Supplementary Information

Multifunctional fluorescence probe for effective visualization, inhibition, and detoxification of *β*-amyloid aggregation *via* covalent binding

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Experimental Section

Materials and measurements.

The reagents and materials used in the experiments were obtained from reagent suppliers, including Sigma-Aldrich (Shanghai), J&K Scientific, Shanghai Aladdin Biochemical Technology, Thermo Fisher Scientific (China), Sangon Biotech (Shanghai). Aβ was purchased from ChinaPeptides with a purity greater than 98%. The reagents used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Beijing Solarbio. The mouse tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Boster Biological Technology co. ltd (Wuhan, China). Paraffin-embedded 8 μm hippocampus brains tissue slices of APP/PS1 transgenic mice (C57BL6, APP/PS1, 12 month old, male) and age-matched wild-type mice (C57BL6, 12 month old, male) were obtained from Xi'an Scintor Medical Technology Co., Ltd. All the animal experiments were conducted in accordance with the protocols approved by the university's institutional animal care and use committee (Shaanxi Normal University). Nuclear magnetic resonance (NMR) spectra were recorded on JEOL 400 MHz NMR spectrometer. A high-resolution mass spectrum (HR-MS) was obtained on Bruker MAXIS. The spectral properties were taken on SHIMADZU UV-2600 spectrophotometer and Hitachi F-7000 spectrophotometers. The absolute quantum yield was measured by Hamamatsu C9920-02G with integrating spheres. The circular dichroism (CD) spectra were taken on a Chirascan CD spectrophotometer. Transmission electron microscopy (TEM) images were obtained on an FEI Tecnai G2 F20 field emission transmission electron

microscope. Confocal laser scanning microscopy (CLSM) images were recorded on Olympus FV1200. In 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) analysis, the absorbance was evaluated by Spectramax M5 microplate reader.

Synthesis of DTB precursor and DTB.

DTB precursor was obtained from 5-bromo-2,2'-bithiophene-5'-carboxaldehyde and [4-(diethylamino)phenyl] boronic acid through Suzuki coupling reaction (Fig. S2). Then, the ethynyl group was introduced through the reaction of ethynylmagnesium bromide and an aldehyde group (Fig. S3-S4).

Under argon atmosphere, 5-bromo-2,2'-bithiophene-5'-carboxaldehyde (1.25 mmol, 0.34 g) and [4-(diethylamino)phenyl]boronic acid (1.26 mmol, 0.24 g) were dissolved in *N*, *N*-dimethylformamide (DMF) and K_2CO_3 aqueous solution (8 M). After $Pd(PPh_3)_4$ was added, the mixture was heated to 80 ^oC for 40 h. After cooling to room temperature, the mixture was extracted with ethyl acetate (EA) and saturated brine, then dried over anhydrous Na_2SO_4. The crude product was purified by column chromatography with petroleum ether (PE)/EA as the eluent to obtain an orange solid (0.15 g, 35%). ^1H NMR of DTB precursor was shown in Fig. S1. ^1H NMR (400 MHz, $CDCl_3$) δ 9.84 (s, 1H), 7.65 (d, J = 4.0 Hz, 1H), 7.46 (d, J = 8.0 Hz, 2H), 7.29 (d, J = 4.0 Hz, 1H), 7.20 (d, J = 4.0 Hz, 1H), 7.08 (d, J = 4.0 Hz, 1H), 6.67 (d, J = 8.0 Hz, 2H), 3.39 (q, J = 8.0 Hz, 4H), 1.19 (t, J = 8.0 Hz, 6H).

Under argon protection, the DTB precursor (0.15 mmol, 51 mg) was dissolved in ethynylmagnesium bromide solution of tetrahydrofuran (THF) and then reacted at 0 ^oC. Saturated NH_4Cl was added to the mixture after 3.5 h. The intermediate was extracted

with EA, dried over anhydrous Na_2SO_4, and concentrated. Then, the intermediate was dissolved in dichloroethane and manganese dioxide (6.9 mmol) was added dropwise, followed by stirring at 0 °C for 8 h. The product was filtered through diatomite, dried over anhydrous $MgSO_4$, concentrated, and purified by column chromatography with PE/EA as the eluent to obtain a dark red solid (0.029 g, 53%). ^{1}H NMR and HR-MS spectra of DTB were shown in Fig. S2 and S3, respectively. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, J = 4.0 Hz, 1H), 7.46 (d, J = 8.0 Hz, 2H), 7.30 (d, J = 4.0 Hz, 1H), 7.16 (d, J = 4.0 Hz, 1H), 7.08 (d, J = 4.0 Hz, 1H), 6.67 (d, J = 8.0 Hz, 2H), 3.40 (q, J = 8.0 Hz, 4H), 3.33 (s, 1H), 1.20 (t, J = 8.0 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.25, 148.85, 147.91, 140.82, 137.60, 137.40, 127.50, 127.33, 127.15, 127.14, 123.47, 123.35, 79.90, 79.10, 29.71, 12.41. HR-MS (ESI): *m/z*: calcd. for $C_{21}H_{19}NOS_2$ H^+ 366.0987 $[M+H]^+$, found 366.0981 $[M+H]^+$.

Preparation of A*β*.

A*β*42 and A*β*40 are two types of A*β*. Although cells produce far more A*β*40 than A*β*42, A*β*42 is more likely to aggregate to form amyloid, which is the major component of senile plaques, indicating that it plays a more important role in the pathogenesis of AD.[1] Therefore, we take A*β*42 as a model to study the interaction between probe and A*β*. A*β* was prepared according to the literature.[2] In general, A*β* lyophilized powder was dissolved at a concentration of 1 mg/mL with hexafluoroisopropanol (HFIP), and ultrasonication was performed on ice for 20 min. After it was completely dissolved, it was shaken at 4 °C for 2 h. The HFIP solution of A*β* was divided into equal volumes and stored at -20 °C after lyophilization. The HFIP-pretreated A*β*42 solid was dissolved

in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM before use and then diluted with 10 mM phosphate-buffered saline (PBS) (pH 7.4) to the desired concentration.

Optical recognition of A*β*.

DTB was diluted to 10 μM with 10 mM PBS (pH 7.4) for the measurement of absorption and fluorescence spectra.

DTB was diluted to 1 μM with 10 mM PBS (pH 7.4), and then incubated with various of different concentrations of A*β* monomer or A*β* aggregate (0, 2, 4, 6, 8, 10, 12, 14, 18, 22, 26, 28, 30, 35, 40, 50 μM) at 37 ^{o}C for 12 h. The A*β* was preincubated for 12 h to form A*β* aggregates. Under the excitation wavelength of 440 nm, the fluorescence emission spectra were measured.

Specificity for A*β* recognition.

After the interfering substance was incubated with DTB (1 μM) for 12 h at 37 ^{o}C, the fluorescence intensity was measured under the excitation wavelength of 440 nm. The interfering substances include amino acids with the concentration of 100 μM (proline, phenylalanine, leucine, glutamic acid, threonine), proteins with the concentration of 50 μg/mL (bovine serum albumin (BSA), hemoglobin, pepsin, lysozyme, concanavalin A and cytochrome C), and thrombin with the concentration of 2.5 μg/mL. [A*β*] = 10 μM.

^{1}H NMR characterization.

2 mM KLVFFAE peptide and 2 mM DTB were dissolved in DMSO-d_6 and shaken overnight at room temperature. And then ^{1}H NMR spectra were measured at 298 K,

313 K, and 323 K respectively. In addition, 2 mM KLVFFAE and 2 mM DTB were used as controls.

Amino-yne click reaction in THF and water.

Iso-propanol-amine (MIPA) was selected to verify the amino-yne click reaction in organic solvent (THF) and aqueous solution (water) for 4 h at RT. In THF, a significant blue shift in absorption spectra and the obvious enhancement of fluorescence occurred. In contrast, there are only negligible changes in both UV-Vis absorption and fluorescence spectra for the reaction in water (Fig. S6). Also, we identified the reaction product by ^{1}H NMR (Fig. S7) and HR-MS (Fig. S8).

Thioflavin T (ThT) assay.

Aβ was diluted to 5 μM with 10 mM PBS (pH 7.4), and then incubated with ThT (10 μM) at 37 ^{o}C for 12 h. Under the excitation wavelength of 410 nm, the fluorescence emission spectra were measured.

SDS-PAGE gel electrophoresis.

Aβ and DTB were incubated for 12 h at 37 ^{o}C with different molar ratios of 1:0, 1:1, 1:5, and 1:10 in PBS buffer. The samples were denatured in protein loading buffer (contains sulfhydryl reducing agent) at 90 ^{o}C for 5 min. After centrifugation, the samples were separated in a commercial precast gel with a concentration of 4-20% and stained with coomassie brilliant blue.

Measurement of CD spectrum.

For the preparation of samples, the PBS solution of Aβ (5 μM) with or without DTB (1 μM) was shaken at 37 ^{o}C for 12 h. The above samples (200 μL) were added to a 1

mm quartz cuvette for scanning (190-260 nm). The bandwidth was 1 nm and the response time was 2 s.

TEM measurement.

The samples (10 μL) to be tested were dropped onto the carbon-coated copper grid for 30 min, washed with ultrapure water (10 μL) 3 times, and then stained with 2% phosphotungstic acid (10 μL) for 10 min. The excess staining solution was removed, and samples were washed with ultrapure water 3 times and finally allowed to dry naturally before imaging. Aβ monomers were preincubated for 12 h to form Aβ aggregates. [DTB] = 1 μM, [Aβ] = 5 μM.

Cytotoxicity assay.

PC-12 cells (rat adrenal medulla chromaffin cells) were cultured in a Dulbecco's modified Eagle's medium (DMEM) containing 15% horse serum, 5% fetal bovine serum (FBS), and 1% streptomycin/penicillin. For the biocompatibility evaluation of DTB, the resuspended PC-12 were seeded on a 96-well plate at a density of 7000 cells per well. After 24 h, the old medium was replaced with a fresh medium containing DTB with different concentrations (0, 0.25, 0.5, 1, 2, 5 μM). After culturing for 48 h, 10 μL MTT in PBS (5 mg/mL) was added to each well. Next, DMSO (200 μL) was added to dissolve the sediment after 4 h incubation and then the absorbance at 570 nm was obtained on the microplate reader.

For the experiment to evaluate the detoxification effect of DTB on Aβ aggregates, PC-12 cells were cultured with DMEM medium containing Aβ (30 μM) alone or Aβ (30 μM)/DTB (1 μM) for 48 h.

Propidium iodide (PI) staining imaging.

After PC-12 cells were seeded in a confocal culture dish and cultured for 24 h, the cells were cultured in the presence of 5 μM Aβ with or without DTB (1 μM) for 24 h. After being washed 2 times with PBS, the cells were stained with 50 μM PI for 45 min in an incubator. After staining, the cells were washed 3 times with PBS before imaging. The excitation wavelength of PI dye is 559 nm and the emission wavelength range is 570-670 nm.

Cellular adherence.

After PC-12 cells were cultured in a confocal dish for 24 h, the medium was replaced with a fresh medium containing Aβ (5 μM)/DTB (1 μM) cultured for different times (1, 4, 6, 8, 12 h) or containing Aβ (5 μM)/ThT (10 μM) and cultured for 12 h. The cells were washed 2 times with PBS before imaging. The excitation wavelength was 405 nm.

Cellular phagocytosis.

BV-2 (mouse microglia) cells were cultured in DMEM containing 10% FBS and 1% streptomycin/penicillin and seeded in a confocal culture dish for 24 h. The old medium was replaced with a fresh medium containing Aβ (5 μM)/DTB (1 μM) or Aβ (5 μM)/ThT (10 μM) and cultured for another 24 h. After removing the medium, the cells were washed twice with PBS before imaging. The excitation wavelength was 405 nm for ThT and 488 nm for DTB, respectively. The emission was collected at 450-500 nm and 550-650 nm for ThT and DTB, respectively.

Measurement of TNF-α level.

BV-2 cells were stimulated with Aβ (5 μM) or Aβ (5 μM)/DTB (1 μM) for 24 h, respectively. The supernatants were collected for the TNF-α assay. TNF-α levels were measured by a mouse TNF-α ELISA kit followed by the instructions provided with the kit.

***In Vitro* fluorescent imaging of brain slices.**

The slices were deparaffinized in xylene (15 min), followed by washing with ethanol (5 min), 50% ethanol (1 min×3) before staining. The slices were incubated in aqueous solution of DTB (100 μM) for 20 min at room temperature and then washed with 40% ethanol. After mounted with cover slips, the brain slices were observed on confocal laser scanning microscopy (Olympus FV1200). To confirm the staining of the plaques, ThS (10 mg/mL) staining was performed in the same slice.

Supporting Figures

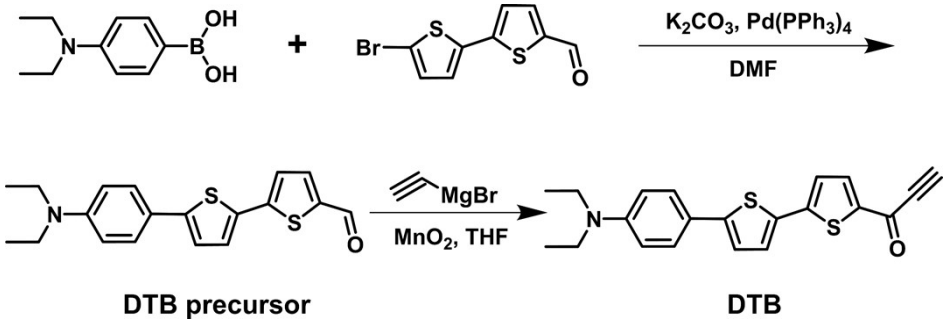


Fig. S1 The synthetic route of DTB.

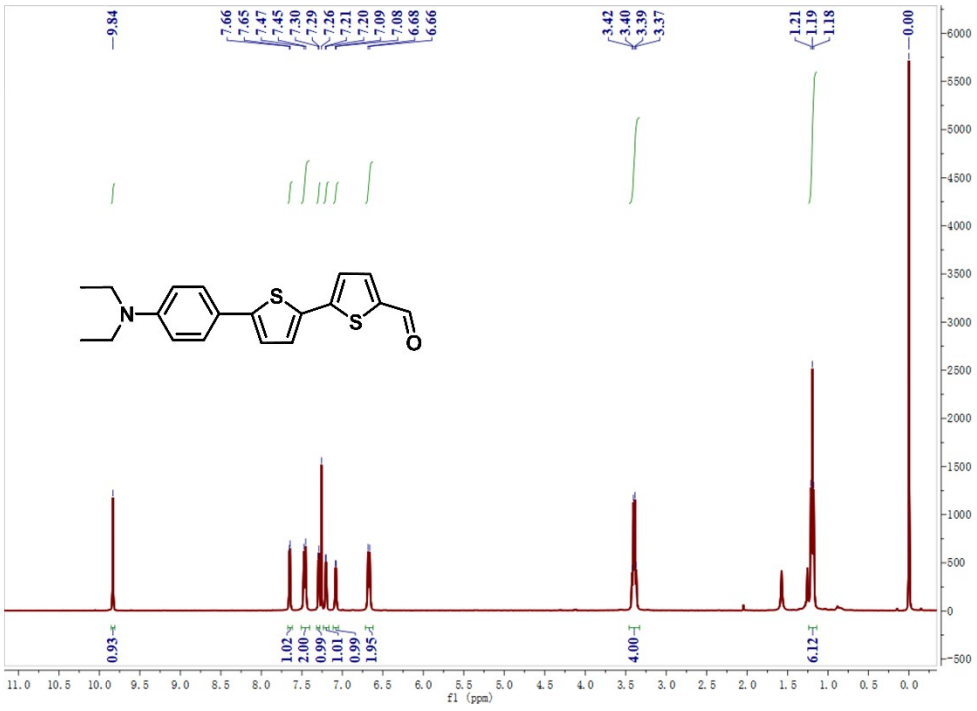


Fig. S2 ^{1}H NMR of DTB precursor.

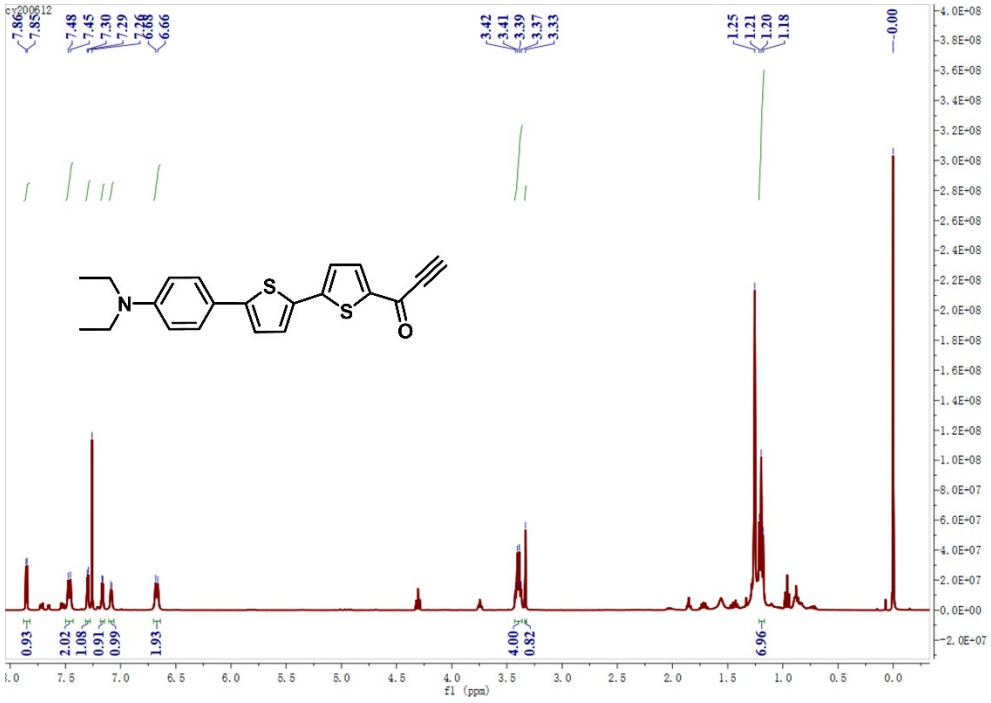


Fig. S3 ^{1}H NMR of DTB.

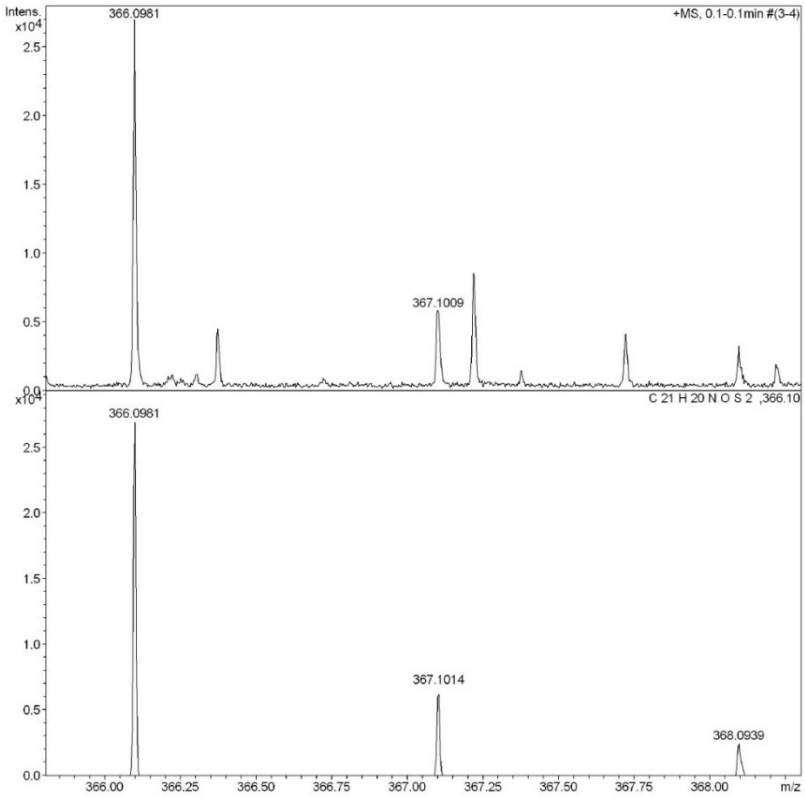


Fig. S4 HR-MS spectrum of DTB.

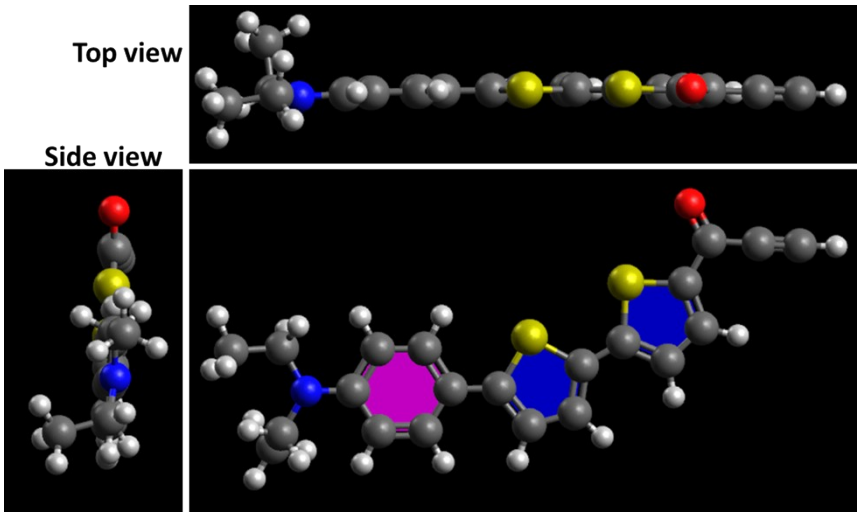


Fig. S5 Optimized geometry of excited state of DTB by TDDFT. The excited state geometry was optimized at Cam-B3LYP/def2-TZVP level with tight convergence criteria by using the ORCA 5.0.2[3] program package. Molecule was visualized with Avogadro 1.2.0 program package.

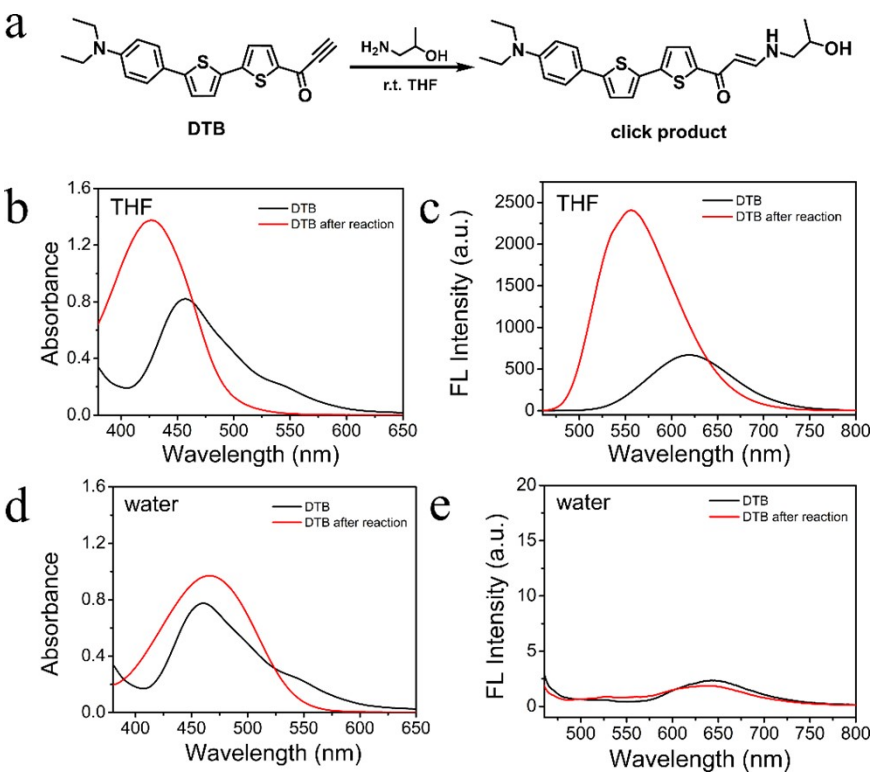


Fig. S6 (a) Click reaction of DTB and MIPA. The absorption spectra (b, d) and emission spectra (c, e) of DTB before and after reaction with MIPA in THF or water. [DTB] = 10 μM, [MIPA] = 100 μM. λ_{ex} = 440 nm.

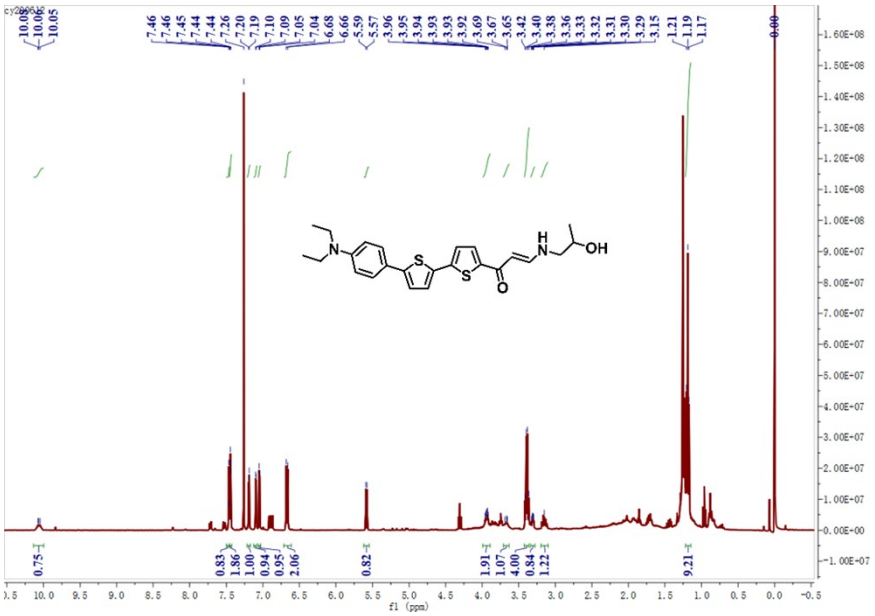


Fig. S7 ^{1}H NMR of the click product of DTB.

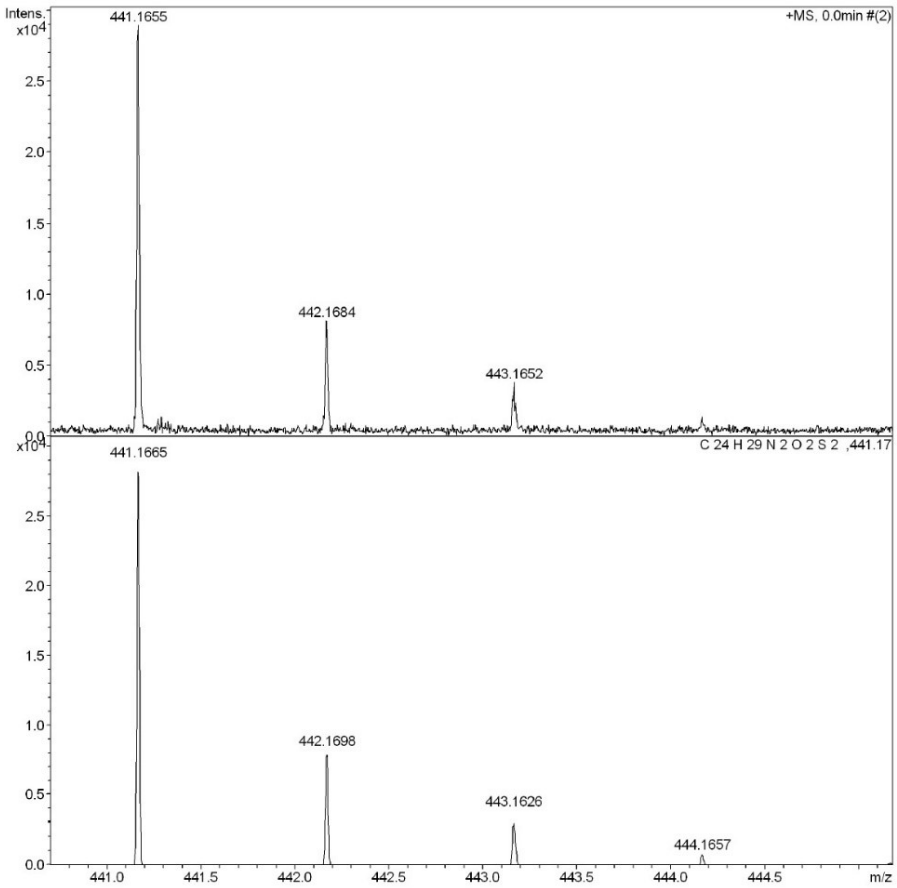


Fig. S8 HR-MS spectrum of the click product of DTB.

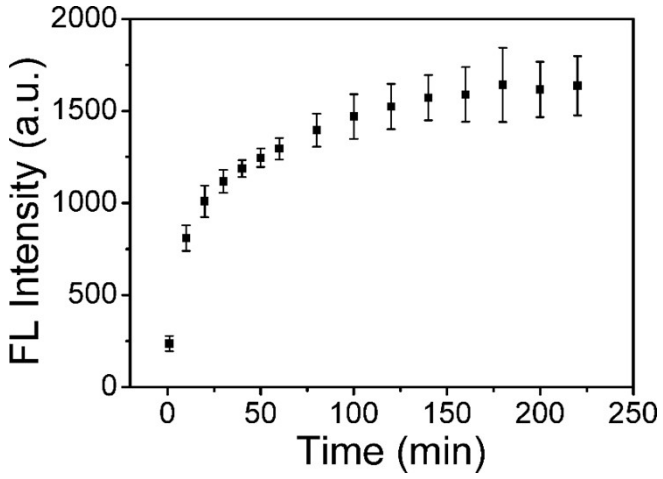


Fig. S9 The fluorescence change of DTB after incubation with Aβ for different time. [DTB] = 1 μM, [Aβ] = 20 μM. λ_{ex} = 440 nm.

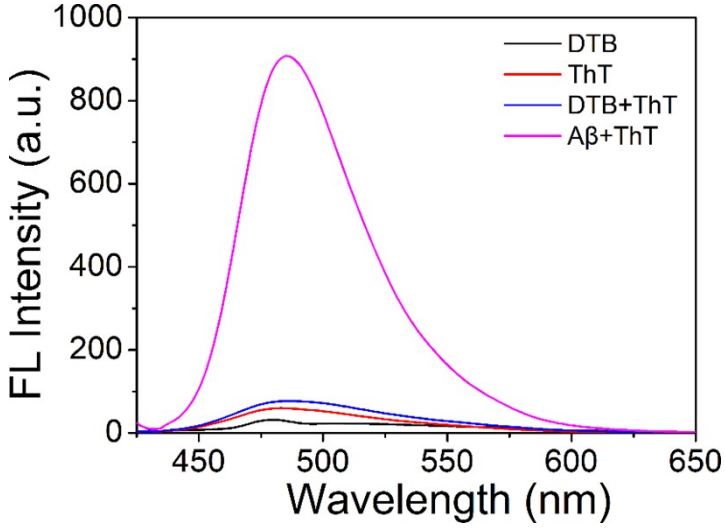


Fig. S10 The formation of Aβ aggregates was confirmed by using ThT. [ThT] = 10 μM, [Aβ] = 5 μM, [DTB] = 1 μM.

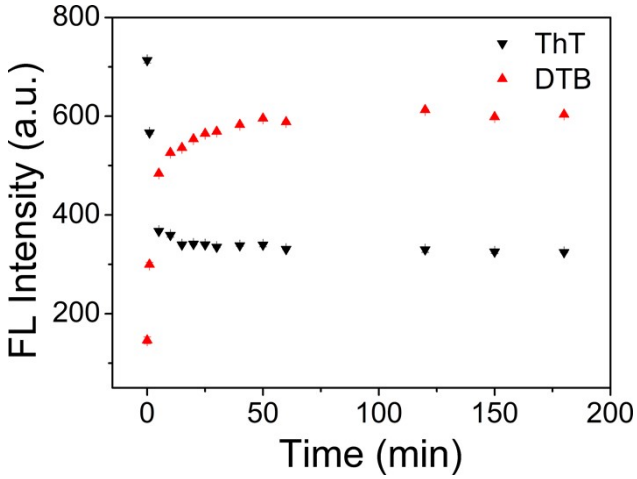
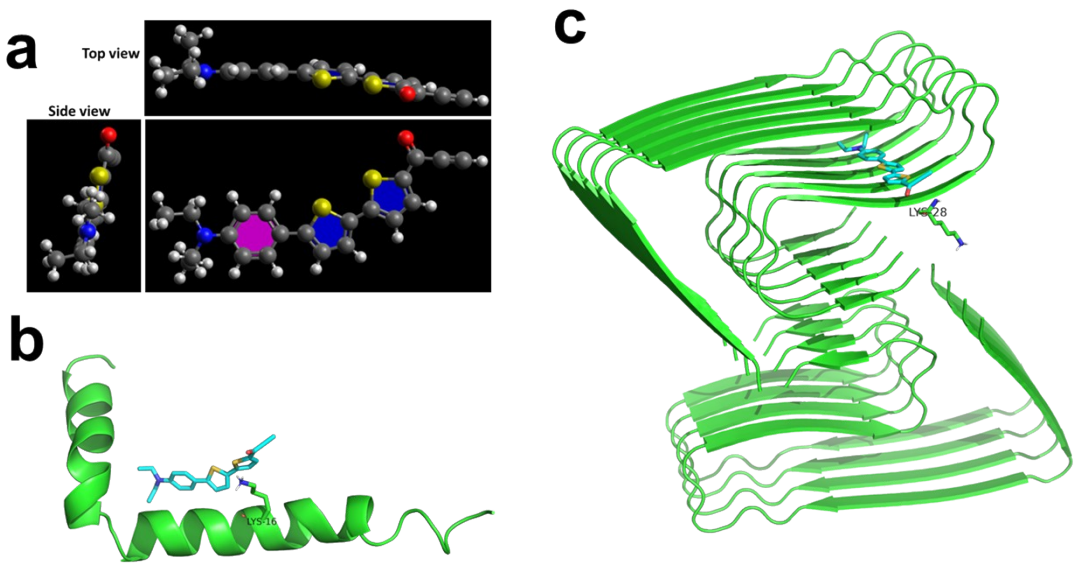


Fig. S11 DTB and ThT fluorescence changes over time after addition of DTB to Aβ/ThT solution that was preincubated for 12 h. [ThT] = 10 μM, [Aβ] = 5 μM.



	Autodock binding energy (kcal/mol)
Aβ42 Monomer (1iyt) +DTB	-5.0
Aβ42 protofibril (5oqv) +DTB	-9.0

Fig. S12 (a) The optimized ground state structure of DTB. The ground state geometry was optimized at PBE0/def2-TZVP level with tight convergence criteria by using the ORCA 5.0.2 program package.[3] Molecules were visualized with Avogadro 1.2.0 program package. (b) the PDB Aβ42 monomer (1iyt) with DTB in the best docking mode. (c) the PDB Aβ42 protofibril (5oqv) with DTB in the best docking mode. Possible reacting lysine residue of DTB were highlighted in stick model (Lys-16 or Lys-28). PDBQT files for receptor and ligand were prepared using AutodockTools 1.5.7.[4] the molecular docking was performed by using Autodock Vina.[5] Molecules were visualized with PyMOL 2.6 (Open-Source Build).

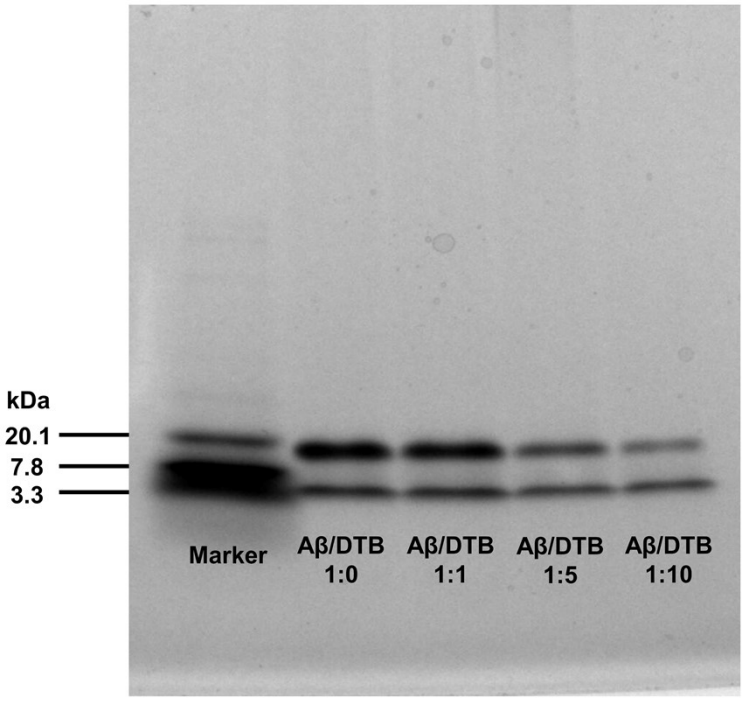


Fig. S13 SDS-PAGE gel electrophoresis of Aβ incubated with DTB at different molar ratios. [Aβ] = 28 μM.

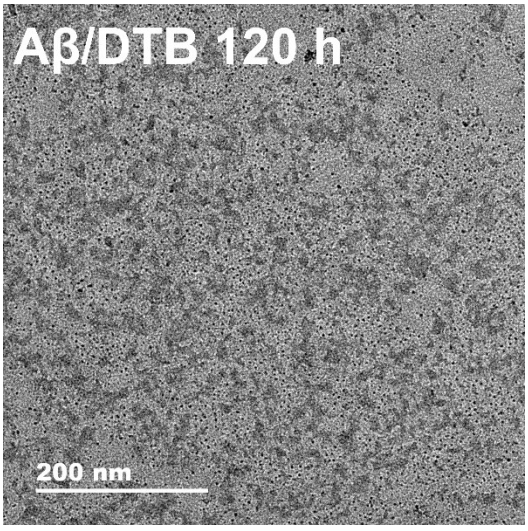


Fig. S14 TEM image of Aβ monomer/DTB incubated for 120 h. [DTB] = 1 μM, [Aβ] = 5 μM. Scale bar is 200 nm.

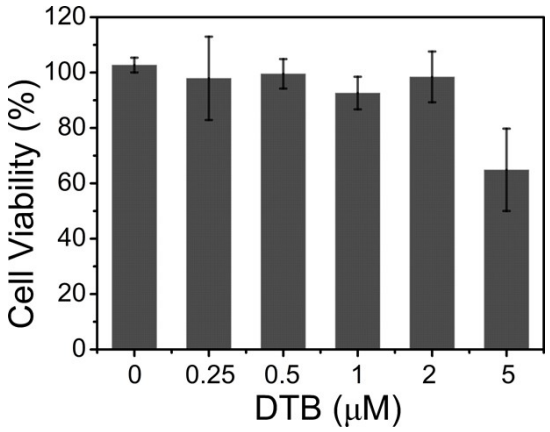


Fig. S15 Cell viability of PC-12 cells after incubated with various concentrations of DTB for 48 h.

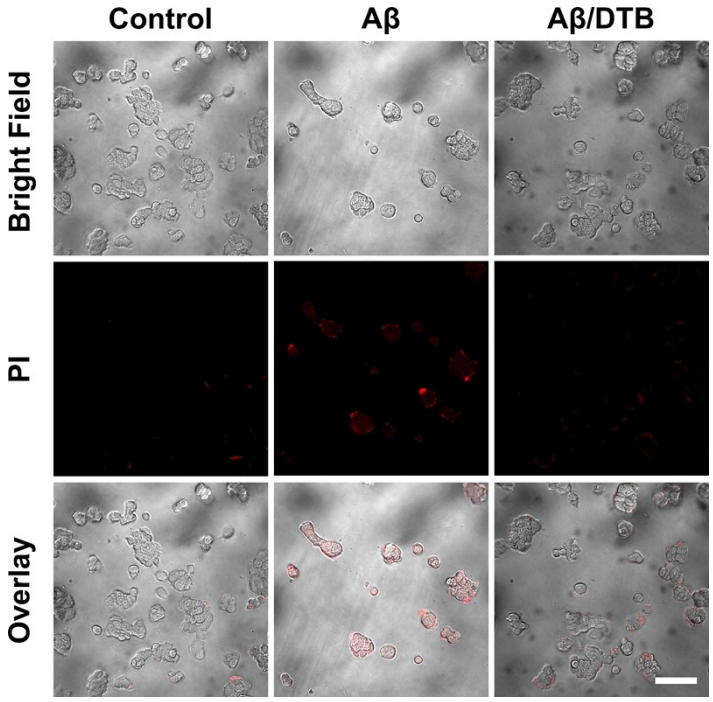


Fig. S16 PI stained images of PC-12 cells after incubated with Aβ or Aβ/DTB for 24 h. [Aβ] = 5 μM, [DTB] = 1 μM, [PI] = 50 μM.For PI: λ_{ex} = 559 nm, λ_{em} = 550-650 nm. The scale bar is 60 μm.

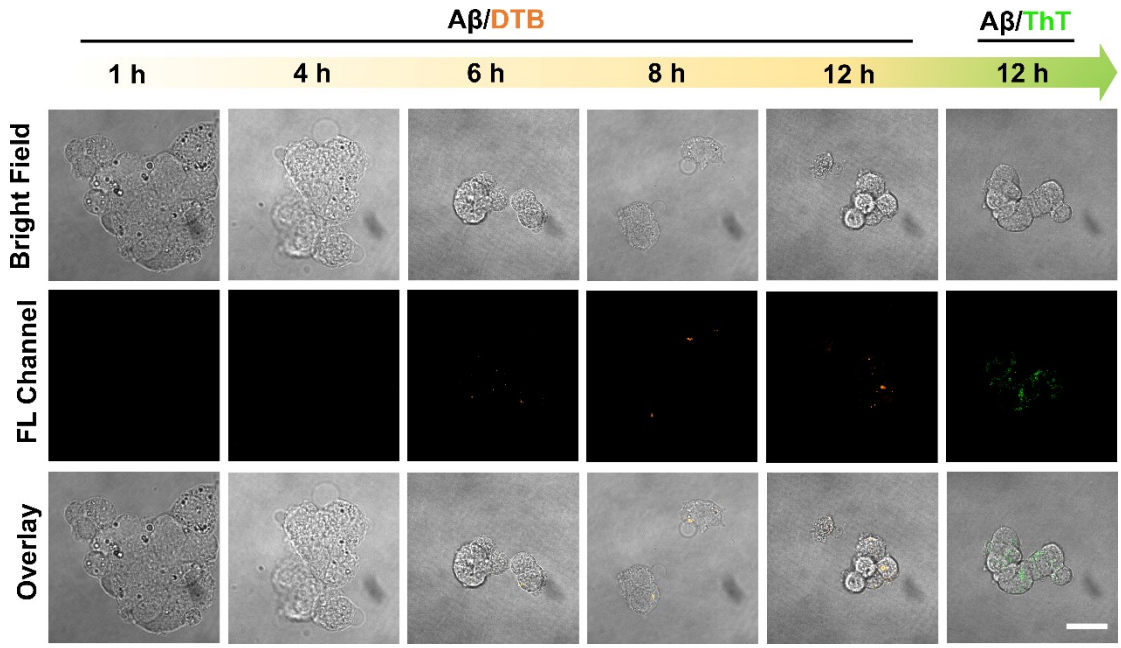


Fig. S17 The CLSM images of Aβ adhesions on PC-12 cell after incubated with DTB or ThT for different times. [Aβ] = 5 μM, [DTB] = 1 μM, [ThT] = 10 μM. The excitation wavelength was 405 nm for ThT, 488 nm for DTB, and collected at 450-500 nm and 550-650 nm for ThT and DTB, respectively. Scale bar is 20 μm.

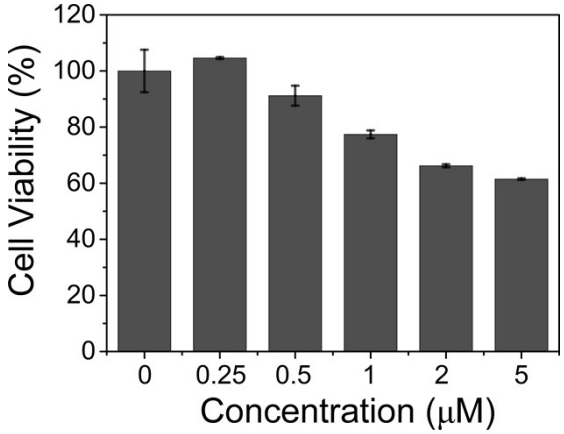


Fig. S18 Cell viability of BV-2 cells after incubated with various concentrations of DTB for 48 h.

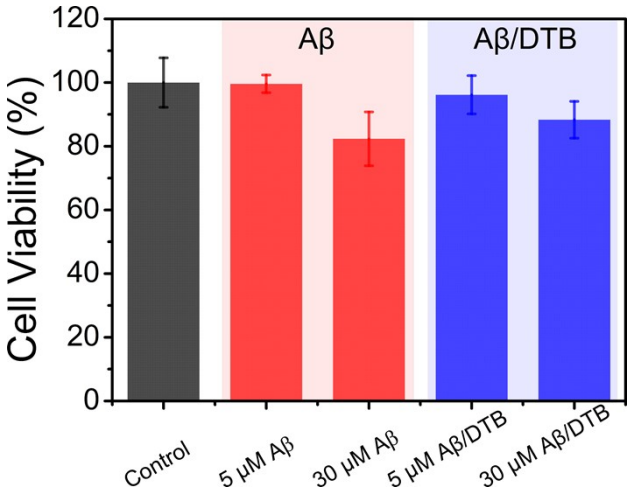


Fig. S19 Cell viability of BV-2 cells after incubated with various concentrations of Aβ and Aβ/DTB for 48 h. [DTB] = 1 μM.

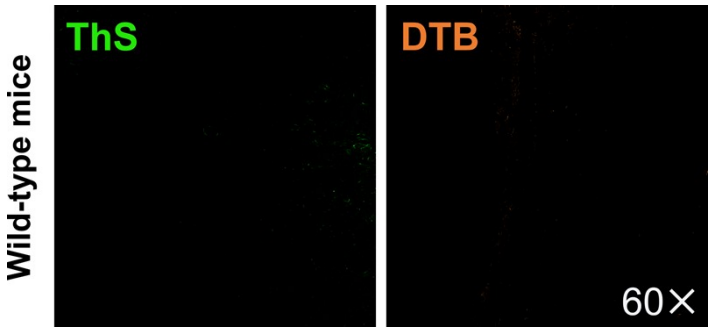


Fig. S20 Histological staining of the brain slices in the hippocampus region from wild-type mice using ThS and DTB, respectively. [ThS] = 10 mg/mL, [DTB] = 100 μM.

References

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