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Supplementary Information

Capturing Protein Droplets: Label-free Visualization and Detection of Protein Liquid-Liquid Phase Separation with an Aggregation-Induced Emission Fluorogen

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Experimental Procedures

Chemicals

Fmoc-amino acids and Rink amide resin were purchased from GL Biochem (Shanghai). 6-maleimidocaproic acid was purchased from HEOWNS. 1,2-bis[4-(3-sulfonatopropoxyl) phenyl]-1,2-diphenylethene (DroProbe) was purchased from AIEgen Biotech Co. Limited. DroProbe is commercially available, and we prepared a stock solution in all the experiments described in this article except when otherwise specified. 5(6)-carboxyfluorescein was purchased from Innochem. Commercially available reagents were used without further purification unless noted otherwise.

Expression, purification of hTau441 and TDP-43

Recombinant hTau441 with His-tag on its N-terminal end was expressed in BL21 (DE3) *E.* coli cells. Bacteria were grown at 37 ℃ overnight and harvested after induction with 1 mM IPTG at 16 ℃ for 16h. Cells were collected by centrifugation and pellets were re-suspended in the lysis buffer (50mM Tris, 150 mM NaCl, 1 mM EDTA, 1mM PMSF, pH 7.4) and lysed by being sonicated on ice. The lysates were centrifuged at 25125g for 60min and the supernatant was collected. The supernatant was boiled for 2 min, then cooled on ice and centrifuged at 25126g for 20 min. The supernatant was collected and applied on nickel-nitrilotriacetic acid (Ni-NTA) affinity column resin with 20 mM competitive imidazole and mixed for 5h. The protein was eluted by concentration-gradient imidazole buffer (from 20 to 300 mM imidazole, 50 mM Tris-HCl and 150 mM NaCl). Then the elution containing hTau441 was then purified by size exclusion chromatography (Superdex 75 10/300 GL) in 15mM Tris buffer, 150mM NaCl, pH 7.4. Purified protein was concentrated and stored in stock solution.

TDP-43（264-414, with His-tag on C-terminal） was expressed in BL21 (DE3) *E.* coli overnight and harvested after induction with 1 mM IPTG at 16 ℃ for 16h. Bacteria were lysed by being sonicated in Buffer A (20 mM Tris-HCl buffer, pH 8, containing 8 M urea, 500 mM NaCl, and 25 mM imidazole). Protein was purified on Ni-NTA affinity column with Buffer A washing, and subsequent elution in Buffer B (20 mM Tris-HCl buffer, pH 8, containing 8 M urea, 200 mM NaCl, and 250 mM imidazole). The elution was further purified by HPLC using a C4 column acetonitrile gradient in water containing 0.05% trifluoroacetic acid. Purified protein was flash frozen and lyophilized.

Single-Cys TDP43 (with tag CELHHHHHH on C-terminal) were generated by site-directed mutagenesis, expressed and purified by Ni-NTA as described above but in the additional presence of 1 mM TCEP as described in a published article.[1]

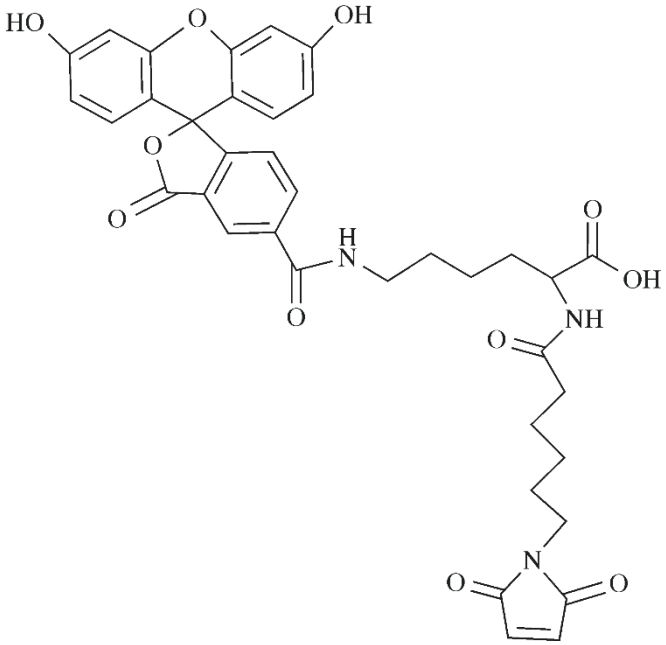
Expression EGPF recombinant U1-70K LC2 (EGFP-LC2)

The vector of EGFP-LC2 (317-407) was received as a gift from Prof. Shizhong Luo's lab (Beijing University of Chemical Technology, China). EGFP-LC2 proteins were expressed in Transetta (DE3) *E.* coli cells. Bacteria were grown at 37 ℃ and harvested after induction with 1 mM IPTG at 16 ℃ for 16h. Then, cells were collected by centrifugation and re-suspended in the lysis buffer (50mM Tris, 150 mM NaCl, 1 mM EDTA, 1mM PMSF, pH 7.4) followed by being sonicated on ice. The lysates were centrifuged at 25125g for 60min and the supernatant was collected. The supernatant was collected and applied on nickel-nitrilotriacetic acid (Ni-NTA) affinity column resin with 20 mM competitive imidazole and mixed for 5h. The protein was eluted by concentration-gradient imidazole buffer (from 20 to 300 mM imidazole, 50 mM Tris-HCl and 500 mM NaCl). EGFP-LC2 were desalted into PBS by desalting column (PD-10 desalting columns contain Sephadex® G-25, GE HealthCare).

Synthesis of FL-labeled hTau441 and TDP43

FL01(Scheme S1, MW: 697.23) was synthesized as normal SPPS methods. The Fmoc-Lys (Mtt)-Wang resin (361 mg) was adopted and mixed with 5 ml piperidine for 20 min with medium shaking to remove Fmoc. Then 6-maleimidocaproic acid (86 mg) were dissolved in DCM with HATU (4 eq.), HOAt (4 eq.), and DIEA (8 eq.) and added into the peptide synthesis vessel with medium shaking for 60 min. Then the resin was treated with 2% TFA in DCM for 30 min to remove Mtt- on the lysine side-chain. 5(6)-carboxyfluorescein(120 mg) was dissolved in DCM with

HATU (4 eq.), HOAt (4 eq.), and DIEA (8 eq.) and added into the vessel with medium shaking for overnight. FL01 was cleaved by a mixture of TFA/ water/ (95/5) and precipitated by cold diethyl ether and purified by HPLC with a C18 column.



Scheme S1 Structure of FL01.

Two native cysteines of hTau441 and single-cysteine variants of TDP43 was adopted to fluorescence labeling in Tris buffer at pH 7.4. Before labeling, hTau441 was treated with TCEP for 1h at 25 ℃. Then 10eq. FL01 was dissolved in 100ul water/acetonitrile (95/5) and added into protein solution for 2h at 25 ℃. Extra FL01 was removed by a desalting column (PD-10 desalting columns contain Sephadex® G-25, GE HealthCare). The labeling efficiency was evaluated by the related concentration of protein and fluorescein.

Sample preparation for phase separation

After purification, EGFP-LC2 protein solution was concentrated into HEPES buffer (pH=7) to prepare a stock solution of concentration of 600 μM and preserved at -80 ℃. Protein concentration was measured by BSA assay. Purified hTau441 protein solution was concentrated into Tris buffer (pH=7) to prepare a stock solution of concentration of 200 μM and preserved at -80 ℃. Protein concentration was measured by BSA assay. Lyophilized TDP-43 protein was dissolved in DMSO to prepare a stock solution of 1mM preserved in -80 ℃. Before every LLPS assay, EGFP-LC2 stock solution and hTau441 stock solution were centrifuged at 8000g for 1min to remove oligomers and precipitates. Before every LLPS assay, TDP-43 stock solution was sonicated for 5min to remove oligomers and precipitates. In confocal-LLPS assay, EGFP-LC2 in stock solution was diluted in 10mM NaCl 15mM HEPES buffer and mixed with 60% PEG as certain volume ratio to certain final EGFP-LC2 concentration. For TDP-43 and hTau441 droplets used for fluorescence imaging by fluorescein, FL-labeled protein and un-label protein were used as 1:20 molar ratio. DroProbe was prepared as stock solution with concentration of 500 μM into Tris buffer and preserved at -20 ℃. The stock solution was sonicated for 5min before use. For DroProbe monitored protein droplets, hTau441 in stock solution was diluted in 10mM/150/250 mM NaCl 15mM HEPES buffer with certain concentration DroProbe, and then mixed with 60% PEG and as certain volume ratio to certain final hTau-441 concentration. For DroProbe monitored protein droplets, TDP-43 and in stock solution was diluted in 150mM NaCl, 10mM Tris buffer with certain concentration DroProbe to a certain final TDP-43 concentration in an experiment.

For droplets fusion characterized by DroProbe, 25 μM unlabelled hTau441 and 20 μM DroProbe were incubated in 15 mM NaCl and 10 mM HEPES buffer with 10% PEG.

For pictures for test tube, the homogenous hTau solution tube containing 10 μM hTau441 and 10 μM DroProbe in 1 M NaCl 10 mM HEPES buffer.The LLPS solution tube contains the same concentration of hTau441 and DroProbe as the homogenous hTau solution tube, but the buffer is 15 mM NaCl, 10 mM HEPES buffer with 30% PEG. DropPobe only tube contained 20 μM DroProbe and the same buffer as the LLPS but no protein.

Fluorescence confocal microscopy of droplets

Image from confocal microscopy was performed with an inverted Carl Zeiss LSM 780 microscope equipped with lasers of 405nm or 488nm excitation. Images were acquired using a Zeiss 63× oil immersion lens. Samples were applied to a glass bottom black wall 384 well plate (Cell-vis) for imaging. All the samples were transferred into plates for imaging immediately after being prepared. Droplets profile analysis was done by using Zen (black edition).

FRAP measurements and repetitive bleaching experiments

FRAP. Samples containing droplets were applied onto microscope slide and covered with a NEO micro cover slide (Matsunami Glass IND LTD). Experiments were performed on a Carl Zeiss LSM 780 microscope equipped with a 63x oil immersion objective. Full-bleach was performed with a pixel resolution 40 nm*40 nm and dimension of 1024*1024. Droplets label with FL- were bleached by a 488 laser at 100% power for 120 iterations, and post bleaching images were taken at 2% laser power at rate of 1.032s. DroProbe monitored droplets were bleached by a 405 nm laser at 100% for 300 iterations, and post bleaching images were taken at 14% laser power at rate of 1.032s. For each time point, at least three droplets were bleached either in each frame or in several frames.

Before repetitive bleaching, 3 images were taken. Repetitive bleaching (by 405 nm laser at 20% power) of the ROI was done after every 4 images was taken (by 405nm 2% power and 488nm 0.4% power). The fluorescence emission intensity of the ROI was monitored for DroProbe at 410-477 nm and FL 510-589 nm.

Turbidity assay

Proteins were prepared as described above. Turbidity was measured by absorption at 350 nm in 384-well plates using a BioTech™ microplate reader. All samples were examined in triplicate (n = 3).

Fluorescence emission spectrum

Proteins were prepared as described above. Fluorescence emission spectrum was measured by excitation at the optimum excitation wavelength at 365nm. The emission spectrum was collected from 400nm to 600nm at 5nm per step. A black well transparent bottom 384-well plate was used in a BioTech™ microplate reader. All samples were examined in triplicate (n = 3). Fluorescence emission spectrum of DroProbe on different glycerol fraction was measured by Edinburgh fluorescence lifetime spectrometry.

Fluorescence lifetime microscopy of droplets

Protein droplets were prepared as described above. Fluorescence lifetime microscopy of droplets were performed on an invert Olymplus FV1200 Confocal microscope with 63x/1.4 oil immersion objective incorporating a FLIM TCSPC system with 405nm laser. The protein droplets were applied onto 384 well plates immediately after the droplets were initiate if not otherwise mentioned. Five ROI region of each lifetime image was randomly selected in the frame and the each τ_{Avr} was calculated using 2 exponential tailfit in the SymPhoTime software. The whole τ_{Avr} of each sample and the standard deviation was calculated from the five ROI. .

Fluorescence lifetime spectrometry

Protein droplets were prepared as described above. Fluorescence lifetime microscopy of droplets were performed on an Edinburgh fluorescence lifetime spectrometry. The raw data was fitted by formula: $I = A+B_1\exp(-t/\tau_1) + B_2\exp(-t/\tau_2) + B_3\exp[-t/\tau_3]$. B_1, B_2 and B_3 represented as the amplitudes of three decay terms, whereas t_1, $t_2$and t_3 are the lifetime components, respectively. The average fluorescence life- time was calculated using the equation : $t_{avg} = B_1t_1 + B_2t_2 + B_3t_3$.

Autofluorescence spectrum and T_m of protein

The autofluorescence spectrum and T_m of protein were measured on a UNit system (Unchained Labs). Samples were prepared with or without DroProbe in 10mM NaCl, 15mM Tris buffer pH 7.4. Original spectrum data was normalized by the maximum emission intensity of the spectrum. The T_m was analyzed by BCM (Barycentric method).

Viscosity and Beer-Lambert plot

The viscosity was measured by Aaton Paar Physica MCR301/302 Rotational Rheometer. Mixtures of glycerol and ethanol were prepared with different glycerol volume fraction. Beer-Lambert plot was obtained by measuring UV-Vis spectrum of DroProbe.

Supplementary Figures

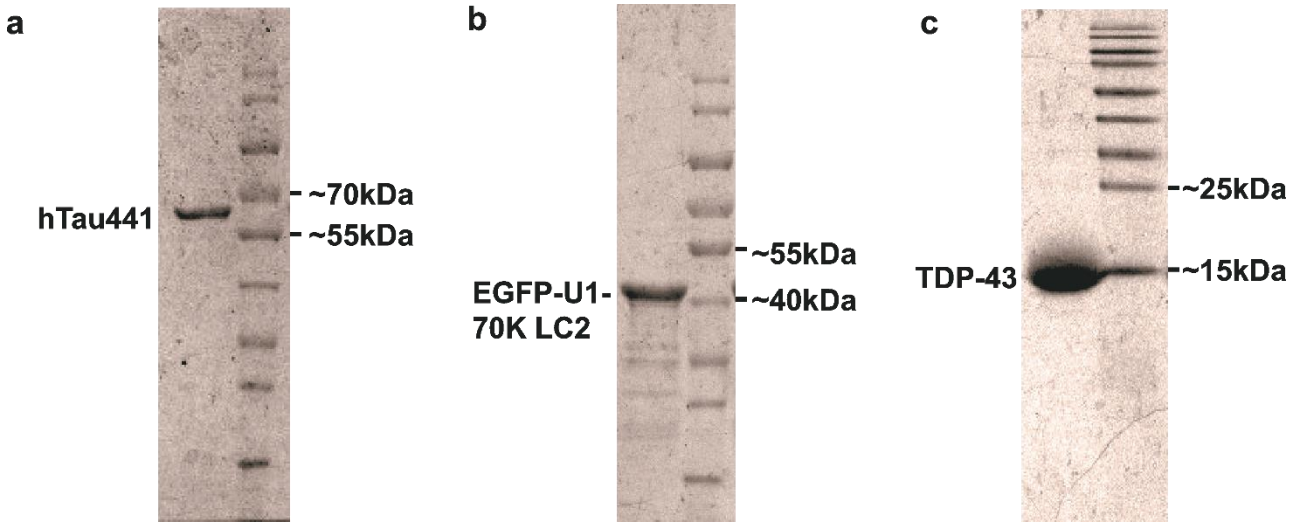


Figure S1 SDS PAGE of purification traces of hTau441(a), TDP-43(b) and EGFP-U1-70k LC2 (c). Line 1 is purified protein, and line 2 is protein ladder.

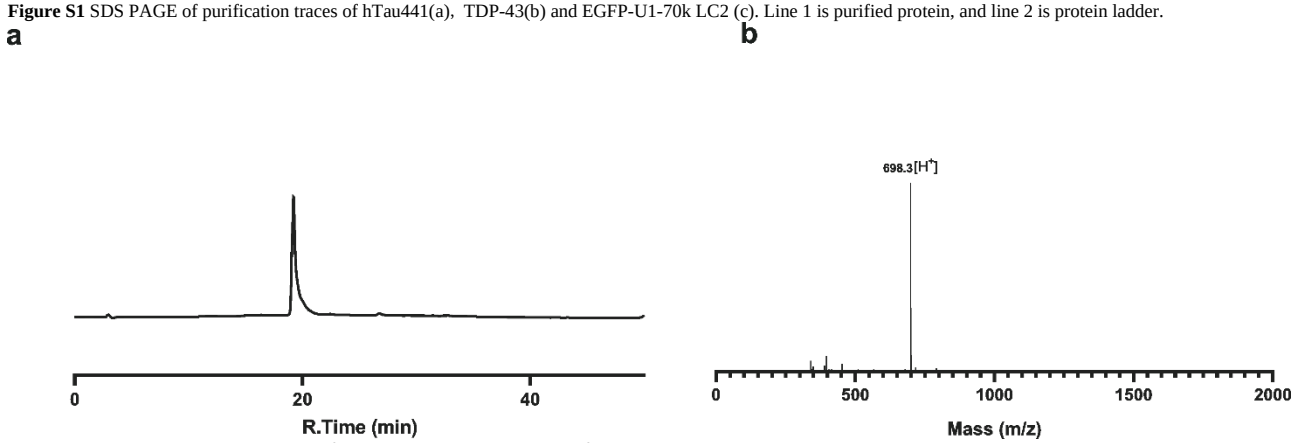


Figure S2 (a) Analytical RP-HPLC trace of FL01 (0-50 min: liner gradient of 0%B to 100%B. A: 100% water +0.06% TFA, B: 20% water +80% acetonitrile +0.06% TFA. All solvents are RP-HPLC grade). (b) ESI mass spectrum of FL01.

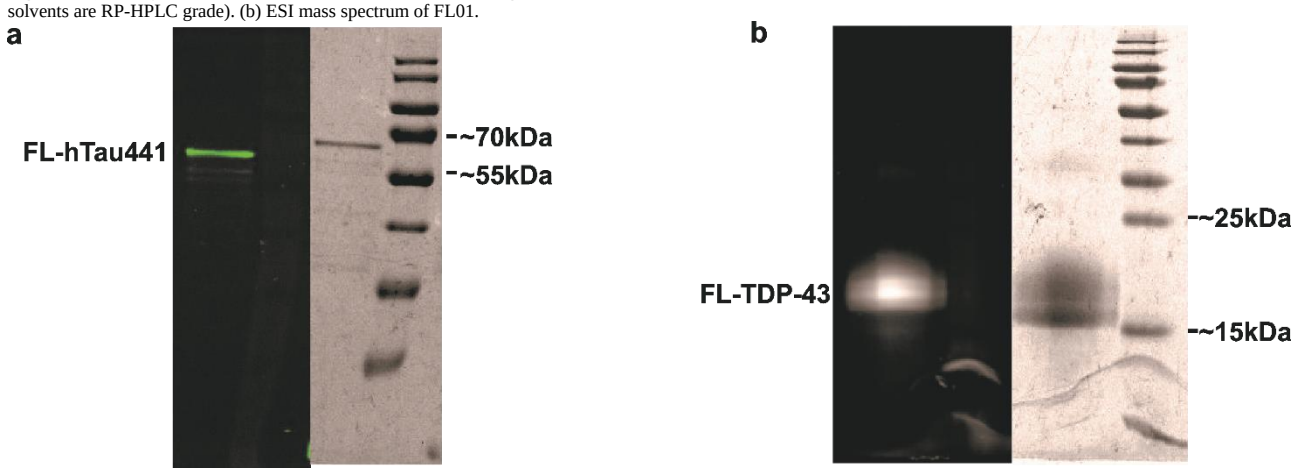


Figure S3 SDS PAGE of purification traces of FL-hTau441(a) and FL-TDP-43(b). Line1 is purified protein, and line 2 is protein ladder.

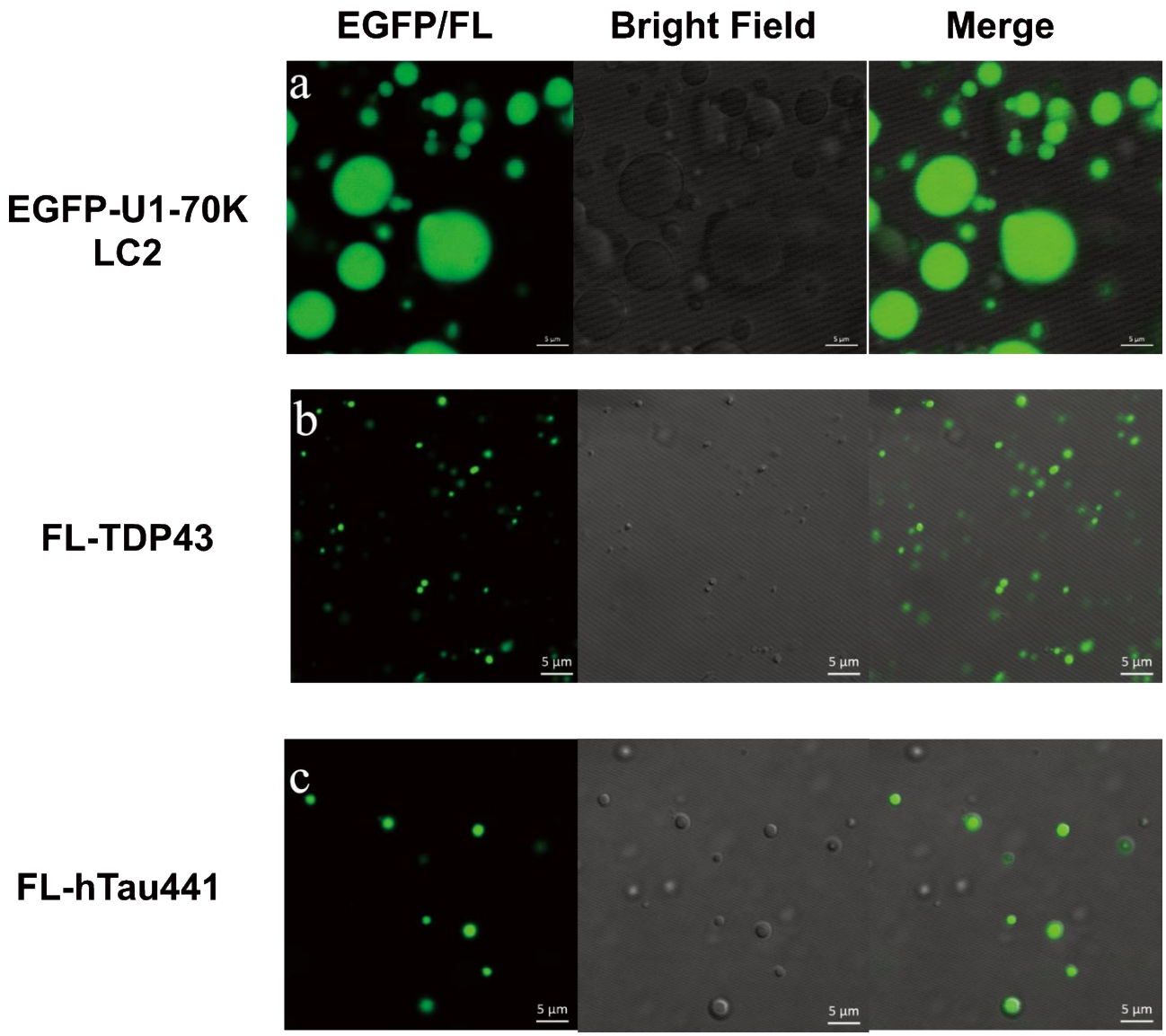


Figure S4 Confocal image of EGFP or fluorescence labelled protein LLPS. (a)200 µM EGFP-LC2 was incubated in 10 mM NaCl and 20% PEG 15 mM HEPES buffer. (b)20 µM FL-TDP-43 was incubated in 150 mM NaCl 10 mM Tris buffer. (c)20 µM FL-hTau441 was incubated in 10 mM NaCl and 10% PEG 15 mM HEPES buffer.

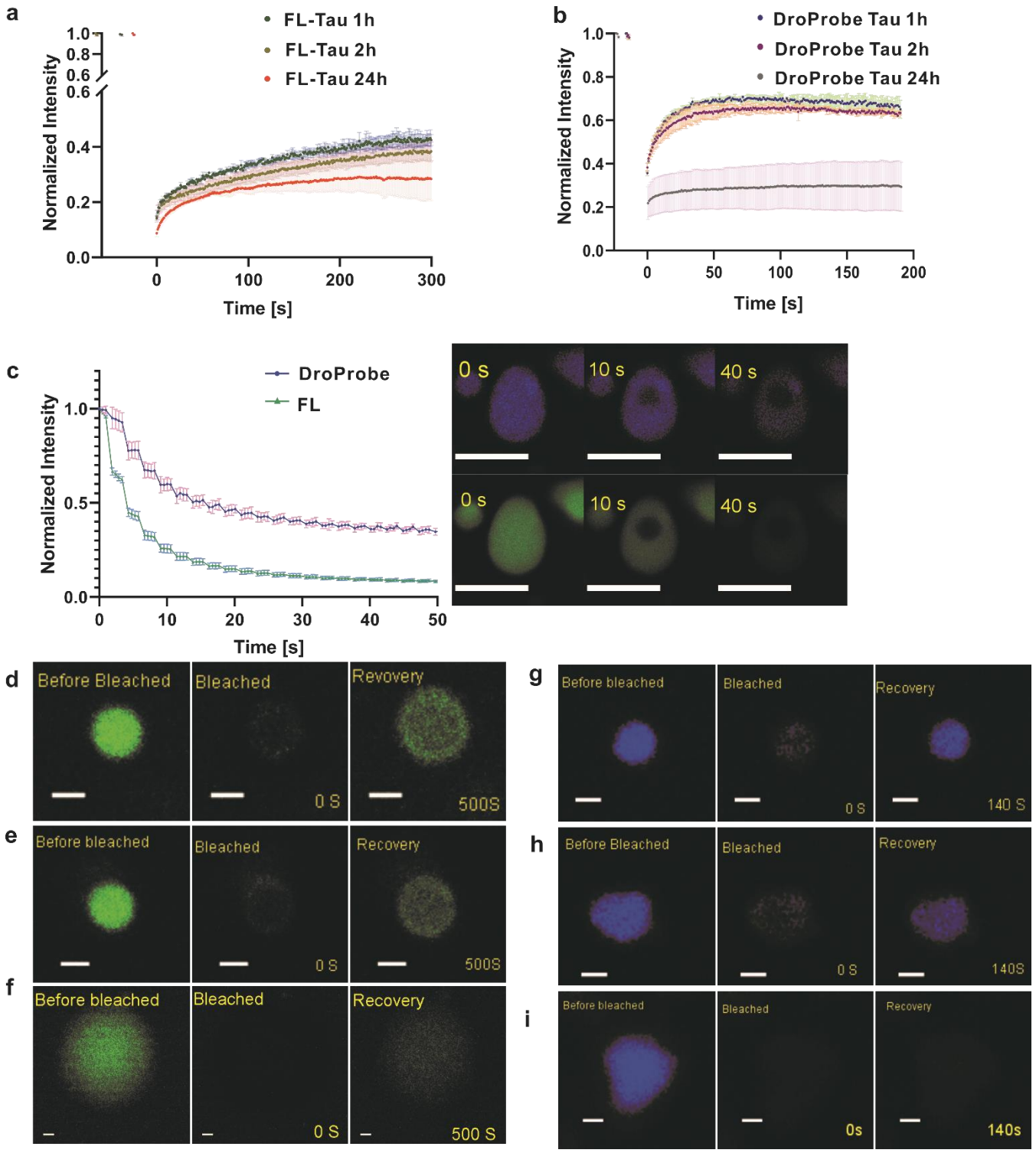


Figure S5 (a) FRAP of FL-hTau441 droplets at different time points. (b) FRAP of DroProbe within hTau441 droplets at different time points. (c) Left: normalized fluorescence intensity of DroProbe and FL under repetitive bleaching. Right: fluorescence images of DroProbe and FL monitoring droplets in 0.486 s, 10.046 s and 40.632 s under repetitive bleaching. Confocal image of the recovery process after photobleaching of the FL-hTau441 (d-f) at 0 h(d), 1h (e) and 12 h(f). Confocal image of the recovery process after photobleaching of hTau441 monitored by DroProbe (g-i) at 0 h(g), 1 h(h) and 12 h(i). The scale bar is 5 μM in c and 1 μM in d-i.

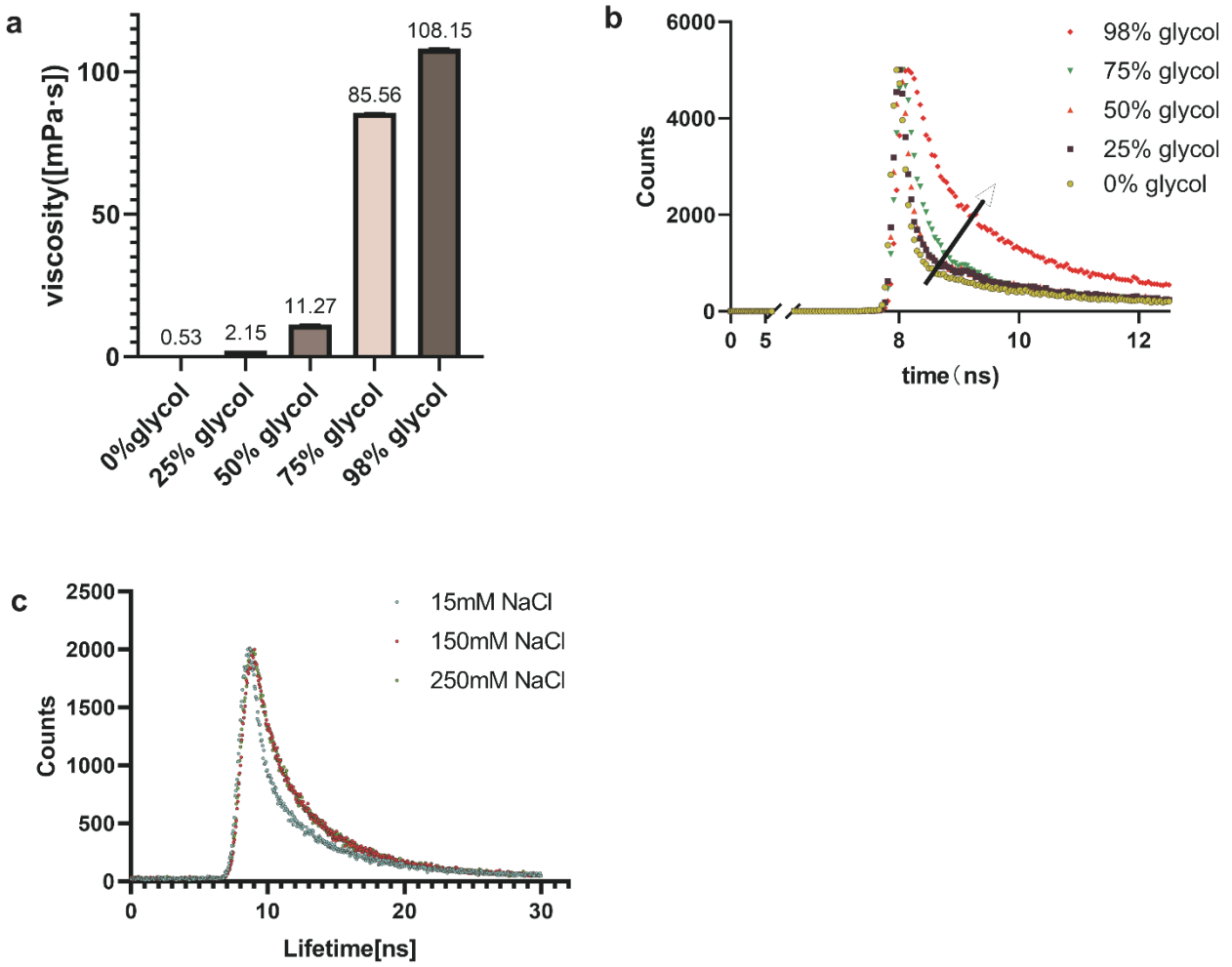


Figure S6 (a) The viscosity of the mixture of methanol and glycerol with different glycerol fraction. (b) Fluorescence lifetime of DroProbe in different glycerol fractions. (c) Fluorescence lifetime spectrum of DroProbe for the hTau441 LLPS formed in 15 mM NaCl, 150 mM NaCl and 250 mM NaCl

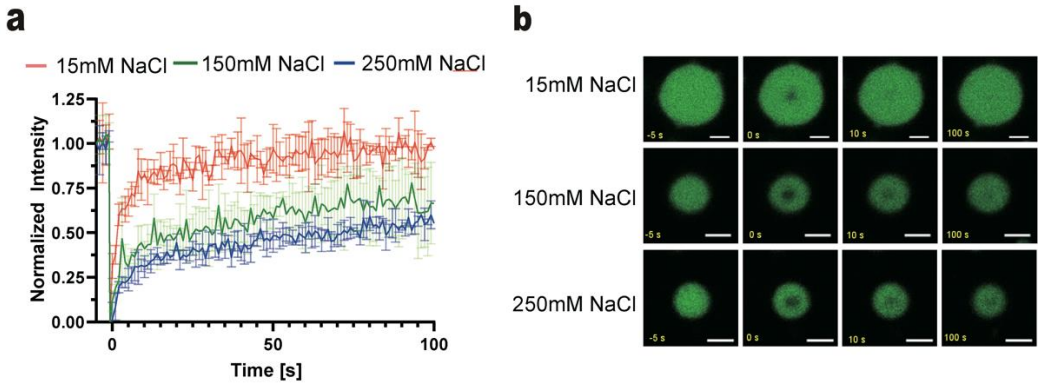


Figure S7 FRAP of Tau LLPS in 15 mM, 150 mM, 250 mM NaCl buffer. (a)Fluorescence intensity recovery of the bleached area. (b). Image of FRAP at different time points.

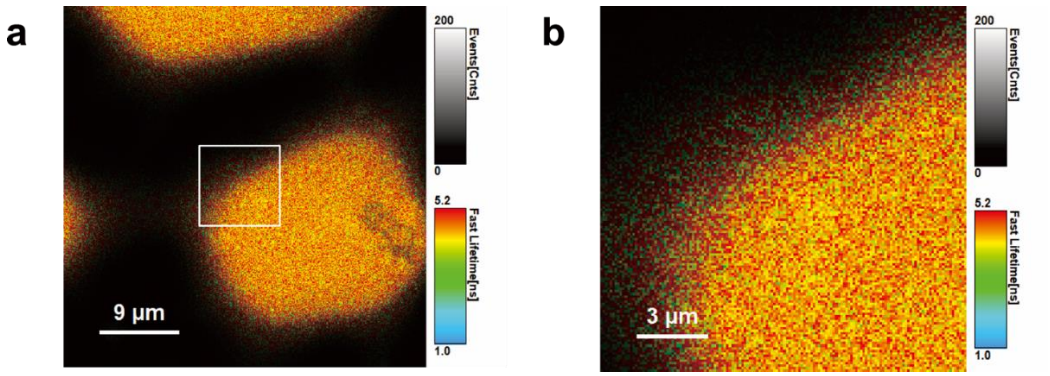


Figure S8 (a) Fluorescence lifetime microscopy image of hTau441 droplets in 15 mM NaCl after 24 h. Mean lifetime of droplets is τ_{Av}= 4.629±0.0128. (b) Fluorescence lifetime microscopy image rectangle ROI of (a).

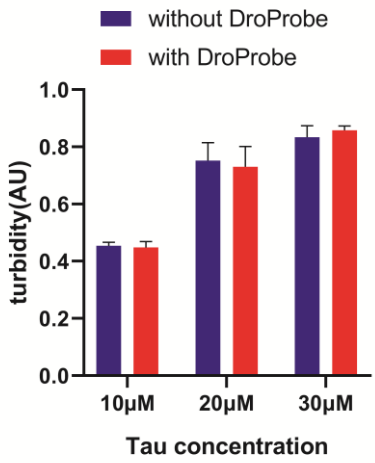


Figure S9. Turbidity of hTau441 LLPS in the presence or absence of DroProbe.

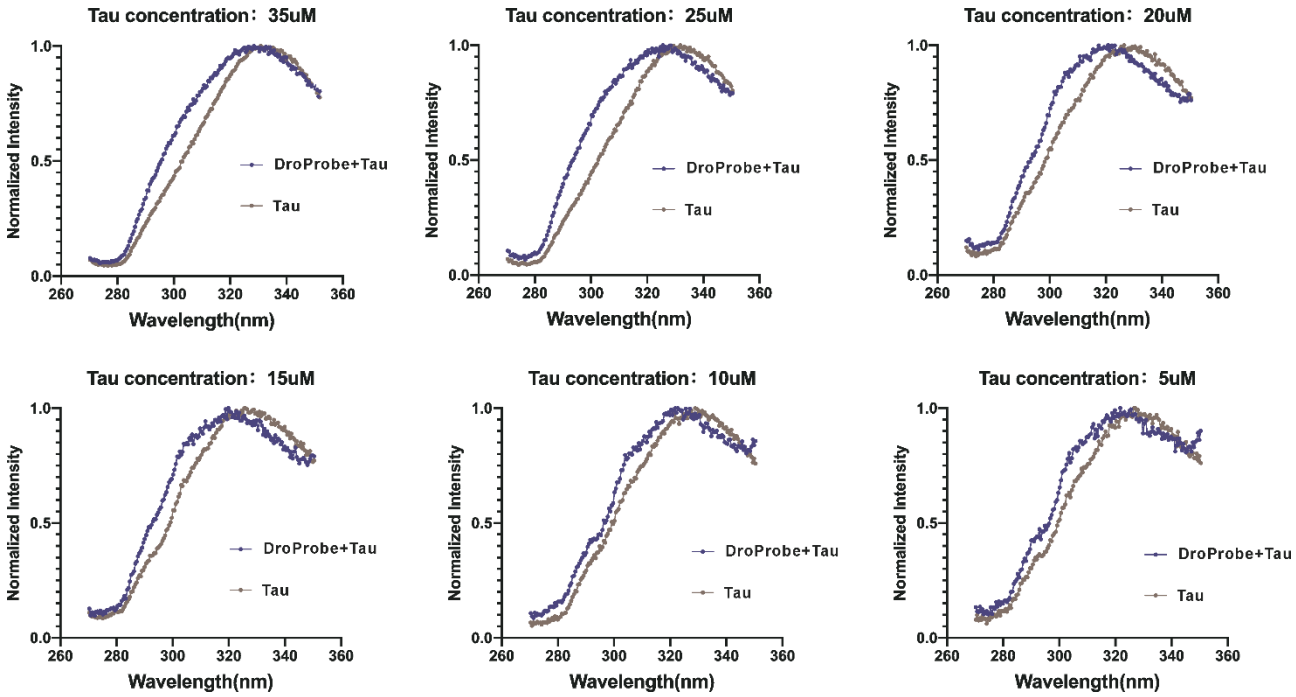


Figure S10 Autofluorescence of hTau441 was found blue-shift by adding DroProbe in all hTau441 concentrations.

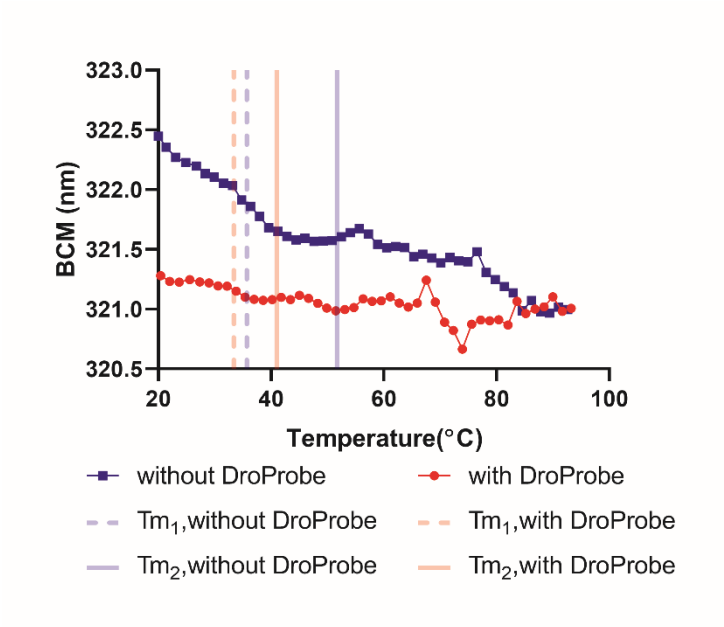


Figure S11. Thermal unfolding states, Tm1 and Tm2, of Tau protein in solution with the absence or presence of DroProbe.

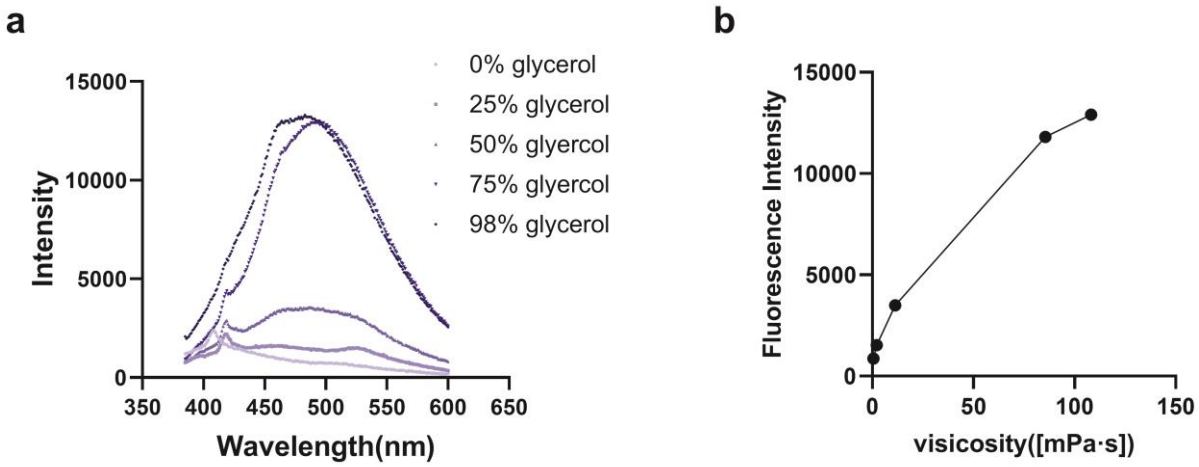


Figure S12 (a) Fluorescence intensity of DroProbe in different glycerol fractions. (b) The fluorescence intensity at 470nm of DroProbe in different glycerol fractions.

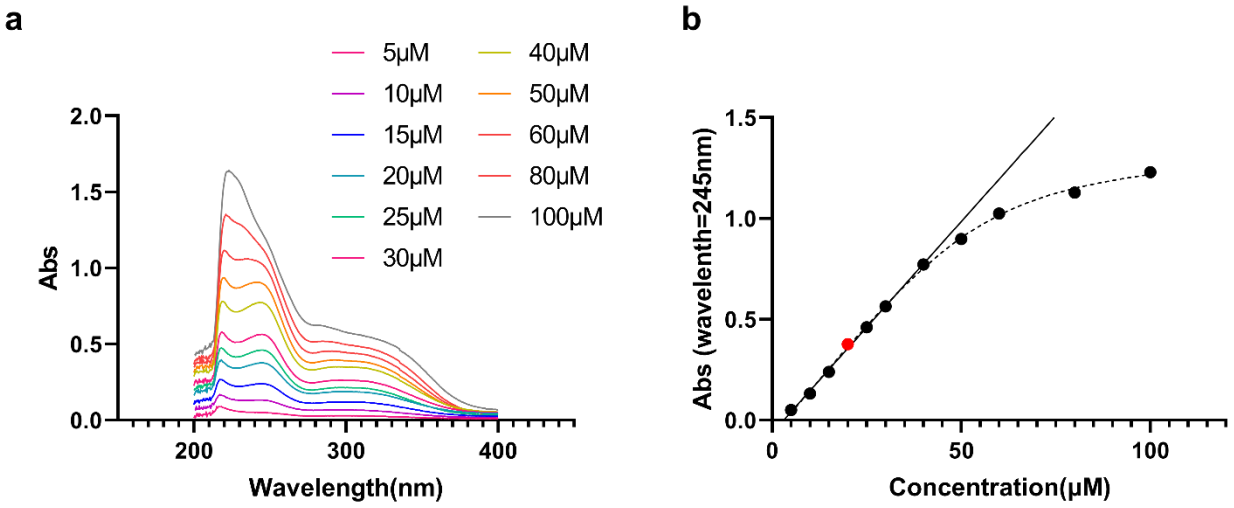


Figure S13 (a) UV-Vis spectrum of DroProbe. (b) Beer-Lambert plot of DroProbe. Our working concentration of 20 μM is within the liner range, indicating the absence of aggregate and dispersion of BSPOTOE

References

[1] Q.-Q. Li, Y.-Y. Luo, Y. Liu, T.-T. Chu, N. Gao, P. Chen, Y.-X. Chen, Y. Li, *Chemical Communications* **2020**. DOI: 10.1039/d0cc01409e, 5370-5373.