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Highly sensitive FRET-FCS detects amyloid β-peptide oligomers in solution at physiological concentrations

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KEYWORDS Alzheimer disease, Amyloid b-peptide, oligomers, Fluorescence Correlation Spectroscopy, Fluorescence Cross-Correlation Spectroscopy, FRET-FCS, Förster Resonance Energy Transfer, Photon Counting Histogram, hydrodynamic radius, diffusion coefficient, radius of gyration, intrinsically disordered proteins, random coil peptides

ABSTRACT Oligomers formed by the amyloid β-peptide (Aβ) are pathogens in Alzheimer disease. Increased knowledge on the oligomerization process is crucial for understanding the disease and for finding treatments. Ideally, Aβ oligomerization should be studied in solution and at physiologically relevant concentrations, but most popular techniques of today are not capable
of such analyses. We demonstrate here that the combination of Förster Resonance Energy Transfer and Fluorescence Correlation Spectroscopy (FRET-FCS) has a unique ability to detect small subpopulations of FRET-active molecules and oligomers. FRET-FCS could readily detect a FRET-active oligonucleotide present at levels as low as 0.5 % compared to FRET-inactive dye molecules. In contrast, three established fluorescence fluctuation techniques (FCS, FCCS and PCH) required fractions between 7 and 11%. When applied to the analysis of Aβ, FRET-FCS detected oligomers consisting of less than ten Aβ molecules which coexisted with the monomers at fractions as low as 2%. Thus, we demonstrate for the first time direct detection of small fractions of Aβ oligomers in solution at physiological concentrations. This ability of FRET-FCS could be an indispensable tool for studying biological oligomerization processes in general, and for finding therapeutically useful oligomerization inhibitors.

Alzheimer disease (AD) affects around 30 million people, and the number is growing as life expectancy increases around the world. Pathologically, the disease is characterized by intracellular tangles formed by the microtubule-associated protein tau, and extracellular fibrillar deposits called amyloid plaques. The fibrils are composed of the amyloid β-peptide (Aβ), a proteolytic product generated from processing of the amyloid precursor protein (APP). A 40 residues long variant (Aβ40) is produced at high levels, (around 80-90% of all Aβ produced), but it is the longer and more hydrophobic 42-residues variant (Aβ42) that is the dominating species in plaques. Most evidence from experiments in vitro and in transgenic mice over expressing Aβ suggest that oligomeric species, and not the fibrils, formed by Aβ are crucial for AD-
The nature of these oligomers is not fully elucidated, and it is important to further characterize them with respect to their structure and size, and how they are formed. Such information would be imperative for the rationale design of compounds aimed at treating AD by interfering with Aβ polymerization.

Ideally, early events of Aβ oligomerization should be studied in solution and at physiologically relevant concentrations. In cerebrospinal fluid (CSF), the Aβ42 levels are around 0.15 nM in healthy controls and 0.06 nM in AD patients, while in the cortex the corresponding buffer soluble levels are approximately 1 nM and 30 nM respectively. Regrettably, the most popular techniques of today are not suitable for studies of sub-µM concentrations, and many of them are off-line techniques. The most common techniques for studying Aβ polymerization are Thioflavine T (ThT) binding, SDS polyacrylamide gel electrophoresis (PAGE) combined with western blot (WB) analysis, and size exclusion chromatography (SEC). However, ThT is less useful for studying early events in the oligomerization process and has limited sensitivity at low concentrations of Aβ. SDS-PAGE may affect oligomerization by the use of SDS, and the equilibrium of the sample by the electrophoresis. In SEC, secondary interaction with the matrix may affect the molecular weight estimation, and the equilibrium is altered during the chromatography. Therefore, there is an urgent need for new methodologies that are capable of shedding light on the early events in Aβ oligomerization.

Fluorescence Correlation Spectroscopy (FCS) and related fluctuation techniques have since the early 1990’s become widely used for analyzing biomolecular interactions and fluctuations, in solution and in living cells. When it comes to analysis of Aβ oligomers in solution, FCS has shown some promise, but these studies report only on large aggregates containing several hundred monomers. For detection of subpopulations of molecules/oligomers which differ
little in size from the dominating population, FCS and fluctuation techniques have clear limitations.

Here we demonstrate that the combination of Förster Resonance Energy Transfer and FCS (FRET-FCS) is highly sensitive when it comes to detection of FRET-active subpopulations. The sensitivity of FRET-FCS was first analysed on a FRET-active oligonucleotide, and thereafter utilized for detection of small subpopulations of Aβ42 oligomers, in samples dominated by monomeric Aβ42.

MATERIALS AND METHODS

Sample preparations

Aβ40-HiLyte488, Aβ40-HiLyte647, Aβ42-HiLyte488 and Aβ42-HiLyte647 purchased from Anaspec, USA, were dissolved in hexafluoro-2-propanol in 1.5 ml plastic tubes (Axygen, maximum recovery) and kept as stock solution at +4 °C. For measurements, a 2 µl pipette tip was first dipped in the Aβ HFIP stock solution and then added directly to the PBS buffer solution on the measurement coverslip, or alternatively 1 µl of stock solution was diluted into 100 µl PBS. For concentrations lower than 200 µM of peptide, measurements were performed on coverslips coated with PLL-PEG (Surface Solutions, Switzerland) in order to minimize adsorption of peptides onto the coverslip surface. Cy5-labeled 36 base pair oligonucleotides and Cy5- and Rhodamine Green-labelled 35 base pair oligonucleotides were purchased from Sigma Aldrich.

Adsorption of Aβ onto glass coverslips
Coverslips were coated with poly-L-lysine-polyethylene glycol (PLL-PEG) in order to reduce adsorption of Aβ onto the glass coverslips. At concentrations of Aβ42 above ~200 nM glass surface treatment was usually not necessary, because the surfaces are then “coated” and saturated by the Aβ peptide itself, without causing significant decrease in the sample concentration.

Furthermore, the adsorption of Aβ42-488 onto the untreated glass coverslips, but not of the dye HiLyte 488, provided a means to estimate the amount of free dye present in the sample. Comparing the initial total fluorescence intensity (from Aβ42-488 + free HiLyte 488 dye) and the final total fluorescence intensity (from free HiLyte 488 alone) when all the Aβ42-488 had adsorbed onto the glass, indicated that about 5 % of the sample consisted of free HL488 dye (Figure S2, right).

**Fluorescence Correlation Spectroscopy (FCS) measurements**

FCS measurements were performed on a home-built instrument for FCCS (Figure S2, left), as has been describe earlier but here with the acousto-optical modulators removed, comprising a HeNe laser (Melles Griot) for the 594 nm line and an Argon laser (Melles Griot) for the 488 nm line. The laser lines are superimposed and reflected via a dichroic mirror (z488/594rpc, Chroma Technology Corp., Rockingham, VT, USA) into an inverted microscope (Olympus IX70), and focused by an objective (Olympus 60 X, NA 1.2, UPlanApo) to generate a dual colour excitation focus with a radius $\omega_0=0.2-0.35 \mu$m. The resulting emission is collected by the same objective, transmitted by the dichroic mirror and thereafter divided by a dichroic beam splitter (620DCXR,Chroma), filtered by emission filters (HQ532/70m, Chroma, for the green emission and HQ700/75, Chroma, for the red emission) and focused onto two pinholes, and finally
detected by single photon counting modules (SPCM-AQR-13/14, PerkinElmer Optoelectronics, Fremont, CA, USA).

Auto- or cross-correlation functions are generated from the collected fluorescence fluctuations from peptides diffusing through the dual laser focus. The FCS- and FCCS-curves were fitted to theoretical models for diffusion of one or two species of different molecular weights in order to obtain the diffusion times.

Even though adsorption of peptides to the glass surface should be minimal at peptide concentrations higher than 200 nM or when coverslips are coated with PLL-PEG, control measurements were always performed at the end of each analysis, especially after the 30 min measurements, to verify that the diffusion time of the sample was 2-2.4 times longer than that of the free dye alone. This ensured that adsorption of peptide to the glass was minimal and did not affect the analysis.

Photons Counting Histogram (PCH) analysis

For PCH analysis, the same instrument is used as for FCS measurement, but instead of analyzing the automatically generated FCS curves, the raw intensity traces are saved for subsequent analysis. The intensity traces consist of the number of detected photons for each time bin. In our measurements the bin time was set to 10 µs, and the number of detected photons per time bin typically varied from 0 to 15. In PCH histograms the logarithm of the y-axis is displayed in order to emphasize small population-differences in bins with larger photon counts. PCH analysis was performed using the free, public domain image processing software ImageJ,
developed at the National Institutes of Health, with a free plugin for PCH analysis developed by Jay Unruh, Stowers Institute for Medical Research.

RESULTS

The formation of aggregates depends on the preparation procedure

During the initial measurements on Aβ42-HiLyte 647 (Aβ42-647) and Aβ42-HiLyte 488 (Aβ42-488) diluted into PBS buffer at concentrations ranging from 10 to 500 nM, aggregates containing several hundred monomers were directly visible in the intensity trace (see SI, Figure S3). We soon found that the formation of aggregates depended on the sample preparation procedure. Since our aim was to analyze the early oligomerization process, i.e. the formation of dimers, trimers, tetramers etc, we developed a preparation protocol which resulted in aggregate-free Aβ solutions (see SI).

In aggregate-free solutions, FCS, FCCS and PCH can only detect Aβ monomers

First, the aggregate-free Aβ solutions were analyzed by FCS, with the hope of detecting the small oligomers by their slightly reduced diffusion coefficient compared to monomers. As a reference for monomeric Aβ, Aβ40 was used which has a lower propensity to form oligomers and aggregates than Aβ42\(^{16}\). However no significant difference in diffusion coefficient between Aβ40 and Aβ42 could be observed (Figure S4 and table S1). Since oligomers may be more likely to form at higher Aβ concentrations, measurements were also performed at 2.5 μM Aβ42. Neither here was any decrease in diffusion coefficient observed (table S1). One possible explanation for the apparent lack of oligomers in the Aβ42 solutions is that the percentage of
oligomers compared to monomers in the samples was too low. Therefore the sensitivity of FCS towards detecting subpopulations of Aβ was investigated, by performing measurements on a Cy5-labeled 36 bp double stranded oligonucleotide to which increasing concentrations of fluorophores was added. These measurements indicated that a sample containing 3% oligomers could be distinguished from a sample containing dye molecules only (Figure S3). However, the size difference between the 36 bp oligonucleotide and dye molecules is large, and the Aβ oligomers, which may contain fewer than ten monomers, will likely have to be present at >10% in order to be detectable, as indicated by studies of the resolution in FCS\textsuperscript{17}.

Next we analyzed the possible presence of Aβ-oligomers using FCCS. FCCS detects the correlated movement of green and red dye molecules, and should therefore be able to detect oligomers which contain both Aβ42-647 and Aβ42-488. FCCS measurements were performed at concentrations varying from 50 nM Aβ42-647 + 20 nM Aβ42-488 up to 4.6 μM Aβ42-647 + 600 nM Aβ42-488 (the Aβ42-647 was kept higher in order to limit the effect of cross-talk). However, zero cross-correlation amplitude was observed in all mixtures, indicating that oligomers were either not present or were below the detection limit (Figure S5).

Also the sensitivity of FCCS was analysed, by measuring on a 35 bp double stranded oligonucleotide labelled with a green dye (Rhodamine Green) and a red dye (Cy5) at each end, to which increasing concentrations of red and green dyes were added (Figure S5). These measurements indicated that the double-labelled oligonucleotide could be detected when present at a relative concentration of 11%, compared to the concentrations of red and green dyes only. Thus, the lack of cross-correlation in the FCCS measurements on Aβ42-647 + Aβ42-488 indicates that oligomers did not exist or were present at fractions below 11%.
In a third approach the possible presence of Aβ-oligomers was investigated using the Photon Counting Histogram, PCH. PCH can detect differences in brightness of the diffusing species and should thereby be able to distinguish the presence of the assumingly brighter Aβ-oligomers. PCH analysis of Aβ42-647 diluted into PBS was performed at concentrations of 30 nM and 220 nM (Figure S6). The histograms could be very well fitted with a single species model, using the same brightness as had previously been obtained from a PCH measurement on the dye HiLyte 647 alone using the same concentrations and excitation powers (Figure S6). Thus, neither PCH analysis of Aβ42-647 could detect the presence of any oligomers under these conditions.

The sensitivity of PCH was estimated by measuring on mixtures of two different fluorophores whose brightness differed by approximately a factor of two. This indicated that the brighter dye could be detected when present at 7 % compared to the less bright dye (Figure S6). Accordingly, in the PCH measurements on Aβ42-647, oligomers were either absent or they were present at fractions lower than 7 %.

FRET occurs in aggregates of Aβ42-647 and Aβ42-488

As described above, the initial measurements on Aβ42-647 and Aβ42-488 resulted in aggregates which were clearly visible in the intensity trace. Since Aβ is a relatively small molecule (the hydrodynamic radius of the Aβ monomer is about 1.6 nm\textsuperscript{18-20}), FRET is likely to occur within aggregates that contain Aβ42-647 and Aβ42-488. The presence of FRET within these aggregates is demonstrated by recording of two consecutive intensity traces: First 488 and 594 nm excitation was used for 30 s, thereafter only 488 nm excitation was used for 30 s. Already during the first 30 s the presence of FRET was indicated by spikes in the red detection channel, while the green detection channel almost completely lacked spikes (Figure 1). During
the second 30 s, as the 594 nm excitation was switched off, the base line in the red detection channel decreased from 300 kHz to 10 kHz. This was expected since the base line corresponds to the fluorescence from Aβ42-647 monomers which are dependent on the 594 nm excitation. In contrast, the aggregates in the red detection channel remained, in frequency as well as in amplitude. This indicates that the FRET was high and did only occur within aggregates (Figure 1).

![Figure 1](image)

**Figure 1.** Red and green fluorescence intensity traces, detected from a sample of 150 nM of Aβ42-647 and 75 nM of Aβ42-488. FRET occurs in aggregates of Aβ42-647 and Aβ42-488 (seen as spikes in the red fluorescence intensity trace). Both the 488 nm- and the 594 nm lasers are activated initially, and after 30 s the 594 nm excitation is turned off.

*FRET-FCS can detect small subpopulations of a FRET-active oligonucleotide*

Having observed FRET in Aβ aggregates, we argued that FRET should be present also in small oligomers of Aβ42-647 and Aβ42-488. We therefore set out to investigate whether FRET-
FCS could be utilized for analyzing the possible presence of small fractions of oligomers in the aggregate-free samples.

The sensitivity of FRET-FCS was investigated by measuring on a specially designed FRET-active oligonucleotide of 25 bp length, into which the dyes Atto 488 and Atto 647N were incorporated into the same strand with 3 bp distance. When this FRET-DNA was excited at 488 nm, equal fluorescence intensities were detected in the green and the red detection channels in our FCS setup.

FRET-FCS measurements were performed on mixtures containing 1 nM of the FRET-DNA and up to 200 nM of green dye and 200 nM of red dye. Even at the highest concentration of red and green dye, corresponding to 0.5% (mol/mol) of FRET-DNA compared to either dye, the presence of FRET-DNA could readily be detected by FRET-FCS (Figure 2, red curve). When FRET-FCS was performed on a sample containing 200 nM of red dye and 200 nm of green dye, but lacking the FRET-DNA, a noisier curve was obtained which indicated the shorter diffusion time of the dyes (Figure 2, black curve). The noisy curve is the result of fluorescence from the green dyes detected by the red detector (cross-talk) as well as fluorescence from the red dyes directly excited by the 488 nm laser.

When the same samples were analysed by standard FCS with respect to changes in diffusion coefficients, the presence of the FRET-DNA could only be distinguished when present at fractions above 5% (Figure S9). This detection limit is in line with that of standard FCS discussed above.
Figure 2. Small fractions of FRET-active molecules can be detected by FRET-FCS. Red curve: FRET-FCS measurements of 1 nM FRET-DNA + 200 nM HiLyte 488 + 200 nM HiLyte 647. Black curve: 200 nM HiLyte 488 + 200 nM of HiLyte 647 without the FRET-DNA. Green curve: A standard FCS curve measured on HiLyte 488 alone showing that the diffusion time of the black curve corresponds to that of free dyes.

*FRET-FCS detects Aβ-oligomers in aggregate-free solutions*

Next FRET-FCS was applied to detect the possible presence of oligomers in aggregate-free samples of Aβ42-647 and Aβ42-488. FRET-FCS curves then readily appeared, with a diffusion time significantly longer than that obtained from standard FCS (Figure 3). The longer diffusion time arises since in FRET-FCS it results from only the diffusion of the FRET-active oligomers (Figure 3, black curve), while in standard FCS it corresponds to the dominating and more highly mobile monomers (Figure 3, blue and green curves).

The mean number of monomers in Aβ-oligomers was estimated accurately by comparing FRET-FCS curves with curves recorded on dye molecules alone (see SI for details).
Figure 3. Detection of oligomers of Aβ42-647 and Aβ42-488 by FRET-FCS. The FRET-FCS curve (black) yields a diffusion time that is longer than that given by the standard FCS curves (blue and green), as expected from the larger size of oligomers compared to monomers.

Thus, in Figure 3, by using that the diffusion time $\tau_{D,FRET-FCS} = 391 \, \mu s$ is 2.23 times longer than the reference diffusion time $\tau_{D,ref} = 176 \, \mu s$ measured on HiLyte 647 alone, we could estimate that the oligomers contained on average $2.23^3 \approx 11$ monomers, assuming that the Aβ-oligomers are spherically shaped and therefore that $\tau_D \propto m^{1/3}$ where $m$ is the particle mass. Other measurements on aggregate-free solutions of Aβ yielded oligomer sizes between eleven and down to three monomers. In one such measurement where the Aβ-oligomers consisted of on average 8 monomers, the oligomer/monomer-fraction was estimated to 2 % (see SI for details).

Finally samples of Aβ42-647 and Aβ42-488 prepared at 145 nM, 255 nM, 580 nM and 2.8 μM were analyzed by FRET-FCS, in order to investigate whether the propensity of Aβ42 to form oligomers varied with concentration. The four samples yielded diffusion times $\tau_D$ of 230, 279, 443 and 339 μs respectively and thus there was a tendency that the average size of oligomers was larger at higher Aβ42 concentrations, even though the largest average oligomer size was
observed at 580 nM and not at 2.8 µM (Figure 4). This is most likely an effect of variations in our procedure for preparing aggregate-free samples (see SI for details).

**Figure 4.** Normalized FRET-FCS curves from measurements on mixtures of Aβ42-647 and Aβ42-488 at varying concentrations.

**DISCUSSION**

The results section showed that FRET-FCS is highly sensitive and able to detect Aβ oligomers consisting of 3-11 monomers at fractions of only a few percent. In contrast, FCS, FCCS and PCH require oligomers to be present at 7-11% and could therefore not detect the Aβ oligomers present in the samples.

Two results indicate strongly that Aβ monomers were the dominating species in the aggregate-free Aβ preparations. First, no cross-correlation was observed in measurements on spike-free mixtures of Aβ42-647 and Aβ42-488 (Figure S5). Thus, even though small percentages of oligomers apparently existed as evidenced by FRET-FCS, the large majority of peptides should
have been in monomeric form. Second, we consistently observed FCS diffusion times of Aβ42-647 that were 2.2 – 2.4 times longer than that of the free dye HiLyte 647. The diffusion coefficient of HiLyte 647 has not been reported previously, but we measured 11 % lower diffusion coefficient for HiLyte 647 compared to Cy5 ($D_{Cy5}=3.6 \cdot 10^{-10} \text{ m}^2/\text{s}$)21, and we therefore use that $D_{HL647}=3.2 \cdot 10^{-10} \text{ m}^2/\text{s}$ at 25 °C. Accordingly the ratios 2.2 to 2.4 indicate a diffusion coefficient for Aβ42-647 in the range $1.33 – 1.45 \cdot 10^{-10} \text{ m}^2/\text{s}$. This agrees with previous experimental estimations of the diffusion coefficient of monomeric Aβ1-40 of $1.4 – 1.5 \cdot 10^{-10} \text{ m}^2/\text{s}$20 and with a detailed analysis yielding $1.52 \cdot 10^{-10} \text{ m}^2/\text{s}$18,19. From the Stokes-Einstein equation the corresponding hydrodynamic radius (also called Stokes radius) for Aβ42-647 equals $R_h=1.7-1.8 \text{ nm}$, and for Aβ1-40 $R_h=1.6-1.7 \text{ nm}$20 and $R_h=1.6 \text{ nm}$18,19. Our value of $R_h=1.7-1.8 \text{ nm}$ should be about 0.1 nm higher due to the attached dye, and should correspond to 1.6-1.7 nm for a non-labelled Aβ1-4222.

When single colour FCS is used for estimating the size of peptides and proteins, it is often assumed that the diffusion coefficient D and the peptide mass m are related by $D \propto m^{-1/3}$23. This holds approximately for folded proteins22 and for oligomers, but it is not valid for single denatured proteins22 or random coil peptides. For random coil peptides, which is the dominating state in freshly diluted aqueous solutions of Aβ40, there is strong support for the relationship $D \propto m^{-0.44}$18. Thus, without knowledge of the molecules’ rigidity the diffusion coefficient alone is an uncertain indicator of the presence of oligomers.

Aβ oligomerization has previously been studied by single colour FCS where a $R_h$ of 1-2 nm was reported24. This is in line with the $R_h$ value presented here for the Aβ monomer, but cannot exclude the additional presence of dimers. Another recent study using FCS reported in contrast a hydrodynamic radius of Aβ1-40 and Aβ1-42 as small as 0.85-0.9 nm25. Such a value deviates
significantly from the above estimates of $R_h$ as well as from other proteins of similar sizes$^{22,26}$, and is only about 50% larger than that of the fluorophore Rhodamine B to which $\alpha\beta$ was coupled ($R_h=0.57$ nm)$^{25}$.

Another measure of protein size is the radius of gyration $R_g$. $R_g$ has been reported for $\alpha\beta$1-40 and $\alpha\beta$1-42 in several studies$^{27-32}$. The $R_g$-values in these reports range from 0.9 nm to 1.3 nm with a mean of 1.14 nm$^{27-32}$. Dividing this value with the mean value of $R_h=1.63$ nm (from our estimate and from refs.$^{18,20}$) gives a ratio of $R_g/R_h=0.70$. Though this value is outside of the region $0.775 < R_g/R_h < 1.51$ commonly stated for particles and large proteins, it is in line with detailed predictions for small proteins and peptides$^{26}$.

FRET and FCS have previously been combined in different forms; for detailed investigation of the effect of FRET on FCCS data$^{33}$, for analyses of conformational fluctuations at single molecule concentrations$^{34}$ or at normal FCS concentrations$^{35,36}$, for the use of pulsed interleaved excitation (PIE)$^{37}$, for analysis of FRET fluctuations within calmodulin$^{38}$, for analysis of mean intensities from donors and acceptors on oligonucleotide drugs using an FCS microscope$^{39}$, and for single-molecule FRET analysis of the packing of DNA into viral capsids$^{40}$. However, FRET-FCS in the form of analysing the red detector-channel FCS curve while exciting only with 488 nm, as utilized in our study, has previously only been employed to analyse the trypsin activity on a FRET-probe$^{41}$. In that study, the ability to detect small fractions of FRET-active species was not investigated, and the signature of a reduced diffusion coefficient of the FRET-active species could not be utilized. Here, we demonstrate that a significant strength of FRET-FCS lies in the combination of a FRET signal and a reduced diffusion coefficient, which gives the sensitivity and specificity to detect rare FRET-active oligomers in a surrounding dominated by FRET-inactive monomers.
CONCLUSIONS

Aggregate-free samples of fluorescently labelled Aβ42 and Aβ40 were prepared and analyzed in solution at physiological concentrations. While the established techniques FCS, FCCS and PCH could not detect any Aβ-oligomers in these preparations, FRET-FCS could readily detect subpopulations of oligomers of Aβ42-647 and Aβ42-488. FRET-FCS detects a signal which is almost undisturbed by the signal from monomers, even when they make up the majority of the sample. FRET-FCS can thereby detect Aβ oligomers consisting of less than ten monomers and present at only a few percent of the concentration of the Aβ monomers. To our knowledge this is the first direct detection of Aβ oligomers in solution at physiological concentrations. FRET-FCS is particularly well suited for detection of such sub-populations of FRET-active species as demonstrated here on Aβ oligomers, and should be of general use for studies of small fractions of protein oligomers at physiological concentrations.

ASSOCIATED CONTENT

Supporting Information. Equations on FCS, details regarding adsorption of Aβ onto coverslips and factors affecting aggregation, additional FCS and FRET-FCS analysis of Aβ. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS
FCS, Fluorescence Correlation Spectroscopy; FCCS, Fluorescence Cross-Correlation Spectroscopy; PCH, Photon Counting Histogram; FRET, Förster Resonance Energy Transfer.

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