Electronic Supplementary Information (ESI)

Development of BODIPY-based fluorescent probe for imaging pathological tau aggregates in live cells

Sungsu Lim[a†], Md Mamunul Haque[ab†], Dongdong Su[cd], Dohee Kim[ae], Jun-Seok Lee[bf], Young-Tae Chang[*cd] and Yun Kyung Kim[*ab]

[a]*Korea Institute of Science and Technology (KIST), Convergence Research Center for Diagnosis, Treatment and Care System of Dementia, Seoul 136-791, South Korea.*
[b]*Biological Chemistry, University of Science and Technology (UST), Daejon 305–333, South Korea.*
[c]*Department of Chemistry & Med Chem Program, National University of Singapore, Singapore 117543, Singapore.*
[d]*Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, Agency for Science, Technology and Research (A*STAR), Singapore 138667, Singapore.*
[e]*Department of Biotechnology, Translational Research Center for Protein Function Control, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, South Korea.*
[f]*Korea Institute of Science and Technology (KIST), Molecular Rocognition Research Center, Seoul 136-791, South Korea.*
Correspondence should be addressed to Young-Tae Chang (email: chmcyt@nus.edu.sg) or Yun Kyung Kim (yunkyungkim@kist.re.kr).
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† Sungsu Lim and Md Mamunul Haque contributed equally to this work.

Materials and methods

General method for probe synthesis

All chemical reagents for the probe synthesis were obtained from Sigma Aldrich, Alfa Aesar, or MERCK, and used without further purification unless otherwise specified. Column chromatography was carried out on Merck Silica Gel 60 (0.040-0.064 mm, 230–400 mesh). Synthetic reactions and analytical characterization were monitored by HPLC-MS (Agilient-1200 series) with a DAD detector and a single quadrupole mass spectrometer (6130 series) with an ESI probe. NMR spectra (600 MHz and 13C-151 MHz) were recorded on Bruker Avance 600 NMR spectrometers. The high resolution electron spray ionization (HR-ESI) mass spectra were obtained on a Bruker micrOTOFQII.

Preparation of protein aggregates

The Tau-K18 fragment, microtubule-binding repeat region of the longest tau isoform (hTau40) was expressed and purified from *E.coli* BL21 (DE3). To induce tau aggregation, purified Tau-K18 protein (0.5 mg/mL) in PBS (pH 7.4) was incubated with 100 μM DTT (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 mg/mL heparin (MW ~18 kDa; Sigma-Aldrich, St. Louis, MO, USA) at 37 ℃ with 220 rpm shaking for 5 days.[1] To induce insulin aggregation, 0.5 mg/mL of insulin (Sigma-Aldrich, St. Louis, MO, USA) in PBS was incubated at 60℃ with shaking 220 rpm for 2 days.[2] For inducing amyloid beta aggregation, 0.5 mg/mL of amyloid beta 1-42 (Aβ42; American peptide, Sunnyvale, CA, USA) in PBS was incubated at 37 ℃ with 220 rpm shaking for 3 days. The aggregation of protein was monitored by ThioflavinS (ThS; Sigma-Aldrich, St. Louis, MO, USA) fluorescence assay. The aggregation mixture of each protein (125 μg/mL in 25 μL PBS) was transferred to a black 384-well plate with 25 μL of PBS containing 2 μM of ThS. Fluorescence intensity was measured at λ_{ex} = 430 nm and λ_{em} = 480-610 nm in a Flexstation2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

In vitro fluorescence screening

To select 'hit' compounds for detecting tau aggregates, aggregation mixture of Tau-K18 (125 μg/mL in 25 μL PBS) was transferred to a black 384-well plate with 25 μL of PBS containing 2 μM of ThS, PBB3 (in house synthesized), curcumin or

BODIPY probes. After 10 min, fluorescence intensity was measured at λ_{ex} = 430 nm and λ_{em} = 480-610 nm for ThS, PBB3 or curcumin signals, at λ_{ex} = 525 nm and λ_{em} = 560-690 nm for BODIPY probes in a Flexstation2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Fluorescence titration and binding assay

For fluorescence titration assay, various concentrations of tau pre-aggregates, tau aggregates, BSA, actin (Sigma-Aldrich, St. Louis, MO, USA) and GAPDH (Sigma-Aldrich, St. Louis, MO, USA) (0-250 μg/mL in 25 μL PBS) was transferred to a black 384-well plate with 25 μL of PBS containing 2 μM of BD-tau or BDNCA318 for 10 min. Then, the fluorescence intensity was measured at λ_{ex} = 525 nm and λ_{em} = 560-690 nm in a Flexstation2 spectrophotometer. To examine binding affinity of probes in diverse proteins, tau pre-aggregates, tau aggregates, Aβ42, Aβ42 aggregates, insulin and insulin aggregates (125 μg/mL in 25 μL) were transferred to a black 384-well plate with 25 μL of PBS containing 2 μM of BD-tau, BDNCA318, ThS, PBB3 or curcumin. After 10 min, the fluorescence intensity was measured at at λ_{ex} = 430 nm and λ_{em} = 480-610 nm for ThS, PBB3 or curcumin, at λ_{ex} = 525 nm and λ_{em} = 560-690 nm for BD-tau and BDNCA318 in a Flexstation2 spectrophotometer.

Quantum yield determination

The quantum yield values for the probes were determined by comparing to rhodamine 123 using a literature procedure [3]. Both probes and rhodamine 123 were excited at the same wavelength (λ_{ex} = 525 nm), measure the integrated emission intensities, and the quantum yield was calculated by the following equation:

$$QY_x = \frac{F_x f_s n_x^2}{F_s f_x n_s^2} QY_s$$

where QY_x is the quantum yield of the experimental probe, QY_s is the quantum yield of the standard (rhodamine 123; 0.89),[4] F is the integrated fluorescence intensity (areas), f is the absorption factor ($f = 1\text{-}10^{-A}$, where A = absorbance), n is the refractive index of the solvent. As DMSO is used for both the experimental probes and standard as solvent, $[n_x^2/n_s^2]$ will be 1.

Fluorescence spectroscopic measurement in diverse solvents

To examine the effect of solvent polarity on probes emission spectra, 15 different solvents with wide range of polarity index (PI; 0 to 9) were used; Hexane, Toluene, Methyl-t-butyl ether, Xylene, Ethyl ether, Dichloromethane, Dichloroethane, Tetrahydrafuran, Chloroform, Ethyl acetate, Acetonitrile, Dimethyl sulfoxide and PBS. All solvents were purchased from Sigma-Aldrich. Each solvents (49 μL) were transferred to a black 384-well plate with a final concentration of 1 μM of BD-tau or BDNCA318 (1 μL of a 50 μM stock solution). Then, the fluorescence emission was measured at λ_{ex} = 525 nm in a Flexstation2 spectrophotometer. Fluorescence intensities at λ_{em} = 590 nm were represented by using heat map (Fig. S4, ESI†).

Hippocampal neuronal cell culture and imaging

Hippocampal neuronal HT22 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5 % CO_2. For treatment of the probes to tau aggregation induced cells, HT22 cells were plated in a black transparent 96-well plate with or without 40 μM forskolin. After 3 days of tau aggregation activation, HT22 cells were treated with 300 nM of BD-tau, BDNCA318, ThS, PBB3 or curcumin for 30 min. After the incubation, the fluorescence responses of the probes were

automatically imaged by using Operetta® (PerkinElmer™) and the fluorescence intensities were analyzed using Harmony 3.1 software (PerkinElmer™). To confirm the tau aggregation activation by forskolin treatment, HT22 cells were fixed and then, immuno-fluorescence stained with anti-phospho-tau (phospho-Ser199) antibody (1:1000, Abcam). Images were obtained by the Operetta®.

Tau-BiFC cell culture and imaging

HEK293 Tau-BiFC cells were grown in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin and 100 μg/mL Geneticin (G418) in humidified atmosphere containing 5% CO_2 at 37 ^{0}C. For investigation of the probes detection to tau aggregates in the living cells, Tau-BiFC cells were plated in a black transparent 96-well plate with or without 40 μM forskolin for 24 hrs. Then, tau-BiFC cells were treated with 250 nM of BD-tau or BDNCA318. After 10 hrs of incubation, the fluorescence responses of tau-BiFC and the probes were monitored by using Operetta® (PerkinElmer™) and quantified by Harmony 3.1 software. For the fluorescence correlation plot between probes signal and tau-BiFC response, fluorescence intensities of probes-stained tau-BiFC cells according to the distance (Pixels) were quantified by Image J software. For fixed- cell analysis with the probes, tau aggregation induced tau-BiFC cells by forskolin were fixed by 3.7 % formaldehyde for 15 min. After washing with PBS, tau-BiFC cells were incubated with 250 nM of BD-tau or BDNCA318 for 8 hrs. Nuclei were counter stained with Hoechst. The fluorescence images were obtained by the Operetta® (PerkinElmer™).

Histology and immunofluorescence analysis

All animal experiments were approved by the Korea Institute of Science and Technology, and the experimental protocols were carried out in accordance with the approved guidelines by Institutional Animal Care and Use Committee of Korea Institute of Science and Technology. The mice expressed the human P301L mutation of the microtubule-associated protein tau gene (MAPT) [5] were used for this experiment. To get brain tissue sections for the test of BD-tau, the mice were perfused with PBS, and then the brains were removed without fixation. The removed brains were embedded with O.C.T. compound (Tissue-TEK), and then cut coronally by 30 μm thickness by using cryotome. The unfixed brain tissue sections were incubated with 5 μM BD-tau. After BD-tau staining, brain tissues were fixed with 3.7% formaldehyde for immuno-staining. After permeabilization with 0.3 % triton-X in PBS, brain tissue sections underwent blocking by 4 % BSA for 1 hr. Anti-tau PHF antibody (AT8, 1:200, Thermo) were treated overnight at 4 °C. Next day, brain tissue sections were stained with Alexa fluor 488-conjugated antibody (1:500, abcam) and nuclei were counter stained with Hoechst. All images were taken by the Operetta® (PerkinElmer™). BD-tau was detected by λ_{ex} = 520-550 and λ_{em} = 560-630.

References

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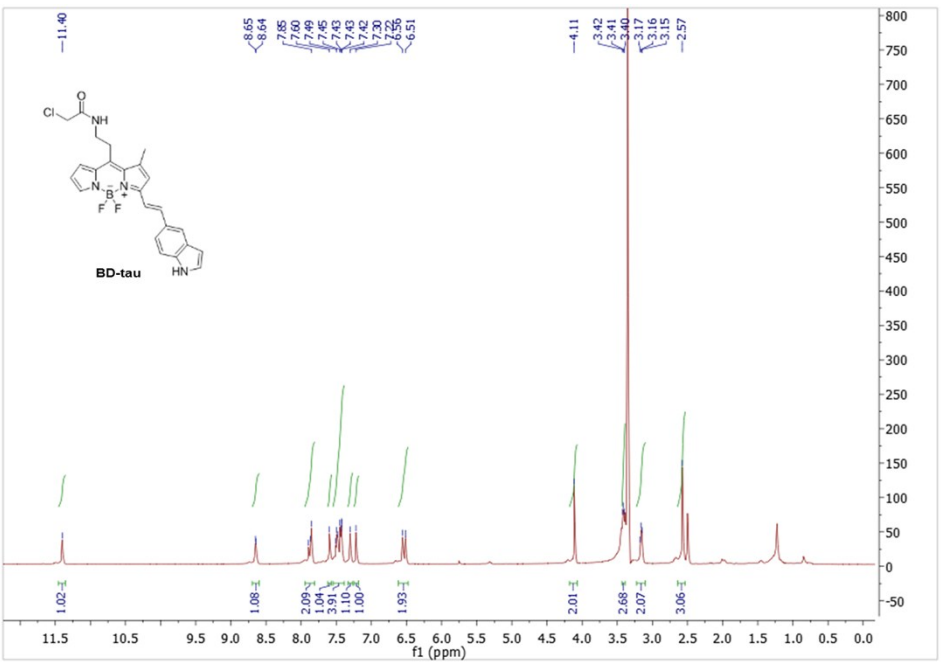
Supplementary Figures

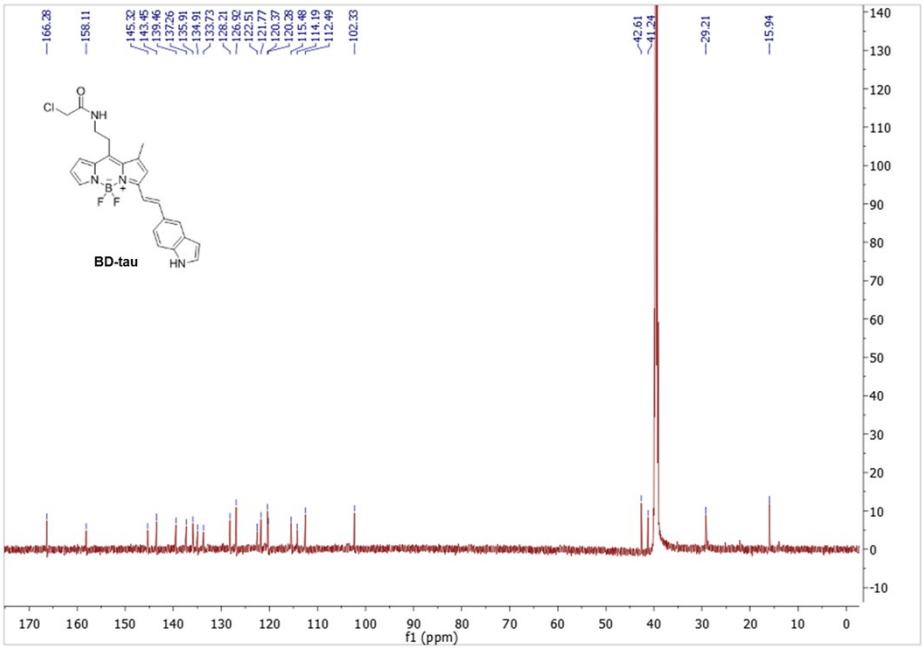
(A) BD-Tau

HRMS m/z ($C_{24}H_{22}BClF_2N_4O$) calculated: 489.1441 (M+Na), found: 489.1436 (M+Na).

^{1}H NMR (600 MHz, DMSO) δ 11.40 (1 H, s), 8.65 (1 H, d, J = 5.3), 7.95 – 7.81 (2 H, m), 7.60 (1 H, s), 7.55 – 7.39 (4 H, m), 7.30 (1 H, s), 7.22 (1 H, s), 6.53 (2 H, d, J = 25.4), 4.11 (2 H, s), 3.43 – 3.39 (2 H, m), 3.23 – 3.10 (2 H, m), 2.57 (3 H, s).

^{13}C NMR (151 MHz, DMSO) δ 166.28, 158.11, 145.32, 143.45, 139.46, 137.26, 135.91, 134.91, 133.73, 128.21, 126.92, 122.51, 121.77, 120.37, 120.28, 115.48, 114.19, 112.49, 102.33, 42.61, 41.24, 29.21, 15.94.



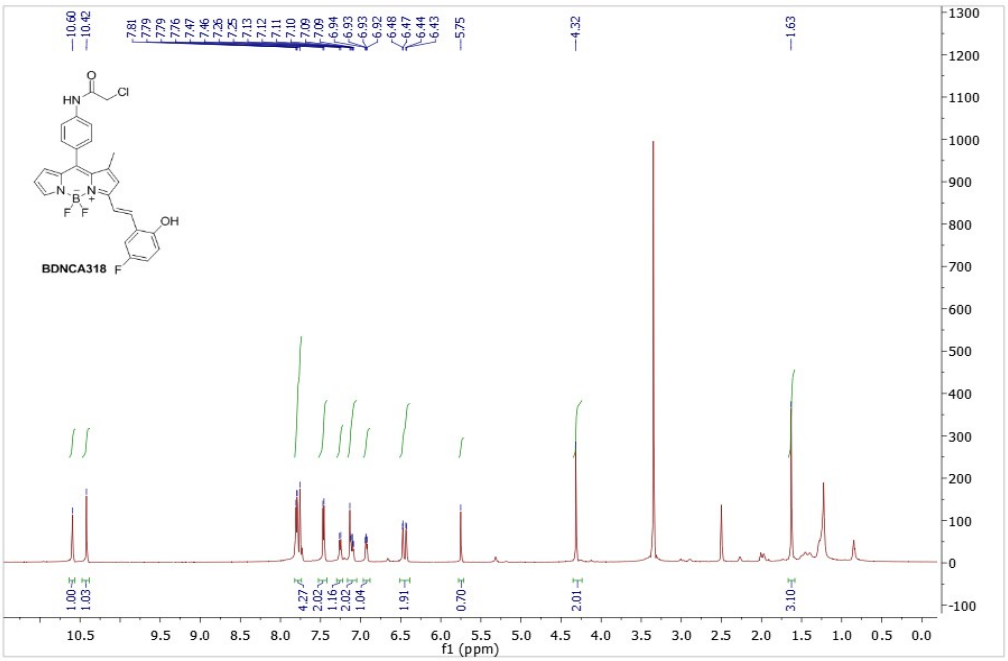


(B) BDNCA318

HRMS m/z ($C_{26}H_{20}BClF_3N_3O_2$) calculated: 532.1187 (M+Na), found: 532.1185 (M+Na).

^{1}H NMR (600 MHz, DMSO) δ 10.60 (1 H, s), 10.42 (1 H, s), 7.79 (4 H, dd, J =15.4, 14.2), 7.46 (2 H, d, J =7.6), 7.25 (1 H, d, J =9.3), 7.16 – 7.05 (2 H, m), 6.93 (1 H, dd, J = 8.6, 4.7), 6.45 (2 H, dd, J =25.3, 2.6), 5.75 (1 H, s), 4.32 (2 H, s), 1.63 (3 H, s).

^{13}C NMR (151 MHz, DMSO) δ 165.05, 158.08, 156.27, 154.71, 153.27, 145.77, 141.37, 139.85, 138.11, 135.87, 134.40, 129.86, 128.48, 126.06, 123.35, 119.88, 119.40, 118.94, 117.88, 117.73, 117.48, 116.48, 114.11, 113.96, 43.64, 15.14.



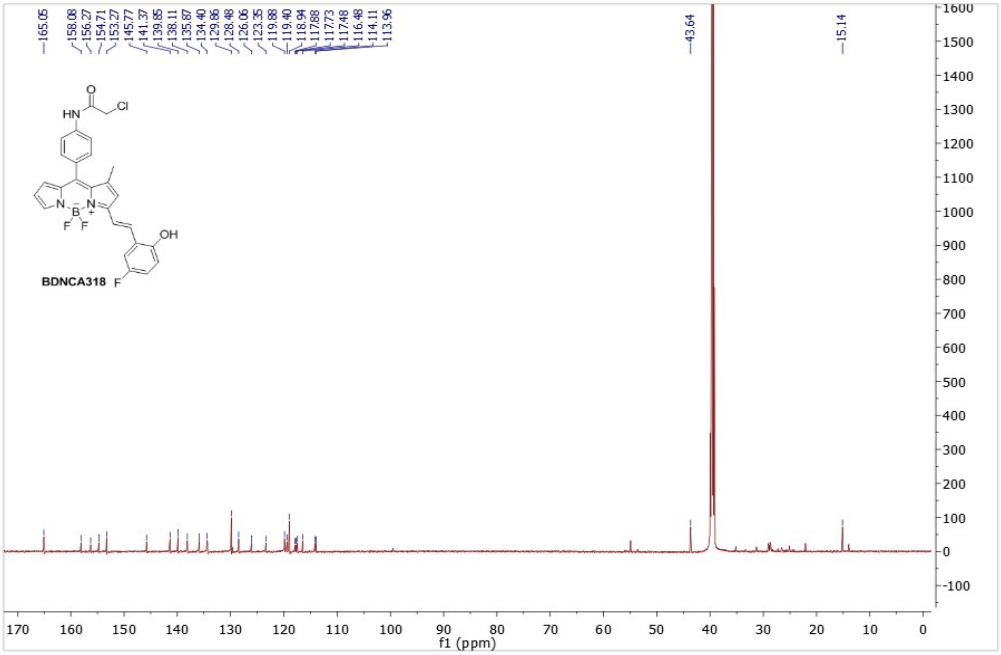


Fig. S1 Characterization of Hit compounds. (A) BD-tau and (B) BDNCA318.

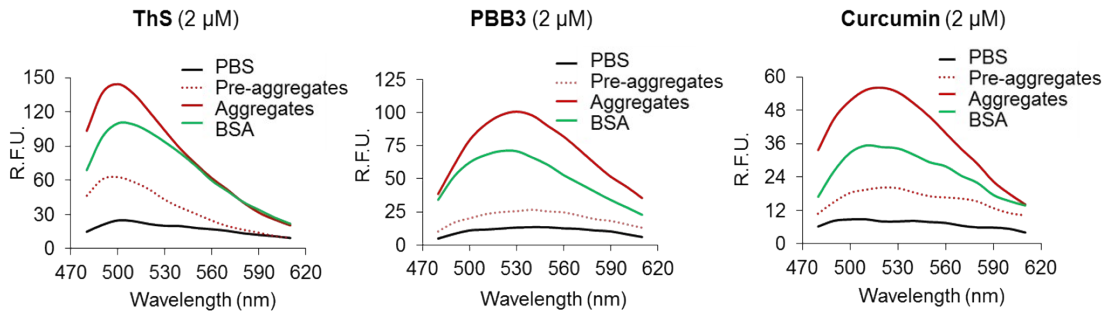


Fig. S2 Emission spectra of ThS, PBB3 and curcumin. Tau pre-aggregates (0.5mg/mL) in PBS was incubated with 100 μM DTT and 0.1 mg/mL heparin at 37 ^{0}C with shaking for 5 days. Then, 125 μg/mL of tau aggregates were mixed with 2 μM of ThS, PBB3 or curcumin, and the fluorescence intensity was measured at an λ_{ex}=430 nm and λ_{em} =480-610 nm.

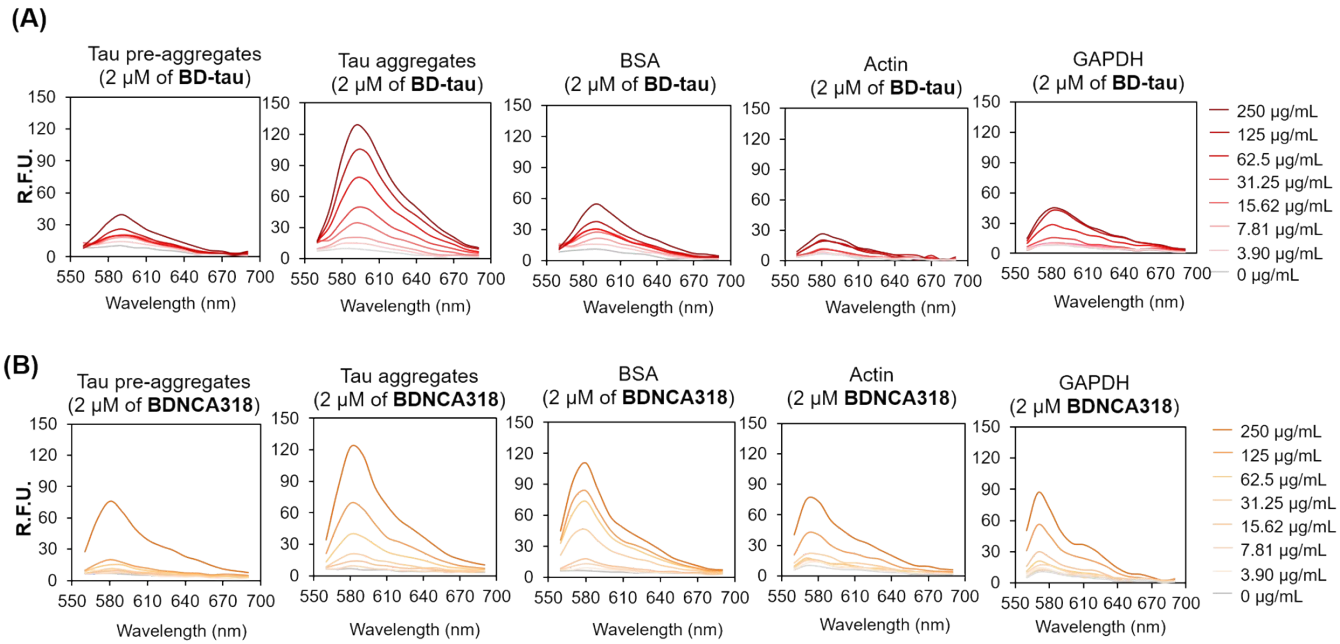


Fig. S3 Fluorescence intensity of 2 μM of BD-tau (A) or BDNCA318 (B) upon interaction with different concentrations of tau pre-aggregates, tau aggregates, BSA, actin or GAPDH (0-250 μg/mL; serial dilution). BD-tau fluorescence intensity increases in the presence of tau aggregates in a concentration-dependent manner. In contrast, BDNCA318 fluorescence intensity increases in the presence of tau aggregates as well as BSA, actin, and GAPDH.

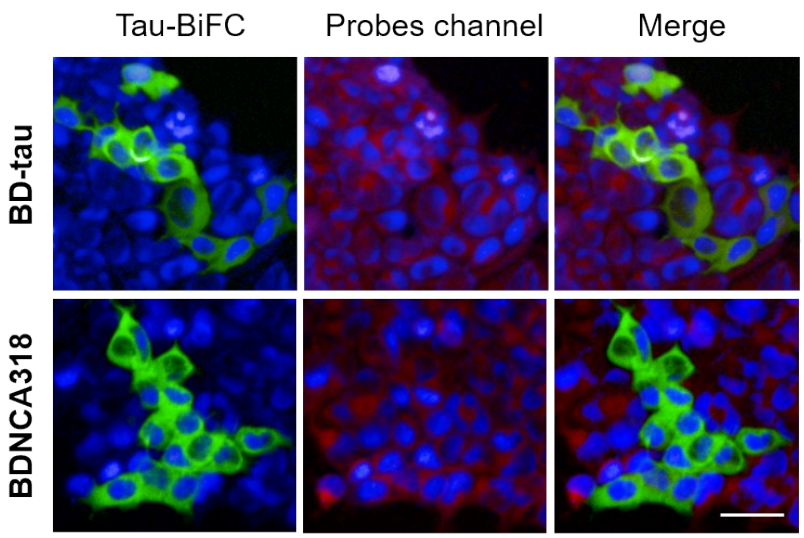


Fig. S4 Fluorescence images of fixed HEK293 Tau-BiFC cells treated with BD-tau or BDNCA318. Tau-BiFC cells were treated with forskolin for 24 hr to induce tau aggregation. Then, cells were fixed, and 250 nM of BD-tau or BDNCA318 were treated for 8 hr. Nuclei were counter stained with Hoechst. Tau-BiFC fluorescence was detected by λ_{ex} = 460-490 nm, λ_{em} = 500-550 nm filters, and BDMCA491 or BDNCA318 was detected by λ_{ex} = 520-550 nm, λ_{em} = 560-630 filters. Scale bar = 50 μm.

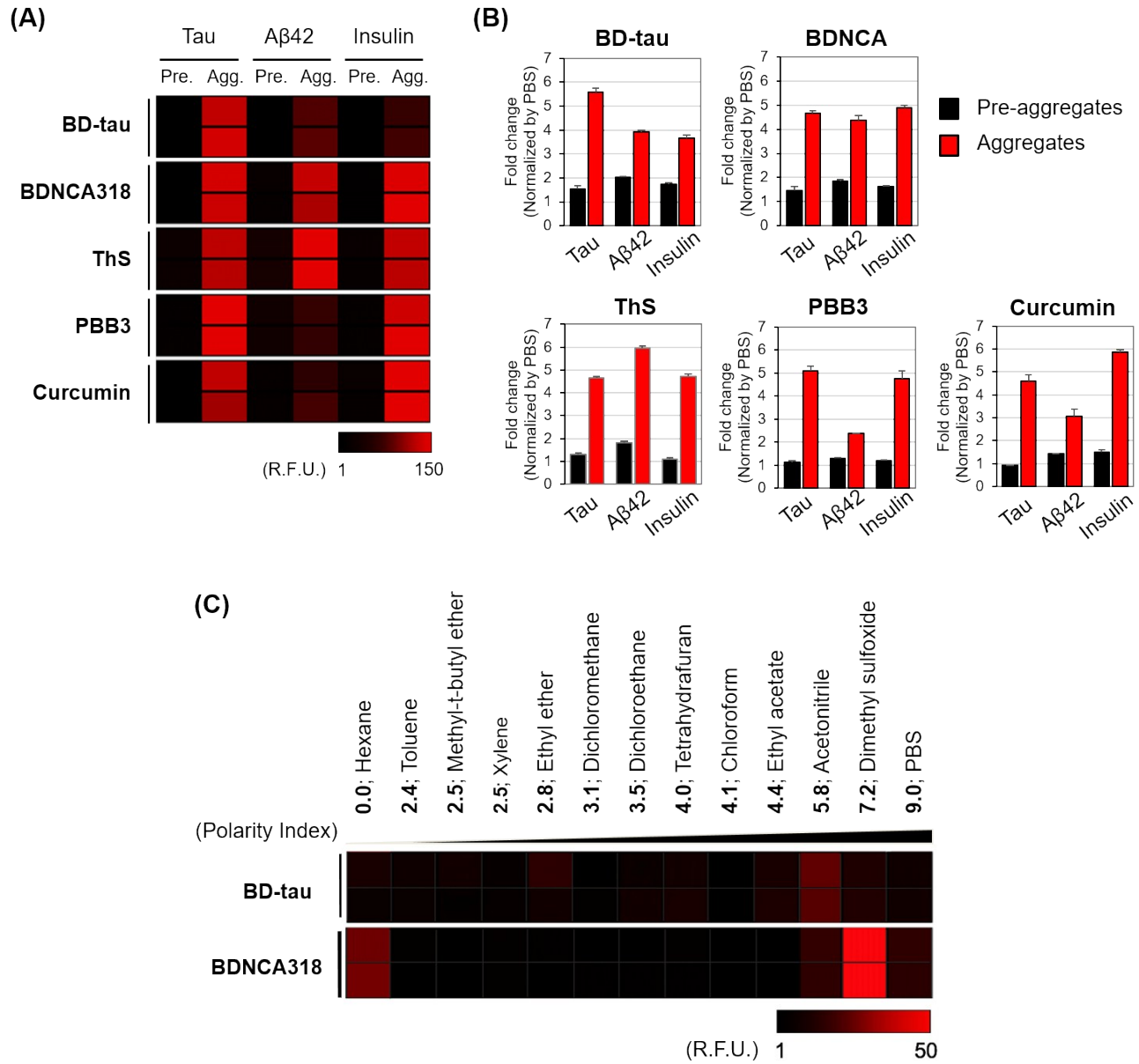


Fig. S5 Selective binding affinity of probes upon interaction of diverse β-sheet proteins. (A-B) 2 μM of BD-tau, BDNCA318, ThS, PBB3 or curcumin was incubated with 125 μg/mL of pre-aggregates (Pre.) or aggregates (Agg.) form of tau, Aβ42, insulin for 10 min. Then, the fluorescence intensity was measured at λ_{ex} = 430 nm and λ_{em} = 480-610 nm for ThS, PBB3 or curcumin, and at λ_{ex} = 525 nm and λ_{em} = 560-690 nm for BD-tau or BDNCA318. The fluorescence intensities at emission maximum were represented by using heat map (A). The black-red color scale of heatmap represents the value of relative fluoresecence unit (R.F.U.). BD-tau's fluorescence was turned on by diverse β-sheet protein aggregates, but BD-tau showed the strongest response to tau aggregates. In comparison, other conventional probes did not show any selectivity to tau aggregates by showing strong responses against amyloid plaque or insulin aggregates. (B) Quantification of probe fluorescence intensity normalized by that of PBS. Error bars represent standard deviation from four repeated experiments. (C) Fluorescence intensity profiles of BD-tau or BDNCA318 in diverse solvents with wide range of polarity index (PI, 0 to 9). BD-tau or BDNCA318 was dissolved in 13 different solvents at a final concentration of 1 μM. Then, the fluorescence emission was measured at λ_{ex} = 525 nm. The balsck-

red color scale of heatmap represents the value of relative fluoresecence unit (R.F.U.). The fluorescence intensity of BD-tau was slightly increased when dissolved in acetonitrile (PI; 5.8). However, there is no clear tendency to correlate the fluorescence intensity with polarity effect.

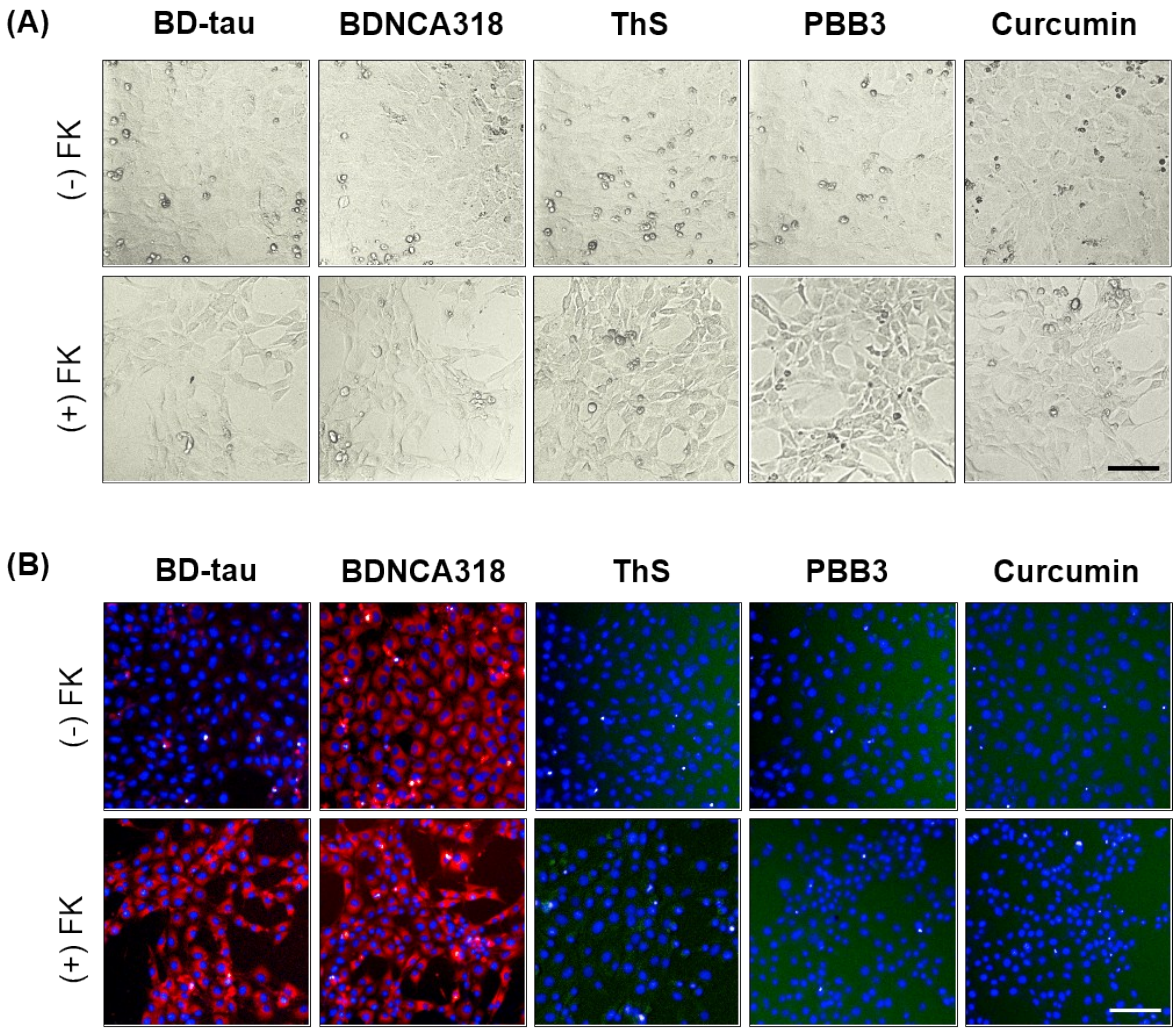


Fig. S6 Bright field (A) and probe/Hoechst (B) images of living HT22 cells incubated with probes in Fig. 2C. Scale bar = 100 μm.