Direct observation of heterogeneous amyloid fibril growth kinetics via two-color super-resolution microscopy

Supplementary Material

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Materials and methods

**Protein labeling.** Monomeric human wild-type α-synuclein was over-expressed and purified as described previously.\(^1\) We choose to place the label at residue 122, located at the C-terminal end of the amino acid sequence of α-synuclein and outside the region which is thought to form the fibrillar structure (the NAC region), in order to prevent a potential effect of the presence of the dye on the kinetics and mechanism of amyloid formation of the protein. No influences are expected on the fibrillar structure and aggregation kinetics for C-terminal labeling, as we have previously shown\(^2\) and resulting from the addition of the fluorophores, as has been confirmed by AFM, electron microscopy (EM) and ThT fluorescence measurements (see below).

The N122C variant of α-synuclein was labeled with maleimide-modified Alexa Fluor\(^{®}\) 647 or Alexa Fluor\(^{®}\) 568 dyes (Invitrogen, Carlsbad, CA, USA) via the cysteine thiol moiety. The labeled protein was purified from the excess of free dye by a P10 desalting column with Sephadex G25 matrix (GE Healthcare, Waukesha, WI, USA), divided into aliquots, flash frozen in liquid \(N_2\), and stored at -80 °C. Each aliquot was thawed immediately prior to use and used only once. The efficiency of the labeling process was checked by mass spectrometry (Mass spectrometry facility, Department of Biochemistry, University of Cambridge), resulting to be higher than 95% for Alexa Fluor\(^{®}\) 647 and 50% for Alexa Fluor\(^{®}\) 568.

**Conversion of monomeric protein to fibrils and two-color seeded assay.** Unlabeled seed fibrils were produced by incubating 500 \(\mu\)l solutions of α-synuclein at concentrations 500-800 \(\mu\)M (in 20 mM phosphate buffer, PB at pH 6.5) for 48 h at 45 °C under strong stirring with a teflon coated stir bar on an RCT basic heat plate (IKA, Staufen, Germany). Alexa\(^{®}\) 568 labeled seed fibrils were prepared as follows: 500 \(\mu\)l of 30 \(\mu\)M sonicated (35 s), unlabeled seed fibrils were incubated with 80 \(\mu\)M of unlabeled monomeric α-synuclein and 3.6 \(\mu\)M of Alexa\(^{®}\) 568 labeled monomeric α-synuclein, in phosphate buffer at pH 6.5 with 0.01% sodium azide at 37 °C under stirring for 48 h. The fibrils formed had lengths of approximately 400 nm, as determined by AFM and were subsequently incubated with monomeric protein at 10 times higher concentration and
at labeling ratio 1:20, labeled with Alexa Fluor® 647 vs. unlabeled monomeric protein. We thus optimized the dye labeling density and found the 1:20 ratio as an optimal one in order to further reduce any potential interference of the labeling with the aggregation process and to improve the localization precision (see section Super-resolution fluorescence microscopy below). This yielded optimum precision at low to moderate laser excitation powers. Finally, the incubation at 37 °C took place in an Eppendorf tube in 20 mM PB and samples at different time points were removed for subsequent microscopy imaging, in the absence of shaking or mixing.

**Thioflavin T kinetic assay.** The elongation of amyloid fibrils of α-synuclein was monitored by following the change of the fluorescence at 480 nm of the amyloid sensitive dye, Thioflavin T (ThT) using an Optima Fluostar platereader (BMG Labtech, Ortenburg, Germany). Samples were prepared, containing 80 µM monomeric α-synuclein, 8 µM sonicated seed fibrils and 20 µM Thioflavin-T.

**Super-resolution fluorescence microscopy by single molecule localization and dSTORM imaging.** Single and two-color super-resolution imaging was performed with a dSTORM microscopy setup, based on a Nikon Eclipse TE 300 inverted widefield microscope and a 100x, 1.49 NA TIRF objective lens (Nikon UK Ltd.). For dSTORM imaging, samples were placed on a glass or quartz coverslip glued to the bottom of an imaging chamber. Photoswitching buffer solution was added which consisted of 100 mM mercaptoethylamine (MEA) in phosphate buffered saline (PBS, pH 7.4), together with a glucose-enzyme oxygen scavenger (40 mg/ml glucose, 50 µg/ml glucose oxidase, 1 µg/ml catalase). The chamber was filled to the top and sealed with a glass coverslip to minimize entrance of oxygen. For the two-color imaging, laser illumination was at 640 nm (Toptica Photonics AG, Graefelfing, Germany) for excitation of the Alexa 647 dye (red channel) and at 561 nm (Oxxius SLIM-561) for excitation of the Alexa 568 dye (green channel). They were collimated and combined by dichroic mirrors and a beam expanding telescope. The laser beams were subsequently focused onto the back focal plane of the objective. A 405 nm (Mitsubishi
Electronics Corp., Tokyo, Japan) laser was used as reactivation source. In order to separate the individual emissions from the two channels (red and green), the fluorescence light in the detection path went through a dichroic filter (Semrock multi-edge filter Di01-R405/488/561/635-25x36 followed by a FF01-446/523/600/677-25 filter, Semrock, Rochester NY, USA) and was subsequently filtered further using band-pass filters (Semrock BP-607/35-25 and BP-642/35-25 for the green and the red channel respectively), before being projected onto a low-noise, highly sensitive electron-multiplying CCD camera (Ixon DV887 ECS-BV, Andor). The excitation intensity was 2 kW/cm\(^2\) for the Toptica laser and 5 kW/cm\(^2\) for the Oxxius laser. The reactivation laser was only turned on when the number of active fluorophores in the field of view was reduced and no spatial drift of the sample was observed during the acquisition time of the two channels. Imaging was performed in TIRF illumination in all cases at the exact centre of an area consisting of 64x64 camera pixels, corresponding to an area on the sample of 10x10 \(\mu m^2\). Typically, 10,000-20,000 fluorescence frames with 10-12 msec exposure time were recorded; the exposure time was matched with the ”on” state of the fluorescent dyes. From each image stack a reconstructed dSTORM image was generated by using in-house developed software\(^3\) based on MATLAB (The MathWork Inc., Natick, USA). The 1:20 labeling ratio (labeled vs. unlabeled protein) was found to be optimum for dSTORM imaging. High labeling density (e.g. 1:1, 1:5, etc) led to frequent mislocalizations or overlapping and thus asymmetric point spread functions (PSFs) which were rejected by the localization algorithm.

**Super-resolution algorithm for localization and visualization.** To obtain super-resolved images of the samples, the raw image data was processed using the Open Source rainSTORM localization microscopy software.\(^3\) To corroborate the robustness of this method, it was confirmed that similar results were obtained using the rapidSTORM software package.\(^4\) The rainSTORM analysis and visualization was as follows: fluorophore positions were determined by applying a ”sparse segmentation and least squares Gaussian fitting” algorithm to the data.\(^5\) Imprecise localizations were excluded by a ”quality-control” process: localizations were rejected if their localization precision, estimated using the Thompson formula,\(^6\) was worse than a user-defined threshold and if
they corresponded to asymmetric, or to too wide or too elliptical PSFs, corresponding to out of focus emission. The accepted localizations were visualized using an Adaptive Gaussian Rendering method. The average localization precision achieved in our imaging was 12 nm.

**Image and Histogram Analysis.** For the two-color localization microscopy images, chromatic aberration between the red and green channels was evaluated using the method described in Ref. ⁷, and found to be small compared with the obtained resolution. Data and two-color co-localization image analysis was performed using our software based on MATLAB ³ to quantify corresponding localization microscopy parameters such as localization precision and standard routine analysis with ImageJ (NIH, Bethesda, Maryland, USA). Cross-talk between the green and the red channel was estimated to be 16% and was subtracted from the images. The time course experiment (for all the different time points) was repeated 3 times. In each repeat dSTORM imaging was performed at 12 different 80 by 80 µm² areas of each sample (placed on the glass coverslip) homogeneously spatially distributed. In total, 53 fibrils were subjected to the analysis. Each Poisson distribution curve was calculated for the mean value derived from the corresponding histogram and its area corresponded to the area under the histogram.

**AFM imaging.** AFM images were acquired using a VEECO Dimension 3100 atomic force microscope (Bruker AXS, Cambridge, UK). The instrument was operated in tapping mode in air using silicon cantilevers with a resonant frequency of 300 kHz, a spring constant of 40 N/m and a tip radius of 10 nm (RTESP, Bruker AXS, Cambridge, UK). Images were collected at a scan rate of 1 Hz. In standard AFM imaging, 5 µL of each fibrillar sample were deposited onto freshly cleaved mica surfaces and left for 2 hours to adsorb. The samples were rinsed with 200 µL of Milli-Q water 5 times and left to dry completely in air before imaging.
Supplementary Figures

The presence of the dye at residue N122 does not affect the elongation of α-synuclein from preformed seeds. The labeled protein α-synuclein N122C follows the same aggregation kinetics as the unlabeled protein, as illustrated by the superposition of the two normalized kinetic traces describing the fibril elongation of the unlabelled and labelled protein in Suppl. Figure S1.

![Supplementary Figure S1](image)

Figure S1: ThT kinetic traces of labeled (with Alexa fluorophore) and unlabeled α-synuclein protein in a seeded assay experiment, showing no difference in the elongation kinetics of the labeled protein.

AFM cannot be reliably used for imaging of distribution of the fibril elongation values. Suppl. Figure S2 shows the time course study with AFM of fibril growth for the same time points as the ones used in the dSTORM experiment. Samples were left to dry before imaging. Fragmentation of fibrils can thus often occur, and also the formation of fibril clusters results in difficulty in length measurements. Additionally, AFM imaging does not allow to distinguish between the seed and the attached monomeric/elongated part of each fibril and thus it is not possible to determine the individual fibril elongation rates.

Seeds catalyse the aggregation process and no end-to-end association is observed in vitro. We incubated monomeric protein labeled with AF647 at 37 °C for 24 h in the absence of seeds, in quiescent conditions and at the same concentration as the one used for the seeded assay. Suppl. Figures S3a) and S3b) show the acquired images from this sample using conventional fluorescence and dSTORM, respectively, where no amyloid fibril was observed. Only certain very small structures pointing to monomers or formation of oligomeric structures can be observed. Additionally,
we investigated whether the seed fibrils could, if incubated under similar conditions as the ones mentioned in the previous experiments, give rise to longer fibrils. This would suggest that seed to seed association and further aggregation is possible. We therefore incubated α-synuclein seeds
labeled with AF568 at the same concentration as in the seeded assay for 24 h at 37 °C. Suppl. Figure S3c) shows the fluorescence image and S3d) shows the corresponding dSTORM image of the same area. In this experiment we confirm that the sample consisting of seeds only is stable over time and no further aggregation occurs. This observation suggests that α-synuclein seed fibrils in the absence of monomeric protein are not able to grow further.
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