Published in final edited form as:

J Phys Chem B. 2009 November 26; 113(47): 15629–15638. doi:10.1021/jp906539k.

Revival of high order fluorescence correlation analysis: generalized theory and biochemical applications

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Abstract

Analysis of high order correlations in fluorescence fluctuation spectroscopy was developed in the late 1980s but since then has been replaced by alternative brightness analysis methods. However, high order correlation has important advantages in many experiments. We present a new cumulant-based formalism of high order correlation that greatly simplifies data analysis. The new formalism is used to derive general expressions for variance of high order correlations that show good agreement with experiment in a model system of fluorescently-labeled DNA oligomers. A simulation of binary systems in which both diffusion time and brightness are varied illustrates clearly that high order analysis has better sensitivity to brightness than fluorescence correlation spectroscopy (FCS). These results have implications for analysis of isomerization reactions and dual-beam FCS with flow. We also demonstrate that high order correlations can detect photobleaching in the observation volume. The application of this formalism to many FCS-based experiments allows more accurate analysis in addition to describing more molecular parameters.

Keywords

fluorescence correlation spectroscopy; high order correlation; macromolecular interactions;
brightness analysis; isomerization reaction; photobleaching

1. Introduction

Fluorescence correlation spectroscopy (FCS) is a method for measuring system properties by analyzing fluctuations of fluorescence from a small observation region.¹ In biochemistry FCS is usually used to measure rotational and translational diffusion coefficients, concentrations and rates of chemical reactions.^{2–4} The development of fluorescent proteins enabled FCS measurements on cells where it can be used to measure cellular concentration of proteins, their aggregation states and mobility. Two main advantages of FCS are that this technique is noninvasive and that it allows kinetic measurements at equilibrium.

There is a large body of work dedicated to improving FCS. A considerable effort goes into experimental development and recent years have witnessed several important achievements. A few notable examples are reduction of detection volume,⁵ beam scanning,⁶ combination of FCS with time-correlated single photon counting⁷, and the development of dual beam^{8–10} and

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Supporting Information Available: Derivation of the theory of two-point correlations of fluorescence signals; outline of variance calculation for high order correlation functions; the procedure followed for correcting the dead time artifacts; factorial cumulants of bivariate distribution expressed in terms of regular cumulants. This information is available free of charge via the Internet at <http://pubs.acs.org>

two-color cross-correlation setups.¹¹ In parallel theoretical efforts have been focused on developing better experimental models that allow description of experimental noise,¹² predict resolving power of the method,¹³ and account for artifacts due to photophysical properties of the dyes.¹⁴ There is one approach, however, that has been largely overlooked but nevertheless is applicable to any FCS study.

The approach that we call High Order FCS (HOFCS) was developed in a series of publications by Palmer and Thompson^{15–17} and has been recently reviewed.¹⁸ The main focus of the original papers was on the application of HOFCS to characterize aggregation states of macromolecules, and the authors used measured time zero values of the autocorrelations for this purpose. In modern literature this goal is usually achieved with some form of photon counting histogram^{19,20} or fluorescence moment analysis.²¹ Mathematically all these methods are equivalent. Several theoretical studies pointed out how time decays of high order autocorrelations can be useful in identifying nonequilibrium processes in biochemistry.^{22–24} Palmer and Thompson also recognized the potential usefulness of temporal decays but this aspect of autocorrelations has never been explored. Here, we show how analysis of the full time course of high order correlations complements a simple autocorrelation function measured in FCS.

This work is intended to serve two purposes. The first is to revive high-order autocorrelation analysis of fluorescence fluctuations. Towards this goal we present a new definition of high order autocorrelation that makes HOFCS theory very concise and therefore simplifies data analysis. We also derive expressions for calculating signal variance in HOFCS that will allow better design of the experiment and therefore should spur widespread use of HOFCS. Our second purpose is to describe applications of HOFCS while focusing specifically on cases where HOFCS has advantages over other fluctuation analysis methods. We first consider the traditional application of HOFCS (characterization of association states) and show in a simulation that HOFCS provides better resolution than FCS when molecules have different brightness. Next, we discuss why HOFCS is essential for correct interpretation of correlation curves in the presence of chemical reactions. We also demonstrate that HOFCS can be used to characterize irreversible processes with photobleaching as an example. Finally, we discuss advantages of HOFCS over other data analysis methods in dual beam experiments with flow.

2. Theory

2.1 First order correlation function

In fluorescence correlation spectroscopy the first order correlation function of the observed light signal, $I(t)$, can be defined as

$$\begin{aligned}
 G_{1,1}(\tau) &= \langle I(t + \tau)I(t) \rangle - \langle I^2(t) \rangle \\
 &= \langle \delta I(t + \tau)\delta I(t) \rangle
 \end{aligned} \tag{1}$$

where $\delta I(t) = I(t) - \langle I(t) \rangle$, and τ is delay time. We note that according to eq 1 the correlation function decays to 0 rather than 1. The more frequently used definition involves normalization by the average value of the signal

$$g_{1,1}(\tau) = \frac{\langle \delta I(t+\tau) \delta I(t) \rangle}{\langle I(t) \rangle^2} \quad (2)$$

Derivation of theoretical expressions describing $g_{1,1}$ for some common experimental configurations can be found elsewhere.²⁵ For molecules undergoing translational diffusion in ideal solution the first order correlation function is given by

$$g_{1,1}(\tau) = \gamma_2 \frac{\sum_{i=1}^M N_i Q_i^2 Y_{1,1}^{(i)}(\tau)}{\left(\sum_{i=1}^M N_i Q_i \right)^2} \quad (3)$$

N_i is the average number of molecules of species i in the beam, Q_i is brightness per molecule, M is the total number of species, and γ_2 is a constant that can be calculated according to the expression

$$\gamma_k = \frac{\int_V W^k(\vec{r}) d\vec{r}}{\int_V W(\vec{r}) d\vec{r}} \quad (4)$$

where V is sample volume and $W(\vec{r})$ is molecule detection function. Finally

$$Y_{1,1}^{(i)}(\tau) = \frac{1}{V_{MDF}} \frac{1}{\gamma_2} \frac{1}{V} \int d\vec{r}_1 \int d\vec{r}_2 W(\vec{r}_1) W(\vec{r}_2) \frac{1}{(4\pi D_i \tau)^{3/2}} \text{Exp} \left(-\frac{|\vec{r}_2 - \vec{r}_1|^2}{4D_i \tau} \right) \quad (5)$$

where D_i is the translational diffusion coefficient of species i . $Y_{1,1}$ has a meaning of two-point correlation for a single molecule since it is equal to the probability of diffusing from point 1 to point 2 weighted by molecule detection function at each point.

2.2. Definition of high order correlation function

The use of high order correlation functions in fluorescence was pioneered by Palmer and Thompson¹⁵ to look at aggregation states of proteins in membranes. They defined the normalized high order correlation function as

$$g_{m,n}(\tau) = \frac{\langle \delta I^m(t+\tau) \delta I^n(t) \rangle - \langle \delta I^m(t) \rangle \langle \delta I^n(t) \rangle}{\langle I(t) \rangle^{m+n}} \quad (6)$$

Theoretical expressions for $g_{m,n}$ have been derived for independent molecules.¹⁵ They depend on a combination of $Y_{k,l}$ where $k+l \leq m+n$, and $Y_{k,l}$ has been extended from the definition given in eq 5 as

$$Y_{k,l}^{(i)}(\tau) = \frac{1}{V_{MDF}} \frac{1}{\gamma_{m+n}} \int_V d\vec{r}_1 \int_V d\vec{r}_2 W^k(\vec{r}_1) W^l(\vec{r}_2) \frac{1}{(4\pi D_i \tau)^{3/2}} \text{Exp}\left(-\frac{|\vec{r}_2 - \vec{r}_1|^2}{4D_i \tau}\right) \quad (7)$$

Therefore, as defined in eq 6, $g_{m,n}$ contains contributions from lower orders of correlation. For independent molecules, however, correlations can only originate from the same molecule and therefore in the theoretical expression for $g_{m,n}$ it is $Y_{m,n}$ (with same indices) that contains new information about the process. For that reason we seek a new definition for high order correlation that would result in the following theoretical expression

$$g_{m,n}(\tau) \propto Y_{m,n}(\tau) \quad (8)$$

The only remaining question is what function of the signal satisfies this relationship. In Supporting Information we show that factorial cumulants of the fluorescence are given by

$$\begin{aligned} \kappa_{[m,n]}(\tau) &= \gamma_{m+n} \sum_{i=1}^M N_i Q_i^{m+n} T^{m+n} Y_{m,n}^{(i)}(\tau) \end{aligned} \quad (9)$$

where γ_{m+n} is calculated according to eq 4. T is the binning time used in calculation. For more information about cumulants of probability distribution, we refer the reader to specialized literature.²⁶ Here we briefly note that cumulants (regular or factorial) are a set of scalar values describing the shape of the distribution in much the same way it is described by moments (mean, variance, etc). The advantage of cumulants in this case comes from the fact that they are additive for a sum of random variables, and therefore contributions of individual molecules add up. The transition from regular cumulants to factorial cumulants takes detector shot noise into account (see Supporting Information). Recently, cumulants of fluorescence intensity were used to describe the histogram of photon counts and take molecular motion into account.^{21,27}

In order to be consistent with eq 2 we also define normalized high order correlation

$$g_{m,n}(\tau) = \frac{\kappa_{[m,n]}(\tau)}{\kappa_{[m,0]} \kappa_{[0,n]}} \quad (10)$$

where $\kappa_{[m,0]}$ and $\kappa_{[0,m]}$ are univariate cumulants (see Supporting Information). The theoretical expression for independent freely diffusing molecules is then given by

$$g_{m,n}(\tau) = \frac{\gamma_{m+n}}{\gamma_m \gamma_n} \frac{\sum_{i=1}^M N_i Q_i^{m+n} Y_{m,n}^{(i)}(\tau)}{\left(\sum_{i=1}^M N_i Q_i^m \right) \left(\sum_{i=1}^M N_i Q_i^n \right)} \quad (11)$$

For a sample with one distinct species ($M = 1$) eq 11 reduces to

$$g_{m,n}(\tau) = \frac{\gamma_{m+n}}{\gamma_m \gamma_n} \frac{1}{N} Y_{m,n}(\tau) \quad (12)$$

The last expression emphasizes similarities with the first order correlation function. Not only do high order correlations decay to 0 but also the time zero value ($g_{m,n}(0)$) is inversely proportional to the number of molecules and is independent of the molecular brightness. The absolute values of $g_{m,n}(0)$ will be different for different orders of correlation due to the prefactor $\gamma_{m+n}/(\gamma_m \gamma_n)$ but their ratios remain the same. Deviation from this behavior reveals heterogeneity in terms of brightness per molecule. In the hypothetical case of uniform illumination all high order correlation functions would have same amplitude as long as the sample was homogeneous.

2.3. Variance of high order correlation functions

Several studies addressed the issue of noise in FCS.^{12,28–31} However, theoretical predictions made in these reports are rather specific to the definition of the correlation function and experimental configuration. We take a different, more general approach to the problem that allows us to calculate variances of correlation functions of arbitrary order. This method, known as calculation of moments of moments,²⁶ assumes that all observations are independent. This assumption is generally speaking violated in an FCS experiment but can be justified and has been successfully used for calculating variances in cumulant analysis of photon counting histogram.^{21,32} In fact, expressions reported previously³² for analysis of two-dimensional histograms require very few modifications to be useful here (see the derivation in Supporting Information).

Based on the general expressions, limiting cases can be readily derived. For example, one often needs an expression for signal to noise ratio (SNR) defined as $A/\text{Var}\{A\}$ where A is the quantity of interest. Such expressions for the amplitudes of high order correlations are shown, considering expressions for one fluorescent species in these specific cases. Neglecting the errors associated with univariate cumulants, SNR of $g_{m,n}$ is defined as

$$SNR_{m,n} = \frac{\kappa_{[m,n]}(\tau)}{(\text{Var}\{\kappa_{[m,n]}(\tau)\})^{1/2}} \Big|_{\tau \rightarrow 0} \quad (13)$$

Assuming that $QT \ll 1$, which is usually warranted by short binning times, T , used in calculation of the correlation functions for short time lags, the results for $g_{1,1}$ and $g_{1,2}$ are provided below

$$SNR_{1,1} = \sqrt{M_{meas}} \gamma_2 QT \sqrt{\frac{N}{\gamma_2 + N}} \quad (14)$$

where M_{meas} is the number of points used in calculation of the time zero value; it is related to the measurement time as $M_{meas} = T_{meas} / T$. Expression 14 has been recently reported.³³ Similarly

$$SNR_{1,2} = \sqrt{M_{meas}} \gamma_3 (QT)^{3/2} \sqrt{\frac{N}{2\gamma_3 + 6\gamma_2 N + 2N^3}} \quad (15)$$

Note two important special cases: high and low concentration limits. The general results with the stated assumptions are

$$SNR_{m,n} \propto \sqrt{M_{meas}} (QT)^{\frac{m+n}{2}} N^{1-\frac{m+n}{2}} \text{ when } N \rightarrow \infty \quad (16)$$

$$SNR_{m,n} \propto \sqrt{M_{meas}} (QT)^{\frac{m+n}{2}} \sqrt{N} \text{ when } N \rightarrow 0 \quad (17)$$

In the high concentration limit, a fluorescence signal becomes Gaussian and therefore correlations other than $g_{1,1}$ tend to 0. When $m = n = 1$ SNR is independent of the number of molecules in the high concentration limit and is proportional to $\sqrt{N}$ in the low concentration limit. These results are in agreement with previous reports of $SNR_{1,1}$.^{28,29}

We emphasize that the approach to noise analysis reported here is made much simpler by the definition given in eq 10. It is possible to take the same approach and calculate standard deviations for correlation functions defined in eq 6 but the results would be far more cumbersome.

2.4. High order correlation functions for a three-dimensional Gaussian beam

Analytical expressions for HOFCS are now derived and used in data analysis. In FCS, the molecule detection function is often considered to be a three-dimensional Gaussian:

$$W(x, y, z) = \text{Exp} \left(-\frac{2(x^2 + y^2)}{w_0^2} - \frac{2z^2}{\alpha^2 w_0^2} \right) \quad (18)$$

where w_0 is the beam waist, and α is beam elongation along the vertical. This approximation allows for a simple analytical expression of two-point correlations:

$$Y_{m,n}(\tau) = \frac{1}{\left(1 + \frac{2mn}{m+n} \frac{\tau}{\tau_D}\right)} \frac{1}{\left(1 + \frac{2mn}{m+n} \frac{\tau}{\alpha^2 \tau_D}\right)^{1/2}} \quad (19)$$

Eq 19 indicates that higher order correlations decay faster than lower order correlations. This observation has been made before,¹⁵ and is not restricted to Gaussian beams. This behavior can be understood since molecule detection efficiency is usually dominated by the central peak and powers of such function decay faster than the function itself. A Gaussian beam model, however, is more specific and suggests that high order correlations are related through scaling of the time axis. A three-dimensional Gaussian beam model also predicts the functional form of the beam shape factors

$$\gamma_m = \frac{1}{m^{3/2}} \quad (20)$$

Generally speaking, the shape of the correlation function decay is relatively insensitive to fine details of molecule detection function. Therefore it is reasonable to think that eq 19 will be sufficient to describe higher order correlations. Eq 20, on the other hand, is known to be incorrect from fluorescence intensity distribution analysis.¹⁹ Therefore we define

$$d_{m,n} = \frac{\gamma_{m+n}}{\gamma_m \gamma_n} \quad (21)$$

and treat $d_{m,n}$ as beam parameters measured in a calibration experiment. The final expression for the high order autocorrelation functions (combining eqs 12, 19, and 21) that will be used to analyze experimental data of a one-component solution is

$$g_{m,n}(\tau) = d_{m,n} \frac{1}{N} \frac{1}{\left(1 + \frac{2mn}{m+n} \frac{\tau}{\tau_D}\right)} \frac{1}{\left(1 + \frac{2mn}{m+n} \frac{\tau}{\alpha^2 \tau_D}\right)^{1/2}} \quad (22)$$

2.5. Chemical reactions in HOFCS

One of the main applications of FCS is measurement of chemical reaction rates at equilibrium. It has been shown³⁴ that for chemical reactions that do not change diffusion properties of the molecules and have rates independent of excitation intensity the second order correlation function can be written as

$$G_{1,1}(\tau) = \gamma_2 X_{1,1}(\tau) Y_{1,1}(\tau) \quad (23)$$

where

$$X_{1,1}(\tau) = \sum_{j,k=1}^M N_j Q_j Q_k S_{jk}(\tau) \quad (24)$$

and $S_{jk}(t)$ is the function that describes the time course of interconversion of species j into k , while Q_j and Q_k are their respective brightnesses. M is still the total number of distinct species but index i has been dropped in eq 24 since all reactants are assumed to have the same diffusion

coefficient. A detailed derivation of eqs 23–24 with some special cases can be found in the original reference.

From eqs 23–24 it is obvious that the part of the correlation function that is due to chemical reactions is independent of observation geometry, and therefore generalization to HOFCS is straightforward. With the assumptions stated above, chemical reactions add a simple factor to the high order correlations

$$\kappa_{[m,n]}(\tau) = \gamma_{m+n} X_{m,n}(\tau) Y_{m,n}(\tau) \quad (25)$$

where

$$X_{m,n}(\tau) = \sum_{j,k=1}^M N_j Q_j^m Q_k^n S_{jk}(\tau) \quad (26)$$

Normalization by univariate cumulants results in

$$g_{m,n}(\tau) = \frac{\gamma_{m+n}}{\gamma_m \gamma_n} \frac{X_{m,n}(\tau) Y_{m,n}(\tau)}{\left(\sum_{j=1}^M N_j Q_j^m \right) \left(\sum_{j=1}^M N_j Q_j^n \right)} \quad (27)$$

Note that the two-point correlation functions defined in eq 27 do not contain any additional information about rates of the chemical reaction compared to regular FCS. However, depending on the relative brightness of the reaction products they will be present in high order correlations to a different extent. Another important observation is that diffusion and chemical reaction decays scale differently among various orders of correlation which may help to distinguish the two processes when they contribute in the same range of lag times.

2.6. HOFCS in the presence of flow

High order correlation functions described above can be readily extended to account for flow through two off-centered volumes, an increasingly popular configuration to study folding of biopolymers³⁵ and separation by capillary electrophoresis³⁶. The modified two-point correlation is

$$Y_{m,n}(\tau) = \frac{1}{\left(1 + \frac{2mn}{m+n} \frac{\tau}{\tau_D}\right)} \frac{1}{\left(1 + \frac{2mn}{m+n} \frac{\tau}{\alpha^2 \tau_D}\right)^{1/2}} \text{Exp} \left[-\frac{\frac{2mn}{m+n} (\tau - \tau_F)^2 \left(\frac{V}{w_0}\right)^2}{1 + \frac{2mn}{m+n} \frac{\tau}{\tau_D}} \right] \quad (28)$$

where τ_F is the transit time between the two beams, and V is the flow velocity. The correlation functions are still given by eq 11.

2.7. Detector artifacts

Two most common artifacts that influence FCS data are afterpulsing and detector dead time. Afterpulses are spurious pulses produced with a low probability after any detected photon, and dead time is a period of time after a detected photon during which the detector is not sensitive to arrival of another photon. In FCS, it is customary to split the signal and calculate cross-correlation function between two detectors. Since the detectors are independent, the observed artifacts are not correlated in the first order approximation. However, since high order correlations involve powers of binned intensity values, cross-correlating the signal as practiced in FCS does not solve the problem. Palmer and Thompson reported expressions that describe correction of moments of fluorescence for dead time artifact.¹⁷ Implementation of these equations is rather cumbersome and depends on the definition of the high order autocorrelation. A simpler method utilizes correction procedures described for univariate histogram of photon counts. We extend these approaches to two dimensions and correct bivariate histogram of photon counts directly and then calculate high order autocorrelations from the corrected histogram (details in Supporting Information). We did not correct high order correlations for afterpulsing since in our experience this artifact contributes only to the first 1–2 μ s of the correlation decay and has a very minor effect on amplitudes of high order correlations.

3. Experimental Methods

3.1. Instrumentation

Evaluation of high order correlation analysis was carried out on a commercially available FCS system (Confocor 2, Zeiss). Fluorescence was excited with 514 nm line of the Argon laser, collected by a 40 $\times$ water immersion objective (NA 1.2), passed through a dichroic beam splitter (HFT 514), a long pass emission filter (LP 530), a 70 μ m pinhole, and collected by an avalanche photodiode. Raw photon records were stored for all measurements (20 repetitions of 30-second measurements). Measurements were carried out at approximately 25 μ W excitation power. This was within the range where counts per molecule depended linearly on the excitation power indicating that saturation and photobleaching artifacts were not significant. The background signal from the buffer solution was not correlated and never exceeded 1 % of the signal.

Photobleaching experiments were carried out on a home-built two-photon excitation system based on the Olympus IX 70 inverted microscope. The excitation laser beam (850 nm) from a titanium sapphire laser (Mira 900, Coherent) was directed into the microscope and focused 100 μ m deep in the sample by a 60 $\times$ water immersion Olympus objective (1.2 NA). We estimate the diameter of the focused laser spot to be 0.4 μ m based on calibration measurements with rhodamine green dye. The fluorescence was collected by the same objective and after passing through an excitation dichroic beam splitter (725DCSPXR, Chroma Technology) and an IR emission filter (E700SP) imaged on a fiber-coupled avalanche photodiode (AQ-131-FC). Raw data files were collected with a Flex card (<http://correlator.com>, FLEX01-12D) operated by a C program (30 repetitions of 60-second measurements).

3.2. Samples

A Rhodamine Green-labeled DNA oligonucleotide of mixed sequence (Integrated DNA Technologies, IA) dissolved in 10 mM Tris (pH 7.5) was used in HOFCS experiments. For photobleaching measurements, a Texas Red-labeled DNA oligonucleotide of mixed sequence (IDT) was used. The photobleaching measurements were carried out in 2.5 mM sodium-cacodylate (pH 7.4), 250 μ M EDTA buffer. All measurements were performed with 8-well cell culture #1 cover glasses (Fisher Scientific).

3.3. Data acquisition and analysis

After the experiment, all data files were processed by an in-house C program to calculate average high order correlation functions. The calculated curves were analyzed in Origin 7.5 (OriginLab, Northampton, MA) by nonlinear least-squares fitting. All fits were weighted by the standard deviations obtained from duplicate experiments. Simulations of high order correlations were carried out in Origin 7.5.

3.4. Calculation of high order autocorrelations

Calculations of the high order autocorrelations utilized the algorithm described previously³⁷ for first order correlation function calculation. Briefly, a correlation function is first computed with the smallest chosen time resolution Δt for time lags $\tau = \Delta t, 2\Delta t, \dots, 2k\Delta t$ ($k = 2^m$). The intensity trace is then coarsened with time resolution $2\Delta t$, and the calculation proceeds for $\tau = 2\Delta t, 4\Delta t, \dots, 2k\Delta t$. This procedure is followed until the desired time range is covered. We adapted this protocol for calculation of bivariate distributions. The advantage of the outlined procedure is that, for any cascade other than the very first one, the lag time is much greater than the current time resolution $n\Delta t$, and one can in essence cover a wide range of lag times with a relatively short binning interval. Correction of the calculated bivariate histogram for dead time through series expansions is described in Supporting Information. Any software algorithm for calculating first order correlation functions can be easily modified to calculate higher order correlations by storing both correlated intensity values rather than calculating their products. The search for correlation is not affected.

Once the bivariate histogram of photon counts is obtained, the bivariate factorial cumulants are calculated. In the first step, unbiased estimators for regular cumulants are calculated according to the published expressions, then converted to factorial cumulants according to eq S21 summarized in Supporting Information. Calculation of univariate factorial cumulants necessary for normalization has been described before²¹ and follows a similar procedure. We employ symmetric normalization that is usually preferred in FCS.³⁸

One may question whether or not a multi-tau algorithm can be applied to calculation of high order autocorrelations, given that for binning time T comparable to the decay time of the process, expressions for factorial cumulants have to be modified via binning functions.²⁷ For one fluorescent species the use of multi-tau algorithm is justified by the fact that the binning functions cancel out due to normalization chosen for $g_{m,n}$. For multiple species there is no simple answer, and work is in progress on the theory that will predict limits of applicability of multi-tau algorithm for use in HOFCS. A practical solution to the problem is to compare results of $g_{m,n}$ calculation with different starting time resolution T_0 . Our experience with this approach is that multi-tau algorithm does not introduce artifacts in the calculated correlation functions as long as a) T_0 is less than 0.1 of the characteristic decay time of the process, and b) for every cascade $T \ll \tau$ is fulfilled.

4. Results

4.1. Three-dimensional Gaussian (3DG) model describes decays but not time zero values of high order autocorrelations

Palmer and Thompson¹⁵ reported time decays of the correlation functions up to order four with their fits to the model that relied on a two-dimensional Gaussian beam approximation. There are several reasons why these results need to be reevaluated. It has been suggested³⁹ that the Gaussian beam approximation is fulfilled much better in 2D experiments, but in solution measurements, diffusion along Z is important. In fact, the observation volume is elongated along the vertical, altering the shape of the correlation function. Finally, from histogram-based brightness analysis it is known that the 3D Gaussian (3DG) model makes inaccurate prediction

of the beam factors γ_m .^{19,39} Therefore we evaluated performance of the 3DG beam model in HOFCS.

The results can be briefly summarized: the 3DG model predicts decays but not time zero values of the correlation functions. Correlation functions obtained with solutions of Rhodamine Green-labeled DNA oligonucleotides showed that when the ratio $\gamma_{m+n}/\gamma_m/\gamma_n$ was calculated using 3DG assumptions, unsatisfactory fits were obtained (data not shown). Instead, beam parameters for each additional order of correlation were designated and measured in a calibration experiment with a known solution (we defined such parameters according to eq 21). When these factors were allowed to vary, fitting quality was improved. Global fit of four correlation functions ($g_{1,1}$, $g_{1,2}$, $g_{1,3}$, and $g_{2,2}$) resulted in $\chi^2 = 1.05$ indicating that the decays of the correlation functions can be described by 3DG model perfectly well. The correlation curves and the residuals are shown in Figure 1; the extracted parameters are $N = 2.35$, $Q = 7.0$ kHz, $\tau_D = 88$ μ s, $d_{1,2} = 2.0$, $d_{1,3} = 2.5$. The values expected for a three-dimensional Gaussian beam are $d_{1,2} = 1.54$ and $d_{1,3} = 1.84$, but this disagreement between theory and experiment is expected based on other brightness analysis methods. Palmer and Thompson made similar observations about 3DG model^{15,17} but we think that our results are more accurate, since our definition of high order autocorrelation does not contain contributions from lower order correlations that can obscure interpretation of the decays.

To conclude this section, we consider an important practical question: how many high order correlations can be realistically estimated from a dataset? The answer is hinted by the *SNR* expressions (eqs 16–17) and depends greatly on the properties of the sample. For a given data collection time, number of molecules, and brightness per molecule, the only way to improve *SNR* is to increase the binning time T . One has to be careful, however, and keep T well below the characteristic decay time; otherwise distortions in the correlation function will result. In general, one can expect to estimate the first four correlation functions from bright fluorophores with binning times of a few microseconds (Figure 1) while for slower decaying processes one should be able to reliably estimate more high order correlations.

4.2. Predicted standard deviation of high order autocorrelation agrees with experiment

Standard deviations measured in the experiment are compared with the theoretical prediction, and results are presented in Figure 2 for four high order autocorrelations ($g_{1,1}$, $g_{1,2}$, $g_{1,3}$, and $g_{2,2}$). The experimental standard deviations for all points were calculated from 20 measurements, each 30 sec long. The theoretical standard deviations were calculated using sample parameters (number of molecules, brightness per molecule, and diffusion time) obtained from the fit of the first order correlation function.

The results of the theoretical calculation based on the moments of moments approach show good agreement with experiment. This finding is very important since understanding of noise in HOFCS allows simulation of realistic correlation curves with incorporated noise to study limitations of the technique. This approach is used to compare resolution of FCS and HOFCS.

This approach to calculating standard deviations does neglect correlations among data points. While this assumption is violated in FCS, it nevertheless leads to very good agreement with experiment. By making this simplifying assumption, we gain generality that allows computation of standard deviations of high order autocorrelations.

4.3. HOFCS provides better resolution than FCS

The ability of HOFCS to resolve multiple components based on diffusion time and brightness is one of the strengths of the method. The approach was first described for FCS analysis,¹³ and here we directly compare high order correlation analysis and regular FCS. First, correlation

curves are simulated for a two-component mixture with components differing in diffusion time and/or brightness. These curves are then analyzed in terms of a (incorrect) one-component model, and resolution of the analysis is judged according to the confidence with which this model can be rejected. Such an approach allows one to establish resolution limits of FCS or HOFCS for a two component mixture.

Three sets of correlation curves were generated where the two components were characterized by brightness ratios of 1, 1.5, and 2. For each set, the diffusion time of the first component (τ_{D1}) was fixed at 300 μ s while the diffusion time of the second component was varied between 0.4 τ_{D1} and 2.5 τ_{D1} . These correlation curves (100 simulations for each set of conditions) were then analyzed in terms of a one component model, and the obtained reduced χ^2 values were used to calculate the probability that this model is incorrect. This procedure relies on F-test and is necessary in order to compare results of FCS and HOFCS since a different number of points is fit in each case. The probability (P) that the two-component model describes the data more accurately is presented in Figure 3. The shapes of the curves that correspond to FCS analysis indicate that this technique is unable to discriminate between two components with similar diffusion constants (ratio of diffusion times close to 1). This result has been obtained before¹³ although details of our calculation are somewhat different (Koppel's standard deviations were used¹³ that tend to overestimate the standard deviation of the correlation function). FCS is not sensitive to brightness and therefore the performance of FCS analysis does not depend on the brightness ratio of the two components (Figure 3, a–c).

On the contrary, results of HOFCS analysis have a striking dependence on brightness ratio, $R = Q_2/Q_1$. As R increases, resolution of the experiment improves as indicated by higher P values for the entire range of diffusion time ratios. An interesting observation is that the minimum of the curve (lowest P) shifts to the left (shorter diffusion time for the bright component) when the brightness ratio is increased. This asymmetry may arise from higher order correlations that have faster decay times and are also dominated by the brighter component.

Another interesting observation is that the HOFCS minimum is slightly broader than that for FCS for $R = 1$. This behavior can be understood since for equal brightness conditions high order correlation curves do not contain new information about the sample. At the same time they decay faster and therefore the informational content per point is reduced.

We would like to note that the trends observed in Figure 3 are general (data not shown). Mainly, greater brightness ratio R benefits HOFCS regardless of the absolute brightness values, and for brighter molecules HOFCS are more useful in agreement with the derived signal-to-noise expressions. Also the resolution limit minimum shifting to the left seems to be a uniform feature.

A natural question may arise as to how HOFCS compares to PCH/FIDA and cumulant analysis. Several considerations favor the use of HOFCS in certain cases. The analysis of higher order autocorrelation is considerably simpler than histogram analysis and unlike the latter preserves dynamic information. PCH/FIDA, on the other hand, provides a more direct way to access brightness information and may be preferred when dynamics is not important. Similar considerations apply to moment or cumulant analysis. Even though correction for diffusion time has been introduced in cumulant analysis, we believe that correlation function methods give superior performance in extracting rates of fluctuation decay, and therefore should be preferred for measuring diffusion coefficients and relaxation rates.

4.4. HOFCS allows complete characterization of two-state isomerization reaction

High order correlation functions have the potential to be useful in studying chemical reactions by FCS. Unlike diffusion components of the correlation function, chemical components decay with the same rate in correlations of all orders. Also the relative contribution of the chemical reaction component to high order autocorrelations will depend on the relative brightness of the reactants.

Consider the simplest isomerization reaction

Such reactions can model two-state folding of biopolymers, transition to triplet state in fluorophores, etc. There are five parameters that describe the fluorescence signal of such a system: number of molecules for each species (C_1 and C_2), brightness of each species (Q_1 and Q_2) and relaxation time T_{kin} . From the first order correlation function one can measure three values (amplitude, baseline, and decay rate of the exponential part of the correlation function). The count-rate contributes another value bringing the number of measured quantities to four. Therefore FCS analysis is sufficient to study the reaction scheme of eq 29 only when one of the parameters is known (for example, $Q_2 = 0$ when transition to the triplet state takes place). Higher order correlations circumvent this limitation. Each higher order correlation contributes another measured value since amplitudes of the exponential decay scale differently depending on relative brightness of the species, but rates and baselines do not.

To illustrate this point we simulated first and second order correlation functions for two sets of parameter values corresponding to the reaction scheme eq 29. In set I, $C_1 = 0.5$, $C_2 = 0.5$, $Q_1 = 10$ kHz, $Q_2 = 30$ kHz, $T_{kin} = 50$ μ s, $\tau_D = 5$ ms while in set II $C_1 = 0.8$, $C_2 = 0.2$, $Q_1 = 25$ kHz, $Q_2 = 0$, $T_{kin} = 50$ μ s, $\tau_D = 5$ ms. It is easily verified that the count-rate for the two reaction schemes is the same (20 kHz). The first order correlation functions are also identical as shown in Figure 4. The second order correlations are different, however, which contributes an additional measurable quantity. More information can be obtained with yet higher order correlations. Therefore, unlike FCS, HOFCS can be used to measure true brightness per molecule and concentrations in isomerization reactions. As noted before, this information is necessary for accurate measurements of forward and reverse rate constants.

4.5. HOFCS detects irreversible processes: example of photobleaching

It was shown theoretically and in simulations by Steinberg²³ that high order autocorrelations can detect irreversible processes. To our knowledge, however, this prediction has never been tested experimentally. Photobleaching is an obvious example of an irreversible transition that a fluorophore can undergo, and here we study the effect of photobleaching on high order autocorrelations.

In Figure 5 we compare two autocorrelations, g_{12} and g_{21} , for Texas Red and Rhodamine Green-labeled DNA molecules. The curves in Figure 5a (Texas Red) were calculated from a data trace obtained under conditions of suspected photobleaching, i.e. at relatively high excitation power (20 mW) and in the absence of antioxidants. The two curves do not overlap, and for all time lags $g_{12} \geq g_{21}$. This is to be expected for excitation-induced irreversible transition since the average photobleached molecule contributes more to the rising front of the fluorescence burst than it contributes to the falling front. Such molecules enter the beam but are destroyed by high excitation power before leaving the observation region. Therefore, the signal due to an individual molecule appears as locally rising on the time scale of the diffusion time causing the asymmetry in high order autocorrelations. The difference between the two

curves divided by the experimental standard deviation is also shown in Figure 5a. This curve emphasizes that the observed differences exceed experimental errors. Similar curves obtained at the same excitation power with Rhodamine Green labeled DNA demonstrate almost perfect overlap (Figure 5b). This observation confirms our interpretation of Figure 5a since Rhodamine Green is expected to be a more photostable dye.⁴⁰

Photobleaching in this case is a local effect observed in the excitation volume only. The overall count rate was not reduced over the course of this experiment because only a small portion of all molecules present in the sample were destroyed. With one-photon excitation and confocal detection, on the other hand, photobleaching in the observation volume is negligible. Therefore g_{12} and g_{21} overlap perfectly well, even though with one-photon excitation there is significant photobleaching in the illuminated region of the sample.

The observation of photobleaching with high order autocorrelations has the potential for avoiding this artifact in FCS. Whereas one would usually carry out several experiments at different excitation powers to identify bias in the correlation function indicative of photobleaching, a single experiment with calculation of high order autocorrelation would suffice.

4.6. HOFCS is especially suitable to dual beam experiments with flow

High order correlation function analysis is particularly useful in the cases when signal is strongly correlated on a particular timescale. This is the case in experiments with two excitation volumes, the experimental geometry used to study flow induced by pressure³⁵ or electric field³⁶ in capillaries. The resulting cross-correlation function calculated from the two observation volumes is dominated by an approximately Gaussian peak at a lag time corresponding to the passage time between the two volumes. Brightness per molecule could be measured by applying one of the commonly used brightness analysis methods to the signal from one of the observation volumes, but any information about cross-correlation is lost in such analysis. High order correlation functions provide an especially valuable alternative in this case.

Consider the situation where two kinds of fluorescent particles are present in solution and assume that these particles display different passage time between the two volumes. An example of such experiment⁴¹ couples FCS with capillary electrophoresis. The resulting correlation functions have two peaks, but interpretation of the relative concentrations is not possible with FCS since knowledge of brightness per molecule is required (separation of fluorescent particles achieved by electrophoresis makes this point visually obvious). In this case HOFCS provides additional information about brightness per molecule and can assign the correct brightness value to each peak

High order correlations will certainly find applications in two-beam FCS coupled with flow. The analysis will be especially valuable when molecules are not fully resolved by electrophoresis giving rise to overlapping peaks. Analysis of high order autocorrelations would then allow resolution of such peaks based on their scaling according to brightness.

5. Conclusions

This paper describes implementation of high order autocorrelation analysis and its biochemical applications. With modern instrumentation, it is possible to measure autocorrelations up to at least order four. The new definition of autocorrelation in terms of cumulants unifies earlier developments in HOFCS^{15–17} and considerably simplifies data analysis so that it is straightforward to write out model HOFCS expressions for arbitrary numbers of fluorescent species and take constant background into account. In addition $g_{m,n}$, as defined in Eq 10, do

not contain contribution from lower order correlations. The shot noise effect that typically appears in discussions of high order autocorrelation is taken into account by considering factorial rather than regular cumulants. The new definitions make accurate predictions of standard deviations in HOFCS. These results will be useful in determining optimal experimental conditions for HOFCS, choosing appropriate collection time to achieve desired signal to noise ratio etc. The simplified S/N expressions can guide intuition when performing HOFCS measurements.

Simulations show clearly that HOFCS is more sensitive than FCS to association states of macromolecules, since HOFCS derives its information from both brightness and diffusion while FCS relies solely on diffusion. In much the same way HOFCS allows measurements of all parameters in a simple two-state isomerization reaction. This application has traditionally been a weak spot of FCS, and additional steady state measurements were required to complement FCS.⁴² With the use of high order autocorrelations the need for such experiments is abolished.

Generally, HOFCS can be applied in any FCS study. Software correlators can be easily modified to calculate high order correlations, and the following analysis is straightforward. HOFCS permits measurements of small differences of diffusion coefficient when it is accompanied by brightness change, such as can occur in biopolymer folding and dynamics. Among natural applications of HOFCS are dual beam experiments with flow.

A demonstration that photobleaching causes asymmetry in high order correlations is, to our knowledge, the first experimental evidence that HOFCS can detect violation of the principle of microscopic reversibility. While this result can be useful in itself for measuring photobleaching and understanding its mechanisms, we suggest that it opens a new avenue for experimental measurements on systems not at equilibrium. It has been debated in the literature that HOFCS can be used to distinguish steady state from equilibrium,²⁴ and our formalism provides a framework for such experiments. Perhaps the most exciting application of HOFCS will be to characterize biochemical systems in steady state

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank W. Tom Stump for maintenance of the confocal/laser system, and Professor Elliot Elson for critical evaluation and helpful comments. This work was supported by the National Institutes of Health (R01GM077231) and in part by a Cori Fellowship to AM.

References and Notes

1. Elson EL, Magde D. *Biopolymers* 1974;13:1–27.
2. Krichevsky O, Bonnet G. *Rep Prog Phys* 2002;65:251–297.
3. Bacia K, Schwille P. *Methods* 2003;29:74–85. [PubMed: 12543073]
4. Haustein E, Schwille P. *Annu Rev Biophys Biomol Struct* 2007;36:151–169. [PubMed: 17477838]
5. Blom H, Kastrup L, Eggeling C. *Curr Pharm Biotechnol* 2006;7:51–66. [PubMed: 16472133]
6. Petrášek Z, Schwille P. *Biophys J* 2008;94:1437–1448. [PubMed: 17933881]
7. Lamb DC, Müller BK, Bräuchle C. *Curr Pharm Biotechnol* 2005;6:405–414. [PubMed: 16248814]
8. Brinkmeier, M. *Fluorescence correlation spectroscopy*. Rigler, R.; Elson, EL., editors. Springer; Berlin: 2001. p. 379–395.
9. Dertinger T, Pacheco V, von der Hocht I, Hartmann R, Grogor I, Enderlein J. *ChemPhysChem* 2007;8:433–443. [PubMed: 17269116]

10. Korlann Y, Dertinger T, Michalet X, Weiss S, Enderlein J. *Opt Express* 2008;16:14609–14616. [PubMed: 18794997]
11. Schwille, P. Fluorescence correlation spectroscopy. Rigler, R.; Elson, EL., editors. Springer; Berlin: 2001. p. 360–378.
12. Saffarian S, Elson EL. *Biophys J* 2003;84:2030–2042. [PubMed: 12609905]
13. Meseth U, Wohland T, Rigler R, Vogel H. *Biophys J* 1999;76:1619–1631. [PubMed: 10049342]
14. Lloyd MD, Shen G. *Curr Pharm Biotechnol* 2006;7:287–301. [PubMed: 16918405]
15. Palmer AG III, Thompson NL. *Biophys J* 1987;52:257–270. [PubMed: 3663831]
16. Palmer AG III, Thompson NL. *Proc Natl Acad Sci USA* 1989;86:6148–6152. [PubMed: 2548201]
17. Palmer AG III, Thompson NL. *Appl Opt* 1989;28:1214–1220.
18. Thompson, NL.; Mitchell, JL. Fluorescence correlation spectroscopy. Rigler, R.; Elson, EL., editors. Springer; Berlin: 2001. p. 438–458.
19. Kask P, Palo K, Ullmann D, Gall K. *Proc Natl Acad Sci USA* 1999;96:13756–13761. [PubMed: 10570145]
20. Chen Y, Müller JD, So PTC, Gratton E. *Biophys J* 1999;77:553–567. [PubMed: 10388780]
21. Müller JD. *Biophys J* 2004;86:3981–3992. [PubMed: 15189894]
22. Steinberg IZ. *Biopolymers* 1987;26:S161–S176. [PubMed: 2437976]
23. Steinberg IZ. *Biophys J* 1986;50:171–179. [PubMed: 3730501]
24. Qian H, Elson EL. *Proc Natl Acad Sci USA* 2004;101:2828–2833. [PubMed: 14970342]
25. Widengren, J.; Mets, Ü. Single molecule detection in solution. Zander, Ch; Enderlein, J.; Keller, RA., editors. Wiley-VCH; 2002. p. 69–120.
26. Kendall, MG.; Stuart, A. The advanced theory of statistics. Vol. 1. MacMillan Publishing; New York: 1977.
27. Wu B, Müller JD. *Biophys J* 2005;89:2721–2735. [PubMed: 16055549]
28. Koppel DE. *Phys Rev A* 1974;10:1938–1945.
29. Qian H. *Biophys Chem* 1990;38:49–57. [PubMed: 2085652]
30. Kask P, Günther R, Azhausen P. *Eur Biophys J* 1997;25:163–169.
31. Wohland T, Rigler R, Vogel H. *Biophys J* 2001;80:2987–2999. [PubMed: 11371471]
32. Wu B, Müller JD. *Biophys J* 2006;91:2687–2698. [PubMed: 16815900]
33. Lowder B, Blair S, Devaux E, Ebbesen TW. *Anal Chem* 2009;81:834–839. [PubMed: 19099408]
34. Palmer AG III, Thompson NL. *Biophys J* 1987;51:339–343. [PubMed: 3828464]
35. Van Orden A, Jung J. *Biopolymers* 2007;89:1–16.
36. Fogarty K, Van Orden A. *Methods* 2008;47:151–158. [PubMed: 18852049]
37. Wahl M, Gregor I, Patting M, Enderlein J. *Opt Express* 2003;11:3583–3591. [PubMed: 19471494]
38. Schätzel K, Drewel M, Stimac S. *J Mod Optics* 1988;35:711–718.
39. Huang B, Perroud TD, Zare RN. *ChemPhysChem* 2004;5:1523–1531. [PubMed: 15535551]
40. Heinze KG, Koltermann A, Schwille P. *Proc Natl Acad Sci USA* 2000;97:10377–10382. [PubMed: 10973482]
41. LeCaptain DJ, Van Orden A. *Anal Chem* 2002;74:1171–1176. [PubMed: 11924980]
42. Bonnet G, Krichevsky O, Libchaber A. *Proc Natl Acad Sci USA* 1998;95:8602–8606. [PubMed: 9671724]

Figure 1.

Fits of four high order correlation functions obtained from RhGreen-labeled DNA to a model describing diffusion of independent molecules in three-dimensional Gaussian beam with adjusted amplitudes. (a) $g_{1,1}$, (b) $g_{1,2}$, (c) $g_{1,3}$, (d) $g_{2,2}$.

Figure 2. Comparison between theoretical (solid circles) and experimental (empty circles) standard deviations of four correlation functions shown in Figure 1 (a) $g_{1,1}$, (b) $g_{1,2}$, (c) $g_{1,3}$, (d) $g_{2,2}$.

Figure 3.

Comparison of resolving power of HOFCS and FCS in a binary mixture of molecules differing in brightness and diffusion coefficient. (a) $Q_2/Q_1 = 1$, (b) $Q_2/Q_1 = 1.5$, (c) $Q_2/Q_1 = 2$. P is the probability that the correct model will be identified based on HOFCS (empty circles) and FCS (solid circles).

Figure 4. First order correlation functions ($g_{1,1}$, lines) are identical for the two sets of parameters describing the isomerization reaction (see text). The time zero values of the second order correlation functions ($g_{1,2}$, symbols) are different imposing a critical constraint on the reaction parameters.

Figure 5.

Asymmetry of high order correlation functions suggests photobleaching in the observation volume. Two high order autocorrelations ($g_{1,2}$, solid line, and $g_{2,1}$, dashed line) are shown for two fluorescently labeled DNA 23-mer oligonucleotides. (a) Texas Red-labeled DNA, (b) Rhodamine Green-labeled DNA.