Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary information for

Millisecond lifetime imaging with a europium complex using a commercial confocal microscope under one or two-photon excitation

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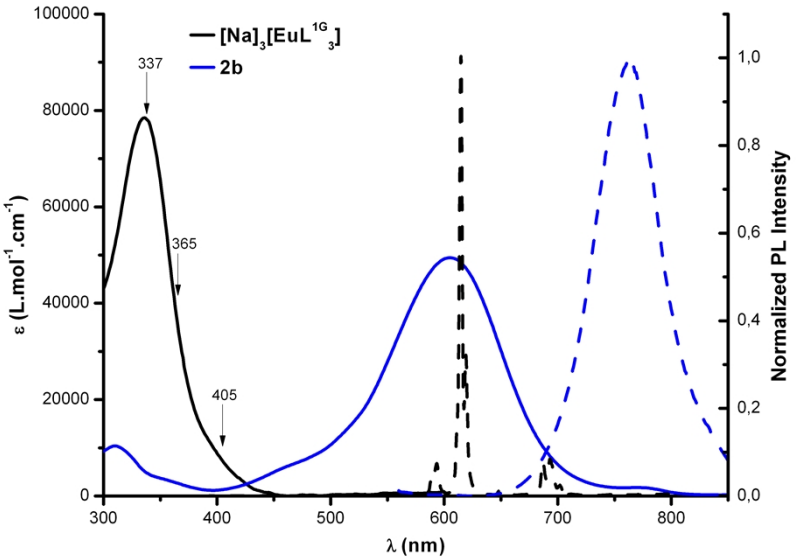


Figure S1. Absorption (plain) and emission (dashed) spectra of $[Na]_3[EuL^{1G}_3]$ and **2b** in water.

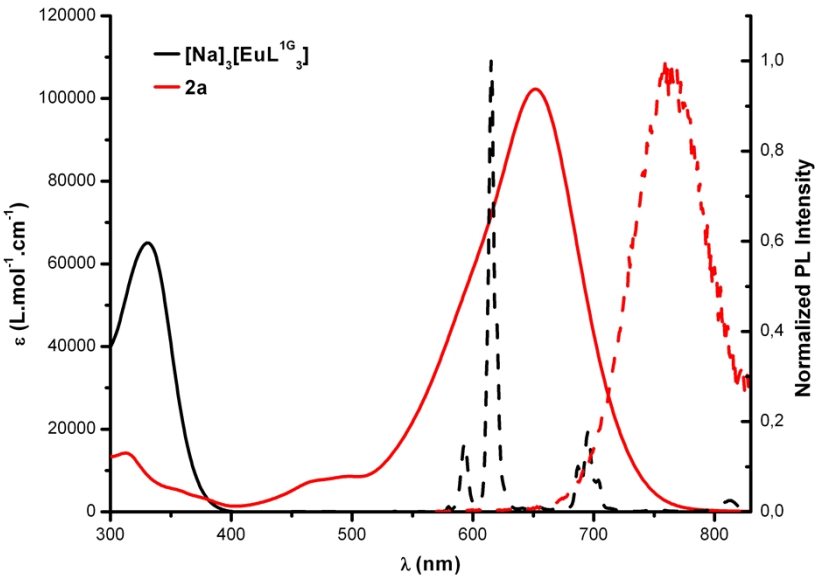


Figure S2. Absorption (plain) and emission (dashed) spectra of $[Na]_3[Eu\mathbf{L}^{1G}{}_3]$ and **2a** in CH_2Cl_2.

Table S1. Quenching of $[Na]_3[Eu\mathbf{L}^{1G}{}_3]$ with **2a** in CH_2Cl_2

2a/$[Na]_3[Eu\mathbf{L}^{1G}{}_3]$ ratio	Eu(III) emission quenching (%)[a]	τ^{Eu} (ms)	η^{FRETb}
0.3	0.10	1.41	0.13
1.1	0.31	1.15	0.29
2.6	0.55	0.86	0.47
4.6	0.71	0.61	0.62
7.7	0.81	0.48	0.70

a. Defined as the ratio between Eu(III) emission in presence and in absence of **2a** measured at the $\Delta J = 2$ transition.

b. $\eta^{FRET} = (1-\tau^{Eu}/\tau_0)$ where τ_0 designed the lifetime in absence of **2a**.

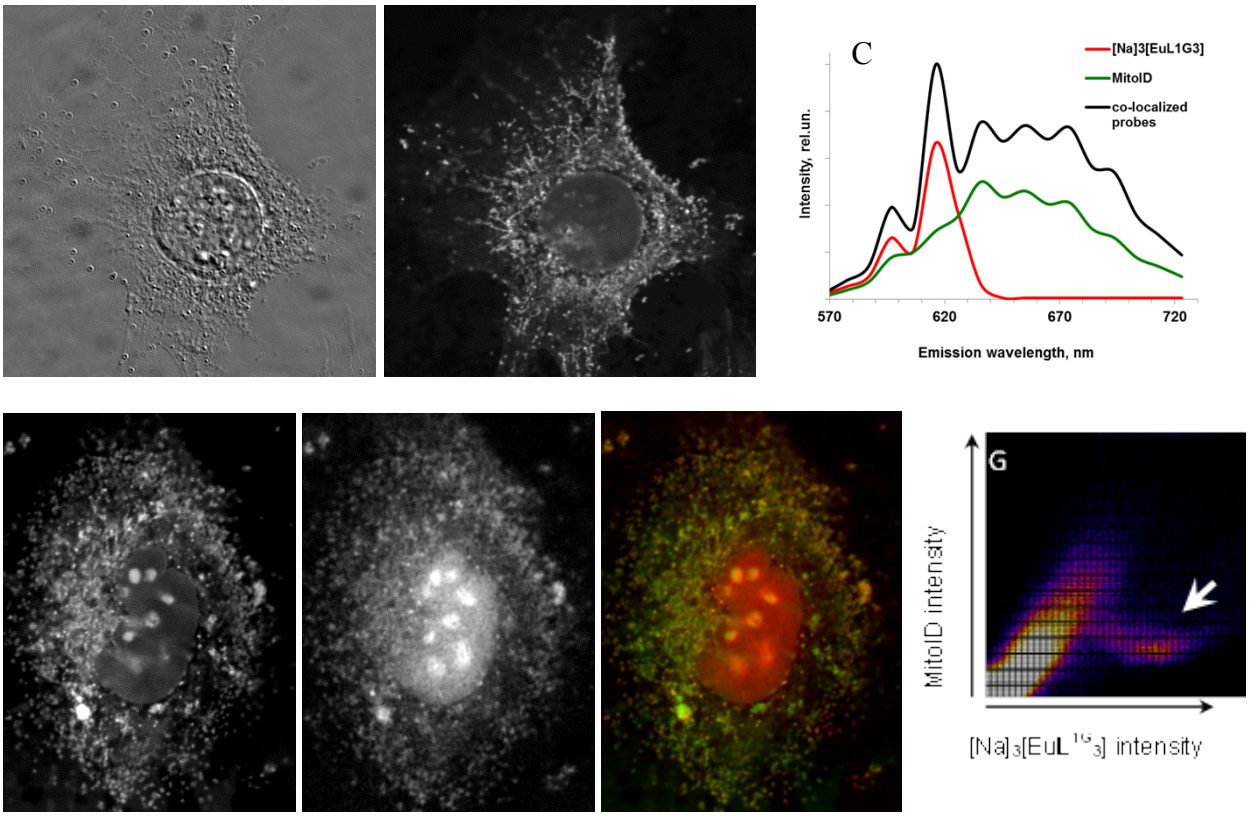


Figure S3. The transmitted light DIC (**A**) and confocal (**B**) images of sub-cellular distribution of the mitochondrial label MitoID. The co-staining of $[Na]_3[Eu\mathbf{L}^{\mathbf{1G}}_3]$ loaded PFA-fixed cells with MitoID (**C-G**). The wide emission spectrum of MitoID overlapped that of europium precluding the use of band-pass detection (**C**). The spectral unmixing confocal microscopy (LSM710) allowed a faithful discrimination of the MitoID (**D**) and $[Na]_3[Eu\mathbf{L}^{\mathbf{1G}}_3]$ (**E**) signals. In spite of lower signal-to-noise ratio of the spectral detection and too fast scanning speed of the confocal microscope (maximum value of 177 µs/pixel), colocalisation of MitoID with $[Na]_3[Eu\mathbf{L}^{\mathbf{1G}}_3]$ (**E**) can be clearly seen in the overlap image (**F**). The positive correlation of both signals is visible in colocalisation scatterplot (**G**) except a region corresponding to nucleoli (arrowhead). One can notice the tendency of MitoID (**D,E** arrowheads) as of $[Na]_3[Eu\mathbf{L}^{\mathbf{1G}}_3]$ and some other tested probes (Acridine Orange, FRET acceptor dyes **2a,b)** to accumulate in the nucleoli. The partition between organelles is however different for each dye and is known to be a complex function of structural symmetry, hydrophobicity and charge of the probe (for review see Horobin *et* al[1]). Scale bars 10 µm.

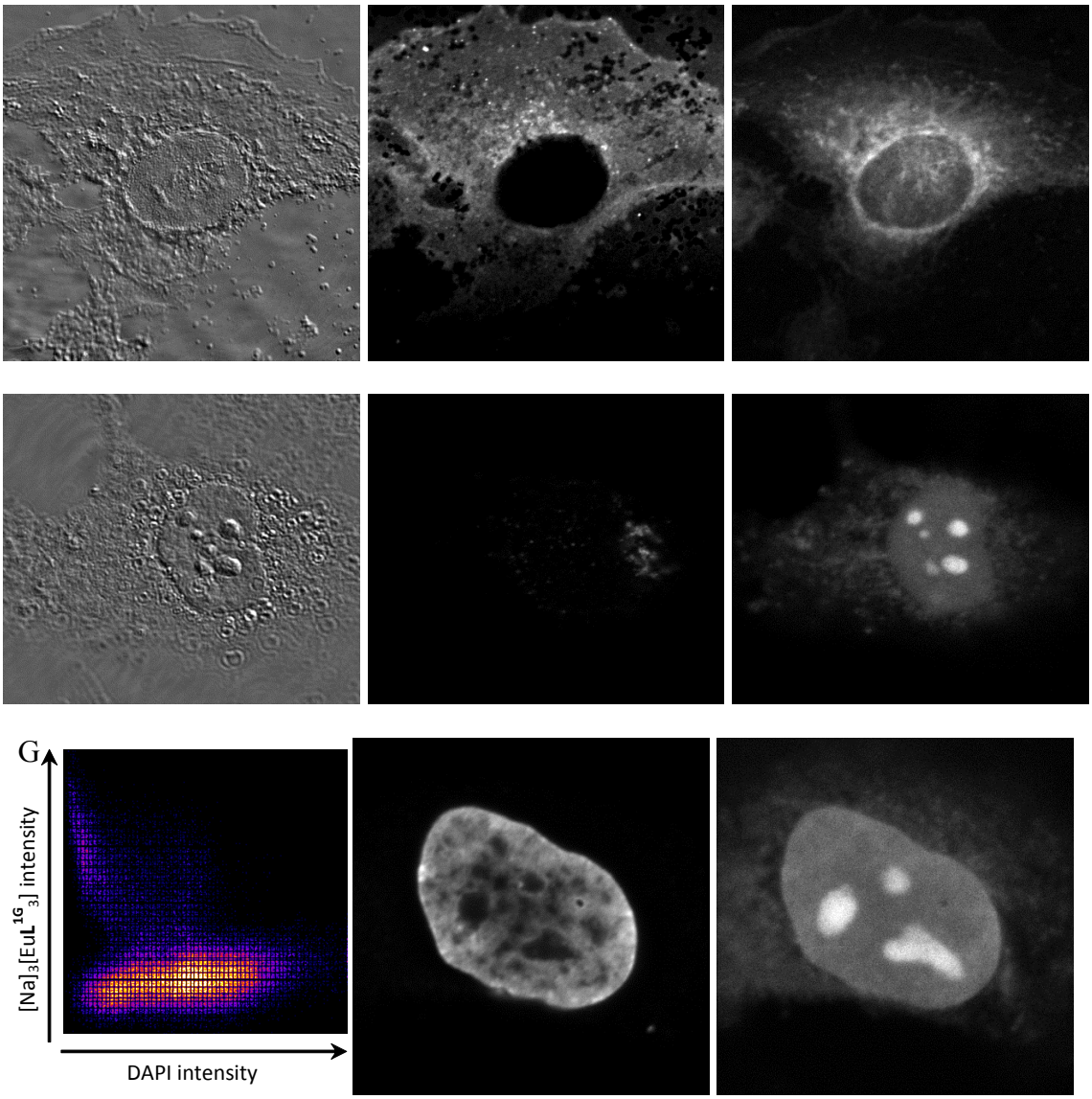


Figure S4. The co-staining of $[Na]_3[Eu\mathbf{L^{1G}}_3]$ loaded fixed cells with the membrane label DiD (**A-C**), the immuno-labelled protein Golgin (**D-F**) and with the DNA intercalator DAPI (**G-I**).**A, D –** transmitted light DIC images, **B,E,H** confocal optical sections, **C,F,I** – $[Na]_3[Eu\mathbf{L^{1G}}_3]$ distribution 2P images. No significant colocalization was detected between these labels and europium complex. The colocalization scatterplot with DAPI in the region of nucleus (**G**) shows the absence of intensity correlation of the chromatin density (**H**) and $[Na]_3[Eu\mathbf{L^{1G}}_3]$ signal (**I**). Scale bars 10 μm.

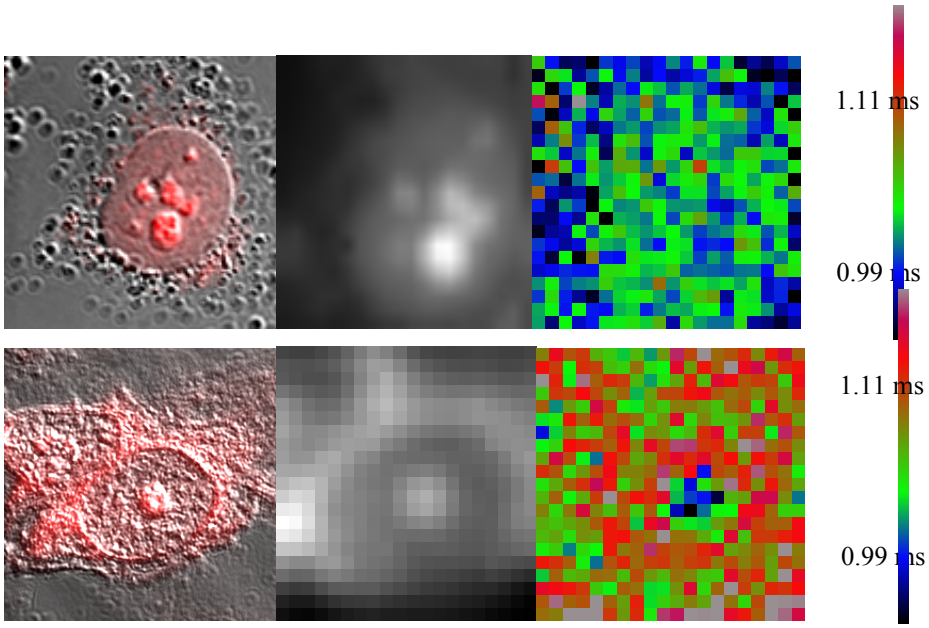


Figure S5. TDS imaging of the fixed cells stained with $[Na]_3[EuL^{1G}_3]$. T24 cells were fixed by PFA (**A**-**C**) or EtOH (**D**-**F**). Transmitted light DIC images (**A**, **D**) are superposed with intensity distribution of Eu. Dashed rectangle shows the region measured by TDS FLIM. Intensity maps of Eu in ROIs measured with the resolution 20x21 pixels (**B**,**E**). Lifetime maps in the ROIs were color coded within the same scale for comparison (**C**,**F**).

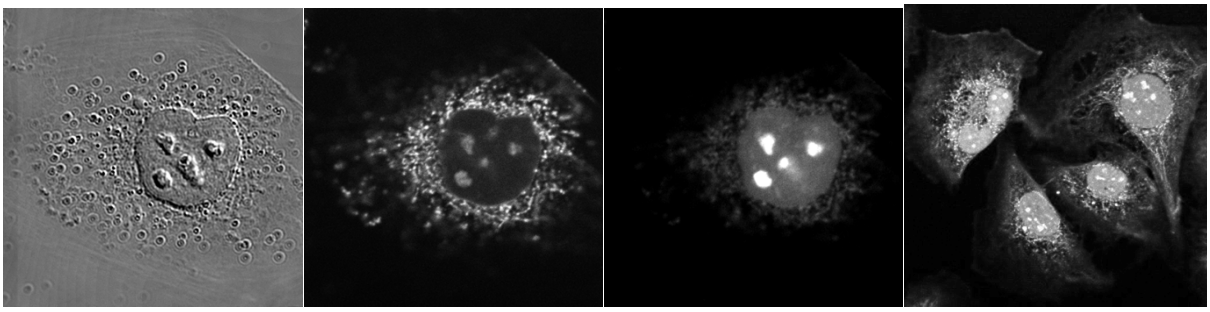


Figure S6. Colocalization of the $[Na]_3[EuL^{1G}_3]$ and **2b** in PFA-fixed cells. **A** – transmitted light DIC image of the fixed cell. **B** – **2b** subcellular distribution shows prominent accumulation in mitochondria, moderate nucleolar and faint chromatin staining, although this diffuse staining could be more pronounced probably depending on the degree of nuclear membrane permeabilization (panel **D**, another experiment). **C** – $[Na]_3[EuL^{1G}_3]$ localization in mitochondria, chromatin and nucleoli is similar to **2b** albeit with completely different partition ratios. The pixels of these three subcellular structures present thus co-localized dyes with variable Donor/Acceptor ratio and are used as for the FRET analysis. Scale bars – 10 μm.

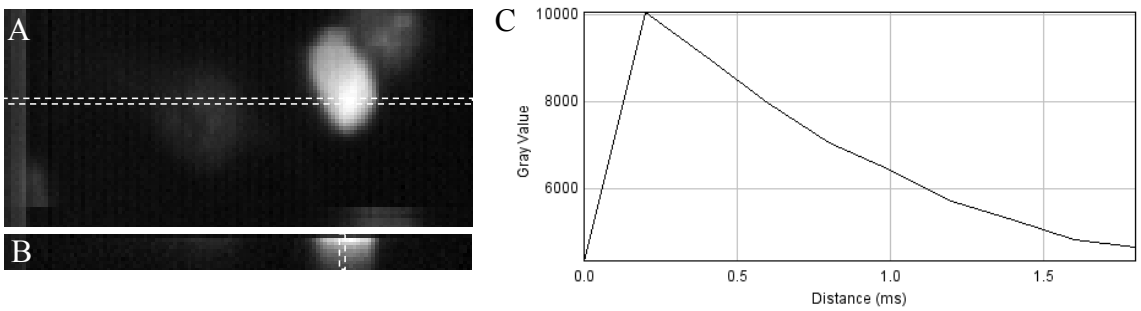
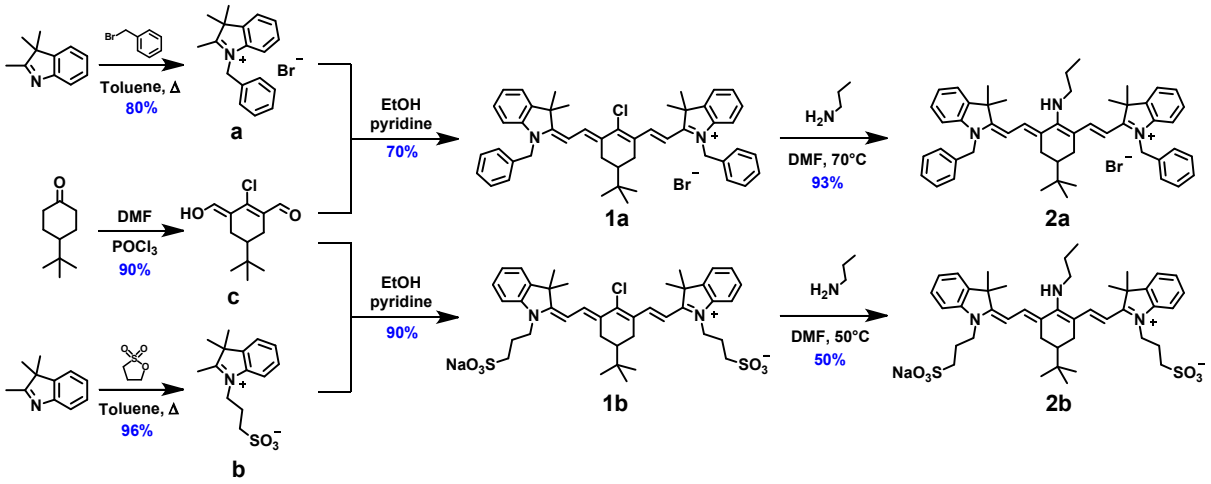
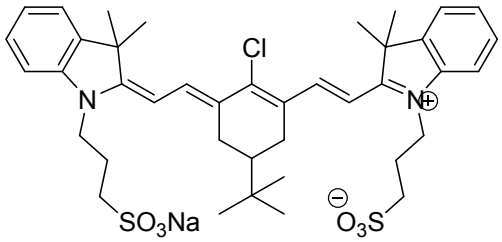


Figure S7. TSLIM imaging in line scanning mode of $[Na]_3[Eu\mathbf{L}^{1G}{}_3]$ in the nucleus of the fixed T24 cell. **A** – 128x60 pixel intensity image. The dashed rectangle represents one of 60 lines, average of 10 repetitions. Scale bars 10 μm. **B** – temporal sampling of the example dashed line outlined in **A**. Ten sequential measurements are presented in ordinate, starting from the one taken before the excitation pulse (top black line). The vertical dashed rectangle outlines luminescence decay in a single pixel. **C** – Intensity profile of the single dashed decay presented in panel **B**.



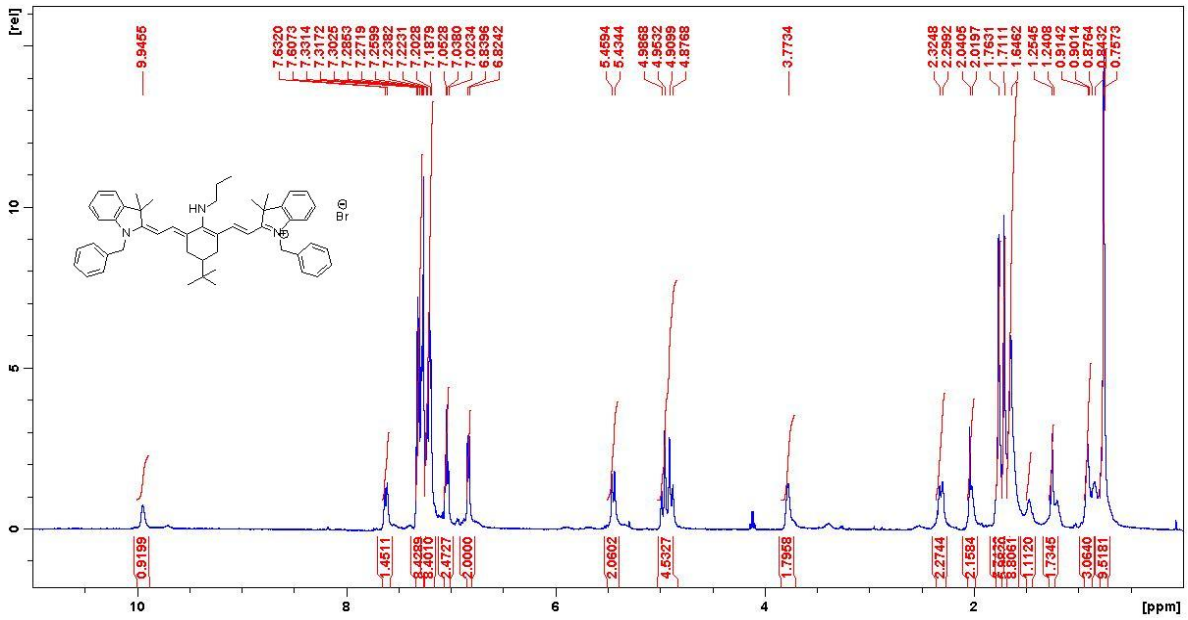
Scheme S1. Synthesis of heptamethines **2a-b**.

Compounds **a**,[2] **b**,[3] **c**,[4] and **1a**[5] were prepared according reported procedures.



Compound 1b. A solution of 74 mg of **c** (0.32 mmol, 1 eq.) and 200 mg of **b** (0.71 mmol, 2.2 eq.) in 10 mL of anhydrous ethanol was added by 0.07 mL of pyridine (0.81 mmol, 2.5 eq.). The solution was stirred for 15h at 80°C. The resulting solution was concentrated under reduced pressure, and the crude residue was submitted to flash chromatography on silica using acetonitrile/methanol/water (85:10:5, R_f = 0.36) as eluent to afford the product as a dark green solid in a 90 % yield (220 mg). ^{1}H NMR (CD_3OD, 500.10 MHz): δ 8.46 (d, $^3J_{trans}$ = 14 Hz, 2H), 7.51 (d, ^3J = 7 Hz, 2H), 7.43-7.41 (m, 4H), 7.29-7.26 (m, 2H), 7.50 (d, $^3J_{trans}$ = 14 Hz, 2H), 4.42 (m, 4H), 3.07 (d, ^2J = 15 Hz, 2H), 2.95 (t, ^3J = 6 Hz, 4H), 2.30-2.24 (m, 6H), 1.75 (s, 12H), 1.54 (m, 1H), 1.14 (s, 9H). ^{13}C NMR (CD_3OD, 125.75 MHz): δ 174.3, 151.3, 145.9, 143.6, 142.7, 129.9, 129.3, 126.4, 123.4, 112.3, 102.6, 49.7, 49.0, 44.4, 46.0, 33.5, 29.0, 28.4, 28.1, 24.2. UV-Vis (H_2O) λ_{max} = 774 nm (ε_{max} = 130000 L.mol^{-1}.cm^{-1}). UV-Vis (CH_3OH) λ_{max} = 782 nm (ε_{max} = 223000 L.mol^{-1}.cm^{-1}). MS (ESI-): $[M]^-$ = 753.2788 (calcd for $C_{40}H_{50}ClN_2O_6S_2^-$: 753.2804).

NMR spectra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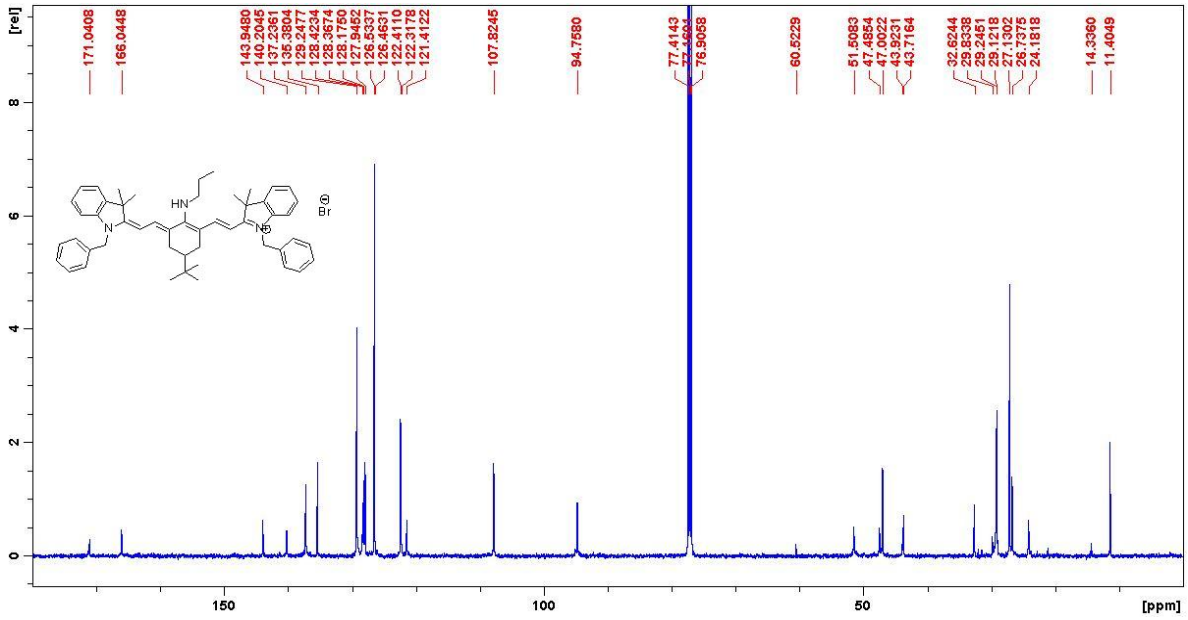
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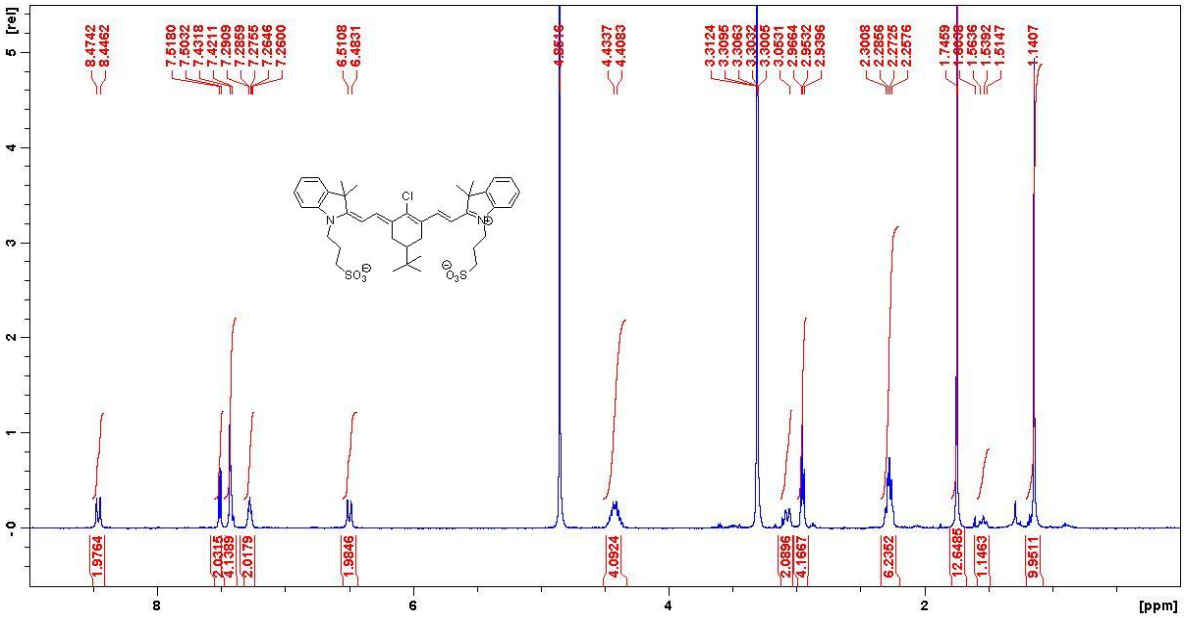
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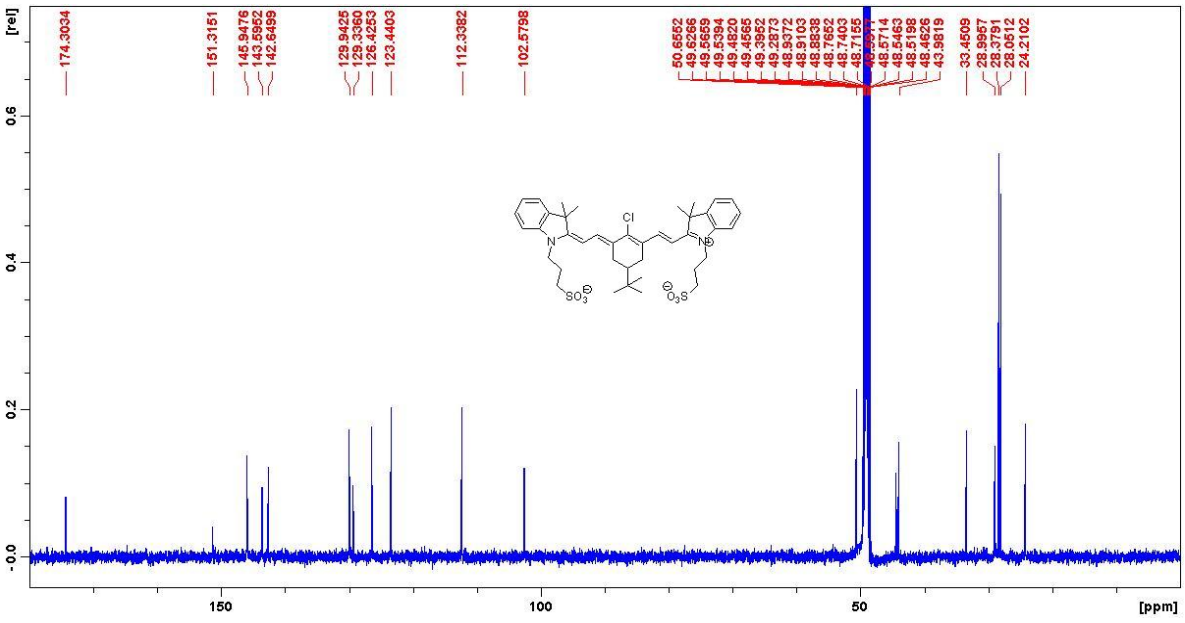
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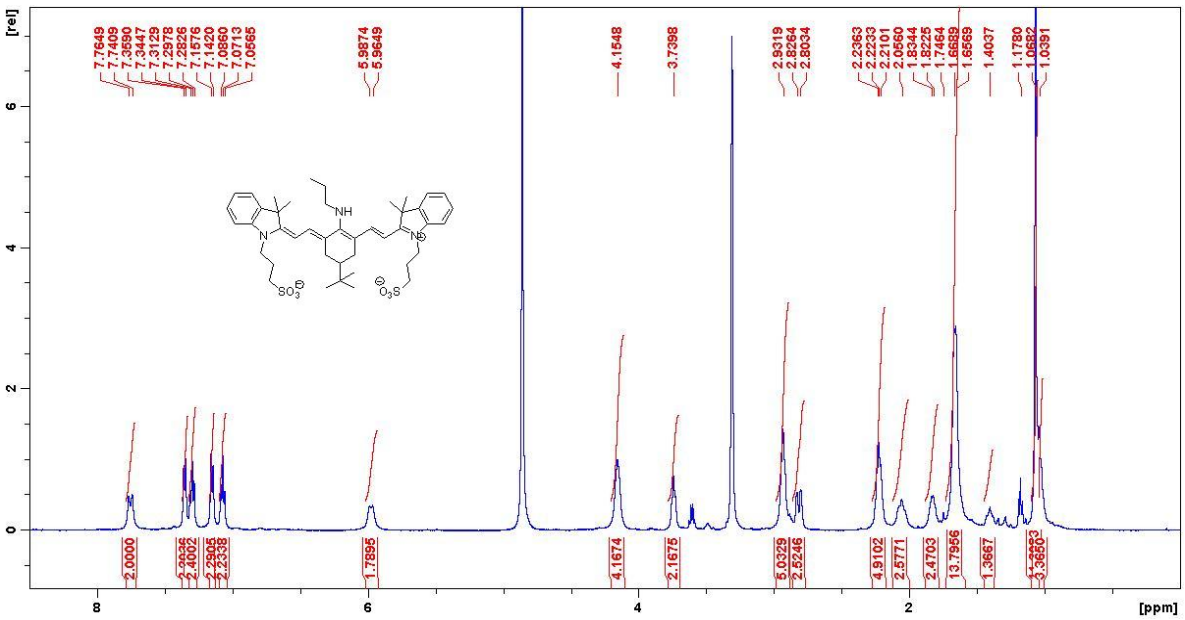
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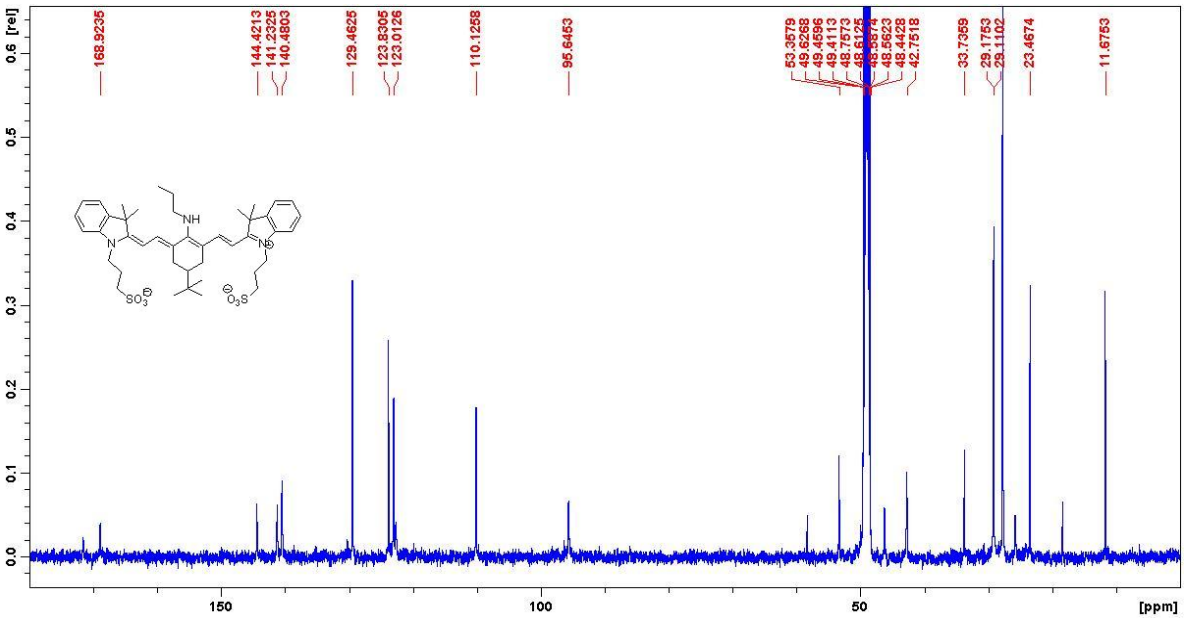
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References

(1) Horobin, R.; Stockert, J.; Rashid-Doubell, F. *Histochem Cell Biol* **2006**, *126*, 165.

(2) Chen, X.; Peng, X.; Cui, A.; Wang, B.; Wang, L.; Zhang, R. *J. Photochem. Photobiol., A* **2006**, *181*, 79.

(3) Flanagan, J. H.; Khan, S. H.; Menchen, S.; Soper, S. A.; Hammer, R. P. *Bioconjugate Chem.* **1997**, *8*, 751.

(4) Reynolds, G. A.; Drexhage, K. H. *J. Org. Chem.* **1977**, *42*, 885.

(5) Bouit, P.-A.; Wetzel, G.; Berginc, G.; Loiseaux, B.; Toupet, L.; Feneyrou, P.; Bretonnière, Y.; Kamada, K.; Maury, O.; Andraud, C. *Chem. Mater.* **2007**, *19*, 5325.