SIMPLE COMPUTER METHOD FOR EVALUATION OF LATERAL DIFFUSION COEFFICIENTS FROM FLUORESCENCE PHOTOBLEACHING RECOVERY KINETICS

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ABSTRACT A method is presented for the analysis of fluorescence photobleaching recovery curves. Based on the simplified kinetic expression of Yguerabide, J., J. A. Schmidt, and E. E. Yguerabide (1982, *Biophys. J.*, 40:69–75), a linearization procedure is described that permits unequivocal determination of all diffusion parameters. The presence of additional membrane flow or multiple diffusion coefficients can easily be detected by this method, and simple corrections for the presence of these alternative recovery processes can be made by the use of a regular mini-computer. The validity of the method is tested on simulated recovery curves, varying the contribution of flow, multiple diffusion coefficients, and statistical noise due to counting error.

Fluorescence photobleaching recovery (FPR) is a powerful technique for measuring lateral diffusion coefficients of lipids and proteins in membranes (see references 1–4 for recent reviews). In the original experimental arrangement that is still the most commonly used for studies on single cells, an attenuated laser beam is focused on a small surface area uniformly labeled with a fluorescent probe, and the fluorescence intensity excited by the beam is monitored as a function of time. After a short 1,000- to 10,000-fold increase in excitation intensity, which results in an irreversible bleaching of fluorophores in the illuminated area, the fluorescence intensity is monitored again with the attenuated beam, and the possible recovery of fluorescence intensity with time is measured in the bleached area. Recovery results either from lateral diffusion of fluorescent molecules or from membrane flow, or from a combination of these two processes (5). In the case of a Gaussian laser beam profile with diffusion occurring from an infinite two-dimensional plane, the recovery of fluorescence intensity with time is given by the following (see reference 6) theoretical equation:

$$f_t = \sum_{j=1}^{m} \phi_j \left(\sum_{n=0}^{\infty} \cdot \frac{(-K)^n \cdot \exp\{-2(t/\tau_F)^2 \cdot n/[1 + n(1 + 2t/\tau_{D,j})]\}}{n![1 + n(1 + 2t/\tau_{D,j})]} \right) \quad (1)$$

in which f_t represents the fluorescence intensity as a function of the time t after photobleaching, relative to the fluorescence intensity before bleaching. K is a bleaching parameter, related to the relative fluorescence intensity immediately after bleaching according to $f_0 = [1 - \exp(-K)]/K$; τ_F is the characteristic time for uniform flow, while ϕ_j is the fraction of fluorophores that undergoes lateral diffusion with a characteristic diffusion time $\tau_{D,j}$. The corresponding lateral diffusion coefficient is given by $D_j = \omega^2/4\tau_{D,j}$ and the rate of uniform flow by $V_0 = \omega/\tau_F$, in which ω is the half-width at e^{-2} height of the Gaussian laser beam at its point of focus on the membrane. In the above equation is has been assumed that the various fractions of fluorescent molecules have similar bleaching characteristics. In the case when $\tau_{D,2,\ldots,m} \gg \tau_{D,1}$, ϕ_1 (or simply ϕ) can be considered as the mobile fraction, and $1 - \phi$ as the immobile fraction of fluorescent molecules in the membrane. In the absence of flow this mobile fraction is given by $\phi = (f_\infty - f_0)/(1 - f_0)$, in which f_∞ represents the relative fluorescence intensity following full recovery after long times.

Three methods are presently available to determine the lateral diffusion coefficient and mobile fraction of a major diffusing component from the kinetics of fluorescence recovery after photobleaching. In the case of a single diffusing component ($m = 1$) and the absence of flow, the recovery curve can be fitted to the parameters K, ϕ, and τ_D according to Eq. 1, by means of multiple regression analysis. This analysis requires much computer time,

however, especially when a regular mini-computer is used. Much more time is needed when an analysis is required for the presence of multiple diffusion coefficients and/or flow, and, in addition, this analysis tends to become ambiguous because of the many variables involved.

Frequently a graphical method is used, in which the values of f_0 and f_∞ are estimated from the recovery curves, and subsequently the half time of recovery $t_{1/2}$, the time required for the fluorescence to reach a value $(f_\infty + f_0)/2$, is determined graphically. According to theoretical considerations $t_{1/2} = \gamma_D \cdot \tau_D$ when recovery merely results from a single diffusing component, and $t_{1/2} = \gamma_F \cdot \tau_F$, in the case of only uniform flow, in which γ_D and γ_F are known functions of K (6). However, this method suffers from a considerable number of drawbacks. First, the value of f_∞ will only be reached after infinite time and is, therefore, generally determined empirically, extrapolating by eye to an asymptotic value, which can easily be obscured by experimental fluctuations and cell movements. Second, the exact value of f_0 is generally also obscured due to the settling time of the detecting system immediately after bleaching. The length of this time period, the delay time, is generally known, but extrapolation of the recovery curve to zero time is hampered by the steepness of this curve in its initial phase. Third, the value of $t_{1/2}$ is obtained from a single point on the curve, and can, therefore, be strongly affected by local fluctuations in the recovery curve. Finally, this method does not correct the recovery kinetics of a major diffusing component for the presence of other diffusing components or flow, and can therefore give rise to considerable errors.

An important contribution towards solving these problems has come from the observation of Yguerabide et al. (7) that in the case of a single diffusion coefficient and the absence of flow the recovery curve for a Gaussian beam profile can be described to a very good approximation by the equation

$$f_t = \frac{f_0 + f_\infty (t/t_{1/2})}{1 + (t/t_{1/2})}. \tag{2}$$

We have confirmed the observation of Yguerabide et al. (7), that this equation describes the recovery curve with an accuracy better than 99% at all points of the curve for bleaching values up to 70%, and better than 99.5% for bleaching up to 60%. This relatively simple equation provides the possibility of linearizing FPR data, which will permit determination of the parameters from all points on the curve. Moreover, deviations from linearity can be indicative for the occurrence of other types of recovery processes. Because f_t is measured as a function of t during an FPR experiment, the three parameters to be determined from the above equation are f_0, f_∞, and $t_{1/2}$. Yguerabide et al. (7) have linearized Eq. 2 by plotting $f_\infty/(f_\infty - f_t)$ vs. t to obtain f_0 and $t_{1/2}$ from the slope and intercept of the best straight line, as determined by linear regression analysis. This approach requires that f_∞ be known; this can be achieved by an iteration procedure to determine the value of f_∞ that gives rise to the best linear plot. In addition, the presence of other recovery processes can be detected using this plot, since it has been demonstrated that flow will cause the plot to deviate positively from a straight line, while multiple diffusion coefficients will cause a negative deviation (7). However, the above method is not well suited for determination of the diffusion coefficient of a major component in the presence of flow or other diffusing components. Under experimental conditions f_∞ is generally unknown, and if flow or multiple diffusion coefficients are involved a linear plot cannot be obtained for any value of f_∞. The value of f_∞ that will give rise to the least deviations from linearity under these conditions, will be strongly affected by the presence of these other recovery processes, and will bear no relationship to the desired f_∞ of the major diffusing component.

In the present paper, we describe a method that permits unequivocal determination of the parameters f_0, f_∞, and $t_{1/2}$ from FPR recovery kinetics on the basis of Eq. 2. Furthermore, it will be demonstrated how the presence of other recovery processes can be detected, and how the recovery kinetics of a major diffusing component can be corrected for concomitant flow or the presence of other diffusing components. In addition, the extent to which noise as a result of counting error will affect determination of the above system parameters has been investigated. We will first consider the situation where recovery occurs according to a single diffusion coefficient in the absence of flow, so that Eq. 2 will be valid. Let t_{ref} be an arbitrary reference time after photobleaching, for which the corresponding relative fluorescence f_{ref} is known from the recovery kinetics. Because f_{ref} is a function of t_{ref} according to Eq. 2, it follows from this equation by elimination of f_0

$$\frac{t - t_{ref}}{f_t - f_{ref}} = \frac{t}{f_\infty - f_{ref}} + \frac{t_{1/2}}{f_\infty - f_{ref}}. \tag{3}$$

Thus, by plotting $(t - t_{ref})(f_t - f_{ref})$ as a function of time t for a known combination of (f_{ref}, t_{ref}), a straight line will be obtained. From the ratio of intercept and slope $t_{1/2}$ can be determined, and from the slope itself $f_\infty - f_{ref}$. Knowing the value of f_{ref} results directly in f_∞. Because f_0 is defined as the relative fluorescence at $t = 0$, it follows from Eq. 3 $f_0 = f_{ref} - (t_{ref}/t_{1/2}) \cdot (f_\infty - f_{ref})$. So by the use of a reference time point the parameters f_0, f_∞, and $t_{1/2}$ can be determined in an unequivocal way. To correct for possible fluctuations in f_{ref}, this analysis can be carried out for various combinations of (f_{ref}, t_{ref}), after which the values obtained for the parameters should be averaged. The strategy used here agrees basically with the theoretical considerations of Koppel (8), namely that any postbleach time can be used as a zero time in the analysis of recovery kinetics.

In the case when the recovery of the major diffusing component is affected by flow or the presence of other diffusing components, the value of f_t will be larger than expected from Eq. 2. These other recovery processes will

therefore cause the plot defined in Eq. 3 to deviate negatively from a straight line. Positive deviation from linearity can be observed if bleaching of the fluorophores takes place during recovery in spite of the attenuated laser intensity. In our experience these deviations from linearity can easily be accounted for by introducing a second-order correction term into Eq. 3, according to

$$\frac{t - t_{ref}}{f_t - f_{ref}} = \frac{t}{f_\infty - f_{ref}} + \frac{t_{1/2}}{f_\infty - f_{ref}} + ct^2. \quad (4)$$

The values of $(t - t_{ref})/(f_t - f_{ref})$ vs. t can now be fitted using second-order regression analysis, as described in detail in reference 9. This analysis results in values of f_0, f_∞, and $t_{1/2}$ characteristic for the recovery of the major diffusing component, corrected for the presence of flow and/or other diffusing components (negative value of c).

In our experimental arrangement we collect 250 data points after photobleaching during a period equal to 10–20 half-times of recovery, after which recovery is 90–99% complete. By definition the midpoint of the bleaching pulse is taken as zero time, and the delay time is defined from zero time to the midpoint of the sample time, corresponding to the first data point. For recovery analysis, five

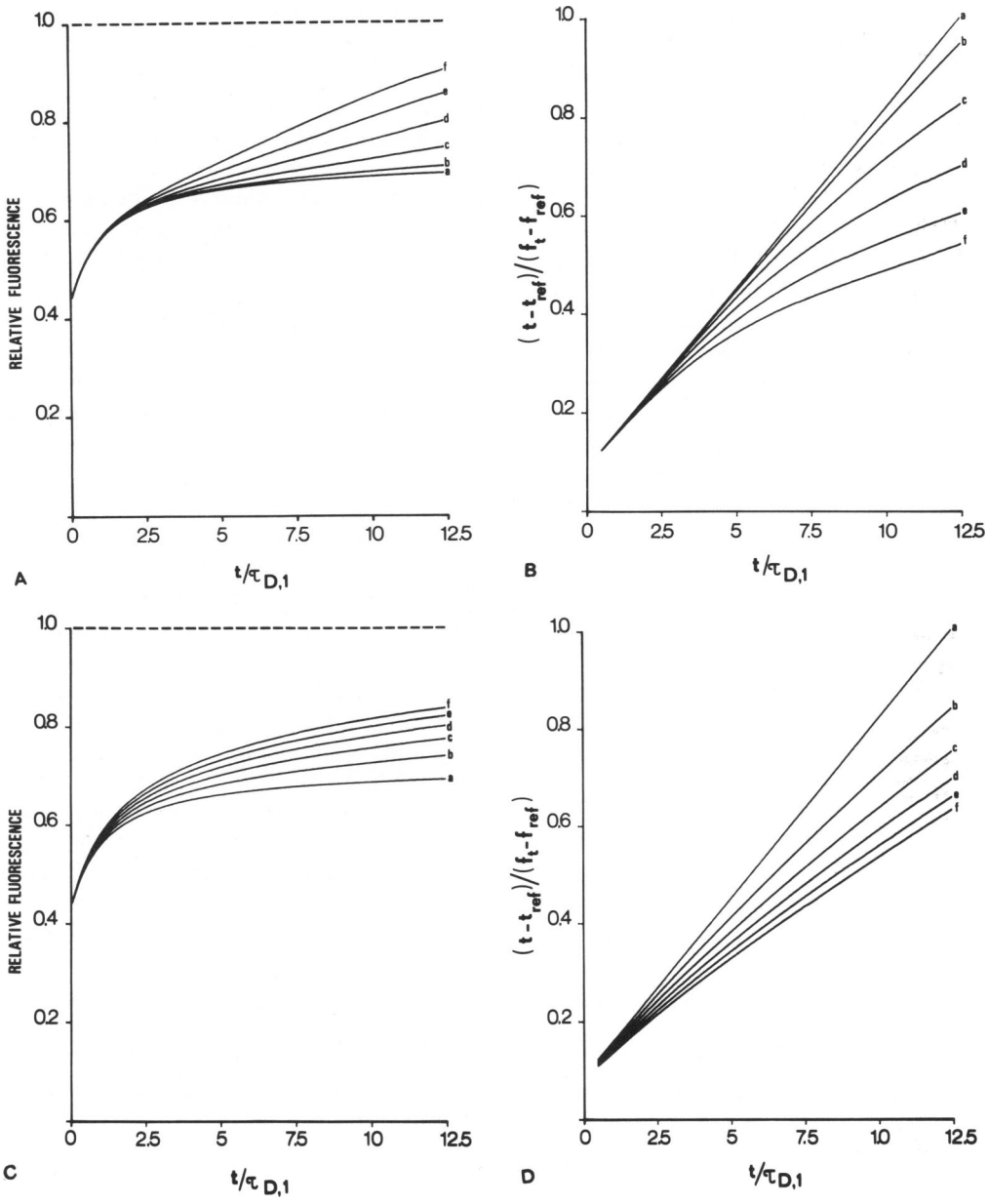

FIGURE 1 FPR curves simulated according to Eq. 1, using $K = 2$ ($\gamma_D = 1.18$; reference 6). *A*, $m = 1$; $\phi_1 = 0.5$. *a*, $\tau_{D,1}/\tau_F = 0$; *b*, 0.02; *c*, 0.04; *d*, 0.06; *e*, 0.08; *f*, 0.10. *B*, same data plotted according to Eq. 3; $t_{ref} = 0.5\ \tau_D$. *C*, $m = 2$; $\phi_1 = \phi_2 = 0.5$; *a*, $\tau_{D,1}/\tau_{D,2} = 0$; *b*, 0.02; *c*, 0.04; *d*, 0.06; *e*, 0.08; *f*, 0.10. *D*, same data plotted according to Eq. 3; $t_{ref} = 0.5\ \tau_D$. Dashed lines in *A* and *C* represent prebleach fluorescence intensity. For simplicity, the data on the *y*-axis in *B* and *D* have been plotted in relative units.

independent reference times are used, all belonging to the first 10 data points after photobleaching, and linear regression analysis is performed starting from the 10th data point after the reference time. Because of the initial steepness of the recovery curve, this avoids f_t becoming close to f_{ref}. To minimize variation in the analysis resulting from noise due to counting error, a smoothing procedure is used setting a value for the relative fluorescence of the nth data point to $f'_n = f_{n-2}/16 + f_{n-1}/4 + 3 \cdot f_n/8 + f_{n+1}/4 + f_{n+2}/16$. Simulated noise-free recovery curves gave identical values of the system parameters with and without the use of this smoothing procedure (data not shown). Analysis is performed over a time period corresponding to a maximum of 15 half times of recovery, after which recovery is 95% complete. Identical weight is given to each data point, which contrasts with the method described by Yguerabide et al. (7). Recovery curves giving rise to a significantly positive value of c (see Eq. 4) are discarded.

To test the validity of the procedure described above, recovery curves were simulated according to Eq. 1 by means of an Apple II microcomputer. Fig. 1 shows simulated recovery curves for a major diffusing component with $K = 2$ and $\phi = 0.5$, values typical for the diffusion behavior of membrane proteins (1–4). In the case of a single diffusing component in the absence of flow (Fig. 1 *A*, line *a*) Eq. 3 yields a straight line (Fig. 1 *B*, line *a*). Values for ϕ and $t_{1/2}$ obtained from this line (Fig. 2; $\tau_{D,1}/\tau_F = 0$) are in good agreement with the values used for simulation of this recovery curve (Fig. 2; dashed lines). With increasing rates of uniform flow (Fig. 1 *A*) the latter plot tends to deviate increasingly from linearity, although relatively little effect is seen in the initial recovery phase (Fig. 1 *B*). When these curves are analysed according to Eq. 4 to correct for the presence of flow, characteristic values of $t_{1/2}$ and ϕ are

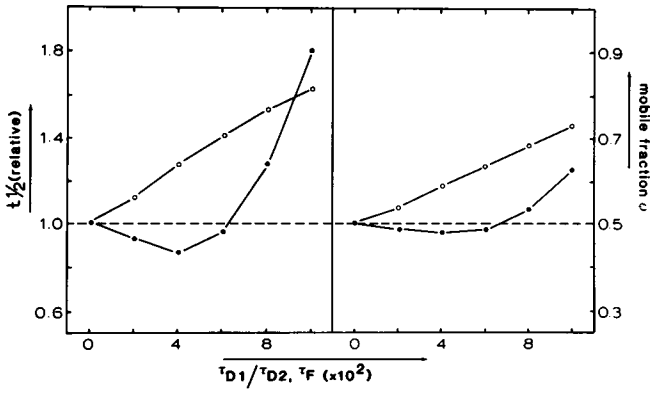

FIGURE 2 Effect of uniform membrane flow (●) and multiple diffusion coefficients (○) on the diffusion kinetics of a major diffusing component. Recovery curves were simulated according to Eq. 1 using the conditions described in the legend of Fig. 1 *A* (●) and *C* (○). Values of f_0, f_∞, and $t_{1/2}$ for the major diffusing component were obtained according to Eq. 4, ϕ was calculated according to $(f_\infty - f_0)/(1 - f_0)$. Dashed lines represent values of ϕ and $t_{1/2}$ ($\gamma_D \cdot \tau_{D,1}$) of the major diffusing component used in the simulation experiment.

obtained for the major diffusing component as shown in Fig. 2. In spite of the presence of flow very similar values for $t_{1/2}$ and ϕ are obtained, if $\tau_{D,1} < 0.06\ \tau_F$. This implies that after correction for flow, curves *a–d* of Fig. 1 *A* all give rise to a recovery curve similar to *a*. The presence of a second diffusion coefficient (Fig. 1 *C*) results in less pronounced deviations from linearity than in the case of membrane flow, but it affects the linearized recovery curves already in their initial phase (Fig. 1 *D*). In general, it can be stated that correction for a second diffusion coefficient is more difficult to obtain than for flow, and depends on the ratio $\tau_{D,2}/\tau_{D,1}$ and ϕ_2/ϕ_1. Fig. 2 shows that in the case $\tau_{D,1} = 0.02\ \tau_{D,2}$ with $\phi_1 = \phi_2 = 0.5$, values obtained for $t_{1/2}$ and ϕ for the major diffusing component are already more than 10% higher than the values used for curve simulation; complete correction can be obtained under these conditions if $\phi_2 < 0.2$ (data not shown). It should be realized, however, that the presence of multiple diffusion coefficients, which are difficult to detect by other means, are clearly indicated in the above analysis because of the deviations from linearity shown in Fig. 1 *D*, but that correction is only partially possible when analyzed according to Eq. 4.

Lipid probes generally diffuse according to a one-component system with almost full recovery (1–4). Besides the possible presence of membrane flow, measurements of lipid lateral mobility can be hampered particularly by the presence of statistical noise due to counting error. If fluorescence intensity is measured by photon counting, the number of fluorescent counts, F, measured during a certain sample time is subject to a standard error equal to $(F)^{1/2}$ (9). Due to the small recovery half time of lipids, short sample times have to be chosen resulting in a relatively high noise level. Fig. 3 *A* shows a simulated recovery curve for a probe with $K = 2$ and $\phi = 0.9$, and a prebleach fluorescence intensity F^0 of 2,500 counts per sample time of $0.05\ \tau_D$. For each fluorescence intensity F ($= f_t \cdot F^0$) calculated according to Eq. 1, a value F' has been generated randomly from a Gaussian distribution according to $F \pm F^{1/2}$. Values for F'/F^0 have been plotted in Fig. 3 *A;* in addition, a delay time has been assumed equal to $0.25\ \tau_D$. Fig. 3 *B* shows this recovery curve plotted according to Eq. 3 after the use of the above mentioned smoothing procedure. The best linear fit through these data points is also indicated. The fitted recovery curve is then generated according to Eq. 2 on the basis of the parameters determined from this straight line, and is shown again in Fig. 3 *A*, extrapolated through the delay time. Fig. 3 *C* shows a similar recovery curve, but this time in the presence of additional membrane flow ($\tau_D = 0.1\ \tau_F$). When plotted according to Eq. 3, the data points show deviation from a linear behavior. Analysis according to Eq. 4, however, results in almost identical values of f_0, f_∞, and $t_{1/2}$ as obtained in the absence of flow, demonstrating that the recovery kinetics of the diffusing probe can be fully

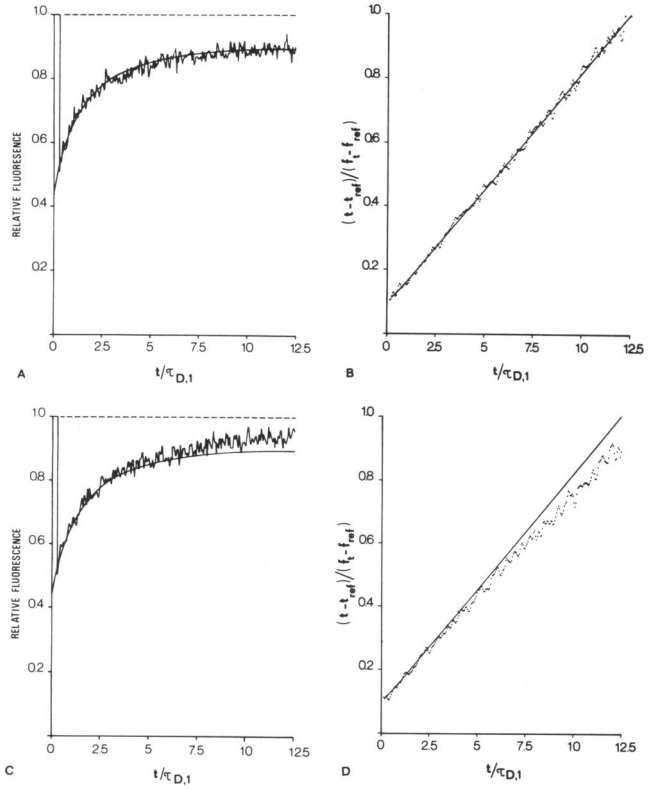

FIGURE 3 FPR curves simulated according to Eq. 1, using $K = 2$. Statistical noise due to counting error was simulated as described in the text. A delay time has been assumed equal to 0.25 τ_D, during which the relative fluorescence has been plotted as 1. Sample time equals 0.05 τ_D. *A*, $m = 1$; $\tau_D/\tau_F = 0$; $\phi = 0.9$; $F^0 = 2{,}500$ counts/sample time. Solid line represents the best fit according to Eq. 2, using the values of f_0, f_∞, and $t_{1/2}$ obtained from *B*. *B*, same data plotted according to Eq. 3 after using the smoothing procedure (see text). Solid line represents best linear fit, from which f_0, f_∞, and $t_{1/2}$ are obtained. t_{ref} used for the plot equals 0.1 τ_D. *C*, $m = 1$; $\tau_D/\tau_F = 0.1$; $\phi = 0.9$; $F^0 = 2{,}500$ counts/sample time. Solid line represents diffusion kinetics of the major diffusing component according to Eq. 2 based on the values of f_0, f_∞, and $t_{1/2}$ obtained from: *D*, same data plotted according to Eq. 3 after using the smoothing procedure (see text). Solid line represents the linear behavior of the major diffusing component according to Eq. 3, after correction for second-order terms according to Eq. 4. t_{ref} used for the plot equals 0.1 τ_D. For simplicity, the data on the *Y*-axis in *B* and *D* have been plotted in relative units.

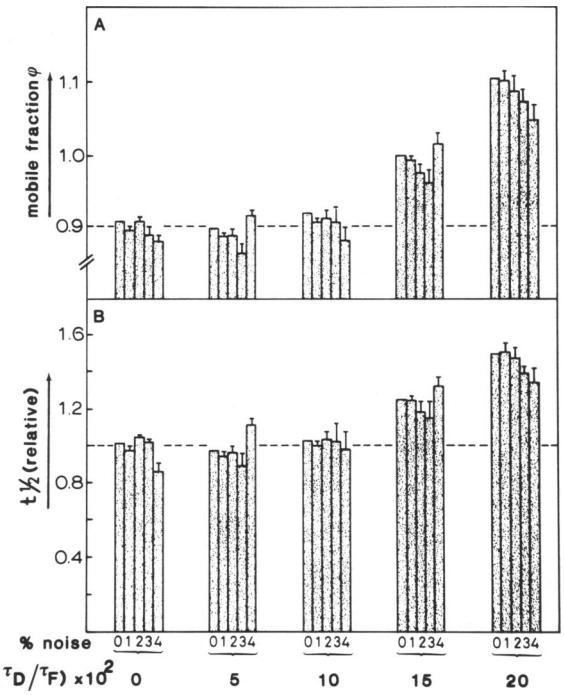

FIGURE 4 Effect of uniform membrane flow and statistical noise due to counting error on the diffusion kinetics of a major diffusing component. FPR curves were simulated according to Eq. 1 using $K = 2$, $m = 1$, $\phi = 0.9$, and various ratios of τ_D/τ_F. Statistical noise due to counting error was simulated as described in the text, using values for F^0 (prebleach intenstiy) of ∞ (0% noise); 10,000 (1%); 2,500 (2%); 1,111 (3%), and 625 (4%) per sample time of 0.05 τ_D. Each curve was simulated in triplicate and analyzed for f_0, f_∞, and $t_{1/2}$ of the major diffusing component according to Eq. 4. Mean and standard error of the mean in the obtained values for $t_{1/2}$ and ϕ are indicated. Dashed line represents values of ϕ and $t_{1/2}$ used for simulation.

corrected for the presence of flow under these conditions. The solid lines in Fig. 3 *C* and *D* are generated from the parameters thus obtained, using Eqs. 2 and 3, respectively, and therefore represent the recovery kinetics of the major diffusing component.

Fig. 4 shows the values of $t_{1/2}$ and ϕ for the major diffusing component from simulated curves ($K = 2$; $\phi = 0.9$) in the presence of various amounts of flow and levels of statistical noise. It is shown that full correction for the presence of flow can be obtained for this type of probe if $\tau_D < 0.1\ \tau_F$, irrespective of the noise level that only affects the standard deviation in the parameters determined. It will be clear that the presence of statistical noise requires the use of more than one reference point in the analysis. So far, we have only used the above method to correct the diffusion kinetics for alternative recovery processes, not to quantify these other processes. We are now using this method routinely for measurements on cells in tissue culture, with good results.

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