Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5479–5483, July 1990
Biophysics

Distribution of molecular aggregation by analysis of fluctuation moments

(occupation number/shot noise/random emission/compound Poisson distribution/fluorescence spectroscopy)

HONG QIAN* AND ELLIOT L. ELSON†

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Saint Louis, MO 63110

Communicated by Howard Reiss, February 6, 1990 (received for review October 5, 1989)

ABSTRACT **The fluorescence from an open volume of a solution of fluorescent molecules fluctuates as the molecules randomly diffuse into and out of the volume. The distribution of degrees of aggregation or polymerization of the fluorescent molecules can be characterized without perturbing the system by measuring either the moments or the amplitude distribution of these fluctuations. We present an experimental verification of this approach applied to simple model systems consisting of solutions of fluorescent particles of well-defined size. We have also characterized the response of the photon-detection device (typically a photomultiplier), which is essential to the analysis of the fluorescence fluctuations, and have compared two methods for determining shot-noise contributions.**

Two methods have recently been introduced to determine the distribution of degrees of polymerization or aggregation of molecules from fluctuations of their fluorescence. This information can be obtained either from the histogram of the amplitudes of the fluctuations (1, 2) or from the distribution moments (3–5). The two approaches are mathematically equivalent (6) but are complementary in practice. Both approaches are extensions of fluorescence correlation spectroscopy (FCS) (7–12). The number of particles in an open measurement volume, the occupation number (ON), is determined from the fluorescence intensity excited by a focused laser beam. To interpret the measurements it is necessary to account both for the effects of shot noise, which stems from the randomness in the emission of fluorescence photons and also photoelectrons in the detector photomultiplier tube (PMT) (5, 13), and for the geometry of the illuminated volume (14, 15). In this paper we apply both approaches to model systems consisting of two kinds of fluorescent beads with different extents of fluorescence emission. The compositions of defined mixtures are compared with those deduced from measurements of the fluctuation moments. It is not now possible to extract the corresponding information directly from the histogram of fluctuations, but the effect of polydispersity on the histogram is clearly seen. In addition, based on its Poisson character, we present an explicit analysis of the contribution of shot noise and consider and compare two different ways to remove this contribution from the measurements.

It is important to distinguish between the thermodynamic and kinetic properties of the ON fluctuations. On average the rates of the fluctuations are determined by the transport coefficients that characterize the system. These kinetic parameters are determined by FCS from the characteristic rates of relaxation of the fluctuations (9–12). The fluctuation amplitudes, however, are determined on average by equilibrium properties such as the mean concentrations of the various species of particles (7, 8). The distribution of degrees of polymerization or aggregation can be determined entirely from the fluctuation amplitudes; no information about the dynamics of the system is needed.

FCS measurements are carried out on systems in an equilibrium state that is unperturbed by the measurements. This is an important advantage in studies of labile, reversible biochemical aggregation or polymerization systems that could be perturbed by measurements under nonequilibrium conditions (e.g., by sedimentation, fractionation, or electron microscopy). Furthermore, the results do not rely on hydrodynamic models, which typically have a weak dependence on particle size.

MATERIALS AND METHODS

Coumarin beads (excitation, 458 nm; emission, 540 nm) with radii 0.115 and 0.05 μm were purchased from Polysciences. At the same bead concentration the fluorescence from the 0.115-μm beads is ≈30 times that of the 0.05-μm beads (see Table 3). Solutions of fluorescent particles or particle mixtures were prepared in chambers of 0.5-inch diameter and 0.015-inch depth (1 inch = 2.54 cm) on serological ring slides (Scientific Products). Slides and coverslips were coated with bovine serum albumin (1 mg/ml) to prevent adsorption of the beads onto the glass surfaces.

The laser microscope (Zeiss Universal) optical system was a modified version of that described previously (16). An argon ion laser (Spectra-Physics, model 164) and a pulse-counting PMT (Hamamatsu, model R943-02) were used as excitation and detection devices. The photoelectric signal was interfaced to a LeCroy 3500 computer (Kinetic Systems, Spring Valley, NY). The ×40, 0.75-n.a. microscope objective lens used in this study provided an in-focus laser beam radius of about 1.4 μm. In the image plane just before the PMT the laser beam radius was 70 μm, and a 200-μm-radius field diaphragm was used to discriminate against fluorescence originating far off focus (14).

Fluorescence from the sample volume was recorded as a sequence of 40,000 intensity measurements, each with a dwell time (integration time) of 10 msec. Under our conditions, the diffusion time for the spheres of 0.115-μm radius was about 200 msec; therefore about 2000 independent fluctuations were in each intensity record. All experiments were carried out using the same excitation intensity and dwell time unless otherwise specified.

Four different samples were prepared: I, a 1:200 dilution 0.115-μm beads; II, a 1:200 dilution 0.05-μm beads; A, a 1:4 mixture of I and II; and B, a 1:9 mixture of I and II.

Basic Theory. Fluctuations of fluorescence intensity are proportional to fluctuations in the number of fluorescent

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FCS, fluorescence correlation spectroscopy; ON, occupation number; PMT, photomultiplier tube.

*Present address: Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229.
†To whom reprint requests should be addressed.

molecules in the observation region and therefore are directly related to the degree of aggregation of fluorophores in the system (1–5, 12, 17, 18). When the number of fluorescent particles is sufficiently low, the distribution of fluorescence intensity fluctuations from a small sample volume is not Gaussian (3). This non-Gaussian distribution contains information about the distribution of fluorescence among the particles and therefore about the degree of aggregation or polymerization of the emitting molecules. The discrete nature of fluorescence photon emission, however, superimposes shot noise on the ideal signal intensity. Further randomness also arises in the PMT. Thus the number of photons, P, emitted per dwell time fluctuates around the ideal fluorescence intensity, $\langle\Phi\rangle$; and the number of photoelectrons, E, detected per dwell time fluctuates around the average photoelectron current excited by the emitted photons that reach the PMT. Therefore, to extract the molecular characteristics of the solution it is necessary to account for the noise from both sources.

In addition to shot noise, it is necessary to account for the three-dimensional shape of the illuminated volume defined by the excitation laser beam with intensity profile $I(r)$. Methods have been introduced using either photon statistics (1, 15) or optical sectioning microscopy (14) to accomplish this.

We denote Φ as the ideal fluorescence intensity and $\Delta\Phi = \Phi - \langle\Phi\rangle$ as the intensity fluctuation. It has been shown (5) that

$$\langle\Phi\rangle = \chi_1 \sum_k q_k \bar{c}_k \quad [1a]$$

$$\langle\Delta\Phi\Delta\Phi\rangle = \chi_2 \sum_k q_k^2 \bar{c}_k \quad [1b]$$

$$\langle\Delta\Phi\Delta\Phi\Delta\Phi\rangle = \chi_3 \sum_k q_k^3 \bar{c}_k, \quad [1c]$$

where $\chi_n = \int I^n(r)dr$ is integrated over the whole sample volume, and q_k and $\bar{c}_k$ are the mean number of fluorescence photons emitted per dwell time per particle and the mean concentration of kth component, respectively.

Since χ_n is proportional to the volume defined by the excitation beam, we define an effective illuminated volume, $v = \chi_1^2/\chi_2$, and denote the mean number of particles in v as $\langle N_k\rangle = v\bar{c}_k = (\chi_1^2/\chi_2)\bar{c}_k$. We can absorb the instrumentation constant χ_2/χ_1, which is proportional to excitation intensity, into q to yield

$$\langle\Phi\rangle = \sum_k q_k\langle N_k\rangle \quad [2a]$$

$$\langle\Delta\Phi\Delta\Phi\rangle = \sum_k q_k^2\langle N_k\rangle \quad [2b]$$

$$\langle\Delta\Phi\Delta\Phi\Delta\Phi\rangle = \sum_k q_k^3\langle N_k\rangle/\gamma, \quad [2c]$$

where $\gamma = \chi_2^2/\chi_1\chi_3$. Although γ can in principle be calculated from the optical characteristics of the microscope, it is best determined empirically (14, 15). Based on previous measurements and a theoretical calculation, we chose $\gamma = 0.5$ for our system (H.Q., unpublished result).

Characterization of Fluorescence Emission and Photon Detection. To obtain the fluorescence fluctuation moments, we must first account for shot noise. By assuming a Poisson distribution of the number of photons emitted by each particle for given excitation intensity and dwell time, we obtain the following relationships between the fluctuations in fluorescence photon counts, P, and in fluorescence intensity, Φ (5, 13, 19):

$$\langle P\rangle = \langle\Phi\rangle \quad [3a]$$

$$\langle\Delta P\Delta P\rangle = \langle\Delta\Phi\Delta\Phi\rangle + \langle\Phi\rangle \quad [3b]$$

$$\langle\Delta P\Delta P\Delta P\rangle = \langle\Delta\Phi\Delta\Phi\Delta\Phi\rangle + 3\langle\Delta\Phi\Delta\Phi\rangle + \langle\Phi\rangle. \quad [3c]$$

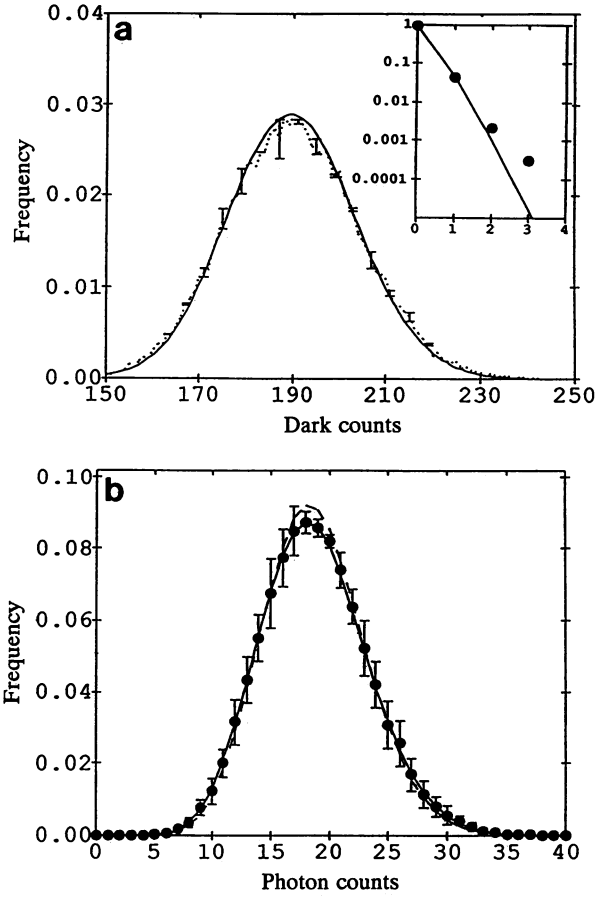

FIG. 1. Characteristics of random photon emission. The histograms present the frequency of measured fluorescence photoelectron counts per 10 msec, E. (*a*) Measured dark count from PMT at 27°C and (*Inset*) at −60°C. The solid curves are Poisson distributions, $\langle E\rangle^n/n!e^{-\langle E\rangle}$, with mean values, $\langle E\rangle$, of 190.1 and 0.047 (*Inset*), respectively. (Note the logarithmic ordinate.) (*b*) Measured photoelectron counts for a stationary fluorescence light source. Calculation of moments yields $\langle E\rangle$ = 18.755 per 10 msec, $\langle(\Delta E)^2\rangle/\langle E\rangle$ = 1.115, and $\langle(\Delta E)^3\rangle/\langle E\rangle$ = 1.35. The solid curve represents a Poisson distribution with mean photon rate = 18.755. The dashed curve represents a compound Poisson distribution for mean photon rate 187 and photoelectric pulse yield ε = 0.1.

Table 1. Characterization of random emission and collection

Distribution	$\langle(\Delta E)^2\rangle/\langle E\rangle$	$\langle(\Delta E)^3\rangle/\langle E\rangle$
Predicted		
Poisson (dark count)	1.0	1.0
Compound Poisson	$1 + \varepsilon$*	$1 + 3\varepsilon + \varepsilon^2$
Observed		
Dark count (−60°C)	1.03	1.08
Dark count (27°C)	1.08 ± 0.03†	1.15 ± 0.24†
Stationary source	1.09 ± 0.03	1.23 ± 0.23

All measurements are the photoelectron readout with 10-msec dwell time.
*ε = 0.1, from Hamamatsu user manual.
†The significant departure of the dark count from unity at room temperature is due to a drift in the mean photon counts.

Table 2. Comparison of second moments, $\langle\Delta\Phi\Delta\Phi\rangle$, by different methods

Method	$\langle\Delta\Phi\Delta\Phi\rangle$			
	I	II	A	B
Subtraction	14,583	77.14	4424	1742
Extrapolation*	14,040	65.25	4302	1716
Extrapolation†	14,370	67.03	4442	1765

Samples I, II, A, and B are described in *Materials and Methods*.
*Curve-fitting to 40 data points of fluorescence autocorrelation function.
†Curve-fitting to initial 10 data points of fluorescence autocorrelation function.

Hence, the moments of photon number fluctuations yield the corresponding moments of Φ by simple subtraction. Conversion of the emitted photons into photoelectrons introduces additional randomness into the signal. The relation between the number of emitted photons, P, and the photoelectron counts, E, follows a Poisson transformation (13), with mean values ε (< 1) for the quantum yield of the PMT:

$$\text{Prob}\{E = n\} = \sum_m [(\varepsilon m)^n/n!]e^{-\varepsilon m}\text{Prob}\{P = m\}. \quad [4]$$

For a stationary light source, the photons arriving at the PMT follow a Poisson distribution: $\langle P\rangle = \langle\Delta P\Delta P\rangle = \langle\Delta P\Delta P\Delta P\rangle$. Therefore, in contrast to the simple Poisson distribution of the background electron current (thermal dark count) of the PMT (Fig. 1), the photoelectrons follow a compound Poisson distribution. This distribution of the photoelectron pulses from a stationary light source yields $\langle\Delta E\Delta E\rangle/\langle E\rangle = 1 + \varepsilon = 1.1$ and $\langle\Delta E\Delta E\Delta E\rangle/\langle E\rangle = 1 + 3\varepsilon + \varepsilon^2 = 1.31$. The right-hand sides of these equations pertain to our PMT, for which $\varepsilon = 0.1$ for visible light (Hamamatsu user manual). These relations are experimentally verified in Table 1 and Fig. 1*b*.

When the fluorescence light source fluctuates, due to fluctuations in fluorophore ON, the relationship between measured photoelectrons, E, and fluorescence intensity, Φ, is obtained from Eqs. **3** and **4**, yielding, for ε small compared to 1 (19),

$$\langle E\rangle = \langle\Phi\rangle \quad [5a]$$

$$\langle\Delta E\Delta E\rangle = \langle\Delta\Phi\Delta\Phi\rangle + \langle\Phi\rangle \quad [5b]$$

$$\langle\Delta E\Delta E\Delta E\rangle = \langle\Delta\Phi\Delta\Phi\Delta\Phi\rangle + 3\langle\Delta\Phi\Delta\Phi\rangle + \langle\Phi\rangle. \quad [5c]$$

Typically, measurements are empirically calibrated by dividing higher moment expressions by some power of the measured mean fluorescence. Hence in Eqs. **5** we have absorbed ε into Φ. Thus, to a first-order approximation, the randomness introduced at the PMT can be neglected (see Eqs. **3**). The practical difference between using a Poisson or a more complicated representation of the relationship between E and Φ is minor. Hence the fluctuation moments of Φ can be obtained from the measured fluctuation moments of E by direct subtraction.

The fluorescence fluctuation moments can also be obtained from the time correlation function of the photon count fluctuations. The moments of the fluorescence intensity fluctuations are obtained by extrapolating the measured and fitted correlation function to zero time (3). The correlation of random emission persists no longer than 10 μsec. Therefore, for transport-dependent fluctuations of the fluorophore ON with characteristic times typically much longer than 10 μsec, the shot noise will be eliminated by the extrapolation. In our experiments the subtraction and extrapolation methods always agree well (Table 2). Note that the time correlation for mixed samples deviates little from the behavior expected for a monodisperse sample (19). Therefore, it would be difficult to obtain the distribution of aggregates from measurements of transport characteristics.

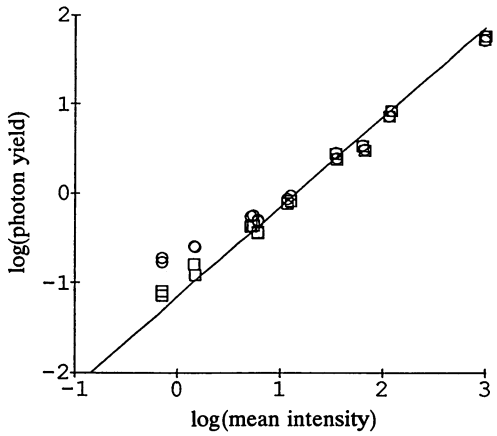

FIG. 2. Proportionality of the photon yield, q, to the mean intensity, $\langle E\rangle$, at different excitation intensities. For a monodisperse solution, $q = \langle E\rangle/\langle N\rangle$ is a linear relationship for a given $\langle N\rangle$. Deviations from linearity occur when the shot-noise contribution becomes comparable to q. The circles are obtained by direct subtraction: $q = \langle\Delta E\Delta E\rangle/\langle E\rangle - 1$. The squares are obtained from the correlation function by extrapolation. The circles begin to deviate from a straight line at about $q = 0.2$ and would reach the plateau $q = 0.1$ when only dark counts are detected (Table 1). The line is represented by $q = 0.07\langle E\rangle$.

Although eliminating the shot noise by subtraction is simple and fast, it fails to yield accurate results when the shot noise is relatively large, i.e., when the photon yield, q (the mean number of photoelectrons per dwell time per fluorescent particle under a given light intensity), is very small. (The yield q is proportional to the PMT and fluorescence quantum yields, absorption coefficient, excitation intensity, and the optical loss.) Taking, for example, the second photocount fluctuation moment from Eq. **5b**, $\langle\Delta E\Delta E\rangle = q\langle N\rangle + q^2\langle\Delta N\Delta N\rangle = q\langle N\rangle(1 + q\langle\Delta N\Delta N\rangle/\langle N\rangle)$. Here N and $\langle N\rangle$ are the ON and its mean value, and $\Delta N = N - \langle N\rangle$. Obviously, the first term on the right is dominant when $q\langle\Delta N\Delta N\rangle/\langle N\rangle << 1$. For a single component system, N follows a Poisson distribution: $\langle\Delta N\Delta N\rangle/\langle N\rangle = 1$. Then, when $q << 1$, the first-order approximation that the photoelectron response be Poissonian is no longer sufficient. Instead, a more accurate representation should be used: $\langle\Delta E\Delta E\rangle/\langle E\rangle = 1 + \varepsilon + q\langle\Delta N\Delta N\rangle/\langle N\rangle$. Hence, if $q\langle\Delta N\Delta N\rangle/\langle N\rangle \approx \varepsilon$, it would be difficult to obtain the ON fluctuation $\langle\Delta N\Delta N\rangle$ from the measured photocount fluctuation moment without an accurate value for ε. In addition, measurement errors will make it even more difficult to extract this relatively small term. Nevertheless, the extrapolation method is still valid even in the presence of a large shot-noise contribution (Fig. 2).

Table 3. Moments from monodisperse samples

Bead radius	m_1	$m_2 \times 10^{-2}$	$m_3 \times 10^{-3}$	$\langle N\rangle$	q	γ
0.115 μm	498 ± 20	6.1 ± 0.2	6.6 ± 0.2	16.7 ± 0.5	29.9 ± 0.4	0.54 ± 0.02
0.05 μm	72.0 ± 3.1	1.40 ± 0.06	0.56 ± 0.08	71.3 ± 3.3	1.02 ± 0.01	0.36 ± 0.02

Columns are defined as follows: $m_1 = \langle\Phi\rangle$; $m_2 = \langle(\Delta\Phi)^2\rangle/\langle\Phi\rangle^2$; $m_3 = \langle(\Delta\Phi)^3\rangle/\langle\Phi\rangle^3$; $\langle N\rangle = 1/m_2$; $q = m_1m_2$; $\gamma = m_2^2/m_3$. Laser excitation-source power was 0.05 W.

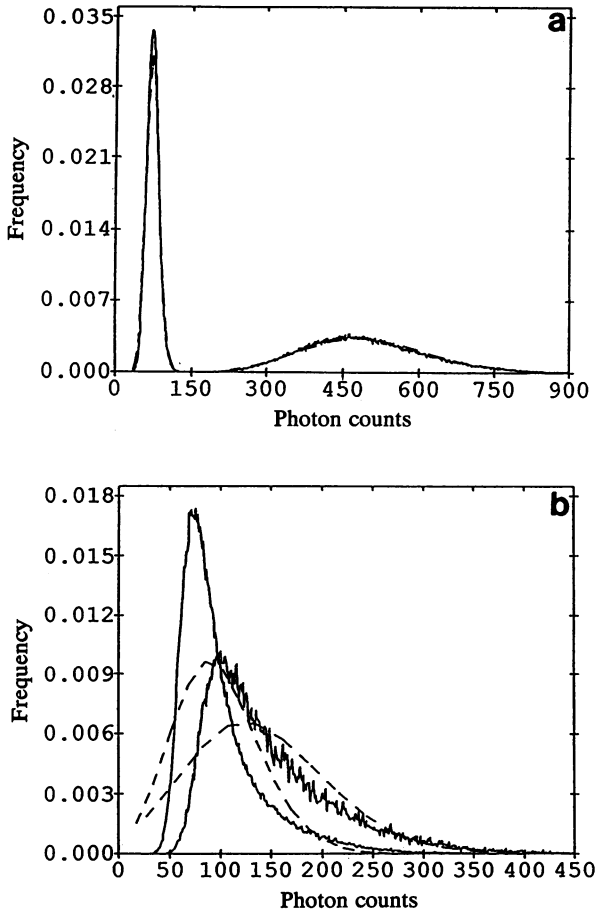

FIG. 3. Fluorescence photon-count histograms for fluorescent bead samples. (*a*) Left curve, 0.05-μm beads; right curve, 0.115-μm beads. (*b*) Data for mixtures A and B (see *Materials and Methods*). Error bars in all four curves are too small to appear in the plot. For a monodisperse solution, the fluorescence photon counts should follow a compound Poisson distribution; for a polydisperse solution, they should deviate from this distribution. All four dashed lines are compound Poisson distributions calculated using the first two moments obtained from the experimental data. In *a*, both curves are in good agreement with a compound Poisson distribution. In *b*, it is clear that both curves are not represented by a compound Poisson distribution, which therefore qualitatively demonstrates polydispersity of these samples.

In a monodisperse system, the mean number of particles in the illuminated volume, $\langle N\rangle$, is determined if both the mean, $q\langle N\rangle$, and the second moment, $q^2\langle N\rangle$, of the fluorescence intensity distribution are known. Moreover, the number of particles in the illuminated volume, Prob$\{N = m\}$, should follow a Poisson distribution (20). For multicomponent systems, however, Prob$\{N = m\}$ is no longer described by a Poisson distribution, and the higher moments provide additional information. Consider, for example, the two-component system in which the effective photon yields of the smaller and larger fluorescent particles are q_1 and νq_1, respectively. Then, from Eqs. 2 the first three moments of the fluorescence intensity fluctuations will be

$$\langle\Phi\rangle = q_1(\langle N_1\rangle + \nu\langle N_2\rangle) \quad [6a]$$

$$\langle\Delta\Phi\Delta\Phi\rangle = q_1^2(\langle N_1\rangle + \nu^2\langle N_2\rangle) \quad [6b]$$

$$\langle\Delta\Phi\Delta\Phi\Delta\Phi\rangle = q_1^3(\langle N_1\rangle + \nu^3\langle N_2\rangle)/\gamma. \quad [6c]$$

Thus, if we knew the values of q_1 and γ, we could determine ν, $\langle N_1\rangle$, and $\langle N_2\rangle$ from measured values of the first three moments of the fluorescence fluctuations.

Table 4. Moments from polydisperse samples

Sample	m_1	$m_2 \times 10^{-2}$	$m_3 \times 10^{-3}$
A	148 ± 5	0.18 ± 0.01	0.089 ± 0.010
B	99.2 ± 1.9	0.17 ± 0.02	0.11 ± 0.02

Conditions were as for Table 3.

RESULTS

To test this approach to characterizing aggregate distributions we have carried out experimental measurements on the homogeneous and mixed bead samples described in *Materials and Methods*.

Analysis of Fluorescence Fluctuations for a System Containing a Single Component. The mean fluorescence intensity and the first two moments of the intensity fluctuations, calculated from the experimental record of photon counts from the two homogeneous samples of beads, are shown in Table 3, where $m_1 = \langle\Phi\rangle$, $m_2 = \langle\Delta\Phi\Delta\Phi\rangle/\langle\Phi\rangle^2$, and $m_3 = \langle\Delta\Phi\Delta\Phi\Delta\Phi\rangle/\langle\Phi\rangle^3$. From the moments are calculated the average number of particles in the illuminated volume, $\langle N\rangle$, the photon yield for each particle, q, and the γ factor. Since the photon yield of the 0.115-μm beads is $\approx$30 times that of the 0.05-μm beads, the former are equivalent to aggregates of 30 of the latter. The distributions of photon counts measured separately for the two types of particles are shown to be well represented by a compound Poisson distribution in Fig. 3*a*.

Analysis of the Fluorescence Fluctuations for a Two-Component Mixture. The results from the two-particle mixtures are shown in Table 4. From Eqs. 6, it may be shown that $\nu = [\gamma\langle(\Delta\Phi)^3\rangle/q_1^3 - \langle(\Delta\Phi)^2\rangle/q_1^2]/[\langle(\Delta\Phi)^2\rangle/q_1^2 - \langle\Phi\rangle/q_1]$. Hence, taking $\gamma = 0.5$ and the value of $q_1 = 1$, as determined previously (Table 3), we obtain ν, $\langle N_1\rangle$, and $\langle N_2\rangle$ (Table 5), in good agreement with the proportions in which the two types of beads were mixed.

The photon-count distributions for samples A and B are shown in Fig. 3*b*. The compound Poisson distribution clearly cannot represent the data. Therefore, we conclude qualitatively that these samples are not monodisperse but have particles with different photon yields, i.e., are mixtures of "aggregates." A formal inversion of the photon count distribution has been derived (21) but has not yet been implemented practically.

DISCUSSION

We have demonstrated that the composition of a mixture of two fluorescent particles can be determined from an analysis of the first three moments of fluorescence ON fluctuations. The experimental system chosen for this study was relatively favorable because the two kinds of particles were highly fluorescent and differed by 30-fold in their fluorescence, corresponding to a mixture of monomers and 30-mers. A similar analysis on less fluorescent and/or more similar particles would require greater measurement accuracy. We have, however, used signal acquisition times of <7 min. Hence accuracy could be improved by extending the period of measurement.

To interpret these measurements it is essential to account for shot noise, which arises both from the quantal nature of fluorescence emission and from the conversion of the emitted light to an electronic signal. The latter noise source can be

Table 5. Compositions of two-component mixtures

	Deduced from moments			Mixture compositions		
Sample	ν	$\langle N_1\rangle$	$\langle N_2\rangle$	ν	n_1	n_2
A	37	44	2.8	30	57	3.3
B	33	50	1.5	30	64	1.7

neglected when the photon yield q is high. Then the difference between the more complicated response function—e.g., a compound Poisson distribution—and a simple Poisson distribution is minor (Fig. 1), and it is possible to use a simple subtraction method to eliminate the shot noise. When q is small it is necessary to use an extrapolation method that requires determination of the initial time course of the higher-order time correlation functions (22, 23).

The fluctuation moments are particularly sensitive to rare large aggregates or noise pulses, which cause the fluorescence to deviate far from its mean intensity. This is because the nth fluctuation moment includes the nth power of these large values, which therefore assume overwhelming weight. This does not occur in the histogram, in which these rare events are located at one extreme of the amplitude axis without distorting the frequency of the more common events. Thus, the moment analysis gives heavier weight to the larger fluctuations, whereas all fluctuations are weighed equally in the histogram analysis. The former can more sensitively detect small amounts of larger aggregates but is more subject to distortion by chance contributions of rare events.

We thank Mr. W. B. McConnaughey for assembling and maintaining our instrument for FCS measurements and Drs. John Cooper, Carl Frieden, and Howard Reiss for their encouragement of this work. This study was supported by National Institutes of Health Grant GM38838.

1. Qian, H. & Elson, E. L. (1988) *Proc. Soc. Photo-Opt. Instrum. Eng.* **909**, 352–359.
2. Qian, H. & Elson, E. L. (1989) *Appl. Polym. Symp.* **43**, 305–314.
3. Palmer, A. G. & Thompson, N. L. (1987) *Biophys. J.* **52**, 257–270.
4. Palmer, A. G. & Thompson, N. L. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6148–6152.
5. Qian, H. & Elson, E. L. (1990) *Biophys. J.* **57**, 375–380.
6. Moran, P. A. P. (1968) *An Introduction to Probability Theory* (Clarendon, Oxford), pp. 62–65.
7. Elson, E. L. & Webb, W. W. (1975) *Annu. Rev. Biophys. Bioeng.* **4**, 311–334.
8. Brenner, S. L., Nossal, R. J. & Weiss, G. H. (1978) *J. Stat. Phys.* **18**, 1–18.
9. Magde, D., Elson, E. L. & Webb, W. W. (1972) *Phys. Rev. Lett.* **29**, 705–708.
10. Elson, E. L. & Magde, D. (1974) *Biopolymers* **13**, 1–27.
11. Magde, D., Elson, E. L. & Webb, W. W. (1974) *Biopolymers* **13**, 29–61.
12. Magde, D., Webb, W. W. & Elson, E. L. (1978) *Biopolymers* **17**, 361–367.
13. Saleh, B. (1978) *Photoelectron Statistics* (Springer, Berlin).
14. Qian, H. & Elson, E. L. (1990) in *Optical Microscopy for Biology*, eds. Herman, B. & Jacobson, K. (Wiley-Liss, New York), pp. 119–130.
15. Palmer, A. G. & Thompson, N. L. (1989) *Appl. Opt.* **28**, 1214–1220.
16. Petersen, N. O., Felder, S. & Elson, E. L. (1986) in *Handbook of Experimental Immunology*, eds. Weir, D. W., Herzenberg, L. A., Blackwell, C. C. & Herzenberg, L. A. (Blackwell, Edinburgh), 4th Ed., Vol. 1, pp. 24.1–24.23.
17. Weissman, M., Schindler, H. & Feher, G. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2776–2780.
18. Petersen, N. O. (1986) *Biophys. J.* **49**, 809–815.
19. Qian, H. (1989) Ph.D. Thesis (Washington Univ., Saint Louis).
20. Landau, L. D. & Lifshitz, E. M. (1969) *Statistical Physics* (Addison-Wesley, Reading, MA), 2nd Ed., p. 357.
21. Qian, H. (1990) *Rev. Sci. Instrum.*, in press.
22. Palmer, A. G. & Thompson, N. L. (1989) *Rev. Sci. Instrum.* **60**, 624–633.
23. Qian, H. (1990) *Biophys. Chem.*, in press.