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TITLE:

Utilizing Time-Resolved Protein-Induced Fluorescence Enhancement to Identify Stable Local Conformations One α-Synuclein Monomer at a Time

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SUMMARY:

Time-resolved single-molecule protein-induced fluorescence enhancement is a useful fluorescence spectroscopic proximity sensor sensitive to local structural changes in proteins. Here we show it can be used to uncover stable local conformations in α-Synuclein, which is otherwise known as globularly unstructured and unstable when measured using the longer range FRET ruler.

ABSTRACT:

Using spectroscopic rulers to track multiple conformations of single biomolecules and their dynamics have revolutionized the understanding of structural dynamics and its contributions to biology. While the FRET-based ruler maps are accurate to inter-dye distances in the 3-10 nm range, other spectroscopic techniques, such as protein-induced fluorescence enhancement (PIFE), report on data mapping to distances between a dye and a protein surface in the shorter 0-3 nm range. Regardless of the method of choice, its use in measuring freely-diffusing biomolecules one at a time retrieves histograms of the experimental parameter yielding separate centrally-distributed sub-populations, where each sub-population represents either a single conformation that stayed unchanged within milliseconds, or multiple conformations that interconvert much faster than milliseconds, and hence an averaged-out sub-population. In single-molecule FRET, where the reported parameter in histograms is the inter-dye FRET efficiency, an intrinsically disordered protein, such as the α-Synuclein monomer in buffer, was previously reported as exhibiting a single averaged-out sub-population of multiple conformations interconverting rapidly. While these past findings depend on the 3-10 nm range of the FRET-

based ruler, we sought to put this protein to the test using single-molecule PIFE, where we track the fluorescence lifetime of Cy3-labeled α-Synuclein proteins one at a time. Interestingly, using this shorter range spectroscopic proximity sensor, Cy3-labeled α-Synuclein exhibits several lifetime sub-populations with distinctly different mean lifetimes that interconvert in 10-100 ms. These results show that while α-Synuclein might be disordered globally, it nonetheless attains stable local structures. In summary, in this work we highlight the advantage of using different spectroscopic proximity sensors that track local or global structural changes one biomolecule at a time.

INTRODUCTION:

Over the past two decades, single-molecule fluorescence-based methods have become a powerful tool for measuring biomolecules[1,2], probing how different bimolecular parameters distribute as well as how they dynamically interconvert between different sub-populations of these parameters at sub-millisecond resolution[3,4,5]. The parameters in these techniques include the energy transfer efficiency in FRET measurements [6,7], fluorescence anisotropy[8,9], fluorescence quantum yields and lifetimes[10,11], as a function of different fluorescence quenching[12] or enhancement[13] mechanisms. One of these mechanisms, better known as protein-induced fluorescence enhancement (PIFE)[14] introduces the enhancement of fluorescence quantum yield and lifetime as a function of steric obstruction to the free isomerization of the fluorophore when in excited-state, caused by protein surfaces in the vicinity of the dye[14-19]. Both FRET and PIFE are considered spectroscopic rulers or proximity sensors since their measured parameter is directly linked to a spatial measure within the labeled biomolecule under measurement. While the FRET efficiency is related to the distance between a pair of dyes within a range of 3-10 nm[20], PIFE tracks increases in fluorescence quantum yields or lifetimes related to the distance between the dye and a surface of a nearby protein in the range of 0-3 nm[19].

Single-molecule FRET has been widely used for providing structural insights into many different protein systems, including intrinsically disordered proteins (IDPs)[21], such as α-Synuclein (α-Syn)[22]. α-Syn can form ordered structures following binding to different biomolecules and under different conditions[23-30]. However, when unbound, the α-Syn monomer is characterized by high conformational heterogeneity with rapidly interconverting conformations[31,32].

The conformations of α-Syn have been studied previously using various different techniques that help in identifying conformational dynamics of such highly heterogeneous and dynamic protein systems[33-39]. Interestingly, single-molecule FRET (smFRET) measurements of α-Syn in buffer reported a single FRET population[40,41] that is an outcome of time-averaging of conformations dynamically interconverting at times much faster than the typical diffusion time of α-Syn through the confocal spot (times as fast as few microseconds and even faster than that, relative to typical millisecond diffusion times)[41,42]. However, using a FRET spectroscopic ruler with the 3-10 nm distance sensitivity sometimes reports only on overall structural changes in a small protein such as α-Syn. Single-molecule measurements utilizing spectroscopic proximity sensors with shorter distance sensitivities have the potential to report on dynamics of local structures. Herein we perform single-molecule PIFE measurements of α-Syn and identify different sub-populations of fluorescence lifetimes mapping to different local structures with transitions between them as

slow as 100 ms. This work summarizes time-resolved smPIFE measurements of freely-diffusing α-Syn molecules one at a time, in buffer and when bound to SDS-based membranes as a short-range single-molecule spectroscopic proximity sensor.

PROTOCOL:

1. Plasmid transformation

1.1. Prepare 0.5 L of SOC medium preparation.

1.1.1. Weigh 10 g of tryptone, 2.5 g of Yeast extract, 0.25 g of sodium chloride (NaCl), 0.1 potassium chloride (KCl).

1.1.2. Add DDW until total volume of 0.5 L.

1.1.3. Adjust to pH 7 by adding sodium hydroxide (NaOH).

1.1.4. Prepare stock aliquots of 100 mL and autoclave.

1.1.5. Before using, add 0.5 mL of sterile magnesium chloride ($MgCl_2$) and 1.8 mL of sterile glucose to 100 mL of SOC.

1.2. Thaw BL21 (DE3) component cells on ice.

1.3. Mix gently the component cells.

1.4. Prepare two 14 mL tubes. Label one as transformation reaction tube and the other one as the control tube.

1.5. Aliquot 100 µL of component cells into the tubes.

1.6. Add 1 µL of plasmid at a concentration of 0.1 ng/µL into the transformation reaction tube.

1.7. Heat SOC medium in 42 °C water path for use in step 1.8.

1.8. Heat-pulse the two tubes in 42 °C water path for a duration of **45 seconds.** (Critical step!)

1.9. Add 900 µL of heated SOC medium to each tube.

1.10. Incubate the tubes at 37 °C for a duration of 45 minutes with shaking at 225 rpm.

1.11. Spread 200 µL of the cells with the transformed plasmid onto one LB (Luria-Bertani)-agar plate containing 100 µg/mL ampicillin, using a sterile cell spreader.

1.12. Spread 200 µL of the cells with the transformed plasmid onto one LB-agar plate without ampicillin, using a sterile spreader **(positive control)**.

1.13. Spread 200 µL of the control cells (not containing a plasmid) onto one LB-agar plate containing 100 µg/mL ampicillin, using a sterile spreader (**negative control)**.

1.14. Incubate the plates overnight at 37 °C.

2. Protein preparation

2.1. Recombinant α-Synuclein expression and purification

2.1.1. Pick a single colony and grow in 100 mL of autoclaved LB (Luria-Bertani) liquid medium containing 100 µg/mL ampicillin at 37 °C **(starter)**.

2.1.2. Prepare four 2 L Erlenmeyer flasks, each containing 1 L of autoclaved LB medium and 100 µg/mL ampicillin **(large inoculation)**.

2.1.3. Measure the optical density (OD) of the cell solution. When the cell density of the starter reaches an $OD_{\lambda=600nm}$ of 0.6-0.7, add 10 mL of starter solution to each liter of LB medium prepared in the previous step.

2.1.4. Grow the bacterial mediums in an incubator shaker at 37 °C and a velocity of 200 rpm.

2.1.5. When the cell density reaches an $OD_{\lambda=600nm}$ of 0.6-0.8, induce protein expression by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) for each liter of growth medium to a final concentration of 1 mM.

NOTE: Dissolve 0.96 g of IPTG in 4 mL of DDW and add 1 mL of the resulting IPTG solution per each L of bacterial growth.

2.1.6. Grow the cells for a duration of 4 hours and collect them by centrifugation in 50 mL tubes at a velocity of 5,170 x g for a duration of 8 minutes.

NOTE: In each round, discard the supernatant, keep the pellet and fill again the same tubes with bacterial growth.

2.1.7. Store the bacterial pellet in deep freeze storage (e.g., -80 °C) for the next day.

2.1.8. The next day, prepare 200 mL of lysis buffer: 40% (w/v) sucrose and buffer A, which includes 30 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT) at pH 8.0.

2.1.9. Add 25 mL of lysis buffer to each tube with bacterial pellet.

2.1.10. Re-suspend the pellet (use sterile plastic Pasteur pipettes) in Lysis buffer (see 2.1.8).

2.1.11. Prepare sterile 250 mL Erlenmeyer flask with a stirrer magnet on ice.

2.1.12. Add 5 mL of lysis buffer, and then transfer the homogeneous re-suspended cells to it.

2.1.13. Stir the cells for a duration of 20 minutes at 200 rpm at room temperature.

2.1.14. Divide the solution into 50 mL tubes and centrifuge at 4 °C for a duration of 30 minutes at a velocity of 17,400 x g.

2.1.15. Discard the supernatant.

2.1.16. Prepare sterile 250 mL Erlenmeyer flask with stirrer magnet on ice.

2.1.17. Add 15 mL of dissolution buffer (90 mL cooled buffer A and 37 µL of $MgCl_2$ dissolved beyond the solubility limit and filtered) into each tube. Use sterile plastic Pasteur pipettes and dissolve the pellet.

2.1.18. When part of the solution becomes homogeneous, move it to a stirred Erlenmeyer flask on ice.

2.1.19. Determine the solution volume in the Erlenmeyer.

2.1.20. Add 10 mg of streptomycin sulfate per each mL of solution (before adding, dissolve streptomycin sulfate in 2 mL of buffer A.

NOTE: This step is performed in order to remove the DNA and ribosomes away.

2.1.21. Stir the solution for a duration of 10-20 minutes at room temperature.

2.1.22. Divide the solution into 50 mL tubes and centrifuge at 4 °C and at a velocity of 20,700 x g for a duration of 30 minutes.

2.1.23. Collect the supernatant and discard the pellet.

2.1.24. Prepare a sterile 250 mL Erlenmeyer flask on ice with a stirrer.

2.1.25. Filter the supernatant using 0.22 µm syringe filter into the clean Erlenmeyer flask.

2.1.26. Determine the solution volume and weigh 0.3 g of ammonium sulfate per each mL of solution.

2.1.27. Gradually, add the ammonium sulfate to the stirred solution.

NOTE: This step is performed to precipitate proteins. When the ammonium sulfate binds the proteins, the solution becomes obscured and blurry, which shows up as white color.

2.1.28. Stir for 30 minutes at room temperature.

2.1.29. Divide the solution to 50 mL tubes and centrifuge at 4 °C and at a velocity of 20,700 g for a duration of 30 minutes. Verify that the centrifuge deceleration rate is not high (~4 m/s^2 would be best)

2.1.30. Carefully discard the supernatant and keep the pellet.

2.1.31. Dissolve the pellet with 20 mL of Buffer A.

2.1.32. Unify all the solutions into one tube, and then measure the UV absorption spectrum of the solution.

NOTE: If the spectrum shows a signature of aggregation (absorption above a wavelength of 300 nm), continue to the next step. If not, skip the next step and move to step 2.1.34.

NOTE: The aggregation index (A.I.) calculated from the UV spectra as $OD_{\lambda=350nm}$/ ($OD_{\lambda=280nm}$ – $OD_{\lambda=350nm}$) ×100.[42]

2.1.33. Use 100 kDa cutoff centriprep tubes and centrifuge at a velocity of 3590 x g at 4 °C for a duration of 15 minutes. Collect the liquid that passed through the filter.

2.1.34. Dialyze the solution at 4 °C overnight, against Buffer A, using dialysis bags with a 3.5 kDa cutoff.

2.1.35. Perform another round of dialysis for a duration of at least 4 hours.

2.1.36. Load the dialyzed sample onto a 1 mL MonoQ anion exchange column. Use 30 mM Tris hydrochloride (HCl) buffer at pH 7.5 as a washing buffer and 30 mM Tris-HCl buffer pH 7.5 with 500 mM NaCl as an elution buffer.

2.1.37. Inject the sample to the column. Then wash with 20 column volumes (CVs) of 0% elution buffer and 100% wash buffer in order to remove the unbound proteins.

2.1.38. Wash with 7 CVs of 30% elution buffer.

2.1.39. Elute the α-Syn protein sample using a gradient that changes from 30% to 100% elution buffer for 30 CVs, at a flow rate of 1.5mL/min.

NOTE: α-Syn elutes at 46-66 CVs at conductivity between 20-24 mS/cm, which is referring to 38-50% elution buffer.

2.1.40. Collect the fractions of the main elution peak. Verify the presence of α-Syn by running samples in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), following staining with either Coomassie Blue or Fast SeeBand staining solution. A band at 15 kDa is expected.

2.1.41. Unify the relevant fractions and dialyze them overnight, against buffer A, using dialysis bags with a 3.5 kDa cutoff at 4 °C.

2.1.42. Prepare aliquots of the protein sample and store them at -20 °C.

2.2. Cy3-labelled α-Synuclein

2.2.1. Reduce the thiol of the cysteine residue in the α-Syn A56C mutant by adding DTT to a final concentration of 2 mM for a duration of 1 hour at room temperature.

2.2.2. Remove DTT by performing two rounds of dialysis using 3.5 kDa cutoff dialysis bags. For the first round, use 30 mM Tris-HCl pH 8.0 and 2 mM EDTA. For the second round, use 50 mM HEPES pH 7.2 and 2 mM EDTA.

2.2.3. Reduce the cysteine residues by adding Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to the protein sample, to a final concentration of 50 µM for a duration of 30 minutes at room temperature.

NOTE: TCEP is a non-sulfhydryl reducing agent, hence it keeps the thiol groups reduced without reacting with the dye. We added 0.5 µL from a stock solution of 1 M TCEP.

2.2.4. Calculate the amounts of protein required for the final reaction volume of 1 mL.

NOTE: Here is an example for the calculation we used:

$$20\ \mu M_{\text{final protein concentration}} \times 1\ mL_{\text{final volume}} = 56\ \mu M_{\text{initial protein concentration}} \times V_{\text{protein to be added}}$$

2.2.5. Calculate the amount of dye required for final reaction volume of 1 mL. The requested dye:protein molar ratio should be at least 3:1.

NOTE: An example for the calculation we used:

$$60\ \mu M_{\text{final dye concentration}} \times 1\ mL_{\text{final volume}} = 370\ \mu M_{\text{initial dye concentration}} \times V_{\text{dye to be add}}$$

2.2.6. Calculate the amount of buffer from the dialysate required to adjust the total reaction volume to 1 mL.

NOTE: Calculation example: $1\ mL - (V_{\text{calculated from step 2.2.4}} + V_{\text{calculated from step 2.2.5}})$

2.2.7. Prepare reaction vial with a magnet on top of a stirrer.

2.2.8. Firstly, add the calculated amount of the protein and the buffer. Afterwards add the calculated amount of the dye (Cy3).

2.2.9. Keep the reaction at room temperature in the dark for a duration of 3-5 hours.

2.2.10. Terminate the reaction by adding 2 mM DTT and continue storing for a duration of 1 hour.

2.2.11. Perform three rounds of dialysis against Buffer A, using dialysis bags with a 3.5 kDa cutoff to remove excess free dye from the solution.

2.2.12. Load the sample on a size exclusion column to further separate the labeled α-Syn from the free dye.

2.2.13. Determine the concentration of pure labeled α-Syn by measuring the absorption of the dye (absorption coefficient for Cy3 is 162,000 $M^{-1}cm^{-1}$).

2.2.14. If needed, concentrate the pure labeled α-Syn solution using a vacuum concentrator (e.g., SpeedVac). Then, perform one round of dialysis against buffer A, using dialysis bags with a 3.5 kDa cutoff and again determine the concentration of the labeled protein.

2.2.15. Prepare aliquots of the labeled protein sample and store them at -20 °C.

3. Measurements

3.1. smPIFE experimental setup

NOTE: Use the following confocal-based setup or similar.

3.1.1. Use a pulsed laser source (in our case, λ= 532 nm picosecond pulsed laser with pulse width of ~100 ps FWHM), operating at a suitable repetition rate (20 MHz in our case) and routed to the SYNC of a time-correlated single photon counting (TCSPC) card, as the source of Cy3 excitation.

3.1.2. Use a dichroic beam splitter with high reflectivity at 532 nm, to separate excitation and scattering from fluorescence.

3.1.3. Use a 100 µm diameter pinhole at the focus of the emitted light, after the collimated emission beam was focused by a lens, and before the emission beam has been re-collimated by another lens.

3.1.4. Use a band pass filter (585/40 nm in our case) to further filter Cy3 fluorescence from other light sources.

3.1.5. Detect the fluorescence using a detector (single-photon avalanche diode or hybrid photomultiplier, in our case) routed (through a 4-to-1 router, in our case) to a TCSPC module (Becker & Hickl SPC-150, in our case).

NOTE: In our case, data acquisition is performed via the VistaVision software (ISS^{TM}) in the time-tagged-time-resolved (TTTR) file format.

3.2. smPIFE sample preparation

3.2.1. Prepare 25 pM Cy3-labeled α-Syn in measurements buffer: 10 mM sodium acetate, 10 mM sodium dihydrogen phosphate, 10 mM glycine pH 8.0, 20 mM NaCl, 10 mM cysteamine and 1 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), in a low protein binding tube.

NOTE: If α-Syn is measured in the presence of SDS vesicles, add the appropriate SDS amount as well.

3.2.2. Rinse an 18-chamber microscopy coverslip slide with 100 µL of 1 mg/mL BSA for a duration of 1 minute, and then remove the bovine serum albumin (BSA).

3.2.3. Add 100 µL of the 25 pM Cy3-labeled α-Syn sample to a chamber in the coverslip slide.

3.2.4. Perform smPIFE measurement of the sample using the described setup, as follows in the next steps.

3.3. smPIFE data acquisition

3.3.1. Use a high numerical aperture water immersion objective lens (in our case, Olympus UPLSAPO 60x NA1.2), and add a drop of ultra-pure water on top of the objective lens.

3.3.2. Fix the coverslip slide in a stage chamber and install it on top of the microscope stage.

3.3.3. Bring the objective lens upwards, until the water droplet on top of the objective lens smears at the bottom of the coverslip slide.

3.3.4. Open the laser shutter and bring the objective lens upwards, while inspecting the pattern on a CCD camera. Observe the Airy rings pattern: the first one represents the focus at the water-glass interface, and then the second one represents the focus at the interface between the glass and the sample solution.

3.3.5. Increase the height of the objective lens by an additional 75 µm, bringing the laser focus deep into the solution, to minimize auto-fluorescence from the glass surface of the coverslip.

3.3.6. Tune the laser power at the objective lens to be ~100 µW.

3.3.7. Start the acquisition of detected photons for a predefined time (2 hours, in our measurements).

NOTE: The majority of the acquisition signal should have a rate (measured in counts per seconds, cps) that is similar to that acquired when measuring just the buffer; the millisecond-binned data should show scarce photon burst events, with the majority of bins having an average rate that is comparable to the typical detector background rate (<1,000 cps, in our case).

4. smPIFE burst analysis

4.1. Raw data conversion

NOTE: The data is usually stored in a binary file with a format that was predefined by the company that manufactures the TCSPC card (.spc files in our case).

4.1.1. Convert the raw data file to the photon-HDF5 universal file format[43], using the software suite *phconvert* (https://github.com/Photon-HDF5/phconvert). Call the raw data file as an input and convert into a raw data .hdf5 file using the appropriate code in the *phconvert* suite (the *Convert ns-ALEX Becker-Hickl files to Photon-HDF5.ipynb* Jupyter notebook, in our case).

NOTE: The conversion into a .hdf5 file includes: (1) determining the relevant photon streams in the raw data (i.e., photon streams registered photons of the relevant detector ID); (2) the relevant photon nanotimes (the photon detection times relative to the excitation SYNC time); and (3) added metadata.

4.2. Burst Search and Selection using *FRETbursts*[44]

NOTE: All of the photon-HDF5 raw data files of the smPIFE measurements, as well as the code summarizing the analysis of the raw data, are stored in Zenodo (https://doi.org/10.5281/zenodo.4587698). All the steps below are detailed and shown within the Jupyter notebooks, also supplied in the Zenodo repository link.

4.2.1. Open the Jupyter Notebooks (within the Anaconda framework, in our case).

4.2.2. Open the notebook *smPIFE-αSyn 56C(Cy3) 25 pM newBuffer (Final notebook).ipynb* (it can be found in the Zenodo link, https://doi.org/10.5281/zenodo.4587698).

4.2.3. Load *FRETbursts*.

4.2.4. Load the photon HDF5 data file.

4.2.5. BG rate assessment: using the histogram of the inter-photon times, calculate the

background (BG) rates for each 30 seconds of data acquisition.

NOTE: The following steps describe the burst search using the sliding window algorithm[45,46,47].

4.2.6. Move a time window of m=20 consecutive photons, one photon at a time.

4.2.7. Collect the photon data if the instantaneous photon rate, $(m/(t_{i+m-1} - t_i))$, is at least F=11 times larger than the BG rate for that period of the data acquisition.

NOTE: A burst is constructed out of all of the consecutive photons that were collected by sliding the window one photon at a time (step 4.2.6.) and agreeing with the photon rate criterion (step 4.2.7).

4.2.8. Calculate the following burst characteristics:
-Burst size: the amount of photons in a burst.
-Burst duration: the time difference between the last and first photon detection times in a burst.
-Burst brightness: the largest value of the instantaneous photon rate in a burst.
-Burst separation: the time interval between consecutive bursts.

NOTE: The following points describe the further burst selection procedure.

4.2.9. Plot the histogram of burst brightness values (the highest instantaneous photon rate in a burst), with the events' axis in logarithmic scale.

4.2.10. Define the burst brightness threshold as the minimal burst brightness value from which the histogram exhibits a decaying pattern.

4.2.11. Select bursts with brightness values larger than the burst brightness threshold.

NOTE: The next steps describe burst mean fluorescence lifetimes.

4.2.12. Plot the histogram of photon nanotimes for all photons in all selected bursts with the photon counts axis in logarithmic scale.

4.2.13. Define the nanotime threshold as the minimal nanotime value from which the histogram of photon nanotimes exhibits a decaying pattern.

4.2.14. Select only photons with nanotimes larger than the nanotime threshold.

4.2.15. Calculate the algebraic average of all selected photon nanotimes.

4.2.16. Subtract the nanotime threshold from the photon nanotime algebraic average. The result is the mean photon nanotime of the burst, which is directly proportional to the mean fluorescence lifetime.

4.2.17. Plot the histogram of all burst mean fluorescence lifetimes. Centrally-distributed sub-populations of fluorescence lifetime may appear. Sub-populations with low value averages represent molecule species with Cy3 that was not sterically obstructed, while sub-populations with higher value averages represent molecule species with Cy3 that was more sterically obstructed.

NOTE: The next steps describe slow between-burst dynamics based on burst recurrence analysis[48]

4.2.18. Plot the histogram of burst separation times, with the separation time axis in logarithmic scale.

NOTE: Two sub-populations of burst separation times will appear:
- A major sub-population with separation times of seconds, representing consecutive bursts originating from different consecutively-measured molecules.
-A minor sub-population with separation times ~<100 ms, representing consecutive bursts both originating from the same molecule, recurring back through the confocal volume.

4.2.19. Select to save all pairs of consecutive bursts that are separated by less than a maximal separation time that defines the same-molecule sub-population (<100 ms, in our case).

4.2.20. Plot a histogram or a scatter plot of the mean fluorescence lifetimes of the first and second bursts for all pairs of bursts that recurred below a certain separation time threshold

RESULTS:

As an IDP, when it is not bound to another biomolecule, α-Syn exhibits structural dynamics between multiple conformations, with transitions at few microseconds[41] and even at hundreds of nanoseconds[42]. When α-Syn crosses the confocal spot, it may undergo thousands of transitions between conformations. Indeed, this was the case when smFRET was used[40, 41]. Here we perform smPIFE measurements in order to test whether α-Syn undergoes local conformational dynamics slower than milliseconds.

The measurement records fluorescence photons emitted from a Cy3 dye, attached to the thiol group of cysteine in the α-Syn A56C mutant. The Cy3 fluorophore can undergo isomerization when in an excited state. However, Cy3 emits a photon when it de-excites from its *trans* isomer. Therefore, if nothing sterically obstructs the Cy3 excited-state isomerization, it will emit few photons on average, but will exhibit a low fluorescence quantum yield and short fluorescence lifetime. However, if the excited-state isomerization of Cy3 is obstructed by, for example, the surface of a nearby protein, the rate of isomerization will decrease, which in turn will lead to more de-excitations from the *trans* isomer, and hence more photons, a higher fluorescence quantum yield and longer fluorescence lifetimes. This is better known as the PIFE effect.

Using smPIFE we measured the mean fluorescence lifetime of Cy3 labeling α-Syn at residue 56

one α-Syn at a time, where the Cy3 dye senses the protein environment around residue 56. The protein was measured at a concentration of 25 pM, in which it is mainly found as a monomer. The results of the smPIFE measurements are shown as histograms of mean fluorescence lifetimes of single α-Syn molecules (**Figure 1**). The mean fluorescence lifetimes can be grouped into two major sub-populations (**Figure 1A**). The first sub-population exhibits short fluorescence lifetimes, with a characteristic fluorescence lifetime of 1.6 ns, representing α-Syn conformational states with no or few protein surfaces found in the vicinity of residue 56. The second sub-population exhibits longer fluorescence lifetimes, with a characteristic fluorescence lifetime of 3.5 ns, representing α-Syn conformational states with more protein surfaces found in the vicinity of residue 56.

It is known that in the presence of ~5-10 mM SDS, the N-terminal and NAC segments of almost all of the α-Syn molecules in solution adopt a helical hairpin structure upon binding to SDS vesicles[41]. Since residue 56 is located within the NAC segment, fluorescence from Cy3 labeling residue 56 is expected to sense a rather uniform microenvironment, since the majority of almost all α-Syn molecules should acquire the vesicle-bound helical hairpin structure. Therefore, we performed similar smPIFE measurements but in the presence of 5 mM SDS as a control, expecting to identify a single population of fluorescence lifetimes. Indeed, these measurements result in a single population of fluorescence lifetimes with a characteristic fluorescence lifetime of 3.1 ns (**Figure 1B**). The ~3 ns characteristic fluorescence lifetime points to a local structure in the vicinity of residue 56, that does not exist in the ~1.5 ns lifetime sub-population of α-Syn in solution, emphasizing the structuring α-Syn undergoes when the helical hairpin is formed and the binding to the SDS vesicle surface has occurred. Interestingly, that single population has a shorter characteristic fluorescence lifetime relative to the ~3.5 ns lifetime sub-population of α-Syn in solution.

The appearance of two distinct centrally distributed sub-populations of single-molecule bursts is a well-known signature of molecular heterogeneity. Since no mixture of separate labeled molecules is involved, both lifetime sub-populations represent two separate species of Cy3 labeling residue 56 in α-Syn (**Figure 1A**). Therefore, the results report dynamic heterogeneity. This is because the parameter reported in the histogram is calculated using all the photons in a burst throughout the few ms duration of the diffusing α-Syn inside the confocal volume. Therefore, Cy3-labeled α-Syn molecules crossed the confocal volume either when exhibiting a short or a long mean lifetime. The transitions between these species must occur slower than the characteristic diffusion times through the confocal spot, hence slower than a few milliseconds. In order to assess this dynamic, we performed burst-recurrence analysis[48]. In short, since we seek to assess dynamics that occur at times longer than the duration of a single-molecule burst, we tested the possibility that a single α-Syn molecule exhibits a change in the Cy3 mean fluorescence lifetime between consecutive crossings of the confocal spot. To do so, we first distinguish between two types of consecutive bursts: i) consecutive bursts of different α-Syn molecules with burst separation times that distribute in seconds, and ii) consecutive bursts of the same α-Syn molecule that recurs in the confocal volume after a burst separation time, at times as slow as ~100 ms (**Figure 2A**). Following the two mean lifetime sub-populations, we chose to inspect pairs of consecutive bursts that are separated by at most 100 ms (**Figure 2A**), where the first out of

the pair of bursts exhibited mean fluorescence lifetime within the short lifetime sub-population (0-2 ns) or within the long lifetime sub-population (>3.5 ns; **Figure 2B**, colored shades). The inspection tests which of the bursts of recurring bursts, represented by the second burst in the pair of consecutive bursts, exhibits mean fluorescence lifetime within the lifetime sub-population opposite to the one in the first burst. One can observe that a fraction of molecules that start as a burst in the short lifetime sub-population recur as a burst outside that range and even within the long lifetime subpopulation (**Figure 2C**), and that a fraction of molecules that start as a burst in the long lifetime sub-population recur as a burst outside that range and even within the short lifetime subpopulation (**Figure 2D**), all within 10-100 ms.

Figure 1: Mean fluorescence lifetime sub-populations of Cy3 labeling residue 56 in α-Syn A56C. mean fluorescence lifetime histograms of freely-diffusing Cy3-labeled α-Syn A56C (at 25 pM) in the absence **(A)** and presence **(B)** of 5 mM SDS.

Figure 2: PIFE burst recurrence analysis shows individual molecules undergo transitions between different average lifetime values within 100 ms. From top to bottom: (**A**) the histogram of separation times between consecutive single-molecule bursts. The orange shade represents separation times between consecutive bursts of recurring molecules, where the first and second bursts arise from the same molecule. (**B**) The mean fluorescence lifetime histogram of all single-molecule bursts. The yellow and green shades represent the range of average lifetime values chosen to represent values within short and long mean lifetime sub-populations, respectively. (**C**) or (**D**). The mean fluorescence lifetime histograms of bursts that were separated from a previous burst by a time within the orange-shaded timescale (in **A**), and where the previous burst had an average lifetime within the range represented by the yellow or green shades, respectively.

DISCUSSION:

Extensive biochemical and biophysical studies were performed to study the structural characteristics of α-Syn and its disordered nature[33-39]. Several works have already utilized freely-diffusing smFRET to investigate the intra-molecular dynamics of the α-Syn monomer free of binding. These works reported the high dynamic heterogeneity of α-Syn, which leads to averaging-out of multiple different structural species within the typical diffusion times through the confocal spot, leading to the appearance of a single FRET population[40,41]. However, one must remember that smFRET measurements report on changes in inter-dye distances occurring within 3-10 nm, a scale characterizing overall structural changes in a small protein such as α-Syn.

We were curious as to what results we might find when using a different fluorescence-based sensor of spatial changes within a protein that is sensitive to local structural dynamics and that has been utilized also at the single-molecule level. smPIFE can track local spatial changes nearby Cy3 labeling a specific amino acid residue in the 0-3 nm range.

In this study, we utilized smPIFE to investigate the dynamics of local structures within α-Syn and more specifically local structure changes nearby the NAC residue 56. The results suggest that the region in the vicinity of residue 56 in α-Syn exhibits a few distinct structural sub-populations that are stable enough thermodynamically to interconvert in as slow as 100 ms, and perhaps even

slower. These sub-populations are identified through the inspection of the mean fluorescence lifetimes of measured Cy3-labeled single α-Syn molecules. In these sub-populations, the longer the characteristic fluorescence lifetime of the sub-population is, the more a protein surface obstructs the excited-state isomerization of Cy3, and hence the closer that protein surface is to that Cy3-labeled residue.

Like other IDPs, α-Syn has been reported to have interactions with other biomolecules, as well as self-association, where in many cases these binding events involve stabilization of a specific structure within the α-Syn subunit[57-60]. Some proteins acquire a specific structure upon binding, via an induced-fit mechanism. However other proteins spontaneously interconvert between several distinct conformations, and the binding event to a specific biomolecule merely stabilizes one of the preexisting conformations. For the latter case, one of the requirements is that the conformation to be stabilized will survive long enough to accommodate the initial binding. Therefore, the longer a structural region in a protein survives, the higher the binding efficiency will be. We suggest that the observed millisecond-stable sub-populations represent the existence of distinct species of local structure in the vicinity of residue 56. These point to different local structure species nearby the middle of the NAC and NTD segments. In a recent work, we report that other Cy3-labeled residues in these segments also exhibit such sub-populations[61]. This result comes to show that the structural dynamics of the free α-Syn monomer can be best described as rapid (few microseconds at most[41]) overall protein dynamics, carrying local structural segments that stay stable for milliseconds. Other IDPs such as Tau and amyloid-β were known to share similar characteristics of carrying a local structured region[49-51].

In addition, smPIFE has been mainly used for studying the interactions between separate biomolecules. Here, we employ smPIFE to investigate PIFE within segments of the same protein. It is important to mention that the majority of previous smPIFE experiments were performed by tracking relative changes in fluorescence intensities of single immobilized Cy3-labeled molecules[13-19,52-54]. While useful for immobilized molecules, this procedure is less informative when measuring freely-diffusing single-molecules. Hwang *et al.* have shown how to measure the PIFE effect also by tracking changes in fluorescence lifetimes, which report directly on the change in the excited-state isomerization dynamics of Cy3[19]. Here we probe the PIFE effect of single diffusing α-Syn via the Cy3 mean fluorescence lifetimes, rather than tracking relative changes in fluorescence intensities. Doing so, we were able to acquire PIFE-related sub-populations despite the short residence time of each single α-Syn molecule in the confocal spot. In fact, the mean fluorescence lifetimes proved to be useful not only in defining PIFE-related sub-populations, but also in assessing slow PIFE dynamics using the burst recurrence analysis framework[48]. However, more work is required in developing procedures that will allow to properly assess faster PIFE dynamics. There are plenty of existing photon statistics tools, utilized to assess rapid FRET dynamics in freely diffusing smFRET experiments, which we intend to repurpose to be used in smPIFE[55,56].

To summarize, in this work we used the relatively new combination of PIFE measurements of freely-diffusing single molecule to identify ms-stable sub-populations of local structures in α-Syn, which were not recovered by smFRET. We employed smPIFE measurements to study α-Syn as a

model IDP and our results extend beyond past findings of α-Syn being globally disordered. The findings suggest α-Syn may carry ms-stable ordered local structures and we hypothesize these local structures may serve a role in binding recognition.

Thus far, smPIFE was used as means to study interactions of Cy3-labeled nucleic acids with their unlabeled protein counterparts[52]. By that, smPIFE was utilized as a powerful tool to sense biomolecular interactions, and hence the natural next step in that direction would be to use it in order to sense protein-protein interactions and their dynamics, one complex at a time.

The power of using a short-range proximity sensor such as smPIFE can assist in identifying stable local structuring within other protein systems considered disordered and structurally unstable. However, the power of using a single-molecule fluorescence-based short proximity sensor does not stop there. There are many biomolecular systems that exhibit short-scale conformational dynamics to facilitate their function, such as many ion channels. We believe smPIFE can serve as a tool complementary to single-molecule FRET, in such cases where the dynamic range of the proximity changes within the protein system does not match the dynamic range of distances FRET can detect and resolve. In summary, we promote the use of smPIFE as a proximity-sensor complementary to single-molecule FRET measurements, in order to cover a wider scale of biomolecular proximities, and perhaps observe clear millisecond-averaged sub-populations of the measured parameters reporting on stable structures that are either local or overall.

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DISCLOSURES:

All authors share no conflict of interest.

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Figure 1

Click here to access/download;Figure;Fig1_PIFE_histogram.png

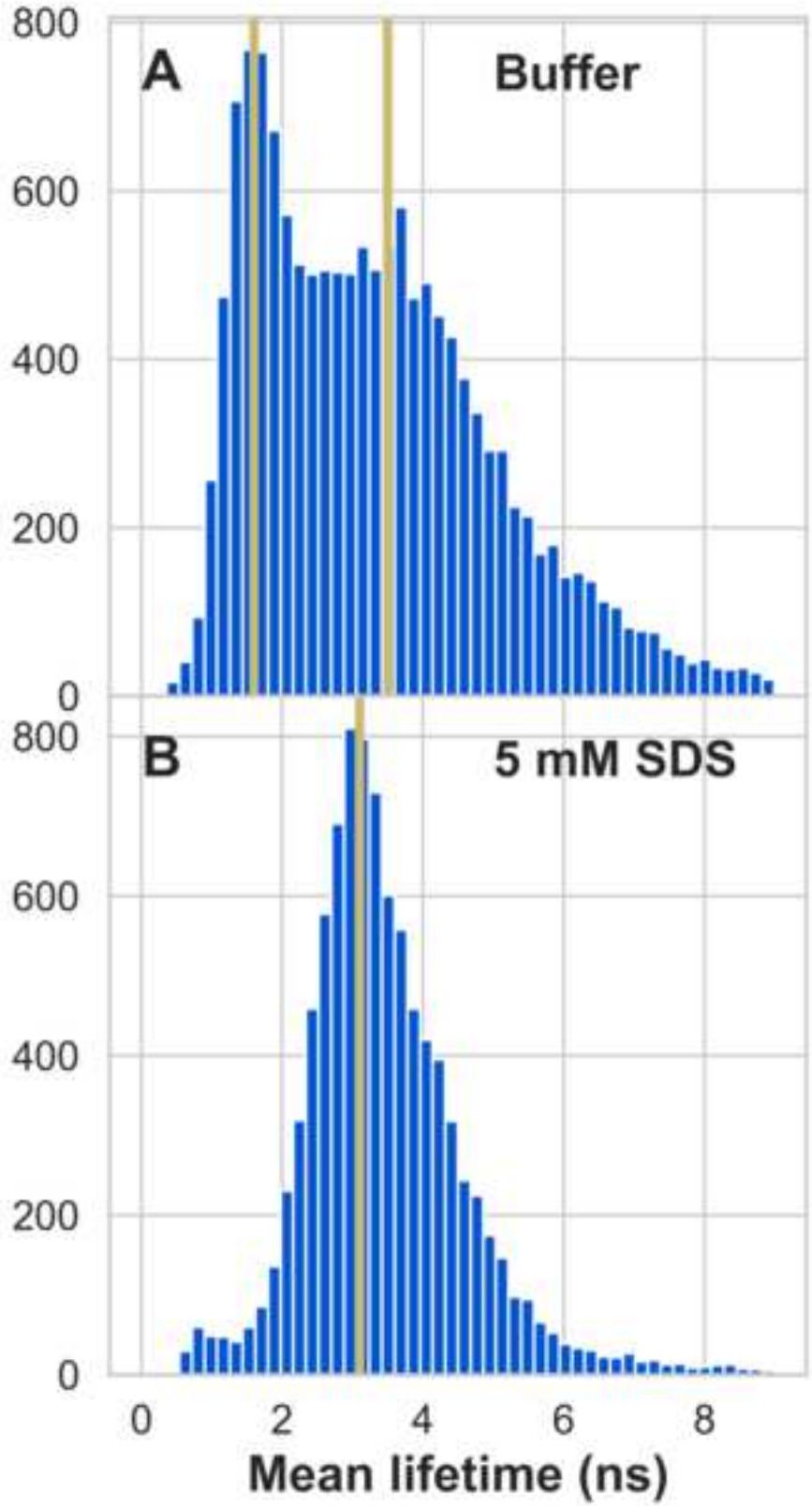
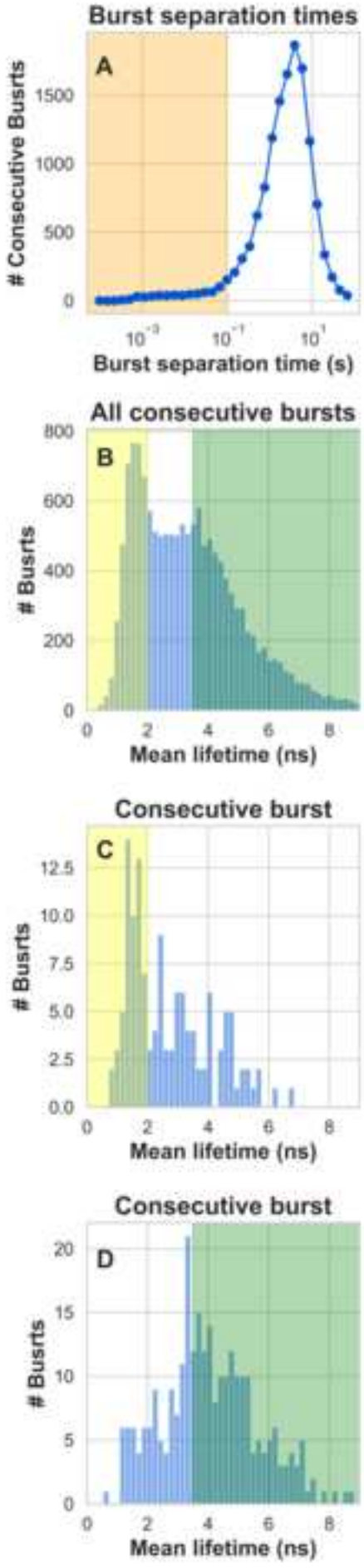


Figure 2

Click here to access/download;Figure;Fig2_PIFE_recurrence_analysis.png



Name of Material/ Equipment	Company	Catalog Number
Amicon Ultra-15 Centrifugal Filter Units	Merc	C7715
ammonium sulfate	Sigma-Aldrich	A4418
BSA	Sigma-Aldrich	A9647
cysteamine	Sigma-Aldrich	30070
dialysis bags - MEGA GeBaFlex-tube	Gene Bio-Application	MEGA320
dithiothreitol (DTT)	Sigma-Aldrich	43815
ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	E5134
Fast SeeBand staining solution	Gene Bio-Application	SB050
Glycine	Sigma-Aldrich	50046
D-Glucose	Sigma-Aldrich	G7021
HEPES	Sigma-Aldrich	54457
HiTrap Desalting 5 mL	Sigma-Aldrich	GE17-1408
6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX)	Sigma-Aldrich	238813
isopropyl β-d-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich	I5502
LB broth	Sigma-Aldrich	L3152
Magnesium chloride	Sigma-Aldrich	63068
MonoQ column	Sigma-Aldrich	54807
protein LoBind tube	Sigma-Aldrich	EP0030108094
Rinse a µ-slide 18	Ibidi	81816
SDS	Sigma-Aldrich	75746
Sodium acetate	Sigma-Aldrich	S2889
Sodium hydroxide	Sigma-Aldrich	S8045
Sodium phosphate monobasic monohydrate	Sigma-Aldrich	71507
Sterile Cell spreaders, Drigalski spatulas	mini-plast	815-004-05-001
streptomycin sulfate	Sigma-Aldrich	S9137
sulfo-Cy3 maleimide	abcam	ab146493
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)	Sigma-Aldrich	75259
Tris-HCl	Sigma-Aldrich	93363
Tryptone	Sigma-Aldrich	T7293
Yeast Extract	Sigma-Aldrich	Y1625

Comments/Description

cutoff: 100 kDa

0.5 mL

Response letter to the Editor and reviewers

We would like to note that all of our responses and all of the text we added or changed in the manuscript are highlighted in cyan, for the convenience of the editor and reviewers.

This is not to be confused with the text that is highlighted in yellow, which is the filmable part of the protocol.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We would like to thank the editors.

2. Please provide an email address for each author.

Where in the text should I provide an E-mail address to each author?

We already provided the E-mail address of the corresponding author in the text, and the addresses of all authors in the journal submission forms online. We believe these should be sufficient

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please avoid making further substeps.

Thank you, we did our best to make the protocol include the minimal amount of sub-steps.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Thank you. We went over the whole protocol and changed it to the imperative tense.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial

language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example Sorvall, centriprep tubes, MonoQ column, HiTrap Desalting, SpeedVac, µ-slide 18 (Ibidi), Olympus UPLSAPO, Jupyter Notebook, Anaconda, VistaVision software (ISSTM), etc.

Commercial language was carefully re-assessed and removed wherever possible, except the following exceptions:

VistaVision software (ISS™). This is mainly because it is a commercial software, and as part of my contact with ISS, I am obliged to mention the setup and software exactly this way. If we omit these phrases, I am open to a lawsuit.

Please note, however, that Jupyter is not considered commercial language of any kind, but rather the actual name of a programming ecosystem, that is part of the open code lingo. Additionally, without this name, "Notebooks" are meaningless. This is why we left the "Jupyter" name in the text. The same is true regarding Anaconda.

Additionally, we prefer to keep writing MonoQ column rather than in the purification part, because there are a variety of ion exchange resins and columns, where each type has specific parameters. The procedure we detailed are specific for using the MonoQ column. Moreover, in the past, we tried a number of ion exchange columns for the purification of αSyn and we have the best results achieved by using the MonoQ column.

SpeedVac is not a commercial name but rather the professional term for a vacuum centrifuge, and hence we continue using it.

In order to promptly respond to this comment, we have now changed the text, so that whenever we mention a part of the optical setup, we refer to the item in general terms, and detail the type of item we used in our setup in parentheses.

6. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points.

The manuscript text format has now been adapted following these requests.

7. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

The centrifuge speed units have now been converted following these requests. Please note that this unit conversion is relevant for centrifugation, and not to shaking, which generally uses rpm as standard units.

8. Please use complete sentences to describe the action being performed.

The sentences have been revised following these requests.

9. Please ensure that each step contains no more than 2-3 actions and no more than 4 sentences.

We made the best we can to adapt the protocol following these requests.

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Additional details were added

11. 1.1 Rationale for using this mutation? How what the transformation performed?

The rationale for using the A56C mutation was that it is situated at the protein segment that was documented in the past as gaining helical structure , and hence would be relevant for conjugating the sCy3 dye to it– this is also referred to in the text.

The transformation part has now been added to the protocol.

How do you check for the correct colonies? By comparing the experimental palte to the controls, negative control:

Bacteria without the plasmid of interest plated in medium with presence on antibiotics (colonies will not grow), and positive control: bacteria with the transformed plasmid plated in medium without antibiotics (plate full of bacteria, no single colonies).

Do you sequence?

As we mention in the acknowledgments, we got the plasmids as a gift from Dr. Elisha Hass Lab. We sequenced the plasmid before performing the transformation and verified that we have the correct gene with mutation at position 56.

Do you perform colony PCR? Please include details.

No need for PCR colonies, since were doing transformation for protein purification and not for DNA/plasmid purification or mutagenesis.

12. 1.1.2: Do you pick single colonies?

Yes.

Do you first make small scale culture before moving on to 4 L batch?

Yes, we corrected this step. Thank you.

13. Please include volume and concentrations of all the solutions/reagents/buffers used in the protocol. e.g., IPTG volume and concentration?

The volumes and concentrations of all solutions/reagents/buffers are now detailed.

14. 1.1.25: How do you check for the salt gradient in this case?

We assume the editors mean to ask a question regarding the salt gradient in the anion exchange chromatography. If indeed this is the case, our answer is that this is trivial and lies at the heart of chromatography. A gradient-based chromatography (any type of chromatography, for that matter) mixes two solutions at continuously changing fractions. In our case, the solutions are buffer without salt, and buffer with salt, and by that, each moment a higher dilution of the salt (hence a higher salt concentration) is introduced to the column. The chromatography controlling software (any such software – very trivial) allows the user to introduce a plan of change in fraction of the two solutions, from a given moment, to the next given moment (say, within an hour, or any other chosen duration), where the fraction of the two solutions will change (say, from 100% solution A to 100% solution B). This is how a chromatography gradient is defined.

Nevertheless, we have now added more details to fully describe the chromatographic gradient used.

15. 1.2.4 - 1.2.6: How do you perform the calculation?

We have now added an example for the calculation.

16. 1.2.12: What do you do after loading?

Running the gradient over the column, and collecting the sample when it elutes.

17. 3.1.1: How do you determine what is relevant in case of photon stream?

The main text describes the burst search and selection algorithm in depth (although it has been covered many times in the last two decades), and this procedure is what filters fluorescence photons from background ones, within the photon stream in the raw data.

18. 3.2: How are each of the steps performed in this case? Please include the actions being performed - Button clicks if any, command lines, etc. If using large scripts, please include it as a supplementary file.

For that purpose, we provided the Jupyter notebooks (see in the supplied links to the Zenodo repository, holding the Jupyter notebook files). The notebooks are very detailed and filled with metadata and explanations. Beyond that, this is standard burst photon statistics, which is described in the text where relevant.

19. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the filmable text **in yellow**, already. Please do not confuse the yellow highlight (filmable content) from the cyan highlight (to show the reviewers the text we revised.

20. Please ensure the results are described in the context of the presented technique, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

This has already been taken care of in the submitted text.

21. Please remove all the figure legends from the figure and include all the Figure Legends together at the end of the Representative Results in the manuscript text.

We would like to thank the editor for pointing this out. We have now removed all figures from the main text, but left the text of the figure legends at the bottom of the manuscript, below the references.

22. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

All of the figures in the manuscript are figures we originally produced. Again, have a look at the Jupyter notebooks, linked in the main text as deposited in the Zenodo repositories.

23. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol.

Added

b) Any modifications and troubleshooting of the technique

All the modifications of smPIFE are thoroughly introduced in the protocol as well as in the Jupyter Notebooks, which even further detail the data analysis (that is already well-described in the main text, regardless).

c) Any limitations of the technique.

These items were already discussed in the original text.

d) The significance with respect to existing methods

These items were already discussed in the original text.

e) Any future applications of the technique

We would like to thank the editor for pointing this out to us. Although future directions of using smPIFE were a part of the submitted text, we have now added additional future directions.

24. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately (all panels combined to form one image file) to your Editorial Manager account.

We have removed all figures from the main text and left the text of the figure legends at the bottom of the main text.

25. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

All trademark and registered symbols have now been removed from the Table of Equipment and Materials as well as sorting all items in the table, as requested.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript covers three procedures: 1) Protein preparation, which includes production of a recombinant protein and labeling this with a fluorescent dye, Cy3. 2) Measurement, which includes confocal microscope experimental setup, sample preparation, and data acquisition. 3) Data

analysis, which includes data conversion, and burst search and selection. These procedures are used to prepare a cysteine-modified alpha-synuclein (A56C) and labeling it with Cy3 for measurement of single bursts of fluorescence whose lifetime and quantum yield depends on the interaction of the dye with the protein surface. The premise is that short lifetimes and low quantum yields correspond to one conformation of the protein while long lifetimes correspond to another. The success depends on two factors: being able to measure the molecules one at a time (giving rise to a burst of fluorescence) in a confocal volume in which it resides less that 100 ms; and being able to select the bursts so that they can be analyzed for their lifetime and their separation in time. The theory is that if you observe one average lifetime, then the conformations exchange during the time spent in the beam, but if two lifetime distributions are observed, then these conformations are longer lived, and presumably represent more stable conformers. Moreover, if the burst are separated by less that about 100 ms, then they are likely to present measurements from the same molecule, so analysis of the lifetime distributions in consecutive burst will give an indication of the timescale of the interconversion. In these experiments, the authors do indeed find two distributions of lifetimes and these can change between consecutive bursts, supporting the theoretical framework and demonstrating that the alpha-synuclein can exist in reasonably stable conformations on the 10-100 ms timescale even though it is an intrinsically disordered protein.

We would like to thank the reviewer for the appreciation of our work.

The protein preparation procedure is described in some detail, the measurement procedure in better detail, and the analysis procedures in great detail. In the opinion of this reviewer, the most important procedures to demonstrate are the measurement and analysis procedures. The reasons for this opinion are that the protein preparation, purification, and labeling will be specific to the protein of choice and presumably the intent here is to allow use of this approach to single molecule protein induced fluorescence enhancement (smPIFE) on any protein of interest, not just alpha-synuclein. Thus the focus should be on that technique, rather than the protein preparation.

Major Concerns:
The protein preparation procedure is riddled with ambiguities with respect to amounts of reagents and volumes used and this must be fixed. For example, it appears that the cell growth is done in 4 L batches, and then centrifuged in 80 50 mL Sorval bottles. Does this make sense? How do you combine the pellets from 80 tubes for freezing? Is 40% sucrose by weight or volume? How much lysis buffer do you use in step 1.1.6 and therefore what volume erlenmeyer do you use in step 1.1.7? How much streptomycin sulfate do you dissolve in a small amount of buffer A in step 1.1.13 in order to get 10 mg per 1 mL solution. At this point it is unclear what the total volume is that is divided into 50 mL tubes in step 1.1.19 and hence it is not clear what is meant by adding 20 mL per 1 L growth in step 1.1.21.
Further, in step 1.2.2. the duration of dialysis is not specified. There is not

enough information to allow the calculations proposed in steps 1.2.4, 1.2.5, 1.2.6 since the desired concentration is not specified.

We ahave now dded details and examples to make it clearer, Thank You.

In section 2.1 the emission pinhole is defined, but the excitation pinhole, which defines the confocal volume and hence the residence time of the molecule during the burst, is not specified.

The confocal volume does not only define the residence time of the molecule per se, but also the residence time that yielded a high enough fluorescence photon rate and signal-to-background ratio, which is well controlled by the emission pinhole. Regardless, the tight focusing of excitation light is achieved by illuminating the back aperture of a high numerical aperture apochromat water objective lens with the collimated laser excitation light. The excitation pinhole that the reviewer is seeking is partly achieved by that. However, even before the laser light reaches the objective lens, it is routed through fiber optics that are narrow enough to induce an excitation pinhole. All of the above are quite trivial confocal microscopy principles, and since the focus of the methods' paper is not relevant here, the setup description we supplied is sufficient. Therefore, although we thank the reviewer for this comment, we decide that adding additional text would not be appropriate in this case. It would be appropriate if the new method had shown new components in the setup itself.

It is hard to evaluate the effectiveness of the Burst Search and Selection procedure without having the software interface available, but it appears to be quite logical and exhaustive.

We would like to thank the reviewer for bringing up this point, however we do refer the reader to FRETbursts, which is also highly documented, years before this submission, and it is outside the scope for this manuscript to re-describe the software. As for the interface, we use Jupyter Notebooks that detail the whole data analysis pipeline, and refer the reader to the Zenodo repositories that contain the notebooks, for everyone to observe them, re-use them, or learn from them. Not only do they detail the analysis, but also provide metadata and descriptions of the step.

Within this description, there is a discussion of a two-dimensional plot to determine whether the lifetimes change between recurring molecules. It would have been good to see an example of this.

We appreciate this point. In fact, Fig. 2 is now the representation of the burst recurrence analysis results, instead of a 2D histogram we initially wanted to show. We decided that since our data includes scarce occurrences of pairs of bursts from the same molecule, a 2D histogram representation will be too sparse and not so informative as to the underlying 10-100 ms dynamics.

It may have been better to have a screen shot of the software interface for the burst selection and analysis processes to give the reader a sense of how easy it is to perform these tasks.

See above response regarding the online deposited Jupyter noteboos. The reviewer is invited to have a deep look at them.

The authors describe the technique as able to "report on data mapping to distances between a dye and a protein surface in the shorter 0-3 nm range". Technically, this is not a ruler in the same sense that FRET is at the longer distances since there is no evidence presented here that there is a spatial relationship between the dye and the protein. Rather, it is an effect that is a result of the protein surface interfering with the rate and extent of conformational change in this particular dye from trans to cis and back again. The language in the manuscript should be changed to reflect this, both in the abstract, the introduction, and the results.

Although the reviewer is correct in the sense that PIFE does not sense point-to-point distances, we clearly state (even in the text the reviewer cites) that it is a one-to-many sensor. We would like to refer the reviewer to the papers we cite when mentioning PIFE as a one-to-many 0-3 nm ruler. This has been shown and quantified already in the cited literature.

Nevertheless, in order to render the language, we have now introduced the more general term, “proximity sensor” for smPIFE, wherever required in the text, without removing overall this discussion about rulers.

As a methodology paper, it might be good to include a more detailed presentation of the interpretation of the data, and the limitations of the method.

The reviewer is of course correct, however both type of content are well covered in the text. Although it is a methodology paper, using smPIFE we recover important biochemical information that is very difficult to be uncovered by other techniques, and hence we believe that the balance between the scientific finding content and the methodology content presented in our manuscript is proper. Shifting the balance the manuscript content even more towards methodology would result in a very technical and dry manuscript. Therefore, we leave the manuscript content and narrative as is.

Minor Concerns:
The manuscript needs some editing for style and clarity.
What is LB in step 1.1.2?

(Luria-Bertani) liquid medium – added to the text.

Reviewer #2:
Manuscript Summary:
This paper focuses on the study of the molecular structure of alpha-synuclein using a novel FRET technique, named PIFE, that has the advantage to be

able to register structural variation of micro domains in proteins, with size smaller than 0-3nm. Both technique and the results are described clearly. The amount of references is appropriate.

We would like to express our gratitude for the positive view the reviewer has shown for our current work

Major Concerns:
The authors should list also the limitations of PIFE besides the advantages, in the introduction or discussion paragraph.

We would like to refer the reviewer back to text, as we already mentioned the limitations in the text.

Minor Concerns:
I would suggest to add the expected volumes, concentrations or calculations in sections 1.2 and 3.2. I mean when the authors say "Calculate" they should also indicate a value as example so the reader or scientist knows the range of values to expect when performing this technique.

We have now added examples for the calculations. Thank You!

Also it would be nice if the authors could comment on the possibility to use this technique in live samples, such as cell cultures as regular FRET methodologies with fluorescent proteins do.

It is complicated to perform PIFE measurements in live cells, due to the different viscosities inside the cells, so PIFE in the cell will report both on macromolecular structural effects and on the different viscosities in the cell. This could have been relevant if biological cells would have exhibited uniform densities, leading to uniform viscosities, which is far from the truth – the cell is highly heterogeneous.