Endogenous α-SYN protein analysis on human brain tissues using single-molecule pull-down assay

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Abstract

Alpha-synuclein (α-SYN) is a central molecule in Parkinson's disease pathogenesis. Despite several studies, the molecular nature of endogenous α-SYN especially in human brain samples is still not well understood due to the lack of reliable methods and the limited amount of bio-specimens. Here, we introduce α-SYN single-molecule pull-down (α-SYN SiMPull) assay combined with *in vivo* protein crosslinking to count individual α-SYN protein and assess its native oligomerization states from biological samples including human *postmortem* brains. A SiMPull assay enables us to count the number of immuno-precipitated proteins, and reveal the stoichiometry of protein complexes by single-molecule fluorescence imaging. This powerful assay can be highly useful in diagnostic applications using various specimens for neurodegenerative diseases including Alzheimer's disease and Parkinson's disease.

Introduction

Alpha-synuclein (α-SYN) is a central molecule in Parkinson's disease (PD) pathogenesis whose aggregates are a major component of Lewy bodies, a pathological hallmark of PD (Spillantini et al., 1997). Both missense point mutations and increased expression of wild-type α-SYN by either multiplication of the *SNCA* genomic locus or other causes including environmental toxins accelerate α-SYN aggregation and its toxicity (Grundemann et al., 2008; Ibanez et al., 2004; Polymeropoulos et al., 1997; Singleton et al., 2003; Vila et al., 2000), suggesting the importance of α-SYN protein levels and oligomeric states in PD pathogenesis.

The characteristics of α-SYN have been mainly studied by using recombinant proteins (Chen et al., 2015; Tuttle et al., 2016; Weinreb et al., 1996; Zijlstra et al., 2012). However, analysis of endogenous α-SYN levels and its aggregation states especially from human brain tissues has been challenging and often reported equivocal results (Bartels et al., 2011; Burre et al., 2013; Dettmer et al., 2013; Fauvet et al., 2012). Especially, selective neuronal loss in the *substantia nigra* (SN) of PD brains severely limits the available dopaminergic neurons compared to control brains (Damier et al., 1999; Dijkstra et al., 2014). Therefore, it is crucial to develop a method capable of quantifying endogenous α-SYN protein levels as well as its aggregation states in biological samples including human *postmortem* brains.

To this end, we sought to develop a specific and sensitive method called 'α-SYN single-molecule pull-down (α-SYN SiMPull) assay' using a recently developed SiMPull method (Aggarwal and Ha, 2016; Jain et al., 2011) together with cell permeable *in vivo* crosslinker, disuccinimidyl glutarate (DSG) (Dettmer et al., 2013). A SiMPull method using

conventional immunoprecipitation combined with single-molecule fluorescence imaging enables rapid and sensitive analysis of protein levels and their stoichiometry at the single-protein resolution (Aggarwal and Ha, 2016; Jain et al., 2014; Jain et al., 2011; Lee et al., 2013; Yeom et al., 2011). In addition, *in vivo* crosslinker increases the stability of endogenous α-SYN protein by preserving their apparent assembly states (Dettmer et al., 2013). Here, we have established α-SYN SiMPull assay, successfully demonstrating that endogenous α-SYN protein levels can be measured at the single-molecule level using only minute amounts of total proteins compared to traditional western blot analysis. Moreover, this method allows us to assess oligomerization states of native α-SYN protein in the cultured cells and the human brain tissues.

Results

Establishing α-SYN SiMPull assay

To achieve specific pull-down of α-SYN protein, we prepared four-antibody system consisting of biotinylated secondary antibody, capturing and detecting primary monoclonal antibodies recognizing different epitopes of α-SYN, and Alexa 647-labeled secondary antibody (Figure 1a) (Jain et al., 2011). With this system, recombinant human α-SYN protein was successfully detected by single-molecule fluorescence microscopy (Figure 1b) (Tang et al., 2017). Next, we tested whether this method is specific enough to selectively capture α-SYN in total cell lysates. For this purpose, we have established α-SYN knockout 293T cells using the CRISPR/Cas9-based genome editing technique. Then, total lysates prepared from α-SYN knockout and overexpressed cells were each tested. The assay successfully pulled down α-SYN from α-SYN overexpressed cell

lysates with high specificity when compared to negligible signals from α-SYN knockout cell lysates (Figure 1c and e). We also applied α-SYN SiMPull to detect endogenous α-SYN from total lysates of wild-type 293T cells, demonstrating that the number of fluorescence spots was increased in a dose-dependent manner (Figure 1d and e).

Analysis of oligomeric states of recombinant α-SYN by α-SYN SiMPull assay

α-SYN oligomerization is strongly implicated in mediating α-SYN toxicity in neurons (Danzer et al., 2007; Outeiro et al., 2008). Therefore, understanding the oligomerization states of α-SYN is important for diagnosing as well as monitoring the progression of PD. To study α-SYN oligomers, first, we adopted Alexa 647-labeled $F(ab')_2$ fragment antibody instead of full IgG to reduce the steric hindrance between antibodies, and used a degree of labeling of ~2.9 to achieve a narrow fluorescence intensity distribution with a negligible amount of unlabeled species (Figure 2 – figure supplement 1a and b). Then SiMPull assay was performed on human α-SYN oligomer prepared by 5-day incubation of recombinant monomer at 37 degrees (Figure 2a-c) (Volles and Lansbury, 2007). We analyzed the fluorescence intensity of the immuno-precipitated molecules, which is proportional to the number of α-SYN in the pulled-down complexes. As we expected, in the oligomeric/fibrillar α-SYN sample, multiple bright spots with various intensity were observed while the monomeric α-SYN sample depicted a narrow distribution centered at low intensity. Interestingly, we also observed fluorescent spots with various shapes, which are larger than the diffraction limit (~ 350 nm) exclusively in oligomeric/fibrillar α-SYN (Figure 2b and c), suggesting that α-SYN SiMPull assay is applicable to morphometric

analysis. Monomeric and oligomeric α-SYN were confirmed using conventional western blot with significantly higher amount of proteins (Figure 2 – figure supplement 2).

Analysis of α-SYN in the cultured cells by α-SYN SiMPull assay

To extend the application of α-SYN SiMPull to analysis of α-SYN oligomeric states in the cells, we tested total lysates from 293T cells overexpressing α-SYN with or without exposure to $FeCl_2$ and a proteasome inhibitor, MG132, which are known to increase aggregation of α-SYN (Hasegawa et al., 2004; Ostrerova-Golts et al., 2000; Takahashi et al., 2007). To stably maintain the native states of α-SYN, *in vivo* protein crosslinking was achieved by DSG followed by cell lysis (Dettmer et al., 2013). α-SYN SiMPull using 40 μL of total lysates (10 ng/μl) demonstrated notably increased population at higher fluorescence intensity in $FeCl_2$ and MG132 treated cells compared to non-treated control (Figure 3a, Figure 3 – figure supplement 1). To further analyze these intensity profiles, fluorescence intensity of a single Alexa 647-labeled $F(ab')_2$ was used as a reference (Figure 2 – figure supplement 1). Assuming it as the intensity of a monomer, we decomposed the intensity profiles of above conditions into monomer and oligomer populations. As shown in Figure 3b and c, oligomeric α-SYN was more pronounced in $FeCl_2$ and MG132 exposed cells (37 %) than non-exposed ones (15 %). The western blot also confirmed that the treatment with $FeCl_2$ and MG132 increased the levels of high-molecular weight α-SYN species (Figure 3 – figure supplement 2). However, we did not observe bright fluorescent spots having different shapes or sizes that were found in recombinant oligomeric/fibrillar α-SYN in $FeCl_2$ and MG132 exposed cells.

Analysis of α-SYN in the human brain tissues by α-SYN SiMPull assay

Lastly, we applied α-SYN SiMPull assay to test human *postmortem* brain samples. The dark pigmented region in the SN of frozen control or PD *postmortem* brain samples that represents remaining dopaminergic neurons was selectively punch-biopsied (~ 10 mg), and then treated with DSG for *in vivo* crosslinking prior to protein extraction (Figure 4a). PD sample showed significant increase in the number of fluorescent spots by 3.3 fold compared to control (Figure 4b). Moreover, the population of oligomeric α-SYN was significantly increased in PD, where it accounted for 56 % of the detected protein, compared to only 23% in control (Figure 4c-e). Neither samples showed different shapes of fibrillar α-SYN. The western blot using significantly higher amount of lysates than SiMPull assay showed prominent levels of high molecular species in PD (Figure 4 – figure supplement 1).

Discussion

A limited number of remaining dopaminergic neurons in the SN of PD brains have been a critical barrier for biochemical analyses of α-SYN in *postmortem* PD brain tissues. Several methods including, but not limited to, western blot have reported controversial results on α-SYN levels as well as its native states with total protein lysates obtained from the SN of human brain tissues (Bartels et al., 2011; Burre et al., 2013; Dettmer et al., 2013; Fauvet et al., 2012). In this study, we employed α-SYN SiMPull assay that enables us to measure endogenous α-SYN protein at the single-molecule level and estimate its oligomeric states using minute amounts of protein lysates. This technique allowed us to

analyze α-SYN in the limited SN region where neuromelanin-positive dopaminergic neurons were spared.

Unlike other methods (Fauvet et al., 2012; Horrocks et al., 2016; Majbour et al., 2016), α-SYN SiMPull assay can be applied regardless of the size or conformation of α-SYN oligomers unless the epitope is concealed. We observed fluorescent spots with various shapes in recombinant oligomeric/fibrillar α-SYN, suggesting that different levels of α-SYN aggregation could be analyzed by morphometric study. However, neither cells nor brain samples gave a similar result. It is possible that the effort to maintain the native states of α-SYN by *in vivo* crosslinking and mild protein extraction procedure is still causing disruption of α-SYN aggregates. Another possibility is that small oligomers are preferably formed while fibrillar α-SYN rarely exists in dopaminergic neurons even in PD.

Here, we assessed monomeric versus oligomeric α-SYN states using a known fluorescence intensity profile of the Alexa 647-labeled $F(ab')_2$. However, for precise stoichiometric analysis of the native states of α-SYN, a quantitative labeling of the primary antibody (Hallam et al., 2015) is desirable along with photobleaching analysis (Jain et al., 2014). This may provide a crucial clue to solve current debate over the oligomeric states of endogenous α-SYN (Burre et al., 2013; Dettmer et al., 2013). Adopting smaller antibodies such as Fab fragment or single-chain variable fragment might be more advantageous to bind each molecule in α-SYN oligomers. We can further reduce protein amounts by imaging a larger area or constructing microfluidic chambers (Kim et al., 2011). Additionally, α-SYN analysis in a single-dopamine neuron could be achieved by *in situ* single cell pull-down assay (Wedeking et al., 2015).

In summary, our α-SYN SiMPull assay will be a powerful tool in quantitation as well as analysis of oligomeric states of various proteins, such as amyloid beta, α-SYN and tau from human *postmortem* brain tissues or cerebrospinal fluids in neurodegenerative diseases including Alzheimer's disease and PD.

Materials and Methods

Preparation of flow chamber for α-SYN SiMPull assay

Quartz slides and glass coverslips were passivated with methoxy polyethylene glycol (mPEG-SVA-5000, Laysan Bio) doped with 2.5 % biotin-PEG (Biotin-PEG-SVA-5000, Laysan Bio) as described (Jain et al., 2012). Each passivated slide and coverslip was assembled into five flow chambers using a double-sided tape. The flow chambers were first wet with 1x phosphate-buffered saline buffer (PBS, pH 7.4), introduced 40 µL of 0.2 mg/mL NeutrAvidin (Thermo Fisher Scientific) in PBS for 5 minutes, then washed with 100 µL of wash buffer (0.1 mg/mL BSA in PBS). Similarly, after introducing 5 µg/ml biotinylated anti-mouse IgG antibody (Abcam, ab97033) and 2.5 µg/ml mouse monoclonal α-SYN antibody (BD Biosciences, 610786), 40 µL of appropriately diluted cell lysates or protein samples incubated overnight with 1.3 µg/ml rabbit monoclonal α-SYN antibody (Epitomics, 2016-1; Abcam, ab51252) were loaded and incubated for 30 minutes on antibody-tethered coverslips. Unbound components were washed out with 100 µL of wash buffer and then 0.7 µg/ml Alexa 647-labeled anti-rabbit full IgG (Invitrogen, A31573) or $F(ab')_2$ fragment antibody (described below) was introduced for 5 minutes. All antibodies were appropriately diluted with 0.1 mg/mL BSA in PBS. To check non-specific

binding of Alexa 647-labeled antibodies, 40 µL of 0.1 mg/mL BSA in PBS was added instead of protein samples and all other steps were kept same as described above.

Single-molecule imaging and spot counting

All imaging was done by a custom-made objective-type total internal reflection fluorescence (TIRF) microscope (Tang et al., 2017) with an imaging buffer containing 0.8% (w/v) dextrose (Sigma), 1 mg/mL glucose oxidase (Sigma), 0.04 mg/mL catalase (EMD Millipore) and 2 mg/mL Trolox (Santa Cruz) to minimize photobleaching of Alexa 647. At least 20 images were acquired at different locations and each imaged area was ~4,700 μm^2. The number and fluorescence intensity of pull-down molecules were analyzed with MATLAB scripts similar to previous studies (Jain et al., 2011; Tang et al., 2017). For analyzing intensities of oligomers/fibrils α-SYN (Figure 2b), the images were segmented with a threshold, and the intensities in the segmented area were summed.

Preparation of Alexa 647-labeled F(ab')$_2$ antibody

Donkey anti-rabbit F(ab')$_2$ fragment antibody (Jackson ImmunoResearch; 711-006-152) was labeled with Alexa 647 NHS ester (Thermo Fisher Scientific) in 100 mM sodium bicarbonate buffer (pH 8.3) for 1 hour at room temperature. After removing unreacted dyes twice with gel filtration columns (NAP-5, GE Healthcare), we measured the absorbance of the labeled antibody with NanoDrop, and calculated its degree of labeling.

Preparation of α-SYN recombinant proteins

To generate α-SYN recombinant proteins, BL21(DE3) *E. coli* strains were transformed with human α-SYN in pT7-7 vector, induced with 1 mM IPTG for 24 hours, and lysed with a lysis buffer (10 mM EDTA, 50 mM Tris pH 8.0, 150 mM NaCl). Then, α-SYN protein was purified with the protocol as described by Volles and Lansbury (Volles and Lansbury, 2007). The purified monomeric α-SYN was dissolved in 1x PBS buffer containing 0.05 % sodium azide. Aliquots were stored at -80 °C. For oligomers/fibrils experiments, monomeric α-SYN was diluted to a concentration of 3 mg/mL and shaken at 200 rpm at 37 °C for 5 days.

Cell culture and generation of α-SYN overexpressed or knockout cells

The HEK 293T cells were grown in DMEM/High glucose medium containing 10 % fetal bovine serum, penicillin (100 U/ml) and streptomycin (50 µg/ml) at 37 °C in a humidified incubator with 5 % CO_2. To overexpress α-SYN, 293T cells were transfected with *N*-terminal flag tagged full-length human α-SYN plasmid using the calcium phosphate transfection method. 36 hours after transfection, cells were collected for α-SYN SiMPull assay and Western blot analyses. To obtain complete α-SYN knockout cells, CRISPR/Cas9 genome editing method was used. 293T cells were co-transfected with CRISPR/Cas9 plasmid together with a cocktail of *SNCA* specific single guide RNAs (sgRNAs) designed using Horizon's free CRISPR guide program (Horizon discovery Ltd., UK), and transfected cells were enriched by flow cytometry sorting using GFP contained in the backbone vector of sgRNA. The sorted cells were genotyped and homozygous α-SYN knockout cells were cultured described as above, then used for α-SYN SiMPull assay. For α-SYN oligomerization-induction in 293T cells, transfected cells with *N*-

terminal flag tagged full-length human α-SYN plasmid were treated with 1 mM $FeCl_2$ (Sigma) for 48 hours. Then 5 μM MG132, proteasome inhibitor (Fisher Scientific) was treated 6 hours prior to collecting cells.

***In vivo* DSG cross-linking and total lysate preparation from 293T cells**

For the preparation of total lysate without DSG treatment in Figure 1, cells were lysed in lysis buffer containing 25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS supplemented with protease inhibitor cocktail (Sigma) on ice for 15 min. The lysed cells were centrifuged at 16,000 x *g* for 15 minutes at 4 °C and the supernatant was collected for the assay.

For *in vivo* cross-linking, cells were first collected in PBS and pelleted by centrifugation. The cell pellets were resuspended in PBS containing 2 mM DSG (disuccinimidyl glutarate) (ProtemChem) with protease inhibitor cocktail (~5 volumes of the pellet) and incubated for 30 minutes at 37 °C with shaking. The reaction was quenched with the addition of 1 M Tris (pH 7.6) to 50 mM final concentration, and incubated for 15 minutes at room temperature. The cross-linked cells were lysed by three cycles of freeze-thaw. The lysed cells were centrifuged at 1,000 x *g* for 10 minutes at 4 °C and the supernatant was collected for α-SYN SiMPull assay and Western blot analyses.

***In vivo* DSG cross-linking and total lysate preparation from postmortem human brain samples**

About 10 mg of SN tissues from freshly frozen control and PD *postmortem* brain samples were precisely isolated by punch biopsy, immediately transferred to 1.5 mL tubes

containing 500 µL of PBS with protease inhibitor cocktail, and then centrifuged 1,500 x *g* for 5 minutes at room temperature. The pellets of intact tissue bits were resuspended in PBS containing 2 mM DSG with protease inhibitor cocktail (~5 volumes of the pellet) and incubated for 30 minutes at 37 °C with shaking, followed by centrifugation at 1,500 x *g* for 5 minutes at room temperature. The supernatant was discarded and the pellets were resuspended in PBS with protease inhibitor cocktail. Then the cross-linked tissues were lysed by three cycles of freeze-thaw and centrifuged at 1,000 x *g* for 10 minutes at 4 °C. The supernatant was collected for α-SYN SiMPull assay and Western blot analyses.

Analysis of α-SYN oligomeric states

Biotinylated rabbit monoclonal antibody (1.3 ng/mL; LSBio, LS-C370648) was introduced on the NeutrAvidin coated PEG surface in the flow chamber, followed by anti-rabbit Alexa 647-labeled F(ab')$_2$ fragment antibody (0.7 µg/mL). In this case, assuming that the fluorescence spot is mostly attributed to a single F(ab')$_2$, its intensity profile was used to extract oligomer population, i.e., $I_{oligo} = I_S - (\alpha_S/\ \alpha_F) \times I_F$, where I_S, I_F, I_{oligo} are intensity profiles of the sample, F(ab')$_2$, and oligomer, respectively, and α_S and α_F are the population value of the sample and F(ab')$_2$ at the first peak.

Western blot

Equal amounts of proteins (0.5 µg of recombinant proteins, 30 µg of 293T cells, and 20 µg of human *postmortem* brain samples) were electrophoresed on 10 % SDS-polyacrylamide gels. The separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane, and blocked with 5 % nonfat skim milk. Mouse monoclonal

α-SYN antibody (BD Biosciences, 610786) was incubated at 4 °C overnight followed by anti-mouse HRP (horseradish peroxidase)-conjugated secondary antibody (Jackson ImmunoResearch, 115-035-146) for chemiluminescent detection.

Statistical analysis

The SiMPull images presented are representative. Statistical analysis was performed with GraphPad Prism v.7 (GraphPad Software) and all data are presented as mean ± standard deviation of at least three independent experiments. An unpaired two-tailed *t* test was used. Values of $p < 0.05$ were considered significant.

Acknowledgements

We thank Vasudha Aggarwal for critically reading manuscript and fruitful discussions. This work was supported by National Institute of Health grant number 5R21NS088923 to Y.S.K. and ORC Mentoring Program Award at University of Central Florida to K.Y.H and Y.S.K.

Competing Interests

The authors declare no competing financial and non-financial competing interests.

Supplementary Material

Figure Supplements:

Figure 2 – Figure supplement 1 and 2

Figure 3 – Figure supplement 1 and 2

Figure 4 – Figure supplement 1

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Figure 1

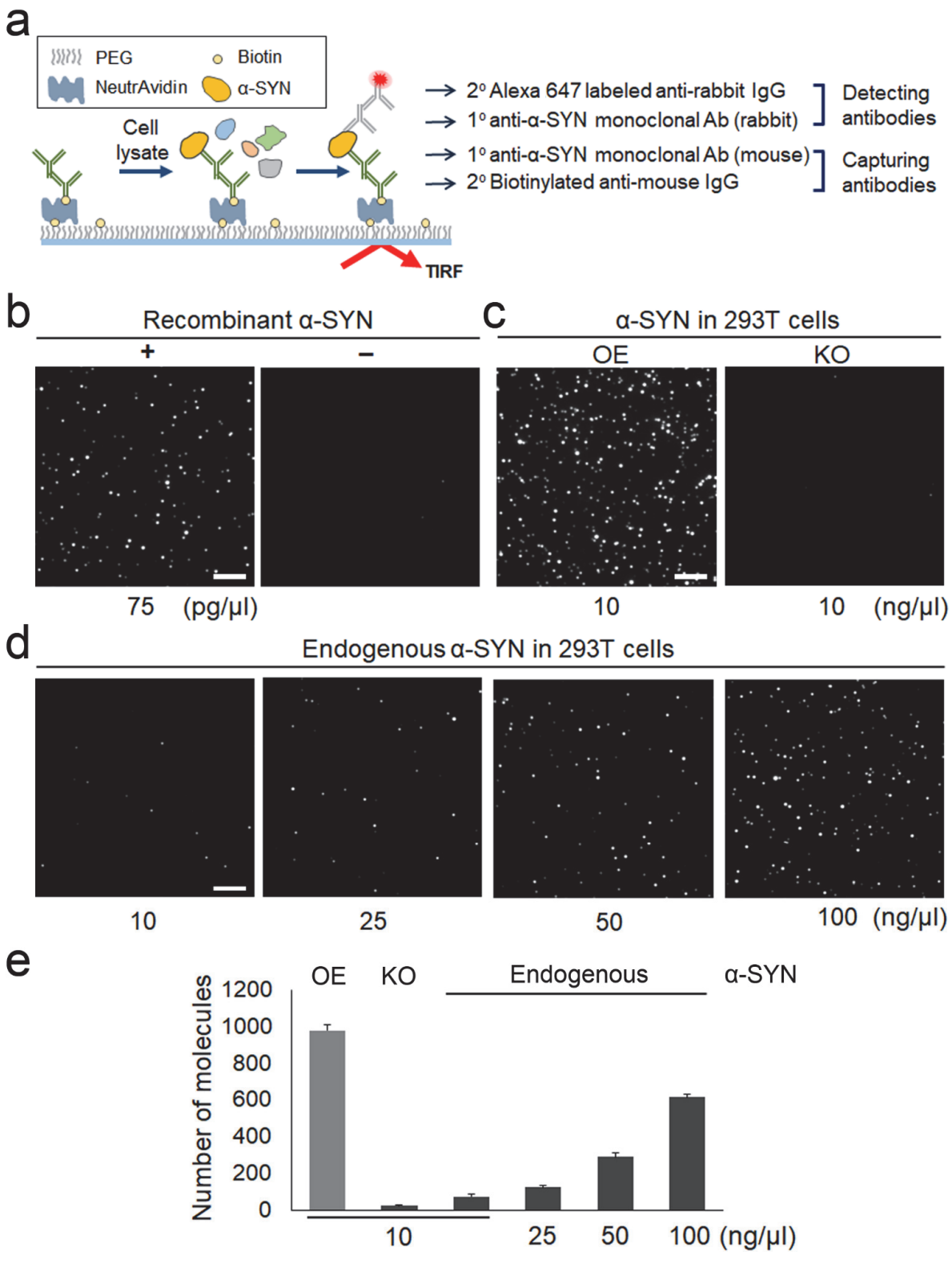

Figure 1. Detection of α-SYN protein using SiMPull assay. (**a**) Schematic diagram of α-SYN SiMPull procedure with four- antibody system. (**b**) Single-molecule images of recombinant human α-SYN protein (75 pg/μl) (left) and non-specific binding of Alexa 647-labeled anti-rabbit IgG (right). (**c**) Single-molecule images of α-SYN taken from total lysates of α-SYN overexpressed (OE) or knockout (KO) 293T cells with 10 ng/μl of total lysates. (**d**) Images of endogenous α-SYN from 293T cells with 10, 25, 50, or 100 ng/μl of total lysates. (**e**) Average number of fluorescent spots of α-SYN molecules per imaging area. More than 20 images were taken and error bars denote standard deviation (s.d.). Scale bar, 5 μm.

Figure 2

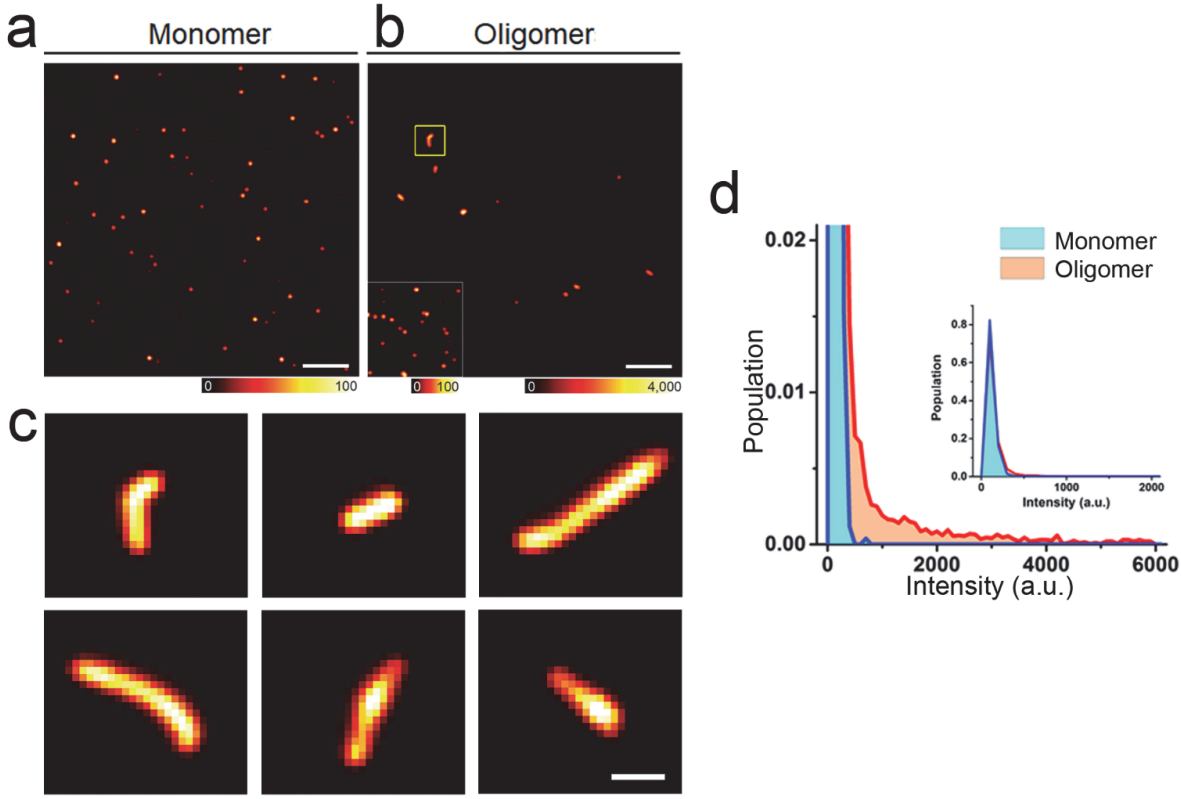

Figure 2. Analysis of oligomeric states of recombinant α-SYN using SiMPull assay. Single-molecule images of monomeric (a) and oligomeric (b) recombinant α-SYN (37.5 pg/ul). Existing monomers among oligomeric α-SYN were displayed on the left corner of (**b**) after intensity adjustment. (**c**) Bright spots with various intensity and shapes in oligomeric/fibrillar α-SYN. The top left is a magnified image of the yellow-boxed area in (**b**), and the others were taken from other images. (**d**) Fluorescence intensity profiles of monomeric (blue) and oligomeric (red) recombinant α-SYN. Scale bar, 5 μm (a,b) and 1 μm (c).

Figure 3

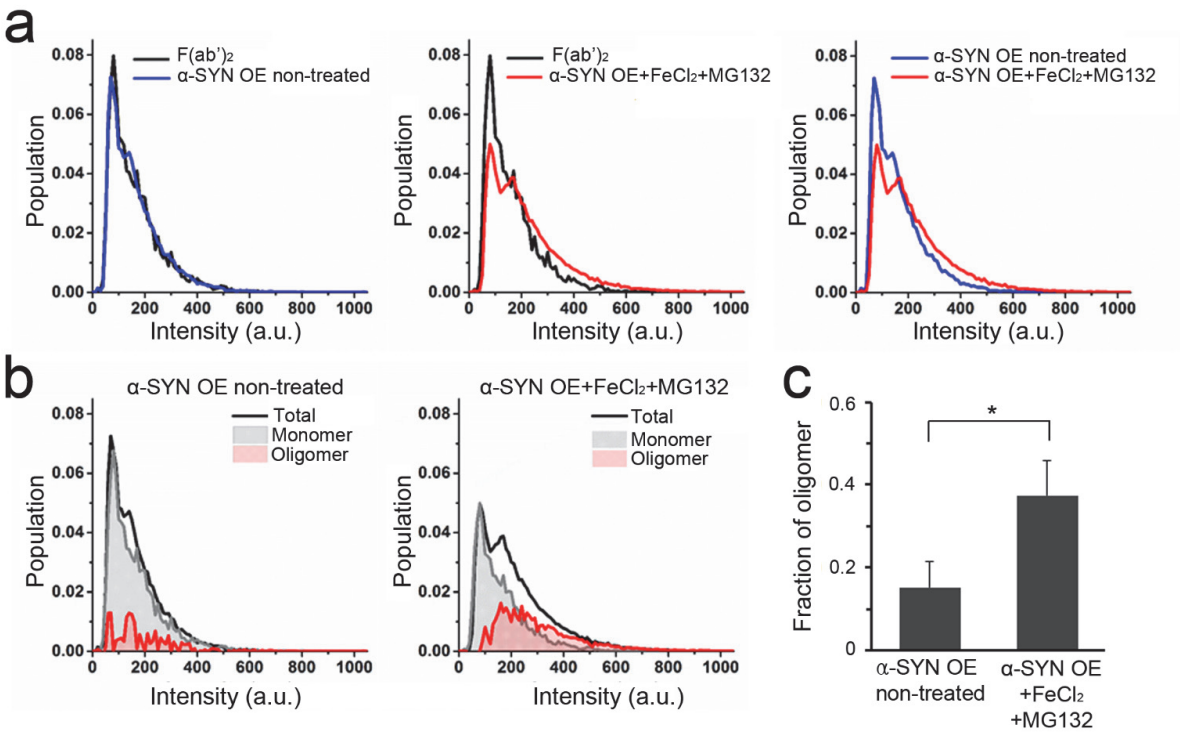

Figure 3. Analysis of oligomeric states of α-SYN from *in vivo* DSG-crosslinked total cell lysates using SiMPull. (**a**) Fluorescence intensity distribution of α-SYN SiMPull assay from α-SYN overexpressed cells with (red) or without (blue) $FeCl_2$ and MG-132 treatment. F(ab')$_2$ denotes Alexa 647-labeled F(ab')$_2$ fragment antibody as a reference of α-SYN monomer. (**b**) Analysis of oligomeric states from the data presented in (**a**). Monomeric (grey) and oligomeric (red) populations were separately plotted. (**c**) Quantitative analysis of the oligomeric states from the data presented in (**b**). Error bars denote standard error of the mean (n = 3). *P< 0.05, by unpaired two-tailed t test. 10 ng/μl of total lysates from *in vivo* DSG-crosslinked 293T cells were used in each assay.

Figure 4

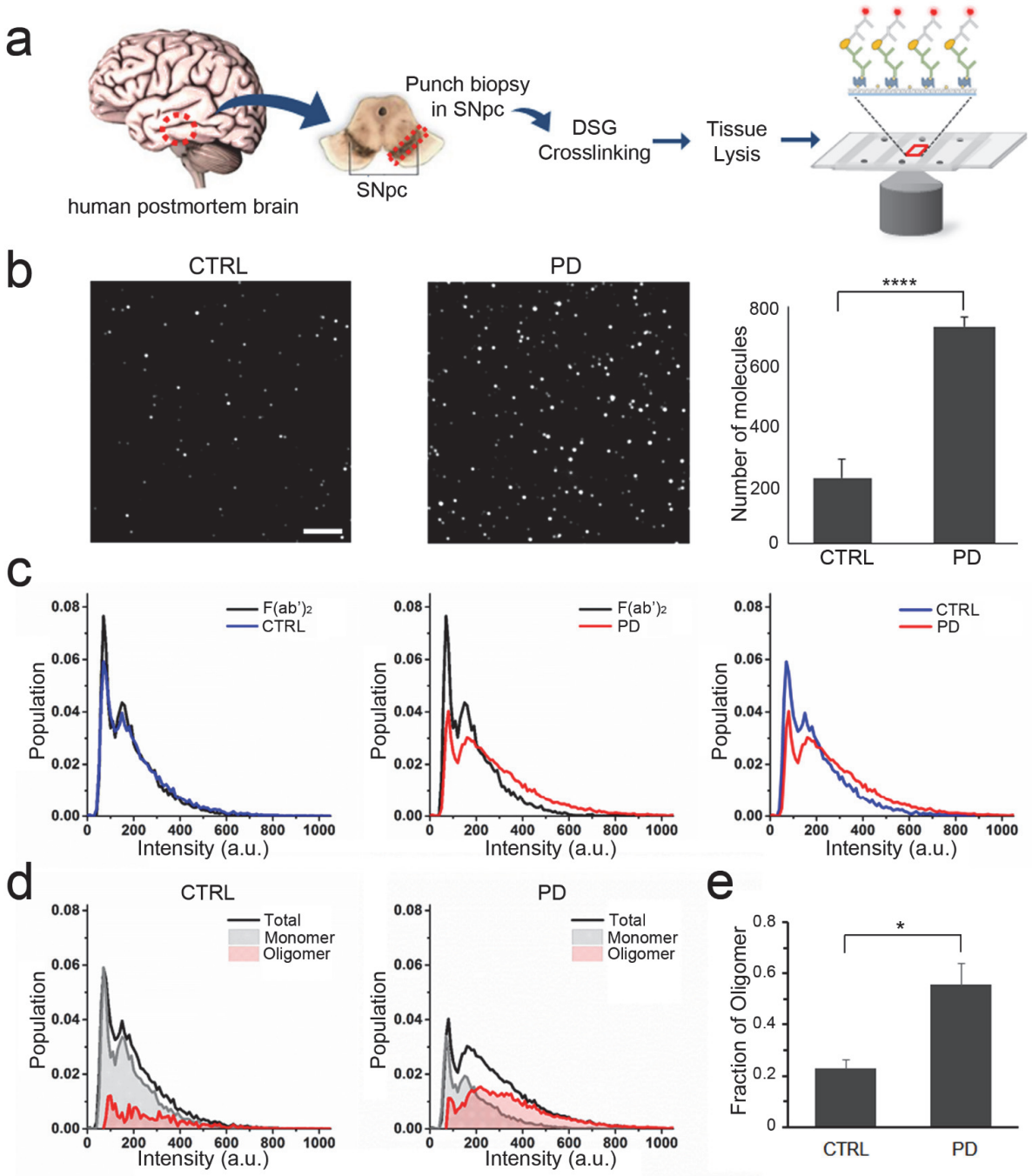

Figure 4. Analysis of oligomeric states of α-SYN from *in vivo* DSG-crosslinked human *postmortem* brain punch biopsy samples using SiMPull. (**a**) Schematic

diagram of α-SYN SiMPull procedure from human *postmortem* brain samples. (**b**) Single-molecule images of α-SYN from control (left) or PD brain samples (middle), and average number of molecules per imaging area (right) taken from 20 images. Scale bar, 5 μm. (**c**) Fluorescence intensity distribution of α-SYN SiMPull assay from control (blue) and PD (red) brain samples plotted with reference intensity profile of $F(ab')_2$ (black). (**d**) Analysis of oligomeric states of control and PD brain samples. Monomeric (grey) and oligomeric (red) populations were separately plotted. (**e**) Quantitative analysis of the oligomeric states from the data presented in (**d**). Error bars denote standard deviation (s.d.) in (**b**) and standard error of the mean (n = 3) in (**e**). *P< 0.05, ****P< 0.0001 by unpaired two-tailed t test. 50 ng/μl of total lysates from *in vivo* DSG-crosslinked human control or PD *postmortem* brain samples were used.

Figure 2 – Figure supplement 1

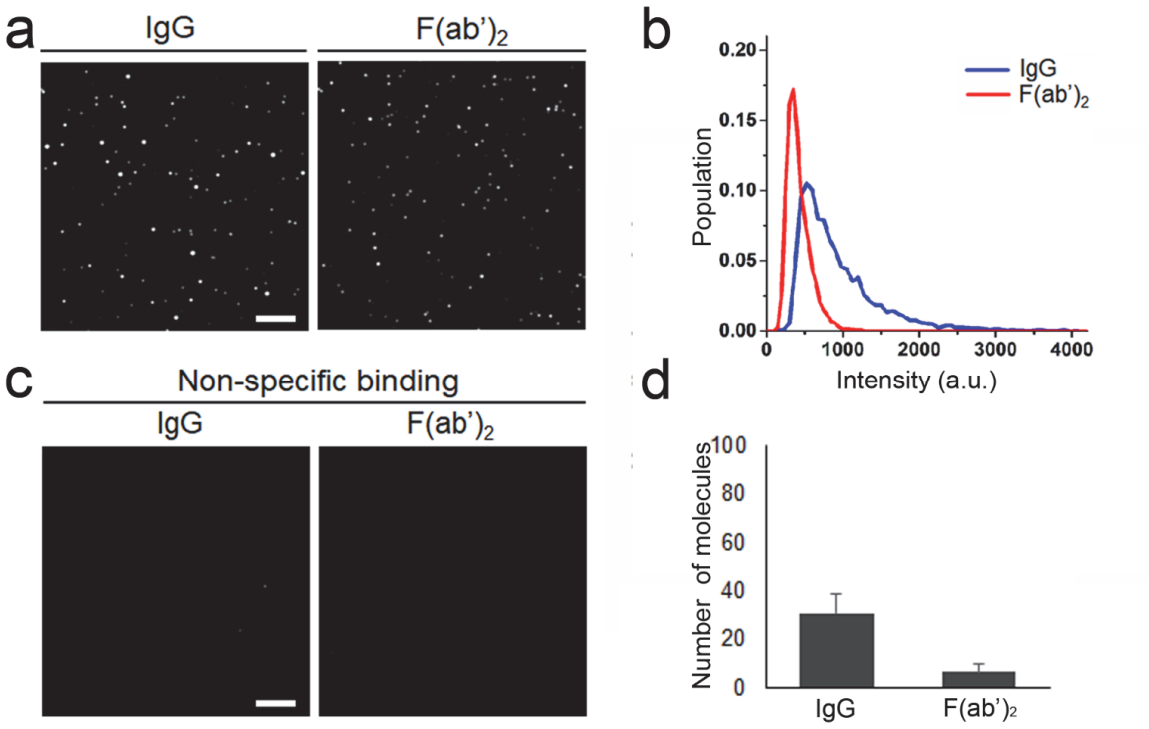

Figure 2 – Figure supplement 1. Fluorescence intensity profiles of Alexa 647-labeled full IgG and F(ab')$_2$ antibodies. Single-molecule images (**a**) and fluorescence intensity distribution (**b**) of Alexa 647-labeled full IgG or F(ab')$_2$ antibodies. Single-molecule images (**c**) and average number of fluorescent spots per imaging area (**d**) of non-specific binding of Alexa 647-labeled IgG or F(ab')$_2$ antibodies. Scale bar, 5 μm. For each sample, 20 images were taken. Error bars denote standard deviation (s.d.).

Figure 2 – Figure supplement 2

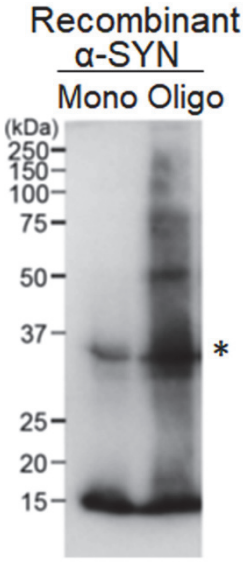

Figure 2 – Figure supplement 2. α-SYN recombinant protein western blot. Western blot for monomeric and oligomeric recombinant α-SYN. The *asterisk* marks a nonspecific band by the antibody. Each 0.5 μg of protein was used.

Figure 3 – Figure supplement 1

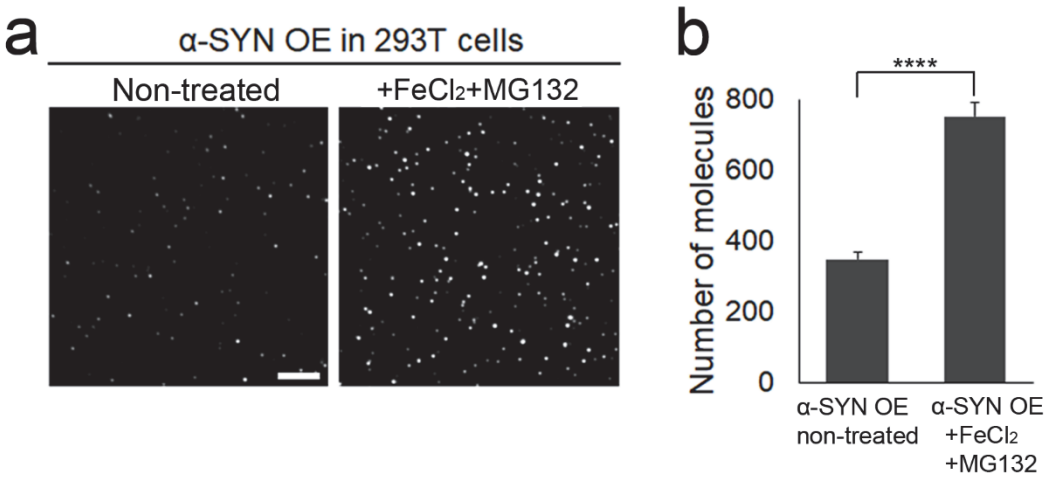

Figure 3 – Figure supplement 1. SiMPull detection and counting of α-SYN from *in vivo* DSG-crosslinked samples. Single-molecule images (**a**) and average number of fluorescent spots per imaging area (**b**) of α-SYN taken from α-SYN overexpressed cells with or without $FeCl_2$ and MG-132 treatment. 10 ng/μl of total lysates were used in each assay. Scale bar, 5 μm. For each sample, 20 images were taken. Error bars denote standard deviation (s.d.). ****P< 0.0001, by unpaired two-tailed *t* test.

Figure 3 – Figure supplement 2

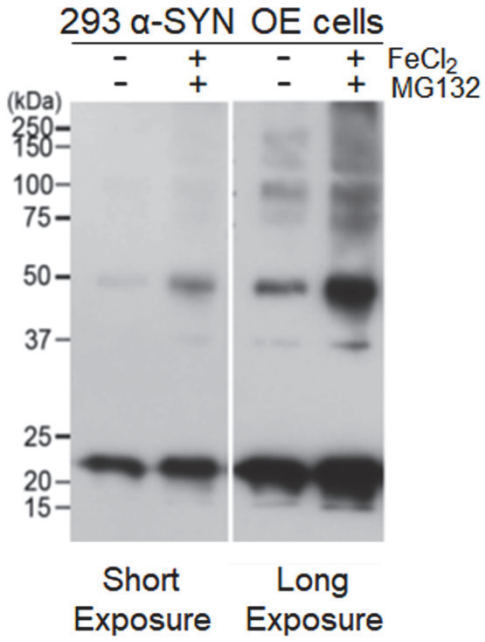

Figure 3 – Figure supplement 2. α-SYN western blot from the cultured cells. α-SYN western blot for *in vivo* DSG-crosslinked α-SYN overexpressed cells with or without $FeCl_2$ and MG-132 treatment. Each 30 μg of total lysates were used.

Figure 4 – Figure supplement 1

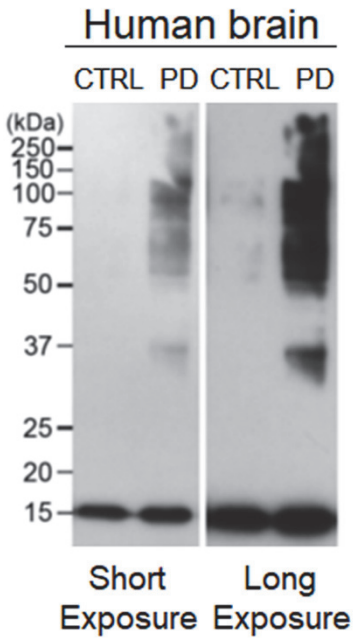

Figure 4 – Figure supplement 1. α-SYN western blot from human postmortem brain samples. α-SYN western blot for *in vivo* DSG-crosslinked human control and PD *postmortem* brain samples. Each 20 µg of total lysates were used.