Electronic Supplementary Information for:

Red turn-on fluorescent probe reveals elevated H_2O_2 in cell anesthesia

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1. Experimental.

Materials and Reagents. 1,1,2-Trimethyl-1h-benzo [e] indole and 4-formylphenylboronic acid were purchased from Energy Chemical. All other chemical reagents are analytical grade. Ultrapure water used in experiment purified in a Milli-Q reference system (Millipore). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), penicillin and streptomycin were bought from Solarbio Co. Ltd, China. Fetal bovine serum was purchased from Zhejiang Tianhang Biotechnology Co. Ltd, China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Beyotime Biotechnology, China. Bicinchoninic acid (BCA) kit was purchased from Nanjing Jiancheng Insititute of Biotechnology, China. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and polyvinylidene fluoride (PVDF) membranes were purchased from Thermo Fisher Scientific, USA. NOX2/gp91 phox Rabbit pAb was bought from Abclonal, Wuhan, China. Anti-β-actin-loading was purchased from Abcam (Cambridge, MA). Goat anti-rabbit IgG H&L (HRP) was bought from Abbkine Scientific Co., Ltd, China.

Characterization Methods. ^{1}H NMR and ^{13}C NMR spectra were measured on Bruker Avance III 400 MHz spectrometer. High resolution electrospray ionization mass spectra (HR-ESI-MS) were measured on AB Sciex TripleTOF 4600 instrument (AB SCIEX, USA). Fluorescence spectra were measured on Hitachi F-7000 spectrofluorimeter (Tokyo, Japan). Ultraviolet absorption spectra were measured on T10CS spectrophotometer (Beijing Puxi, China). The fluorescence images of cells were obtained by Leica TCS SP8 confocal laser scanning microscope (Germany) with a 63×oil objective lens and an optical section of 0.5 μm. The image processing was acquired by the software of the corresponding instrument (Leica

Application Suite). The absorbance for MTT analysis was measured on a multifunctional microplate reader (Multiskan go, Thermo, USA).

Synthesis of Probe LJ-1.

Compound **2**: It was synthesized by borrowing from our previous method with slight modifications (*Chem. Commun.*, 2019, *55*, 7410; *Chem. Commun.*, 2021, *57*, 4376). Firstly, 1,1,2-trimethyl-1H-benz[e]indole (**1**, 500.0 mg, 2.40 mmol) and methyl iodide (200 μL, 3.21 mmol) were dissolved in acetonitrile (10 mL) and refluxed at 82 ℃ for 12 h. After the reaction, it was cooled to room temperature and the organic layer was removed under vacuum. Then, the crude product was separated with CH_2Cl_2/MeOH (v/v, 50:1) by silica gel column chromatography to obtain compound **2** (yield: 70%).

Probe **LJ-1**: Compound **2** (250 mg, 1.07 mmol), 4-formylphenylboronic acid (**3**, 187 mg, 1.29 mmol) and sodium acetate (375.5 mg, 1.61 mmol) were dissolved in acetic anhydride (10 mL) and stirred for 2 h at 80 °C. After completion of the reaction, 40 mL of dichloromethane was added, and sufficient volume of water (40 mL × 3) was added for extraction. The organic phase was collected and dried with anhydrous magnesium sulfate to remove the residual water, and concentrated under reduced pressure. The crude product was separated by CH_2Cl_2/MeOH column chromatography (v/v, 50:1) to obtain compound **4** as yellow solid (yield: 56%). The ^{1}H NMR, ^{13}C NMR spectra and HRMS of **LJ-1** are shown in Figures S1, S2 and S3 respectively. ^{1}H NMR (400 MHz, CD_3OD, 298 K): δ 8.50 (d, J = 16.4 Hz, 1H), 8.42 (d, J = 8.4 Hz, 1H), 8.25 (d, J = 8.8 Hz, 1H), 8.17 (d, J = 8.2 Hz, 1H), 8.07-7.99 (m,3H), 7.83-7.71 (m, 5H), 4.33 (s, 3H), 2.10 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d_6, 298 K): δ 183.23, 152.29, 139.91, 138.78, 136.26, 135.16, 133.79, 131.38, 130.51, 129.54, 128.93, 127.76, 127.11, 123.71, 113.88, 113.64, 54.40,

35.79, 25.46. HR-ESI-MS (m/z) calcd for $C_{23}H_{23}BNO_2^+$ $[M]^+$: 356.1816, found 356.1770 (Fig. S3).

General Procedure for Spectroscopic Detection of H_2O_2. The stock solution of probe was 1 mM dissolved in DMSO. The final test mixed solution contained probe stock solution (10 μL) and PBS buffer (10 mM, pH 7.4, 990 μL). The fluorescence intensity was measured at λ_{ex} = 515 nm and the emission wavelength was 570 nm. The voltage was 700V and the emission slit widths were 5 nm. The selectivity testing solutions were prepared by dissolving or diluting each of them in deionized water. Peroxynitrite ($ONOO^-$) was produced by the reaction of $NaNO_2$ with hydrogen peroxide in an acidic solution. Singlet oxygen ($^1O_2$) was obtained by the reaction of NaClO with H_2O_2. The reaction of Fe^{2+} with H_2O_2 generated Hydroxyl radical (·OH). Superoxide ($O_2^{\bullet-}$) stock solution was prepared by dissolving potassium superoxide in DMSO.

Determination of the detection limit. The limit of detection (LOD) was calculated based on the following equation:

$$LOD = 3\sigma/k$$

Where σ represents the standard deviation and k represents the slope of the titration spectra curve among the limited range.

Cell culture. PC12 cells were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences and cultured in DMEM supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, 10% FBS, and stayed in an incubator with an atmosphere of 10% CO_2 /90% air at 37°C.

MTT assay. For the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, PC12 cells (1×10^4 cells/well) were seeded in 96-well plates and cultured for 24 h. Then,

except control group, cells were incubated with the probe with final concentrations of 5, 10, 20, 40, 80, 160 μM for another 24 h. Then the cell viability was detected. Briefly, 20 μl MTT solution of 5 mg/mL was mixed with PBS, and added into each well and incubated for another 4 h. Then DMEM in each well was discarded and 150 μL DMSO was added. To fully dissolve the crystallization, plates were shaken for 10 minutes. The optical density (OD) of each well was measured at 490 nm wavelength on the microplate reader.

Calculation of inhibition rate: inhibition rate (%)=(1 - Mean OD value of each drug group/ Mean OD value of control group)×100%. The experiment was repeated for 6 times, and the half inhibition concentration (IC_{50}) was calculated.

Measurement of H_2O_2 in vivo. In this study, the concentration of ketamine was selected according to references (Daiying Zuo, Feng Sun, Jiahui Cui, Yumiao Liu, Zi Liu, Xuejiao Zhou, Zengqiang Li & Yingliang Wu, *Scientific Reports*, 2017, 5; 7(1): 10523; Xue-song Wang, Long-cheng Li, Xue Zhang, Jin Gao, *Human and Experimental Toxicology*, 2021; 40: S519-S529). Firstly, the action of different concentrations of ketamine on the release of H_2O_2 was detected. PC12 cells were divided into 3 groups: control group (same volume of saline), ketamine groups (final concentrations of 10 and 100 μM respectively). Cells were seeded into laser confocal dishes and added different concentration of ketamine after 24 h. Then cells were cultured for another 12 h. The probe solution (final concentration was 10 μM and prepared with serum-free medium) was added 40 min before observation. Cells were washed three times with PBS, and the relative amount of cell fluorescence was observed immediately with a laser confocal microscope, then the relative fluorescence intensity was analyzed.

Secondly, effect of different action duration of ketamine on the release of H_2O_2 was detected.

According to the previous results, the dose of ketamine was designed at a concentration of 10 and 100 μM, and the action duration was 3, 6 and 12 h respectively. The same method was used to compare the relative amount of H_2O_2.

Determination of NOX2 protein expression by Western blotting assay. PC12 cells were divided into the following groups: control group and ketamine groups (final concentration of 10 μM acting for 3 h, 6 h and 12 h and final concentration of 100 μM acting for 3 h, 6 h and 12 h respectively). Cells were seeded in 6-well plates, and ketamine was added after 24 h. Then total protein was extracted, and the protein concentration was quantified using a BCA kit. The target protein was separated via SDS-PAGE, and transferred to a PVDF membrane. The PVDF membrane was blocked in skim milk powder at room temperature and then incubated with the primary antibody anti-NOX2 at 1/200 and anti-β-actin-loading control at 1μg/mL at 4 ℃ overnight. Then the membrane was washed and cultured with goat anti-rabbit IgG H&L (HRP) at 1/5000 at room temperature for 1 h. Results were visualized using a Luminescent Image Analyzer. Protein expression was quantified by Multi gauge V3.0 software (Fujifilm, Co., Tokyo, Japan). The gray value ratio of the target protein to β-actin was calculated as the relative content of target protein. The experiment was repeated three times.

Statistics. Data were expressed as mean ± SD or RSD and the GraphPad Prism V6.0 software (GraphPad Software, Inc., USA) was used. Multiple comparisons were evaluated by one-way analysis of variance (ANOVA). Student *t* test was applied for examining the statistical significance between groups. $P < 0.05$ was considered statistically difference.

2. ^{1}H NMR, ^{13}C NMR and HRMS for LJ-1

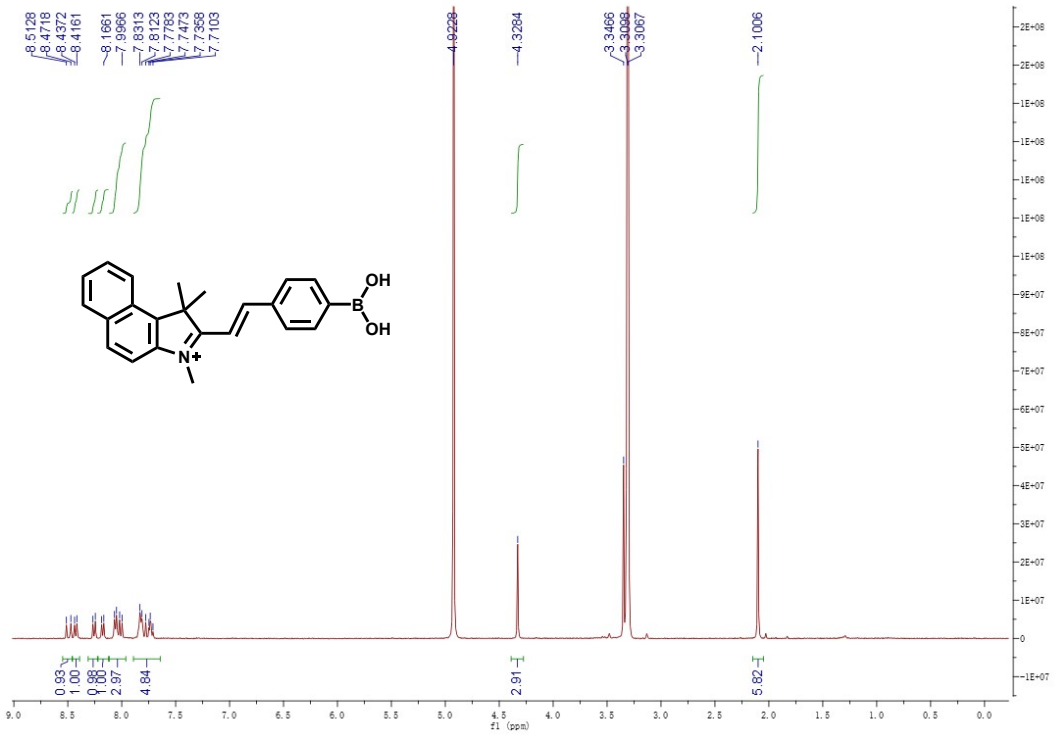

Fig. S1. ^{1}H NMR spectrum of **LJ-1** (400 MHz, CD_3OD, 298 K).

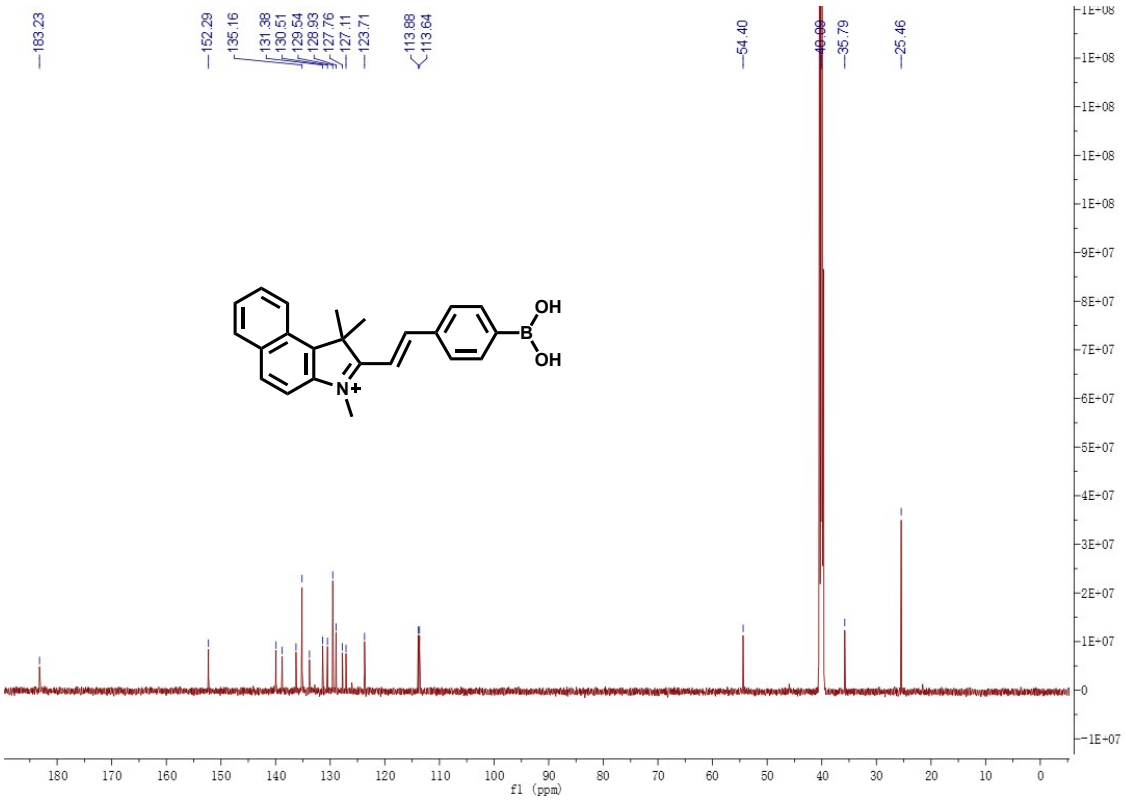

Fig. S2. ^{13}C NMR spectrum of **LJ -1** (151 MHz, DMSO-d_6, 298 K).

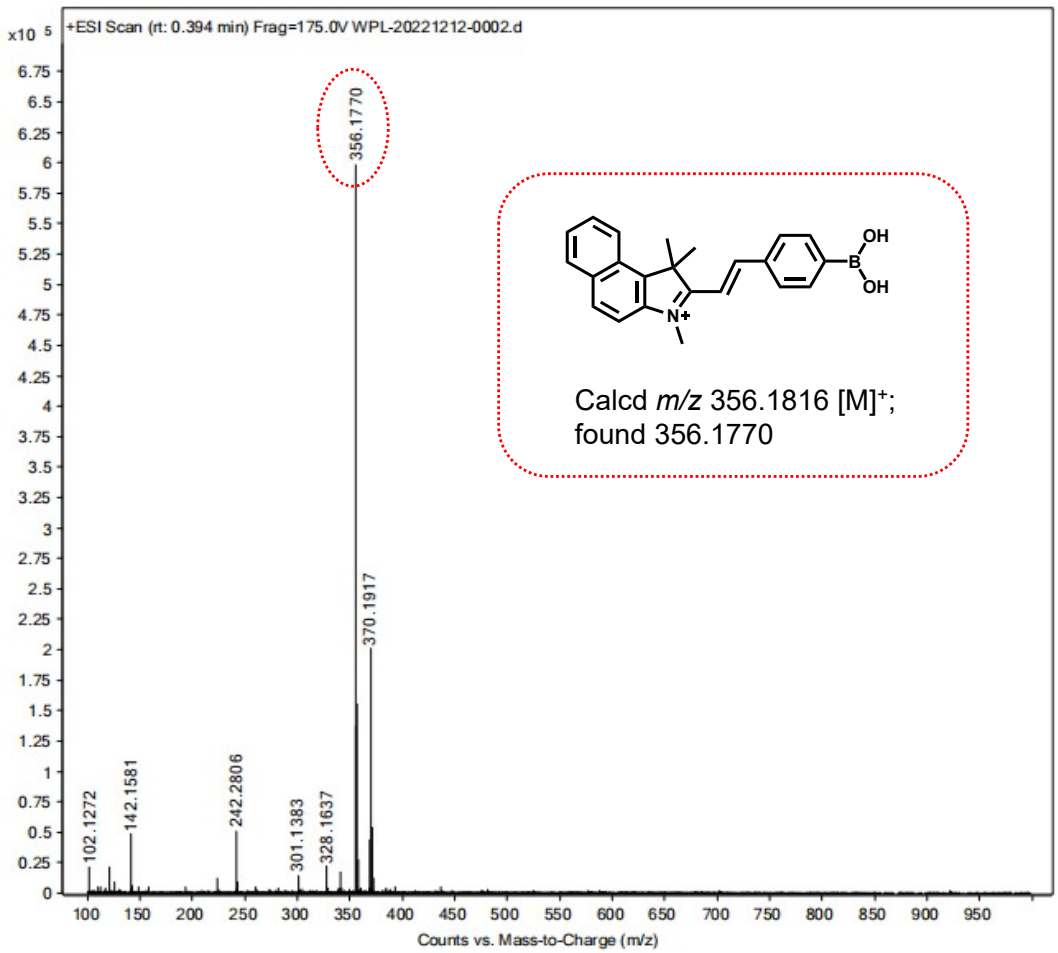

Fig. S3. HRMS result of probe **LJ-1**.

3. Water-Solubility.

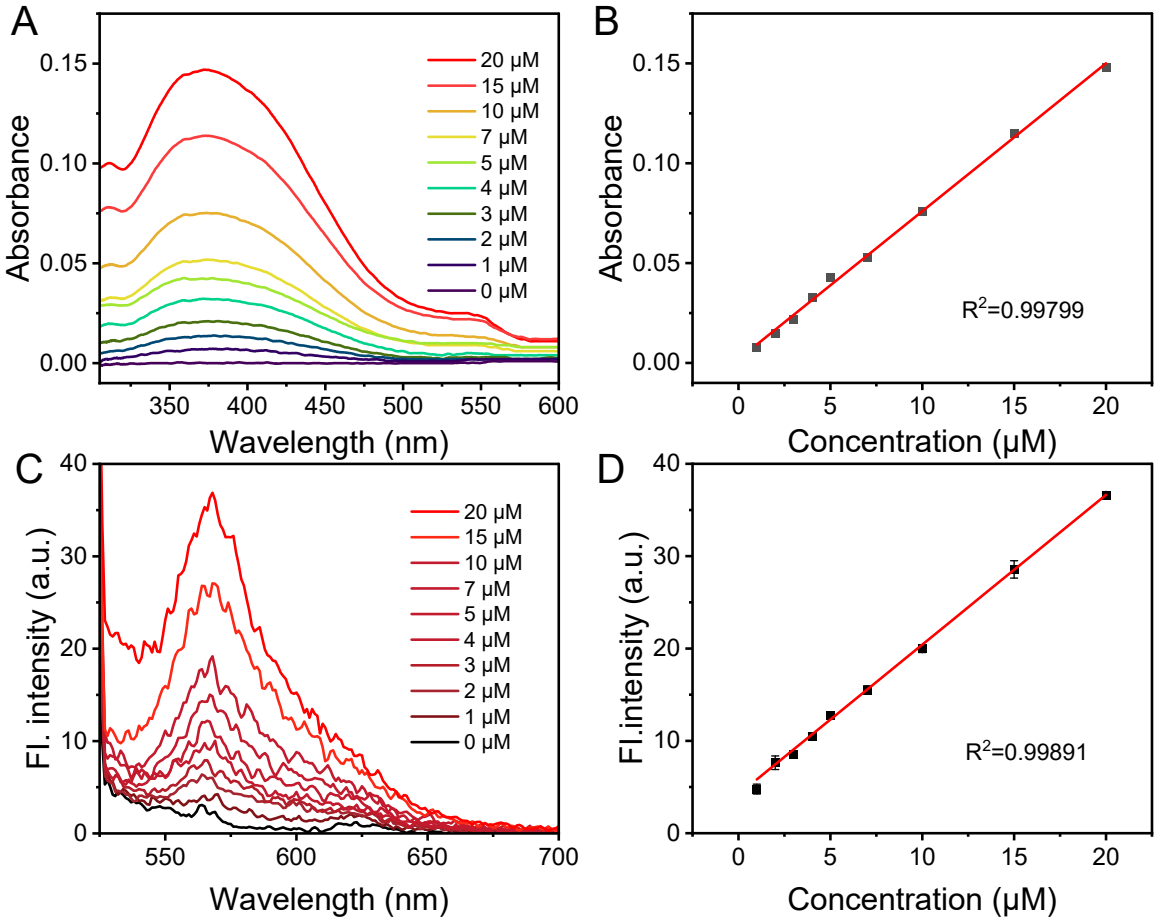

Fig. S4. (A) UV-Vis spectra and (C) Fluorescence spectra of different concentration of **LJ-1** in PBS. Linear relationship of (B) the absorbance at 372 nm and (D) the absorbance at 570 nm with the concentrations of **LJ-1**. $\lambda_{ex/em}$ = 515/570 nm. Slits: 5/5 nm. Error bars indicate relative standard deviations (RSD), n = 3.

4. Reaction mechanism studies

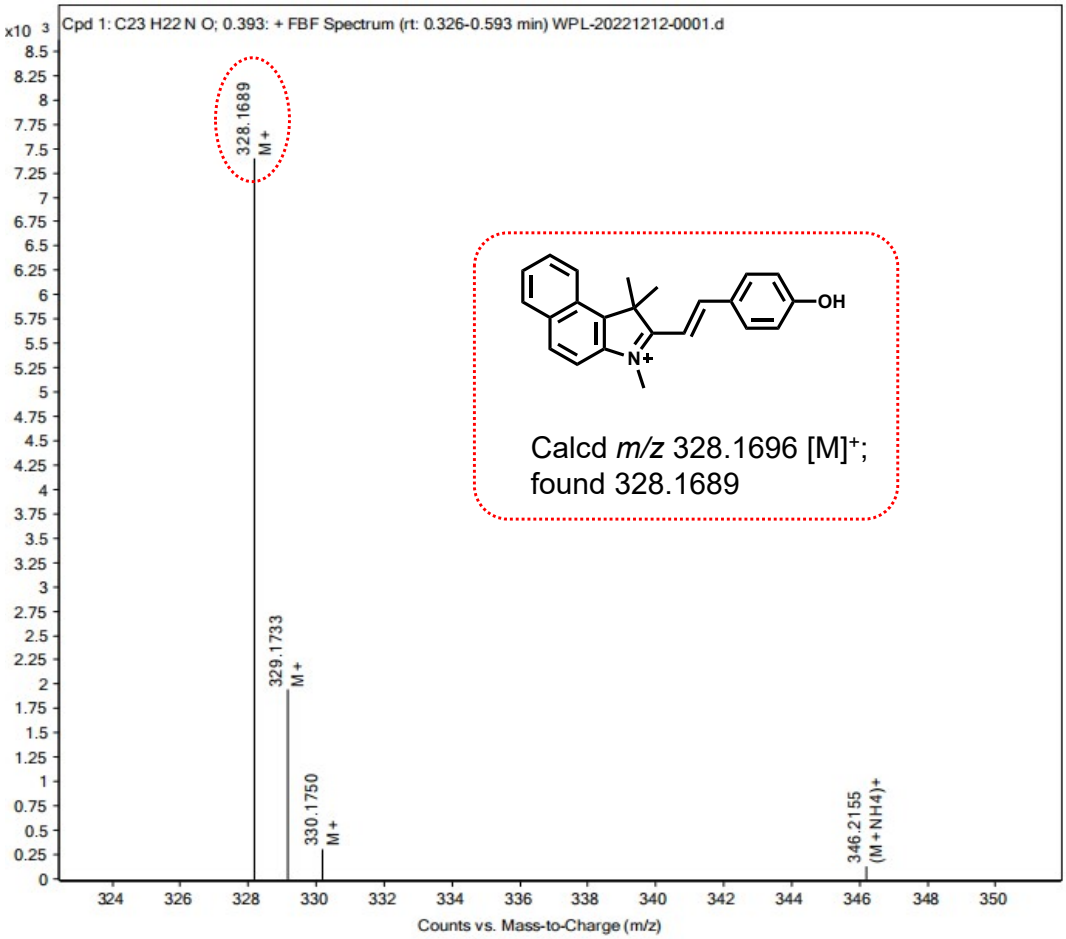

Fig. S5. HRMS result of reaction mixture of probe **LJ-1** with H_2O_2.

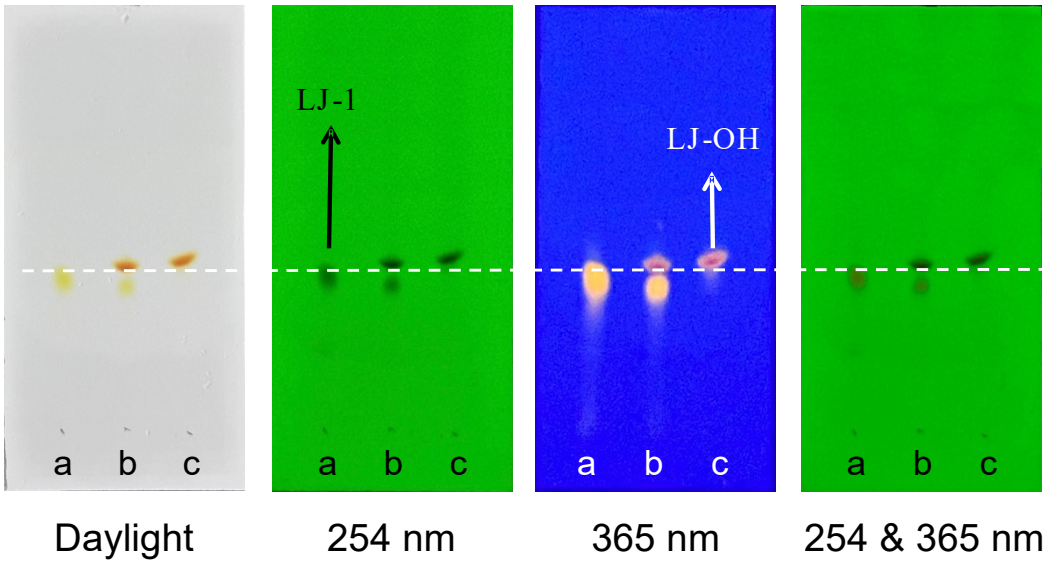

Fig. S6. The reaction of the probe **LJ-1** after addition of H_2O_2 for 40 min was indicated by TLC under different lights from left to right: daylight, 254 nm, 365 nm and the mixed light of 254 nm

and 365 nm. Letters a-c in the TLC plates represent: (a) **LJ-1** (10 μM), (b) sample from the reaction mixture (10 μM of **LJ-1** and 200 μM of H_2O_2), (c) reference sample of **LJ-OH** (10 μM). Reaction solution: CH_2Cl_2/MeOH=20/1 (v/v).

5. Optimization of experimental conditions

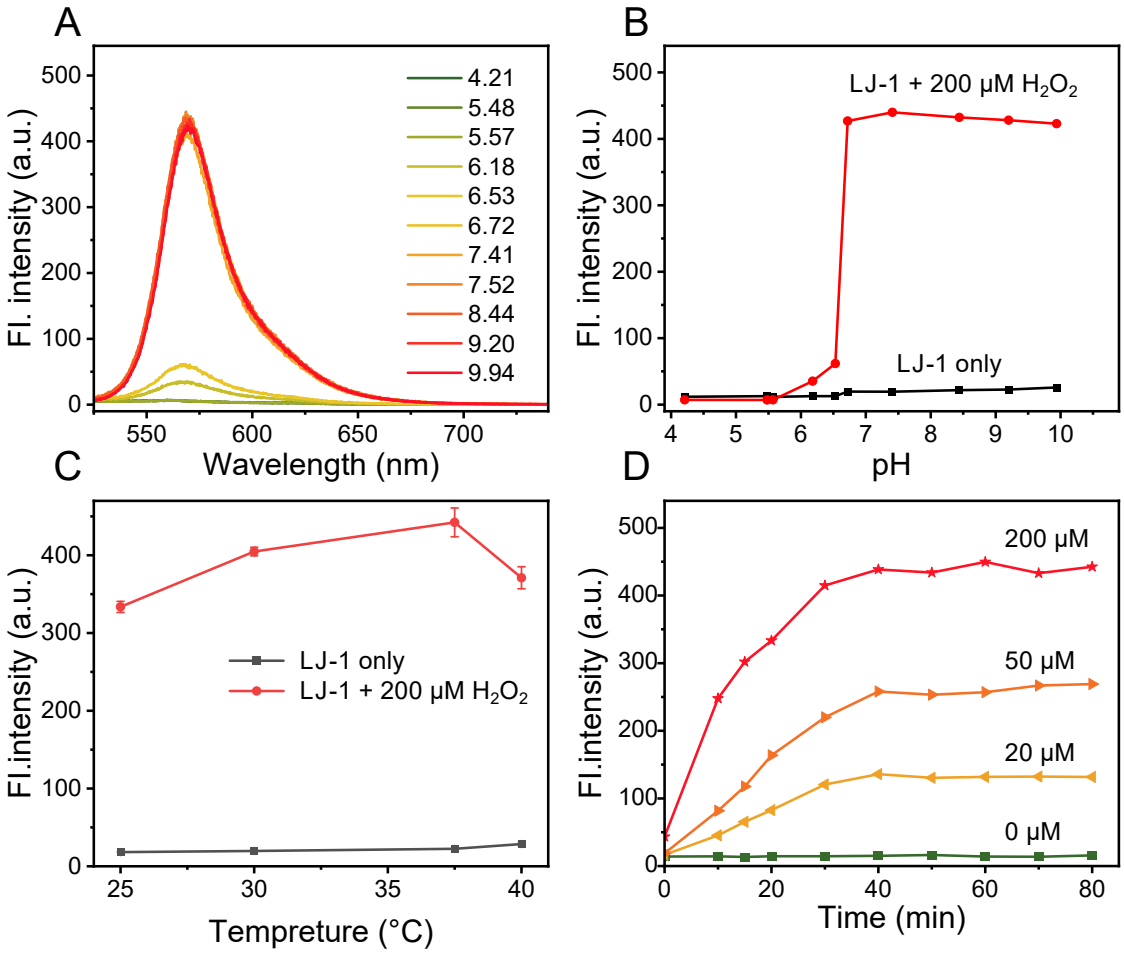

Fig. S7. (A) Fluorescence intensity of probe **LJ-1** (10 μM) at different pH values (4.21–9.94) in presence of H_2O_2 (200 μM) in the phosphate buffer. (B) Fluorescence intensity changes of probe **LJ-1** (10 μM) at 570 nm in the absence and presence of H_2O_2 (200 μM) under different pH conditions. (C) Thermal stability of **LJ-1**(10 μM) and the reaction system (10 μM of LJ-1 and 200 μM of H_2O_2) at a temperature range of 25−40 °C. (D) The fluorescence intensity changes of probe **LJ-1** toward different concentrations of H_2O_2 at different response time. $\lambda_{ex/em}$ = 515/570 nm. Slits: 5/5 nm. Error bars indicate relative standard deviations (RSD), n = 3.

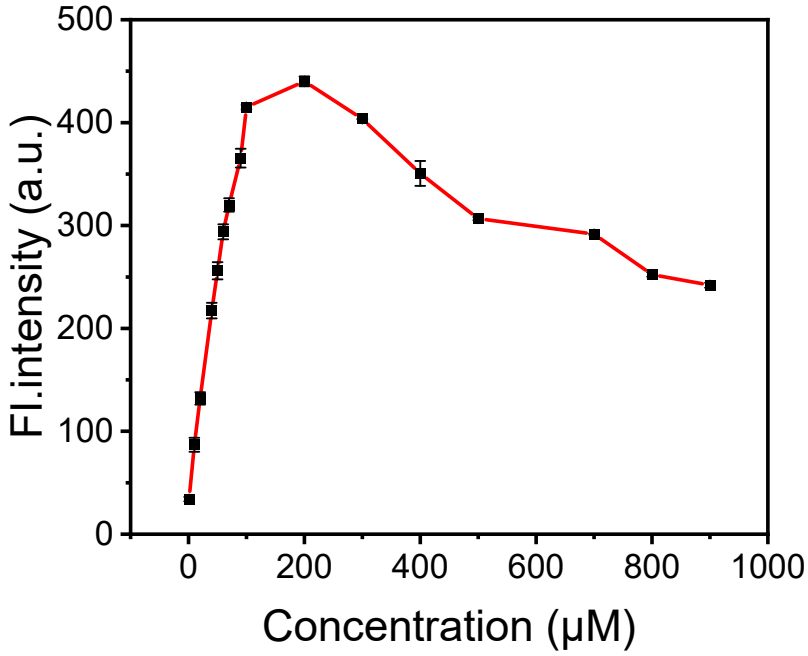

Fig. S8. Fluorescence response of probe **LJ-1** (10 μM) at 570 nm as a function of concentration of H_2O_2 for 40 min. $\lambda_{ex/em}$ = 515/570 nm. Slits: 5/5 nm. Error bars indicate relative standard deviations (RSD), n = 3.

6. Selectivity Evaluation

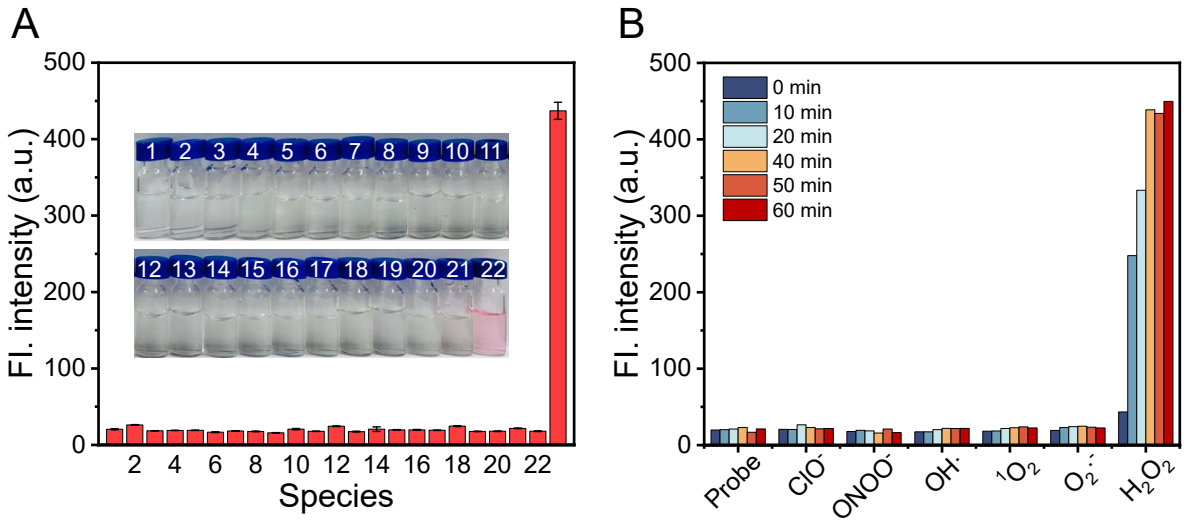

Fig. S9 (A) Probe **LJ-1** (10 μM) fluorescence response to potential representative species (200 μM): (1) blank, (2) ·OH, (3) $O_2^{\cdot-}$, (4) $^1O_2$, (5) ClO^-, (6) $ONOO^-$, (7) Ca^{2+}, (8) Fe^{3+}, (9) Mg^{2+}, (10) Na^+, (11) SO_2, (12) GSH, (13) Leu, (14) Cys, (15) Ala, (16) Ser, (17) Hcy, (18) Arg, (19) NADH, (20) N-acetyl-Cys, (21) NO_2^-, (22) H_2O_2 in PBS buffer (pH 7.4) during a 40 min incubation at 37 °C. Inset: the photograph of the corresponding color change. (B) Fluorescence emission bar diagram of probe **LJ-1** (10 μM) upon the addition of other ROSs with time lapse. $\lambda_{ex/em}$ = 515/570 nm. Slits: 5/5 nm. Error bars indicate relative standard deviations (RSD), n = 3.

7. Inhibition Test

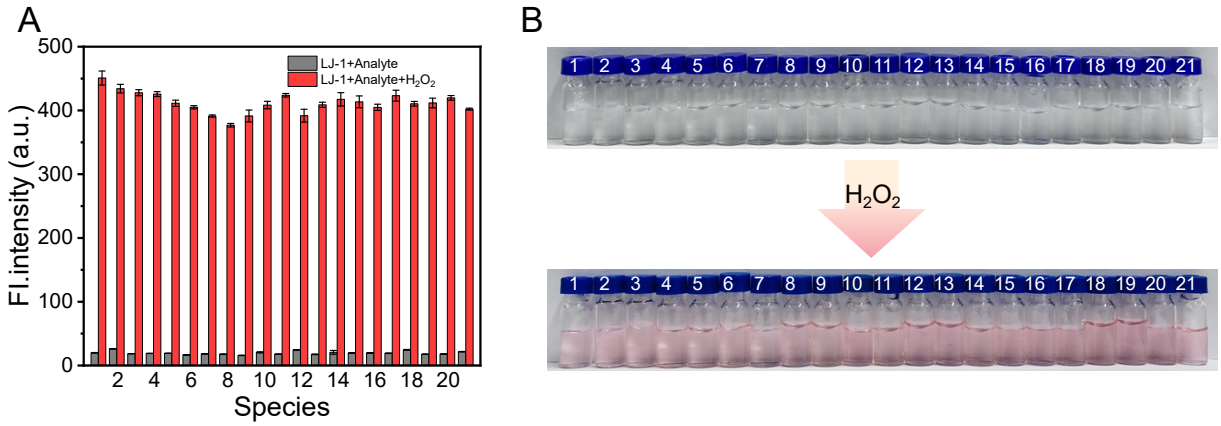

Fig. S10. (A) Fluorescence intensity change of **LJ-1** (10 μM) upon addition of various species (200 μM). For each: (1) blank, (2) •OH, (3) O_2•$^-$, (4) $^1O_2$, (5) ClO^-, (6) $ONOO^-$, (7) Ca^{2+}, (8) Fe^{3+}, (9) Mg^{2+}, (10) Na^+, (11) SO_2, (12) GSH, (13) Leu, (14) Cys, (15) Ala, (16) Ser, (17) Hcy, (18) Arg, (19) NADH, (20) N-acetyl-Cys, (21) NO_2^-. (B) The photograph of the corresponding color change after reaction with H_2O_2 (200 μM) for 40 min. $\lambda_{ex/em}$ = 515/570 nm. Slits: 5/5 nm. Error bars indicate relative standard deviations (RSD), n = 3.

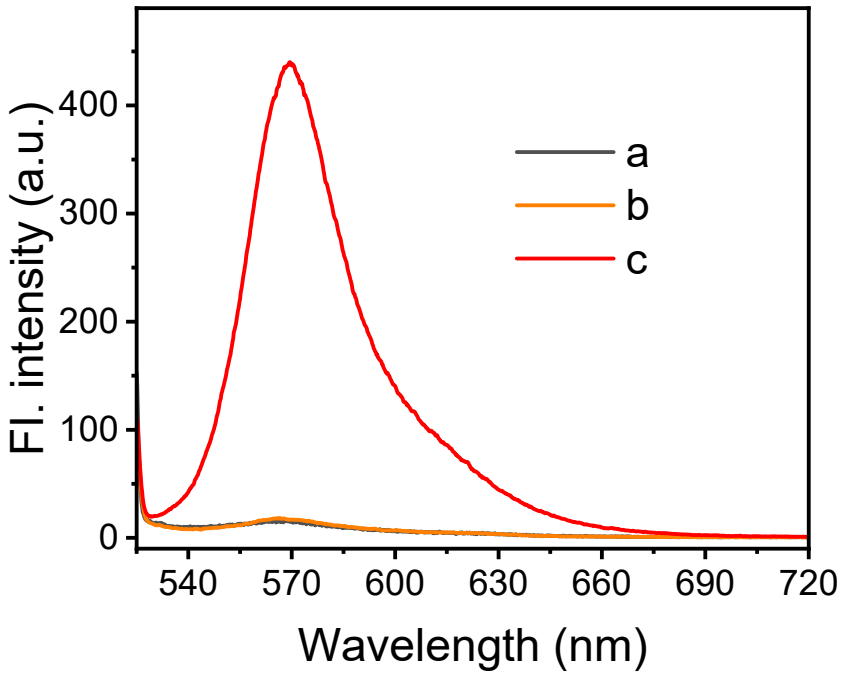

Fig. S11. Fluorescence alterations of probe **LJ-1** following the addition of the H_2O_2 scavenger MnO_2: (a) **LJ-1** (10 μM); (b) **LJ-1** (10 μM) pretreated with MnO_2 (10 mg/mL) for 3 min, then the presence of H_2O_2 (200 μM) for 40 min; (c) **LJ-1** (10 μM) in the presence of H_2O_2 (200 μM) for 40 min. $\lambda_{ex/em}$ = 515/570 nm. Slits: 5/5 nm.

8. Effect of fluorescent probe on cell viability

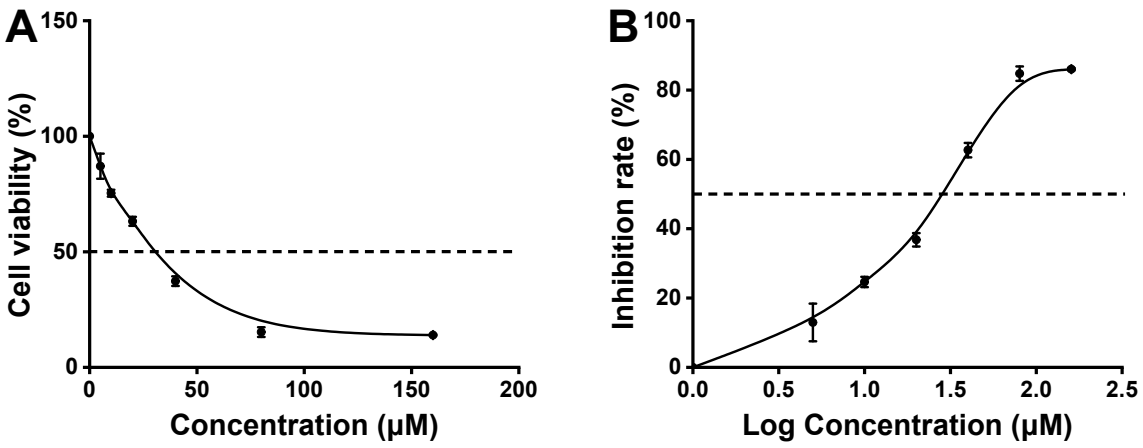

Fig. S12. The cell viability (A) and inhibition rate (B) of fluorescent probe to PC12 cells. Error bars indicate standard deviations (SD), n = 6.

9. Confocal fluorescence images in cells

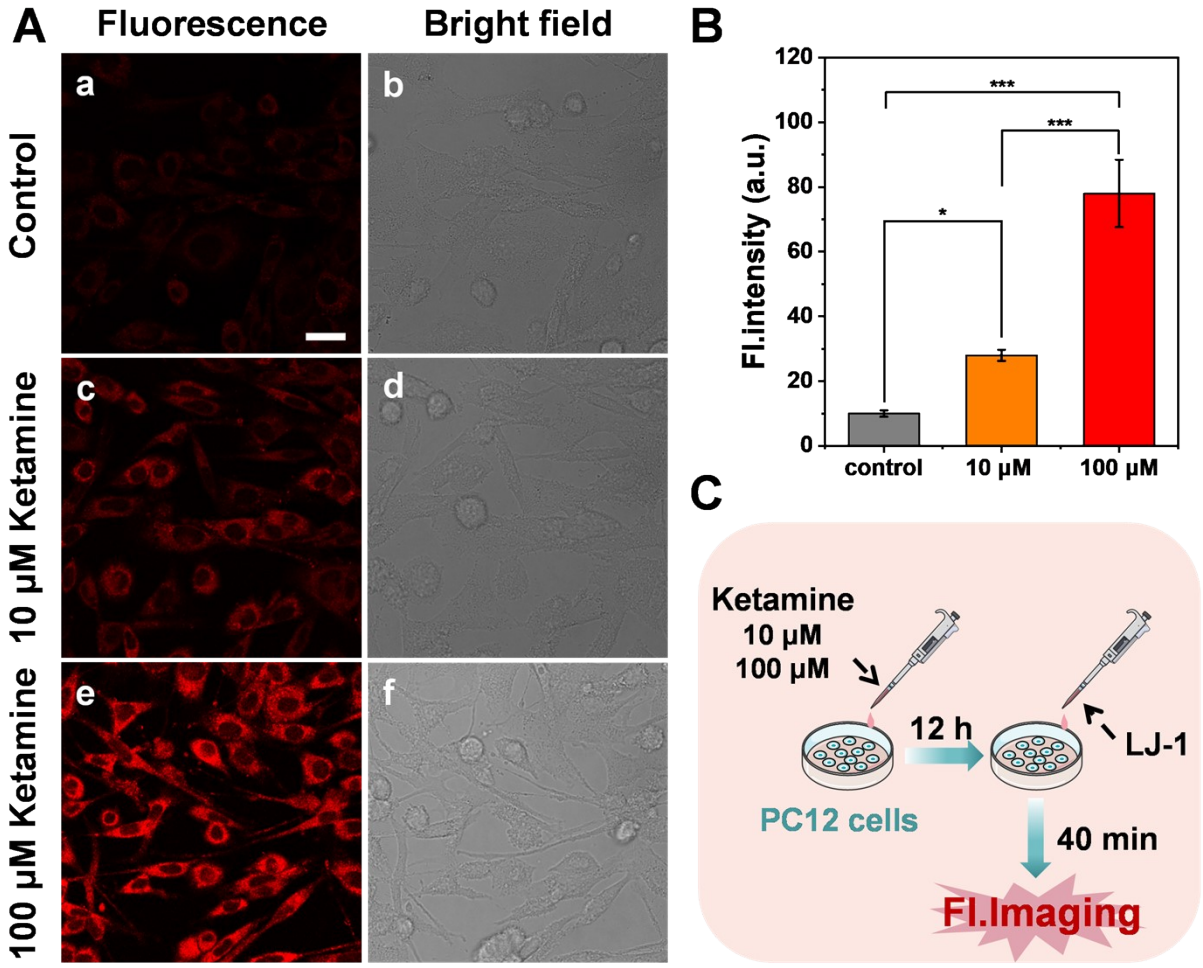

Fig. S13. (A) Fluorescence imaging of endogenous H_2O_2 in PC12 cells: (a-b), cells treated with probe **LJ-1** (10 μM) for 40 min only; (c-d), cells successively treated with ketamine (10 μM) for 12 h and probe **LJ-1** (10 μM) for 40 min; (e-f), cells successively treated with ketamine (100

μM) for 12 h and probe **LJ-1** (10 μM) for 40 min. (B) Bar graphs representing the fluorescence intensities of a, c and e in (A). (C) Schematic diagram of the experiments to observe how fluorescence changes after adding ketamine (10 μM, 100 μM) in the PC12 cells for 12h, respectively. Error bars indicate relative standard deviations (RSD), n = 3, *(p<0.05) and ***(p<0.001). λ_{ex} = 514 nm; λ_{em} = [579nm - 707nm] nm. Scal bar: 24 μm.

10. Original images of western blotting assay

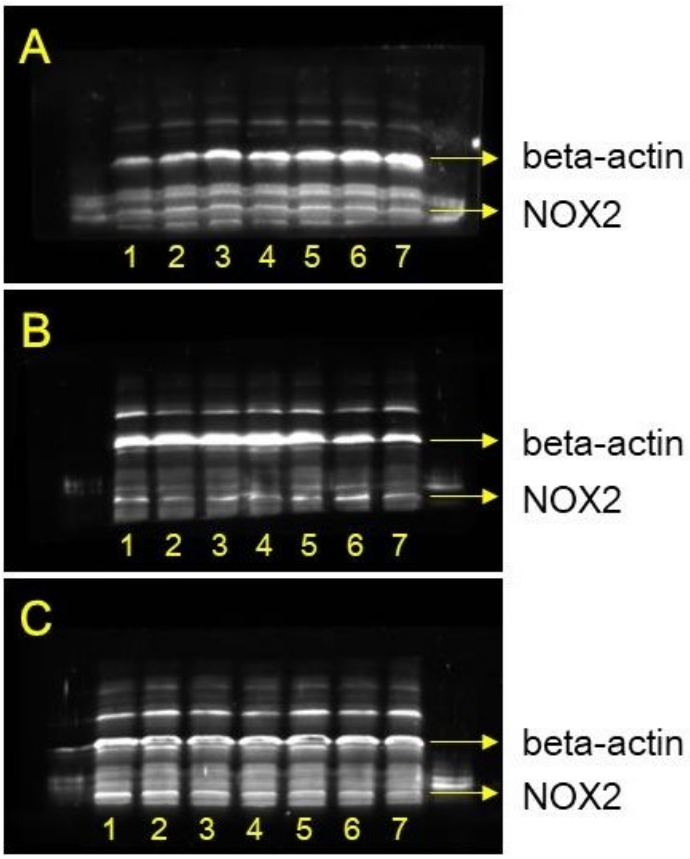

Fig. S14. Original images of Western blotting assay for 3 times: (A), (B) and (C). Band (1): control. Band (2): ketamine (10 μM) acting for 3 h. Band (3): ketamine (10 μM) acting for 6 h. Band (4): ketamine (10 μM) acting for 12 h. Band (5): ketamine (100 μM) acting for 3 h. Band (6): ketamine (100 μM) acting for 6 h. Band (7): ketamine (100 μM) acting for 12 h.