Supporting information

Solvatochromic Nile Red Probes with FRET Quencher Reveal Lipid Order Heterogeneity in Living and Apoptotic Cells

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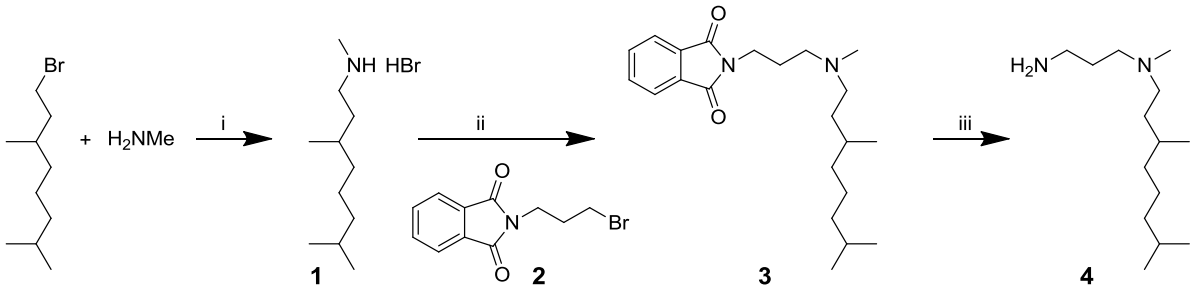
Methods

All chemicals and solvents for synthesis were from Sigma-Aldrich, Alfa Aesar or TCI Europe. NMR spectra were recorded at 20°C on Bruker Avance III 400 MHz spectrometer. Mass spectra were obtained using an Agilent Q-TOF 6520 mass spectrometer.

Synthesis bNR10S probe and bQ10S quencher



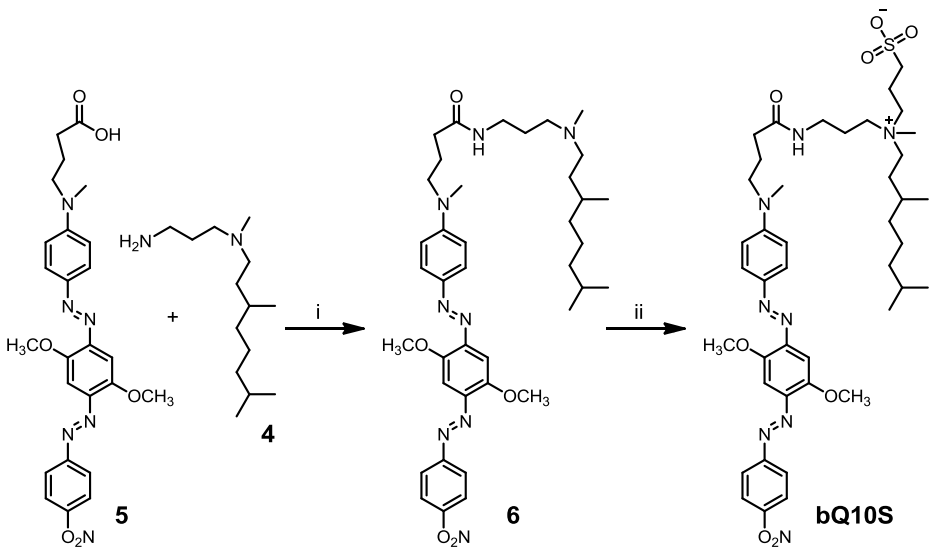
Scheme S1. Synthesis of the intermediate amines for bNR12S and bQ10S. (i) THF, RT overnight ; (ii) K_2CO_3, DMF, heating (110°C) 2h (40%) ; (iii) Hydrazine, EtOH, reflux 3h (82%).

N,3,7-trimethyloctan-1-amine hydrobromide (1). To the solution of H_2NMe (2.5eq, 25 mL) in THF, 1-bromo-3,7-dimethyloctane (1.05eq, 4 mL) was added slowly. The mixture was stirred overnight at room temperature. The solvent was removed under vacuum to give the desired product as a white precipitate. The residue was used directly in the next step without further purification ^{1}H RMN (400 MHz, dioxane) δ 3.33 (s, 2H), 2.88 (m, 2H), 2.55(t, J = 5.2Hz, 3H), 2.37 (q, J = 6 Hz, 2H), 1.62-1.07 (m; 10H), 0.85 (d, J = 6.8Hz, 9H). HR-LC-MS: m/z $C_{11}H_{25}N$ Calcd. 171.1987, found 171.1983.

2-(3-((3,7-dimethyloctyl)(methyl)amino)propyl)isoindoline-1,3-dione (3). N^1-(3,7-dimethyloctyl)-N,3,7-trimethyloctan-1-amine hydrobromide **1** (1.05eq, 1.1 g) and N-(3-bromopropyl)phthalimide **2** (1eq, 1.0 g) were dissolved in DMF then, K2CO3 (4eq, 2.16 g) was added to the reaction. The mixture was then heated to 110°C for 2h. The solvent was removed under vacuum and, after addition of water, the

product was extracted by EtOAc three times and washed with water. Then, it was purified by column chromatography using CH_2Cl_2/MeOH (9:1) as eluent to obtain the desired product as yellowish oil. ^{1}H RMN (400 MHz, $CDCl_3$) δ 7.82 (q, J = 2.8 Hz, 2H), 7.69 (q, J = 2.8 Hz, 2H), 3.71 (t, J = 7.2 Hz, 2H), 2.48(m, 2H), 2.38 (m, 2H), 2,23 (s, 3H), 1.89 (quin, J = 7.6Hz, 2H) 1.55-1.02 (m, 10H), 0.83 (d, J = 6.4Hz, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.36, 133.89, 132.16, 123.19, 77.33, 77.21, 77.01, 76.69, 55.65, 55.06, 41.68, 39.26, 37.33, 36.35, 31.26, 27.94, 25.91, 24.66, 22.70, 22.60, 19.70. HR-LC-MS: m/z $C_{22}H_{34}N_2O_2$ Calcd. 358.2620, found 358.2632.

N^1-(3,7-dimethyloctyl)-N^1-methylpropane-1,3-diamine (4). to a solution of 2-(3-((3,7-dimethyloctyl)(methyl)amino)propyl)isoindoline-1,3-dione (1eq, 100 mg) in EtOH, ninhydrine (1.5eq, 21 µL) was added and the mixture was heated to reflux for three hours. The crude product was dissolved in CH_2Cl_2 and washed by a solution of NaOH 1M three times (100 mL). The solvent is removed under vacuum to obtain the crude product as yellowish oil. The residue was used directly in the next step without further purification.

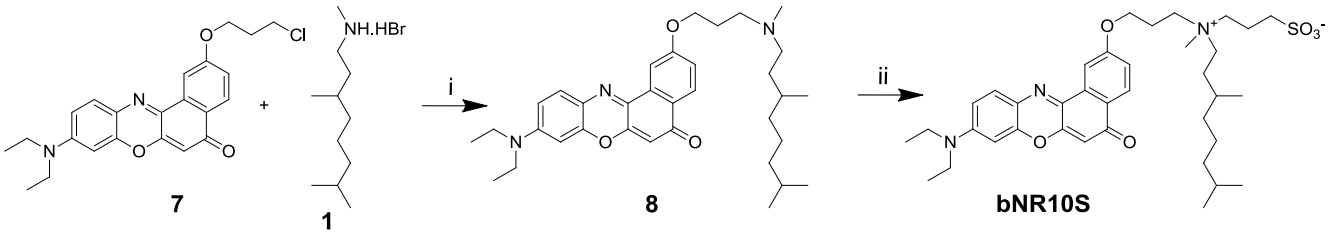


Scheme S2. Synthesis of bQ10S quencher. (i) DMF/CH_2Cl_2, HBTU, HOBT, DIEA, rt 6h (45%); (ii) 1,3-propanesultone, K_2CO_3, CH3CN, reflux 48h (51%).

4-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)-N-(3-((3,7-dimethyloctyl)(methyl)amino)propyl)butanamide (6). 4-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanoic acid (**5**) (1eq, 20mg), N1-(3,7-dimethyloctyl)-N1-methylpropane-1,3-diamine (**4**) (1.5eq, 14 mg), BOP (1.1eq, 19mg), HOBT (1.38eq, 8mg), DIEA (5eq, 35 µL) were dissolved in a mixture of DMF/CH_2Cl_2 (1:1; 2 mL) and let at room temperature for 6h. Then the crude product was purified on preparative TLC using MeOH/CH_2Cl_2 (1:9) and then EtOAC/CH_2Cl_2 (2:8) as eluents to obtain a dark purple crystalline powder. ^{1}H RMN (400 MHz, $CDCl_3$) δ 8.33 (d, J = 8.8 Hz, 2H), 8.01 (d, J = 8.8 Hz, 2H), 7.9 (d, J = 8.8 Hz, 2H), 7.45 (d, J = 15.6 Hz, 2H), 6.75 (d, J = 8.8 Hz, 2H), 4.06 (s, 3H), 4.01 (s, 3H), 3.49 (t, J = 7.2 Hz, 2H), 3.33 (q, J = 6Hz, 2H), 3.07 (s, 3H), 2.53 (m, 2H), 2.43 (m, 2H), 2.26 (s, 3H) 2.2 (t, J = 6.8Hz, 2H) 1.96 (m, 2H), 1.70 (m, 2H),

1.50 (m, 2H), 1.35-1.04 (m, 9H), 0.85 (d, J = 7.6 Hz, 3H), 0.83 (d, J = 6.8 Hz, 6H). HR-LC-MS: m/z $C_{39}H_{56}N_8O_5$ Calcd. 716.9125, found 716.9145.

3-((3-(4-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)butanamido)propyl)(3,7-dimethyloctyl)(methyl)ammonio)propane-1-sulfonate (bQ10S). 4-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)-N-(3-((3,7-dimethyloctyl)(methyl)amino)propyl)butanamide (**6**) (1eq, 6.7 mg), 1,3-propanesultone (3eq, 2.4 µL) and K_2CO_3 (4eq, 5 mg) were mixed in CH3CN (5 mL). The reaction mixture was heated to reflux for 48h and the crude product was purified by preparative TLC using MeOH/CH_2CL_2 (2:8) as eluent. A dark purple crystalline powder was obtained. ^{1}H RMN (400 MHz, $CDCl_3$) δ 8.34 (d, J = 9.2 Hz, 2H), 8.01 (d, J = 8.8 Hz, 2H), 7.87 (d, J = 8.8 Hz, 2H), 7.44 (d, J = 14.4 Hz, 2H), 6.81 (d, J = 8.4 Hz, 2H), 4.05 (s, 3H), 4.00 (s, 3H), 3.49 (t, J = 7.2 Hz, 2H) 3.6 (br, 2H), 3.07 (s, 3H), 2.98 (s, 3H), 2.89 (br, 2H), 2.36 (m, 2H), 2.31 (m, 2H), 2.17 (br, 2H), 1.96 (m, 2H), 1.59 (m, 2H), 1.52-1.04 (m, 16H),0.87 (d, J = 6 Hz, 3H), 0.82 (d, J = 6.8 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.43, 153.67, 152.32, 150.87, 148.40, 146.79, 144.29, 142.05, 126.21, 124.72, 123.50, 111.53, 101.00, 100.22, 56.89, 56.81, 51.98, 48.49, 38.97, 38.48, 36.86, 34.17, 32.90, 31.92, 30.97, 29.69, 29.35, 29.25, 29.14, 29.03, 27.89, 24.57, 22.96, 22.66, 22.52, 22.34, 19.23, 14.10. HR-LC-MS: $C_{42}H_{62}N_8O_8S$ Calcd. 838.4411, found 838.4482.



Scheme S3. Synthesis of bNR10S. (i) KI, K2CO3, CH3CN, reflux 48h (55%); (ii) 1,3-propanesultone, K2CO3, CH3CN, reflux 24h (49%).

9-(diethylamino)-2-(3-((3,7-dimethyloctyl)(methyl)amino)propoxy)-5H-benzo[a]phenoxazin-5-one (8). 2-(3-Chloropropoxy)-9-(diethylamino)-5H-benzo[a]phenoxazin-5-one (**7**)(*1*) (1eq, 100 mg) was dissolved in CH_3CN (~15 mL) and then N^1-(3,7-dimethyloctyl)-N^1-methylpropane-1,3-diamine (**1**) (2.2eq, 156.57 mg), KI (0.5eq, 20 mg) and K2CO3 (2.5eq, 84 mg) were added to the solution. The reaction mixture was heated to reflux for 48h. After evaporation of the solvent, the crude product was purified by column chromatography using CH_2Cl_2/MeOH (95:5 then 9:1) as eluent. A dark red crystalline powder was obtained. ^{1}H RMN (400 MHz, $CDCl_3$) δ 8.19 (d, J= 8.8 Hz, 1H), 8.03 (d, J = 2.4 Hz, 1H), 7.58 (d, J = 9.2 Hz, 1H), 7.14 (dd, J = 8.8, 2.8 Hz, 1H), 6.63 (dd, J = 8.8, 2.8 Hz, 1H), 6.43 (d, J = 2.8 Hz, 1H), 6.27 (s, 1H), 4.22 (t, J = 6.4 Hz, 2H), 3.44 (q, J = 6.8 Hz, 4 H), 2.66 (m, 2H), 2.47 (m, 2H), 2.33 (s, 3H), 2.08 (quin, J = 6.4 Hz, 2H) 1.58-1.05 (m, 16H), 0.86 (d, J = 6.4 Hz, 3H), 0.82 (d, J = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 183.27, 161.63, 152.06, 150.72, 146.86, 140.06, 134.08, 131.06, 127.75, 125.68, 124.70, 118.26, 109.49, 106.66, 105.31, 96.32, 77.22, 66.49, 55.81, 54.20, 45.07, 42.07, 39.26, 37.34, 31.30, 27.94, 26.79, 24.69, 22.70, 22.60, 19.73, 12.62. HR-LC-MS: m/z $C_{34}H_{47}N_3O_3$ Calcd. 545.3617, found 545.3612.

3-((3-((9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl)oxy)propyl)(3,7-dimethyloctyl)(methyl)ammonio)propane-1-sulfonate (bNR10S). 9-(Diethylamino)-2-(3-((3,7-dimethyloctyl)(methyl)amino)propoxy)-5H-benzo[a]phenoxazin-5-one (**8**) (1eq, 60 mg) was dissolved in CH3CN (5 mL) and then 1,3-propanesultone (2.7eq; 32.9 µL) and K_2CO_3 (2eq, 30.4 mg) was added to the solution. The reaction mixture was refluxed for 48h. After solvent evaporation, the crude product was purified by column chromatography using CH_2Cl_2/MeOH (9:1 then 8:2) as eluent. A dark red crystalline powder was obtained. ^{1}H RMN (400 MHz, $CDCl_3$) δ 7.94 (d, J = 8.4 Hz, 1H), 7.61 (d, J = 1.6 Hz, 1H), 7.26 (d, J = 8.8 Hz, 1H), 6.98 (dd, J = 9.2, 2 Hz, 1H), 6.35 (dd, J = 9.2, 2 Hz, 1H), 6.12 (d, J = 2 Hz, 1H), 5.94 (s, 1H), 4.11 (br, 2H), 3.67 (br, 5H), 3.55 (br, 2H), 6.29 (q, J = 6.4Hz, 4H), 3.22 (s, 3H), 2.99 (m, 2H),2.27 (m, 4H), 1.66 (m, 1H), 1.55-1.18 (m, 8H), 1.14 (t, J = 7.2 Hz, 6H), 1.02 (m, 2H), 0.86 (d, J = 6 Hz, 3H), 0.74 (d, J = 6 Hz; 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.69, 163.00, 160.65, 151.83, 150.84, 146.55, 138.76, 133.79, 131.10, 127.60, 125.52, 124.74, 117.85, 109.83, 106.63, 104.66, 95.94, 90.75, 64.71, 63.75, 62.62, 48.83, 47.61, 45.06, 39.01, 37.04, 36.99, 30.96, 28.97, 27.88, 24.60, 22.66, 22.54, 19.22, 18.82, 12.68. HR-LC-MS: m/z $C_{38}H_{55}N_3O_6S$ Calcd. 667.3655, found 667.3646.

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

Large unilamellar vesicles (LUVs) were obtained by the extrusion method as previously described.(*2*) 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). LUVs were labeled by adding aliquots (generally 2 µL) of probe stock solutions in dimethyl sulfoxide to 1-mL solutions of vesicles to obtain a 0.4 µM final probe concentration (final DMSO volume <0.25%). 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. Concentration of the lipids was generally 200 µM.

Giant unilamellar vesicles (GUVs) were generated by electroformation in a home-built liquid cell (University of Odense, Denmark), using previously described procedures.(*3*) 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, thermostated 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 0.1 µM final probe concentration (final DMSO volume <0.25%). Marker of Ld phase NBD-DOPE was added before the electroformation at the probe/ lipid ratio 1/500 (total lipid).

Cell Lines, Culture Conditions, and Treatment. HeLa and 293 T 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. CHO cells were cultured in Ham's F-12 Nutrient Mixture (F-12 Nut Mix (1X) + 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. A cell concentration of 5–10 x 10^4 cells ml^{-1} was maintained by removal of a portion of the culture and replacement with fresh medium 3 times per week. For apoptosis studies, cells were treated with 25 µM IPA-3 (Sigma-Aldrich) for 48 h in a humidified incubator with 5% CO2 atmosphere at 37 °C, as it was described elsewhere (*4*). To verify the apoptosis induction, the cells treated with an apoptosis-inducing agent IPA-3 were stained using GFP-labeled Annexin V, and monitored with Beckman Coulter Epic XL flow cytometer. To get sufficiently high signal 25000 events were counted per sample. Obtained data was analyzed with FCS express software.

In fluorescence spectroscopy experiments, cells were detached by trypsinization. DMEM medium was first removed from the culture dish, and cells were washed two times with DPBS. Trypsin 10x (Lonza) solution was diluted 10 times by DPBS and incubated with the cells at 37 °C for 4 min. The solution of trypsinized cells was then diluted by DPBS, transferred to Falcon tubes and centrifuged at 900 rpm for 5 min. The washing procedure was repeated one more time with HBSS solution. To stain the cell suspension with NR12S or bNR10S probes, an appropriate aliquot of its stock solution in DMSO was added to 0.5 mL of HBSS buffer, and after vortexing the solution was immediately added to 0.5 mL of the cell suspension to obtain a final probe concentration of 40 nM (<0.25% DMSO) and a cell concentration of 5×10^5-10^6 cells/mL. This low concentration of NR12S was necessary to minimize any possible artifacts related to the probe excess.(*5*) In the case of bNR10S, this concentration was also appropriate, as our tests in three cell lines showed that the spectral shifts due to excessive binding of the probe are observed only above 40 nM (Fig. S8). It should be noted that only freshly prepared solutions of the probe in HBSS should be used (<1 min) for cell staining, because of the slow aggregation of the probe in water. Before measurements, the cell suspension with probe was incubated for 7 min at room temperature in the dark. In spectroscopy experiments, the quencher (bQ10S) can be added directly from the stock solution (at an appropriate concentration) into the cell sample.

For microscopy studies, HeLa cells were seeded onto the 2-well Lab-Tek chambers at the density of 75×10^3 cells/well. After washing the cells with Ringer's solution, a freshly prepared solution of probes in Ringer's solution was added to the cells (< 0.25% DMSO volume) and incubated for 7 min in the dark with 5% CO2 atmosphere at 37 °C. Final probe concentration was 40 nM. bQ10S was added the same way; an appropriate aliquot of its stock solution in DMSO was added to 200µL of Ringer's solution, and after vortexing the solution was immediately added to the cell sample.

Fluorescence Spectroscopy and Microscopy. Absorption spectra were recorded on a Cary 4 spectrophotometer (Varian) and fluorescence spectra on a Fluorolog (Jobin Yvon, Horiba) spectrofluorometer. Fluorescence emission spectra were systematically recorded at 520 nm excitation wavelength at room temperature, unless otherwise indicated. All the spectra were corrected from the fluorescence of the corresponding blank (suspension of cells or lipid vesicles without the probe). The analysis of the fluorescence spectra in order to recalculate the partition of the probes between Lo and Ld phases was done as described in the supplementary information.

Fluorescence microscopy experiments of giant vesicles were performed by using a Leica TSC SPE confocal microscope. For ratiometric imaging excitation was provided by 488 nm, while the emission was split on two channels: "green" (550-580 nm) and "red" (590-620 nm). The images were processed with a

homemade program under ImageJ that generates a ratiometric image by dividing the image of the green 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. To image selectively the Nile Red probes (NR12S and bNR10S) and NBD-DOPE without significant cross-talk, the former were excited at 561 nm and detected at 570-650 nm, while the latter was excited at 488 nm and detected at 500-520 nm.

Fluorescence ratiometric imaging of giant vesicles were 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 (*6*). Two-photon excitation was provided by a titanium-sapphire laser (Tsunami, 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 50 s with an excitation power around 30 mW (λ) 830 nm) at the sample level. Images corresponding to the green and red channels were recorded simultaneously using a dichroic mirror (Beamsplitter 585 DCXR) and two APDs. The experiments were done at 25 °C.

Confocal microscopy experiments with HeLa cells were performed by using ZEISS LSM 710-FCS with HXC PL APO 63x/1.40 OIL CS objective at 37°C, 5% CO_2 and high humidity. The excitation was provided by 488 nm Argon laser. Emission was spitted on two channels: “green” (550-580 nm) and “red” (590-620 nm). For full quality information from the cells the transmission channel was applied. The images were processed with the Image J software.

To obtain ratiometric images a plug-in for Image J, prepared by Romain Vauchelles, was used. It generates a ratiometric image by dividing the image of the short-wavelength band by that of the long-wavelength band. 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.

Binding of probes to lipid vesicles

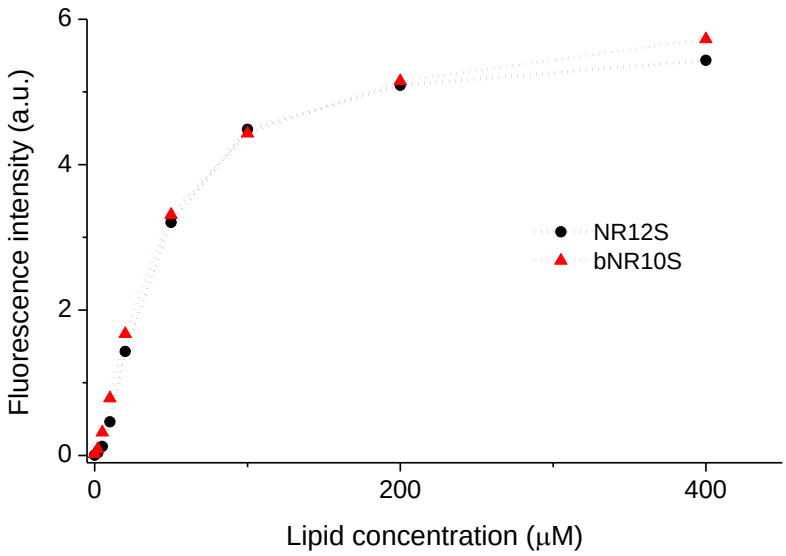


Figure S1. Fluorescence intensity at the emission maximum (607 nm) of NR12S and bNR10S in DOPC vesicles (LUVs) as a function of lipid concentration.

Analysis of probe flip-flop

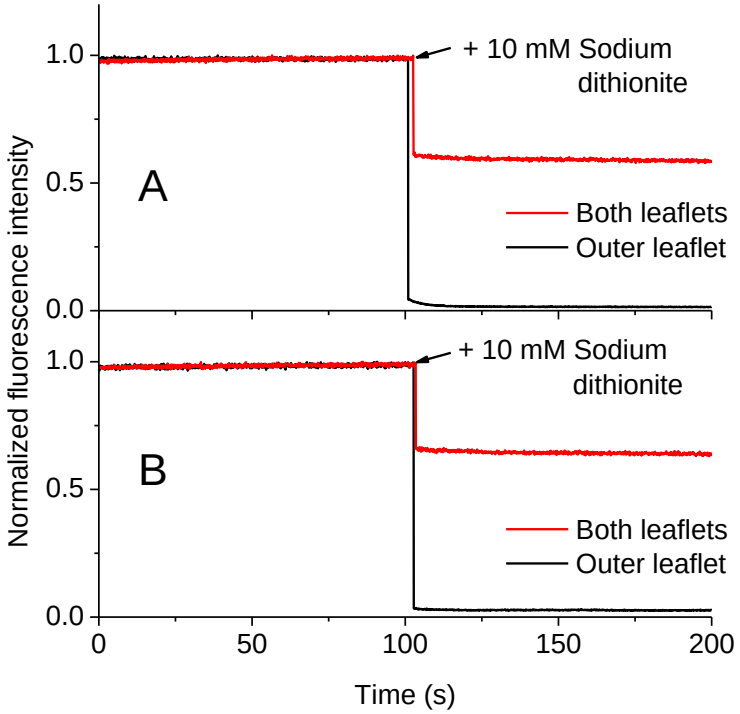


Figure S2. Flip-flop studies of NR12S and bNR10S. Effect of sodium dithionite addition on the fluorescence intensity of NR12S (A) and bNR10S (B) added to LUVs in two ways: before LUVs preparation (expected both leaflets staining) and after the LUVs preparation (expected outer leaflet staining) with 1h incubation at rt.

Binding kinetics to model membranes

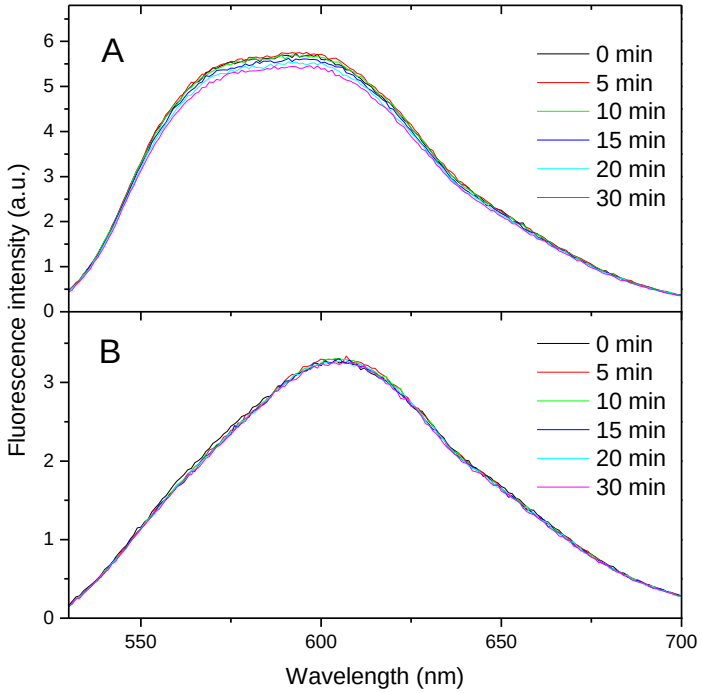


Figure S3. Fluorescence spectra of NR12S (A) and bNR10S (B) recorded at different time after addition of the probes to the mixture of lipid vesicles presenting Lo and Ld phases (Lo/Ld ratio is 10/1). The time 0 corresponds to recording rapidly after the probe addition (approximate delay time is 30 s).

Analysis of the probe partitioning

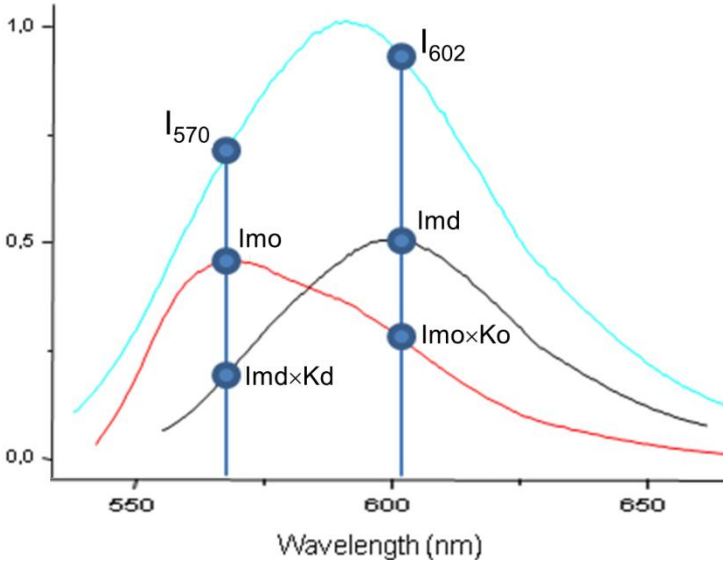


Figure S4. Presentation of the spectrum of the probe in the mixture of phases as an overlap of two spectra in the individual Lo (red) and Ld (black) phases.

It is possible to calculate the partition ratio of a probe between two types of phases, by calculating the contribution of the probe signal from each phase into final spectrum in the phase mixtures. Assuming that the observed spectrum in the mixture of phases is a sum of two spectra in the Lo and Ld phases, the fluorescence intensity at 570 nm (maximum for Lo phase) and 602 nm (maximum for Ld phase) of the spectrum, $I_t(570)$ and $I_t(602)$, respectively, can be expressed as follows:

$$I_{570} = Imo + Imd * K_d \qquad (1)$$

$$I_{602} = Imd + Imo * K_o \qquad (2)$$

Where *Imo* and *Imd* are intensities at the maximum in Lo (570 nm) and Ld (602 nm) phases,

K_d and K_o are coefficients obtained from individual spectra of a probe in pure Lo and Ld phases as follows: $Ko = I_{602}(Lo) / I_{570}(Lo) = 0.773$; $Kd = I_{570}(Ld) / I_{602}(Ld) = 0.683$, where $I_{602}(Lo)$ and $I_{570}(Lo)$ are intensities at 602 and 570 nm for the spectrum in pure Lo phase, while $I_{570}(Ld)$ and $I_{602}(Ld)$ are the intensities at 570 and 602 nm for the spectrum in pure Ld phase.

The solution of the equations (1) and (2) gives:

$$Imd = \frac{I_{602} - I_{570} * K_O}{1 - K_D * K_O} \qquad (3)$$

$$Imo = I_{570} - Imd * K_D \qquad (4)$$

Thus, using equations (3) and (4), we could recalculate the pick intensities of the spectra in Lo and Ld phases that contribute to the spectrum in the mixture. These intensities provide direct information about the partition ratio of the probe between Lo and Ld phases (mixed at 1/1 ratio):

$$\frac{[Probe]_{Ld}}{[Probe]_{Lo}} = \frac{Imd}{Imo} * \frac{I_{570}(Lo)}{I_{602}(Ld)} \qquad (5)$$

Equation (5) converts the ratio Imo/Imd in the partition ratio by taking into account the intensity ratio of the spectra in pure Lo and Ld.

Cellular data

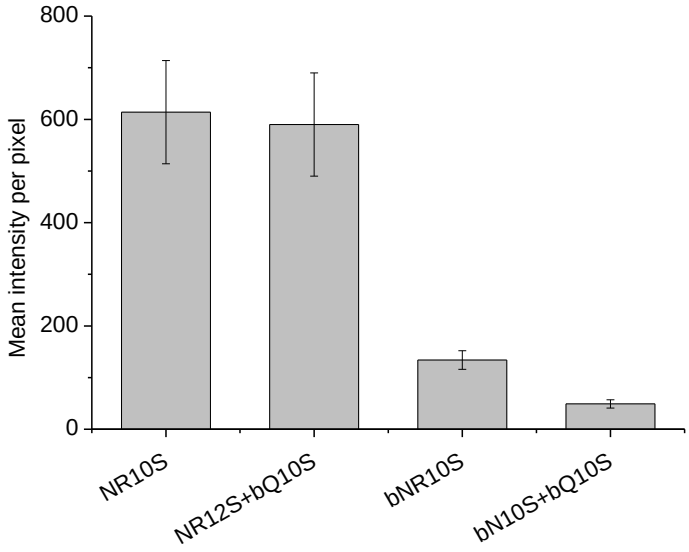


Figure S5. Mean fluorescence intensity of NR12S and bNR10S probes in HeLa cells without and with bQ10S quencher. The analysis was done using confocal images from Fig. 4.

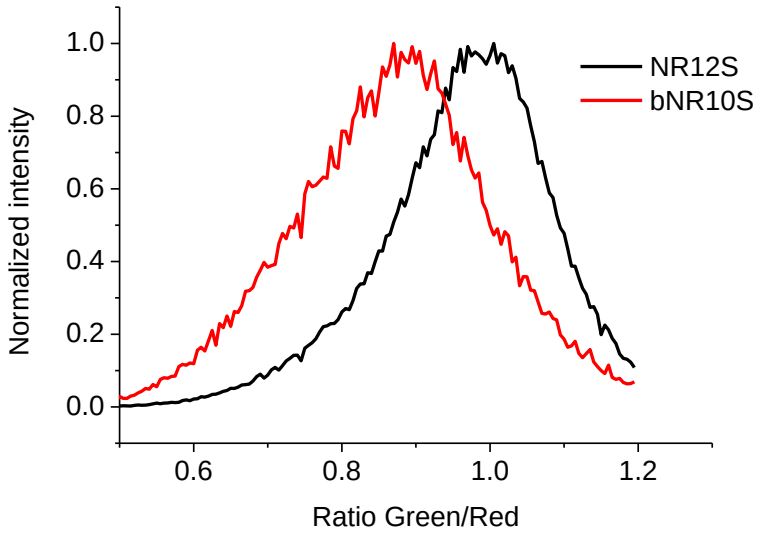


Figure S6. Distribution of the intensity ratio at the Green to Red channels for HeLa cells stained with NR12S and bNR10S probes obtained from analysis of corresponding ratiometric images in Figs. 4E,F. The normalized intensity represents the total intensity of all pixels with a given Green to Red ratio.

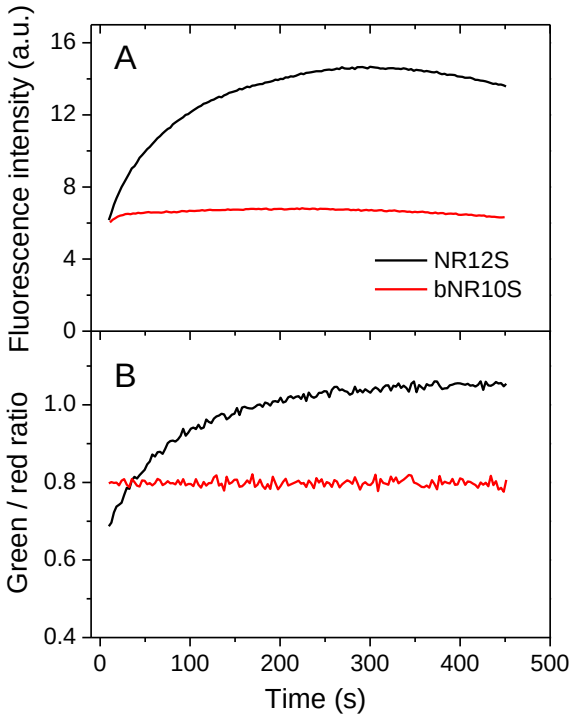


Figure S7. Binding kinetics of NR12S and bNR10S probes to HeLa cells followed from fluorescence intensity (A) and the green/red ratio (B). The intensity was a sum of intensities at the green (570 nm) and the red (602 nm) channels. Excitation wavelength was 520 nm. The intensity ratio green/red was measured between 570 nm (green) and 602 nm (red) channels.

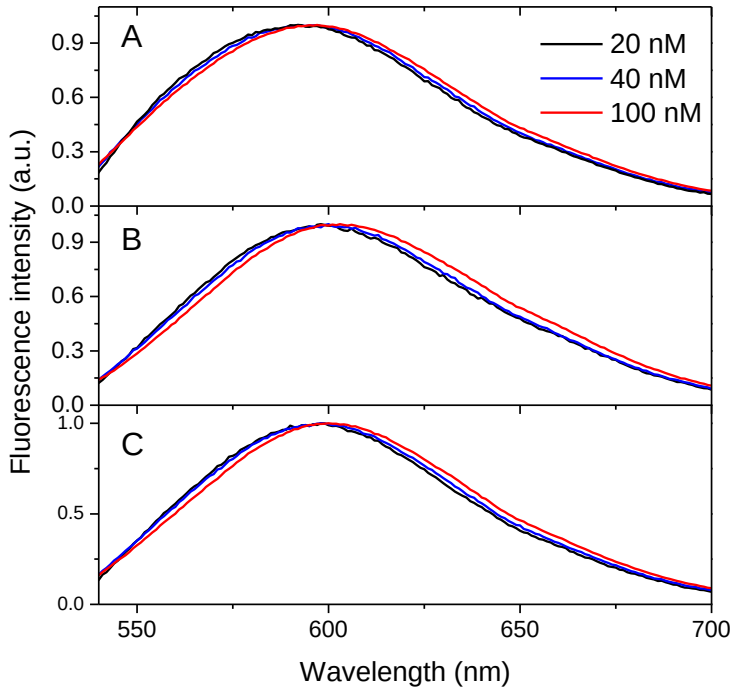


Figure S8. Normalized fluorescence spectra of bNR10S in suspensions of HeLa (A), CHO (B) and T293 (C) cells at different probe concentrations. The spectral shift to the red at 100 nM for all three cell lines shows the beginning of the excessive probe binding.

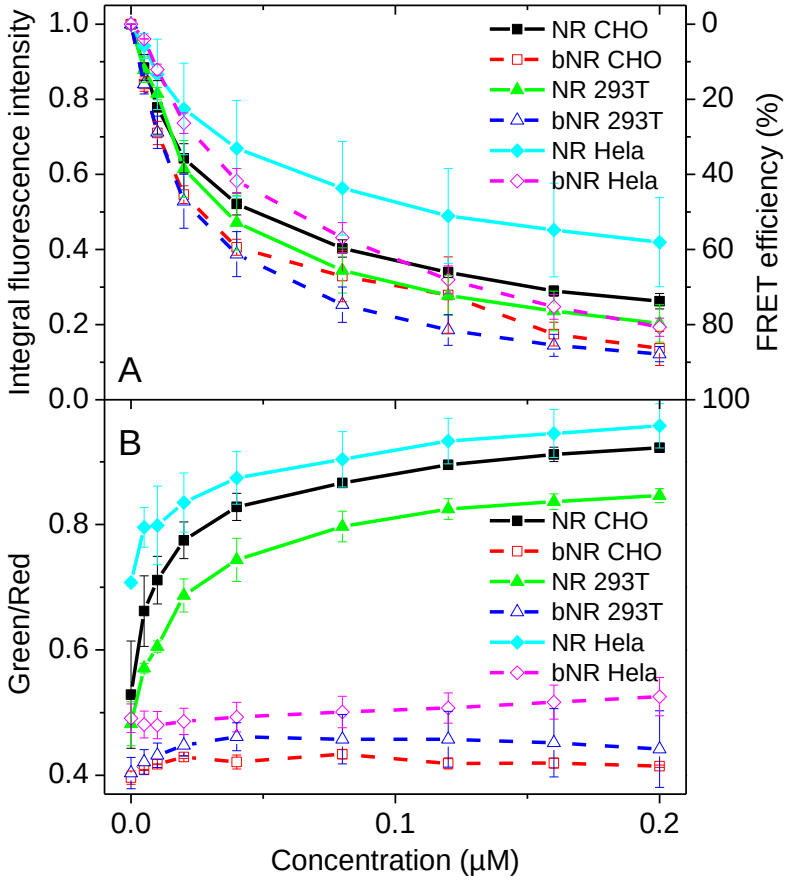


Figure S9. Relative integral intensity with FRET efficiency (A) and Green/Red Ratio of NR12S and bNR10S in different cell lines as a function of bQ10S quencher concentration.

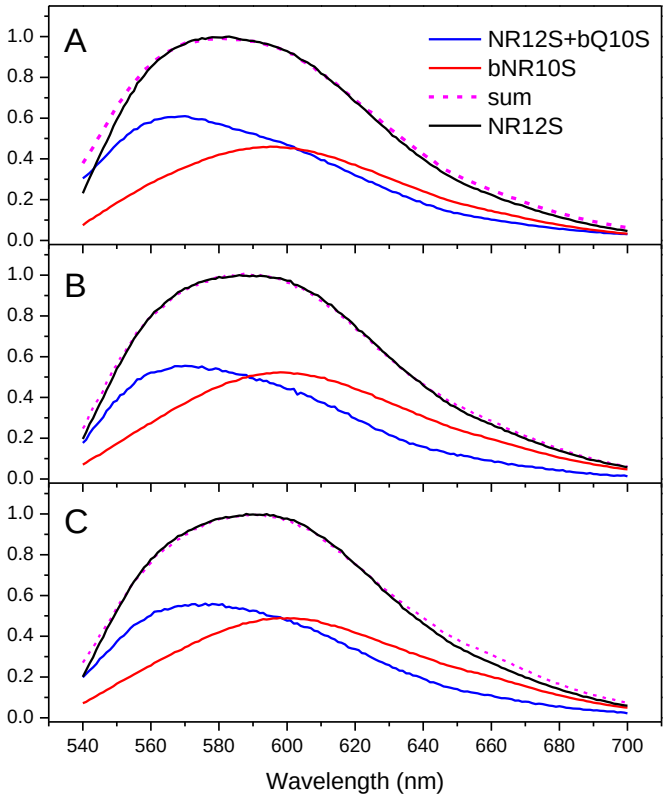


Figure S10. Fluorescence spectra of NR12S (black) in HeLa (A), CHO (B) and T293 (C) cells and a linear a combination (sum, magenta) of spectra of bNR10S, (specific to Ld phase, red), and NR12S with BQ10S quencher (specific to Lo phase, blue). The relative intensities of the blue and the red spectra were obtained by iteration until the best correspondence of their sum with the real spectra of NR12S is achieved.

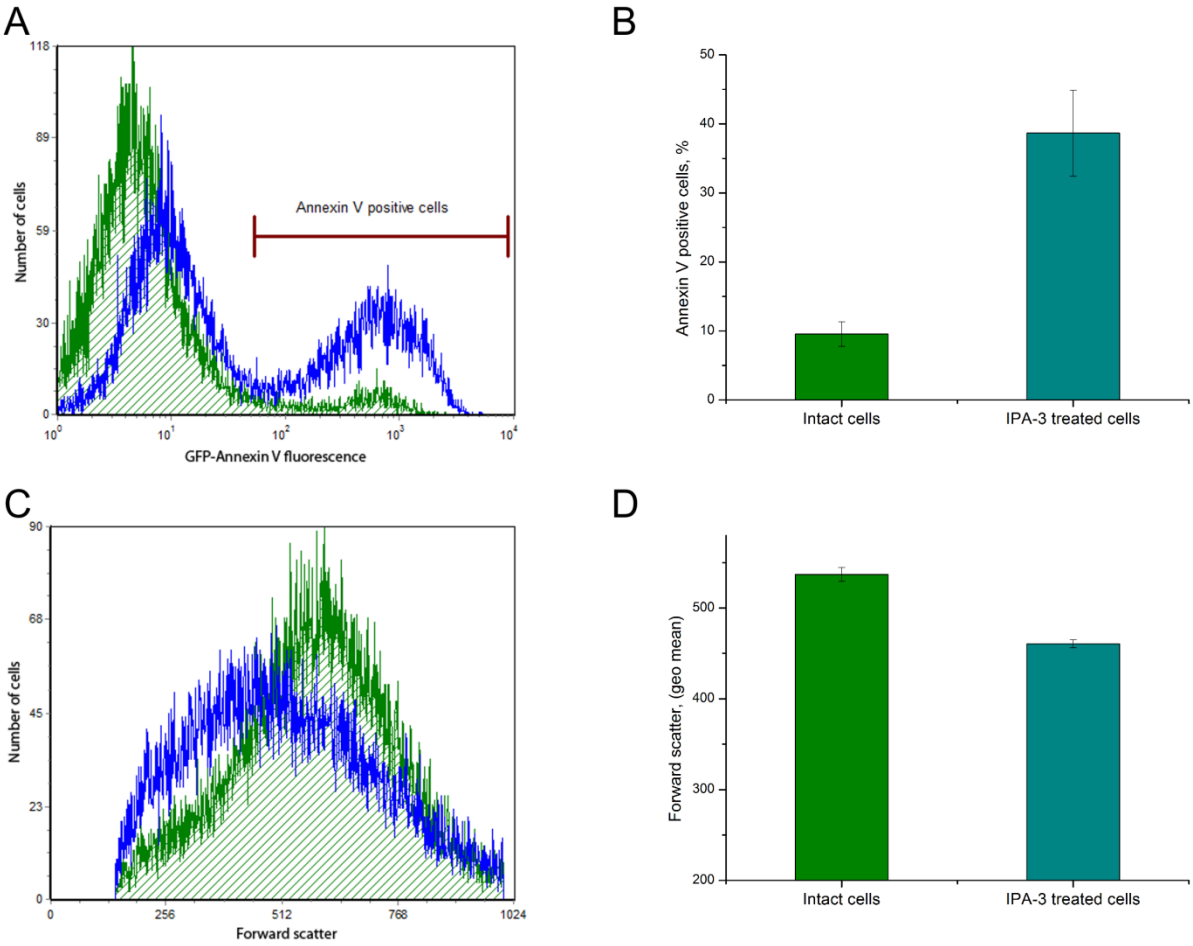


Figure S11. The flow cytometry data of intact and IPA-3 treated HeLa cells stained with GFP-labeled Annexin V. (A, C) Original histograms of GFP-Annexin V fluorescence intensity (A) and the forward scatter of HeLa cells following exposure for 48 hours to complete DMEM media without (green line) and with (blue line) 25 μM IPA-3. (B, D) Arithmetic means ± SEM (n = 4-8) of Annexin V-positive HeLa cells (B) forward scatter (D) following exposure for 48 hours to complete DMEM media without (green bar) or with (blue bar) IPA-3.

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