Bright Fluorogenic Squaraines with Tuned Cell Entry for Selective Imaging of Plasma Membrane vs Endoplasmic Reticulum

Mayeul Collot, Rémy Kreder, Anatoliy L. Tatarets, Leonid D. Patsenker, Yves Mely and Andrey Klymchenko

Supplementary Information

Materials and methods

All chemicals and solvents for synthesis were from Sigma-Aldrich.

Lipid Vesicles.

Dioleoylphosphatidylcholine (DOPC) and cholesterol were purchased from Sigma-Aldrich. Bovine brain sphingomyelin (SM) was from Avanti Polar Lipids (Alabaster, AL).

Large unilamellar vesicles (LUVs) were obtained by the extrusion method as previously described.[1] Briefly, a suspension of multilamellar vesicles was extruded by using a Lipex Biomembranes extruder (Vancouver, Canada). The size of the filters was first 0.2 µm (7 passages) and thereafter 0.1 µm (10 passages). This generates monodisperse LUVs with a mean diameter of 0.11 µm as measured with a Malvern Zetamaster 300 (Malvern, U.K.). LUVs were labeled by adding aliquots (generally 2 µL) of probe stock solutions in dimethyl sulfoxide to 1-mL solutions of vesicles. Since the probe binding kinetics is very rapid, the fluorescence experiments were performed a few minutes after addition of the aliquot. A 20 mM phosphate buffer, pH 7.4, was used in these experiments. Concentrations of the probes and lipids were generally 0.2 and 200 µM, respectively.

Giant unilamellar vesicles (GUVs) were generated by electroformation in a home-built liquid cell (University of Odense, Denmark), using previously described procedures.[2] 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 to give the final suspension of GUVs used in microscopy experiments. The staining of GUVs was performed by addition of an aliquot of the probe stock solution in DMSO to obtain a 0.2 µM final probe concentration (final DMSO volume <0.25%).

Cell Lines, Culture Conditions, and Treatment.

HeLa cells were cultured in Dulbecco's modified Eagle medium (D-MEM, Low glucose, +GlutaMAX, Gibco-Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Lonza), and 1% antibiotic solution (penicillin–streptomycin, Gibco-invitrogen) in a humidified incubator with 5% CO2/95% air atmosphere at 37 °C. CEM-SS lymphocytes (ATCC) were cultured in PRMI 1640 (Gibco-Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Lonza), and 1% antibiotic solution (penicillin–

streptomycin, Gibco-invitrogen) in a humidified incubator with 5% CO_2 atmosphere at 37°C. The cell concentration of 5–10 $\times$ 10^4 cells ml^{-1} was maintained by removal of a portion of the culture and replacement with fresh medium 3 times per week.

In fluorescence spectroscopy experiments, CEM-SS cells were also washed twice with HBSS using centrifugation before addition of the probes. To stain the cell suspension with SQ8S, SQ12S or dSQ12S probes, an appropriate aliquot of its stock solution in DMSO was added to the lymphocytes (10^6 per mL) in HBSS buffer, to obtain a final probe concentration of 20 nM. Spectroscopic measurements were performed under stirring.

For the microscopy studies, attached HeLa cells were washed two times by gentle rinsing with HBSS. A freshly prepared solution of SQ8S, SQ12S or dSQ12S in HBSS was then added to the cells to a final concentration of 20 nM or 1 nM. Staining of the cells with commercial probes DiD (Life Technologies), Vybrant ™ DiD (Life Technologies) and CellVue® Claret (Sigma-Aldrich) were done in the same way, unless indicated. In the case of CellVue® Claret, "diluant C", provided within the kit, was used as buffer for cell rinsing and staining, while HBSS was used for comparison. Imaging was performed directly without washing step after 5-10 min.

Fluorescence Spectroscopy and Microscopy.

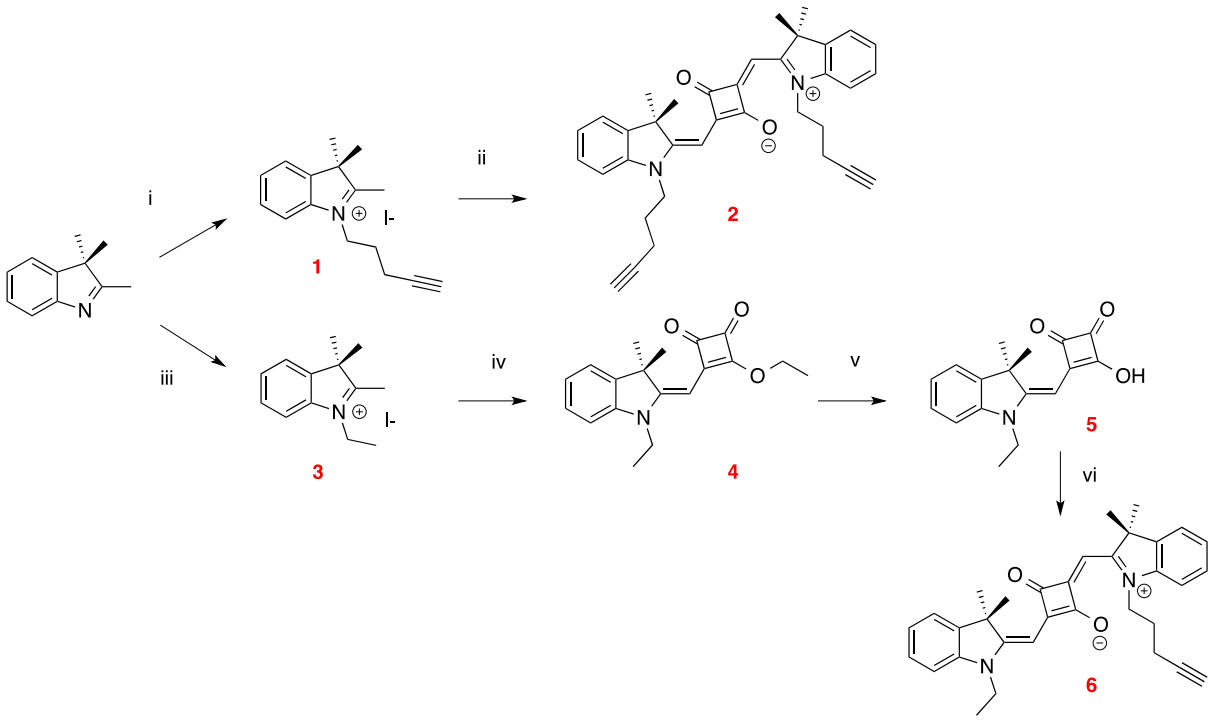
Absorption spectra were recorded on a Cary 4 spectrophotometer (Varian) and fluorescence spectra on a Fluoromax 4 (Jobin Yvon, Horiba) spectrofluorometer. Fluorescence emission spectra were systematically recorded at 600 nm excitation wavelength at room temperature, unless indicated. All the spectra were corrected from wavelength-dependent response of the detector. The fluorescence of the corresponding blank suspension of cells or lipid vesicles without the probe was subtracted from these spectra.

Fluorescence microscopy of GUVs was performed by using a home-built two-photon laser scanning setup based on an Olympus IX70 inverted microscope with an Olympus 60x 1.2NA water immersion objective. Two-photon excitation was provided by an InSight DeepSee laser (Spectra Physics), and photons were detected with Avalanche Photodiodes (APD SPCM-AQR-14-FC, Perkin-Elmer) connected to a counter/timer PCI board (PCI6602, National Instrument). Imaging was carried out using two fast galvo mirrors in the descanned fluorescence collection mode. Typical acquisition time was 50s with an excitation power around 30 mW (830 nm). Images corresponding to the blue and red channels were recorded simultaneously using a dichroic mirror (Beamsplitter 585 DCXR) and two APDs. The images were processed with a homemade program under ImageJ that generates a ratiometric image by dividing the image of the blue channel by that of the red channel. For each pixel, a pseudocolor scale is used for coding the ratio, while the intensity is defined by the integral intensity recorded for both channels at the corresponding pixel.

Confocal microscopy experiments were performed by using a Leica TCS SPE-II with HXC PL APO 63x/1.40 OIL CS objective. For imaging DAPI, the excitation wavelength of the laser was 405 nm, while the detection range was set between 430 and 480 nm. For imaging WGA-Alexa 488 and ER tracker green, the excitation wavelength was 488 nm, while the detection range was set between 495 and 550 nm. For imaging squaraine and DiD probes, the excitation wavelength was 632 nm, while the emission was set between 640 and 750 nm. The images were processed with the ImageJ software.

Synthesis of membrane probes

1. Squaraine derivative, SQ12S, dSQ12S and SQ8S.



Scheme 1. Synthesis of « clickable » Squaraine dye. i) 5-chloro-1-pentyne, KI, CH_3CN, 85°C, 48h (20%) ; ii) Squaric acid, pyridine, 100°C, 12h (10%) ; iii) idoethane, CH_3CN, 85°C, 48h (97%) ; iv) Diethyl squarate, Et_3N, EtOH, 80°C, 6h (59%) ; v) NaOH (40%) EtOH, 80°C, 20 min; vi) **1**, toluene, n-butanol, 110°C, 18h (76%)

2,3,3-trimethyl-1-(pent-4-yn-1-yl)-3H-indol-1-ium (1)

To a suspention of Potassium iodide (7.100 g, 43.03 mmol, 2.2 eq) in CH_3CN (25 mL) was added 5-chloro-1-pentyne (2.000 g, 19.50 mmol, 1 eq), the yellow mixture was stirred at 50°C. After 10 min, 2,3,3-trimethylindolenine (3.100 g, 19.50 mmol, 1 eq) was added dropwise and the reaction was refluxed overnight. The mixture was then cool down to room temperature and the inorganic salts were filtrated. The solvent was removed under vacuum and the crude product was dissolved into a small quantity of acetone. The mixture was then precipitated in cold ether, three times. Dark purple crystal were obtained, yield 20%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.79 – 7.72 (m, 1H), 7.57 – 7.47 (m, 3H), 4.87 – 4.77 (m, 2H), 3.15 (s, 3H), 2.44 (td, *J* = 6.4, 2.6 Hz, 2H), 2.19 (p, *J* = 6.5 Hz, 3H), 2.05 (t, *J* = 2.6 Hz, 1H), 1.61 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 196.53, 141.57, 141.07, 130.20, 129.64, 123.27, 115.58, 81.99, 71.14, 54.77, 48.86, 26.59, 23.28, 17.36, 16.18. HRMS (ES+) Calc. for $C_{16}H_{20}N$ $[M]^+$ 226.1590, found 226.1590.

4-((3,3-dimethyl-1-(pent-4-yn-1-yl)-3H-indol-1-ium-2-yl)methylene)-2-((3,3-dimethyl-1-(pent-4-yn-1-yl)indolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate (2)

2,3,3-trimethyl-1-(pent-4-yn-1-yl)-3H-indol-1-ium (1) (200 mg, 0.566 mmol, 2 eq) and 3,4-dihydroxycyclobut-3-ene-1,2-dione (32 mg, 0.283 mmol, 1 eq) were dissolved in pyridine (5 mL), the mixture was heated overnight at 100°C (12/16h). The solvent was then removed under vacuum and

the crude product was washed with HCl 1M solution, three times, to remove the pyridine. The product is purified by column chromatography with CH_2Cl_2/MeOH (98/2) as eluent. Dark blue-green solid was obtained, yield 10%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.30 – 7.22 (m, 4H), 7.11 – 7.02 (m, 4H), 5.91 (s, 2H), 4.09 (s, 4H), 2.28 (td, *J* = 6.7, 2.6 Hz, 4H), 2.08 (t, *J* = 2.6 Hz, 2H), 1.98 (p, *J* = 6.9 Hz, 5H), 1.71 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.16, 179.99, 170.12, 142.44, 142.05, 127.86, 123.85, 122.31, 109.41, 86.70, 82.66, 77.24, 70.19, 49.35, 42.30, 27.13, 25.65, 16.14. HRMS (ES+) Calc. for $C_{36}H_{36}N_2O_2$ $[M+H]^+$ 529.2850, found 529.2843.

1-ethyl-2,3,3-trimethyl-3H-indol-1-ium (3)

2,3,3-trimethylindolenine (1 eq ; 5.1 g) and idoethane (3 eq ; 14.8 g) were dissolved in CH_3CN (75 mL), the reaction was heated up to 85°C (reflux) under stirring for 48H. The solvent was then removed under vacuum and the crude product was dissolved in a small amount of acetone. The mixture is then precipitated in cold ether, three times to obtain the maximum of product. Pink crystals were obtained, yield 97%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.67 (tdd, *J* = 4.3, 3.7, 2.9, 2.1 Hz, 1H), 7.57 – 7.48 (m, 3H), 4.71 (q, *J* = 7.5 Hz, 2H), 3.10 (s, 3H), 1.58 (d, *J* = 11.5 Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 195.42, 141.72, 140.63, 130.18, 129.61, 123.38, 115.33, 54.67, 45.56, 23.13, 16.99, 13.52. HRMS (ES+) Calc. for $C_{13}H_{18}N$ $[M]^+$ 188.1434, found 188.1439.

(E)-3-ethoxy-4-((1-ethyl-3,3-dimethylindolin-2-ylidene)methyl)cyclobut-3-ene-1,2-dione (4)

1-ethyl-2,3,3-trimethyl-3H-indol-1-ium (3) (2.20 g, 6.984 mmol, 1.2 eq), diethyl squarate (1.00 g, 5.882 mmol, 1 eq) and Et_3N (1.6 g, 15.88 mmol, 2.7 eq) were dissolved in EtOH (20 mL). The mixture was refluxed for 6h. The solvents were then removed under vacuum and the crude product was purified by column chromatography with CH_2Cl_2/MeOH (95/5) as eluent. Dark orange solid was obtained, yield 59%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.23 – 7.18 (m, 2H), 7.01 (td, *J* = 7.5, 1.0 Hz, 1H), 6.82 (dd, *J* = 8.3, 1.0 Hz, 1H), 5.34 (s, 1H), 4.83 (q, *J* = 7.1 Hz, 2H), 3.82 (q, *J* = 7.2 Hz, 2H), 1.55 (s, 6H), 1.47 (t, *J* = 7.1 Hz, 3H), 1.27 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.53, 187.48, 187.43, 173.78, 167.97, 142.14, 141.07, 127.77, 122.69, 122.03, 108.12, 80.94, 69.89, 47.98, 37.66, 26.89, 15.93, 11.35. HRMS (ES+) Calc. for $C_{19}H_{21}NO_3$ [M] 311.1521, found 311.1509. Data in accordance with the literature.[3]

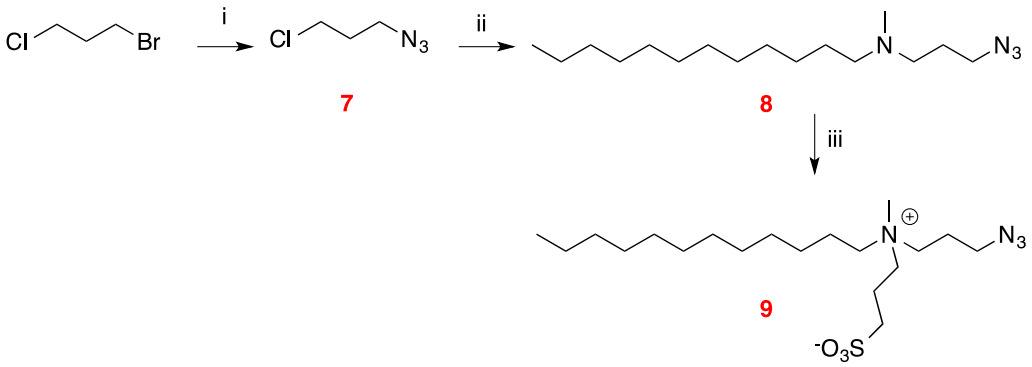
(E)-3-((1-ethyl-3,3-dimethylindolin-2-ylidene)methyl)-4-hydroxycyclobut-3-ene-1,2-dione (5)

(E)-3-ethoxy-4-((1-ethyl-3,3-dimethylindolin-2-ylidene)methyl)cyclobut-3-ene-1,2-dione (4) (400 mg, 3.215 mmol, 1eq) was dissolved in EtOH (5mL) and the reaction was refluxed before 300 µL of a NaOH 40% solution was added to the mixture, the heating was kept for 5 more min. The reaction was then cooled down to RT and IR-120-H^+ resin was added until a neutral pH was reached. The resin was removed by filtration and the solvent under vacuum. The crude product was used directly into the next step without further purification.

2-((3,3-dimethyl-1-(pent-4-yn-1-yl)indolin-2-ylidene)methyl)-4-((1-ethyl-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene)-3-oxocyclobut-1-en-1-olate (6)

(E)-3-((1-ethyl-3,3-dimethylindolin-2-ylidene)methyl)-4-hydroxycyclobut-3-ene-1,2-dione (5) (200 mg, 0.706 mmol, 1 eq) and 2,3,3-trimethyl-1-(pent-4-yn-1-yl)-3H-indol-1-ium (1) (249 mg, 0.706 mmol, 1 eq) were dissolved in 8 mL of a mixture of toluene/n-butanol (1/1). The reaction was equipped with a Dean-Stark apparatus and was refluxed for 18h. After 5 min the solution turns green and after one hour turns blue-green. The solvent was removed under vacuum and the crude product was purified by column chromatography with EtOAc/MeOH (95/5) as eluent. Blue-green powder was obtained,

yield 76%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.32 – 7.21 (m, 4H), 7.12 – 7.01 (m, 3H), 6.94 (d, *J* = 7.9 Hz, 1H), 5.94 – 5.83 (m, 2H), 4.03 (s, 4H), 3.42 (s, 1H), 2.28 (td, *J* = 6.7, 2.6 Hz, 2H), 1.98 (p, *J* = 6.9 Hz, 2H), 1.72 (s, 12H), 1.33 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.36, 180.35, 179.37, 170.09, 169.68, 142.33, 141.92, 127.81, 123.85, 123.65, 122.37, 122.29, 109.23, 86.40, 82.71, 77.33, 77.22, 77.02, 76.70, 70.13, 50.83, 49.43, 49.23, 42.23, 38.48, 27.18, 26.96, 25.62, 16.14, 12.05. HRMS (ES+) Calc. for $C_{33}H_{35}N_2O_2$ $[M+H]^+$ 491.2693, found 491.2685.



Scheme 2. Synthesis of the "clickable" anchor. i) NaN_3, DMF, 25°C, 20h (86%); ii) N-methyldodecylamine, CH_3CN, 80°C, 6h (38%); iii) 1,3-propanesultone, K_2CO_3, CH_3CN, 90°C, 12h (61%).

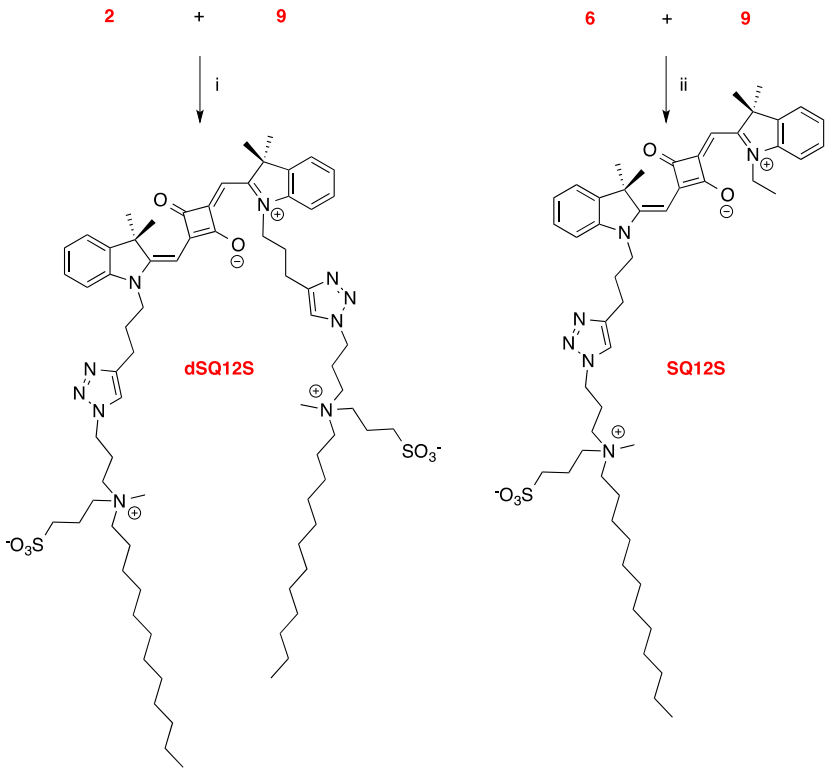
1-azido-3-chloropropane (7) was synthesised according to a published procedure.[4]

N-(3-azidopropyl)-N-methyldodecan-1-amine (8)

1-azido-3-chloropropane (7)(0.602 g, 5.016 mmol, 1 eq) and dodecyl(methyl)amine (1.000 g, 5.016 mmol, 1eq) were dissolved in 15 mL of CH_3CN. The solution was then heated at 80°C in a sealed flask (to avoid the evaporation of 7) for 6h. The solvent was removed with the help of a rotary evaporator and the crude product was purified by column chromatography with CH_2Cl_2/MeOH (5 to 10 % of MeOH) as eluent. A yellowish oil was obtained, yield 38%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 3.28 (t, *J* = 6.8 Hz, 2H), 2.38 (t, *J* = 7.2 Hz, 2H), 2.33 – 2.24 (m, 2H), 2.17 (s, 3H), 1.71 (p, *J* = 6.9 Hz, 2H), 1.41 (q, *J* = 7.5, 7.0 Hz, 2H), 1.20 (d, *J* = 7.8 Hz, 18H), 0.86 – 0.75 (m, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 57.92, 54.57, 49.64, 42.15, 31.91, 29.66, 29.63, 29.60, 29.53, 29.34, 27.51, 27.30, 26.80, 22.68, 14.09. **HRMS (ES+)** Calc. for $C_{16}H_{35}N_4$ $[M+H]^+$ 283.2856, found 283.2852.

3-((3-azidopropyl)(dodecyl)(methyl)ammonio)propane-1-sulfonate (9)

N-(3-azidopropyl)-N-methyldodecan-1-amine (8) (500 mg, 1.770 mmol, 1eq) was dissolved in CH_3CN (15 mL), then 1,3-propanesultone (1.100 g, 8.851 mmol, 5 eq) was added to the mixture. The reaction was then heated to reflux for 12h. The white solid that formed was filtered off and washed several times with CH_3CN and dryed. A white powder was obtained, yield 61%. ^{1}H NMR (400 MHz, Methanol-d_4) δ 3.46 – 3.36 (m, 4H), 3.31 – 3.24 (m, 2H), 2.96 (s, 3H), 2.77 (t, *J* = 6.7 Hz, 2H), 2.13 – 2.00 (m, 2H), 1.97 – 1.86 (m, 2H), 1.75 – 1.60 (m, 2H), 1.36 – 1.13 (m, 18H), 0.80 (t, *J* = 6.8 Hz, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 61.98, 61.43, 58.96, 48.65, 48.13, 47.57, 31.89, 29.58, 29.46, 29.40, 29.31, 29.15, 26.39, 22.67, 22.29, 22.27, 19.13, 14.10. **HRMS (ES+)** Calc. for $C_{19}H_{41}N_4O_3S$ $[M+H]^+$ 405.2894, found 405.2899.



Scheme 3. Synthesis of dSQ12S and SQ12S. i) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, dioxane/water (3/1), RT, 30 min (52%) ; ii) $CuSO_4 \cdot 5H_2O$, sodium, dioxane/water (3/1), RT, 30 min (82%).

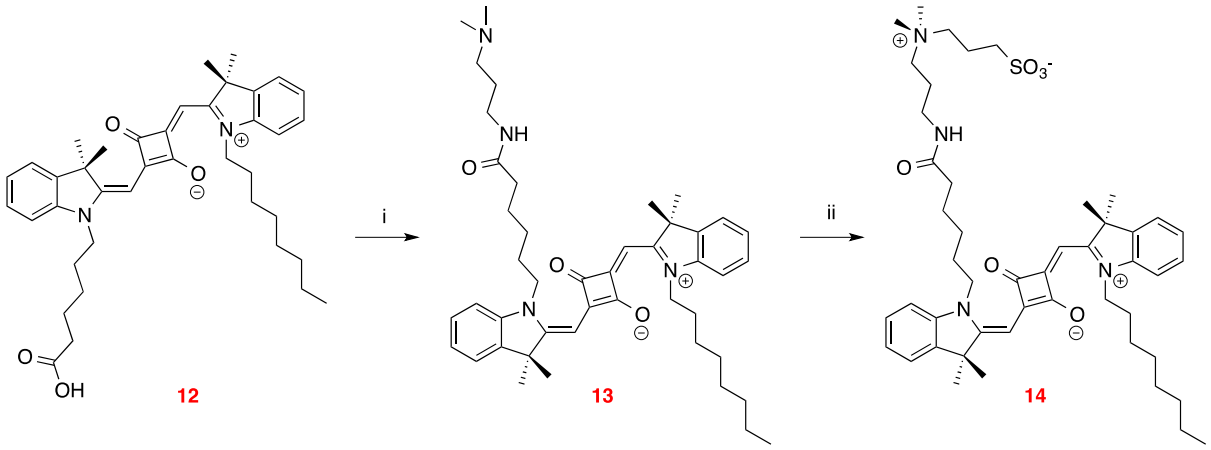
"Click" coupling was performed according to the following protocol: The appropriate squaraine (2) or (6) (1 eq) and compound (9) (respectively 2.2 and 1.2 eq) were dissolved in a mixture dioxane/water (3/1). $CuSO_4 \cdot 5H_2O$ and sodium ascorbate were dissolved in an eppendorf with water and the solution was mixed until the mixture turn yellow/orange. The content of the eppendorf was then added to the mixture and the reaction was stirred at room temperature for 30 min. The solvent was then removed under vacuum and the crude product was purified by gel filtration chromatography Sephadex® LH-20 with MeOH/CH_2Cl_2 (1/1) as eluent. Blue-green powder, yield 82% (SQ12S (11)) and 52% (bSQ12S (10)).

bSQ12S : ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.37 (d, *J* = 7.4 Hz, 2H), 7.27 (t, *J* = 7.6 Hz, 2H), 7.17 (d, *J* = 7.9 Hz, 2H), 7.11 (t, *J* = 7.4 Hz, 2H), 5.80 (s, 2H), 4.45 (t, *J* = 6.5 Hz, 4H), 4.11 (s, 4H), 3.48 – 3.36 (m, 4H), 3.35 – 3.25 (m, 4H), 2.95 (s, 6H), 2.84 – 2.78 (m, 4H), 2.74 (t, *J* = 6.5 Hz, 4H), 2.40 – 2.28 (m, 4H), 2.20 – 2.08 (m, 4H), 2.06 – 1.98 (m, 4H), 1.65 (s, 12H), 1.60 – 1.46 (m, 6H), 1.28 – 1.05 (m, 40H), 0.78 (t, *J* = 6.7 Hz, 6H). **HRMS (ES+)** Calc. for $C_{74}H_{118}N_{10}O_8S_2$ $[M+2H]^{2+}$ (m/z) 669.4282, found 669.4289.

The amount of product did not allow the measurement of a sufficiently resolved ^{13}C NMR spectra.

SQ12S : ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.90 (s, 1H), 7.35 – 7.20 (m, 4H), 7.09 (q, *J* = 7.3 Hz, 2H), 7.01 (d, *J* = 7.9 Hz, 1H), 6.96 (d, *J* = 7.9 Hz, 1H), 5.99 (s, 1H), 5.83 (s, 1H), 4.60 (s, 2H), 4.04 (s, 4H), 3.60 (d, *J* = 77.4 Hz, 4H), 3.23 – 3.13 (m, 2H), 3.11 (s, 3H), 2.93 – 2.82 (m, 2H), 2.80 (t, *J* = 7.0 Hz, 2H), 2.62 – 2.49 (m, 2H), 2.21 – 2.12 (m, 4H), 1.70 (s, 12H), 1.57 (s, 2H), 1.33 (t, *J* = 7.2 Hz, 3H), 1.26 – 1.06 (m, 20H), 0.78 (t, *J* = 6.9 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 179.20, 178.88, 170.17, 169.72, 146.41,

142.25, 141.91, 127.92, 127.86, 123.85, 123.81, 122.97, 122.42, 122.21, 109.70, 109.22, 86.43, 86.26, 62.22, 61.19, 58.92, 49.33, 49.29, 48.63, 47.46, 46.78, 42.83, 38.48, 31.86, 29.55, 29.44, 29.28, 29.16, 27.08, 26.96, 26.41, 26.13, 23.14, 22.81, 22.65, 22.30, 19.02, 14.10, 12.03. **HRMS (ES+)** Calc. for $C_{52}H_{74}N_6O_5S$ 894.5441, found 909.5306 ($M+CH_3$).



Scheme 4. Synthesis of **SQ8S**. i) 3-(Dimethylamino)-1-propylamine, BOP, HOBt, DIEA, DMF/CH_2Cl_2 (1/1), RT, overnight (64%) ii) 1,3-propanesultone , K_2CO_3, CH_3CN, 80°C, overnight (62%).

Compound **12** was purchased from SETA BioMedicals. ^{1}H NMR (400 MHz, DMSO-d_6), δ, ppm: 7.51 (2H, d, 7.2 Hz, aromatic C*H*), 7.28–7.38 (4, m, aromatic C*H*), 7.16 (2H, t, 6.9 Hz, aromatic C*H*), 5.80 (1H, s, C*H*), 4.07 (4H, br. s, NCH_2), 2.19 (2H, t, 6.9 Hz, CH_2COOH), 1.69–1.77 (4H, m, CH_2), 1.67 (12H, s, (CH_3)$_2$), 1.50–1.60 (2H, m, CH_2), 1.27–1.44 (6H, m, CH_2), 1.22 (6H, s, CH_2), 0.82 (3H, t, 7.2 Hz, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6), δ, ppm: 180.6 (CO-squaraine), 178.5 (C=N), 174.3 (COOH), 169.0 (C-squaraine), 142.2 (aromatic C), 141.4 (aromatic C), 127.9 (aromatic C), 123.6 (aromatic C), 122.2 (aromatic C), 110.3 (aromatic C), 86.0 (-CH=), 48.7 (*C*(CH$_3$)$_2$), 42.9 (NCH$_2$), 33.7 (*C*H$_2$), 31.1 (*C*H$_2$), 28.6 (*C*H$_2$), 28.5 (*C*H$_2$), 26.5 (*C*H$_2$), 26.3 (*C*H$_2$), 26.1 (*C*H$_2$), 25.9 (*C*H$_2$), 24.3 (*C*H$_2$), 22.0 (C(*C*H$_3$)$_2$), 13.9 (*C*H$_3$).

2-((3,3-dimethyl-1-octylindolin-2-ylidene)methyl)-4-((1-(6-((3-(dimethylamino)propyl)amino)-6-oxohexyl)-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene)-3-oxocyclobut-1-en-1-olate (13)

4-((1-(5-carboxypentyl)-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene)-2-((3,3-dimethyl-1-octylindolin-2-ylidene)methyl)-3-oxocyclobut-1-en-1-olate (12) (1 eq ; 25mg) , BOP (1.1 eq ; 19.5mg), HOBt (1.38 eq ; 8 mg) and DIEA (5 eq ; 26 mg) are dissolved in a mixture of DMF/CH_2Cl_2 (1/1) (4mL). 3-(Dimethylamino)-1-propylamine (1.5 eq ; 6.2 mg) is added to the reaction and this one is keep under stirring at room temperature overnight. The solvent is then removed under vacuum and the crude product is purified by preparative TLC with CH_2Cl_2/MeOH/NH_3 (7.5/2/0.5) as eluent. Blue-green powder, yield 64 %.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.55 (s, 1H), 7.29 (m, 2H), 7.24 (tt, *J* = 7.7, 1.5 Hz, 2H), 7.08 (td, *J* = 7.4, 3.4 Hz, 2H), 6.92 (d, *J* = 7.9 Hz, 2H), 5.89 (s, 1H), 5.85 (s, 1H), 3.91 (s, 4H), 3.30 (q, *J* = 6.2 Hz, 2H), 2.45 (t, *J* = 6.9 Hz, 2H), 2.27 (s, 6H), 2.22 – 2.14 (m, 2H), 1.71 (d, *J* = 2.7 Hz, 12H), 1.44 (q, *J* = 7.4 Hz, 2H), 1.39 – 1.14 (m, 18H), 0.80 (t, *J* = 6.8 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 182.49, 179.24, 178.83, 173.09, 170.19, 170.09, 142.28, 127.85, 123.83, 123.77, 122.35, 109.47, 109.47, 109.33, 86.57, 86.34, 57.56, 49.30, 44.74, 43.81, 43.05, 38.10, 36.31, 31.76, 29.70, 29.35, 29.17, 27.07, 27.05, 26.25, 26.05, 25.89, 24.96, 22.61, 14.08. **HRMS (ES+)** Calc. for $C_{45}H_{63}N_4O_3$ $[M+H]^+$ 707.4895, found 707.4896.

3-((3-(6-(2-((3-((3,3-dimethyl-1-octylindolin-2-ylidene)methyl)-2-oxido-4-oxocyclobut-2-en-1-ylidene)methyl)-3,3-dimethyl-3H-indol-1-ium-1-yl)hexanamido)propyl)dimethylammonio)propane-1-sulfonate (14)

2-((3,3-dimethyl-1-octylindolin-2-ylidene)methyl)-4-((1-(6-((3-(dimethylamino)propyl)amino)-6-oxohexyl)-3,3-dimethyl-3H-indol-1-ium-2-yl)methylene)-3-oxocyclobut-1-en-1-olate (13) (1 eq ; 8.8 mg) is dissolved in CH_3CN (5 mL), 1,3-propanesultone (2 eq; 3.1mg) and K_2CO_3 (2eq, 3.4 mg) have been added to the mixture. The reaction is heated to 90°C overnight under stirring. The solvent is then removed under vacuum and the crude product is purified by preparative TLC CH_2Cl_2/MeOH/NH_3 (6/3/1) as eluent. Blue-green powder, yield 62%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.96 (s, 1H), 7.32 – 7.20 (m, 4H), 7.04 (td, *J* = 7.4, 4.9 Hz, 2H), 6.91 (dd, *J* = 16.6, 8.0 Hz, 2H), 5.85 (s, 1H), 5.80 (s, 1H), 3.88 (s, 4H), 3.65 (s, 2H), 3.47 (s, 2H), 3.31 (s, 2H), 3.12 (s, 6H), 2.87 (s, 2H), 2.27 – 2.15 (m, 4H), 2.02 (s, 2H), 1.71 – 1.66 (m, 12H), 1.42 – 1.15 (m, 18H), 0.79 (t, *J* = 6.3 Hz, 3H). **HRMS (ES+)** Calc. for $C_{48}H_{69}N_4O_6S$ $[M+H]^+$ 829.4932, found 829.4947.

The amount of product did not allow the measurement of a sufficiently resolved ^{13}C NMR spectra.

Absorption and emission spectra of the squaraine probes in organic solvents.

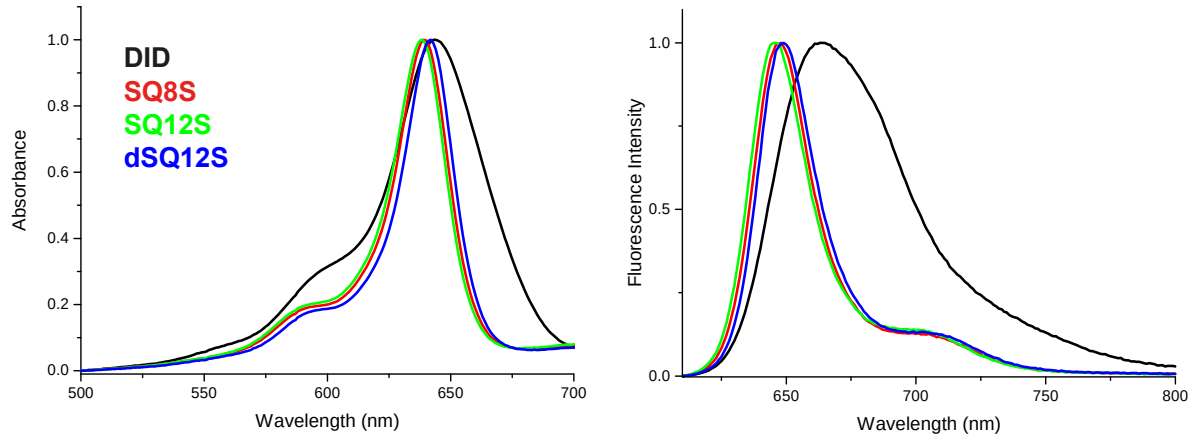


Figure S1. Absorption (left) and emission (right) spectra of the probes in dioxane.

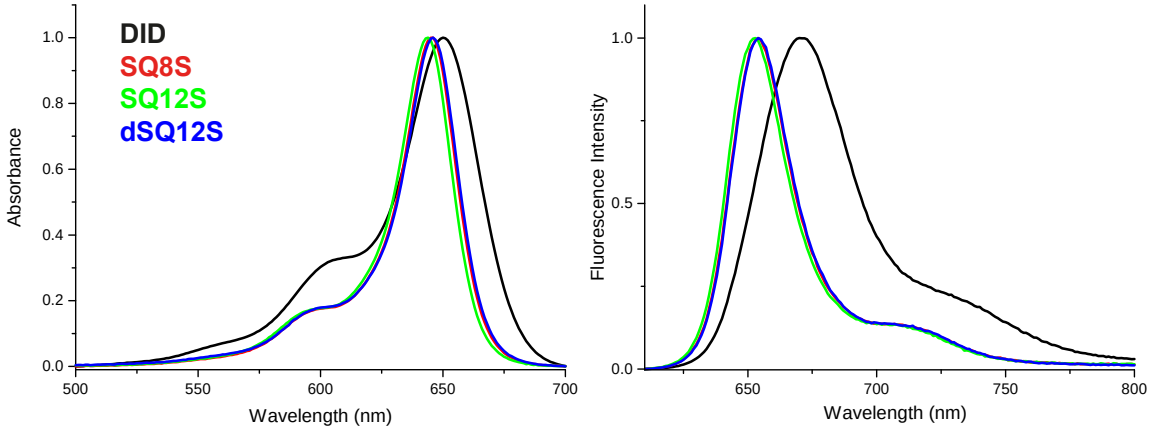


Figure S2. Absorption (left) and emission (right) spectra of the probes in DMSO.

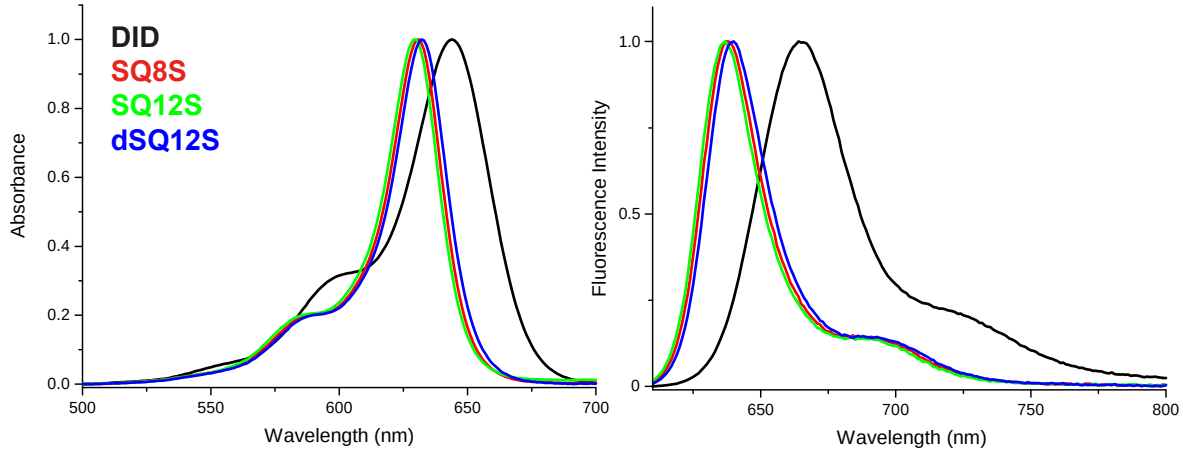


Figure S3. Absorption (left) and emission (right) spectra of the NIR sensors in MeOH.

Imaging giant vesicles stained with the probes using polarized two-photon excitation light

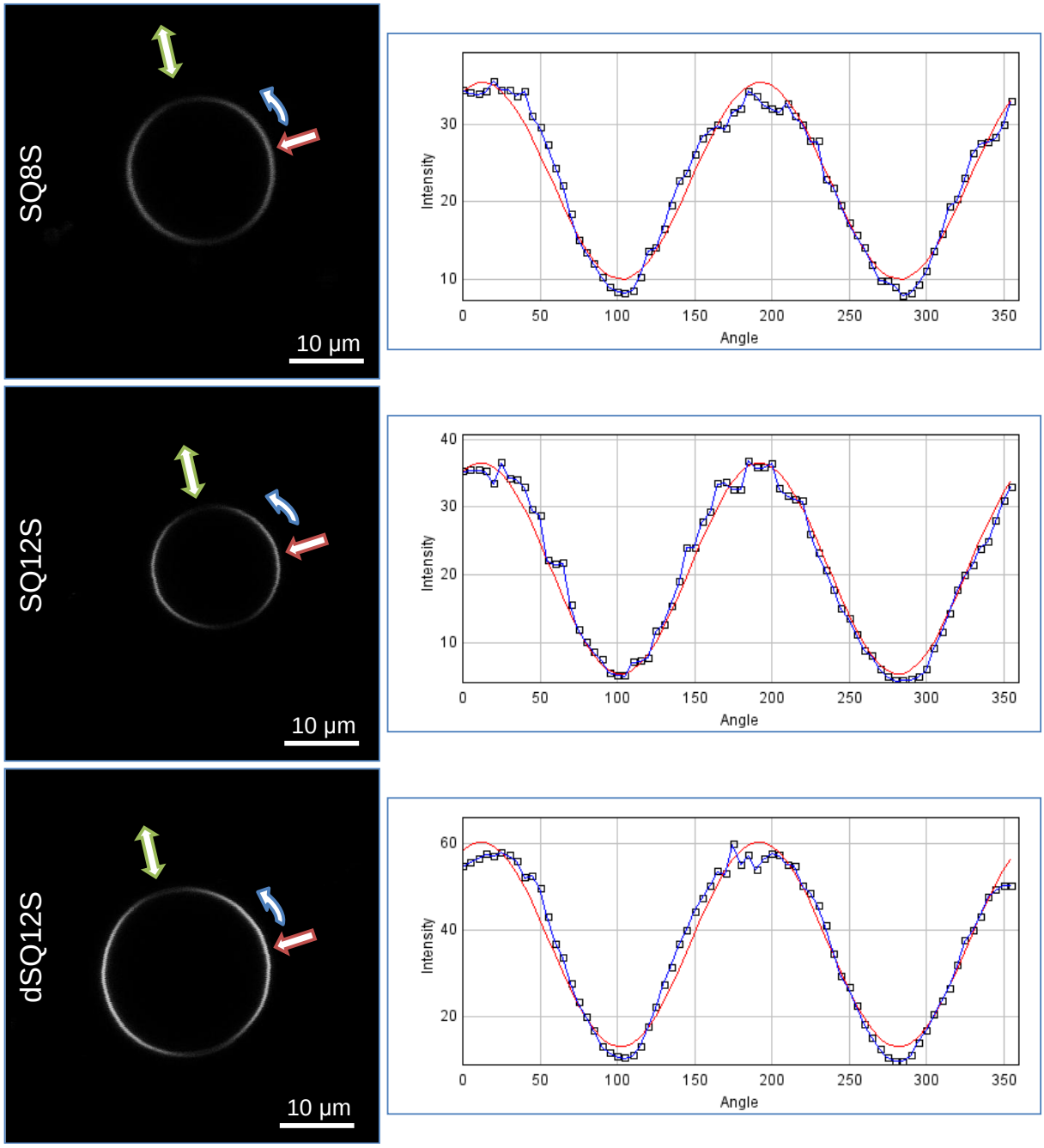


Figure S4. Left: Two-photon excited fluorescence image of DOPC giant vesicles (GUV) in the presence of 50 nM of the squaraine probes. Red arrow represents the 0° angle, the blue arrow depicts the direction of analysis for the intensity profile. The double arrow (green) is the direction of polarization of the excitation light. Right: corresponding fluorescence intensity alone the GUVs membrane as a function of angle with respect to the arrow in red. The fitted data is the red curve. The excitation wavelength was 830 nm.

Kinetics of probe binding to lymphocytes

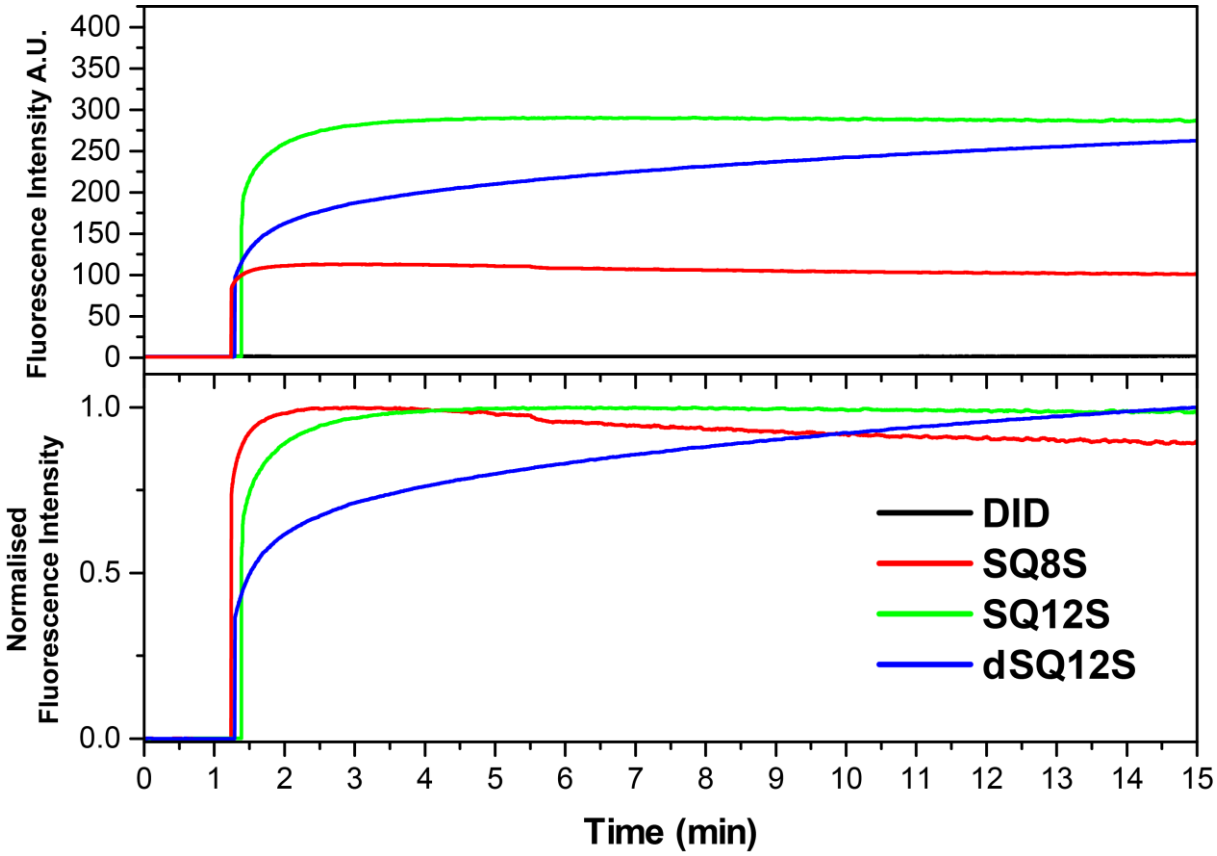


Figure S5. Evolution of fluorescence intensity at 650 nm (excitation at 635 nm) of the probes in the presence of 1 million lymphocytes/mL. The probes were added after 100 s to a stirred solution of lymphocytes. Final concentration of probes was 20 nM.

Evolution of the membrane staining over 30 min using the squaraine probes.

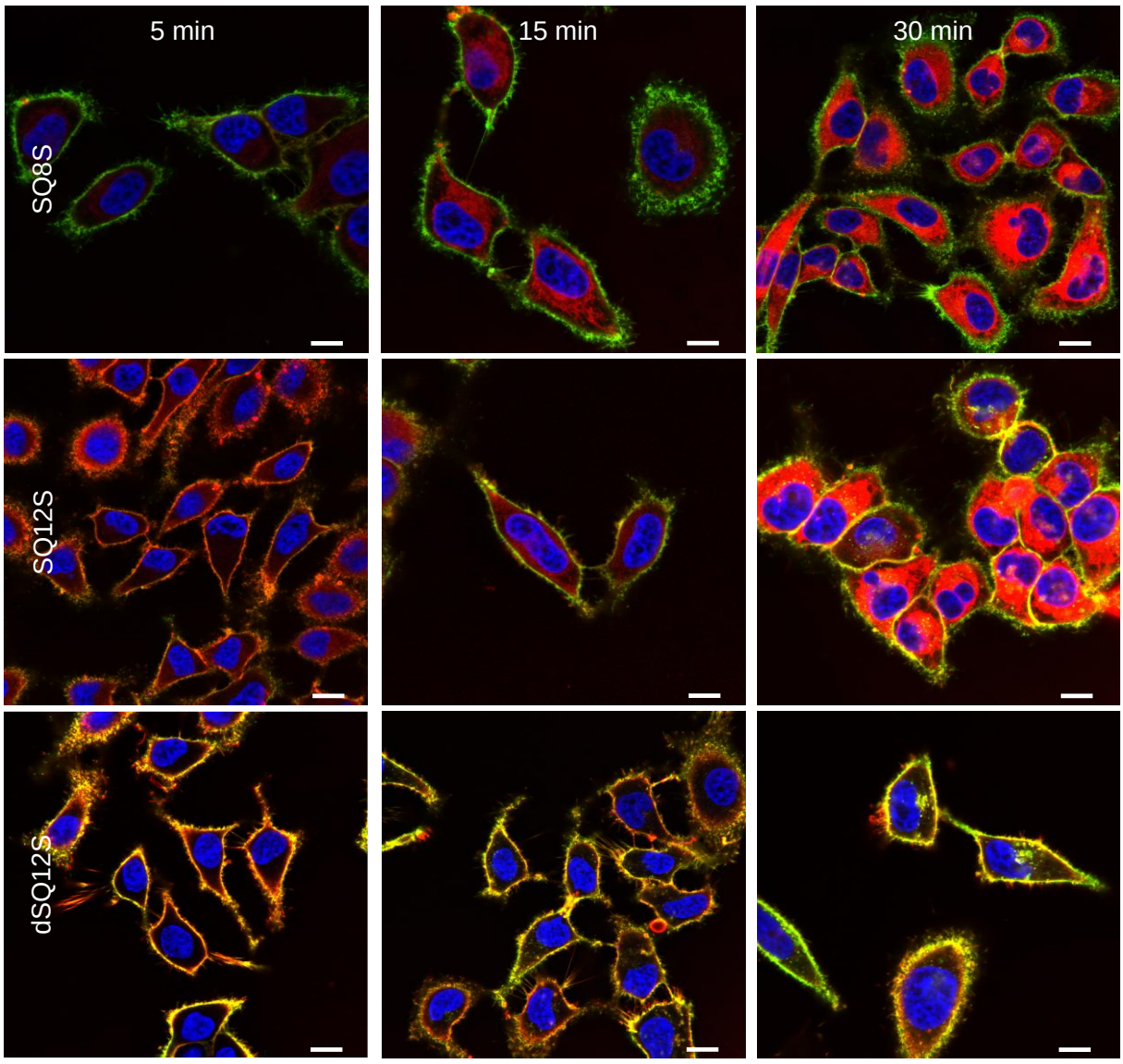


Figure S6. Laser scanning confocal microscopy images of HeLa cells incubated with the squaraine probes (20 nM) without washing, at different incubation times (red). Nucleus was stained by Hoechst (2 μg.mL^{-1}) (blue) and the plasma membrane was stained with WGA-488 (5 μg.mL^{-1}) (green). Scale bar is 10 μm.

Staining of the cell membranes using the squaraine probes at 1 nM.

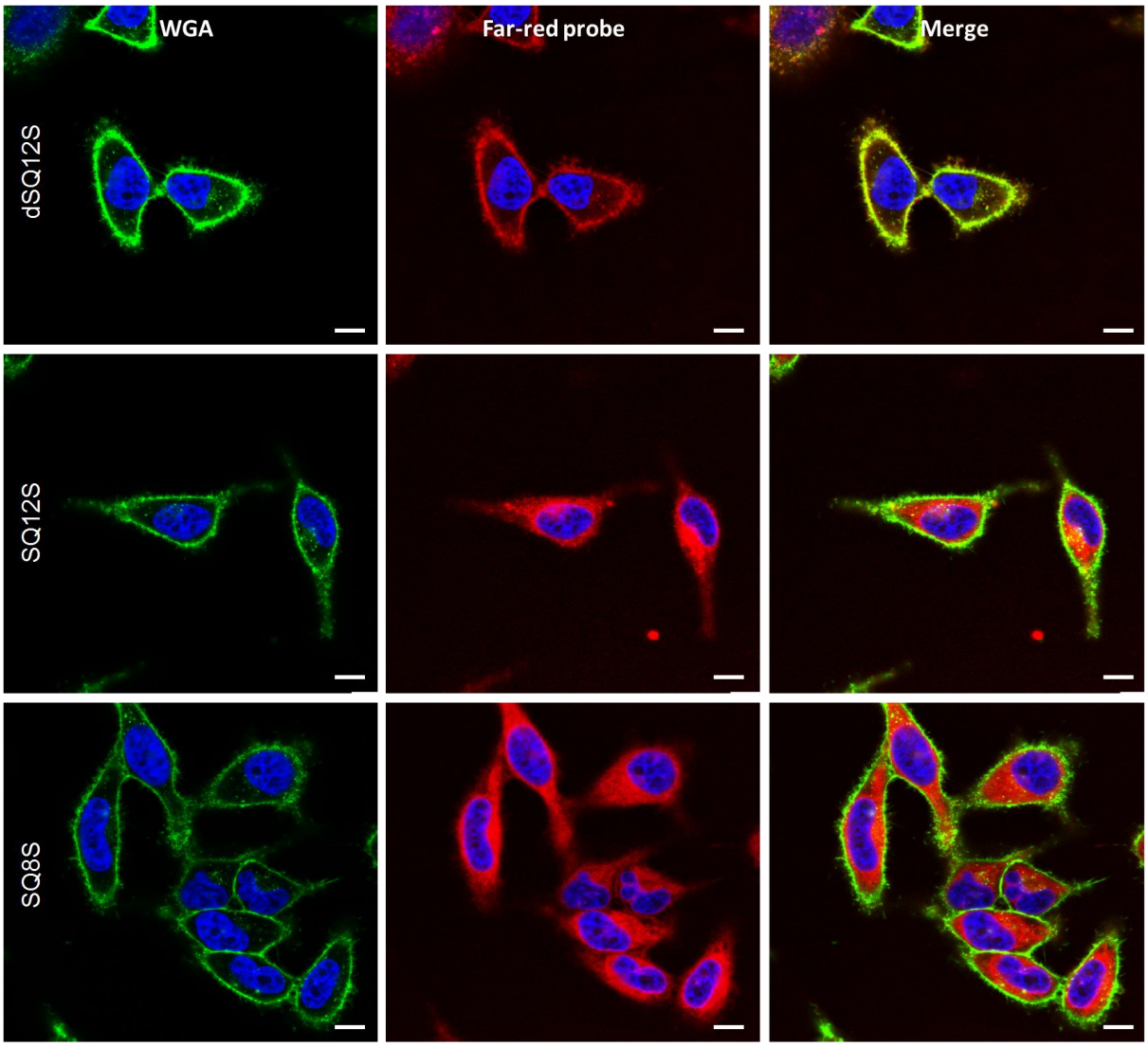


Figure S7. Laser scanning confocal microscopy images of HeLa cells incubated for 1 hour with the probes at 1 nM without washing (red). Nucleus was stained by Hoechst (2 µg.mL-1) (blue) and the plasma membrane was stained with WGA-488 (5 µg.mL-1) (green). Left images are the WGA-488 and Hoechst channel, middle images are the squaraine probes and Hoechst channel, and right images are the merge of the three channels. Scale bar is 10 µm.

Identification of the organelles stained by SQ8S using ER-tracker.

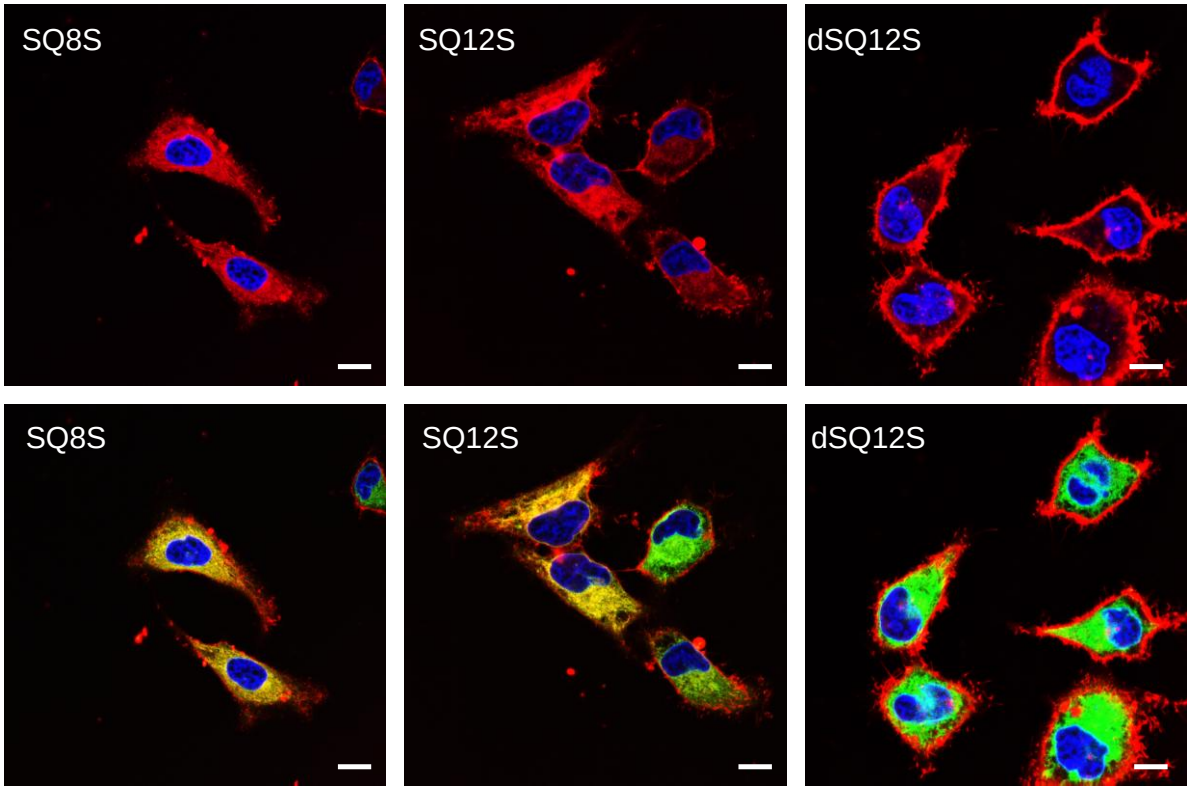


Figure S8. Laser scanning confocal microscopy images of HeLa cells incubated for 30 minutes with 20 nM of the squaraine probes (red) without washing. Nucleus was stained by Hoechst (2 $\mu g.mL^{-1}$) (blue). Top images blue and red channel. Bottom images: merge of the blue, green and red channel for colocalisation with ER-tracker green (750 nM). Scale bar is 10 µm.

Localisation of DiD in HeLa cells.

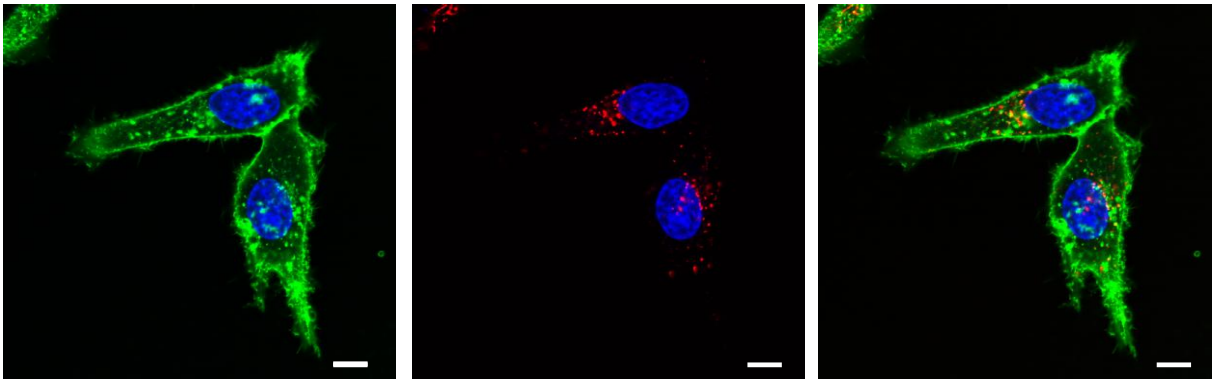


Figure S9. Laser scanning confocal microscopy images of HeLa cells incubated for 1 hour with DiD (20 nM) without washing (red). Nucleus was stained by Hoechst (2 μg.mL^{-1}) (blue) and the plasma membrane was stained with WGA-488 (5 μg.mL^{-1}) (green). Scale bar is 10 μm. Left image is the WGA-488 and Hoechst channel, middle image is the squaraine probes and Hoechst channel, right image is the merge of the three channels. Scale bar is 10 μM. Laser power for the DiD probe was increased compared to the squaraine dyes in order to be able to localize the dye.

Comparison of the squaraine probe dSQ12S with commercial probes Vybrant™ DiD and CellVue® Claret for cell membrane staining.

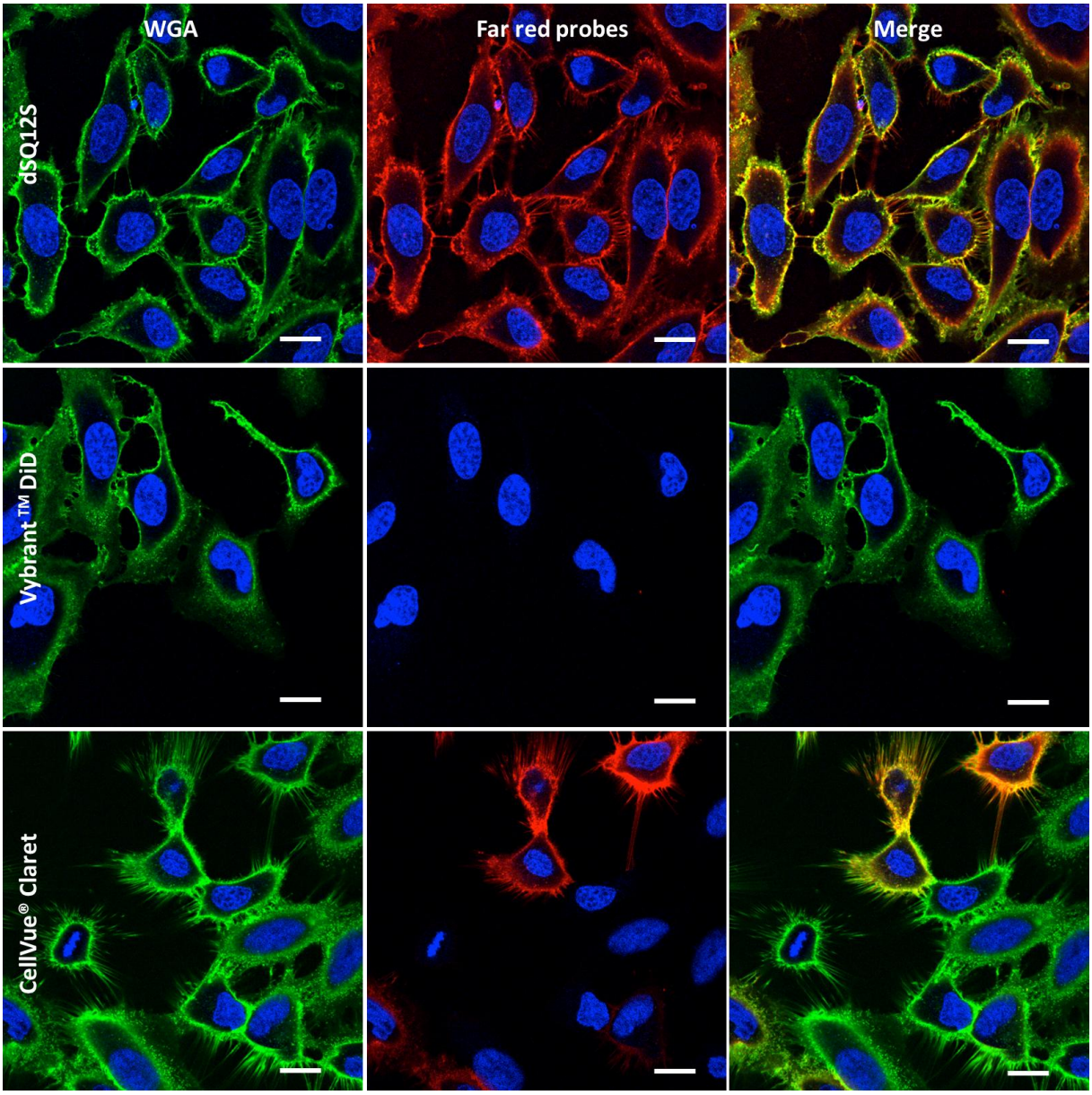


Figure S10. Laser scanning confocal microscopy images of HeLa cells in presence of 20 nM far-red membrane probes (in HBSS for dSQ12S and Vybrant™ DiD and in "diluant C" for CellVue® Claret) without washing (red) after 10 minutes. Nucleus was stained by Hoechst (2 $mg.mL^{-1}$) (blue) and the plasma membrane was stained with WGA-488 (5 $mg.mL^{-1}$) (green). Left images are the WGA-488 and Hoechst channels, middle images are the far red probes and Hoechst channels, right images are the merge of the three channels. Scale bar is 20 µm.

Staining of the cell membranes using Vybrant™ DiD at high concentration (2 mM).

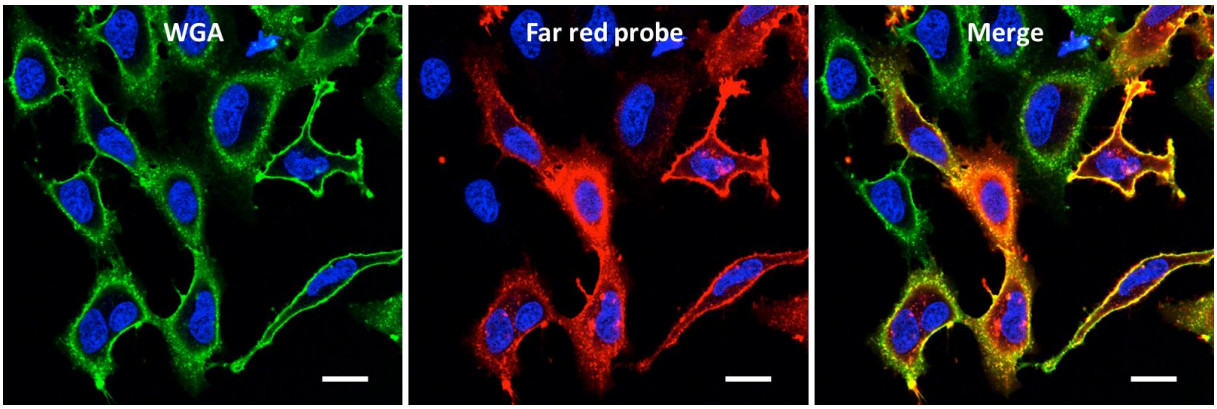


Figure S11. Laser scanning confocal microscopy images of HeLa cells in presence of 2 mM Vybrant™ DiD in HBSS without washing (red) after 10 minutes. Nucleus was stained by Hoechst (2 mg.mL^{-1}) (blue) and the plasma membrane was stained with WGA-488 (5 mg.mL^{-1}) (green). Left image is the WGA-488 and Hoechst channel, middle image is the far red probes and Hoechst channel, right image is the merge of the three channels. Scale bar is 20 µm.

Negative effect of HBSS on cell staining by CellVue® Claret.

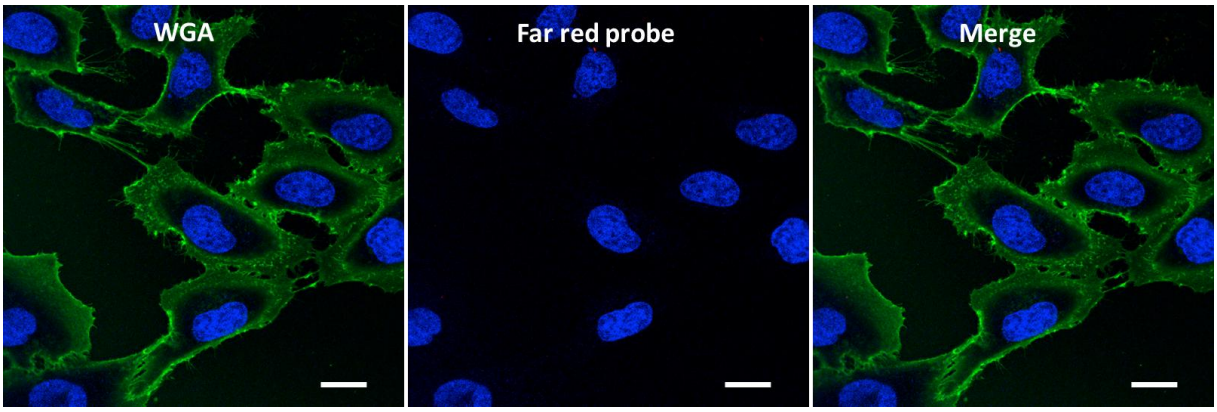


Figure S12. Laser scanning confocal microscopy images of HeLa cells in presence of 20 nM CellVue® Claret in HBSS without washing (red) after 10 minutes. Nucleus was stained by Hoechst (2 $mg.mL^{-1}$) (blue) and the plasma membrane was stained with WGA-488 (5 $mg.mL^{-1}$) (green). Left image is the WGA-488 and Hoechst channel, middle image is the far red probes and Hoechst channel, right image is the merge of the three channels. Scale bar is 20 µm.

Staining of endoplasmic reticulum using sub-nanomolar concentration of SQ8S.

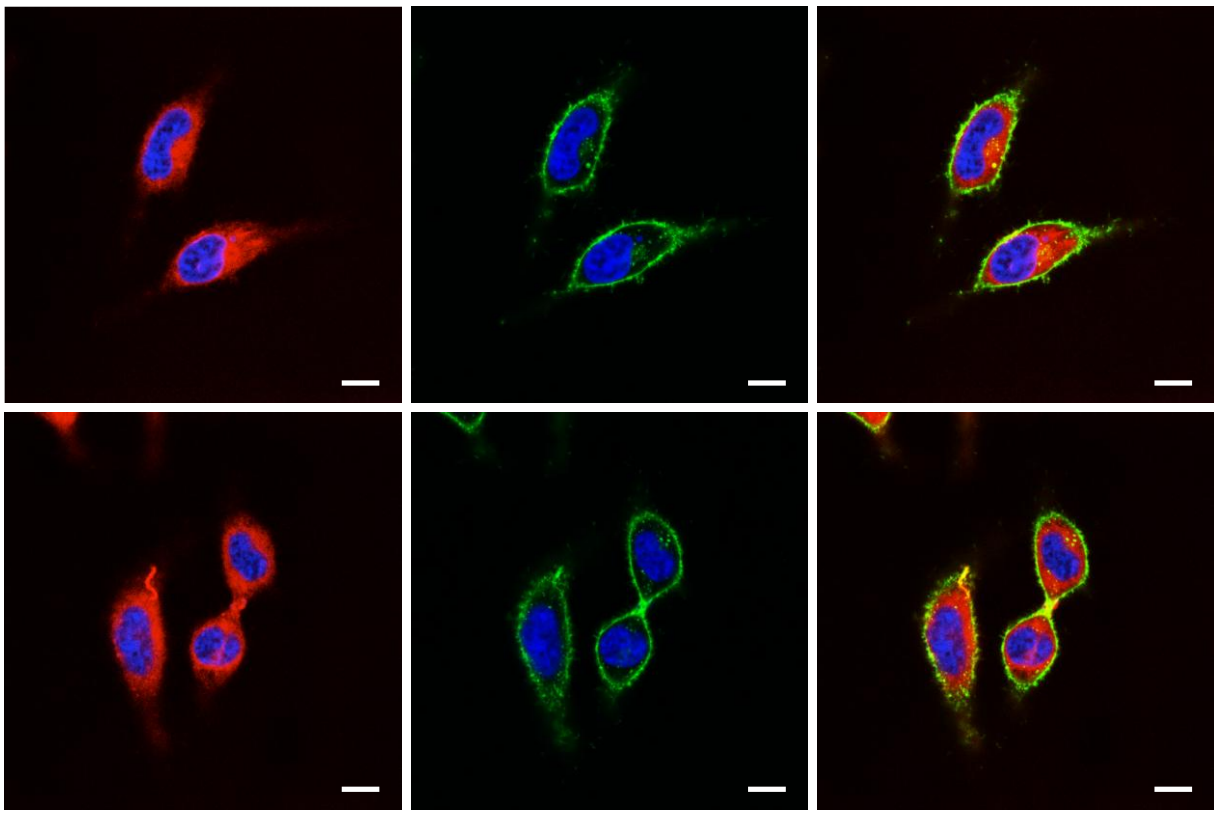


Figure S13. Laser scanning confocal microscopy images of HeLa cells incubated with the SQ8S at a concentration of 0.5 nM without washing (red). Nucleus was stained by Hoechst (2 µg.mL-1) (blue) and the plasma membrane was stained with WGA-488 (5 µg.mL-1) (green). Left images are the SQ8S probe and Hoechst channel, middle images are the WGA-488 and Hoechst channel and right images are the merge of the three channels. Scale bar is 10 µm.

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