Biophysical Chemistry, 38 (1990) 49–57
Elsevier

BIOCHE 01459

On the statistics of fluorescence correlation spectroscopy

Hong Qian

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

Received 14 February 1990
Accepted 20 March 1990

Diffusive occupation number fluctuation; Random variable; Time sequence statistics; Shot noise; Gaussian process

I present a detailed statistical analysis of fluorescence correlation spectroscopy (FCS) which is a natural extension of an early work. This analysis more realistically takes account of the following issues. (1) A spatial Gaussian laser excitation of fluorescence, (2) the effect of a small number of fluorescent molecules in the observation volume, (3) the shot noise effect due to random emission of fluorescent photons, and (4) a hyperbolic form for the fluorescence autocorrelation function obtained in the case of diffusion. Based on these assumptions, the results differ from the earlier work in several respects, in particular, the dependence of the signal-to-noise ratio on sample concentration and the understanding of shot noise in fluorescence fluctuation moments.

1. Introduction

In fluorescence correlation spectroscopy (FCS), a technique that can be used to determine the properties of molecular transport and chemical reaction, spontaneous concentration fluctuations of fluorescent molecules in a small region of the sample are measured in terms of fluctuations of fluorescent intensity induced by focused excitation laser. At equilibrium each spontaneous microscopic fluctuation is stochastic in nature, but the average time course is well dictated by the phenomenological dynamics of the system, i.e., diffusion, flow or drift, and/or chemical conversion [1,2]. In FCS, statistical analysis, via a fluorescence fluctuation autocorrelation function, is used to derive macroscopic phenomenological parameters, i.e., the diffusion coefficient, drift or flow rate, and/or chemical rate constant, from the repetitive measurement of microscopic fluctuations. Therefore, it is essential to understand the statistial accuracy of FCS in order to obtain optimal experimental performance and design. This paper is an extension of the early work of Koppel [3]. Detailed analysis is developed for a much more realistic diffusion problem. Most of the mathematics involved in the derivations has been omitted and the focus is mainly on the physical interpretations and implications of the results.

The main differences between the present results and those of the earlier work are as follows: (1) An arbitrary spatial profile of the excitation laser, a Gaussian beam in particular, has been considered, rather than uniform illumination. It is essential to use a focused laser beam to obtain a small illumination volume, but a focused laser beam has inevitably a nonuniform intensity profile [4]. (2) A more realistic hyperbolic time correlation for diffusion is used instead of an exponential correlation function. It will be shown that this has a significant theoretical consequence on the

Correspondence address: H. Qian, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, U.S.A.

convergence rate of the signal-to-noise ratio in the experimentally measured autocorrelation function. The hyperbolic function is the correct form of fluorescence correlation function for simple diffusion [5]. (3) Shot noise is discussed rigorously. This mathematical formalism is necessary to accomodate recent work using high-order moments to characterize molecular aggregation [6,7]. (4) It is not assumed that the fluorescence intensity is a Gaussian random process. The non-Gaussian behaviour of the fluorescence intensity is also essential for the analysis of higher fluctuation moments [6]. The assumption of Gaussian fluorescence intensity is equivalent to assuming that the number of particles in the observation region, m, goes to ∞, thereby eliminating the characteristics of the fluctuation statistics of interest in polydisperse system. As pointed out by Palmer and Thompson [6], under these conditions, the capability of high-moments analysis diminishes.

The most important results are: (1) It is demonstrated that the measured photocounts are related to the fluorescence intensity by a Poisson transformation. This relationship is independent of the details of the statistical distribution of fluorescence intensity and its spatial characteristics, i.e., the Poisson nature of shot noise is generic, and is robust against the details of the nature of the fluorescence fluctuation and the optical instrument. Therefore, correction of the shot noise effect, either by the direct Poisson method [8] or by correlation function extrapolation [6], can be accomplished independently of the measurement system. (2) The leading asymptotic term for convergence of the experimentally calculated autocorrelation function to its expectation is on the order of $\ln N/N$ rather than $1/N$, where N is the total number of repetitive measurements. (3) The previously asserted lack of dependence of the signal-to-noise ratio of the autocorrelation function on sample concentration [3] is shown to be valid only at high sample concentrations, and a more general but complex relation is developed. (4) Illustration is given of how to exclude shot noise completely, by extrapolation of the measured high-order fluctuation correlation function, with properly chosen time arguments.

2. Photocounts as a stochastic process

Let us consider a single fluorescent particle diffusing freely under a given excitation light intensity profile $I(\boldsymbol{r})$, where $\boldsymbol{r}$ can be two-dimensional on a membrane surface, or three-dimensional in a bulk solution. The particle is undergoing random diffusion, and its position $\boldsymbol{r}$ is best characterized by a diffusion equation. As a function of position $\boldsymbol{r}$, the fluorescence intensity itself is a random process. Furthermore, even with constant fluorescence intensity, the number of detected photons or photocounts, within a defined counting interval, varies due to the nature of fluorescence random emission, thereby causing shot noise. To derive the stochastic characteristic of the detected photocounts in terms of the diffusive motion of the underlying particles is the main theme of this section. We begin by considering single particle diffusion in a sample volume V; then the case of M identical but independent particles is dealt with; and finally, we let both M and sample volume $V \to \infty$, but hold M/V $(=\bar{c})$ constant. This line of derivation is valid for an ideal solution, where intermolecular interactions are negligible.

2.1. The photocounts from a single diffusive particle

If we denote the random process $\boldsymbol{p}(t)$ as the detected photocounts emitted from a single fluorescent particle which is confined within a volume V, diffusing freely and excited by a laser with intensity profile $I(\boldsymbol{r})$, we have:

$$\operatorname{Prob}\{\boldsymbol{p}=k\} = \int_V [\lambda T I(\boldsymbol{r})]^k/k! \exp[-\lambda T I(\boldsymbol{r})] \times P(\boldsymbol{r})\, \mathrm{d}\boldsymbol{r} \qquad (1)$$

where the λ is the photon yield of the particle per unit time under unit excitation and T the photon counting time interval for each measurement (dwell time). The number of fluorescent photons emitted under constant excitation is assumed to be a Poisson distribution [9]. The probability distribution, $P(\boldsymbol{r})$, of position $\boldsymbol{r}$ is uniform and equals $1/V$ for a particle at equilibrium in free space. The joint probability for $\boldsymbol{p}(t)$ at t_1 and t_2 is

$$\mathrm{Prob}\{k,t_1;h,t_2\} = \mathrm{Prob}\{k,0;h,t_2-t_1\} = \mathrm{Prob}\{k,0;h,\tau\}$$
$$= \begin{cases} \int\int P(\boldsymbol{r}_1)\dfrac{[\lambda TI(\boldsymbol{r}_1)]^k}{k!}e^{-\lambda TI(\boldsymbol{r}_1)}\,\mathrm{Prob}\{\boldsymbol{r}_2\,|\,\boldsymbol{r}_1,\tau\} \\ \quad\times\dfrac{[\lambda TI(\boldsymbol{r}_2)]^h}{h!}e^{-\lambda TI(\boldsymbol{r}_2)}\,\mathrm{d}\boldsymbol{r}_1\,\mathrm{d}\boldsymbol{r}_2 \\ \quad(\text{for } \tau = t_2 - t_1 \neq 0) \\ \delta k\int P(\boldsymbol{r})\dfrac{[\lambda TI(\boldsymbol{r})]^k}{k!}e^{-\lambda TI(\boldsymbol{r})}\,\mathrm{d}\boldsymbol{r} \\ \quad(\text{for } \tau = t_2 - t_1 = 0) \end{cases} \tag{2}$$

we use the transition probability of $\boldsymbol{r}$ appropriate for a diffusion process:

$$\mathrm{Prob}\{\boldsymbol{r}_2\,|\,\boldsymbol{r}_1,\tau\} = (1/4\pi\mathrm{D}\tau)\times\exp\left[-(\boldsymbol{r}_1-\boldsymbol{r}_2)^2/4D\tau\right]$$

Use of the transition probability for free space here is justified, since the observation volume is always a negligible fraction of the total sample volume, and hence the boundary of the sample chamber does not effect the diffusion of the particle.

2.2. The Poisson transformation and shot noise

Now let us consider the effect of shot noise. To demonstrate the shot noise effect on the measured fluorescence intensity, the following equation, which is equivalent to eq. 1, is important:

$$\mathrm{Prob}\{p=k\} = \int_0^\infty (x)^k/k!\,\exp(-x)\times\mathrm{Prob}\{\Phi=x\}\,\mathrm{d}x \tag{1'}$$

where Φ is the ideal, shot noise free, fluorescence intensity, defined as $\Phi = \lambda TI(\boldsymbol{r})$, a function of the random variable $\boldsymbol{r}$ ($0 \leq \Phi \leq \infty$). The mathematical proof is straightforward and is omitted here. However, it is worth noting that:

$$P(\boldsymbol{r})\,\mathrm{d}\boldsymbol{r} = \mathrm{Prob}\{\Phi = \lambda TI(\boldsymbol{r})\}\,\mathrm{d}(\lambda TI(\boldsymbol{r}))$$

Eq. 1′ has a strong physical interpretation: when the fluorescence intensity $\Phi = x$, then the probability of detecting k photons is given as a Poisson distribution with x as mean. In other words, the probability distribution of the detected photocounts p is simply a Poisson transformation of the distribution of Φ, independent of the detailed form of $\mathrm{Prob}\{\Phi = x\}$. From eq. 1′, it is easy to verify that the factorial moments of p, $\langle p!/(p-k)!\rangle$, are related to the ordinary moments of Φ, $\langle\Phi^k\rangle$, as follows:

$$\langle p!/(p-k)!\rangle = \langle\Phi^k\rangle \tag{3}$$

This result has previously been derived for uniform illumination (see ref. 3, and references cited therein). Therefore, irrespective of the detailed form of fluorescence intensity, Φ, i.e., the motion of the underlying particle $P(\boldsymbol{r})$ and the functional form of the excitation profile $I(\boldsymbol{r})$, eqs 1′ and 3 are valid.

Since eq. 2 is discontinuous at $\tau = 0$, care must be exercised to extend eq. 3 to a time correlation. The discontinuity in eq. 2 arises from the fact that the time correlation for the photon emission can be neglected in our studies. This is a good approximation, since the correlation time in the emission is approx. 10^{-14} s (shot noise) and the correlation time of diffusion in which we are interested is about 10^{-4} s. Therefore, the emission is random on this time scale, and the shot noise is correlated neither with itself in any measurement time scale, nor with particle motion. Shot noise contributes only when $\tau = 0$, and decays to zero when $\tau \neq 0$.

2.3. The Gaussian laser excitation profile

We have demonstrated that the photon count distribution, p, and fluorescence intensity distribution free of shot noise, Φ, are related by a Poisson transformation. Now we derive the dependence of the moments of the fluorescence intensity distribution on the excitation profile. If we denote $x_k = \int_V I^k(\boldsymbol{r})\,\mathrm{d}\boldsymbol{r}$, we can calculate factorial moments of p from eq. 1, $\langle p!/(p-k)!\rangle = (\lambda T)^k x_k/V$. Furthermore, for a two-dimensional Gaussian excitation profile $I(\boldsymbol{r}) = I(0)\exp(-2r^2/\omega^2)$ [5], we have $x_k = \pi\omega^2 I^k(0)/2k$. If we denote $\Omega = \pi\omega^2/V = \pi\omega^2/(\pi R^2)$ and $q = \lambda TI(0)$, then:

$$\langle p!/(p-k)!\rangle = (\lambda T)^k x_k/V = \Omega q^k/2k \tag{3'}$$

This is similar to eq. 19 in Koppel's paper [3], where a uniform disk excitation is assumed so that $x_k = \pi\omega^2 I^k(0)$. Eq. 3′ can be rewritten as follows:

$$\langle \boldsymbol{p} \rangle = \Omega q/2$$
$$\langle \boldsymbol{p}^2 \rangle = \Omega[q^2/4 + q/2]$$
$$\langle \boldsymbol{p}^3 \rangle = \Omega[q^3/6 + 3q^2/4 + q/2]$$
$$\langle \boldsymbol{p}^4 \rangle = \Omega[q^4/8 + q^3 + 7q^2/4 + q/2]$$

where q is the photon yield, the mean photon counts per fluorescent molecule for the given dwell time T and excitation intensity $I(0)$ [7]. Those terms with low power of q are the contributions from shot noise. Obviously, when $q \gg 1$, the effects of shot noise can be neglected [10].

The time correlations can be calculated from the joint probabilities. For example,

$$\begin{aligned}\langle \boldsymbol{p}(t_1)\boldsymbol{p}(t_2)\rangle &= (\Omega q^2/4)g_1(t_2 - t_1)\\ &\quad + (\Omega q/2)\delta_{t_1,t_2}\end{aligned}$$

$$\begin{aligned}\langle \boldsymbol{p}(t_1)\boldsymbol{p}(t_2)\boldsymbol{p}(t_3)\rangle &= (q^3/6)g_2(t_2 - t_1, t_3 - t_2)\\ &\quad + (\Omega q^2/4)\delta_{t_1,t_2}g_1(t_3 - t_2)\\ &\quad + (\Omega q^2/4)\delta_{t_2,t_3}g_1(t_2 - t_1)\\ &\quad + \Omega(q^2/4 + q/2)\delta_{t_1,t_2}\delta_{t_2,t_3}\end{aligned}$$

$$\begin{aligned}&\langle \boldsymbol{p}(t_1)\boldsymbol{p}(t_2)\boldsymbol{p}(t_3)\boldsymbol{p}(t_4)\rangle\\ &\quad = (\Omega q^4/8)g_3(t_2 - t_1, t_3 - t_2, t_4 - t_3)\\ &\qquad + (\Omega q^3/6)\delta_{t_1,t_2}g_2(t_3 - t_2, t_4 - t_3)\\ &\qquad + (\Omega q^3/6)\delta_{t_2,t_3}g_2(t_2 - t_1, t_4 - t_3)\\ &\qquad + (\Omega q^3/6)\delta_{t_3,t_4}g_2(t_3 - t_2, t_2 - t_1)\\ &\qquad + (\Omega q^2/4)\delta_{t_1,t_2}\delta_{t_3,t_4}g_1(t_3 - t_2)\\ &\qquad + (\Omega q^3/6)\delta_{t_1,t_2}\delta_{t_2,t_3}g_2(0, t_4 - t_3)\\ &\qquad + (\Omega q^2/4)\delta_{t_1,t_2}\delta_{t_2,t_3}g_1(t_4 - t_3)\\ &\qquad + (\Omega q^3/6)\delta_{t_2,t_3}\delta_{t_3,t_4}g_2(t_2 - t_1, 0)\\ &\qquad + (\Omega q^2/4)\delta_{t_2,t_3}\delta_{t_3,t_4}g_1(t_2 - t_1)\\ &\qquad + \Omega(q^3/6 + q^2 + q/2)\delta_{t_1,t_2}\delta_{t_2,t_3}\delta_{t_3,t_4}\\ &\qquad \ldots\end{aligned}$$

Here the g_k are normalized, multiple-time-point, high-order correlation functions. The delta function δ_{t_1,t_2} is equal to unity when $t_1 = t_2$ and equals zero when $t_1 \neq t_2$, indicating the discontinuities. There will be no shot noise contribution if and only if $t_1 \neq t_2$ and $t_2 \neq t_3$ $(t_1 \leq t_2 \leq t_3)$. If two time arguments are equal but not the third, there will still be some shot noise contribution [10].

2.4. The photocounts from M independent particles

For a system consisting of M identical, independent particles, the total detected photocounts, $\boldsymbol{P}$, is the sum of individual $\boldsymbol{p}$s:

$$\boldsymbol{P} = \boldsymbol{p}_1 + \boldsymbol{p}_2 + \boldsymbol{p}_3 + \ldots + \boldsymbol{p}_M.$$

Hence,

$$\langle \boldsymbol{P} \rangle = M\langle \boldsymbol{p} \rangle = Mq\Omega/2 = mq/2.$$

Here we let $M \to \infty$, $\Omega \to 0$, and $M\Omega \to m$ which is the mean number of particles in the $\pi\omega^2$ region. When $\Omega \to 0$, all the term Ω^2, Ω^3,... can be omitted when compared to Ω, therefore $\langle(\boldsymbol{p} - \langle \boldsymbol{p} \rangle)^k\rangle = \langle(\Delta \boldsymbol{p})^k\rangle \simeq \langle \boldsymbol{p}^k \rangle$, since $\langle \boldsymbol{p}^i \rangle^j \propto \Omega^j$. We use the notation $o(\Omega)$ to indicate all terms of order higher than Ω, hence $o(\Omega)/\Omega \to 0$ as $\Omega \to 0$. Therefore,

$$\langle(\Delta \boldsymbol{P})^2\rangle = M\langle(\Delta \boldsymbol{p})^2\rangle = m[q^2/4 + q/2]$$

$$\begin{aligned}\langle(\Delta \boldsymbol{P})^3\rangle &= M\langle(\Delta \boldsymbol{p})^3\rangle\\ &= m[q^3/6 + 3q^2/4 + q/2]\end{aligned}$$

$$\begin{aligned}\langle(\Delta \boldsymbol{P})^4\rangle &= M\langle(\Delta \boldsymbol{p})^4\rangle + 3M(M-1)\langle(\Delta \boldsymbol{p})^2\rangle^2\\ &= m[q^4/8 + q^3 + 7q^2/4 + q/2]\\ &\quad + 3\langle(\Delta \boldsymbol{P})^2\rangle^2\\ &\quad \ldots\end{aligned}$$

Similar results are well known from a study of photoelectron statistics [9]. Also,

$$\langle \Delta \boldsymbol{P}(t_1)\Delta \boldsymbol{P}(t_2)\rangle = M\langle \boldsymbol{p}(t_1)\boldsymbol{p}(t_2)\rangle \tag{4a}$$

$$\langle \Delta \boldsymbol{P}(t_1)\Delta \boldsymbol{P}(t_2)\Delta \boldsymbol{P}(t_3)\rangle = M\langle \boldsymbol{p}(t_1)\boldsymbol{p}(t_2)\boldsymbol{p}(t_3)\rangle \tag{4b}$$

$$\begin{aligned}&\langle \Delta \boldsymbol{P}(t_1)\Delta \boldsymbol{P}(t_2)\Delta \boldsymbol{P}(t_3)\Delta \boldsymbol{P}(t_4)\rangle\\ &\quad = M\langle \boldsymbol{p}(t_1)\boldsymbol{p}(t_2)\boldsymbol{p}(t_3)\boldsymbol{p}(t_4)\rangle\\ &\qquad + \langle \Delta \boldsymbol{P}(t_1)\Delta \boldsymbol{P}(t_2)\rangle\langle \Delta \boldsymbol{P}(t_3)\Delta \boldsymbol{P}(t_4)\rangle\\ &\qquad + \langle \Delta \boldsymbol{P}(t_1)\Delta \boldsymbol{P}(t_3)\rangle\langle \Delta \boldsymbol{P}(t_2)\Delta \boldsymbol{P}(t_4)\rangle\\ &\qquad + \langle \Delta \boldsymbol{P}(t_1)\Delta \boldsymbol{P}(t_4)\rangle\langle \Delta \boldsymbol{P}(t_2)\Delta \boldsymbol{P}(t_3)\rangle\end{aligned} \tag{4c}$$

In eq. 4c, the first term on the right is proportional to m, all the others, to m^2. Therefore, when $m \to \infty$, the first term is negligible and eq. 4c becomes eq. 32 in Koppel's paper [3]. This result is the characteristic property of a Gaussian random process [11]. However, non-Gaussian behaviour, i.e., small m, has been demonstrated to be essential for the high-moment analysis of molecular aggregation [6,7], and therefore, is not negligible in general.

As demonstrated before, we cannot obtain higher moments of fluorescence fluctuations free of shot noise directly from the high moments of the measured photon counts. One conventional method to correct the shot noise is by calculating the time correlation function. Since the shot noise contributes only to $\tau = 0$ in our measured fluorescence fluctuation correlation function, we can extrapolate the time correlation to zero time to obtain the shot noise free fluorescence intensity moments. For a single-component system, the functional forms of correlation functions higher than order two can be calculated analytically, although the calculations are tedious but straightforward. The first-order correlation function has the form [5]:

$$G_1(\tau_1) = (\pi\omega^2/2)[2(1+\tau_1/\tau_d)]^{-1}$$

the second- and third-order correlation functions are [8]:

$$G_2(\tau_1,\tau_2) = (\pi\omega^2/2)[4(1+\tau_1/\tau_d)(1+\tau_2/\tau_d) - 1]^{-1}$$

$$G_3(\tau_1,\tau_2,\tau_3) = (\pi\omega^2/2)[8(1+\tau_1/\tau_d)(1+\tau_2/\tau_d) \times (1+\tau_3/\tau_d) - 2(\tau_1+\tau_3)/\tau_d - 4]^{-1}$$

where $\tau_d = \omega^2/4D$, $t_4 \geq t_3 \geq t_2 \geq t_1 \geq t_0 = 0$, and $\tau_i = t_i - t_{i-1} \geq 0$. In our laboratory, we calculate $g_2(\tau,\tau) = (\pi\omega^2/2)[4(1+\tau/\tau_d)^2 - 1]^{-1}$ and $g_3(\tau,\tau,\tau) = (\pi\omega^2/8)[2(1+\tau/\tau_d)^3 - (1+\tau/\tau_d)]^{-1}$. When we extrapolate to zero time, shot noise is completely eliminated. On the other hand, extrapolation based on the functional forms of $G_2(0,\tau)$ and $G_3(0,\tau,0)$ is not totally shot noise free (see section 4). Fig. 1 shows an example of these FCS correlation functions for molecular diffusion. It is interesting to note that the half decay time is shorter for higher order correlation functions. Thus, the higher order correlation function emphasize smaller spatial frequencies of the diffusion.

3. The statistical analysis of FCS

As we stated in the foregoing, the FCS analysis is a statistical method. Therefore, even with infinitely precise measurements of the fluorescence intensities, uncertainties in the experimental autocorrelation function are expected. The experimental autocorrelation function is calculated from averaging a sequence of repetitive intensity measurements. Therefore, the uncertainties, or more formally the statistical variance, are inversely proportional to the number of repetitions.

3.1. The statistics of a time sequence

We denote the mean of N repetitive experimental measurements,

$$\boldsymbol{S}_1 = (\boldsymbol{P}_1 + \boldsymbol{P}_2 + \boldsymbol{P}_3 + \ldots + \boldsymbol{P}_N)/N$$

Thus $\langle \boldsymbol{S}_1 \rangle = \langle \boldsymbol{P} \rangle$, independent of N. It is well known that if all the measurements $\boldsymbol{P}_k$ are independent, then the variance of $\boldsymbol{S}$:

$$\langle \Delta \boldsymbol{S}_1^2 \rangle = \langle \Delta \boldsymbol{P}^2 \rangle / N$$

However, since the experimentally collected data are a time sequence, usually they are not totally statistically independent, hence the time correlation plays an important role in the statistics. If these $\boldsymbol{P}$ are measured successively with time interval, T, less than the characteristic correlation time, they are necessarily correlated with each other, thus,

$$\langle \Delta \boldsymbol{S}_1^2 \rangle = \langle \Delta \boldsymbol{P}^2 \rangle \left\{ 1/N + (2/N) \sum_{k=1}^{N-1} (1 - k/N) \times g_1(k) \right\}$$

If $\int_0^\infty g_1(t)\,\mathrm{d}t = \tau < +\infty$, then $\langle \Delta S_1^2 \rangle \sim (1 + 2/\sigma)\langle \Delta P^2 \rangle / N + o(1/N)$. Here $\sigma = T/\tau$. When σ is small, $\langle \Delta S_1^2 \rangle \sim \langle \Delta P^2 \rangle / (\sigma N/2)$, the reduction by a factor of $\sigma/2$ indicating that only those measurements with time separation longer than 2τ can be treated as independent measurements.

Unfortunately, the time correlation of FCS for diffusion is hyperbolic which does not satisfy the above condition ($\int_0^\infty g_1(t)\,\mathrm{d}t < +\infty$). Actually, $\int_0^{NT} g(t)\mathrm{d}t = \int_0^{NT} \mathrm{d}t/(1 + t/\tau_\mathrm{d}) \sim \ln(\sigma N) \to \infty$ [5], and this time correlation is so persistant that:

$$\begin{aligned}\left\langle \Delta S_1^2 \right\rangle &= 2\langle \Delta\Phi^2 \rangle \ln(\sigma N)/(\sigma N) + \langle \Delta P^2 \rangle / N \\ &\quad - 2\langle \Delta\Phi^2 \rangle_0 / (\sigma N) + o(1/N) \\ &\qquad (\sigma = T/\tau_\mathrm{d}) \qquad (5)\end{aligned}$$

here $\langle \Delta\Phi^2 \rangle = \lim_{t \to 0} \langle P(0)P(t) \rangle$, the second moment of fluorescence intensity. (Therefore, if there is shot noise in P, then $\langle \Delta P \Delta P \rangle = \langle \Delta\Phi\Delta\Phi \rangle + \langle P \rangle$ [7].) Although eq. 5 does go to zero, the rate is much slower than the conventional $1/N$. When $N \to \infty$, the leading term is $\ln N/N$ rather than $1/N$. On the other hand, the second term can be neglected only when $\ln(\sigma N) \gg 1$ which is an experimental condition rarely realized. In the following analysis, we will keep both the $\ln(N)/N$ and $1/N$ terms, and neglect only terms $o(1/N)$. A rough estimation of eq. 5 can be obtained easily based on a continuous calculation which does not include the shot noise contribution:

$$\begin{aligned}\left\langle \Delta S_1^2 \right\rangle &= \langle \Delta\Phi\Delta\Phi \rangle / (NT)^2 \int_0^{NT} \mathrm{d}t_1 \int_0^{NT} \mathrm{d}t_2 \\ &\quad \times g_1(|t_2 - t_1|)\end{aligned}$$

For the higher moments

$$\left\langle \Delta S_1^3 \right\rangle \sim 9\langle \Delta\Phi^3 \rangle (\ln(\sigma N)/2(\sigma N))^2 \quad N \to \infty$$

$$\left\langle \Delta S_1^4 \right\rangle \sim 12\langle \Delta\Phi^4 \rangle (\ln(\sigma N)/(\sigma N))^3 \quad N \to \infty$$

They are all on the order of $o(1/N)$, and so can be neglected when compared with $\langle \Delta S_1^2 \rangle$.

3.2. Autocorrelation of a fluorescence time sequence in FCS

In the FCS experiments, we calculatexxxxx

$$S_2(\nu) = \sum_{k=1}^{N} P_k P_{k+\nu}/N - \left(\sum_{k=1}^{N} P_k/N \right)^2$$

$$\begin{aligned}\langle S_2(\nu) \rangle &= \langle P(0)P(\nu T) \rangle - \left\langle S_1^2 \right\rangle \\ &= \langle P(0)P(\nu T) \rangle - \left\langle (\Delta S_1)^2 \right\rangle - \langle S_1 \rangle^2 \\ &= \langle \Delta P(0)\Delta P(\nu T) \rangle - \left\langle (\Delta S_1)^2 \right\rangle\end{aligned}$$

It is interesting to note that $\langle S_2(\nu) \rangle$ itself depends on N, in contrast to $\langle S_1 \rangle = \langle P \rangle$, and is always less than the true value $\langle \Delta P(0)\Delta P(\nu T) \rangle$. The approach to the true value, with increasing N, is not 'symmetric' (see section 4).

When $N \to \infty$, the variance of $S_2(\nu)$ is:

$$\begin{aligned}&\langle (\Delta S_2(\nu))^2 \rangle \\ &= (1/N^2) \sum_k \sum_h \langle \Delta P_k \Delta P_{k+\nu} \Delta P_h \Delta P_{h+\nu} \rangle \\ &\quad - \langle \Delta P_k \Delta P_{k+\nu} \rangle^2 + 2\left\langle (\Delta S_1)^2 \right\rangle \langle \Delta P_k \Delta P_{k+\nu} \rangle \\ &\quad + o(1/N) \qquad (6)\end{aligned}$$

$$= \begin{cases} \left\{ \begin{aligned} & mq^4\left[(p+1)\ln(r_1(p-r_2-1)/r_2 \right. \\ & \left. /(p-r_1-1))/(r_1-r_2)/2 - \ln(p)\right] \\ & /(4\sigma N) + mq^4 \ln(2p\sigma N/(2p-1)) \\ & /(8p^2\sigma N) + m^2q^4 \ln(\sigma N)/(4p\sigma N) \\ & -1/(\sigma N)\left[mq^4/(8p^2) + m^2q^4/(4p) - m^2q^4 \right. \\ & \left. \times \ln(p/(2-p))/(p-1)/16 - m^2q^4/8\right] \\ & +(1/N)\left[mq^4/8/(2p-1) + mq^3 \right. \\ & \left. /(4p-1) + mq^3/(4p^2-1) + mq^2/(4p)\right] \\ & + m^2q^4/16 + m^2q^4/(8p) + m^2q^4/(16p^2) \\ & + m^2q^3/4 + m^2q^3/(4p) + m^2q^2/4 + m^2q^2 \\ & /4/(2p-1) + o(1/N) \qquad (\mathit{for}\ \nu \neq 0) \end{aligned} \right. \\ \left\{ \begin{aligned} & (mq^4/8)\ln(2\sigma N)/(\sigma N) + (mq^3/2) \\ & \times \ln(4\sigma N/3)/(\sigma N) + \left[mq^2/2 + m^2q^4/4 \right. \\ & \left. + m^2q^3/2\right] \ln(\sigma N)/(\sigma N) - \left[mq^4/8 \right. \\ & \left. + mq^3/2 + mq^2/2 + m^2q^3/2\right]/(\sigma N) \\ & + \left[mq^4/8 + mq^3 + 7mq^2/4 + mq/2 \right. \\ & \left. + m^2q^4/4 + m^2q^3 + m^2q^2\right]/N \\ & + o(1/N) \qquad (\mathit{for}\ \nu = 0) \end{aligned} \right. \end{cases}$$

where $p = 1 + \sigma\nu$, and r_1 and r_2 are the roots of the quadratic equation $2r^2 - 2\sigma\nu r - 2\sigma\nu - 1 = 0$.

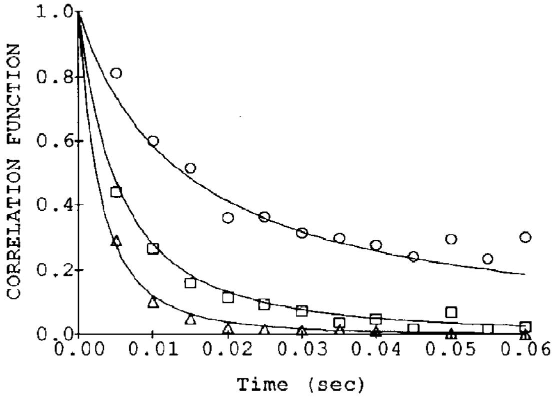

Fig. 1. Normalized higher order fluorescence correlation functions from a fluorescent labeled protein sample: $g_1(\tau)$ (○), $g_2(\tau,\tau)$ (□), and $g_3(\tau,\tau,\tau)$ (△). Lines: $[1+\tau/0.014]^{-1}$, $3[4(1+\tau/0.014)^2-1]^{-1}$, and $[2(1+\tau/0.014)^3-(1+\tau/0.014)]^{-1}$.

The signal-to-noise ratio in the measurement is defined as $\langle S_2\rangle/\sqrt{\langle(\Delta S_2(\nu))^2\rangle}$ [3].

When $q\rightarrow\infty$, the shot noise effect can be neglected and there will be no discontinuity at $\nu=0$:

$$\left\langle(\Delta S_2(\nu))^2\right\rangle$$
$$=\left(mq^4/8\right)\,\ln(2\sigma N)/(\sigma N)+\left(m^2q^4/4\right)$$
$$\times\ln(\sigma N)/(\sigma N)-mq^4/8\sigma N+\left[mq^4/8\right.$$
$$\left.+m^2q^4/4\right]/N+o(1/N)$$

Otherwise, the shot noise contributes additionally to the variance of S_2, the second moment of the measured photon counts.

When $m\rightarrow\infty$, the m terms can be neglected when compared to the terms of order of m^2. Therefore, $\langle(\Delta S_2(\nu))^2\rangle\propto m^2$, and since $\langle S_2(\nu)\rangle\propto m$, the signal-to-noise ratio, $\langle S_2\rangle/\sqrt{\langle(\Delta S_2(\nu))^2\rangle}$, is independent of m, the number of particles in the observation region, but depends only on q, the photon yield for a single particle. This is the result Koppel [3] obtained by assuming that fluorescence intensity is a Gaussian random process. Thus, the independence of the signal-to-noise ratio from sample concentration is merely a limited case of $m\rightarrow\infty$. More quantitatively, when $m\rightarrow\infty$,

$$\left\langle(\Delta S_2(\nu))^2\right\rangle$$
$$=m^2q^4\ln(\sigma N)/(4\sigma N)+(1/N)\left[m^2q^4/4\right.$$
$$\left.+m^2q^3/2+m^2q^2/2\right]$$

therefore,

$$\langle S_2\rangle/\sqrt{\left\langle(\Delta S_2(\nu))^2\right\rangle}$$
$$=2q(\sigma N)^{1/2}/\sqrt{q^2\ln(\sigma N)+\sigma q^2+2\sigma q+2\sigma}$$

which is the same as Koppel's eq. 40 [3].

3.3. A dimensional scaling analysis of signal-to-noise ratio

The task of obtaining quantitatively the analytic result for the signal-to-noise ratio is tedious, and the result is not transparent. Here, we have employed a simple, comprehensible scaling argument to demonstrate, semi-quantitatively, the above result. It is easy to verify without detailed calculation that the terms involved in eq. 6 have the forms m^iq^j ($i=1,2$; $j=1$–4). For simplicity, we retain only the highest and lowest order terms and neglect the others. Hence,

$$\left\langle(\Delta S_2(\nu))^2\right\rangle\sim\left(mq^2+mq^4+m^2q^2+m^2q^4\right)/N$$

where we have ignored q^3 terms. Therefore, if $m\gg1$, $\langle(\Delta S_2(\nu))^2\rangle\sim(m^2q^2+m^2q^4)/N$. Since $\langle S_2\rangle=mq^2$, then the signal-to-noise ratio $(S/N)\sim qN^{1/2}/\sqrt{1+q^2}$. Thus, when q increases, the (S/N) approaches the stochastic limitation at which the signal-to-noise ratio is limited solely by the stochastic nature of the fluctuation measurements. On the other hand, if $m\ll1$,

$$(S/N)\sim q(mN)^{1/2}/\sqrt{1+q^2}\,.$$

This is to say, when both m and q are small, the (S/N) does depend on the magnitude of signal (mq^2), but when $q\gg1$, it depends on $(mN)^{1/2}$, the number of particles in the observation region. It is worth noting that the relative fluctuation $\sqrt{\langle\Delta P\Delta P\rangle}/\langle P\rangle^2\sim(mN)^{-1/2}$.

4. Discussion

4.1. The shot noise and high moments

The moments of the fluorescence intensity distribution can provide information about molecular aggregation [7,12]. However, the moments of the photon counts which are experimentally measured have contributions from the shot noise due to the random emission of fluorescent photons. To estimate correctly the moments, two methods have been introduced. One of these procedures involves the fitting of the time correlation function and extrapolating to time zero [6,10,13]. This method is time-consuming, and requires extensive calculation and curve fitting. Moreover, it is necessary to know the form of the correlation function a priori. Also, when this method is applied to moments higher than order 2, there are additional discontinuities at $t_1 = t_2$. For example,

$$\lim_{t\to 0}\langle \boldsymbol{P}(0)\boldsymbol{P}^2(t)\rangle \neq \lim_{\substack{t_1,t_2\to 0\\ t_1\neq t_2}}\langle \boldsymbol{P}(0)\boldsymbol{P}(t_1)\boldsymbol{P}(t_2)\rangle$$

The right-hand side is what we need, but the left-hand side tends to overestmate the third moment, since there is still a shot noise contribution.

The alternative approach is to eliminate directly the shot noise effect from moments of photon count distribution according to eq. 3. It has been shown that when shot noise is overwhelming, this method will not provide an accurate estimation of moments of fluorescence intensity, and the extrapolation can provide better results.

4.2. Knowing $\langle \boldsymbol{P}\rangle$ a priori

The following discussion is of interest from a theoretical point of view. In Koppel's paper [3], beside defining $\boldsymbol{S}_2 = \Sigma^N \boldsymbol{P}_k^2/N - (\Sigma^N \boldsymbol{P}_k/N)^2$, he has defined an alternative form $\boldsymbol{S}_2' = \Sigma^N \boldsymbol{P}_k^2/N - \langle \boldsymbol{P}\rangle^2$. Here, we need to know the exact value of $\langle \boldsymbol{P}\rangle$ a priori. One surprising result is that the signal-to-noise ratio for $\boldsymbol{S}_2'$ is actually greater than $\boldsymbol{S}_2$. This anti-intuitive conclusion results from an inappropriate definition of $\boldsymbol{S}_2'$. The moment by definition is $\langle(\boldsymbol{P} - \langle \boldsymbol{P}\rangle)^2\rangle$, and this form is mathematically equal to $\langle \boldsymbol{P}^2\rangle - \langle \boldsymbol{P}\rangle^2$. Therefore, if we know $\langle \boldsymbol{P}\rangle$, we should construct the experimental estimation $\boldsymbol{S}_2'' = \Sigma_k^n(\boldsymbol{P}_k - \langle \boldsymbol{P}\rangle)^2/n$, rather than $\boldsymbol{S}_2'$. Obviously, $\langle \boldsymbol{S}_2''\rangle = \langle(\Delta \boldsymbol{P})^2\rangle$, and $\langle(\Delta \boldsymbol{S}_2'')^2\rangle = (1/N^2)\Sigma_k\Sigma_h\langle(\Delta \boldsymbol{P}_k)^2(\Delta \boldsymbol{P}_h)^2\rangle - \langle(\Delta \boldsymbol{P})^2\rangle^2$. This is even smaller than $\langle(\Delta \boldsymbol{S}_2)^2\rangle = (1/N^2)\Sigma_k\Sigma_h\langle(\Delta \boldsymbol{P}_k)^2(\Delta \boldsymbol{P}_h)^2\rangle - \langle(\Delta \boldsymbol{P})^2\rangle^2 + 2\langle \Delta \boldsymbol{S}_1^2\rangle\langle(\Delta \boldsymbol{P})^2\rangle - \langle(\Delta \boldsymbol{S})^2\rangle^2$, therefore the extra information helps. The estimation for the time correlation, $\nu \neq 0$, has a similar conclusion.

4.3. The asymmetric fluctuation of $\boldsymbol{S}_2$

As mentioned previously, the estimation $\boldsymbol{S}_2$ approaches $\langle \Delta \boldsymbol{P}^2\rangle$ in an asymmetric fashion:

$$\begin{aligned}\langle \boldsymbol{S}_2\rangle &= \langle(\Delta \boldsymbol{P})^2\rangle - \left\langle(\Delta \boldsymbol{S}_1)^2\right\rangle \\ &= \langle(\Delta \boldsymbol{P})^2\rangle - 2\langle(\Delta \boldsymbol{P})^2\rangle_0 \ln N/(\sigma N) - \\ &\quad \langle(\Delta \boldsymbol{P})^2\rangle/N + \langle(\Delta \boldsymbol{P})^2\rangle_0/(\sigma N) + o(1/N)\end{aligned}$$

In our experimental studies, due to the limitation of the measuring equipment, we usually have a series of measurements of $\boldsymbol{S}_2(\nu, N)$. It is important to note that simply averaging $\langle \boldsymbol{S}_2(\nu, N)\rangle$ is not correct due to this asymmetry. The correct approach is to average $\langle \boldsymbol{S}_2 + \boldsymbol{S}_1^2\rangle$ and $\langle \boldsymbol{S}_1\rangle^2$, then

$$\langle \boldsymbol{P}(0)\boldsymbol{P}(\nu T)\rangle - \langle \boldsymbol{P}\rangle^2 = \left\langle \boldsymbol{S}_2 + \boldsymbol{S}_1^2\right\rangle - \langle \boldsymbol{S}_1\rangle^2.$$

The wrong method always underestimates the $\langle \Delta \boldsymbol{P}(0)\Delta \boldsymbol{P}(t)\rangle$. Also, due to this asymmetry, for any finite N, the experimentally measured $\boldsymbol{S}_2(\nu)$ cannot be expected to yield the second fluctuation moment, $\langle \Delta \boldsymbol{P}(0)\Delta \boldsymbol{P}(\nu T)\rangle$, precisely.

4.3. The Gaussian assumption for the fluorescence intensity

Gaussian behaviour arises only when $m \to \infty$, a consequence of the central limit theorem. In the case where $m \gg 1$, Koppel [3] has predicted independence of the signal-to-noise ratio from the sample concentration. Most FCS experiments are performed at dilute concentration, however. Recently, attempts to characterize molecular aggregation from high moments of fluorescence intensity have been carried out. The high-order moments do contain novel information about a polydisperse system [6,7]. The non-Gaussian feature of the fluorescence intensity in FCS is inherent and differs from light scattering measurements, where large number of scatterers ensure the Gaussian behavior of scattering light.

Acknowledgements

The author would like to thank his advisor, Dr Elliot Elson, for many years of guidance, encouragement, and help, particularly for numerous discussions and suggestions in the course of this work. This study was supported by NIH grant GM38838.

Glossary

Symbol	Definition
p	photon counts as a random variable, due to emission of single fluorescent particle
P	photon counts as a random variable, due to emission from M independent fluorescent particles
Φ	fluorescence intensity which is proportional to the number of particles in the observation region
T	experimental time duration for integrating each measurement of photon counts
q	mean photon counts per particle
m	average number of particles in the $\pi\omega^2$ region
N	number of data points
τ_d	characteristic time constant for diffusion in FCS experiments
σ	$=T/\tau_d$
S_1	experimental estimate of $\langle P \rangle$
$S_2(\nu)$	experimental estimate of $\langle \Delta P(0) \Delta P(\nu T) \rangle$

References

1 E.L. Elson and W.W. Webb, Annu. Rev. Biophys. Bioeng. 4 (1975) 311.
2 E.L. Elson, Annu. Rev. Phys. Chem. 36 (1985) 379.
3 D.E. Koppel, Phys. Rev. A 10 (1974) 1938.
4 H. Kogelnik and T. Li, Appl. Opt. 10 (1966) 1550.
5 E.L. Elson and D. Magde, Biopolymers, 13 (1974) 1.
6 A.G. Palmer and N.L. Thompson, Biophys. J. 52 (1987) 257.
7 H. Qian and E.L. Elson, Biophys. J. 57 (1990) 375.
8 H. Qian, Ph.D. Thesis, Washington University, St. Louis, MO (1989).
9 B. Saleh, Photoelectron Statistics (Springer, Berlin, 1978).
10 A.G. Palmer and N.L. Thompson, Rev. Sci. Instrum. 60 (1989) 624.
11 W. Feller, An introduction to probability theory and its applications (Wiley, New York, 1957).
12 H. Qian and E.L. Elson, Proc. Soc. Photo-opt. Instrum. Eng. 909 (1988) 352.
13 R.D. Icenogle and E.L. Elson, Biopolymers 22 (1985) 1919.