Molecular Brightness Characterization of EGFP In Vivo by Fluorescence Fluctuation Spectroscopy

Yan Chen, Joachim D. Müller, QiaoQiao Ruan, and Enrico Gratton
Department of Physics, University of Illinois at Urbana Champaign, Urbana, Illinois 61801 USA

ABSTRACT We characterize the molecular properties of autofluorescence and transiently expressed EGFP in the nucleus and in the cytoplasm of HeLa cells by fluorescence correlation spectroscopy (FCS) and by photon counting histogram (PCH) analysis. PCH has been characterized and applied in vitro, but its potential for in vivo studies needs to be explored. Thus, this study mainly focuses on the characterization of PCH analysis in vivo. The strength of PCH lies in its ability to distinguish biomolecules by their molecular brightness value. Because the concept of molecular brightness is crucial for PCH analysis, we study the molecular brightness of EGFP and determine the statistical accuracy of its measurement under in vivo conditions. We started by characterizing the influence of autofluorescence on EGFP measurements. We found a molecular brightness of EGFP that is a factor of 10 higher than the brightness of the autofluorescence. Moment analysis demonstrates that the contribution of autofluorescence to fluorescence fluctuation experiments is negligible at EGFP concentrations of one protein per excitation volume. The molecular brightness of EGFP measured in the nucleus, the cytoplasm, and in vitro are identical and our study demonstrates that molecular brightness is a very stable and predictable quantity for cellular measurements. In addition to PCH, we also analyzed the autocorrelation function of EGFP. The diffusion coefficient of EGFP is a factor of 3 lower in vivo than compared to in vitro, and a simple diffusion process describes the autocorrelation function. We found that in the nucleus the fluorescence intensity is stable as a function of time, while measurements in the cytoplasm display fluorescence intensity drifts that complicate the data analysis. We introduce and discuss an analysis method that minimizes the influence of the intensity drifts on PCH analysis. This method allows us to recover the correct molecular brightness of EGFP even in the presence of drifts of the fluorescence intensity signal. We found the molecular brightness of EGFP to be a very robust parameter, and anticipate the use of PCH analysis for the study of oligomerization processes in vivo.

INTRODUCTION

Understanding cellular function at the molecular level is one of the holy grails of biophysics, yet we lack essential experimental tools to observe cells with the necessary level of detail. Most techniques are based on biochemical essays, which destroy the cell to isolate its components, to infer from subsequent analysis the functional state of cells. It would be far superior to use spectroscopic techniques tailored to investigate cellular activity in vivo. Fluorescence techniques have always played an important role in cellular studies. The availability of green fluorescent protein (GFP) and its various mutants revolutionized our possibilities of studying cellular processes in vivo (Tsien, 1998). Molecular biology allows the construction of fusion proteins by genetically tagging GFP to a protein of interest. The fluorescence of the GFP-tagged fusion protein gives information about gene expression and localization inside cells. However, information regarding protein concentration, diffusion processes, and biochemical interactions is difficult to obtain with the standard techniques.

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Address reprint requests to Dr. Yan Chen, Dept. of Physics, University of Minnesota, 116 Church St., SE, Minneapolis, MN 55455. Tel.: 612-626-8684; Fax: 612-624-4578; E-mail: chen@physics.umn.edu.

J. D. Müller's present address is Dept. of Physics, University of Minnesota, 116 Church St., SE, Minneapolis, MN 55455. E-mail: mueller@physics.umn.edu.

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Fluorescence correlation spectroscopy (FCS) promises to develop into a quantitative tool for the in vivo characterization of absolute concentrations, molecular interactions, and kinetic processes, such as diffusion and chemical reactions. Webb and co-workers originally introduced the technique of FCS in 1972 (Magde et al., 1972). Their work was soon followed by others (Ehrenberg and Rigler, 1974; Koppel, 1974; Aragon and Pecora, 1975). Subsequent integration of confocal and two-photon techniques significantly increased the signal-to-noise ratio of FCS and reduced the measured volume element to less than one femtoliter (Rigler et al., 1993; Koppel et al., 1994; Qian and Elson, 1991; Berland et al., 1995). With these improvements, it is now possible to routinely measure on the single molecule level. FCS has been used to determine translational and rotational diffusion (Borejdo, 1979; Fahey et al., 1977; Ehrenberg and Rigler, 1974), chemical reactions (Thompson and Axelrod, 1983; Icenogle and Elson, 1983; Bonnet et al., 1998; Haupts et al., 1998; Starr and Thompson, 2001), flow rates (Magde et al., 1978; Gosch et al., 2000), aggregate formation (Qian and Elson, 1990a; Palmer and Thompson, 1989; Petersen et al., 1993; Berland et al., 1996), and triplet state parameters (Widengren et al., 1994). FCS has developed into a powerful tool for the characterization of biomolecules in vitro.

FCS combines a number of unique features that make it extremely attractive for intracellular applications. First, FCS determines kinetic processes from equilibrium fluctuations, thus no external perturbation is required to obtain kinetic information. Second, the extreme sensitivity of FCS allows

the detection of processes at the single molecule level. FCS measurements in vivo with two-photon excitation were first demonstrated by Berland et al. (1995). The potential of FCS for cellular applications has been explored further using one-photon and two-photon excitation by characterizing autofluorescence and various fluorescent probes (Brock et al., 1998; Schwille et al., 1999a). FCS has been applied to study the hybridization of oligonucleotides (Politz et al., 1998), GFP fusion proteins (Brock et al., 1999), diffusion processes of EGFP (Wachsmuth et al., 2000), substrate and neuron interactions (Boonen et al., 2000), and active protein transport through plastid tubules (Kohler et al., 2000).

FCS uses the autocorrelation function to characterize intensity fluctuations and is extremely powerful for characterizing dynamic properties of fluorescent molecules, such as chemical reactions or diffusion coefficients. FCS is also able to resolve a mixture of fluorescent species by differences in their diffusion coefficient. However, resolving species by their diffusion coefficients lacks sensitivity when the molecular weight of two species differs by less than a factor of 5 to 8 (Meseth et al., 1999). Therefore, the autocorrelation approach fails in separating certain mixtures, such as a mixture of dimers and monomers, where the molecular weight ratio between the two species is only a factor of 2. To address this shortcoming of the traditional FCS approach we developed a different method for separating species based on molecular brightness rather than molecular weight. To extract the molecular brightness from fluctuation experiments, we developed a theory to analyze the probability distribution of the photon counts that is experimentally determined by the photon counting histogram (PCH). The distribution of photon counts is uniquely described by two parameters for each fluorescent species: the molecular brightness of the particle and the average number of particles within the observation volume (Chen et al., 1999b). The photon counting histogram of multiple independent species is the convolution of the photon counting histogram of each individual species. Successful resolution of the histogram into its components is largely a matter of signal statistics (Müller et al., 2000).

The concept of PCH is quite straightforward. A particle with a given brightness produces a characteristic intensity fluctuation as it traverses through the observation volume. If another particle with a higher molecular brightness enters the observation volume, then a stronger intensity fluctuation of the fluorescence signal occurs. The statistics of the intensity amplitudes capture the distribution of molecular brightness values and their recurrence frequency. Thus, the amplitude statistics provide a quantitative description of the molecular brightness values of the particles together with their respective concentrations. A recent study demonstrates that even without any previous knowledge about sample parameters, PCH analysis is capable of resolving a brightness ratio of 2 from a single measurement (Müller et al., 2000). Thus, PCH has the sensitivity to resolve a mixture of monomers and dimers, because a dimer appears twice as bright as the monomer. Another analysis technique, which is similar to PCH, has been described in the literature and has been applied to study DNA hybridization and cleavage (Kask et al., 1999).

So far, PCH has been applied in vitro to probe hormone receptors and the formation of oligomer-polymer complexes (Margeat et al., 2001; Van Rompaey et al., 2001). In addition, PCH and FCS analysis were combined to investigate the properties of luminescent nanoparticles, which are promising candidates for future photostable biolabels (Akcakir et al., 2000). Here we are interested in the potential of PCH analysis for cellular applications. We are primarily concerned with the accuracy of determining the molecular brightness under in vivo conditions. In addition, we perform autocorrelation analysis to recover the diffusion coefficient. Both PCH and autocorrelation analyses use exactly the same experimental data, but each technique focuses on a different property of the stochastic signal. While the autocorrelation function is a measure of the time-dependent decay of the fluctuations to their equilibrium value, the photon counting histogram captures the amplitude distribution of these fluctuations. Thus, autocorrelation and PCH analysis are complementary techniques.

Experiments that extend FCS and PCH into the cellar environment face a few challenges not encountered by typical in vitro applications. A prominent difference between in vitro and in vivo studies is the presence of cellular molecules with intrinsic fluorescence. This autofluorescence adds a background contribution to any fluorescence measurement in the cellular environment. To address this issue, we characterize the molecular brightness and number of autofluorescence molecules in the cytoplasm and the nucleus of HeLa cells, which allows us to quantify the influence of the autofluorescence on fluorescence fluctuation measurements. This characterization of the autofluorescence is a prerequisite for quantitative studies of fluorescent biomolecules under in vivo conditions.

We also measured the green fluorescent protein, EGFP, in the cell nucleus and the cytoplasm. EGFP has become a very important intracellular fluorescent marker and has been used for many fusion protein systems. Since each protein of interest will carry one EGFP tag, it provides a very convenient method for studying molecular oligomerization using fluctuation experiments to detect the changes in molecular brightness. We are interested in identifying the accuracy of in vivo measurement with autocorrelation and PCH analysis. Specifically, three parameters, the molecular brightness, the number of molecules per excitation volume, and the diffusion coefficient of EGFP are determined under in vivo conditions. This knowledge allows judging which fluctuation experiments are feasible under in vivo conditions.

MATERIALS AND METHODS

Instrumentation

The instrumentation for two-photon fluorescence fluctuation experiments is similar to that described by Chen et al. (1999a) with few modifications. A mode-locked Ti:sapphire laser (Mira 900, Coherent, Palo Alto, CA) pumped by an intracavity doubled Nd:YVO_4 laser (Coherent Inc., Santa Clara, CA) was used as the two-photon excitation source. The experiments were carried out using a Zeiss Axiovert 135 TV microscope (Thornwood, NY) with a 63× Plan Apochromat oil immersion objective (N.A. = 1.4). An excitation wavelength of 895 nm was used for all measurements. The fluorescence was collected by passing through standard blue glass filters CM500 (Chroma Technology, Brattleboro, VT). The average power at the sample was 1.75 mW for all measurements. Photon counts were detected with an avalanche photodiode (APD) (Perkin-Elmer, model: SPCM-AQ-141, Vaudrevil, Canada). The data acquisition time for individual measurements is ~50 s. The output of the APD unit, which produces TTL pulses, was directly connected to a home-built data acquisition card, and sampled at either 1 MHz or 20 kHz (Eid et al., 2000). The recorded photon counts were stored and later analyzed with programs written for PV-WAVE (Version 6.21, Visual Numerics, Inc., Boulder, CO) and with LFD Globals Unlimited software (Champaign, IL).

Sample preparations and measurements

His-tagged EGFP was prepared according to Patterson et al. (1997). Protein was dialyzed with PBS buffer and its purity was checked with SDS gel stained with Coomassie Brilliant Blue. The concentration of protein was determined by absorption measurement using an extinction coefficient of 53,000 M^{-1} cm^{-1} at 489 nm.

HeLa cells were maintained in 10% fetal calf serum and DMEM (without phenol red) media. Transfections of HeLa cells were carried out by comixing EGFP plasmid, (Clontech, Palo Alto, CA) with effectene transfection reagent (Qiagen, Valencia, CA). Cells were kept around 60% confluency on the day of transfection. The transfection procedure strictly followed the manufacturer's instructions. We found that the expression level of EGFP is reduced to levels accessible to FCS measurements by using a linearized plasmid. Cells were typically measured 24 h after transfection and the growth medium was exchanged with PBS buffer.

HeLa cells were cultured in 8-well chambers (Naglenunc International, Rochester, NY), which were mounted on the stage of the microscope. The microscope is equipped with DIC optics and a CCD camera, which is mounted at one of the exit ports of the microscope. The CCD camera is used to position a cell and control the focus of the optics into the nucleus or cytoplasm for the FCS studies. After aligning the sample and instrument properly, the microscope is switched from bright-field illumination to two-photon microscopy for the fluorescence fluctuation measurements.

Data analysis

The autocorrelation function, $g(\tau)$, of an FCS experiment is calculated from the photon counts by,

$$g(\tau) = \frac{\langle F(t) \cdot F(t+\tau)\rangle - \langle F(t)\rangle^2}{\langle F(t)\rangle^2}, \qquad (1)$$

where $F(t)$ represents the detected photon counts at time t, and τ is the lag time. The angle brackets represent the time average. Experimental autocorrelation functions, $g(\tau)$, were fitted to theoretical functions using a Gaussian-Lorentzian beam profile (Berland et al., 1995) to recover the diffusion coefficient and the number of molecules.

The theoretical description of PCH for a single species has been described in the literature (Chen et al., 1999b; Müller et al., 2001). The experimentally determined histogram of photon counts is uniquely described by a theoretical distribution function $\Pi(k; \bar{N}, \varepsilon)$. Two parameters, the molecular brightness ε (in counts per second per molecule, cpsm) and the average number of molecule per excitation volume $\bar{N}$ describe the experimental histogram of a single species. The product of the molecular brightness ε and the number of molecules $\bar{N}$ gives the average intensity (in counts per second). The photon-counting histogram of a mixture of species is simply the convolution of the histograms of the individual species. It is possible to successfully resolve the individual species without any additional information about the sample mixture by PCH analysis, if the molecular brightness contrast is sufficient (Müller et al., 2000).

RESULTS

Autofluorescence of the nucleus

We characterized the feasibility of quantitative fluctuation measurements inside of living cells by two-photon excitation at 895 nm. Quantitative measurements in cells are complicated by the ubiquitous presence of autofluorescence. Of all cellular compartments the autofluorescence intensity is weakest in the nucleus. However, the fluorescence intensity is not the most crucial parameter for fluorescence fluctuation experiments. The same fluorescence intensity could be the result of many dim autofluorescent particles or a few bright particles. Both scenarios influence fluctuation measurements of fluorescent probes, such as GFP, in very different ways. Thus, we start by characterizing the molecular brightness of the autofluorescence. High excitation powers within cells not only lead to photobleaching, but also to oxidative-stress, which damage the cell (Konig et al., 1996). Therefore, we only used very low excitation powers, which were typically around 1.75 mW at the sample. We observed no photobleaching or oxidative-stress in any of our measurements. Cells that looked healthy and spread nicely as judged by wide field illumination were selected for the measurements. Fig. 1 *A* displays a representative photon-counting histogram of the autofluorescence signal from the cell nucleus. The average intensity of this measurement is 1500 cps. PCH analysis recovers a molecular brightness for the autofluorescence of 2700 cpsm and a concentration of 0.56 molecules in the excitation volume of the instrument. A number of different molecular species inside of cells contribute to the autofluorescence signal. Thus, the parameters recovered from the single-species PCH analysis are a characterization of the heterogeneous autofluorescence mixture in terms of an effective molecular brightness ε_A and an effective number of molecules $\bar{N}_A$. The intrinsic fluorescence intensity inside the nucleus is usually stable as a function of time (Fig. 1 *A*, *inset*). To determine the properties of a cell population and the signal variations that occur from cell to cell, we measured a total of 27 cells. We did not observe strong intensity variations within this cell population. Fig. 2 *A* displays the distribution of molecular brightness values, ε_A, for the cell population. Table 1 summarizes the average brightness, $\langle\varepsilon_A\rangle$, and the average number of molecules, $\langle\bar{N}_A\rangle$, of the

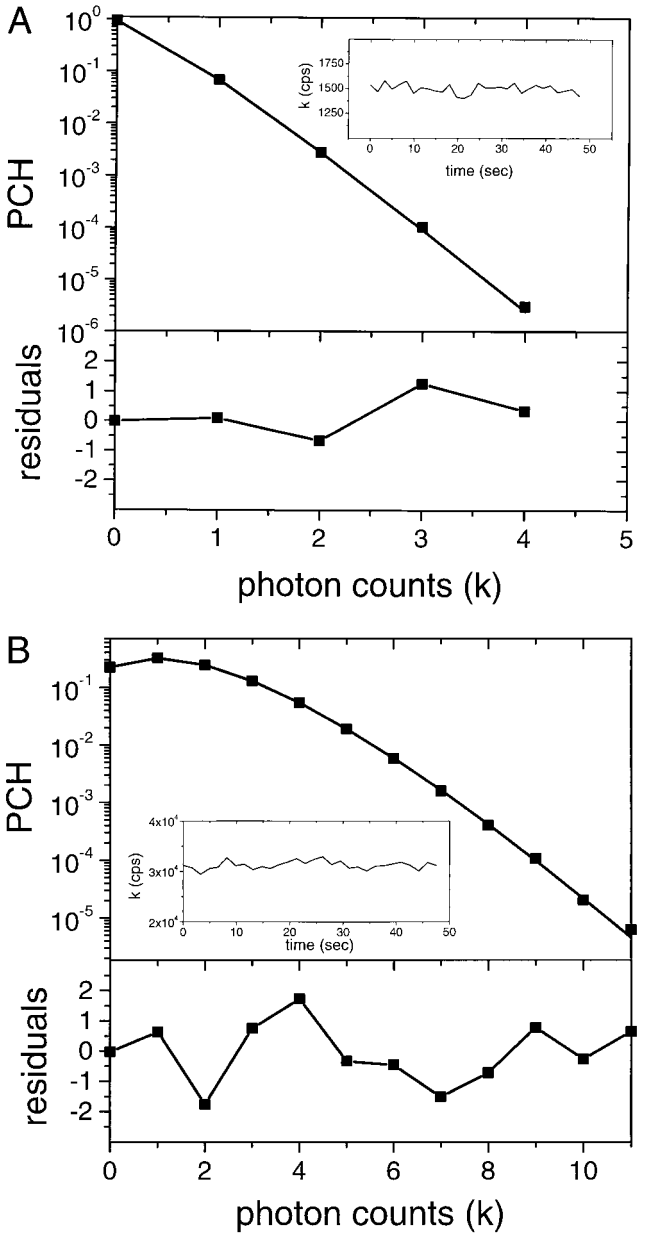

FIGURE 1 Representative photon counting histogram (PCH) of the autofluorescence (*A*) and EGFP (*B*) from the nucleus. The power at sample was <2 mW. The solid line represents a fit to a single species model. The fit recovers a particle number $\bar{N}_A$ of 0.56 and a brightness ε_A of 2700 cpsm for the autofluorescence. For EGFP the particle number $\bar{N}$ is 1.3 and the molecular brightness ε is 24,000 cpsm. The inset displays the average photon counts as a function of time on the second timescale.

nuclear autofluorescence of cells measured. The average brightness of the autofluorescence is 2500 cpsm, with a standard deviation of 450 cpsm. The average concentration is 0.7 molecules per excitation volume, with a standard deviation of 0.19.

The observed variations of the molecular brightness and the concentration within the 27 cells measured could be the result of individual differences experienced from cell to cell, or could reflect the heterogeneous nature of the cell nucleus. To differentiate between these two scenarios, we performed a series of measurements on a single cell nucleus while randomly varying the location of the laser spot between each measurement. This experiment probes the heterogeneity experienced within a single cell. We recovered an average molecular brightness of 2400 cpsm, with a standard deviation of 430 cpsm. The average particle concentration was 0.80, with a standard deviation of 0.20.

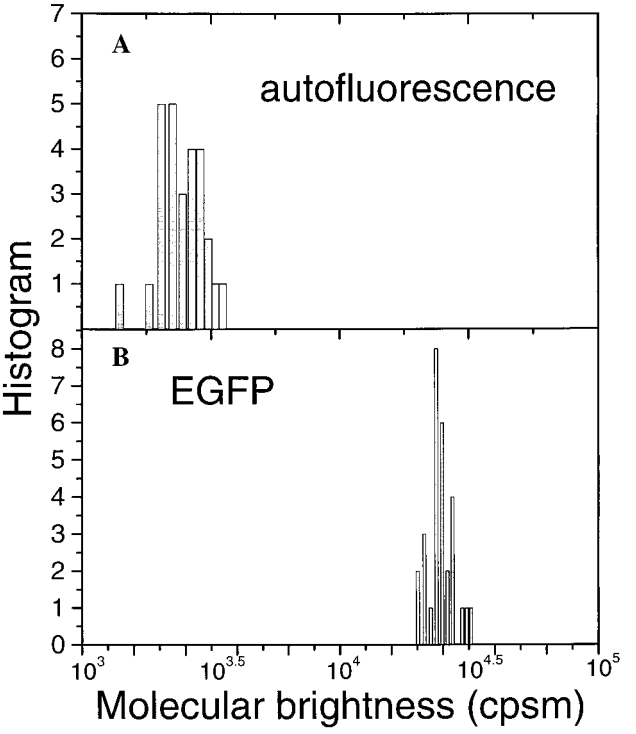

FIGURE 2 Molecular brightness distribution of the autofluorescence and EGFP in the nucleus from a total of 29 cells. The average molecular brightness of the autofluorescence is 2490 cpsm with a relative standard uncertainty of 18%. The average molecular brightness of EGFP is 25,000 cpsm with a relative standard uncertainty of 12%.

EGFP inside the nucleus

Transient transfection of HeLa cells with EGFP plasmid results in a very heterogeneous cell population with widely

TABLE 1 PCH analysis of the autofluorescence from the nucleus and the cytoplasm of HeLa cells

Autofluorescence	Nucleus (27 cells)	Cytoplasm (27 cells)	Nucleus (22 position in one cell)	Cytoplasm (22 position in one cell)
$\langle\varepsilon_A\rangle$ (cpsm)	2490 ± 450	2100 ± 430	2360 ± 430	2480 ± 470
$\langle\bar{N}_A\rangle$	0.70 ± 0.19	1.40 ± 0.41	0.80 ± 0.20	1.50 ± 0.46

The average and standard deviation of the molecular brightness and the number of molecules of autofluorescence were determined from a total of 27 cells and from 22 randomly sampled locations within the same cell.

TABLE 2 PCH and autocorrelation analysis of EGFP in vivo and in vitro

EGFP	Nucleus (29 cells)	Cytoplasm (29 cells)	Nucleus (22 position in one cell)	Solution (4 positions)
$\langle\varepsilon\rangle$ (cpsm)	25000 ± 2900	24800 ± 4200	25500 ± 2400	25500 ± 600
$\langle D\rangle$ (μm^2/s)	23.5 ± 5.3	25.2 ± 4.5	28.6 ± 6.6	78.0 ± 6.4

The average and standard deviation of the molecular brightness and the diffusion coefficient were determined from 29 cells, from 22 randomly sampled positions within a single cell nucleus, and for a solution of EGFP.

varying concentrations of expressed EGFP protein. The expression level varies by several orders of magnitude. Because the average autofluorescence inside the nucleus is ~1500 cps under our experimental conditions, we arbitrarily set a lower threshold of 6000 cps. Cells with a fluorescence intensity above this threshold were considered as successfully transfected and were selected for the experiments. We measured a total of 29 transfected cells. Fig. 1 *B* displays a typical measurement of photon counts analyzed by PCH. The inset of Fig. 1 *B* shows the average intensity trace as a function of time. The fluorescence intensity inside the nucleus usually is stable as a function of time, similar to the observation made when the nuclear autofluorescence was measured. PCH analysis recovered a molecular brightness value of 24,000 cpsm and 1.3 molecules of EGFP for this particular measurement. Fig. 2 *B* shows the distribution of the molecular brightness for EGFP from a total of 29 cells. Table 2 summarizes the statistics from the cell measurements. The average molecular brightness value for EGFP is 25,000 cpsm, with a standard deviation of 2900 cpsm. The distribution of the concentration of EGFP, however, is very wide, because of the nature of transient transfection. The numbers varied from 0.26 to 15 molecules per excitation volume for the cell population measured. For each determination of the molecular brightness and the concentration of EGFP, we took the autofluorescence into account by including a second species in the PCH analysis using the average molecular brightness, $\langle\varepsilon_A\rangle$, and concentration, $\langle\bar{N}_A\rangle$, obtained from the nuclear autofluorescence analysis. To determine and contrast the variations within the cell population from the heterogeneity experienced within a single cell, we performed a series of measurements in the nucleus of a single transfected cell. For each measurement the laser was refocused to a different location within the nucleus. With this procedure a total of 22 randomly selected locations were measured. We obtained an average molecular brightness value of 25,500 cpsm and a standard deviation of 2400 cpsm.

We compared the molecular brightness of EGFP in the nucleus with in vitro measurements of purified EGFP. To keep the conditions between in vitro and in vivo measurements as similar as possible, we exchanged the extracellular medium with a solution that contained ~7 nM of EGFP and measured

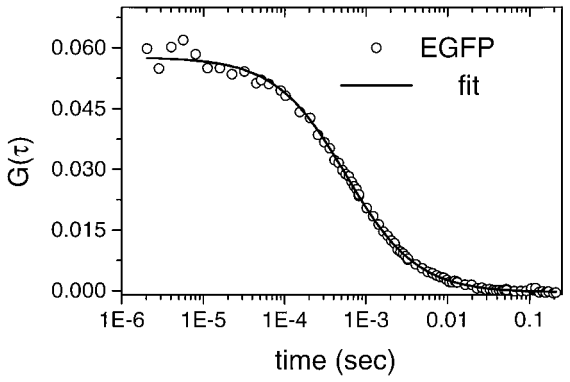

FIGURE 3 Autocorrelation function of EGFP inside the nucleus. The autocorrelation function (*circles*) was fit to a model of a single, freely diffusing species (*solid line*). The recovered diffusion coefficient is 24.6 μm^2/s. The fluctuation amplitude corresponds to 1.3 EGFP molecules in the excitation volume.

the solution close to the surface of the coverslip. This experimental setup allowed us to compare in vivo and in vitro measurements side by side without changing the sample between measurements. Samples with <20% cell confluency were chosen and in vitro measurements were performed in an area not covered by cells. We obtained a molecular brightness of 25,500 ± 600 cpsm for EGFP in solution.

The same experimental data sets used for PCH analysis were also used to calculate the autocorrelation function. We recovered the diffusion coefficient of EGFP in the nucleus by fitting the experimental autocorrelation function. Fig. 3 depicts a typical autocorrelation curve together with the best fit to a model of a single diffusing species. Because we have access to the complete sequence of photon counts we are able to calculate the experimental uncertainty of the autocorrelation function and evaluate the goodness of fit for each data set. Among the 29 cells measured, only one autocorrelation curve displayed a slight misfit. The reduced χ^2 of all other autocorrelation curves is close to one. Thus, no statistically compelling reason to invoke a more complex diffusion model is warranted by our data. Table 2 summarizes the statistics for the diffusion coefficient of EGFP inside the nucleus. The average diffusion coefficient of 23.5 μm^2/s with a standard deviation of 5.3 μm^2/s was determined from a total of 29 transfected cells. The diffusion coefficient obtained from a single cell at 22 different locations is 28.6 μm^2/s with a standard deviation of 6.6 μm^2/s. The diffusion coefficient of EGFP in aqueous solution is 78 μm^2/s, with a standard deviation of 6.4 μm^2/s. Thus, the diffusion of EGFP is slowed down by approximately a factor of 3 in the nucleus as compared to the aqueous solution.

Autofluorescence from the cytoplasm

The cytoplasm is a more challenging environment for fluorescence fluctuation measurements than the nucleus. The autofluorescence properties are quite heterogeneous in the

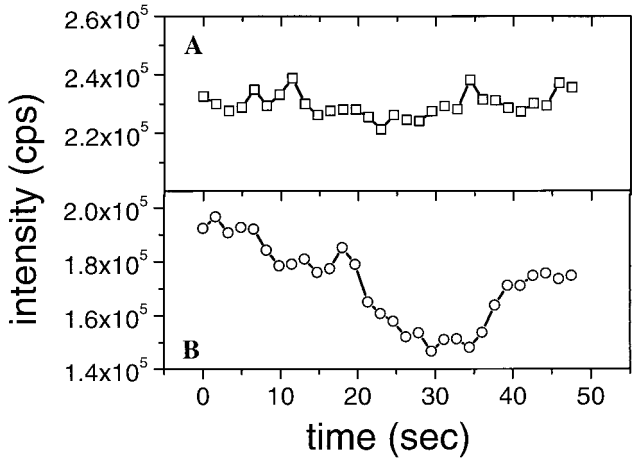

FIGURE 4 The autofluorescence intensity in the cytoplasm as a function of time. (*A*) The fluorescence intensity is stable over the measurement period; (*B*) the fluorescence intensity drifts as a function of time.

cytoplasm. Typically, a strong autofluorescence intensity is observed at the exterior of the nuclear envelope. The autofluorescence intensity becomes more homogeneous and less strong as one approaches the edge of a cell. Fluctuation analysis requires a stable intensity signal, thus we choose to perform measurements in the cytoplasm while avoiding the region close to the nuclear envelope. Even so, we still observed intensity variations on the time scale of seconds inside the cytoplasm, which is in contrast to the stability of the fluorescence intensity in the nucleus. Table 1 summarizes the results from measurements on 27 cells by PCH analysis. The average molecular brightness, ε_A, of the autofluorescence in the cytoplasm is 2100 cpsm, with a standard deviation of 430 cpsm. We recovered the average number of autofluorescent particles, $\bar{N}_A$, as 1.4 with a standard deviation of 0.4. Thus, the concentration of autofluorescent molecules in the cytoplasm is a factor of two higher than in the nucleus. The values of the molecular brightness in the cytoplasm and the nucleus are very similar. We also performed multiple measurements in the cytoplasm of a single cell at 22 different locations. We recovered an average brightness value ε_A of 2500 cpsm, with a standard deviation of 470 cpsm and an average particle concentration $\bar{N}_A$ of 1.5 molecules per excitation volume, with a standard deviation of 0.46.

EGFP inside the cytoplasm

Similar to the measurements of the autofluorescence from the cytoplasm, we also observed intensity fluctuations on the time scale of seconds for measurements of EGFP in the cytoplasm. The relative stability of the intensity signal depends on the spatial location within the cell. While a stable intensity signal was obtained for some measurements, other measurements showed strong intensity variations. Fig. 4 exemplifies this behavior by displaying the time-dependent intensity for two different measurements in the cytoplasm. The intensity of the first experiment (Fig. 4 *A*) is stable, while the second experiment shows strong fluctuations on the time scale of seconds (Fig. 4 *B*).

TABLE 3 PCH and autocorrelation analysis by the local and integral method

Cytoplasm	$\bar{N}_{local}$	ε_{local} (cpsm)	D_{local} ($\mu m^2/s$)	$\bar{N}_{integral}$	$\varepsilon_{integral}$ (cpsm)	$D_{integral}$ ($\mu m^2/s$)
EGFP (stable intensity)	8.6	26,600	19.3	8.4	27,400	17.4
EGFP (intensity drifts)	6.6	26,000	19.2	4.1	42,000	16.0

The particle number $\bar{N}$, the molecular brightness ε, and the diffusion coefficient of EGFP were recovered by either local or integral analysis of the PCH and autocorrelation function.

Traditionally, data analysis is performed on the entire data record of an experiment, which in our case typically lasted 45 s. We will refer to this type of data analysis as integral analysis. However, the fluorescence intensity in our experiment experiences fluctuations on the time scale of seconds. These intensity variations confound the interpretation of the data after applying the integral analysis tools. To deal with the signal variations we propose to analyze the data on time scales shorter than the typical time scale of the intensity drifts. This procedure should minimize the influence of the signal variations on the analysis of the data. Here, we break the data record of an experiment into small data sets of $\sim$1.5 s and perform the analysis on each short data set to extract the particle concentration $\bar{N}$, the molecular brightness ε, and the diffusion coefficient D of each segment. In the last step, the average of each parameter over all segments is determined to get the final parameters, $\bar{N}_{local}$, ε_{local}, and D_{local}. We use the subscript "local" to denote that the parameters are the average values from the analysis of many short data segments.

Table 3 compares the molecular brightness, the number of molecules, and the diffusion coefficient recovered by the integral and local analysis method. When the average intensity is stable (Fig. 4 *A*) the two methods recover, within experimental error, identical values for the particle concentration, the brightness, and the diffusion coefficient. When the intensity drifts (Fig. 4 *B*), the particle concentration and the brightness recovered by both methods differ by $>60\%$, while the diffusion coefficient is essentially the same. Integral analysis leads to an overestimation of the molecular brightness, while local analysis recovers the correct value of the molecular brightness of EGFP.

We characterized the molecular brightness, number of molecules, and diffusion coefficient of EGFP inside the cytoplasm by using local analysis. Table 2 summarizes the results obtained from the analysis of a total of 29 cells. The average molecular brightness of EGFP inside the cytoplasm

is 24,800 cpsm, with a standard deviation of 4200 cpsm. The average diffusion coefficient of EGFP is 25.2 $\mu m^2/s$, with a standard deviation of 4.5 $\mu m^2/s$. Thus, the diffusion coefficient of EGFP in the cytoplasm is about a factor of 3 smaller than compared to an aqueous solution.

DISCUSSION

The sensitivity of fluorescence combined with the information content of noise measurements makes fluorescence correlation spectroscopy a promising tool for quantitatively studying the properties of biomolecules. After FCS was introduced in 1972, researchers applied FCS to probe cellular systems in vivo (Elson et al., 1976; Koppel et al., 1976). Because of the limitations of available instrumentation, these studies have focused on membrane systems where the excitation volume is reduced to two dimensions. After the introduction of confocal and two-photon microscopy a number of groups applied FCS to measure living cells directly. Previous work has focused on the autocorrelation function to analyze in vivo measurements; in this paper we specifically look at the accuracy and reproducibility of determining the molecular brightness by PCH analysis. The molecular brightness is a crucial parameter for resolving molecular aggregates by PCH analysis (Müller et al., 2000).

To perform quantitative measurements in vivo it is important to deal with the autofluorescence contribution. Autofluorescence introduces a background signal that in many cases has to be taken properly into account to recover quantitative information about cellular processes. Some properties of the autofluorescence have been characterized by autocorrelation analysis using one-photon and two-photon excitation (Brock et al., 1998; Schwille et al., 1999b). Here we are interested in the effective molecular brightness and effective number of molecules of autofluorescent molecules that are present in the nucleus and cytoplasm of HeLa cells. These two quantities of the autofluorescence are the determining factors that influence the fluctuation amplitude of a fluorescent biomolecule measured by PCH and FCS in the presence of autofluorescence.

The autofluorescence molecules are very heterogeneous in terms of their fluorescence makeup. Studies performed on cultured mammalian cell lines demonstrate that most of the cell fluorescence arises from reduced pyridine nucleotides, flavins, and lipofuscin (Aubin, 1979; Benson et al., 1979; Croce et al., 1999; Andersson et al., 1998). The two-photon cross-section of FMN and NADH has been determined in vitro (Xu et al., 1996). NADH can be effectively excited from 700 to 800 nm, while FMN has a very broad excitation spectrum and can be excited from 700 to 1000 nm. The excitation spectrum of lipofuscin by two-photon excitation is not known. For one-photon excitation, lipofuscin has an excitation maximum at 355 nm and an emission maximum at 432 nm (Hegedus et al., 1982). Consequently, at our excitation wavelength, $\lambda = 895$ nm, we excite flavins, but not reduced pyridine nucleotides and lipofuscin. The quantum yield of flavin depends on the local environment of the fluorophore, which adds to the molecular brightness heterogeneity. In addition, each autofluorescent species could be present in the form of small aggregates or oligomers with varying numbers of fluorophores, which further contributes to the complexity of the molecular brightness of the autofluorescence. This complexity of the autofluorescence signal allows us only to talk about an effective molecular brightness and an effective number of autofluorescent molecules. These parameters are not physical properties of autofluorescent molecules, but rather specify the collective properties of the heterogeneous autofluorescence signal. However, the effective parameters are completely sufficient to characterize the influence of the autofluorescence on fluorescence fluctuation measurements. In principle, PCH analysis could resolve individual autofluorescent species if they differ sufficiently in their molecular brightness. In our measurement, we used very low excitation power to avoid light-induced oxidative-stress in the living cells (Konig et al., 1996; Brock et al., 1998). The low molecular brightness values set a practical limit for resolving individual species by PCH analysis (Müller et al., 2000). Thus, we use an effective molecular brightness and an effective number of particles to characterize the autofluorescence.

The ratio of the molecular brightness of autofluorescence in the cytoplasm with respect to the one in the nucleus is 0.84, which is based on the average of 27 cells (Table 1). Given the uncertainty in the molecular brightness of the cell population of ~20%, there is no significant difference in the brightness of autofluorescence between the cytoplasm and the nucleus. We arrived at the same conclusion when we compared measurements taken at 22 different positions within the same cell. We recovered virtually identical molecular brightness values for the autofluorescence in the cytoplasm and nucleus. A further characterization of the molecular brightness would require the use of a higher excitation power to resolve individual species that contribute to the autofluorescence.

The particle number of autofluorescence molecules in the cytoplasm is almost a factor of 2 higher than in the nucleus. We stress that we have measured at selected locations in the cytoplasm, while avoiding granular or vesicle-like structure as judged by bright-field imaging. The cytoplasm has many locations with very strong autofluorescence (data not shown), which are very hard to take into account during data analysis because they contain very bright and slowly moving particles. Nevertheless, our data indicate that the higher autofluorescence intensity in the cytosol originates from apparently twice as many autofluorescent molecules as are present in the nucleus. This is an encouraging result, because the contribution of a fluorescent species to the total fluctuation amplitude scales with the square of the molecular brightness, but is only proportional to the number of

molecules (Chen et al., 2000). Thus, the contribution of the autofluorescence in the cytoplasm to the fluctuation amplitude is only twice as high as the contribution from the nuclear autofluorescence. The effective concentration of autofluorescent molecules is 13 nM in the nucleus and 23 nM in the cytoplasm, if we assume an excitation volume of 0.1 fl. Brock et al. (1998) arrived at a concentration of 0.3–15 nM of autofluorescent molecules in the cytoplasm by using one-photon excitation at a wavelength of 532 nm. They measured the emission spectrum and attributed the autofluorescence to flavins. However, it is difficult to directly compare these results with our study because different cell lines, excitation wavelengths, and power levels were used.

The molecular brightness value of a fluorescence probe depends on the excitation power and the optical transmission of the instrument. Variation of the two-photon excitation power quadratically changes the molecular brightness. However, the molecular brightness ratio of two fluorescent probes is independent of excitation power as long as there are no unwanted photo-effects, such as saturation or bleaching. We performed our measurements at a very low excitation power (~1.75 mW). Although it is possible to successfully perform intracellular measurement at much higher powers with two-photon excitation (Schwille et al., 1999b), we choose to use a very moderate power to minimize any unwanted photo-effects. It is known that cells are sensitive to the excitation light (Konig et al., 1996). High excitation power not only leads to photobleaching, but can also dramatically increase the autofluorescence of cells. The cellular response to laser light is currently not well understood. When we performed multiple measurements on a single cell, it took 3½ h from start to finish. Thus, to be on the safe side and avoid artifacts, we decided to use a very low power to measure the molecular brightness of the autofluorescence and EGFP. We also measured initially at an even lower power (around 1.2 mW at the sample). We recovered a molecular brightness of EGFP of 11,000 cpsm, and for the autofluorescence of 1200 cpsm. As expected, the molecular brightness values of the autofluorescence and of EGFP scale quadratically with two-photon excitation power. However, since the molecular brightness of the autofluorescence is only 1000 cpsm at 1.2 mW, the signal statistics are not very good and background counts could potentially influence the accuracy of the molecular brightness statistics. Thus, we used a slightly higher power of 1.75 mW for our measurements.

The inverse relationship between the number of molecules $\bar{N}$ and the fluctuation amplitude sets an upper concentration limit for FCS experiments. Thus, it is important to limit the number of molecules inside the excitation volume to yield observable fluorescence fluctuations. The concentration of EGFP by transient transfection is not well-regulated. Typically, either cells fail to express EGFP or express it at concentrations too high for optimal FCS experiments.

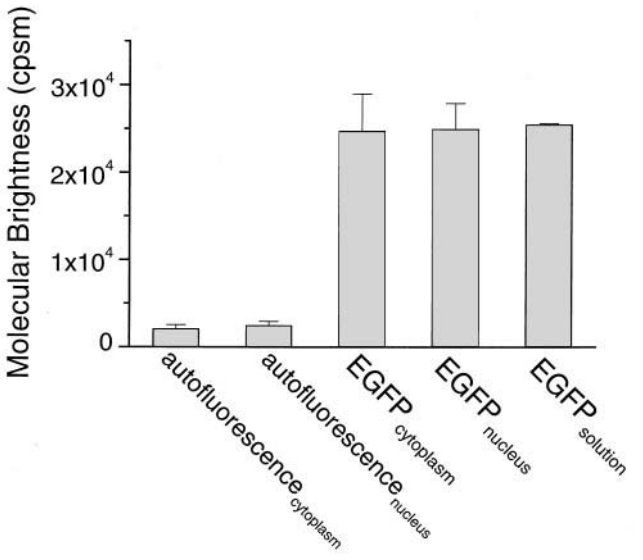

FIGURE 5 Comparison of the molecular brightness values of autofluorescence and EGFP molecules in the cytoplasm and the nucleus. The molecular brightness of autofluorescence is 2490 cpsm in the nucleus and 2100 cpsm in the cytoplasm. The molecular brightness of EGFP is 25,000 cpsm in the nucleus, 24,800 cpsm in the cytoplasm, and 25,500 cpsm in solution.

By cutting the plasmid we found that the expression levels of EGFP decrease to the concentration range accessible by FCS. The signal-to-background ratio of the fluorescence sets the lower concentration limit for fluorescence fluctuation experiments. The sample properties are difficult to extract from the fluorescence fluctuations once the fluorescence intensity decreases close to the background level. We therefore set a limit of 6000 cps in our case as the lower intensity limit. Cells with fluorescence intensities higher than the threshold were selected for measurements. In the nucleus, the number of EGFP molecules that we measured ranged from 0.26 to 15, which corresponds to a concentration range of 4–250 nM.

We observed the same molecular brightness in the cytoplasm (ε = 24,800 cpsm), in the nucleus (ε = 25,000 cpsm), and in vitro (ε = 25,500 cpsm). The pH in the cytoplasm is ~7.2, and since the pK_a of EGFP is ~6.15 (Kneen et al., 1998; Llopis et al., 1998), we do not expect quenching of its fluorescence due to a pH effect. However, the cytoplasm contains organelles, such as lysosomes, that have pH values of ~5. Thus, if we perform measurements in one of these organelles, we expect to recover a lower molecular brightness for EGFP. Among the 29 measurements we performed on the cytoplasm, we did not see a clear indication of a reduced molecular brightness. Thus, we conclude that none of the measurements was performed at the location of a lysosome.

Fig. 5 compares the molecular brightness of EGFP and autofluorescence from the nucleus and the cytoplasm. The

molecular brightness of the autofluorescence is an order of magnitude less than the brightness of EGFP. Thus, under most circumstances the autofluorescence does not contribute significantly to the fluorescence fluctuations observed for the EGFP protein. A quick way to judge the influence of the autofluorescence on fluctuation measurements of EGFP is to consider the first two moments of the photon counts (Qian and Elson, 1990b; Chen et al., 2000),

$$\langle k \rangle = \varepsilon_A \bar{N}_A + \varepsilon_{EGFP} \bar{N}_{EGFP} = \varepsilon_{app} \bar{N}_{app}, \quad (2)$$

$$\frac{\langle k^2 \rangle}{\langle k \rangle^2} = \gamma \frac{\bar{N}_A \varepsilon_A^2 + \bar{N}_{EGFP} \varepsilon_{EGFP}^2}{\varepsilon_A \bar{N}_A + \varepsilon_{EGFP} \bar{N}_{EGFP}} = \gamma \frac{1}{\bar{N}_{app}}. \quad (3)$$

The molecular brightness of the autofluorescence (ε_A) and of the EGFP protein (ε_{EGFP}), together with the number of molecules of autofluorescence ($\bar{N}_A$) and the number of EGFP molecules ($\bar{N}_{EGFP}$), are the four parameters that determine the first two moments. Analysis of in vivo measurements of transfected cells by a single species model leads to an apparent molecular brightness (ε_{app}) and an apparent number of molecules ($\bar{N}_{app}$). Therefore, we arrive at an expression for ε_{app} and $\bar{N}_{app}$ in terms of the number of molecules ($\bar{N}_A$, $\bar{N}_{EGFP}$) and the brightness (ε_A, ε_{EGFP}) of the autofluorescence and the EGFP proteins,

$$N_{app} = \frac{(\bar{N}_A \varepsilon_A + \bar{N}_{EGFP} \varepsilon_{EGFP})^2}{\bar{N}_A \varepsilon_A^2 + \bar{N}_{EGFP} \varepsilon_{EGFP}^2}, \quad (4)$$

$$\varepsilon_{app} = \frac{\bar{N}_A \varepsilon_A^2 + \bar{N}_{EGFP} \varepsilon_{EGFP}^2}{\bar{N}_a \varepsilon_a + \bar{N}_{EGFP} \varepsilon_{EGFP}}. \quad (5)$$

The deviation between the EGFP parameters, ε_{EGP} and $\bar{N}_{EGP}$, and the apparent parameters recovered from the actual measurements is determined by Eqs. 4 and 5. Under our experimental conditions, the molecular brightness of EGFP is a factor of 10 higher than the molecular brightness of autofluorescence. Consequently, if the number of EGFP molecules is larger or equal to one molecule in the excitation volume, then the deviation between the apparent and the underlying EGFP parameters is less than the experimental error of the cellular measurements, and no corrections accounting for the autofluorescence are necessary. However, if the number of EGFP molecules falls below one molecule in the excitation volume, the influence of the autofluorescence becomes significant and needs to be taken into account during data analysis.

The molecular brightness (ε_A) and the number of molecules ($\bar{N}_A$) that characterize the autofluorescence of an individual cell transfected by EGFP are not known to us. Thus, we used the average values of the molecular brightness and the number of molecules, from the autofluorescence of 27 cells, to correct the EGFP measurements. One could use either Eqs. 4 and 5 to calculate the corrected parameters of EGFP from the experimental values, or directly use PCH analysis with two species, where one species is fixed to the autofluorescence parameters. Both methods give identical results. As expected, attempts to correct the parameters of EGFP from cells with fluorescence intensities of >25,000 cps did not change the experimental values of ε_{EGFP} and $\bar{N}_{EGFP}$. However, if not corrected, data from cells with lower fluorescence intensities resulted in a reduced molecular brightness ε_{EGFP} of EGFP, as apparent from Eq. 5. We corrected these data sets by taking the autofluorescence contribution into account and were able to recover the same molecular brightness for EGFP as for the cells with high expression levels.

Because of the low excitation power used, the autocorrelation curves obtained for autofluorescence are very noisy. Consequently, we did not attempt to fit the autocorrelation curves of the autofluorescence. For EGFP measurements, we analyzed the autocorrelation functions and recovered the diffusion coefficient. The diffusion of EGFP is slowed down by a factor of 3 in in vivo measurements as compared to in vitro measurements. These numbers are in good agreement with previous FRAP measurements (Lang et al., 1986; Luby-Phelps et al., 1986; Kao et al., 1993; Seksek et al., 1997; Swaminathan et al., 1997).

We found it sufficient to describe the autocorrelation data by a single species undergoing Brownian motion, without the need to invoke anomalous diffusion models. It is not yet resolved which diffusion model is proper to describe measurements in cells. Simple diffusion of EGFP in cells has been observed (Schwille et al., 1999a), while another study found anomalous diffusion behavior (Wachsmuth et al., 2000). Although one can certainly argue that the studies use very different cell lines (HeLa versus AT-1), at present the cause for the differences in diffusion behavior is not apparent. FCS experiments rely on the autocorrelation function to extract information on kinetics and sample composition. Thus, it will be extremely important to thoroughly characterize the properties of the autocorrelation function under in vivo conditions, so that it becomes clear what information can be extracted reliably by autocorrelation analysis from cell data. Our measurements show simple diffusion for EGFP in cells, which indicates that EGFP does not interact significantly with other molecular components within the cell. Thus, EGFP seems to be a very good fluorescent marker for in vivo fluctuation studies of fusion proteins, because EGFP does not seem to play an active role in the cellular environment.

Fluctuation experiments are generally performed on systems at equilibrium or under steady-state conditions. If the system is not stationary, it becomes exceedingly difficult to analyze the fluctuations, because most analysis tools require a stationary system. The intensity fluctuations in cells can be nonstationary, which show up in the intensity trace of the experimental data. Data taken in the nucleus display a stable intensity level, and no signal variations due to the cell are observed (Fig. 1 *B*, *inset*). The fluorescence intensity of the data from the cytoplasm shows signal variations (Fig. 4 *B*).

The intensity fluctuations in the cytoplasm display no systematic trend. The signal can be stable for some time, but then drifts to a different level. These intensity drifts take place on the timescale of a few seconds. This type of intensity fluctuations will influence PCH and autocorrelation analysis differently. Autocorrelation analysis separates the fluorescence fluctuations from EGFP molecules passing through the laser beam, which has a typical timescale of 1 ms, from the slow intensity fluctuations, which happen on the timescale of seconds. The slow intensity fluctuations only influence the shape of the autocorrelation function on the second timescale. In a crude approximation the slow intensity fluctuations affect the early timescales of the autocorrelation function by adding an offset value. Thus, integral or local autocorrelation analysis of the fluctuation data recovers rather similar diffusion coefficients.

In the case of PCH analysis the slow intensity fluctuations will add to the intrinsic fluctuations from EGFP molecules passing through the excitation volume. PCH does not discriminate among the timescales of the intensity fluctuations, but rather looks at them as an integral quantity. Thus, integral PCH analysis of the cytoplasmic data incorporates the fluctuations from EGFP molecules and the slow intensity fluctuations, which lead to an overestimation of the fluctuation amplitude. To separate the fast intensity fluctuations caused by the diffusing EGFP molecules from the slow intensity fluctuations from the cell we performed a local PCH analysis, where small data segments were analyzed individually. The fluorescence intensity of the short data segment is relatively stable compared to the total intensity trace of the data. Thus, much less slow fluctuations add to the local PCH analysis, which allowed us to recover the correct molecular brightness of EGFP in cells even in the presence of slow intensity fluctuations.

Our in vivo study shows approximately a 20% uncertainty in recovering the molecular brightness (ε) and the number of molecules ($\bar{N}$) for the autofluorescence, and 10–20% uncertainty in recovering the molecular brightness of EGFP measurements by PCH analysis. Autocorrelation analysis of EGFP measurements also results in a 20% uncertainty in the diffusion coefficient of the protein. We determined the statistics of in vivo fluctuation experiments both by analysis of data taken from a population of cells and from the repeated measurements on a single cell. Both methods led to the same uncertainty in recovering parameters from the in vivo measurements. Thus, measurements of multiple cells do not lead to larger uncertainties than measurements within a single cell. In vitro measurements of EGFP result in a statistical accuracy of 2% for PCH analysis and an uncertainty of 8% in the diffusion coefficient by autocorrelation analysis. In vivo measurements carry a larger uncertainty in the fit parameters as compared to in vitro measurements, which reflects the complications for fluorescence fluctuation measurements due to the cellular measurements.

To test the applicability of PCH for in vivo studies we characterized the molecular brightness, which is the crucial parameter for PCH analysis. The molecular brightness values of EGFP in the cytoplasm, in the nucleus, and in aqueous solution are identical. The molecular brightness seems to be a very robust quantity and therefore presents a good marker for in vivo studies. The diffusion coefficient in cells, however, differs from the value in aqueous solution and depends on the cellular environment. The possibility of anomalous diffusion in cells is another issue that complicates data analysis. Thus, it appears that the molecular brightness is a more unambiguous parameter than the diffusion coefficient. Our study demonstrates that the molecular brightness of EGFP is about an order of magnitude stronger than the autofluorescence. Because we used a very broad band-pass filter for collecting the fluorescence, the brightness ratio is not yet optimized. The use of optical filters that are optimized for the in vivo detection of EGFP will further improve the brightness ratio between EGFP and the autofluorescence (Niswender et al., 1995).

In this study we demonstrate that PCH has the capability to analyze the fluorescence of EGFP in the presence of autofluorescence with single molecule sensitivity. The uncertainty in the molecular brightness of these measurements is ~10% in the nucleus and ~20% in the cytoplasm. The observation of oligomer formation in vivo is a very attractive application of PCH analysis. For example, if two EGFP-fusion proteins associate to form a dimer, then the molecular brightness of the dimer is a factor of two larger than that of the corresponding monomer. PCH analysis in vitro has the sensitivity to separate the dimer from the monomer. The molecular brightness of EGFP together with its uncertainty, which we characterized in this study, allows us to judge the sensitivity of the PCH algorithm to separate an oligomeric mixture in vivo. We predict that the statistics of PCH analysis allow the resolution of monomeric and dimeric fusion proteins in living cells. The formation of oligomeric complexes is an enormously important regulation and activation mechanism in cells. The promise to observe oligomerization events by PCH opens exciting new possibilities to study cellular processes, and is a step toward the goal of directly observing biochemistry in living cells.

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