Supporting Information

Fluorescence labeling methods influence the aggregation process of α-syn in vitro differently.

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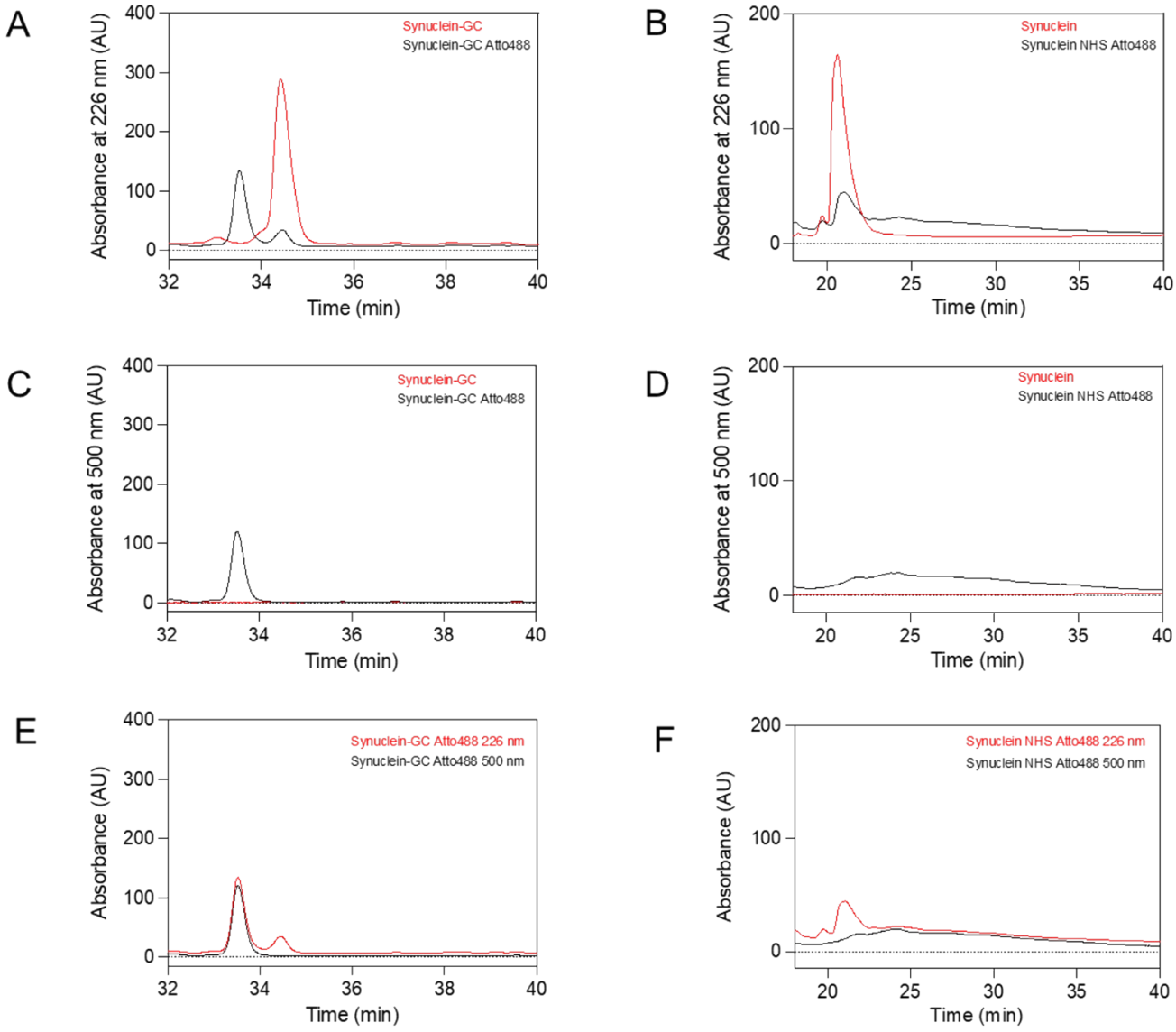


Figure S1. RP-HPLC analysis comparing the monomer before and after the labeling reaction. Left - α-synGC (red line) and α-synGC post-reaction (black line) profiles at 226 nm (A) and 500 nm (C). In E), the profiles at 226 (red line) and 500 nm (black line) of α-synGC post-reaction have been overlapped to highlight the conjugated species. Right - Synuclein (red line) and Synuclein post-reaction (black line) profiles at 226 nm (B) and 500 nm (D). In F), the profiles at 226 (red line) and 500 nm (black line) of Synuclein post-reaction have been overlapped to better visualize the conjugated species. As expected, the NHS reaction generates a heterogeneous population of Atto-synuclein species making very difficult the calculation of the number of dye molecules per protein (Suppl. Fig.1 B-D-F). On the other hand, we were able to calculate an approximated value of the overall labelling efficiency; in both the cases ~90% of the monomers are labeled. Considering this result and the procedure employed for samples preparation (see Materials and Methods) we consider a ratio between labeled and unlabeled monomers of 1:22 for both the dyes employed.

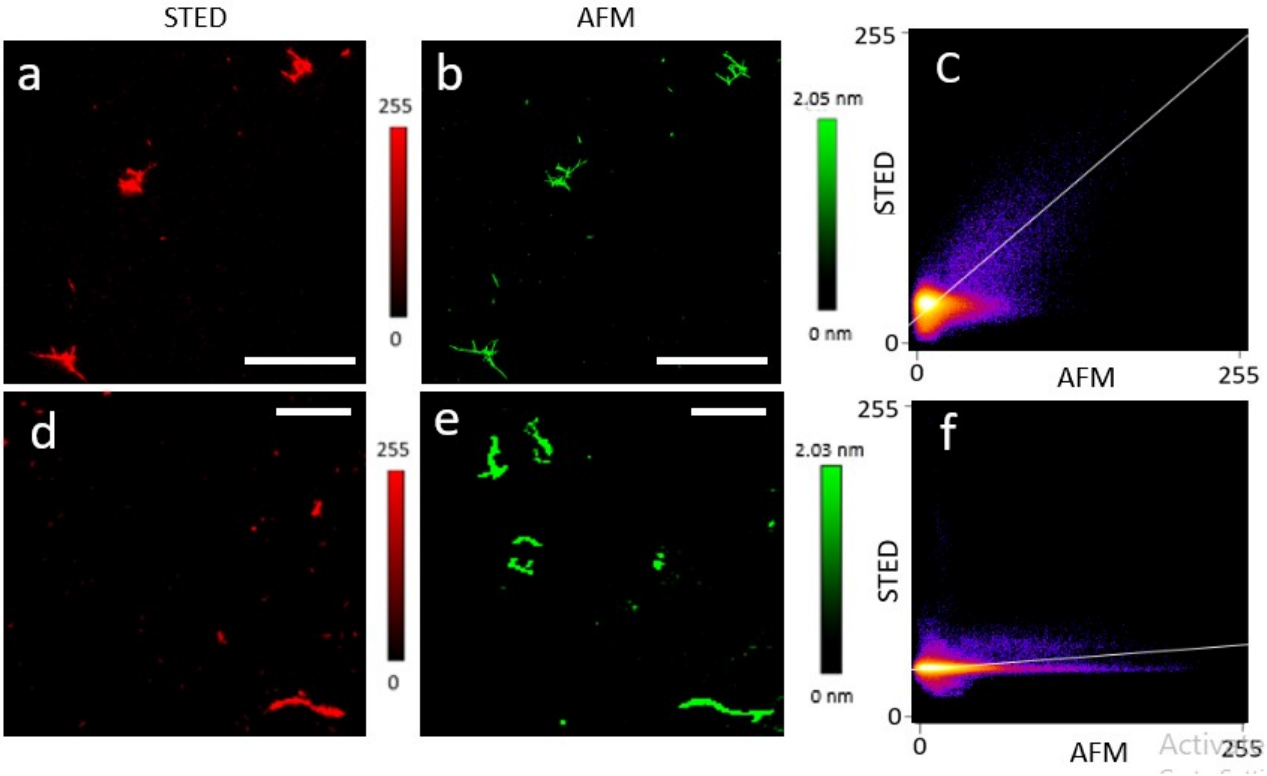
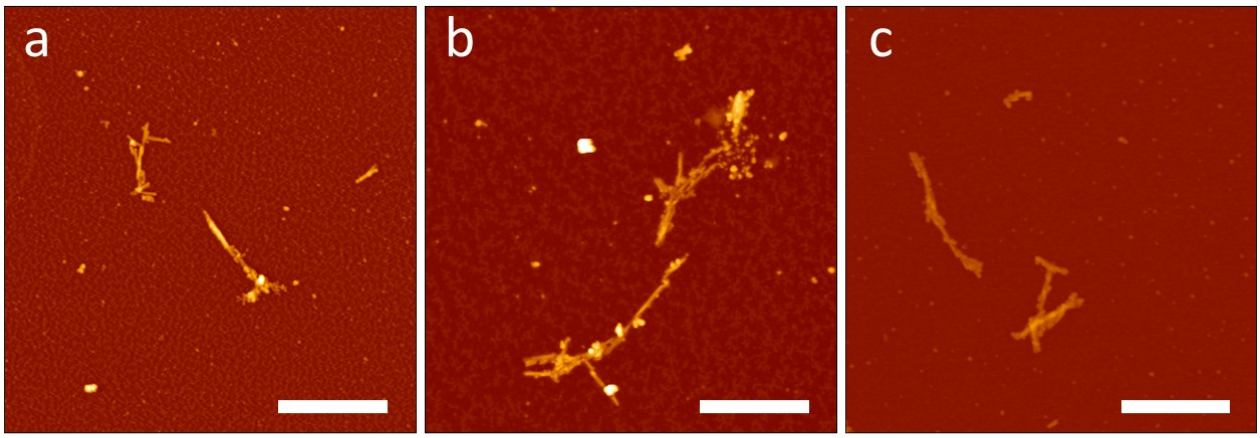


Figure S2. Representative co-localization analysis between STED (left) and AFM (right) images correspond to α-syn fibrils site-specific labeled with ATTO488 with a functional maleimide (a,b), and with ATTO 488-NHS (d,e) at a dye-to-protein ratio of 1:22. 2D intensity histograms (c,f) were obtained by using the co-localization function of ImageJ (NIH, Bethesda, MD, USA). The white line is representing the Mander's coefficient (see Materials and Methods). Phasor cloud is an additional tool to get separated intensity channels. The phasor plots c and f are slightly not symmetric with respect to the diagonal due to the different resolution of the two techniques. The phasor plot associated with the image on the sample labeled with ATTO 488-NHS shows a tail strongly elongated towards the green channel, this large asymmetry is not only due to the different spatial resolution of the STED and AFM techniques but to the lack of fluorescent aggregates. Scale bar: (a,b) 5 μm, (d,e) 1 μm.



	Thickness, h (nm)	Width, W (nm)	$(W_m/W_t)_{nom}$; $(W_m/W_t)_{min}$
unlabeled	9.7±2.3	62± 13	1.6 ; 1.1
ATTO488-maleimide	9.6±3.5	64±12	1.6 ; 1.2
ATTO 488-NHS	6.7±1.7	47±5	1.5 ; 1.0

Figure S3. Representative AFM images froorm the aggregation of unlabeled (a), ATTO488-maleimide (b), and ATTO488-NHS (c) labeled α-syn. Scale bars: 1 µm. The Z-range is 50 nm for all the images. The lower contrast between fibrils and substrate displayed in c is due to the lower fibrils thickness. Table indicates fibrils thickness and width and the ratio W_m/W_t, where W_m is the measured width and W_t the theoretical width considering the fibril thickness and considering the fibril as a cylinder (circular cross-section). The relation $W_t=2\sqrt{2hR}$ can be derived from geometrical considerations. R is the tip radius. Two different R has been considered, basedding on manufacturer specifications. The nominal R is 20 nm and the maximum 60 nm. From this two values $(W_m/W_t)_{nom}$ and $(W_m/W_t)_{min}$ were calculated. The W_m/W_t is one in the case of a cylindrical shape, while it is > in the case of flattened fibrils. In this case fibrils are just slightly flattened

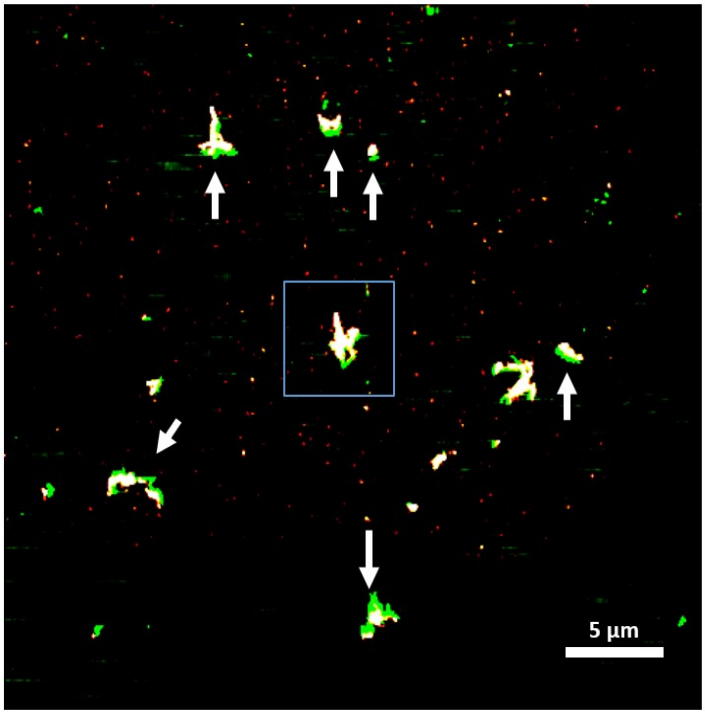


Figure S4. Representative correlative AFM-STED image of α-syn fibrils at a dye-to-protein ratio of 1:22. The ratio of co-localization is 0.73. In this case, co-localization ratio is affected by optical aberrations. This effect is generally evident on larger fields of view. Due to the very small size of fibrils, a misalignment in the order of tens of nanometers results in a significant decrease in the co-localization ratio that affects the average values of the whole dataset. Looking at the arrows it is clear that the mismatch is not due to an image shift but to a distortion induced by the optical system on the image periphery. On the upper part of the image, the fluorescence is up-shifted with respect to the AFM topography, on the contrary, on the lower part the fluorescence is slightly down-shifted. A good overlap is obtained in the central area. The scale bar is 5 µm.

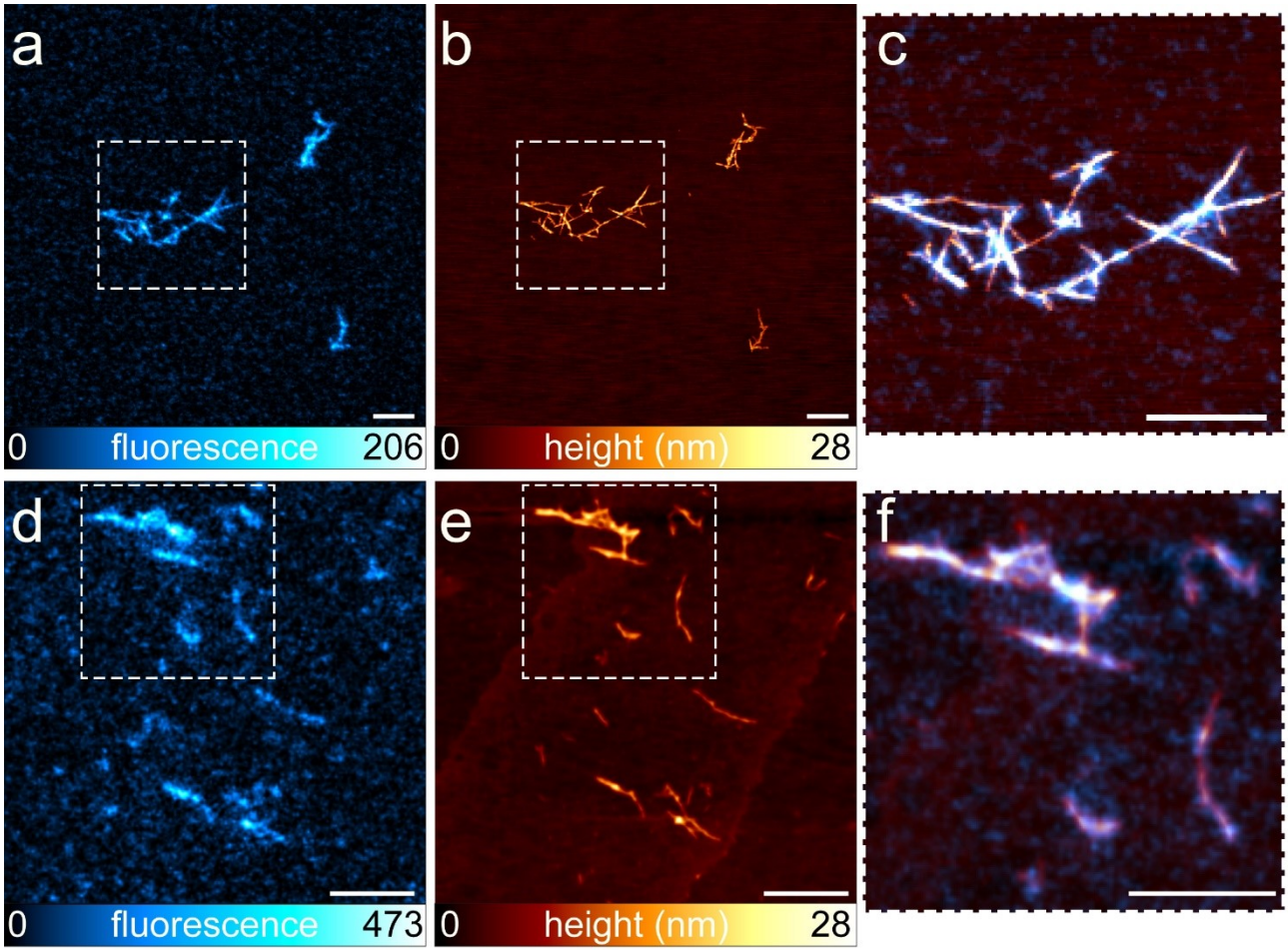


Figure S5. α-syn fibrils site-specific labeled with ATTO488 with a functional maleimide at a dye-to-protein ratio of 1:22 visualized by STED microscopy (a, d), AFM (b, e), and the correlative AFM-STED microscopy in a zoomed area of the image (c, f). Fibrils appear entirely and homogeneously labeled (c). Scale bars: 1 µm (a-c).

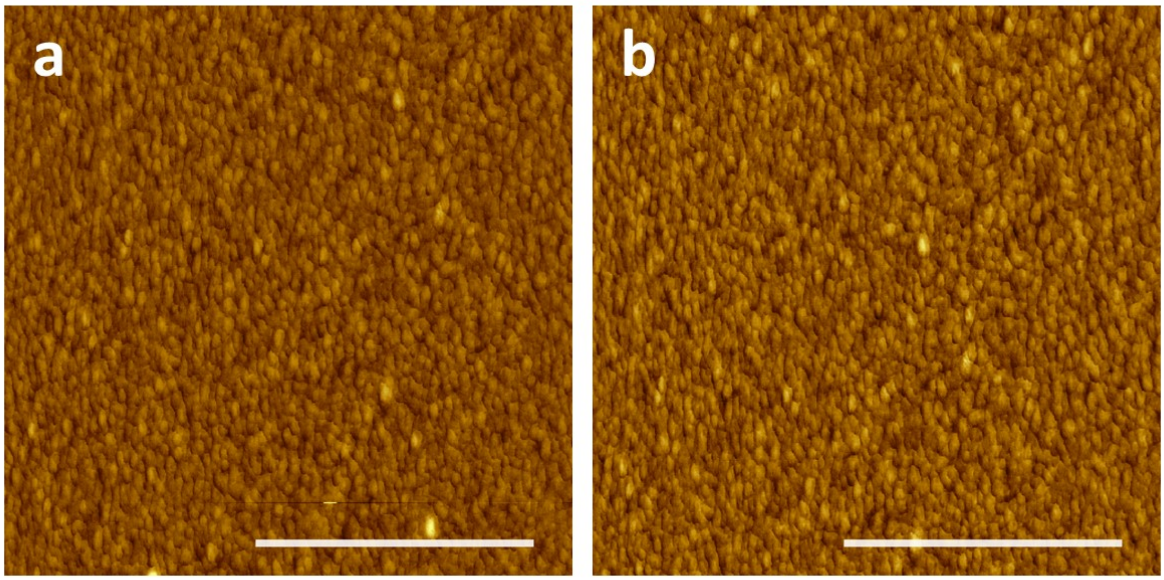


Figure S6. Tapping mode AFM images of α-syn before (a) and after the labelling with ATTO488 NHS. Sample preparation was the same as described in Materials and Methods for the samples after aggregation (50 times dilution, 20 minutes of incubation time), but these samples have been prepared on freshly cleaved mica substrates. A large amount of globular objects is present, while the absence of any fibrillar aggregate is confirmed. Scale bar is 500 nm, the Z-range is 3.5 nm.

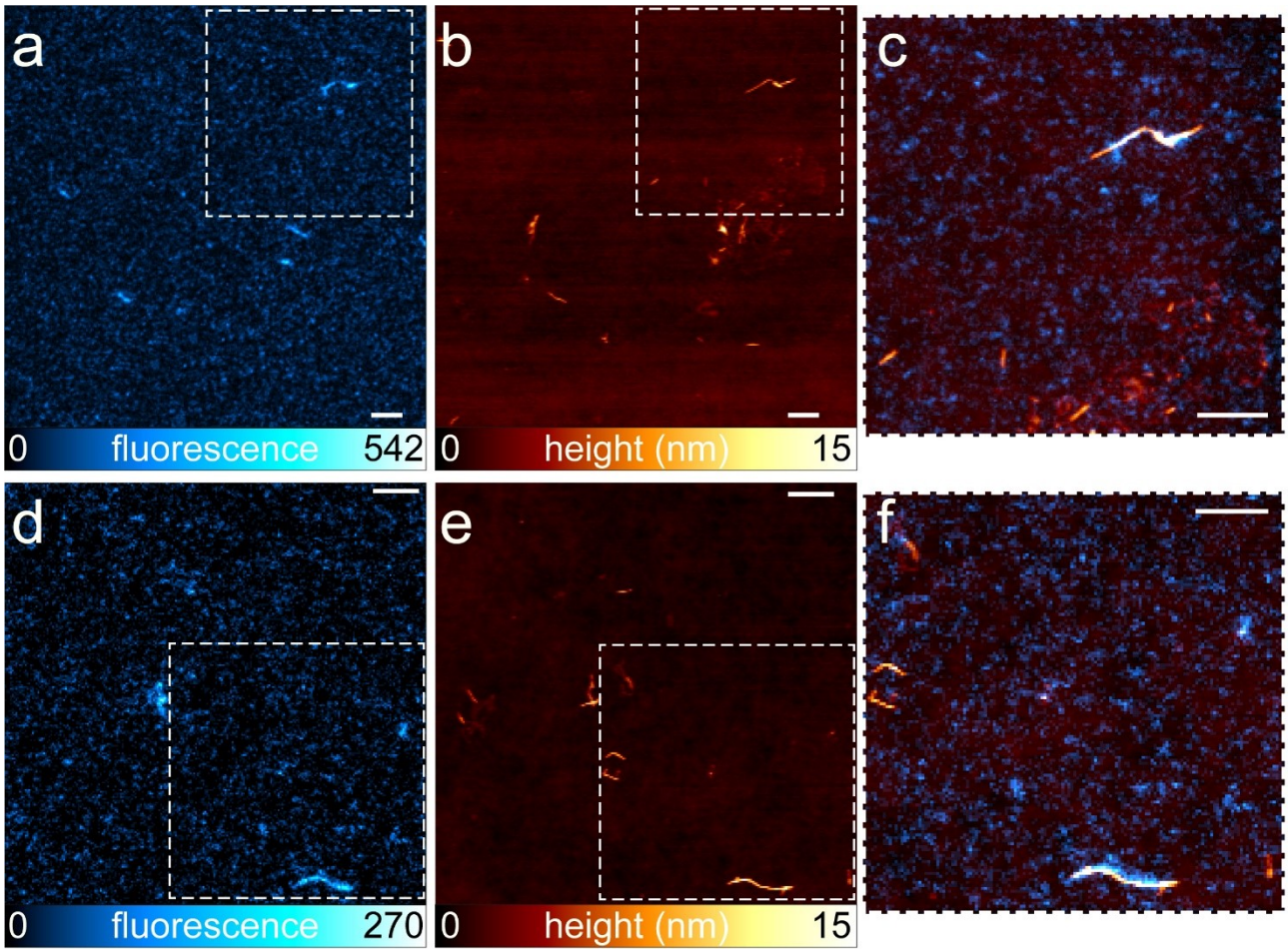


Figure S7. α-syn fibrils labeled with ATTO488 NHS at a dye-to-protein ratio of 1:22. STED image (a), AFM image (b), and the correlative AFM-STED image obtained in the area of the inset (c). A large population of fibrils is not fluorescent, being visible in the AFM images only. The scale bar is 1 μm (a–c).

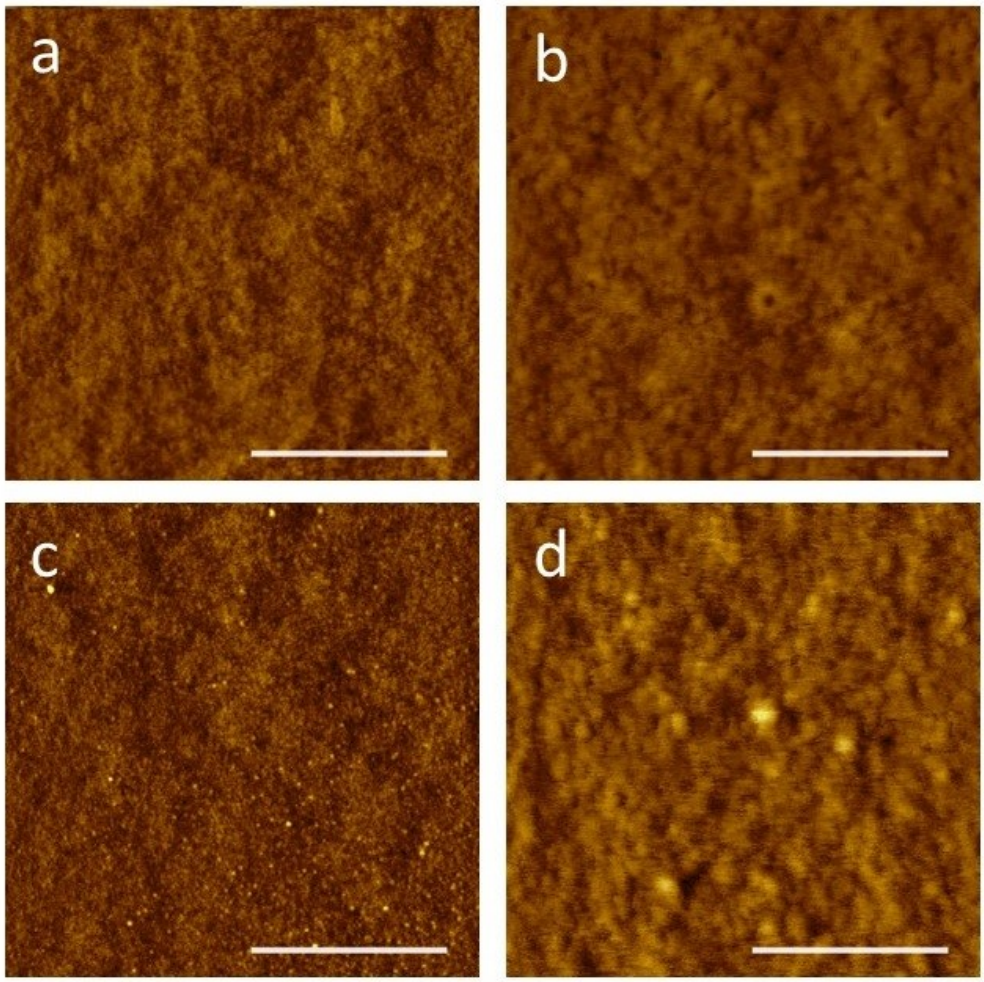


Figure S8. Tapping mode AFM images of bare glass substrates (a,b) and aggregated α-syn solution deposited on the same type of glass substrate following the procedure described in Materials and Methods section (c,d). Scale bars are 1 μm (a,c) and 200 nm (b,d), the Z-range is 2 .5 nm for all the images. The average roughness (R_a), calculated on images with a size of 500 nm x 500 nm, was 130±20 nm for bare glass and 160±40 for the sample after α-syn deposition. Since we excluded areas with fibrils, we concluded that the change is due to the presence of other materials, hence, monomeric α-syn or small non-fibrillar aggregates. Small roundish features protruding of less than 2 nm form the surface are clearly visible in c and d. These small objects are hardly distinguishable at the resolution and vertical scale employed for correlative microscopy images, for this reason some fluorescent spots have not a clear correspondence in the AFM images.