Size analysis of polyglutamine protein aggregates using fluorescence detection in an analytical ultracentrifuge

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Running title: Sedimentation analysis using fluorescence detection

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SUMMARY

Defining the aggregation process of proteins formed by poly-amino acid repeats in cells remains a challenging task due to a lack of robust techniques for their isolation and quantitation. Sedimentation velocity methodology using fluorescence detected analytical ultracentrifugation is one approach that can offer significant insight into aggregation formation and kinetics. While this technique has traditionally been used with purified proteins, it is now possible for substantial information to be collected with studies using cell lysates expressing a GFP-tagged protein of interest. In this chapter, we describe protocols for sample preparation and setting up the fluorescence detection system in an analytical ultracentrifuge to perform sedimentation velocity experiments on cell lysates containing aggregates formed by poly-amino acid repeat proteins.

1. INTRODUCTION

The biochemical characterization of protein aggregates formed by tandem amino acid repeats is challenging due to the size and often insoluble nature of the aggregates, as well as the lack of robust techniques for their separation and quantitation. Recent developments in analytical ultracentrifugation methods, however, have opened the way for more precise measurements of such aggregates. The analytical ultracentrifuge has had a long history in studies of protein self-assembly. Sedimentation velocity and equilibrium experiments on the analytical ultracentrifuge have been widely used for characterizing protein complexes, protein-protein interactions [1-3], and more specifically for the analysis of amyloid oligomers and fibrils [4]. The development of a commercial fluorescence detection system (FDS) for the analytical ultracentrifuge has further extended the sensitivity and specificity of these techniques [5,6]. The FDS is well suited for exploring the sedimentation behavior of fluorescently-labeled proteins over a broad range of concentrations. Of particular interest is the development of green fluorescence protein (GFP) constructs as *in vivo* fluorescence tags, permitting the use of sedimentation velocity studies and fluorescence detection for the analysis of protein aggregation and fibril formation in cell lysates and *ex vivo* [7].

A particularly useful feature of the FDS is the capacity to study how proteins aggregate in the context of their natural environment, such as in cells or in extracellular fluids provided the solutions are not (overly) turbid. Early published work with the FDS showed that GFP could be detected specifically in solutions containing high concentrations of a “background” protein (bovine serum albumin), which demonstrated the capacity for its application in complex solutions [5]. FDS was also shown to be useful for the study of fluorescent “tracers” in serum and *E.coli* lysate and for monitoring antibody binding to antigens [6]. More recently, sedimentation velocity experiments using the FDS has been used to monitor the aggregation kinetics of huntingtin protein in mammalian cell lysates [7], successfully resolving three differently-sized populations that exist in cells.

The present chapter will present protocols for sample preparation and setting up the FDS unit in the analytical ultracentrifuge for analysis. The concept and theory behind sedimentation velocity experiments on the analytical ultracentrifuge has been detailed in reviews elsewhere [8,4,9] and will only be perfunctorily reviewed here. Briefly, the sedimentation velocity technique monitors the radial concentration distributions (boundaries) of solutes during movement through a centrifugation field in a sample compartment over a period of time. Figure 1 illustrates a typical sedimentation velocity experiment showing a sample

compartment containing the protein sample. At the start of the experiment (t0), the protein fluorescence intensity along the distance of the cell is uniform. At various times (i.e., t1, t2) of sedimentation, at constant speed and temperature, the boundary moves down the cell as the solute is depleted from the sample meniscus. Macromolecules of different size and shape will yield a different rate of sedimentation and pattern of sedimentation. The overall sedimentation profile can thus be fitted to known sedimentation models to yield a size distribution plot that describes the size and proportion of all the species in a sample solution. Use of SEDFIT software to perform experimental data analysis and model fitting has been detailed elsewhere and the reader is again directed to them [10,9,11].

2. MATERIALS

1. Mammalian cells expressing the protein of interest tagged to GFP. (More details for the design of the system are explained in Section 3.1)

2. Phosphate-buffered saline

3. Liquid nitrogen or a dry/ice ethanol bath

2. TX buffer : 20 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 150 mM NaCl, 1% w/v Triton-X100, benzonase nuclease (Merck, Germany) and a complete EDTA-free protease inhibitor cocktail tablet (Roche Applied Science). For bead lysis, the buffer can omit Triton X-100.

2. 27 Gauge needle and syringe.

3. 0.5 mm Zirconia/Silica beads (DainTree Scientific, Australia) in vials with an O-ring and a screw-cap.

4. Fluorescence plate reader

3. Bicinchoninic acid kits (eg from Thermo Scientific).

4. Sucrose.

5. Digital refractometer.

6. Beckman XL-I or XL-A analytical ultracentrifuge equipped with an AVIV Biomedical (Lakewood, NJ) fluorescence-detection system (FDS). See **Note 1**.

7. Heavy mineral oil (FC43 oil, Fluorinert).

3. METHODS

The presently available commercial FDS is equipped with a 488 nm excitation laser, with emission from the sample directed through a pair of 505 nm cut-off filters and into a photomultiplier tube for signal digitization. Thus, the FDS is suitable for detection of dyes such as fluorescein and GFP, as well as other fluorophores with compatible excitation and emission properties.

3.1. Tag the protein of interest with GFP or an appropriate fluorophore

The fluorescence properties of GFP are well suited for tracking proteins expressed in mammalian cells using the FDS. Other fluorescent proteins – notably cyan fluorescent protein (CFP) – are not sufficiently bright when used in mammalian cell lysates with the standard FDS optics. However, CFP is adequate for detection of purified proteins using the FDS, albeit with far less sensitivity than GFP. It remains to be tested how well other variants such as yellow fluorescent protein (YFP) work. An important consideration is that a monomeric form of GFP is used, such as EGFP containing the A206K mutation, since many early GFP derivatives are known to oligomerize [12]. We have found that the Emerald form of GFP, which contains the mutations S72A, N149K, M153T, I167T, A206K on the original EGFP [13], is a bright and particularly useful construct for sedimentation analysis using the FDS.

3.2. Protein expression.

1. As a starting point for relatively abundantly expressed proteins (e.g. using a cytomegalovirus promoter to drive high yielding expression), about 2.5×10^6 transiently transfected mammalian cells, or a confluent 25 cm^2 plate, should be sufficient to prepare 2-4 samples for sedimentation analysis using total cellular protein concentrations of 0.2-0.5 mg/mL. We generally use Neuro2A cells for expressing polyglutamine proteins, but have found that higher levels of expression can be obtained with HEK293 cells.
2. Harvest cells expressing the fluorescent protein of interest by scraping culture flasks in phosphate-buffered saline.
3. Pellet harvested cells by centrifuging at approximately 250 g for 5 minutes.
4. Snap-freeze the pellet in liquid nitrogen or a dry/ice ethanol bath at -80ºC until lysis to avoid protein degradation.

3.3. Triton X-100-mediated cell lysis.

1. To lyse cells, thaw the cell pellets on ice. The following steps should be performed on ice with ice-cold buffers. Lyse cells by extrusion through a 27 Gauge needle in TX buffer.
2. Snap-freeze using liquid N_2 and store at -80ºC until further use.

3.4. Mechanical cell lysis.

Some weaker self-associations in protein aggregates may be perturbed by the presence of Triton-X100. In this case, we have alternatively used 0.5 mm Zirconia/Silica beads to lyse cells mechanically. This alternative protocol is detailed below.
1. Resuspend cell pellet in TX buffer lacking Triton-X100.
2. Once resuspended, divide cells into two 0.5 mL aliquots. Add each to separate 2 mL polystyrene screw cap vials containing an approximate 0.5 mL volume of beads. For efficient lysis, a similar ratio of beads to sample and a similar ratio of bead/sample to air is essential.
3. Vortex the samples 6 times for 45 seconds each, with intervals of 30 seconds on ice. Samples should be kept on ice to avoid heating of the sample.
4. Transfer lysate into 1.5 mL microcentrifuge tubes using a 27 Gauge needle to avoid transfer of the beads.
5. Snap-freeze the samples and store at -80ºC until further use.

3.5. Standardization of experimental parameters including lysate total protein concentrations.

1. If the protein (or oligomer) of interest is known to be entirely soluble, then spin the samples at 5,000 g for 30 minutes to reduce baseline turbidity. If the protein of interest is suspected to form large aggregates or complexes, or it is not known whether it does or not, do not spin the crude lysate as this may remove larger complexes from the sample and bias the analysis.

2. Measure the fluorescence of the supernatant (or whole cell lysate) using a fluorescence plate reader.

3. After matching the samples based on fluorescence, as an optional second control, expression levels can be matched/validated for degradation via Western blot quantitation.

4. A useful internal control for influences of lysate components on sedimentation behavior when comparing two or more proteins/samples is to analyze different experimental samples in exactly the same lysate concentrations. This can be established simply by determination and standardization of total cellular protein concentration (e.g. bicinchoninic acid protein

assays) and by adjusting the transfections to obtain standardized levels of protein-GFP expression or by diluting lysates with lysates from untransfected cells. See **Note 2.**

3.6 Viscosity adjustment with sucrose

A key step in the resolution of the very large inclusions formed by huntingtin and other poly-amino-acid repeat proteins is the addition of sucrose in the sample to increase its viscosity. This enables slower movement of inclusions through the sample compartment in the ultracentrifuge and allows their sedimentation to be monitored. For inclusions formed by huntingtin and other poly-amino acid repeat proteins, we have found that a final concentration of 2 M sucrose (≈ 55 % w/w) works well. See **Notes 3 and 4**.

1. Prepare an approximately 3M sucrose stock by weighing out 51.35 g of sucrose and topping up to 50 mL with purified water in a small glass beaker (preferably 100 mL capacity).
2. Place on a heating block and heat to 200 ºC for 10 min with constant stirring.
3. Transfer the dissolved solution to a 50 mL tube and leave to cool to room temperature.
4. Measure the refractive index of the sucrose solution using a digital refractometer. See **note 5**. The refractive index (RI) can be used to precisely calculate the molarity (M) of the solution and the subsequent weight and volume of the amount of sucrose solution required to create a final concentration of 2 M sucrose using the following formulae [14]:

$$Molarity\ (M) = 21.019RI - 28.036 \quad (1)$$

$$Density\ of\ sucrose\ (\rho) = 0.1257M + 1.0017 \quad (2)$$

$$Volume\ of\ sucrose\ to\ get\ final\ 2M = \left(\frac{2}{M}\right) * total\ volume \quad (3)$$

$$Weight\ of\ sucrose\ to\ get\ final\ 2M = Volume\ of\ sucrose * density\ of\ sucrose \quad (4)$$

5. Use the volume to calculate how much TX buffer is required to get to a final volume of 500 uL. Weigh out the sucrose solution (the solution at this stage is too viscous to pipet accurately) into 1.5 mL microcentrifuge tubes.
6. Aliquot the correct amounts of TX buffer into each microcentrifuge tube and add the corresponding amount of untransfected and fluorescent sample. In the case of 2M sucrose samples, layer the sample carefully on top of the sucrose.
7. When all components have been added, mix the samples gently using a 1000 uL pipette. Mix by stirring the sample with the pipette tip. Do not suck up any sample as it is very viscous when unmixed and will stick in the pipette tip. Subsequently, thoroughly resuspend

the sample by turning it upside down and flicking the sample repeatedly. Samples are now ready for analytical ultracentrifugation with the FDS.

3.7. Sedimentation analysis using the FDS

Depending on the nature of the aggregates, it may be necessary to perform a number of complementary sedimentation velocity experiments at different rotor speeds to capture the sedimentation properties of all the species of interest present in cells. See **Note 4**. Monomers (eg masses of ~40 kDa) and low mass oligomers in cell lysate are resolved by standard high speed experiments (50,000 rpm) and without using sucrose. Low speed experiments (3,000 rpm) capture the sedimentation of material up to about 5000 S in cell lysate, which is equivalent to masses on the scale of millions Da. A feature of polyglutamine aggregates is that substantially larger material may also be present. In this case, addition of 2 M sucrose significantly slows sedimentation rates and has been useful to monitor material up to about 320,000 S using a rotor speed of 3000 rpm [7]. Such material is sufficiently large (eg 1.5-8 μm in size) to move in and out of the laser focal point (~10 μm) and this behaviour appears as apparent noise in the data [7]. While this profoundly lowers the capacity for quantitative analysis of such large material, approximate sizes can be still be estimated. Comparison of the combinatorial sedimentation velocity experiments enables proportions of the different molecular forms to be calculated from the experimental data, effectively enabling aggregation kinetics to be tracked in cells.

1. Load 50 μL of FC43 heavy mineral oil into each compartment of the cell centerpiece using gel-loading tips. Heavy oil is required because scans to the base of the cell (>7.1 cm for a double-sector cell) suffer from significant attenuation in fluorescence intensity as the base is approached. This detection artifact is caused by the cell window holder and screw ring blocking an increasing proportion of the cone-shaped laser beam as it moves towards the cell bottom. Without the use of heavy oil, large aggregates that have sedimented to the bottom of the cell at early scan times may not be observed.
2. Layer 350 μL of sample on top of the heavy mineral oil in each sector of the cell centerpiece using gel-loading tips.
3. Seal the filling holes with provided plastic spacers and screws.
4. Insert and align the cells into the rotor with the filling holes facing the center of the rotor.
5. Install the Aviv FDS unit into the ultracentrifuge following Aviv-provided instructions.
6. Allow the vacuum in the rotor chamber to fall below 100 μm before turning on the laser.

7. Switch on the laser, initiate the provided AOS software and start the rotor spinning at 3000 rpm. See **note 6.**

8. When the laser is locked and the magnet angle of the rotor has been established, check the fluorescence signal of the samples, and adjust the gain setting for the photomultiplier tube accordingly. In our experience, data with the highest signal to noise ratio and most useful for sedimentation analysis is obtained when the gain is set to provide intensities above 400 counts (arbitrary units) and below 3000 counts. Detection of signal intensities is set to cut off at ~4000 counts to avoid saturation of the detector system.

9. Set the experimental parameters to the desired rotor speeds, temperature and running time. For monitoring large aggregates and inclusions in sucrose, a rotor speed of 3000 rpm is typically used. For oligomeric species, buffer that does not contain sucrose and a rotor speed of 3000 rpm is employed.

Figure 2A illustrates an example of the sedimentation profile of a GFP-tagged polyglutamine peptide (72Q) in 2M sucrose. This sample was spun at 3000 rpm where only very large aggregates and inclusions sediment. Approximately 50% of the total fluorescence signal is sedimenting under these conditions, indicating that about half of the sample consists of very large aggregates. In contrast, at a centrifugal speed of 50,000 rpm and with no sucrose in solution (Fig. 2B), very large aggregates and inclusions sediment to the bottom almost immediately and do not yield a detectable sedimentation profile. The remaining monomers and small oligomers sediment detectably at this speed.

10. Analysis of experimental data is performed using SEDFIT software (by Peter Schuck), which can be downloaded for free from http://www.analyticalultracentrifugation.com. See **notes 7-10.** To account for differences in solvent density and viscosity, sedimentation coefficients (S) can be converted in terms of a standard solvent (water at 20 ºC) ($S_{20,w}$) using the following formula:

$$S_{20,w} = S_{observed}\left(\frac{\eta_{T,w}}{\eta_{20,w}}\right)\left(\frac{\eta_s}{\eta_w}\right)\left(\frac{1-\bar{\upsilon}\rho_{20,w}}{1-\bar{\upsilon}\rho_{T,s}}\right) \quad (5)$$

where $\eta_{T,w}$ and $\eta_{20,w}$ are the viscosities of water at the experimental temperature and at 20 ºC respectively, η_s and η_w are the viscosities of the solvent and water respectively at a common temperature, υ is partial specific volume, $\rho_{20,w}$ is the density of water at 20 ºC and $\rho_{T,s}$ is the density of solvent at experimental temperature. Further details on experimental analysis can be obtained from the reviews stated in the Introduction. Estimations of sucrose viscosities to calculate η_s at high concentrations and different temperatures can be obtained in [15], and the densities $\rho_{T,s}$ from equation (2). The other values for equation (5) can be calculated in the

program Sednterp (by John Philo), which can be downloaded for free from http://www.jphilo.mailway.com/download.htm. From our example in Fig 2B, fits to this data yielded a size distribution plot of a predominant single species at 2.3 S, consistent with a GFP-poly 72Q protein (Figure 2C).

4. NOTES

1. The rotor to be used for the XLI is an An-Ti60 rotor, which hold sample containers ("cells"). Cells should be of a velocity type such as double-sector charcoal-epon centerpieces and either quartz or sapphire windows. A Delrin 5-sector calibration cell manually filled with fluorescein is used for automated calibration of the radial position and angular location of the cell compartments. Operator control of the FDS (including control of the ultracentrifuge itself) is achieved using Advanced Operating Software (Aviv Biomedical).

2. If samples with similar total protein concentrations but vastly different levels of fluorescent protein are being examined, the samples can be matched to allow accurate comparison between samples by measuring the fluorescence associated with 100 μL of 1 g/L cell lysate in a 96-well plate total protein in a plate-reader, followed by dilution of these samples with untransfected cell lysate to yield a similar fluorescence level. This step is necessary if protein expression varies significantly between samples. The final sample volume for experiments with the FDS experiments should be 500 uL with a minimum loading volume of 350 uL.

3. It is important to ensure that all sedimentation experiments are run under exactly the same conditions of temperature, buffer density and viscosity. Experimentally, this often requires using pre-chilled rotors and cells assembled ahead of time, and up to two hours of temperature equilibration in the ultracentrifuge prior to analysis. We typically perform our experiments at 11 ºC, which is a compromise between cooler temperatures to minimize sample degradation and the practical operation of the instrument under defined conditions. It may also be necessary to standardize the density and viscosity of the buffer/lysate using densitometer and viscometer measurements – sucrose densities and viscosities at high concentrations can be challenging to determine using standard sources, such as Sednterp. Reference [15] provides a handy source of viscosity versus sucrose concentration and temperatures. There are also multi-channel cells available that would allow multiple sample conditions to be performed in one run.

4. Lysates of certain cell types may contain components that exhibit intrinsic fluorescence. Control experiments should, therefore, be conducted to determine whether fluorescence and/or sedimentation of lysate components are observed using the FDS. Lysates of the cells used in our experiments (mouse Neuro2A cells and HEK293 cells) overexpressing a non-fluorescently tagged control protein yielded negligible fluorescence and no observable moving boundaries in control experiments [7].

5. When preparing sucrose solutions, we have observed that the larger the beaker, the faster the sucrose will re-crystallize, resulting in a non-homogenous solution. A convenient way to check for this is by dipping a pipette tip in the solution and letting a drop of the solution fall on a paper tissue. Then smooth out the drop using a gloved finger and feel for any inconsistencies or granular material.

6. Initiation of the FDS instrument entails firing of the laser and subsequent laser-power lock, after which the angular location of the calibration strip is acquired. Once this is achieved, emission gain settings and experiment conditions can be adjusted by the operator. These processes require a rotor speed of 3000 rpm and can take from ~10-30 minutes to complete. Large aggregates, such as inclusions and amyloid fibrils, may sediment significantly at 3000 rpm; thus, minimizing this period is particularly important. While laser-power lock and angular position settings are automated by the AOS software, time can be saved in setting of the fluorescence gains using prior knowledge of the approximate fluorescence yields of samples and which samples are matched. In addition, previously saved experimental method conditions may be used. It is also worth noting that firing of the laser requires a centrifuge chamber vacuum of below 150 microns. Thus, it is recommended that the vacuum be allowed to equilibrate to below this value with the rotor stationary.

7. Sedimentation profiles obtained by fluorescence detection do not yield a visible sample meniscus. This is in contrast to data obtained with absorbance detection optics, which yield a spike at the sample meniscus position due to light deflection at the air-water interface. An accurate determination of the sample meniscus position is important for calculating the rate of movement of experimental sedimentation boundaries. To define the meniscus position, a small amount of light mineral oil (~5 μl) containing 0.1% (w/v) 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503, Invitrogen) may be layered on top of the sample [16]. One potential problem, however, is that BODIPY may bind the protein of interest and co-sediment with the protein. Alternatively, the meniscus position can be empirically fitted in the SEDFIT analysis software without the use of extraneous dye. We

have found that experimental fitting of the meniscus in the software yields good fits to experimental data in most cases.

8. The baseline of all FDS fluorescence scans is offset from zero due to the presence of a small ‘dark count’ signal. The magnitude of this offset scales with gain settings and can be manually estimated for each experiment from the fluorescence signal obtained within the air-gap above the solution column of each sector.

9. Due to the sensitivity of the FDS and the very low concentrations of fluorescent sample needed, one potential problem is the detection of fluorescent material adhering to the cell windows, thereby contributing to noise in the sedimentation data. This only occurs when using purified protein (i.e. non-cell lysates). This problem can be resolved by including carrier proteins such as bovine serum albumin or hen egg white lysozyme in the sample. The effect of carrier proteins on the labeled protein of interest can be checked by performing sedimentation experiments using varying concentrations of carrier protein. Conversely, using high concentrations of fluorescent sample may result in an inner filtering effect where signal intensity no longer scales linearly with sample concentration. The extent of inner filtering in a sample can be established by a titration curve of the sample over the range of concentrations of interest.

10. An upwards-sloping plateau is sometimes observed in the sedimentation profile. This has been attributed to small errors in the tracking of the laser beam such that the focal point of the beam shifts during the scan or is too close the sector walls, resulting in systematic intensity variation [6]. The effects of this on accurate sedimentation analysis can be mitigated by correcting for time-independent (TI) noise in the data fitting software (SEDFIT). Similarly, drifts in the baseline over time (radial-independent noise (RI)) can also be corrected in SEDFIT.

11. One caveat for sedimentation analysis of biologically complex solutions - especially very concentrated solutions typical of biological fluids - is the inherent large effects on the sedimentation of the protein of interest from non-ideality or protein-protein interactions. Nevertheless, with careful controls much information can still be gleaned from experiments performed in biological fluids, and our previous studies have shown the system to be robust for examination of proteins expressed in mammalian cells when compared to the purified recombinant counterpart [7]. A useful control is to perform an experiment on the purified recombinant protein and compare the results to those in cell lysate. Nevertheless, deviations

in s values from non-ideality should not change broader conclusions about heterogeneity between vastly different sized aggregate species commonly seen in amyloid-like aggregation kinetics [7]. A further note is that at high centrifugal speeds, sucrose will co-sediment with the protein of interest and form gradients in the sample container, potentially resulting in non-ideal sedimentation behavior. We have only employed sucrose when using centrifugal speeds of 3000 rpm.

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FIGURE LEGENDS

Fig. 1. Sedimentation velocity experiment in an analytical ultracentrifuge. Fluorescently-labelled proteins in a sample container are spun at an appropriate speed. Arrows indicate the direction of the centrifugal force. At initial time (t0), all the molecules are homogenously distributed at each point along the sample container ("cells") and the fluorescence intensity at each point after the meniscus is equal. After certain times of centrifugation (t1, t2), the protein molecules sediment towards the bottom of the cell, manifesting in a moving fluorescence concentration boundary along the length of the cell compartment.

Fig. 2. Sedimentation analysis of GFP-tagged polyQ72 peptide. (A) The sample was in TX buffer containing 2 M sucrose, and the centrifugation speed used was 3000 rpm. Under such conditions, monomers and oligomers do not sediment (manifest in the non-moving bottom 50% of the profile). Very large aggregates (inclusions) sediment (manifest in the upper 50% of the profile). The first scan during the centrifugation experiment is shown in red, with subsequent scans shown in black, then grayscales. (B) The sample is now in TX buffer without sucrose and the centrifugation speed used was 50,000 rpm. Under these conditions, very large aggregates sediment immediately to the bottom of the cell and is not detected by the instrument. The remaining smaller monomers sediment detectably and yield a sedimentation profile. (C) Fitting of the experimental sedimentation profile in (B) to a sedimentation model with no assumptions of heterogeneity (c(s)) yields a size distribution plot indicating a predominant single species at ~ 2.3 S.

Figure 1

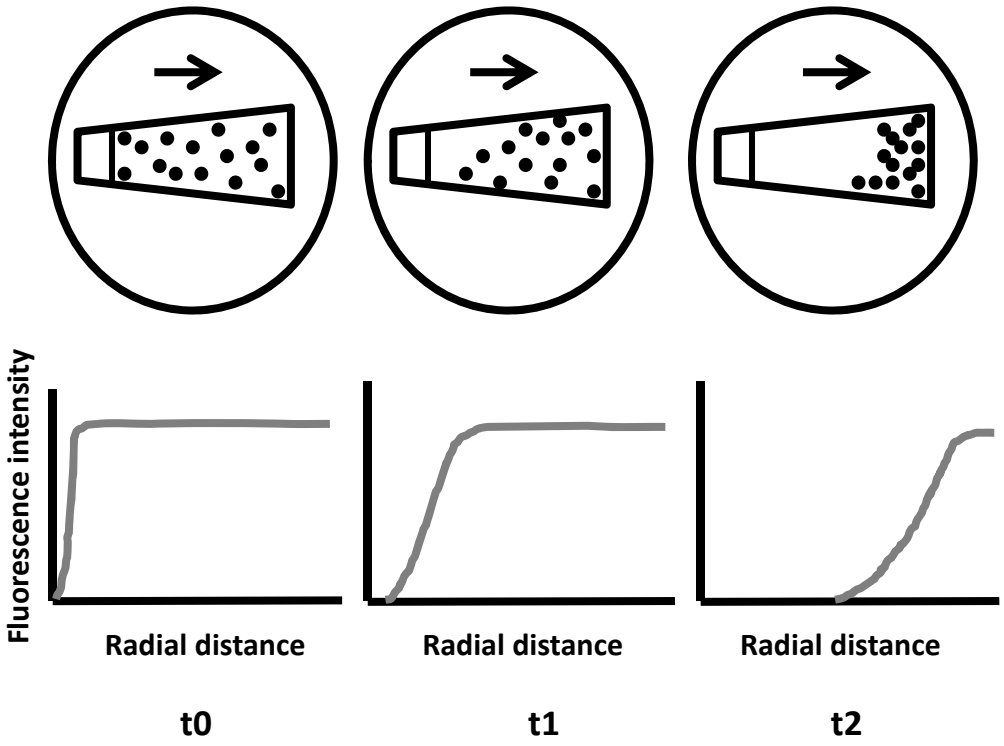
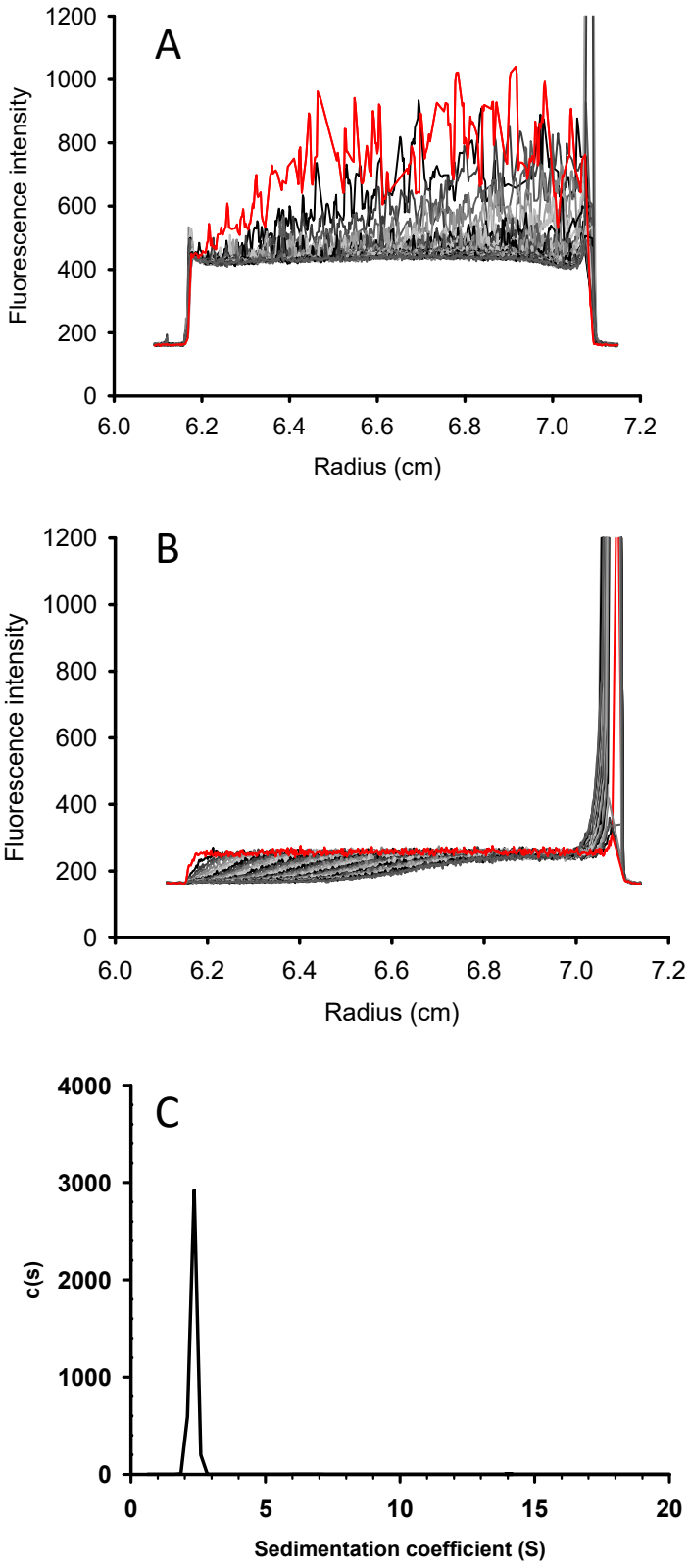

Fluorescence intensity
Radial distance
Radial distance
Radial distance
t0
t1
t2

Figure 2


A
Fluorescence intensity
1200
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0
6.0
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Radius (cm)
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c(s)
4000
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2000
1000
0
0
5
10
15
20
Sedimentation coefficient (S)

Author/s:
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