# THEORY OF SAMPLE TRANSLATION IN FLUORESCENCE CORRELATION SPECTROSCOPY

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ABSTRACT New applications of the technique of fluorescence correlation spectroscopy (FCS) require lateral translation of the sample through a focused laser beam (Peterson, N. O., D. C. Johnson, and M. J. Schlesinger, 1986, *Biophys. J.*, 49:817–820). Here, the effect of sample translation on the shape of the FCS autocorrelation function is examined in general. It is found that if the lateral diffusion coefficients of the fluorescent species obey certain conditions, then the FCS autocorrelation function is a simple product of one function that depends only on transport coefficients and another function that depends only on the rate constants of chemical reactions that occur in the sample. This simple form should allow manageable data analyses in new FCS experiments that involve sample translation.

## INTRODUCTION

Fluorescence correlation spectroscopy (FCS) is a technique for measuring chemical reaction rates, diffusion coefficients, and flow rates of fluorescent or fluorescent-labeled molecules in a system at equilibrium (Elson, 1985; Elson and Magde, 1974; Magde et al., 1974). In FCS, an open volume containing a small number of fluorescent molecules is illuminated with a focused laser beam. The measured fluorescence fluctuates as molecules move through the illuminated volume and/or undergo transitions between states with different absorptivities, fluorescence quantum efficiencies, or fluorescence collection efficiencies. Rates of transport and chemical reaction are obtained from the time autocorrelation function of the fluctuations in fluorescence. In particular, FCS has been applied to the kinetics of binding of the dye ethidium bromide to DNA (Magde et al., 1974; Icenogle and Elson, 1983*a*,*b*; Magde et al., 1972; Sorscher et al., 1980), to the motion of myosin fragments during actin-activated ATPase (Borejdo, 1979), to the assumption of different orientations by myosin subfragment 1 in contracting muscle fibers (Borejdo et al., 1979), to fluorescence immunoassays (Briggs et al., 1981; Nicoli et al., 1980), to immunoglobulin surface-binding kinetics (Thompson and Axelrod, 1983), to the diffusion of 3,3′-dioctadecylindocarbocyanine iodide in water-ethyl alcohol solutions and in black lipid membranes (Koppel et al., 1976), to the determination of the molecular weights of DNA molecules (Weissman et al., 1976), and to the measurement of the sizes of focused laser beams (Sorscher and Klein, 1980).

In one of the promising new applications of FCS, called "Scanning FCS" (Peterson, 1986; Peterson et al., 1986), a laser beam is focused to a small spot on a membrane that contains or has bound fluorescent-labeled molecules in aggregates. As the membrane is translated laterally through the focused laser beam, the measured fluorescence fluctuates as different aggregate-containing regions are illuminated. The extrapolated time-zero value of the temporal autocorrelation of fluorescence fluctuations is related to the distribution of aggregate sizes in the membrane. This new application of FCS has recently been used to examine virus glycoprotein aggregation on cell surfaces (Peterson et al., 1986).

In addition, sample translation is sometimes necessary in an FCS experiment, even if molecular aggregates are not under study. For example, the signal-to-noise ratio of an FCS autocorrelation function increases with the number of fluoresced photons collected per correlation time per molecule (Koppel, 1974). For many samples, particularly those in which lateral diffusion is slow, the incident light intensity that would be required to compute an acceptably accurate autocorrelation function in an experimentally reasonable time photobleaches the fluorescent molecules during data collection. One method of avoiding this difficulty is to translate the sample, the exciting laser beam, or the observation aperture, during data collection. In this case, the theoretical interpretation of the autocorrelation function must include the additional effect of sample translation on the fluorescence fluctuations.

Experimental FCS studies have relied on theoretical predictions of the form of the autocorrelation function in the presence of transport and chemical reaction (Elson, 1985; Elson and Magde, 1974; Aragón and Pecora, 1976;

Magde, 1977; Thompson et al., 1981; Thompson, 1982). General theoretical expressions for the form of the fluorescence fluctuation autocorrelation function for samples containing molecules that are undergoing lateral diffusion, rotational diffusion, and reaction have been derived (Elson, 1985; Aragón and Pecora, 1976). However, the form of the FCS autocorrelation function in the presence of uniform translation has been examined in detail only when coupled with lateral diffusion (Magde et al., 1978), or a two-state isomerization (Elson, 1986), but not with a general chemical reaction. In this paper, we consider this latter most general case. As shown earlier, in general (Elson, 1985; Elson and Magde, 1974; Aragón and Pecora, 1976), transport and reaction terms are coupled and the autocorrelation function has a complicated dependence on the eigenvalues and eigenfunctions of a matrix whose elements depend on the reaction rates and transport coefficients. However, we show here that if (*a*) diffusion is slow, or (*b*) the diffusion coefficients of all fluorescent species are equal, then the autocorrelation function quite simply separates into a known transport factor that depends on the flow rate and the diffusion coefficient, and another factor that depends only on the reaction rate constants.

## CONCENTRATION CORRELATION FUNCTIONS

The autocorrelation function of temporal fluctuations in fluorescence is determined by the correlation functions of concentration fluctuations in the $i$th chemical species at positions $\mathbf{r}$ with concentration fluctuations in the $j$th chemical species at positions $\mathbf{r}'$ and times $t$ earlier, denoted by (Elson and Magde, 1974):

$$f_{ij}(\mathbf{r}, \mathbf{r}', t) = \langle \delta C_i(\mathbf{r}, t) \delta C_j(\mathbf{r}', 0) \rangle, \quad i, j = 1 \text{ to } N. \tag{1}$$

In Eq. 1, $\delta C_i(\mathbf{r}, t)$ is the temporal fluctuation of the concentration $C_i(\mathbf{r}, t)$ of molecules in the $i$th state at position $\mathbf{r}$ and time $t$ from its average value $\langle C_i \rangle$, or

$$\delta C_i(\mathbf{r}, t) = C_i(\mathbf{r}, t) - \langle C_i \rangle. \tag{2}$$

$N$ is the number of chemical species present, $\langle\ \rangle$ denotes an ensemble average, and we consider two-dimensional samples such that $\mathbf{r} = x\hat{\mathbf{i}} + y\hat{\mathbf{j}}$. In the presence of translation or diffusion an open volume is observed. This means that concentration fluctuations are correlated at the same time only between identical species and at the same position (Elson and Magde, 1974), so that

$$f_{ij}(\mathbf{r}, \mathbf{r}', 0) = \langle C_i \rangle \, \delta(\mathbf{r} - \mathbf{r}') \delta_{ij}, \tag{3}$$

where $\delta(\ )$ is a Dirac delta function and $\delta_{ij}$ is the Kronecker delta.

The concentration of molecules in the $i$th state at position $\mathbf{r}$ and time $t$ is determined by the following equation (Elson, 1985):

$$\frac{\partial}{\partial t} C_i(\mathbf{r}, t) = -V \frac{\partial}{\partial y} C_i(\mathbf{r}, t) + \sum_{j=1}^{N} T_{ij} C_j(\mathbf{r}, t) + D_i \nabla^2 C_i(\mathbf{r}, t), \tag{4}$$

where $V$ is the rate of translation (occurring along the y-axis), $T_{ij}$ are kinetic coefficients, $D_i$ is the diffusion coefficient of a molecule in the $i$th state, and we have assumed linearized chemical kinetic terms because the concentration fluctuations are small (Elson, 1985).[1] At equilibrium, the concentration of each species is constant, so that

$$\sum_{j=1}^{N} T_{ij} \langle C_j \rangle = 0, \quad i = 1, N. \tag{5}$$

Using Eqs. 1, 2, and 5 in Eq. 4, one finds that (Elson, 1985)

$$\frac{\partial}{\partial t} \phi_{ik} = \sqrt{-1} q_y V \phi_{ik} + \sum_{j=1}^{N} T_{ij} \phi_{jk} - q^2 D_i \phi_{ik}, \tag{6}$$

where the variable $\mathbf{r}$ has been Fourier transformed to a variable $\mathbf{q}$, and the transform of $f_{ij}(\mathbf{r}, \mathbf{r}', t)$ is denoted by $\phi_{ij}(\mathbf{q}, \mathbf{r}', t)$. The initial conditions for Eq. 6 are the Fourier transforms of the initial conditions in Eq. 3.

If $D_i = D$ for all values of $i$, including the special case of $D = 0$, then the function $\phi_{ik}$ separates quite simply into a product of one function $S_{ik}(t)$ that depends on the values of $T_{ik}$ but not on $D$ or $V$, and another function that depends only on $D$ and $V$ but not on the $T_{ik}$. In particular, the solutions to Eq. 6 with the proper initial conditions are

$$\phi_{ik} = \alpha(\mathbf{q}, \mathbf{r}', t) S_{ik}(t), \tag{7}$$

where

$$\alpha(\mathbf{q}, \mathbf{r}', t) = \exp\left[i\mathbf{q} \cdot (\mathbf{r}' + \hat{\mathbf{j}} V t) - q^2 D t\right]/2\pi, \tag{8}$$

and the $S_{ik}(t)$ are the solutions to the following set of equations and initial conditions:

$$S_{ik}(t) = \sum_{j=1}^{N} T_{ij} S_{jk}(t),$$
$$S_{ik}(0) = \langle C_i \rangle \delta_{ik}. \tag{9}$$

Inverse transforming Eq. 7 gives

$$f_{ij}(\mathbf{r}, \mathbf{r}', t) = A(\mathbf{r}, \mathbf{r}', t) S_{ij}(t), \tag{10}$$

[1] By starting with Eq. 4, we have not considered the effect of translational (Thompson and Burghardt, 1985) or rotational (Shoup et al., 1981) diffusion on the chemical kinetic rates and/or rate equation. Similarly, we have not considered processes such as coupled bulk diffusion, association/dissociation at sites on a surface, and surface diffusion (Thompson et al., 1981).

where

$$A(\mathbf{r}, \mathbf{r}', t) = \exp\left[-|\mathbf{r} - \mathbf{r}' - \hat{\mathbf{j}}Vt|^2/(4Dt)\right]/(4\pi Dt). \quad (11)$$

In the limit where $D$ approaches zero,

$$f_{ij}(\mathbf{r}, \mathbf{r}', t) \rightarrow \delta(x - x')\,\delta(y - y' - Vt)\,S_{ij}(t). \quad (12)$$

## FLUORESCENCE FLUCTUATION AUTOCORRELATION FUNCTION

The normalized autocorrelation function of fluorescence fluctuations $G(t)$, for a planar sample, is given by (Elson and Magde, 1974)

$$G(t) = \frac{\sum_{i,j=1}^{N} Q_i Q_j \iint I(\mathbf{r})\, I(\mathbf{r}')\, f_{ij}(\mathbf{r}, \mathbf{r}', t)\, \mathrm{d}^2r\, \mathrm{d}^2r'}{\left[a \sum_{i=1}^{N} Q_i \langle C_i \rangle\right]^2}, \quad (13)$$

where the $Q_i$ are dimensionless constants that are proportional to the products of the absorptivities, quantum efficiencies, and experimental fluorescence collection efficiencies of molecules in the $i$th states; $I(\mathbf{r})$ is proportional to the intensity profile of the exciting light and the transmission function of the observation aperture; the integrals are over all two-dimensional space; and

$$a = \int I(\mathbf{r})\, \mathrm{d}^2r. \quad (14)$$

Using Eqs. 3 and 10 in Eq. 13, we find that $G(t)$ is given by

$$G(t) = G(0)\, X(t)\, H(t), \quad (15)$$

where

$$X(t) = \sum_{i,j=1}^{N} Q_i\, Q_j\, S_{ij}(t) \Big/ \sum_{i=1}^{N} Q_i^2 \langle C_i \rangle, \quad (16)$$

$$H(t) = (1/b) \iint I(\mathbf{r})\, I(\mathbf{r}')\, A(\mathbf{r}, \mathbf{r}', t)\, \mathrm{d}^2r\, \mathrm{d}^2r', \quad (17)$$

$$b = \int I^2(\mathbf{r})\, \mathrm{d}^2r, \quad (18)$$

and

$$G(0) = \frac{b \sum_{i=1}^{N} Q_i^2 \langle C_i \rangle}{\left[a \sum_{i=1}^{N} Q_i \langle C_i \rangle\right]^2}. \quad (19)$$

Thus, the autocorrelation function is a simple product of two functions, $X(t)$ and $H(t)$, normalized so that $X(0) = H(0) = 1$. $X(t)$ depends only on the kinetics of transition between the different molecular states. $H(t)$ has been calculated previously; it depends only on the illumination/observation profile $I(\mathbf{r})$, the diffusion coefficient $D$, and the translation rate $V$. For a Gaussian-shaped illumination profile of $1/e^2$-radius $s$ and a planar sample (Magde et al., 1978),

$$H(t) = [1 + (t/\tau_d)]^{-1} \exp\left[-(t/\tau_t)^2/(1 + t/\tau_d)\right], \quad (20)$$

where $\tau_d = s^2/4D$ and $\tau_t = s/V$.

The result in Eq. 15 means that, if certain constraints on the $D_i$ are satisfied, then (*a*) the autocorrelation function can readily be known for any reaction mechanism, by solving Eqs. 9 and inserting them into Eq. 16; and (*b*) the characteristic decay times of $H(t)$ due to translation, $\tau_t$, and diffusion, $\tau_d$, must be greater than the characteristic decay times of $X(t)$ for the reaction rates $T_{ij}$ to be measurable.

## SOLUTION FOR A TWO-STATE ISOMERIZATION

The simplest kinetic scheme is a two-state isomerization:

$$C_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C_2. \quad (21)$$

In this scheme, the T-matrix is given by

$$\mathrm{T} = \begin{pmatrix} -k_1 & k_2 \\ k_1 & -k_2 \end{pmatrix} \quad (22)$$

and the solutions to Eqs. 9 are

$$\begin{aligned} S_{11} &= \langle C_1 \rangle [k_2 + k_1 \exp(-Rt)]/R \\ S_{12} &= \langle C_2 \rangle k_2 [1 - \exp(-Rt)]/R \\ S_{21} &= \langle C_1 \rangle k_1 [1 - \exp(-Rt)]/R \\ S_{22} &= \langle C_2 \rangle [k_1 + k_2 \exp(-Rt)]/R, \end{aligned} \quad (23)$$

where $R = k_1 + k_2$.

The FCS autocorrelation function is found by using Eqs. 23 in Eq. 16 with $N = 2$. We find that

$$\begin{aligned} X(t) = {} & [R(Q_1^2\langle C_1 \rangle + Q_2^2\langle C_1 \rangle)]^{-1} \\ & \cdot [Q_1^2\langle C_1 \rangle k_2 + Q_2^2\langle C_2 \rangle k_1 \\ & + Q_1 Q_2(\langle C_1 \rangle k_1 + \langle C_2 \rangle k_2) \\ & + [Q_1^2\langle C_1 \rangle k_1 + Q_2^2\langle C_2 \rangle k_2 \\ & - Q_1 Q_2(\langle C_1 \rangle k_1 + \langle C_2 \rangle k_2)] \exp(-Rt)] \end{aligned} \quad (24)$$

and

$$G(0) = \frac{b[Q_1^2\langle C_1 \rangle + Q_2^2\langle C_2 \rangle]}{a^2[Q_1\langle C_1 \rangle + Q_2\langle C_2 \rangle]^2}. \quad (25)$$

Examination of Eq. 24 shows that $X(0) = 1$, as it was defined, but that $X(t)$ does not approach zero when $t$ approaches $\infty$, as one might expect. If either $D$ or $V$ is unequal to zero, $H(t)$ does approach zero as $t$ approaches $\infty$ and consequently the correlation function $G(t)$ approaches zero as expected. If $V$ and $D$ both approach zero, the decay of $H(t)$ becomes much slower than that of

$X(t)$ and the decay time will exceed the experimental correlation time. In this case, an open volume is not observed, Eq. 3 does not hold, and the results of the previous sections are not applicable.

## EFFECTS OF ROTATIONAL DIFFUSION

The autocorrelation function for a system undergoing reaction, lateral diffusion, and rotational diffusion has been derived previously by Aragón and Pecora, 1976. Their results can be extended to include translation by adding a flow term to their rate equation. This changes the definition of the matrix whose eigenvalues and eigenvectors determine the autocorrelation function.

In general the rotational terms couple to the other processes and the autocorrelation function does not separate; however, two sets of conditions exist which lead to simplification. For many systems, rotational diffusion is much faster than the other processes being considered. In such cases, the rotational terms in the autocorrelation function decay much faster than the remaining terms and the effect of rotation can be ignored on a slower time scale. The resultant autocorrelation function is then determined by lateral diffusion, translation, and reaction only, and the results of the above sections apply.

If rotational diffusion is not negligible on the time scale of interest, the autocorrelation function still separates if the rotational diffusion coefficients are the same for each species present, in addition to the limitations on lateral diffusion coefficients discussed above. In this case an additional multiplicative factor due to rotation appears in the autocorrelation function.

Both of these simplifying cases have been discussed in more detail elsewhere (Aragón and Pecora, 1976). It is noted that the autocorrelation function for the isomerization reaction considered by Aragón and Pecora can be altered to include translation by replacing $H_A(t)$ in their Eq. 6.11 with $H(t)$ from our Eq. 20.

## DISCUSSION

We have theoretically obtained certain conditions under which, in the presence of sample translation, an FCS autocorrelation function separates into a simple product of one function that depends only on transport coefficients and another function that depends only on chemical kinetic rate constants. We have found, first, that if rotational diffusion is fast and the processes giving rise to fluorescence fluctuations on a slower time scale are sample translation, translational diffusion, and chemical reaction, then, if the translational diffusion coefficients of all fluorescent species are equal, the FCS autocorrelation function assumes a simple separated form. Although the restriction that the translational diffusion coefficients of the fluorescent species are equal rules out some interesting systems (e.g., aggregation reactions or the binding of a small ligand to a large multivalent substrate), it does include two rather general special cases: (*a*) systems in which only one species is fluorescent or in which the fluorescence of all species but one is negligible (e.g., single-site ethidium bromide binding to DNA); and (*b*) systems in which there is no translational diffusion (e.g., a contracting skeletal muscle fiber in which different chemical "states" refer to different orientational distributions of fluorescent-labeled myosin [Borejdo et al., 1979; Burghardt et al., 1983] or a sample in which nondiffusing elements undergo isomerization reactions). We have found, second, that if rotational diffusion is not negligible on the time scale of interest, the autocorrelation function still assumes the simple separated form if the rotational diffusion coefficients are all equal and the translational diffusion coefficients are all equal. The simple separated form for the FCS autocorrelation function should allow manageable data analyses in some FCS experiments that involve sample translation.

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