Electronic Supplementary Information
A lysosome targetable fluorescent probe for endogenous imaging of hydrogen peroxide in living cells

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Procedure for cell imaging.
Instruments and experimental procedures

General information

All reagents were purchased from Aldrich and were used without further purification. HPLC grade Acetonitrile was used in UV-vis and fluorescence studies. UV-vis spectra were recorded on a SHIMADZU UV-2450 spectrophotometer, with a quartz cuvette (path length 1 cm). The cell holder was thermostatted at 25 °C. The fluorescence spectra were recorded with a SHIMADZU 5301 PC spectrofluorimeter. Elemental analysis was done using a Flash EA 1112 CHNS/O analyzer from Thermo Electron Corporation. $^1$H spectra were recorded on a JEOL-FT 400 MHz NMR Spectrometer using DMSO-$d_6$ as solvent. Data are reported as follows: chemical shift in ppm ($d$), multiplicity ($s$ = singlet, $d$ = doublet, $t$ = triplet, $m$ = multiplet, br = broad singlet), coupling constants $J$ (Hz), integration and interpretation. Fluorescence quantum yields were determined by using optically matching solution of fluorescein ($\Phi_{fr} = 0.95$ in aqueous NaOH) as standard at an excitation wavelength of 490 nm and quantum yield is calculated using the equation:

$$\Phi_s = \Phi_f \times \frac{1-10^{\Delta A_r}}{1-10^{\Delta A_s}} \times \frac{N^2_r}{N^2_s} \times \frac{D_s}{D_r}$$

$\Phi_s$ and $\Phi_f$ are the radiative quantum yields of sample and the reference respectively, $A_s$ and $A_r$ are the absorbance of the sample and the reference respectively, $D_s$ and $D_r$ the respective areas of emission for sample and reference. $L_s$ and $L_r$ are the lengths of the absorption cells of sample and reference respectively. $N_s$ and $N_r$ are the refractive indices of the sample and reference solutions (pure solvents were assumed respectively).

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**Procedure for sensing**

UV-vis and fluorescence titrations were performed on 5.0 µM solution of ligand in H₂O (DMSO used only solubilisation of probe LyNC; buffered with MES, pH = 5) mixture. Hydrogen peroxide (H₂O₂), tert-butyl hydroperoxide (TBHP), and hypochlorite (OCl⁻) were delivered from 30%, 70%, and 5% aqueous solutions, respectively. Hydroxyl radical (•OH) and tert-butoxy radical (•O'Bu) were generated by reaction of 100 µM Fe²⁺ with 100 µM H₂O₂ or 100 µM TBHP, respectively. Nitric oxide was used as DEA·NONOate which was source of NO). In titration experiments, each time a 3 ml solution of ligand was filled in a quartz cuvette (path length, 1 cm) and spectra were recorded after the addition of appropriate analyte.
Synthetic routes and characteristic data

Synthesis of probe **LyNC**

A mixture of 4-bromo-1,8-naphthalic anhydride (500 mg, 1.818 mmol) and dopamine hydrochloride (859 mg, 4.545 mmol) with few drops of Et₃N in Ethanol (10 mL) was stirred at reflux. After overnight, the reaction mixture was cooled down to room temperature, and the solvent was evaporated in vacuum. The crude mixture was purified by column chromatography and obtained as yellow solid (410 mg, 54% yield). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 8.81 (s, 1 H), 8.66 (s, 1 H), 8.56 (t, $J = 8.0$ Hz, 2 H), 8.33 (d, $J = 8.0$ Hz, 1 H), 8.21 (d, $J = 8.0$ Hz, 1 H), 7.98 (t, $J = 12$ Hz, 1 H), 6.67–6.61 (m, 2 H), 6.49 (d, $J = 8.0$ Hz, 1 H), 4.15 (t, $J = 8.0$ Hz, 2 H), 2.73 (t, $J = 8.0$ Hz, 2 H). ESI MS (ES+): m/z Calcd for C$_{20}$H$_{14}$BrNO$_4$: 433.9998 [M+Na]$^+$; found: 434.0097.

**Synthesis of probe LyNC:**

4-(2-Aminoethyl)morpholine (700 μL, 4.86 mmol) was added to a solution of compound 2 (200 mg, 0.486 mmol) in dimethyl sulfoxide (5 mL), and the reaction mixture was stirred overnight at 90–100 °C. After reaction complete, reaction mixture was cooled to room temperature and poured into cold water. The compound was purified by silica gel column chromatography get yellowish brown compound of **LyNC** (98 mg, 53% yield). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 8.61 (br, 1 H), 8.46 (m,1 H), 8.41 (m, 1 H), 8.24 (d, $J = 8$ Hz, 1 H), 7.76 (t, $J = 8$ Hz, 1 H), 6.78 (d, $J = 8$ Hz, 1 H), 6.61 (s, 1H), 6.59 (d, $J = 8$ Hz, 1 H), 6.44 (d, $J = 8$ Hz, 1 H), 4.1 (m, 2 H),
3.87 (br, 2 H), 3.56 (t, 4 H), 3.17 (br, 4 H), 2.67 (m, 4 H); \(^{13}\)C NMR (100 MHz, DMSO-d\(_6\)) \(\delta\) 164.33, 162.98, 157.57, 152.52, 150.76, 145.60, 143.92, 136.75, 136.59, 133.68, 131.44, 130.98, 129.54, 124.58, 121.95, 120.25, 119.44, 116.37, 115.80, 66.62, 61.93, 53.64, 50.04, 42.47, 33.50. ESI MS (ES+): m/z Calcd for C\(_{25}\)H\(_{27}\)N\(_3\)O\(_5\): 462.2023 [M+H]; found: 462.2054.

**Table S1:** Comparison with recent reports in the literature

<table>
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<tr>
<th>Journal</th>
<th>Fluorescence response and medium</th>
<th>Detection limit</th>
<th>Fast response</th>
<th>Exogenous Detection</th>
<th>Endogenous detection</th>
<th>Tissue imaging in variable depth</th>
<th>Detection In vivo models</th>
</tr>
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<tbody>
<tr>
<td>Present Manuscript</td>
<td>Turn-On and 0.5% DMSO used as co-solvent buffer with PBS, pH = 7.4</td>
<td>0.22 (\mu)M</td>
<td>Moderate</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td>Scientific Reports</td>
<td>Turn-On and In PBS (pH 7.4) solution containing 1% DMF</td>
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<td>NO</td>
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<td>Anal. Chem. 2016, 88, 1455–1461</td>
<td>Turn-On and in DMSO/phosphate buffer (1:99 v/v, 20 mM, pH 7.4)</td>
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<td>Adv. Mater. 2016, 28, 8755–8759</td>
<td>Ratiometric and in PBS buffer,</td>
<td>3.15X 10 (^{-7}) M</td>
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<td>Yes</td>
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<td>Chem. Sci., 2016, 7, 6153–6159</td>
<td>Turn-On and ratiometric (10 mM PBS, pH 8.0 and pH 7.4)</td>
<td>80 nM and 120 nM</td>
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<td>Anal. Chem., DOI: 10.1021/acs.analchem.6b00654</td>
<td>Turn-On and at pH 5.0 (acetate buffer)</td>
<td>0.23 (\mu)M</td>
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<td>Biosensors and Bioelectronics, 2016, 79, 237–243,</td>
<td>Turn-On and PBS buffer, pH 7.4, containing 50% DMF as a co-solvent.</td>
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<td>Chem. Commun., 2015, 51, 3641–3644</td>
<td>Ratiometric and 90% H(_2)O–THF</td>
<td>Yes</td>
<td>Yes</td>
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Figure S1: Mass spectra of compound 2
Figure S2: $^1$H NMR spectra of probe LyNC (400 MHz)

Figure S3: $^{13}$C NMR spectra of probe LyNC (100 MHz)
**Figure S4:** Mass spectra of probe LyNC

**Figure S5:** UV-vis spectra of probe LyNC in H₂O buffered with MES, pH = 5 with the addition of hydrogen peroxide (H₂O₂).
Figure S6: $^1$H NMR spectrum of probe LyNQ (aromatic region)

Figure S7: Mass spectrum of probe LyNQ
Figure S8: Fluorescence emission spectra of probe LyNC (5.0 µM) and presence of H$_2$O$_2$ (0-220 µM) in different pH values (pH 4.9 to 7.4).

Figure S9: Time dependent fluorescence enhancement of probe LyNC in H$_2$O buffered with PBS, pH = 7.4 with the addition of hydrogen peroxide (H$_2$O$_2$).
To determine the detection limit, fluorescence titration of probe LyNC with H₂O₂ was carried out by adding aliquots of H₂O₂ solution (in equiv.) and the fluorescence intensity as a function of H₂O₂ added was then plotted. From this graph the concentration at which there was a sharp change in the fluorescence intensity multiplied with the concentration of probe LyNC gave the detection limit. Equation used for calculating detection limit (DL):

\[ DL = CL \times CT \]

CL = Conc. of Ligand; CT = Conc. of Titrant at which change observed.

Detection limit (DL) of H₂O₂ with Probe LyNC:

Thus; \( DL = 5 \times 10^{-6} \times 0.045 \)

\[ = 0.225 \times 10^{-6} \text{ M} \]

\[ = 0.22 \mu \text{M} \]

**Figure S10:** Showing the fluorescence intensity of Probe LyNC at 536 nm as a function of H₂O₂ concentration (equiv.) in H₂O buffered with PBS, pH = 7.4, \( \lambda_{\text{ex}} = 450 \text{ nm} \).
Figure S11: Fluorescence bar diagram of probe LyNC (5.0 µM) upon the addition of biothiols (1 mM for GSH and 0.5 mM for cysteine and homocysteine). Slit width: 5/3; ($\lambda_{ex} = 450$ nm).

Figure S12: Absorption spectra of probe LyNC calculated with time-dependent density functional theory (TDDFT) at the B3LYP/6-31G level using Gaussian 09.
Figure S13: Frontier molecular orbital (MO) of LyNC and LyNQ calculated with density functional theory (DFT) at the B3LYP/6-31G level using Gaussian 09.
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Figure S15: Represents the cell viability of the C6 glioma cells treated with probe LyNC determined using MTT assay.
Figure S16: Histogram depicting the relative expression of the probe LyNC in different treatment groups in C6 cell lines.

Figure S17: Histogram depicting the relative expression of the probe LyNC in different treatment groups in BV-2 cell lines.
Figure S18: Representing the colocalization of the probe LyNC + H₂O₂ (100 μM) with (i) LysoTracker and (ii) MitoTracker in the C6 glioma cells.

Figure S19: Depicts the histogram representing the change in relative expression of the probe LyNC in different treatment groups of tissue imaging. Images were taken using A1R Nikon confocal laser scanning Microscopes.
**Figure S20:** (A) Optical Sectioning of the 40 μm rat hippocampal slice labelled with 5.0 μM of LyNC along Z-axis from top to bottom (10X magnification). (a) Brain sections were incubated with 5.0 μM probe for 1 hr. (b) Probe LyNC pre-treated sections were exposed to 3 mM H₂O₂ for another 1 hr exogenously. (c) Sections treated with 3 mM of inhibitor i.e., TEMPO for 1 hr followed by treatment with 5.0 μM probe for 1 hr. (B) The graph showing the change in fluorescent intensity with change in depth at the interval of 5.0 μm along the z axis among different treatment groups.
**Procedure of cell imaging**

**Cell culture and treatments**

C6 glioma and BV-2 microglial (Resident macrophage of brain) cell lines were obtained from NCCS, Pune and NBRC, Manesar, respectively. These Cell lines were maintained in DMEM medium supplemented with 10% FBS, 1X PSN antibiotic solution at 37°C in humid environment containing 5% CO₂. Cells were seeded in 12 well plates and 90 mm petri dishes at a cell density of 10,000 cells/ml for fluorescence imaging.

**Treatment and Fluorescence Detection in the Cells:** For the purpose of this study, six groups were chosen as follows for the fluorescence detection:

(I) Control group.

(II) Unstimulated cells exposed to probe LyNC (2.0 μM) for 60 min.

(III) Unstimulated cells treated with probe LyNC (2.0 μM) for 60 min followed by washing with 1X and exposed to H₂O₂ (100.0 μM) for 60 min.

(IV) For the detection of endogenous H₂O₂, LPS activated C6 glial cells (2μg/ml for 24 hrs) and BV-2 microglial cells (100 ng/ml for 24 hrs) were exposed to probe LyNC (2.0 μM) for 60 min.

**For Fluorescence Detection:** Firstly, cells were washed with 1X PBS thrice for 5 minutes each and fixed with acetone: methanol in 1:1 ratio for 10 min. After fixing cells were washed with 1X PBS for 5 min followed by mounting on the slides using antifading medium. Images were taken with A1R Nikon Laser Scanning Confocal microscope at 488 nm channel.

**MTT Assay**

Cytostatic activity of compounds was determined by using MTT assay which is based on the reduction of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, yellow in color) into formazan (blue color) by mitochondrial succinate dehydrogenase. C6 and BV2 cells (5000 cells/100uL/well) were incubated in 96 well plate for 24 hours. Cells were treated with 100 μL of different concentration of test compound for 30 minutes in CO₂ incubator. After 30 mins of treatment period, 100 μL of freshly prepared MTT solution was added in each well. Medium was removed after the incubation period of 2-4 hours followed by the addition of 200 μL of DMSO to dissolve the formazan crystals. Absorbance was taken at 595 nm by an ELISA Plate Reader (Biotek Synergy HT). Untreated cells were taken as control. All the experiments were performed in triplicate. Cytostatic activity of compounds was determined by using given
**Statistical Analysis**

Values are expressed as mean standard error of mean (SEM). SigmaStat for Windows (v3.5) was adopted to analyze the results by using a one-way ANOVA test to determine the significance of the mean values. Values of p<0.05 were considered to be statistically significant.

**Tissue Imaging**

Formal permission to conduct animal experiments was obtained from the Institutional Animal Ethical committee, Reg. No. of Animal house: 226/CPCSEA. All animal experimental protocols were performed in accordance with the guidelines of ‘Animal Care and Use’ laid down by Institutional Animal Ethical Committee, Guru Nanak Dev University. A Wistar strain rat was decapitated and its brain was carefully dissected. The freshly dissected brain was submerged in cryomatrix inside the mold and then snap-frozen in chilled isopentane for 5 min. Then, the cryomatrix-embedded brain was carefully removed from the mold, mounted, and thick coronal sections (40 μm) were cut directly on the microscopic glass slide by using a freezing cryomicrotome. For the analysis, the sections were divided into four groups: (1) the brain sections were incubated with probe LyNC alone (5 μM) for 1 h at 37 °C; (2) the sections were pretreated with probe LyNC and then exposed to an exogenous H₂O₂ source for 1 h; (3) the sections were pretreated with probe LyNC and then treated with TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl, ROS) scavenger for 1 h. After treatment, images were recorded by using an A1R Nikon Laser Scanning Confocal microscope at 10X magnification at the 488 nm channel. Optical sectioning was performed to observe the changes in fluorescent intensity on changing the depth along the z axis at thickness intervals of 5.0 μm.

**Imaging of Nematodes**

For the purpose of this study, three groups were chosen as follows for the fluorescence detection: (I) Nematodes exposed to probe LyNC (10.0 μM) for 3 hrs.
(II) Nematodes treated with probe LyNC (10.0 μM) for 3hrs followed by washing with 1X and exposed to H₂O₂ (0.5 and 1 mM) for 6 hrs.

(III) TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl, ROS scavenger) (1.0 mM) treated nematodes are exposed with probe LyNC (10.0 μM) followed by washing with 1X buffer and then exposed to H₂O₂ (0.5 mM) for 6 hrs.

Images were taken with A1R Nikon Laser Scanning Confocal microscope at 488 nm channel.