Supporting Information

Mitochondria-targeted two-photon fluorogenic probe for dual-imaging viscosity and H$_2$O$_2$ level in Parkinson's disease models

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General Procedures

Unless otherwise noted, all chemicals were purchased from commercial suppliers and used without further purification. All reactions were carried out under a dry nitrogen protection. Reaction progress was monitored by TLC on pre-coated silica plates and spots were visualized by UV light or iodine. Silica gel 60 (200-300 mesh, Silicycle) was used for column chromatography. N, N-Dimethylformamide (DMF) and Dichloromethane (CH$_2$Cl$_2$, DCM) were distilled over CaH$_2$. Petroleum ether (PE, 60-90°C), DCM, Ethyl acetate (EA) and Methanol (MeOH) were used as eluents for Flash column chromatography with Merck silica gel (0.040-0.063). $^1$H and $^{13}$C NMR spectra were acquired over Bruker DRX500 spectrometer in CDCl$_3$ or DMSO-$d_6$ at 25°C. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane (Si(CH$_3$)$_4$ = 0.00 ppm) or residual solvent peaks (CDCl$_3$ = 7.26 ppm, DMSO-$d_6$ = 2.50 ppm, MeOD = 3.31 ppm). $^1$H NMR coupling constants ($J$) are reported in Hertz (Hz) and multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublet). Mass spectra were recorded on a Finnigan LCQ mass spectrometer, a Shimadzu LC-IT-TOF spectrometer. Absorption spectra and fluorescence spectra were recorded using BioTek Cytation 5 Cell Imaging Multi-Mode Reader. All the measurements were performed at room temperature. All images were acquired on Zeiss LSM880 NLO (2+1 with BIG) Confocal Microscope System equipped with objective LD C-Apochromat 63x/1.15 W Corr M27, cell incubator with temperature control resolution ±0.1°C, 405 nm Diode laser, Argon ion laser (458, 488 and 514 nm), HeNe laser (543 and 594 nm), and a 633 nm laser, with 8 channels AOTF for simultaneous control of 8 laser lines. Ultrapure water was used to prepare all aqueous solutions.

Experimental Section

\[
\begin{align*}
\text{HO} & \quad \text{Br} \quad \text{N} \quad \text{Br} \\
\text{N} & \quad \text{CN} \\
1 & \quad \text{Br} \quad \text{N} \quad \text{CN} \\
2 & \quad \text{CHO} \quad \text{CN} \\
3 & \quad \text{pre-Mito} \\
\end{align*}
\]

Scheme S1. Compounds 1-3 were synthesized according to reported literatures.$^{[1, 2]}$ Synthesized road of pre-Mito.

Reagents and conditions. i) Na$_2$S$_2$O$_5$, Me$_2$NH, H$_2$O, 165°C, 6 h, 80%; ii) CuCN, pyridine, 220°C 2 h, 70%; iii) DIBAL, toluene, -78°C, 30 min; room temperature, 4 h, 75%; iv) potassium tert-butoxide, dry DMF, 4-picline, 70%.

The response of Mito-LX to viscosity changes

The solvents were obtained by mixing methanol-glycerol systems in different proportions. Measurement was carried out with a NDJ-8S rotational viscometer, and each viscosity value was recorded. The solutions of Mito-LX with different viscosity were prepared by adding the stock
solution (1.0 mM) to 10 mL of solvent mixture (methanol-glycerol solvent systems) to obtain the final concentration of the Mito-LX (10 μM). These solutions were sonicated for 5 min to eliminate air bubbles. After standing for 1 h at a constant temperature, the solutions were measured using a microplate reader (BioTek, USA) with an excitation of 485 nm.

**The effect of pH**

Different values of pH (2 to 12) of PBS buffer were prepared with different concentrations of hydrochloric acid and sodium hydroxide solution with ultrapure water. The fluorescence emission spectrum at 590 nm of the probe Mito-LX (10 μM) with/without 10 equiv H₂O₂ were measured after incubating for 2 h in various pH solutions with an excitation of 420 nm.

**Preparation of various ROS and RNS species**

ONOO⁻: To a vigorously stirred solution of NaNO₂ (0.6 M, 10 mL) and H₂O₂ (0.7 M, 10 mL) in deionized H₂O at 0°C was added HCl (0.6 M, 10 mL), immediately followed by the rapid addition of NaOH (1.5 M, 20 mL). Excess hydrogen peroxide was removed by passing the solution through a short column of MnO₂. The concentration of ONOO⁻ was determined by UV analysis with the extinction coefficient at 302 nm (ε = 1670 M⁻¹ cm⁻¹). Aliquots of the solution were stored at -20°C for use.

NO: A solution of the H₂SO₄ (3.6 M) was added dropwise into a stirred solution of NaNO₂ (7.3 M). The emitted gas was allowed to pass through a solution of NaOH (2 M) first and then deionized H₂O to make a saturated NO solution of 2.0 mM.

¹O₂: NaMoO₄ (10 mM) and H₂O₂ (10 mM) were prepared in PBS (10 mM, pH 7.4). Equal aliquots of these solutions were then mixed to yield ¹O₂ of 5 mM.

H₂O₂ and ClO⁻: H₂O₂ and NaClO solution were prepared by diluting commercial H₂O₂ and NaClO solutions with PBS (10 mM, pH 7.4) to make 10 mM stock solutions.

•OH: •OH was generated by Fenton reaction. To a solution of H₂O₂ (1.0 mM, 1.0 mL) in PBS (10 mM, pH 7.4) was added FeSO₄ solution (1.0 mM, 100 µL) at ambient temperature (stock solution 0.1 mM).

ROO•: ROO• was generated from 2,2'-azobis(2-amidinopropane) dihydrochloride, which was dissolved in PBS (10 mM, pH 7.4) 1 h before use to make a stock solution of 10 mM.

**Quantum Yield and two-photon absorption cross-section measurement**

Quantum yield was determined using courmain-6 as a standard. Fluorescence quantum yield was determined by the following equation:

\[
\Phi_X = \frac{\Phi_{ST} \cdot A_{ST} \cdot F_X \cdot \eta X^2}{A_X \cdot F_{ST} \cdot \eta ST^2}
\]

(1)

Φ is the quantum yield; A is the absorbance at the excitation wavelength (A was kept at ≤0.05 during fluorescence measurements to avoid self-quenching), F is the fluorescence intensity at the excitation
wavelength; $\eta$ is the refractive index of the solvent. The subscripts ST and X refer to the standard and unknown respectively. Quantum yield of coumarin-6 is 0.8 in ethanol. $^4\Phi$ of Mito-LX was 0.10 and $\Phi$ of pre-Mito was 0.49 in PBS (contains 30% DMSO with $pH = 8.0$).

The two-photon cross-section ($\sigma$) and action cross-section ($\Phi \sigma$) was measured by using the two-photon induced fluorescence measurement technique with the following equation.

$$\sigma_s = \sigma_r \frac{F_s \Phi_s G \eta_r}{F_r \Phi_r C \eta_s}$$  \hspace{1cm} (2)

The subscripts “s” and “r” stand for the sample and reference molecules respectively. $F$ is the integrated fluorescence intensities measured at the same power of the excitation beam. $\Phi$ is the fluorescence quantum yield. $\eta$ is refractive index. The number density of the molecules in the solution was denoted as $C$. $\sigma_r$ is the two-photon cross section of the reference molecule. The two-photon cross sections ($\Phi$) of Mito-LX and pre-Mito were calculated to be 854.89 GM and 1166.99 GM, respectively.

![Fig. S1. UV-vis absorption of Mito-LX (10 μM) in the solution with different ratios of methanol (M)-glycerin (G).](image1)

![Fig. S2. Fluorescence intensity at 730 nm of Mito-LX (10 μM) in 90% glycerin/methanol with different pH values. $\lambda_{ex} = 480$ nm.](image2)
Fig. S3. Fluorescence spectra of Mito-LX (10 μM) in 10% glycerin/methanol with different temperatures. λ<sub>ex</sub> = 480 nm.

Fig. S4. Fluorescence intensity at 730 nm of Mito-LX (10 μM) in PBS buffer (containing 30% DMSO) with different pH values. λ<sub>ex</sub> = 480 nm.

Fig. S5. Fluorescence spectra of Mito-LX (10 μM) in PBS buffer (containing 30% DMSO, pH = 8.0) with different temperatures. λ<sub>ex</sub> = 480 nm.
**Fig. S6.** Fluorescence intensity at 730 nm of pre-Mito (10 μM) and Mito-LX (10 μM) in the solution with different ratios of methanol (M)-glycerin (G), $\lambda_{ex} = 480$ nm.

**Fig. S7.** Fluorescence intensity of pre-Mito (10 μM) in the solution with different ratios of methanol (M)-glycerin (G), $\lambda_{ex} = 380$ nm.

**Fig. S8.** Fluorescence intensity at 585 nm of pre-Mito (10 μM) in PBS buffer (containing 30% DMSO) with different pH values. $\lambda_{ex} = 380$ nm.
Fig. S9. Fluorescence spectra of pre-Mito (10 μM) in different temperature of PBS buffer (containing 30% DMSO, pH = 8.0). $\lambda_{ex} = 420$ nm.

Fig. S10. DFT optimized structure of Mito-LX (A, B) and pre-Mito (C, D) in ground state and excited state. In the ball-and-stick representation, hydrogen, carbon, nitrogen, oxygen and boron atoms are colored in white, gray, blue, red and pink, respectively.

The method for determining the limit of detection (LOD)

First the calibration curve was obtained from the plot of fluorescence intensity at 585 nm, as a function of the $\text{H}_2\text{O}_2$ concentration. The regression curve equation was then obtained for the lower concentration part.

$$I_{585} = 117.49 + 3757.7[H_2O_2] \quad (R = 0.9863)$$

The detection limit = $3 \times \sigma / k$

Where $k$ is the slope of the curve equation, and $\sigma$ represents the standard deviation for the probe Mito-LX solution’s fluorescence intensity in the absence of $\text{H}_2\text{O}_2$.\textsuperscript{[5]}

$$\text{LOD} = 3 \times 6.2328 / 3757.7 = 4.98 \text{ nM}$$
Fig. S11. Multi-recorded fluorescence spectra of blank measurement. Insert: the data of standard deviation (σ) of blank measurement from fluorescence spectra. λ<sub>ex</sub> = 420 nm.

Fig. S12. UV-vis absorption of Mito-LX (10 μM) with H<sub>2</sub>O<sub>2</sub> (100 μM) in different time in PBS buffer (containing 30%DMSO, pH = 8.0).

Fig. S13. Effect of pH on the fluorescence intensity at 585 nm of the Mito-LX (10 μM) in the absence and presence of H<sub>2</sub>O<sub>2</sub>. λ<sub>ex</sub> = 420 nm.

(B) Fluorescence intensities of Mito-LX (10 µM) treated with various species in the presence of H$_2$O$_2$ in PBS buffer. PBS buffer contains 30% DMSO with the pH = 8; $\lambda_{ex}/\lambda_{em} = 420/585$ nm.

Fig. S15. (A) TP fluorescence intensity at 720 nm of Mito-LX (2 µM) in different ratios of methanol (M) and glycerol (G) solution. $\lambda_{ex} = 800$ nm; (B) TP fluorescence intensity at 585 nm of Mito-LX (2 µM) w/o H$_2$O$_2$ and the reference (Flu1$^{[12]}$, 10 µM). $\lambda_{ex} = 760$ nm.
Table S1. Properties of the representative developed fluorescent viscosity and H$_2$O$_2$ probes and the probe Mito-LX.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Probe</th>
<th>Dual channel</th>
<th>λ (nm)</th>
<th>Detection limit (M)</th>
<th>Emission shift (nm)</th>
<th>Imaging mode</th>
<th>Imaging application</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>This work</td>
<td>Mito-LX</td>
<td>H$_2$O$_2$ and viscosity</td>
<td>420/585</td>
<td>$4.97 \times 10^{-9}$</td>
<td>165/ 245</td>
<td>TP</td>
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<tr>
<td>1</td>
<td>Mito-VH</td>
<td>H$_2$O$_2$ and viscosity</td>
<td>400/510</td>
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<td>110/ 107</td>
<td>OP</td>
<td>HeLa cells</td>
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<tr>
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<td>Lyso-HP</td>
<td>H$_2$O$_2$</td>
<td>474/550</td>
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<td>76</td>
<td>TP</td>
<td>HeLa cells, tissue</td>
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<tr>
<td>3</td>
<td>TPNR-H$_2$O$_2$</td>
<td>H$_2$O$_2$</td>
<td>560/860</td>
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<td>300</td>
<td>TP</td>
<td>MCF-7, RAW 264.7 cells,</td>
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<td>Lyso-B</td>
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<td>36</td>
<td>TP</td>
<td>HepG2 cells, HeLa cells</td>
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<tr>
<td>5</td>
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<td>--</td>
<td>70</td>
<td>TP</td>
<td>HeLa cells, rat liver, zebrafishes</td>
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<tr>
<td>6</td>
<td>MHC-V1</td>
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<td>--</td>
<td>34</td>
<td>OP</td>
<td>HeLa cells</td>
</tr>
</tbody>
</table>

Noted: OP, one-photon; TP, two-photon.

Fig. S16. The proposed mechanism of Mito-LX in the detection of H$_2$O$_2$. $^1$H NMR spectra of Mito-LX (14 mM) obtained during the titration with H$_2$O$_2$ (140 mM) in CDCl$_3$. (Blue) $^1$H NMR spectrum of Mito-LX; (Green) $^1$H NMR spectrum of Mito-LX treated with H$_2$O$_2$ for 2 h and then purification; (Red) $^1$H NMR spectrum of pure pre-Mito.
Fig. S17. Cell viability of HepG2 incubated with *Mito-LX* at different concentrations.

Fig. S18. Fluorescence spectra of probe *Mito-LX* (10 µM) in the absence and presence of nystatin (10 µM) in PBS buffer (pH = 8.0, containing 30% DMSO) buffer at 37°C for 30 min. $\lambda_{ex} = 480$ nm.

Fig. S19. (A) Confocal fluorescence images of live zebrafishes incubated with *Mito-LX* (10 µM) without/with NAC (2 µM). (B) Relative fluorescence intensity of images (A). $\lambda_{ex} = 405$ nm for OPFM, $\lambda_{ex} = 760$ nm for TPFM; PMT = 550-620 nm; Scale bar = 400 µm.
Fig. S20. (A) Confocal fluorescence images of live zebrafishes incubated with Mito-LX (10 μM). (B) Relative fluorescence intensity of images (A). Green channel: $\lambda_{ex} = 405$ nm for OP, $\lambda_{ex} = 760$ nm for TPFM; PMT = 550-620 nm; Red channel: $\lambda_{ex} = 488$ nm for OPFM, $\lambda_{ex} = 800$ nm for TP; PMT = 710-740 nm, Scale bar = 400 μm.

Fig. S21. Z-scan of two-photon fluorescence images of live zebrafish pretreated with Mito-LX (10 μM) incubation at depths of approximately 0 to 280 μm with a magnification of 10 μm. $\lambda_{ex} = 800$ nm for TPFM; PMT = 710-750 nm; Scale bar = 400 μm.
**Fig. S22.** $^1$H NMR spectrum of compound 1.

**Fig. S23.** $^1$H NMR spectrum of compound 2.
Fig. S24. $^1$H NMR spectrum of compound 3.

Fig. S25. $^1$H NMR spectrum of pre-Mito.
Fig. S26. $^{13}$C NMR spectrum of pre-Mito.

Fig. S27. $^1$H NMR spectrum of Mito-LX.
Fig. S28. $^{13}$C NMR spectrum of Mito-LX.

Fig. S29. HRMS of Mito-LX.
Reference