CHARACTERIZATION OF THE EQUILIBRIUM DISTRIBUTION OF POLYMER MOLECULAR WEIGHTS BY FLUORESCENCE DISTRIBUTION SPECTROSCOPY (THEORETICAL RESULTS)

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SYNOPSIS

A method is presented to characterize the molecular weight distribution of a polymer composed of fluorescent monomers in solution. This method, which we call "Fluorescence Distribution Spectroscopy" (FDS), measures the amplitude of fluctuations of the total number of polymer subunits in a well defined small open volume. The number of subunits is measured in terms of the total fluorescence emission from a volume defined by a laser beam. FDS is essentially an equilibrium measurement which does not depend on the time course of the flucutations or on any dynamical properties of the system. We present a statistical relationship between the measured subunit number fluctuations and the distribution of polymer molecular weights. The relationship is illustrated with computer simulations of an application to an equilibrium aggregation. This method does not perturb the measured system and therefore has the the notable advantage being applicable to labile or reversible polymerization or aggregation systems and therefore should be particularly useful in biological studies.

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INTRODUCTION

Fluctuation spectroscopies are promising techniques to study the dynamics of macromolecules [1]. One of these methods, fluorescence correlation spectroscopy (FCS) [2,3], records spontaneous fluctuations of the number of fluorophores in a small observation region defined by a laser beam in terms of measured fluctuations of fluorescence photon counts. Since the number of fluorescent molecules in the open region changes more rapidly the faster they diffuse (or drift or flow), the time correlation of the fluctuation is entirely determined by the transport properties of the molecules and the size of the observation region. Therefore, FCS can measure transport properties from the time course of the fluorescence fluctuation autocorrelation function [2,3].

Application of this approach to a solution of fluorescence labeled polymer molecules can yield information in addition to transport properties. One can obtain the distribution of degrees of polymerization from a statistical analysis of the distribution of fluorescence photon counts. We have developed this idea in a new method which we call Fluorescence Distribution Spectroscopy (FDS). FDS is an equilibrium analysis which is independent of the transport properties of the polymer molecules. The theory is formalized by assuming the measured fluctuations are statistically independent of each other. Many previous techniques to measure polymer molecular weights are based on the dependence of transport properties on polymer molecular size and must be interpreted in terms of some hydrodynamic model [4]. Other methods require polymer fractionation techniques which cannot be applied to labile biological polymers [5,6]. Ideally, FDS can provide a perturbation-free measurement of the distribution of polymer molecular weights in a reversible polymerization system in equilibrium.

THE BASIC THEORY

The fluorescence measurement provides directly a record of emitted photon counts, which yields a histogram of the number of photons recorded in each counting interval. This probability distribution of photon counts results both from the random emission characteristic of individual fluorophores and from the random number of fluorophores in the observation region due to concentration fluctuations. For each single fluorophore, the random emission follows the Poisson distribution [7] so that the probability that n photons are recorded in a counting interval is:

$$\text{Prob}\{P=n\} = \lambda^n e^{-\lambda}/n! \tag{1}$$

where λ is the mean emission photon count per fluorophore and per counting interval under the given excitation light intensity. We assume that each subunit (monomer) is labeled with one fluorophore and that its optical properties are not affected by polymerization, that the polymer solution is dilute, and that polymer molecules diffuse independently. Then, the relationship between the measured distribution of photon counts, denoted by P_P, and the fluorophore number distribution, P_N, has the form of a Poisson transform,

$$P_P(n) = \sum_{m=0}^{\infty} P_N(m) \frac{(\lambda m)^n}{n!} e^{-\lambda m} \tag{2}$$

Next, we show that P_N is, in turn, related to the polymer size distribution by deriving the generating function for the fluorophore number distribution $Q_N(x) = \Sigma_n P_N(n)x^n$. First consider a solution containing only i-mers. Let A_i denote the average number of i-mers in the observation region. Since the i fluorophores on an i-mer diffuse as one unit, the distribution of i-mer fluorophores in the observation region will be, again, a Poisson distribution. Hence the probability that there are n i-mers and therefore ni monomers in the sample region is

$$\text{Prob}\{N=ni\} = (A_i)^n \exp[-A_i]/n! \tag{3}$$

Note that only i, 2i, 3i $\cdots$ can be observed. The corresponding generating function is

$$Q(x) = \Sigma_i\, x^{ni}\text{Prob}\{N=ni\}$$

$$= \exp\{-A_i(1-x^i)\} \tag{4}$$

The generating function for the entire system, Q_N, is just the product of the generating functions for each of the i-mer size classes [8],

$$Q_N(x) = \exp\{-A_1(1-x)\}\exp\{-A_2(1-x^2)\}\cdots \tag{5}$$

or

$$\ln Q_N(x) = -\sum_{i=1}^{\infty} A_i(1-x^i) = -A_0 + \sum_{i=1}^{\infty} A_i x^i \tag{6}$$

where A_0 is the mean total number of polymer molecules in the observation region.

Now it is possible to derive the distribution of polymers sizes from $P_N(n)$. It is readily shown that

$$A_0 = \sum_{n=1}^{\infty} A_n = -\ln Q_N(0) = -\ln P_N(0) \tag{7}$$

$$A_t = \sum_{n=0}^{\infty} nA_n = \frac{Q_N'(1)}{Q_N(1)} \tag{8}$$

where A_0 and A_t are the means of the total number of aggregates and of subunits respectively in the observation region. We obtain the distribution of the i-mers, A_i, by expanding $Q_N(x)$ in a power series to yield:

$$A_1 = \frac{P_N(1)}{P_N(0)} \tag{9a}$$

$$A_2 = \frac{P_N(2)}{P_N(0)} - \frac{1}{2}\frac{P_N(1)^2}{P_N(0)^2} \tag{9b}$$

$$A_3 = \frac{P_N(3)}{P_N(0)} - \frac{P_N(1)P_N(2)}{P_N(0)^2} + \frac{1}{3}\frac{P_N(1)^3}{P_N(0)^3} \tag{9c}$$

$\cdots$

A mathematical solution for the inversion of the Poisson transformation (2) would allow a direct calculation of $P_N(n)$ from the experimentally measurable histogram $P_P(m)$. Some general discussion of generating functions can be found in references [4] and [10].

If we denote $Q_P(x)=\Sigma_n P_P(n)x^n$, the generating function of distribution $P_P(n)$, it is easy to verify that:

$$Q_P(x) = Q_N[e^{-\lambda(1-x)}] \qquad (10)$$

Therefore, we have

$$\begin{aligned} \ln Q_P(x) &= \ln Q_N[e^{-\lambda(1-x)}] \\ &= -A_0 + \Sigma_{n=1}\{A_n e^{-\lambda(1-x)n}\} \end{aligned} \qquad (11)$$

or, if we let $y=e^{-\lambda(1-x)}$, then,

$$-A_0 + \Sigma_{n=1}\{A_n y^n\} = \ln Q_P(1+\ln y/\lambda) \qquad (12)$$

REAL AND SIMULATED DATA

An example of an FCS/FDS experiment on a suspension of 0.1 μm beads highly labeled with coumarin is presented in Figure 1. The direct fluorescence fluctuation record is shown in Part A. The fluctuation autocorrelation function is shown in Part B. This autocorrelation function yields a value of 83 msec for the time correlation of diffusion of the beads. It should be emphasized, however, that the dynamics of bead motion play no role in the FDS results apart from specifying how the time parameters of the measurement should be set. The total data recording period must be long enough to include a sufficiently large number of fluctuations to guarantee statistical accuracy. Also the counting interval must be short enough relative to the characteristic diffusion time so that all statistically relevant fluorescence fluctuations are recorded, but as long as possible within this limit to maximize the photon counts for each data point. Part C presents the distribution of photon counts per counting interval, i.e., the distribution, P_P. In a polydisperse system information about the distribution of polymer or aggregate sizes can be obtained from this histogram by FDS analysis. In this example of a monodisperse system the measured distribution is well fitted by the expected compound Poisson distribution.

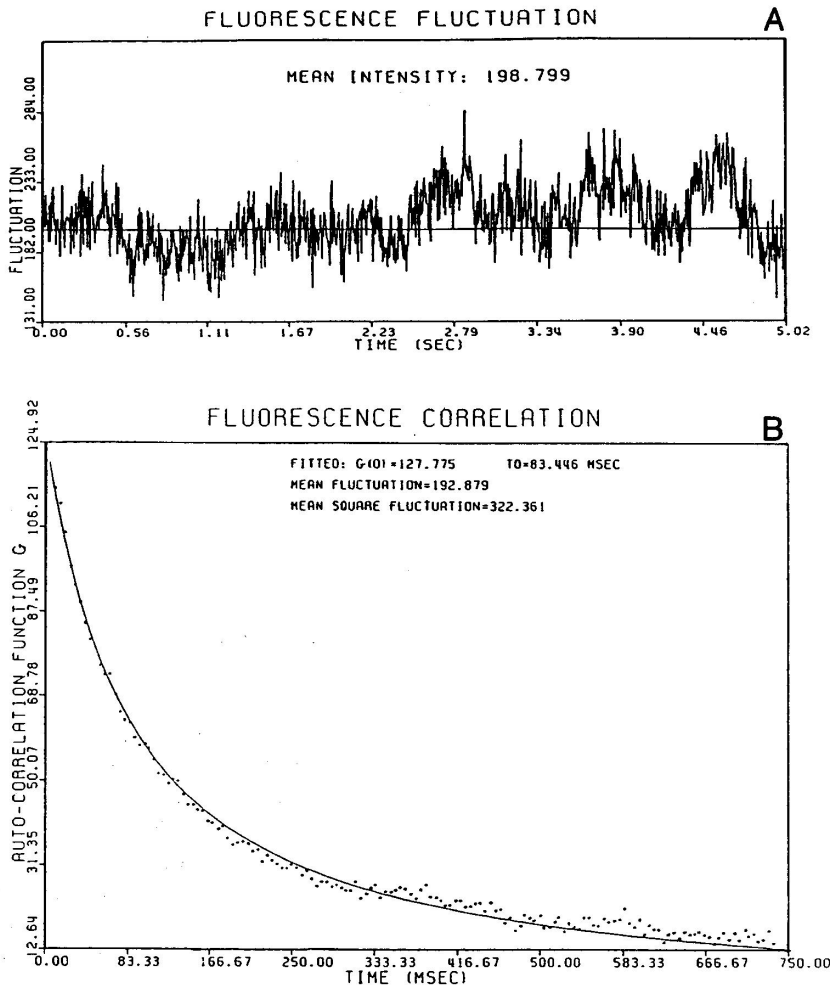
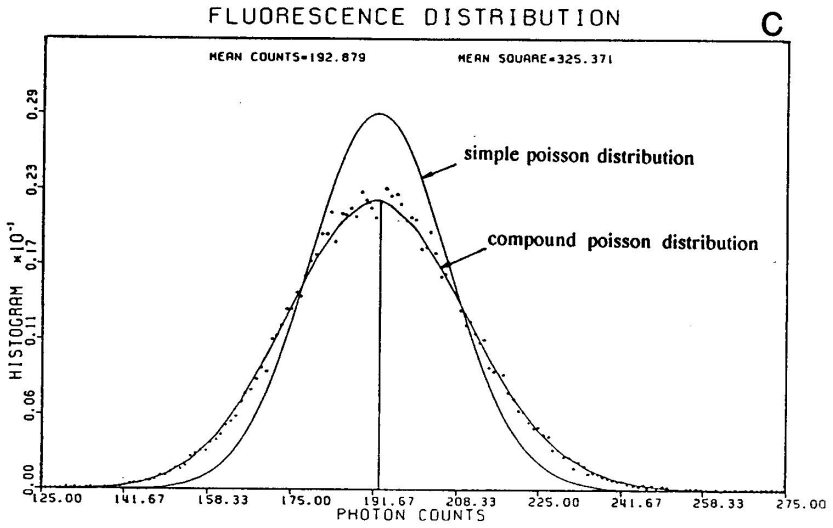


FIG. 1. An example of an FCS/FDS Experiment. (A) Digital photon count of the fluorescence emitted from a ω^2 = 10-15 μm^2 subregion of a much larger sample chamber which contains a solution of fluorescent beads. The integration time interval is 0.005 sec, and the experiment lasts about 4 minutes. These beads are used to simulate aggregates. (B) Fluorescence fluctuation autocorrelation function of the data from Part A. By FCS analysis the time correlation determines a characteristic time T_d which is used to calculate mean diffusion constant of the particles by formula $D=\omega^2/4T_d$ (reference 2). (C) The distribution of photon counts per 5 msec counting interval, P_P. FDS analysis yields information about the distribution of aggregates from this histogram. In this monodisperse sample, the photocount distribution is expected to be a compound poisson distribution. A simple poisson distribution does not fit the data.



The mathematical relation between $\{A_i\}$ and P_P, derived in terms of a generating function, is complex. Hence we demonstrate the expected capabilities of FDS by a computer simulation. Figure 2 compares the distributions P_P and P_N for a mixture of 5-mers and 25-mers (Part B) with a superposition of the histograms obtained from 5-mers and 25-mers separately (Part A). It is clear that random emission smears the fluorophore number distribution. That is, mathematically, $<\Delta P \Delta P> > <\Delta N \Delta N>$. Therefore, the inverse Poisson transformation serves as a "filter" to sharpen the distribution. In some favourable conditions, however, even the photocount distribution can give qualitative results. For example, Figure 2 demonstrates that two peaks can be resolved in the simulated photon count distribution from a mixture of 5-mers and 25-mers.

DISCUSSION

Ideally the determination of a polymer size distribution by FDS would invert the Poisson transform of Equation (2) to obtain the

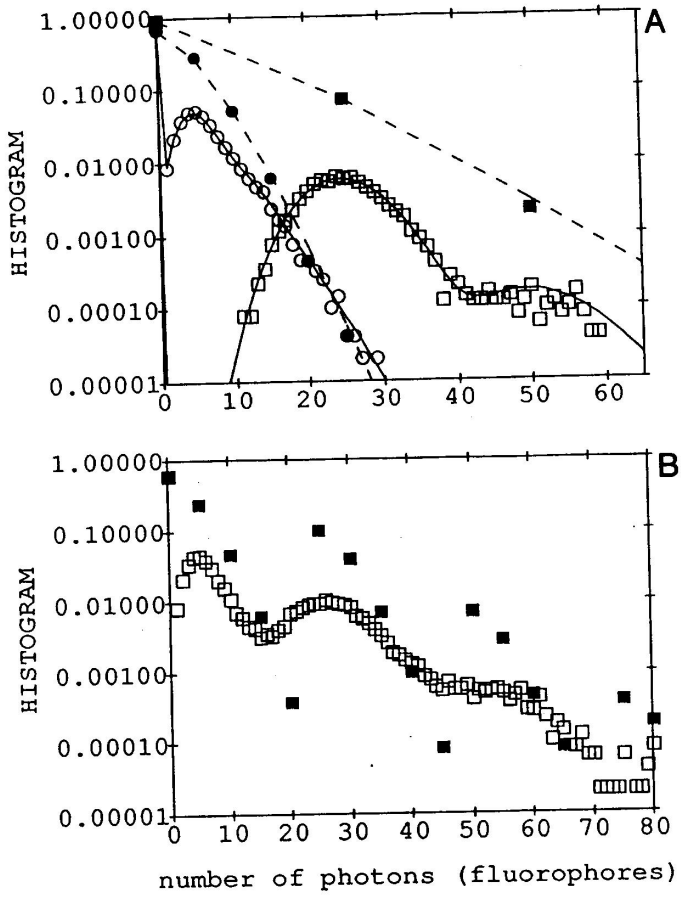


FIG. 2. Computer simulation of an FDS Experiment. The simulation randomly distributes n particles in a circle with a radius of 7.07 units. The subregion under observation has a radius of 1 unit. For each particle in the observation subregion, a Poisson random number generator simulates the photon emission according to the given number of fluorophores (m) on the particle (The mean number of photons emitted per fluorophore per time interval is set to 1). After recording the photon count the particles are randomly rearranged and the process is repeated. (A, upper) The fluorophore distributions P_N for two different examples, n=20,m=5 and n=4,m=25, represented by filled circles and squares, respectively. The open circles and squares are distributions of photon counts, P_P. Solid and dashed lines are expected theoretical curves. (B, lower) The fluorophore (filled squares) and photon (open squares) distributions for a mixture of 20 pentamers and 8 25-mers. There are peaks in P_P at 5 and 25. If the P_P data (open squares) were "filtered" by an inverse Poisson transformation, the P_N distribution (filled squares) would result.

fluorophore number distribution P_N from the experimentally measured photocount distribution function P_P. Then, the size distribution, A_i, could be derived from P_N as demonstrated in Equations 9 (a-c). A closely related method to characterize molecular aggregation from the moments of the photocount distribution has also been developed recently [11,12]. It is obvious that a two component system can be characterized by the first three moments, and a three component system can be characterized by the first four moments. This is analogous to Equation 9 which shows that the first three terms of P_N are required to determine A_2 and the first four terms of P_N, to determine A_3. In experimental work we have noticed that the moment analysis method is extremely sensitive to the presence of aggregates, but is also very sensitive to external noise, which can strongly perturb the measurement. In contrast the FDS method is relatively insensitive to the external noise and therefore simpler to accomplish experimentally, but also does not so sensitively detect aggregates as the moment analysis.

The statistical analysis we present here can be applied in studies other than fluorescence spectroscopy. Any physical or chemical measurements which are proportional to the total number of monomers in a well defined small volume can be analysed in the same fashion. For example, the total mass or the total absorbance of a well defined aerosol of fine droplets can be analysed as above. Similarly scattered light could be used to replace fluorescence with this approach. But the relationship between light scattered and molecular weight is complex for large particles.

An important feature of FDS is its independence of the dynamic properties of the aggregates. It invokes no hydrodynamic model, and is purely an equilibrium analysis. In principle, FDS requires each succesive measurement of concentration to be independent. This is usually not true in an actual experiment due to slow diffusion. But it is obvious that if the total integration time is sufficiently long, the approach is still valid.

CONCLUSIONS

FDS is a statistical equilibrium analysis which yields the polymer molecular weight distribution. It can also be used to characterize molecular aggregations. This method is perturbation-free and relatively model independent. FDS undoubtedly has potential applications in polymer chemistry and biochemistry.

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