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Statistical accuracy in fluorescence correlation spectroscopy*

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Fluorescence correlation spectroscopy is a promising technique for the study of chemical kinetics and diffusion processes in small, well-defined sample volumes. Laser-induced fluorescence is used as a specific highly sensitive probe of concentration, permitting the analysis of the characteristic time dependence of spontaneous concentration fluctuations. We present here a quantitative analysis of the statistical and systematic errors inherent in such an experiment. Theoretical expressions are derived for the signal-to-noise ratio for various operational definitions of the photocount correlation signal. It is shown that the critical parameter governing the statistical accuracy of the experiment is not the total number of photocounts per correlation time, but (when background fluorescence is negligible) the photocounts per correlation time, *per fluorescent molecule*, a parameter independent of the number of fluorescent molecules in the sample volume. Comparisons are made with parallel results for laser-light scattering experiments derived by other authors. Finally, we consider the effects of background fluorescence, photolysis, and other deviations from ideality.

I. INTRODUCTION

Fluorescence correlation spectroscopy (FCS)[1-3] shows great promise as a tool for the study of chemical kinetics and *in situ* diffusion processes. It is one of a general class of proposed concentration correlation spectroscopy techniques.[2] The common basis of these techniques is the use of specific physical probes as continuous monitors of the concentrations of specific chemical components in small well-defined sample volumes. Autocorrelation functions of the monitor signals are used to characterize the time dependence of spontaneous fluctuations of the number of molecules in the sample volume. An effective concentration monitor should be specific and highly sensitive. Fluorescence scores high in both regards and was used by Magde, Elson, and Webb[1,3] to study the thermodynamic fluctuations about equilibrium, due to chemical reaction and diffusion, in solutions of ethidium bromide and DNA. FCS has also been applied to studies of membrane structure,[4] via measurements of diffusion coefficients of fluorescent probes incorporated into lipid bilayer membranes. Molecular number fluctuations can also be studied by Rayleigh light scattering,[5-8] resonance Raman scattering, and with monitors of such properties as conductivity[9] and optical absorption and rotation.

In the typical FCS experiment, a small sample volume is illuminated with cw laser radiation. This can be a closed volume, perhaps a single living cell, or an open volume, defined, in part, by the laser beam itself. After appropriate optical filtering to eliminate incident laser light, the emitted fluorescence is detected by a photomultiplier tube, and analyzed with an analog photocurrent or digital photocount correlator.

The instantaneous fluorescence is a chaotic Gaussian field. The power spectrum is sufficiently broad, however, ($\Delta\omega \simeq 10^{14}$ sec^{-1}), that field correlations persist only over immeasurably short times (~10^{-14} sec). Thus, for a fixed concentration of fluorescent molecules, the fluorescence intensity integrated over any practical measuring interval is constant and directly proportional to that concentration. While there are no spatial field correlations, the fluorescence intensity, due to the nature of the number fluctuation phenomenon itself, is coherent over the full 4π sr of emission. Thus the well-designed FCS experiment collects the fluorescence over as large a solid angle as possible. Figure 1 reproduces the schematic of the optical apparatus of the pioneering FCS experiment of Magde, Elson, and Webb.[3]

FCS experiments are difficult in practice, because the fluorescence fluctuations are small relative to the average intensity. To achieve adequate signal-to-noise ratios, Magde, Elson, and Webb[1,3] needed data integration times of many hours. It is the purpose of this paper to calculate theoretical expressions for the photocount correlation signal-to-noise ratio for the fluorescence experiment, and, in so doing, gain further insight into the processes involved and develop general guidelines for the achievement of optimal experimental results. We will thus be able to see the manner in which such related parameters as laser intensity, beam diameter, sample volume, concentration of molecules, number of molecules, and fluorescence photon counting rate affect the statistical accuracy of the experiment. In the appropriate limits (see Sec. III C), it is expected that the results of this paper can be applied to other types of number fluctuation

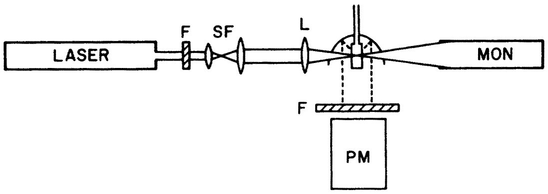

FIG. 1. Schematic of the optical apparatus for an FCS experiment, reproduced from Ref. 3. The symbols are: F, colored filter; SF, spatial filter; L, lens; MON, laser intensity monitor; PM, photomultiplier. The sample cell and the parabolic fluorescence-collecting mirror are also shown.

experiments as well.

Comparisons are made with parallel results, derived elsewhere,[10-14] for the optical fields of laser light-scattering experiments. In these light-scattering experiments, the intensity fluctuations are not due to number fluctuations, but arise as a result of fluctuations in the relative phases, at the detector, of the light from different scattering centers.

II. DEFINITIONS

The most efficient way to characterize the time dependence of optical intensity fluctuations is with digital photocount autocorrelation. We will restrict ourselves to "full" or "unclipped" correlation in which one determines experimental estimates of the twofold correlation function,

$$G(\tau) \equiv \langle n(0)n(\tau)\rangle . \tag{1}$$

Here $n(t)$ is the number of detected photons or photocounts in a counting interval centered at time t and the angular brackets indicate an ensemble or infinite-run time average. A "signal," in this experiment, is appropriately defined as the correlation level above the accidental correlation background,

$$S(\tau) \equiv G(\tau) - \langle n\rangle^2 ; \tag{2}$$

and the signal-to-noise ratio has the form

$$S/N \equiv S(\tau)/\{\mathrm{var}[\hat{S}(\tau)]\}^{1/2} , \tag{3}$$

where $\hat{S}(\tau)$ is the experimental estimate of $S(\tau)$ and $\mathrm{var}[\hat{S}(\tau)]$ is its variance.

An experiment consists of a series of N consecutive counting intervals of period T. We assume throughout that T is adjusted to be much less than the correlation time. The jth interval centered at jT is characterized by the number of photocounts $n(jT)$. With such a counting scheme, one can compute the experimental estimates of $G(\tau)$,

$$\hat{G}(mT) \equiv N^{-1} \sum_{i=1}^{N} n(iT)n(iT + mT) , \tag{4}$$

for a series of different delay times, $\{mT\}$, simultaneously. There are several possible operational definitions of $\hat{S}(\tau)$. We consider three below:

$$\text{(i)} \quad \hat{S}^{(\mathrm{i})}(mT) \equiv \hat{G}(mT) - \hat{G}(MT) . \tag{5}$$

We choose here a value of $M \gg m$ so that $G(MT) \simeq \langle n\rangle^2$. At the same time, we require $N \gg M$.

$$\text{(ii)} \quad \hat{S}^{(\mathrm{ii})}(mT) = \hat{G}(mT) - (\hat{n})^2 , \tag{6}$$

where

$$\hat{n} \equiv N^{-1} \sum_{i=1}^{N} n(iT) \tag{7}$$

is the experimental estimate of $\langle n\rangle$.

$$\text{(iii)} \quad \hat{S}^{(\mathrm{iii})}(mT) = \hat{G}(mT) - \langle n\rangle^2 . \tag{8}$$

The variance of the form (i) is a good measure of the relative scatter of different points on the measured correlation function. More than any other, this form tells us how good the data "look." Form (ii) requires the auxiliary measurement of the quantity $\hat{n}$. This gives, as we shall see, a decrease in the signal variance. One can measure $\hat{n}$, not $\langle n\rangle$, but form (iii) is included for the purpose of discussion. One might think that, given the exact value of $\langle n\rangle$, form (iii) would give the best results. This is not the case, however. For the intensity statistics we consider below, the variance of $\hat{S}^{(\mathrm{iii})}(mT)$ can be several orders of magnitude larger than those of $\hat{S}^{(\mathrm{i})}(mT)$ and $\hat{S}^{(\mathrm{ii})}(mT)$.

III. CALCULATING SIGNAL VARIANCE

There are three basic steps in the calculation of $\mathrm{var}[\hat{S}(mT)]$. First, $\mathrm{var}[\hat{S}(mT)]$ is written in terms of photocount correlation functions. Next, the photocount correlation functions are written in terms of correlation functions of the detected light intensity. These procedures are considered below in a fashion similar to the treatment of Ref. 11. Finally, these intensity correlation functions are evaluated for the particular field statistics of interest.

A. Photocount correlation functions

For each of the three definitions of $\hat{S}(mT)$, a stochastic variable $x(t)$ can be defined to transform the variance to the form

$$\mathrm{var}[\hat{S}(mT)] = \mathrm{var}\left(N^{-1} \sum_{i=1}^{N} x(iT)\right) . \tag{9}$$

Thus we have[15]

$$\mathrm{var}[\hat{S}(mT)] = N^{-2} \sum_{i,j} \langle x(iT)x(jT)\rangle - N^{-2}\Big(\sum_i \langle x(iT)\rangle\Big)^2$$
$$= N^{-1}\mathrm{var}[x] + 2N^{-1}\sum_{k=1}^{N-1} [\langle x(0)x(kT)\rangle - \langle x\rangle^2] \times [1 - kN^{-1}] . \tag{10}$$

For forms (i) and (iii), we have directly

$$\mathrm{var}[\hat{S}^{(\mathrm{i})}(mT)] = \mathrm{var}\Big(N^{-1}\sum_{i=1}^{N} n(iT)[n(iT+mT) - n(iT+MT)]\Big) , \tag{11}$$

so that

$$x^{(\mathrm{i})}(iT) \equiv n(iT)[n(iT+mT) - n(iT+MT)] ; \tag{12}$$

and

$$\mathrm{var}[\hat{S}^{(\mathrm{iii})}(mT)] = \mathrm{var}[\hat{G}(mT)] , \tag{13}$$

so that

$$x^{(\mathrm{iii})}(iT) \equiv n(iT)n(iT+mT) . \tag{14}$$

For form (ii), keeping terms to first order in $\langle n\rangle - \hat{n}$,

$$\mathrm{var}[\hat{S}^{(\mathrm{ii})}(mT)] = \mathrm{var}[\hat{G}(mT) - 2\langle n\rangle\hat{n}] , \tag{15}$$

so that

$$x^{(\mathrm{ii})}(iT) \equiv n(iT)[n(iT+mT) - 2\langle n\rangle] . \tag{16}$$

Thus, for example, combining Eqs. (10) and (12), we get

$$\begin{aligned}\mathrm{var}[\hat{S}^{(\mathrm{i})}(mT)] = N^{-1}\{&\langle n^2(0)n^2(mT)\rangle + \langle n^2(0)n^2(MT)\rangle - 2\langle n^2(0)n(mT)n(MT)\rangle - [\langle n(0)n(mT)\rangle - \langle n\rangle^2]^2\} \\ &+ 2N^{-1}\sum_{k=1}^{N-1}\{\langle n(0)n(mT)n(kT)n(kT+mT)\rangle + \langle n(0)n(MT)n(kT)n(kT+MT)\rangle \\ &- \langle n(0)n(mT)n(kT)n(kT+MT)\rangle - \langle n(0)n(MT)n(kT)n(kT+mT)\rangle - [\langle n(0)n(mT)\rangle - \langle n\rangle^2]^2\} ,\end{aligned} \tag{17}$$

where we have taken N to be sufficiently large that the end effects (expressed in the factor $[1 - kN^{-1}]$) are negligible.

B. Intensity correlation functions

The development up to this point has been sufficiently general that it holds for any periodically sampled stationary random variable. We must now relate the photocount autocorrelation functions of the above expressions to the correlation functions of $I(t)$, the detected light intensity integrated in space and time over the counting interval centered at time t. We can do this by using the fact that the factorial moments of n,

$$F_l(n) \equiv \langle n!/(n-l)!\rangle , \tag{18}$$

are proportional to the ordinary moments of I,

$$F_l(n) = \langle I^l\rangle \tag{19}$$

(see Ref. 16, for example), where, for simplicity and without loss of generality, we have set the proportionality constant to unity. Thus, for example,

$$G(mT) = \langle n(0)n(mT)\rangle = \langle I(0)I(mT)\rangle , \tag{20}$$

$$\langle n(0)[n(0)-1]n(mT)[n(mT)-1]\rangle = \langle I^2(0)I^2(mT)\rangle , \tag{21}$$

$$\langle n(0)[n(0)-1]n(mT)\rangle = \langle I^2(0)I(mT)\rangle , \tag{22}$$

so that

$$\langle n^2(0)n^2(mT)\rangle = \langle I^2(0)I^2(mT)\rangle + \langle I^2(0)I(mT)\rangle + \langle I(0)I^2(mT)\rangle + \langle I(0)I(mT)\rangle . \tag{23}$$

Similarly,

$$\langle n(0)n(mT)n(kT)n(kT+mT)\rangle = \langle I(0)I(mT)I(kT)I(kT+mT)\rangle + \delta_{m,k}\langle I(0)I(mT)I(2mT)\rangle . \tag{24}$$

C. Fluorescence intensity statistics

For a large number of fluorescent molecules, as a direct consequence of the central limit theorem,

$$\delta I(t) \equiv I(t) - \langle I\rangle \tag{25}$$

is a real Gaussian variable. Defining

$$f(\tau) \equiv \langle \delta I(0)\delta I(\tau)\rangle / \langle(\delta I)^2\rangle , \tag{26}$$

we have

$$\langle I(0)I(\tau)\rangle = \langle I\rangle^2[1 + \beta f(\tau)] , \tag{27}$$

$$S(\tau) = \langle n\rangle^2 \beta f(\tau) , \tag{28}$$

where

$$\beta = \langle(\delta I)^2\rangle / \langle I\rangle^2 \tag{29}$$

is the normalized variance of I. For an open sample volume defined within a much larger total volume, it is clear that[2,5]

$$\beta = \mathfrak{N}^{-1} , \tag{30}$$

where $\mathfrak{N}$ is the average number of fluorescent molecules in the sample volume (e.g., within the $1/e^2$ points of the Gaussian intensity profile of a focused laser beam). For an evenly illuminated closed system, with number fluctuations arising from spontaneous chemical transformations between a fluorescent and nonfluorescent state,

$$\beta = p\mathcal{N}^{-1}, \tag{31}$$

where p is the average fraction of potentially fluorescent molecules in the nonfluorescent state. These expressions for β are derived assuming that the background or "blank" fluorescence is negligible. The effects of blank fluorescence and other deviations from ideality are discussed in Sec. VIII.

In a Gaussian process, all second- and higher-order correlation functions can be expressed as sums of products of the first-order function. For example,

$$\begin{aligned}\langle \delta I(t_1)\delta I(t_2)\delta I(t_3)\delta I(t_4)\rangle &= \langle \delta I(t_1)\delta I(t_2)\rangle\langle \delta I(t_3)\delta I(t_4)\rangle + \langle \delta I(t_1)\delta I(t_3)\rangle\langle \delta I(t_2)\delta I(t_4)\rangle + \langle \delta I(t_1)\delta I(t_4)\rangle\langle \delta I(t_2)\delta I(t_3)\rangle \\ &= \langle I\rangle^4\beta[f(|t_2-t_1|)f(|t_4-t_3|) + f(|t_3-t_1|)f(|t_4-t_2|) + f(|t_4-t_1|)f(|t_3-t_2|)]\,. \end{aligned}\tag{32}$$

Thus the field statistics are completely determined with a specified $\langle I\rangle$, β, and $f(\tau)$.

The results below were calculated for an exponential signal, i.e.,

$$f(\tau) = e^{-\Gamma\tau}, \tag{33}$$

but are expected to be qualitatively correct for other forms as well. One would have an exponential function, for example, with a chemical reaction in a closed uniformly illuminated volume.

IV. RESULTS

Combining Eqs. (3), (10), (12), (14), (16), (28), and (33), applying the properties illustrated by Eqs. (19) and (32), it is straightforward (but tedious) to show that for the first two forms of $\hat{S}(\tau)$, denoted, respectively, by $a=\text{i}$ or ii,

$$\begin{aligned}\operatorname{var}[S^{(a)}(mT)] = {}& \left(\langle n\rangle^4\beta^2N^{-1}\frac{(1+e^{-2\Gamma T})(1+\delta_{a,i}+e^{-2\Gamma mT})+2m(1-e^{-2\Gamma T})e^{-2\Gamma mT}}{1-e^{-2\Gamma T}}\right) \\ & +2\langle n\rangle^3\beta N^{-1}(1+\delta_{a,i}+e^{-2\Gamma mT})+\langle n\rangle^2N^{-1}(1+\delta_{a,i}+\beta e^{-\Gamma mT}),\end{aligned}\tag{34}$$

where

$$\begin{aligned}\delta_{a,i} &\equiv 1 \quad \text{for } a=\text{i} \\ &\equiv 0 \quad \text{for } a=\text{ii}\,.\end{aligned}\tag{35}$$

Here again, β is the normalized variance of the fluorescence intensity and Γ is the decay rate (inverse correlation time) of the assumed exponential correlation function. [See Eqs. (29) and (33).] Thus, with $\beta \ll 1$, in the limit $\Gamma T \ll 1$,

$$(S/N)_m^{(a)} = \langle n\rangle\beta(N\Gamma T)^{1/2}e^{-\Gamma mT}\{\langle n\rangle^2\beta^2[1+\delta_{a,i}+(1+2m\Gamma T)e^{-2\Gamma mT}]+2\Gamma T\langle n\rangle\beta(1+\delta_{a,i}+e^{-2\Gamma mT})+(1+\delta_{a,i})\Gamma T\}^{-1/2}. \tag{36}$$

Similarly,

$$\begin{aligned}\operatorname{var}[\hat{S}^{(\text{iii})}(mT)] = {}& 2\langle n\rangle^4\beta N^{-1}(1+3e^{-\Gamma T})(1-e^{-\Gamma T})^{-1}+\{\langle n\rangle^4\beta^2N^{-1}(1-e^{-2\Gamma T})^{-1} \\ & \times[(1+e^{-2\Gamma mT})(1+e^{-2\Gamma T})+2m(1-e^{-2\Gamma T})e^{-2\Gamma mT}]\} \\ & +2\langle n\rangle^3N^{-1}[2+\beta(1+4e^{-\Gamma mT}+e^{-2\Gamma mT})]+\langle n\rangle^2N^{-1}(1+\beta e^{-\Gamma mT}),\end{aligned}\tag{37}$$

and, with $\beta \ll 1$, in the limit $\Gamma T \ll 1$,

$$\left(\frac{S}{N}\right)_m^{(\text{iii})} = \frac{\langle n\rangle\beta(N\Gamma T)^{1/2}e^{-\Gamma mT}}{(8\langle n\rangle^2\beta+4\Gamma T\langle n\rangle+\Gamma T)^{1/2}}\,. \tag{38}$$

For ease of comparison, we summarize these results in the limit of zero delay time ($m\Gamma T \to 0$):

$$\left(\frac{S}{N}\right)_{m\to 0}^{(\text{i})} = \frac{\langle n\rangle\beta(N\Gamma T)^{1/2}}{(3\langle n\rangle^2\beta^2+6\Gamma T\langle n\rangle\beta+2\Gamma T)^{1/2}}\,; \tag{39}$$

$$\left(\frac{S}{N}\right)_{m\to 0}^{(\text{ii})} = \frac{\langle n\rangle\beta(N\Gamma T)^{1/2}}{(2\langle n\rangle^2\beta^2+4\Gamma T\langle n\rangle\beta+\Gamma T)^{1/2}}\,; \tag{40}$$

$$\left(\frac{S}{N}\right)_{m\to 0}^{(\text{iii})} = \frac{\langle n\rangle\beta(N\Gamma T)^{1/2}}{(8\langle n\rangle^2\beta+4\Gamma T\langle n\rangle+\Gamma T)^{1/2}}\,. \tag{41}$$

V. DISCUSSION

We see directly from Eq. (36) that, for all combinations of counting rates and delay times,

$$(S/N)_m^{(ii)} > (S/N)_m^{(i)} \,. \tag{42}$$

Thus, as anticipated, the auxiliary measurement of $\hat{n}$ has provided an increase in the signal-to-noise ratio. This advantage of $\hat{S}^{(ii)}(mT)$ is relatively small, but not insignificant: To achieve the same statistical accuracy, measurements of $\hat{S}^{(i)}(mT)$ need experimental run times 50–100% longer.

For $\langle n\rangle$ sufficiently small, the variance of all three forms is dominated by the $\langle n\rangle^2$ terms and

$$(S/N)_m \sim N^{1/2}\langle n\rangle\beta e^{-\Gamma m T} \,. \tag{43}$$

This is the so-called Poisson noise limit, the limit in which the variance of $\hat{S}(mT)$ is dominated by the statistical noise of the detection process. Note, however, that "sufficiently small" has a different meaning for $(S/N)_m^{(iii)}$ than it has for forms (i) and (ii). In case (iii) we require $\langle n\rangle \ll 1$, while in (i) and (ii) we only need $\langle n\rangle \ll \beta^{-1}$.

The major contribution to $\mathrm{var}[\hat{G}(mT)]$ is the uncertainty of the background. Thus while the variance of a single point of the measured correlation function ($\mathrm{var}[\hat{S}^{(iii)}(mT)] = \mathrm{var}[\hat{G}(mT)]$) can be quite large, the relative scatter between different points ($\mathrm{var}[\hat{S}^{(i)}(mT)] = \mathrm{var}[\hat{G}(mT) - \hat{G}(MT)]$) will be substantially smaller, as will the variance of a signal [form (ii)], which takes advantage of the strong correlation between $\hat{G}(mT) - G(mT)$ and $(\hat{n})^2 - \langle n\rangle^2$. In the calculation of the variances of forms (i) and (ii), terms of order of $\langle n\rangle^4\beta$ and $\langle n\rangle^3$, which (for $\langle n\rangle > 1$) dominate the variance of $S^{(iii)}(mT)$, cancel out exactly to zero. As a result, for $\langle n\rangle > 1$, $(S/N)_m^{(i)}$ and $(S/N)_m^{(ii)}$ are greater than $(S/N)_m^{(iii)}$ by a factor of $\sim\beta^{-1/2}$ to $\sim\langle n\rangle^{1/2}$, whichever is smaller.

In the signal-to-noise ratio of forms (i) and (ii), $\langle n\rangle$ and β appear only as the product $\langle n\rangle\beta$. This is undoubtedly the most important result of this paper. This means, recalling Eqs. (30) and (31), that the critical parameter in the fluorescence correlation experiment is not the total counting rate, but the counting rate per fluorescent molecule, a quantity independent of the total number of molecules (see Sec. VIII, however). This predicted independence of signal-to-noise ratio from the sample concentration has been observed[3] in analog photocurrent correlation experiments. Similarly, it is not the total incident laser power that is important, but the incident power per unit area. When $\langle n\rangle\beta \simeq 1$, we are well within the optimal high-counting-rate limit, where the noise is due solely to the stochastic nature of the intensity fluctuations, and for forms (i) and (ii) [see Eqs. (39) and (40)],

$$(S/N)_{m\to 0} \sim (N\Gamma T)^{1/2} \,. \tag{44}$$

The factor $N\Gamma T$ is the ratio of the total experiment run time (NT) to the correlation time of the fluorescence intensity (Γ^{-1}).

VI. VARIANCE OF $\hat{\Gamma}$

The principal goal of the calculations for laser light-scattering experiments[10-14] was the determination of the variance of $\hat{\Gamma}$, the experimental estimate of Γ, determined with an appropriate least-squares data analysis (see also Ref. 17). That has not been the case in this paper, inasmuch as an exponential signal is not as universally applicable in fluorescence correlation experiments as it is for laser light scattering. An exponential $f(\tau)$ was chosen here mainly for computational convenience. Nevertheless, it is worthwhile here to examine $\mathrm{var}\hat{\Gamma}$ in the low-counting-rate limit to define explicitly the parameter of merit governing the success of the experiment as a whole.

We consider a two-parameter least-squares fit with $\hat{\Gamma}$ and $\hat{C}$ defined to have the values that minimize the sum $\sum_m [\hat{S}(mT) - \hat{C}e^{-\hat{\Gamma} m T}]^2 W_m$, where

$$W_m \propto [\mathrm{var}\,\hat{S}(mT)]^{-1} \tag{45}$$

are appropriately chosen weight factors. $\mathrm{var}\,\hat{\Gamma}$, in general, is a function not only of $\mathrm{var}[\hat{S}(mT)]$, but $\mathrm{covar}[\hat{S}(mT), \hat{S}(m'T)]$ as well.[14] Assuming independent signal errors,

$$\mathrm{covar}[\hat{S}(mT), \hat{S}(m'T)] = \mathrm{var}[\hat{S}(mT)]\delta_{m,m'} \,, \tag{46}$$

an assumption valid only in the low-counting-rate limit, we have, adapting Eq. (36) of Ref. 11,

$$\frac{\Gamma}{(\mathrm{var}\,\hat{\Gamma})^{1/2}} = \left\{[\langle (m\Gamma T)^2\rangle_{\mathrm{av}} - (\langle mT\rangle_{\mathrm{av}})^2] \times \sum_m \left[\left(\frac{S}{N}\right)_m\right]^2\right\}^{1/2} , \tag{47}$$

where

$$\langle g(m\Gamma T)\rangle_{\mathrm{av}} \equiv \frac{\sum_m g(m\Gamma T)[(S/N)_m]^2}{\sum_m [(S/N)_m]^2} \,. \tag{48}$$

In the low-counting-rate limit, with

$$[(S/N)_m^{(ii)}]^2 = N\langle n\rangle^2\beta^2 e^{-2\Gamma m T} \,, \tag{49}$$

with a large number of correlation channels, so that,

$$\Gamma T \sum_m g(m\Gamma T)e^{-2\Gamma m T} = \int_0^\infty g(x)e^{-2x}\,dx \,, \tag{50}$$

we find

$$\frac{\Gamma}{(\mathrm{var}\,\hat{\Gamma})^{1/2}} = 2^{-3/2}\frac{\langle n\rangle\beta}{\Gamma T}(N\Gamma T)^{1/2} \,. \tag{51}$$

Thus, the critical parameter is $\langle n\rangle\beta/\Gamma T$, the number of photocounts per fluorescent molecule per correlation time.

VII. COMPARISON WITH LIGHT SCATTERING

It is of some interest to compare the results calculated here with parallel results derived for the optical fields of laser light-scattering experiments. We first consider $(S/N)_m^{(G)}$, the signal-to-noise ratio with a Gaussian optical field, the field characteristic of an important class of "homodyne" scattering experiments. (Note that here the *field* is Gaussian distributed. For the FCS experiment we considered a Gaussian-distributed *intensity*.) The signal now has the form

$$S^{(G)}(\tau) = \langle n\rangle^2 f_c(A)\,|g^{(1)}(\tau)|^2\,, \tag{52}$$

where

$$g^{(1)}(\tau) \equiv \frac{\langle E_s^*(0)E_s(\tau)\rangle}{\langle |E_s|^2\rangle} \tag{53}$$

is the normalized first-order correlation function of the scattered field and $f_c(A)$, the normalized variance of the intensity, is a function of the degree of spatial coherence of the field across the detection area A.[18-20] Signal-to-noise calculations have been performed for detection areas much less than a coherence area A_c, in which case, $f_c(A) = 1$. Choosing $|g^{(1)}(\tau)|^2 = e^{-\Gamma\tau}$, with $\hat{S}^{(G)}(mT)$ in a form closely analogous to form (ii) of this paper, we have [Ref. 11, Eq. (22)]

$$\begin{aligned}(S/N)_m^{(G)} = \langle n\rangle(N\Gamma T)^{1/2}e^{-\Gamma m T}\\ \times\{\langle n\rangle^2[1+8e^{-\Gamma m T} - (5+2m\Gamma T)e^{-2\Gamma m T}]\\ +2\Gamma T\langle n\rangle(1+e^{-m\Gamma T})^2 + \Gamma T(1+e^{-\Gamma m T})\}^{-1/2}\end{aligned} \tag{54}$$

and

$$\left(\frac{S}{N}\right)_{m\to 0}^{(G)} = \frac{\langle n\rangle(N\Gamma T)^{1/2}}{(4\langle n\rangle^2 + 8\Gamma T\langle n\rangle + 2\Gamma T)^{1/2}}\,. \tag{55}$$

Equations (54) and (55) are remarkably similar in form to Eqs. (36) and (40), only now, instead of a factor of $\langle n\rangle\beta$, we have just $\langle n\rangle$. This shows that the fluorescence correlation experiment can, in principle, be just as fast and efficient as the more conventional laser light-scattering experiment, but the photocount counting rate must be larger by the factor β^{-1}.

For a large detection area, the integrated intensity of the scattered field $I_s(t)$ is the sum of many independent contributions from each of the different coherence areas, and

$$f_c(A) \propto 1/\mathfrak{N}_c\,, \tag{56}$$

where

$$\mathfrak{N}_c = A/A_c \tag{57}$$

is the number of coherence areas present. For $\mathfrak{N}_c \gg 1$, by the central limit theorem, $\delta I_s(t)$ is Gaussian distributed, exactly analogous to the fluorescence statistics considered above. Thus the results and conclusions established above for FCS can be adapted and applied to this case as well. Whereas for FCS the critical parameter was shown to be the photocounts/molecule/correlation time, we now have $\langle n\rangle/\mathfrak{N}_c\Gamma T$, the photocounts/coherence area/correlation time, a factor independent of the number of coherence areas. Adapting Forrester's description[18] of a coherence area, we can formulate a general parameter of merit: the number of photocounts/"degree of freedom."

The same analogy can be extended to "heterodyne" light-scattering experiments as well. In this technique, the scattered field $E_s(t)$ is mixed at the detector with a large constant component of coherent reflected laser light, $E_c(t)$. The total intensity (for $A \ll A_c$) has the form

$$I_H(t) = |E_c(t) + E_s(t)|^2\,, \tag{58}$$

so that, if $|E_c|^2 \gg |E_s(t)|^2$,

$$I_H(t) \simeq |E_c|^2 + 2\,\mathrm{Re}[E_c^*(t)E_s(t)]\,. \tag{59}$$

Thus, if $E_s(t)$ is a complex Gaussian variable, $\delta I_H(t) = 2\,\mathrm{Re}[E_c^*(t)E_s(t)]$ is still another real Gaussian variable, exactly analogous to Eq. (25). The analogy is complete, with the following equivalent factors:

$$f(\tau) \rightarrow |g^{(1)}(\tau)|\,, \tag{60}$$

$$\beta \rightarrow 2\langle |E_s|^2\rangle/\langle I_H\rangle = 2\langle n_s\rangle/\langle n_H\rangle\,, \tag{61}$$

$$\langle n\rangle\beta \rightarrow 2\langle n_s\rangle\,. \tag{62}$$

Thus, the above results can also be applied to the heterodyne spectroscopy technique. Signal-to-noise calculations have been performed[21] for the heterodyne experiment, but only in the low-counting-rate ($\langle n_s\rangle \ll 1$) limit.

VIII. PRACTICAL CONSIDERATIONS

The general approach to a successful FCS experiment is to maximize $\langle n\rangle\beta$ within the limits imposed by such effects as background or "blank" fluorescence, systematic fluctuations, photolysis, and photon counting deadtimes. We can characterize the intensity of the constant background fluorescence as that of an equivalent number of fluorescent molecules, $\mathfrak{N}'$. Similarly, the systematic fluctuations, principally those from incident laser fluctuations, can be characterized by their normalized variance β'. Equation (30) now takes the form

$$\beta = \mathfrak{N}/(\mathfrak{N} + \mathfrak{N}')^2, \tag{63}$$

where $\mathfrak{N}$ again is the number of fluorescent molecules of interest. Thus

$$\langle n \rangle \beta \propto \mathfrak{N}/(\mathfrak{N} + \mathfrak{N}') \tag{64}$$

is not strictly independent of $\mathfrak{N}$, making it advantageous to have $\mathfrak{N} \gg \mathfrak{N}'$. $\mathfrak{N}$ can be adjusted by varying the concentration and/or sample volume, under the constraint that β be much greater than β'. Otherwise, the number fluctuations of interest will be obscured by the systematic effects. For a fixed $\mathfrak{N}'$, β is at its maximum value of $(4\mathfrak{N}')^{-1}$ when $\mathfrak{N}$ equals $\mathfrak{N}'$. If this isn't much greater than β', the experiment is in trouble.

$\langle n \rangle \beta$ can be increased by increasing the laser intensity, but only to a limited extent. At high intensities, laser-induced photochemistry can make measurements impossible. If $\langle n \rangle \beta \gtrsim 1$, there is no need to increase the intensity further. The ratio of the quantum efficiencies of fluorescence and photolysis determines whether a sufficiently high counting rate can be obtained.

If it is possible to do so, it is often advantageous to adjust the correlation time (Γ^{-1}) of the experiment. A chemical relaxation time can be changed, for example, by changing the sample concentration or viscosity. In a diffusion experiment one can adjust the viscosity or the laser beam diameter. What is optimal depends upon the interrelationships between the different parameters of a particular experiment. For example, if the counting rate $c \equiv \langle n \rangle / T$ and β are held constant, and T is scaled so that $\gamma \equiv \Gamma T$ remains fixed, $(S/N)^{(ii)}_{m \to 0}$ is a maximum when

$$\Gamma^2 = 2c^2\beta^2\gamma. \tag{65}$$

In a diffusion experiment,[2] however, the correlation time equivalent to Γ^{-1} is proportional to the square of the beam diameter. Therefore, if the total laser power is kept constant, the number of photocounts/molecule/correlation time is independent of the beam diameter. It is thus advantageous in this case to go to as small a beam diameter (as short a correlation time) as possible.

In the best-designed experiment, $\mathfrak{N}'$ will still have a value of many thousands. Thus even at its maximum of $(4\mathfrak{N}')^{-1}$, β is extremely small, as we have assumed throughout our calculations. This necessitates extremely high counting rates, so that detection dead times can become important. Therefore, our principal concern is how these dead times affect the form of the signal $S(\tau)$. For a constant intensity, the single interval counting distribution is normally Poisson. The presence of a dead time τ_D after each detected photon produces the following modification[22]:

$$\frac{(I)^n}{n!} e^{-I} \rightarrow \frac{(I)^n}{n!} e^{-I}[1 + n(I - n + 1)(\tau_D/T) + \theta(\tau_D/T)^2], \tag{66}$$

where T again is the width of the counting interval. Keeping first-order terms, we can extend this to the two-interval photocount probability distribution function:

$$\begin{aligned} p(n_1, n_2) = \Big\langle \frac{(I_1)^{n_1}}{n_1!} \frac{(I_2)^{n_2}}{n_2!} \exp[-(I_1 + I_2)] \\ \times \Big[1 + \Big(\frac{\tau_D}{T}\Big)[n_1(I_1 - n_1 + 1) \\ + n_2(I_2 - n_2 + 1)]\Big]\Big\rangle, \end{aligned} \tag{67}$$

where the angular brackets signify an averaging over the joint distribution $p(I_1, I_2)$. Then

$$\begin{aligned} \langle n_1, n_2 \rangle &= \sum_{n_1, n_2} p(n_1, n_2) n_1 n_2 \\ &= \langle I_1 I_2 \rangle - (\tau_D/T) \langle I_1 (I_2)^2 + I_2 (I_1)^2 \rangle, \end{aligned} \tag{68}$$

so that with

$$\begin{aligned} \langle I(0) I^2(\tau) \rangle &= \langle I(\tau) I^2(0) \rangle \\ &= \langle I \rangle^3 \{1 + \beta[1 + 2f(\tau)]\} \end{aligned} \tag{69}$$

and

$$\langle n \rangle = \langle I \rangle [1 - (\tau_D/T) \langle I \rangle (1 + \beta)], \tag{70}$$

$$S(\tau) \simeq \langle n \rangle^2 (1 - 2\tau_D \langle n \rangle / T) \beta f(\tau). \tag{71}$$

Thus, to first order, the net effect is just a decrease in the apparent value of β.

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[1] D. Magde, E. Elson, and W. W. Webb, Phys. Rev. Lett. 29, 705 (1972).

[2] E. Elson and D. Magde, Biopolymers 13, 1 (1974).

[3] D. Magde, E. Elson, and W. W. Webb, Biopolymers 13, 29 (1974).

[4] T. J. Herbert, W. W. Webb, and E. L. Elson (private

communication).
[5] D. W. Schaefer and B. J. Berne, Phys. Rev. Lett. 29, 475 (1972).
[6] D. W. Schaefer, Science 180, 1293 (1973).
[7] D. W. Schaefer, G. Banks, and S. Alpert, Nature 248, 162 (1974).
[8] D. H. Johnson and W. W. Webb (private communication).
[9] G. Feher and M. Weissman, Proc. Nat. Acad. Sci. USA 70, 870 (1973).
[10] V. Digiorgio and J. B. Lastovka, Phys. Rev. A 4, 2033 (1971).
[11] E. Jakeman, E. R. Pike, and S. Swain, J. Phys. A 4, 517 (1971).
[12] H. C. Kelly, IEEE J. Quantum Electron. 7, 541 (1971).
[13] A. J. Hughes, E. Jakeman, C. J. Oliver, and E. R. Pike, J. Phys. A 6, 1327 (1973).
[14] B. E. A. Saleh and M. F. Cardoso, J. Phys. A 6, 1897 (1973).
[15] W. B. Davenport and W. L. Root, *Random Signals and Noise* (McGraw-Hill, New York, 1958).
[16] C. D. Cantrell, Phys. Rev. A 1, 672 (1970).
[17] D. E. Koppel, J. Chem. Phys. 57, 4814 (1972).
[18] A. T. Forrester, Am. J. Phys. 24, 192 (1956).
[19] E. Jakeman, C. J. Oliver, and E. R. Pike, J. Phys. A 3, L45 (1970).
[20] D. E. Koppel, J. Appl. Phys. 42, 3216 (1971).
[21] E. Jakeman, J. Phys. A 5, 149 (1972).
[22] I. DeLotto, P. F. Manfredi, and P. Principi, Energia Nucleare 11, 557 (1964).