Supplementary Information

A red-shifted donor-acceptor hemicyanine-based probe for mitochondrial pH in live cells

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Material and Methods:
All the chemicals and reagents employed in this work for synthetic purposes were from Sigma-Aldrich, and they were all used exactly as the supplier provided. Using a Milli-Q instrument from Millipore, water was purified. Sigma-Aldrich provided all of the analytical or HPLC grade solvents needed for the synthesis of fluorescent dyes or spectroscopic analysis. Supplements and media for cell culture acquired from Invitrogen.

Instrumentation:
Bruker NMR instruments operating at 600 and 800 MHz were used to gather ^{1}H NMR and ^{13}C NMR spectra. Mass spectrometry data were obtained with an electrospray ionisation (ESI) mass spectrometer in the positive ionisation mode on Thermo Scientific LTQ Orbitrap XL. The buffer's pH was measured using a Eutech Instrument pH 2700 model metre. Using a Shimadzu UV-visible spectrophotometer (U2600), absorption spectra were recorded. A Horiba fluorolog spectrofluorometer with a 3 nm slit for excitation and emission was used to obtain the fluorescence spectra. The CCK-8 assay's absorbance was measured using a Thermo-varioskanlux multimode microplate reader. A laser scanning confocal fluorescence microscope (Olympus FV-3000-IX-81) was used to acquire the fluorescence images.

Preparation of Britton Robinson (BR) buffer:
The BR buffer is a universal pH buffer, and it consists of 40 mM glacial acetic acid (CAS no. 64-19-7), 40 mM orthophosphoric acid (CAS no. 7664-38-2), and 40 mM boric acid(CAS no. 10043-35-3)[39].This BR solution was titrated with 1M NaOH to make buffers of the desired pH.

Synthesis of Mito-pH2 dye: To 1g of 1,1,2-trimethyl-1H-benzo[e]indole [marked as 1, Scheme-1] 0.5 g of methyl iodide was added in the presence of acetonitrile and continuously stirred at 80˚C overnight. The obtained product from the overnight reaction 1,1,2-trimethyl-1H-benzo[e]indolium iodide (1 equivalent) was mixed with syringaldehyde [marked as 2, Scheme-1] (1 equivalent) along with 5.0 ml ethanol. Later, 0.5 ml of piperidine was added and refluxed for 4 hours at 80˚C. After cooling to room temperature, a brown solid precipitate was obtained. The precipitated brown solid was filtered and purified by column chromatography using Dichloromethane/Methanol as eluent to yield a glittery brown solid (Mito-pH2 dye). -Thin Layer Chromatography silica gel 60G F_{254} plates analysis showed a single fluorescent spot under the UV light after the completion of reaction. The scheme-1 outlines the synthetic pathway for the fluorescent probe Mito-pH2.

^{1}H NMR spectroscopy of Mito-pH2 dye dissolved in deuterated DMSO and data have been recorded with residual DMSO-d5 (H) as a reference, whose position was set to 2.5 ppm. Mito-pH2 showed peaks at 7.51, and 8.44 ppm with J-J coupling constant (Karplus equation) = 16.2

Hz (Figure S1) which confirmed that the molecule exists in the trans-configuration. **^{1}H NMR** (600 MHz, DMSO-d_6) δ 8.44 (d, J = 16.2 Hz, 1H), 8.42 (d, J = 8.2 Hz, 1H), 8.28 (d, J = 8.8 Hz, 1H), 8.21 (d, J = 8.3 Hz, 1H), 8.07 (d, J = 8.8 Hz, 1H), 7.81 (t, 1H), 7.71 (t, J = 7.6 Hz, 1H), 7.59 (s, 2H), 7.51 (d, J = 16.2 Hz, 1H), 4.25 (s, 3H), 3.93 (s, 6H), 2.02 (s, 6H). **^{13}C NMR** (150MHz, DMSO-d_6) 182.3, 153.7, 148.8, 142.7, 139.9, 137.7, 133.4, 131.2, 130.8, 128.8, 127.4, 127.2, 125.08, 123.4, 113.5, 109.6, 56.9, 56.5, 53.8, 35.0, 25.9 (Figure S2). M/Z was calculated for $C_{25}H_{26}NO_3$ $[M]^+$ 388 and found using ESI mass-spectrometry determination 388.1907 (Figure S3). The melting point of Mito-pH2 dye was found to be 259 ˚C.

Spectroscopic analysis:
In HPLC grade DMSO, a Stock Solution of Mito-pH2 dye (1 mM) was made. Analyte stock solutions were made with Milli-Q water. The Mito-pH2 dye was diluted to 2 μM in a 40 mM BR buffer solution to perform spectral measurements. Every time, a quartz cuvette with a 10 mm optical path length was filled with 600 μl of the dye solution. Every spectroscopic experiment was carried out at ambient temperature.

UV-Visible absorption and fluorescence spectra measurement:
Mito-pH2 (2.0 μM) was dissolved in the varying pH buffer solution (pH 3.0-9.0). The absorption and fluorescence spectra were recorded immediately under different excitation wavelengths. For the interference study, various metal ion (K^+; Ni^{2+}; Zn^{2+}; Fe^{2+}; Ca^{2+}; Mn^{2+}; Mg^{2+}; Co^{2+}; Cu^{2+}; Ba^{2+}; Na^+; Li^{2+}; SO_4^{2-}; CO_3^{2-}; SO_3^{2-}; Br^-; N_3^- and NO_2^-) were mixed with Mito-pH2 (2.0 μM) in Phosphate Buffer Saline (PBS), then the fluorescence spectra were measured. To check the effect of organic sulphur, increasing concentrations of cysteine were added to Mito-pH2 solution and fluorescence were recorded.

Quantum yield measurement:

The quantum yield (Φ) of Mito-pH2 dye was determined using a relative method with Rhodamine 6G[1] (Φ = 0.95 in ethanol) and Cresyl Blue[2] (Φ = 0.54 in methanol) as standards. Mito-pH2 dye was prepared in cell culture grade MilliQ water, while Rhodamine 6G and Cresyl Blue were prepared in ethanol and methanol, respectively. For Mito-pH2, three solutions were prepared with absorbance values between 0.01 and 0.1 at the excitation wavelengths of 465 nm and 585 nm. Fluorescence spectra were recorded for each solution using a 10 mm pathlength cuvette, with excitation and emission ranges of 465 nm/500-700 nm and 585 nm/600-700 nm, respectively. Similar procedures were followed for the standard dye solutions, preparing them with absorbance between 0.01 and 0.1 at their respective excitation wavelengths. The quantum yield was calculated using the following equation.

$$\Phi_x = \Phi_{st}\left(\frac{A_{st}}{A_x} \times \frac{F_x}{F_{st}} \times \frac{\eta_x^2}{\eta_{st}^2}\right) \qquad (Eq.\ 2)$$

Where x and st stand for Mito-pH2 and standard respectively. Φ = Quantum Yield, A = absorbance value at λ_{max}. F = Integrated fluorescence intensity and η = refractive index.

Cell culture and confocal live-cell fluorescence imaging:
NIH-3T3 (ATCC catalog # CRL-1658) mouse embryonic fibroblast cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum

(FBS) with 1% Penicillin Streptomycin (10^3 U/mL) solution in a humidified incubator containing 5% CO_2 at 37 °C. Before the imaging experiment, cells were seeded in a 35mm glass bottom dish with 10mm micro-well, #1 cover glass fibronectin-coated confocal dishes. For experiments, cells were used between passages #6-18. The imaging of the live NIH-3T3 cells was carried out by a laser scanning confocal fluorescence microscope, using 60X oil immersion objective Olympus FV-3000- IX-81. The Mito-pH2 dye fluorescence images were recorded with band path 580–620 nm upon excitation at 561 nm.

Cytotoxicity using WST-8 assay and Trypan blue:

NIH-3T3 cells (100µl) were seeded at a density of 10,000 cells per well in a 96-well plate and incubated in a cell culture incubator at 37 °C with 5% CO_2 for 24 hours. Subsequently, the cells were carefully washed with DPBS to eliminate any dead or unattached cells. Following the wash, the cells were treated with Mito-pH2 dye at various concentrations (0, 100, 300, 500, 2000, 5000nM) and incubated for an additional 24 hours in the cell culture incubator. After this incubation period, the culture medium containing different concentrations of the dye was aspirated, and a mixture of 100µl of fresh culture media and 10µl of CCK-8 reagent was added to each well. The cells were then incubated for an additional two hours at 37 °C. After this incubation, absorbance at 450 nm was measured using a multimode microplate reader.

For Trypan blue assay NIH-3T3 cells were seeded at a density of 10,000 cells per well in a 96-well plate and incubated in a cell culture incubator at 37 °C with 5% CO2 for 24 hours. Subsequently, thecells were carefully washed with DPBS to eliminate any dead or unattached cells. After 24 hours of culturing cells were carefully washed and different concentration (100, 300, 500, 2000 and 5000 nM) of Mito-pH2 were added with fresh medium. Again cells were cultured for 24 hours and aspirated by PBS. 1 part of 0.4% trypan blue and 1-part cell suspension were mixed (dilution of cells). The mixture was incubated for 3 minutes at room temperature. A small volume (5µl) of the trypan blue-cell mixture was loaded onto a haemocytometer. Viable (unstained) and non-viable(stained) cells were counted under a binocular microscope. The percentage of viable cells was calculated as follows:

$$Viable\ cells\ (\%) = \frac{total\ no.\ of\ viable \frac{cells}{ml}}{total\ no.\ of \frac{cells}{ml}} X100$$

pK_a determination using absorbance spectroscopy:

$$pH = pK_a + log_{10}\frac{[In^-]}{[HIn]} \qquad (Eq.\ 3)$$

The above-mentioned Henderson-Hasselbalch (Eq. 3) used to calculate the pK_a of Mito-pH2 dye by measuring the absorbance intensities of the ratiometric dye at two different wavelengths. The Mito-pH2 dye exhibits absorbance at two distinct wavelengths, 465 nm (denoted by λ_1, protonated form) and 585 nm (denoted by λ_2, deprotonated form). The ratio of $[In^-]/[HIn]$ can be re-written as below[40]

$$\frac{[In^-]}{[HIn]} = \frac{A_{\lambda 2(measured)} - A_{\lambda 2(min)}}{A_{\lambda 1(measured)} - A_{\lambda 1(min)}} * \frac{A_{\lambda 1,\, lowest\ pH}}{A_{\lambda 2,highest\ pH}} \qquad (Eq.\ 4)$$

Insert the ratio of $[In^-]/[HIn]$ in Henderson-Hasselbalch equation (Eq. 3). Finally, the pH values are plotted against the $\log([In^-]/[HIn])$ values. The pK_a of the Mito-pH2 is the point at which the line intersects the pH axis.

Co-localisation study:

For the mitochondria co-localisation study, NIH-3T3 cells were co-stained and incubated for 30 mins at 37 °C inside the cell culture incubator (5% CO_2). The culture media containing Mito Tracker Green (MTG), a commercial mitochondria-specific dye (300 nM) and Mito-pH2 (500 nM). After washing two times with PBS, the fluorescence images were recorded respectively with band path 580–620 nm upon excitation at 561 nm (Mito-pH2 dye) and band path 500- 520 nm upon excitation at 488 nm (MTG). Pearson's correlation coefficient (PCC) was calculated using the JACoP plugin of ImageJ.

Image analysis of confocal micrographs:

For the quantification of mitochondria stained with Mito-pH2 in NIH3T3 cells using a confocal laser scanning microscope, a series of image processing steps were applied to obtain accurate measurements. We used our previously reported semi-automatic image analysis pipeline for quantifying mitochondrial fluorescence of mito-pH2. The workflow implemented[18] is summarized here:

1. Initial image conversion: The original 16-bit raw image was converted to an 8-bit image in ImageJ/Fiji.
2.Apply adaptive thresholding: Apply an adaptive threshold (plugin available in Fiji) to the 8-bit image. Choose the "Weighted Mean" method, and settings for block size and C-value adapted per set of images.
3. Binary image generation: The binary image was further processed by subtracting 254 from all pixel values, resulting in a binary mitochondria mask. This manipulation ensured that mitochondrial pixels were represented as 1, while the background was set to 0.
4. Region-of-interest (ROI) selection: To quantify the mitochondria, a manual ROI selection was performed. Using the Polygon Selection tool, the cell boundary was traced by creating a series of connected points. This ensured that the ROI enclosed the entire cell of interest.
5. Analyze particles within ROI: Go to "Analyze" > "Analyze Particles." In the dialog box, set the "Minimum size" to 5 and "Maximum size" to "Infinity" (to capture all potential mitochondria). Click "Add" to include identified particles in the ROI manager.
6. Transfer ROI to original Image: Switch back to the original 16-bit image for quantification purposes.
7. Mitochondria quantification: Transfer ROI to 16-bit image. Utilize the ROI manager to transfer the selected ROI containing mitochondria from the binary image to the original 16-bit image.
8. Measure the ROI: Click on the "Measure" button to obtain quantitative data on the selected ROI.

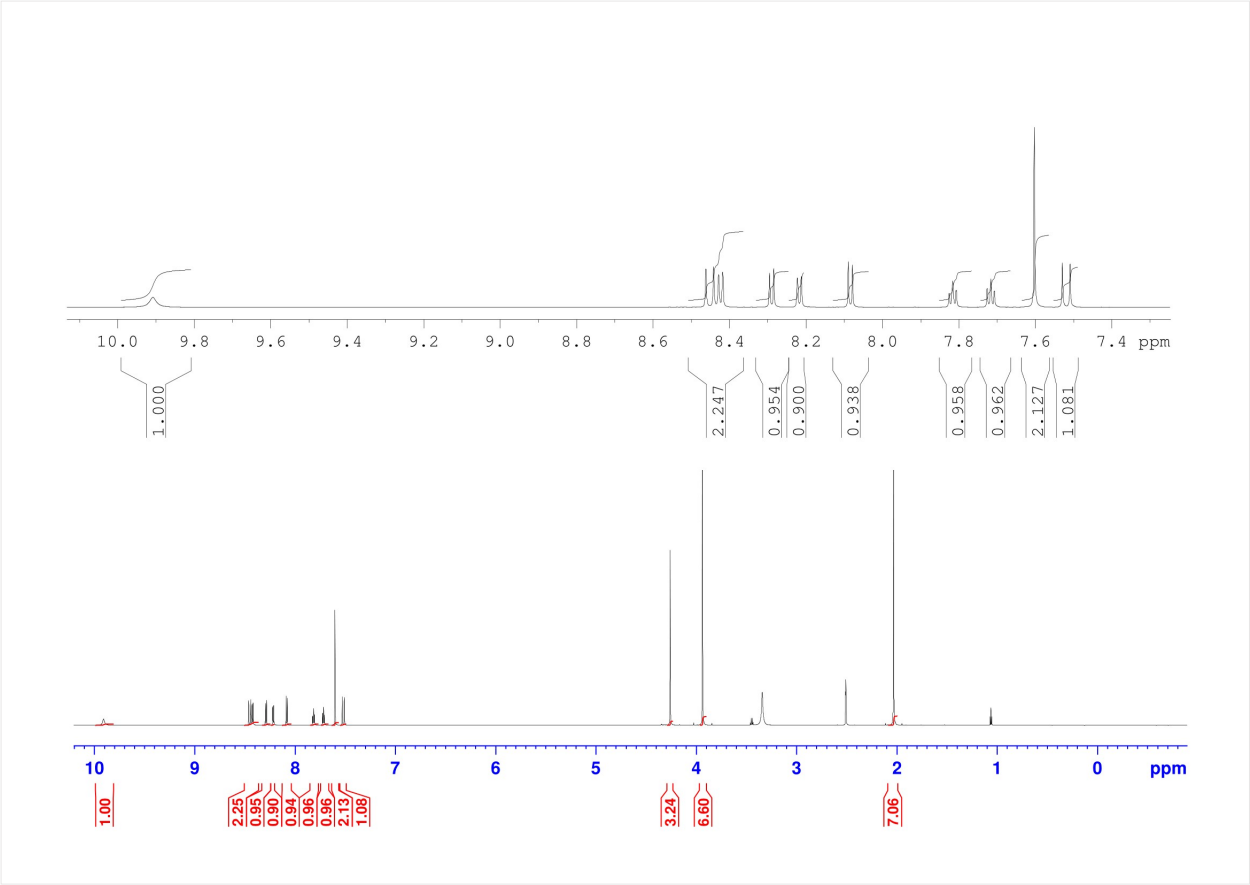


Figure S1. ^{1}H NMR spectrum of Mito-pH2 dye in DMSO-d_6 solution.

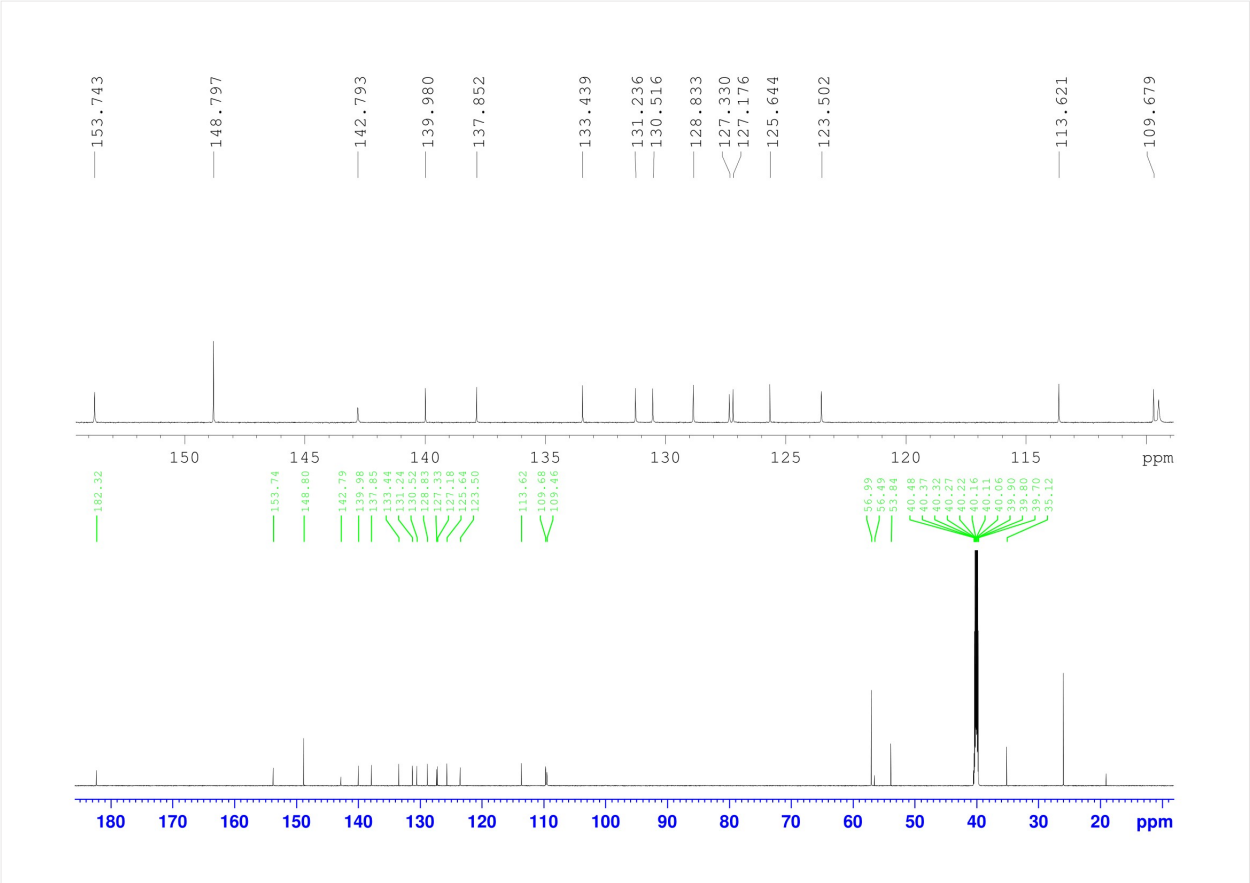
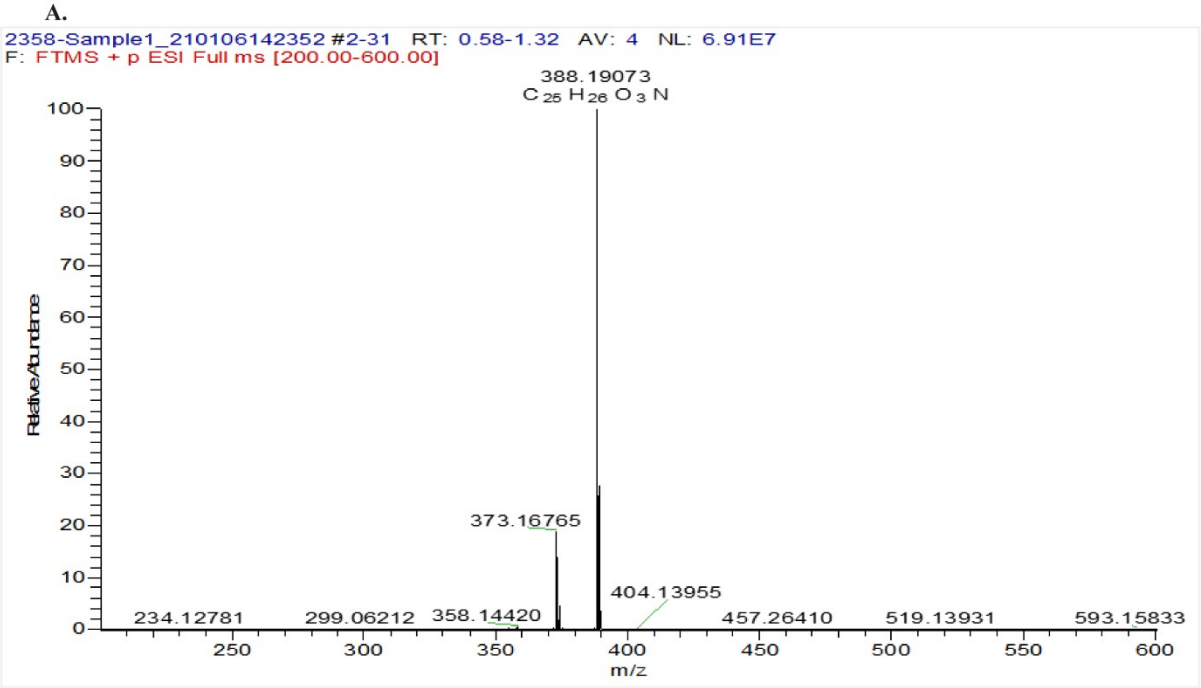


Figure S2. ^{13}C-NMR spectrum of Mito-pH2 dye in DMSO-d_6 solution.

A.



B.

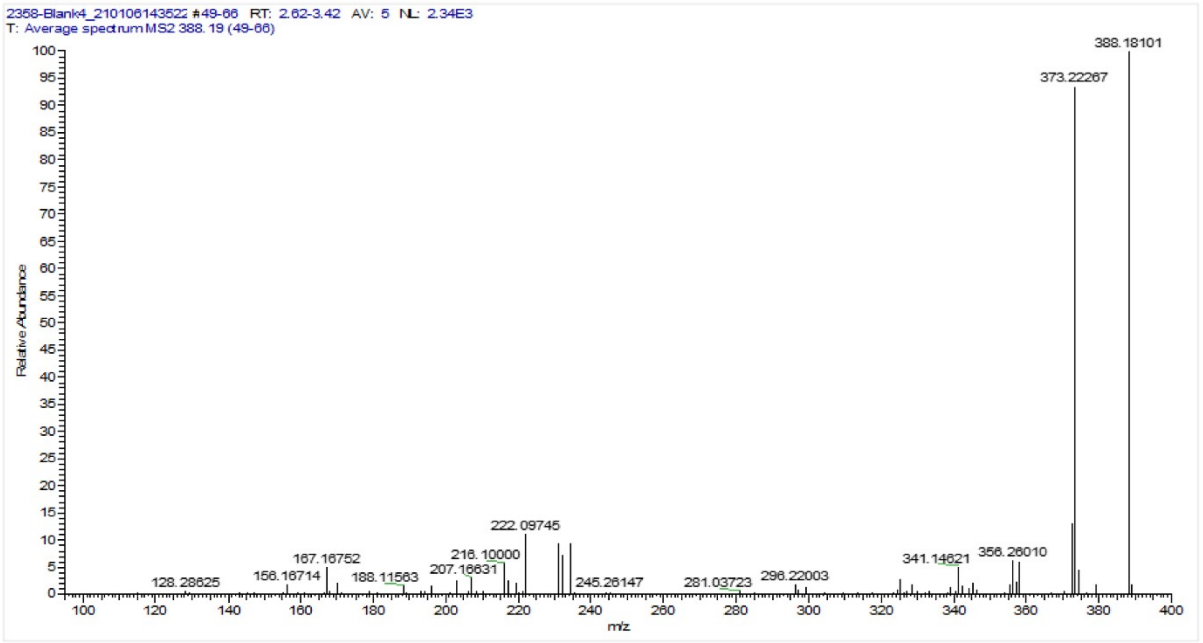


Figure S3.A) High-resolution electrospray ionisation mass spectrum of Mito-pH2 dye. B) MS/MS of Mito-pH2 dye.

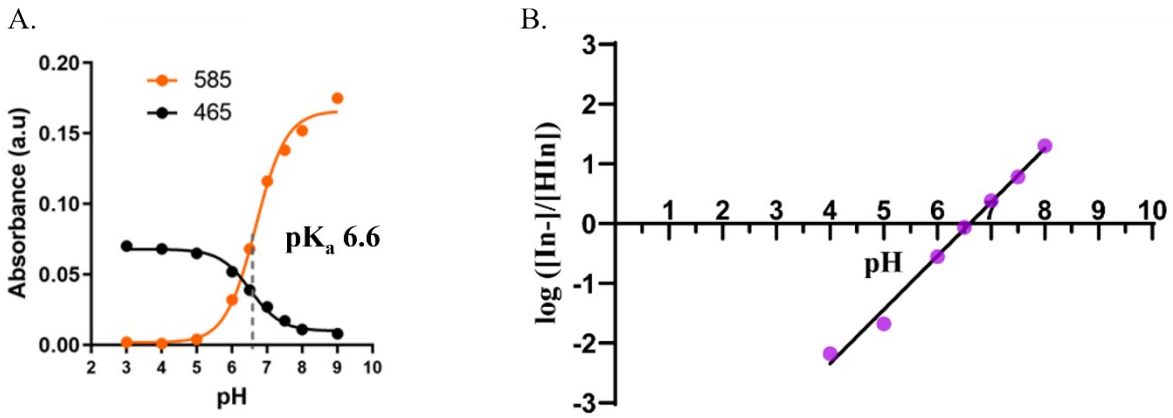


Figure S4. A) Absorbance intensities are used to calculate the pK_a value because absorbance is devoid of any excited state complexities. The solid black and orange curve was obtained by sigmoidal fitting. The pK_a value computed using the Handerson-Hasselbach equation yielded a value of 6.6. B) Plot of Mito-pH2 of pH vs. log [In^-/HIn]. The x-intercept is the pK_a 6.6 value of Mito-pH2 with R^2 = 0.99.

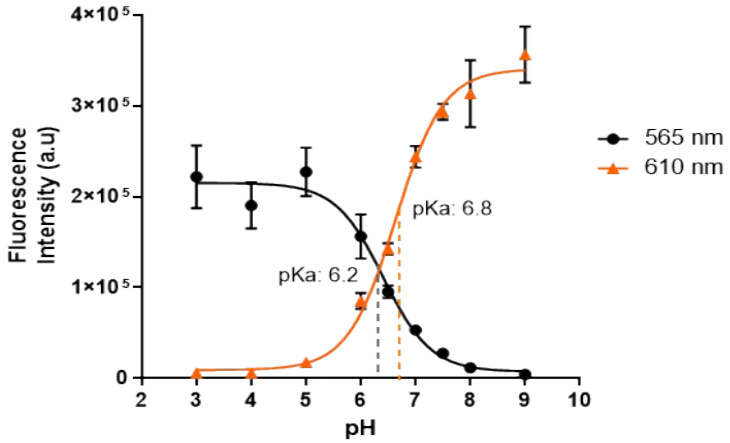


Figure S5: pKa calculations from fluorescence data. Shown here is the fluorescence intensities of the emission bands from the protonated (565 nm) and deprotonated (610 nm) species as a function of pH. pKa from the fluorescence data was calculated using the Handerson-Hasselbach equation.

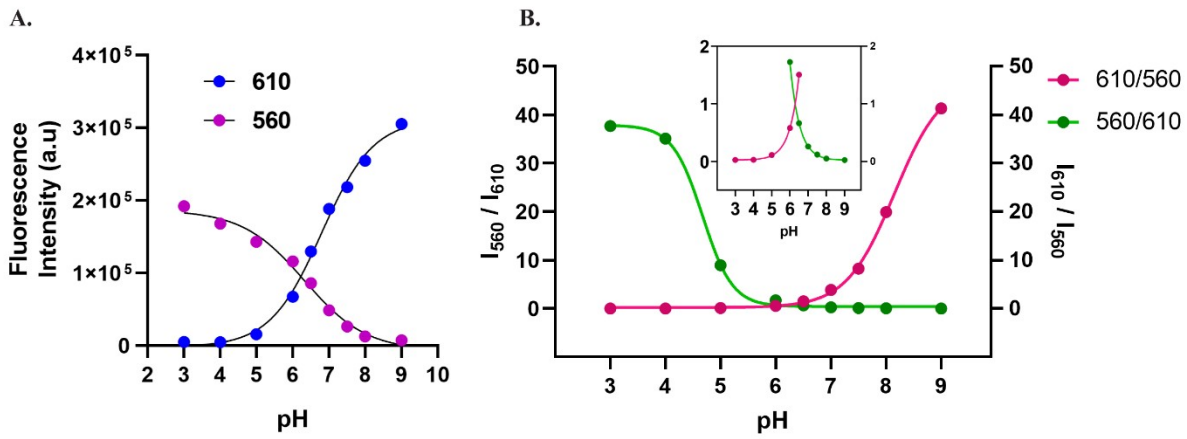


Figure S6. (A) Individual fluorescence intensities of Mito-pH2 at 560 nm (λ_{ex}=465 nm) and 610 nm (λ_{ex}=585 nm) are plotted against pH. (B) Ratiometric Fluorescence Signals vs pH, the left Y-axis represents the I_{560}/I_{610} ratio, while the right Y-axis represents the I_{610}/I_{560} ratio.

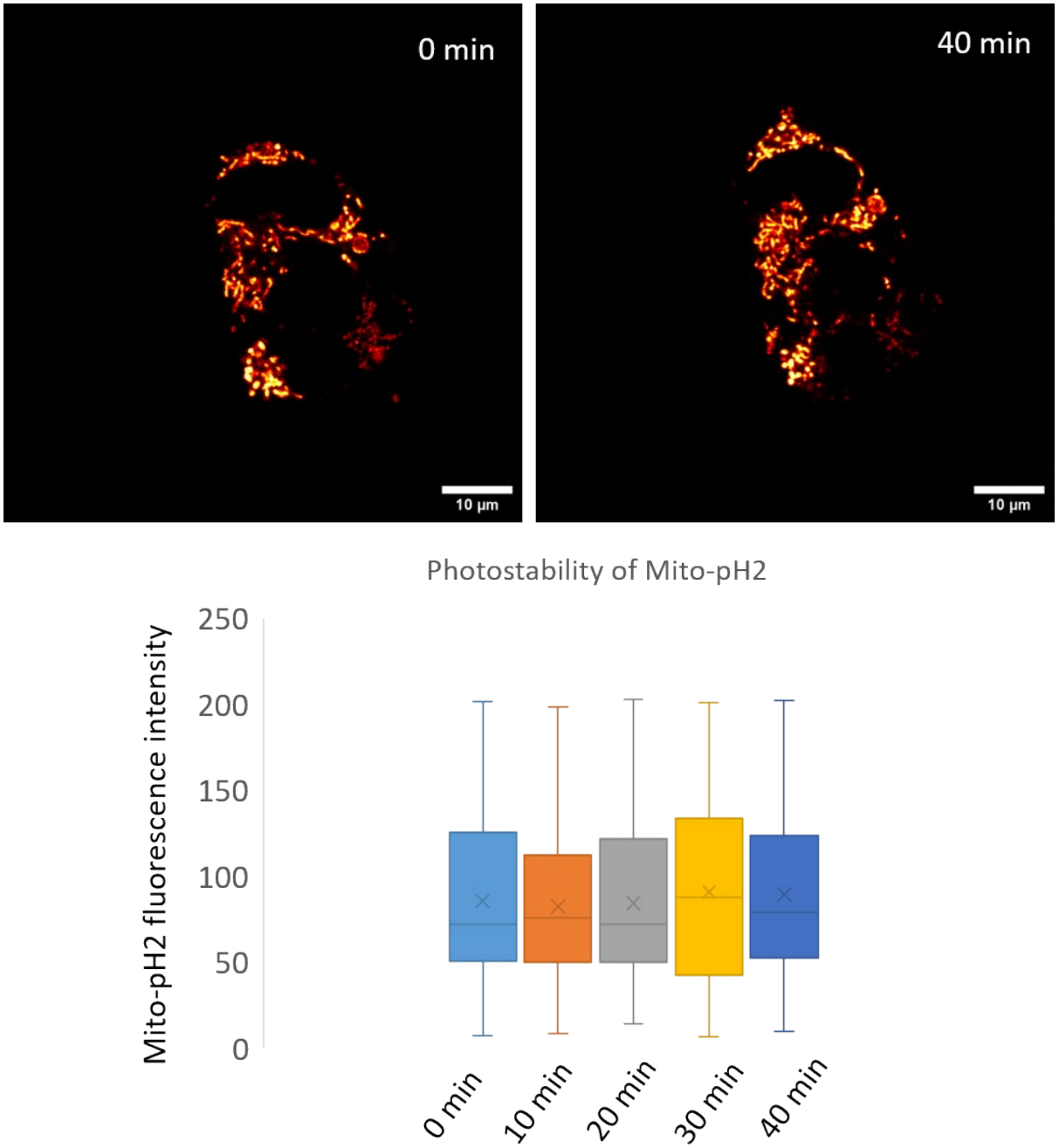


Figure S7: Dye photostability under imaging conditions. Upper panel shows two representative confocal micrographs of Mito-pH2 stained mitochondria from live NIH 3T3 cells at 0 min and after 40 min of exposure to 561 nm laser. No significant changes in dye fluorescence is seen, clearly indicating dye photostability under imaging conditions. Fluorescence intensity of Mito-pH2 stained cells at different time points i.e. 0,10, 20, 30 and 40 mins. Dye stained-cells are under continuous exposure of 561 nm laser for 40 min. Intensity of mitochondrial fluorescence was measured using Mitochondrial Analyzer, a plugin in ImageJ

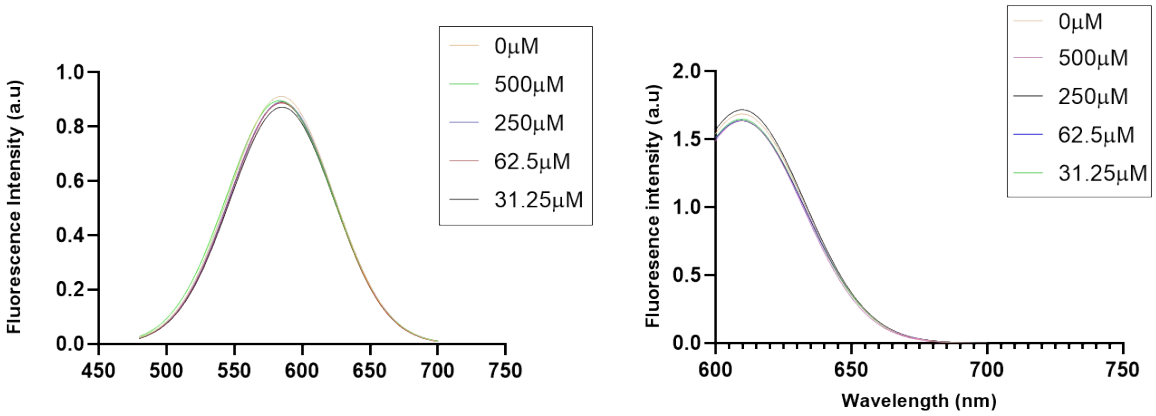


Figure S8: Emission spectra of Mito-pH2 dye solution with increasing concentrations (0 µM, 500 µM, 250 µM, 62.5 µM, 31.25 µM) of cysteine. The spectra were recorded at two excitation wavelengths: 465 nm (left) and 585 nm (right) specific for protonated and deprotonated species respectively.

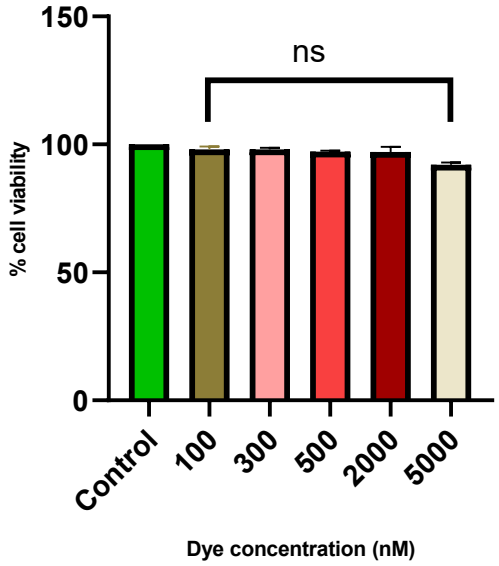


Figure S9: Cytocompatibility of Mito-pH2 using Trypan blue assay. The viability of NIH3T3 cells treated with various concentrations of Mito-pH2 assessed using the Trypan blue assay. Statistical analysis using a non-parametric t-test (n = 3) indicates no significant changes in cell viability with increasing dye concentrations up to 5 µM, with a significance level of $P > 0.05$.

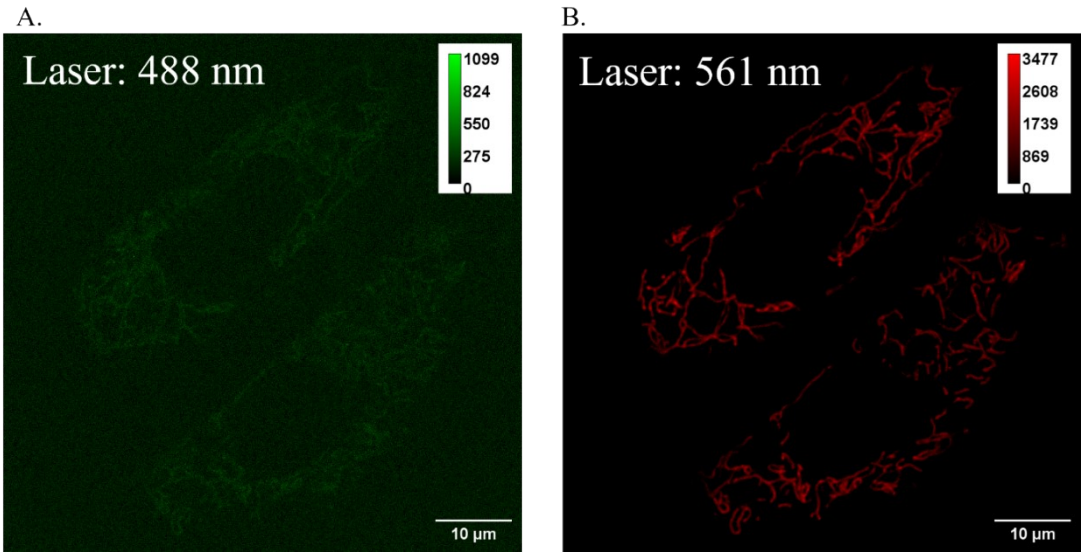


Figure S10. Confocal fluorescence images of NIH3T3 cells stained with 500 nM Mito-pH2 and treated with sodium acetate. (A) green channel: λ_{ex}= 488 nm, λ_{em}= 550-585 nm (B) red channel: λ_{ex}= 561 nm, λ_{em}= 580-620 nm.

References

1. B. Ehrenberg, V. Montana, M. D. Wei, J. P. Wuskell and L. M. Loew, *Biophys. J.*, 1988, **53**, 785–794.

2. P. Luo, M. Wang, W. Liu, L. Liu and P. Xu, *Molecules*, 2022, **27**, 7750.