

CONTINUOUS FLUORESCENCE MICROPHOTOLYSIS BY A $\sin^2 kx$ GRATING

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Received 9 October 1985; in final form 2 December 1985

A method is derived to evaluate the fluorescence intensity which is observed in photobleaching experiments involving a *time-independent* $\sin^2 kx$ grating. This grating corresponds to a local (position x) rate constant $r \sin^2 kx$ of photobleaching. Application of the method demonstrates how such experiments can yield information on lateral diffusion in artificial and biological membranes. A simple analytical approximation for the fluorescence intensity which holds for a time period less than $10/r$ is provided.

1. Introduction

Much information on the transport of biological materials has come from the photobleaching method, often referred to as fluorescence microphotolysis (FM) or fluorescence recovery after photobleaching (FRAP) [1–6]. This method has served to monitor lateral diffusion of lipids and proteins in artificial [7–9] and cellular [1,3,7,10] membranes as well as transport across membranes [10].

To apply the photobleaching method the material of interest (membrane protein or lipid) is labelled by suitable fluorescent dyes. The dyes have the property that radiation produces fluorescence as well as a photo-reaction resulting in a *non-fluorescent* product. The photobleaching method employs irradiation beams of various geometries and intensities. The observable is the total fluorescence of the irradiated dyes. The spatial inhomogeneity (geometry) of the irradiation beam induces a dependence on the lateral diffusion. However, to obtain information on the lateral diffusion from the time-dependence of this observable one needs to solve a reaction–diffusion equation that connects lateral diffusion, photobleaching and fluorescence. In this Letter we provide the necessary solution for the case of lateral diffusion of a dye-labelled material in a membrane probed by light with the intensity profile $r \sin^2 kx$. Such an intensity pattern can be produced by fluorescence excitation in the membrane

plane with total internal reflexion [11,12].

The apparatus for this method is described in ref. [12]. It involves a membrane mounted on a fixed stage. Into the membrane plane are coupled by means of a pair of prisms the splitted beams of a laser light source. The beams are guided along the plane by total internal reflection. In the area of intersection of the focused beams the $\sin^2 kx$ intensity pattern is generated holographically with a periodicity of 2–10 μm depending on the in-plane angle between the two beams.

In the conventional application of the photobleaching method [5] a very high initial intensity of the laser light is chosen to produce a spatially periodic perturbation of the concentration of the dye. The equilibration of this concentration profile is then monitored by means of a second laser beam with the same spatial periodicity (grating). The intensity of this beam is low to avoid bleaching. In the following we consider a variant of this method in which a single *time-independent* light intensity is chosen. The advantage of this observation, termed continuous fluorescence microphotolysis, has been discussed and demonstrated experimentally [7].

The reason why continuous fluorescence microphotolysis is most suitable for the method of total internal reflexion interference photobleaching is due to the difficulty connected with the holographic generation of the $\sin^2 kx$ intensity pattern. The stability of this

pattern over the length scale of the wavelength of the light is technically very difficult. In this respect it is advantageous if a perturbation of the holographic pattern due to the switching of the light intensities can be avoided [13].

2. Reaction-diffusion description of the experiment

The relevant property of the dye-labelled material in the photobleaching experiment is their time-dependent concentration profile $p(x, t)$. This quantity is governed by the reaction-diffusion equation

$$\partial_t p(x, t) = (D\partial_x^2 - r \sin^2 kx) p(x, t) \quad (1a)$$

subject to the initial condition

$$p(x, t=0) = 1. \quad (1b)$$

The fluorescence signal which we seek to evaluate as the observable of the photobleaching experiment is

$$S(t) = N \int_0^\lambda dx \sin^2 kx p(x, t), \quad (2a)$$

where $\lambda = \pi/k$ and N is the normalization constant defined through the initial value

$$S(t=0) = 1. \quad (2b)$$

The solution is periodic with spatial period λ , a property which follows from the identity $\sin^2 kx = \frac{1}{2} [1 - \cos(2kx)]$. The solution should reach its maximum value at $x = 0$ since at this position the light intensity vanishes and, therefore, no bleaching occurs. Hence, the solution obeys the boundary condition

$$\partial_x p(x, t) = 0, \quad x = 0, \lambda. \quad (3)$$

One can expand this solution in the Fourier series

$$p(x, t) = \sum_{n=0}^{\infty} A_n(t) \cos(2nkx). \quad (4)$$

The expansion coefficients A_n are determined through (1a) and (1b). Inserting (4) into (1a) yields after some algebra exploiting the orthogonality properties of $\cos(2nkx)$ over the interval $[0, \lambda]$

$$\partial_t A_0 = r'(A_1 - 2A_0), \quad (5a)$$

$$\partial_t A_1 = -D'A_1 + r'(2A_0 - 2A_1 + A_2), \quad (5b)$$

$$\begin{aligned} \partial_t A_n &= -n^2 D'A_n + r'(A_{n-1} - 2A_n + A_{n+1}), \\ n &= 2, 3, \dots, \end{aligned} \quad (5c)$$

where $r' = \frac{1}{4}r$ and $D' = 4Dk^2$. The initial condition (1b) corresponds to

$$A_n(t=0) = \delta_{n0} \quad (5d)$$

and the observable (2a), (2b) is

$$S(t) = A_0(t) - \frac{1}{2}A_1(t). \quad (5e)$$

Eqs. (5a)–(5c) may be cast into the vector representation

$$\partial_t A = LA, \quad (5f)$$

where A represents the vector with components A_n and L represents the linear operator on the rhs of (5a)–(5c).

3. Limits of fast and slow diffusion

The evolution equation (5) identifies two relevant rate constants which govern the dynamics of the photobleaching experiment under consideration: the rate constant r' of photobleaching and the rate constant D' of diffusional relaxation over the interval $[0, \lambda]$. In case $D' \gg r'$ the dye-labelled material diffuses so fast that the photobleaching process cannot produce any gradients in the concentration profile. In this case

$$S(t) = \exp(-\frac{1}{2}rt). \quad (6)$$

In the opposite case $D' \ll r'$ the mobility of the dye-labelled material can be neglected and the observable is

$$S(t) = N \int_0^\lambda dx \sin^2 kx \exp(-rt \sin^2 kx). \quad (7a)$$

At very long times $t > 1/D'$ one expects, however, a deviation from this time dependence. Expression (7a) can be related to an integral representation of the modified Bessel functions $I_n(z)$ and one can derive [14]

$$S(t) = \exp(-\frac{1}{2}rt) [I_0(\frac{1}{2}rt) - I_1(\frac{1}{2}rt)]. \quad (7b)$$

The asymptotic behaviour of this function is [14]

$$S(t) \simeq (2\pi)^{-1/2} (\frac{1}{2}rt)^{-3/2} + O((\frac{1}{2}rt)^{-5/2}), \quad (7c)$$

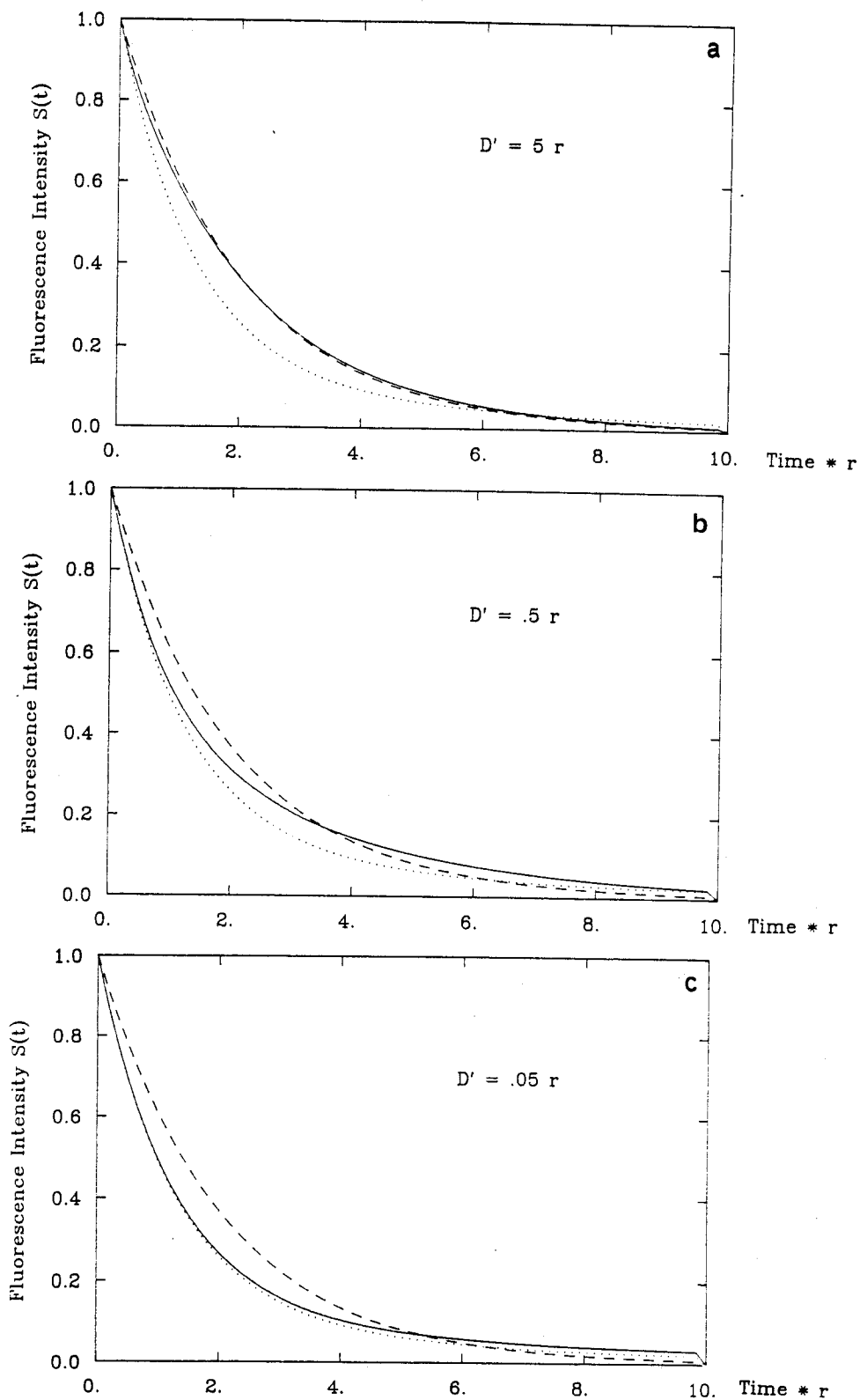


Fig. 1. Comparison of the fluorescence intensity $S(t)$ calculated according to eqs. (8) and (9) (—) with the intensity for fast (---) and slow (..) diffusion; (a) for $D' = 5r$, (b) for $D' = 0.5r$, (c) for $D' = 0.05r$.

which demonstrates that the observable decays asymptotically more slowly in the case of slow diffusion than in the case of fast diffusion. This behaviour originates from the slow bleaching of dyes in the vicinity of $x = 0$ where bleaching is slow.

At short times the functional behaviour of (7b) is [14]

$$S(t) \approx 1 - \frac{3}{4}rt + O((\frac{1}{2}rt)^2). \quad (7d)$$

This demonstrates that the initial decay of the observable $S(t)$ is faster in the slow-diffusion limit (slope $-\frac{3}{4}r$) than in the limit of fast-diffusion (slope $-\frac{1}{2}r$). The limiting behaviour of the observable $S(t)$ is presented (together with observables for intermediate D' values) in fig. 1 demonstrating what has been discussed above.

The important conclusion reached from eqs. (6) and (7) is that in the limit of fast and slow diffusion the observable $S(t)$ becomes *independent* of the diffusion coefficient. In section 4 we will show that these limits of a D -insensitive observable is assumed for $D' > 5r$ and for $D' < 0.05r$.

4. Numerical solution

We did not succeed to obtain an analytical solution of eq. (5). In lieu of such solution we present in this section a numerical algorithm which rapidly produces a reliable numerical solution. The algorithm suggested here discretizes the time variable, i.e. chooses the instances $t = j\tau, j = 0, 1, \dots$ and also neglects high Fourier components by assuming $A_n = 0$ for $n > N$ (in our calculations we assumed $N = 50$). The algorithm is then cast into the vector equation

$$A[(j+1)\tau] - A(j\tau) = \frac{1}{2}\tau L \{A[(j+1)\tau] + A(j\tau)\}, \quad (8a)$$

where $A^T = (A_0, A_1, A_2, \dots, A_N)$ and where L is defined as in (5f). To ensure the numerical stability of the algorithm one needs to represent the coefficients $A_n(t)$ on the rhs of (5a)–(5c) by their time average $\frac{1}{2}\{A_n[(j+1)\tau] + A_n(j\tau)\}$. This procedure is commonly referred to as the implicit method [15]. Because of the tridiagonal form of the operator L eq. (8a) corresponds to a linear three-term recursion equation

$$-\alpha_n A_{n-1}[(j+1)\tau] + \beta_n A_n[(j+1)\tau] - \gamma_n A_{n+1}[(j+1)\tau] = \delta_n, \quad (8b)$$

in which the coefficients on the lhs are time-independent:

$$\alpha_n = \frac{1}{8}r\tau, \quad n = 0, 1, 2, \dots, \quad (8c)$$

$$\beta_0 = 1 + \frac{1}{4}r\tau, \quad \beta_n = \beta_0 + \frac{1}{2}n^2 D'\tau, \quad n = 1, 2, \dots, \quad (8d)$$

$$\gamma_0 = 0, \quad \gamma_1 = \frac{1}{4}r\tau, \quad \gamma_n = \frac{1}{8}r\tau, \quad n = 2, 3, \dots \quad (8e)$$

and

$$\delta_0 = A_0(j\tau) + \frac{1}{8}r\tau [A_1(j\tau) - 2A_0(j\tau)], \quad (8f)$$

$$\delta_1 = A_1(j\tau) + \frac{1}{8}r\tau [2A_0(j\tau) - 2A_1(j\tau) + A_2(j\tau)] - \frac{1}{2}D'\tau A_1(j\tau), \quad (8g)$$

$$\delta_n = A_n(j\tau) + \frac{1}{8}r\tau [A_{n-1}(j\tau) - 2A_n(j\tau) + A_{n+1}(j\tau)] - \frac{1}{2}n^2 D'\tau A_n(j\tau), \quad n = 2, 3, \dots \quad (8h)$$

Eqs. (8) can then be employed to evaluate recursively the vectors $A(j\tau), j = 1, 2, \dots$ starting from $A^T(0) = (1, 0, 0, \dots)$. For this purpose one needs to solve the inhomogeneous linear equation (8b). The following scheme suggested in ref. [15] is most reliable. One first determines recursively

$$E_0 = \alpha_0/\beta_0, \quad F_0 = \delta_0/\beta_0, \quad (9a)$$

$$E_n = \alpha_n/(\beta_n - \gamma_n E_{n-1}), \quad (9b)$$

$$F_n = (\delta_n + \gamma_n F_{n-1})/(\beta_n - \gamma_n E_{n-1}) \quad (9c)$$

for $n = 1, 2, \dots, N$ and evaluates then sequentially

$$A_N[(j+1)\tau] = F_N, \quad (9d)$$

$$A_n[(j+1)\tau] = E_n A_{n+1}[(j+1)\tau] + F_n, \quad n = N-1, N-2, \dots, 0. \quad (9e)$$

Fig. 1 presents the resulting observable for three different D' values, $D' = 5r, 0.5r$ and $0.05r$. The diagrams show that for a given r value, i.e. for a given light intensity, there exists only a certain window of D' values, namely $[0.05r, 5r]$, in which the observation is D' -sensitive. In this range of D' values $S(t)$ shifts from the slow-diffusion limit (7) to the high-dif-

fusion limit (6). For larger and smaller D' values the observable $S(t)$ becomes independent of D' . Actually for smaller D' values the convergence to (7) at long times is slower than indicated here. However, since $S(t)$ is small at long times the observation may not be able to resolve accurately the long-time decay of $S(t)$. Hence, this slow convergence should not be of practical importance.

We may note that an alternative numerical solution could have been developed by discretizing the time as well as the space variable of the reaction-diffusion equation (1). The structure of the resulting equation, an inhomogeneous three-term linear recursion, corresponds to that of eq. (8) and, hence, the algorithm of eqs. (8) and (9) can be applied to its solution. About 100 spatial points should be chosen for an accurate solution which makes this solution more time consuming than the one adopted here which involves a Fourier transformation. However, in case the light intensity profile bears higher Fourier components, as for a square wave, eq. (1) should be solved directly, i.e. without Fourier transformation, by the algorithm presented.

The numerical solution developed here can also be applied to describe very accurately the conventional photobleaching experiment involving two light intensities. The first strong intensity phase lasting from $t = 0$ to $t = t_1$ is to be described exactly as done above. This yields the vector $A(t_1)$ which is then to be used as the initial condition replacing (5d) in the description of the second weak intensity phase. The time-integration of $A(t)$ follows again eqs. (8) and (9) and yields $S(t)$. This calculation avoids an approximation of the concentration profile after the strong intensity phase and does not neglect the bleaching during the weak-intensity phase.

5. Solution by a generalized moment expansion

In this section we show how an approximate solution of eq. (5) can be obtained by means of a generalized moment expansion. This method which is presented in detail in ref. [8] describes the observable in terms of a multi-exponential decay. For a demonstration we restrict the approximation in the following to a bi-exponential description

$$s(t) = a_1 \exp(-\lambda_1 t) + a_2 \exp(-\lambda_2 t). \quad (10)$$

The coefficients $a_{1,2}$ and $\lambda_{1,2}$ are determined such that $s(t)$ reproduces the following generalized moments of the exact observable

$$\mu_0 = S(t=0),$$

$$\mu_1 = -\partial_t S(t=0), \quad (11b)$$

$$\mu_2 = \partial_t^2 S(t=0), \quad (11c)$$

$$\mu_{-1} = \int_0^\infty dt S(t). \quad (11d)$$

These moments assume the values

$$\mu_0 = 1, \quad \mu_1 = 3r', \quad \mu_2 = r'(10r' + D'),$$

$$\mu_{-1} = 1/2r'. \quad (12a)$$

The first three identities can be derived from the formal solution of eq. (5)

$$S(t) = (1, -0.5, 0, 0, \dots) \exp(Lt) e_0 \quad (13)$$

and the last identity from inserting into (11d) the identity

$$S(t) = -(2/r) \partial_t A_0, \quad (14)$$

which follows from (5a) and (5e). The vector e_0 in (13) defined through $e_0^T = (1, 0, 0, \dots)$ represents the initial condition (5d). Knowledge of the moments (11a)–(11d) allows one to determine the coefficients and decay constants of (10) according to the algorithm suggested by the following equations:

$$x = \mu_0^2 - \mu_1 \mu_{-1}, \quad y = \mu_1 \mu_0 - \mu_2 \mu_{-1},$$

$$z = \mu_1^2 - \mu_2 \mu_0, \quad (15a)$$

$$\lambda_1 = [y + (y^2 - 4xz)^{1/2}] / 2x,$$

$$\lambda_2 = [y - (y^2 - 4xz)^{1/2}] / 2x, \quad (15b)$$

$$a_1 = (\lambda_2 \mu_1 - \mu_2) / \lambda_1 (\lambda_2 - \lambda_1),$$

$$a_2 = (\lambda_1 \mu_1 - \mu_2) / \lambda_2 (\lambda_1 - \lambda_2). \quad (15c)$$

Fig. 2 compares the resulting approximation with the exact observable for $D' = 0.05r$. In case of larger D' values the accuracy of the approximation improves. This implies that the approximate $s(t)$ can be em-

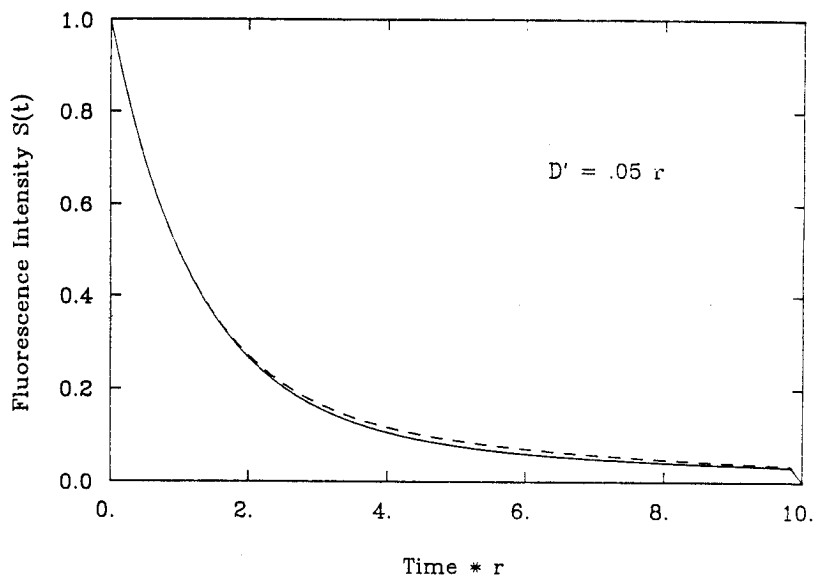


Fig. 2. Comparison of the fluorescence intensity $S(t)$ for $D' = 0.05r$ evaluated by means of eqs. (8) and (9) (—) and approximated by means of eqs. (10), (12) and (15) (---).

ployed safely in the relevant D' interval $[0.05r, 5r]$ and in the relevant time interval $[0, 10/r]$. If one applies the approximation for smaller D' values the approximation becomes less accurate. This is no disadvantage since in this range the observation is not yielding information on D' anyway.

To extend the approximation (10), (12), (15) to longer times one may consider a moment expansion involving the moments μ_{-1} to μ_4 following the method suggested in ref. [8]. However, this approximation will not be very accurate either at very long times. A long-time approximation by means of a generalized moment expansion cannot be constructed. The reason for this calamity is that the observable for small D' values decays nearly as $t^{-3/2}$. As a result the long-time moments

$$\mu_{-n} = \frac{1}{(n-1)!} \int_0^{\infty} dt t^{n-1} S(t) \quad (16)$$

do not exist for $n > 1$ and, hence, a moment expansion reproducing further long-time moments does not exist.

6. Summary

We have developed a numerical procedure which de-

termines the fluorescence intensity observable in a photobleaching experiment involving a *time-independent* grating with a local rate constant of photobleaching $r \sin^2 kx$. We have demonstrated thereby that the corresponding observation is sensitive to diffusion coefficients in the range $0.05r < 4Dk^2 < 5r$. We have also provided a simple approximation which holds in this range of diffusion constants for times up to $10/r$.

Acknowledgement

The topic of this paper was suggested to the author by H. Gaub and H. McConnell. The author thanks both of them for fruitful discussion and for the information on the feasibility of the experiment discussed here. This work was supported by the Deutsche Forschungsgemeinschaft (Schu 523).

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