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Supporting Information

Long-term STED imaging of amyloid fibers with exchangeable Thioflavin T

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Materials and methods

Chemicals

ThT was purchased from Sigma-Aldrich and used after purification by double recrystallization from water. ThT concentrations were assigned by absorbance with ε_{412nm} = 36,000 M^{-1} cm^{-1}. AF488 NHS ester (Lumiprobe) was used as received. PBS powder for preparing 0.01 M solutions at pH 7.4 was purchased from Sigma-Aldrich.

Protein expression and amyloid fibrils preparation

Plasmids pT7.7 containing the sequences of ΔN and wild type α-synuclein were produced as described in the literature[1,2] and transformed into *Escherichia coli* BL21 cells. Bacteria were grown in LB (Lysogeny broth medium, Broth Miller) supplemented with ampicillin (100 µg/mL, Fisher Chemical). Protein expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Quimigen) cultured at 37 °C for 4 h, and cells were harvested by centrifugation (4,600 xg, 15 min, room temperature). The cell pellet was resuspended in 10 mM 2-amino-2-(hydroxymethyl) propane-1,3-diol pH 8.0 (Tris-HCl, VWR), supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA, Fisher BioReagents) and 1 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF, VWR). Cells were lysed by sonication and pelleted by centrifugation (11,000 xg, 60 min, room temperature). The recovered supernatant was heated for 20 min at 90 °C and centrifuged (17,100 xg, 20 min, 4 °C). The supernatant was precipitated with 60 % NH_4SO_4 (PubChem, 3 h, 4 °C) and centrifuged again. The pellet was resuspended in 25 mM Tris-HCl pH 8.0 and loaded onto a 5 mL HiTrap Q-Sepharose column on an FPLC (fast protein liquid chromatography, ÄKTAFPLC, GE Healthcare). Proteins were eluted with a 0 to 600 mM NaCl (VWR) gradient, and fractions containing the proteins were concentrated by ultrafiltration and loaded onto a Superdex-200 16/60 chromatography column, previously equilibrated with 25 mM Tris-HCl pH 7.5. The eluted proteins were concentrated and stored at −80 °C. Protein concentrations were estimated from the absorbance at 274 nm with an extinction coefficient of 4,215 M^{-1} cm^{-1} for ΔN and 5,620 M^{-1} cm^{-1} for α-synuclein.

Fiber formation was promoted by incubating 200 µM of the purified proteins in 25 mM Tris pH 7.4, containing 5 mM $MgCl_2$ (AppliChem), 150 mM NaCl (AppliChem), 5 mM KCl (AppliChem), and 0.01 % sodium dodecyl sulfate (SDS, AppliChem) at 37 °C and shaking at 800 rpm for 24 h.

AF488 labeling of ΔN fibers

AF488 NHS ester was dissolved in DMSO and slowly added to a solution of preformed ΔN fibers (20 µM) in PBS pH 8.5 to a final label concentration of 3 µM. The solution was gently stirred for 3 hours at room temperature and in the dark. The unbound dye was removed by centrifugation using 100 kDa Amicon Ultra 0.5 mL centrifugal filters (Merck Millipore, 2000 xg, 45 min, 4 °C).

Spectroscopic measurements

Absorption, and fluorescence excitation and emission spectra of free ThT and amyloid-bound ThT in PBS solutions were performed in Cary 50 (Varian) and Fluoromax-4 (Horiba), respectively, using 1 cm pathlength quartz cuvettes (Hellma) at room temperature.

Sample preparation

Glass coverslips (#1.5, Thermo Scientific) were cleaned by successive steps of 15 min of sonication in spectroscopic grade acetone (VWR), followed by alkaline detergent (Hellmanex), 0.5 M NaOH solution and MilliQ water, and finally dried with N_2. Coverslips were functionalized with poly-L-lysine (0.1 % w/v Sigma-Aldrich) by depositing 20 µl solution for 60 min, washed with MilliQ water and dried with N_2. Amyloid fibers were deposited for 30 min on coverslips, washed and dried as described above. Recrystallized ThT was dissolved in PBS (0.8 – 2 µM) just before the experiments, added on the deposited fibers, incubated for 15 – 20 min in the dark and imaged.

Imaging and data analysis

Confocal and STED images were acquired using commercial Leica TCS SP8 STED 3X microscope (Leica Microsystems) equipped with a 100x/1.4 NA oil immersion objective (HCX PL APO OIL CS2). A pulsed white light laser (Leica Microsystems) was used for excitation at 470 nm (50 – 60 %, laser intensity 8 – 8.8 kW/cm^2, repetition rate: 80 MHz) and a continuous wave laser at 592 nm (70 %, laser intensity 16.4 GW/cm^2) for depletion. Laser powers were measured using a slide power meter sensor (PM160, Thorlabs) at the position of the sample. A Hybrid Detector (HyD, Leica Microsystems) with a detection window set to 480 – 550 nm was used with a gate of 0.8 ns with respect to the excitation pulse. Pixel size was set to 23 – 38 nm and the pinhole was set to 1.0 airy units. Confocal and STED images (1024 x 1024 pixels) were recorded with bidirectional exposure at 400 Hz and using 1x and 6x line accumulation, respectively. For continuous STED experiments, the pixel dwell time was 600 ns and a total time per frame of about 7 seconds.

Raw images were analyzed using the open-source Fiji (imagej.net/Fiji). To help visualization, a Gaussian smoothing with 0.8 pixel radius was applied. For cross-section analysis, raw data were fitted to a Gaussian or Lorentzian function for confocal and STED images, respectively, using OriginPro 8.6. To extract the fluorescence intensities for the photobleaching curves, 3 ROIs of 10 x 10 µm^2 containing fibers were selected from the raw image, and their total intensity values averaged. Background was subtracted using the average intensity values from 3 ROIs with no fibers and intensities were normalized to the first frame. Each value in the photobleaching curves represents the mean of at least three replicates of 44-frame series. A single exponential decay was used to fit the data using GraphPad 5.0.

Supporting Figures

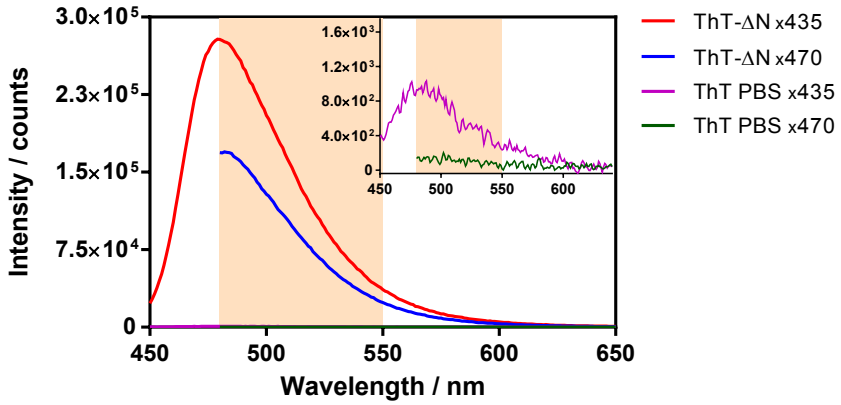

Figure S1. Fluorescence properties of free and amyloid-bound ThT upon illumination at 435 nm, where both free and bound molecules strongly absorb, and at 470 nm, as used in imaging experiments, to predominantly excite the bound labels. Irradiation at longer wavelengths allows to essentially suppress the residual fluorescence that arises from the excitation of free fluorophores (see inset) while maintaining strong emission intensity from the bound labels. The fluorogenic properties of ThT and the bathochromic shift are valuable properties that greatly minimize background fluorescence signal. The fluorescence intensity was integrated over the range 480-550 nm, corresponding to the detection window used in imaging experiments (colored in light orange).

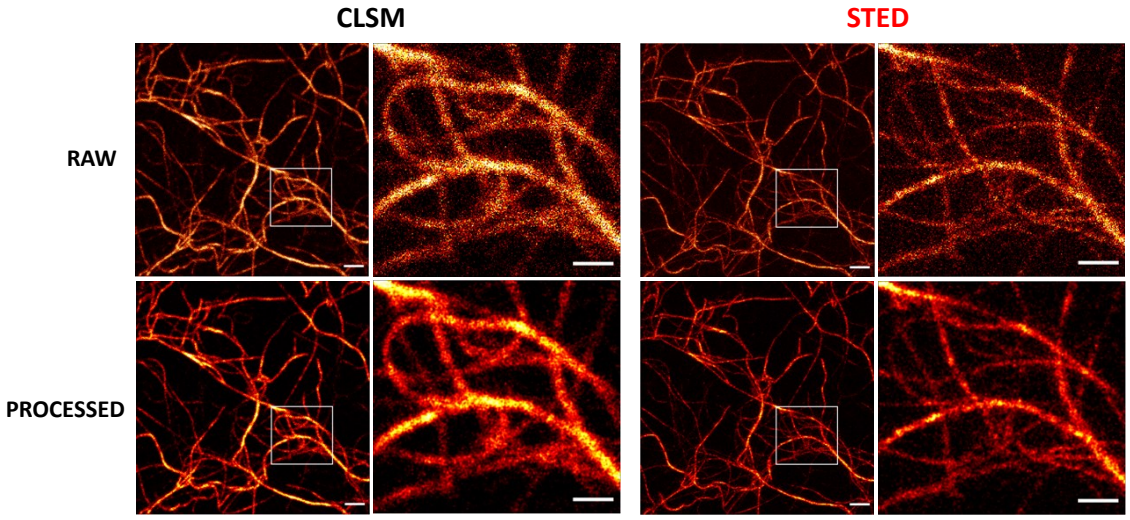

Figure S2. Image processing of confocal and STED images of amyloid ΔN fibers labeled with 2 µM ThT. Raw and processed examples of full and magnified ROIs are shown for comparison, applying a Gaussian smoothing with 0.8 pixel radius. Scale bars are 2 µm and 1 µm for full and zoomed-in images, respectively.

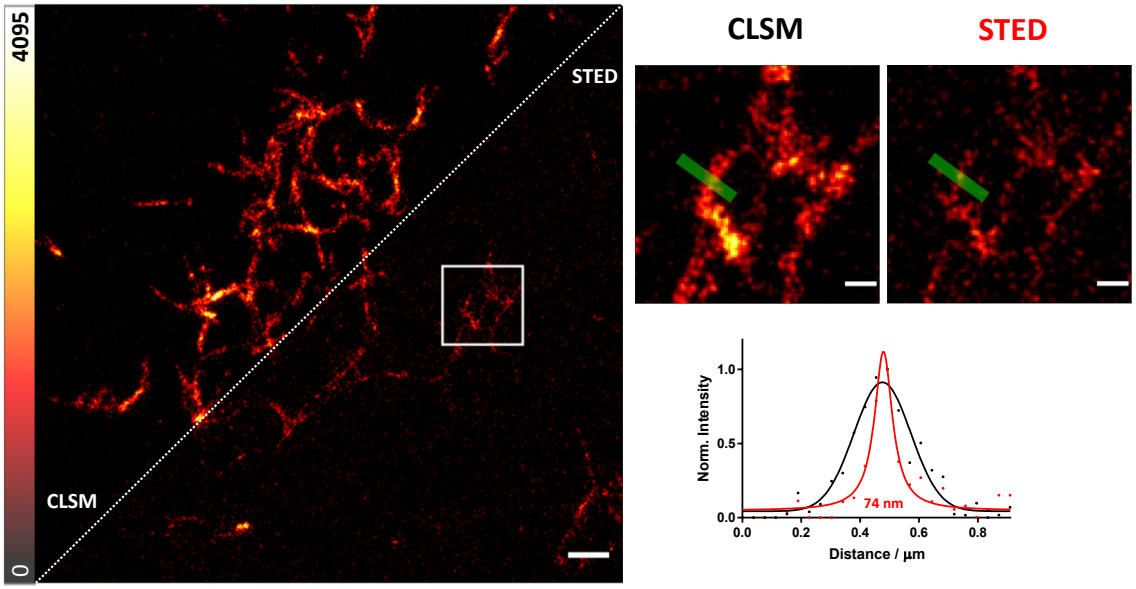

Figure S3. Comparison of CLSM and STED images of wild type α-synuclein fibers labeled with ThT (2 µM). Line profiles from the green cross-sections show the improvement in resolution and structures not resolvable in the confocal image (black and red lines for CLSM and STED images, respectively). Line profiles were taken from raw images and averaged over a width of 6 pixels. Scale bars are 2 µm and 0.5 µm for full and magnified images, respectively.

References

1 H. Liu and J. H. Naismith, *BMC Biotechnol.*, 2008, **8**, 91.

2 B. Sot, A. Rubio-Muñoz, A. Leal-Quintero, J. Martínez-Sabando, M. Marcilla, C. Roodveldt and J. M. Valpuesta, *Sci. Rep.*, 2017, **7**, 1–12.