Electronic Supplementary Material (ESI) for Analyst.

Mega-Stokes Pyrene Ceramide Conjugates for STED Imaging of Lipid Droplets in Live Cells.

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Supplementary Information

Experimental Section

Materials and Instrumentation

All chemical reagents were purchased from Sigma Aldrich (Ireland) and used without further purification unless otherwise stated. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), brain sphingomyelin (BSM), cholesterol (Chol) and N-heptadecanoyl-D-erythro-sphingosine C17 Ceramide were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Nile Red technical grade was purchased from Sigma Aldrich (Ireland).

Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz or 600 MHz Bruker spectrometer respectively and the solvent stated. The spectra were processed using Bruker Topspin NMR software. High Resolution Mass Spectrometry (HR-MS) was carried out at the Mass Spectrometry facility, University College Dublin. MALDI-ToF mass spectrometry was performed on MALDI-Q-ToF Premier instrument at Trinity College Dublin. Elemental analysis was carried out using an Exeter Analytical CE 440 elemental analyser at the Microanalytical Laboratory, University College Dublin. Analytical HPLC was performed on a Varian 940-LC Liquid Chromatograph using an Agilent Pursuit XRs 5C-C18 column (4.6x250 mm) and the solvent system stated. Flow rates were kept at 1.6 mL/min and run times were 20 minutes. PDAD was used for peak detection and the analysis was followed by monitoring 280 nm and 430 nm channels. UV – Vis absorption spectra were recorded on Varian Cary 50 spectrometer. Samples were analysed in Hellma quartz fluorescence cuvettes, with a path length of 1 cm, and spectral range of 330 – 800 nm unless otherwise stated. Background measurements were carried out at room temperature prior to each measurement. Emission Spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer with excitation and emission slit widths stated. All analyses were carried out using quartz cuvettes and background correction was applied prior to measurement. Fluorescence lifetime measurements were carried out using a PicoQuant FluoTime 100 Compact FLS TCSPC system using a 450 nm pulsed laser source generated from a PicoQuant PDL800-B box. The instrument response function was determined using Ludox colloidal silica solution. Lifetime decay plots were analysed using PicoQuant TimeHarp software. The goodness of each fit to exponential decay kinetics was assessed from chi – squared values (where χ^2 <1.3) and visual inspection of residuals.

Preparation of supported lipid bilayers

The lipid bilayers were prepared by a hybrid method using Langmuir-Blodgett – Vesicle Fusion methods inspired by Basit et al[1]. The lipid bottom layer is formed by transferring a lipid monolayer by Langmuir-Blodgett technique (KSV Nema). The surface pressure was controlled by a Wilherm platinum balance. Lipid solution was spread at the sub-phase surface (MiliQ water, pH 7.4) and left to evaporate for about 15 min. The lipid monolayer was transferred at 32mN/m. To prepare liposomes lipids dissolved in chloroform were mixed to PyLa-C17Cer at ratio of 5000:1 mol and dried gently against nitrogen flow. Dried lipids were suspended in PBS buffer by vortex agitation, then liposomes were prepared by lipid extrusion (Avanti Lipids) with 0.1um PC membrane. Liposomes composed of raft composition were extruded at 50^{o}C and kept at this temperature to assure proper lipid fluidity. After liposomes introduction to the microfluidic chamber vesicle's solution were left to react for 30 min. To remove any residual liposomes, the vesicle solution is purged with PBS buffer.

Fluorescence lifetime correlation spectroscopy

Diffusion coefficients for labelled supported lipid bilayers were obtained using fluorescence lifetime correlation spectroscopy, FLCS performed on a Microtime 200 system (Picoquant GmBH, Berlin, Germany). The system consists of an inverted microscope model Olympus X1-71 with an Olympus UPlanSApo 60x/1.2 water immersion

objective. The fluorophores were excited with a 440 nm PicoTA from Toptica (Picoquant). The laser was directed onto 440/532rpc dichroic mirror and focused on the aforementioned objective. The sample fluorescence was collected through the same objective and filtered by the dichroic mirror and by a 460 nm interference filter. The sample fluorescence was passed through a 50 µM pinhole onto a Single Photon Avalanche Diode (SPAD). The autocorrelation functions (ACFs) were fit using SymphoTime 2.3 software (Picoquant GmBH, Berlin, Germany) to the following equation (1):

$$G(\tau) = \frac{1}{N(1-T)}\left[1 - T + Te^{\left(-\frac{\tau}{\tau_T}\right)}\right]\left[1 + \left(\frac{\tau}{\tau_D}\right)^{\alpha}\right]^{-1} \quad (1)$$

Where G(τ) is the autocorrelation function of fluorescence fluctuations; N is the average number of diffusing fluorophores in the effective volume; τ is the delay time; T is the fraction of molecules in the triplet state; α is the anomalous parameter; τD is the diffusion time of the molecules and τ_T is the decay time for the triplet state. The bilayer was located by scanning the microscope along the z-axis to identify peak fluorescence intensity. A minimum of nine measurements were taken and any outliers were disregarded. Autocorrelation curves were fit to a 2D model to determine the diffusion coefficients. All lipid bilayers used in the FLCS experiments were labelled with PyLa-C17Cer at a concentration of 10 nM. The confocal volume was determined by prior calibration using Atto 425, fitting the resulting autocorrelation function using the known diffusion coefficient of the dye at 20 °C. The free diffusion of Atto 425 was calculated with equation 2 following the water viscosity correction[2] :

$$D(T) = D(25℃)\frac{T}{298.15K}\frac{8.9.10^{-4}\,Pa.s}{\eta(T)} \quad (2)$$

Where D is the dye diffusion and η is the viscosity of water.

Fluorescence lifetime imaging (FLIM) of Lipid Bilayers

Fluorescence lifetime imaging was carried out using a PicoQuant 100 system attached to Leica TSP inverted (DMi8) confocal microscope using a 100x oil immersion objective. Each sample was acquired for 120 s with a 512 x 512 resolution. A 405 nm pulsed laser was used to excite the dyes in supported lipid bilayers and in live cell samples. Data was analysed using PicoQuant Symphotime software.

Cell culture

HeLa cells, a cervical cancer cell line, were cultured. The media used to culture the cells was MEM media supplemented with 2% L-glutamine, 1% MEM non-essential amino acid solution, 10% foetal bovine serum and 1% penicillin-streptomycin and grown at 37°C with 5% CO_2. Cells were harvested or split at 90% confluency using 0.25% trypsin for 5 minutes at 37°C.

Confocal microscopy for cell imaging

HeLa cells were seeded at 1.5 x 10^5 cells in 2 mL culture media on poly-L-lysine coated, #1.5 coverslips in a 6-well plate and left for 48 h at 37°C under 5% CO_2. PyLa and PyLa-C17Cer were added to the wells in cell media to give a final concentration of 2 µM (final DMSO concentration of 0.5%), and were incubated

for 2 h at 37°C with 5% CO_2. Prior to imaging, the compounds were removed and the cells were washed once with PBS supplemented with 1.1 mM $MgCl_2$ and 0.9 mM $CaCl_2$. The cells were imaged live using a Leica TSP DMi8 confocal microscope with a 100X oil immersion objective lens. A heated box covered the stage to keep the temperature at 37°C. A 405 nm laser was used to excite the compounds, and a 520-620 nm filter was used to collect the emission.

For real-time imaging, cells were seeded at 1.5 x 10^5 cells in 2 mL culture media on 35 mm high precision glass-bottom dishes (Ibidi, Germany) as described previously. PyLa and PyLa-C17Cer were added to Leibovitz media to give a final concentration of 2 µM and was added to live cells and imaged immediately using Time Series mode to capture images every 1 minute over a 25-minute period. To assess the mode of uptake, cells were prepared as mentioned above and incubated with the compounds at 4°C for 4 h. Cells were washed with PBS (supplemented with 1.1 mM $MgCl_2$ and 0.9 mM $CaCl_2$) and imaged immediately.

To induce lipid droplet production, HeLa cells were seeded and incubated as described previously. TNF-α (Bio-techne Ltd) was added to the cells to give a final concentration of 10 ng/mL in Hela media, and cells were incubated for 16 h in the absence of light at 37°C and 5% CO_2. PyLa-C17Cer (2 µM) was added to the cells for 2 h prior to imaging.

Phototoxicity

HeLa cells were seeded at 1.5 x 10^5 on 35 mm glass bottom culture dishes and incubated for 48 h. The media was removed and cells were washed with PBS supplemented with 1.1 mM $MgCl_2$ and 0.9 mM $CaCl_2$ and transferred to a 37°C heated stage. 2 µM of PyLa-C17Cer in HeLa media for 2 h. Prior to imaging, the dye was removed, cells were washed with supplemented PBS, and DRAQ 7 (1:100) was added to the live cells. The Time Series mode on a Leica TSP DMi8 confocal microscope was used. DRAQ 7, which stains the nucleus of dead cells, was excited at 633 nm and collected between 635 -720 nm. Cells were scanned continuously using the PyLa-C17Cer settings (0.06 mW/cm^2) for 5-minute intervals, imaging with the DRAQ 7 settings between intervals to assess viability. For the control, cells were set up as described above, and DRAQ 7 (1:100) in HeLa media was added to the live cells. They were scanned continuously using the Pyrene settings and imaged after each interval to assess for DRAQ 7 uptake.

Cytotoxicity Studies

HeLa cells were seeded in 96-well plates (Sarstedt flat-bottom cell+ culture plate) at 1 × 10^4 in 100 µL media for 24 h at 37°C with 5% CO_2. PyLa and PyLa-C17Cer were added for 20 hours at 37°C with 5% CO_2. Final dye concentrations were 100, 50, 20, 10, 5 and 1 µM. The Alamar blue assay (Promocell GmbH) was used to measure cell viability by the addition of 10 µL resazurin reagent and cells were incubated for 7 h at 37°C in the dark. Absorbance was measured using a Tecan 96-well plate reader at 570 nm and 600 nm (corrected for background subtraction).

FLIM of live HeLa Cells

HeLa cells were prepared and stained as described for confocal imaging. Live FLIM images were acquired using SymphoTime 200, Picoquant, attached to a Leica TSP DMi8 confocal microscope using a 100x oil immersion objective and heated stage set to 37°C. Each FLIM image was acquired for 2 minutes with 512 x 512 resolution. A 405 nm laser was used to excite the sample, and emission collected using a 520 – 620 nm filter. The data was

analysed using PicoQuant Symphotime software. Lifetimes were fit to a mono-exponential decay for PyLa, and bi-exponential decay for PyLa-C17Cer, until a X^2 value of 0.9-1.1 was achieved.

STED imaging of Live HeLa cells

HeLa cells were seeded at 1.5 x 10^5 cells in 2 mL culture media on poly-L-lysine coated, #1.5 coverslips in a 6-well plate and left for 24 h at 37°C under 5% CO_2. TNF-α was added to the cells to give a final concentration of 10 ng/mL in Hela media, and cells were incubated for 16 h in the absence of light at 37°C and 5% CO_2. PyLa-C17Cer (2 μM) was added to the cells for 2 h prior to imaging. The dye was removed and the cells were washed x2 for 2 minutes with supplemented PBS. To acquire STED images, a Leica DMI8 confocal system with STED lasers was used. A 405 nm laser (PicoQuant) was used to excite PyLa-C17Cer, collecting the emission between 520 -620 nm, using a 100X oil immersion objective. Images were scanned at 2408 x 2408 resolutions, using a scan speed of 0.032 frame/s. A line accumulation of 6 was used to eliminate as much background as possible. A time gating system was used to remove any autofluorescence and was set to 1.5 -12 ns. A 660 nm depletion laser was used to acquire the live cell STED images. Images were deconvolved using Huygens Professional software. All data and FWHM analysis were carried out on raw images before deconvolution process using Image J and OriginPro.

Photostability

HeLa cells were prepped for STED as described above and stained with either PyLa-C17Cer (2 μM) or Nile Red (1 ng/mL). The samples were imaged using the optimum STED settings. A 405 nm laser was used to excite Pyla-C17Cer, collecting the emission between 520 - 620 nm, and a 560 nm white light laser was used to excite Nile Red collecting the emission between 580 – 670 nm with a 100X oil immersion objective. The STED 660 nm at 0.05 W was used for both samples. The images were acquired at 1024 x 1024 resolutions every 1 minutes for 30 minutes at a pixel dwell time of 2.43 μs. The emission intensity of a selected area in both samples was measured at each time interval over the 20 minutes and plot to show stability over time.

Computational Studies

The quantum chemical calculations were undertaken using the Gaussian 16 programme suite.[3] The B3LYP hybrid functional was used with a range of basis sets as indicated.[4-5] Initial structures were obtained from molecular mechanics methods. The polarisable continuum model[6-7] was used to mimic the solvent environment. GaussView 3.0[8] was used to visualise the electron density difference maps and the AOMix package was used to simulate the UV/visible spectra.[9-10] Band structures used Gaussian functions with a width at half height of 3000 cm^{-1}.

Synthesis and Structural Characterisation

Synthesis of pyrene-1-carboxylic acid (2)

Synthesised according to a reported method.[43] Yield: brown solid, 0.671 g, (2.51 mmol, 57 %).

^{1}H NMR (400 MHz, DMSO-d^6) (ppm): 9.23 (d, J = 9.4, 1H); 8.62 (d, J = 8.2, 1H); 8.41 (dd, J = 8.1, 1.7, 2H); 8.37-8.33 (m, 3H); 8.26 (d, J = 9, 1H); 8.16 (t, J = 7.6, 1H).

Synthesis of 3,6,8-(4-(dimethylamino)phenyl)pyrene-1-carboxylic acid (PyLa) (4)

Over a period of 15 min bromine (1.32 g, 8.2 mmol) was added dropwise to a solution of pyrene-1-carboxylic acid (2) (0.517 g, 2.1 mmol) in nitrobenzene (40 mL) at 160 °C under vigorous stirring. The reaction mixture was kept at 160 °C for 5 h, after which it was allowed to cool to room temperature. The precipitate was filtered off and washed with EtOH and dried under vacuum overnight. The reaction afforded 3,6,8-tris-bromopyrene-1-carboxylic acid **(2)** as a yellow powder (0.852 g, 1.76 mmol, 84 %). Due to its low solubility, the product was used in the next step without further purification or characterisation. Following this, **(2)** (0.169 g, 0.35 mmol), 4-(dimethylaminophenyl) boronic acid (0.348 g, 2.11 mmol), $Pd(PPh_3)_4$ (0.070 g, 0.061 mmol) and K_2CO_3 (0.5 g, 3.6 mmol) were added to a nitrogen saturated mixture of toluene (10 mL) and ethanol (5 mL). The reaction mixture was then heated to reflux under nitrogen for 24 h. After the mixture was allowed to cool to room temperature, diluted with dichloromethane (150 mL) and was then washed with water (3 x 50 mL). The precipitate was collected by filtration and purified on silica gel by column chromatography using dichloromethane/methanol (9:1) to afford PyLa **(4)** as an orange powder (0.091 g, 0.2 mmol, 57 %).

^{1}H NMR (600 MHz, DMSO-d_6) (ppm): 13.30 (s, 1H); 9.12 (d, J = 9.6, 1H); 8.45 (s, 1H); 8.34 (d, J = 9.6, 1H); 8.28 (d, J = 9.4, 1H); 8.17 (d, J = 9.4, 1H); 7.93 (s, 1H); 7.56-7.49 (m, 6H); 6.94 (dd, J = 8.5, 6H); 3.02 (s, 6 H); 3.018 (s, 6 H); 3.00 (s, 6 H).

^{13}C NMR (150 MHz, DMSO-d_6): 171.76, 150.09, 149.95, 136.93, 136.72, 136.52, 129.19, 128.97, 128.80, 128.59, 128.55, 127.56, 127.35, 127.18, 127.13, 126.43, 126.08, 124.99, 124.45, 123.47, 112.85, 112.83, 112.81

HR-MS (ESI-TOF) m/z: calculated for $C_{41}H_{37}N_3O_2$ 604.2964 found 604.2964 ([M$^+$])

Synthesis of 3,6,8-tris-(4-(dimethylamino)phenyl)pyrene-1-C17-Ceramide (PyLa-C17Cer) (5)

To a stirred solution of PyLa **(4)** (27 mg, 0.045 mmol,) and 4-(dimethylaminopyridine) (0.55 mg, 0.0045 mmol) in DCM (2 mL), a solution of C17 ceramide (50 mg, 0.09 mmol) in DCM (2 mL) was added. The reaction mixture was stirred for 5 min at room temperature. A solution of DCC (10.22 mg, 0.049 mmol) in DCM (2 mL) was added and the mixture was stirred at room temperature overnight. The crude mixture was concentrated to dryness via vacuum. The crude product was purified by chromatography on preparative silica tlc using DCM/MeOH (9:1) to afford a yellow powder (6.7 mg, 0.005 mmol, 8.6 %).

^{1}H NMR (600 MHz, $CDCl_3$) (ppm): 9.11 (d, J = 11, 1H); 8.54 (s, 1H); 8.44 (d, J = 10.5, 1 H); 8.34 (d, J = 10.3, 1H); 8.20 (d, J = 6.8, 1H); 8.05 (s, 1H); 7.58-7.51 (m, 6H); 6.91 (d, J = 9.2, 6H); 6.07 (d, J = 7.92, 1H); 5.79-5.74 (m, 1H); 5.58-5.54 (m, 1H); 4.76 (q, J = 6.4, 1 H); 4.53 (dd, J = 11.6, 4.7, 1H); 4.47-4.43 (m, 1H); 4.31-4.25 (s, 1H); 3.02 (s, 6 H); 3.016 (s, 6 H); 3.00 (s, 6 H); 2.2-2.17 (m, 2H); 2.02-1.95 (m, 2H); 1.29-1.17 (m, 48H); 0.88-0.84 (m, 7H).

MALDI TOF MS LD+ calculated for $C_{75}H_{101}N_4O_4$: 1136.8082 found 1136.8058 ([M$^+$])

Characterisation Data:

Pyrene-1-carboxylic acid (2).

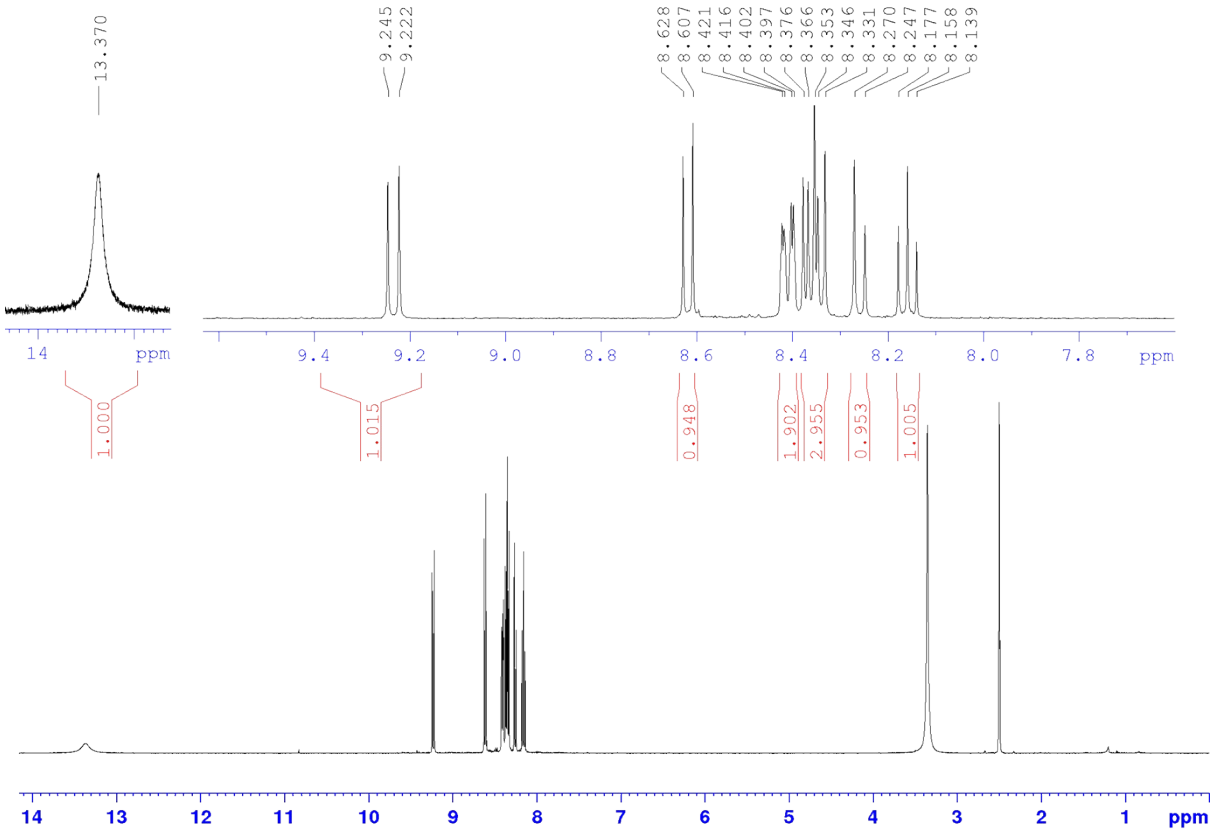


Figure S1 ^{1}H NMR Spectrum (400 MHz) of (**2)** in DMSO-d^{6}.

3,6,8-tris-(4-(dimethylamino)phenyl)pyrene-1-carboxylic acid (PyLa) (4).

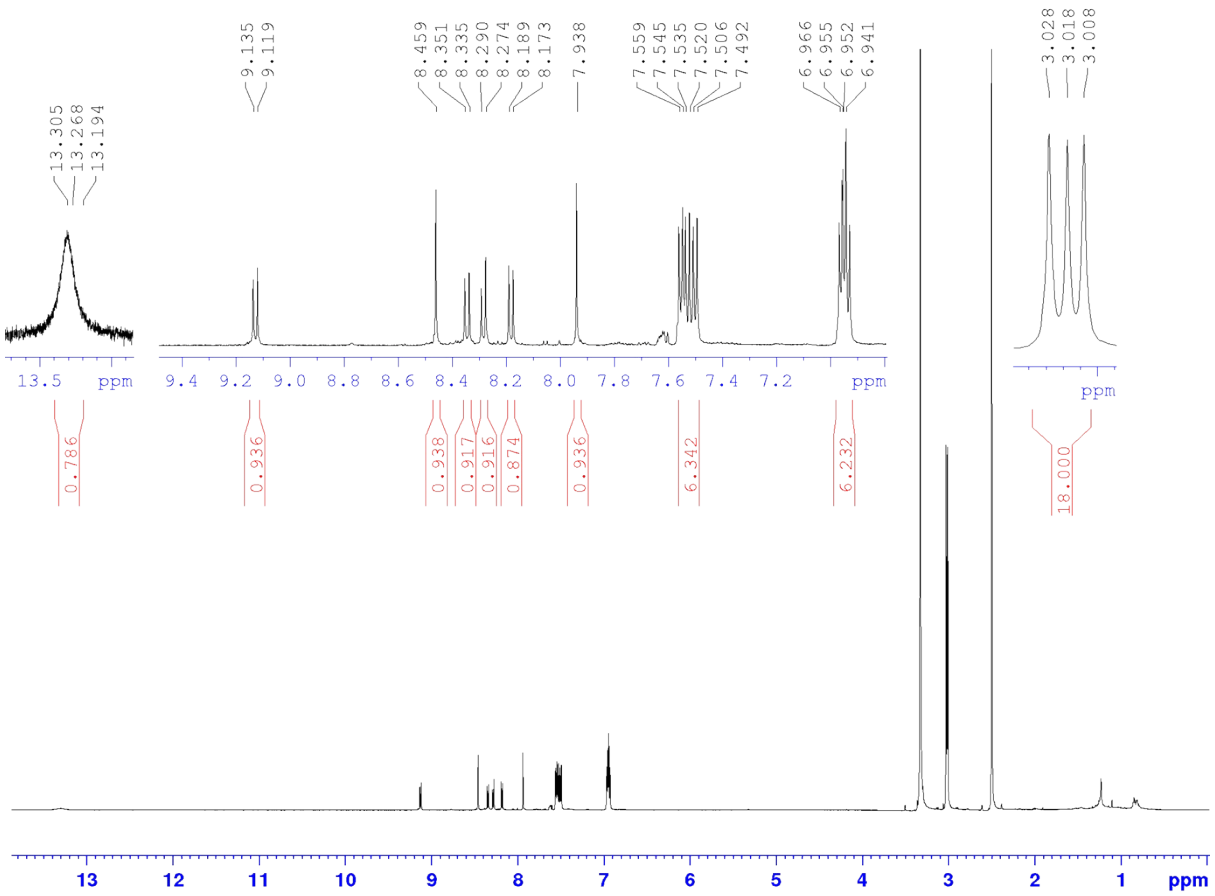


Figure S2 ^{1}H NMR Spectrum (600 MHz) of (**4**) in DMSO-d^{6}.

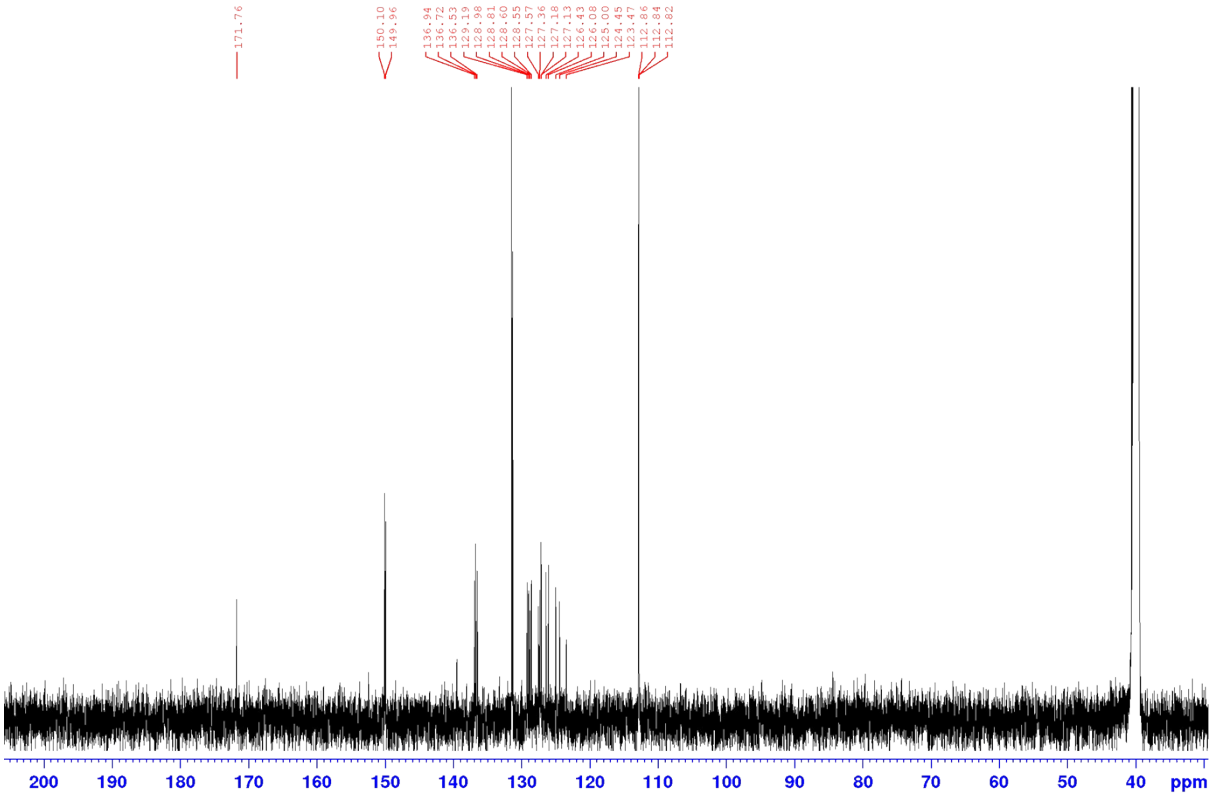


Figure S3 ^{13}C NMR Spectrum (150 MHz) of (**4**) in DMSO-d^6.

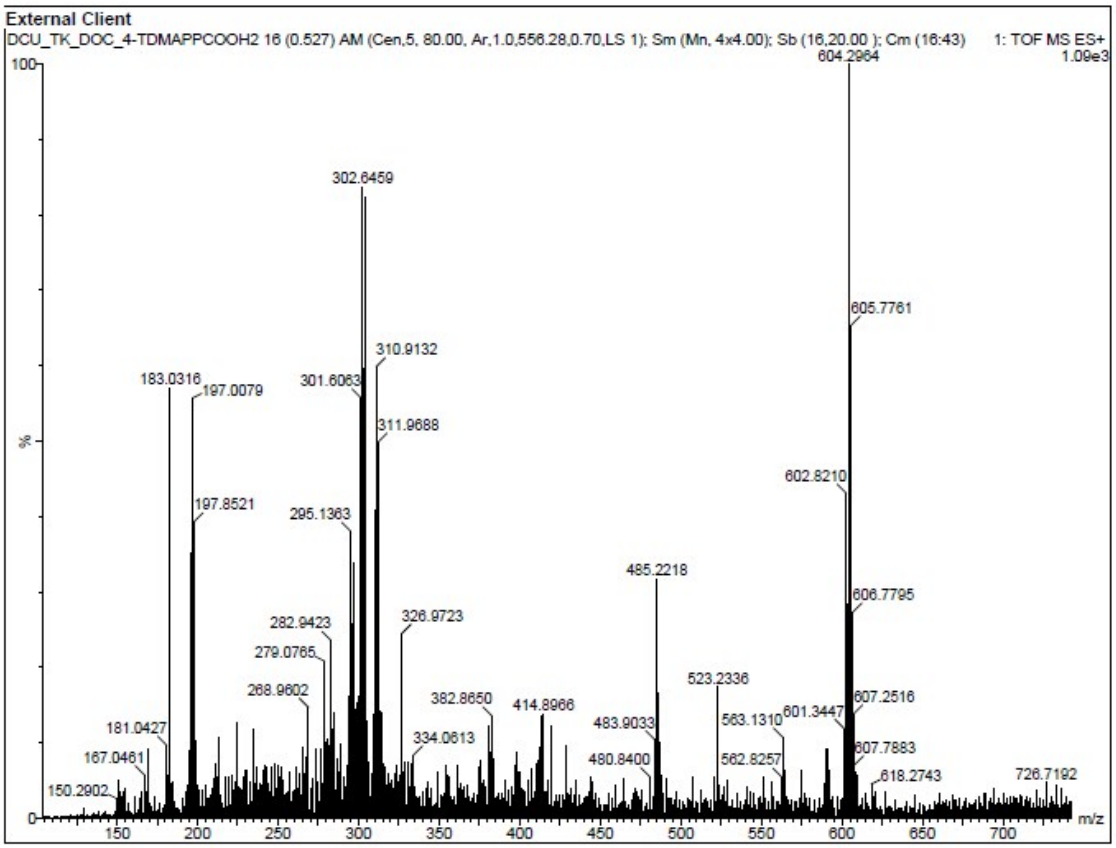


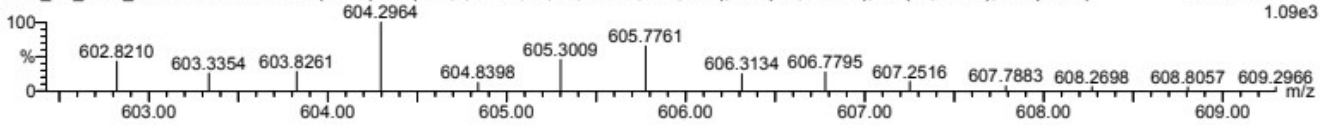
Figure S4 HR-MS (ESI-QTOF): Single Mass Analysis of **(4)** indicating [M]+

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 70.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
4 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

External Client
DCU_TK_DOC_4-TDMAPPCOOH2 16 (0.527) AM (Cen,5, 80.00, Ar,1.0,556.28,0.70,LS 1); Sm (Mn, 4x4.00); Sb (16,20.00); Cm (16:43) 1: TOF MS ES+ 1.09e3



Minimum:				-1.5		
Maximum:		200.0	5.0	70.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
604.2964	604.2964	0.0	0.0	24.5	1	C41 H38 N3 O2

Figure S5 HR-MS (ESI-QTOF): Single Mass Analysis of **(4)** indicating [M]+.

3,6,8-tris-(4-(dimethylamino)phenyl)pyrene-1-C17-Ceramide (PyLa-C17Cer) (5).

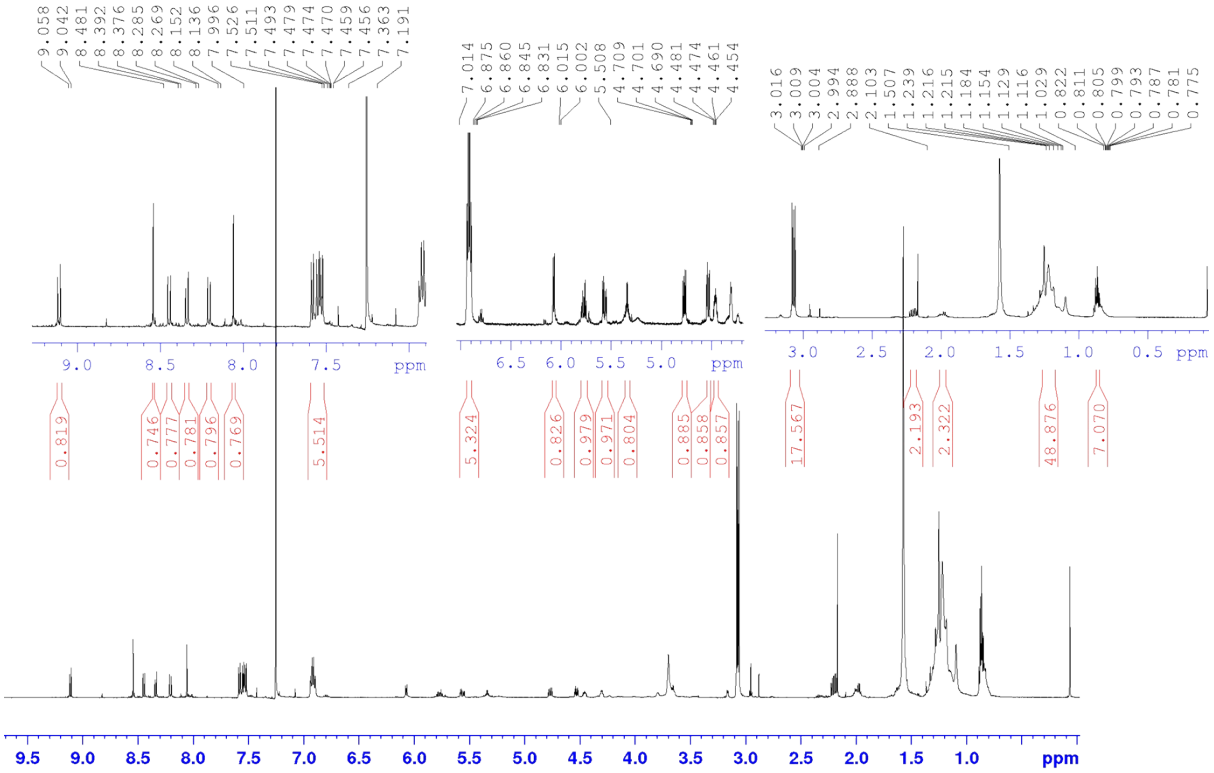


Figure S6 ^{1}H NMR Spectrum (600 MHz) of (**5**) in $CDCl_3$.

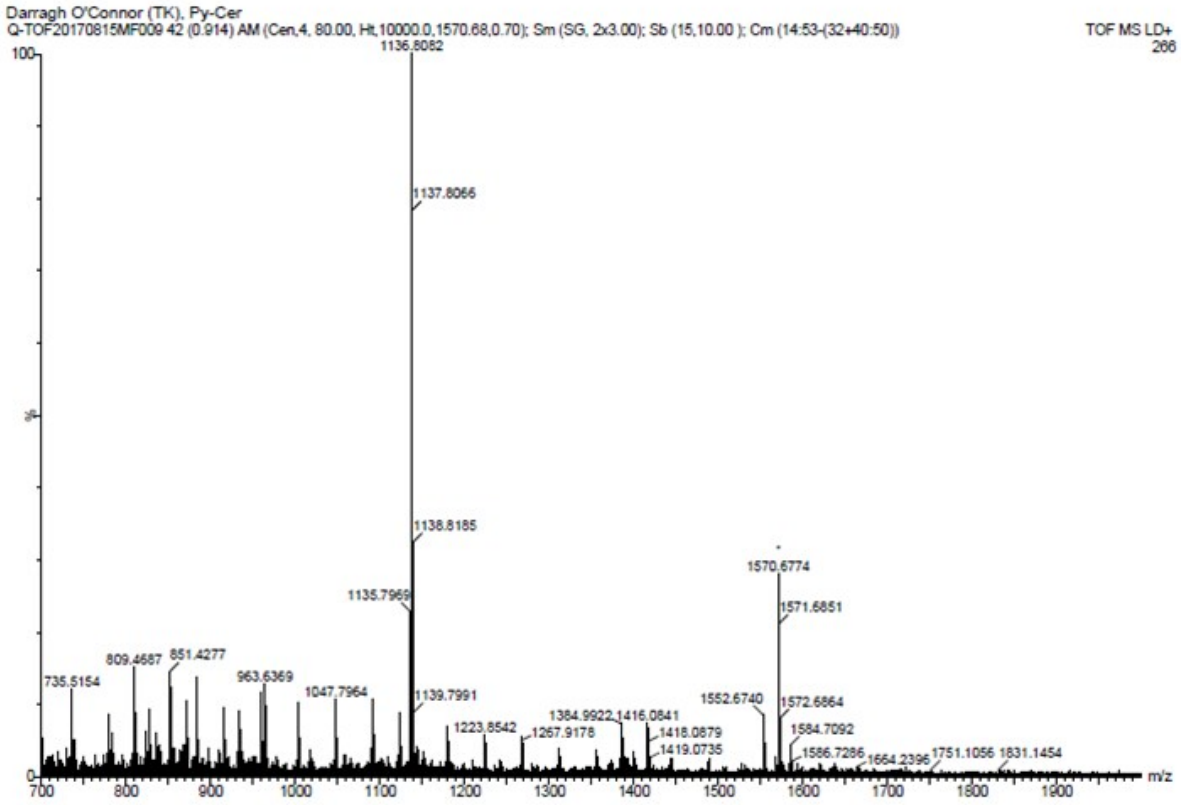


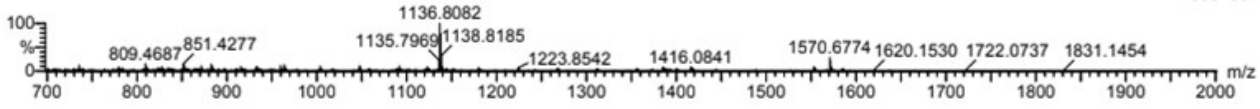
Figure S7 MALDI-TOF: Single Mass Analysis of **(5)** indicating [M]+.

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 50.0 PPM / DBE: min = -1.5, max = 1000.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 5

Monoisotopic Mass, Odd and Even Electron Ions
11 formula(e) evaluated with 1 results within limits (up to 10 closest results for each mass)
Elements Used:
C: 0-76 H: 0-104 N: 0-4 O: 0-4

Darragh O'Connor (TK), Py-Cer
Q-TOF20170815MF009 42 (0.914) AM (Cen,4, 80.00, Ht,10000.0,1570.68,0.70); Sm (SG, 2x3.00); Sb (15,10.00); Cm (14:53-(32+40:50))
TOF MS LD+
2.66e+002



Minimum:				-1.5			
Maximum:		5.0	50.0	1000.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
1136.8082	1136.8058	2.4	2.1	27.0	55.9	0.0	C76 H104 N4 O4

Figure S8 MALDI-TOF: Single Mass Analysis of **(5)** indicating [M]+.

HPLC Analysis

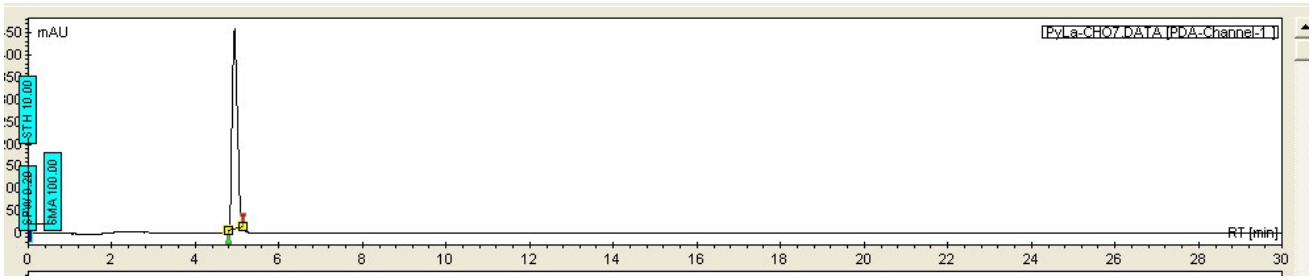


Figure S9 HPLC of reference compound **(1)** using C18 reverse phase column, flow rate 1.0 mL/min, run time 30 min. Wavelengths 394 nm and 430 nm. Solvent ACN TFA 0.1 %.

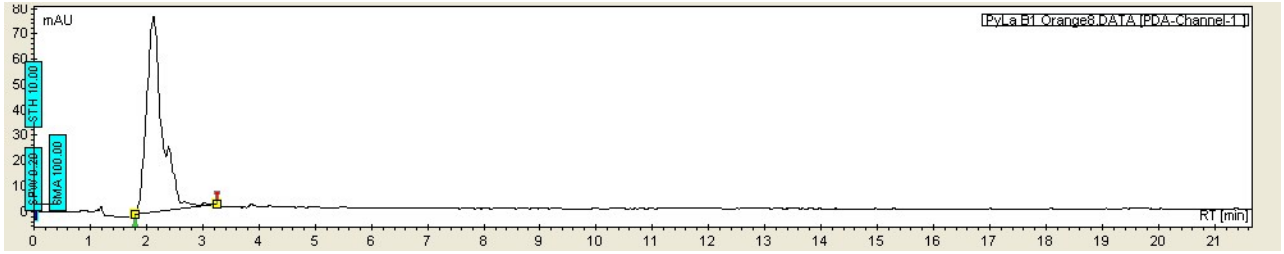


Figure S10 HPLC of **(3)** using C18 reverse phase column, flow rate 1.0 mL/min, run time 20 min. Wavelengths 394 nm and 430 nm. Solvent ACN TFA 0.1 %.

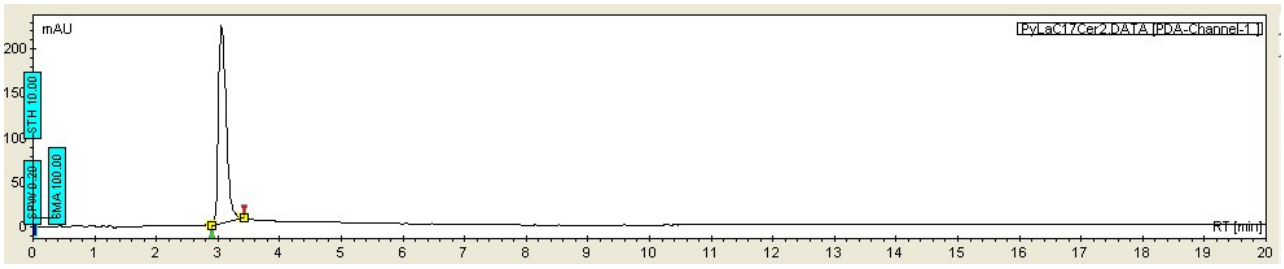


Figure S11 HPLC of **(5)** using C18 reverse phase column, flow rate 1.0 mL/min, run time 20 min. Wavelengths 394 nm and 430 nm. Solvent ACN 100 %.

Photophysical Characterisation

Table S1. Summary of photophysical data

Compound	ϕ_{fl}	Solvent	λ_{abs} (nm) (ε = x 10^3 M^{-1} cm^{-1})	λ_{em} (nm) (ε_r)*	τ_{lum} (ns) ± SD
PyLa	0.40	DCM	431 (20.55)	552 (9.08)	4.42 ± 0.01
	[a]	Acetone	426 (12.57)	580 (20.7)	3.34 ± 0.02
	[a]	MeOH	396 (23.38)	525 (33.0)	3.54 ± 0.01
	0.49	Acetonitrile	399 (28.19)	520 (37.50)	3.85 ± 0.04
	[a]	H_2O	413 (0.79)	557 (78.54)	0.38 ± 0.01
PyLa-C17Cer	0.10	DCM	427 (13.74)	562 (9.08)	4.34 ± 0.00
	[a]	Acetone	428 (4.61)	592 (20.7)	2.66 ± 0.17
	[a]	MeOH	416 (4.70)	510 (33.0)	2.60 ± 0.28
	0.08	Acetonitrile	423 (9.60)	582 (37.50)	2.98 ± 0.02
	[a]	H_2O	417 (0.76)	557 (78.54)	0.84 ± 0.006

Solutions of PyLa and PyLa-C17Cer were were absorbance matched at 0.4 (a.u.) and emission spectra were obtained using excitation slit width 5 nm and emission slit width 2.5 nm. Lifetime data was recorded in triplicate at concentrations of (10 μM) and curves conformed to tailfit criteria of $\chi^2 < 1.3$. Quantum yields were obtained using Atto 425 in water (ϕ_{fl} = 0.90) with slit widths 2.5 nm as a standard. [a]Not measured. *Dielectric constants of solvents at 20 °C.

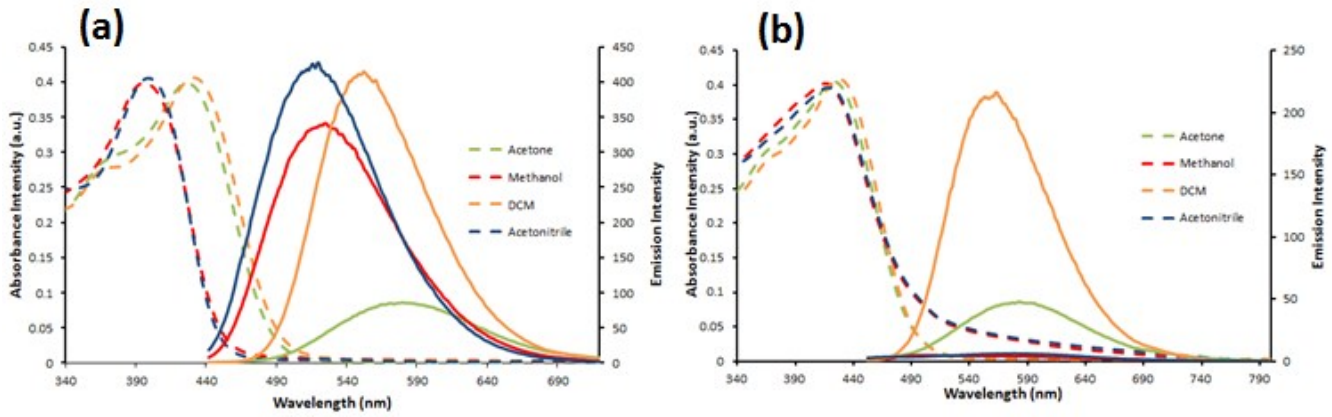


Figure S12 (a) Solvent dependence absorbance and emission spectra for PyLa (absorbance matched at 0.4 a.u. excitation slit width 5 nm, emission slit width 2.5 nm). (b) Solvent dependence absorbance and emission spectra for PyLa-C17-Cer (absorbance matched at 0.4 a.u. excitation slit width 5 nm, emission slit width 2.5 nm).

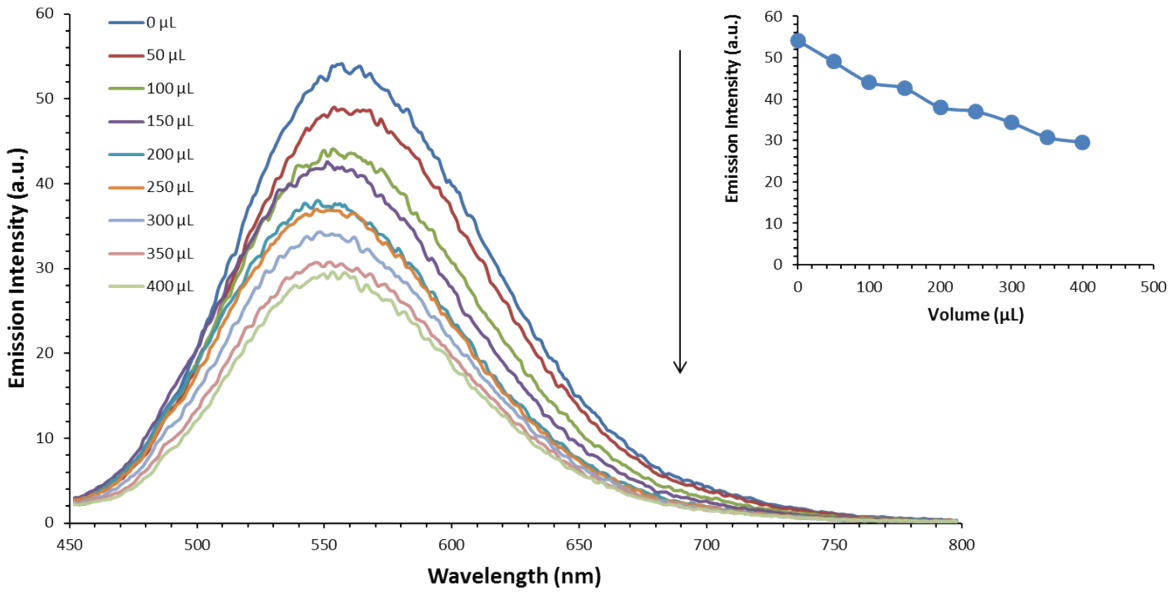


Figure S13 Emission spectra of PyLa **(4)** (50 µM) in MeOH with the addition of 50 µL water titrations. Excitation slit width 5 nm and emission slit width 2.5 nm were used for emission intensity measurements. Emission intensity decreases by 45 % following addition of water (21 mol % water to MeOH). Inset shows maximum emission versus volume of water added.

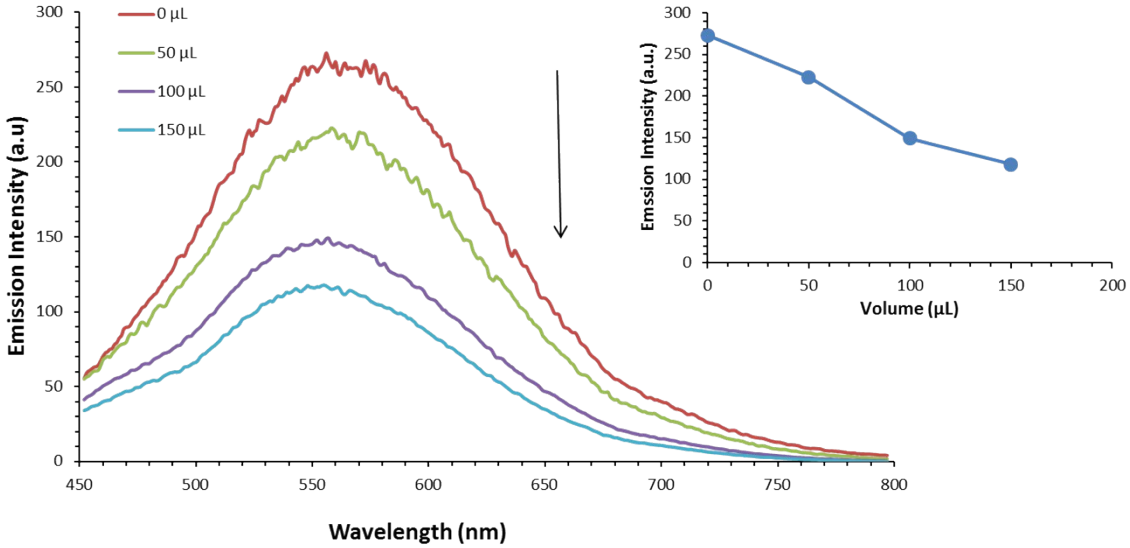


Figure S14 Emission spectra of PyLa-C17Cer **(5)** (50 µM) in MeOH with the addition of 50 µL water titrations. Excitation and emission slit widths 10 nm were used for emission intensity measurements. Emission intensity decreases by 58 % following addition of water (11.36 mol % water to MeOH). Inset shows maximum emission versus volume of water added.

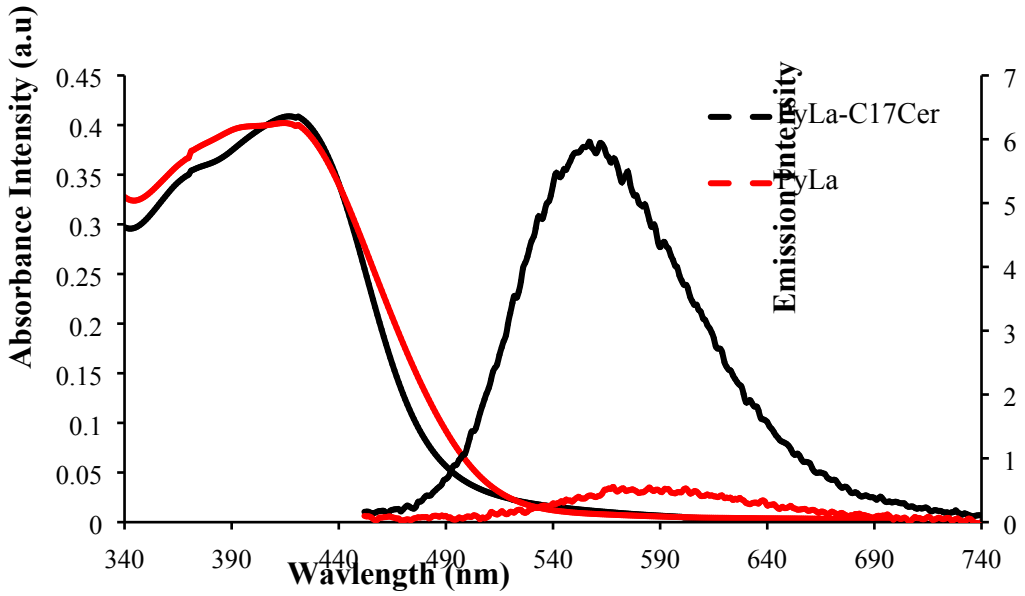


Figure S15 Absorbance and emission spectra of PyLa **(4)** and PyLa-C17Cer **(5)** in water absorbance matched at 0.4 absorbance intensity. Emission spectra were obtained with excitation slits widths set to 5 nm and emission slit widths set to 2.5 nm. Emission spectra show both dyes exhibit extremely weak emission in aqueous media.

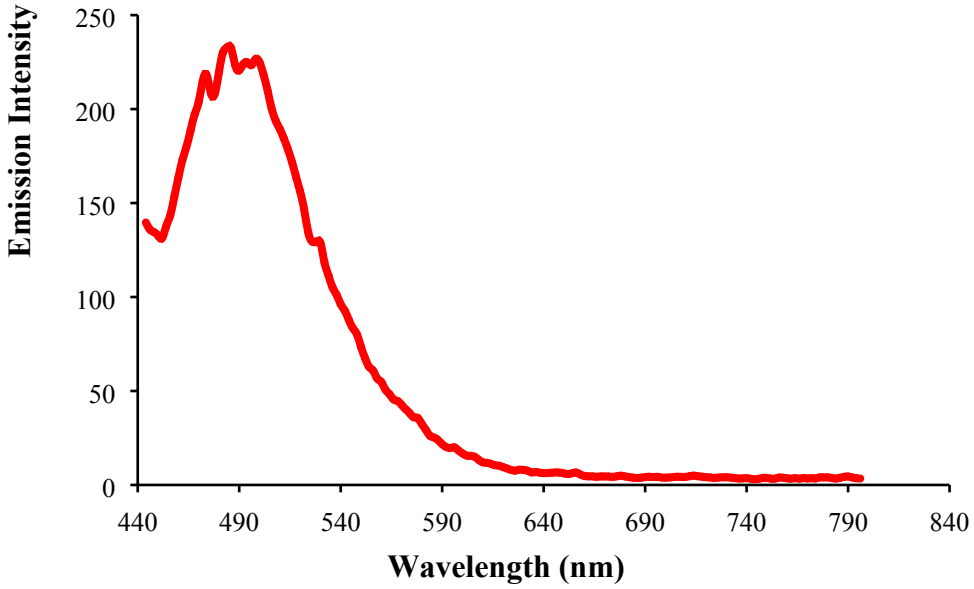


Figure S16 77K emission spectrum of PyLa **(4)** in butyronitrile 1 µM. Sample was excited at 420 nm using an excitation slit width of 5 nm and an emission slit width of 10 nm.

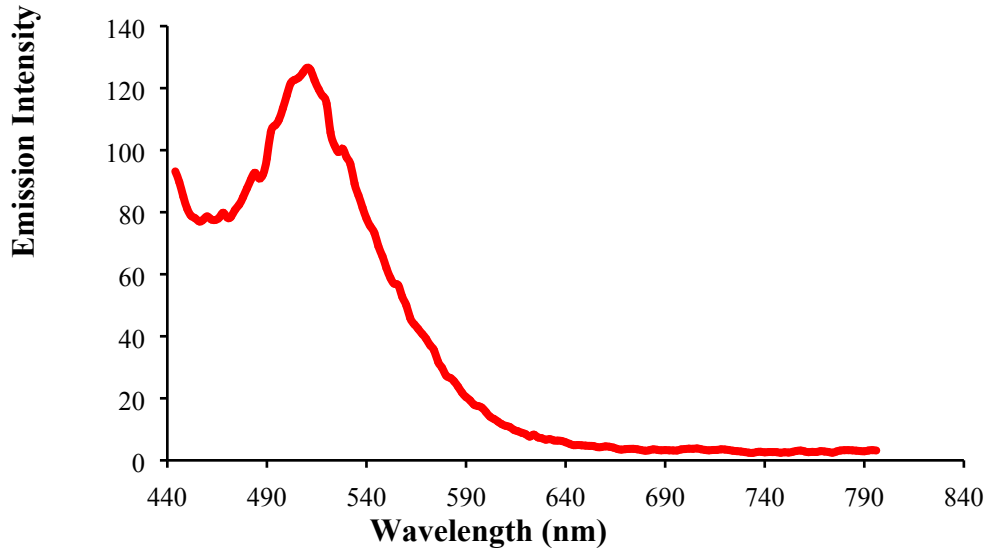


Figure S17 77K emission spectrum of PyLa-C17Cer **(5)** in butyronitrile 1 µM. Sample was excited at 420 nm using an excitation slit width of 5 nm and an emission slit width of 10 nm.

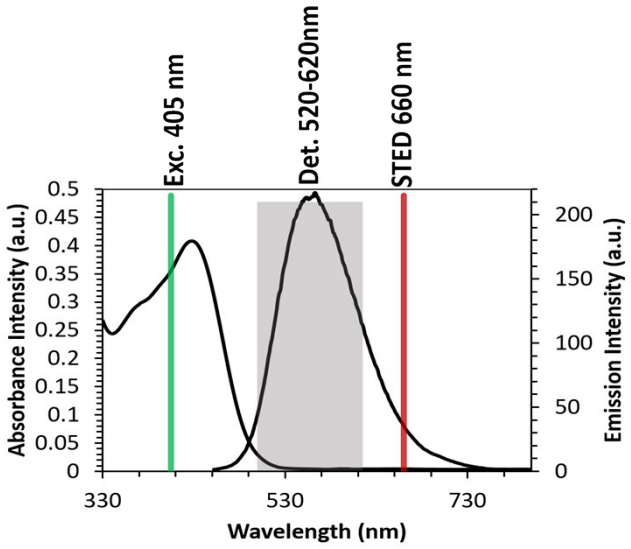


Figure S18 Absorbance and emission spectra of PyLa-C17Cer in DCM. The confocal excitation 405 nm (—) and STED 660 depletion laser (—).

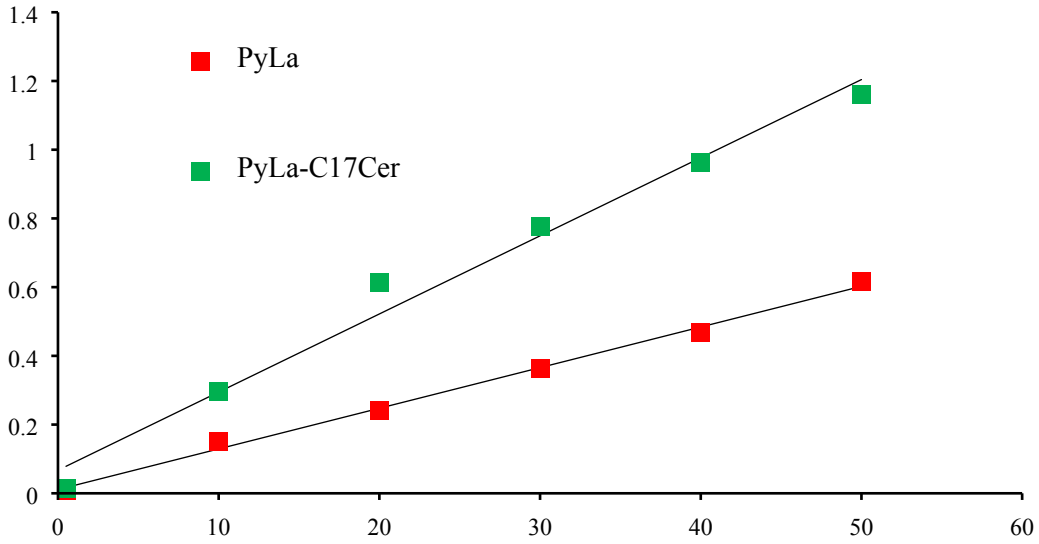


Figure S19 Concentration dependent absorption of PyLa (-) and PyLa-C17Cer (-) in dichloromethane using concentrations ranging between 0.5 – 50 µM. The graph shows that PyLa **(4)** and PyLa-C17Cer **(5)** does not exhibit any aggregation up to concentrations of 25 times greater than imaging concentration (2 µM).

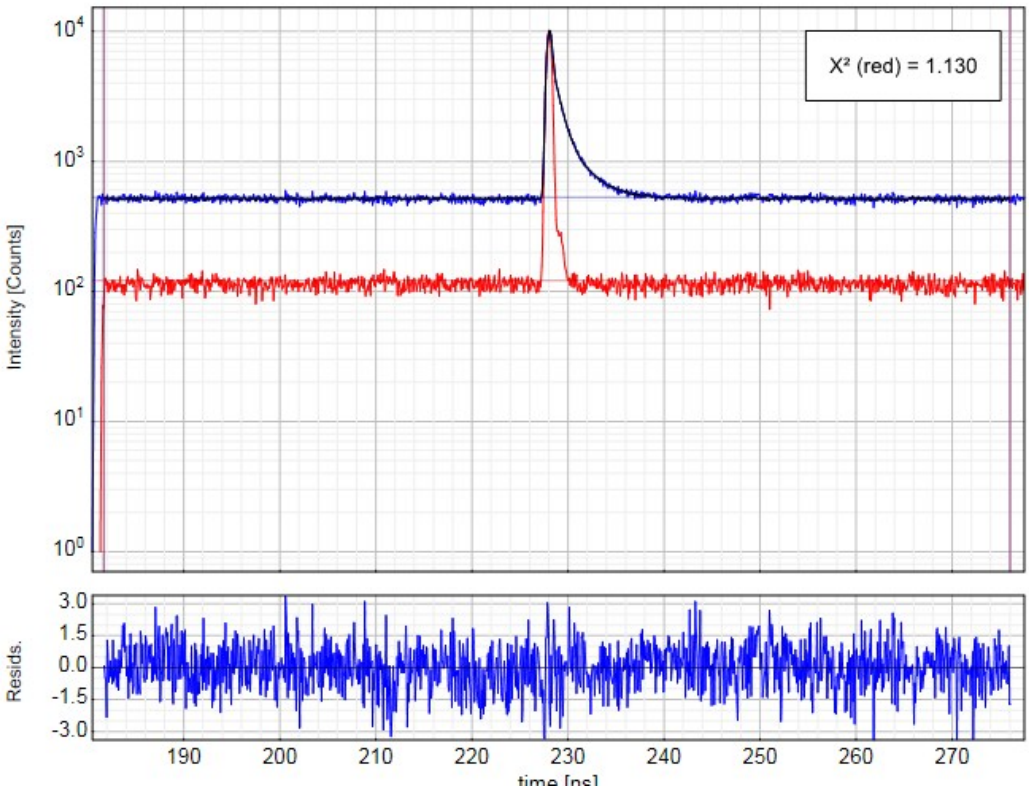


Figure S20 Time correlated Single Photon counting trace for PyLa-C17Cer in Acetonitrile (10 µM) with IRF (red) at room temperature.

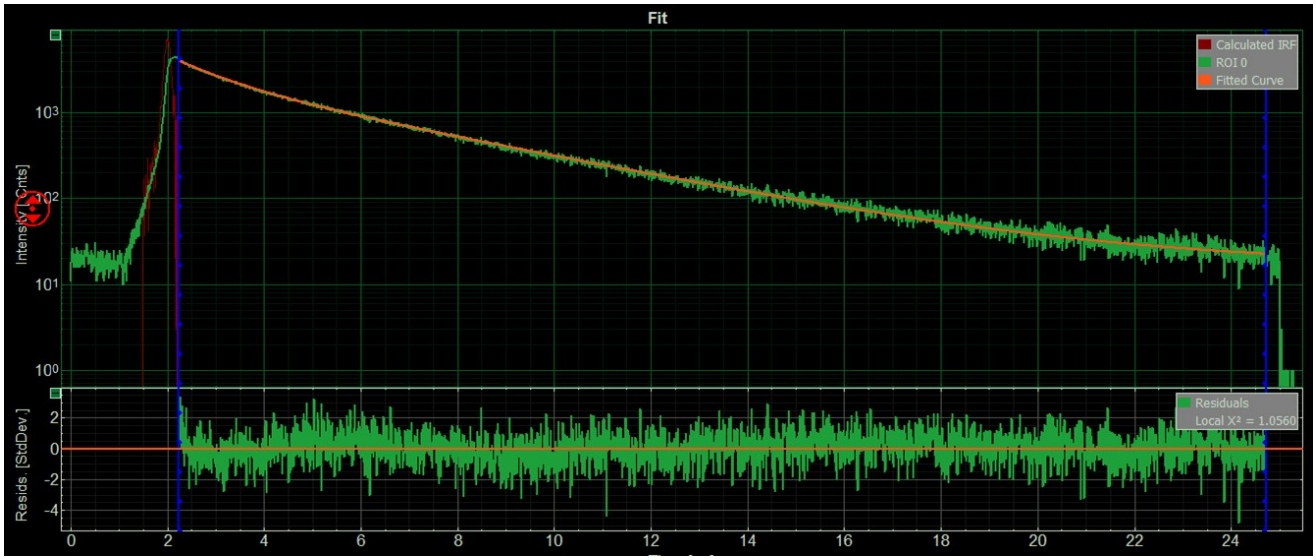


Figure S21 Fluorescent lifetime imaging decay for PyLa in lipid bilayers of composition DOPC/SM/Chol 4:4:2 mol % (10 µM).

Cell Studies

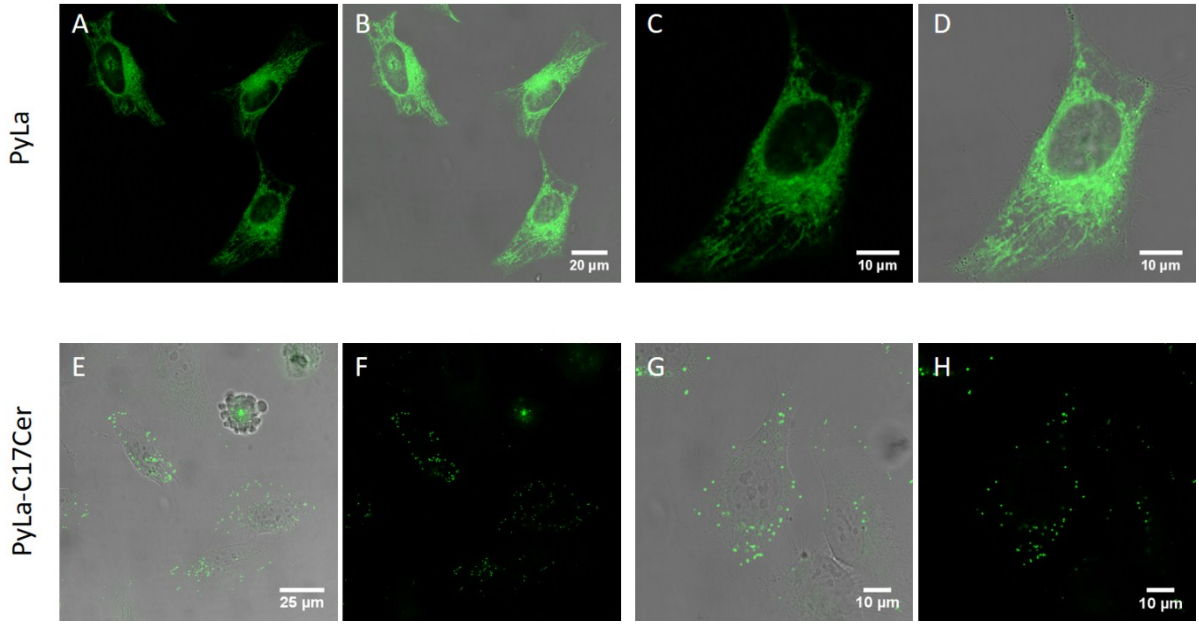


Figure S22 Live HeLa cells incubated with 2 µM PyLa(D) and PyLA-C17Cer (E-H) for 1 h, where A, B, E, F show a group of cells, and C,D,G,H show a single cell, showing the distribution. Ex 405 nm, Em 520-620 nm.

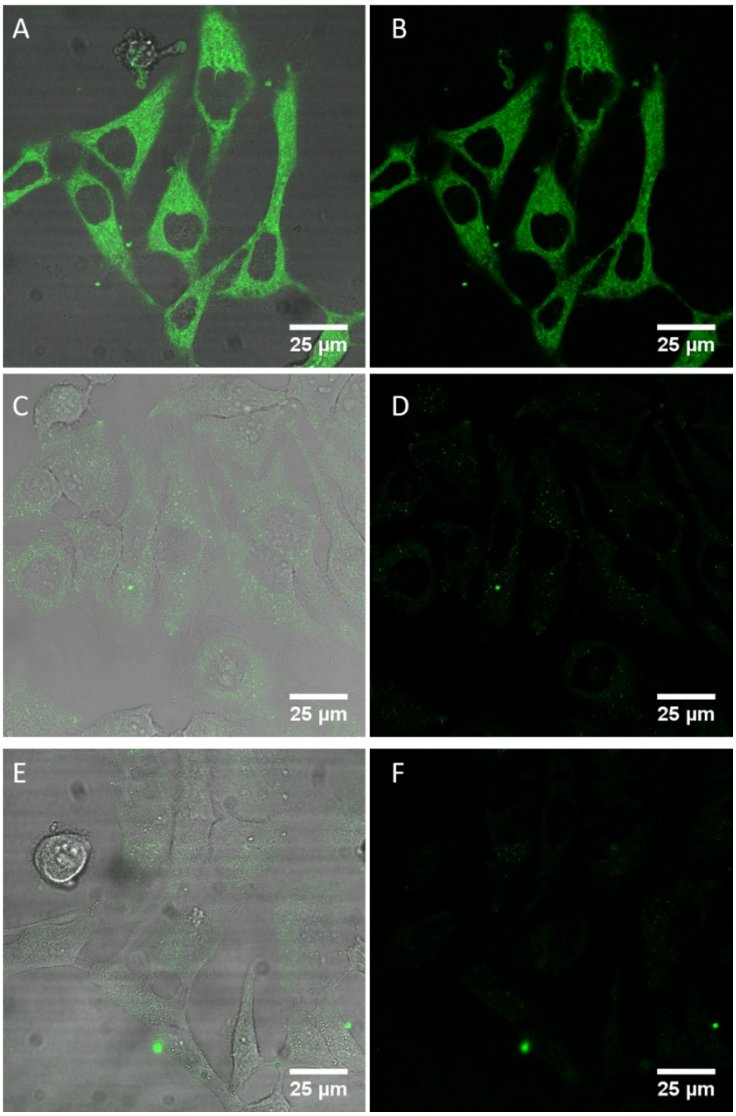


Figure S23 Uptake at 4 °C in live HeLa cells. Cells were incubated at 4 °C for 1 h, then 2 µM PyLa (A,B) and PyLa-C17Cer (C,D) was added and incubated at 4 °C for 4 h prior to imaging. HeLa cells were treated with TNF-a for 16 h, then incubated at 4 °C with PyLa-C17Cer (E,F).

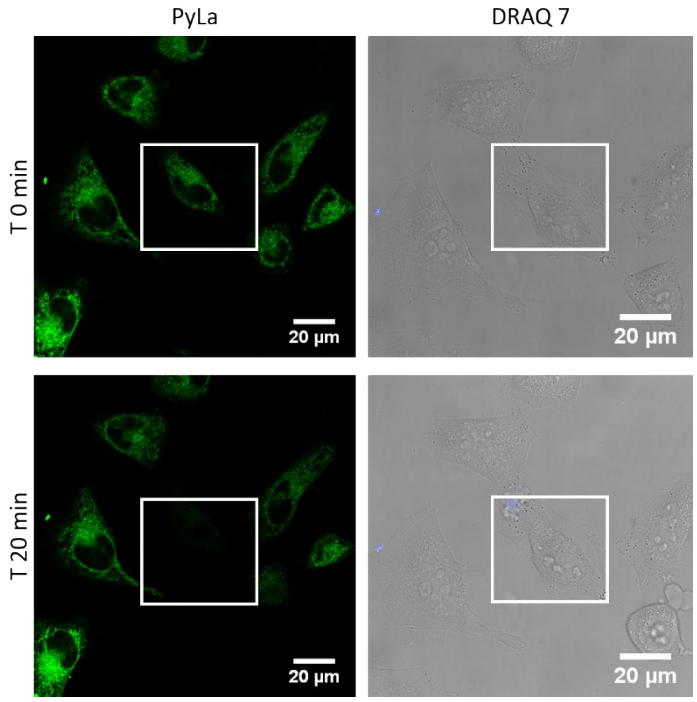


Figure S24 Phototoxicity of PyLa in live Hela cells. Cells were stained with PyLa (2 µM, 2 h). The cell highlighted in the white box was continuously scanned for 5 minutes exciting at 405 nm (0.06 mW/cm^2) then imaged using the DRAQ7 settings (Ex 633 nm, Em 635-720 nm) to determine viability for a total of 20 minutes. An overlay of the **4** channel and DRAQ 7 channel at T 0 minutes and T 20 minutes shows the distribution of the dyes in the cells.

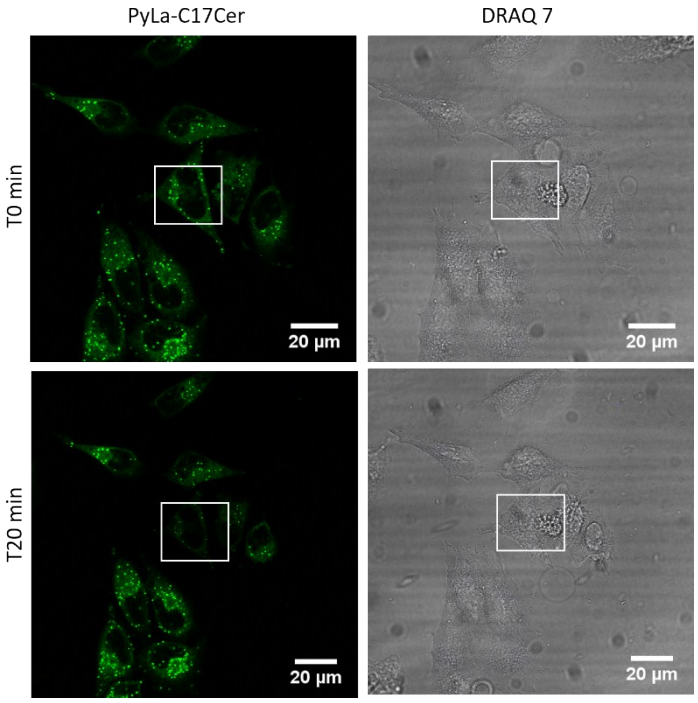


Figure S25 Phototoxicity of PyLa-C17Cer in live Hela cells. Cells were stained with (2 µM, 2 h). The cell highlighted in the white box was continuously scanned for 5 minutes exciting at 405 nm (0.06

mW/cm^2), then imaged using the DRAQ7 settings (Ex 633 nm, Em 635-720 nm) to determine viability for a total of 30 minutes. An overlay of the PyLa-C17Cer channel and DRAQ 7 channel at T 0 minutes and T 20 minutes shows the distribution of the dyes in the cells.

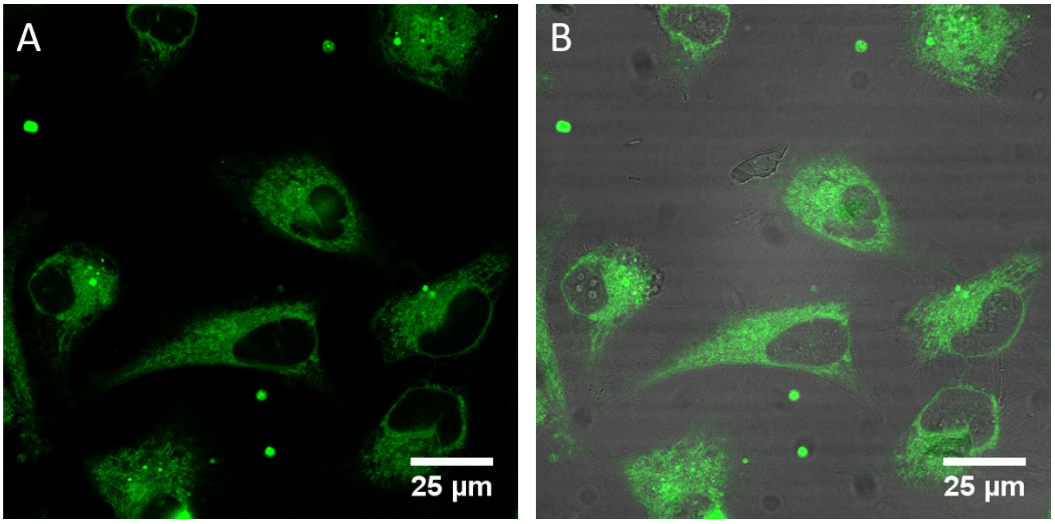


Figure S26 Live Hela cells treated with TNF-α for 16 h. PyLA (2 µM) was added to cells and incubated for 2 h at 37 °C. Cells were washed and imaged using at 405 nm laser to excite PyLa, and the emission was collected at 520-620 nm. PyLa does not enter and stain LDs.

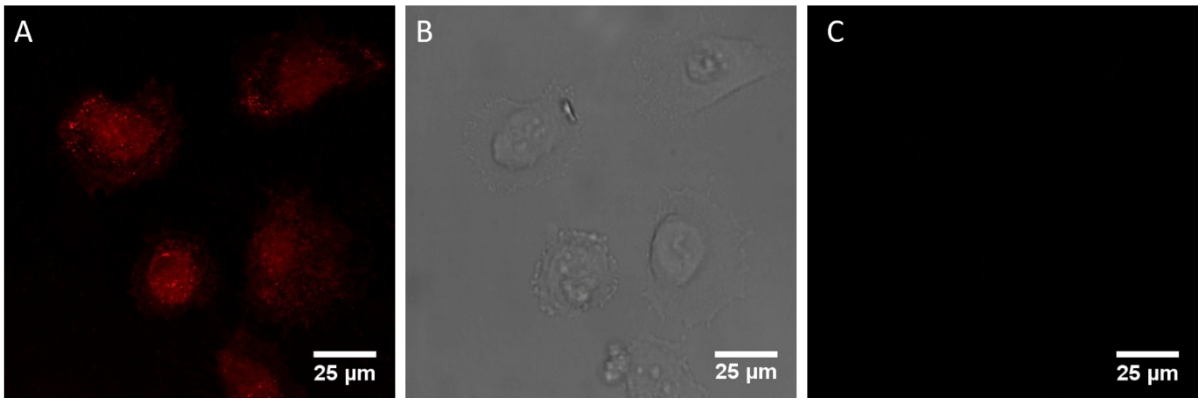


Figure S27 Control HeLa cells stained with Nile Red only (1 ng/mL, 20 minutes and washed) (A) (Ex 552, Em 560–700 nm), and imaged using Pyrene settings (Ex 405, Em 520-620 nm), to confirm no cross-talk was occurring between the 2 dyes, shown in (B) and (C) where no emission was recorded at the Pyrene settings.

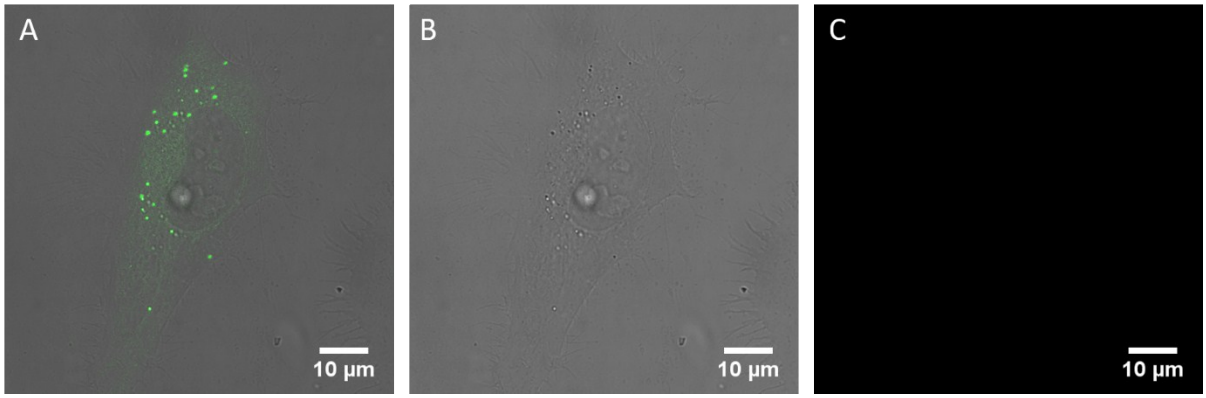


Figure S28 Control HeLa cells stained with PyLa-C17Cer (2 µM, 2 h and washed) (A) (Ex 405, Em 520-620 nm), and imaged using Nile Red settings (Ex 552, Em 560–700 nm), to show no cross talk between the 2 dyes, shown in (B) and (C) where no emission was recorded at the Pyrene settings.

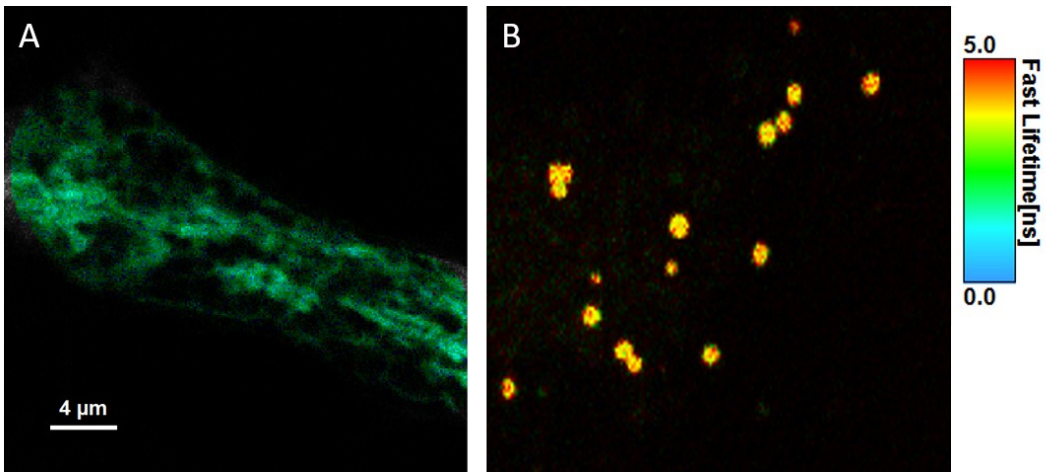


Figure S29 False-colour FLIM of a region of the cytoplasm stained with PyLa (A) and a group of lipid droplets in a live HeLa cell stained with PyLa-C17Cer (B). Cells were incubated with 2 µM PyLa (A) or PyLa-C17Cer (B) for 2 h, then washed with PBS before carrying out FLIM. (Ex 405, Em 520-620 nM).

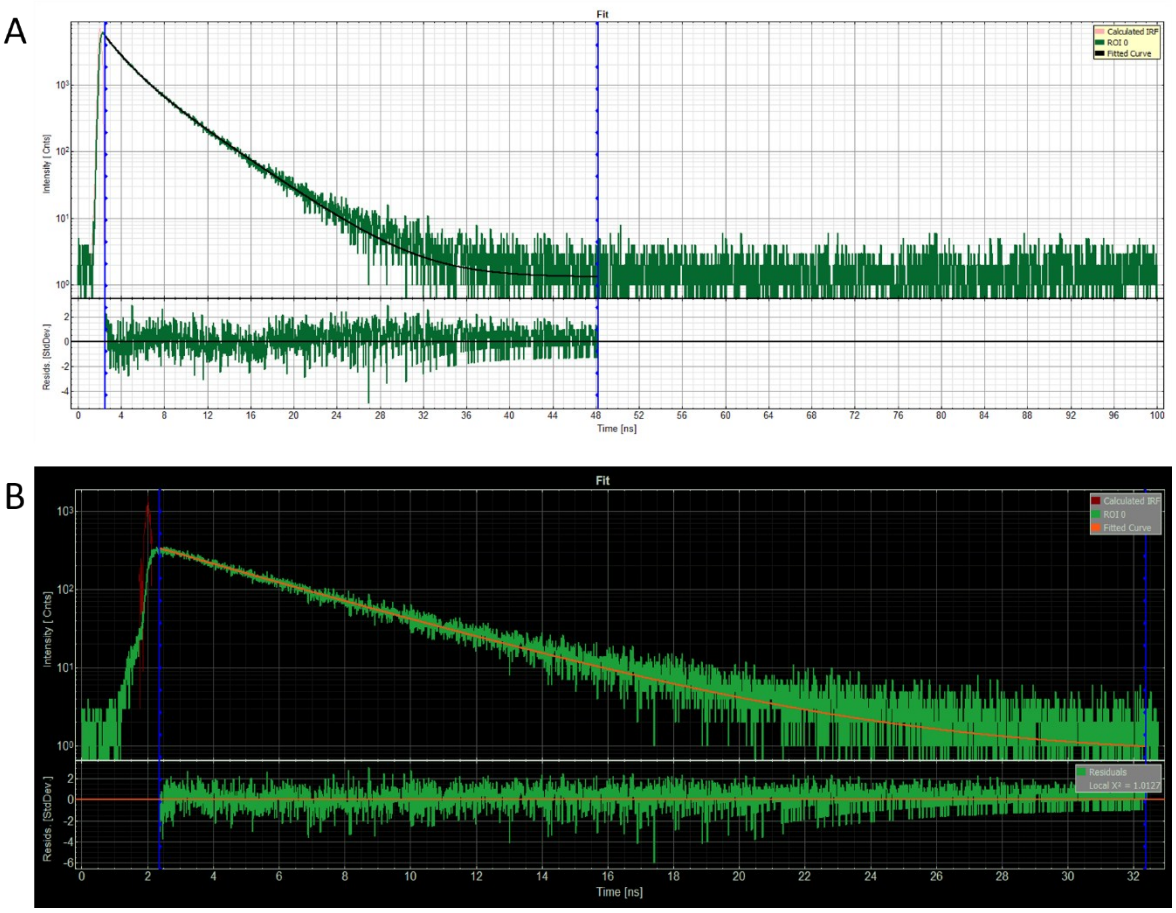
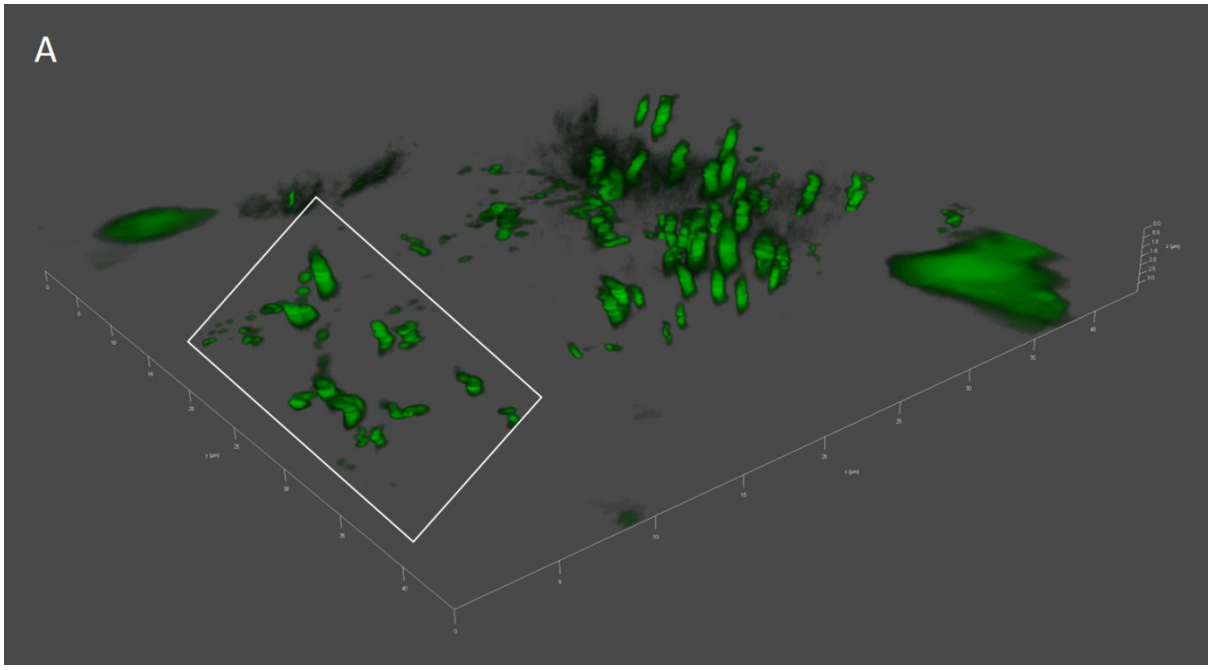


Figure S30 FLIM lifetime decays for PyLa (A) and PyLa-C17Cer (B) in live HeLa cells. Ex 405, Em 520-620 nm.



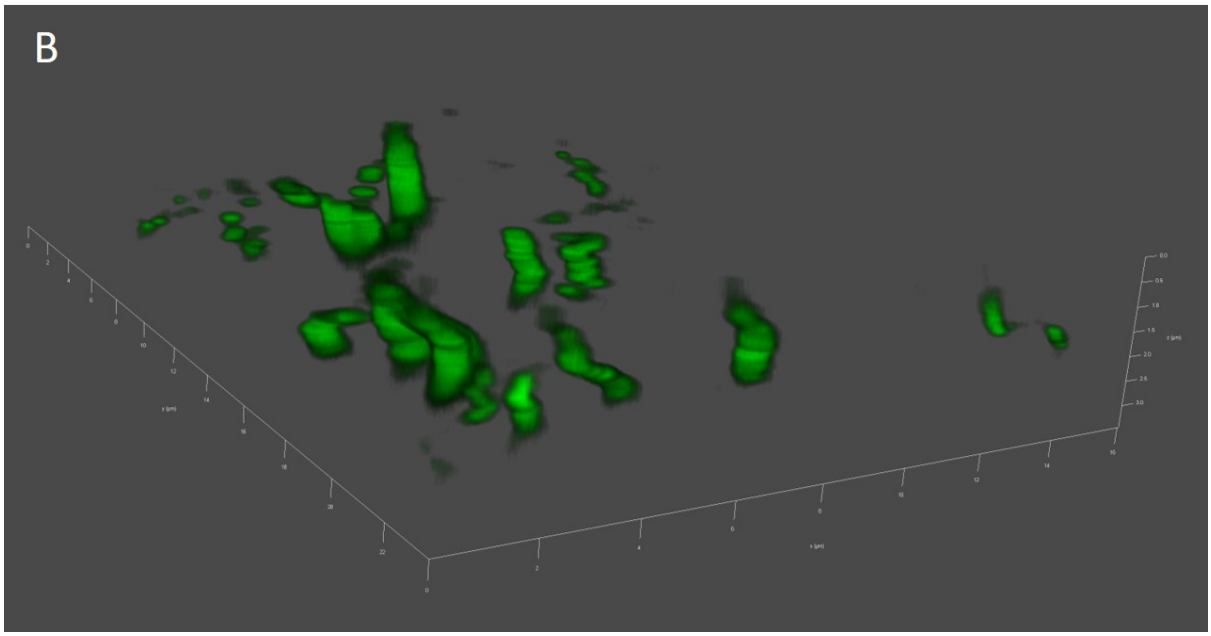
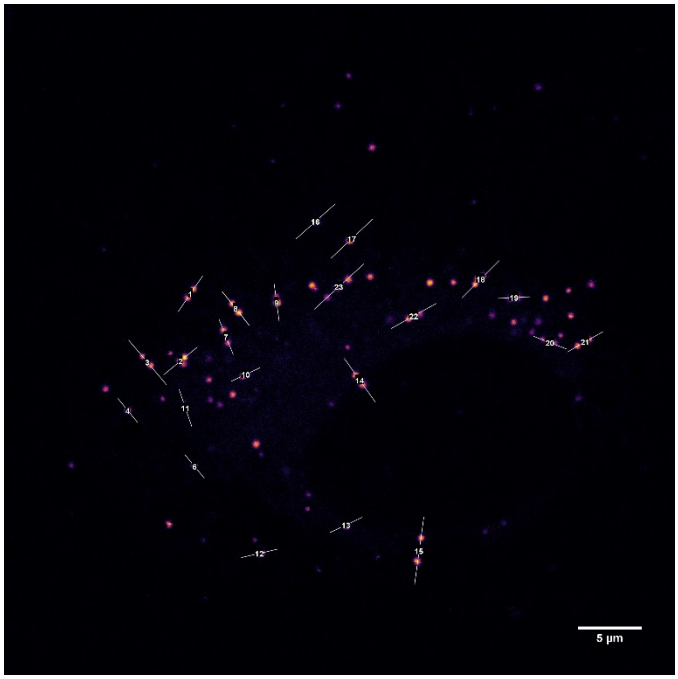


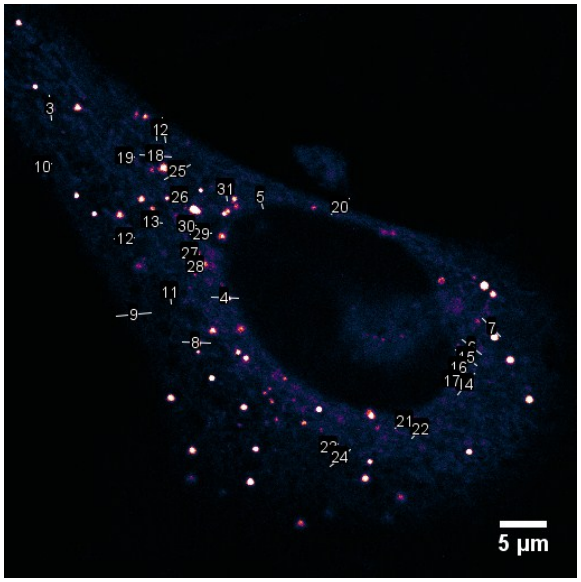
Figure S31 Corresponding 3D confocal z-stack of PyLa-C17Cer in live HeLa cells to the 3D FLIM z-stack video/image. The distribution of PyLa-C17Cer throughout a single HeLa cell (A), and the region of droplets chosen for 3D FLIM (B) shown in Figure 5 (C) in the main text.



ROI	Confocal	STED
1	0.435	0.383
	0.440	0.383
2	0.864	0.339
		0.385
3	0.474	0.426
	0.372	0.294
4	0.455	0.411
5	0.522	0.422
	0.425	0.405
6	0.423	0.345
7	0.460	0.424
	0.477	0.408
8	0.522	0.422
	0.457	0.405
9	0.932	0.385
		0.446
10	0.659	0.366
		0.331
11	0.468	0.415
12	0.476	0.449
13	0.409	0.347
14	0.560	0.435
	0.492	0.427
15	0.453	0.382
	0.548	0.424
16	0.518	0.409
17	0.556	0.465
18	0.556	0.336
		0.405
		0.352
19	0.962	0.399
		0.479
20	0.702	0.308
	0.292	0.338
		0.307
21	0.446	0.421
	0.411	0.384
22	0.464	0.411
	0.410	0.411
Average	0.516	0.396
Standard Deviation (±)	0.144	0.044

Figure S32 Cell. 1 Raw data full width half maxima (FWHM) values of a live Hela cell stained with PyLa-C17Cer (2 µM, 2 h), where region of interest (ROI) is a lipid droplet, and the FWHM value for confocal

and STED of each lipid droplet. Data was obtained using Image J, and FWHM was worked out using Origin Pro 2016 on a STED image before deconvolution.



ROI	Confocal	STED
1	0.802	0.341
		0.450
2	0.088	0.048
	0.719	0.283
3	0.645	0.275
	0.412	0.475
4	0.559	0.378
5	0.512	0.319
6	0.515	0.404
7	0.861	0.389
		0.328
8	0.582	0.313
9	0.591	0.358
10	0.458	0.341
11	0.518	0.421
12	0.520	0.430
13	0.608	0.315
14	0.608	0.450
15	0.530	0.448
16	0.782	0.383
		0.376
17	0.723	0.421
18	0.508	0.360
19	0.413	0.355
20	0.61	0.405
21	0.584	0.372
22	0.485	0.358
Average	0.561	0.365
Standard Deviation (±)	0.152	0.080

Figure S33 Cell 2 Raw data full width half maxima (FWHM) values of a live Hela cell stained with PyLa-C17Cer (2 µM, 2 h) and the corresponding cell, where region of interest (ROI) is a lipid droplet, and the FWHM value for confocal and STED of each lipid droplet. Data was obtained using Image J, and FWHM was worked out using Origin Pro 2016 on a STED image before deconvolution.

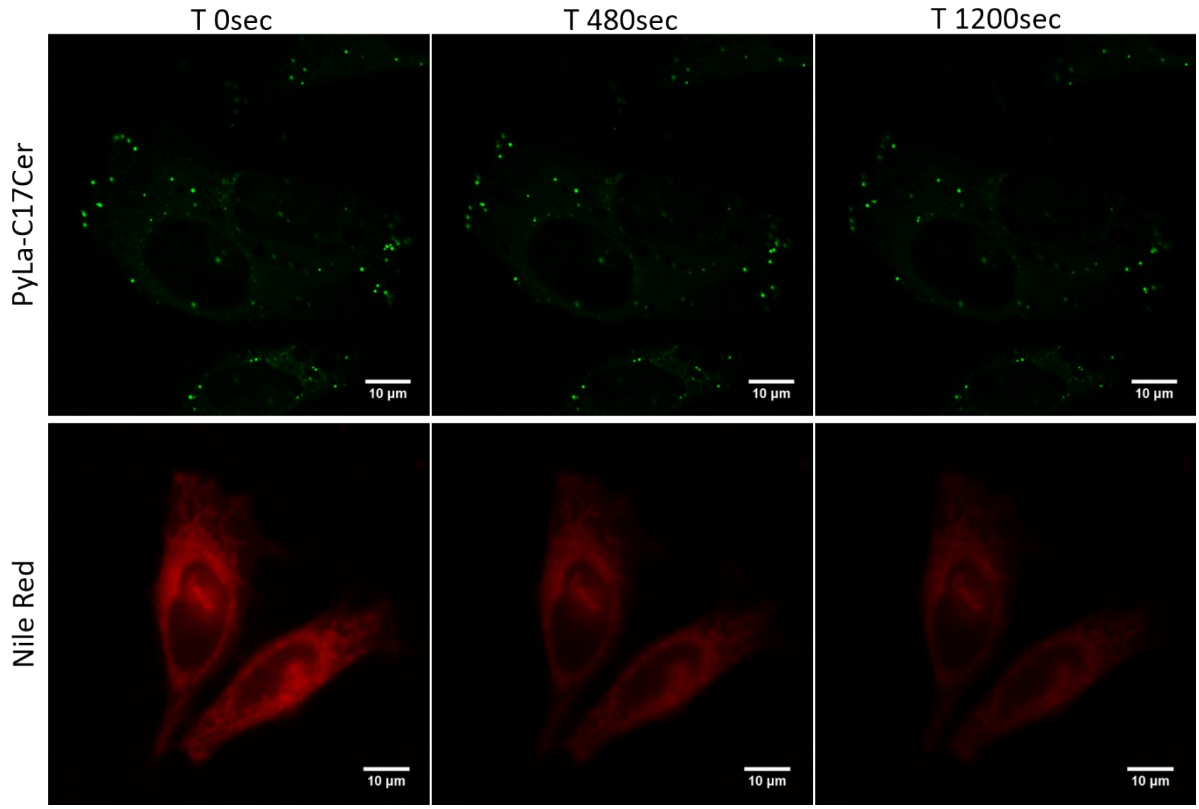


Figure S34 STED photostability of live HeLa cells stained with PyLa-C17Cer, and Nile Red. A 405 nm laser was used to excite PyLa-C17Cer, collecting the emission between 520 -620 nm, and a 560 nm white light laser was used to excite Nile Red collecting the emission between 580 – 670 nm. The STED 660 nm at 0.03 W/cm^2 was used for both samples. The images were acquired at 1024 x 1024 resolutions every 1 minutes for 30 minutes at a pixel dwell time of 2.43 µs. The emission intensity of a selected area in both samples was measured at each time interval over the 20 minutes and plot to show stability over time.

References

(1) Basit, H.; Gaul, V.; Maher, S.; Forster, R. J.; Keyes, T. E. Aqueous-Filled Polymer Microcavity Arrays: Versatile & Stable Lipid Bilayer Platforms Offering High Lateral Mobility to Incorporated Membrane Proteins. *Analyst* **2015**, *140* (9), 3012–3018.

(2) Müller, C. B.; Loman, A.; Pacheco, V.; Koberling, F.; Willbold, D.; Richtering, W.; Enderlein, J. Precise Measurement of Diffusion by Multi-Color Dual-Focus Fluorescence Correlation Spectroscopy. *EPL (Europhysics Letters)* **2008**, *83* (4), 46001.

(3) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A. V.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Sonnenberg, J. L.; Williams-Young, D.; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery Jr, J.A. ; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J. J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Keith, T.

A.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; Fox, D. J. *Gaussian 16*, Revision A.03; Gaussian, Inc., : Wallingford CT, 2016.

(4) Becke, A. D., Density-Functional Thermochemistry 3. The Role of Exact Exchange. *J. Chem. Phys.* **1993,** *98* (7), 5648-5652.

(5) Lee, C. T.; Yang, W. T.; Parr, R. G., Development of the Colle-Salvetti Correlation-Energy Formula into a Functional of the Electron-Density. *Phys. Rev. B* **1988,** *37* (2), 785-789.

(6) Tomasi, J.; Mennucci, B.; Cammi, R., Quantum mechanical continuum solvation models. *Chem. Rev.* **2005,** *105* (8), 2999-3093.

(7) Cossi, M.; Barone, V.; Cammi, R.; Tomasi, J., Ab initio study of solvated molecules: A new implementation of the polarizable continuum mode. *Chem. Phys. Lett.* **1996,** *255*, 327-335.

(8) *GaussView 3.0*, 3.0; GaussView 3.0, Gaussian Inc., Pittsburgh, PA, USA: 2003.

(9) Gorelsky, S. I. *AOMix program,* http://www.sg-chem.net/, version 6.88; University of Ottawa: Ottawa 2013.

(10) Gorelsky, S. I.; Lever, A. B. P., Electronic structure and spectra of ruthenium diimine complexes by denisty functional theory and INDO/S. Comparison of the two methods. *J. Organomet. Chem.* **2001,** *635*, 187-196.