Fluorescence Anisotropy UnCOVERS Changes in Protein Packing with Inclusion Growth in a Cellular Model of Polyglutamine Aggregation

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ABSTRACT: The aggregation of polyglutamine-rich proteins is closely linked with numerous neurodegenerative disorders. In pathological and cellular models, the appearance of protein-rich inclusions in cells acts as a read out of protein aggregation. The precise organization of aggregated protein in these inclusions and their mode of growth are still poorly understood. Here, fluorescence anisotropy-based measurements have been used to probe protein packing across inclusions of varying brightness, formed by a monomeric enhanced green fluorescent protein (mEGFP)-tagged polyglutamine model peptide in cells. High-resolution, confocal-based steady-state anisotropy measurements report a large depolarization, consistent with extensive homo-Förster (fluorescence) resonance energy transfer (FRET) between the sequestered mEGFP-tagged protein molecules. An inverse correlation of fluorescence anisotropy with intensity is seen across inclusions, which becomes emphasized when the observed fluorescence anisotropy values of inclusions are corrected for the fluorescence contribution of the diffusible protein, present within and around smaller inclusions. Homo-FRET becomes enhanced as inclusion size increases. This enhancement is confirmed by two-photon excitation-based time-resolved fluorescence anisotropy decay measurements, which also suggest that the mEGFP-tagged protein molecules are arranged in multiple ways within inclusions. Bright inclusions display faster FRET rates with a larger number of mEGFP moieties participating in homo-FRET than faint inclusions do. These results are consistent with a model in which the protein is more closely packed in the brighter inclusions. In such a possible mechanism, the higher packing density of protein molecules in brighter inclusions would suggest that inclusion growth could involve an intermolecular compaction event within the inclusion, as more monomers and aggregates are recruited into the growing inclusion.

Protein aggregation and inclusion body formation by a set of polyglutamine (poly-Q)-expanded proteins are known to be associated with a number of neurodegenerative disorders.1,2 Huntingtin is one such poly-Q-expanded, aggregation-prone protein that has been shown to form aggregates in vitro, in cells, and in animal models.3−6 The aggregation propensity of huntingtin protein and the frequency of inclusions observed in patients have been shown to be dependent on the length of the poly-Q segment.7,8 Huntingtin has been shown to form a wide variety of aggregate-related structures, ranging from large fibrils to smaller aggregates and soluble oligomers in vitro.9−12 Under in vitro conditions, aggregation has been described as a nucleation-based model, with fibrils and large aggregates as the final end products.9,13 The aggregation mechanism becomes altered as a function of poly-Q repeat length and of the protein sequence flanking the poly-Q segment.11,14 In cells, expression of poly-Q-expanded huntingtin (Qn >35−40) results in the accumulation of the protein in large inclusions.8 The truncated N-terminal fragment of huntingtin (containing the poly-Q region) shows the remarkably fast appearance of inclusions.15 The prominent inclusions formed by huntingtin proteins are “insoluble protein deposits” that have been termed IPOD’s.16 The appearance of these inclusions seems to correlate well with cellular survival as opposed to toxicity, thus suggesting a protective role for these inclusions in cells.17 It appears that diffusible and soluble oligomers, which are formed prior to or escape inclusion body formation, may be responsible for cellular toxicity.18−20 Understanding the formation and dynamics of aggregate-rich inclusions is important for understanding fully the cellular pathophysiology of poly-Q-associated toxicity and could further suggest means of mitigating the aggregate-associated toxicity.

Several models for describing inclusion growth have been proposed. In one model, an inclusion grows by sequestration of passively diffusible monomeric protein, present in the vicinity of the inclusion.21 The second mechanism envisages inclusion growth as an aggregation of aggregates. In this scenario, small aggregates formed initially at multiple locations in cells coalesce to form inclusion bodies, at a given site in the cells. Huntington inclusions have been suggested to form mainly by the latter mechanism, wherein small aggregates are actively transported along microtubules to the site of inclusion body formation.22 In this regard, huntingtin inclusions resemble a class of inclusions

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known as aggresomes, formed by several other aggregation-prone proteins. The mechanism of conversion of smaller aggregates into a large inclusion, as well as the organization of these aggregates within inclusions, is not well understood. A direct probe for looking at interaggregate interaction is required to understand the packing organization of protein molecules in inclusions.

Poly-Q tract-containing aggregation-prone proteins are typically large, varying in size from the 364-residue ataxin-3 to the 3144-residue huntingtin. The observation that for all these proteins, aggregation is driven by the poly-Q tract when it exceeds a certain length has led to the widespread use of the protein to study poly-Q inclusions inside cells. Although it is possible that the GFP tag may modulate the ability of the poly-Q tract to aggregate in inclusion bodies, just as the protein sequence it replaces could, GFP-tagged poly-Q constructs retain an important property of the original proteins from which the poly-Q tracts are derived: their ability to form inclusions depends on the length of the poly-Q tract. For example, the construct 74Q-GFP, which has the N-terminal fragment of huntingtin exon 1 containing a pathogenic poly-Q length of 74 glutamine residues tagged with GFP (Figure 1A), forms inclusions, while the construct 23Q-GFP, containing the shorter nonpathogenic poly-Q length of 23 glutamine residues, does not form inclusions in cells.

The ability to use GFP-tagged poly-Q tracts to study inclusion body formation has allowed the widespread use of fluorescence or Förster resonance energy transfer (FRET) measurements to report on inclusion body formation. For example, hetero-FRET-based measurements have suggested that huntingtin molecules are organized in the proximity of each other in inclusions. Hetero-FRET measurements are easier to interpret when the molecules are organized in smaller clusters. In larger clusters, the precise relationship between the cluster size and the extent of FRET observed becomes complex, as FRET can occur in multiple ways, and because the donor- and acceptor-tagged fluorophores separated by short distances (homo-FRET). Homo-FRET leads to a sharp reduction in the observed fluorescence anisotropy value, which is proportional to the extent of FRET and therefore provides a direct measure of the packing density with which GFP-tagged protein molecules are organized. Thus, anisotropy measurements are ideal for looking at large clusters of closely organized molecules and for probing a number of protein aggregates and oligomers.

In this study, high-resolution confocal microscopy-based, steady-state fluorescence anisotropy imaging was conducted on inclusions formed by an mEGFP-tagged, poly-Q-containing peptide from the huntingtin protein in mammalian cells. These measurements were complemented with high-resolution two-photon, time-resolved fluorescence anisotropy decay measurements. Steady-state anisotropy measurements showed that the fluorescence anisotropy values in inclusions are much lower than in diffusely expressing (aggregate-free) regions. The extent of the reduction in anisotropy values was found to correlate inversely with the fluorescence intensity of the inclusions and can be attributed to homo-FRET. In the time-resolved anisotropy measurements too, bright inclusions showed a higher degree of homo-FRET than the fainter inclusions. The protein molecules in the larger and brighter inclusions appear to be more closely packed and are arranged in larger clusters, compared to the protein molecules in smaller and relatively fainter inclusions. As the relative amount of aggregated protein is greater in large inclusions, it appears that the packing density of the aggregated protein is considerably higher in the larger inclusions. Inclusion growth appears to be accompanied by an increase in protein packing density.

MATERIALS AND METHODS

Recombinant DNA Constructs. Plasmids encoding truncated huntingtin exon 1 with polyglutamine tracts with lengths of 23 (normal) and 74 (pathogenic) residues were used for protein expression in cells (Figure 1A). Both constructs contained amino acid residues 8–57 of normal human huntingtin protein in the pEGFP C-1 vector. The N-terminal EGFP moiety in both constructs was further converted to monomeric form (mEGFP) by introducing the A206K mutation, using site-directed mutagenesis. mEGFP (pEGFP C-1) was also expressed alone in cells, as an independent control for anisotropy measurements.

Cell Culture and Transfection. HEK-293T (Lenti-X, Clontech) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, high glucose, Gibco) with 10% fetal bovine serum (FBS, Gibco) and grown in 5% CO2 at 37 °C. Cells were plated and grown overnight on poly-D-lysine (PDL, Sigma)-coated coverslip bottom dishes before being transfected. Transfections were conducted with Fugene 6/HD reagents (Roche), as per the manufacturer’s recommended protocols. Approximately 200 to 250 ng of plasmid DNA was used to transfect the cells in each coverslip dish, in a volume of 200–250 μL. Cells were imaged in Dulbecco’s phosphate-buffered saline (DPBS, Gibco) with calcium, magnesium, and glucose, or in HEPES-buffered Hank’s Balanced Salt Solution (HBSS, pH 7.4, Gibco).

Steady-State Anisotropy (SSA) Imaging. Steady-state anisotropy imaging was conducted using a LSM 5 LIVE microscope (Zeiss Confocal Systems, Jena), custom designed for measuring steady-state fluorescence emission anisotropy. A high-NA objective (63×, 1.4NA) combined with high zoom settings (pixel size of 100 nm) was used to capture high-resolution images. Emitted fluorescence from samples was split into parallel and perpendicular polarized fluorescence by a polarizing beam splitter. Individual polarized emissions were passed through slits to achieve confocality (slit width of ~1.19 airy units, z slice thickness of <1 μm). Polarized emissions were further filtered by matched 495–555 nm band-pass filters and were focused onto two linear CCD detectors. Cells were imaged with a low laser power to avoid photobleaching due to imaging. All images acquired were averages of two frames. For all imaging experiments, fluorescein at pH 11 (1 mM NaOH) was used as the G-factor control (explained below), while a recombinant EGFP solution (pH 8, Tris) was imaged as an anisotropy control.

The imaging of a large number of inclusion-containing cells encompassing a wide range of intensity values was conducted under different imaging conditions (Figure 2C). Briefly, the camera gain, scan speed (exposure time), and laser power were adjusted to obtain sufficiently bright images of each inclusion. Images of the fluorescein solution were also acquired under these different imaging conditions. The relative intensity of the fluorescein solution was used as a reference for normalizing across images of different inclusions.

The LSM DUO-SCAN module attached to LSM 5 LIVE was used for conducting photobleaching along with SSA imaging. Here SSA images were acquired with the line-scanning LIVE
module, while precisely patterned photobleaching was achieved by the point-scanning DUO module. For photobleaching of inclusions, a modified fluorescence recovery after photobleaching (FRAP)-like strategy was used in a repeated manner.\textsuperscript{40} Briefly, images of inclusion-containing cells were first acquired in the anisotropy imaging mode. This was followed by a short pulse (<2 s) of iterative photobleaching in a region containing the entire inclusion. The photobleached inclusion was imaged again after a gap of 10 s, to avoid any contribution of reversible photobleaching. To achieve a sufficiently reduced intensity (<25% of prebleach intensity) in inclusions, repeated cycles of imaging and photobleaching were conducted.

A modified fluorescence loss in photobleaching (FLIP)-like strategy was employed for reducing the contribution of diffuse monomeric protein from the observed anisotropy of aggregated protein.\textsuperscript{40} Briefly, an inclusion-containing cell was imaged in anisotropy mode (as described above). A small region (3–4 μm\(^2\) wide) away from the inclusion was iteratively photobleached for 10–20 s. The same inclusion-containing cell was imaged again after 60 s, allowing bleached molecules present outside the inclusion to become equilibrated with the diffusible protein present within the inclusion.

**Generation of Whole Frame SSA Images.** All image analysis was conducted with FIJI/ImageJ.\textsuperscript{41} Parallel and perpendicular polarization images were aligned with the help of the “Descriptor Based Registration” plugin.\textsuperscript{42} Images of subresolution (~200 nm wide) fluorescent beads acquired under similar imaging conditions were used as reference images for alignment. Aligned images were buffer corrected, followed by nearest neighbor averaging (mean filter with a one-pixel radius). To remove instrument bias between the two detectors at a pixel by pixel level, a G factor correction was applied to the images. Fluorescein is a small fluorescent molecule that tumbles very fast with a rotational correlation time of ~120 ps, resulting in a nearly complete depolarized emission during the imaging time window (pixel dwell time of >20 μs). The G factor image refers to a correction image obtained by dividing the parallel intensity image with the perpendicular intensity image of a dilute solution of fluorescein.

\[ G = \frac{I_P}{I_L} \text{(fluorescein)} \]  

(1)

Parallel and perpendicular intensity images of a fluorescein solution were also buffer corrected and nearest neighbor filtered, as in the case of the cell images, before the G factor image was created. A precise G factor image was made by averaging a stack of five to seven individual G factor images.

This G factor correction was applied to all perpendicular polarization images of cells and control fluorophore (GFP) solutions. Whole frame SSA images (r) were thus calculated as follows:

\[ r = \frac{I_P - GI_L}{I_P + 2GI_L} \]  

(2)

and total intensity (\(I_{\text{Total}}\)) images were calculated as

\[ I_{\text{Total}} = I_P + 2GI_L \]  

(3)

Total intensity images were thresholded to highlight the regions of interest, i.e., whole cells when protein expression was diffuse and inclusions for aggregate-containing cells. The same threshold binary maps were applied to the corresponding anisotropy images. In the photobleaching assay, each round of photobleaching reduces the intensity values of some regions around the inclusion to below the set threshold value (0.1 times the prebleach peak intensity pixel value in Figure 3A). The intensity and anisotropy information in these regions thus is filtered from the image, which leads to the impression that the inclusion has become smaller after photobleaching. Anisotropy images were pseudocolored to an appropriate scale (Royal LUT, FIJI). On the basis of the anisotropy images of the EGFP solution, all the analysis of cells was limited to a region at the center of the image frame (central 350 pixel × 450 pixel region out of the 512 pixel × 512 pixel frame) where the anisotropy variation remained minimal and constant across measurements. The mean SSA of the EGFP solution measured at the center of the frame was 0.252 ± 0.005. Fluorescein solution images acquired under similar imaging conditions were used as reference images for correcting the total intensity images of cells against nonuniform illumination across the image frame.

**Analysis of the Whole Frame SSA Images.**

**Diffuse Regions.** The average SSA of nonaggregated 74Q-mEGFP protein, with or without small aggregates present elsewhere in expressing cells, was compared to the average anisotropies of mEGFP and 23Q-mEGFP proteins expressed in cells, as follows (Figure 1C). Anisotropy values from 50 small regions of 5 pixel × 5 pixel widths (0.25 μm\(^2\) wide) were recorded from each cell, and a distribution of anisotropy values was generated for each construct. The individual distributions were fit to a Gaussian function, providing an average value of anisotropy (with the standard deviation) for each construct.

**Inclusions.** The average steady-state intensity and anisotropy values of aggregated 74Q-mEGFP protein were compared for a large number of inclusion-containing cells as follows (Figure 2C). The average intensity and anisotropy values from a small, 3 pixel × 3 pixel (0.09 μm\(^2\)) wide region at the center of each inclusion were recorded for a number of inclusion-containing cells. The width of the chosen region was kept deliberately small, to compare even the smallest of inclusions, with a size comparable to the point spread function (PSF) of the objective.

**Intensity–Anisotropy Distribution of Inclusions and Two-Component Model Fitting.** For each inclusion-containing cell, a rectangular region containing the aggregated protein was selected from the total intensity and its respective anisotropy image. The chosen regions were sufficiently wide to span a small margin of diffuse, nonaggregated protein present around the inclusions. For each region, intensity and anisotropy values from all pixels were recorded. The intensity values were further divided into 8–15 small bins of equal intensity intervals. The intensity and anisotropy values of all pixels contained in the individual intensity bins were averaged, to obtain single mean values of intensity and anisotropy, with their respective standard deviations. The mean values of anisotropy were plotted against their intensity values, for all intensity bins. To reduce noise, low-intensity regions were omitted (if required). The intensity–anisotropy distributions of faint and small inclusions were analyzed by the two-component model (see below).

The two-component model presumes that the inclusion-containing region of a cell is composed of aggregated protein (A) and diffuse protein (D). For a mixture of fluorophores, the total intensity of the mixture is given by the linear sum of the intensities of the individual components. The total anisotropy value observed for such a mixture is given by the weighted sum of the individual anisotropies of the two components, weighted by their fractional intensity contributions toward the observed total intensity. Thus, the observed steady-state intensity (\(I_{\text{Total}}\)) and
anisotropy (r_{Total}) value from a given part of an inclusion can be represented as
\[ I_{Total} = I_D + I_A \] (4)
and
\[ r_{Total} = \frac{I_D r_D + I_A r_A}{I_{Total}} \] (5)
where \( I_D \) and \( I_A \) are the apparent intensities of the diffusible and aggregated protein present in any given part of the inclusion, respectively, and \( r_D \) and \( r_A \) are the corresponding values of the fluorescence anisotropies.

Upon rearrangement of eqs 4 and 5, the observed anisotropy in a given part of an inclusion is described by
\[ r_{Total} = r_A + \frac{I_D (r_D - r_A)}{I_{Total}} \] (6)

Equation 6 represents a linear sum of two terms; hence, for a given fixed value of \( r_{Total} \), a solution with highest values of \( r_D \) and \( I_D \) used would yield a lowest value of \( r_A \). Thus, the predicted value of aggregate anisotropy (\( r_A \)), with the assumptions used (see Results), represents the lowest possible value, capable of explaining the observed distribution. The actual value of aggregate anisotropy could be anywhere within the interval of the observed value (upper bound) and the predicted value (lower bound).

**Time-Resolved Anisotropy (TRA) Decay Measurements.** Time-resolved anisotropy measurements from cells were taken using a LSM 510 Meta confocal microscope (Zeiss Confocal Systems) with a 63× (NA 1.4) objective.43 A Mai-Tai Ti-Sapphire (Spectra physics) mode-locked laser tuned at 920 nm was used to achieve two-photon excitation of mEGFP. To achieve a single G factor across measurements, the excitation beam was parked at the center of the frame, and all measurements were made at this point. The emitted photons were directed to the nondescanned port by a 680 nm reflector (Zeiss) and were further filtered by an EGFP emission filter. The emitted photons were then separated into parallel and perpendicular polarized components by a polarization beam splitter and were collected simultaneously by two R3809U MCP photomultiplier tubes (Hamamatsu). TCSPC traces were acquired with a SPC-830 (Becker and Hickl) card working in “Stop-Start” mode. All traces were acquired at an 80 MHz excitation repetition rate with an acquisition time resolution of 12.2 ps per channel. To satisfy TCSPC criteria, and to avoid any pulse pile-up effect, emitted photons were acquired at a count rate of <0.2 MHz. Colloidal gold particles dried on a coverslip were used to measure the instrument response function (IRF). A dilute solution of fluorescein at pH 11 was used for measuring the G factor, and an EGFP solution was used as an anisotropy control. The instrumentation easily allowed the resolution of the fast TRA decay of fluorescein [100–140 ps decay constant (data not shown)]. The measured TRA decay constants of fluorescein and EGFP were found not to significantly vary across experiments.

**Analysis of TRA Decay Traces.** Individually polarized fluorescence traces (parallel and perpendicular intensity decays) for all samples were first aligned with each other along their rising edges and were background corrected. The channel with peak counts in similarly aligned IRF traces was used as the start channel for all decays (\( t_0 \)). The G factor value was calculated by matching the tails of the two polarized traces of fluorescein solution, at long time points (2 ns onward). Time-dependent anisotropy, \( r(t) \), was calculated for each sample using the following equation:
\[ r(t) = \frac{I_G(t) - GI_A(t)}{I_G(t) + 2GI_A(t)} \] (7)

Similarly, a fluorescence lifetime (total intensity) trace, \( I(t) \), was generated as
\[ I(t) = I_G(t) + 2GI_A(t) \] (8)

The observed TRA traces showed a minor distortion in the form of an apparent fast decay at the start of all traces, which presumably represents an effect of the IRF. The full width at half-maximum (fwhm) of the IRFs for both detectors was <50 ps. Hence, the calculated TRA traces were directly comparable after the initial 60–100 ps. For a simple comparison, the observed TRA traces were fit directly to nonlinear, least-squares, sum of exponential decay models, based on the following equation:
\[ r(t) = r_0 \sum_{i=1}^{n} A_i e^{-t/\tau_i} \] (9)
and
\[ \sum_{i=1}^{n} A_i = 1 \] (10)
where \( n = 1, 2, and 3 \) for one-, two-, and three-exponential decays, respectively.

Here \( r_0 \) refers to the apparent initial anisotropy, observed at time \( t_0 \). \( \tau_i \) and \( A_i \) represent the individual apparent anisotropy decay time and the corresponding amplitude, respectively.

As mentioned above, because of initial IRF distortion, the initial parts of all traces (60–120 ps) were not fitted. The intensity values in the total intensity traces were normalized to the intensity in the first channel of the given trace, from where the TRA traces were to be fitted. These normalized intensity traces were then used as the weights for fitting the corresponding anisotropy decay traces. TRA traces from mEGFP-expressing cells were fitted to a single-exponential decay model, and a range of apparent \( r_0 \) values were recovered by back extrapolating fits to time \( t_0 \). The \( r_0 \) values remained between 0.32 and 0.35 for mEGFP- and diffuse 74Q-mEGFP-expressing cells. These values were used as a reference for describing TRA traces from 74Q-mEGFP inclusion-containing cells. In the three-exponential fitting for TRA traces of inclusions, the longest correlation time was constrained to be >40 ns. This number is not reliable as the amplitude of the rotational component was very low. The actual slow rotational correlational time is likely to be much higher (>100 ns). All fitting analyses were conducted using SigmaPlot (Systat).

**RESULTS**

In this study, intracellular inclusions formed by an mEGFP-tagged poly-Q-expanded peptide were examined by fluorescence anisotropy-based measurements. For observing rapid and efficient inclusion body formation in cells, an N-terminal fragment of huntingtin exon 1 containing a pathogenic poly-Q length of 74 glutamine residues (74Q) was expressed in HEK-293T cells (Figure 1A).28 A similar peptide with a shorter nonpathogenic poly-Q length of 23 glutamine residues (23Q) was used as a nonaggregating control protein. HEK-293T cells were used for transient expression because of the relative ease of transient transfections and high protein expression levels. HEK-
measurements taken with high-NA objectives show significant depolarization and compression of the fluorescence anisotropy scale because of the mixing of individual polarized light components. Steady-state anisotropy (SSA) imaging was conducted using a line-scanning confocal (slit-based) microscope, which allowed fast imaging with a sufficiently large anisotropy scale (Materials and Methods). Time-resolved anisotropy (TRA) measurements were taken using two-photon excitation of the mEGFP moieties. Two-photon excitation expands the scale of observed anisotropy; hence, some of the loss in the dynamic range of fluorescence anisotropy measurements due to the use of a high-NA objective is partially compensated for. It is important to note that while the use of a high-NA objective compresses the dynamic range, the determination of decay rate constants is unaffected.35

Steady-State Fluorescence Anisotropy Profiles of Cells Expressing mEGFP-Tagged Poly-Q-Containing Peptides.

Figure 1B shows representative whole cell SSA images of cells expressing the three mEGFP-tagged proteins, with diffuse expression of the individual proteins. The fluorescence anisotropy of protein molecules in different regions of cells expressing any of the three proteins appeared to be similar. The average value of fluorescence anisotropy observed in regions of cells showing diffuse (inclusion-free) expression of 74Q-mEGFP was compared with that in the cells expressing 23Q-mEGFP or mEGFP-alone (Materials and Methods and Figure 1C). As expected, the SSA values measured from mEGFP-alone-expressing cells were fairly high (r_{average} ~ 0.27). Similar anisotropy values were observed in cells expressing 23Q-mEGFP and diffuse 74Q-mEGFP. The similarity in the fluorescence anisotropy values suggests that the observed anisotropy values are dominated by the large (240 residues) mEGFP moiety; linkage of either poly-Q-containing peptide to the mEGFP moiety does not change the latter’s anisotropy.

A similar analysis was conducted with cells containing 74Q-mEGFP inclusions (Materials and Methods). Figure 2A shows representative steady-state intensity and anisotropy images of
different inclusion-containing cells, arranged in an increasing order of intensity. All inclusions that showed high fluorescence intensity displayed fluorescence anisotropy values that were significantly lower than values in nearby diffuse regions. The fluorescence intensity appears to be highest, and the fluorescence anisotropy appears to be lowest at approximately the center of each inclusion (Figure 2B). The bright inclusions displayed SSA values much lower than the relatively faint inclusions; the center of inclusion 4 in Figure 2A displayed the highest intensity value and the lowest fluorescence anisotropy value among the four inclusions. The fluorescence anisotropy is seen to increase from inclusion 4 to inclusion 1, correlating inversely with size and intensity.

Figure 2C illustrates this inverse correlation quantitatively for a large number of inclusions, spanning a wide intensity range (see Materials and Methods). The SSA values observed at the centers of inclusions were significantly lower than the average SSA value displayed by cells devoid of large inclusions (Figure 2C). The SSA values observed for the bright inclusions were one-half to nearly one-third of those observed for aggregate-free diffuse regions, signifying the extensive depolarization observed in inclusions. It should be noted that the spread in SSA values at any given intensity value is small compared to the range of SSA values seen across inclusions.

Homo-FRET Is Responsible for the Observed Low Steady-State Anisotropy Values in Inclusions. The possibility that the low fluorescence anisotropy observed in the 74Q-mEGFP inclusions is indeed due to homo-FRET was tested by a photobleaching-induced anisotropy recovery assay. Photobleaching brings about a reduction in the density of active fluorophores, thus effectively decreasing the number of fluorophore pairs involved in FRET, which leads to an increase in fluorescence anisotropy. Figure 3 shows the change in the SSA of an inclusion upon photobleaching. Photobleaching across the entire inclusion was conducted in intermittent pulses alternated with imaging cycles (Figure 3A). It is seen that the fluorescence anisotropy of the entire inclusion increases with a concomitant photobleaching-induced reduction in intensity. Figure 3B shows how the SSA value measured at the center of the inclusion, increases with a decrease in intensity at the center of the inclusion. The data in Figure 3 validate the interpretation that the cause of depolarization is strong homo-FRET between the closely packed mEGFP moieties in the inclusions.

Estimating the Actual SSA Values of Aggregated Protein within an Inclusion. Inclusion body formation by 74Q-mEGFP involves a time-dependent sequestration of diffuse protein molecules into the inclusions (Figure S1 of the Supporting Information). At an early time point of the observation, when an inclusion body is small and faint (Figure 4),
scenarios, it is important to know the actual value of the aggregate anisotropy. To differentiate between these two scenarios, it is important to know the actual value of the fluorescence anisotropy for the aggregated protein in an inclusion. The impact of the presence of diffusible protein within and around the aggregated material on the observed values of anisotropy for any given inclusion can be estimated by a simple two-component model (see eq 6 in Materials and Methods).

The two-component model is based on several assumptions. (1) It assumes that the measurements of intensity ($I_{\text{total}}$) and anisotropy ($r_{\text{total}}$) in each image pixel contain the contributions of the aggregate intensity ($I_A$) and anisotropy ($r_A$) as well as of the diffusible protein intensity ($I_D$) and anisotropy ($r_D$) (see eqs 4 and 5 of Materials and Methods). (2) It is assumed that the value of $r_D$ is constant throughout the inclusion, implying that if there is any heterogeneity in $r_A$ values within an inclusion it can still be represented by an average $r_A$ value. This assumption appears to be reasonable given the spatial resolution of our measurements is such that any spatial heterogeneity in $r_A$ values within an inclusion is not directly measurable; hence, the simplest possible assumption is made regarding the structure (and hence $r_A$) of the aggregate within an inclusion. (3) It assumes that the value of $r_D$ is the same throughout the inclusion and is the same as that of the diffusible protein present just outside the periphery of the inclusion because it is this protein that diffuses into the inclusion. Hence, the value of $r_D$ was fixed at the constant value obtained for diffuse 74Q-mEGFP (Figure 1C). (4) It is also assumed that $I_D$ is uniform throughout the inclusion and in the immediate vicinity of the inclusion, which implies that the diffusible protein has equilibrated throughout just outside the inclusion. It should be noted that this assumption would not be valid if the packing density varies drastically throughout the inclusion, which might lead to a diffusion gradient within the inclusion, or if binding of the diffusible protein to the aggregate occurs faster than the fluorescence lifetime (nanoseconds), which is unlikely.

The two-component model was first tested on faint small inclusions. Figure 4A shows that correlation of fluorescence anisotropy with intensity within a faint inclusion. It should be noted that faint inclusions are small (diameter of <2 μm), and because the z slice thickness of the confocal microscope is ~1 μm, the measurement would include contribution from diffusible protein present immediately below and above the inclusion. It is seen that the data are described well by eq 6 based on the two-component model. Importantly, this agreement of the data with the minimalistic two-component model suggests that the assumptions on which the model is based are reasonable at least for faint inclusions. The two-component model was found to also describe well the SSA–intensity correlation of inclusions of intermediate brightness (see below).

The fit to eq 6 predicts the value of $I_D$ for diffusible protein present within and immediately above and below the inclusion and also predicts the value of $r_D$. It should be noted that the value predicted for $r_D$ is a lower bound to its true value because of the assumption that the value of $I_D$ is uniformly high throughout the inclusion, as high as that present in the immediate vicinity of the inclusion. Figure 4B shows curves simulated on the basis of the two-component model, in which a given amount of diffusible protein is mixed with an increasing amount of aggregated protein. As expected, the fluorescence anisotropy of the simulated curve approaches the actual value of aggregate anisotropy, when the contribution of diffusible protein is lowest.

The Aggregate Anisotropy Value Predicted by the Two-Component Model Represents a Lower Bound on the Actual Value of Aggregate Anisotropy. Figure 5 shows a faint inclusion-containing cell (prebleach case), with the inclusion-containing region highlighted by a blue square. The
The pixel-wise distribution of this region fits well to eq 6 of the two-component model (Figure 5B). In the fit, the value of $r_D$ was fixed to the average SSA value observed in diffusely expressing regions of the inclusion-free cells (Figure 1C). The value of $r_A$ was predicted to be 0.196 (Figure 5B), and on the basis of the assumptions of the model, this value represents the lowest possible value of aggregate anisotropy that is capable of explaining the data. It should be noted that when the value used for $r_D$ was varied over a small range, the value obtained for $r_A$ was not affected significantly. It was important to further validate the two-component model for inclusions of faint and intermediate brightness by determining whether the value predicted for $r_A$ by use of the model does indeed match the actual anisotropy value of the aggregated protein within the inclusion.

A photobleaching-based perturbation methodology was used for this purpose (Materials and Methods). A small region in a faint inclusion-containing cell, localized away from the inclusion, was repeatedly photobleached (Figure 5A), to reduce the fluorescence contribution of the diffusible protein present within the inclusion. The postbleach steady-state intensity image in Figure 5A, taken after the bleached diffusible protein had equilibrated throughout the cell, shows that a major fraction of the diffusible protein pool was photobleached, as indicated by the loss of

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**Figure 4.** Contribution of diffusible protein to the observed fluorescence anisotropy value of an aggregate-rich inclusion. (A) Steady-state intensity and anisotropy images (pseudocolored) of an inclusion-containing region of a cell (3.8 μm × 3.8 μm). The plot shows the raw distribution of intensity and anisotropy values obtained from all pixels within the region (blue circles). The raw distribution was binned into narrow intensity bins, and their average intensity and anisotropy values are shown as red circles. The green line through the binned data points is a fit of the data to eq 6. (B) Schematic that describes the equilibration of diffusible protein present outside and within the inclusion. Because of simple diffusion, the concentration of diffusible protein present within an inclusion cannot exceed that in the bulk cytoplasmic pool. The plot shows simulated values of anisotropy, obtained by mixing a fixed amount of diffusible protein with an increasing concentration of aggregated protein, in a defined volume. The black and red lines indicate the anisotropy values of the diffuse and aggregated proteins used for simulation. The amount of diffusible protein was varied from 5% (yellow) to 10% (green) to 20% (blue) to 50% (cyan) (represented as the percent fraction occupied by the diffusible pool, at the highest-intensity value).

**Figure 5.** Anisotropy value of aggregated protein obtained from the two-component model-based analysis represents a lower limit to the actual value. (A) Steady-state intensity images of an inclusion-containing cell, before and after the fluorescence of the diffusible pool in the cell had been reduced by continuous photobleaching of a small region, denoted by the green square, outside of the inclusion. The insets in both images depict magnified steady-state anisotropy images of the aggregate-containing region of the cell (blue and red square), before and after diffusible protein outside the inclusion had been photobleached. The anisotropy images have been smoothed by a 1 pixel radius to improve the visual contrast. (B) Binned pixel-wise intensity−anisotropy distributions of the inclusion-containing region before (blue squares) and after (red squares) diffusible protein outside the inclusion had been photobleached. The cyan line represents the fit to eq 6. The dashed pink line at $r = 0.196$ denotes the value of aggregate anisotropy ($r_A$) for the inclusion obtained from the fit. The intensity values for both distributions have been normalized to the mean intensity value of the brightest bin before the commencement of photobleaching. The scale bar is 5 μm.
intensity around the inclusion. The intensity in the inclusion-containing region was also found to decrease, signifying a loss of diffusible protein present within and around the inclusion. Figure 5B shows the binned pixel-wise distributions of intensity and anisotropy values for the inclusion-containing region, before and after the photobleaching event. The observed fluorescence anisotropy value in the postbleach inclusion-containing region is clearly lower than that in the prebleach case. The loss of both fluorescence intensity and anisotropy in the inclusion, after the photobleaching event, occurs because the diffusible protein detected originally with the inclusion has now been replaced by the photobleached diffusible protein from the cytoplasm. The observed loss of intensity and anisotropy cannot be attributed to direct photobleaching of the inclusion or a significant aggregation of bleached monomers into the inclusion (which is very unlikely to have happened within 1–2 min, the duration of the experiment), as in either of these cases, the SSA of the inclusion would have increased post-photobleaching (Figure 3).

It should be noted that the SSA of the inclusion-containing region decreases after photobleaching and is only slightly higher than the predicted (lower bound) value of SSA for the aggregated protein, indicating the reliability of the two-component model.

**Large and Bright Inclusions Have Significantly Lower Steady-State Anisotropy Values, Compared to Those of Faint Inclusions.** Figure 6A shows how well the two-component model describes the pixel-wise intensity–anisotropy distributions of inclusions, of varying brightness. It is seen that the intensity–anisotropy correlation for an inclusion of intermediate brightness is also described well by the model. The observed and predicted values for the intermediate inclusion are found to be lower than those for the faint inclusion but are higher than the observed value for the bright inclusion.

Figure 6A also shows that the two-component model does not adequately describe the intensity–anisotropy correlation of a bright inclusion, whose observed SSA values are well-separated from those of the faint inclusion. For the bright inclusion, the observed value of anisotropy at the brightest region of the inclusion was found to be lower than the value of anisotropy for the aggregated protein predicted by the two-component model. It appears that the assumptions of the two-component model are not valid for the large and bright inclusions. In particular, the assumption that the concentration of the diffusible protein within such an inclusion is uniform throughout the inclusion and the same as that at its periphery is unlikely to be valid because of the tight packing within the large inclusion as suggested by its low observed anisotropy values. It is more likely that a concentration gradient of diffusible protein exists within the inclusion. Second, the application of the two-component model depends on the proper measurement of intensity. The intensity value observed at any point in an image represents a three-dimensional convolution of the point spread function (PSF) of the microscope with the amount of fluorophore. Because of poor z-resolution...
resolution and imperfect confocality (slits), intensity measurements within a large inclusion could be skewed because of the contribution of aggregated protein present above and below the z slice. For smaller inclusions of faint and intermediate brightness, whose dimensions are comparable to the z slice thickness of the confocal microscope, the out-of-plane intensity contribution will be smaller, and the measurement will be less skewed. Hence, the two-component model works for smaller inclusions in predicting the anisotropy of the aggregated protein, but not for the large and bright inclusions. Nevertheless, it can be argued that because the large and bright inclusions have intensity values much higher (by 10−20-fold) than that of the diffusible protein present in the proximity, the fluorescence anisotropy value observed for a bright inclusion is more likely to be close to the actual fluorescence anisotropy of the aggregated protein present in the inclusion (Figure 4B).

Figure 6B shows the spread of the $r_A$ values obtained by fitting the individual pixel-wise distributions of 25 faint inclusions (left panel) and 15 intermediate inclusions to eq 6 (middle panel). The $r_A$ values obtained for the faint and intermediate inclusions were compared with the fluorescence anisotropy values observed in multiple bright inclusions. The data in Figure 6 show that the $r_A$ values for the faint inclusions fall within a rather broad range (0.13−0.19) and are significantly higher than the measured fluorescence anisotropy values for bright inclusions, which are dispersed over a fairly narrow range (0.1−0.118). Because the value of $r_A$ obtained from the model is likely to be a lower bound (see above), the actual value of aggregate anisotropy in faint inclusions would likely be significantly higher than that in bright inclusions. The observed and predicted $r_A$ values for inclusions with intermediate intensity values are contained within the range of 0.12−0.16. Most of these values are higher than the anisotropy values observed for bright inclusions but lower than the observed anisotropy values of the majority of faint inclusions and the predicted anisotropy values of a few faint inclusions. Because the predicted value of $r_A$ represents a lower bound on the actual value of aggregate anisotropy, the calculated $r_A$ values suggest a gradual decrease in anisotropy because of gradual enhancement of homo-FRET, with an increase in the size and brightness of the inclusions.

TRA Decay Traces from Inclusions Display Multi-exponential Decay Components. TRA decay measurements were conducted for two reasons. (1) A change in anisotropy that can be attributed to homo-FRET will manifest itself as a fast component in the TRA decay. When present, this fast homo-FRET-allowed component will be independent of diffusible monomer concentration, which will not show homo-FRET. (2) The TRA measurements kinetically resolve the contribution of homo-FRET from that of rotational depolarization, as homo-FRET occurs over a much shorter time scale (within the first few nanoseconds of the decay), as compared to the much slower rotational depolarization (typically >10 ns for EGFP). Thus, TRA decay measurements can reveal directly the mechanism by which emitted fluorescence is depolarized during the fluorescence lifetime of an excited fluorophore.

Figure 7 shows the TRA decays of diffusible and aggregated 74Q-mEGFP protein in cells. Each TRA measurement was made from a single point in a cell by accumulating the emitted photons for 30−60 s (see Materials and Methods). The TRA decay from a cell diffusely expressing the 74Q-mEGFP protein is very similar to that from an mEGFP-alone-expressing cell (Figure 7A) and is described well by a single-exponential decay, signifying the slow rotational tumbling of the molecules in the cytoplasm (see Materials and Methods). The slightly slower tumbling of 74Q-mEGFP compared to that of mEGFP is caused by the extra length of the poly-Q peptide (~100 amino acid residues) added to the mEGFP moiety. Figure 7B shows a representative TRA trace from a bright and large 74Q-mEGFP inclusion. It shows a significant loss of anisotropy within the first 2 ns of the decay, compared to the TRA trace of the diffusible protein in an
aggregate-free cell. This is typical for homo-FRET-dependent depolarization. The TRA trace appears to be relatively flat after the initial $2-3 \text{ ns}$ of the decay, because the aggregated protein undergoes very slow rotational depolarization. The TRA decay curves from inclusions could not be described as single-exponential decays and were therefore analyzed as multi-exponential decays (Figure 7B). A two-exponential fit approximated the decay trace better than a single-exponential fit but also failed to fully describe the observed decay curve (Figure 7B). A three-exponential fit was found to describe the observed trace well and yields very fast and fast anisotropy decay times of $\sim 150 \text{ ps}$ and $1.15 \text{ ns}$, respectively, for the TRA trace shown. The third component represents the slow tumbling of the aggregated protein and is slower than $40 \text{ ns}$. The very fast and fast decay components are also jointly responsible for $>80\%$ of the observed decay. This suggests that the majority of the mEGFP moieties in 74Q-mEGFP inclusions participate in homo-FRET. The very fast and fast decay components observed here are within the range ($80 \text{ ps}$ to $2.4 \text{ ns}$) of the homo-FRET-dependent TRA decay time constants observed previously in other GFP-based studies.33,31

Bright Inclusions Display a Higher Degree of Homo-FRET Than Fainter Inclusions. Figure 8 compares TRA decays from large and bright inclusions to those from relatively faint and small inclusions. The TRA decay trace from the fainter inclusion shows a considerable loss of anisotropy within the first $2 \text{ ns}$ of decay, similar to the TRA decay trace from a bright inclusion (Figure 8A), but the extent of relative depolarization achieved in the first $2-3 \text{ ns}$ is considerably smaller than that from a bright inclusion. This is highlighted by the high residual fluorescence anisotropy value at these time points of TRA decay of the faint inclusion compared to that of the bright inclusion (Figure 8A). The slope of the initial decrease in fluorescence anisotropy is also less steep in the TRA trace from the faint inclusion when compared to that from the bright inclusion. Figure 8B compares the very fast and fast anisotropy decay components, and their joint amplitudes, for multiple bright and faint inclusions. The observed very fast and fast anisotropy decay times are comparatively shorter for the bright inclusions. The combined amplitudes of the very fast and fast anisotropy decay times for bright inclusions also appear to be higher in comparison. This is indicative of a higher extent of homo-FRET in brighter inclusions.

## DISCUSSION
In any given cell, an individual 74Q-mEGFP inclusion grows over time in both size and fluorescence intensity; it transforms from a small and faint inclusion into a large and bright inclusion (Figure S1 of the Supporting Information). However, because of the stochasticity of the aggregation process,52 different cells commence inclusion formation at different times, so that at any given time point, different cells show inclusions that vary in size as well as in the intensity of the fluorescence of the mEGFP tag attached to the poly-Q tract. To study how the packing of aggregated protein within an inclusion changes with inclusion growth, both the growth of a smaller inclusion into a larger inclusion can be followed over time in a cell or inclusions of varying size and brightness present at the same time point in different cells can be directly compared by measurement of their SSA values. The latter strategy was followed in this study, because probing the same cell over a long time ($\sim 5 \text{ h}$ or more) taken for a small inclusion to become a larger one can cause photobleaching-induced variations in their SSA values.

It should be noted that the analysis of the SSA measurements on an inclusion yields a value for $r_A$ that represents an average over the inclusion and does not take into account any heterogeneity in $r_A$ values, in different regions of the inclusion. A comparison of such averaged $r_A$ values across inclusions masks any differences in heterogeneity in the nature of internal packing with which different aggregates may be organized within an
mEGFP moieties in the 74Q-mEGFP aggregate are comparable to the Förster radius of the mEGFP adduct, which is ∼4.6 nm, and that the mEGFP moieties are organized in the proximity of each other within relatively large clusters within the inclusion. The value of aggregate anisotropy can be correlated directly with the extent of homo-FRET; the larger the extent of homo-FRET, the lower the anisotropy of an aggregate.

**TRA Measurements Confirm the Occurrence of Homo-FRET and Reveal the Complexity of Aggregate Structure.** When identical fluorophores are assembled in the proximity of each other, the occurrence of homo-FRET results in not only a decrease in SSA values but also the presence of initial fast depolarization in TRA decays. The observation of an initial fast depolarization, within the first 2–3 ns, in the TRA decay curves of 74Q-mEGFP inclusions (Figures 7B and 8A) is, therefore, a direct evidence of the occurrence of strong homo-FRET within the inclusions. In the large and bright inclusions, the fluorescence anisotropy value has decreased to less than one-third of the initial value ($r_0 \sim 0.32$) within the first 2 ns of the TRA decay, when homo-FRET is complete. Such a large change in fluorescence anisotropy has not been observed in other EGFP-based polarization studies, suggesting that the 74Q-mEGFP molecules are very closely organized in large clusters. Although it may be possible to relate the cluster size with the extent of homo-FRET observed, it is not so straightforward for mEGFP-labeled proteins, but it would appear that the cluster size of 74Q-mEGFP molecules undergoing homo-FRET in large inclusions is at least greater than 3.

Rather surprisingly, the homo-FRET component of the TRA decay curves cannot be described as a single-exponential decay. Instead, the homo-FRET-related anisotropy decay appears to occur with two rates. The very fast and fast TRA decay rates of the mEGFP moieties in the inclusions appear to be an outcome of the stoichiometry and geometry of fluorophore arrangements or simply a feature associated with large cluster sizes. The two decay times are likely to represent at least two distinct FRET regimes, which may differ in either interfluorophore distances and/or relative fluorophore orientations or may simply point toward an underlying heterogeneity with which different aggregates may be packed within an inclusion. Currently, it is not possible to interpret the complex homo-FRET component of the decay quantitatively, but the apparent rates and amplitudes can be used to draw qualitative conclusions.

The homo-FRET-associated decay rates observed in bright inclusions are relatively faster than those observed in faint inclusions (Figure 8). The total amplitude of the homo-FRET component of the TRA decay that is over within 2–3 ns (Figure 8) is greater for the large inclusions than for the small inclusions. This result indicates that the cluster of 74Q-mEGFP molecules undergoing homo-FRET is greater in the large than in the small inclusions. It should be noted that the greater the number of molecules in a given cluster, the greater the overall probability of occurrence of homo-FRET. The TRA decay measurements suggest that a bright inclusion contains a greater concentration of aggregated 74Q-mEGFP molecules, and these molecules are more closely packed, when compared with a relatively faint inclusion. Because a faint inclusion represents an early precursor of a bright inclusion, it appears that the growth of an inclusion may be associated with a gradual increase in the packing density of the aggregating protein.

**The Organization of Protein Molecules in 74Q-mEGFP Inclusions Changes Gradually with Growth.** The extent of homo-FRET-allowed depolarization in the inclusions depends
on the number of 74Q-mEGFP molecules in the proximity (the size of the cluster)\textsuperscript{30} the distances separating the mEGFP adducts, and the relative orientations of the mEGFP adducts with respect to each other. The SSA measurements indicate that the extent of homo-FRET is enhanced as small and faint inclusions transform gradually into larger and brighter inclusions (see Results). The enhancement of homo-FRET in the large and bright inclusions as compared to the smaller and fainter inclusions is also supported by the observation that the rates of fast depolarization observed in the TRA measurements (Figure 8) are faster in the former than in the latter. Thus, the changes in anisotropy correlated with inclusion brightness, observed in this study, could result from changes in the 74Q-mEGFP packing density but could also arise from changes in the relative orientations of the mEGFP adducts. It is also possible that the enhancement of the homo-FRET rates may itself be a manifestation of increased cluster size,\textsuperscript{30,58} which itself implies an increase in the level of protein packing, so that more fluorophores can undergo FRET with each other. In either case, the enhancement of homo-FRET would be caused by structural changes occurring within the inclusion as a small and faint inclusion grows into a larger and brighter inclusion.

The large change in the extent of depolarization observed as the small inclusions grow into large inclusions (see Results) makes it unlikely that the decrease in anisotropy can be accounted for by only a change in the relative fluorophore dipole orientation,\textsuperscript{60} without a concomitant decrease in interfluorophore separation or an increase in cluster size. It is of course possible that in the large and bright inclusions, the mEGFP moieties, which are large barrel-shaped proteins (2.4 \text{nm} in width and 4.2 \text{nm} in length),\textsuperscript{34} are clustered in the closest possible contact\textsuperscript{35} in a set of specific nonrandom orientations that allow for high homo-FRET efficiency as well as extent of depolarization. It has been argued that the interactions between mEGFP barrels in the proximity can specify a set of specific nonrandom orientations.\textsuperscript{57} The lower homo-FRET efficiency as well as the much lower level of depolarization seen in the smaller inclusions suggests that the set of specific nonrandom fluorophore orientations that are present in the large inclusions are different in the smaller inclusions. This is likely because the mEGFP moieties have not yet come into the closest possible contact specified by interactions between the mEGFP barrels. In other words, in this scenario, the mEGFP moieties would have to be more separated in the small inclusions than in the bright inclusions. Further, it is possible that just as the presence of the mEGFP tag may possibly affect the aggregation of 74Q-mEGFP (as described above), the presence of the rest of the structure in intact huntingtin protein would also modulate the aggregation process of the poly-Q tract that drives aggregation. These effects will have to be investigated in future studies.

Thus, while it is likely that changes in the relative orientations between the 74Q-mEGFP molecules may occur as the inclusion grows with addition of more 74Q-mEGFP protein, it is unlikely that these changes occur without a concomitant decrease in the distances separating the mEGFP adducts or an increase in the relative cluster size available for homo-FRET. Hence, it is likely that the structural changes in the aggregate that occur with inclusion growth (see above) are accompanied by an increase in packing density. Indeed, the observation that the intensity of an inclusion increases by nearly 10-fold (Figure S1 of the Supporting Information), a factor too large to be attributed to imperfect confocality (see above), as it grows from small to large, supports the possibility that inclusion growth is accompanied by an increase in packing density.

**The Organization of mEGFP-Tagged Aggregated Protein Molecules in Inclusions and the Mode of Inclusion Growth.** A simple model\textsuperscript{21} of inclusion bodies in mammalian cells envisages them to be assemblies of aggregates.
In the simplest of such cases, the inclusion may represent a region of higher aggregate concentration, without any interaggregate association [bright inclusion (Figure 9)]. In this study, the homo-FRET measurements suggest the possibility that as small inclusions transform into large inclusions, changes in the packing of the 74Q-mEGFP molecules occur throughout the inclusion, including the center. The results are consistent with a model in which inclusion growth is accompanied by an increase in packing density. Speculatively, the growth of an inclusion may occur by integration of individual aggregate structures at the molecular level [bright inclusion (Figure 9)], especially at the center of the inclusion. In such a possible scenario, an internal rearrangement of aggregate structure would have to occur after the association of the aggregates to form inclusions, and the larger the inclusion, the greater the internal structural rearrangement leading to a higher packing density at the inclusion center. It is possible that the increase in cluster size at the center of inclusions, which accompanies inclusion growth, may be a signature of such an internal structural rearrangement. It is also to be noted that if an inclusion were to form by simple juxtaposition of smaller aggregates, then it is possible that homo-FRET will increase and anisotropy will decrease, but only at the interaggregate interface present at the periphery of the inclusion. Accordingly, a decrease in anisotropy would not be observed at the center of the inclusion, contrary to the observation made in this study (Figures 2 and 6).

At the other extreme, inclusion growth might occur by addition of monomeric protein. If monomeric protein were to add only at the periphery of a growing inclusion, then this addition would have to be accompanied by internal packing rearrangement as described above for aggregate growth occurring by the assembly of smaller aggregates. Larger inclusions have been shown to have distinct cores, and for such inclusions, recruitment of new protein has been shown to happen only at the periphery, which is less densely packed. It is possible, however, that monomeric protein can diffuse into the center and other parts of a small inclusion that is packed loosely, and its integration into the inclusion there leads to an increase in the level of homo-FRET and an increase in packing density. The stage at which the interior of an inclusion becomes sufficiently tightly packed for monomeric protein to be prevented from diffusing into it remains to be determined. In future studies, it would be interesting to study inclusion body formation as a crossover from diffusion-limited aggregation to a more compact growth.

**Biological Relevance of This Study.** This study, utilizing fluorescence anisotropy as an elegant probe for examining structural variations between homo-oligomeric aggregates, has suggested that as inclusions grow inside cells, structural changes occur within them that affect internal packing and that packing density increases within the core. The TRA measurements have revealed heterogeneity in aggregate structures, which might explain the observation that poly-Q aggregates have heterogeneous molecular interactions with associated proteins. It has been proposed that the presence of inclusion bodies may be toxic because they can sequester other proteins whose functions are important for cellular survival. Indeed, large inclusion bodies have been shown to bind transcription factors but only at their periphery. This study suggests that at early stages of inclusion body growth, before they become too densely packed, small inclusions may be more capable of sequestering functionally important protein or may interact with different sets of proteins compared to large inclusions. Indeed, it has been suggested that early inclusions, and not late and large inclusions, along with the diffusible protein may be the principal toxic forms.

The homo-FRET-based assay described here for studying aggregate growth will be useful in designing high-throughput screening assays for testing the ability of different molecules to induce or prevent inclusion growth. It is known that protein aggregation is strongly affected by the conditions of aggregation, and this may possibly affect the underlying pathogenesis. For instance, aggregates formed by osmotic stress seem to differ from those induced by aging. Homo-FRET-dependent analysis may represent an attractive way of comparing such structures, providing information at the level of molecular arrangement.

**ASSOCIATED CONTENT**

Supporting Information

Representative images of a cell, showing the growth of a poly-Q inclusion with time (Figure S1), and the method used for following inclusion growth. This material is available free of charge via the Internet at http://pubs.acs.org.

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**ABBREVIATIONS**

mEGFP, monomeric enhanced green fluorescent protein; poly-Q, polyglutamine; FRET, Förster (fluorescence) resonance energy transfer; FRAP, fluorescence recovery after photobleaching; FLIP, fluorescence loss in photobleaching; SSA, steady-state anisotropy; TRA, time-resolved anisotropy; 74Q-mEGFP (poly-Q74) and 23Q-mEGFP (poly-Q23), mEGFP-tagged huntingtin exon 1 fragment with 74 and 23 glutamine residues, respectively; I and r, intensity and anisotropy, respectively; D and A, diffusible and aggregated protein, respectively; TCSPC, time-correlated single-photon counting.

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