Photochemical Rewriting

Basic method: A triangle is invisibly baked (1° under non-moving) over a small area of the sample—a typically a cell or a non-vacuum surface. The recovery of the fluorescence is assumed to be due to 2D diffusion, or some localized monomolecular process (electrophoresis, etc.)

Required: 1. Photochemical baking
2. In culture, the solution with the dye to fluorescence
3. No exchange with any solution—place through

THEORY

Theory

Baking process is assumed to follow a first order process:

\[ \frac{dC(r,t)}{dt} = -k I(t)C(r,t) \]

Can use of unbleached fluorescence at position \( r \) at time \( t \). Bleaching parameter = \( k = \frac{Q}{I_0} \) amount of bleaching over time.
Figure 8: Optics and electronics of the model system test. Bleaching laser (Spectra-Physics, Mountain View, Calif., model 164) power was 1 W. The rhodamine 6G (Fisher Scientific Co., Pittsburgh, Pa.) aqueous solution concentration for all experiments was $4.6 \times 10^{-6}$ M, contained in a rectangular Suprafil quartz cuvette (C) of 100 μm path length. The photomultiplier (PM) was an EMI 9658 R. The barrier filter was saturated aqueous potassium dichromate solution.

assures that the amount of fluorophore bleached during the observation period will be no more than 1% of that bleached initially. Bleaching time $t$ was always less than $1/10$ of $t_0$. The beam profile was obtained by measuring the laser light transmitted past a razor blade edge as it translates across the beam at the sample position; spot size was varied by vertical adjustment of the focusing beam lens relative to the sample position.

The fluorescence decay of R6G in H$_2$O and 1:1 glycerol-H$_2$O in a closed uniformly illuminated volume was measured and confirmed to be approximately exponential with only a negligible amount of dark recovery. This test is important because the theoretical treatment presented here is not applicable if significant chemical recovery of fluorescence occurs in the time scale of the experiment or if the bleaching reaction is complex. Since reversibility of photobleaching is common and depends upon solvent effects, impurities, and local environment of the fluorophore, it must be checked under the conditions of the intended experiment. For example, in experiments in single living cells an entire cell should be uniformly bleached so that possible fluorescence recovery due to chemical reversibility can be observed in the absence of recovery due to transport.

Results

Table 1: shows the computed $D$ values of several runs of various $K$, $w$, and published viscosity values. The average diffusion constant $D$ for R6G in H$_2$O is $(1.2 \pm 0.2) \times 10^{-4}$ cm$^2$/s; in 1:1 (vol) glycerol-H$_2$O, $D$ is $(0.26 \pm 0.09) \times 10^{-4}$ cm$^2$/s. The uncertainties are based upon reproducibility. The ratio of the two values is approximately equal to the ratio of viscosities of the two solvents, as expected. The scaling of $t_0$ with $w^2$ is correct to within the uncertainty; there is no apparent systematic dependence on $K$.

The data was fit to the theory by the log-log method since recovery was usually less than 100% complete due to dye adhering to the glass surfaces. Fig. 9 shows the match of theory to experiment for a typical run. The fit is very close, but there is a consistent
I(r) is bleaching intensity (for a laser, usually) at point r.

The initial core of anIRg net (samples at time t=0) is given by:

\[ C(r,0) = C_0 \exp\left[-\alpha T I(r)\right] \]

This is the concentration at the beginning of the reaction after a bleach point T that is short compared to the reaction time. C0 is the initial core of the bleached point (assumed to be uniform) to points in the bleaching.

The function for \( F(r) \) describes an intensity profile as written in the text below.

Gaussian
\[ F(r) = \frac{P_0}{\pi \omega^2} e^{-r^2/\omega^2} \]

Where \( r = \frac{x - x_0}{\omega} \) is the distance; \( P_0 = \) the laser power.

Circle shift
\[ I(r) = \frac{P_0}{2\pi\omega^2} r \geq 2\omega \]

\[ I(1) = 0 \quad \text{for} \quad r > 2\omega \]
The differential eq for lateral transport of a single species with diffusion constant $D$ and uniform flow with velocity $v$, in the $x$ direction is given by

$$\frac{2 C(r,t)}{2t} = D \nabla^2 C(r,t) - v \frac{\partial C(r,t)}{\partial x}$$

**Boundary conditions**

**At $r = \infty$,** $C(\infty, t) = C_0$.

**At $r = 0$,** $C(0, t = 0) = C_0 \exp(-2I(r))$.

The fluorescence intensity along a line $t = 0$ (after bleaching plane) is given by

$$F(t) = \frac{\gamma}{4\pi} \int_0^\infty I(r) k_C(r,t) \, dr$$

$\gamma$ = product of all quantum efficiency of light absorption. $A$ is local attenuation factor

$$F \propto \frac{\gamma}{4\pi} \int_0^\infty I(r) k_C(r,t) \, dr$$

The $\gamma$ factor changes with $\theta$ with $K$.

For gaussian intensity distribution $F$ also with $K$.
tion of recovery. Before the beginning of bleaching, $F_k = qP_0C_e/A$. The initial fluorescence $F_k(0)$ after bleaching depends upon beam profile but not the mode of recovery:

$$F_k(0) = (qP_0C_e/A)K^{-1}(1 - e^{-K}),$$

for a Gaussian beam;

$$F_k(0) = (qP_0C_e/A)e^{-K},$$

for a uniform circular disc. Eqs. 7 and 8 show how the $K$ value of a recovery curve can be uniquely determined from its $t = 0$ point. A convenient way of displaying fluorescence recovery curves is in fractional form $F_k(t)$ defined as follows:

$$F_k(t) = [F_k(t) - F_k(0)]/[F_k(\infty) - F_k(0)].$$

In the following paragraphs only the essential results, solutions for $F_k(t)$ or $f_k(t)$ are presented; outlines of the derivations are displayed in the Appendix.

**Diffusion (V = 0)**

**Gaussian Intensity Profile.** The closed form solution is

$$F_k(t) = (qP_0C_e/A)vK^{-v}\Gamma(v) P(2K | 2v),$$

where $v = (1 + 2t/\tau_D)^{-1}$; $\tau_D = w^2/4D$, the "characteristic" diffusion time; and $I(v)$

![Figure 2](image-url)

**Figure 2** Normalized fluorescence recovery $F_k(t)$ vs. $t/\tau_D$ for diffusion, with Gaussian beam, for various values of $K$.

![Figure 3](image-url)

**Figure 3** Fractional fluorescence recovery $f_k(t)$ vs. $t/\tau_D$ for diffusion, with a Gaussian beam, for various values of $K$. Note that the relationship between $t_{1/2}$ and $\tau_D$ depends upon $K$.
For uniform circle intensity profile. Results for diffusion and flow are shown in Fig 4 (TRANS). Note flow pattern has a **dog leg** shape.

**Application:**

**Summary – Show TRANS**

Note that some fractions of fluorophores are immobilized, i.e. **incomplete recovery**.

Other cases (dil, a molecular probe) has complete and uniform recovery after bleaching.

**Mention:** Hackenbrock has used this method to study diffusion of the electron transport complex in mitochondria. UNC, Chapel Hill.
is the gamma function. The $\chi^2$-probability distribution $P(2K \mid 2\nu)$ is tabulated in ref. 11 (p. 978). Fluorescence $F_K(t)$ and the related fractional fluorescence $f_K(t)$ are plotted in Figs. 2 and 3, respectively, as function of $t/\tau_D$ for various $K$ values. For large $K$, where $P(2K \mid 2\nu)$ approaches one, Eq. 10 reduces to a simple approximate form:

$$F_K(t) = (qP_cC_a/A) K^{-1/2} \Gamma(\nu).$$

(11)

to within 1% accuracy for $K \geq 4$ and $t/\tau_D \geq 0.25$.

A series solution for $F_K(t)$ valid for all $K$ and $t$ is:

$$F_K(t) = (qP_cC_a/A) \sum_{n=0}^\infty [(-K)^n/n!]\left[1 + n(1 + 2t/\tau_D)^{-1}\right].$$

(12)

For $K \ll 1$, this assumes the simple form

$$F_{K=1}(t) = (qP_cC_a/A) [1 - K/2(1 + t/\tau_D)].$$

(13)

Uniform Circle Profile. For this case, the fractional fluorescence recovery $I_a(t)$ is independent of the bleaching parameter $K$. A solution in the form of an integral over a $P^\infty$ function (12) can be derived, but the following series solution is much more convenient for numerical evaluation:

$$I_a(t) = 1 - (\tau_D/t) \exp(-2\tau_D/t)[I_a(2\tau_D/t) + I_2(2\tau_D/t)]$$

$$+ 2 \sum_{k=0}^\infty \frac{(-1)^k(2k + 2)!(k + 1)!(\tau_D/t)^{k+2}}{(k!)(k + 2)!}.$$  

(14)

where $I_a$ and $I_2$ are modified Bessel functions and $\tau_D = w^2/4D$. In Fig. 4 we plot $I_a(t)$ vs. $t/\tau_D$.

**Figure 4.** Fractional fluorescence recovery $I_a(t)$ vs. $t/\tau_D$ for diffusion (——) and for flow (---). with a uniform circular disc beam.
Figure 1. Photobleaching recovery curves of L6 myoblasts. (a) Myoblasts labeled with dil: \( D = 9.2 \times 10^{-9} \) cm²/sec (for beam radius = 4 μm); 95% fluorescence recovery. (b) Myoblasts labeled with TNP and rhodamine-labeled antibodies against TNP: \( D = 1.9 \times 10^{-10} \) cm²/sec (for beam radius = 1.1 μm); 54% fluorescence recovery. Note that the time for fluorescence recovery varies inversely as the square of the beam radius (Axelrod et al., 1976a). (Reprinted from Schlessinger et al., 1977a; copyright © 1977 by the American Association for the Advancement of Science.)

The curve for dil in the plasma membrane of an L6 (rat myoblast) cell. The curve behaves according to theory (Axelrod et al., 1976a) for diffusion with a coefficient \( D = 9.2 \times 10^{-9} \) cm²/sec. (Measurements on other cells—including rat peritoneal mast cells, chicken and rat embryo fibroblasts, chicken and rat embryo myoblasts and myotubes, mouse peritoneal macrophage, and 3T3 cells—also yield diffusion coefficients near \( 10^{-8} \) cm²/sec.) Measurements of dil diffusion in L6 and other types of cells usually show complete recovery of the fluorescence in the bleached region to the level prior to bleaching. Therefore, the lipid bilayer exists as a continuous membrane matrix over distances substantially greater than 1 μm (the largest beam radius used), without being significantly partitioned into closed regions. When incomplete recovery is observed in the first bleach, labeling of internal cell structures is also seen. This suggests that immobile dil has been internalized. Cross-linking of membrane proteins does not affect the dil mobility, nor does treatment by azide (which poisons oxidative metabolism), by colchicine (which disrupts microtubule assembly), or by cytochalasin B (which, among other effects, disrupts some microfilaments).

The uniformity of the behavior observed for dil indicates that the factors controlling its lateral mobility vary little among cells of different types and under different conditions. Hence it appears that, to the extent that dil mobility measures a property which might be termed the “fluidity” of the membrane lipid bilayer, that fluidity is a fairly uniform property of cells. Other measures of membrane fluidity have been obtained by methods that detect microscopic rotational motions of spin-labeled (Hubbell & McConnell, 1969) or fluorescence-labeled (Shinitzky et al., 1971) probe molecules. In contrast to these methods, which primarily detect motions over ranges of molecular dimensions, FPR experiments observe lateral motions over macroscopic distances in the range of microns. The relationship between the membrane properties measured by microscopic and macroscopic transport is still unclear; for example, compartmentation could restrict the latter without affecting the former. Detailed experimental comparisons are needed to clarify this matter.

The lateral mobility of components of L6 plasma membranes labeled with fluorescein isothiocyanate or 2,4,6-trinitrobenzene sulfonate is both far slower and more heterogeneous than that of the lipid probe. Mostly these reagents label surface proteins (Edidin, Zagansky, and Lardner, 1976; Comoglio et al., 1975), although membrane components other than proteins may also be labeled to some extent. Surface molecules labeled with trinitrophenyl (TNP) groups were marked by rhodamine-labeled antibody directed against TNP. Diffusion coefficients of molecules marked in either way were close to \( 2 \times 10^{-10} \) cm²/sec (Schlessinger et al., 1977a). Use of the antibody-labeling procedure verified that we were observing cell surface molecules, since the antibody molecules should not permeate the plasma membrane. Comparable results were previously obtained by Edidin, Zagansky, and Lardner (1976) using a similar approach. The similarity in the diffusion coefficients measured for the two types of labels indicates that the hydrodynamic resistance to the motion of the antibody molecule had little effect on the mobility of the surface component to which it was bound. This is not surprising, since in free solution the mobility of an antibody molecule should be 100-1,000 times greater than that measured for the labeled surface components. Figure 1b presents a recovery curve from a cell labeled with TNP and anti-TNP antibodies. The measured diffusion coefficient is \( D = 1.9 \times 10^{-10} \) cm²/sec. A noteworthy feature of this curve is that the fluorescence at long times recovered only about 50% of its value before bleaching. This is in marked contrast to the behavior of dil and is most simply interpreted as an indication that a fraction of
derived from microscopic motions of lipids suggests that the same forces limit both. It may be misleading nevertheless to describe the factors limiting the mobility as a viscosity. It is not certain that fluid-dynamic factors limit the lateral mobility even of so relatively simple a molecule as dil. Interaction of authentic lipids and dil with hydrophobic membrane proteins could also limit their rate of motion.

Evidence for the immobilization of membrane lipids by hydrophobic proteins has been obtained in studies using spin-labeled fatty acids (Jost et al., 1973). The experimental results were interpreted in terms of a boundary layer of lipid strongly absorbed to the protein. The formation of a lipid boundary layer could influence the mobility of both the lipid and the protein. To study this phenomenon, it would be desirable to use fluorescence-labeled lipids that retain their capacity for binding to membrane proteins. Moreover, the diffusion of dil in black lipid model membranes is approximately tenfold faster than in plasma membranes (Fahey et al., 1977). This comparison is clouded, however, by doubts about the adequacy of black lipid membranes as models of biological membranes (P. Fahey and W. Webb, personal communication). It is also important to realize that dil may not participate in some interactions that involve biological lipids (e.g., interactions of glycolipids through their carbohydrate moieties).

Proteins present a more complex picture. They occur in both mobile and immobile states. We have considered two proteins that form specific structures on the cell surface in which the constituent molecules are essentially immobile. The mechanisms by which these structures develop and the forces that maintain them pose an interesting problem for further research. It seems likely that they are different for CSP and AChR. Furthermore, in contrast to CSP, AChR is also present on the cell surface in a diffuse patternless distribution that contains both mobile and immobile molecules. In this respect the diffuse AChR resembles other randomly distributed membrane proteins, even of defined specificity such as the IgE-Fc receptor, which are present in both mobile and immobile states. The physiological significance and structural explanation of these immobile proteins is now unknown, as are the forces that determine the diffusion rates of the mobile proteins. It seems that the apparent viscosity of the lipid bilayer may be too.