THEORETICAL ANALYSIS OF FLUORESCENCE PHOTOBLEACHING RECOVERY EXPERIMENTS

D. M. SOUMPASIS
Institute for Theoretical Physics, Freie Universität Berlin, D-1000 Berlin 33, Federal Republic of Germany

ABSTRACT We derive an exact closed formula for the fluorescence recovery curve measured in fluorescence photobleaching recovery experiments employing uniform circular laser beams. In contrast to the expression used currently, this result is very simple and free of mathematical drawbacks, thus facilitating the quantitative analysis of experimental data.

INTRODUCTION

The fluorescence photobleaching recovery (FPR) technique (also abbreviated FRAP; fluorescence recovery after photobleaching) has become a standard method in studies of the translational dynamics of membrane components. In addition to the original version of the technique (1, 2), which is the one most commonly used in several laboratories, two refined variants have been recently reported (3, 4) that are better suited to examine some special cases such as anisotropic diffusion and the superposition of flow and diffusional processes.

In this communication we are solely concerned with one theoretical aspect of the original FPR version, namely, the calculation of the fluorescence recovery curve for uniform circular beam profiles because, in this case, the formula in use seems to cause some technical trouble. In order to establish a connection between our work and the results of Axelrod et al. (1) (to be called I) and to facilitate understanding, we first briefly review the basic features of the method and then proceed with the calculations using the same symbols as in I.

REVIEW OF FPR THEORY

The fluorescently labeled membrane component to be studied is initially assumed to be uniformly distributed in the membrane, represented by an infinite plane. An intense, focused laser pulse of appropriate wavelength bleaches a small circular spot (typically 8 μm in radius) on the membrane. Subsequently, the same beam is attenuated and monitors the reappearance of fluorescence within the circle due to the arrival (via diffusion and/or flow) of unbleached molecules. The so-called fluorescence recovery curve $F_K(t)$ (fluorescence intensity vs. time after bleaching) thereby recorded contains all the information needed to analyze the transport process quantitatively. Restricting ourselves to the case most commonly occurring in practice (namely, pure isotropic diffusion), we have (Eq. 6 of I)

$$F_K(t) = \frac{q}{A} \int I(r)\, C_K(r, t)\, \mathrm{d}^2 r, \tag{1}$$

where q is the product of all quantum efficiencies of laser light absorption, emission, and detection, A is the attenuation factor of the beam during fluorescence recovery, $I(r)$ is the intensity profile of the bleaching pulse in the plane of the membrane, and the origin of polar coordinates is taken to be the center of the bleached circle. $C_K(r, t)$, the number concentration of unbleached molecules at radial distance r and time t, must be a solution of the diffusion equation with circular symmetry and must satisfy the boundary condition

$$C_K(\infty, t) = C_o \tag{2a}$$

and the initial condition

$$C_K(r, 0) = C_o \exp\,[-\alpha T I(r)]. \tag{2b}$$

$\alpha I(r)$ is the rate constant of the first-order irreversible photobleaching reaction, and T is the width of the bleaching laser pulse, which is much smaller than any characteristic transport time. The parameter K,

$$K = \alpha T I(0) \tag{3}$$

is called the bleaching parameter and measures the amount of bleaching induced. The data are more conveniently displayed in the form of fractional fluorescence recovery curves $f_K(t)$ defined by Eq. 9 of I:

$$F_K(t) = \frac{F_K(t) - F_K(0)}{F_K(\infty) - F_K(0)}. \tag{4}$$

As seen from Eqs. 1–4, the explicit form of $F_K(t)$ depends on the intensity profile of the laser beam $I(r)$. For Gaussian beams, $F_K(t)$ is expressed in a closed form involving well tabulated special functions (Eq. 10 of I) and will not be considered here.

FLUORESCENCE RECOVERY CURVE FOR UNIFORM CIRCULAR BEAMS

Technical problems arise when one analyzes the data of experiments employing uniform circular disk profile beams (Vaz, W., and T. Jovin, personal communication and unpublished results). In this case, the relevant formula for $I(r)$ and $f_K(t)$ given in I (Eqs. 4 and 14) are

$$I(r) = \begin{cases} P_o/\pi w^2 & r \le w \\ 0 & r > w, \end{cases} \tag{5}$$

$$f_k(t) = 1 - \frac{\tau_D}{t}\exp(-2\tau_D/t)[\mathbf{I}_0(2\tau_D/t) + \mathbf{I}_1(2\tau_D/t)] + 2\sum_{k=0}^{\infty}\frac{(-1)^k(2k+2)!\,(k+1)!}{(k!)^2[(k+2)!]^2}\left(\frac{\tau_D}{t}\right)^{k+2}. \tag{6}$$

$\mathbf{I}_o$ and $\mathbf{I}_1$ are modified Bessel functions, $\tau_D = w^2/4D$ is the characteristic diffusion time, w is the radius of the circular beam, D is the diffusion coefficient, and P_o is the total laser power. Due to the singularity at $t = 0$, the formula in Eq. 6 is inconvenient for numerical work, particularly in this sort of time regime: ($t < 0.1\ \tau_D$) and cannot be easily used as an aid in transport diagnostics (e.g., in order to decide whether one or several diffusing components are present). For these reasons we decided to reconsider the problem and derive a simpler formula.

Instead of considering the diffusion of unbleached molecules into the circular spot (i.e., calculating $C_K[r, t]$), it is slightly more convenient to consider the diffusion of bleached molecules out of the spot, i.e., to calculate their concentration $\overset{*}{C}_K(r, t)$ where

$$C_K(r, t) + \overset{*}{C}_K(r, t) = C_o \tag{7}$$

expresses the fact that the total concentration of labeled molecules remains constant. The boundary and initial conditions (Eqs. 2a and 2b) now become

$$\overset{*}{C}_K(\infty, t) = 0 \tag{8a}$$

and

$$\overset{*}{C}_k(r, 0) = \begin{cases} C_0(1 - \exp - K) & r \le w \\ 0 & r > w \end{cases} \tag{8b}$$

A convenient integral representation of $\overset{*}{C}_K(r, t)$ (5) is

$$\overset{*}{C}_K(r, t) = \frac{1}{2Dt}\int_0^{\infty} dr' r' \overset{*}{C}_K(r', 0)\exp\left(-\frac{r^2 + r'^2}{4Dt}\right)\mathbf{I}_o\left(\frac{rr'}{2Dt}\right). \tag{9}$$

Using the well known (6) integral identities

$$\frac{1}{2\gamma}\exp\left(-\frac{\alpha^2 + \beta^2}{4\gamma}\right)\mathbf{I}_\nu\left(\frac{\alpha\beta}{2\gamma}\right) = \int_0^{\infty} ds\, s\, \mathbf{J}_\nu(s\alpha)\mathbf{J}_\nu(s\beta)\exp - \gamma s^2 \tag{10}$$

for $\gamma = Dt$, $\nu = 0$, $\alpha = r$, $\beta = r'$ and

$$\int_0^{w} r'\, \mathbf{J}_o(sr')\, dr' = \frac{w}{s}\mathbf{J}_1(ws), \tag{11}$$

where the $\mathbf{J}_\nu$ are Bessel functions in conjunction with Eqs. 8b and 9, we arrive at

$$\overset{*}{C}_K(r, t) = wC_o(1 - \exp - K)\int_0^{\infty} ds \exp - \gamma s^2\, \mathbf{J}_o(sr)\mathbf{J}_1(ws). \tag{12}$$

This expression is now used, together with Eqs. 7, 5, 3, 1, and the identity 11 to yield, after some straightforward manipulation, the fluorescence recovery curve

$$F_K(t) = F_- - 2[F_- - F_o(K)]\int_0^{\infty}\frac{dx}{x}\mathbf{J}_1^2(x)\exp - \delta(t)\,x^2, \tag{13}$$

where $\delta(t) = \gamma(t)/w^2 = Dt/w^2$, $F_- = q/AP_oC_o$, and $F_o(K) = F_-\exp - K$ are the fluorescence intensities before and just after bleaching, respectively.

The integral appearing in Eq. 13 may be expressed in terms of a certain generalized hypergeometric series by means of a known integral representation of the latter (7). However, this result is not particularly useful because we want to have an expression as simple and as compact as possible and not another series representation. This may be accomplished as follows.

Differentiating Eq. 13 with respect to time we obtain

$$\frac{dF_k(t)}{dt} = \frac{2D}{w^2}[F_- - F_k(0)]\int_0^{\infty} dx\, xJ_1^2(x)\exp - \delta(t)x^2 = [F_- - F_k(0)]\frac{1}{t}\exp(-2\tau_D/t)\quad \mathbf{I}_1(2\tau_D/t). \tag{14}$$

The second equality is a consequence of identity 10. We now integrate Eq. 14 from t to ∞ and obtain finally

$$\begin{aligned} F_k(t) &= F_k(\infty) - [F_- - F_k(0)] \\ &\quad \cdot \int_t^{\infty}\frac{dt'}{t'}\exp(-2\tau_D/t')\,\mathbf{I}_1(2\tau_D/t') \\ &= F_k(\infty) - [F_- - F_k(0)] \\ &\quad \cdot \int_0^{2\tau_D/t}\frac{d\zeta}{\zeta}\exp(-\zeta)\,\mathbf{I}_1(\zeta) \\ &= F_k(\infty) - [F_- - F_k(0)] \\ &\quad \cdot \{1 - \exp(-2\tau_D/t)[\mathbf{I}_o(2\tau_D/t) \\ &\quad + \mathbf{I}_1(2\tau_D/t)]\}. \end{aligned} \tag{15}$$

Assuming as usual full recovery (i.e., $F_K(\infty) = F_-$), we obtain the fractional recovery curve defined by Eq. 4 in the simple closed form

$$f(t) = \exp(-2\tau_D/t)[\mathbf{I}_o(2\tau_D/t) + \mathbf{I}_1(2\tau_D/t)]. \quad (16)$$

Notice that $f(t)$ is independent of the bleaching parameter K. A short time expansion of $f(t)$ is easily derived by means of the large argument asymptotic expansions of the I_ν in Eq. 16, namely,

$$f(t) = \left(\frac{t}{4\pi\tau_D}\right)^{1/2}\left[2 - \frac{1}{2}\left(\frac{t}{4\tau_D}\right) - {}^3\!/_{16}\left(\frac{t}{4\tau_D}\right)^2 - {}^{15}\!/_{64}\left(\frac{t}{4\tau_D}\right)^3 - \ldots\right] t \ll \tau_D. \quad (17)$$

The ascending power series expansion of the right-hand side of Eq. 16 leads to the long time behavior

$$f(t) = 1 - \tau_D/t + (\tau_D/t)^2 - \ldots t \gg \tau_D. \quad (18)$$

The easiest (but not necessarily most accurate) way to determine τ_D (i.e., D too) is that proposed in I. The numerical solution of the equation $F(t_{1/2}) = {}^1\!/_2$ yields

$$D = 0.224\, w^2/t_{1/2}, \quad (19)$$

which is identical to Eq. 19 of I. Alternatively, τ_D may be extracted from a least squares fit of the experimental data to expression 16, or if sufficiently accurate short- and/or long-time data are available from suitable plots based on Eqs. 17 and/or 18.

Experimental FRAP data (Vaz, W., and T. Jovin, personal communication and unpublished results) of the fluorescent lipid analogue NBD-C_{14}PE in DMPC multibilayers (see [8] for abbreviations) are displayed in Fig. 1 together with the theoretical curve Eq. 16 ($\tau_D = 4.92\ s$, $D = 3.9 \cdot 10^{-8}\ cm^2\ s^{-1}$). In this case, the single component diffusion picture seems to describe the situation quite well. This is not always true. For example, it is observed that the

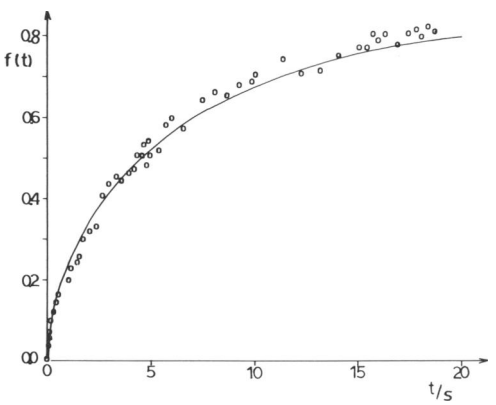

FIGURE 1 Fluorescence recovery curve of the lipid analogue NBD-C_{14}PE in DMPC multibilayers. O, T = 24.9°C and w = 8.8 μm; ——, experiment by Vaz and Jovin (personal communication and unpublished results); theory (Eq. 16 with $D = 3.9 \cdot 10^{-8}\ cm^2\ s^{-1}$ $\tau_D = 4.92\ s$ from Eq. 19).

lateral motion of both lipids (8) and proteins (9) in gel-state bilayers is better described by two diffusing populations, a fast and a slow one, with D's differing by at least an order of magnitude, and fractions θ_F and θ_S varying with temperature, type of host lipid, etc.

If both components have identical photobleaching characteristics, diffuse isotropically and independently, and if recovery is complete, analysis along the line described above yields the fluorescence recovery curve

$$f(t) = f_F(t) + \theta_F[f_s(t) - f_F(t)], \quad (20)$$

where $f_F(t)$ and $f_S(t)$ are given by Eq. 16 with τ_D^F and τ_D^S, respectively.

Provided that one has accurate enough experimental data, the three parameters θ_F, τ_D^F, τ_D^S, can be determined from computer fitting of the data to Eq. 20. A simple method to decide whether the one component isotropic diffusion picture is valid or not is to determine the apparent D (or τ_D) via several alternative routes. Say, from Eq. 19 and

$$D = 0.7386\, w^2/t_{3/4} \quad (21)$$

or Eqs. 17–19. Whenever single component isotropic diffusion prevails, the results should be identical within experimental errors.

I am indebted to Drs. W. Vaz and T. Jovin for drawing my attention to this problem and sending me their unpublished experimental data and to Dr. J. Schlessinger for many stimulating discussions and warm hospitality while I was visiting the Weizmann Institute of Science.

I also wish to thank the European Molecular Biology Organization for financial assistance through a research fellowship.

Received for publication 24 March 1982 and in revised form 20 July 1982.

REFERENCES

1. Axelrod, D., D. E. Koppel, J. Schlessinger, E. Elson, and W. W. Webb. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J.* 16:1055–1069.
2. Koppel, D. E., D. Axelrod, J. Schlessinger, E. Elson, and W. W. Webb. 1976. Dynamics of fluorescence marker concentration as a probe of mobility. *Biophys. J.* 16:1315–1329.
3. Smith, B. A., and H. M. McConnell. 1978. Determination of molecular motion in membranes using periodic pattern photobleaching. *Proc. Natl. Acad. Sci. USA.* 75:2759–2763.
4. Koppel, D. E. 1979. Fluorescence redistribution after photobleaching: a new multipoint analysis of membrane translational dynamics. *Biophys. J.* 28:281–291.
5. Carslaw, H. S., and J. C. Jaeger. 1959. Conduction of Heat in Solids. Oxford University Press, London. 353ff.
6. Magnus, W., F. Oberhettinger, and R. P. Soni. 1966. Formulas and Theorems for the Special Functions of Mathematical Physics. Springer-Verlag, Berlin-Heidelberg-New York. 86, 93.
7. Gradshteyn, I. S., and I. W. Ryzhik. 1965. Table of Integrals and Products. Academic Press, Inc., New York. 718.
8. Derzko, Z., and K. Jakobson. 1980. Comparative lateral diffusion of fluorescent lipid analogues in phospholipid multibilayers. *Biochemistry* 19:6050–6057.
9. Vaz, W., H. Kapitza, J. Stümpel, E. Sackmann, and T. Jovin. 1981. Translational mobility of glucophorin in bilayer membranes of dimyristoylphosphatidylcholine. *Biochemistry.* 20:1392–1395.