Supporting Information

for

A Novel NIR Fluorescent Probe for Copper(II)imaging in Parkinson's disease Mouse Brain

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1. Experimental General

Instruments and Materials. Unless otherwise stated, all reagents and chemicals were purchased from commercial suppliers and used as received. Double distilled water was used in all spectroscopic studies. NMR spectra were collected on a Bruker nuclear magnetic resonance 400 instrument. Mass spectra were obtained on a Thermo Scientific DSQ II MS spectrometer (for low-resolution mass spectrometry) and Waters Xevo G2-XSQTOF (for high-resolution mass spectrometry, HR-MS). UV-Vis and fluorescence spectra were obtained with a Shimadzu UV-2600 UV-Vis spectrophotometer and a Hitachi F-4600 fluorescence spectrophotometer, respectively. A TCS SP8 Laser Scanning Confocal Microscopy (Leica, Germany) was used for cell imaging experiments. Mice imaging experiments were measured on an instrument of PerkinElmer IVIS Lumina Series III. The zebrafish embryos were purchased from Shanghai FishBio Co., Ltd.

The partition coefficient between water and oil

The shaking method was used to calculate the oil-water partition coefficient. In the n octanol/PBS buffer system, the n-octanol and HEPES (v/v = 1:1) were shaken in a shaker for 24 h, and then placed in a centrifuge for 0.5 h to separate the two phases (2500 rpm). The upper layer is the n-octanol organic phase, and the lower layer is the PBS aqueous phase. We prepared a probe solution with a pre-saturated PBS buffer solution. The initial absorption (Abs_1) was detected by a UV-1750 spectrometer. An equal volume of probe solution was mixed with pre-saturated n-octanol and placed in a regular shaker at room temperature for 24 h, and then placed in a centrifuge for 0.5 h to separate the two phases (2500 rpm). The absorption (Abs_2) of the probe in the PBS buffer layer was determined by a UV-1750 spectrometer, and the oil-water partition coefficient was calculated according to the following equation.

$$\mathrm{Log}P = \log\left[(Abs_1 - Abs_2)/Abs_2\right]$$

Establishment of MPTP induced PD model

All animal experiments procedures were approved by the Animal Experiment Center of Hubei University of Chinese Medicine and followed institutional guidelines. Normal 8 weeks male C57BL/6J mice (weighing 20 g) were purchased from *Three Gorges*

University. The mice were randomly divided into two groups: control group and model group(n = 6). After a week of adaptation feeding and behavioral training, the model group received intraperitoneal injections of MPTP (30 mg/kg) for 9 consecutive days, with a dosage of 0.01 ml/g body weight once daily, while the control group received injections of an equal volume of saline. After the final administration, the mice underwent behavioral tests including pole climbing, hanging, open field, and novel object recognition. Whole brains of the mice were collected for TH immunofluorescence staining. Timeline illustration of the experimental procedure was shown in **Figure S13**. Motor coordination was assessed by the pole test, the suspension test, open field test, and the novel object recognition.

Behavioral assay

To evaluate the effects of MPTP-induced motor deficits in mice, the rotarod, pole and open-field tests were used to measure the motor impairments of the animals. All mice were trained before the start of experiments, and mice that were unable to pass the baseline behavioural performance were excluded

Pole test: The mouse is placed head-up on top of a rough-surfaced iron rod (diameter 1.5 cm, height 60 cm), and the time it takes for the mouse to turn around and touch the front paws to the ground is recorded. The test is repeated 3 times at 15-minute intervals, and behavior changes are evaluated based on the average of the test results.

Suspension test: Both front paws of the mouse are simultaneously suspended at the midpoint of a horizontal wire (80 cm*40 cm), and the time it takes for the mouse to crawl from the midpoint to either end point is observed (including the time for all four paws to grasp the wire). The test is repeated 3 times at 15-minute intervals, and behavior changes are evaluated based on the average of the test results.

Open Field Test: The mouse is placed in the center of an open field (40×40×40 cm) and allowed to freely move for 10 minutes, while its movement trajectory is recorded. Between each experiment, the field is thoroughly cleaned with alcohol, and a tracking system (Noldus Etho Vision XT15) is used to record the trajectory and measure the mouse's activity. We track the time the mouse spends in the central zone, total distance moved, and average speed.

Novel Object Recognition: The mouse is initially allowed to adapt to the experimental apparatus without objects for 10 minutes, followed by 6 minutes of familiarization with two identical objects. One of the identical objects is then replaced with a different object, and placed in the apparatus with the objects equidistant from the side walls. The mouse is placed in the apparatus with its back facing the object, and the time the mouse spends exploring each object (by touching with its mouth or nose) is recorded using tracking software.

TH immunofluorescent staining

The brain tissues were harvested and fixed using 4% paraformaldehyde, then were cut into 4 μm coronal sections. Slices were then deparaffinized with xylene and ethanol, sealed with PBS containing 0.5% TritonX-100 and 5% BSA for 30 min, and incubated with the TH antibody (mouse anti-TH Ab, 1:500, ab137869, abcam) at 4 °C overnight. Then, the excess antibody was washed off with PBS, after which slices were incubated with the secondary antibody (1:200) for 1h at room temperature. Finally, the slides were incubated with diaminobenzidine (DAB) for 5 min. The TH immunofluorescent stained slices were imaged by a fluorescent inverted microscope (IX73, Olympus) and the number of TH-positive cells in the SN and STR region was analyzed by Image J software.

Intracranial injection

C57 and PD mice were anaesthetised with 1% pentobarbital sodium and fixed on a stereotaxic apparatus. After exposing the cranium of the mice, the fontanel of the mouse brain was positioned, and then a 0.5 mm hole was made at the lower right side of the fontanel using a hand-held cranial drill (RWD). A microsyringe was used to slowly inject **DDAO-Cu** (2.5 μM, 2 μL).

2. Structure Characterization Data and Synthetic Route

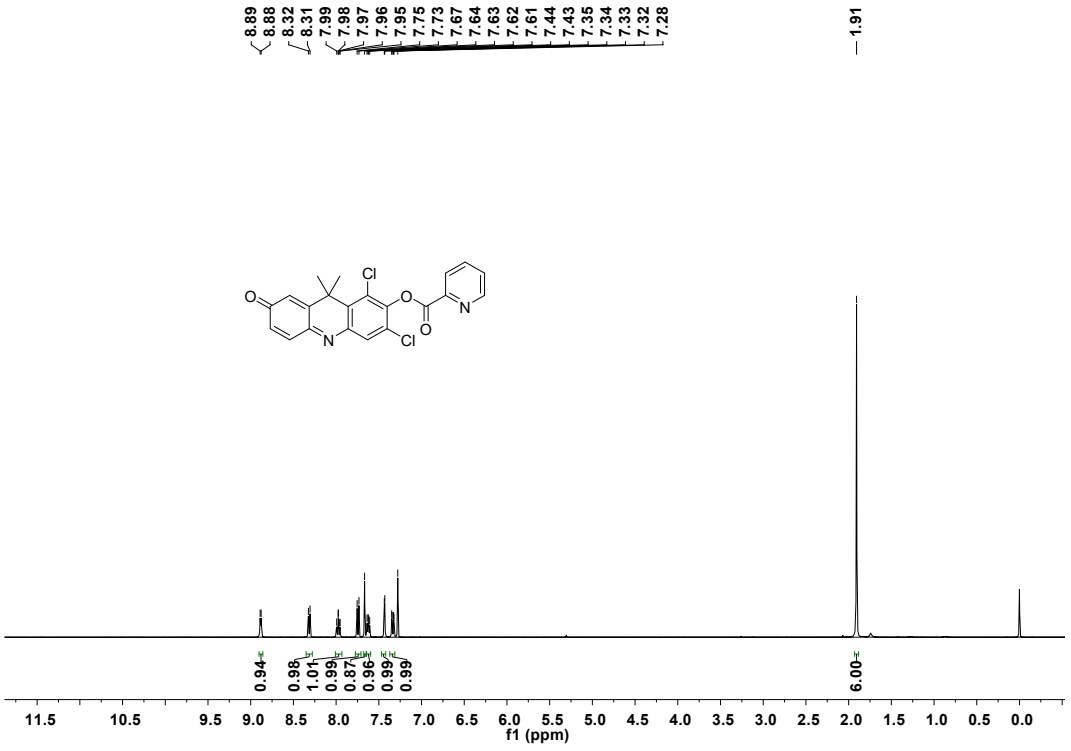


Figure S1. ^{1}H NMR spectrum of **DDAO-Cu** in $CDCl_3$

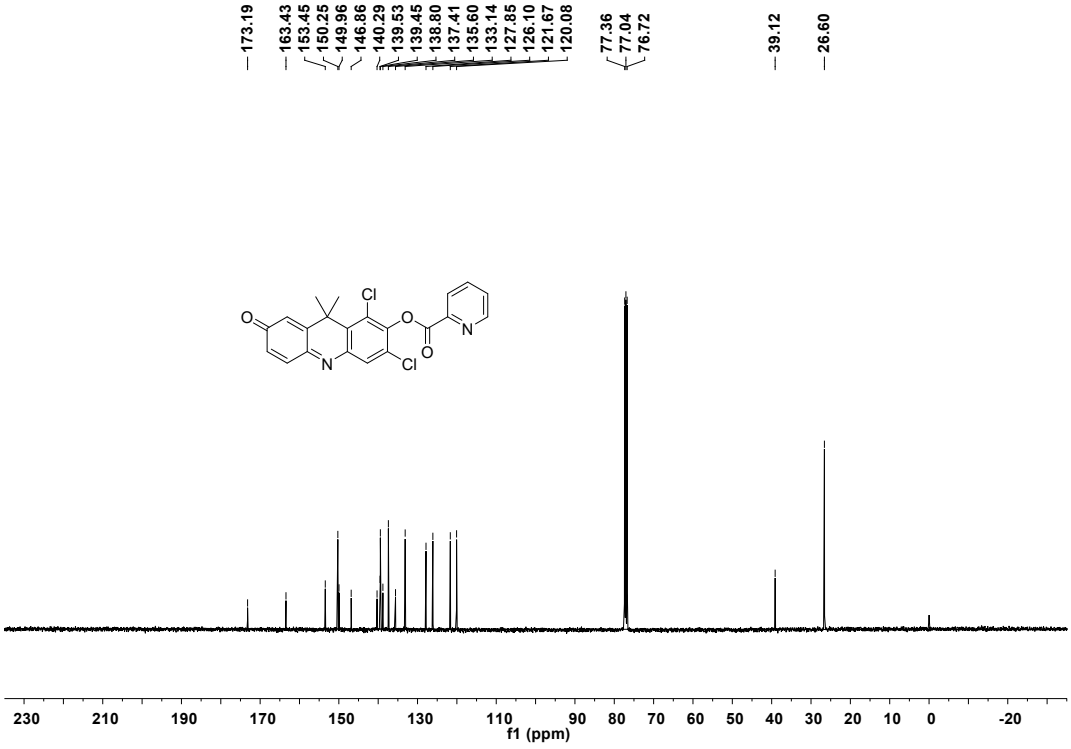


Figure S2. ^{13}C NMR spectrum of **DDAO-Cu** in $CDCl_3$

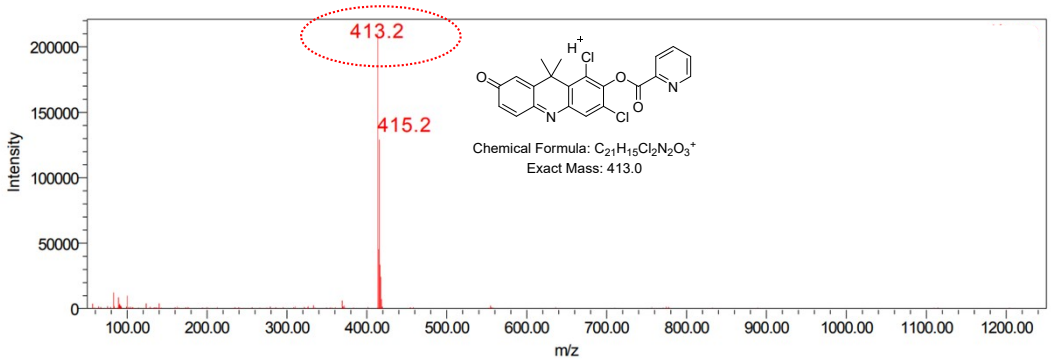


Figure S3. LC-MS spectrum of **DDAO-Cu**

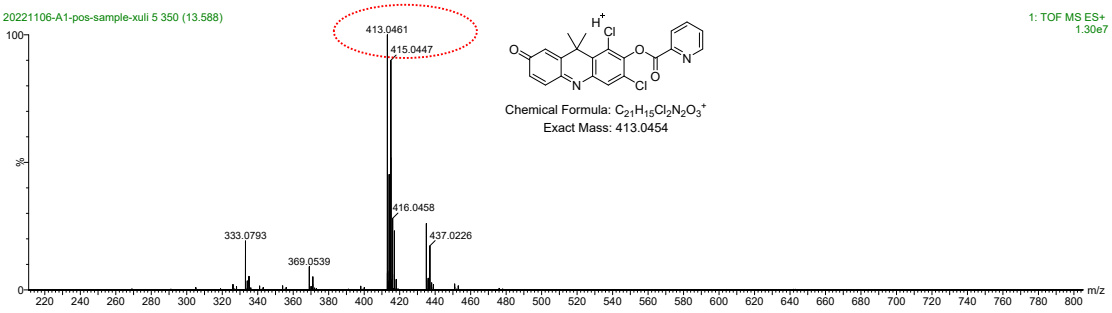
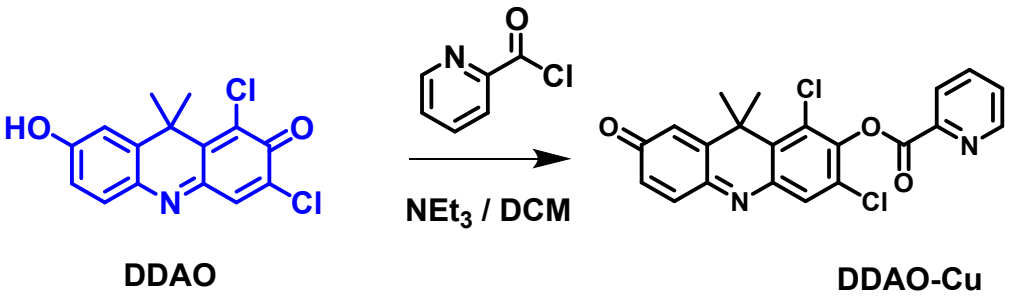


Figure S4. HPLC-MS spectrum of **DDAO-Cu**



Scheme S1: Synthetic route for probe **DDAO-Cu.**

3. Additional Experiments and Spectra

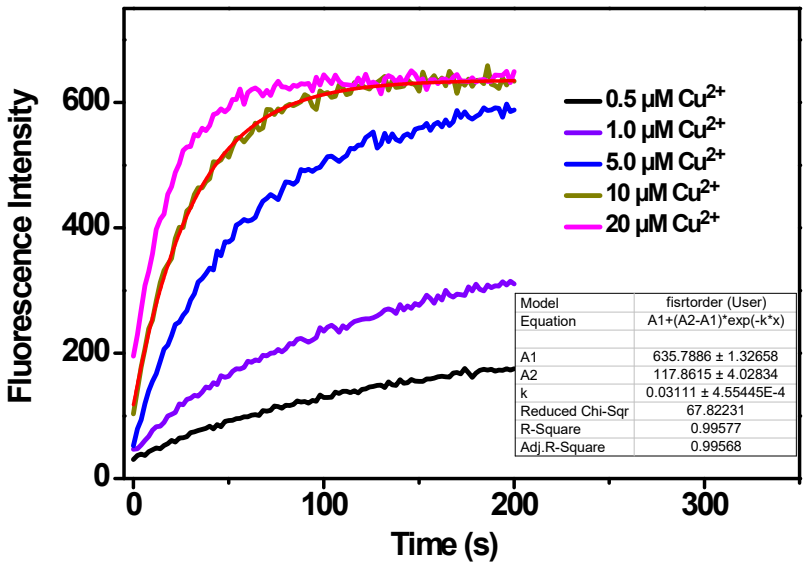


Figure S5. Fluorescent kinetics of probe **DDAO-Cu** (5 μM) upon addition of various concentrations of Cu^{2+} in HEPES buffer (10 mM, pH 7.4,) at 37 °C. The fluorescence was observed at 662 nm with excitation of 602 nm, slit width: d_{ex} = 2.5 nm, d_{em} = 5 nm. The red line shows the curve fitting of the kinetic data with a first-order equation (see the Table inserted), which gives a rate constant (k_{obs}) to be about 0.031 s^{-1} for the reaction between **DDAO-Cu** (5 μM) and Cu^{2+}(10 μM).

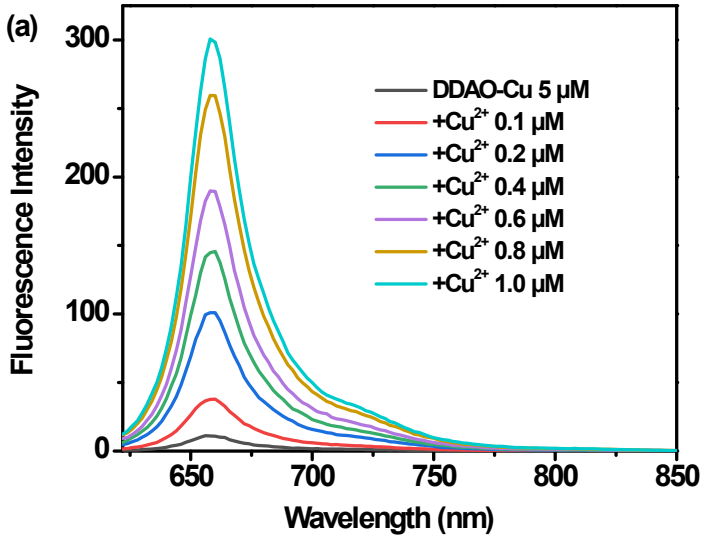


Figure S6. Fluorescence responses of **DDAO-Cu**(5 μM) upon addition of Cu^{2+} (0.1-1.0 μM). Each data was collected after 3 min of incubation. λ_{ex}= 602 nm.d_{ex}=2.5 nm, d_{em}=5 nm.

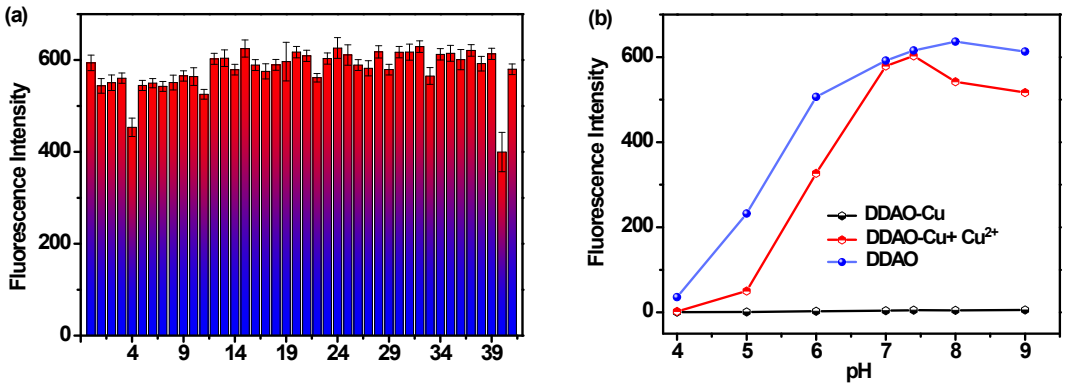


Figure S7. (a) Fluorescence intensity (662 nm) changes of Cu^{2+}(10 μM) with **5 μM DDAO-Cu** in the prescent of other analytes (100 μM unless stated otherwise) in HEPES buffer (pH 7.4,10 mM) at 37°C for 3 min. Analytes: (0) None，(1) Mn^{2+}, (2)Li^{+}, (3)Sr^{2+}, (4)Al^{3+}, (5)Sn^{2+}, (6)Cd^{2+}, (7)Ni^{2+}, (8)Zn^{2+}, (9)Fe^{2+}, (10)Fe^{3+}, (11)Hg^{2+}, (12)Na^{+}, (13)K^{+}, (14)Ca^{2+}, (15)Ba^{2+}, (16)NH_4^{+}, (17)SO_4^{2-}, (18)NO_3^{-}, (19)HSO_3^{-}, (20)CO_3^{2-}, (21)HCO_3^{-}, (22) F^{-}, (23) Cl^{-}, (24) Br^{-}, (25) I^{-}, (26)$S_2O_3^{2-}$, (27)$C_2O_4^{2-}$, (28)$C_3H_5O_3^{-}$, (29)AcO^{-}, (30)Leu, (31)Thr, (32)Gly, (33)Try, (34)Ser, (35)Tyr, (35)Phe, (37)Met, (38)Hcy, (39)Cys, (40)GSH(5 mM), (41)HS^{-}. λ_{ex}= 602 nm.d_{ex} =2.5 nm, d_{em}= 5 nm. (b) Effects of pH values on the fluorescence responses of **DDAO** (5 μM), **DDAO-Cu** (5 μM) to Cu^{2+} (10 μM) in HEPES buffer (10 mM, pH = 7.4) at 37 °C. The data represent the fluorescence intensity at 662 nm. λ_{ex} = 602 nm, d_{ex} =2.5 nm, d_{em} = 5 nm.

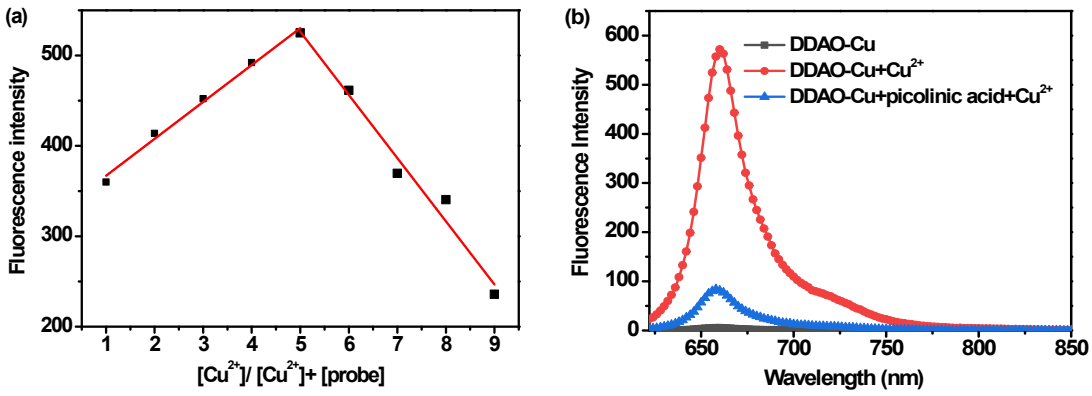


Figure S8. (a) Job's plot of **DDAO-Cu**. The [probe]+[Cu^{2+}] concentration was constant at 10 μM. (b) Fluorescence spectra of **DDAO-Cu**(5 μM) in the absence and presence

of picolinic acid (50 μM) upon addition of Cu^{2+} in HEPES buffer (pH 7.4, 10 mM) at 37°C for 3 min. λ_{ex}= 602 nm. d_{ex}=2.5 nm, d_{em}=5 nm.

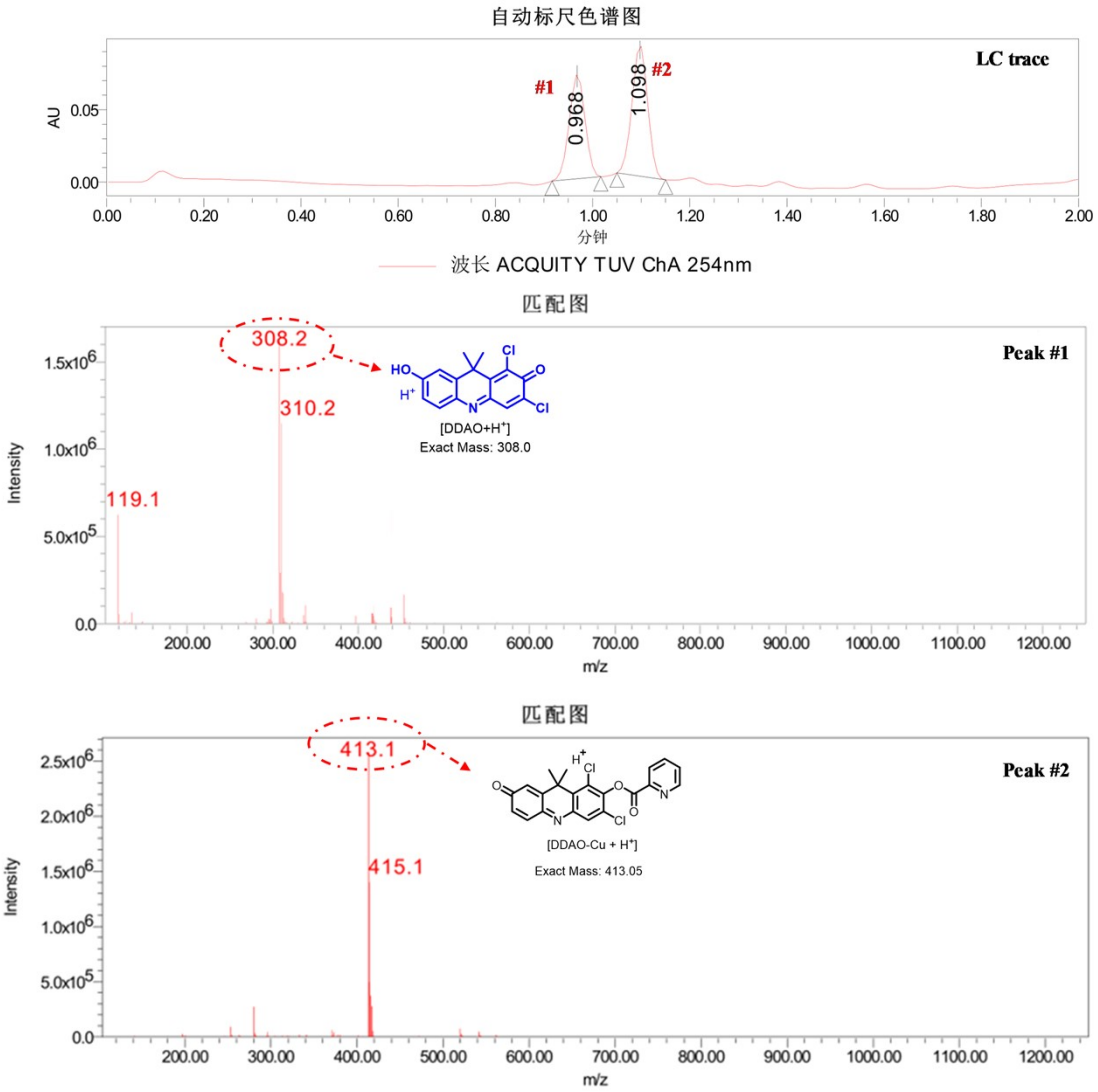
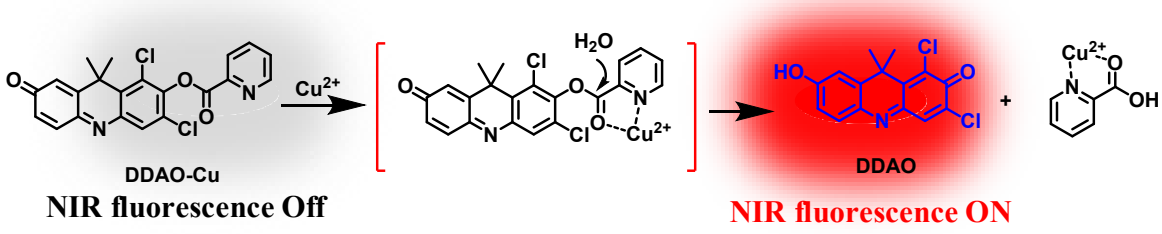


Figure S9. Low resolution mass spectrum of the reaction mixture (30 μM **DDAO** + 10 μM Cu^{2+}) in HEPES buffer (10 mM, pH 7.4). The peak at m/z =308.2can be assigned to the produced **DDAO** (calcd.308.0) and the peak at m/z = 413.1can be assigned to **DDAO-Cu** (calcd. 413.0).



Scheme S2: The Mechanism of **DDAO-Cu** detection of Cu^{2+}.

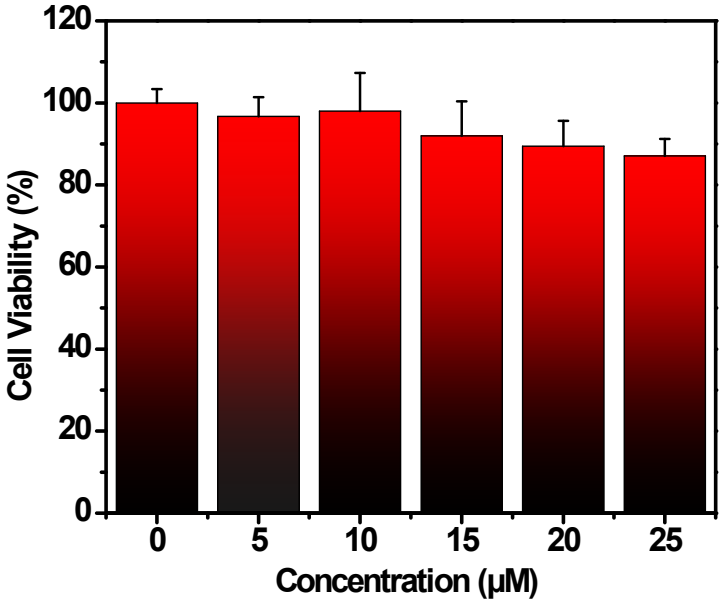


Figure S10. A MTT assay shows the percentage of viable PC12 cells after treatment with different concentrations of probe **DDAO-Cu** for 24 hours.

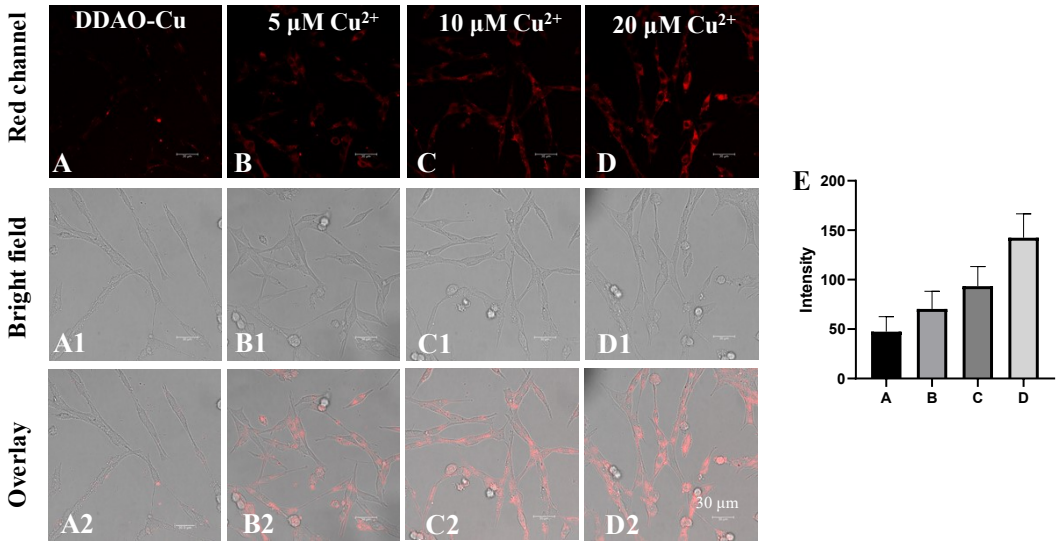


Figure S11. Imaging of exogenous Cu^{2+} in PC12 cells with 5 μM **DDAO-Cu**. (A) Cells were stained with **DDAO-Cu** for 20 min as control. (B-D) Cells were preincubated with 5, 10 and 20 μM of Cu^{2+} for 30 min and then with **DDAO-Cu** for 20 min, respectively. (E) The fluorescence intensity of A-D. Red channel ($\lambda_{ex}/\lambda_{em}$ = 561/620-680 nm)

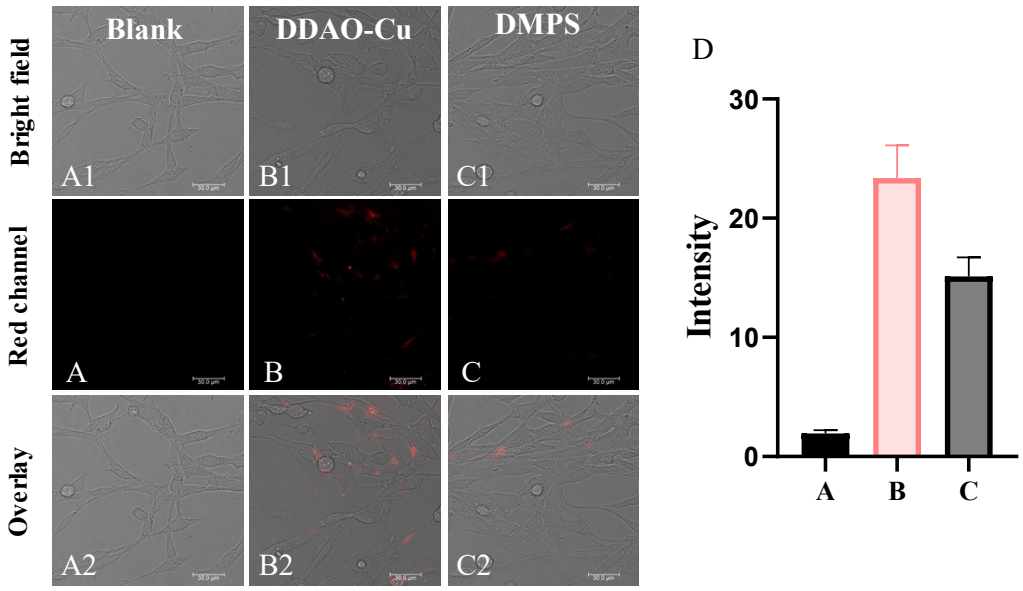


Figure S12. Imaging of endogenous Cu^{2+} with probe **DDAO-Cu (**5 μM) in PC12 cells. (A): Cell blanks. (B): Cells were incubated with probe **DDAO-Cu** (5 μM) for 20 min (C): Cells were incubated with DMPS (300 μM) for 30 min, and then with probe **DDAO-Cu** (5 μM) for 20 min; (D) The fluorescence intensity of A-C. Red channel ($\lambda_{ex}/\lambda_{em}$ = 561/620-680 nm)

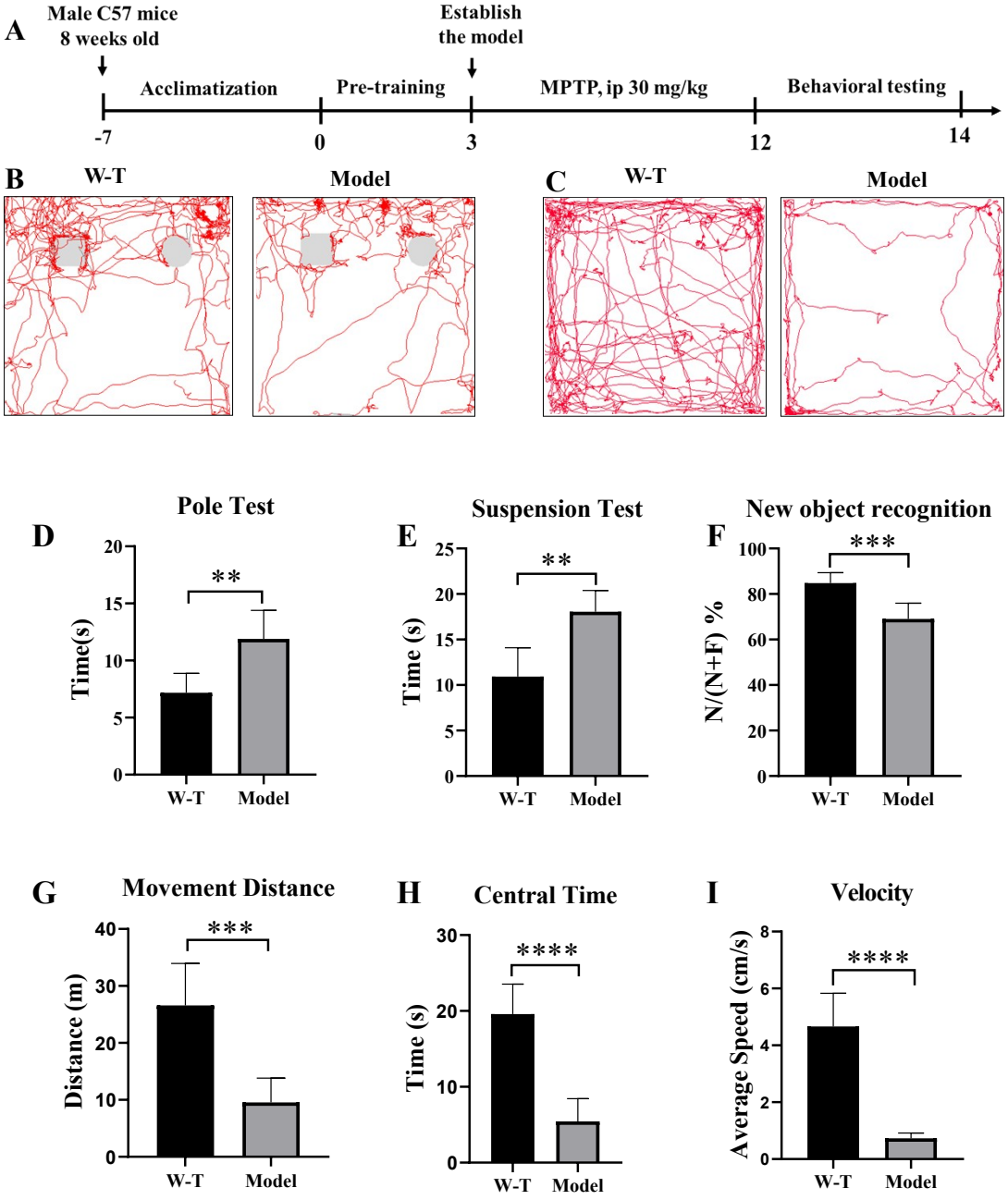


Figure S13. (A) Timeline illustration of the experimental procedure.(B) Representative trajectory plots of overall movements in the new object recognition. (C) Representative trajectory plots of overall movements in the open field test (D) Time to crawl in the pole test. (E) Time to crawl in the suspension test. (F) Exploration index in the new object recognition. (G-I) Movement distance, central time and velocity in the open field test. n = 6, *P＜0.05, **P＜0.01, ***P＜0.001, ****P＜0.0001.

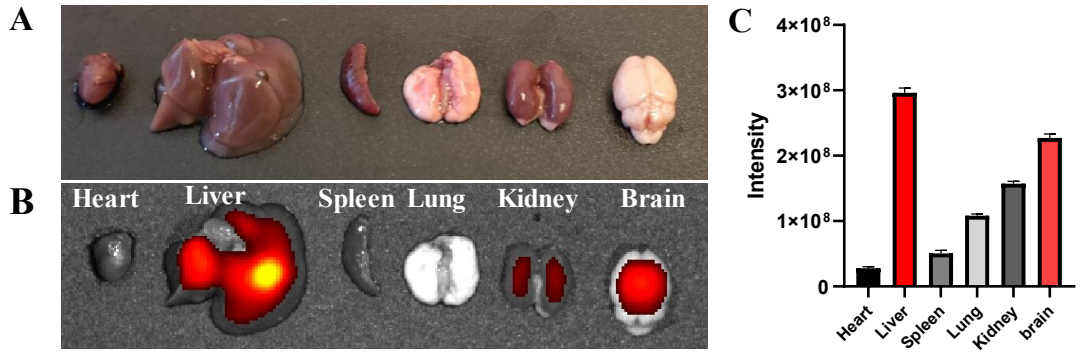


Figure S14. Ex vivo images(A) and fluorescence images(B) of the different organs of W-T mice, including brain, kidney, lung, spleen, liver, heart, and tumor, which were sacrificed at 40 min postinjection with **DDAO-Cu.** (C) the fluorescence intensity of B. $\lambda_{ex}/\lambda_{em}$ = 600/660 nm.

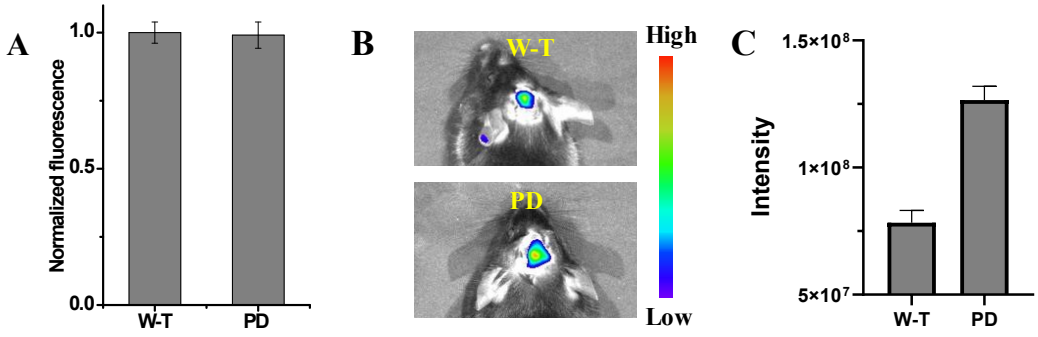


Figure S15. (A) Normalized flurescence intensity at 662 nm of **DDAO-Cu** in HEPES buffer (pH 7.4, 10 mM, containing 10% mouse serum) at 37 ℃ for 10 min. (B)Imaging of C57 W-T mice and PD mice after brain injection of **DDAO-Cu** (2.5 μM, 2 μL) for 5 min, respectively. (C) the fluorescence intensity of B. $\lambda_{ex}/\lambda_{em}$ = 600/660 nm.