Supplementary information

The fluorescence detection of autophagosomes in live cells under starvation using core-substituted naphthalene diimide probes

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-500 007, Telangana, India.

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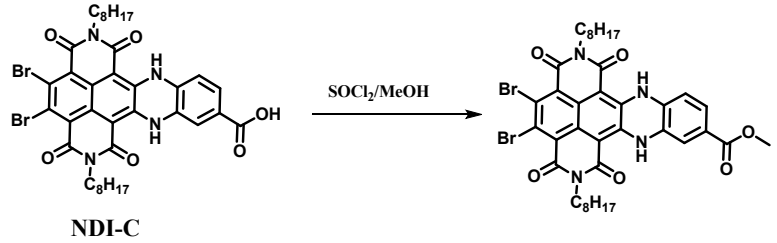
Experimental Section.



Scheme S1. Synthesis of Core **NDI-C** ester

General Material and Methods:

1,4,5,8 Tetracarboxylic naphthalene dianhydride, octylamine, 3, 4-diaminobenzoic acid, acetic acid (AcOH), bromine (Br_2) and dimethylformamide (DMF) were purchased from Sigma Aldrich (Bangalore, Karnataka, India) and are used without further purification, unless otherwise specified. UV-vis absorption spectra were recorded by UV-vis-1800 schimadzu

spectrophotometer. Fluorescence emission spectra were obtained on Cary eclipse fluorescence spectrophotometer Agilent technologies.

Synthesis of NDI-C[2]

Tetrabromo di-octyl naphthalenediimide (cNDI-Br) were synthesised by reported method,[1] (200 mg, 0.24 mmol) were added in 10 mL of dry DMF with vigorous stirring for 10 min in which added 3,4-diaminobenzoic acid (45 mg, 0.29 mmol) then above reaction mixture was subjected to reflux 3h during this color of the reaction mixture turns from wine red to dark blue and progress of reaction was monitored by TLC. The reaction mixture was cooled after completion of reaction at room temperature. Solvent was removed under reduced pressure by rota evaporator. The reaction mixture was reprecipitated from methanol, filtered and washed with methanol (Several times). The crude compound **NDI-C** was obtained as a violet coloured solid which was further purified by column chromatography (DCM: MeOH; 5%), 130 mg, 66% yield. M.P: >300 ˚C; **FT-IR** (KBr cm^{-1}): 2921, 2852, 2954, 2921, 2852, 1693, 1633, 1582, 1501, 1453; **^{1}H NMR** (DMSO-d_6, 300 MHz) **:** δ 13.12 (s, 2H), 7.80-6.76 (d, 1H), 7.30-7.21 (s, 1H), 6.60-6.54 (d, 1H), 6.30-6.16 (s, 2H,br), 4.12-4.09 (t, 4H), 1.70-1.56 (m, 4H), 1.43-1.25 (m, 20H), 0.90-0.82 (t, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz)**:** failed due to limited solubility; **[ESI-Mass] *m/z*:** $C_{37}H_{40}Br_2N_4O_6$: $[M]^+$:796, $[M+2H]^+$: 798.

Synthesis of NDI-C ester:

NDI-C (50 mg, 0.0628 mM) in 5 mL of dry MeOH was stirred at room temperature which is insoluble then dropwise addition of thionyl chloride (1 mL excess) at 0 ˚C for further 10 mins then keep stirring for 6 h at ambient temperature reaction monitored by TLC, solvent was evaporated under reduced pressure by rota evaporator remaining residue titrated by MeOH and filtered crude solid purified by column chromatography [DCM: hexane (7:3)] faint green solid 65 mg; 78% yield . **M.P:** >300˚C; **FT-IR** (KBr cm^{-1}): *v* 3430, 2921, 2851,1689, 1624, 1582,1501, 1452, 1261, 1231,1205,1098, 1013, 910, 790, 618; **^{1}H NMR** ($CDCl_3$,

300 MHz)**:** δ 13.11 (s, 2H), 7.68-7.66 (d, 1H), 7.51 (s, 1H), 6.86-6.84 (d, 1H), 4.13-4.10 (t, 4H), 3.91 (s, 3H), 1.69-1.65 (m, 4H) 1.25 (m, 20H),0.89-0.86 (t, 6H); ^{13}C NMR ($CDCl_3$, **125** MHz)**:** δ: 165.2, 164.4, 159.9, 147.6, 127.7, 124.4, 123.9, 119.0, 117.4, 52.4, 41.4, 34.8, 31.4 30.1, 29.6, 27.2, 22.6, 14.1; [**ESI-Mass]** ***m/z*:** $C_{38}H_{42}Br_2N_4O_6$: $[M]^+$: 809.6; **HRMS** [**ESI-Mass]** ***m/z*:** calcd for $C_{38}H_{43}Br_2N_4O_6Na$: $[M+H+Na]^+$ calc.: 832.2411, found: 832.2386.

BIOLOGICAL ASSAY

Materials and Methods

Cell culture. The human Skin melanoma cells (SKMEL2) were maintained in DMEM. (Life Technologies, Inc., USA) supplemented with 2 mM glutamine and 10% (v/v) heat-inactivated fetal bovine serum at 37 ˚C in the presence of 5% CO_2. Unless otherwise mentioned, SKMEL 2 cells were used in all the studies. All the cells were maintained in drug-free media for at least 2 weeks before microscopy experiments.

After 48 h of growth of cells, the medium was removed from wells and the cells were washed with DPBS (Life Technologies, Inc., USA). A **NDI-C** was added to the each well to get a final concentration 20 uM/mL. The cells were incubated for 20 min at room temp. and used for Microscopy. The images of cells with **NDI-C** were recorded after 20 min. 1M Sod. phosphate buffer (pH 6) was used for the increase in the pH of the intracellular components in cells after 20 min of preincubation at 37 °C. The images were recorded with the help of Cy5 light cube in EVOS Fl microscopy (Life Technologies, Inc., USA).

Chemicals and Buffers.

The stock solution of 5 mM **NDI-C** was prepared in dimethyl sulfoxide. The loading buffer was prepared by dilution of the carboxyl **NDI-C** stock solution in Phosphate buffer of the required pH to a final concentration of 20 μM; the buffer was stored on ice and protected from the light.

Fluorescence spectra of the probe:

pH_i determination.

For every measurement a cell sample corresponding to 1 mL at 0.25 OD_{660} was collected by centrifugation at 13,000 rpm for 5 min and resuspended in 250 µL of the loading buffer. After incubation at 28 °C for 11 min on a shaker, the cells were collected by centrifugation at 13,000 rpm for 5 min and resuspended in 250 µL of McIlvaine buffer, pH 3.0 if not otherwise stated. The samples were put on ice and immediately analyzed by flow cytometry. For the overall experiments, the samples were protected from light in order to guarantee the stability of the probe.

The growth medium was removed from the cell culture plates. The cells were washed twice with DPBS before the exposure to **NDI-C.** The **NDI-C** was added to the wells and the images recorded at each 20 min interval by Cy5 light cube in EVOS Fl microscopy (Life Technologies, Inc., USA).

Effect of external pH on pH_i.

The cells were grown and harvested in exponential and stationary phases as described above. Then the cells were stained in phosphate buffer at pH 4.5 following the protocol previously described. The stained cells were then divided and incubated for 20 min in phosphate buffers of different pH values between 3 and 7.0 after a washing step in the same buffer of incubation. The experiment was performed in duplicate. A NucBlue® Live ReadyProbes® Reagent was added to the wells for the staining of the live cells.

Flow cytometric analysis and transportation of NDI-C in cells.

The 48 h grown Skin melanoma cells were trypsinized with 0.25% trypsin and centrifuged at 2000 rpm for 2 min. 1 mL of the cells were exposed to 1 uM ouabain and incubated at 37 °C for 20 min. The cells with or without ouabain were stained with **NDI-C** and PI. 5 µL of **NDI-C** and 1 µL of the 100 µg/mL PI working solution were added to each 100 µL of cell suspension. After 20 min incubation at room temp, 100 µL of phosphate buffer (pH 4.5) was

added to the tube. After gentle mixing the cells were kept on ice and analysed by Attune flow cytometry. This reading was considered as zero h reading.

The probe was excited by using a 488 nm (20 mW) laser, while the fluorescence emission was measured through a 690/50 band-pass filter (BL3 parameter) and a 660 /20 pass filter (RL1 parameter). All data were acquired in a linear mode. Threshold settings were adjusted so that the cell debris was excluded from the data acquisition. A total of 10,000 cells were measured for every sample. Data analysis was performed with Attune cytometric V2.1 software.

Supporting Figures

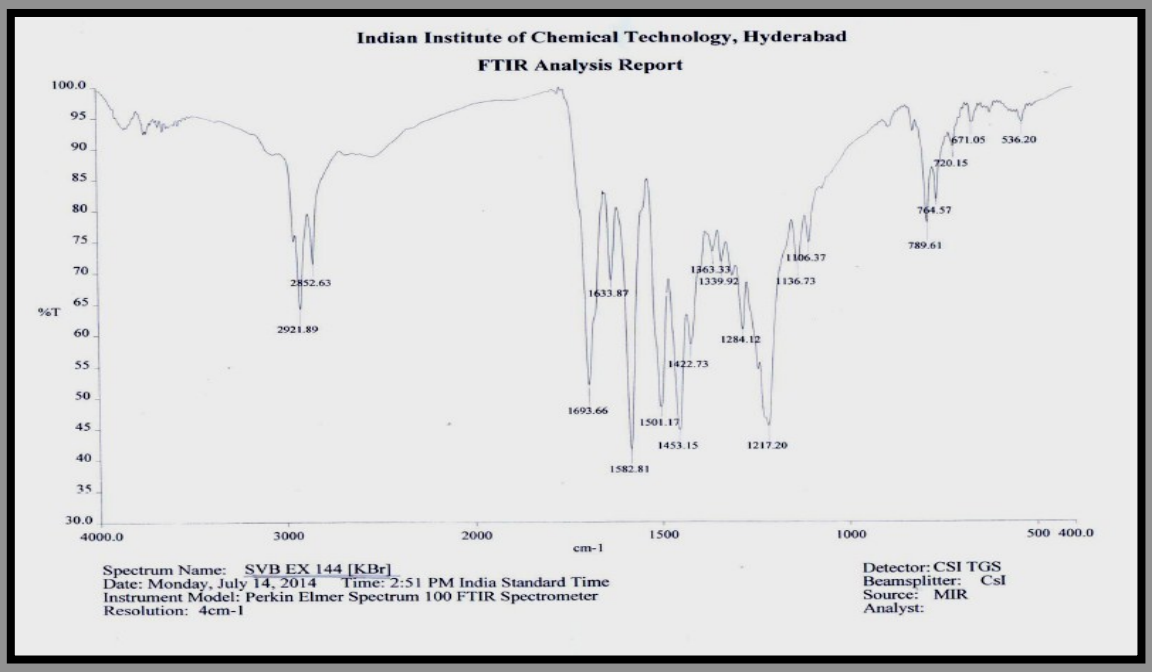


Figure S1. FT-IR of **NDI-C**

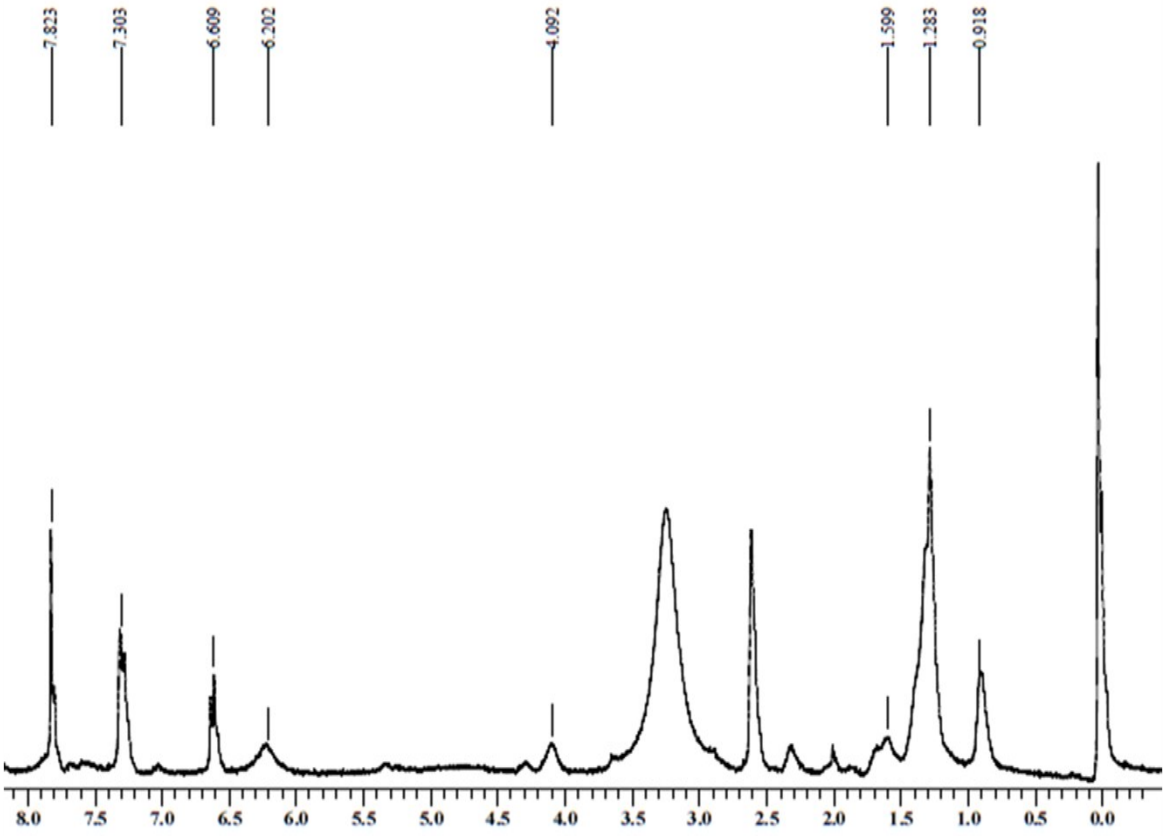


Figure S2. ^{1}H NMR of **NDI-C.**

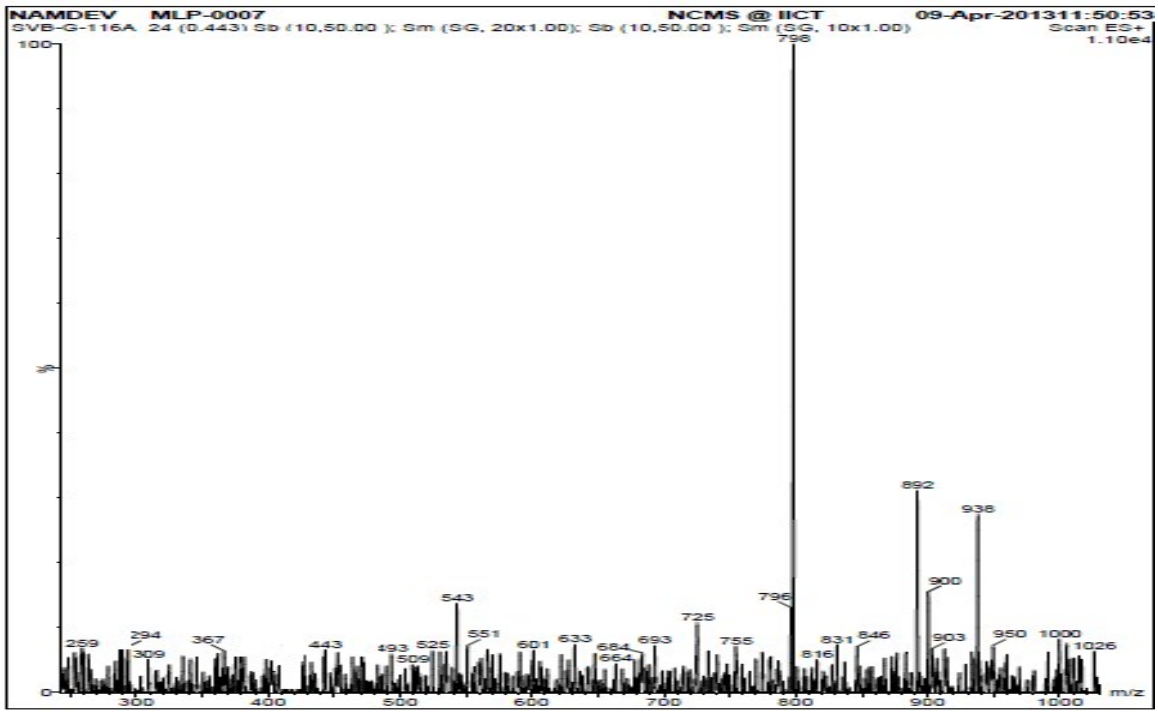


Figure S3. Positive ESI-MS of **NDI-C**

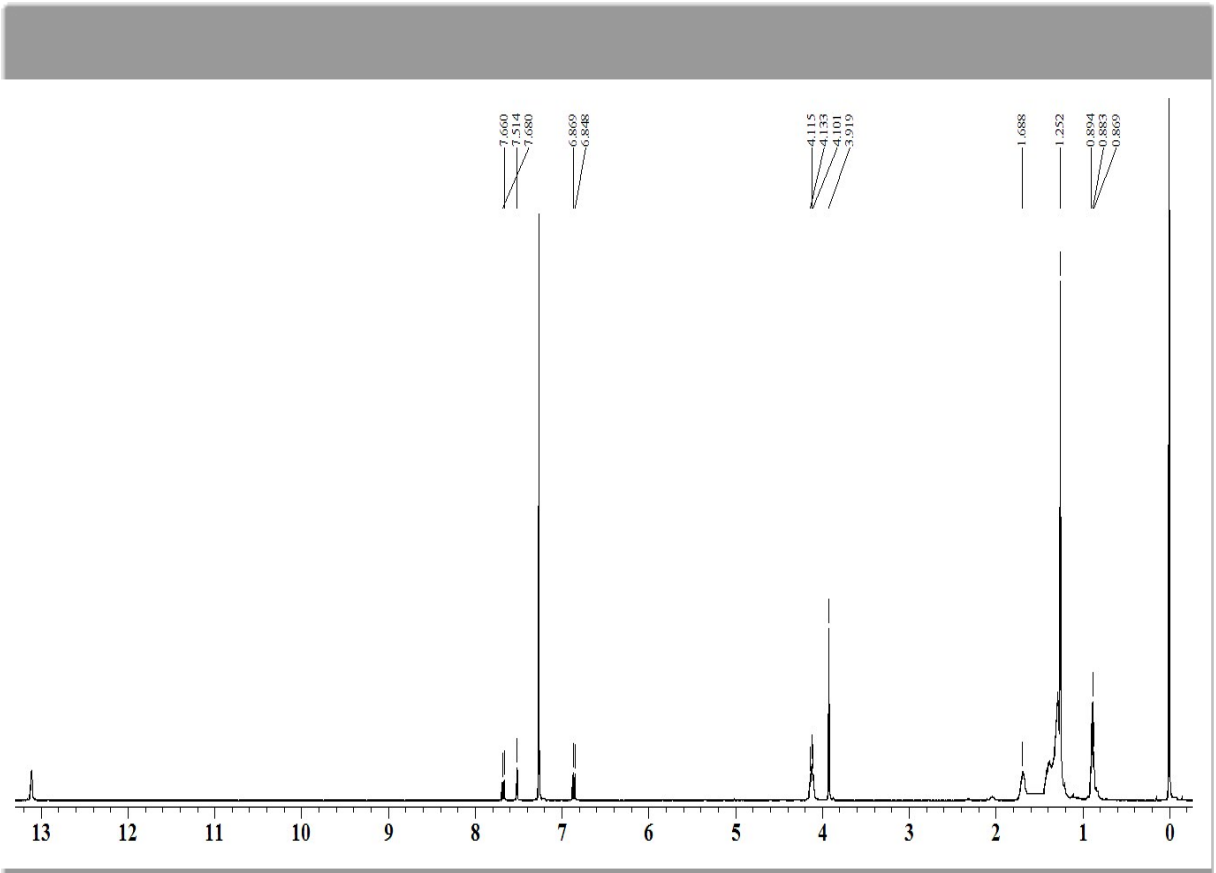


Figure S4. ^{1}H NMR of **NDI-C** ester

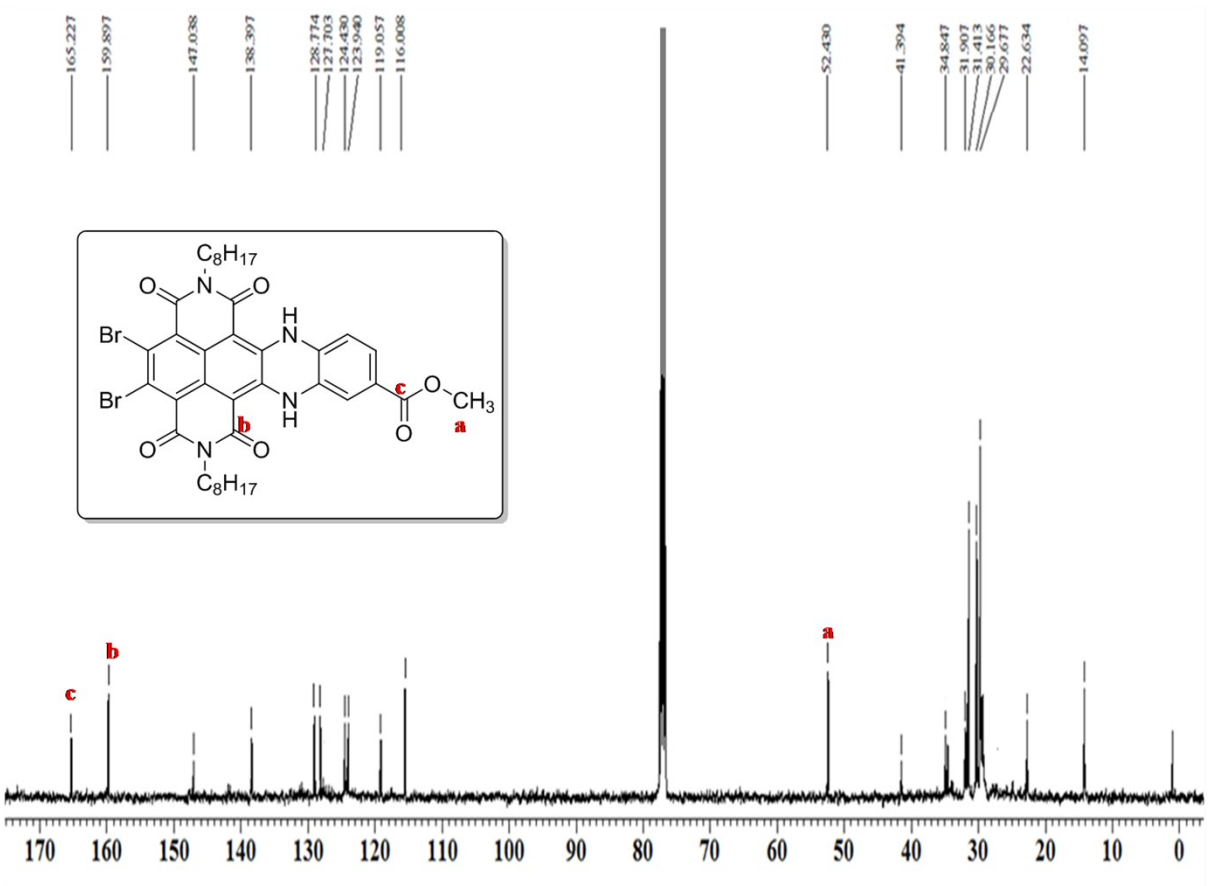


Figure S5. ^{13}C NMR of **NDI-C** ester.

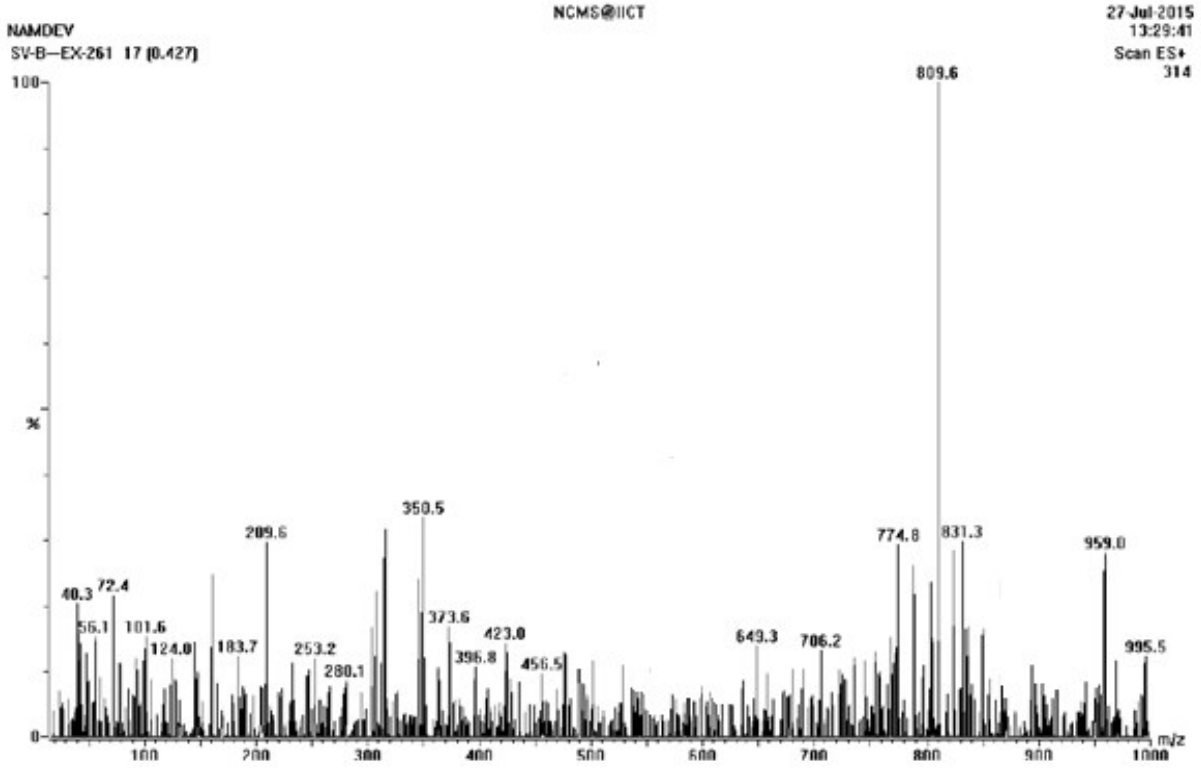


Figure S6. ESI-MS of **NDI-C** ester

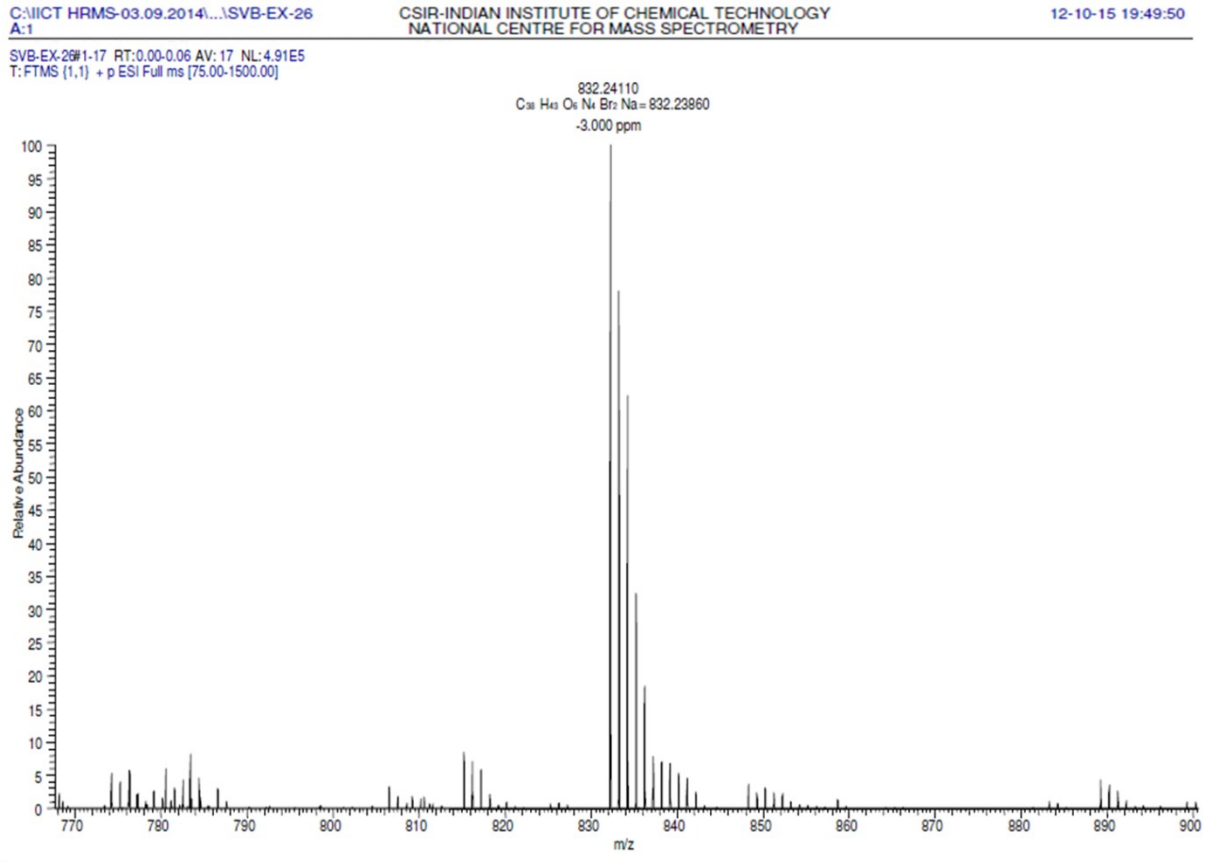


Figure S7. ESI-MS of **NDI-C** ester

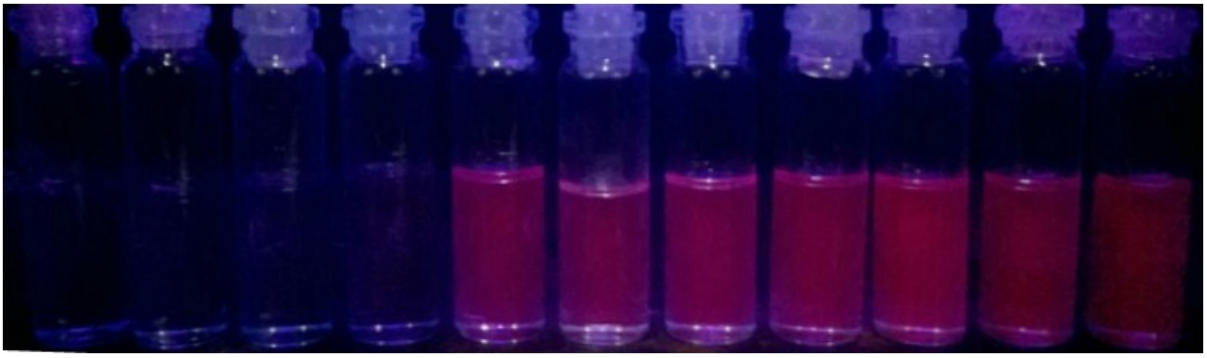


Figure S8. Naked-eye colorimetric change in **NDI-C** (5 x 10^{-4} M) solution from left to right upon increasing changes in pH.

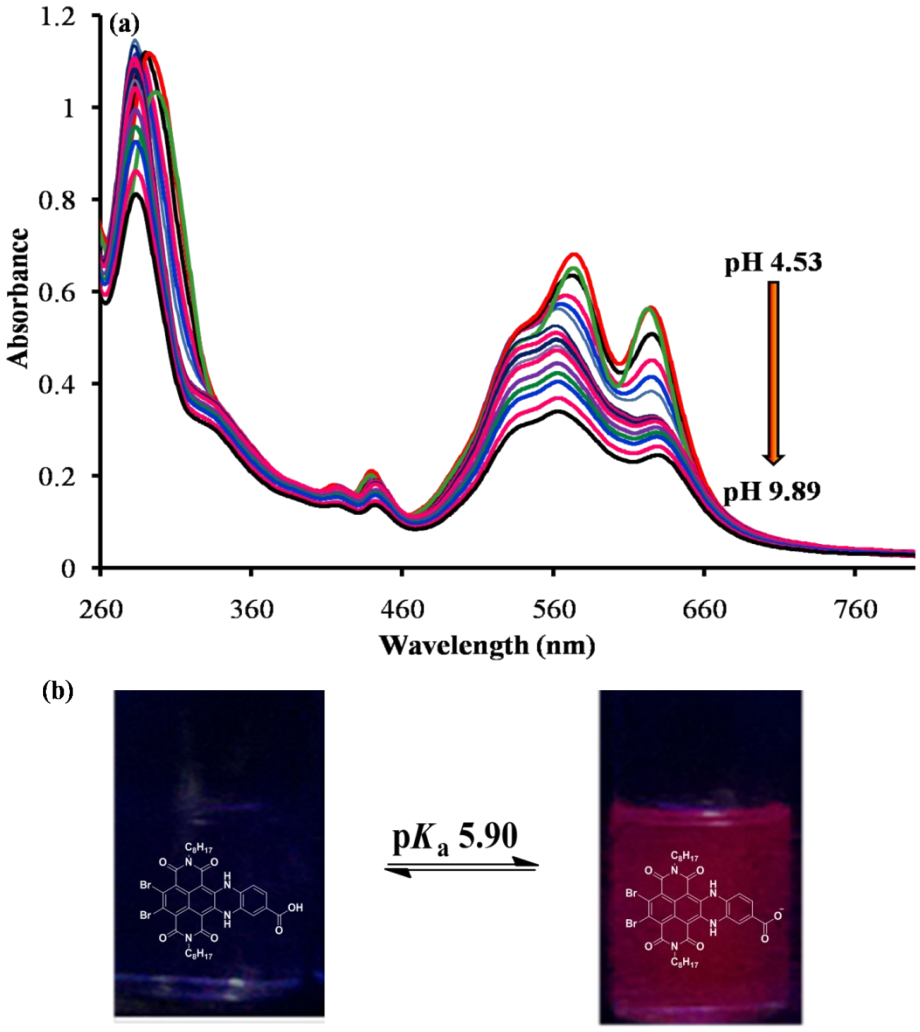


Figure S9. (a) Absorption spectra of **NDI-C** (5 x 10^{-5} M) in Britton-Robinson buffer (buffer/DMSO 8:2) at various pH; (b) It shows color change of **NDI-C** solution at pK_a = 5.90.

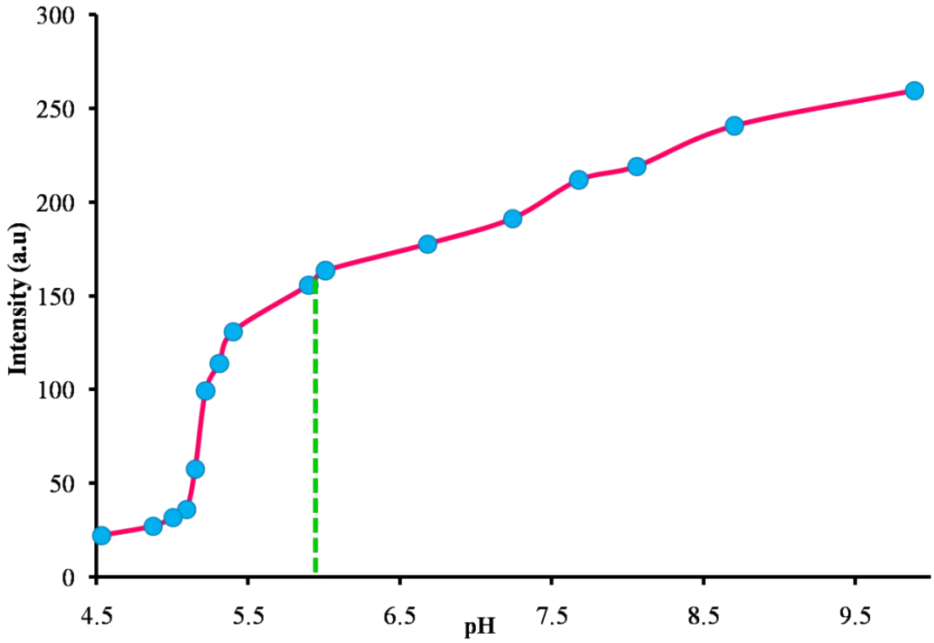


Figure S10. Fluorescence response of **NDI-C** (5 x 10^{-5} M) at 615 nm various pH in [Britton Robinson buffer: DMSO (8:2)]

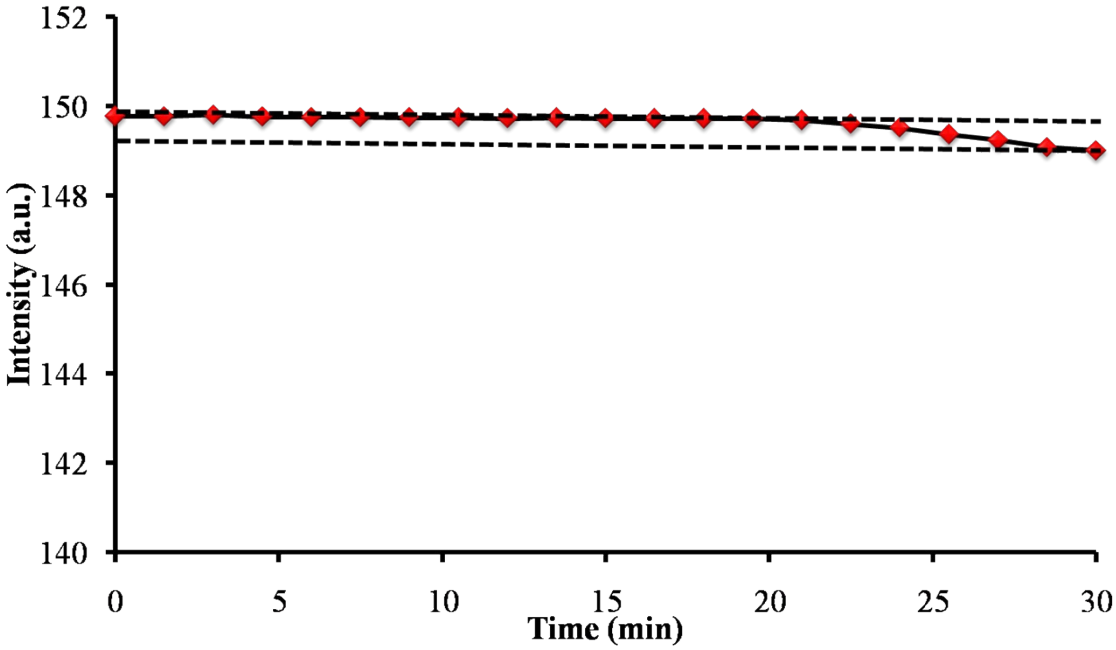


Figure S11. Fluorescence intensity (λ_{ex} = 572 nm) at 612 nm at pH 5.90 of **NDI-C** (5x10^{-5} M) verses time in minutes.

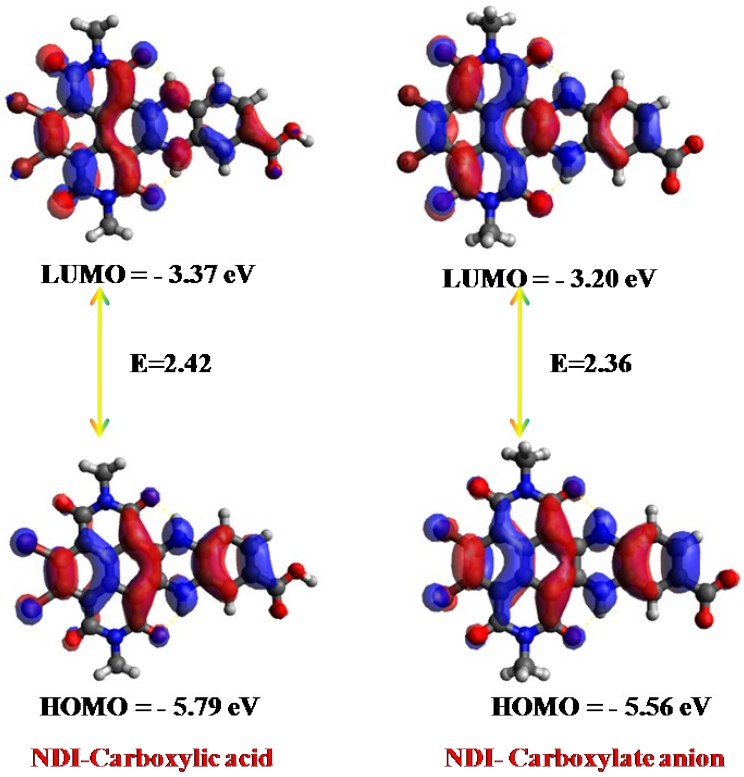


Figure S12. Frontier molecular orbitals of **NDI-C** and **NDI-C** anion.

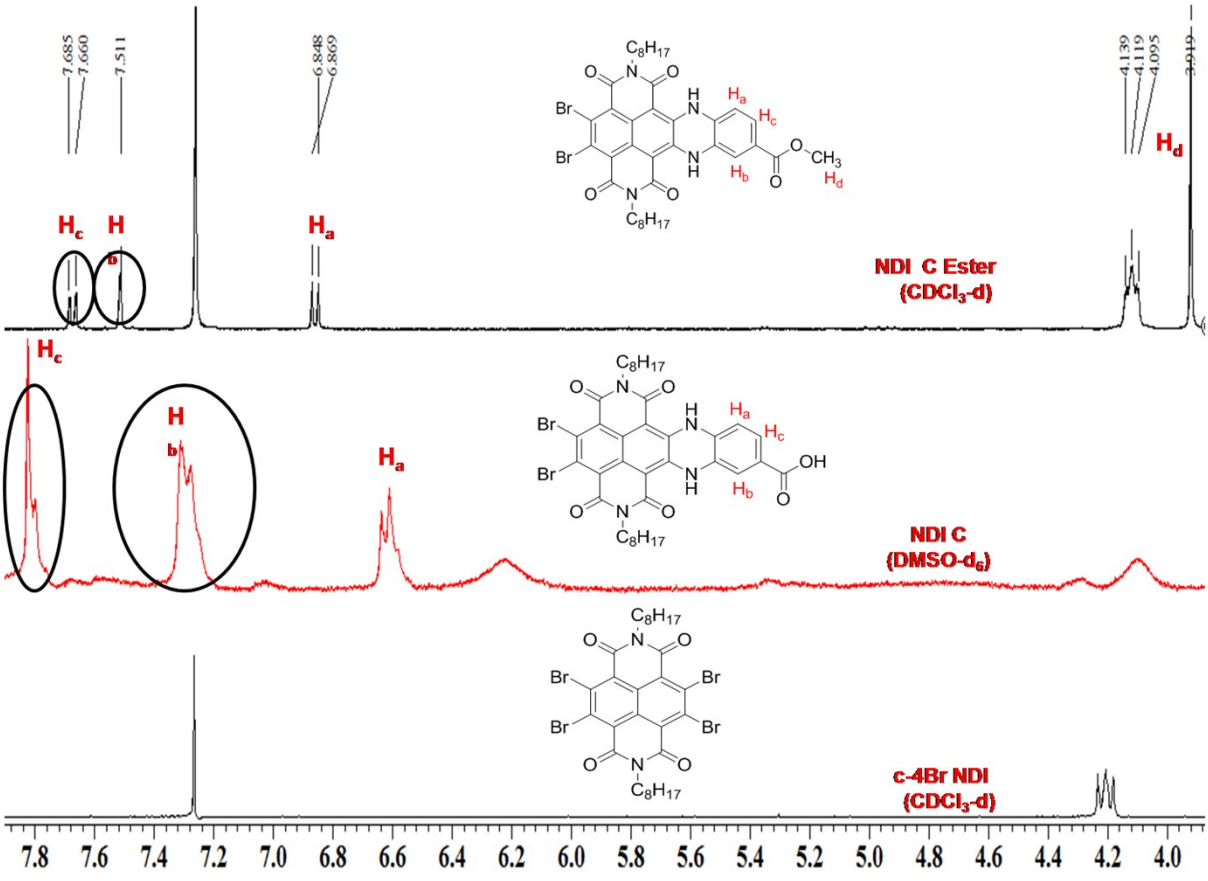


Figure S13. [1]H NMR of **c-4Br NDI**, **NDI-C**, **NDI-C** ester respectively.

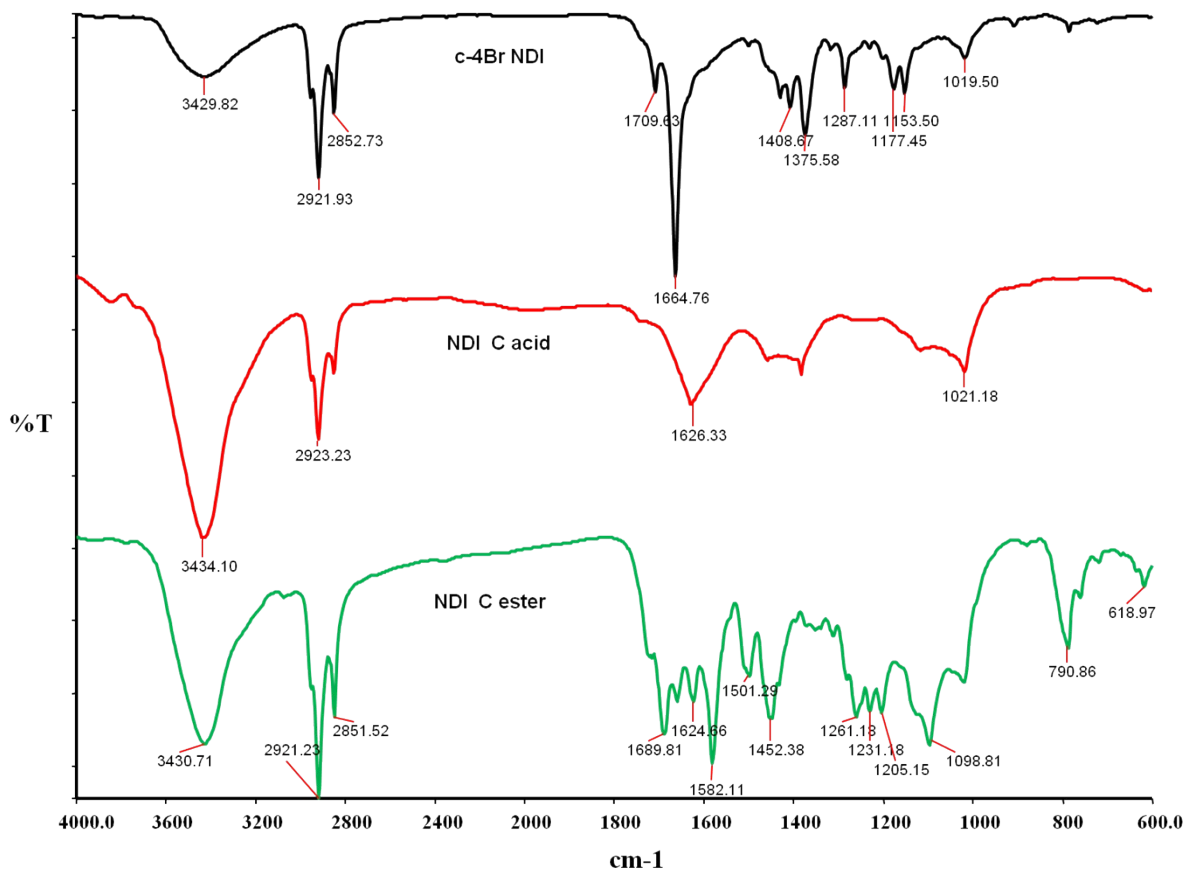


Figure S14. IR of **c-4Br NDI**, **NDI-C**, **NDI-C** ester respectively.

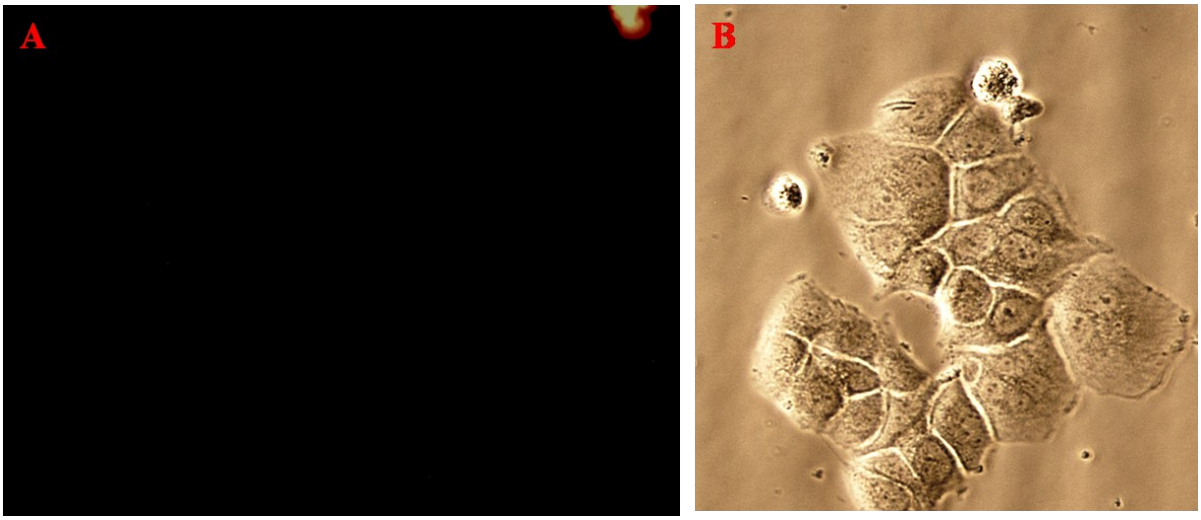


Figure S15. Fluorescence imaging of NDI-C in Skin melanoma cells. The image acquired at A) Cy5 light cube and B) transmitted mode with phase contrast objective after 40 min incubation in a pH 7 phosphate buffer.

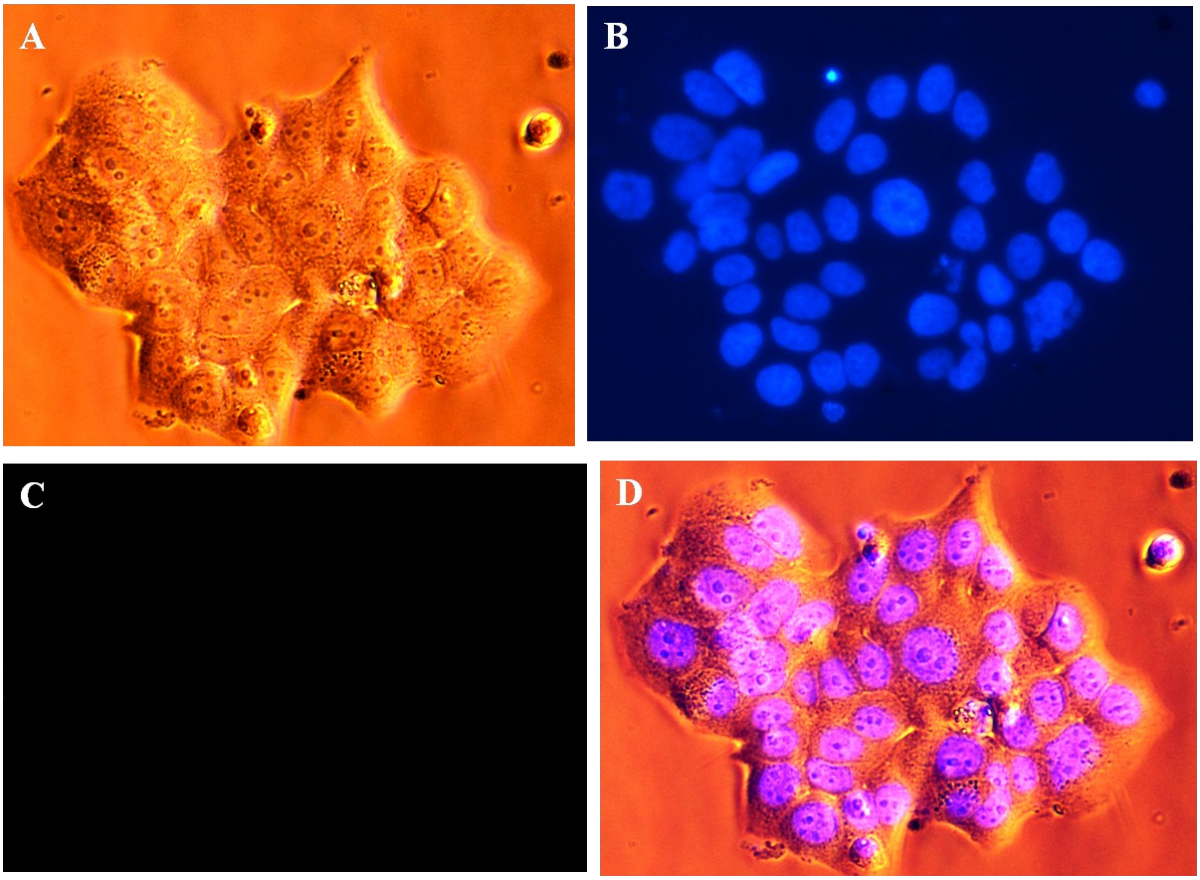


Figure S16. Fluorescence imaging of NDI-C in Skin melanoma cells. NucBlue® Live ReadyProbes® Reagent was added to the wells for the staining of the live cells. The images acquired separately at DAPI light cube, Cy5 light cube and transmitted mode with phase contrast objective. Where A) transmitted light image after 40 mins; B) NucBlue Stained nuclei at DAPI Light cube; C) after 40 min incubation Cy5 light cube in a pH 7.2 phosphate buffer; D) RGB image after 40 min of incubation in a pH 7.2 phosphate .

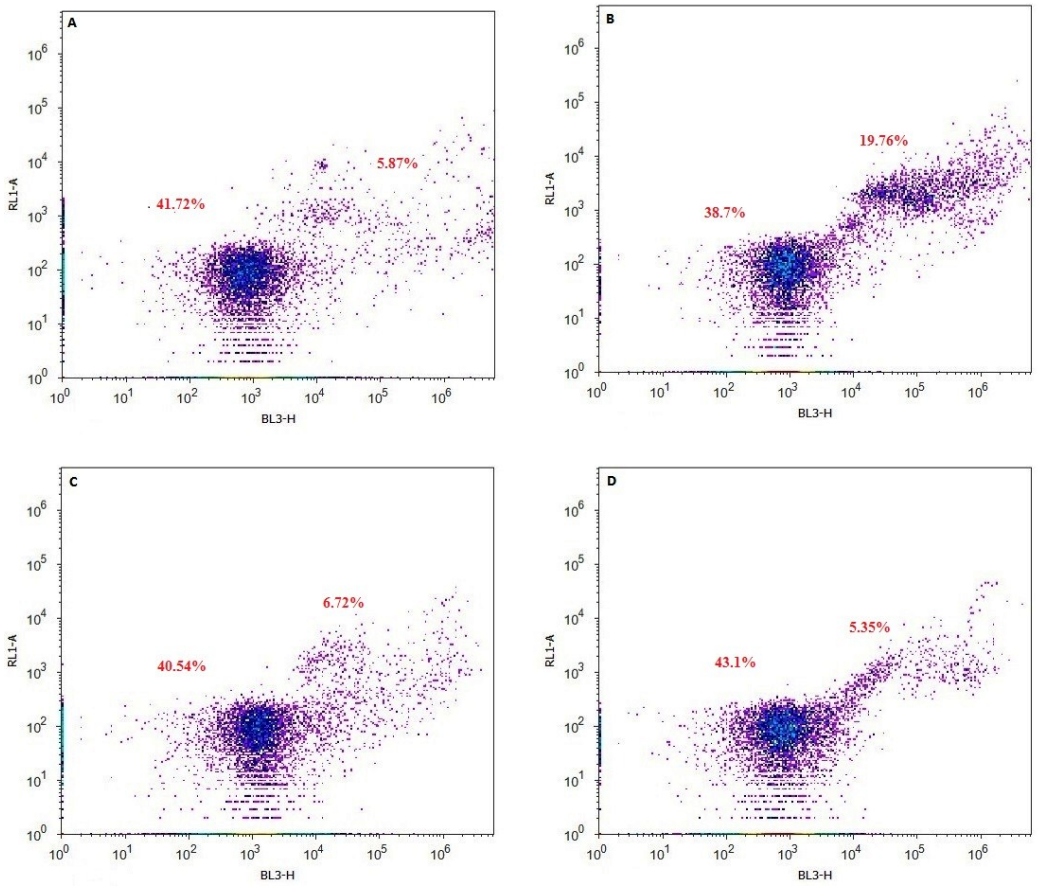


Figure S17. Flow cytometry analysis of **NDI-C** in Skin melanoma cells. Where A) cells without Ouabain and at zero h, B) cells without Ouabain and after 20 min incubation. C) cells with Ouabain at zero h, D) cells with Ouabain and after 20 min incubation.

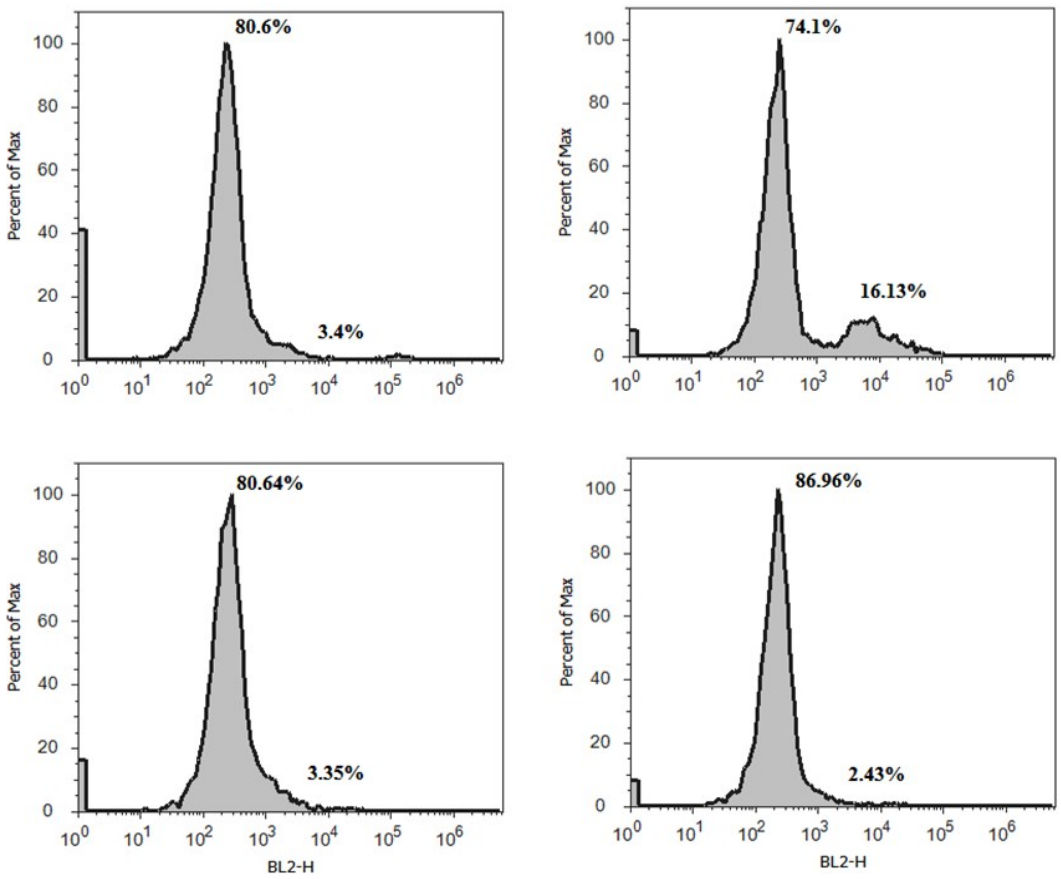
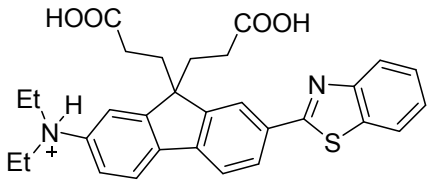
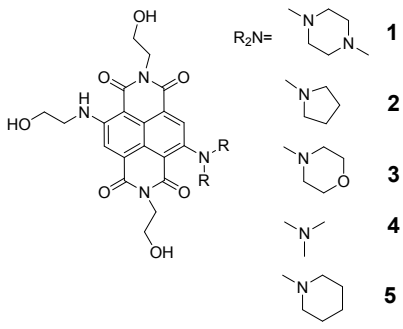


Figure S18. Histogram of flow cytometry analysis of **NDI-C** in skin melanoma cells. Where A) cells without Ouabain and at zero h, B) cells without Ouabain and after 20 min incubation. C) cells with Ouabain at zero h, D) cells with Ouabain and after 20 min incubation.

Table S1 List of chromophores with pH range with plausible applications in cell line.

Sr. No.	pH Sensor	Study	References
1		Near-neutral fluorescent pH indicator with p*K*a of 6.96. Its dispersion in cell cytosol was good, and showed low cytotoxicity. The compound works as an excellent one-photon ratiometric fluorescent pH indicator.	3
2		This NDI pH sensor is a useful tool for both the selective staining of acidic vesicular organelles and Sensitive for changes in the pHi associated to treatment induced cell stress. In addition, possible applications were demonstrated of similar NDI-based pH sensors. It monitors the pH of the extracellular environment. This probe is useful to distinguish tumor from normal tissue *in vivo*.	4

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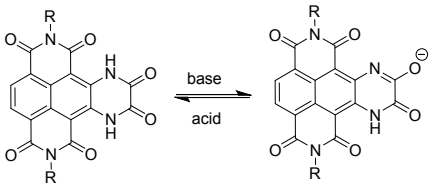
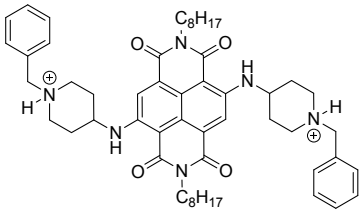


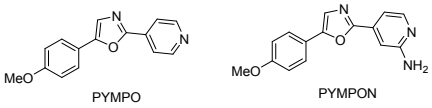
These NDIs described as an efficient fluorescent pH sensors in water. Furthermore, it was reported that these are very cytotoxic towards the colon cell line HT29, with an EC50 of 300 nM respectively, 5

4



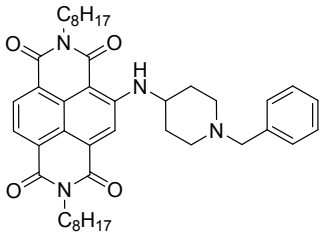
Amino core-substituted naphthalene diimide probe is reported for the investigation of H^+ sensing. The optical output change in optical output in both absorption and emission was also reported. 6

5



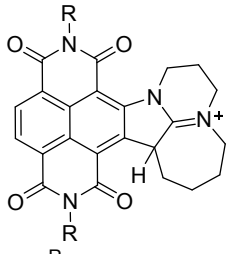
PYMPO and PYMPON were reported as pH values measurements in the pH 3–8 range by a ratiometric method. Authors demonstrated that it could find that PYMPON was useful as applications as intracellular meters. 7

6



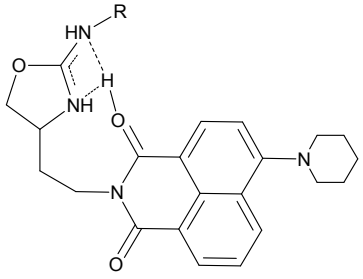
This core-substituted naphthalene diimide (cNDI) probe is sensitive to both H^+ and solvent polarity has been demonstrated. 8

7



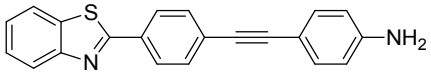
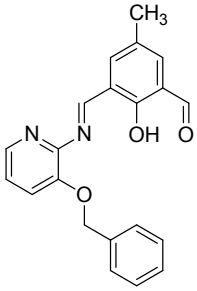
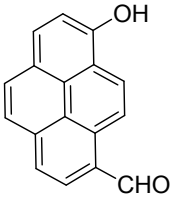
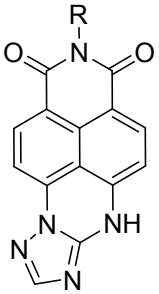
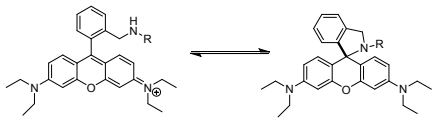
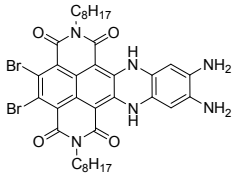
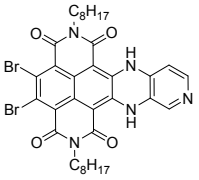
NDI-DBU is a new pH-sensitive fluorophore derived from the NDI compound was demonstrated. It was observed that the strong ICT interaction between the dialkylamino group and NDI core was suppressed by the protonation of **NDI-DBU**. 9

8



Such pH probes might have potential in biological or supramolecular systems.

10

9		The reported fluorescent pH sensor was employed under strongly acidic conditions based on the ICT effect. It exhibit high stability, sensitivity and selectivity. This probe can be employed to detect acidity within the pH range 0.5–2.5.	11
10		This probe is highly selective fluorescent pH sensor in a Britton Robinson buffer at 25 ^{0}C. It exhibits a 250-fold increase in fluorescence emission intensity within the pH range of 4.2 to 8.3. The pKa observed for this probe is 6.63. This pKa is appropriate for the biological organelles.	12
11		The NGI fluorescent probe was used to calibrate the cytosol pH value in living cells.	13
12		This probe play important role in the pH 5-8 range in aqueous solution. Presence of long chain effect for mapping the pH change in both the extracellular microenvironment and the inner cells.	14
13		A series of rhodamine-base analogues were synthesized from rhodamine B for the fluorescence based detection of pH. The probe **3** has been employed to be the most biologically relevant probe, because of its dynamic response in the "acidic window".	15
14		This pH sensor was employed to detect rough endoplasmic reticulum.	16
15		The probe is very sensitive to HCl, The long-wavelength absorption of the probe and emission close to the infrared may lead to employ this probe as a pH indicator for bioprocessing, medicinal and photonic materials.	17

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