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Supplementary Materials for

AFM-STED correlative nanoscopy reveals a dark side in fluorescence microscopy imaging

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This PDF file includes:

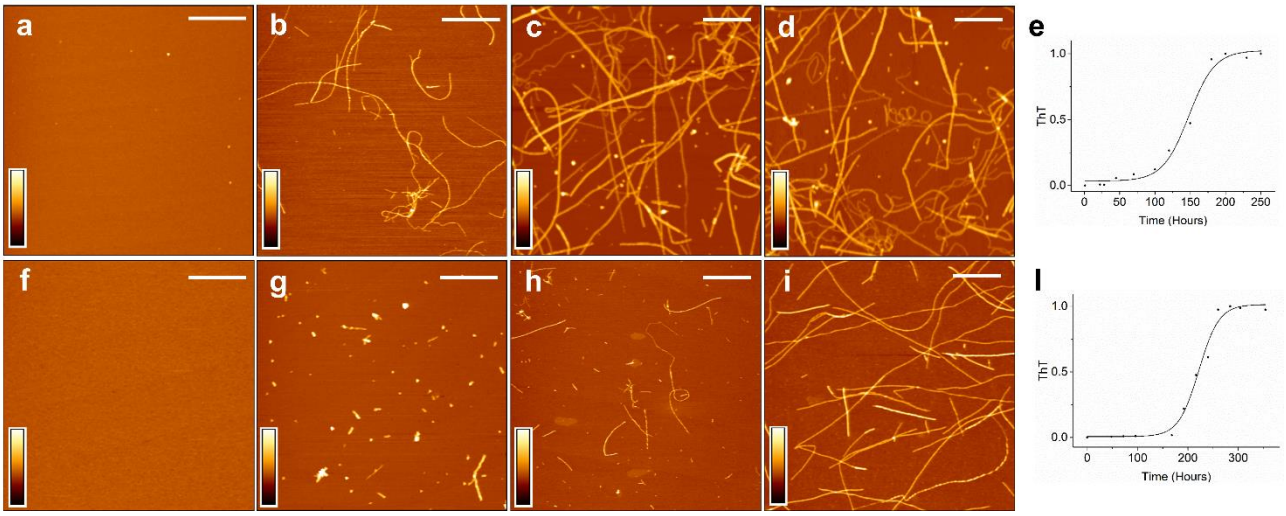

Fig. S1. AFM and ThT study of insulin aggregation process. AFM was used to follow the aggregation of insulin and to characterize the morphology of the fibrils. Aggregation of unlabelled insulin at (**a**) 0 day, (**b**) 6 days, (**c**) 12 days (**d**) 14 days. Surface coverage data: 0.4% (a), 7.5% (b), 32.1% (c), 33% (d). Aggregation of Insulin 1:19 dye-to-protein ratio at (**f**) 0 day, (**g**) 6 days, (**h**) 12 days (**i**) 14 days. (a, b, f, g), 15 nm (c, d, h, i). Surface coverage data: 0.1% (f), 2.5% (g), 6% (h), 22% (i). Scale bars: 2 µm. Vertical color scale: 7 nm. The aggregation of unlabeled (**e**) and labeled insulin at a dye to protein ratio of 1:19 (**l**) was also followed by ThT fluorescence assay. The presence of the dye induces a slight increase of the lag phase time. However, after 14 days (336 h) ThT fluorescence has already reached the plateau for both. All the correlative measurements derived by samples at 14 days of aggregation.

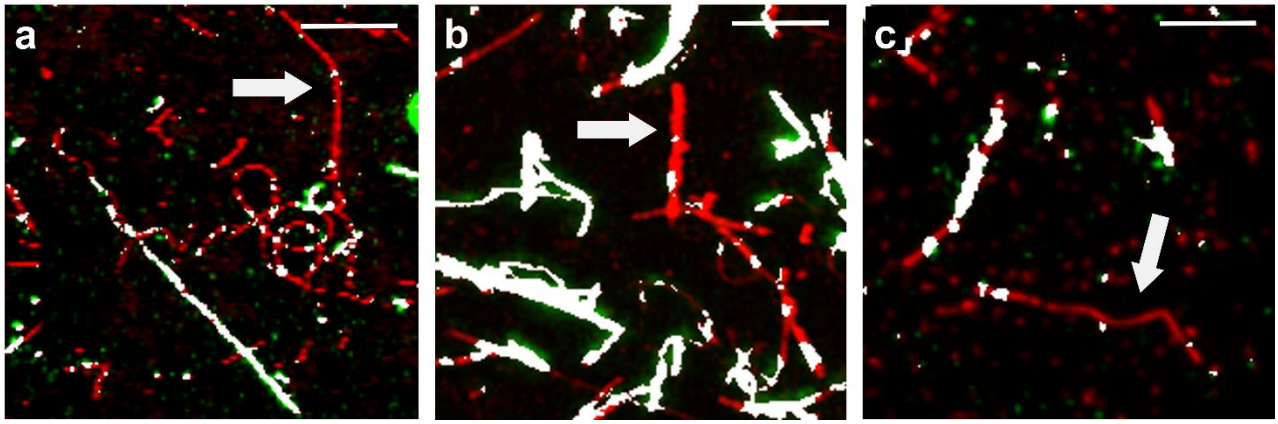

Fig. S2. Correlative images of insulin fibrils on a smaller area. The single labelled/unlabelled fibrils are clearly displayed in correlative images on smaller area. (**a**) 1:19, (**b**) 1:99 and (**c**) 1:499. Scale (a) 2 µm (b, c) 1 µm

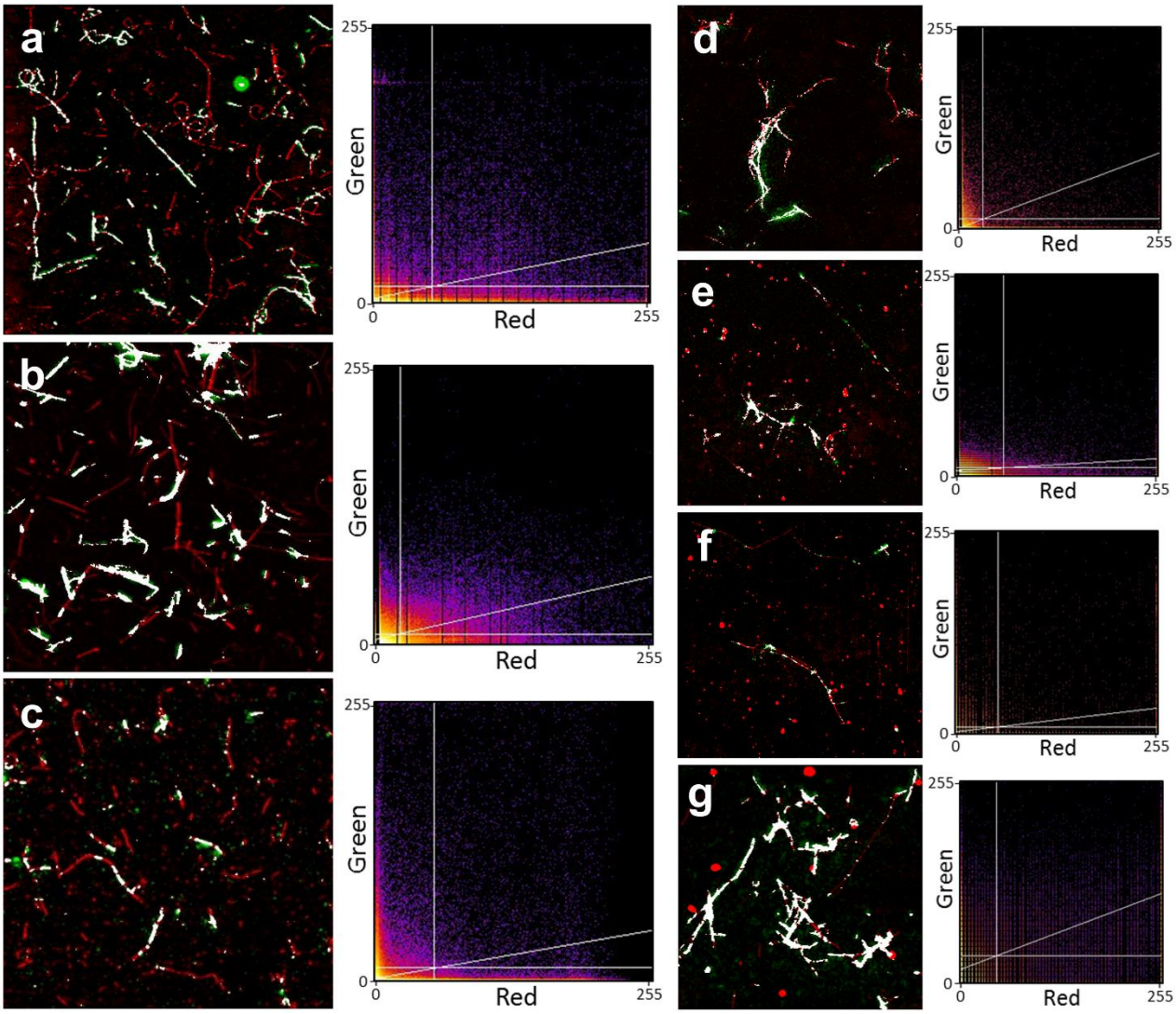

Fig. S3. Colocalization analysis between AFM and STED images. Scatter plots. Images a-c are the same shown in Figs. 2 and 3 in the main text. Images d-g are shown in Fig. 4 in the manuscript. Correlative images are presented side-by-side with the correspondent scatter plot obtained by using the colocalization function of ImageJ (NIH, Bethesda, MD, USA). See Materials and Methods for further details.

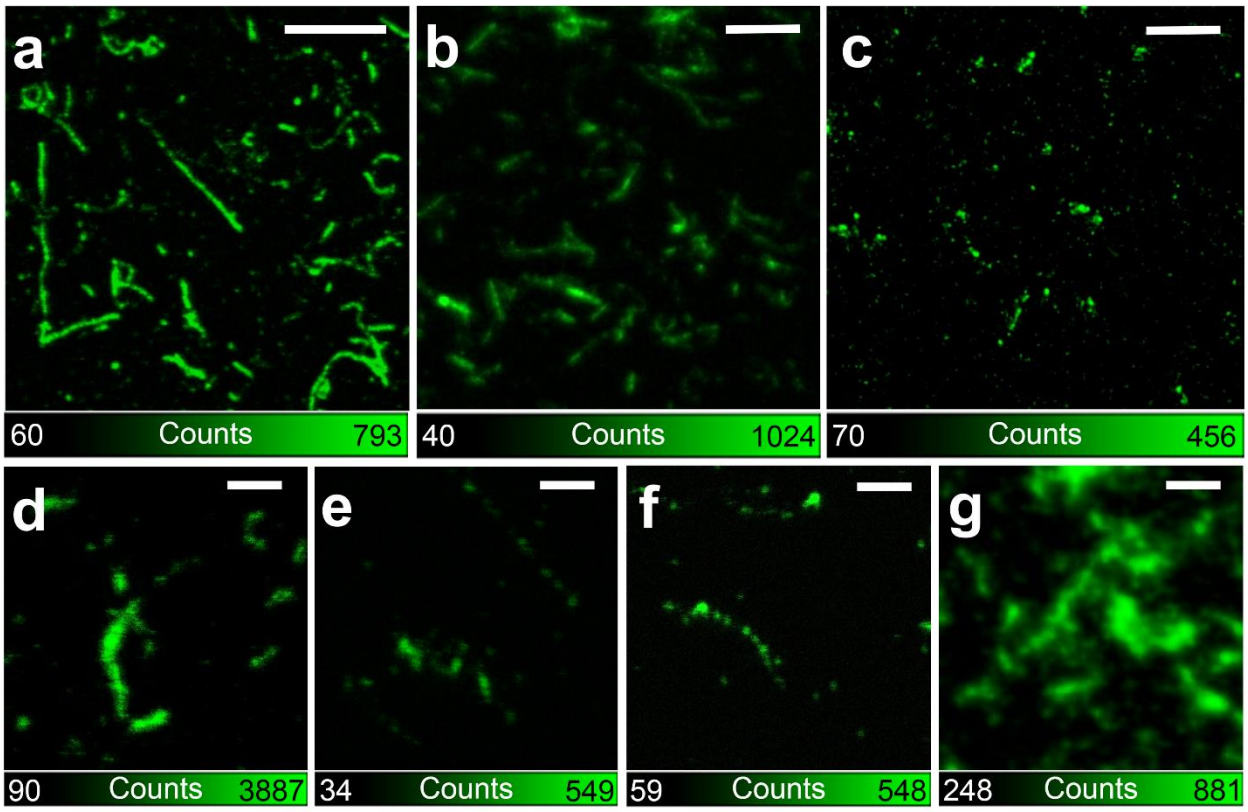

Fig. S4. Confocal microscopy images. Confocal microscopy images of amyloid fibrils from insulin (**a-c**) and $A\beta_{(1-42)}$ (**d-f**), and $A\beta_{(1-40)}$ (**g**). In particular the images derive from 1:19 samples (a, d, g), 1:99 (b, e) , 1:499 (c, f). These images were acquired on the exact same area of the STED images shown in the manuscript, acquired before STED image acquisition. These images demonstrate that the areas without fluorescent signals are not due to the photobleaching effect. Scale bar: 5 µm (a), 2 µm (b-g).

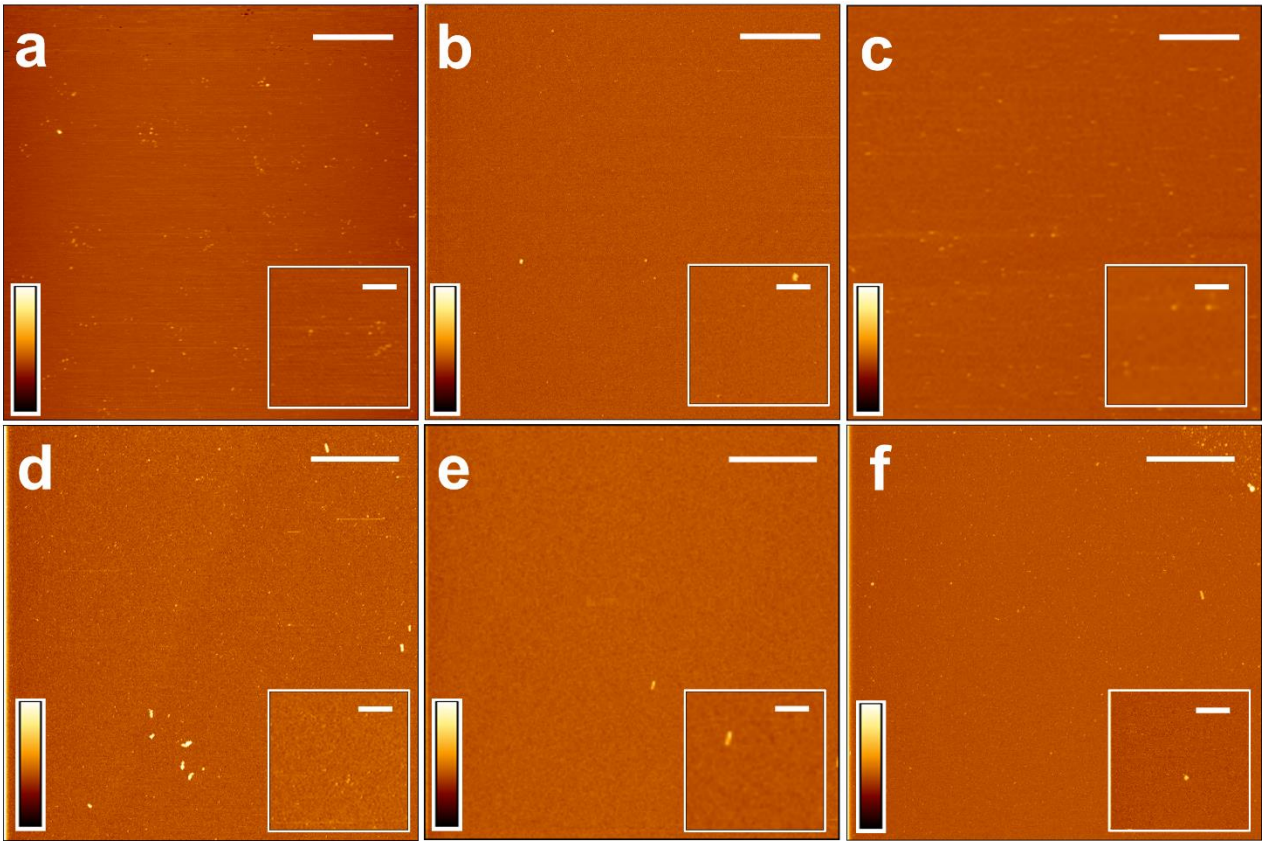

Fig. S5. Aβ after resuspension of the aggregation medium. AFM was used to control every step during the time of aggregation. $A\beta_{(1-42)}$ (**a**) unlabelled, (**b**) 1:19, (**c**) 1:99, (**d**) 1:499. $A\beta_{(1-40)}$ (**e**) unlabelled and (**f**) 1:19. Scale bar: 1 µm; inset 200 nm. Vertical color scale: 0-15 nm. Prefibrillar aggregates are not present at the beginning of the aggregation process, confirming by surface coverage data. In particular: 0.5% (a), 0.6% (b), 1% (c), 0.8% (d), 0.4% (e), 0.7% (f) surface coverage data.

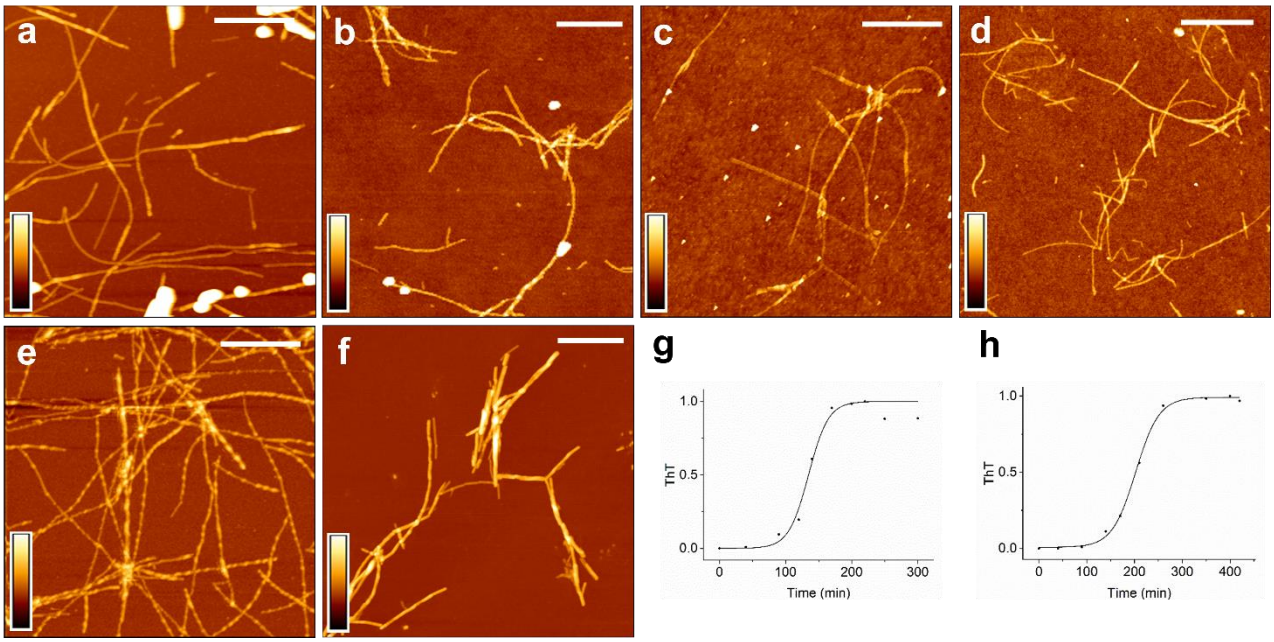

Fig. S6. Amyloid aggregates from Aβ peptides. In particular. $A\beta_{(1-42)}$ (**a**) unlabelled, (**b**) 1:19, (**c**) 1:99, (**d**) 1:499 after 4 h of aggregation. $A\beta_{(1-40)}$ (**e**) unlabeled and (**f**) 1:19 after 1 day of aggregation. The aggregation of unlabeled (**g**) and labeled $A\beta_{(1-42)}$ at a dye to protein ratio of 1:19 (**h**) was also followed by ThT fluorescence assay. Scale bar: 1 µm. Vertical color scale: (a) 10 nm (b-f) 20nm. Surface coverage data: 17.3% (a), 7.3% (b), 6.1% (c), 7% (d), 20.2% (e), 12.2% (f).

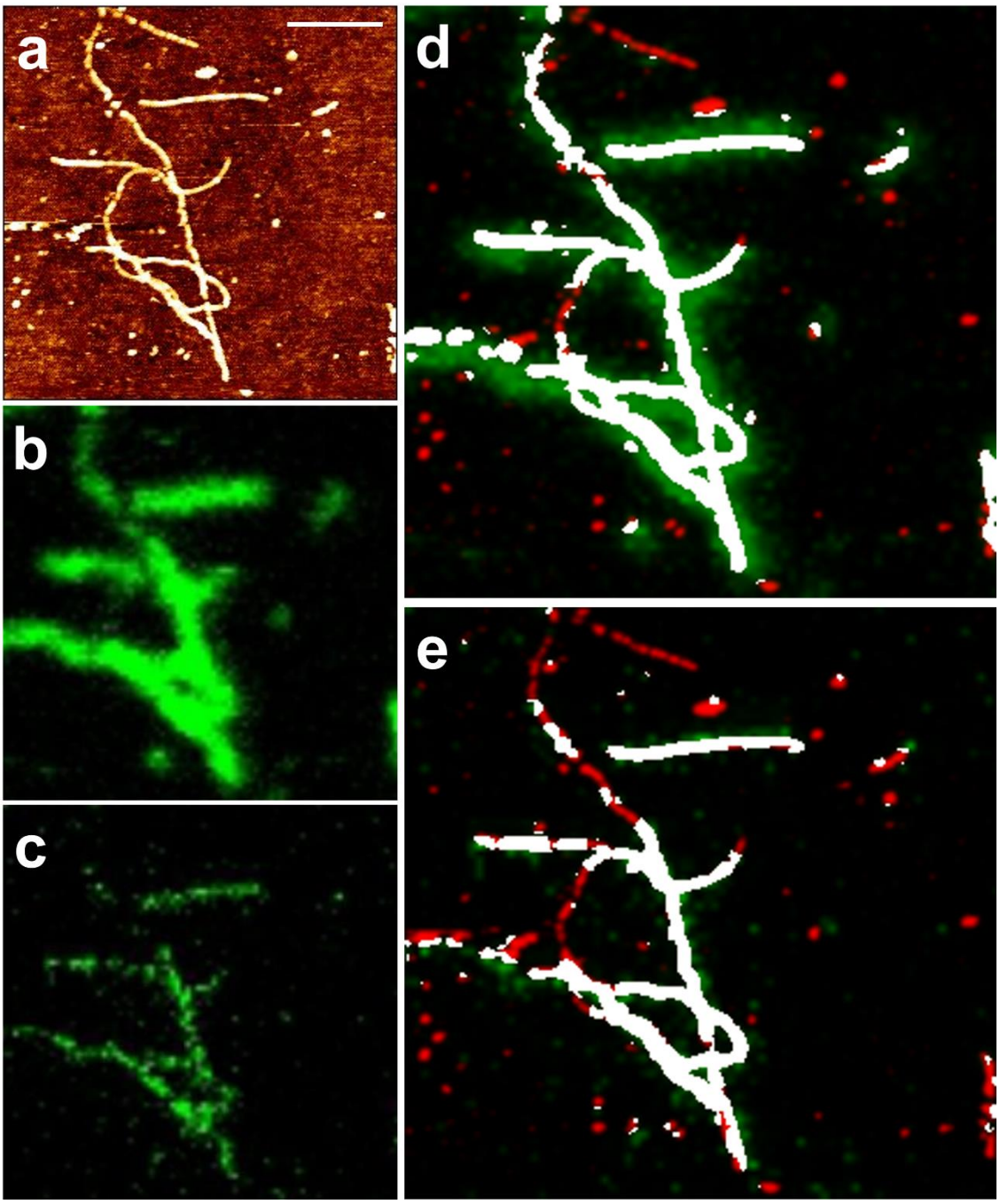

Fig. S7. Example of correlation between AFM-confocal and AFM-STED images. Insulin fibril 1:19 dye-to-protein ratio. AFM (**a**), Confocal (**b**) and STED (**d**) were analysed by using the Colocalization function by ImageJ (NIH, Bethesda, MD, USA). The ratio of co-localization is the ratio between the number of co-localized pixels (white pixels in d and **e**) and the total number of pixel that composed the fibrils in the AFM images. Scale bar: 1µm.