Supporting Information

Different Conformational Subensembles of the Intrinsically Disordered Protein α-Synuclein in Cells

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Intermolecular FRET of αS on vesicles

The FRET observed on single vesicles in the SH-SY5Y cells does not result from a single labeled αS molecule. Multiple FRET labeled αS proteins are bound to the vesicles. Close proximity of the labeled αS proteins therefore possibly also results in intermolecular FRET. To test if the observed high FRET on vesicles was a result of intermolecular rather than intramolecular FRET the cells were microinjected with a 1:1 mixture of αS-AF488 and αS-AF568. Note that in this experiment the fluorophore concentration was kept constant, the concentration of protein is twice as high as in the experiments with double labeled protein. As observed for the FRET labeled protein (Figure 3) the FRET index distribution of the mixture of proteins differed between the cytoplasm and on vesicles (Figure S1). Following expectations intermolecular FRET was higher on vesicles. However, the FRET index distributions for intermolecular FRET are shifted to much lower values compared to intra-molecular FRET. The observed intermolecular FRET index values deviate from zero for a number of reasons. 1) When αS-AF488 and αS-AF568 share the excitation volume, direct excitation of the αS-AF568 amounts to approximately 5% of the αS-AF488 excitation. This results in a non-zero FRET index. 2) The cellular autofluorescence mainly contributes to the red (acceptor) channel. This also increases the FRET index. 3) When labeled proteins are bound to a vesicle a small amount of FRET is expected due to intermolecular proximity. Taking this into account we therefore conclude that the FRET data presented in Figure 3 results from intra-molecular FRET. The observed FRET is a result of a specific protein conformation rather than a signature of inter-αS-interactions.

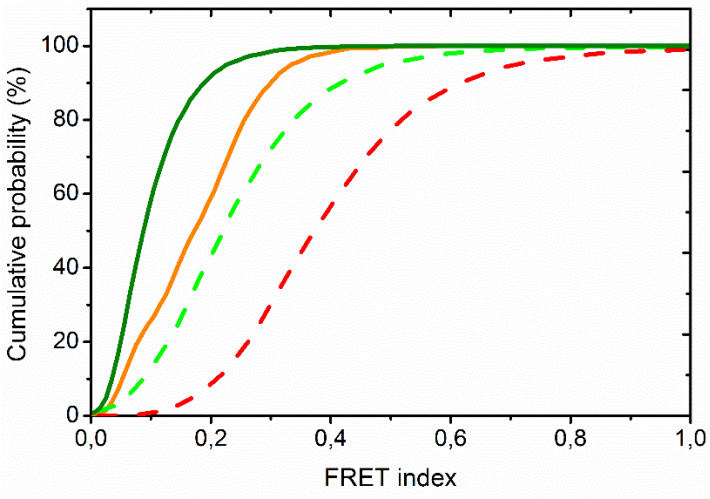

Figure S1: Comparison of intra- and intermolecular FRET in SH-SY5Y cells microinjected with appropriately labeled αS in a cumulative histogram. As observed for intra-molecular FRET (dashed lines, red: on vesicles, green: in cytoplasm), the intermolecular FRET index distributions observed in cells in which both αS-AF488 and αS-AF568 were injected also differs between the cytoplasm (dark green) and on vesicles (orange). The intermolecular FRET index distributions are shifted to lower FRET index values.

Materials and Methods

Cell culture and membrane labeling: The SH-SY5Y cells (ATCC, USA) were grown in proliferation medium DMEM-F12 GlutaMAX™ supplemented with 10% heat inactivated FBS, 1% non-essential amino acids, 10 mM HEPES buffer, and 1% Penicillin/Streptomycin, all obtained from Gibco® (Invitrogen, USA). For labeling the membranes of the SH-SY5Y cells, 5.0 µg/ml wheat germ agglutinin (WGA) tagged with the fluorophore AlexaFluor®647 (AF647) (Invitrogen, USA), was incubated with living cells for 2 hours. The labeled cells were used for membrane colocalization imaging after microinjection of AlexaFluor®488 (AF488)

labeled αS. After microinjection experiments, cells were washed with PBS (3x) and fixed in 3.7% paraformaldehyde/PBS solution for 10 minutes at room temperature (RT). After washing with PBS, samples were mounted using mounting medium (ibidi, Germany).

Preparation of FRET labeled αS: An αS modification in which the amino acids at position 9 and 69 were replaced with cysteines was prepared and labeled as described before[1]. In short, the cysteines were reduced with 1 mM DTT, then an equimolar concentration of the maleimide derivative of AF488 was added to 0.5 ml of 200 µM αS(9C/69C). After 1 hour incubation at RT, the AF488 labeled αS(9C/69C) was desalted using a Zeba Spin desalting column (Pierce Biotechnology) to remove unreacted AF488 and DTT, followed by immediate incubation with 330 mg prewashed Thiopropyl Sepharose 6B (GE Healthcare Life Sciences). This step separates αS(9C/69C) labeled with two AF488 dyes from protein containing one or zero labels. The single-labeled and/or unlabeled αS that was bound to the column was subsequently eluted using 10-15 ml of 10 mM Tris-HCl, pH 7.4 buffer, containing β-mercaptoethanol. After pooling the eluted fractions were concentrated to a volume of about 0.5 ml and desalted. Then a 2-3x molar excess of AF568 was added. After incubation for 1 hour at RT, free dye was removed using two desalting steps and the solution was filtered through a Microcon YM100 filter (Millipore, Bedford, MA). The stoichiometry of the dyes on the protein (AF488:AF568) was obtained from UV/Vis absorption spectra (Nanodrop, Thermofisher, USA) using the known extinction coefficients of the dyes. The ratio between AF488 and AF568 on αS(9C/69C) was determined to be 0.83:1. The excess of AF568 agrees with the removal of αS containing two AF488 dyes during the labeling procedure. This procedure ensured that the final sample only contained the AF488 and AF568 FRET labeled αS and some αS with two AF568.

Preparation of labeled αS: A cysteine mutant of αS (αS18C) was used to prepare single labeled αS. In short, the cysteines were reduced with 1 SmM DTT, immediately after removal of DTT an excess concentration of the maleimide derivative of either AF488 or AF568 was added to 0.5 ml of 200 µM αS. After 1 hour incubation at RT, the labeled αS was desalted using a Zeba Spin desalting column (Pierce Biotechnology) to remove unreacted dyes. The concentrations of the labeled proteins were determined using UV/Vis absorption spectra (Nanodrop, Thermofisher, USA) using the known extinction coefficients of the dyes and the protein.

Preparation of FRET labeled αS(9C/69C) *fibrils:* FRET labeled fibrils were prepared as described elsewhere[2], with minor modifications. In short, a final concentration of 100 µM αS (99 µM wild type αS + 1 µM AF488 and AF568 FRET labeled αS) was incubated in 400 µl 10 mM Tris-HCl buffer (pH 7.4) in 2 ml Lo-Bind round bottom centrifuge tubes (Eppendorf, Germany) in an incubator (Termaks, Norway) at 37 °C with 500 rpm shaking (Shaker, Titramax100, Heidolph, Germany) for 7 days. Then the content of the tube was slightly concentrated by centrifugation at 3000 × g for 10 minutes, and 20 µl from the bottom of the tube was deposited on a clean coverslip for imaging.

Microinjection setup: Microinjections were performed using a FemtoJet® (Eppendorf, Germany), equipped with a manual hydraulic 3D micromanipulator (Narishige, Japan). For injection, pre-pulled borosilicate glass micropipettes with an inner filament and inner diameter of 400 nm (WPI, USA) were used. In order to decrease the adherence of the cells to the surface of the capillaries, capillaries were UV-O_3 cleaned prior to use. The microinjections were performed using micropipettes back filled with 300 nM FRET labeled αS (in 10mM Tris, pH 7.4) and monitored on a Nikon TE2000 microscope (Nikon, Japan). For performing microinjections, an injection pressure, a constant pressure, and a duration of the injection of 150 hPa, 15hPa, and 0.1 second were used respectively.

In order to find back the position of the microinjected cells, the cells were seeded on 35 mm imaging dishes with a glass bottom imprinted with 50 μm grids (ibidi, Germany). The glass bottom dishes were coated manually with collagen IV prior to seeding. The injections were done after the cells reached a confluency of 80-90%.

Chow et al. used comparable microinjection settings (capillaries with an inner diameter of 500 nm, an injection pressure and a duration of the injection equal to 150 hPa and 0.1 second) and reported that these settings resulted in an injection volume of 500 fl per delivery[3]. Assuming a similar injection volume in our setup, we diluted 2% w/v FluoSpheres™ (100 nm diameter yellow-green fluorescent nano-beads; Invitrogen, USA), equivalent to a concentration of $3.6x10^{13}$ particles/ml according to the manufacturer's description, 15,000 times in 10 mM Tris, pH 7.4 to obtain ~1 particle per 500 fl injection volume with the settings used. The resulting dilution was microinjected in cells and resulted in the expected Poisson distribution of the number of beads per injection, confirming that the volume of injection in our experiments is ~500 fl. Although the microinjection resulted in inflation of the cell, the cells rapidly recovered to their original shape and volume.

Imaging setup and configurations. Fluorescence images were taken using an ultrasensitive custom-built inverted confocal microscope, described in detail elsewhere[4] with minor modifications. In short, as excitation source, a pulsed diode laser operating at 485 nm at a repetition rate of 20 MHz (PDL800-D, PicoQuant, Germany) was used. The sample was excited and the emission was collected through the same microscope objective (Plan Apo VC, 60X, 1.2NA, Nikon). A dichroic mirror (405/488/594nm BrightLine® triple-edge laser-flat Dichroic Beamsplitter, Semrock, USA) was used to reflect the excitation light towards the objective, and to transmit the emitted light towards the detectors. The remaining excitation light in the detection path was suppressed with a long pass filter (RazorEdge®, 488 nm, Semrock, USA) and a StopLine®, 488/14 nm filter (Semrock, USA). The emission was separated into two spectral channels with a 585 nm dichroic beam splitter (T585lpxr , Chroma, USA): a green channel for detection at wavelengths shorter than 561 nm (RazorEdge®, 561 nm short pass, Semrock, USA) and a red channel for detection from 590 nm to 770 nm (590 nm long pass, Olympus, Japan in combination with a BrightLine®, 770nm short pass, Semrock, USA). The emission was spatially filtered using a 15 μm pinhole and was focused onto a single photon avalanche diode (MPD-5CTC, PicoQuant, Germany), connected to a photon counting module (PicoHarp300, PicoQuant, Germany).

Microinjected cell samples were imaged at a rate of 1 ms per pixel at a low excitation power of ~80 W/cm^2, to prevent bleaching. To collect enough photons, the intensities in each channel

were summed over 5 consecutive images for each area of Sest. Fibrils were imaged with the same settings, but with only one scan per area of interest. Autofluorescence of the SH-SY5Y cells, the non-treated cells, was imaged with a higher excitation power of ~800 W/cm^2.

FRET analysis. To determine the FRET indices for membrane bound αS, regions of interest (ROIs) with an average of ~25 pixels/punctum, were defined around the high intensity puncta. These ROIs enclosed the fluorescent puncta identified in both channels, and the corresponding red to green ratio was calculated. For cytoplasmic αS, ROIs with an average of 267 pixels/ROI were drawn. The ROIs in the cytoplasm were selected to include as much diffuse signal in the cytoplasm of the cells as possible, while excluding any signal from the high intensity puncta. The histograms of the FRET index are based on average values per ROI. In the cumulative histograms the FRET index of individual pixels in the ROIs was considered and cumulated.

Membrane colocalization: As an excitation source for AlexaFluor® 647 (AF647) dye, a pulsed diode laser operating at 640 nm at a repetition rate of 20 MHz (LDH-D-C-640, PicoQuant, Germany) was used. The emission filters for the green and red channels were changed to a 550 nm band pass (BrightLine®, 550/88 nm, Semrock, USA), and a 664 nm long pass (RazorEdge®, 664 nm long pass, Semrock, USA) filter, respectively. The imaging was done sequentially using 485 nm (~80 W/cm^2) and 640 nm (~1 W/cm^2) excitation light sources. Then the puncta which appeared in the red channel image with 485 nm excitation, were photobleached by maximum power of the 640 nm laser (~1000 W/cm^2). After photobleaching of the acceptor, another set of images were sequentially obtained with the 485 nm and 640 nm excitation light sources.

Primary neuronal cells and staining: Cells were obtained from the cortical cerebral hemispheres of newborn (P1) Wistar rat pups as described elsewhere[5]. The isolated cells were allowed to adhere to polyethylenimine coated coverslips (Sigma-Aldrich, USA) and cultured in 900 µl serum and antibiotics-free R12 medium[6]. All research involving animals has been conducted according to Dutch law (as stated in "Wet op de dierproeven"), and approved by DEC, the Dutch Animal Use Committee.

The cell samples were stained using standard immunostaining protocols. In brief, after fixation and permeabilization, the autofluorescence was quenched and aspecific binding sites were blocked. For staining of αS a primary mouse anti-αS (121-125) (Santa Cruz) antibody was used in combination with an AF555 labeled secondary goat anti-mouse antibody (Invitrogen, USA). For actin staining, cells were incubated with phalloidin-Alexa Fluor®488 (Invitrogen, USA) and nuclear counterstaining was performed with DAPI. Samples were mounted in mounting medium (ibidi, Germany).

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