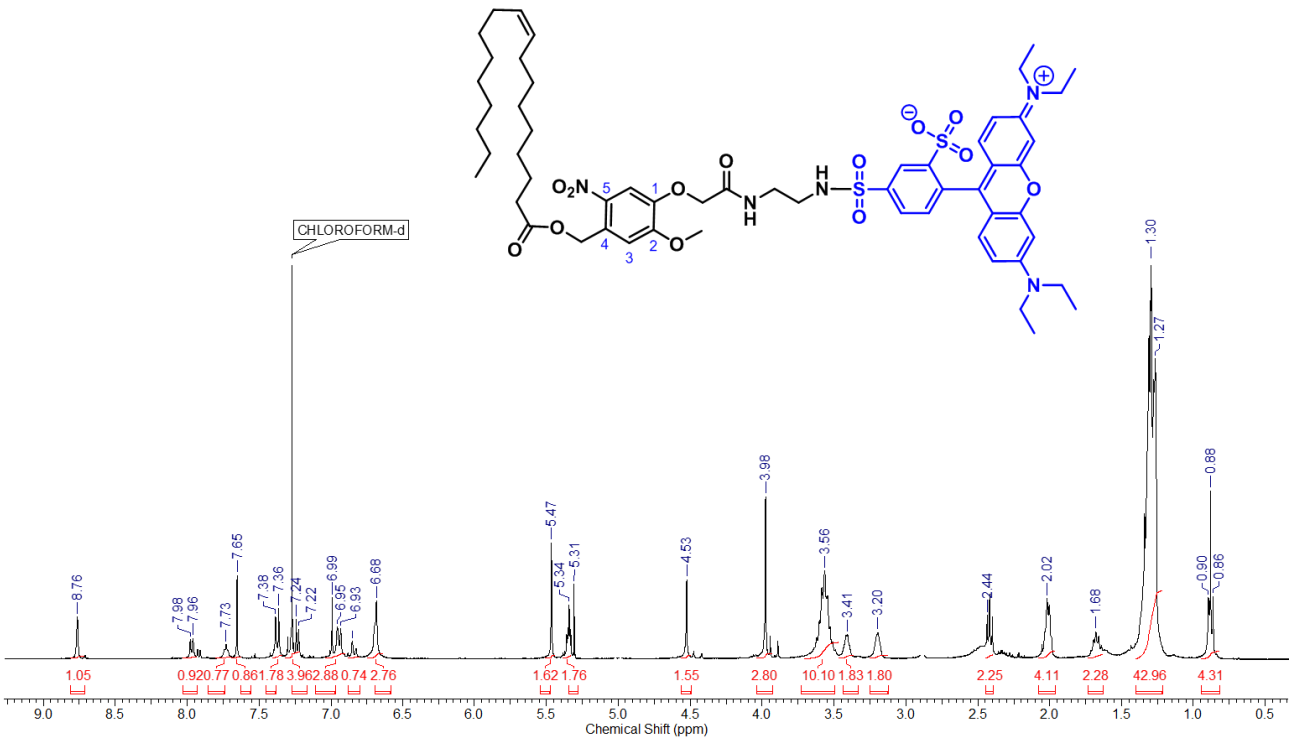


Fig. S16 ^{1}H NMR spectrum of **Compound IMC2-OA** in $CDCl_3$

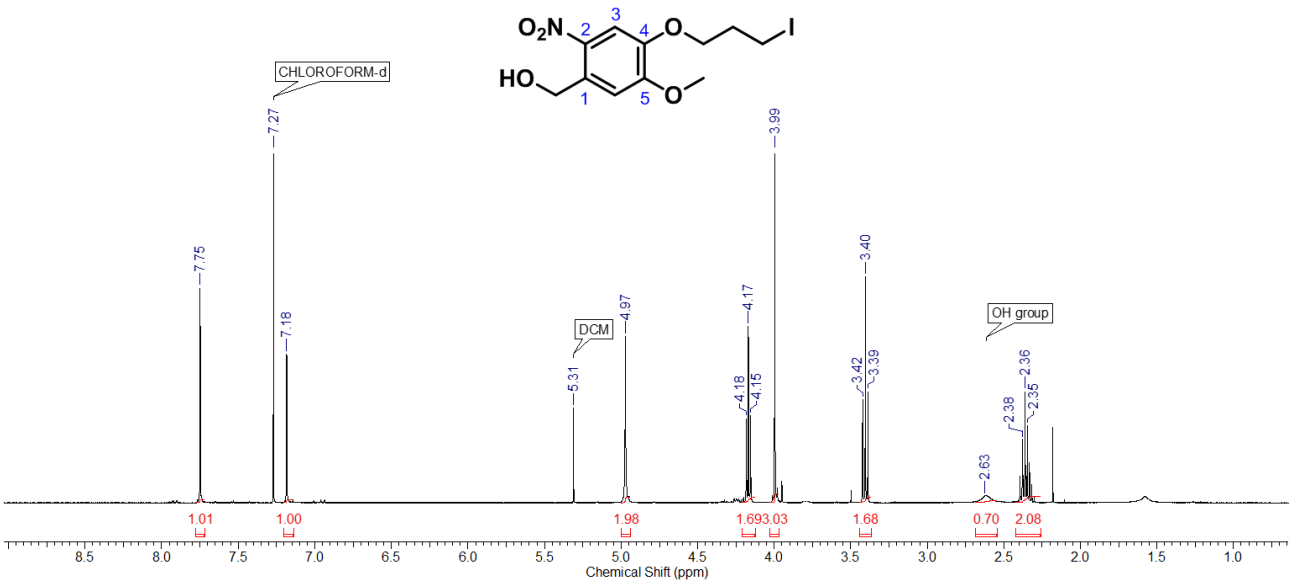


Fig. S17 ^{1}H NMR spectrum of **Compound 4** in $CDCl_3$

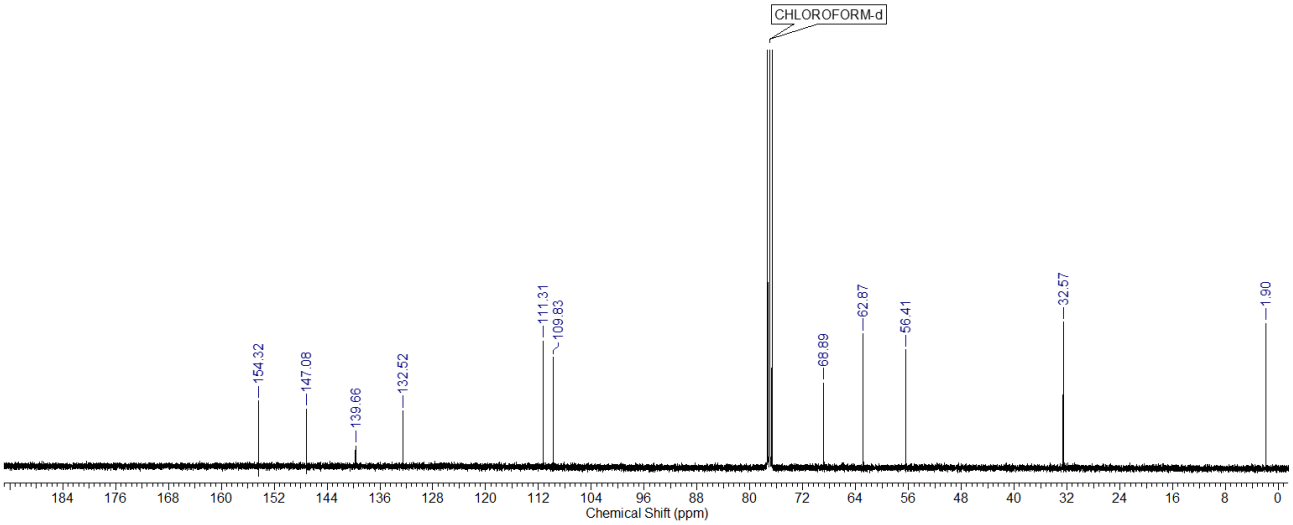


Fig. S18 ^{13}C NMR spectrum of **Compound 4** in $CDCl_3$

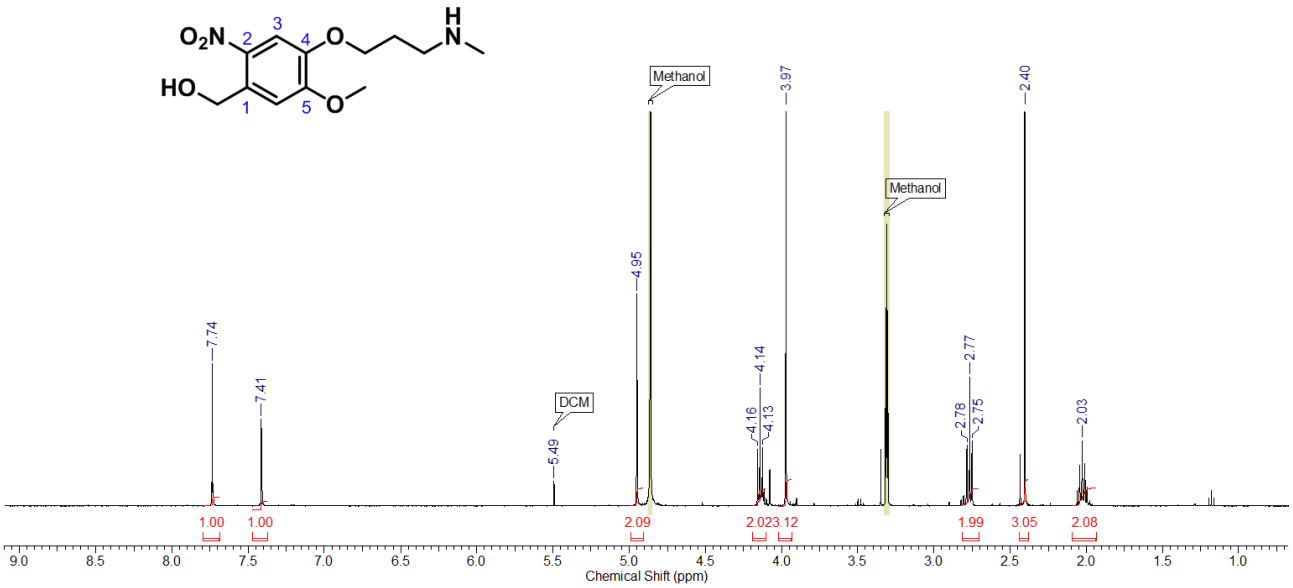


Fig. S19 ^{1}H NMR spectrum of **Compound 5** in CD_3OD

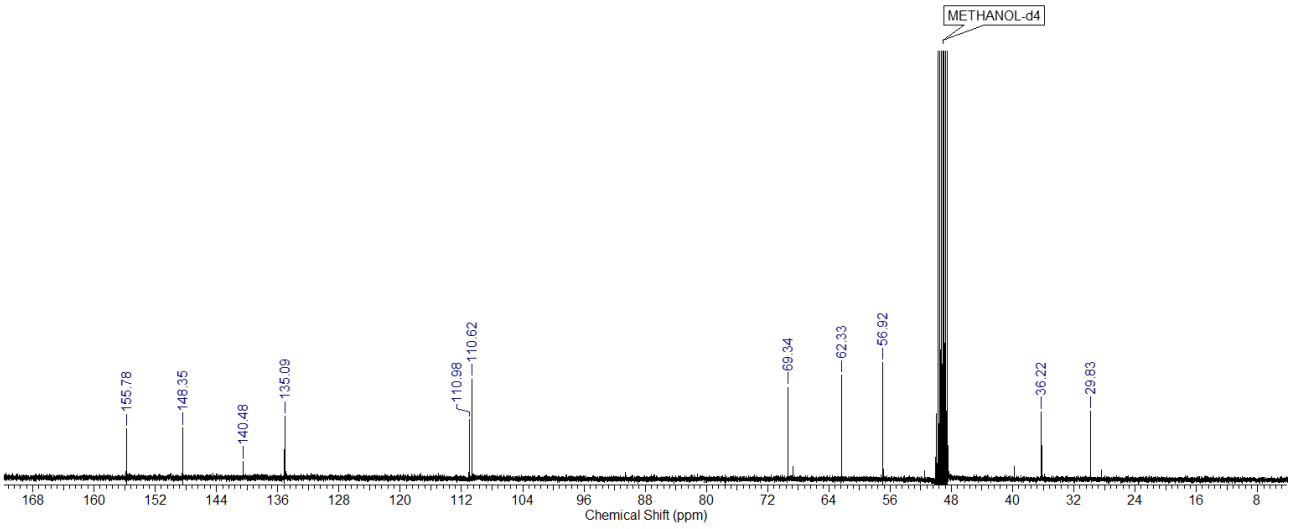


Fig. S20 ^{13}C NMR spectrum of **Compound 5** in CD_3OD

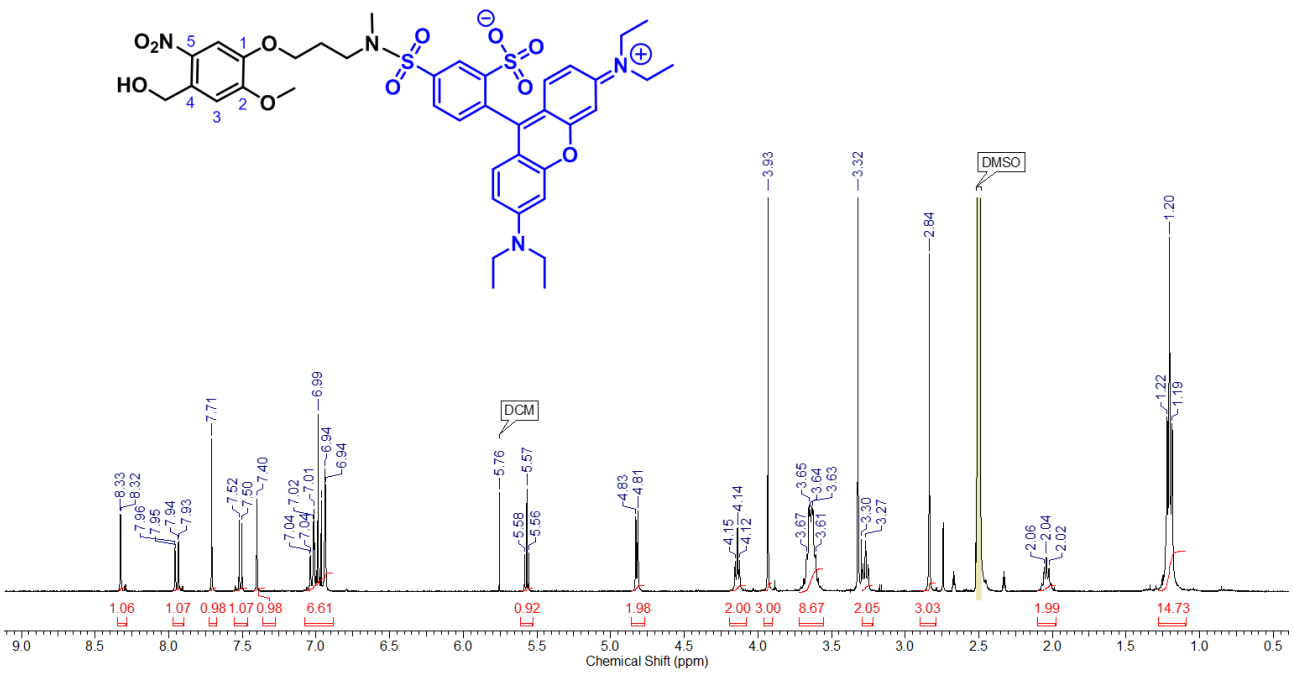


Fig. S21 ^{1}H NMR spectrum of **Compound 6** in DMSO-d_6

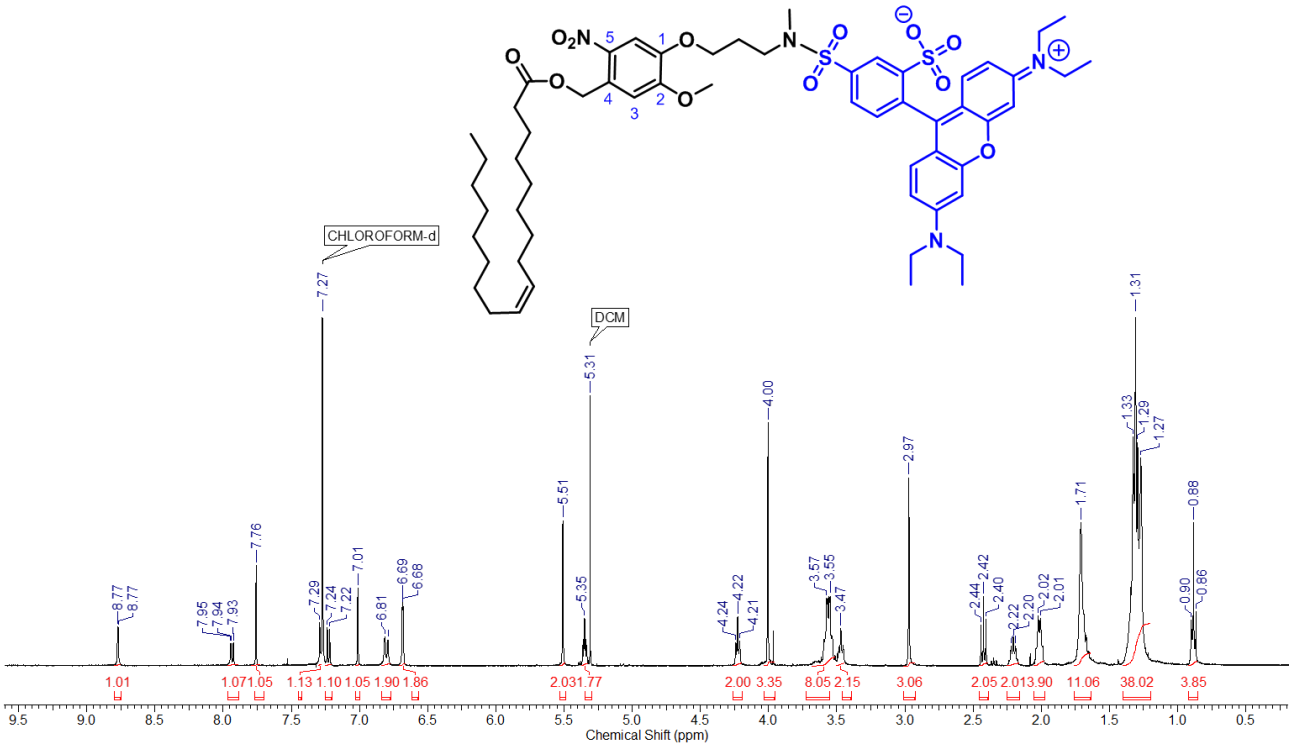


Fig. S22 ^{13}H NMR spectrum of **IMC-OA** in $CDCl_3$.

6. References

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