.

Supporting Information

A Peptide-Based Fluorescent Sensor for Anionic Phospholipids

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1. General Procedures and Materials

Unless otherwise mentioned all the chemicals used were of analytical grade, procured from commercial sources and used without further purification. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC), L-α-phosphatidylinositol-4,5-bisphosphate, ammonium salt, brain, porcine, (PIP2), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol), sodium salt (PG), and L-α-phosphatidylinositol, sodium salt (PI) were purchased from Avanti Polar Lipids. For solid-phase peptide synthesis, resins, amino acids, and activating reagent 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide (HATU) were procured from Novabiochem (Merck Millipore). Acrylodan, fluorescent organelle markers, and Alexa-647-Annexin-V were purchased from Molecular Probes, Thermo Fisher Scientific. Piperidine, N, N-dimethylformamide (DMF), methanol, chloroform, and acetonitrile were purchased from Sigma-Aldrich. N, N-diisopropylethylamine (DIPEA) was obtained from TCI Chemicals. All the chemicals used in cell culture were procured from Gibco. Water required for the experiments described in the paper was deionized using a Milli Q Integral 3 water purification unit (Millipore Corp. Billerica, MA, USA).

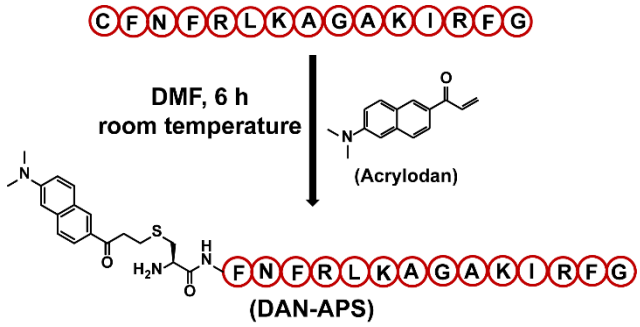
Purification of crude unlabeled peptide and **DAN-APS** after synthesis, were all performed in semi-prep high-performance liquid chromatography (HPLC) system (Prominence, Shimadzu Corp.). Low-resolution mass spectral analyses for characterization and checking purity of unlabeled peptide and **DAN-APS** were performed on a liquid chromatography-mass spectrometer (LCMS-2020, Shimadzu Corp.) with an electro spray ionization probe (LC-ESI-MS). Further mass spectrometric characterization of the unlabeled peptide and **DAN-APS** was performed in a MALDI-TOF system (Bruker UltrafleXtreme, Bruker Daltonics). For determining the concentration of **DAN-APS**, UV-visible absorption spectrum was recorded in an absorption spectrophotometer (JASCO Inc.). **DAN-APS** aliquots were dried on a vacuum concentrator (Eppendorf Concentrator Plus, Eppendorf AG, Germany). Size distribution of SUVs after their preparation in laboratory was determined using Dynamic Light Scattering (DLS) (DynaPro, Protein Solutions, Wyatt Technology Corp., USA). For measurement of *in vitro* fluorescence response of **DAN-APS** a FluoroLog-3 spectrofluorometer (Horiba Jobin Yvon) was used. For fluorescence confocal imaging of GUVs and HeLa cells with **DAN-APS** and other fluorescent markers an LSM 710 confocal microscope (Zeiss) and a 63X oil immersion objective (Zeiss) were used. For processing and analysis of confocal microscopy images Fiji (ImageJ, NIH, USA) software was used.

2. Peptide Synthesis:

Synthesis of CFNFRLKAGAKIRFG peptide sequence was carried out using solid-phase peptide synthesis on H-Rink amide resin (ChemMatrix, 35-100 mesh, loading 0.6 mmol/g resin) on a home-built peptide synthesizer.[1] For each amino acid coupling step on the resin, a solution of N-terminal Fmoc protected amino acid (4 eq., 0.24 mmol), HATU (4 eq., 0.24 mmol) and DIPEA (5 eq, 0.3 mmol) in DMF were mixed with 100 mg resin and taken inside a custom-made reactor placed inside a water bath set at 70 °C. After each coupling step the excess amino acid was washed by a steady flow of DMF at flow rates 5 mL/min and 20 mL/min, successively, followed by the N-terminal deprotection using 50% piperidine in DMF solution at 20 mL/min. The washing of excess amino acid as well as the deprotection of Fmoc group was monitored in a photo diode array detector connected with the reactor by setting the monitored absorption wavelength at 301 nm. Both the Fmoc deprotection step followed by the coupling of appropriate amino acid were repeated successively till the synthesis of the desired sequence of the peptide was ensured. After the completion of synthesis of peptide, the resin beads were washed with DMF and methanol and dried overnight under vacuum. The peptide from the resin beads were cleaved by treating the beads with a mixture of trifluoroacetic acid, triisopropylsilane, phenol, water, ethanedithiol, and thioanisole (80: 2.5: 5: 5: 2.5: 5) for 5 h. The resin beads were removed from the mixture carefully and the solution was concentrated under a steady flow of nitrogen. Crude peptide was precipitated from the solution by addition of cold methyl tert-butyl ether. The crude peptide was then dried, characterized by MALDI-TOF-MS and taken forward for dye labeling after purification on HPLC.

3. Synthesis of DAN-APS:

For attachment of the acrylodan dye to the CFNFRLKAGAKIRFG peptide sequence, purified peptide (3 mg, 0.0017 mmol) was dissolved in anhydrous DMF and mixed with acrylodan (0.45 mg, 0.002 mmol, 1.2 equivalent) solution in DMF (scheme S1). The mixture was allowed to stir for 6 h at room temperature in dark. Then water was added to quench the reaction and the mixture was directly taken forward for HPLC purification. The purified fractions collected from HPLC were characterized using LC-ESI-MS and MALDI-TOF-MS. The purified peptide was dissolved in water to prepare the stock solution for carrying out *in vitro* and in cell studies.



Scheme S1: DAN labelling of CFNFRLKAGAKIRFG to synthesize **DAN-APS**.

4. Purification of CFNFRLKAGAKIRFG and DAN-CFNFRLKAGAKIRFG (DAN-APS) Peptides:

Both the unlabeled and DAN labeled CFNFRLKAGAKIRFG peptide sequences were purified using a semi-prep HPLC equipped with a Phenomenex Luna C18 (10 µm, 250 x 10 mm) semi-prep column. A gradient

of acetonitrile (0.1% trifluoroacetic acid) and water (0.1% trifluoroacetic acid) was used for the elution of the peptides observing the eluent absorption at 220 nm and 380 nm simultaneously. After elution, the fractions containing the peptides, were identified using MALDI-TOF-MS. Identified fractions were dried under vacuum on a vacuum concentrator. Purity of the fractions containing peptides was confirmed by LC-ESI-MS equipped with Agilent Eclipse Plus C18 (5 µm, 250 x 4.6 mm) column using a gradient of acetonitrile (0.01% formic acid) and water (0.01% formic acid) as mobile phase.

5. HPLC and LC-ESI-MS Gradient Details and Characterization of DAN-APS:

HPLC Purification:

Solvent: Solvent A: water with 0.1% trifluoroacetic acid; solvent B: acetonitrile with 0.1% trifluoroacetic acid.

Gradient: 0-5 min: 5% solvent B; 5-50 min: 5-100 % solvent B; 50-55 min: 100% solvent B; 55-60 min: 5% solvent B.

Column: Phenomenex Luna C18 250 x 10 mm.

LC-ESI-MS Characterization:

Solvent: Solvent A: water with 0.01% formic acid; solvent B: acetonitrile with 0.01% formic acid.

Gradient: 0-5 min: 5% solvent B; 5-50 min: 5-100 % solvent B; 50-55 min: 100% solvent B; 55-60 min: 5% solvent B.

Column: Agilent Eclipse Plus C18 250 x 4.6 mm.

DAN-APS: DAN-CFNFRLKAGAKIRFG

Molecular weight: 1952.4 (Calc. for $C_{95}H_{142}N_{26}O_{17}S$)

MALDI-TOF-MS, m/z: 1952.3 $(M)^+$

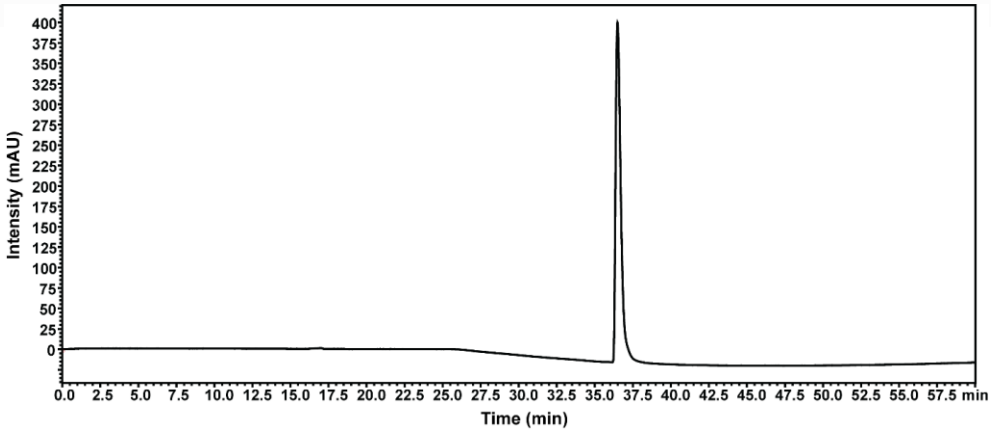


Figure S1: LC elution trace of **DAN-APS** at 380 nm.

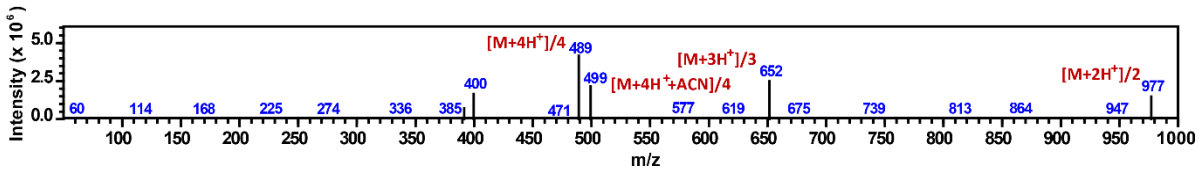


Figure S2: ESI-MS (+ve mode) of peak (37 min) corresponding to the elution of **DAN-APS** in LC.

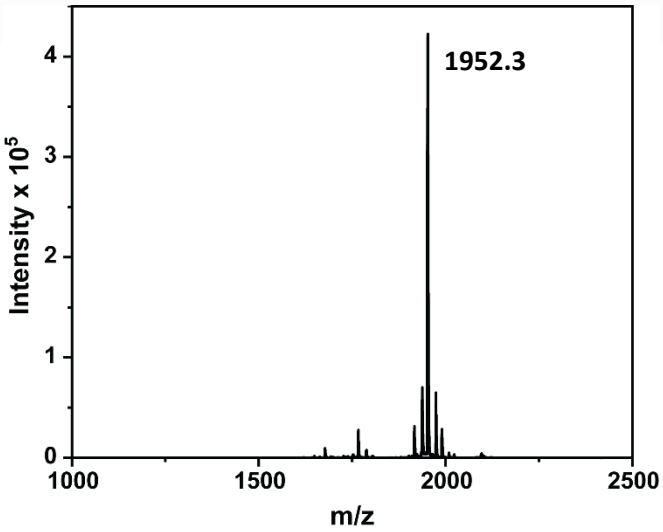


Figure S3: MALDI-TOF Spectrum of **DAN-APS**.

6. UV-Visible Absorption of DAN-APS:

To determine the concentration of the stock solution of **DAN-APS** (10 µL) was diluted by adding water (190 µL). Absorption of diluted solution of **DAN-APS** was measured in an absorption spectrophotometer. We used the absorption coefficient of Prodan (molar extinction coefficient of Prodan at 365 nm = 14500 M^{-1} cm^{-1}), another similar polarity sensitive fluorophore, for calculating the concentration of **DAN-APS**, because in **DAN-APS**, the cysteine conjugated DAN structurally resembles Prodan.[2] Hence the absorption of **DAN-APS** at 365 nm, was used to calculate the concentration of diluted and the stock solution of the sensor.

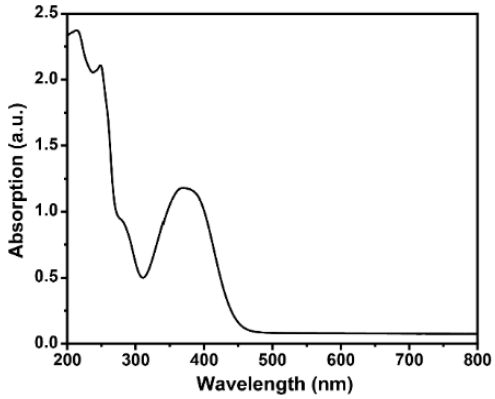


Figure S4: UV-Visible absorption spectrum of **DAN-APS** dissolved in water.

7. Determination of Partition Coefficient (log P) Value of DAN-APS:

For determination of log P value of **DAN-APS**, 20 µL of an aqueous stock (500 µM) of the sensor was diluted to 400 µL in deionized water. Absorption of 200 µL of the solution was recorded. The rest 200 µL of the solution was mixed with 200 µL 1-octanol and the mixture was shaken for 30 min and the mixture was allowed to stand for 2 h for separation of aqueous and octanol layer. After that the aqueous layer was carefully taken out and the absorption of the aqueous layer was recorded. Appropriate solvents were used for baseline measurements. The absorbance value of **DAN-APS** at 365 nm, recorded at the beginning of the experiment was considered as absorbance value of aqueous layer at t = 0 h and the absorbance value of the aqueous layer of **DAN-APS** at 365 nm, separated from octanol, was considered as absorbance value at t = 2.5 h. These values were used to calculate the log P value of DAN-APS following the equation:

$$\log P = \log [(\text{Absorbance at 0 h} - \text{Absorbance at 2.5 h}) / \text{Absorbance at 2.5 h}]$$

8. Preparation of Small Unilamellar Vesicles (SUVs):

A previously standardized method in our laboratory was followed for preparation of SUVs.[3-5] The detailed protocol for the preparation is described below.

Solid phospholipid stocks, stored at -20 °C, were dissolved in HPLC grade chloroform (for PS, PI, PG, PE, PA, PC) or in a mixture of chloroform: methanol: water (20:9:1) (for PIP2) and mixed in clean glass vials in appropriate molar ratio by vigorous vortexing. The quantities of individual lipids and corresponding volumes transferred from stocks for preparing the lipid mixtures are provided in Table S1. After mixing, solvent from the lipid mixtures was evaporated under steady flow of nitrogen and further dried under vacuum overnight to obtain the lipid films. The dried lipid films were hydrated by addition of aqueous buffer (pH 7.4) containing Na-HEPES (20 mM), NaCl (100 mM) followed by thorough vortexing, such that the total phospholipid concentration was maintained at 1 mg/mL. Then the hydrated lipid films were made to undergo 5 successive cycles of freezing at -196 °C and thawing at 37 °C. SUVs of uniform diameter were formed from the non-uniform multilamellar lipid films via extrusion by passing the aqueous lipid suspensions through polycarbonate membranes of 100 nm pore diameter (Whatman, GE Healthcare) at 25 °C. The formation of the SUVs of around 100 nm diameter was confirmed by DLS. The concentration of the phospholipids in the SUVs was determined by estimating the total phosphate content of the vesicles by a previously reported procedure.[6, 7]

Supplementary Table S1: Volumes of phospholipid stocks used for preparation of SUVs.

Mol% PS	PS Stock	PC Stock	PS Taken (mg) (vol from stock)	PC Taken (mg) (vol from stock)	Total phospholipid (mg)
1	0.65 mg PS dissolved in 650 µL $CHCl_3$ (M.W. 783.988)	4.40 mg PC dissolved in 440 µL $CHCl_3$ (M.W. 770.123)	0.003 (3 µL)	0.247 (24.7 µL)	0.25
2			0.005 (5 µL)	0.245 (24.5 µL)	0.25
5			0.013 (13 µL)	0.237 (23.7 µL)	0.25
10			0.025 (25 µL)	0.225 (22.5 µL)	0.25
15			0.038 (38µL)	0.212 (21.2 µL)	0.25

20			0.05 (50 µL)	0.200 (20 µL)	0.25
25			0.063 (63 µL)	0.187 (18.7 µL)	0.25
30			0.076 (76 µL)	0.174 (17.4 µL)	0.25
35			0.089 (89 µL)	0.161 (16.1 µL)	0.25
Mol% PG	**PG Stock**	**PC Stock**	**PG Taken (mg) (vol from stock)**	**PC Taken (mg) (vol from stock)**	**Total phospholipid (mg)**
1	2.97 mg PG dissolved in 1188 µL $CHCl_3$ (M.W. 770.99)	2.25 mg PC dissolved in 225 µL $CHCl_3$ (M.W. 770.123)	0.003 (1.2 µL)	0.247 (24.7 µL)	0.25
2			0.005 (2.0 µL)	0.245 (24.5 µL)	0.25
5			0.013 (5.2 µL)	0.237 (23.7 µL)	0.25
10			0.025 (10 µL)	0.225 (22.5 µL)	0.25
15			0.038 (15.2 µL)	0.212 (21.2 µL)	0.25
20			0.050 (20 µL)	0.200 (20.0 µL)	0.25
25			0.063 (25.2 µL)	0.187 (18.7 µL)	0.25
30			0.075 (30 µL)	0.175 (17.5 µL)	0.25
35			0.088 (35.2 µL)	0.162 (16.2 µL)	0.25
Mol% PA	**PA Stock**	**PC Stock**	**PA Taken (mg) (vol from stock)**	**PC Taken (mg) (vol from stock)**	**Total phospholipid (mg)**
1	0.5 mg PA dissolved in 500 µL $CHCl_3$ (M.W. 706.2)	7.00 mg PC dissolved in 700 µL $CHCl_3$ (M.W. 770.123)	0.003 (3 µL)	0.247 (24.7 µL)	0.25
2			0.005 (5 µL)	0.245 (24.5 µL)	0.25
5			0.012 (12 µL)	0.239 (23.9 µL)	0.251
9			0.021 (21 µL)	0.229 (22.9 µL)	0.25
14			0.033 (33µL)	0.220 (22.0 µL)	0.253
17			0.040 (40 µL)	0.211 (21.1 µL)	0.251
25			0.060 (60 µL)	0.187 (18.7 µL)	0.247
31			0.074 (74 µL)	0.180 (17.4 µL)	0.254
36			0.087 (87 µL)	0.170 (16.1 µL)	0.257
Mol% PI	**PI Stock**	**PC Stock**	**PI Taken**	**PC Taken**	**Total phospholipid (mg)**

			(mg) (vol from stock)	(mg) (vol from stock)	
1	1.00 mg PI dissolved in 400 µL $CHCl_3$ (M.W. 902.1)	5 mg PC dissolved in 500 µL $CHCl_3$ (M.W. 770.123)	0.003 (1.2 µL)	0.247 (24.7 µL)	0.25
2			0.006 (2.4 µL)	0.244 (24.5 µL)	0.25
5			0.014 (5.6 µL)	0.236 (23.6 µL)	0.25
9			0.026 (10.4 µL)	0.224 (22.4 µL)	0.25
13			0.037 (14.8 µL)	0.213 (21.2 µL)	0.25
17			0.047 (18.8 µL)	0.200 (20.0 µL)	0.25
23			0.065 (26.0 µL)	0.185 (18.5 µL)	0.25
29			0.079 (31.6 µL)	0.171 (17.1 µL)	0.25
33			0.092 (36.8 µL)	0.158 (15.8 µL)	0.25
Mol% PIP2	**PIP2 Stock**	**PC Stock**	**PIP2 Taken (mg) (vol from stock)**	**PC Taken (mg) (vol from stock)**	**Total phospholipid (mg)**
1	0.2 mg PIP2 dissolved in 200 µL $CHCl_3$ (M.W. 1096.38)	5 mg PC dissolved in 500 µL $CHCl_3$ (M.W. 770.123)	0.004 (4.0 µL)	0.246 (24.6 µL)	0.25
2			0.007 (7.0 µL)	0.243 (24.3 µL)	0.25
3			0.011 (11.0 µL)	0.239 (23.9 µL)	0.25
5			0.017 (17.0 µL)	0.233 (23.3 µL)	0.25
9			0.031 (31.0 µL)	0.219 (21.9 µL)	0.25
13			0.044 (44.0 µL)	0.206 (20.6 µL)	0.25
17			0.055 (55.0 µL)	0.195 (19.5 µL)	0.25
Mol% PE	**PE Stock**	**PC Stock**	**PE Taken (mg) (vol from stock)**	**PC Taken (mg) (vol from stock)**	**Total phospholipid (mg)**
1	1.00 mg PE dissolved in 400 µL $CHCl_3$ (M.W. 718)	5 mg PC dissolved in 500 µL $CHCl_3$ (M.W. 770.1)	0.002 (0.8 µL)	0.248 (24.7 µL)	0.25
2			0.005 (2.0 µL)	0.245 (24.5 µL)	0.25
5			0.011 (4.4 µL)	0.239 (23.9 µL)	0.25
9			0.021 (8.4 µL)	0.229 (22.9 µL)	0.25
17			0.039 (15.6 µL)	0.211 (21.1 µL)	0.25
23			0.055 (22.0 µL)	0.195 (19.5 µL)	0.25

29			0.068 (27.2 µL)	0.182 (18.2 µL)	0.25

9. Size Distributions of Phospholipid SUVs as Determined from Dynamic Light Scattering:

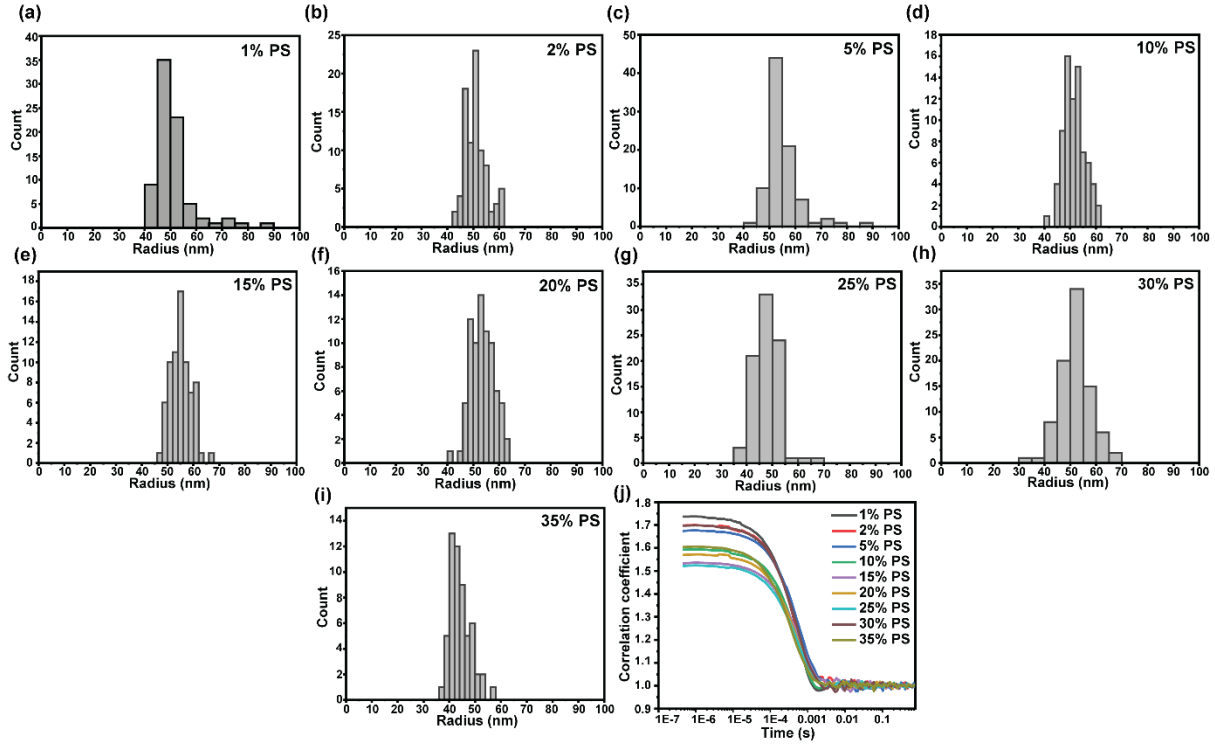


Figure S5: Size distribution (hydrodynamic radius) of SUVs prepared with different mol percent of PS in PC (plots a to i) that were used for the fluorescence titration experiments. Corresponding autocorrelation coefficient plots are also provided for each type of SUV (plot j).

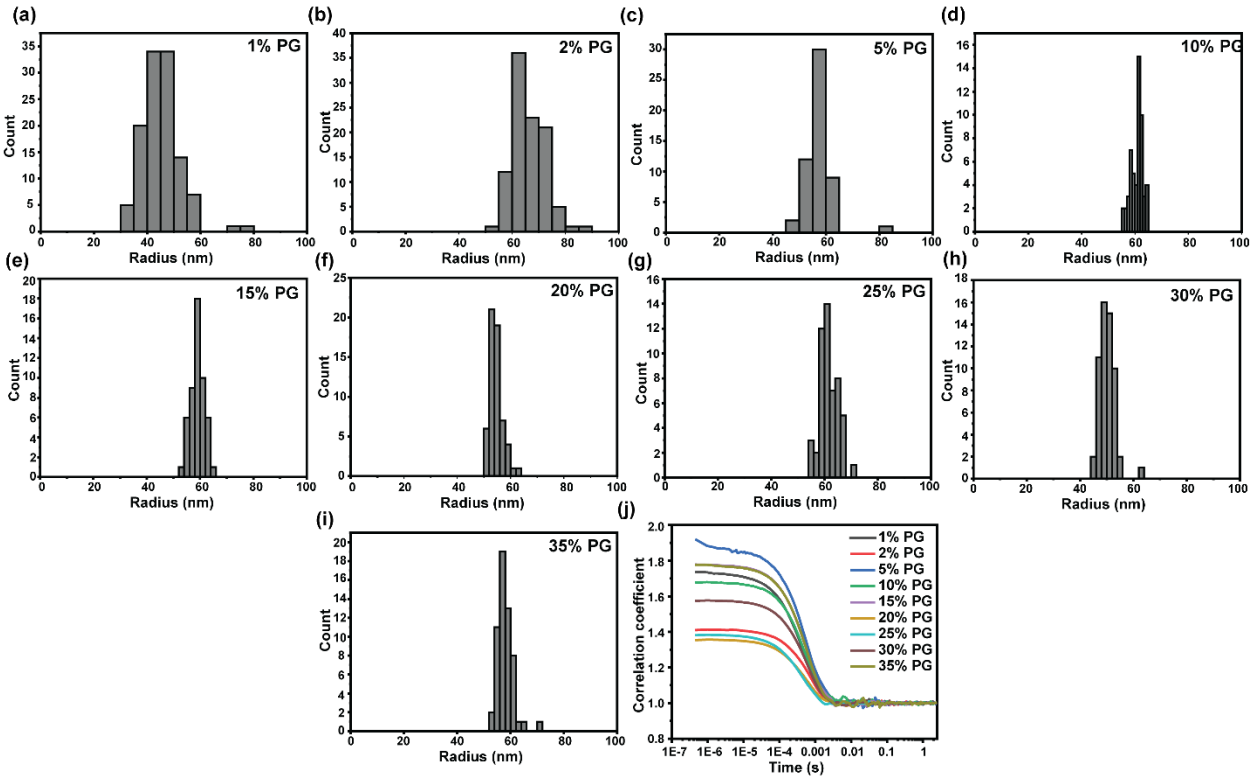


Figure S6: Size distribution (hydrodynamic radius) of SUVs prepared with different mol percent of PG in PC (plots a to i) that were used for the fluorescence titration experiments. Corresponding autocorrelation coefficient plots are also provided for each type of SUV (plot j).

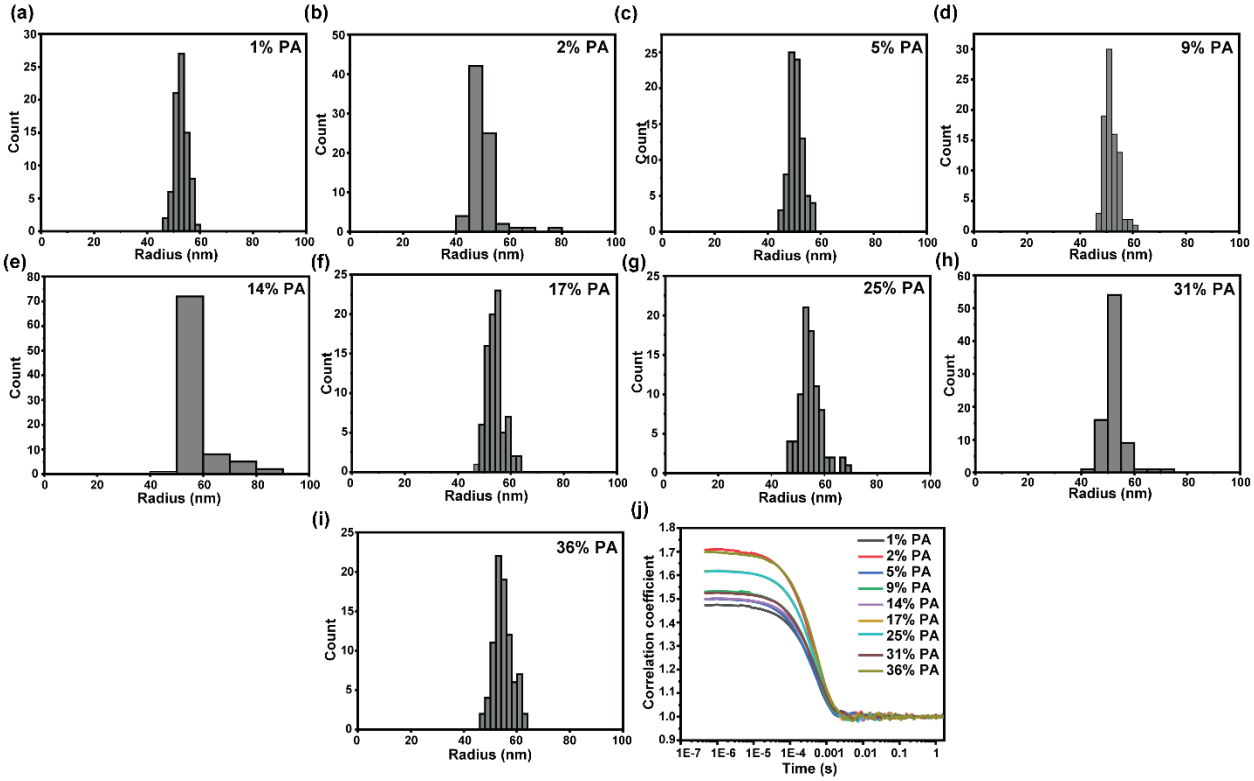


Figure S7: Size distribution (hydrodynamic radius) of SUVs prepared with different mol percent of PA in PC (plots a to i) that were used for the fluorescence titration experiments. Corresponding autocorrelation coefficient plots are also provided for each type of SUV (plot j).

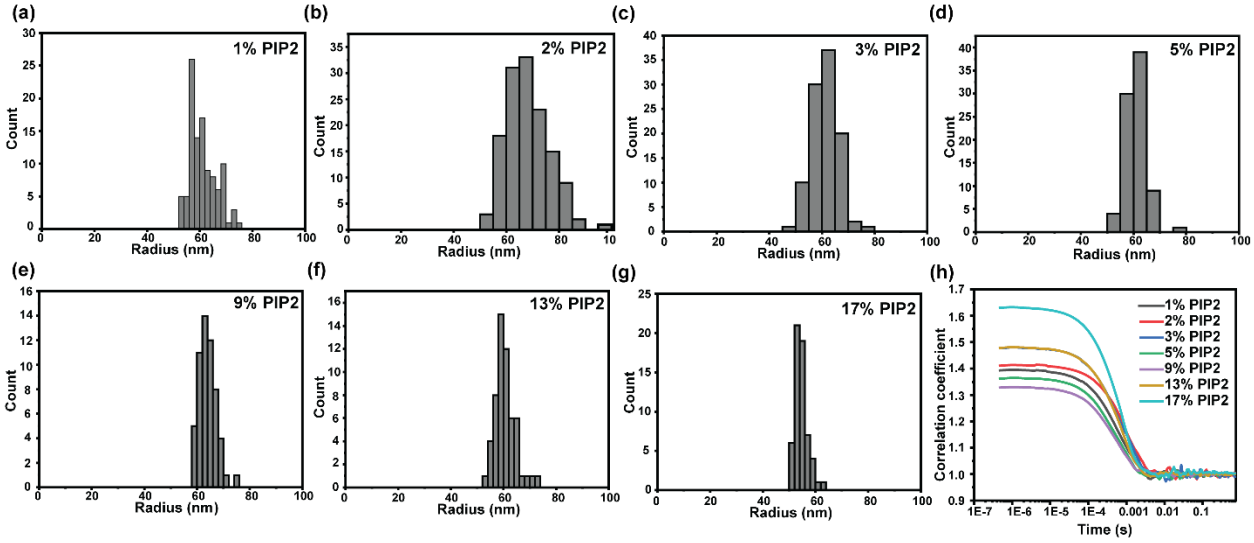


Figure S8: Size distribution (hydrodynamic radius) of SUVs prepared with different mol percent of PIP2 in PC (plots a to g) that were used for the fluorescence titration experiments. Corresponding autocorrelation coefficient plots are also provided for each type of SUV (plot h).

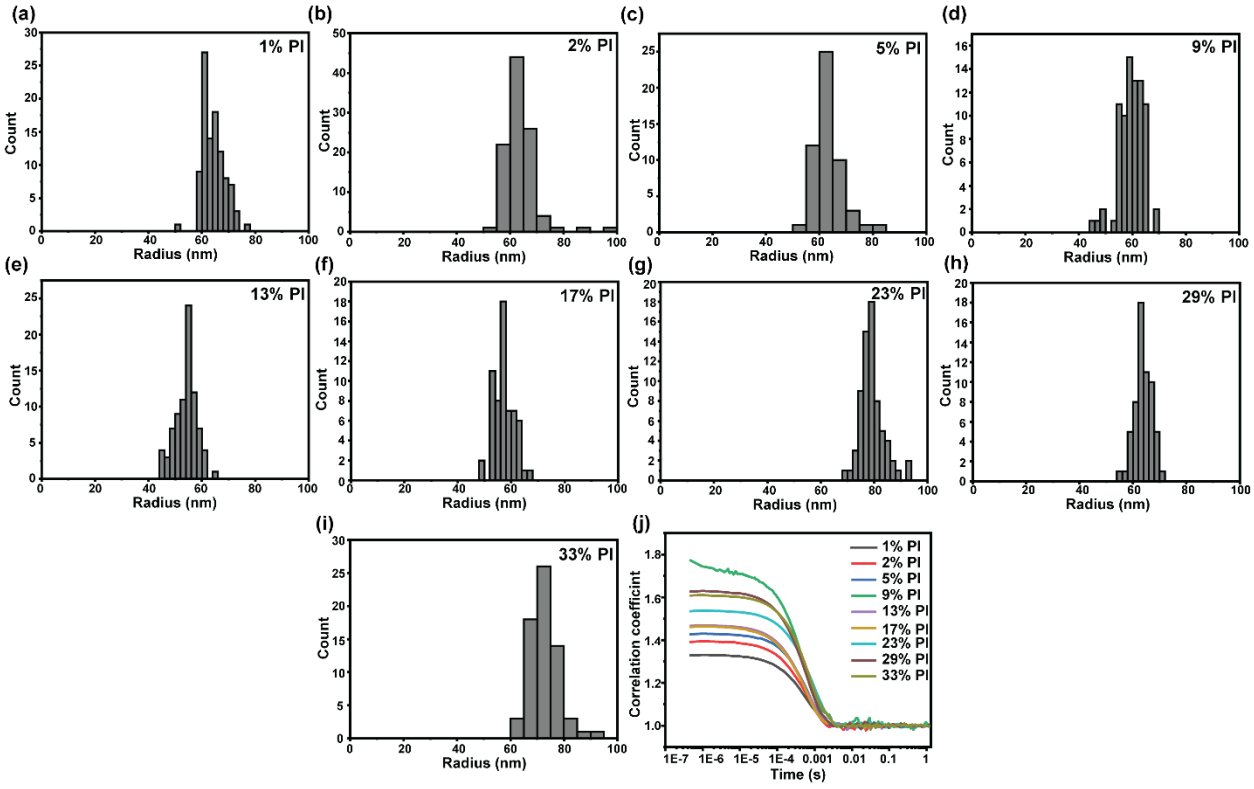


Figure S9: Size distribution (hydrodynamic radius) of SUVs prepared with different mol percent of PI in PC (plots a to i) that were used for the fluorescence titration experiments. Corresponding autocorrelation coefficient plots are also provided for each type of SUV (plot j).

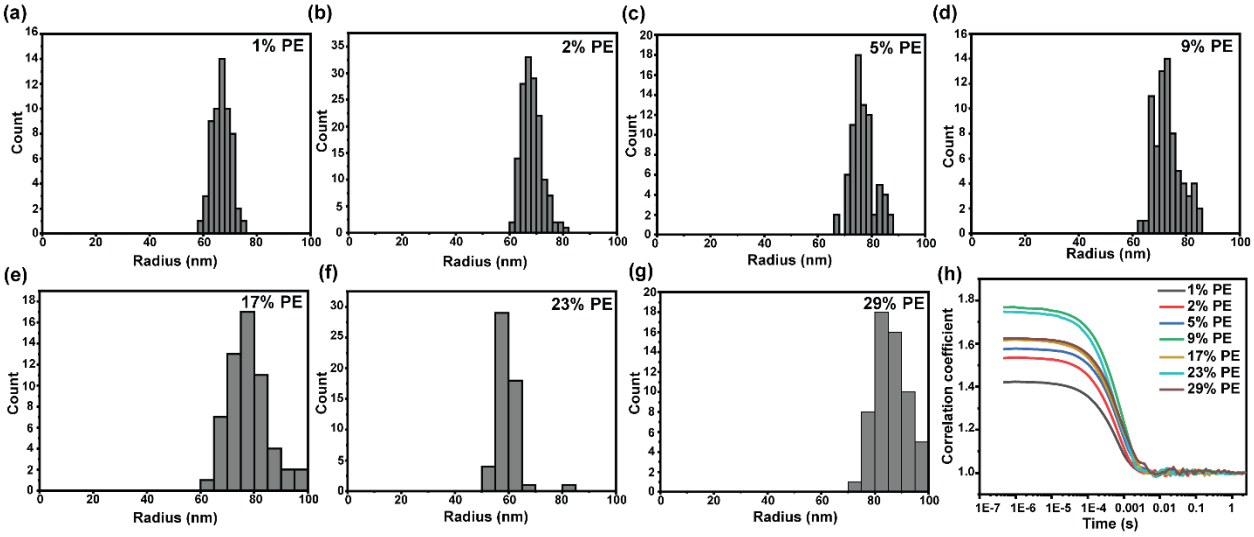


Figure S10: Size distribution (hydrodynamic radius) of SUVs prepared with different mol percent of PE in PC (plots a to g) that were used for the fluorescence titration experiments. Corresponding autocorrelation coefficient plots are also provided for each type of SUV (plot h).

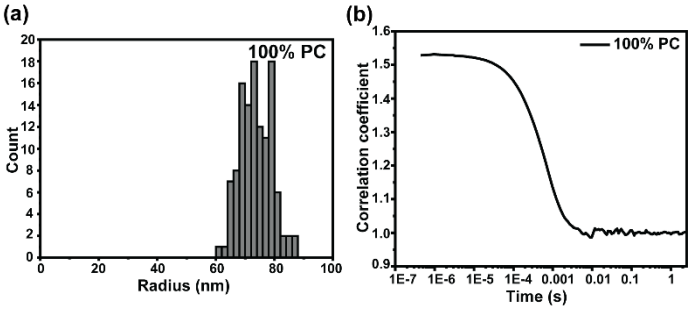


Figure S11: Size distribution (hydrodynamic radius) of SUVs prepared with PC (plot a) that were used for fluorescence titration experiments. Corresponding autocorrelation coefficients plot are also provided for each type of SUVs (plot b).

Supplementary Table S2: Mean radius and standard deviation of SUVs.

	1%	**2%**	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**	**35%**
PS	51.37± 7.89	51.04± 4.18	55.00 ± 6.46	51.58 ± 4.08	55.12 ± 4.04	53.48± 4.32	48.03± 4.70	52.042± 6.15	44.28± 4.06
PG	45.30± 7.23	66.25± 6.18	57.50± 4.55	60.80± 2.25	58.99± 2.55	54.62± 2.31	61.65± 3.38	50.11± 2.90	57.94± 2.94
	1%	**2%**	**5%**	**9%**	**13%**	**17%**	**23%**	**29%**	**33%**
PI	64.37± 4.02	63.66± 5.86	63.77± 5.40	59.61± 4.58	54.05± 4.04	57.48± 3.69	79.17± 4.29	63.86± 3.17	72.95± 5.20
	1%	**2%**	**5%**	**9%**	**14%**	**17%**	**25%**	**31%**	**36%**
PA	52.94± 2.34	49.98± 4.93	50.60± 2.66	51.85± 2.70	58.02± 10.12	53.94± 3.25	54.72± 4.20	52.39± 4.13	54.69± 3.50
	1%	**2%**	**5%**	**9%**	**--**	**17%**	**23%**	**29%**	**--**
PE	66.83± 3.29	68.24± 3.72	76.64± 4.38	73.21± 5.07	--	78.48± 8.34	59.58± 4.31	87.44± 8.06	--

	1%	**2%**	**3%**	**5%**	**9%**	**13%**	**17%**
PIP2	61.25± 5.11	68.36± 8.06	60.91± 4.81	60.89± 4.17	64.04± 3.23	60.45± 3.83	54.62± 2.31

	100%
PC	73.92±4.99

Supplementary Table S3: Polydispersity indices of SUVs.

	1%	**2%**	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**	**35%**
PS	0.28	0.20	0.20	0.21	0.20	0.20	0.24	0.29	0.43
PG	0.13	0.12	0.19	0.11	0.12	0.15	0.17	0.16	0.11
	1%	**2%**	**5%**	**9%**	**13%**	**17%**	**23%**	**29%**	**33%**
PI	0.15	0.18	0.27	0.15	0.11	0.27	0.21	0.37	0.15
	1%	**2%**	**5%**	**9%**	**14%**	**17%**	**25%**	**31%**	**36%**
PA	0.15	0.38	0.21	0.21	0.22	0.19	0.17	0.26	0.21
	1%	**2%**	**5%**	**9%**	**--**	**17%**	**23%**	**29%**	**--**
PE	0.19	0.15	0.15	0.17	--	0.15	0.15	0.17	--

	1%	2%	3%	5%	9%	13%	17%
PIP2	0.20	0.24	0.14	0.35	0.47	0.19	0.20
				100%			
				0.21			
PC							

10. Measurement of Fluorescence Response of DAN-APS in Presence of SUVs:

Constant volume *in vitro* fluorescence titrations were performed in aqueous buffer (pH 7.4) containing Na-HEPES (20 mM), NaCl (100 mM) at 25 °C. Different SUV dispersions containing mixture of PC and 1, 2, 5, 10, 15, 20, 25, 30 and 35 mol% PS were prepared maintaining total phospholipid concentration 1 mg/mL in each case, following the protocol mentioned in section 8, SI. For measuring the fluorescence response of **DAN-APS** to increasing PS, different solutions were prepared with sensor (1.5 µM) and SUVs with different PS mol%. Total phosphate concentration and volume of the mixtures were kept at 20 µM and 200 µL, respectively. Fluorescence spectra of different solutions of **DAN-APS** and SUVs were recorded on a spectrofluorometer by exciting the sensor at 380 nm. For determining the value of dissociation constant (K_d) of the sensor for PS, ratio of fluorescence emission intensities at 450 nm and 525 nm ($F_{450\ nm}/F_{525\ nm}$) for individual solutions were plotted against the corresponding PS concentrations. The response curve obtained was fitted to equations 5 and 6, section 11, SI to obtain the K_d value. Identical titrations were repeated for other physiologically relevant phospholipids such as PG, PIP2, PE, PA, PI and K_d values were determined for each phospholipid.

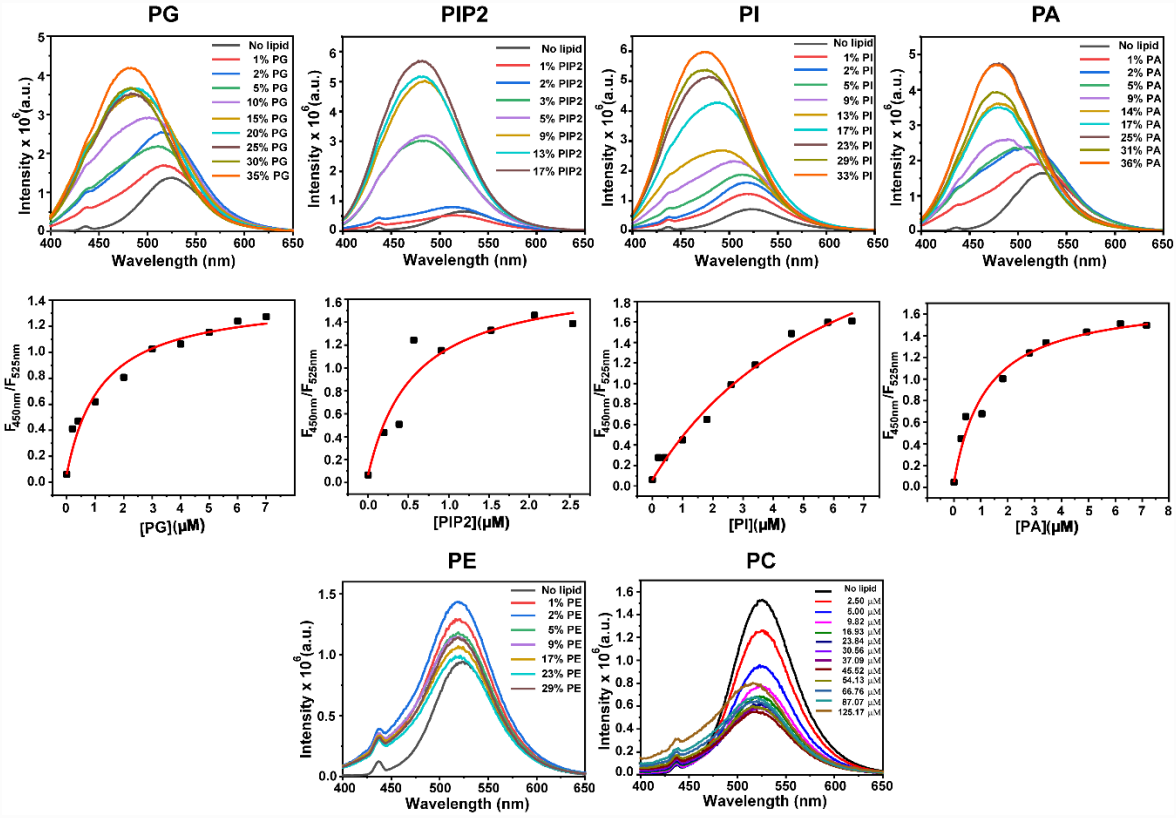


Figure S12: *In vitro* fluorescence response of **DAN-APS** with physiologically relevant phospholipids. **Top Panel:** Fluorescence emission spectra of individual solutions consisting of **DAN-APS** (1.5 μM) and anionic phospholipids mixed with PC such that the total phosphate concentration remained 20 μM for each composition of the individual phospholipids. **Middle Panel:** Ratiometric response of **DAN-APS** against individual anionic phospholipid concentrations. The response curve has been fitted using the equation 6, section 11. **Bottom Panel:** Fluorescence emission spectra of individual solutions consisting of **DAN-APS** (1.5 μM) at different compositions of PE in PC (left) and different concentrations of 100% PC SUVs (right).

11. Determination of Binding Constant of DAN-APS with Different Phospholipids using Constant Volume Fluorescence Titration Data:

We fitted the ratiometric response of **DAN-APS** for different phospholipids based on the ratiometric fitting equation by Grynkiewicz et al.[8] Emission maxima of **DAN-APS** shifts from 525 nm for the unbound sensor in aqueous buffered solution to around 480 nm when it binds to PS or other anionic phospholipids. For measuring the ratiometric response of the sensor for its binding to phospholipids, we chose the intensities at two wavelengths: 525 nm, representative of unbound and bound sensor and 450 nm, representative of mostly the membrane bound population of the sensor. We assumed fluorescence intensities at 525 nm and 450 nm would be contributed from both free and bound population of the sensor and the contributions were proportional to the concentrations of the free (c_f) and membrane bound (c_b) sensor. Hence, we introduced four proportionality constants $S_{f,525}$, $S_{b,525}$, $S_{f,450}$, $S_{b,450}$.

Also, assuming 1:1 binding of the sensor with phospholipid membranes, the concentrations of free and membrane bound sensor should follow the following relationship:

$$c_b = c_f[PS]/K_d \tag{1}$$

Fluorescence intensities at 525 nm and 450 nm would be following:

$$F_{525} = S_{b,525}\, c_b + S_{f,525}\, c_f \tag{2}$$

$$F_{450} = S_{b,450}\, c_b + S_{f,450}\, c_f \tag{3}$$

Hence, the ratio of intensities at 450 and 525 nm,

$$R = \frac{F_{450}}{F_{525}} = \frac{S_{b,450}\, c_b + S_{f,450}\, c_f}{S_{b,525}\, c_b + S_{f,525}\, c_f}$$

Or,
$$R = \frac{S_{b,450}(\frac{c_f[PS]}{K_d}) + S_{f,450} c_f}{S_{b,525}(\frac{c_f[PS]}{K_d}) + S_{f,525} c_f}$$

Or,
$$R = \frac{S_{b,450}(\frac{[PS]}{K_d}) + S_{f,450}}{S_{b,525}(\frac{[PS]}{K_d}) + S_{f,525}}$$

Or,
$$S_{b,450}\ [PS] + S_{f,450}\ K_d = R(S_{b,525}\,[PS] + S_{f,525}\ K_d)$$

Or,
$$[PS](S_{b,450} - RS_{b,525}) = K_d(RS_{f,525} - S_{f,450})$$

Or, $$[PS] = \frac{K_d(RS_{f,525} - S_{f,450})}{S_{b,450} - RS_{b,525}}$$

Or, $$[PS] = \frac{K_d(R - \frac{S_{f,450}}{S_{f,525}})}{(\frac{S_{b,450}}{S_{b,525}} - R)} \times \frac{S_{f,525}}{S_{b,525}} \quad (4)$$

When lipid is not added to the sensor solution then, $[PS] = 0$, $R = R_{min}$

Then, $$R_{min} - \frac{S_{f,450}}{S_{f,525}} = 0$$

Or, $$R_{min} = \frac{S_{f,450}}{S_{f,525}}$$

At saturating lipid concentrations, $R = R_{max}$

Then, $$\frac{S_{b,450}}{S_{b,525}} - R_{max} = 0$$

Or, $$R_{max} = \frac{S_{b,450}}{S_{b,525}}$$

From equation 4, $$[PS] = K_d'(\frac{R - R_{min}}{R_{max} - R}), \quad \text{where } K_d' = K_d(\frac{S_{f,525}}{S_{b,525}})$$

Rearranging equation 4, $$R = R_{min} + \frac{R_{max} - R_{min}}{1 + \frac{K_d'}{[PS]}}$$

Also, equation 2, in absence of lipid, $c_b = 0$ and $F_{f,525} = S_{f,525}\, c_f$

Or, $$S_{f,525} = \frac{F_{525}}{c_f}$$

Again, at saturating lipid concentrations, $c_f = 0$ and $c_b = c_f$

So, $$F_{b,525} = S_{b,525}\, c_f$$

Hence, $$\frac{S_{f,525}}{S_{b,525}} = \frac{(\frac{F_{f,525}}{c_f})}{(\frac{F_{b,525}}{c_f})}$$

Or, $$\frac{S_{f,525}}{S_{b,525}} = \frac{F_{f,525}}{F_{b,525}}$$

Therefore, dissociation constant $$K_d = \frac{K_d'}{(\frac{S_{f,525}}{S_{b,525}})} = \frac{K_d'}{(\frac{F_{f,525}}{F_{b,525}})} \quad (5)$$

Where K_d' is determined from

$$\frac{F_{450}}{F_{525}} = (\frac{F_{450}}{F_{525}})_{min} + \frac{(\frac{F_{450}}{F_{525}})_{max} - (\frac{F_{450}}{F_{525}})_{min}}{1 + \frac{K_d'}{[PS]}} \quad (6)$$

This implies that the dissociation constant (K_d) for the binding of the sensor with a phospholipid can be determined by dividing the apparent dissociation constant (K_d') obtained by fitting the ratiometric titration plot by the ratio of intensities of free and bound sensor at 525 nm.

12. Determination of Quantum Yield of DAN-APS and PS-Bound DAN-APS:

Quantum yields of **DAN-APS** in absence and presence of PS SUVs were estimated with respect to quinine sulfate in 0.1 M H_2SO_4 as standard.[9] The concentrations of solutions used for the standard were 0 µM (blank), 4 µM, 5 µM, 8 µM, and 10 µM. The quantum yield calculations were done based on the reported quantum yield of quinine sulfate at 350 nm which was 0.58. For the estimation of the quantum yield of **DAN-APS** in absence and presence of PS SUVs the following solutions were prepared: 0 µM (blank), 2 µM, 3 µM and 4 µM solutions of only **DAN-APS**; 0 µM (blank), 2 µM, 3 µM and 4 µM solutions of **DAN-APS** mixed with 15% PS-PC SUVs (80 µM total phosphate concentration) in Na-HEPES (20 mM), NaCl (100 mM) (pH 7.4). Absorption spectra were recorded for all solutions. Fluorescence spectra (λ_{ex}: 375 nm, λ_{em}: 400 to 650 nm for **DAN-APS** and λ_{ex}: 350 nm, λ_{em}: 360 to 560 nm for quinine sulfate) of all solutions were recorded in identical slit width (1 nm x 1 nm). Integrated area for individual fluorescence spectrum was plotted against the absorbance (at 375 nm for **DAN-APS** and at 350 nm for quinine sulfate) of each solution. The plot of integrated fluorescence intensity versus absorbance for each fluorophore was fitted to a linear fit. The ratio of the slopes of free **DAN-APS** and SUV bound **DAN-APS** with quinine sulfate was used to calculate the quantum yield of free **DAN-APS** and SUV bound **DAN-APS** using the following equation:

$$\phi_{DAN-APS} = \phi_{std} \text{ X } \left(\frac{Slope_{DAN-APS}}{Slope_{std}}\right) \text{ X } \left(\frac{\eta_{DAN-APS}}{\eta_{std}}\right) \quad (7)$$

where ϕ_{std}, $\phi_{DAN\text{-}APS}$ are the quantum yields of the standard and **DAN-APS**, either free or SUV bound; $Slope_{std}$ and $Slope_{DAN\text{-}APS}$ are the slopes of the fits obtained from the integrated fluorescence versus absorbance plots of standard and **DAN-APS**, either free or SUV bound; η_{std}, $\eta_{DAN\text{-}APS}$ are the refractive indices of solvents of standard fluorophore and **DAN-APS**. For the estimation of quantum yield of free and SUV bound **DAN-APS**, the ϕ_{std} = 0.58, η_{std}, $\eta_{DAN\text{-}APS}$ = 1.33 were used.

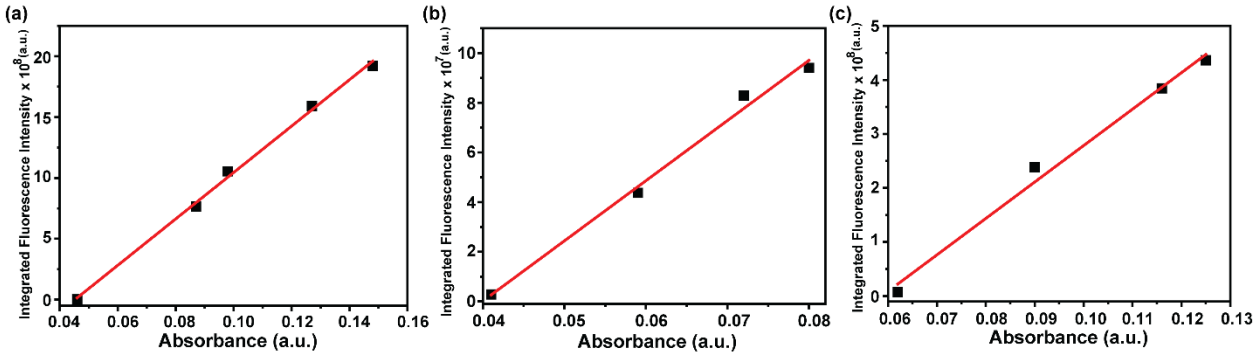


Figure S13: Integrated fluorescence vs absorbance plots of (a) quinine sulfate in 0.1M H_2SO_4 (b) **DAN-APS** and (c) **DAN-APS** in presence of PS SUVs. Linear fits (red line) of the data points in the plots yielded slopes **1.91 x 10^{10}**, **2.42 x 10^9** and **6.44 x 10^9** for quinine sulfate, **DAN-APS**, and **DAN-APS** in presence of PS SUVs, respectively.

13. Fluorescence Response of DAN-APS in Presence of Soluble Inositol Phosphates and Cys-PSBP-6:

Constant volume *in vitro* fluorescence titrations were performed in Na-HEPES (20 mM), NaCl (100 mM), pH 7.4 at 25 °C. For testing the effect of soluble phosphorylated carbohydrates on fluorescence response of **DAN-APS** towards PS SUVs, different mixtures were prepared with sensor (1.5 µM) and 25% PS SUVs with either of the inositol phosphates, IP3 (40 µM), or IP4 (40 µM) or IP6 (40 µM). Total phospholipid concentration and volume of the mixtures were kept at 20 µM and 200 µL, respectively. Fluorescence spectra of different mixtures of **DAN-APS**, SUVs, and phosphorylated carbohydrates were recorded on a spectrofluorometer by exciting the samples at 380 nm. Ratiometric response of different mixtures ($F_{450\ nm}/F_{525\ nm}$) were plotted for comparison. The data were averaged over at least three independent experiments.

For testing the reversibility of the fluorescence response of **DAN-APS**, a competition titration of the PS bound sensor was carried out with fluorophore unlabeled peptide, **Cys-PSBP-6** (CFNFRLKAGAKIRFG). First, the fluorescence spectra of solutions of **DAN-APS** and **DAN-APS** with 10 mol% PS were recorded. The **Cys-PSBP-6** peptide was then titrated into the solution containing **DAN-APS** and 10% PS.

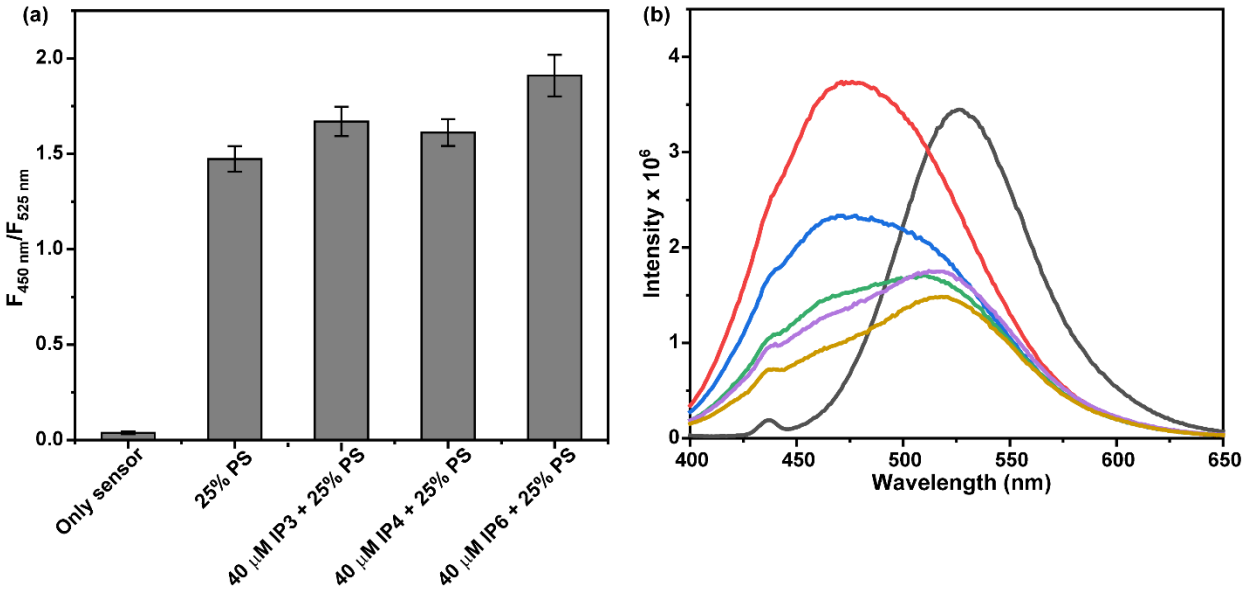


Figure S14: (a) Comparison of in vitro ratiometric fluorescence response of: only **DAN-APS**; **DAN-APS** in presence of 25% PS (total phospholipid 20 µM); **DAN-APS** and 25% PS (total phospholipid 20 µM) along with either IP3 (40 µM), or IP4 (40 µM), or IP6 (40 µM) in 20 mM Na-HEPES, 100 mM NaCl (pH 7.4). (b) Competition titration of PS bound **DAN-APS** with fluorophore unlabeled peptide, **Cys-PSBP-6**. Fluorescence emission spectra (λ_{ex}: 380 nm) of solutions containing **DAN-APS** (1.5 µM) in 20 mM Na-HEPES, 100 mM NaCl (pH 7.4), with either no lipid (black) or SUVs containing 10 mol % PS in PC (red, total phospholipid 20 µM). Fluorescence spectra after addition of **Cys-PSBP-6** solution into the solution containing **DAN-APS** and 10 mol % PS in PC SUVs, with final concentrations of **Cys-PSBP-6** being 9 µM (blue), 21 µM (green), 31 µM (purple), 41 µM (yellow).

14. Measurement of Fluorescence Response of DAN-APS in Presence of SUVs in Cell Imaging Media:

Constant volume *in vitro* fluorescence titrations were performed in DMEM with no phenol red and serum (pH 7.4) at 25 °C. Different SUV dispersions containing mixtures of PC and 2, 5, 10, 15, 20, 25 mol% PS in PC were prepared maintaining total phospholipid concentration 1 mg/mL in each case, following the protocol mentioned is section 8, SI. For measuring the fluorescence response of **DAN-APS** to increasing PS, different solutions were prepared with sensor (1.5 µM) and SUVs with different PS mol%. Total phospholipid concentration and volume of the mixtures were kept at 20 µM and 200 µL, respectively. Fluorescence spectra of different solutions of **DAN-APS** and SUVs as well as only DMEM media were recorded on a spectrofluorometer by exciting the samples at 380 nm.

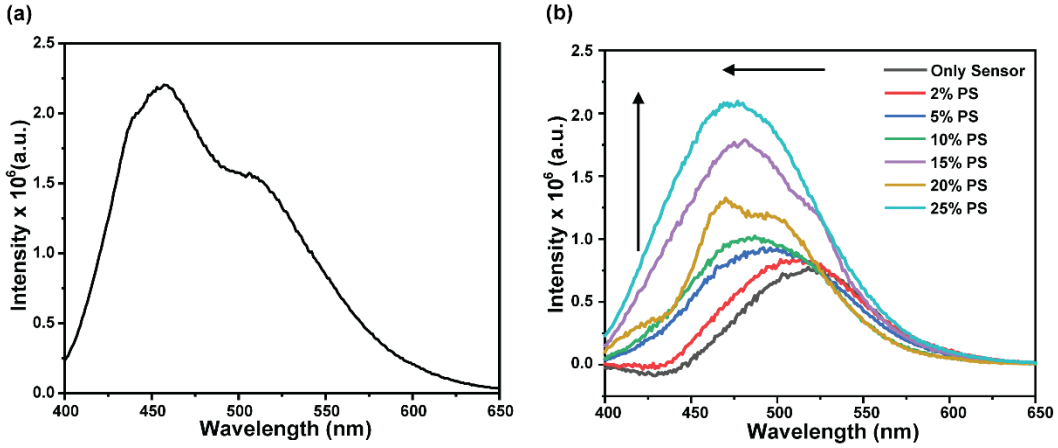


Figure S15: Fluorescence response of **DAN-APS** with PS in DMEM, pH 7.4, with no phenol red and serum. (a) Fluorescence emission spectrum (λ_{ex}: 380 nm) of only DMEM, with no phenol red and serum. (b) Fluorescence emission spectra (λ_{ex}: 380 nm) of individual solutions containing **DAN-APS** (1.5 µM) in DMEM, pH 7.4, with no phenol red and serum added with either no lipid or SUVs containing 2 mol%, 5 mol%, 10 mol%, 15 mol%, 20 mol%, 25 mol% PS mixed with PC such that the total lipid concentration remained 20 µM. Fluorescence spectra of each solution were plotted after subtracting the fluorescence contribution of media. Vertical and horizontal arrows indicate the enhancement in the intensity and blue shift in the spectra of the sensor with increasing PS concentrations, respectively.

15. Preparation of Giant Unilamellar Vesicles (GUVs):

GUVs were prepared according to a previously reported method.[10] Aliquots of PS and PC stocks, stored at -20 °C, were dissolved in HPLC grade chloroform and mixed in clean glass vials in appropriate molar ratio by vigorous vortexing. The lipid concentration was maintained at 1 mg/mL in chloroform. Lipid mixture solution (5 µL) was placed and smeared on top of a previously formed polyvinyl alcohol (PVA) film (5% w/w, 20 µL) on cover-slips kept on a hot plate, set at 80 °C. After the solvent of the lipid mixture readily evaporated leaving the dried lipid film on the PVA film, it was carefully scraped off the cover-slips with a needle and immersed in aqueous buffer (pH 7.4, 200 µL) containing Na-HEPES (20 mM), NaCl (100 mM), **DAN-APS** (1.5 µM) and left undisturbed for 30 min. The GUVs formed on the PVA film were released into the buffer through pipette aspiration and the buffer containing the GUVs was transferred into an 8 well glass bottomed imaging chamber (NUNC Lab-Tek, Thermo Fisher Scientific). The floor of the imaging chamber was previously coated with bovine serum albumin (1 mg/mL) for 1 h to stabilize GUVs in the chambers for imaging.

16. Confocal Fluorescence Imaging of GUVs:

Imaging was performed in a fluorescence confocal microscope powered with a tunable multiphoton excitation laser (Mai-Tai, Spectra-Physics), and images were collected in the internal detector channel using 63X oil immersion objective. GUVs were allowed to settle down to the bottom of the chambers and located by monitoring the transmission images in the microscope. The sensor **DAN-APS** was excited at 780 nm via two photon excitation and the emission was collected at blue channel (420-460 nm). Laser power and detector gain were adjusted to avoid signal saturation at the GUVs containing highest PS levels (16 % PS-PC). The images of GUVs containing different levels of PS were acquired at identical laser power and detector gain. Analysis of the GUV images were performed using the Fiji ImageJ software. The fluorescence intensities along the perimeters of the GUVs were measured using a Concentric Circles plugin obtained from http://rsb.info.nih.gov/ij/plugins.

17. Mammalian Cell Culture:

HeLa cells were cultured in Dulbecco's modified eagle's medium (DMEM, Sigma-Aldrich) supplemented with Fetal Bovine Serum (10%, Gibco), Penicillin (50 units/mL, Gibco) and Streptomycin (50 µg/mL, Gibco) in T25 culture flasks at 37 °C under humidified air containing 5% CO_2. For passaging and plating cells, the cells were detached from T25 flasks by adding 1x Trypsin solution.

18. Cell Viability Experiments:

For testing the toxicity of **DAN-APS** on HeLa cells, cells were seeded and grown for at least 16 h in 96 well plates (cell density 10000 cells per well) in DMEM media with phenol red and supplemented with Fetal Bovine Serum. Solutions of different concentrations (1.75 µM, 3.5 µM, 7 µM, 15 µM) of the sensor were prepared from stock in DMEM, pH 7.4, with no phenol red and serum. For incubating the cells with **DAN-APS**, growth media from the wells was removed and replenished with **DAN-APS** solutions (100 µL per well). The incubation with the sensor was continued for 15 min, 30 min and 1 h at 37 °C in an incubator. As control, cells were incubated with media containing no sensor for designated time durations. After incubation, the media containing the sensor was removed and the wells were filled with MTT solution (0.5 mg/mL, 180 µL per well) in DMEM, pH 7.4, with no phenol red and serum. MTT treated cells were kept at 37 °C incubator for at least 3 h for the formation of formazan crystals. Then the media from each well was removed and the wells were refilled with DMSO (150 µL per well) and the plates were shaken for 5 min to dissolve the crystal. Absorption values at 570 nm of the resultant purple solutions were recorded for each well. As background control, absorbance of only DMSO was also recorded. Cell viability was calculated as follows:

% Cell viability = [(Experimental value − Background control) / (Control − Background control)] x 100.

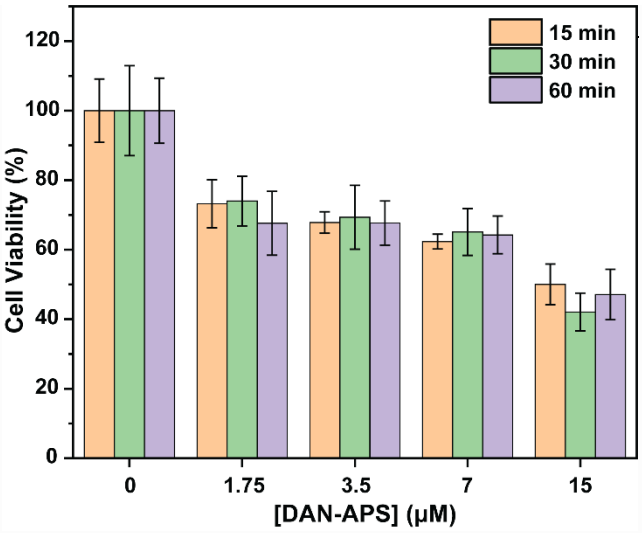


Figure S16: Cell viability in the presence of DAN-APS at different concentrations (0, 1.75, 3.5, 7 and 15 µM) in HeLa cells after 15 min, 30 min, and 1 h of incubation at 37 °C in DMEM, pH 7.4, with no phenol red and serum. Percentage of viable cells was determined using MTT assay. Error bars represent the standard deviation of three replicate experiments.

19. <u>Imaging Anionic Phospholipids in Live Cells with DAN-APS:</u>

HeLa cells, plated on glass bottomed imaging plates, were serum starved for 4-5 h prior imaging. After removing the nutrient media, cells were washed with DMEM (without phenol red and serum) and incubated with sensor solution (7 µM) in the same media for 15 min. After incubation, the sensor solution was removed, cells were washed and taken for imaging. The cells were imaged in confocal microscope under similar set up, as described in section 16. For the excitation of **DAN-APS,** 780 nm multiphoton laser was used and the emission was collected at green (560-590 nm) and blue (420-460 nm) channels simultaneously. Z series was obtained with a spacing 1.00 µm. The analyses of the cell images were performed using ImageJ software.

For incorporating **DAN-APS** into live HeLa cells through lipofection, the sensor was mixed with lipofectamine 3000 reagents (Thermo Fisher Scientific), L3000 and P3000. **DAN-APS** (10 µg) was first mixed with L3000 (0.5 µL) and P3000 (1 µL) in Opti-MEM (30.5 µL) media and kept undisturbed for 15 min at room temperature. Nutrient media of the cells was removed and cells were washed with Opti-MEM media and then incubated with the lipofection mixture along with an additional 75 µL of Opti-MEM media. The cells were kept at 37 °C in the CO_2 incubator for 4.5 h. After incubation, the lipofection mixture from the cells was removed and the cells were washed with DMEM (without phenol red and serum) and taken forward for imaging in the same media.

20. DLS measurements of DAN-APS in Cell Culture Media:

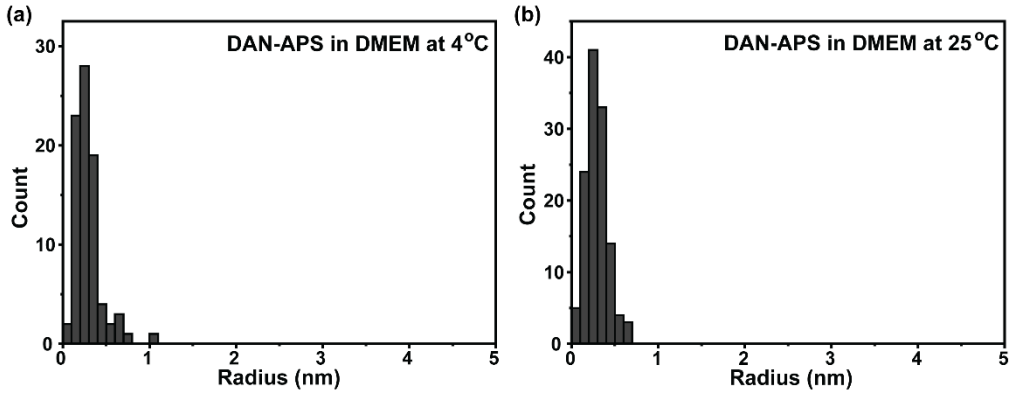


Figure S17: Size distribution (hydrodynamic radius) plots obtained from DLS of **DAN-APS** solutions (7 µM) in serum-free DMEM media, pH 7.4, at (a) 4 °C and (b) 25 °C.

21. Flow Cytometry Experiments:

Around 400K HeLa cells were seeded in polylysine coated plastic petri-plates and allowed to grow for 16 h. For inducing apoptosis, cells from selected plates were treated with Cisplatin (20 μM) for 24 h. After induction of apoptosis, both Cisplatin untreated viable (control) and apoptotic cells were harvested via treatment of 1x trypsin solution in PBS followed by incubation with either **DAN-APS** (7 μM, 15 min) or Anx V-AF 647 (5 μL, 15 mins) in HEPES (10 mM), NaCl (140 mM) and $CaCl_2$ (2.5 mM) at 25 °C. For Co-incubation of **DAN-APS** with Anx V-AF 647, cells were first incubated with Anx V-AF 647 (5 μL, 15 mins), washed and further incubated with **DAN-APS** (7 μM, 15 min) under identical buffer composition. After incubation the cells were washed and taken forward for flow cytometry measurements. Before measurements were recorded, the cell suspension was passed through a strainer to separate cell debris. Excitation sources and emissions used for the fluorophores were 405 nm/ 450 nm (for **DAN-APS**) and 640 nm / 670 nm (for Anx V-AF647). Flow cytometry measurements were recorded in a BD LSR Fortessa system and the data were analyzed in FlowJo software. At least two independent experiments were performed for **DAN-APS** and AnxV-AF647 incubated samples.

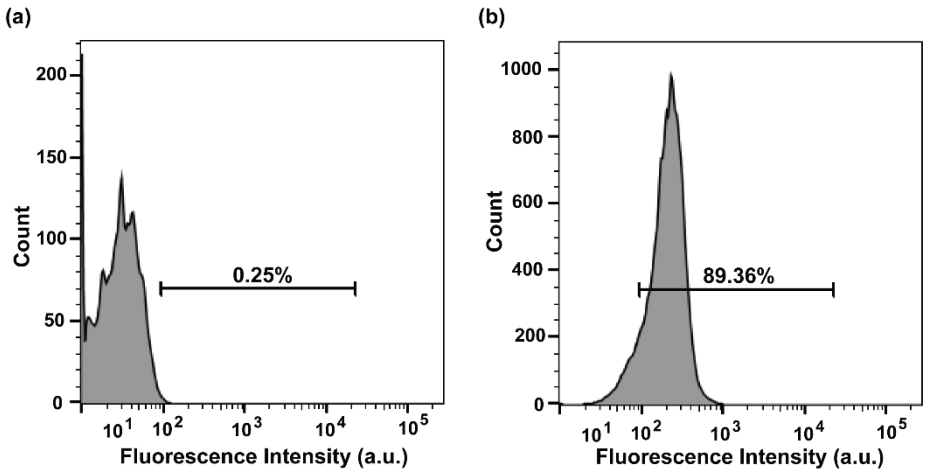


Figure S18: Flow cytometry results indicating the uptake of **DAN-APS** by viable cells. Histograms represent the analysis of viable HeLa cells either with no incubation (a) or incubated with **DAN-APS** (7 μM, 15 min, (b) at 37 °C in 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$. For flow cytometry experiments, excitation sources/ emission filters used: **DAN-APS** (405 nm/450 nm). Cell population is plotted against mean fluorescence intensities of **DAN-APS**.

22. z-stack Confocal Fluorescence Images of DAN-APS in Living HeLa Cells

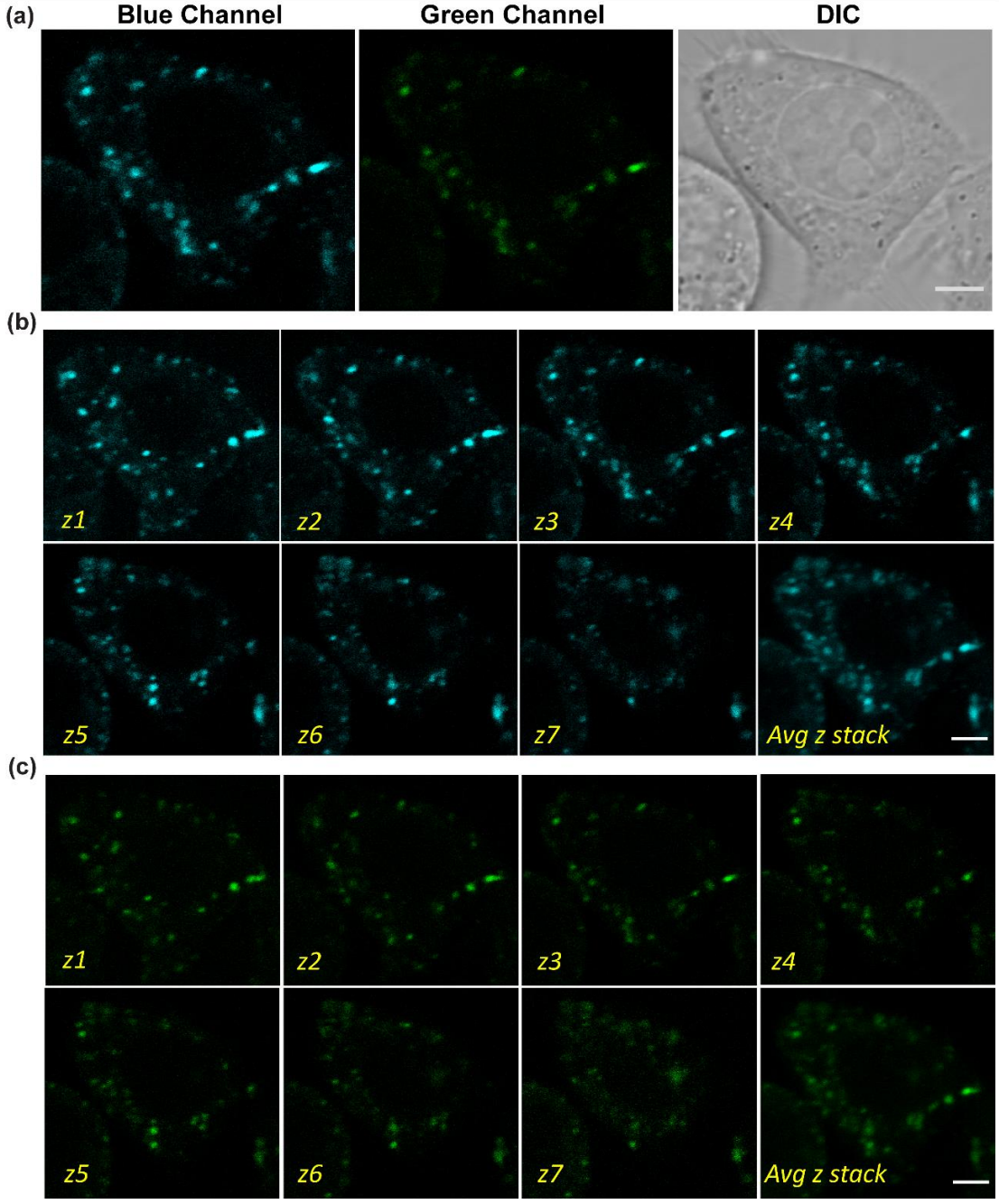


Figure S19: (a) Representative confocal single z-plane images of living HeLa cells along with the transmission image. Cells were incubated with **DAN-APS** (7 µM, 15 mins) at 37 °C in serum-free DMEM media, pH 7.4. **DAN-APS** was excited with a two-photon laser (λ_{ex}: 780 nm) and the fluorescence emission at blue channel (λ_{em}: 420 nm - 460 nm) and green channel (λ_{em}: 560 nm - 590 nm) were collected simultaneously. Representative single z-plane confocal images of same HeLa cells (z1 to z7, 1 µm apart)

along with average z projection of the planes, in blue channel (b) and green channel (c), depicting cell permeability of **DAN-APS**. Scale bar, 5 µm.

23. Fluorescence Spectrum of DAN-APS in Presence of Lipofectamine Reagents

Constant volume in vitro fluorescence titrations were performed in Na-HEPES (20 mM), NaCl (100 mM) (pH 7.4) at 25 °C. For testing the influence of lipofectamine reagents on fluorescence response of **DAN-APS**, a mixture was prepared with sensor (1.5 µM) and L3000 and P3000 (1 µL each). Volume of the mixture, diluted with buffer, was 200 µL. Fluorescence spectra of **DAN-APS** solution and the mixture of **DAN-APS** and lipofectamine reagents were recorded on a spectrofluorometer by exciting the samples at 380 nm. Ratiometric response ($F_{450\ nm}/F_{525\ nm}$) was plotted for comparison.

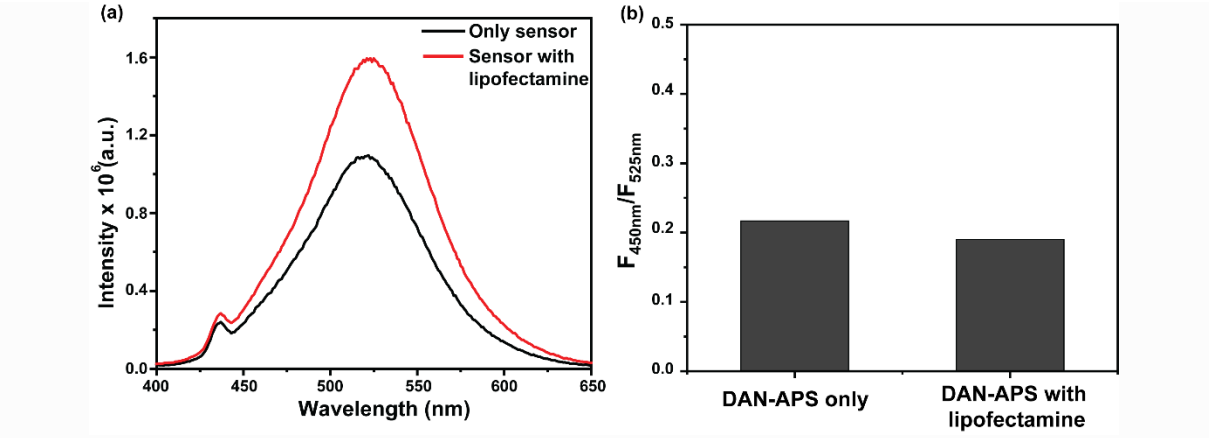


Figure S20: (a) *In vitro* fluorescence emission spectra (λ_{ex}: 380 nm) of **DAN-APS** (1.5 µM) with or without presence of lipofectamine 3000 reagents (1 µL L3000, 1 µL P3000) in Na-HEPES (20 mM), NaCl (100 mM), pH 7.4. (b) Bar plot comparing the ratiometric response of **DAN-APS** in presence and absence of lipofectamine 3000 reagents, as calculated from the fluorescence emission spectra.

24. Colocalization Studies of DAN-APS with Organelle Trackers:

For carrying out the colocalization studies of **DAN-APS** with different organelle trackers, serum starved Hela cells, plated on glass bottomed imaging plates, were first incubated with solutions of organelle trackers (LysoTracker Red, Dextran-Alexa Fluor 546, MitoTracker Red, CellMask Green from Thermo Fisher Scientific) followed by incubation with **DAN-APS** (7 µM, 15 min) at 37 °C. Incubation time of 30 min was used for LysoTracker Red (200 nM) and MitoTracker Red (200 nM), 4 h for Dextran-Alexa Fluor 546 (10 µM), and 15 min for CellMask Green (1x), as per the protocols recommended by the manufacturers. Following organelle tracker incubation, the cells were incubated with **DAN-APS** and washed with DMEM (without phenol red and serum) buffer. For imaging, the cells incubated with either LysoTracker Red, Dextran-Alexa Fluor 546, or MitoTracker Red were irradiated with 561 nm laser and the emission was collected in the region 570 nm-650 nm. The cells incubated with CellMask Green were irradiated with 488 nm and the emission was collected in the region 500 nm-550 nm. For the excitation of **DAN-APS**, 780 nm multiphoton laser was used and the emission was collected at the blue (420-460 nm) channel. Colocalization analysis was performed using Fiji (ImageJ) software. For analysis, blue channel images of the cells were colocalized with the corresponding marker channel images.

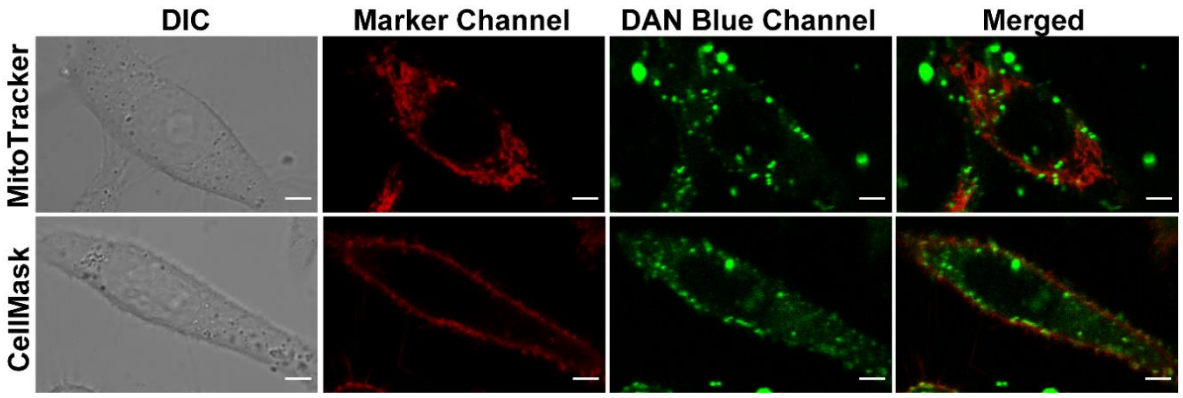


Figure S21: Representative DIC and confocal single z-plane images of live HeLa cells incubated with MitoTracker (200 nM, 30 min) or CellMask (1x, 15 min) followed by incubation with **DAN-APS** (7 µM, 15 min) at 37 °C in DMEM, pH 7.4, with no phenol red and serum, and washed. The cells were irradiated with λ_{ex}: 561 nm (for MitoTracker) or λ_{ex}: 488 nm (for CellMask) and two photon laser (λ_{ex}: 780 nm) for **DAN-APS**. The fluorescence emissions at marker channel (for MitoTracker λ_{em}: 570 nm - 650 nm or for CellMask, λ_{em}: 500 nm - 550 nm) and blue channel for **DAN-APS** (λ_{em}: 420 nm - 460 nm) were collected successively. Scale bar, 5 µm. The colocalization of organelle trackers (false color red) with **DAN-APS** (false color green) is depicted by yellow spots in merged panel.

25. Colocalization Studies of DAN-APS with TopFluor PS:

Cells were cooled on ice and then incubated with TopFluor PS (2.7 µM, pre-cooled on ice) for 15 min. Next, the TopFluor PS solution was removed and the cells were washed with DMEM (37 °C, no phenol red and serum) media. Then the cells were incubated with **DAN-APS** (7 µM, 15 min) in DMEM (no phenol red and serum) at 37 °C in the CO_2 incubator and finally washed again before imaging. The cells were irradiated with 488 nm (TopFluor PS) and 780 nm (**DAN-APS**) successively and the emissions were collected at green (500 nm-550 nm) and blue (420 nm-460 nm) channels.

26. Colocalization Studies of DAN-APS with Lactadherin C2-RFP:

Lactadherin C2-RFP (Lact C2-RFP) protein was expressed in HeLa cells via transfection of the Lact C2-RFP plasmid in live HeLa cells. Transfection of Lact C2-RFP plasmid in HeLa cells was performed through lipofection technique. For lipofection, Lipofectamine 3000, from Thermo Fisher Scientific was used and the recommended protocol was followed. Briefly, a mixture of Lipofectamine 3000 and plasmid in Opti-MEM media was prepared with the following volumes of reagents: Lipofectamine L3000 (1.5 µL), P3000 (2 µL), plasmid (2 µL, concentration 527 ng/ µL) and Opti-MEM media (50 µL). The mixture was allowed to stand for 15 min before incubating the cells. Growth medium of the live HeLa cells seeded in glass bottomed petri plate was removed and washed with Opti-MEM media. Then the Lipofectamine 3000 reagent mixture was added to the cells. 20-25 µL of Opti-MEM media was added to the cells additionally to ensure the cells remain submerged in the media. The cells were kept at 37 °C in the CO_2 incubator for incubation with the Lipofectamine 3000 mixture for 6-7 h. After incubation, the Lipofectamine 3000 mixture was removed, cells were washed with DMEM-FBS growth media and were kept at 37 °C for the expression of Lact C2-RFP protein for 3 days.

After 3 days the cells were incubated with **DAN-APS** (7 µM, 15 min) in DMEM (no phenol red and serum) at 37 °C in the CO_2 incubator and finally washed again before imaging. The cells were irradiated with 561 nm (Lact C2-RFP) and 780 nm (**DAN-APS**) successively and the emission was collected at red (570 nm-700 nm) and blue (420 nm-460 nm) channels.

27. <u>Bleed-Through Control Experiments:</u>

For checking the bleed through fluorescence contribution of **DAN-APS** into emission of different organelle trackers, and also from the organelle trackers into **DAN-APS** emission, individual plates of serum starved HeLa cells were incubated with solutions of either organelle trackers (LysoTracker Red, MitoTracker Red, Dextran-Alexa Fluor 546, 37 °C), or TopFluor PS (0 °C) or with **DAN-APS** (7 µM, 15 min, 37 °C). The cells incubated with the organelle trackers were irradiated with the respective excitation laser followed by 780 nm excitation. The emissions were collected at respective marker channels as well as at blue channel (λ_{em}: 420 nm - 460 nm for λ_{ex}: 780 nm) successively (Figure S23).

The cells incubated with only **DAN-APS** were irradiated with 488 nm, 561 nm, 633 nm and 780 nm lasers successively and the emissions were collected at λ_{em}: 500 nm - 550 nm (for λ_{ex}: 488 nm), λ_{em}: 570 nm - 650 nm (for λ_{ex}: 561 nm), λ_{em}: 650 nm - 700 nm (for λ_{ex}: 633 nm) and λ_{em}: 420 nm - 460 nm (for λ_{ex}: 780 nm), Figure S22.

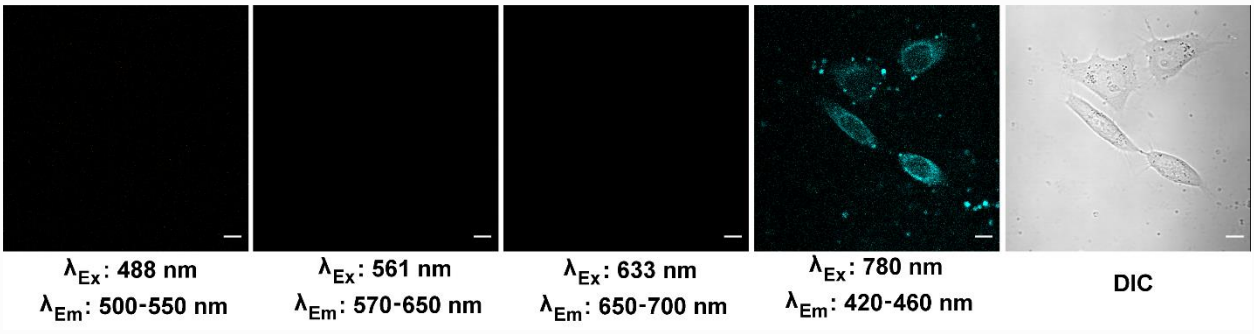


Figure S22: Representative confocal single z-plane and DIC images of live HeLa cells incubated with **DAN-APS** (7 µM, 15 min) in DMEM (pH 7.4), with no phenol red and serum, and washed. Scale bar, 10 µm.

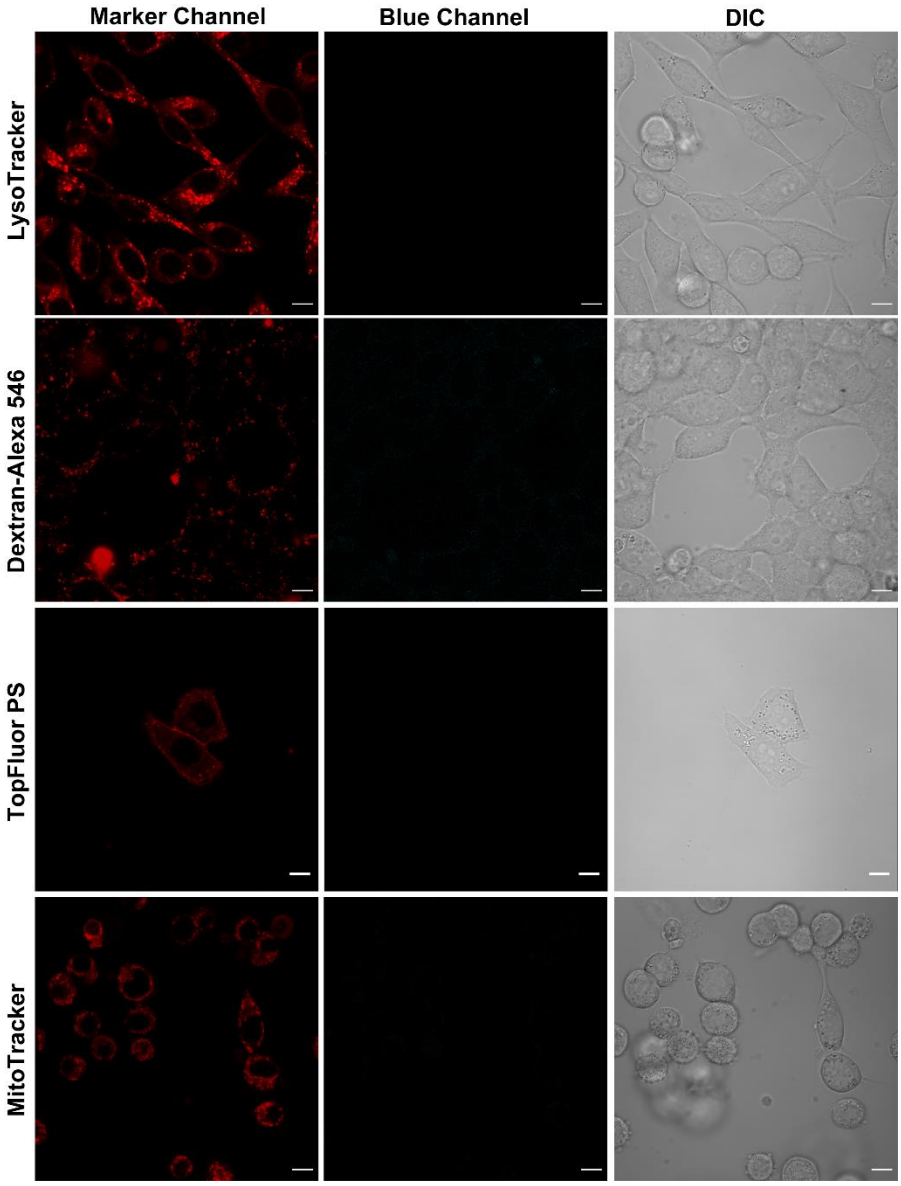


Figure S23: Representative confocal single z-plane and DIC images of live HeLa cells incubated with LysoTracker (200 nM, 30 min), Dextran-Alexa Fluor 546 (10 µM, 4 h), TopFluor PS (2.7 µM, 15 min, 0 °C) or MitoTracker (200 nM, 30 min) in DMEM (pH 7.4), no phenol red and serum, and washed. The cells were irradiated with λ_{ex}: 561 nm (for LysoTracker, MitoTracker and Dextran-Alexa 546) or λ_{ex}: 488 nm (for TopFluor PS) and two photon laser (λ_{ex}: 780 nm); the fluorescence emissions at marker channel (for LysoTracker, MitoTracker and Dextran-Alexa 546, λ_{em}: 570 nm - 650 nm, for λ_{ex}: 561 nm or for TopFluor PS, λ_{em}: 500 nm - 550 nm, for λ_{ex}: 488 nm) and blue channel (λ_{em}: 420 nm - 460 nm, for λ_{ex}: 780 nm) were collected successively. Scale bar, 10 µm.

28. Details of Colocalization Analysis:

Colocalization analysis for LysoTracker Red, Dextran Alexa 546, and TopFluor PS:

Image processing for colocalization analysis was performed using Fiji (ImageJ). For analysis, marker channel and blue channel (**DAN-APS**) images of the cells were considered. Before analysis, the background fluorescence for both the channels were subtracted and fluorescence counts of individual channels were normalized by multiplication. Threshold values for the analysis were obtained by running Coloc 2 plugin in Fiji. The threshold values obtained from the Coloc 2 analysis were used to decide on the threshold values for the calculation of Mander's (tM1 and tM2) coefficients using the JACoP plugin. The threshold values were selected to be as close as possible to the numbers obtained from the Coloc 2 analysis. Three independent experiments were analyzed to obtain average tM1 and tM2 values.

Colocalization analysis for Lactadherin C2-RFP:

Image processing for colocalization analysis was performed using Fiji (ImageJ). For analysis, marker channel and blue channel (**DAN-APS**) images of the cells were considered. Before analysis, the background fluorescence for both the channels were subtracted. Since, Lactadherin C2-RFP was transfected into cells not all cells expressed the protein. Hence, regions of interest (ROI) were selected to include cells expressing Lactadherin C2-RFP. The intensities in the Lactadherin C2-RFP channels and the **DAN-APS** blue channels within each ROI were normalized by multiplication. The selected ROIs were then analyzed by the Coloc 2 plugin to obtain the Mander's (tM1 and tM2) coefficients. Three independent experiments were analyzed to obtain average tM1 and tM2 values.

29. Comparison of Confocal Images of Cells Incubated with Lipofected DAN-APS with Cells Incubated with TopFluor PS and Lact C2-RFP

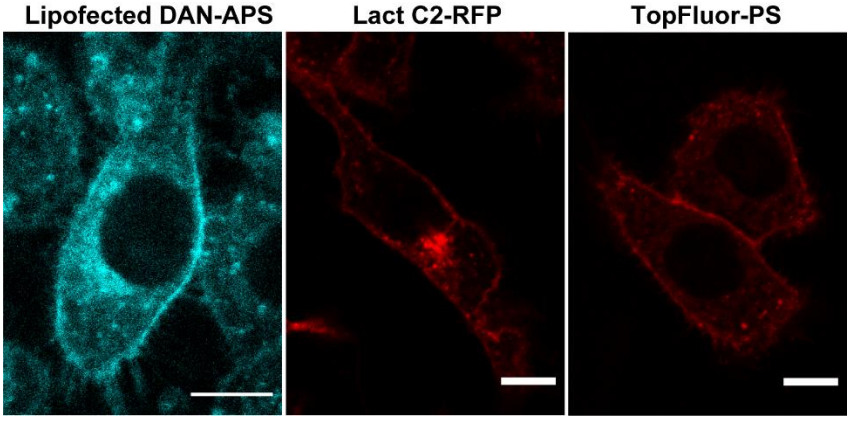


Figure S24: Representative confocal single z-plane images of Live HeLa cells depicting plasma membrane and intracellular staining with either lipofected **DAN-APS** (10 μg) or expressible Lactadherin C2-RFP or with TopFluor PS (2.7 μM, 15 min, 0 °C). The cells were irradiated with either λ_{ex}: 561 nm (for Lactadherin C2-RFP) or λ_{ex}: 488 nm (for TopFluor PS) or two photon laser (λ_{ex}: 780 nm) for **DAN-APS**. The fluorescence

emissions at marker channel (for Lactadherin C2-RFP, λ_{em}: 570 nm - 650 nm or for TopFluor PS, λ_{em}: 500 nm - 550 nm) and blue channel for **DAN-APS** (λ_{em}: 420 nm - 460 nm) were collected. Scale bar, 10 µm.

30. Imaging Apoptotic Cell Membranes with DAN-APS:

HeLa cells were incubated with Cisplatin (20 µM) for 24 h or H_2O_2 (0.2 mM) for 4 h in complete media to induce apoptosis. Apoptotic cells were washed and incubated with commercial Annexin V-Alexa Fluor 647 solution (5 µL, Anx V-AF 647, Invitrogen, Thermo Fisher Scientific, incubation time 15 min) diluted into a buffer (150 µL) containing HEPES (10 mM), NaCl (140 mM) and $CaCl_2$ (2.5 mM) at 25 °C. The cells were subsequently washed and incubated again with **DAN-APS** sensor (7 µM, 15 min) in buffer (200 µL) containing HEPES (10 mM), NaCl (140 mM) and $CaCl_2$ (2.5 mM) at 25 °C. For confocal microscopy imaging of cells, Anx V-AF 647 was excited using 633 nm laser and for **DAN-APS**, 780 nm two photon excitation laser was used.

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