

Surface Binding Rates of Nonfluorescent Molecules May Be Obtained by Total Internal Reflection with Fluorescence Correlation Spectroscopy

Dear Sir:

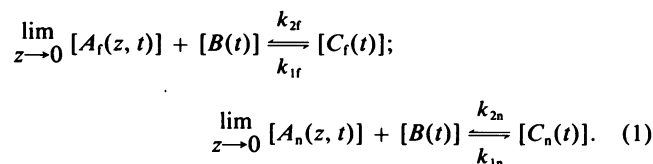
The combination of total internal reflection and fluorescence correlation spectroscopy (TIR/FCS) as a technique for measuring the binding and unbinding rates of solute molecules at a surface has recently been described (Thompson et al., 1981). In TIR/FCS, fluorescent-labeled molecules are in dynamic equilibrium between bulk-solubilized and surface-bound states. The molecules fluoresce only inside a thin layer next to the surface, which is illuminated by the evanescent wave of a totally internally reflected laser beam. The fluorescence originating from a small area of the liquid/solid interface, defined by an image plane aperture of a fluorescence microscope, fluctuates in time as individual molecules enter and leave this area through surface binding and unbinding. Under appropriate conditions, the experimental autocorrelation function of the fluorescence fluctuations is a function of the surface reaction on and off rates. TIR/FCS has already been used to study the nonspecific adsorption of rhodamine-labeled immunoglobulins on quartz (Thompson and Axelrod, 1981). The technique is potentially capable of studying the binding of solute molecules not only to bare quartz, but also to a large variety of surface coatings such as polymers, phospholipids, and specific receptor molecules or ligands.

It would appear that the technique is limited to those solute molecules that either autofluoresce or are labeled with a fluorescent dye. This letter suggests a method whereby TIR/FCS can be employed to measure the adsorption rates of nonfluorescent solute molecules. In this more complicated version of TIR/FCS, nonfluorescent solute molecules and a fluorescent analogue compete for binding at the same surface sites, and the entire process is in chemical and thermodynamic equilibrium. The size and shape of the autocorrelation function of fluorescence fluctuations arising from the binding and unbinding of fluorescent molecules will be determined by the on and off rates of both the nonfluorescent and fluorescent species. If the surface reaction rates of the fluorescent species are known from TIR/FCS experiments performed in the absence of the nonfluorescent species, then the surface reaction rates of the nonfluorescent species can be measured from TIR/FCS autocorrelation functions obtained while both species compete for the surface sites.

The theory outlined in this letter also suggests an experimental method whereby TIR/FCS on systems with only one chemical species in solution can be made more widely applicable. Usually, a high solute concentration will insure that the autocorrelation function depends only on the surface kinetic rates and not the bulk diffusion coefficient (Thompson et al., 1981; p. 449). Unfortunately, high solute concentrations also nearly saturate the surface sites so that the relative size of spontaneous fluctuations in the number of occupied sites (and hence the relative size of the fluorescence fluctuations) is small. These two requirements narrow the range of applicability for TIR/FCS. However, if the solute concentration is high but only a small fraction of the molecules are actually fluorescent-labeled, the problem can be

overcome. The following theory for competition between fluorescent and nonfluorescent species applies to a partially fluorescent-labeled population of solute molecules.

In both cases, fluorescent and nonfluorescent molecules of concentrations A_f and A_n freely diffuse in solution and react with surface sites of concentration B to form fluorescent and nonfluorescent complexes of concentrations C_f and C_n . Coordinate z is the perpendicular distance from the surface to a point in solution, t is the time, k_{1f} and k_{2f} are the on and off rates of the fluorescent molecules, and k_{1n} and k_{2n} are the on and off rates of the nonfluorescent molecules. Throughout, $\delta X \equiv X - \langle X \rangle$ denotes the fluctuation from equilibrium value $\langle X \rangle$, for X equal to A_f , A_n , B , C_f , C_n or measured fluorescence F . Brackets $\langle \rangle$ denote a thermodynamic ensemble average. The process is represented by the following equations:



The equilibrium constants of the coupled reactions are

$$\kappa_f = k_{1f}/k_{2f} = \langle C_f \rangle / \langle A_f \rangle \langle B \rangle;$$

$$\kappa_n = k_{1n}/k_{2n} = \langle C_n \rangle / \langle A_n \rangle \langle B \rangle. \quad (2)$$

The measured fluorescence $F(t)$ is proportional to the surface concentration of the fluorescent molecules $C_f(t)$. The autocorrelation $G(t)$ of concentration fluctuations in C_f and in fluorescence F , and the cross-correlation $H(t)$ of concentration fluctuations in C_n and C_f are normalized as

$$G(t) \equiv \langle \delta C_f(t) \delta C_f(0) \rangle / \langle C_f \rangle^2 = \langle \delta F(t) \delta F(0) \rangle / \langle F \rangle^2$$

$$H(t) \equiv \langle \delta C_n(t) \delta C_f(0) \rangle / \langle C_f \rangle^2. \quad (3)$$

The fluorescent and nonfluorescent surface concentrations are determined by the following coupled differential equations:

$$dC_f/dt = k_{1f}B \left[\lim_{z \rightarrow 0} A_f \right] - k_{2f}C_f;$$

$$dC_n/dt = k_{1n}B \left[\lim_{z \rightarrow 0} A_n \right] - k_{2n}C_n. \quad (4)$$

The concentrations in Eqs. 4 can be rewritten in terms of the equilibrium concentration values and fluctuations from these values. The total concentration of surface sites (bound and unbound) remains exactly constant during an experiment, such that $\delta B = -\delta C_f - \delta C_n$. After applying the equilibrium relationships Eq. 2 and eliminating terms proportional to the product of two fluctuations, the resulting equation is multiplied by $\delta C_f(0)$, a thermodynamic ensemble average is taken, and the result is divided by $\langle C_f \rangle^2$.

For high enough bulk concentrations and fast enough bulk diffusion, a fluctuation in surface concentration rapidly dissipates away from the surface immediately after desorption. This is a special (but experimentally accessible) case called the "reaction limit" (see Thompson et al., 1981) in which the equation solutions do not depend on the bulk diffusion coefficient. In this case, cross-correlations of concentration fluctuations δA_f and δA_n with fluctuations δC_f on the surface persist only for times much less than the characteristic autocorrelation time of fluorescence fluctuations arising from fluctuations in surface concentration. Therefore, for time scales of interest,

$$\lim_{z \rightarrow 0} [\langle \delta A_f(z, t) \delta C_f(0) \rangle] = \lim_{z \rightarrow 0} [\langle \delta A_n(z, t) \delta C_f(0) \rangle] = 0. \quad (5)$$

With these considerations, Eqs. 4 lead to

$$\begin{aligned} dG/dt &= -[k_{1f}\langle A_f \rangle + k_{2f}]G - [k_{1f}\langle A_f \rangle]H \\ dH/dt &= -[k_{1n}\langle A_n \rangle + k_{2n}]H - [k_{1n}\langle A_n \rangle]G. \end{aligned} \quad (6)$$

Assuming a constant total number of surface binding sites N [including fluorescent complexes $N_f(t)$, nonfluorescent complexes $N_n(t)$, and unoccupied sites $N_b(t)$], we define β as the average fraction of sites which are unbound at equilibrium and γ as the average fraction of bound sites which are fluorescent at equilibrium, so that

$$\begin{aligned} \beta &= \langle N_b \rangle / [\langle N_b \rangle + \langle N_f \rangle + \langle N_n \rangle] \\ \gamma &= \langle N_f \rangle / [\langle N_f \rangle + \langle N_n \rangle]. \end{aligned} \quad (7)$$

The mean number of fluorescent complexes $\langle N_f \rangle$, the mean square fluctuation in the number of sites which are occupied by fluorescent solute molecules $\langle \delta N_f^2 \rangle$, and the correlation between number fluctuations in the nonfluorescent and fluorescent surface-bound molecules at the same time $\langle \delta N_n \delta N_f \rangle$ are (Feher and Weissman, 1973)

$$\begin{aligned} \langle N_f \rangle &= \gamma(1 - \beta)N \\ \langle \delta N_f^2 \rangle &= \langle N_f \rangle [1 - \langle N_f \rangle / N] = N\gamma(1 - \beta) [1 - \gamma(1 - \beta)] \\ \langle \delta N_n \delta N_f \rangle &= [\langle (\delta N_f + \delta N_n)^2 \rangle - \langle \delta N_f^2 \rangle - \langle \delta N_n^2 \rangle] / 2 \\ &= -N\gamma(1 - \gamma)(1 - \beta)^2. \end{aligned} \quad (8)$$

The initial conditions of Eqs. 6 are thus

$$\begin{aligned} G(0) &= \langle \delta N_f^2 \rangle / \langle N_f \rangle^2 = \frac{1}{N} \left[\frac{1}{\gamma(1 - \beta)} - 1 \right] \\ H(0) &= \langle \delta N_n \delta N_f \rangle / \langle N_f \rangle^2 = \frac{-1}{N} \left[\frac{1 - \gamma}{\gamma} \right]. \end{aligned} \quad (9)$$

The solution for $G(t)$ from Eqs. 6 with initial conditions Eqs. 9 is:

$$\begin{aligned} \frac{G(t)}{G(0)} &= [(1 + c) \exp(at/2) \\ &+ (1 - c) \exp(-at/2)] [\exp(-bt/2)] / 2 \end{aligned} \quad (10)$$

where

$$\begin{aligned} a &= [k_{1f}\langle A_f \rangle - k_{1n}\langle A_n \rangle + k_{2f} - k_{2n}]^2 \\ &+ 4k_{1f}\langle A_f \rangle k_{1n}\langle A_n \rangle]^{1/2} \\ b &= k_{1f}\langle A_f \rangle + k_{1n}\langle A_n \rangle + k_{2f} + k_{2n} \\ c &= [k_{1n}\langle A_n \rangle - (2\epsilon + 1)k_{1f}\langle A_f \rangle \\ &+ k_{2n} - k_{2f}] / a \\ \epsilon &= H(0)/G(0) = -(1 - \gamma)(1 - \beta) / [1 - \gamma(1 - \beta)]. \end{aligned} \quad (11)$$

When $\langle A_n \rangle = 0$ ($\gamma = 1$), $G(t)$ in Eq. 10 reduces to the usual form for TIR/FCS, $G(t) \rightarrow [\beta/N(1 - \beta)] \exp(-k_{2f}t/\beta)$.

If the bulk concentrations are low enough so that $\kappa_f\langle A_f \rangle \approx \kappa_n\langle A_n \rangle \approx 0$, then the decay rate of $G(t)$ is k_{2f} . Under these conditions, only a small percentage of the surface sites are occupied, the activities of the two molecular types are uncoupled, and the shape of the autocorrelation function does not depend on k_{1n} or k_{2n} . If, on the other hand, the bulk concentrations are high enough to nearly saturate the surface sites, such that $k_{1f}\langle A_f \rangle$, $k_{1n}\langle A_n \rangle \gg k_{2f}$, k_{2n} , $\gamma \neq 1$, and $\gamma \neq 0$, then

$$\begin{aligned} G(t) &\rightarrow \frac{1}{N} \left[\frac{1 - \gamma}{\gamma} \right] \exp \left[- \frac{k_{1f}\langle A_f \rangle k_{2n} + k_{1n}\langle A_n \rangle k_{2f}}{k_{1f}\langle A_f \rangle + k_{1n}\langle A_n \rangle} t \right] \\ &= \frac{1}{N} \left[\frac{1 - \gamma}{\gamma} \right] \exp \left[-t \left/ \left(\frac{\gamma}{k_{2n}} + \frac{1 - \gamma}{k_{2f}} \right) \right. \right]. \end{aligned} \quad (12)$$

Given the values of k_{1f} and k_{2f} from previous TIR/FCS experiments on the same surface but with no nonfluorescent competitor, and autocorrelation functions for at least two sets of bulk concentrations $\langle A_f \rangle$ and $\langle A_n \rangle$, then the values of k_{1n} and k_{2n} can be obtained using Eq. 12. This means that the kinetic rates of a nonfluorescent species can be measured by observing the fluctuations due to a competing fluorescent species.

If the fluorescent and nonfluorescent species are identical except that the former is conjugated to a small unobtrusive dye, then the kinetic rates of the two binding processes should be equal ($k_{1f} = k_{1n} \equiv k_1$, $k_{2f} = k_{2n} \equiv k_2$), and $G(t)$ in Eq. 10 reduces to

$$\begin{aligned} G(t) &\rightarrow \frac{1}{N} \left[\frac{1}{\gamma(1 - \beta)} - 1 \right] [(1 - \gamma - \epsilon\gamma) \\ &+ (\gamma + \epsilon\gamma)] \exp[-k_1(\langle A_f \rangle + \langle A_n \rangle)t] \exp(-k_2t). \end{aligned} \quad (13)$$

Thus, mixing labeled and unlabeled solute while keeping β constant does not interfere with the capability of TIR/FCS to measure k_1 and k_2 . $G(0)$ is roughly the square of the fraction with which the signal $F(t)$ fluctuates due to the surface dynamics of the fluorescent molecules, and must be larger than the square of the fraction with which $F(t)$ fluctuates due to other noise sources (such as uncorrected instabilities in the intensity of the incident laser beam or local vibrations of the experimental apparatus). In our laboratory, a $G(0)$ value as large as 0.001 is sufficient. By reducing γ (i.e., decreasing the ratio of fluorescent to nonfluorescent solute), $G(0)$ can be made quite large, even for a small β (see Eq. 9). The competition approach thereby makes feasible experiments performed near surface saturation. The failure of a mixture to behave according to Eq. 13 indicates that labeled solute binds and unbinds with kinetic rates different than those of the unlabeled solute.

The original description of TIR/FCS also introduces a companion technique, total internal reflection with fluorescence photobleaching recovery (TIR/FPR) (Thompson et al., 1981; Burghardt and Axelrod, 1981). A TIR/FPR recovery curve obtained during fluorescent/nonfluorescent competition reveals information only about the equilibrium constant of the nonfluorescent species rather than its kinetic rates.

TIR/FCS measures the kinetics of fluorescent-labeled solubilized biomolecules which are associating with and dissociating from a two-dimensional target, while requiring no extrinsic perturbation from chemical or thermodynamic equilibrium and no spectroscopic change between the free and adsorbed states. Under the conditions described in this letter, the solute molecules need not even be fluorescent-labeled, as long as a fluorescent competitor exists. Virtually nothing inherent to the technique need interfere with the process under study, and the major limitations are in the details of the biochemistry of a particular system and in the rate of data collection and analysis.

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