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Enhancing Lateral Resolution Using Two-Colour Direct Stochastic Optical Reconstruction Microscopy to Unravel Synaptic Tau Pathology in Alzheimer's Disease

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Keywords: Alzheimer's disease | array tomography | super resolution microscopy | tau

ABSTRACT

Aims: In Alzheimer's disease (AD), the pathological accumulation of tau in synapses contributes to synapse dysfunction and loss. However, the small and complex structure of synapses limits the investigation when using conventional techniques. In this work, we describe the combination of array tomography (AT) with two-colour direct stochastic optical reconstruction microscopy (dSTORM) to enhance lateral resolution for resolving synaptic terminals in human postmortem brain.

Methods: We applied this combination to study synapses in brain samples (from biopsy and postmortem) from healthy subjects and pathological synaptic tau (aggregates and oligomers) in samples from AD patients.

Results: AT combined with dSTORM allowed the characterisation of the orientation and shape of the synaptic terminals and the synaptic cleft. In addition, this combination confirmed the presence of oligomeric tau in synaptic terminals in AD.

Conclusions: Overall, we found that the combination of AT and two-colour dSTORM provides optimal resolution to detect pathological synaptic tau and its spatial relationship with presynaptic and postsynaptic terminals.

List of Abbreviations: AD, Alzheimer's disease; AT, array tomography; A β , amyloid- β ; CEM, conventional epifluorescence microscopy; CM, conventional microscopy; DLB, dementia with Lewy bodies; dSTORM, direct stochastic optical reconstruction microscopy; FFPE, formalin-fixed paraffin-embedded; HC, healthy control; NFTs, neurofibrillary tangles; PMI, postmortem interval; PSD95, postsynaptic density 95; SAD, sporadic Alzheimer's disease; SMLM, single molecule localisation microscopy; SYPH, synaptophysin; TIRF, total internal reflection fluorescence. Sònia Sirisi and Alberto Lleó contributed equally.

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Summary

- Combining array tomography with two-colour direct stochastic reconstruction microscopy provides optimal resolution to detect pathological synaptic tau in human AD brains.
- The combination allows the study of the spatial relationship between pathological tau with presynaptic and postsynaptic terminals.
- These innovative techniques deliver crucial insights into the study of intact human synapses and shed light on the importance of synaptic pathology in AD.

1 | Introduction

Alzheimer's disease (AD) is characterised by the presence in the brain of extracellular aggregation of Amyloid- β (A β) peptides, which form amyloid plaques, and the intracellular deposition of hyperphosphorylated tau, which forms neurofibrillary tangles (NFTs). Although the burden of NFTs correlates with neuronal loss and clinical severity, it does not account for total neuronal loss [1–3], suggesting the potential involvement of soluble tau species or other factors in the pathophysiology [4]. Synapse loss postmortem is known to correlate with cognitive decline in AD [5, 6]. However, the small and complex structure of synapses poses a significant challenge to elucidating their role using conventional techniques. Traditional biochemical, genetic, structural and imaging methods have yielded valuable insights into biological processes; nevertheless, they fall short in offering a detailed molecular perspective on the nanometre scale organisation [7]. Advances in microscopy techniques in the last decades have opened a window to investigate synaptic pathology in neurodegenerative diseases. We and others have used array tomography (AT), a novel microscopy technique especially suited for visualising synapses in human brain and for investigating synaptic aggregates in patients with AD and dementia with Lewy bodies (DLB) [8–11]. AT offers an increased spatial resolution by combining ultrathin sectioning of the tissue with immunofluorescence, allowing high-resolution quantitative analysis of different ultrastructures, such as synapses or pathological

aggregates in DLB and AD [9, 12, 13]. The small size of synaptic aggregates has led other groups to look for microscopy techniques with higher resolution. In a previous study, we combined AT with two-colour direct stochastic optical reconstruction microscopy (dSTORM), a single molecule localisation microscopy (SMLM) technique, capable of detecting interactions within nanometre scale [14]. Using this combination, we showed that oligomeric, misfolded and phosphorylated tau species are found at synaptic terminals of AD brains even in areas with low NFT burden [11]. In the present work, we describe the full capabilities of the combination of AT with dSTORM to achieve enhanced lateral resolution for resolving synaptic terminals in human postmortem brain samples from healthy subjects and synaptic tau oligomers in samples from AD patients. The enhancement of the lateral resolution enables a more precise study of the spatial relationship between pathological proteins and the synaptic terminals, which could provide crucial insights into synaptic pathology in neurodegenerative diseases.

2 | Materials and Methods

2.1 | Protocol Approval, Patient Consent and Postmortem Human Brain Samples

The study was approved by the local ethics committee of Hospital de Sant Pau, Barcelona. All research was performed in accordance with relevant guidelines and regulations. All brain tissue samples were obtained from patients after they or their legal representatives gave written-informed consent for the use of their brain tissue and medical records for research purposes, as approved by the Ethics Committee of the Hospital Clínic-FRCB-IDIBAPS, in accordance with the Declaration of Helsinki. Neuropathological examination was performed according to standardised protocols at the Neurological Tissue Bank of the Hospital Clínic-FRCB-IDIBAPS, a brain bank with expertise in neurodegenerative diseases. Cortical biopsies were obtained from neurosurgical procedures directed at removing deep brain tumours from patients at the Hospital de la Santa Creu i de Sant Pau (Barcelona, Spain). All samples were processed using previously described methods [15] and generally recommended [8]. The demographic and clinical data of the cohort are outlined in Table 1.

TABLE 1 | Demographic and clinical data of the cohort.

Cases No.	Clinical diagnosis	Gender	Age at biopsy or age of death	PMI (h)	Region	Braak NFT stage	Study
1	HC (biopsy) ^a	F	62	NA	Temporal	NA	1, 2
2	HC (biopsy) ^a	M	46	NA	Temporal	NA	1, 2
3	HC	F	83	7.5	Frontal	II	1, 2
4	HC	F	89	8.8	Frontal	I	1, 2
5	SAD	F	90	18.0	Frontal	V	1, 2
6	SAD	M	64	17.9	Frontal	VI	1, 2, 3, 4
7	SAD	F	82	13.8	Frontal	IV	1, 2, 3, 4

Abbreviations: F, female; HC, healthy control; M, male; PMI, postmortem interval; SAD, sporadic Alzheimer's disease.

^aCortical brain biopsy. Studies: (1) AT+conventional epifluorescence microscope, (2) AT+dSTORM, (3) Formalin-fixed paraffin-embedded + Confocal, and (4) formalin-fixed paraffin-embedded + dSTORM.

2.2 | Immunofluorescence Staining

Formalin-fixed paraffin-embedded (FFPE) sections from the frontal cortex were deparaffinised and rehydrated following standard procedure [16]. Then, the antigen retrieval step was performed. Next, sections were blocked with 0.2% bovine serum albumin for 1 h, followed by incubation of the samples overnight with primary antibodies diluted 1:500 at 4°C (anti-Synaptophysin, AF5555, R&D Systems; anti-PSD95, 3450, Cell Signaling; anti-AT8, 90206, Innogenetics). The next day, AlexaFluor secondary antibodies diluted 1:1000 were incubated for 1 h at room temperature (Donkey anti-Rabbit, Alexa Fluor 488, A21206, ThermoFisher; Donkey anti-Mouse Alexa Fluor 488, A21202, ThermoFisher; Donkey anti-Goat Alexa Fluor 647, A21447, ThermoFisher). After washing with PBS, the sections were incubated with Sudan Black B (199664, Sigma-Aldrich) for 5 min and washed with PBS again to remove any precipitates that may have been generated. For visualisation of the nuclei, the samples were incubated with Hoechst 1:5000 (33258, Life Technologies). Finally, the sections were mounted on slides with Immu-Mount mounting medium (Thermo Scientific™). Images were recorded by Leica inverted fluorescence confocal microscope (Leica TCS SP5-AOBS, Wetzlar, Germany), and cover slides were viewed with HCX PL APO 63X/1.4 NA IMM Corr objective. Fluorescent images were acquired in a scan format of 1024×1024 pixels, and a 3× scanner zoom factor was used (0.4-μm pixel size). A pulsed 405-nm laser was used for Hoechst visualisation, collecting images in a spectral range of 450 to 460 nm.

2.3 | AT and Image Acquisition

The AT protocol was applied using previously described methods [10, 16]. Briefly, Lowicryl Resin white embedded brain tissue blocks were sectioned using a diamond knife (Diatome, United Kingdom) generating ribbons of 70-nm-thick consecutive sections. Then, ribbons were incubated with Tris-Glycine solution at room temperature for 5 min and blocked for 30 min with cold water fish blocking buffer (G7041, Sigma-Aldrich). Sections were incubated overnight with primary antibodies diluted 1:50 at 4°C (anti-Synaptophysin, AF5555, R&D Systems; anti-PSD95, 3450, Cell Signaling; anti-T22, ABN454-I, Millipore). After TBS washes, secondary antibodies diluted 1:50 were applied for 30 min at room temperature (Donkey anti-Rabbit, Alexa Fluor 488, A21206, ThermoFisher; Donkey anti-Mouse Alexa Fluor 488, A21202, ThermoFisher; Donkey anti-Goat Alexa Fluor 647, A21447, ThermoFisher). Sections were washed with TBS and stained with Hoechst diluted 1:100 (33258, Life Technologies) for 5 min for nuclei visualisation. Finally, coverslips were mounted on microscope slides with Immu-Mount mounting medium (9990402, EpreDia). Images of three regions of interest were obtained on at least 12 consecutive 70-nm-thick serial sections using the nuclei as a guide with a fully automated epifluorescence upright microscope (BX51, Olympus) with a 63×1.2 NA Plan Apochromat objective.

2.4 | Direct STORM Acquisition (dSTORM) and Image Reconstruction

For image reconstruction using dSTORM, sections 70 nm (AT) and 2 μm (FFPE) thick were stained as aforementioned using the same primary and secondary antibodies, Hoechst dye diluted

1:100 (33258, Life Technologies) was used to visualise the nuclei (Figure 1A), and then, the coverslips were mounted with OXEA buffer (STORM buffer) [17]. To pinpoint individual fluorophores within the sample, fluorescent dyes go through a switching cycle, transitioning between an 'on' and 'off' state known as 'blinking'. During this phenomenon, most dyes remain inactive, whereas a few of them randomly activate, allowing for their individual identification. This cyclic process repeats multiple times, enabling the identification of the emitting fluorophore in each successive cycle (Figure 1B). STORM images were acquired using a Nikon N-STORM system configured for total internal reflection fluorescence (TIRF) imaging. Laser inclination was tuned to adjust focus and maximise the signal-to-noise ratio. AlexaFluor-647 was excited illuminating the sample with the 647 nm (20% of 160 mW) and AlexaFluor-488 with the 488 (40% of 80 mW) laser line built into the microscope. Fluorescence was collected using a Nikon 100×, 1.49 NA oil immersion objective and passed through a quad-band pass dichroic filter (97335 Nikon). For each channel, 10,000 frames at 20-ms integration time were acquired. Images were recorded onto a 256×256-pixel region of a Hamamatsu ORCA Flash 4.0 CMOS camera (0.16-μm pixel size). Also, using the 405-nm laser, a low-resolution image of the nucleus was taken and subsequently utilised for 3D image alignment. STORM images were analysed with the STORM module of the NIS element Nikon software (Figure 1C) [18].

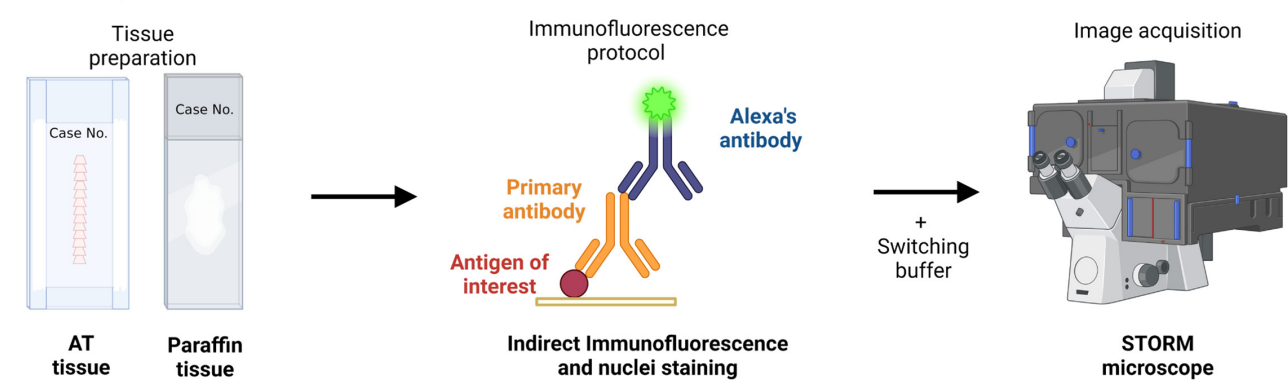
2.5 | Image Analysis

Image analysis for both AT and AT+dSTORM images was performed using ImageJ (US National Institutes of Health, Bethesda, Maryland, United States), Matlab software (Mathworks Inc., MA, United States) and Python (Centrum voor Wiskunde en Informatica Amsterdam, the Netherlands). The scripts have been deposited at https://github.com/MemoryUnitSantPau/Synseg_py with the names density, distance and shape, and segmentator. Images from serial sections were stacked, aligned and segmented using a semiautomated mean local threshold. The nonspecific staining (not present in at least two consecutive sections) was considered background and removed. Three-dimensional reconstructions of representative synapses were generated using the ImageJ Volume Viewer plug-in with tricubic smooth interpolation. To assess the distance between centroids, first, stacks of protein images are loaded and processed frame by frame. Each frame undergoes thresholding to eliminate intensity values below 20% of the maximum intensity of that frame. Subsequently, the frames are labelled to extract their properties, utilising the 'spicy' and 'scikit image' libraries. The detected object's shape is parametrised using 'diplib' and 'cv2' measurement tools obtaining measurements about the roundness, circularity and eccentricity, among other characteristic values. The distance between centroids is then computed using the Pythagorean theorem. Finally, to calculate the synaptic cleft width, the pixels of both proteins are listed, and for each pair of points, the distance is calculated, and the minimum is recognised.

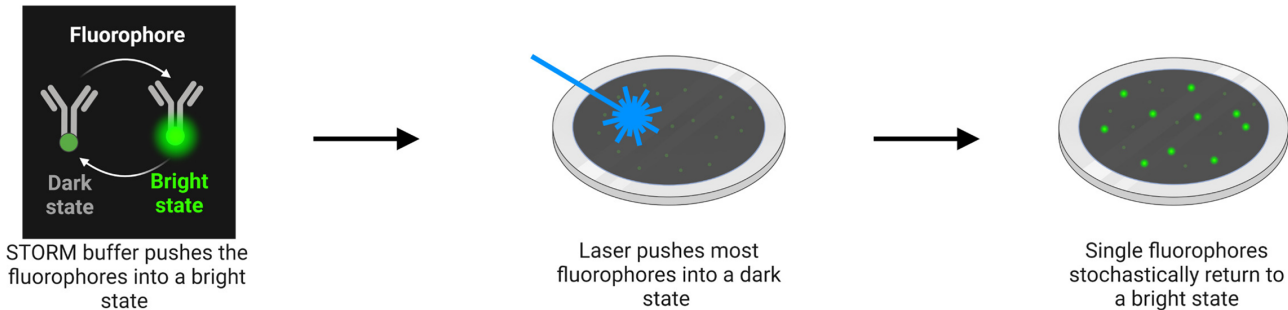
2.6 | Statistical Analyses

Statistical analyses were performed in R Version 4.3.1. We excluded data points from the analysis that exceeded three times the interquartile range. We evaluated the distribution

A. TECHNIQUE OVERVIEW



B. STORM BUFFER PRINCIPLE



C. STORM SET-UP

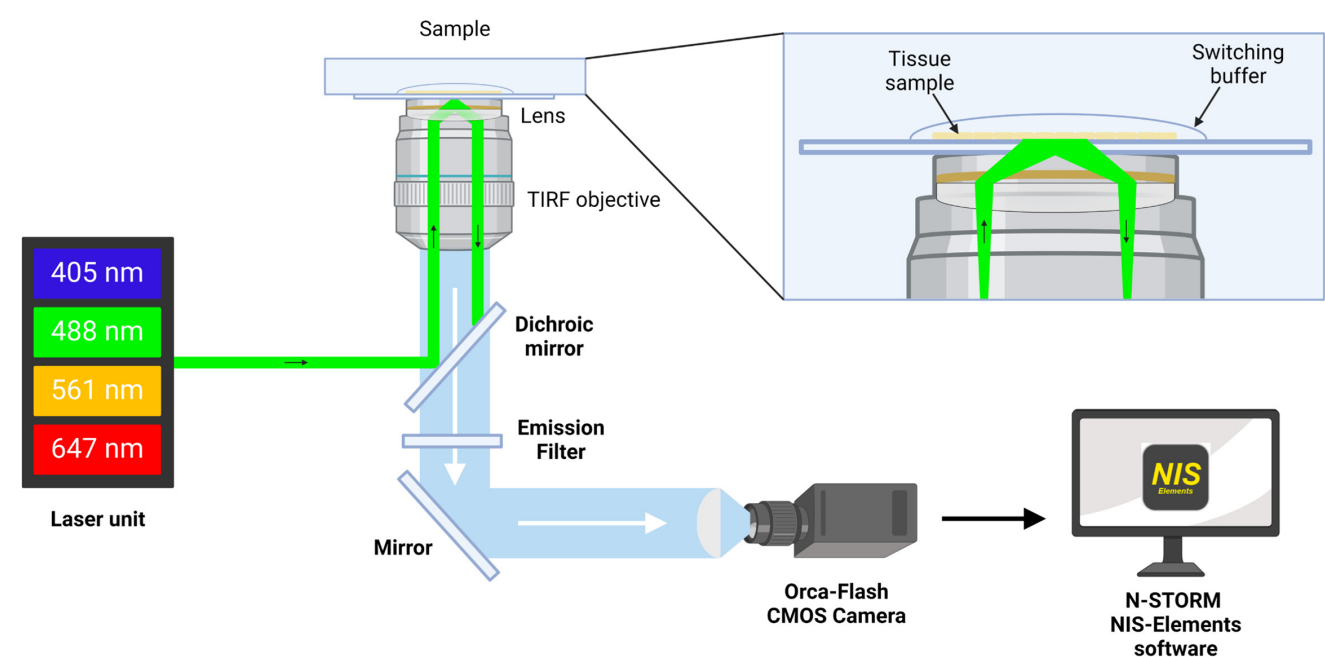


FIGURE 1 | Visual summary of dSTORM imaging. (A) Technique overview showing the three main steps: tissue preparation, immunofluorescence of the samples and image acquisition with a two-colour STORM microscope. (B) Schematic representation of the STORM buffer principle and the stochastic activation of fluorophores. (C) The STORM microscope has a laser unit with four channels, allowing the acquisition of two-colour images as well as recognition of the nuclei of the field of interest for reconstruction using the STORM module of the NIS element Nikon software for analysis.

of our data (Shapiro–Wilk, $p < 0.05$) before performing a Mann–Whitney test to assess the differences between techniques when measuring the object dimension and distance of the synaptic proteins. To evaluate the contribution of PMI

in the synaptic cleft width, we used a linear regression with the PMI as a covariate. Differences in area, length and other measurements of synaptic proteins were evaluated with Wilcoxon's test.

3 | Results

3.1 | Enhancement of Lateral Resolution With dSTORM to Detect Tau Aggregates in Formalin-Fixed Paraffin-Embedded Brain Samples

To compare the resolution achieved using confocal microscopy and dSTORM, we immunostained FFPE samples from patients with SAD with a phosphorylated tau marker (p-tau, AT8) to detect neurofibrillary tangles and tau immunoreactive neurites. The samples were imaged with confocal microscopy (Figure S1A) and subsequently with dSTORM, resulting in a significant improvement in lateral resolution (Figure S1B). This enhanced resolution revealed the discontinuous nature of tau+ neurites in contrast to the classical continuous appearance observed with conventional microscopy (CM) due to light diffraction. As the thickness of the FFPE sample is greater than the diffraction limit of the microscope, resolution along the *z* axis is poor in FFPE tissue and degrades faster than on the XY plane [12]. As a result, it was not possible to visualise the interaction of tau aggregates with synaptic terminals using either imaging technique.

3.2 | Enhancement of Lateral Resolution With dSTORM to Visualise Single Synapses in AT Samples

We next investigated the potential of the combination of dSTORM with our classical AT protocol [8, 10, 13] to visualise synaptic pairs in human brain samples. As previously described, we selected 70-nm-thick AT samples from an HC (frontal cortex) and immunostained them using an antisynaptophysin (SYPH) antibody, as a presynaptic marker, and anti-PSD95 (PSD) antibody, as a postsynaptic marker (Figure 2A). Using fluorescence microscopy, synaptic pairs appear as puncta with a distance between the presynaptic and postsynaptic centroids within a 0.5- μ m range [11]. The resolution obtained with AT allows the 3D reconstruction of single synapses, which enables the localisation of presynaptic and postsynaptic structures separately (Figure 2B) and the presence of synaptic aggregates [8, 10]. We next performed the same experimental design combining AT with two-colour dSTORM. The dSTORM image is the final reconstruction of 10,000 frames per channel. The improved resolution achieved with AT + dSTORM allowed detailed visualisation of the presynaptic and postsynaptic terminals and the synaptic cleft (Figure 2C,D). In addition, the combination allows a better characterisation of the orientation and shape of the synapse. This result indicates that the combination of AT and dSTORM offers increased properties for the characterisation of the synapses in the healthy human brain.

3.3 | Assessing Protein–Protein Distances, Synaptic Cleft Size and Object Dimensions With AT + dSTORM

The improved lateral resolution of AT + dSTORM allowed a precise characterisation of the synaptic compartments. This allowed the quantification of the distances between the presynaptic and postsynaptic structures. We performed image

binarisation with a preestablished threshold (Figure 2E), resulting in increased accuracy for distance calculations. We analysed over 400 single synapses (200 for each technique) using frontal cortex AT samples from HC ($n=2$). We observed a significant difference in the median distance between centroids when comparing conventional epifluorescence microscopy with respect to dSTORM (409 ± 80 vs. 190 ± 70 nm, $p < 0.01$) (Figure 2F). The use of AT + dSTORM revealed that the area of PSD95 labelling is almost twice as large as that of SYPH labelling (Figure 2H). Furthermore, using the combination AT + dSTORM, we quantified the width of the synaptic cleft, revealing a median value of 64 ± 59 nm. This allowed us to discriminate objects without overlap at a distance as small as 8 nm (Figure 2G). To confirm that the values obtained with AT-STORM were not influenced by the PMI or the AT protocol, we also measured the synaptic cleft in samples obtained from surgical cortical biopsies ($n=2$). We did not observe significant differences in synaptic cleft distance between post-mortem AT controls and cortical biopsy samples (Figure S2). In addition, PMI was added as a covariate in the linear regression model, showing no significant contribution (adj. r^2 : -1.032 ; p value: 0.82). This result supports that the PMI does not affect the measurements of the synapses using AT.

3.4 | AT + dSTORM Detects Oligomeric Tau in Synapses in AD Brains

We next confirmed our previous result using AT + dSTORM, showing the presence of tau oligomers at the synapse using the T22 antibody (Figure 3A) [11]. We expanded the number of frontal cortex samples of SAD patients to characterise synaptic tau aggregates ($n=3$). We observed that the centroid of the presynaptic oligomeric tau immunoreactivity was closer to the centroid of the presynaptic terminal (SYPH) when compared to the immunoreactivity observed in the postsynaptic density (PSD95, Figure 3B). Furthermore, we characterised the morphology of the aggregates, their area, eccentricity and length (Figure 3C–E). Oligomeric tau immunoreactivity was larger than the synaptic markers (SYPH and PSD95), but there were no significant differences between them. When evaluating the eccentricity of the three proteins, both SYPH and T22 were significantly more circular than PSD95 ($p < 0.05$). Furthermore, we measured the length of the major axis of oligomeric tau immunoreactivity present in the synapse, and we did not detect significant differences (median length of 315.27 ± 117.69 nm in the presynaptic compartment and of 332.43 ± 163.59 nm in the postsynaptic compartment). Finally, of a total of 200 tau+ synapses analysed in three SAD cases, tau oligomers were found more frequently in presynaptic terminals than in postsynaptic terminals.

4 | Discussion

In this work, we describe the potential of the combination of AT and two-colour dSTORM to study synaptic tau pathology in AD brains. Combining these two microscopy techniques, we achieved nanoscale resolution that allows an optimal visualisation of single synapses and confirms the presence of oligomeric forms of tau at synaptic compartments in human brain.

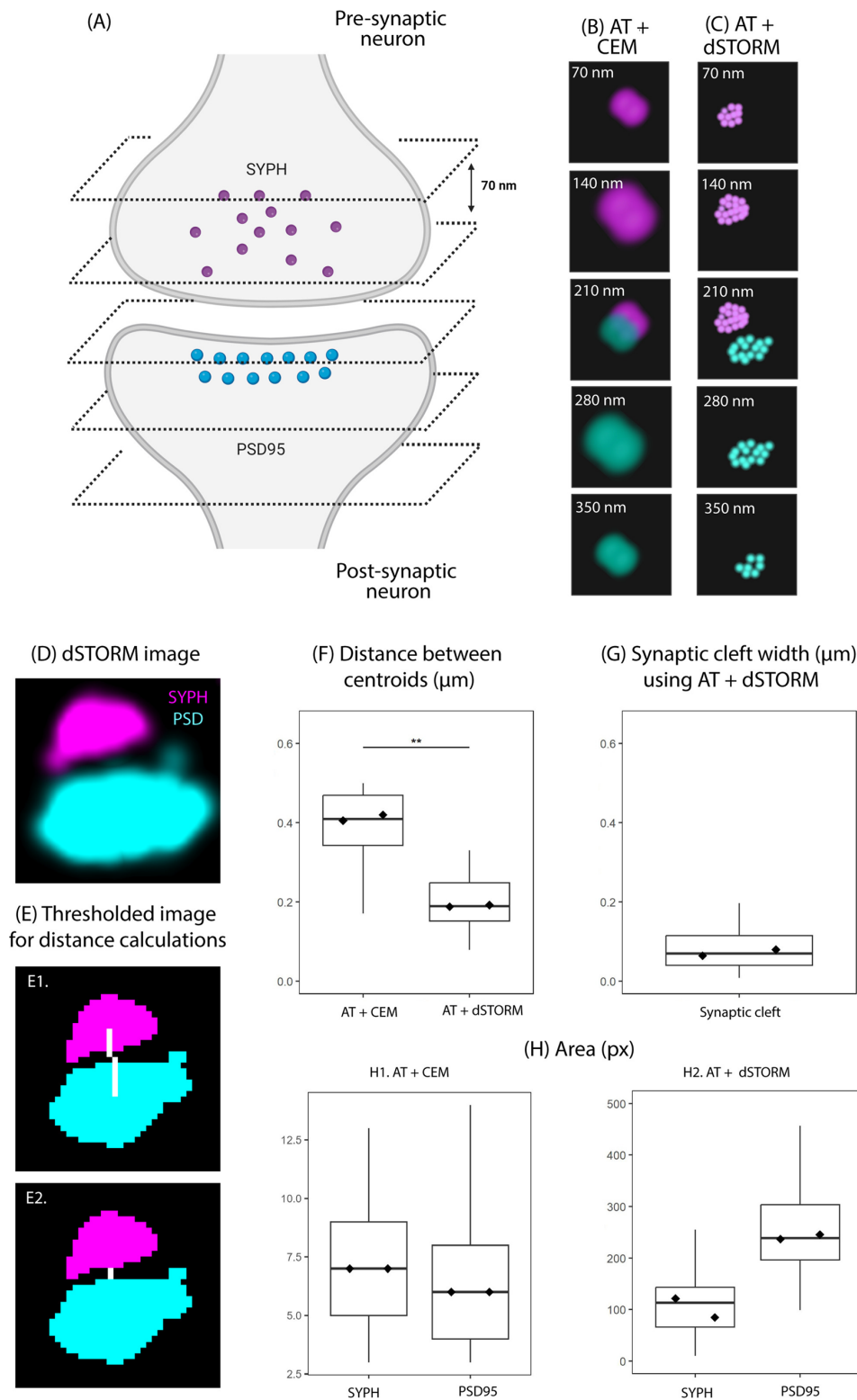


FIGURE 2 | Imaging single synapses from human brain. (A) Graphical representation of 70-nm consecutive sections, immunolabelled with antibodies raised against SYPH and PSD95. The production of ultrathin ribbons enables the subsequent reconstruction of the ultrastructure of the brain tissue. (B, C) Graphical synaptogram comparing the resolution of AT + conventional epifluorescence microscopy (CEM) (B) and AT + dSTORM (C). (D) dSTORM image of a single synapse as a result of the rendering process. (E) Thresholded image used for measuring the distance between centroids (E1, white diagonal represents 101.84 nm) and the width of the synaptic cleft (E2, white line represents 21.5 nm). (F) The centroid's distance obtained with AT samples and conventional epifluorescence microscope and in combination with dSTORM. (G) The median value of synaptic cleft width using the AT + dSTORM images and the dispersion of the data represented in the boxplot. Each point corresponds to a single synapse. Error bars indicate medians with standard deviations. ** $p < 0.01$. (H) Area quantifications (pixels) of objects using (H1) AT samples with CEM and (H2) AT with dSTORM. The boxplot represents the distribution of all data points, whereas the statistical analysis was performed on the mean values for each case, which are displayed as diamonds. Error bars indicate medians with standard deviations.

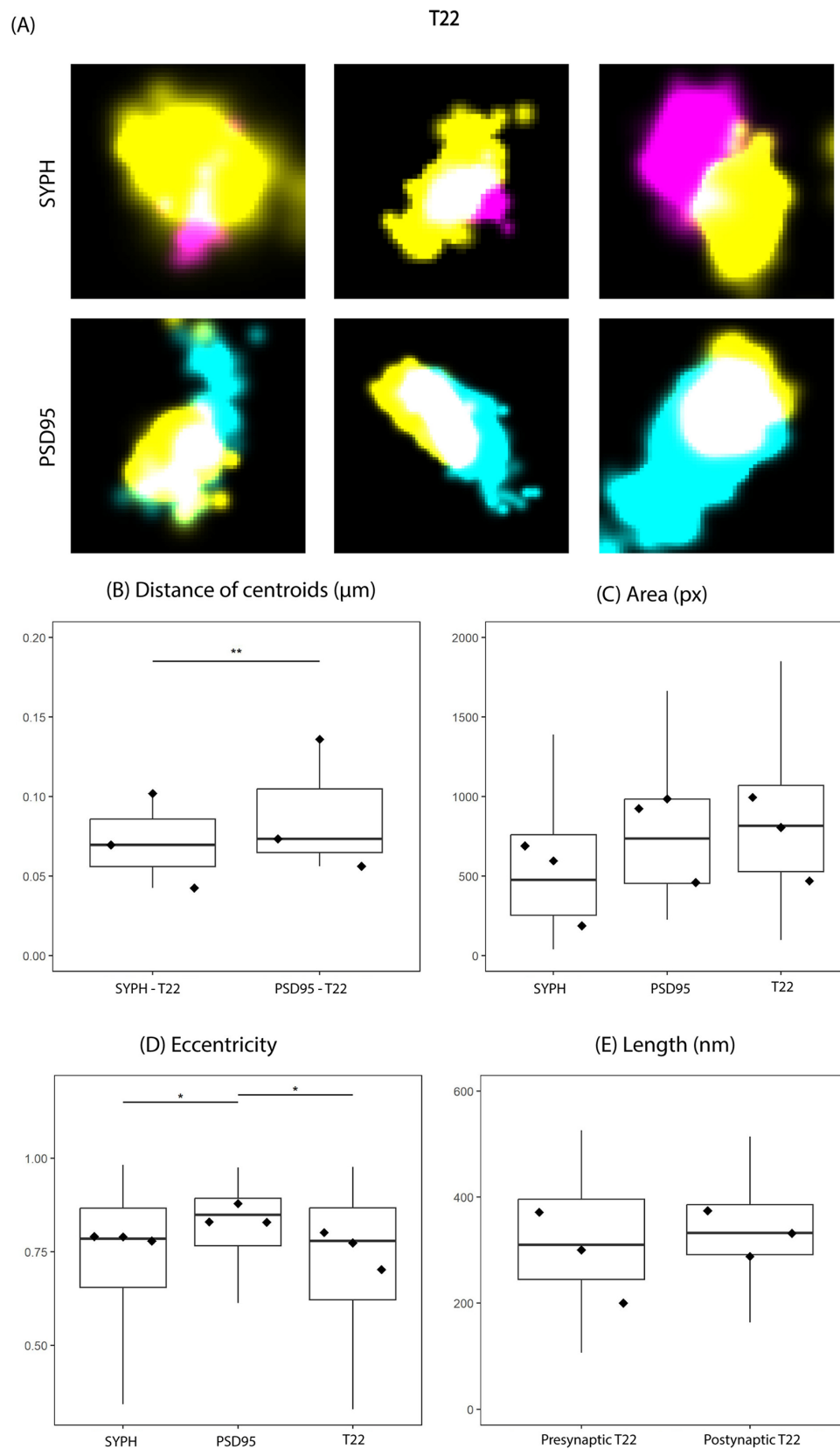


FIGURE 3 | Assessment of the presence of oligomeric tau in pre and postsynaptic compartments. (A) Representative images of oligomeric tau (T22, yellow) in the presynaptic compartment (SYPH, magenta) and the postsynaptic compartment (PSD95, cyan). (B) Distance calculation between centroids of tau aggregates and synaptic compartments. Evaluation of (C) area in pixels and (D) eccentricity of the three markers. (E) Measurement of the length of the major axis in nanometres of T22 oligomers in the two synaptic compartments. The boxplot represents the distribution of all data points, whereas the statistical analysis was performed on the mean values for each case, which are displayed as diamonds. Error bars indicate medians with standard deviations. * $p < 0.05$, ** $p < 0.01$.

dSTORM is an SMLM that consists of photo-switchable fluorescent molecules that stochastically turn on and off within a diffraction-limited area at different times, preventing their signals from overlapping significantly. When isolated in space, the position of each fluorophore can be determined by localising the centre positions of their signal. dSTORM can currently achieve a lateral resolution of 10–30 nm [19]. dSTORM offers some advantages over other traditional techniques, such as transmission electron microscopy, especially in terms of sample preparation and multiple targets [13].

Several studies have reported the presence of pathological tau at the synapse in the AD brain [20–25]. Pathological tau at the synapse is believed to contribute to synaptic dysfunction and loss in AD [26]. In this study, we assessed the presence of synaptic *p*-tau aggregates (AT8) in SAD frontal cortex FFPE samples using dSTORM. The increased lateral resolution revealed a discontinuous nature of tau+ neurites. To investigate the relationship between synapses and the oligomeric forms of tau in AD, we next applied the combination of AT and two-colour dSTORM. Using both techniques, we were able to visualise and identify with high precision the presynaptic and postsynaptic terminals. In addition, we were able to visualise the synaptic cleft with a median width of approximately 64 nm, which closely approximates the range reported by electron microscopy of 20–50 nm [27–30]. This combination also allowed us to observe the orientation in space and the morphology of synaptic terminals. Our results were also validated in cortical biopsy samples, indicating that PMI or the AT sample preparation protocol does not affect synaptic ultrastructure.

There is increasing evidence that oligomeric forms of tau are one of the early events in AD pathology and the most synaptotoxic tau species, contributing to synaptic dysfunction and loss [23, 24, 31–33]. Here, using AT with two-colour dSTORM, we were able to detect tau oligomers (using T22 antibody) colocalising with presynaptic and postsynaptic markers (SYPH and PSD95, respectively) confirming the presence of this pathological form of tau at the synapse. T22 immunoreactivity was found more frequently in presynaptic terminals in the frontal cortex, consistent with our previous results in the temporal and primary visual cortex [11]. Taking advantage of the improved resolution of AT+dSTORM, we defined the characteristics of eccentricity and length, with results similar to those seen in primary cell cultures [34]. Taken together, this study establishes the combination of AT+dSTORM as an optimal technique to study synaptic tau aggregates, paving the way for the study of other proteins that are toxic to the synaptic compartment.

This study has limitations. First, dSTORM requires long acquisition times and extensive postprocessing. Second, the intrinsic composition of the sample tends to distort and blur the single-molecule emission patterns [35]. To overcome this distortion, TIRF microscopy is used, wherein the laser is tilted to an angle at which the sample is illuminated in an exceptionally thin axial region [36]. This allowed us to obtain an enhanced spatial resolution. Finally, the study is limited in sample size due to the scarcity of the AT samples and the findings need to be replicated in additional cases including different subtypes of AD.

5 | Conclusions

In this study, we demonstrate that the combination of AT with two-colour dSTORM offers an improvement of the lateral resolution suitable to investigate synaptic pathology in the human AD brain. These novel techniques offer valuable information for the study of intact human synapses and provide insight into the relevance of synaptic pathology in AD.

Author Contributions

É.S.-A., S.P., T.S.-J., S.S. and A.L. designed the research. S.T., C.Q.-S., À.B., L.M.-P. and I.A. collected and prepared the samples. É.S.-A., B.M.-L. and S.T. performed the experiments. É.S.-A., J.A.E., J.P. and M.C.-C. designed the scripts and analysed the data. J.F., O.B., S.P., S.S. and A.L. supervised the study. All authors read and approved the final manuscript.

Acknowledgements

The authors have nothing to report.

Ethics Statement

Approval was obtained from the ethics committee at Hospital de la Santa Creu i Sant Pau and Hospital Clínic- Fundació Recerca Clínic Barcelona - Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The image analysis scripts are freely available on GitHub (https://github.com/MemoryUnitSantPau/Synseg_py). The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

References

1. A. Serrano-Pozo, M. P. Frosch, E. Masliah, and B. T. Hyman, "Neuropathological Alterations in Alzheimer Disease," *Cold Spring Harbor Perspectives in Medicine* 1, no. 1 (2011): a006189, <https://doi.org/10.1101/cshperspect.a006189>.
2. P. V. Arriagada, J. H. Growdon, E. T. Hedley-Whyte, and B. T. Hyman, "Neurofibrillary Tangles but Not Senile Plaques Parallel Duration and Severity of Alzheimer's Disease," *Neurology* 42, no. 3 (1992): 631.
3. C. M. Wischik, C. R. Harrington, and J. M. D. Storey, "Tau-Aggregation Inhibitor Therapy for Alzheimer's Disease," *Biochemical Pharmacology* 88, no. 4 (2014): 529–539.
4. T. Gómez-Isla, R. Hollister, H. West, et al., "Neuronal Loss Correlates With but Exceeds Neurofibrillary Tangles in Alzheimer's Disease," *Annals of Neurology* 41, no. 1 (1997): 17–24.
5. C. Escartin, J. Herms, E. Capetillo-Zarate, J. Subramanian, J. C. Savage, and M. È. Tremblay, "Synaptic Loss in Alzheimer's Disease: Mechanistic Insights Provided by Two-Photon In Vivo Imaging of Transgenic Mouse Models," *Frontiers in Cellular Neuroscience* 14 (2020): 592607.
6. M. Sheng, B. L. Sabatini, and T. C. Südhof, "Synapses and Alzheimer's Disease," *Cold Spring Harbor Perspectives in Biology* 4, no. 5 (2012): 10.
7. S. V. Paeon, P. R. Nicovich, M. Mollazade, T. Tabarin, and K. Gaus, "Clus-DoC: A Combined Cluster Detection and Colocalization Analysis

- for Single-Molecule Localization Microscopy Data,” *Molecular Biology of the Cell* 27, no. 22 (2016): 3627–3636.
8. M. Colom-Cadena, J. Pegueroles, A. G. Herrmann, et al., “Synaptic Phosphorylated α -Synuclein in Dementia With Lewy Bodies,” *Brain* 140, no. 12 (2017): 3204–3214.
9. M. Querol-Vilaseca, M. Colom-Cadena, J. Pegueroles, et al., “Nanoscale Structure of Amyloid- β Plaques in Alzheimer's Disease,” *Scientific Reports* 9, no. 1 (2019): 5181.
10. P. Ferrer-Raventós, D. Puertollano-Martín, M. Querol-Vilaseca, et al., “Amyloid Precursor Protein β CTF Accumulates in Synapses in Sporadic and Genetic Forms of Alzheimer's Disease,” *Neuropathology and Applied Neurobiology* 49, no. 1 (2023): e12879.
11. M. Colom-Cadena, C. Davies, S. Sirisi, et al., “Synaptic Oligomeric tau in Alzheimer's Disease—A Potential Culprit in the Spread of tau Pathology Through the Brain,” *Neuron* 111, no. 14 (2023): 2170–2183. e6.
12. K. D. Micheva and S. J. Smith, “Array Tomography: A new Tool for Imaging the Molecular Architecture and Ultrastructure of Neural Circuits,” *Neuron* 55, no. 1 (2007): 25–36.
13. K. R. Kay, C. Smith, A. K. Wright, et al., “Studying Synapses in Human Brain With Array Tomography and Electron Microscopy,” *Nature Protocols* 8, no. 7 (2013): 1366–1380.
14. S. Herbert, H. Soares, C. Zimmer, and R. Henriques, “Single-Molecule Localization Super-Resolution Microscopy: Deeper and Faster,” *Microscopy and Microanalysis: The Official Journal of Microscopy Society of America, Microbeam Analysis Society, Microscopical Society of Canada* 18 (2012): 1419–1429.
15. T. J. Montine, C. H. Phelps, T. G. Beach, et al., “National Institute on Aging-Alzheimer's Association Guidelines for the Neuropathologic Assessment of Alzheimer's Disease: A Practical Approach NIH Public Access,” *Acta Neuropathologica* 123, no. 1 (2012): 1–11.
16. D. Y. Mason, K. Micklem, and M. Jones, “Double Immunofluorescence Labelling of Routinely Processed Paraffin Sections,” *Journal of Pathology* 191 (2000): 452–461.
17. N. Olivier, D. Keller, P. Gönczy, and S. Manley, “Resolution Doubling in 3D-STORM Imaging Through Improved Buffers,” *PLoS ONE* 8, no. 7 (2013): 1–9.
18. D. van der Zwaag, N. Vanparijs, S. Wijnands, R. de Rycke, B. G. de Geest, and L. Albertazzi, “Super Resolution Imaging of Nanoparticles Cellular Uptake and Trafficking,” *ACS Applied Materials & Interfaces* 8, no. 10 (2016): 6391–6399.
19. J. Tam and D. Merino, “Stochastic Optical Reconstruction Microscopy (STORM) in Comparison With Stimulated Emission Depletion (STED) and Other Imaging Methods,” *Journal of Neurochemistry* 135, no. 4 (2015): 643–658.
20. B. G. Perez-Nievas, T. D. Stein, H. C. Tai, et al., “Dissecting Phenotypic Traits Linked to Human Resilience to Alzheimer's Pathology,” *Brain* 136, no. Pt 8 (2013): 2510–2526.
21. L. Zhou, J. McInnes, K. Wierda, et al., “Tau Association With Synaptic Vesicles Causes Presynaptic Dysfunction,” *Nature Communications* 8, no. 1 (2017): 15295, <https://doi.org/10.1038/ncomms15295>.
22. J. McInnes, K. Wierda, A. Snellinx, et al., “Synaptogyrin-3 Mediates Presynaptic Dysfunction Induced by Tau,” *Neuron* 97, no. 4 (2018): 823–835.e8.
23. C. A. Lasagna-Reeves, D. L. Castillo-Carranza, U. Sengupta, et al., “Identification of Oligomers at Early Stages of Tau Aggregation in Alzheimer's Disease,” *FASEB Journal* 26, no. 5 (2012): 1946–1959.
24. D. L. Castillo-Carranza, J. E. Gerson, U. Sengupta, M. J. Guerrero-Muñoz, C. A. Lasagna-Reeves, and R. Kaye, “Specific Targeting of tau Oligomers in Htau Mice Prevents Cognitive Impairment and tau Toxicity Following Injection With Brain-Derived tau Oligomeric Seeds,” *Journal of Alzheimer's Disease* 40, no. s1 (2014): S97–S111.
25. C. A. Lasagna-Reeves, D. L. Castillo-Carranza, U. Sengupta, A. L. Clos, G. R. Jackson, and R. Kaye, “Tau Oligomers Impair Memory and Induce Synaptic and Mitochondrial Dysfunction in Wild-Type Mice,” *Molecular Neurodegeneration* 6, no. 1 (2011): 1–14.
26. M. Wu, M. Zhang, X. Yin, et al., “The Role of Pathological tau in Synaptic Dysfunction in Alzheimer's Diseases,” *Translational Neurodegeneration* 10 (2021): 45.
27. X. Yang and W. Annaert, “The Nanoscopic Organization of Synapse Structures: A Common Basis for Cell Communication,” *Membranes* 11, no. 4 (2021): 248.
28. B. Zuber, I. Nikonenko, P. Klauser, D. Muller, and J. Dubochet, “The Mammalian Central Nervous Synaptic Cleft Contains a High Density of Periodically Organized Complexes,” *Proceedings of the National Academy of Sciences of the United States of America* 102, no. 52 (2005): 19192–19197.
29. B. High, A. A. Cole, X. Chen, and T. S. Reese, “Electron Microscopic Tomography Reveals Discrete Transcleft Elements at Excitatory and Inhibitory Synapses,” *Frontiers in Synaptic Neuroscience* 7, no. 6 (2015): 9.
30. K. Zheng, T. P. Jensen, L. P. Savtchenko, J. A. Levitt, K. Suhling, and D. A. Rusakov, “Nanoscale Diffusion in the Synaptic Cleft and Beyond Measured With Time-Resolved Fluorescence Anisotropy Imaging,” *Scientific Reports* 7, no. 1 (2017): 1–10.
31. G. Niewiadomska, W. Niewiadomski, M. Steczkowska, and A. Gasiorowska, “Tau Oligomers Neurotoxicity,” *Life (Basel, Switzerland)* 11, no. 1 (2021): 28.
32. Z. Berger, H. Roder, A. Hanna, et al., “Accumulation of Pathological tau Species and Memory Loss in a Conditional Model of Tauopathy,” *Journal of Neuroscience* 27, no. 14 (2007): 3650–3662.
33. J. E. Gerson and R. Kaye, “Formation and Propagation of Tau Oligomeric Seeds,” *Frontiers in Neurology* 4 (2013): 93.
34. E. Dimou, T. Katsinelos, G. Meisl, et al., “Super-Resolution Imaging Unveils the Self-Replication of tau Aggregates Upon Seeding,” *Cell Reports* 42, no. 7 (2023): 112725.
35. P. Codron, F. Letournel, S. Marty, et al., “STochastic Optical Reconstruction Microscopy (STORM) Reveals the Nanoscale Organization of Pathological Aggregates in Human Brain,” *Neuropathology and Applied Neurobiology* 47, no. 1 (2021): 127–142.
36. K. N. Fish, “Total Internal Reflection Fluorescence (TIRF) Microscopy,” *Current Protocols in Cytometry* 12, no. 18 (2009).

Supporting Information

Additional supporting information can be found online in the Supporting Information section.