

## Supporting information

# Tailor-made switchable solvatochromic probes for live-cell super-resolution imaging of plasma membrane organization

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## Materials and Methods

**Synthesis and characterization of probes.** General methods and materials. All the reagents were purchased from Sigma-Aldrich or Alfa Aesar or TCI and used as received. MilliQ-water (Millipore) was used in all experiments. NMR spectra were recorded at 20°C on a BrukerAvance III 400 spectrometer. Mass spectra were obtained using an Agilent Q-TOF 6520 mass spectrometer. Absorption and emission spectra were recorded on an Edinburgh FS5 spectrofluorometer equipped with a thermostated cell compartment. Fluorescence quantum yields were measured using Nile Red (NR) in methanol ( $\lambda_{\text{ex}} = 520$  nm,  $\text{QY}_{\text{ref}} = 38\%^{[1]}$ ) as a reference.

**Ethyl 4-((3-methoxyphenyl)amino)butanoate (1a).** 5 g of m-anisidine were mixed with 12.34 g (2.2 eq.) of freshly ground  $\text{K}_2\text{CO}_3$  and 1.35 g (0.2 eq.) of KI in 40 ml of dry DMF, after that 7.93 g of ethyl 4-bromobutanoate was added and the mixture was stirred at 60 °C for 6h (control by TLC) under Ar atmosphere. After the reaction the solvent was evaporated *in vacuo*, the solid residue was dissolved in  $\text{Et}_2\text{O}$  and washed with water and brine. The organic phase was dried over  $\text{Na}_2\text{SO}_4$  and the solvent was evaporated *in vacuo*. The crude product was purified by column chromatography ( $\text{SiO}_2$ ; Hept:EtOAc 3:1, dry deposit on celite). The product is to be kept under Ar atmosphere due to oxidation. Compound **1a**: yield 7.69g (79.8 %) as colourless oil.  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  ppm 1.23 – 1.30 (td,  $J=7.0$ , 1.0 Hz, 3 H) 1.95 (quin,  $J=7.0$  Hz, 2 H) 2.43 (t,  $J=7.3$  Hz, 2 H) 3.17 (t,  $J=7.0$  Hz, 2 H) 3.78 (s, 3 H) 4.15 (q,  $J=7.3$  Hz, 2 H) 6.17 (t,  $J=2.4$  Hz, 1 H) 6.23 (ddd,  $J=8.1$ , 2.4, 0.8 Hz, 1 H) 6.27 (ddd,  $J=8.1$ , 2.4, 0.8 Hz, 1 H) 7.08 (t,  $J=8.1$  Hz, 1 H). **MS (ESI),  $m/z$ :**  $[\text{M}+\text{H}]^+$  calcd for  $\text{C}_{13}\text{H}_{20}\text{NO}_3^+$ , 238.14; found, 238.14.

**Ethyl 4-(ethyl(3-methoxyphenyl)amino)butanoate (2a).** 7.68 g of compound **1a** were mixed with 15.17 g (7.82 mL, 3 eq.) of ethyl iodide and 11.18 g (3 eq.) of freshly ground  $\text{K}_2\text{CO}_3$  in 40 mL of dry DMF. The reaction was stirred at 50 °C for 20h (control by TLC) under Ar atmosphere. After the reaction, the solvent was evaporated *in vacuo*, the solid residue was dissolved in DCM and washed with water and brine. The organic phase was dried over  $\text{Na}_2\text{SO}_4$  and the solvent was evaporated *in vacuo*. The crude product was purified by column chromatography ( $\text{SiO}_2$ ; Hept:EtOAc 4:1, dry deposit on celite). The product is to be kept under Ar atmosphere due to oxidation. Compound **2a**: yield 6.31g (73.5 %) as colourless oil.  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  ppm 1.16 (t,  $J=7.0$  Hz, 3 H) 1.28 (t,  $J=7.2$  Hz, 3 H) 1.93 (quin,  $J=7.3$  Hz, 2 H) 2.36 (t,  $J=7.3$  Hz, 2 H) 3.28 - 3.33 (m, 2 H) 3.36 (q,  $J=7.0$  Hz, 2 H) 3.81 (s, 3 H)

4.16 (q,  $J=7.0$  Hz, 2 H) 6.22 - 6.30 (m, 2 H) 6.33 - 6.36 (m, 1 H) 7.13 (t,  $J=8.0$  Hz, 1 H). **MS (ESI),  $m/z$ :**  $[M+H]^+$  calcd for  $C_{15}H_{24}NO_3^+$ , 266.17; found, 266.17.

**4-(ethyl(3-hydroxyphenyl)amino)butanoic acid (3a).** 5.46 g of compound **2a** were mixed with 40 mL of  $HBr_{(conc)}$  and the mixture was refluxed for 8h (control by TLC). After the reaction the solvent was evaporated *in vacuo*, followed by heating to 100 °C under high vacuum for 3h to evaporate the traces of solvent. Compound **3a**: yield 5.60 g (98.0 %) as a colourless solid.  **$^1H$  NMR (400 MHz, Methanol- $d_4$ )  $\delta$  ppm** 1.16 (t,  $J=7.3$  Hz, 3 H) 1.51 - 1.76 (m, 1 H) 1.76 - 2.03 (m, 1 H) 2.34 - 2.47 (m, 2 H) 3.60 - 3.70 (m, 5 H) 6.99 (dd,  $J=8.0, 2.3$  Hz, 1 H) 7.03 (q,  $J=2.3$  Hz, 1 H) 7.04 (dt,  $J=4.5, 1.0$  Hz, 1 H) 7.06 (dt,  $J=4.5, 1.0$  Hz, 1 H) 7.44 (t,  $J=8.0$  Hz, 1 H). **MS (ESI),  $m/z$ :**  $[M]^+$  calcd for  $C_{12}H_{17}NO_3^+$ , 223.12; found, 223.12.

**General procedure for 3-(alkylammonio)propane-1-sulfonate (6a and 6b),** adapted from elsewhere.<sup>[21]</sup> 4g of corresponding alkylamine were mixed with 1.2 eq. of 1,3-propanesultone in 30 mL of dry acetone. The reaction was refluxed under Ar atmosphere for 24h. After the reaction, the crude product was filtered off, washed 2 times with acetone and recrystallized from ethanol.

Compound **6a**: yield 4.58 g (69.2 %) as a white solid.  **$^1H$  NMR (400 MHz, DMSO- $d_6$  (60 °C))  $\delta$  ppm** 0.88 (t,  $J=6.8$  Hz, 3 H) 1.24 - 1.35 (m, 18 H) 1.57 (quin,  $J=7.6$  Hz, 2 H) 1.97 (quin,  $J=6.4$  Hz, 2 H) 2.66 (t,  $J=6.4$  Hz, 2 H) 2.88 (t,  $J=7.3$  Hz, 2 H) 3.06 (t,  $J=6.4$  Hz, 2 H). **MS (ESI),  $m/z$ :**  $[M]^+$  calcd for  $C_{15}H_{32}NO_3S^+$ , 306.21; found, 306.21. Compound **6b**: yield 6.56 g (61.4 %) as a white solid.  **$^1H$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  ppm**  $\delta$  ppm 0.89 (t,  $J=7.3$  Hz, 3 H) 1.33 (sxt,  $J=7.4$  Hz, 2 H) 1.49 - 1.58 (m, 2 H) 1.93 (quin,  $J=6.8$  Hz, 2 H) 2.62 (t,  $J=6.7$  Hz, 2 H) 2.88 (t,  $J=7.7$  Hz, 2 H) 3.04 (t,  $J=6.8$  Hz, 2 H) **MS (ESI),  $m/z$ :**  $[M]^+$  calcd for  $C_{15}H_{32}NO_3S^+$ , 194.09; found, 194.08.

**4-(ethyl-5-oxo-5H-benzo[a]phenoxazin-9-yl)amino)butanoic acid (5a).** 2.87 g of 4-(ethyl(3-hydroxyphenyl)amino)butanoic acid (**3a**) were dissolved in 10 mL of water together with 2 mL of  $HCl_{(conc)}$  at r.t. After this the mixture was cooled to 0 °C and a solution of 1.33 g (1.1 eq.) of  $NaNO_2$  in 10 mL of water was added dropwise at 0 °C. The reaction mixture was stirred for 1.5h at 0 °C, after which the water was removed by liophilization. The product, 4-(ethyl(3-hydroxy-4-nitrosophenyl)amino)butanoic acid (**4a**), was used for the next step without further purification.

3.24 g of compound **4a** were mixed with 2.78 g (1.5 eq.) of 1-naphtol and 15 ml of dry DMF. The mixture was stirred at 80 °C for 5h. After the reaction the solvent was evaporated *in vacuo*, and the residues were dissolved in DCM and filtered through  $SiO_2$ . After this, the solvent was evaporated *in vacuo*. In order to purify the compound, a methyl ester of compound **5a** was formed by an addition of 1.54 g (0.94 mL) of  $SOCl_2$  dropwise to the solution of the crude **5a** in 40 mL of MeOH at 0 °C. After 1h of stirring at 0 °C, the mixture was stirred for 20h at r.t., afterwards the solvent was evaporated *in vacuo*. The methyl ester of **5a** was purified by column chromatography ( $SiO_2$ ; Hept:EtOAc 1:1, dry deposit on celite).  **$^1H$  NMR (400 MHz,  $CDCl_3$ )  $\delta$  ppm** 1.26 (t,  $J=7.0$  Hz, 3 H) (quin,  $J=7.4$  Hz, 2 H) 2.38 - 2.47 (m, 2 H) 3.39 - 3.52 (m, 4 H) 3.73 (s, 3 H) 6.39 (s, 1 H) 6.50 (d,  $J=2.8$  Hz, 1 H) 6.69 (dd,  $J=9.0, 2.8$  Hz, 1 H) 7.61 (d,  $J=9.0$  Hz, 1 H) 7.65 (td,  $J=7.8, 1.4$  Hz, 1 H) 7.74 (dt,  $J=7.8, 1.40$  Hz, 1 H) 8.30 (dd,  $J=7.8, 1.3$  Hz, 1 H) 8.65 (dd,  $J=7.9, 0.9$  Hz, 1 H). **MS (ESI),  $m/z$ :**  $[M+Na]^+$  calcd for  $C_{22}H_{20}N_2O_4$ , 413.16; found, 413.15.

On the next step, 370 mg of methyl ester of **5a** were dissolved in 15 mL of 20%  $HCl_{(aq)}$  and the mixture was stirred for 15h at 90 °C (control by TLC). After the reaction the solvent was removed *in vacuo* and the solid residue was twice washed with water on a filter. Compound **5a**: overall yield 345 mg (7.7 %) as dark red solid.  **$^1H$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  ppm** 1.16 (t,  $J=6.9$  Hz, 3 H) 1.81 (quin,  $J=7.5$  Hz,

2 H) 2.35 (t,  $J=7.3$  Hz, 2 H) 3.44 (quin,  $J=7.5$  Hz, 2 H) 3.52 (t,  $J=6.9$  Hz, 2 H) 6.32 (s, 1 H) 6.76 (s, 1 H) 6.91 (d,  $J=9.4$  Hz, 1 H) 7.63 (d,  $J=8.5$  Hz, 1 H) 7.72 (t,  $J=7.5$  Hz, 1 H) 7.81 (t,  $J=7.5$  Hz, 1 H) 8.13 (d,  $J=7.8$  Hz, 1 H) 8.56 (d,  $J=7.8$  Hz, 1 H).  $^{13}\text{C}$  NMR (101 MHz, DMSO- $d_6$ )  $\delta$  12.73 (CH<sub>3</sub>) 22.75 (C<sub>al</sub>) 31.04 (C<sub>al</sub>) 41.28 (C<sub>al</sub>) 45.45 (C<sub>al</sub>) 49.81 (C<sub>al</sub>) 78.58 (C<sub>al</sub>) 96.77 (C<sub>ar</sub>) 104.87 (C<sub>ar</sub>) 111.19 (C<sub>ar</sub>) 123.86 (C<sub>ar</sub>) 124.23 (C<sub>ar</sub>) 125.18 (C<sub>ar</sub>) 125.47 (C<sub>ar</sub>) 130.44 (C<sub>ar</sub>) 131.45 (C<sub>ar</sub>) 132.05 (C<sub>ar</sub>) 132.08 (C<sub>ar</sub>) 135.43 (C<sub>ar</sub>) 146.95 (C<sub>ar</sub>) 174.66 (C<sub>ar</sub>) 182.03 (C<sub>ar</sub>) 192.67 (s, COOH) 217.84 (s, 1 C<sub>ar</sub>). MS (ESI),  $m/z$ : [M+Na]<sup>+</sup> calcd for C<sub>22</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub>Na, 399.14; found, 399.13.

**General procedure for 3-(N-alkyl-4(ethyl(5-oxo-5H-benzo[a]phenoxazin-9-yl)amino)butanamido)propane-1-sulfonate (NR12A and NR4A).** 30 mg of compound **5a** were dissolved in 2 mL of dry DMF together with 32.9 mg (1.05 eq.) of HATU and 20.6 mg (28  $\mu\text{L}$ , 2 eq.) of DIPEA. After 5 minutes, a solution of 1.05 eq. of the corresponding 3-(alkylammonio)propane-1-sulfonate (**6a** or **6b**) and 20.6 mg (28  $\mu\text{L}$ , 2 eq.) of DIPEA in 2 mL of dry DMF was added and the mixture was stirred for 24h at r.t. under Ar atmosphere (control by TLC). After the reaction the solvent was evaporated *in vacuo* and the crude product was purified by gradient column chromatography (SiO<sub>2</sub>; DCM:MeOH 88:12 to DCM:MeOH 85:15 for **NR12A** and DCM:MeOH 85:15 to DCM:MeOH 82:18 for **NR4A**). Compound **NR12A**: yield 29.9 mg (47.2 %) as dark red solid.  $^1\text{H}$  NMR (400 MHz, Methanol- $d_4$ )  $\delta$  ppm 0.81 - 0.92 (m, 3 H) 1.11 - 1.33 (m, 19 H) 1.34 - 1.40 (m, 4 H) 1.47 - 1.62 (m, 2 H) 1.91 - 2.00 (m, 2 H) 2.00 - 2.10 (m, 2 H) 2.41 - 2.58 (m, 2 H) 2.78 - 2.89 (m, 2 H) 3.36 - 3.58 (m, 6 H) 6.23 (s, 1 H) 6.55 (dd,  $J=8.8, 2.5$  Hz, 1 H) 6.83 (ddd,  $J=12.0, 9.3, 2.5$  Hz, 1 H) 7.50 (d,  $J=9.3$  Hz, 1 H) 7.57 - 7.66 (m, 1 H) 7.66 - 7.76 (m, 1 H) 8.13 (d,  $J=7.8$  Hz, 1 H) 8.53 (d,  $J=7.8$  Hz, 1 H).  $^{13}\text{C}$  NMR (101 MHz, Methanol- $d_4$ )  $\delta$  ppm 15.29 (CH<sub>3</sub>) 15.71 (CH<sub>3</sub>) 16.99 (C<sub>al</sub>) 19.91 (C<sub>al</sub>) 21.32 (C<sub>al</sub>) 26.24 (C<sub>al</sub>) 26.70 (C<sub>al</sub>) 27.12 (C<sub>al</sub>) 28.22 (C<sub>al</sub>) 32.98 (C<sub>al</sub>) 33.08 (C<sub>al</sub>) 33.15 (C<sub>al</sub>) 33.27 (C<sub>al</sub>) 35.58 (C<sub>al</sub>) 46.38 (C<sub>al</sub>) 58.42 (C<sub>al</sub>) 99.98 (C<sub>ar</sub>) 108.04 (C<sub>ar</sub>) 114.78 (C<sub>ar</sub>) 127.40 (C<sub>ar</sub>) 128.84 (C<sub>ar</sub>) 129.18 (C<sub>ar</sub>) 133.36 (C<sub>ar</sub>) 135.02 (C<sub>ar</sub>) 135.21 (C<sub>ar</sub>) 135.96 (C<sub>ar</sub>) 141.93 (C<sub>ar</sub>) 150.61 (C<sub>ar</sub>) 155.78 (C<sub>ar</sub>) 156.50 (C<sub>ar</sub>) 176.93 (C<sub>ar</sub>) 187.84 (C<sub>ar</sub>). MS (ESI),  $m/z$ : [M]<sup>-</sup> calcd for C<sub>37</sub>H<sub>50</sub>N<sub>3</sub>O<sub>6</sub>S<sup>-</sup>, 664.34; found, 664.34. Compound **NR4A**: yield 32.8 mg (60.5 %) as dark red solid.  $^1\text{H}$  NMR (400 MHz, Methanol- $d_4$ )  $\delta$  ppm 0.96 (dt,  $J=9.1, 7.4$  Hz, 3 H) 1.21 (t,  $J=7.0$  Hz, 3 H) 1.30 - 1.41 (m, 4 H) 1.51 - 1.61 (m, 2 H) 1.82 - 1.97 (m, 2 H) 1.97 - 2.12 (m, 2 H) 2.39 - 2.57 (m, 2 H) 2.79 - 2.89 (m, 2 H) 3.34 - 3.57 (m, 6 H) 6.11 (d,  $J=2.0$  Hz, 1 H) 6.42 (dd,  $J=9.2, 2.7$  Hz, 1 H) 6.72 (td,  $J=8.8, 2.7$  Hz, 1 H) 7.37 (dd,  $J=9.2, 3.4$  Hz, 1 H) 7.52 - 7.59 (m, 1 H) 7.64 (td,  $J=7.5, 1.3$  Hz, 1 H) 8.05 (dd,  $J=7.8, 1.00$  Hz, 1 H) 8.38 - 8.44 (m, 1 H).  $^{13}\text{C}$  NMR (101 MHz, Methanol- $d_4$ )  $\delta$  15.39 (CH<sub>3</sub>) 15.73 (CH<sub>3</sub>) 16.83 (C<sub>al</sub>) 19.92 (C<sub>al</sub>) 21.36 (C<sub>al</sub>) 26.67 (C<sub>al</sub>) 27.18 (C<sub>al</sub>) 28.24 (C<sub>al</sub>) 33.27 (C<sub>al</sub>) 34.70 (C<sub>al</sub>) 46.39 (C<sub>al</sub>) 58.42 (C<sub>al</sub>) 99.86 (C<sub>ar</sub>) 107.95 (C<sub>ar</sub>) 114.70 (C<sub>ar</sub>) 127.32 (C<sub>ar</sub>) 128.76 (C<sub>ar</sub>) 129.08 (C<sub>ar</sub>) 133.27 (C<sub>ar</sub>) 134.93 (C<sub>ar</sub>) 135.14 (C<sub>ar</sub>) 135.84 (C<sub>ar</sub>) 141.79 (C<sub>ar</sub>) 150.44 (C<sub>ar</sub>) 155.62 (C<sub>ar</sub>) 156.32 (C<sub>ar</sub>) 176.84 (C<sub>ar</sub>) 187.68 (C<sub>ar</sub>). MS (ESI),  $m/z$ : [M]<sup>+</sup> calcd for C<sub>29</sub>H<sub>34</sub>N<sub>3</sub>O<sub>6</sub>S<sup>+</sup>, 552.22; found, 552.21.

**Preparation of liposomes.** All types of LUVs used were prepared by the following procedure. A stock solution of corresponding lipid(s) in chloroform was placed into a round-neck flask, after which the solvent was evaporated *in vacuo* and phosphate buffer (20 mM, pH 7.4) was added. After all the solid was dissolved a suspension of multilamellar vesicles was extruded by using a Lipex Biomembranes extruder (Vancouver, Canada). The size of the filters was first 0.2  $\mu\text{m}$  (7 passages) and thereafter 0.1  $\mu\text{m}$  (10 passages). This generates monodisperse LUVs with a mean diameter of 0.12  $\mu\text{m}$  as measured with a

Malvern Zetamaster 300 (Malvern, U.K.). Phospholipid:Cholesterol molar ratio in case of DOPC/Chol and Sphing/Chol was 1:0.9.

**Preparation of giant unilamellar vesicles (GUVs).** GUVs were prepared by electroformation in a home-built liquid cell (University of Odense, Denmark), using previously described procedure.<sup>[3]</sup> A 0.1 mM solution of lipids in chloroform was deposited on the platinum wires of the chamber, and the solvent was evaporated under vacuum for 30 min. The chamber, thermostatted at 55 °C, was filled with a 300 mM sucrose solution, and a 2-V, 10-Hz alternating electric current was applied to this capacitor-like configuration for *ca.* 2 h. Then, a 50 µL aliquot of the obtained stock solution of GUVs in sucrose (cooled down to room temperature) was added to 200 µL of 300 mM glucose solution containing 200 nM of the probe (obtained by adding corresponding dye stock solution in DMSO to glucose solution, final DMSO concentration <0.32%) to give the final suspension of stained GUVs used in microscopy experiments.

**Preparation of supported lipid bilayers.** Supported lipid bilayers were prepared by vesicle fusion of small unilamellar vesicles (SUVs) in a similar fashion as the previous work.<sup>[4]</sup> Briefly, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and sphingomyelin were purchased from Avanti Polar Lipids (850375C, 860062C). Cholesterol was purchased from Sigma (C8667). Each lipid was separately dissolved in chloroform. To prepare DOPC SUVs, the DOPC stock solution was transferred to a pre-cleaned round-bottom flask, and chloroform was evaporated under nitrogen flow. For ternary mixture SUVs, the lipid solutions were mixed in a pre-cleaned round-bottom flask at the designated ratios, and chloroform was evaporated under nitrogen flow. The lipid cake was rehydrated with ~60 °C milli-Q water to the concentration of ~2 mg/mL, then sonicated at ~60 °C for ~40 min until clear SUV suspension was formed. To form a supported lipid bilayer, the SUV suspension and buffer solution (150 mM NaCl, 10 mM HEPES, 3 mM CaCl<sub>2</sub>) were gently added to a pre-cleaned, hydrophilic coverslip at 1:4 ratio. The coverslip was incubated at ~60 °C for ~15 min, and then was thoroughly rinsed with Dulbecco's Phosphate Buffered Saline (DPBS). The lipid bilayer was imaged with 10 nM NR12A, NR4A, NR12S, or Nile Red in DPBS.

**Cell Lines, Culture Conditions, and Treatment.** HeLa cells (ATCC CCL-2) and KB ATCC CCL-17) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco Invitrogen), supplemented with 10% fetal bovine serum (FBS, Lonza), 1% l-Glutamine (Sigma Aldrich) and 1% antibiotic solution (penicillin-streptomycin, Gibco-Invitrogen) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were seeded onto a chambered coverglass (IBiDi) at a density of 5×10<sup>4</sup> cells/well 24 h before the microscopy measurement. For conventional microscopy experiments, the attached HeLa cells in ABIDI dishes were washed twice with warm Dulbecco's Phosphate buffered saline (DPBS, Dominique Dutscher), after that 1 mL of 20 nM dye solution in DPBS was added and the cells were incubated for 10 min. at r.t. Concentrations used for photobleaching experiments were 20 nM for NR4A, NR12S, and NR; 7 nM for NR12A. For super-resolution measurements, COS-7 (ATCC CRL-1651) and HeLa (ATCC CCL-2) cells were maintained in DMEM supplemented with 10% FBS in 5% CO<sub>2</sub> at 37 °C. For imaging, cells were plated on 18-mm #1.5 glass coverslips. When cells reached ~50% confluency after 24~48 hr, the coverslip was mounted on a metal holder (CSC-18; Bioscience Tools) in the imaging media (L15 with 20 mM HEPES). Cells were imaged with 10 nM NR4A or Nile Red, or 20 nM NR12S in the imaging media at room temperature. For fixed cell experiments, cells were fixed in 3% paraformaldehyde + 0.1%

glutaraldehyde in DPBS for 20 min at room temperature. After fixation, the sample was treated with freshly prepared 0.1% NaBH<sub>4</sub> in DPBS for 5 min, and thoroughly washed with DPBS three times.

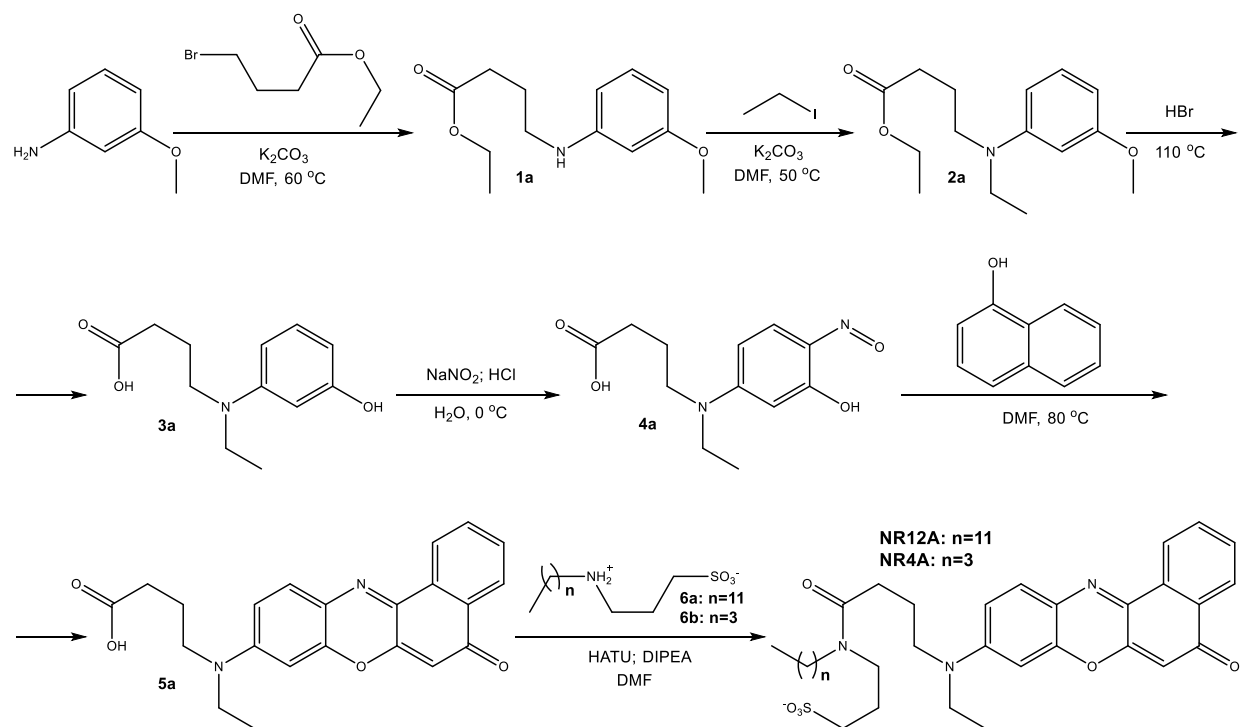
**Fluorescence microscopy.** Cellular imaging of cells was performed using Nikon Ti-E inverted microscope, equipped with CFI Plan Apo ×60 oil (NA = 1.4) objective, X-Light spinning disk module (CREST Optics) (in case of confocal imaging) and a Hamamatsu Orca Flash 4 sCMOS camera with a bandpass filter 593 ± 20 nm (Semrock FF01-593/40-25). The excitation in confocal mode was provided by a 532 nm diode laser (OXXIUS). The exposure time in confocal mode was set to 500 ms per image frame. For photobleaching experiments and widefield imaging, the excitation was provided by light emitting diode (SpectraX) at 550 nm. The 550-nm light power was set to 4.33 mW at the sample level. The diameter of the wide-field illumination spot was 420 μm, according to a photobleaching test, so that the power density used at 550 nm was 3.14 W·cm<sup>-2</sup>. The exposure time in widefield mode was set to 500 ms per image frame. Photobleaching tests were performed by illuminating the samples continuously over 60 seconds in widefield mode, followed by imaging immediately after the illumination and after incubation for 10 min at r.t. All the images were recorded using NIS Elements and then processed using Fiji software.

Confocal imaging of GUVs was performed on a Leica TSC SPE confocal microscope with HXC PL APO 63x/1.40 OIL CS objective. The excitation light was provided by a 488 nm laser while the fluorescence was detected at two spectral ranges: 550–600 nm (I<sub>550-600</sub>) and 600–650 nm (I<sub>600-650</sub>) in a sequential mode with rapid alternation of the detection wavelengths to minimize drift effect. Then, an average of 4 to 5 images in each channel was used. All the detection parameters at each channel were kept constant, while the laser excitation power was adjusted to achieve good signal for each sample. The laser power settings (% of maximal intensity) were: 30% for DOPC GUVs with all probes, 10% for SM/Chol and DOPC/SM/Chol GUVs with NR12A, NR12S and Nile Red and 100% for SM/Chol and DOPC/SM/Chol GUVs with NR4A. The ratiometric images were generated by using special macros under ImageJ (developed by Romain Vauchelles) that divides the image of the I<sub>550-600</sub> channel by that of the I<sub>600-650</sub> channel. For each pixel, a pseudo-color scale is used for coding the ratio, while the intensity is defined by the integral intensity recorded for both channels at the corresponding pixel.

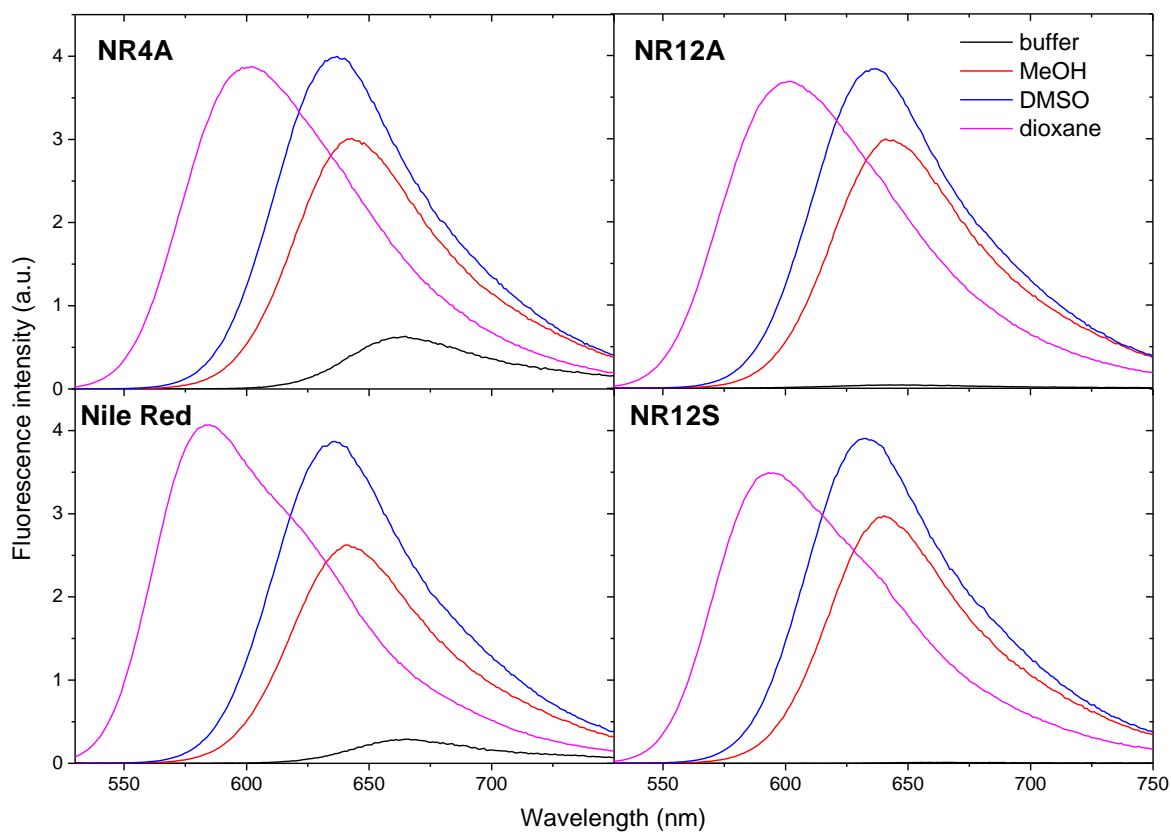
**3D-PAINT and SR-PAINT super-resolution microscopy.** 3D-PAINT and SR-PAINT imaging were performed with a home-built setup based on a Nikon Ti-E inverted fluorescence microscope, as previously described.<sup>[4]</sup> Briefly, a 561-nm laser (Coherent) was coupled to the back focal plane of an oil-immersion objective lens (Nikon CFI Plan Apochromat lambda 100x, NA1.45). A translational stage shifted the laser to the edge of the objective, thus illuminating ~1 μm into the sample at a typical power density of ~2 kW/cm<sup>2</sup>. For 3D-PAINT, emission was filtered by a bandpass filter (ET605/70m, Chroma), and passed through a cylindrical lens ( $f = 1000$  mm) to achieve astigmatism for axial localization.<sup>[5]</sup> Final image was projected onto an EM-CCD camera (iXon Ultra 897, Andor), which operated at 110 frames per second for 256x256 pixels for ~50,000 frames, or 200 frames per second for 256x128 pixels for ~100,000 frames. For SR-PAINT, emission was filtered with a long-pass filter (ET575lp, Chroma) and a short-pass filter (FF01-758/SP, Semrock), and cropped at the image plane of the microscope camera port to a width of ~4 mm. The cropped intermediate image was collimated by an achromatic lens ( $f = 80$  mm), and was split into two paths at a 50:50 beam splitter (BSW10, Thorlabs). In Path 1, emission was

focused by an achromatic lens ( $f = 75$  mm) onto one-half of an EM-CCD camera. In Path 2, emission was dispersed by an equilateral calcium fluoride prism (PS863, Thorlabs) before being focused by an achromatic lens ( $f = 60$  mm) onto the other half of the same camera. Wavelength calibration was performed using fluorescent beads and narrow bandpass filters, as previously described.<sup>[6]</sup> The acquired single-molecule data were analyzed as described previously,<sup>[4,6]</sup> with time sequences of PAINT images generated using single-molecule data accumulated every  $\sim 1$  min.

## Supporting figures

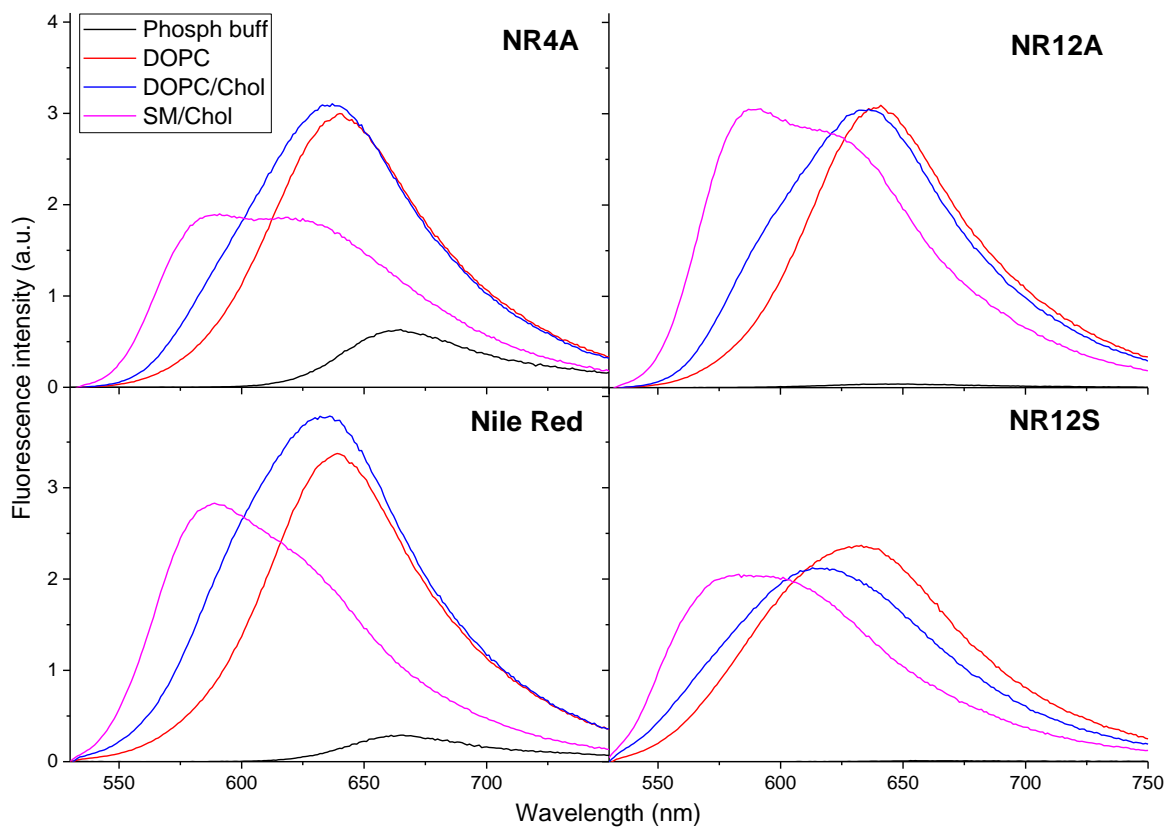


**Fig. S1.** Synthesis scheme for NR12A and NR4A membrane probes.

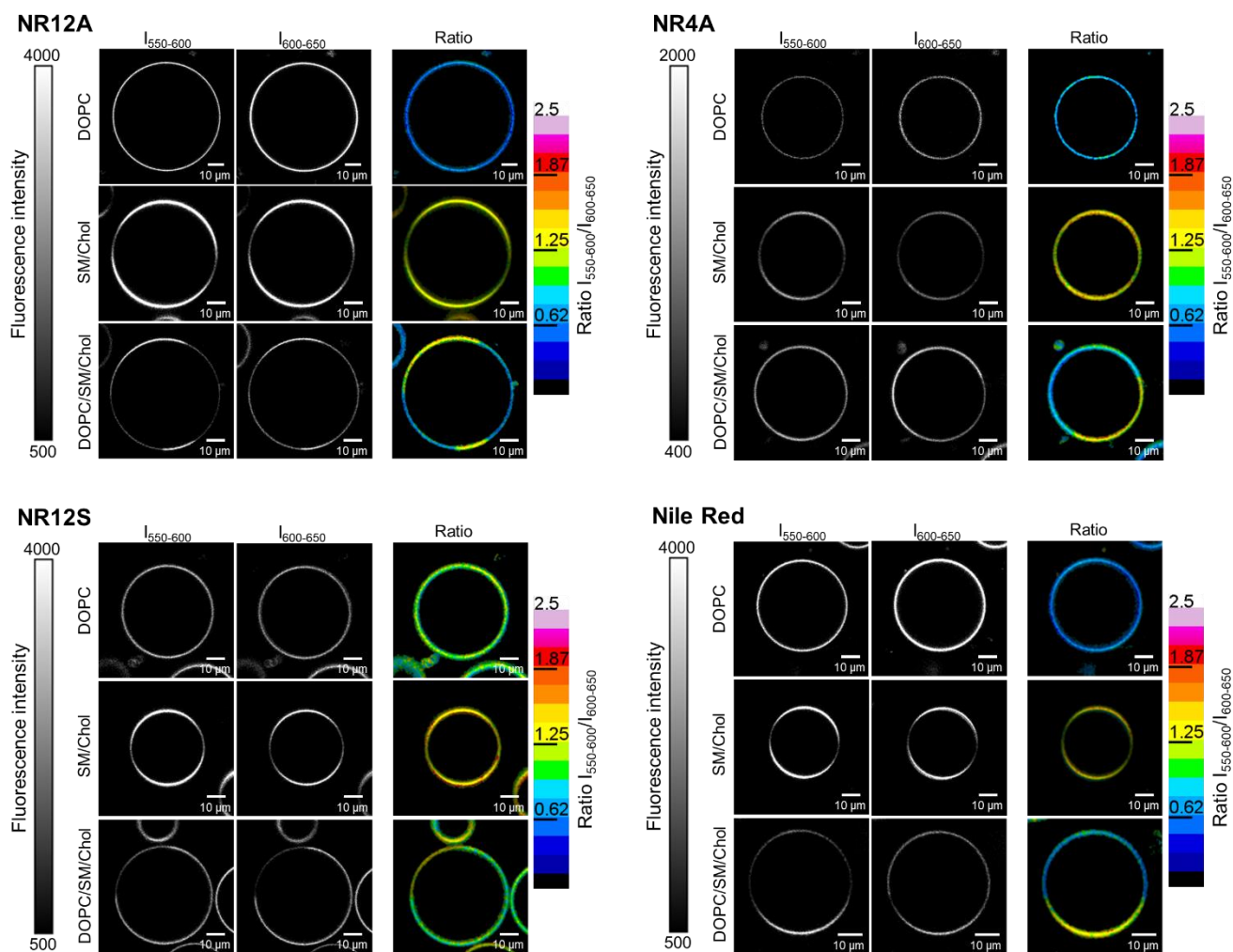


**Fig. S2.** Fluorescence emission spectra of membrane probes NR4A, NR12A, Nile Red, and NR12S in different solvents. Probe concentration 2  $\mu$ M. Excitation wavelength was 520 nm. All the spectra were corrected via dividing by absorbance at the excitation wavelength.

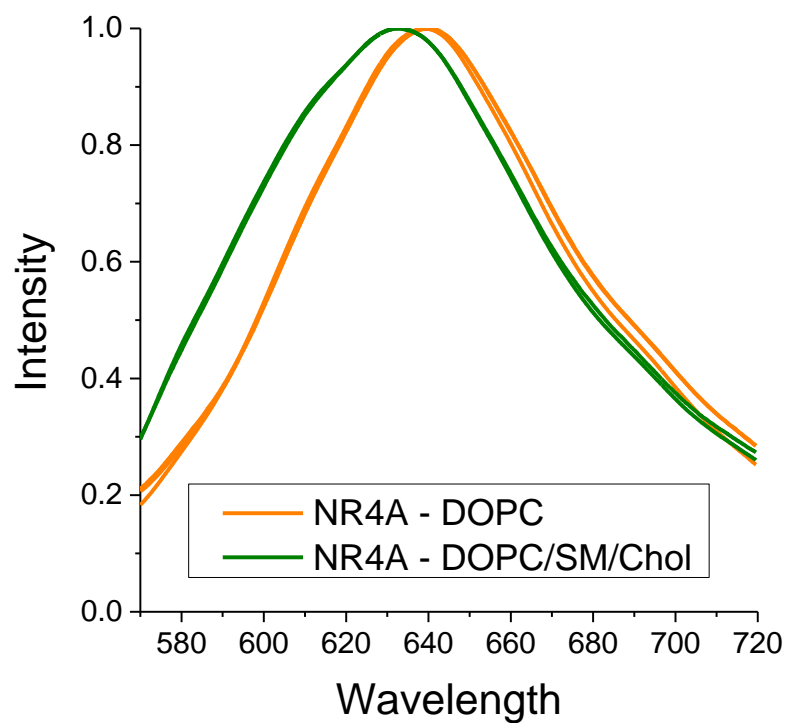




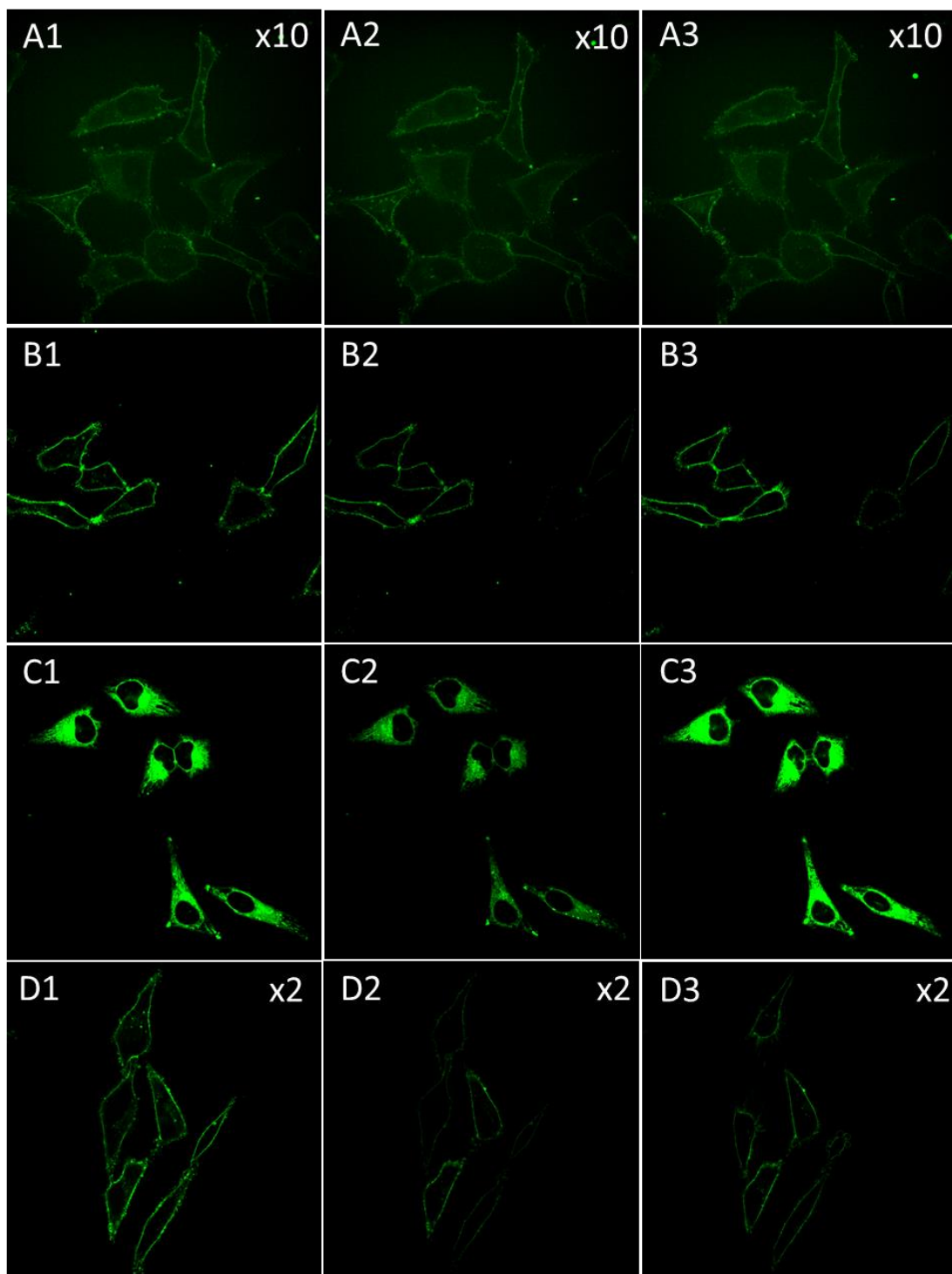
**Fig. S3.** Fluorescence emission spectra of membrane probes NR4A, NR12A, Nile Red, and NR12S in phosphate buffer and liposomes (LUVs) of different composition. Probe concentration 2  $\mu\text{M}$ , lipid concentration 200  $\mu\text{M}$  (in 20 mM phosphate buffer, pH 7.4). Excitation wavelength was 520 nm. All the spectra were corrected via dividing by absorbance at the excitation wavelength.



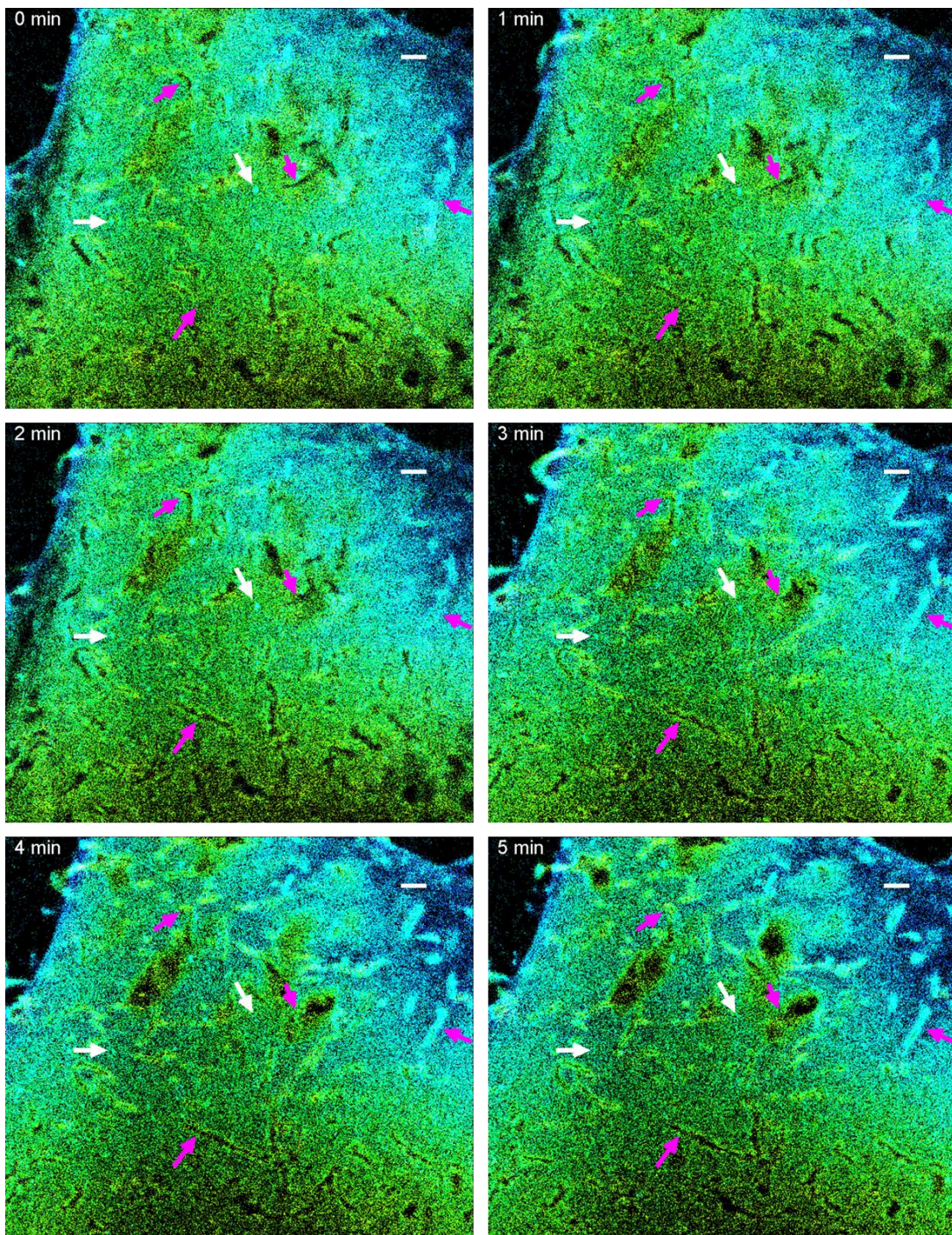
**Fig. S4.** Confocal microscopy images of giant vesicles (GUVs) of different compositions (DOPC; SM/Chol, 2/1; and DOPC/SM/Chol, 1/1/0.7) stained with NR12A, NR4A, NR12S, and Nile Red. Probe concentration was 200 nM (in 300 mM glucose solution). Excitation wavelength was 488 nm. Ratiometric images were created by dividing the intensity of  $I_{550-600}$  channel by that of the  $I_{600-650}$  channel for each pixel. The laser power settings (% of maximal intensity) were: 30% for DOPC GUVs with all probes, 10% for SM/Chol and DOPC/SM/Chol GUVs with NR12A, NR12S and Nile Red, and 100% for SM/Chol and DOPC/SM/Chol GUVs with NR4A.



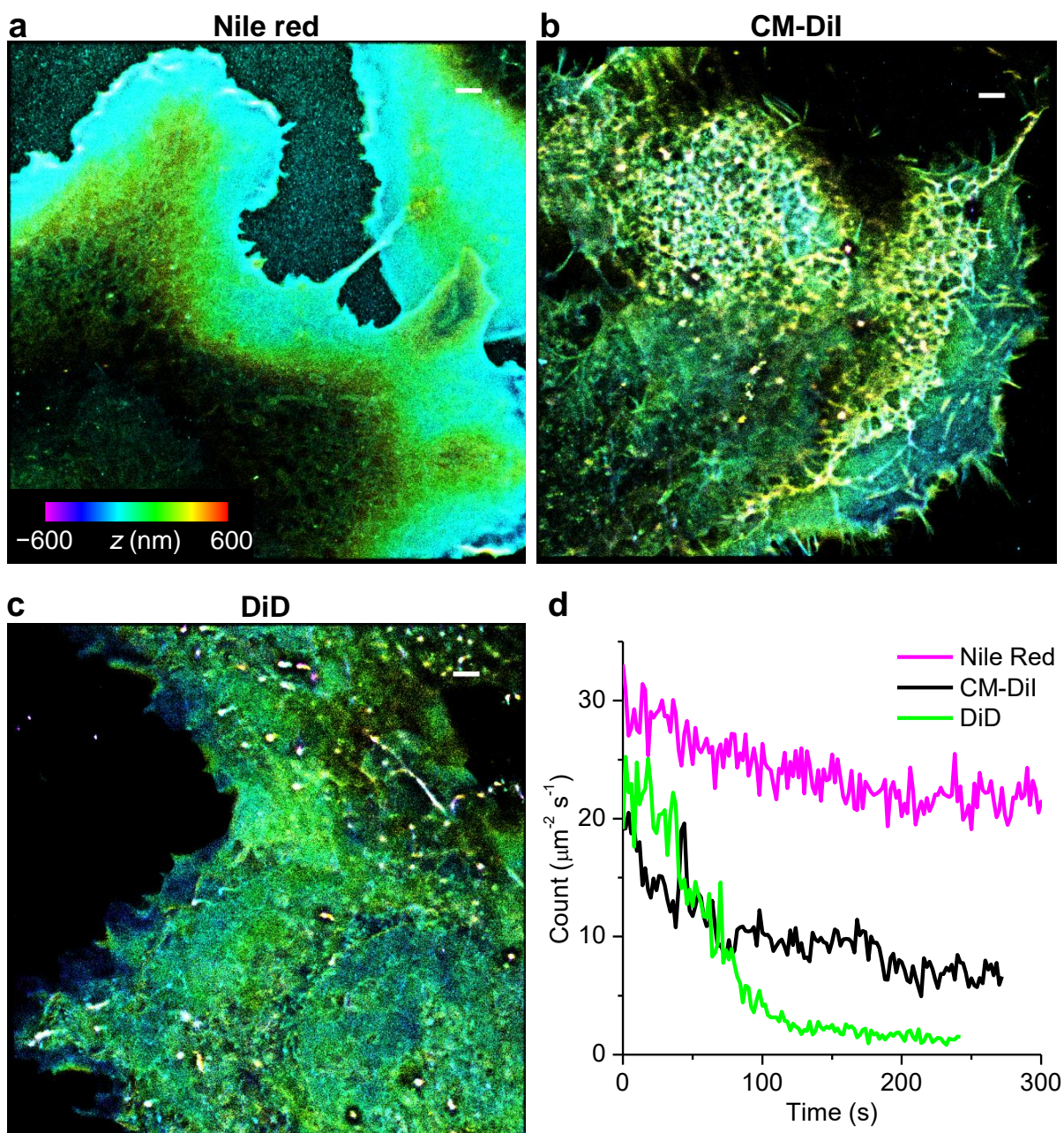
**Fig. S5.** Single-molecule spectroscopy results for NR4A (10 nM) in supported lipid bilayers. Averaged single-molecule spectra of NR4A on the DOPC bilayer (orange) and the bilayer composed of ternary lipid mixture (green).



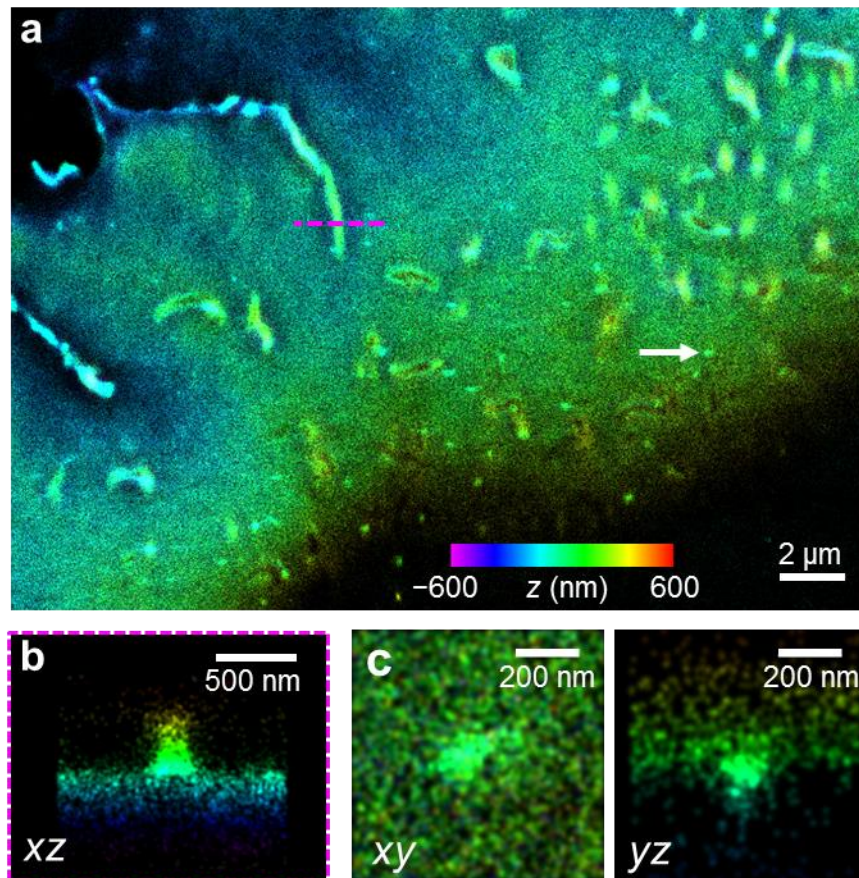
**Fig. S6.** Spinning disk confocal microscopy images of HeLa cells stained with NR4A, NR12A, Nile Red and NR12S before illumination (A1, B1, C1, D1), immediately after 60 seconds of illumination with 550 nm light at  $3.14 \text{ W}\cdot\text{cm}^{-2}$  (A2, B2, C2 and D2, correspondingly), and after illumination followed by 10 min of incubation at r. t. (A3, B3, C3 and D3, correspondingly). Dye concentration 20 nM.



**Fig. S7.** (a) A time sequence of the 3D-PAINT data for the NR4A-stained plasma membrane of the HeLa cell shown in Fig. 2e-j, presented for single-molecule images accumulated every 1 min. Scale bar: 1  $\mu\text{m}$ . Magenta arrows point to notable structural changes in the observed nanoscale protrusions. White arrows point to two apparent endocytosis sites that moved into the cell during the recording timeframe.



**Fig. S8.** 3D-SMLM images of live COS-7 cells stained with Nile Red (a), CM-DiI (b), and DiD (c). (a) Nile Red imaging was done by including 10 nM of the probe in the imaging media (L15 with 20 mM HEPES). (b,c) CM-DiI and DiD imaging was done by incubating the cells in 10  $\mu\text{M}$  of the probes for 20 min, and then the probes were washed out. The imaging buffer contained an oxygen scavenger and 25 mM cysteamine to assist photoswitching. During imaging, a 405 nm laser was applied at  $\sim 0.1 \text{ W/cm}^2$  to photoswitch dark molecules to the emitting state. All three probes permeated the plasma membrane and stained internal structures including ER, mitochondrial, and nuclear membranes. (d) Time-dependent counts of single molecules detected per  $\mu\text{m}^2$  per second for the three probes. Single-molecule images were recorded at 110 frames per second. This showed that DiD did not photoswitch well under this condition.



**Fig S9.** (a) Zoom-in 3D PAINt image of the top plasma membrane of a COS-7 cell stained by NR4A at 10 nM. (b) Virtual cross-section in the  $xz$  plane along the magenta dash line in (a). (c) Zoom-in in-plane ( $xy$ ) and vertical ( $yz$ ) images of the endocytosis site marked by the white arrow.

## Supporting tables

**Table S1.** Absorption and emission band maxima and quantum yield values for membrane probes in solvents and lipid vesicles.

Solvent or medium	Probe name											
	NR12A			NR4A			Nile Red			NR12S		
	$\lambda_{\text{max}}$ Abs	$\lambda_{\text{max}}$ Em	QY (%)	$\lambda_{\text{max}}$ Abs	$\lambda_{\text{max}}$ Em	QY (%)	$\lambda_{\text{max}}$ Abs	$\lambda_{\text{max}}$ Em	QY (%)	$\lambda_{\text{max}}$ Abs	$\lambda_{\text{max}}$ Em	QY (%)
DOPC LUVs	552	641	51.1±1.3	552	640	53.0±3.3	549	639	55.4±2.4	530	633	47.6±2.1
DOPC/Chol LUVs	551	636	47.1±0.7	553	637	47.7±3.1	554	636	71.4±7.8	526	613	44.6±4.6
SM/Chol LUVs	546	592	39.4±5.4	552	591	41.4±2.3	541	589	52.0±5.1	510	583	42.7±4.6
PBS	537	648	0.096±0.018	586	665	10.1±2.3	586	666	4.7±0.7	521	655	0.097±0.025
MeOH	553	641	44.2±0.8	549	643	45.0±2.5	551	641	38.0±1.3	553	640	43.8±1.0
DMSO	555	637	71.1±2.5	553	637	75.0±2.6	550	336	71.1±2.5	550	633	74.2±2.6
Dioxane	526	602	69.8±2.4	526	602	74.6±2.6	520	585	74.1±2.6	520	593	67.8±2.3

<sup>a</sup> Probe concentration was 2  $\mu\text{M}$ . Total phospholipid concentration for all liposomes was 200  $\mu\text{M}$  (phosphate buffer 20 mM, pH 7.4). Excitation wavelength was 520 nm. Errors are standard deviation of the mean (n = 3).



**Table S2.** Ratios between fluorescence intensities at 640 nm and 591 nm (for NR12A, NR4A, Nile Red) or 633 nm and 583 nm (for NR12S) and full width at half maximum values for membrane probes in lipid vesicles of different composition.<sup>a</sup>

	<b>NR12A</b>	<b>NR4A</b>	<b>Nile Red</b>	<b>NR12S</b>
	<b>I<sub>591</sub>/I<sub>640</sub></b>	<b>I<sub>591</sub>/I<sub>640</sub></b>	<b>I<sub>591</sub>/I<sub>640</sub></b>	<b>I<sub>583</sub>/I<sub>633</sub></b>
<b>DOPC</b>	0.253±0.003	0.261±0.012	0.276±0.008	0.500±0.015
<b>DOPC/Chol</b>	0.552±0.015	0.490±0.026	0.565±0.022	0.758±0.029
<b>SM/Chol</b>	1.48±0.02	1.27±0.14	1.55±0.10	1.44±0.09
	<b>FWHM (nm)</b>	<b>FWHM (nm)</b>	<b>FWHM (nm)</b>	<b>FWHM (nm)</b>
<b>DOPC</b>	76.8±0.2	77.5±0.1	76.8±0.1	99.5±0.2
<b>DOPC/Chol</b>	90.3±0.4	87.5±0.9	90.9±0.7	104.0±0.8
<b>SM/Chol</b>	91.5±4.5	102.8±8.2	89.9±5.8	100.3±6.5

<sup>a</sup>Errors are standard deviation of the mean (n = 3).

## Supporting Movie

**Movie S1.** Time-laps recordings for 1 s of single-molecule raw images (110 frames per second) taken in DOPC supported bilayers with 10 nM of NR12A, NR4A, NR12S or Nile Red in DPBS. Scale bars: 2  $\mu\text{m}$ .

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