Lysosome Specific Near-Infrared Fluorescent Probe for the *in vitro* Cancer Cell Detection and Non-Invasive *in vivo* Imaging

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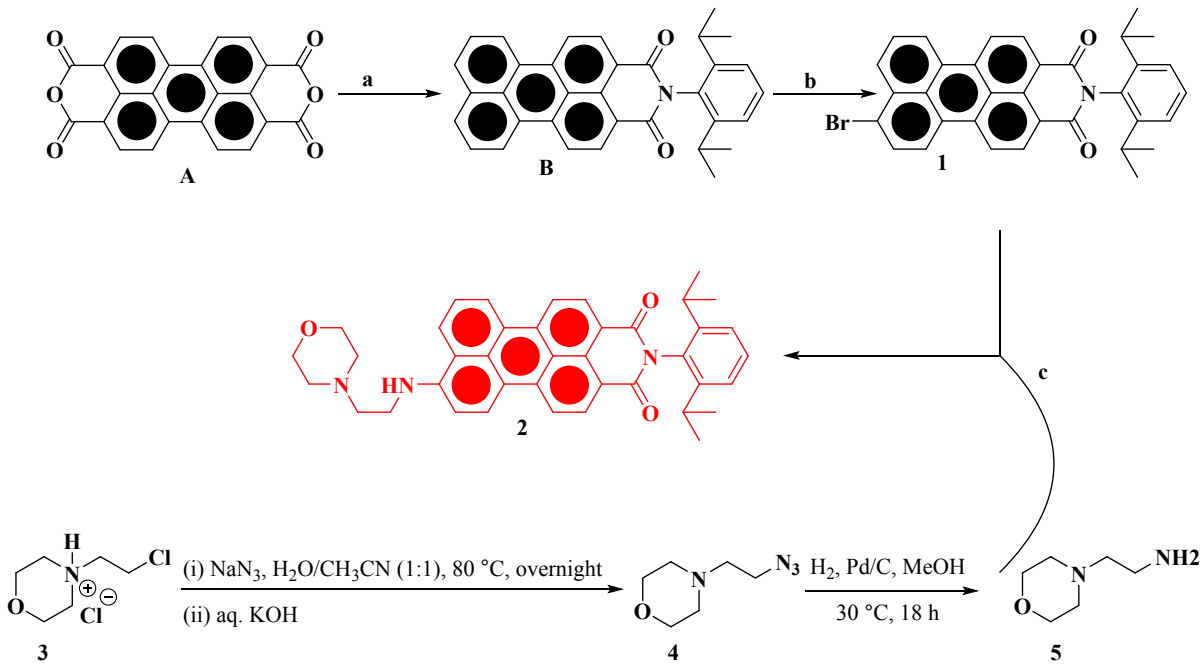
1. Materials and methods:

All reagents were purchased from Sigma Aldrich and used without further purification. Acetonitrile and dichloromethane were distilled from CaH_2 before use. ^1H NMR spectra were recorded on a BRUKER- AV 400 and 500 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 7.26 ppm). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (Hz). ^{13}C NMR (175 MHz) spectra were recorded on a BRUKER-AV 500 MHz Spectrometer with complete proton decoupling. Chemical shifts are reported in ppm from tetramethylsilane with the

solvent resonance as the internal standard (deuterochloroform: 77.0 ppm). UV/vis absorption spectra were recorded on an Agilent Cary 5000 UV-Vis-NIR UV/vis spectrophotometer, fluorescence emission spectra were recorded on the Agilent Cary Eclipse Fluorescence Spectrophotometer, FT-IR spectra were recorded on a Thermo Nicolet Nexus 670 FT-IR and HRMS spectra were recorded on a TOF MS ES+ mass spectrometer. Cytotoxicity were recorded on Synergy™ H1 is a configurable multi-mode microplate reader. Confocal images were recorded on Nikon Ti Eclipse confocal microscope. Chromatographic purification was done with 100-200 mesh silica gel (Merck). For reaction monitoring, precoated silica gel 60 F254 TLC sheets (Merck) were used. Cell culture media and all the other materials required for culturing were obtained from Gibco, USA

2. Preparation and Characterization.

The perylene monoimide dye N-(2,6-di-isopropyl)perylene-3,4- dicarboximide (**B**) was synthesized in one step following the literature procedure.[1] Then, the corresponding 9-Bromo compound (**1**) was prepared by the reaction of compound B with Br_2 in chlorobenzene at room temperature for 10 min. Subsequently 2-morpholinoethan-1-amine was installed on compound **1** to get the desired compound **2**. All the compound were cauterized by ^1H NMR, ^{13}C NMR and mass spectrometry.

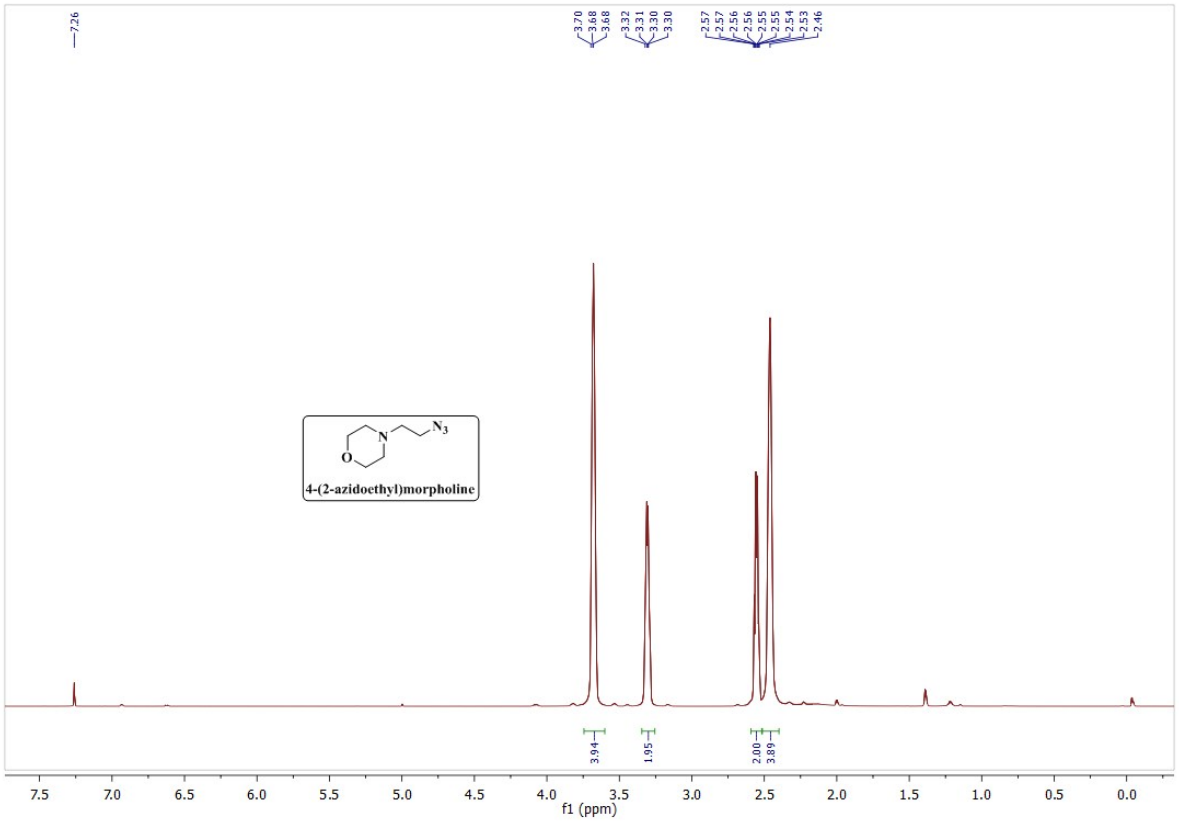


Reagents and conditions: (a) imidazole, 2,6-diisopropylaniline, $Zn(OAc)_2$, 190 °C, 24h. (b) Br_2, chlorobenzene, rt, 10 min, (c) 2-morpholinoethan-1-amine, NaO^tBu, Tris (dibenzylideneacetone) dipalladium(0), (2,2′-bis(diphenylphosphino)-1,1′-binaphthyl), toluene, reflux 12h.

Scheme S1. Synthesis of the Probe **2**

2.1. Synthesis of *4-(2-azidoethyl)morpholine:*

4-(2-chloroethyl)morpholine.HCl salt (500 mg, 2.7 mmol) was dissolved in H_2O/CH_3CN (1:1). Then NaN_3 (436 mg, 6.7 mmol) was added to it and the reaction mixture was refluxed for overnight at 80 ^oC. Then the reaction mixture was allowed to cool to room temperature and was basified with 2M KOH solution followed by extracted with diethyl ether. The organic layer was collected and dried over anhydrous Na_2SO_4. The solvent was evaporated to dryness to get yellow liquid (331 mg, 79 % of yield). ^1H NMR (500 MHz, $CDCl_3$) δ = 3.74 – 3.60 (m, 4H), 3.31 (dd, J = 7.6, 3.8 Hz, 2H), 2.59 – 2.52 (m, 2H), 2.46 (s, 4H), IR (cm^{-1}): 2100 cm^{-1}; ESI-MS: $[M+H]^+$ cal. for $C_6H_{13}N_4O$ 157.11, found 157.05.



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MASS REPORT

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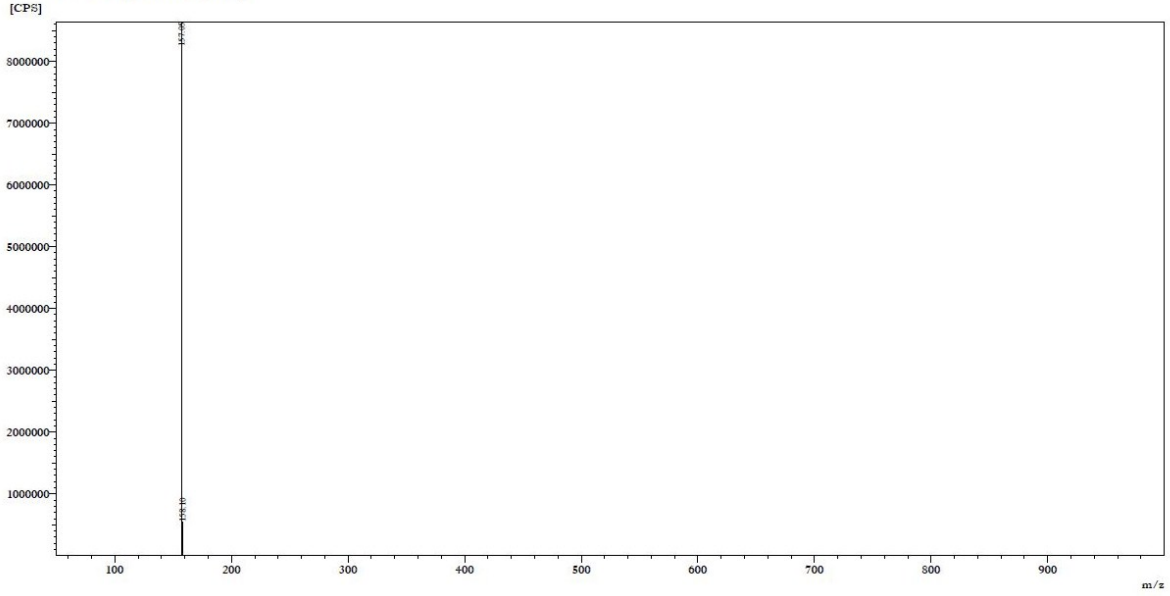
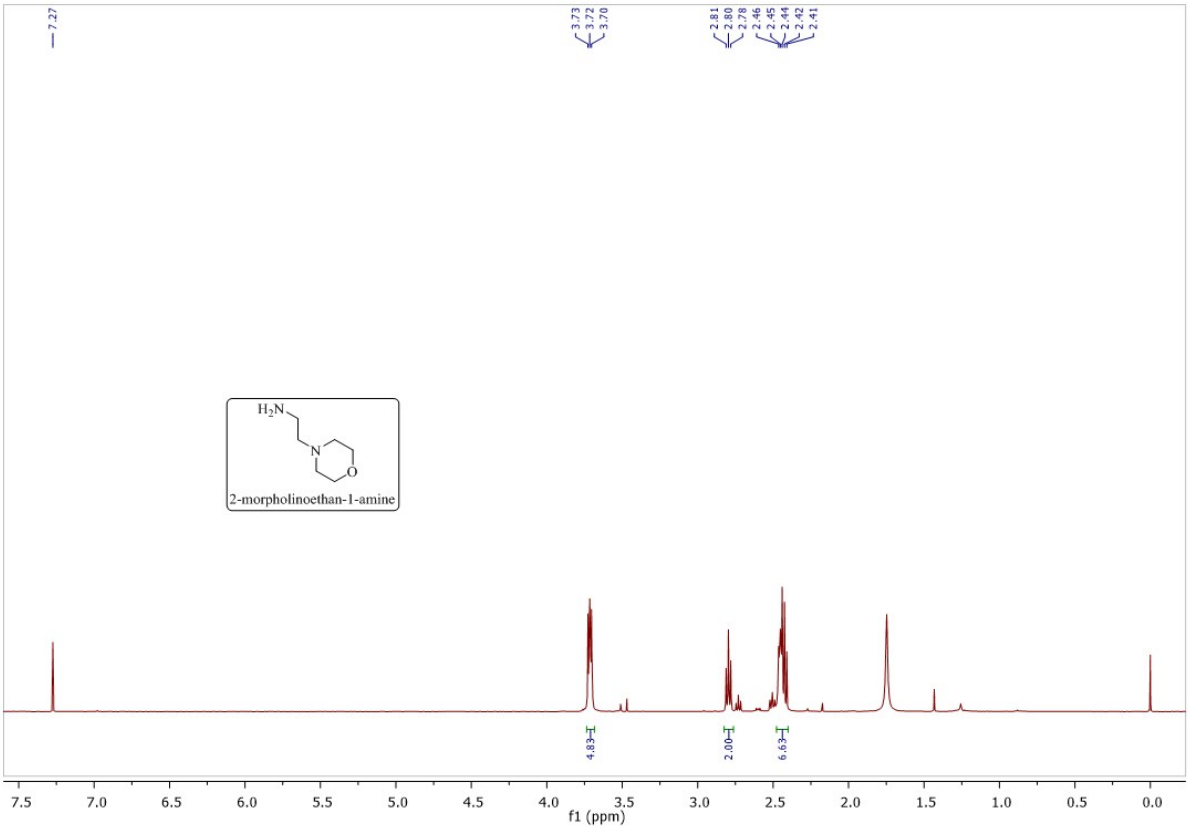
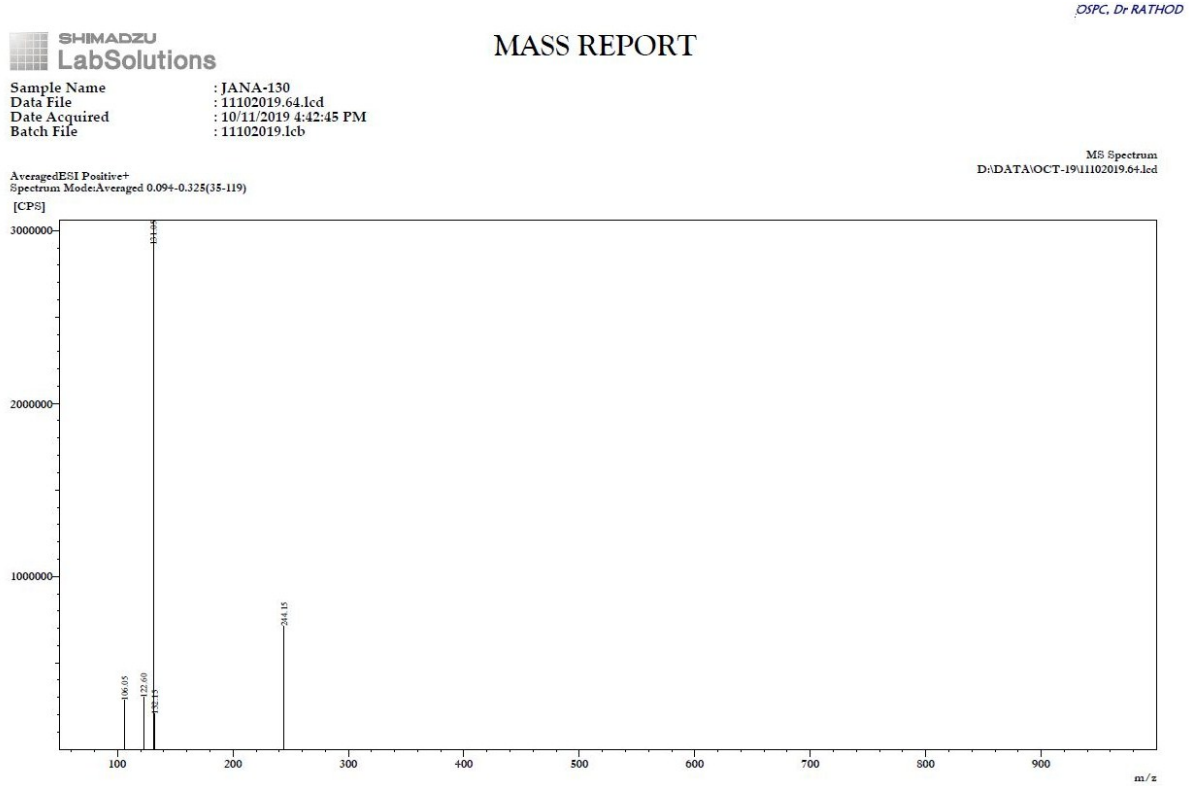


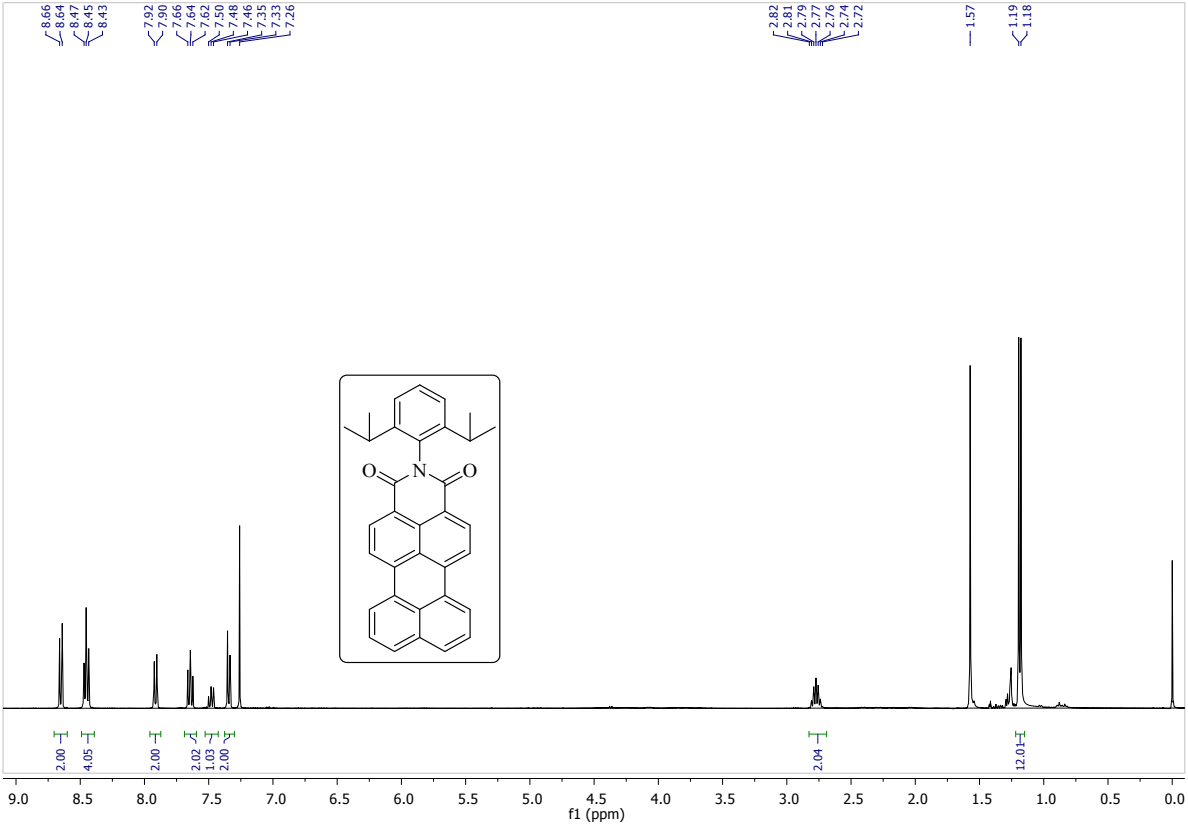
2.2. **Synthesis of *2-morpholinoethan-1-amine:*** In a dry two neck round bottom flask 4-(2-azidoethyl)morpholine (200 mg, 1.3 mmol) was taken under N_2 atmosphere in 10 mL of dry methanol. Then 10 mol% of Pd/C (13 mg, 0.13 mmol) was added to the reaction mixture under N_2 atmosphere followed by insertion of a needle connected to hydrogen (H_2) balloon. The reaction mixture was left for stirring at 30 °C for 18 h. After the completion of the reaction H_2 balloon was removed and reaction mixture was filtered through celite with 20 mL of methanol. Filtrate was concentrated under rotary evaporator and the mass obtained was again dissolved in ethyl acetate followed by filtration through celite to get the charcoal free yellow oil (128 mg, 77% yield) which was used for the next reaction without purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.75 – 3.68 (m, 4H), 2.80 (t, *J* = 6.1 Hz, 2H), 2.48 – 2.39 (m, 6H); IR (cm^{-1}): 3420, 1666, 1299, and 1115; ESI-MS: $[M+H]^+$ cal. for $C_6H_{15}N_2O$ 131.12, found 131.05.





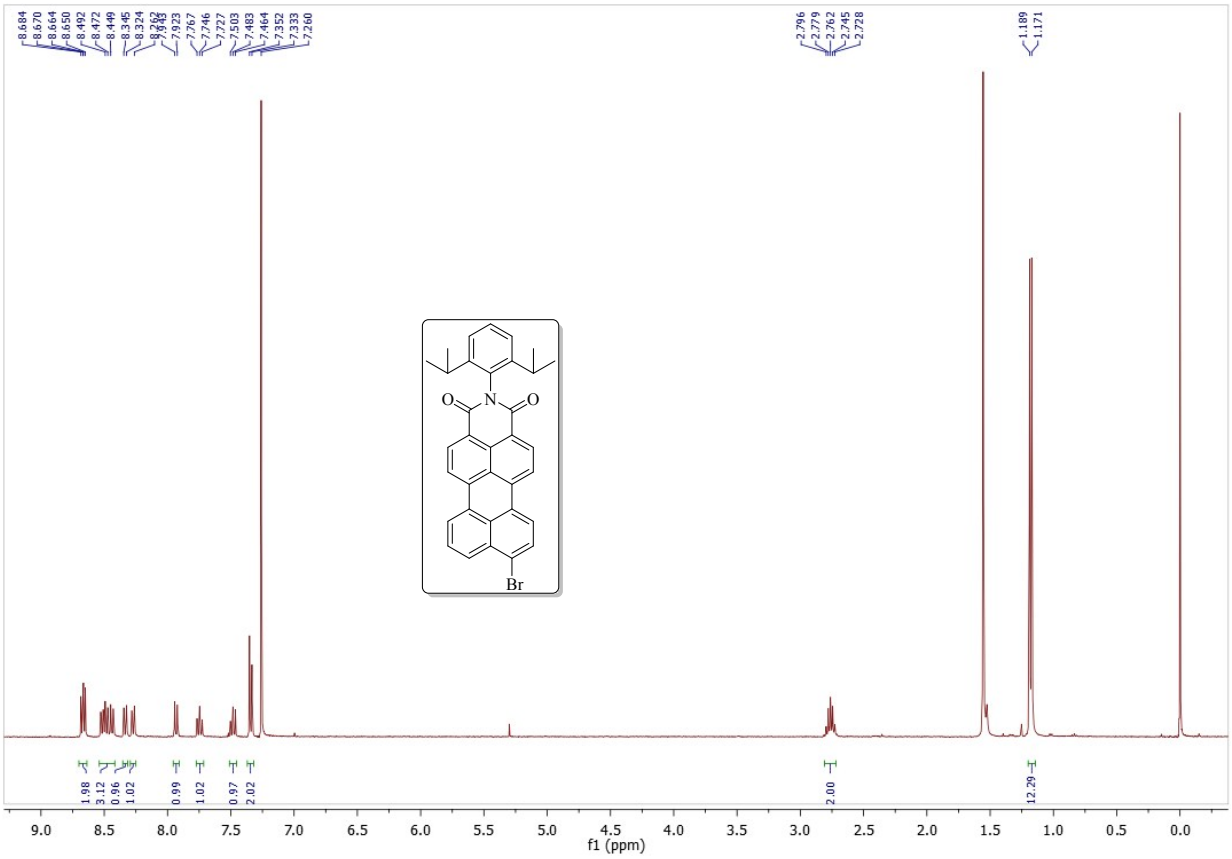
2.3. Synthesis of *N-(2,6-di-isopropylphenyl)perylene-3,4-dicarboximide:* A mixture of perylene-3,4,9,10-tetracarboxilic dianhydride (1.8 g, 4.65 mmol), imidazole (9.3 g, 137.3 mmol), zinc acetate dihydrate (0.660 g, 3.6 mmol), water (4 mL, 225 mmol) and 2,6-

diisopropylaniline (0.451 g, 2.55 mmol) was heated in a 100 mL autoclave at 190 °C for 24 h. After completion of the reaction, the reaction mixture was washed out from the reaction vessel with ethanol followed by concentrated HCl was added and ethanol was evaporated. The brown red solid was collected by vacuum filtration, which was dried and purified by column chromatography using chloroform over silica gel yielding 1.425 g (65%) as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ= 8.65 (d, *J* = 8.1 Hz, 2H), 8.45 (t, *J* = 7.3 Hz, 4H), 7.91 (d, *J* = 8.0 Hz, 2H), 7.64 (t, *J* = 7.8 Hz, 2H), 7.53 – 7.43 (m, 1H), 7.34 (d, *J* = 7.8 Hz, 2H), 2.77 (m, 2H), 1.19 (d, *J* = 6.9 Hz, 12H); IR (cm^{-1}): 1654.



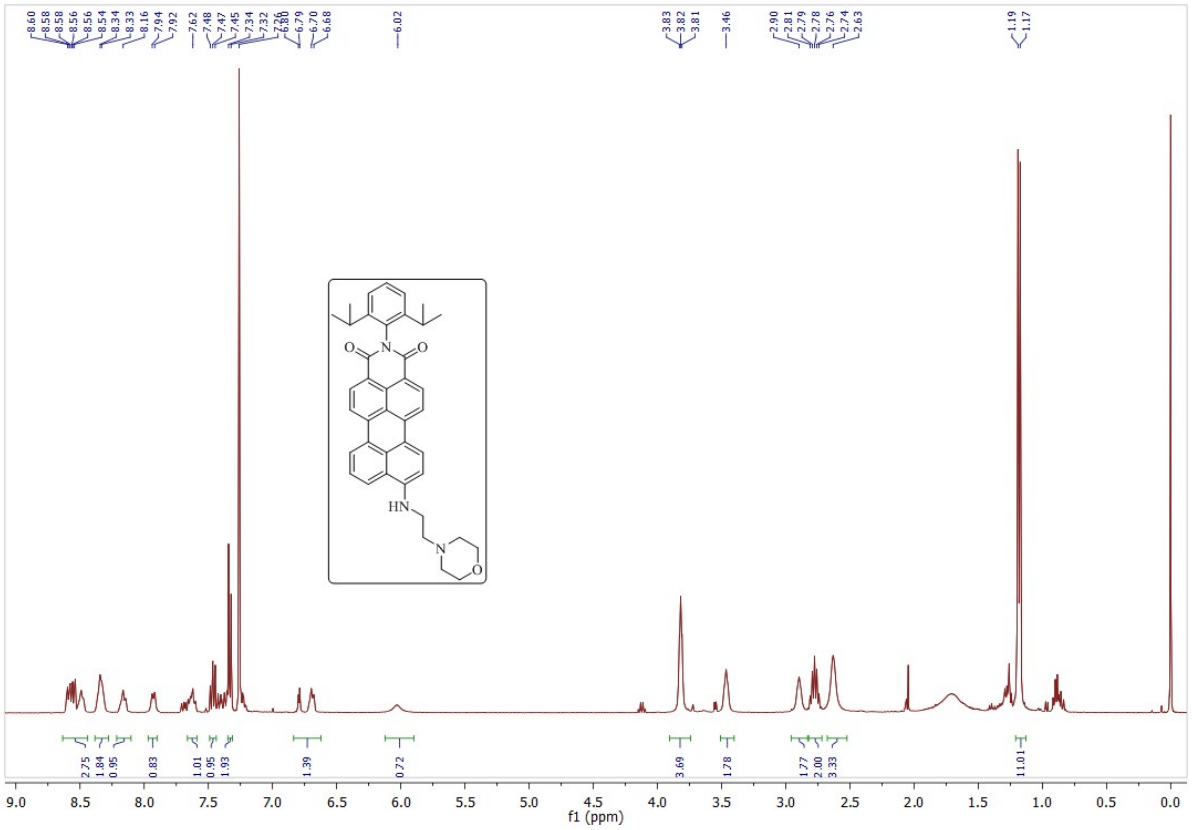
2.4. Synthesis of *N-(2,6diisopropylphenyl)9bromoperylene3,4dicarboximide (1):*

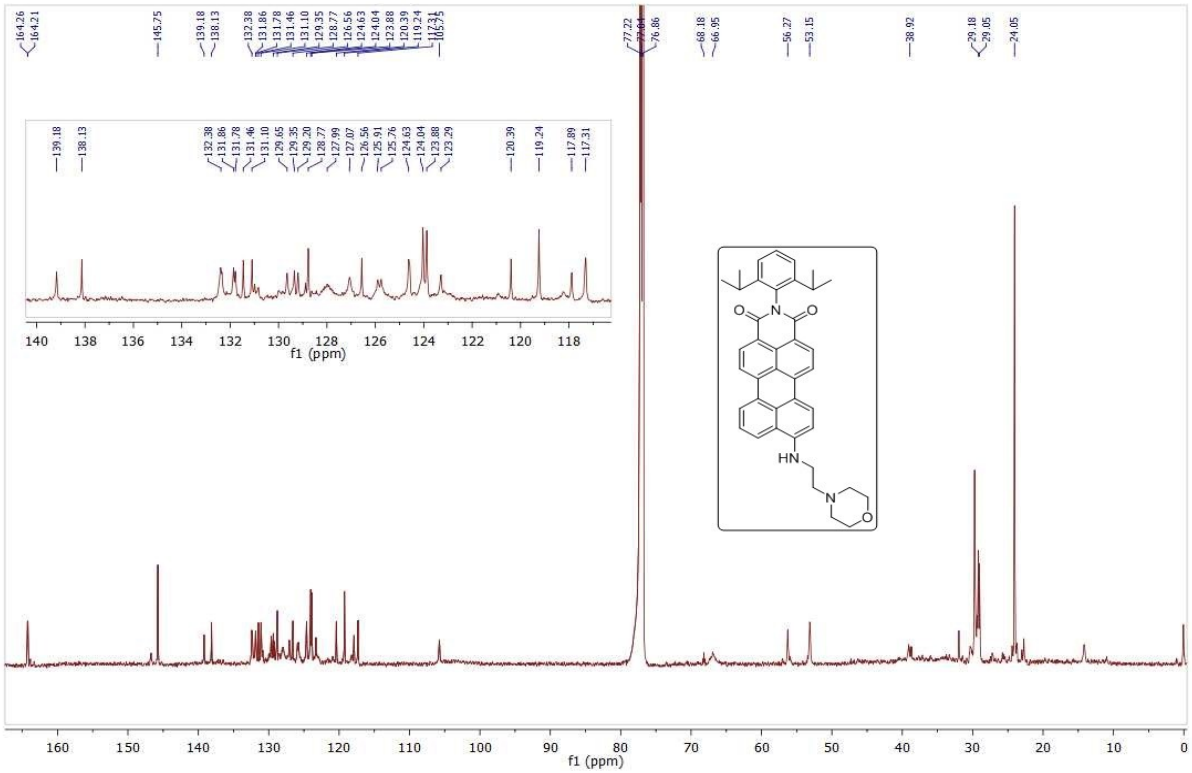
N-(2,6-di-isopropylphenyl)perylene-3,4-dicarboximide (1.0 g, 2.07 mmol) was dissolved in 100 mL chlorobenzene followed by Br_2 (0.24 mL, 22.4 mmol) was added via syringe, and the reaction mixture was stirred for 10 min at rt. The reaction mixture was washed with 100 mL sodium thiosulfate solution twice and with 100 mL water once. The organic layer was concentrated on a rotary evaporator. The crude reaction mixture was purified via column chromatography using toluene as the eluent, yielding 1.033 g (89%) as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.68 (d, *J* = 5.9 Hz, 1H), 8.66 (d, *J* = 5.9 Hz, 1H), 8.52 (d, *J* = 7.7 Hz, 1H), 8.48 (d, *J* = 8.1 Hz, 1H), 8.44 (d, *J* = 8.1 Hz, 1H), 8.33 (d, *J* = 8.5 Hz, 1H), 8.27 (d, *J* = 8.2 Hz, 1H), 7.93 (d, *J* = 8.1 Hz, 1H), 7.75 (t, *J* = 8.0 Hz, 1H), 7.50 – 7.45 (m, 1H), 7.34 (d, *J* = 7.8 Hz, 2H), 2.76 (m, 6.8 Hz, 2H), 1.18 (d, *J* = 6.9 Hz, 12H); IR (cm^{-1}): 1658.



2.5. Synthesis of *2-(2,6-diisopropylphenyl)-8-((2-morpholinoethyl)amino)-1H-benzo[10,5]anthra[2,1,9-def]isoquinoline-1,3(2H)-dione:* In a flame dry round bottom flask $Pd_2(dba)_3$ (8 mg) and 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (BINAP) (26 mg) was taken in 2 mL of dry toluene under N_2 atmosphere and the mixture was stirred for 30 min under heating condition. Then the bromide **1** (50 mg) was added to the aliquot followed by addition of 2-morpholinoethan-1-amine (13 mg) and 1.5 equiv potassium tert-butoxide (26 mg). The reaction mixture was refluxed at 110 ˚C for 4 hour. A deep blue color appeared with the indication of the formation of desired amine compound. The progress of the reaction was monitored by TLC using the mobile phase of 5 % of MeOH in DCM. After the completion of the reaction, it was filtered through celite. To the filtrate ethyl acetate was added and washed with water (25 mL X 2) and brine (25 mL). Organic layer was collected and dried over anhydrous sodium sulfate. Solvent was evaporated to dryness under rotary evaporator to get the crude mass. The crude was column chromatographed on silica gel of M 230-400 to get the compound **2** (35 mg, 70% of isolated yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.60 – 8.53 (m, 2H), 8.47 (d, 1H), 8.34 (d, *J* = 4.5 Hz, 2H), 8.15 (d, *J* = 7.9 Hz, 1H), 7.93 (d, *J* = 7.8 Hz, 1H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.47 (t, *J* = 7.8 Hz, 1H), 7.33 (d, *J* = 7.8 Hz, 2H), 6.69 (d, *J* = 7.9 Hz, 1H), 6.03 (s, 1H), 3.89 – 3.76 (m, 4H), 3.46 (s, 2H), 2.90 (s, 2H), 2.78 (m, 2H), 2.63 (s, 4H), 1.18 (d, *J* = 6.8 Hz, 12H). ^{13}C NMR (175 MHz, $CDCl_3$) δ 164.26, 164.21, 145.75, 139.18, 138.13, 132.38, 131.86, 131.78, 131.46, 131.10, 129.65, 129.35, 129.20, 128.77, 127.99, 127.07, 126.56, 125.91, 125.76, 124.63, 124.04, 123.88, 123.29, 120.39, 119.24, 117.89, 117.31, 105.75, 68.18, 66.95, 56.27, 53.15, 38.92, 29.18, 29.05,

24.05. IR (cm^{-1}): 3422, 1654, ESI-HRMS: cal. for $C_{40}H_{40}N_3O_3$ $[M+H]^+$ = 610.3070, found 610.3067.





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MASS REPORT

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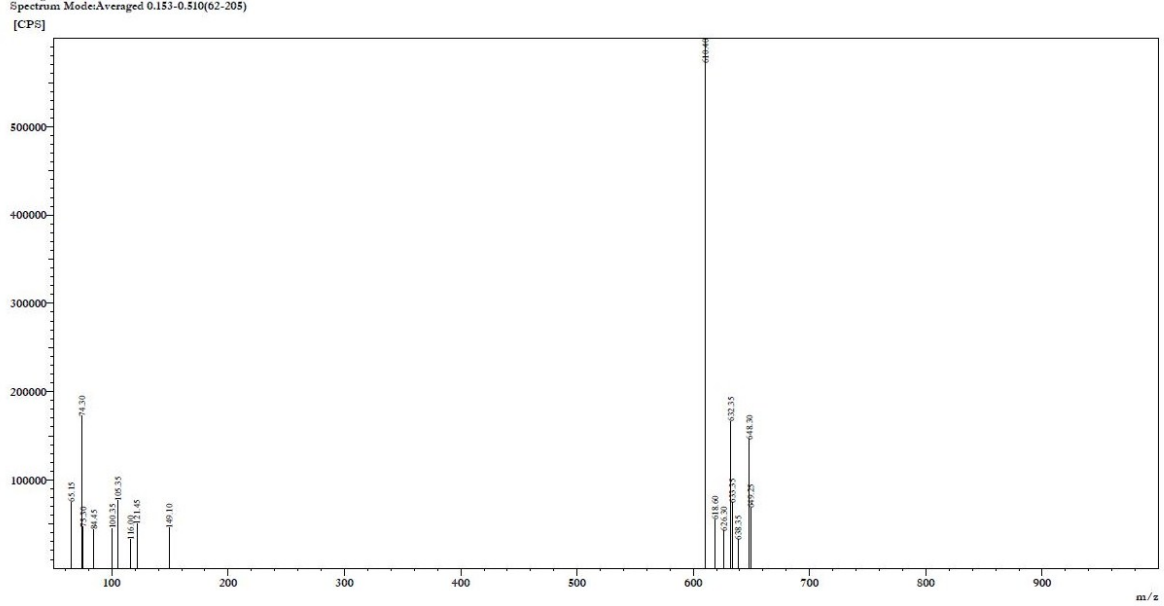
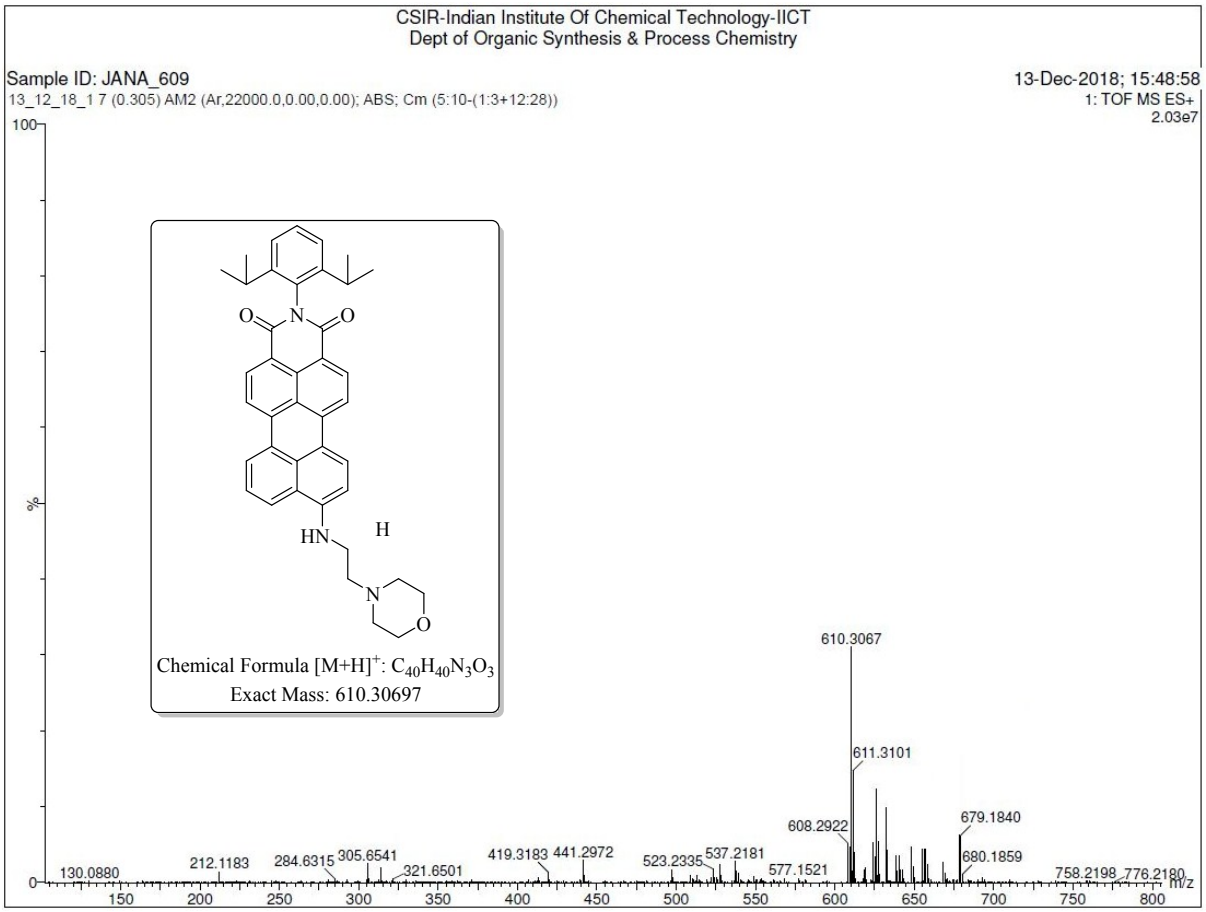


HRMS



Single Mass Analysis
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Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
13 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-40 H: 0-40 N: 0-3 O: 0-3 Na: 0-1

Minimum:				-1.5				
Maximum:		5.0	5.0	50.0				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
610.3067	610.3070	-0.3	-0.5	22.5	131.8	n/a	n/a	C40 H40 N3 O3

HPLC analysis reveals the purity of the probe **2** is more than 96%.

SHIMADZU LabSolutions **Analysis Report**

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Batch Filename	: Rakesh batch .lcb		
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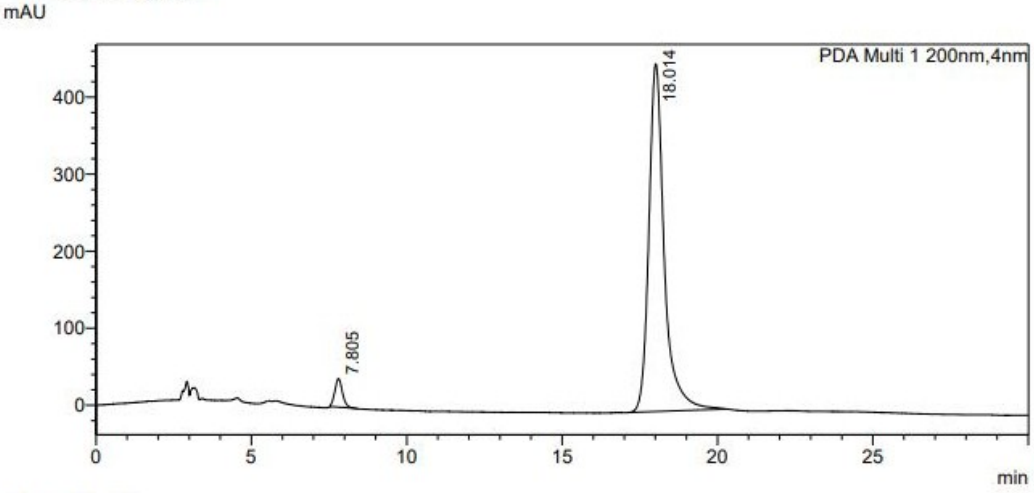

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PDA Ch1 200nm

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2	18.014	15548701	451248	92.415	96.093
Total		16180967	488284	100.000	100.000

3. Photophysical properties of Probe 2

The absorption and emission spectra of the probe **2** (1×10^{-5} M) was recorded in different solvent systems (both organic solvents and in binary mixture of acetonitrile in different percentage of water). The pH-dependent emission spectra of probe **2** were measured in various pH (5% acetonitrile (v/v) in phosphate buffer in MES) starting from pH 3 to 8. Emission spectra were recorded on exciting the molecule at 610 nm in every case.

4. Cell Culture

Cancerous cell lines MCF-7 (Michigan Cancer Foundation-7), Gl-261(Glioma-261), RKO (rectal carcinoma), U87 (Uppsala 87 Malignant Glioma), CT26 (mus musculus colon carcinoma) and the non cancerous cell line CHO (Chinese Hamster Ovary) and HEK-293 (Human embryonic kidney 293) cells were cultured in DMEM (Dulbecco Modified Eagle Medium) media (Gibco) supplemented with 10% (v/v) fetal bovine serum, 1% L-glutamine, 1% non-essential amino acid, 1% penicillin, and 1% streptomycin. All cells were maintained in a humidified 5% CO_2 incubator at 37 °C.

5. Cytotoxic studies using MTT assay

The *in vitro* cytotoxic studies of probe **2** have been carried out using the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5–diphenyltetrazolium bromide) assay. Briefly, cancerous cell line MCF7 and GL261 as well as normal cell line CHO were grown separately in their log phase. Cells were seeded in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) for 8 h. Different concentrations (1.25-40µm) of probe **2** in HEPES buffer containing 5% DMSO were added and incubated at 37 °C in 5% CO_2 for different time intervals (24h, 48h and 72h). Then cytotoxicity was measured using the MTT assay. It was evident from the MTT assay data that the probe **2** showed no toxicity to the corresponding cell lines (**Figure: S1a-c**).

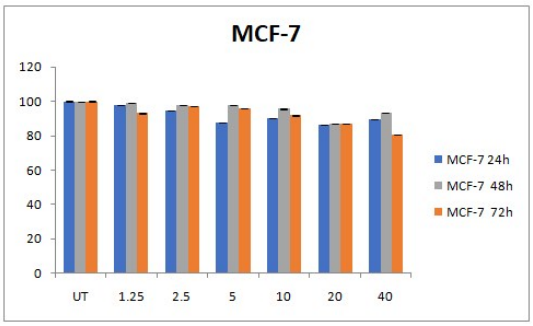


Figure S1a. Comparative cell viability study of probe **2** on MCF7 cell line in different time intervals 24h, (blue bar) 48h, (grey bar) and 72h. (orange bar) at different concentrations.

Figure S1b Comparative cell viability study of probe **2** on GL261 cell line in different time intervals 24h, (blue bar) 48h, (grey bar) and 72h. (orange bar) at different concentrations.

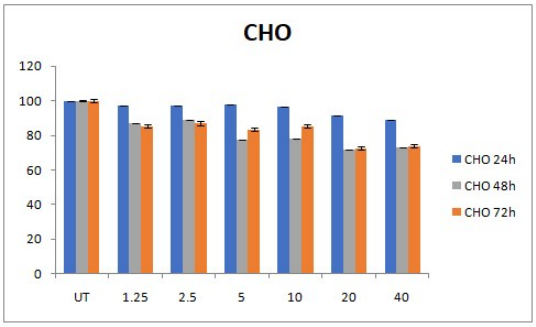


Figure S1c. Comparative cell viability study of Probe **2** on CHO cell line in different time intervals 24h, (blue bar) 48h, (grey bar) and 72h. (orange bar) at different concentrations.

6. Cellular uptake study using confocal microscopy:

The degrees of cellular uptake for the Probe **2** in cancer cells and non cancerous cells were studied qualitatively by confocal microscopy. Briefly, MCF-7, U87, CT26, RKO, GL261, CHO, and HEK-293 cells were seeded (5 x $10^{4)}$ in 35 mm cover glass with 1 mL of growth medium for 12 h than cell were washed with 1x PBS (3×1 mL) and treated with probe **2** (2 μM) for 4 h. cells were washed with 1x PBS (3×1 mL) then fixed with 4% paraformaldehyde and cell nuclei were stained with DAPI. Mount on cover slip onto glass slide and images were recorded by confocal microscopy (Nikon Ti Eclipse). Representative images are shown in the **Figure S2**.

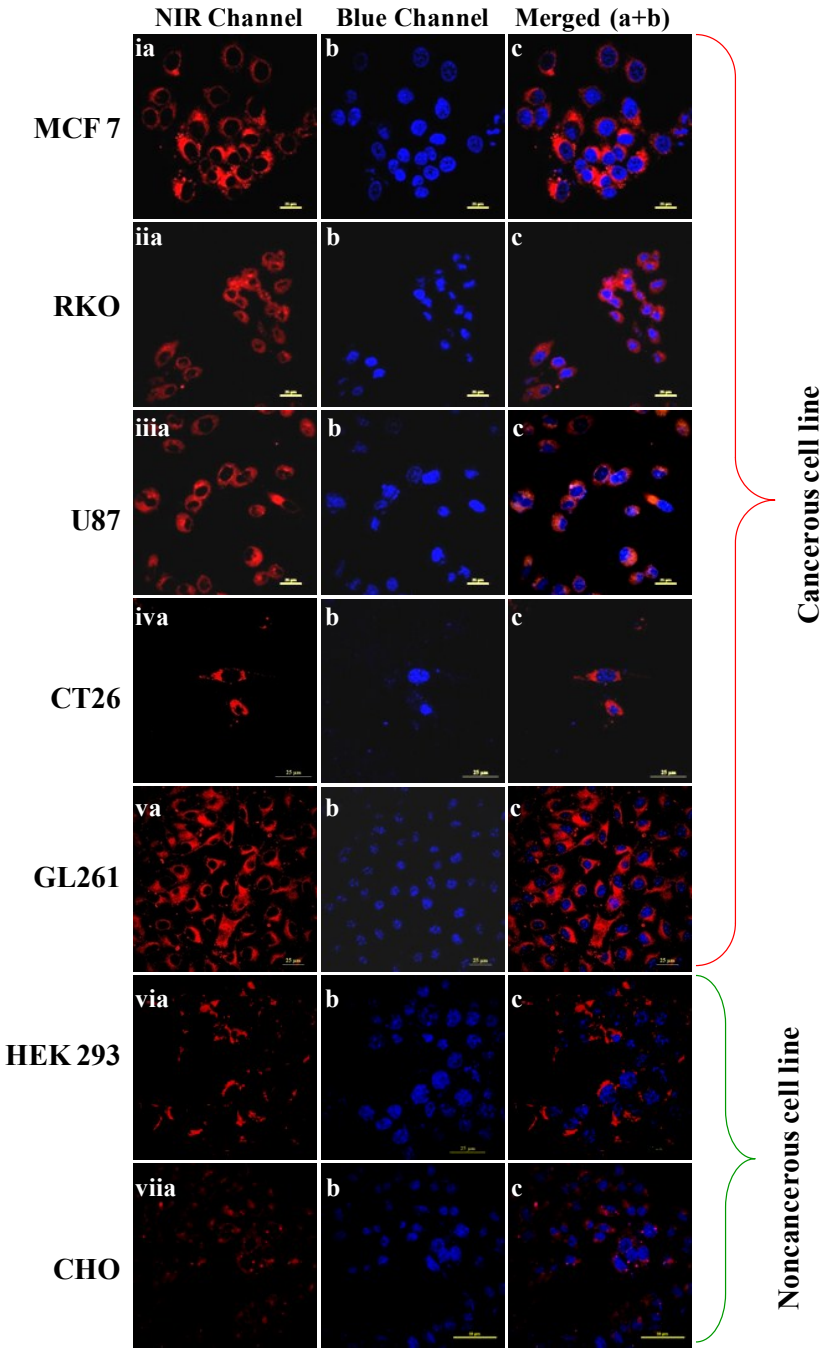


Figure S2. CLSM images of (i) MCF-7, (ii) RKO, (iii) U87, (iv) CT26, (v)GL261, (vi) HEK 293 and (vii) CHO cell lines incubated with probe **2** and DAPI: (a) NIR channel, (b) blue channel and (d) merged images. Scale bar 25µm. All images were taken keeping the microscopic parameter the same.

7. Intra cellular distribution of probe 2 in MCF 7 cell line:

Intra cellular localization of the probe **2** by cancerous MCF-7 cell line was monitored by confocal laser scanning microscopy (CLSM). The cells were cultured following the standard protocols and was incubated with the probe **2** (2 µM) under 5% CO_2 at humified conditions at 37 °C for 4 h. Thereafter, cells were fixed in paraformaldehyde for 15 min and after washing three times with PBS, cell nucleus were stained with 4,6-diamidino-2-phenylindole (DAPI) and the lysosome and mitochondria were stained separately with lysotracker green and mytotracker green respectively. Imaging was done by Nikon confocal microscope (Eclipse Ti-E) using the respective filter.

Table S1. Pearson's correlation coefficient (Rr), overlap coefficient (R), overlap coefficients k1 and k2, and colocalization coefficients M1 and M2 were calculated on the set of computer-simulated images shown in the manuscript.

Fig No.	Pearson's correlation coefficient (Rr)	Overlap coefficient (R)	Overlap coefficients k1	Overlap coefficients k2	Colocalization coefficients M1	Colocalization coefficients M2
Fig 2.ie	0.888	0.887	0.713	1.105	1.00	0.916
Fig 2.iie	0.649	0.467	1.013	0.744	0.991	0.416
Fig 2.iiie	0.940	1.026	0.872	0.964	0.994	0.568
Fig 2.ive	0.547	0.654	0.900	0.475	1.000	0.999
Fig 5.if	0.764	0.808	0.550	1.186	0.978	0.999
Fig 5.iif	0.814	0.832	0.586	1.182	0.922	0.998
Fig 5.iiif	0.781	0.786	0.446	1.383	0.924	0.990
Fig 5.ivf	0.803	0.834	0.541	1.30	0.888	0.996
Fig 5.vf	0.844	0.856	0.851	0.861	0.995	0.994
Fig 6.if	0.965	0.966	1.017	0.917	0.944	0.999
Fig 6.iif	0.968	0.971	0.956	0.987	0.958	0.995
Fig 6.iiif	0.956	0.958	0.869	1.056	0.920	0.996

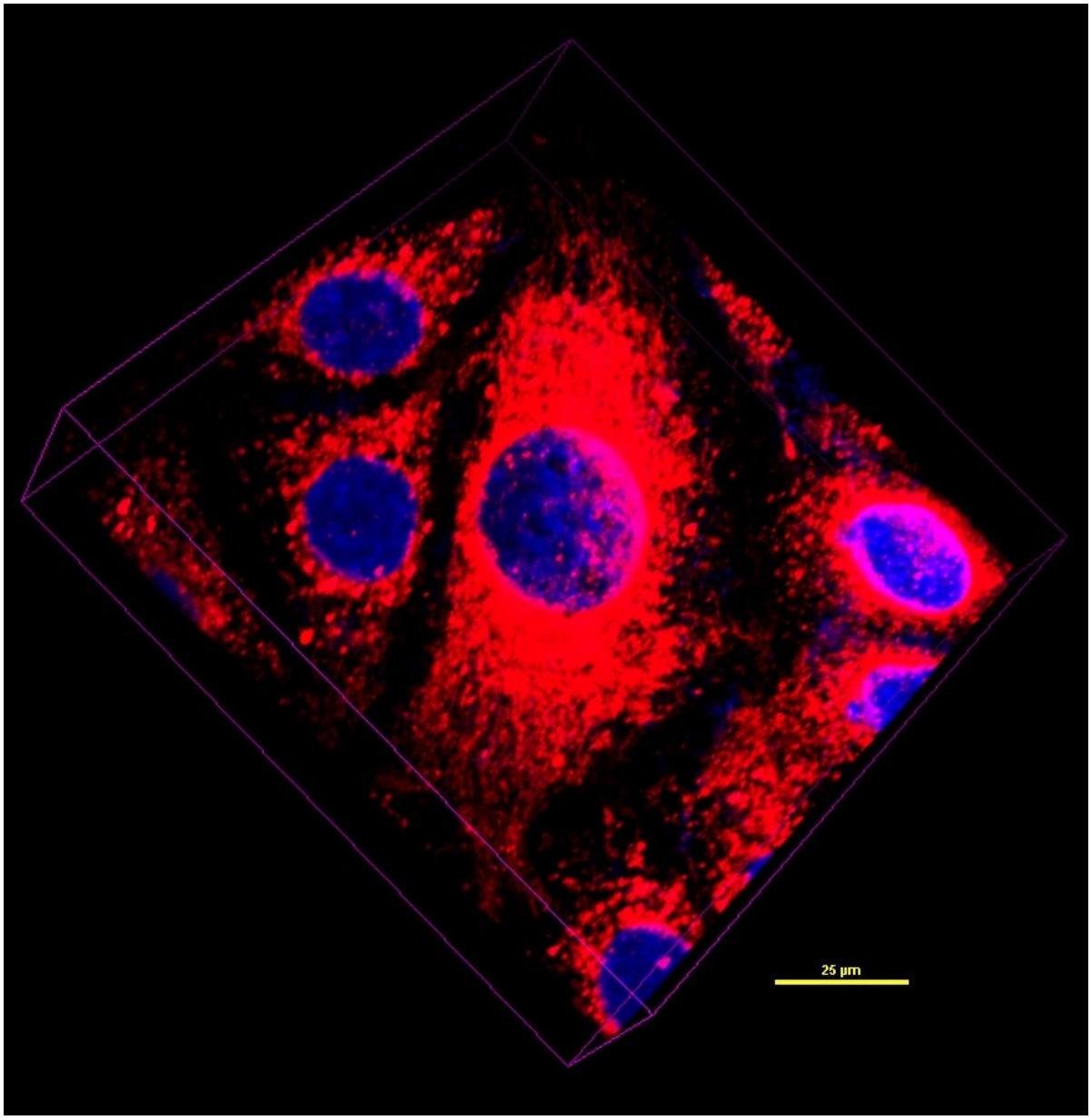


Figure S3. Confocal Z-stack image of fixed MCF-7 cell (cells incubated with the probe **2** and DAPI): Image was rotated and optically sectioned along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2**. The image shows the probe **2** is distributed in the cytoplasm of the cell.

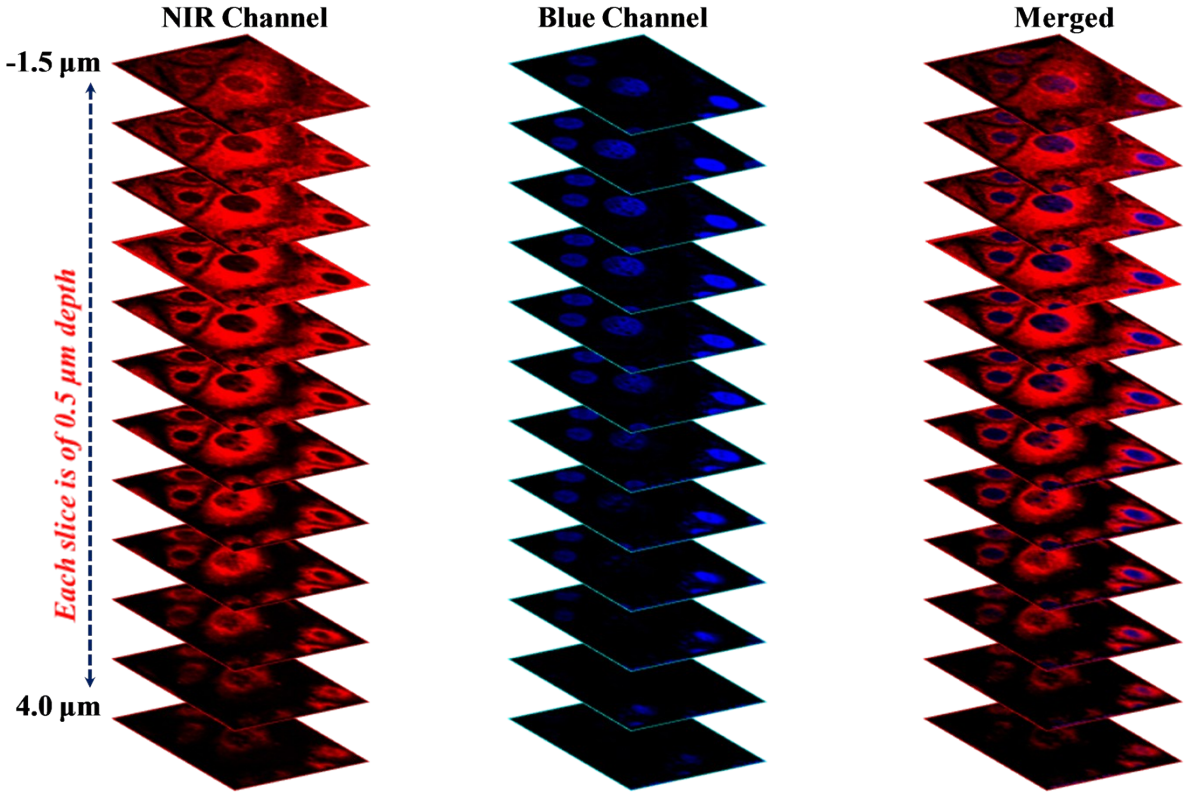


Figure S4. Confocal Z-stack images of MCF-7 cell (cell lines incubated with the probe **2** and DAPI): Image was rotated and optically sectioned (each section depth is 0.5µm) along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2.** (a) NIR, (b) green, (c) blue channel and (d) merged images. From the images it can be clearly seen that the probe **2** is mostly located in the cytoplasm of the cell.

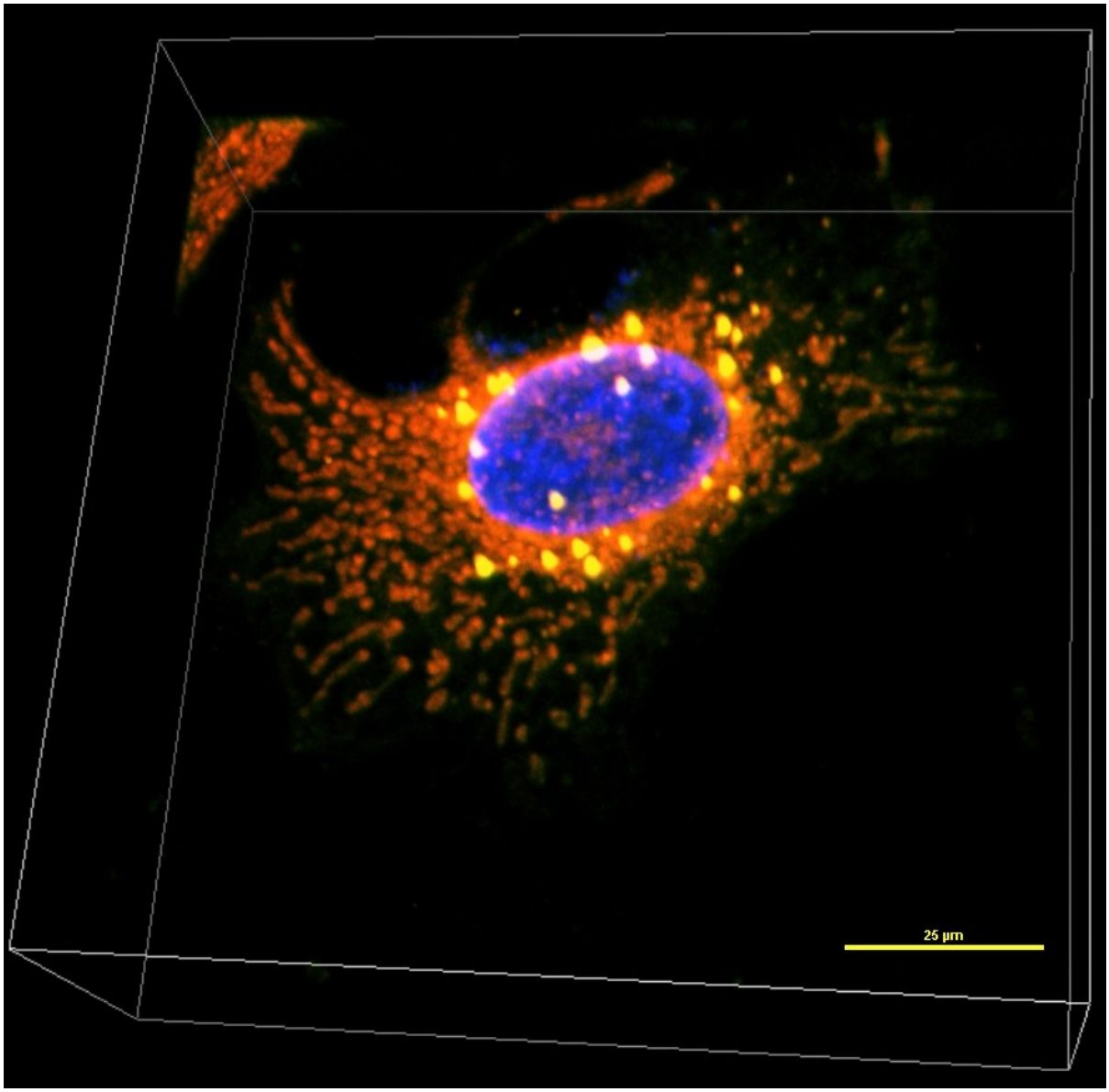


Figure S5. Confocal Z-stack images of MCF-7 cell (cells were incubated with the probe **2**, DAPI, and lysotracker green): Image was rotated and optically sectioned along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2.** From the images it can be clearly seen that both the probe **2** and the lysotracker green are co-localized in the lysosome.

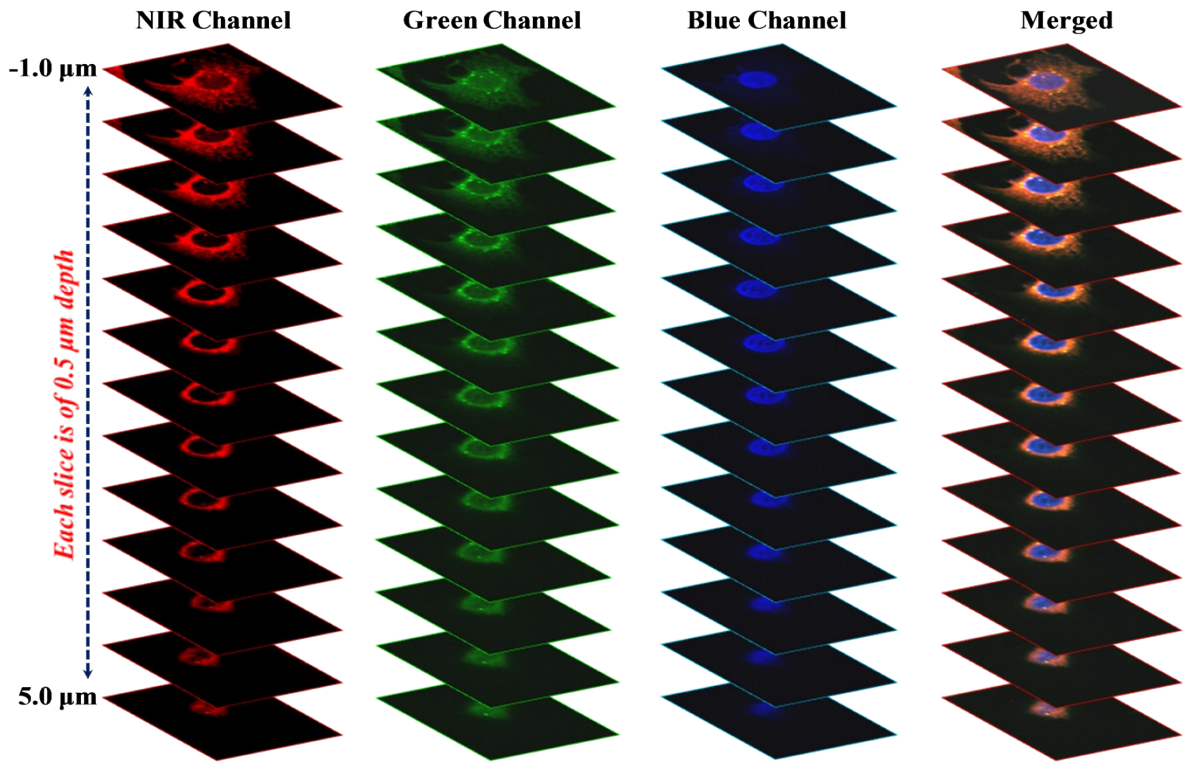


Figure S6. Confocal Z-stack images of MCF-7 cell (cell lines incubated with the probe **2**, DAPI, and lysotracker green): Image was rotated and optically sectioned (each section depth is 0.5µm) along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2.** (a) NIR, (b) green, (c) blue channel and (d) merged images. From the images it can be clearly seen that both the probe **2** and the lysotracker green are co-localized in the lysosome.

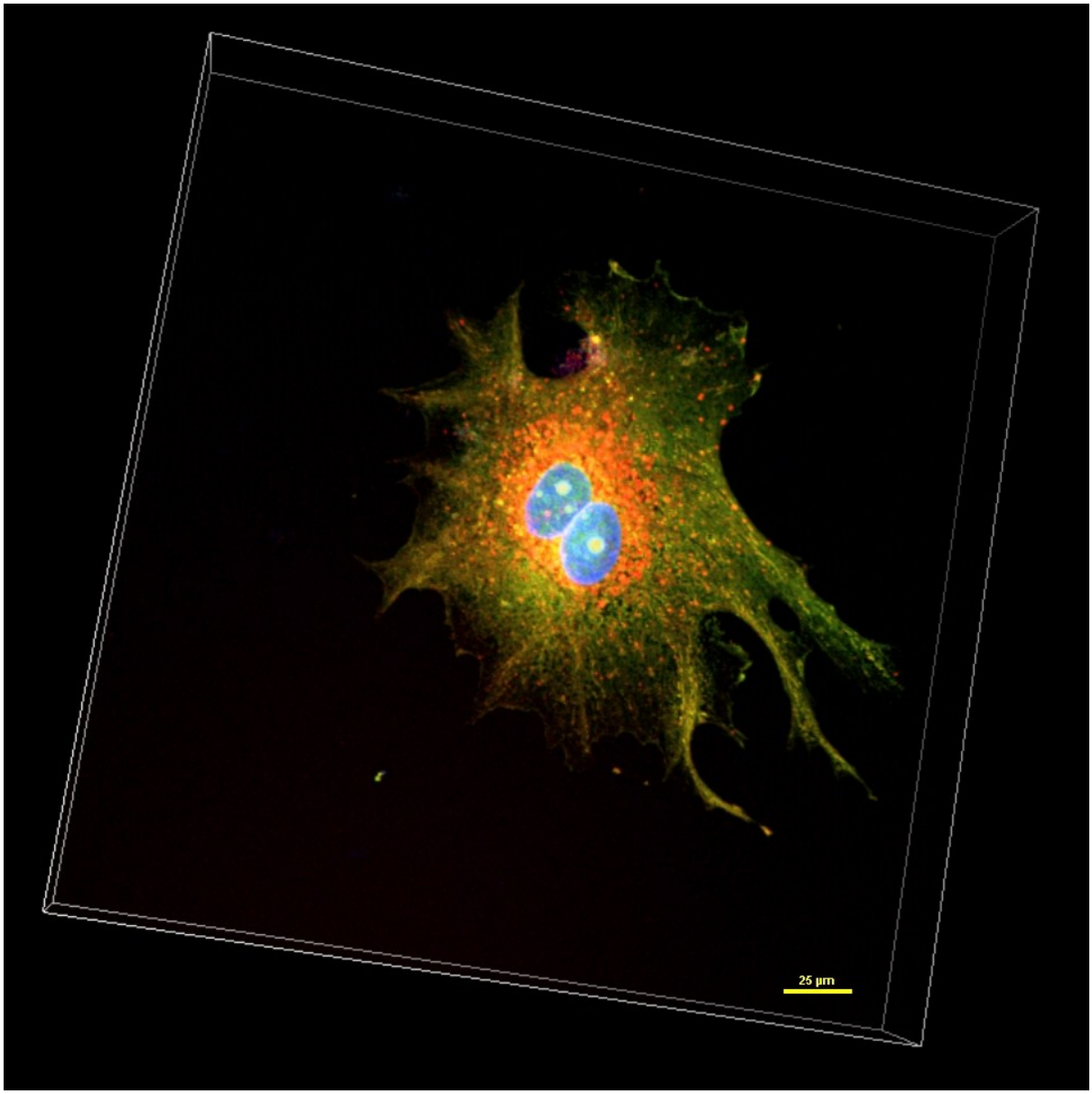


Figure S7. Confocal Z-stack images of MCF-7 cell (cell lines incubated with the probe **2**, DAPI, and mitotracker green): Image was rotated and optically sectioned along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2.** From the images it can be clearly seen that the probe **2** and the mytotracker green are located separately in the cell.

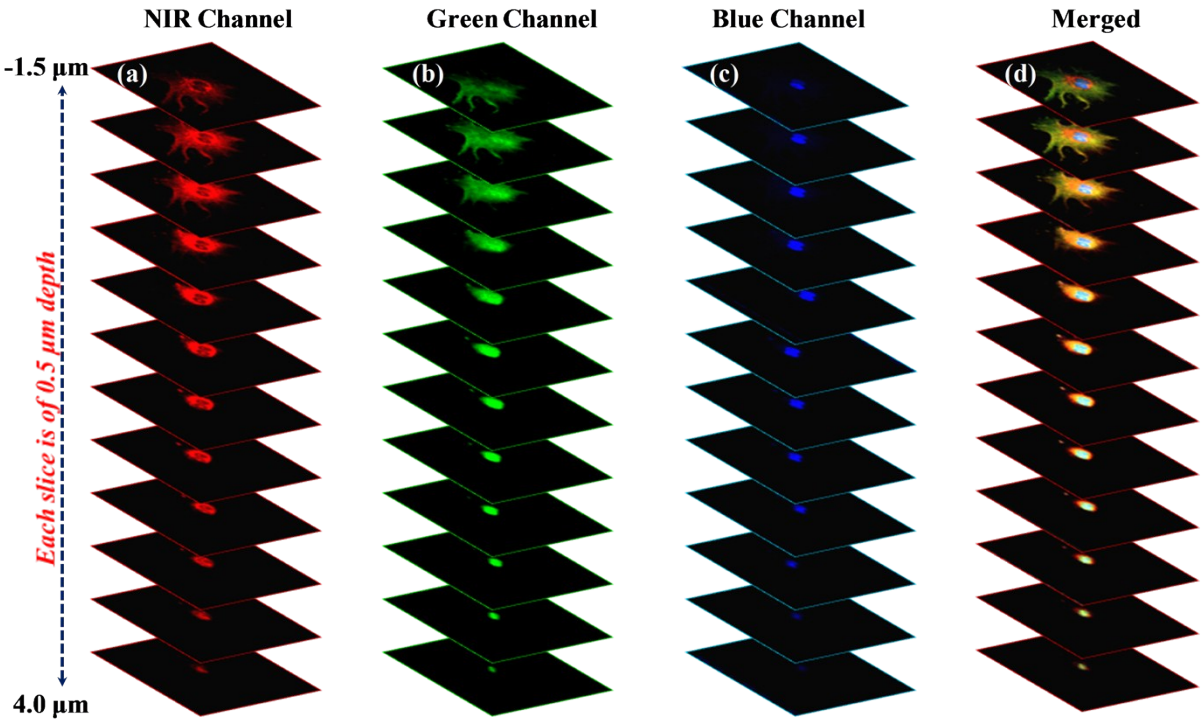


Figure S8. Confocal Z-stack images of MCF-7 cell (cell lines incubated with the probe **2**, DAPI, and mitotracker green): Image was rotated and optically sectioned (each section depth is 0.5µm) along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2.** (a) NIR, (b) green, (c) blue channel and (d) merged images. From the images it can be clearly seen that the probe **2** and the mytotracker green are located separately in the cell.

8. Intra cellular distribution of probe 2 in CHO cell line:

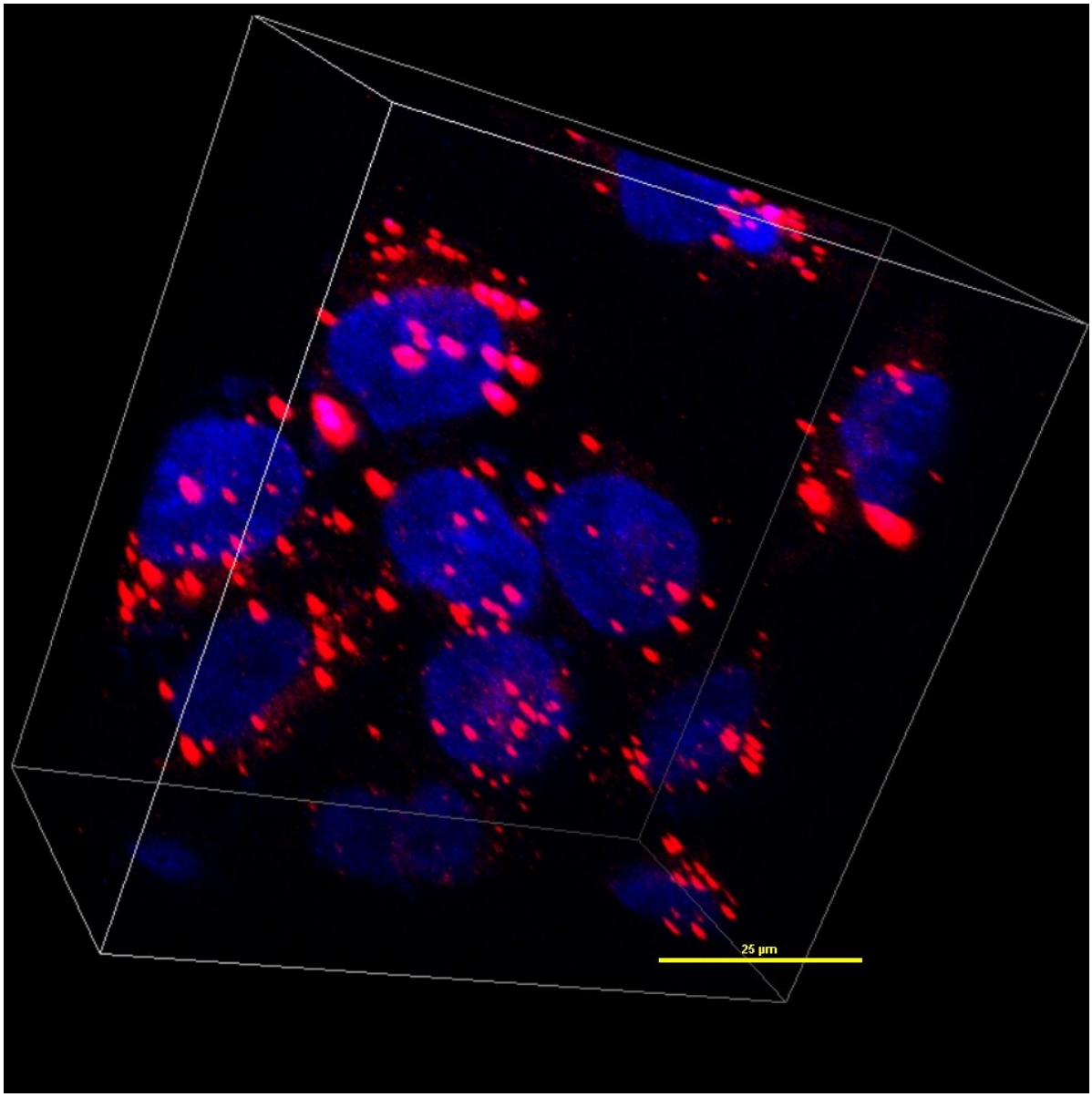


Figure S9. Confocal Z-stack image of fixed CHO cell (cells incubated with the probe **2** and DAPI): Image was rotated and optically sectioned along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2**. The image shows the probe **2** is distributed in the cytoplasm of the cell.

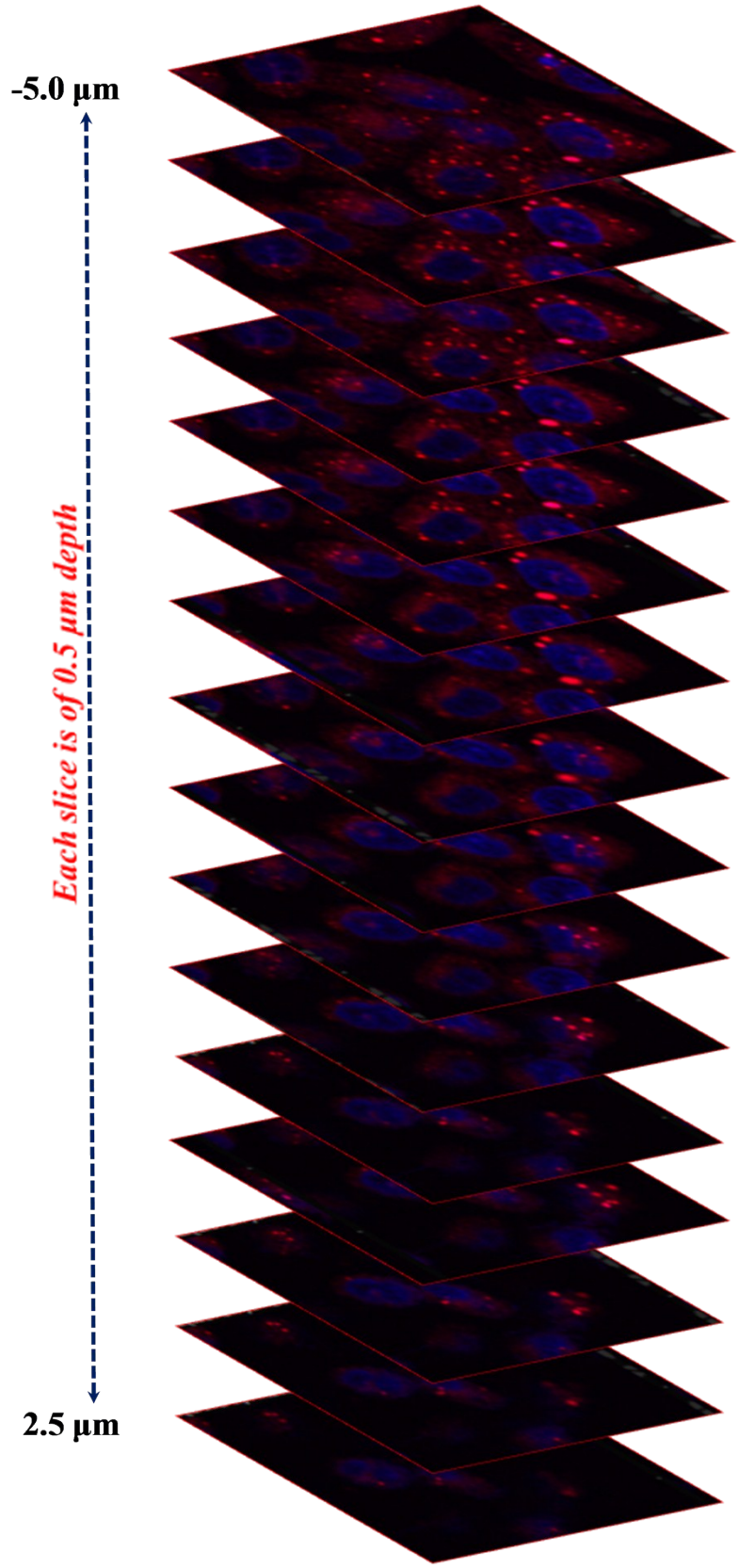


Figure S10. Confocal Z-stack images of CHO cell (cell lines incubated with the probe **2** and DAPI and: Image was rotated and optically sectioned (each section depth is 0.5µm) along the Z-axis to have a better visualization of the cellular distribution of the probe **2**.

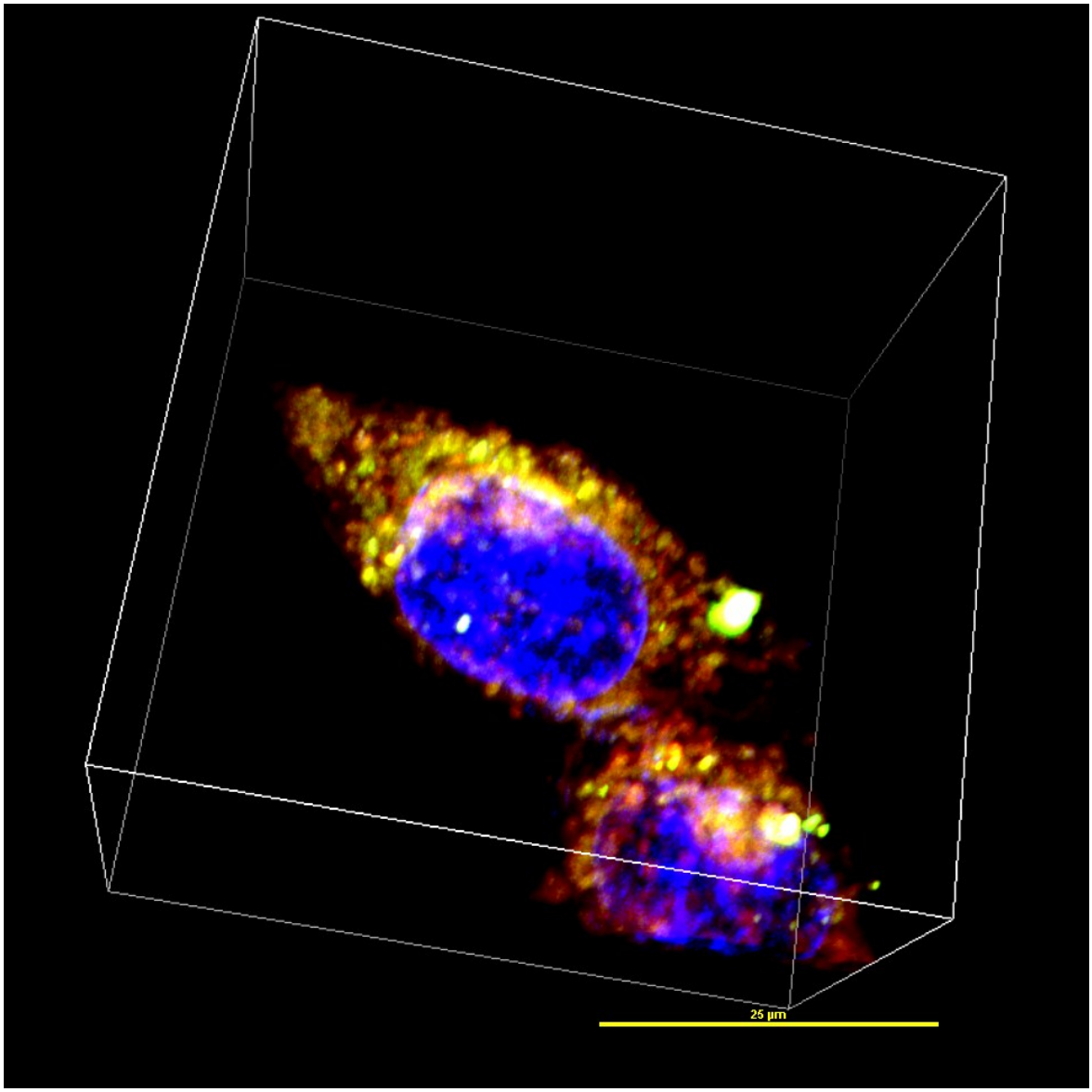


Figure S11. Confocal Z-stack images of CHO cell (cells were incubated with the probe **2**, DAPI, and lysotracker green): Image was rotated and optically sectioned along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2.** From the images it can be clearly seen that both the probe **2** and the lysotracker green are co-localized in the lysosome.

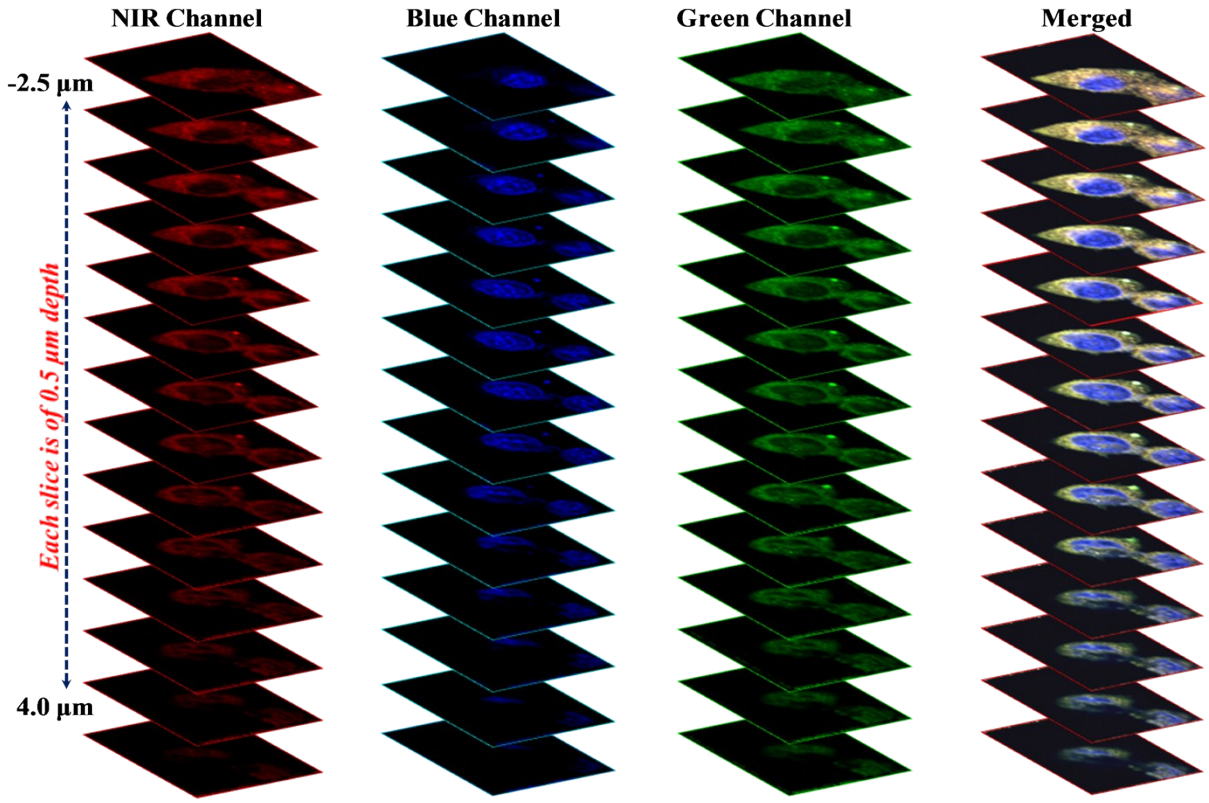


Figure S12. Confocal Z-stack images of CHO cell (cell lines incubated with the probe **2**, DAPI, and lysotracker green): Image was rotated and optically sectioned (each section depth is 0.5µm) along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2.** (a) NIR, (b) blue, (c) green channel and (d) merged images.

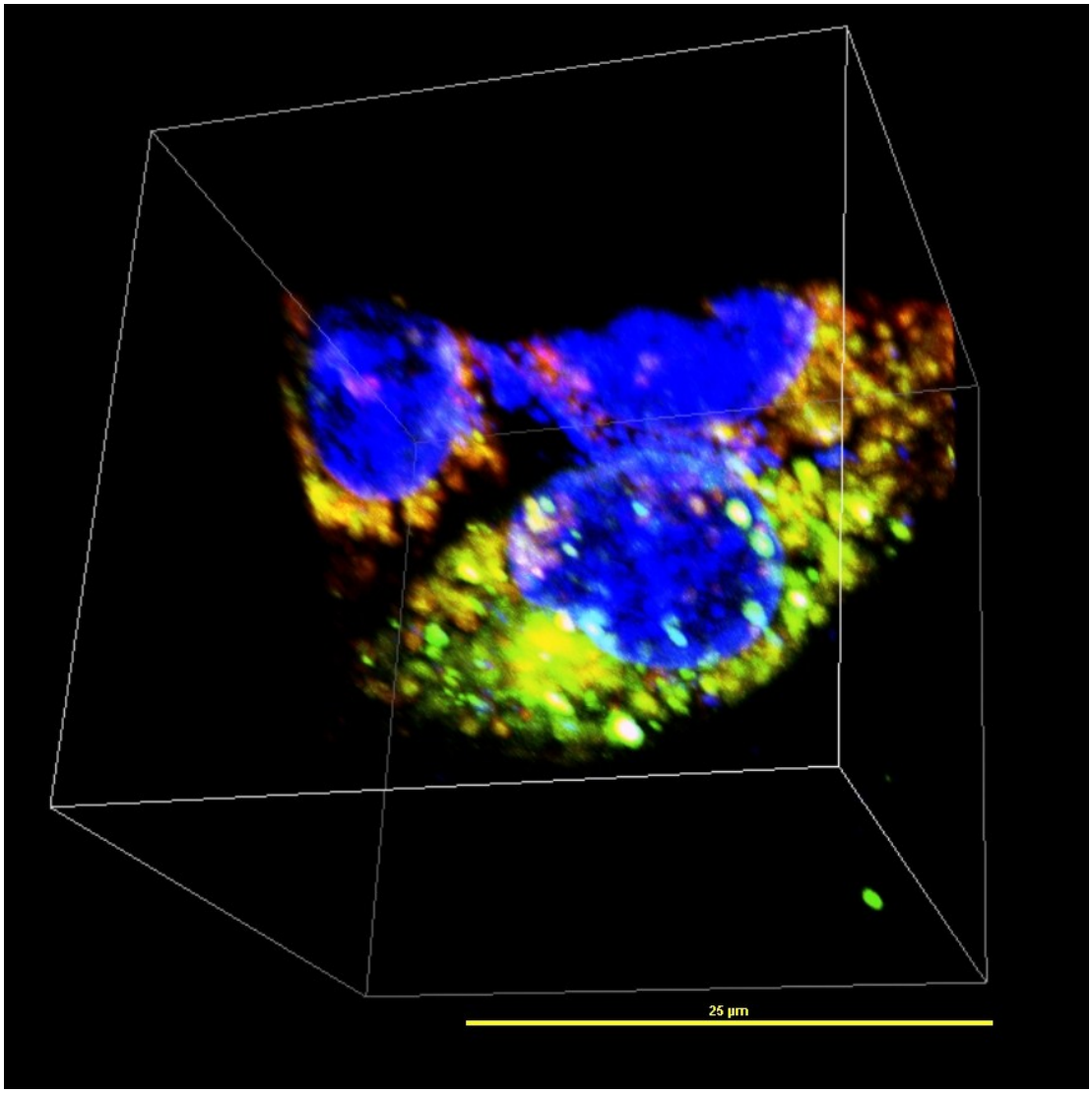


Figure S13. Confocal Z-stack images of CHO cell (cell lines incubated with the probe **2**, DAPI, and mitotracker green): Image was rotated and optically sectioned along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2.** From the images it can be clearly seen that the probe **2** and the mytotracker green are located separately in the cell.

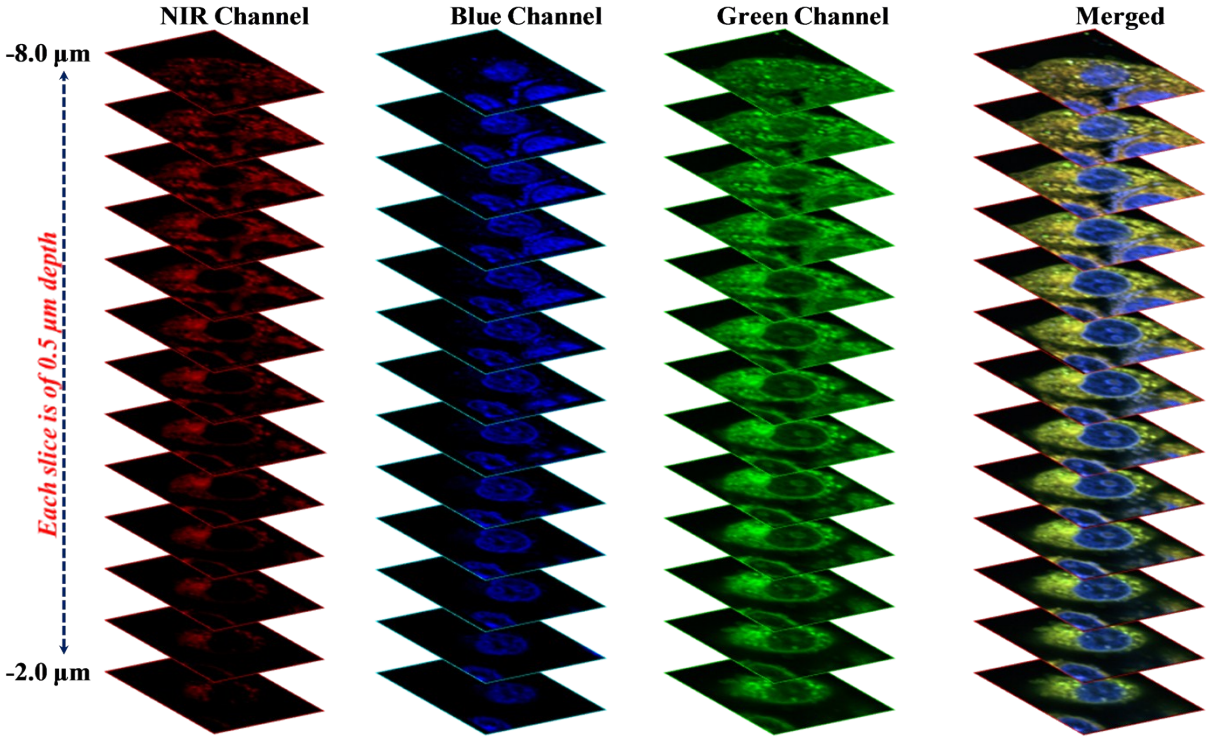


Figure S14. Confocal Z-stack images of CHO cell (cell lines incubated with the probe **2**, DAPI, and mitotracker green): Image was rotated and optically sectioned (each section depth is 0.5µm) along the Z-axis to have a better visualization of the intra cellular distribution of the probe **2.** (a) NIR, (b) blue, (c) green channel and (d) merged images.

9. Live Cell Imaging

MCF 7 cells (5×10^4) were grown in sterilized 35 mm cover glass-bottom (SPL life sciences co. Ltd. Korea) confocal dishes containing DMEM supplemented with 10% fetal bovine serum at 5% CO_2 environment at 37˚C to control and create the appropriate environment for imaging in healthy live cells. After confluence, cells were washed with 1x PBS (3×1 mL) then treated with probe **2** (2 µM). After 2 h of incubation cells were washed thoroughly with PBS (3×1 mL) followed by 1 mL fresh culture media was added to the well and cells were observed with confocal microscope (Nikon Ti Eclipse).

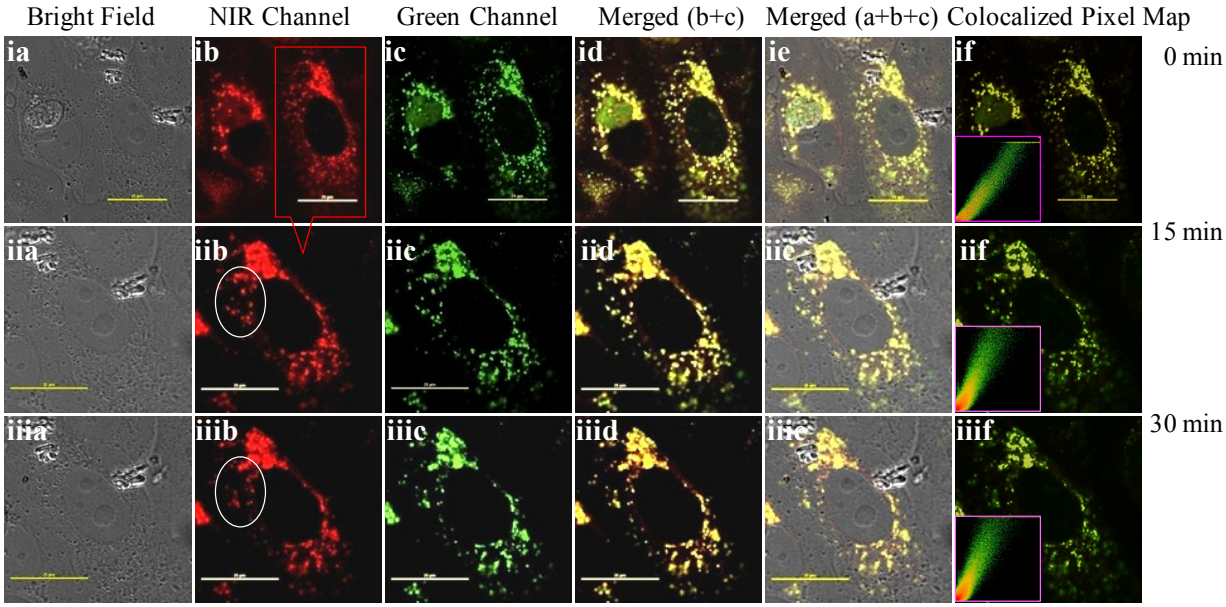


Figure S15. CLSM images of live MCF7 cells: cells were incubated with 2 and LTG for 4 h. Imaging was done in every 15 min starting from 0 min to 30 min. (a) bright field, (b) NIR, (c) green channel, (d) merged of NIR and green channel, (e) merged of a, b and c channel (f) colocalization pixel map of corresponding NIR and green channel. Inset of (1f-vf) is the corresponding scatter plot: concentrated pixels along the diagonals indicate high degree of colocalization of probe 2 and LTG. Scale bar 25μm.

10. Cellular Uptake study with Flow Cytometry

The degree of cellular uptake was studied quantitatively using flow cytometry. Cancerous MCF-7 and noncancerous CHO cells were cultured separately in 6 well plates at a density of $1X10^5$ cells per well for 8 h. The cells were then incubated with Probe **2** (2 μM) for three different time intervals (0, 2 and 4 h respectively). Cells were harvested by trypsinization, washed with PBS (3×1 mL) and were analysed using flow cytometer (BD FACS canto II) under PE channel. The shift of the fluorescently labelled cells was compared with untreated cells using FCS software.

11. In vivo experiment:

Female C57BL/6J mice (6−8 week old, each weighing 20−22 g) were procured from the animal house of our Institute. All animals were maintained in filtered-top autoclavable cages provided with sterilized water, food, and bedding. All the experiments were conducted in accordance with the protocols approved by our Institutional Animal Ethics committee vide approval (IICT/IAEC/44/2018) of the Indian Institute of Chemical Technology (CSIR-IICT), Hyderabad, India.

11.1. *In vivo* bio-distribution studies of probe 2 using C57BL/6J mice models:

After successful *in vitro* studies of the probe **2** we were interested to evaluate applicability of the same for the in vivo deep tissue imaging in C57BL/6J mice model by the means of in vivo NIR imaging technique. In vivo imaging studies have been conducted in accordance with the guidelines from the CPCSEA, New Delhi after approval of Institutional Animal Ethics Committee (IAEC) on animal care with approved protocols. The probe **2** was

intravenously injected to rodents at intervals of 0, 8, 16 and 24 hours to a smaller group of 4-5 animals at a dose of 4mg/kg (in PBS solution) of body weight of mice. Animals were Euthanized 8, 16 and 24h after the injection, and dissected. The organs of interest (usually: liver, spleen, kidney, brain, heart and tumor, etc.) are placed in pre-weighed containers, then into an in-vivo imager device that measures NIR emission. The results give a dynamic view of how the probe **2** moves through the animal.

11.2. Immunohistochemistry:

4-6 weeks old C57BL/6J mice were treated with **2** at two different concentrations (4 and 16 mg/kg respectively) and in a control experiment mice were treated with Hanks'balanced salt solution (HBSS) by i.v. injection in every alternative day for a period of 4 weeks. After completion of the treatment five major organs i.e. heart, kidney, lungs, liver and spleen were harvested from the treated and controlled mice and were fixed in 10% (w/v) neutral buffered formalin and the fixed tissues were embedded in paraffin from which 4 µm sections were prepared. Hematoxylin and eosin (H&E) staining experiment was carried out following the standard protocol. Briefly, sections were immersed in the filtered Harris Hematoxylin for 10 seconds and washed with running water followed by it was immersed in EOSIN stain for ~30 seconds and the sections were again washed with running water. Subsequently all the sections were dehydrate in ascending percentage of alcohol solutions (50%, 70%, 80%, 95% x 2 and 100% x 2) in Columbia staining dish. Finally, sections were cleared with xylene (3-4x) in Columbia staining dish and mounted on coverslip onto the section on glass slide with Permount. The images were acquired with an optical microscope.

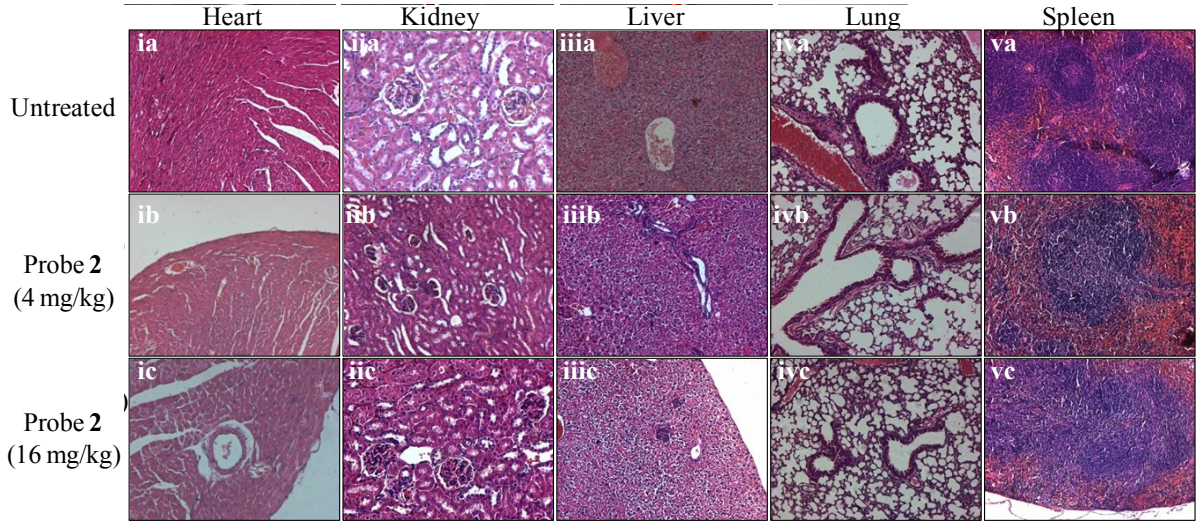


Figure S16. i-v(a-c): HE stained tissue sections of different organs harvested from the mice with and without treatment with the probe **2**.

12. pH dependent ratiometric cell imaging

12.1. pH dependent ratiometric cell imaging on fixed MCF 7 cells:

Cells ($5x10^4$) were grown in sterilized 35 mm cover glass-bottom (SPL life sciences co. Ltd. Korea) confocal dishes containing DMEM supplemented with 10% fetal bovine serum and

5% CO_2 environment at 37°C. After confluence cells were washed with 1x PBS (3×1 mL) and treated with probe 2. After 4 h of incubation cells were fixed with 4% paraformaldehyde and washed thoroughly with PBS (3×1 mL) then 1 mL fresh media of pH 7.4 was added to the well and cells were imaged with confocal microscope (Nikon Ti Eclipse). Afterwards media was discarded and washed with PBS followed by fresh culture media of pH 4.5 was added and kept for 20 min followed by images were taken with confocal microscope keeping all the microscopic parameter the same.

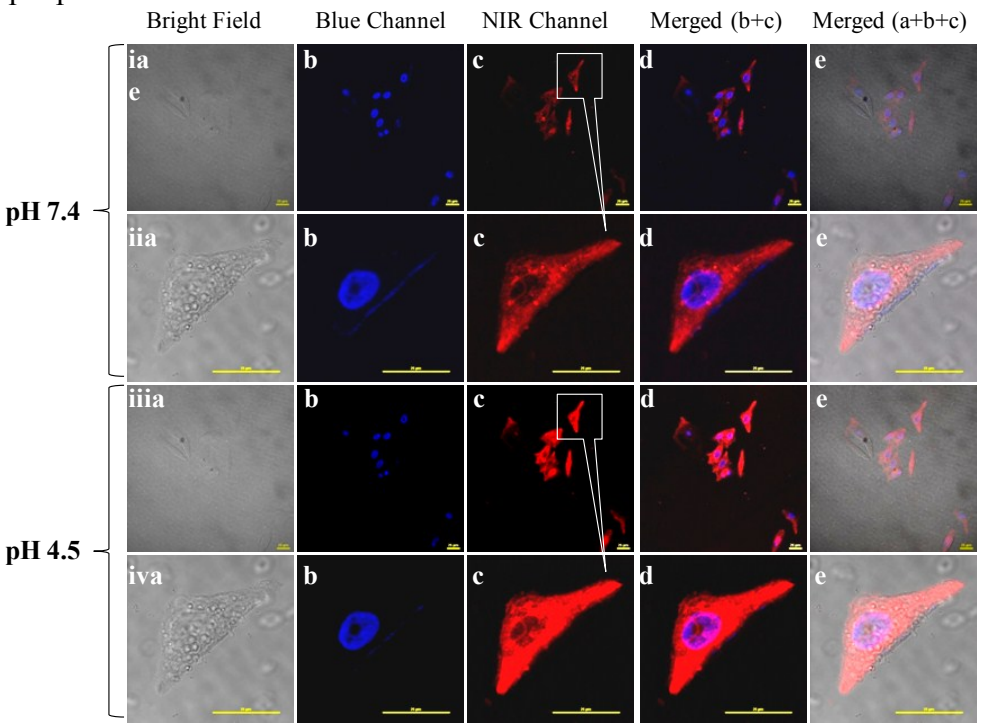


Figure S17. CLSM images of MCF-7 cells incubated with 2: (ia-e and iia-e) at pH 7.4. (iiia-e and iv a-e) The same cell imaged after media changed to pH 4.5, keeping all the microscopic parameter the same. Scale bar 25µm.

12.2. pH dependent ratiometric cell imaging on live MCF 7 cells:

MCF-7 cells ($5x10^4$) were grown in sterilized 35 mm cover glass-bottom (SPL life sciences co. Ltd. Korea) confocal dishes containing DMEM supplemented with 10% fetal bovine serum and 5% CO_2 environment at 37°C to create the appropriate environment for imaging in healthy live cells. After confluence cells were washed with 1x PBS (3×1 mL) and treated with probe 2. After 4 h of incubation cells were washed thoroughly with PBS (3×1 mL) 1 mL fresh media of pH 7.4 was added to the well and cells were imaged with confocal microscope (Nikon Ti Eclipse). Afterwards media was discarded and washed with PBS followed by fresh culture media of pH 4.5 was added and waited for 20 min followed by image was taken with confocal microscope keeping all the microscopic parameter the same.

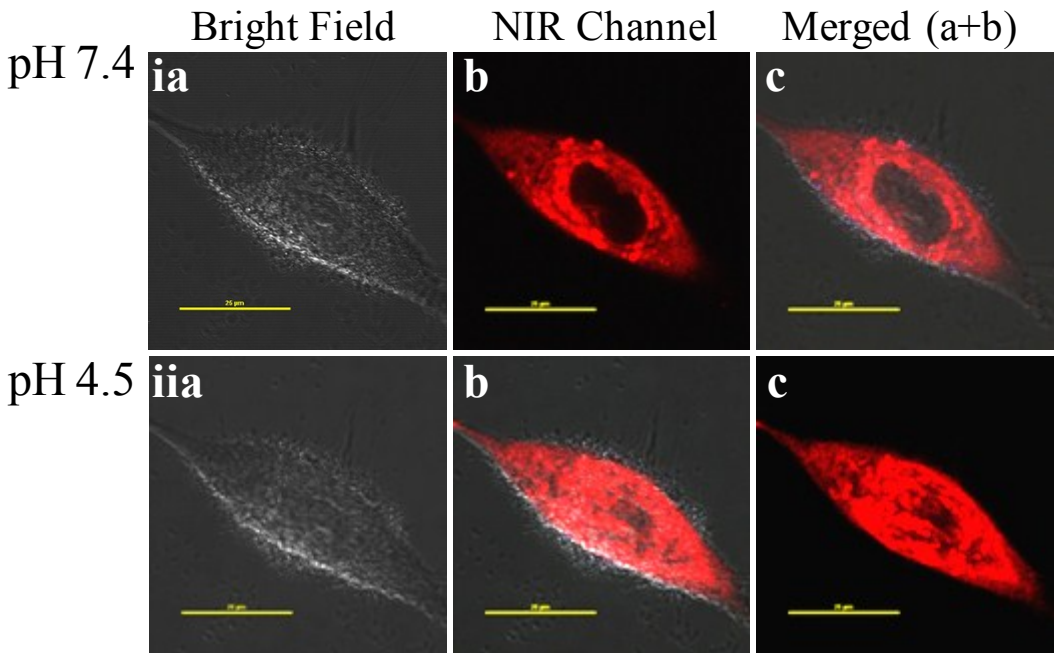


Figure S18. CLSM images of live MCF-7 cells incubated with **2**: (ia-c) at pH 7.4. (iia-c) The same cells imaged after media changed to pH 4.5, keeping all the microscopic parameter the same. Scale bar 25μm.

13. Photostability of probe 2:

MCF-7 cells were incubated with the probe **2** and the lysotracker green for 4 h followed by media was discarded and cells were fixed with paraformaldehyde (4%) and washed two times with PBS. Cell nucleus was stained with DAPI. CLSM images was taken continuously for 8 min keeping the laser on which revealed fast photo-bleaching of lysotracker green compared to the probe **2** (**Figure S11**). Therefore, the probe 2 can be an ideal candidate for the longer acquisition of bioanalytical and biomedical imaging.

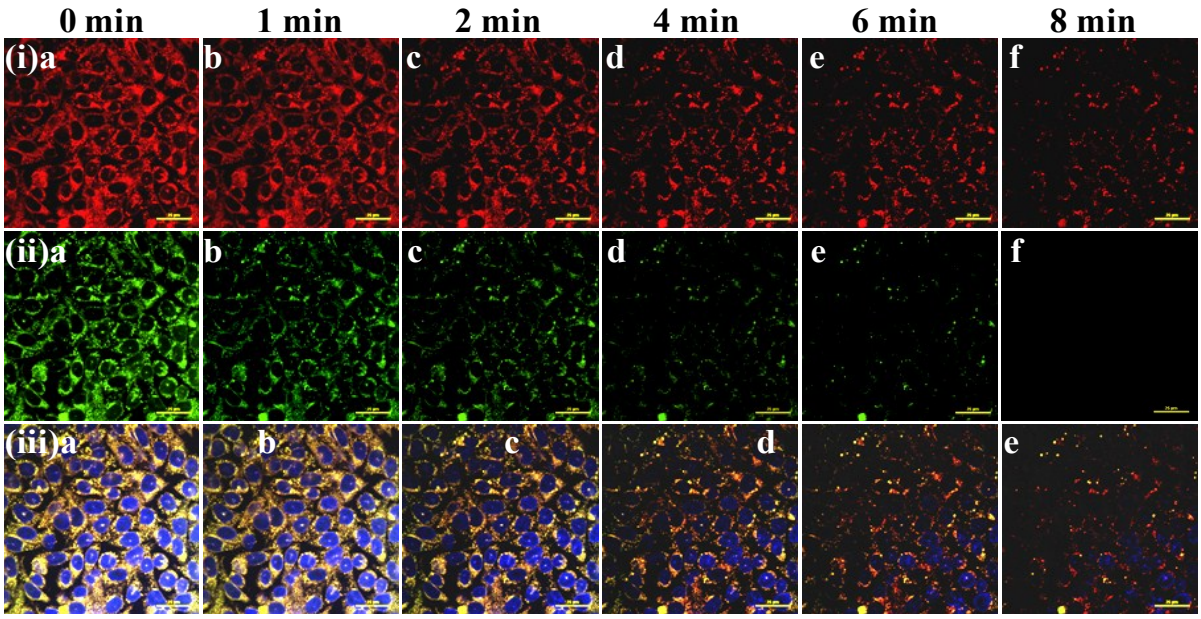


Figure S19. CLSM images of fixed MCF-7 cells incubated with 2 and lysotracker green: (i) NIR emission from the probe 2, (ii) emission from the lysotracker green and (iii) merged images of (i) and (ii). Scale bar 25 μm.

14. Particle size analysis:

Since the probe **2** is hydrophobic in nature so in aqueous media it forms nanoparticles. We performed dynamic light scattering (DLS) analysis to determine the particle size. The hydrodynamic diameter of freshly prepared nanoparticles 2 at ph 7.4 was of 184 nm (**Fig S20, S21**). We also have measured the zeta potential of freshly prepared nanoparticles 2 which was -2.8 mV (**Fig S22, S23**).

Whereas, the hydrodynamic diameter of nanoparticles of **2** at pH 5 was found to be 58 nm (**Fig S24, S25**) which clearly supports our hypothesis of protonation of morpholine N atom and thereby dissolution of the aggregates.

New Size Template

Created by: acer
Created at: 24-Sep-19 11:56:41 AM

Anton Paar

General

Measurement name	MOR ONP SIZE in pH 7.4	**User**	
Method		**Time**	
Status	Succeeded	**Instrument type**	
Measurement type	Particle size		

Settings

Measurement cell	Disposable	**Equilibration time**	0h 01m 00s
Angle	Back scatter	**Analysis model**	General
Target temperature	25.0 °C	**Cumulant model**	Advanced

Material

Name	New material	**Refractive index Laser 1**	1.0000
Absorption Laser 1	0.0000		

Solvent

Name	PBS tablet	**Refractive index**	1.3300
Viscosity	0.8882 mPa.s		

Data output

Hydrodynamic diameter	184.09 nm	**Polydispersity index**	15.9 %
Intercept $g1^2$	0.6826	**Mean intensity**	289.2 kcounts/s
Filter optical density	1.8500	**Baseline**	0.998
Focus position	-3.7 mm	**Angle used**	Back scatter
Processed runs	10	**Transmittance**	97.3 %
Diffusion Coefficient	- $\mu m^2/s$		

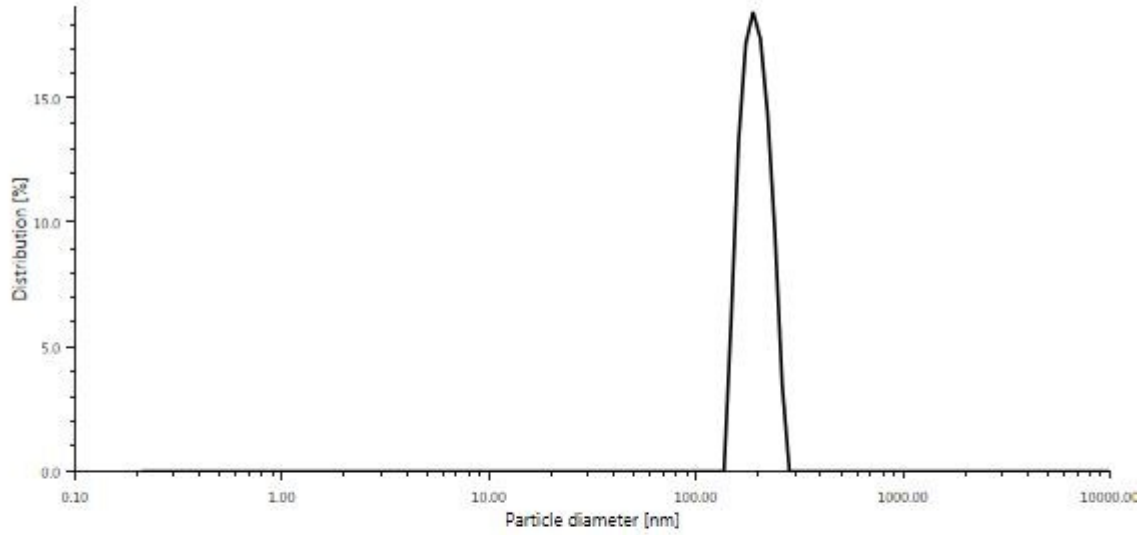
Figure S20: Hydrodynamic diameter of freshly prepared nanoparticles 2 at pH 7.4.

New Size Template

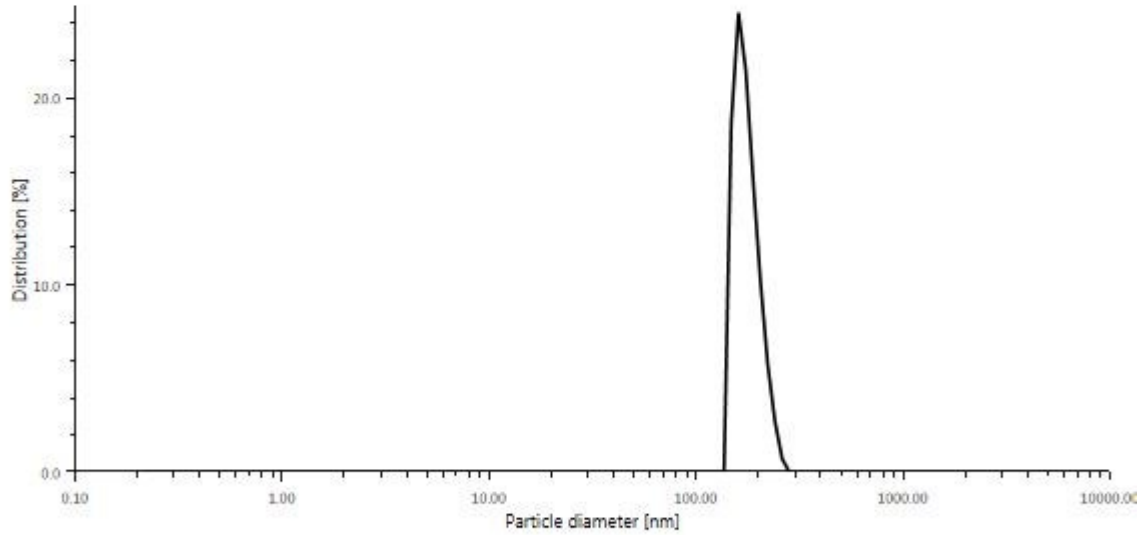
Created by: acer
Created at: 24-Sep-19 11:56:41 AM

Anton Paar

Particle size distribution by intensity



Particle size distribution by number



Particle size distribution peaks

Weighting model	Peak 1 [nm]	Peak 2 [nm]	Peak 3 [nm]
Intensity	195.05	-	-
Volume	187.75	-	-
Number	176.56	-	-

Kalliope version: 1.4.4
Serial number: 81962693

Measurement completed successfully
Page 2 of 3

Figure S21: Particle size distribution of freshly prepared nanoparticles 2 at pH 7.4.

Standard report

Created by: acer
Created at: 24-Sep-19 11:59:02 AM

Anton Paar

General

Measurement name	MOR ORG NP Zeta pH 7.4	**User**	
Method		**Time**	
Status	Succeeded	**Instrument type**	
Measurement type	Zeta potential		

Settings

Measurement cell	Omega Cuvette	**Equilibration time**	0h 01m 00s
Approximation	Smoluchowski	**Target temperature**	25.0 °C
Adjustment mode	Automatic	**Voltage**	200.0 V
Debye factor	1.5		

Quality

Run mode	Manual	**Number of runs**	100

Solvent

Name	Water	**Refractive index**	1.3303
Viscosity	0.8903 mPa.s	**Relative permittivity**	78.37

Data output

Mean zeta potential	0.0 mV	**Electrophoretic mobility**	-0.0011 µm*cm/Vs
Distribution peak value	-2.8 mV	**Filter optical density**	2.1639
Processed runs	100	**Conductivity**	0.001 mS/cm
Adjusted voltage	200.0 V	**+/- Standard deviation**	0.2 mV
Mean intensity	692.2 kcounts/s	**Transmittance**	86.5 %

Figure S22: Zeta potential of freshly prepared nanoparticles 2 at pH 7.4.

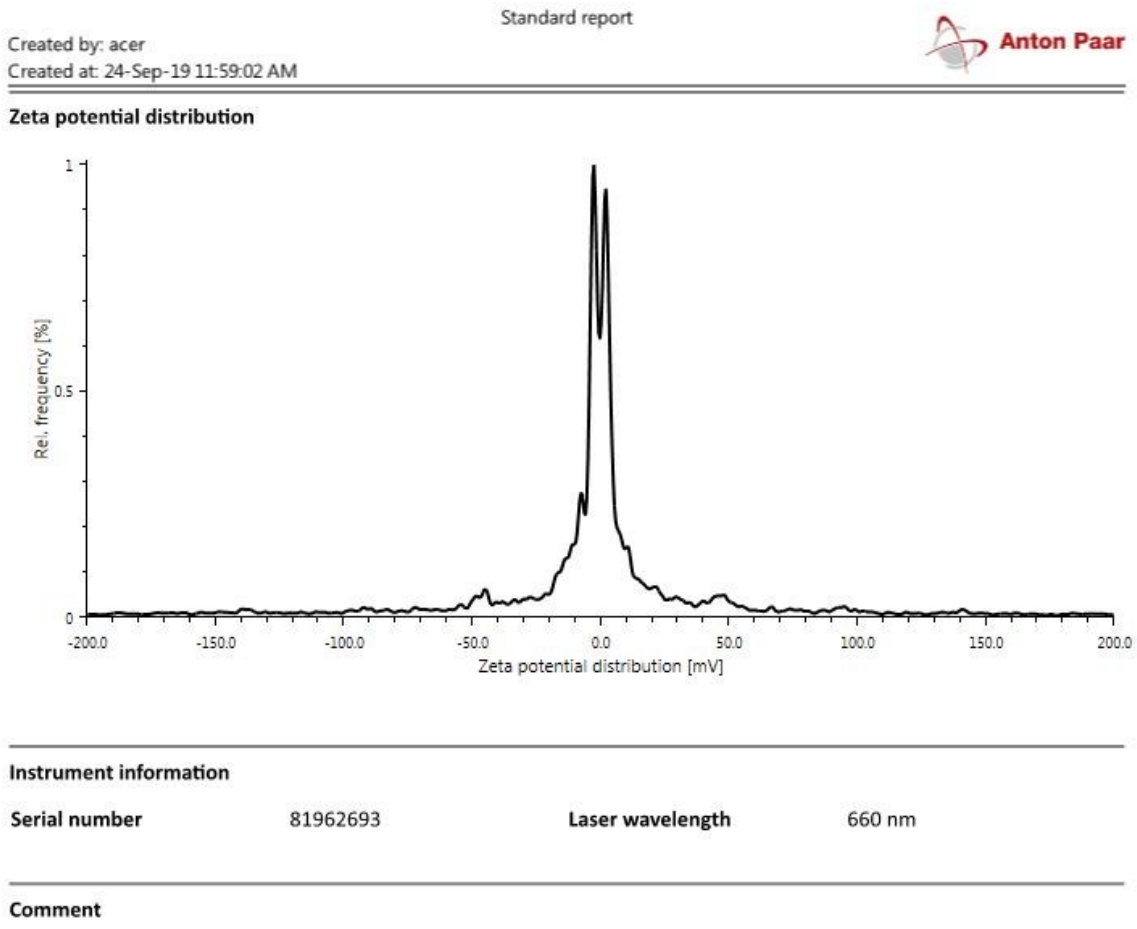


Figure S23: Zeta potential distribution of freshly prepared nanoparticles 2 at pH 7.4.

New Size Template

Created by: acer
Created at: 24-Sep-19 12:06:16 PM

Anton Paar

General

Measurement name	30 min MOR pH 5	**User**	
Method		**Time**	
Status	Succeeded	**Instrument type**	
Measurement type	Particle size		

Settings

Measurement cell	Disposable	**Equilibration time**	0h 01m 00s
Angle	Automatic	**Analysis model**	General
Target temperature	25.0 °C	**Cumulant model**	Advanced

Material

Name	New material	**Refractive index Laser 1**	1.0000
Absorption Laser 1	0.0000		

Solvent

Name	Water	**Refractive index**	1.3303
Viscosity	0.8903 mPa.s		

Data output

Hydrodynamic diameter	58.01 nm	**Polydispersity index**	121.5 %
Intercept $g1^2$	782.6758	**Mean intensity**	0.2 kcounts/s
Filter optical density	0.0000	**Baseline**	1.000
Focus position	0.6 mm	**Angle used**	Side scatter
Processed runs	10	**Transmittance**	101.2 %
Diffusion Coefficient	8.5 $\mu m^2/s$		

Figure S24: Hydrodynamic diameter of the nanoparticles of 2 at pH 5.

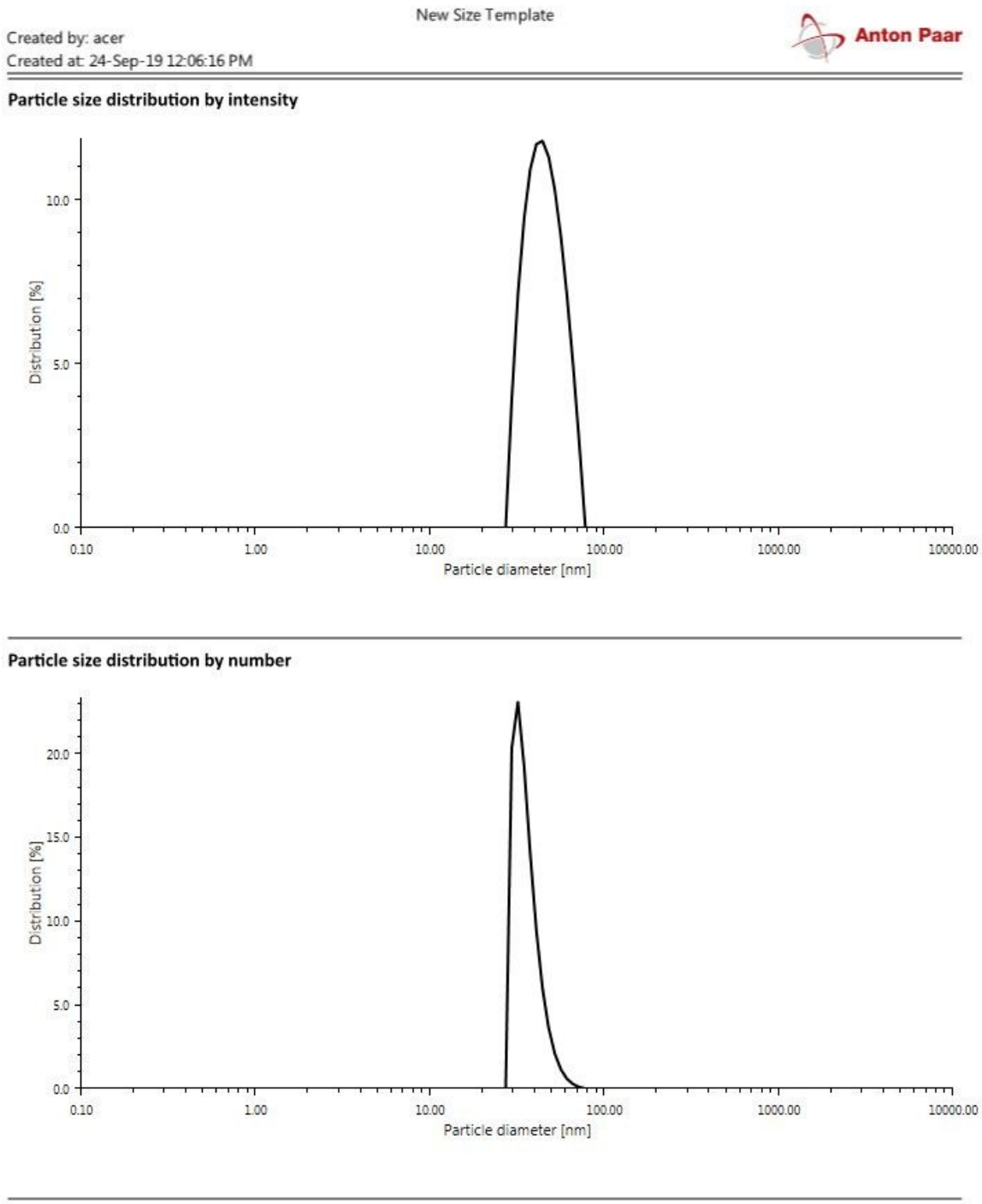


Figure S25: Particle size distribution of the nanoparticles of 2 at pH 5.

Standard report

Created by: acer
Created at: 24-Sep-19 12:13:52 PM

Anton Paar

General

Measurement name	Zeta mor ph-5	**User**	
Method		**Time**	
Status	Succeeded	**Instrument type**	
Measurement type	Zeta potential		

Settings

Measurement cell	Omega Cuvette	**Equilibration time**	0h 01m 00s
Approximation	Smoluchowski	**Target temperature**	25.0 °C
Adjustment mode	Automatic	**Voltage**	200.0 V
Debye factor	1.5		

Quality

Run mode	Manual	**Number of runs**	100

Solvent

Name	Water	**Refractive index**	1.3303
Viscosity	0.8903 mPa.s	**Relative permittivity**	78.37

Data output

Mean zeta potential	0.0 mV	**Electrophoretic mobility**	-0.0012 µm*cm/Vs
Distribution peak value	-2.2 mV	**Filter optical density**	2.7151
Processed runs	100	**Conductivity**	22.191 mS/cm
Adjusted voltage	48.7 V	**+/- Standard deviation**	0.3 mV
Mean intensity	692.2 kcounts/s	**Transmittance**	1.4 %

Figure S26: Zeta potential of nanoparticles 2 at pH 5.

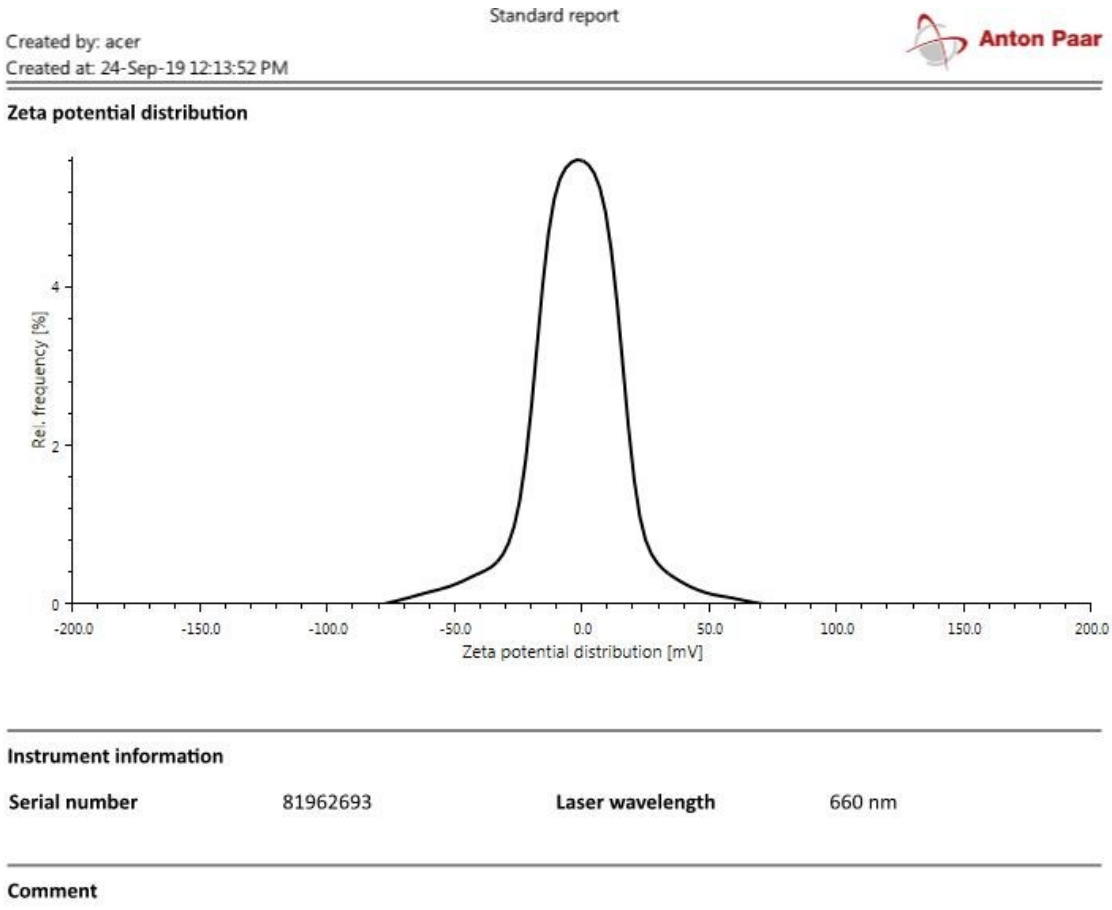


Figure S27: Zeta potential distribution of nanoparticles 2 at pH 5.