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Detection of Protein Aggregation using Fluorescence Correlation Spectroscopy

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Abstract

Protein aggregation is a hallmark of neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and so on. To detect and analyze soluble or diffuse protein oligomers or aggregates, fluorescence correlation spectroscopy (FCS), which can detect the diffusion speed and brightness of a single particle with a single molecule sensitivity, has been used. However, the proper procedure and know-how for protein aggregation detection have not been widely shared. Here, we show a standard procedure of FCS measurement for diffusion properties of aggregation-prone proteins in cell lysate and live cells: ALS-associated 25 kDa carboxyl-terminal fragment of TAR DNA/RNA-binding protein 43 kDa (TDP25) and superoxide dismutase 1 (SOD1). The representative results show that a part of aggregates of green fluorescent protein (GFP)-tagged TDP25 was slightly included in the soluble fraction of murine neuroblastoma Neuro2a cell lysate. Moreover, GFP-tagged SOD1 carrying ALS-associated mutation shows a slower diffusion in live cells. Accordingly, we here introduce the procedure to detect the protein aggregation via its diffusion property using FCS.

Introduction

Protein aggregations involving neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Huntington's disease, and so on[1] are known to be toxic and would disturb protein homeostasis (proteostasis) in the cells and organs, that could then lead to aging[2]. The clearance of protein aggregation is expected as a therapeutic strategy; however, chemicals that prevent protein aggregation formation and degrade protein aggregates (e.g., small molecules or drugs) have not been established yet. Moreover, how protein aggregation exerts toxicity remains elusive. Therefore, to promote research projects related to protein aggregation, it is important to introduce high throughput procedures to simply detect protein aggregation. Protein aggregation detection using antibodies recognizing the conformation of protein aggregation and aggregation-specific fluorescent dye has been widely used[3].

However, it is difficult to detect the aggregation, especially in live cells using such classical procedures.

Förster resonance energy transfer (FRET) is a procedure to detect protein aggregation and structural change. However, FRET is unable to analyze protein dynamics (e.g., diffusion and oligomerization of protein in live cells)[3]. Therefore, we introduce here a simple protocol to detect protein aggregation in solution (e.g., cell lysate) and live cells using fluorescence correlation spectroscopy (FCS), which measures the diffusion property and brightness of fluorescent molecules with single molecule sensitivity[4]. FCS is a photon-counting method by using a laser scan confocal microscope (LSM). Using a highly sensitive photon-detector and calculation of autocorrelation function (ACF) of photon arrival time, the pass through time and brightness of the fluorescent molecules in the detection volume is measured. The diffusion slows with an increase of molecular weight; thus, intermolecular interaction can be estimated using FCS. Even more powerfully, an increase in the brightness of the fluorescent molecule indicates homo-oligomerization of the molecules. Therefore, FCS is a powerful tool to detect such protein aggregation.

Protocol

1. Materials and reagents

1. Use pyrogenic free solutions and medium for cell culture (**Table 1**).
2. Prepare solutions for the biochemical experiment using ultrapure water and use as DNase/RNase free.
3. Select an appropriate FBS for the cell culture with a lot check process. Since the selected FBS lot changes regularly, the catalog and lot number for FBS cannot be represented here.
4. Plasmid DNA
    1. Prepare pmeGFP-N1[5] for eGFP monomer expression; pmeGFP-C1-TDP25[6] for GFP-TDP25 expression; pmeGFP-N1-SOD1-G85R[7] for SOD1-G85R-GFP expression; and pCAGGS[8] as a carrier.
    NOTE: meGFP is a monomeric variant carrying A206K mutation of enhanced GFP (eGFP). TDP25 is an ALS-associated C-terminal fragment of TDP-43. SOD1-G85R is an ALS-associated mutant of SOD1.
5. Cell strain
    1. Use murine neuroblastoma Neuro2a cells.
    NOTE: Neuro2a cells have high transfection efficiency and highly express exogenous proteins. For TDP25 expression, cell strains having high expression efficiencies such as Neuro2a or HEK293 cells are required. In HeLa cells, TDP25 was not able to be efficiently expressed.

2. Cell culture and transfection

1. Prepare a 100 mm plastic dish growing Neuro2a cells semi-confluently in normal growth medium.
NOTE: It is necessary to incubate at 37 °C for approximately 48-72 hours after the previous seeding until semi-confluency is reached.
2. Remove the medium.
3. Add 0.5 mL of trypsin-EDTA solution into the cell-culture dish and incubate them at 37 °C for 1 min.
4. Add 9.5 mL of normal growth medium into the dish and suspend the detached cells.

5. Using Trypan blue to stain the dead cells, count the number of cells using a cell counter or manually. Then dilute the cells in culture medium (1.0×10^5/mL).
6. Add 2 mL of cell suspension into a 35 mm plastic dish for cell lysis or a glass base dish for live-cell measurement.
7. Incubate the dish at 37 °C for 1 day.
8. Start the following preparations 15 minutes before the day of the transfection.
9. Prepare two 1.5 mL tubes and add 100 µL of Opti-MEM I to each tube.
10. In the first tube, mix 1.0 µg of plasmid DNA (Solution A). In the second tube, mix 2.5 µL of Lipofectamine 2000 (Solution B).
    NOTE: To maintain transfection efficiency, keep the total DNA amount the same. To reduce the expression level for FCS measurement in live cells, the fraction amount of plasmid DNA for protein expression should decrease (e.g., 0.2 µg of pmeGFP-N1-SOD1-G85R and 0.8 µg of pCAGGS mixture). Moreover, keep the same ratio between the volume of Lipofectamine 2000 and the amount of plasmid DNA.
11. Gently mix Solution A and B by adding one into either tube, and then incubate it for 1 min at room temperature (Solution C).
12. Add the Solution C to the culture medium; and incubate the cells for 24 h at 37 °C.

3. Cell lysis & medium exchange

1. Check the GFP expression using a routine microscope.
2. Remove the medium in the dish.
3. Add 2 mL of PBS at 25 °C to wash out the medium. Remove the PBS.
4. Place the dish on an aluminum plate on top of the crushed ice.
    NOTE: We use a 1 mm thick aluminum plate cut in a Sunday tool store or commercially available one as laboratory equipment.
5. Add 200 µL of lysis buffer at 4 °C. Shake the dish mildly so that the buffer is evenly distributed at the bottom of the dish.
6. Scrape the dish using a cell scraper and recover the lysate with undissolved cell debris in a new 1.5 mL tube.
7. Centrifuge the solution at 20,400 x *g* for 5 minutes at 4 °C.
8. Recover the supernatant in a new 1.5 mL tube and keep it at 4 °C or on ice.
    NOTE: Do not freeze the lysate.
9. For live cell measurements, replace the medium before the measurement. Check the cell attachment using a phase-contrast microscope. Confirm expression using a fluorescence microscope.

4. Fluorescence correlation spectroscopy (FCS) calibration

1. Start the system and run the operation software. Turn on the $Argon^+$ laser (458/477/488/514 nm). Stabilize the system at least for 30 min.
2. Set up the optical path: Main beam splitter: HFT488; Dichroic mirror: NFT600; Fluorescence barrier filter: a bandpass filter 505-540 (BP505-540); Detector: avalanche photodiode (APD)).
3. Set the pinhole size by entering the value directly (66 µm; 1 airy unit).
4. Add the Rhodamine 6G (Rh6G) solution into a well of the cover glass chamber on the stage.

5. Add the immersion ultrapure water on the objective. Do not use the immersion oil.
6. Set the the chamber on the microscope stage. Move the stage to the appropriate position.
7. Focus on the upper glass surface by measuring the scattered light from the glass surface. After lowering the objective lens to the bottom, turn the focus knob clockwise to adjust.
   NOTE: Check the turning direction of the focus knob because it is opposite between those made in Japan and Germany.
8. Raise the focal point 200 µm above the upper glass surface to observe the inside of the solution.
9. Click the **Count rate** and monitor the photon counts rate.
10. Gradually increase the acousto-optic tunable filters (AOTF) value (e.g., excitation laser transmission) so that the count rate value is more than 10 kHz.
11. Click the **Pinhole Adjustment** and open the pinhole adjustment wizard. Find the pinhole position with the highest (a peak) number of photons in both the x- and y-axis.
12. Turn the correction ring of the objective lens so that the counts per molecule (CPM) value is the highest.
    NOTE: Since the thickness of the cover glass of the chamber specified here is 0.12-0.17 mm, the correction ring is at around its minimum or just a few turns from it, where the CPM is at its maximum.
13. Decrease the AOTF value gradually so that the CPM value becomes 2-3 kHz.
14. Acquire the autocorrelation function (ACF) of Rh6G for 90 s.
15. Once measurement is complete, click **Fit** to perform curve fitting analysis.
16. Select a model for one-component three-dimensional (3D) diffusion with a triplet state.
    NOTE: The Rh6G is monodisperse and one-component diffuse with a triplet state.
17. Set the fitting start time by moving the red line. Click **Fit All** and check the fit deviation. After performing the curve fitting, make sure the diffusion time (DT) and the structure parameter (SP) are roughly in the range of 20-30 µs and 4-8, respectively.
18. Remember the structural parameter value. Use the SP value for curve fitting analysis of all ACFs measured on the same day, under the same optical conditions, and using the same type of glass base dish or chambered cover glass set on the microscope stage.

5. FCS measurement in cell lysate

1. Prepare the cell lysates (see above).
2. Place the lysate on the cover glass chamber. Place a lid to avoid drying and the stage lid for light shading.
3. Set the acquisition condition, laser power, measurement time, and repetitions.
4. Click **Count rate** and adjust the AOTF value of the laser so that the CPM value becomes more than 1 kHz.
5. Perform a trial measurement for 1 min. Check whether the calculated ACF shows a positive amplitude and smooth decay.
6. Perform the main measurement for 5 min.
   NOTE: The total measurement time is gradually increased until the shape of the ACF does not change even if the measurement time is increased.

7. Click Fit to perform curve fitting analysis.
8. Select a model for two-component 3D diffusion with a triplet state. Remember to enter the SP value and change its setting to "Fixed" not "Free" before clicking Fit all.
9. Export the fitted table as a tab-delimited text file.
10. If necessary, export the ACF and Count rate record as a tab-delimited text file.

6. FCS measurement in live cells

1. Replace the medium with a fresh one before the measurement.
2. Use a heat stage incubator. Set the cell-culture dish on the microscope stage.
3. Confirm the focus and position using the confocal micrope. Select the measuring cell. Zoom in and adjust the cell position. Acquire fluorescence images of a GFP-expressing cell using the slow scanning speed mode.
4. Select an FCS-measuring position using **Position** in the "FCS" section.
5. Select at least one FCS measurement point using a crosshair (**Figure 1**).
6. Measure the ACF for 1 min.
   NOTE: Because fluorescent proteins tend to be photobleached in live cells due to slow movement compared to the solution, the measurement time should be minimum. It is generally difficult to obtain ACF in the cell as smoothly as solution measurements because prolonged measurement will lead to photobleaching of fluorescent proteins.
7. Click **Fit** to perform curve fitting analysis using a model for two-component 3D diffusion with a triplet state.
   NOTE: For live cell measurements, a model for two-component 3D diffusion with a triplet state would be better because it is empirically difficult to decrease the chi-square value with a one-component 3D diffusion model because of the existence of various mobile components in the live cell. But even using a model for three-component 3D diffusion, the diffusion components are usually not separated compared to using the model for two-components.

Representative Results

We performed FCS measurement of GFP-TDP25 in cell lysate and SOD1-G85R-GFP in live cells. In both cases, a positive amplitude and smooth ACFs were able to be acquired. We have shown that a portion of GFP-TDP25 expressed in Neuro2a cells was recovered in the soluble fraction under the indicated condition[6]. In the soluble fraction of the cell lysate, extremely bright fluorescence molecules were detected in the photon count rate record using FCS (**Figure 2A**, top, arrow). Such "spikes (also called burst)" were not observed in GFP monomers and monodisperse chemical fluorescent dye solution, suggesting that the spikes indicate oligomeric proteins[9]. Curve fitting analysis using a model assuming two-component 3D diffusion with a triplet state showed that the fast diffusing molecules (DT_{Fast} = 186 µs) were ~90% and the remaining 10% was 2.3 ms (**Figure 2A**, bottom; and **Table 2**).

During the SOD1-G85R-GFP measurement in live cells, the photon count rate record showed a gradual decrease, suggesting its photobleaching in the detection volume (**Figure 2B**, top). Although the contribution of the photobleaching was shown in a longer than 1 s range in the ACF, a positive amplitude and smooth decay of the ACF were observed (**Figure 2B**, bottom). Curve fitting analysis

using a model assuming two-component 3D diffusion with a triplet state showed that the fast diffusing molecules (DT_{Fast} = 397 µs) were ~93.4% and the remaining 6.6% was 12.3 ms (**Table 2**). ALS-linked mutation, G85R, in SOD1 did not allow a dramatic difference in diffusion property compared to wild type. However, proteasome inhibition decreased the diffusion rate only in the G85R mutant of SOD1 in the cytoplasm[7].

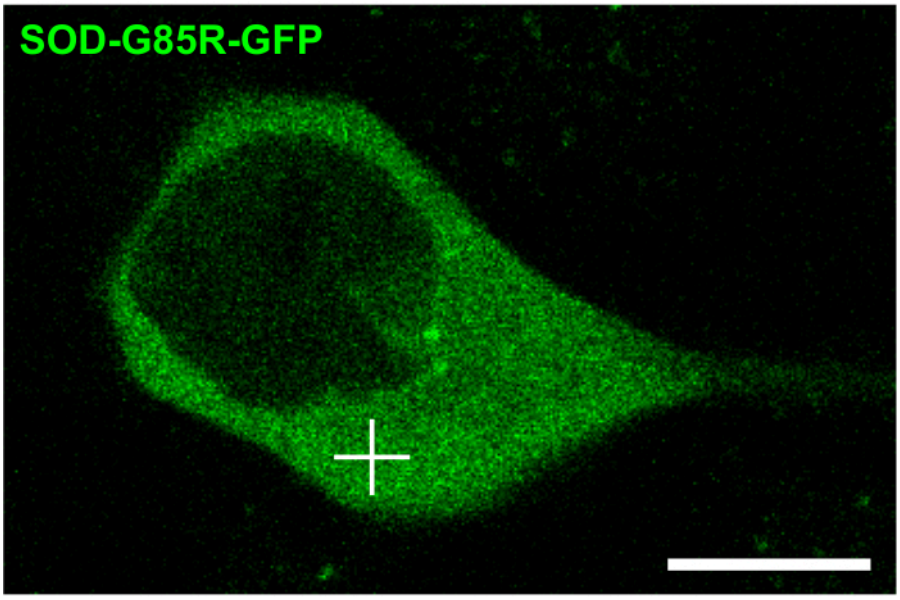

Figure 1: Confocal fluorescent image of Neuro2a cells expressing SOD1-G85R-GFP. Confocal fluorescent image of Neuro2a cells expressing SOD1-G85R-GFP. The crosshair indicates the FCS measurement position in the cytoplasm. Bar = 10 µm. Please click here to view a larger version of this figure.

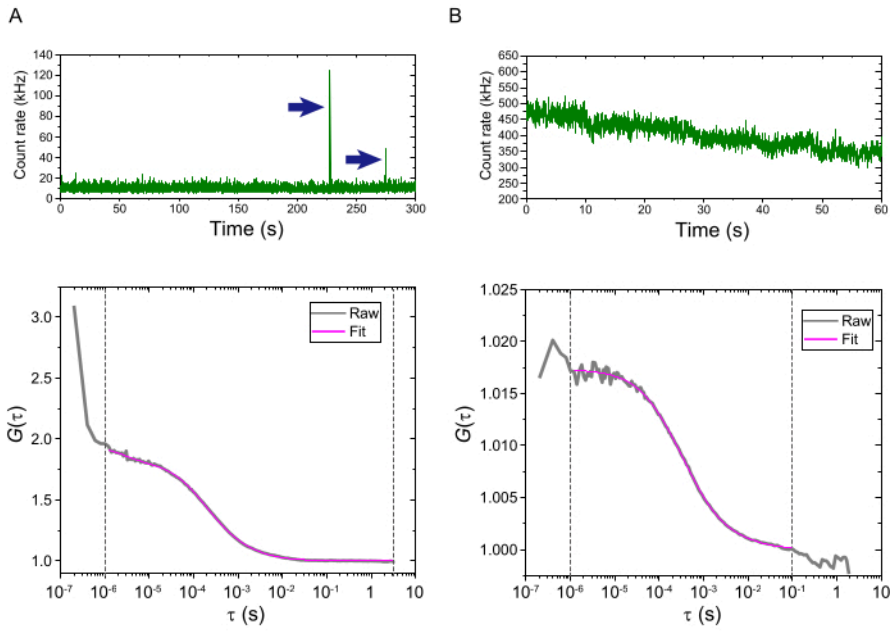

Figure 2: Typical FCS results and fitted curves for the autocorrelation functions. (**A** and **B**) Top: Recorded photon count rate in the FCS measurement time range (Green line). Bottom: Calculated autocorrelation functions (ACFs; Raw, grey lines) and fitted ACF curves using a model for two-component three-dimensional diffusion with a triplet state (Fit, magenta lines). *G*(τ) for Y-axis indicates the amplitude of ACFs at time τ sec. for X-axis. Dot lines show fitting start and end time points. Dark blue arrows show the spike with extremely bright proteins passing through the detection volume (i.e., soluble oligomers/aggregates). Please click here to view a larger version of this figure.

Name of Material/Equipment	Components	Comments/Description
0.1 mM Rhodamine 6G solution.		
1 M HEPES-KOH pH 7.5		
2 M Sodium chloride (NaCl)		
Lysis buffer	50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1% Triton X-100, 1× protease inhibitor cocktail	Protease inhibitor cocktail should be mixed just before the cell lysis.
Normal growth medium	DMEM supplemented with 10% FBS and 100 U/mL penicillin G and 100 mg/mL Streptomycin	Lot check for FBS should be required.
Phosphate buffer saline (PBS)	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4	

Table 1: Solution compositions

	CPM (kHz)	DT_{Fast} (ms)	Fast component (%)	DT_{Slow} (ms)	Slow component (%)
GFP-TDP25 in lysate	7.9	186	89.7	2.3	10.3
SOD1-G85R-GFP in live cell	6.3	397	93.4	12.3	6.6

Table 2: Typical fitted values for the autocorrelation functions

Fitted values for the autocorrelation functions are represented in **Figure 2** using a model for two-component three-dimensional diffusion with a triplet state. Counts per molecule (CPM), fast and slow diffusion time (DT_{Fast} and DT_{Slow}, respectively), and their components (Fast and Slow component) are represented.

Discussion

Regarding system calibration before measurements, the same glasswares as the one used to measure the sample should be used (e.g., the 8-wells cover glass chamber for cell lysate and the 35-mm glass base dish for live cells). Because of the adsorption of Rh6G on the glass, its effective concentration may sometimes decrease. If so, a highly concentrated Rh6G solution such as 1 µM should be used just for the pinhole adjustment. Extremely high photon count rates must be avoided to protect the detector (e.g., more than 1000 kHz). Moreover, the concentrated solution is not appropriate

for the acquisition of autocorrelation function (maintain less than 100 kHz). The calibration of pinhole position is important. If the optical system is used frequently, it is rare for dramatical dislocation of the pinhole; thus, using "Fine" at the beginning is often no problem to find the appropriate position. If no peak position of the count rates using "Fine" is found, use the "Coarse" to move the pinhole from one end of the range of motion to the other before the position-finding using "Fine". If the amplitude of the ACF was flat, check whether the focus and position are appropriate.

After the Rh6G measurement and its fitting, if the DT and/or SP is out of the indicated range, re-try the pinhole and correction ring adjustment. When still showing out of the range, we recommend contacting the manufacturer's support as it is likely due to suspected defection of the optical system. Using the measured DT and SP in addition to the known diffusion coefficient of Rh6G (414 $\mu m^2/s$) in water, the effective beam waist can be calculated because DT is dependent on the beam waist[10]. Moreover, as the SP is the ratio of beam waist and the height of the effective detection volume, the detection volume can be calculated. The volume calculation is required to determine absolute concentration. More description of the principle and troubleshooting during the calibration is also available in protocols as reported previously[11,12,13].

Some spikes were observed in the FCS measurement of GFP-TDP25 in cell lysate (**Figure 2A**, top). We show that such spikes were observed in FCS measurement of aggregate-prone huntingtin including expanded polyglutamine repeats labeled with GFP or yellow fluorescent protein (YFP) (HttQ78 and HttQ143)[9]; it thus suggests soluble oligomers/aggregates of GFP-TDP25 in the soluble fraction of the cell lysate. But such spike population was rare; thus, the ACF and its curve-fitting result may not include a major contribution of such spikes. A possible reason for only a small amount of aggregates being included in the soluble fraction is likely because TDP25 is highly aggregation-prone and its aggregates are fractionated in the insoluble fraction as shown using a fractionation of the cell lysate followed by western blotting detection[6]. The phenomena of the tendency to recover to the insoluble fraction of aggregation-prone protein have been often observed[6,9]. Such soluble oligomers/aggregates cannot be easily detected using conventional biochemical methods such as SDS-PAGE followed by western blotting; thus, FCS has an advantage. However, to analyze multi-components using conventional FCS is difficult because it measures the average population. More developmental procedures combined with Bayesian nonparametric analysis would determine the multi-components in the sample without any assumptions for the components[14].

The diffusion time in live cells was relatively slow compared to the cell lysate. Since viscosity in the cell is known to be higher than that in solutions such as PBS and detergent-containing buffer[15], the diffusion time in live cells theoretically gets 2.5-3 times longer. Due to this slow diffusion in live cells comparing in solution, photobleaching of fluorescent tags can often be caused. To correct the photobleaching effect on ACFs, some procedures such as exponential decay assumption[16] and noise filtering using wavelet function have been proposed[17]. Although such corrections are believed to be effective, it has still not been so simple for general users because it requires programming skills. Moreover, in live cell measurements, the fitting range should be determined more carefully so that the chi-square value of the fitted curve becomes small. As shown in **Figure 2B**, the range where G(τ) was less than 1 was excluded from the fitting range. Alternatively, more

photostable fluorescent tags are required to analyze slowly diffusing/moving molecules. GFP is easy to use as a labeling tag, and its stability is well, but it is often photobleached during FCS measurements. To overcome this photostable property, HaloTag with tetramethylrhodamine (TMR) as a chemical fluorescent dye has been available[18]. However, incomplete labeling by the fluorescent ligands (e.g., TMR-ligand) and trapped dye pools are problematic issues for specific labeling of proteins of interest[19]; thus, exogenous expression of protein-of-interest tagged with fluorescent proteins would be the first choice for fluorescent labeling in live cells.

There are many kinds of fluorescent proteins, as well as chemical fluorescent dyes; however, as shown in this article, monomeric enhanced GFP (meGFP) is an easy-to-use tag for FCS as well as other fluorescence microscopy because its biochemical and fluorescence properties are well known. Therefore, in our studies, meGFP is the first choice and generally used to label the aggregation-prone proteins for FCS measurements[6,7,9].

Disclosures

These authors have no conflicts of interest.

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