LETTER TO THE EDITOR

Criticality of Beam Alignment in Fluorescence Photobleaching Recovery Experiments

Dear Sir:

Fluorescence photobleaching recovery (FPR; Axelrod et al., 1976) techniques are now used routinely to measure rates of diffusion of specific components of cell and model membranes. Our own FPR studies (Leuther et al., 1979) have led us to ask whether diffusion constants obtained by this method have not exhibited more variation than the methodology inherently requires and, if so, what might cause such variation. We conclude that experimenters using this technique should be made aware of an experimental cause of potentially major systematic error and irreproducibility in FPR measurements of diffusion constants. This cause is inadequate superimposition of the bleaching and interrogation light spots on the membrane, thus leading to artificially long fluorescence recovery times. We will discuss why this superimposition is difficult to achieve experimentally but must first develop quantitatively the consequences of bleaching and interrogation spot misalignment on measured diffusion constants. A closed-form solution can be obtained in the experimentally-relevant case of a Gaussian beam and diffusive recovery, provided that consideration be restricted to relatively low extents of bleaching. For higher extents of bleaching, the same general results must still be valid.

The solution is obtained by considering Axelrod and co-workers' (1976) expression for fluorescence recovery after bleaching as expressed in their Eq 6. In our derivation, all terms will have, unless otherwise stated, the same meanings as in this previous work. Three steps lead to the desired results. First, one passes to rectangular coordinates centered on the bleaching spot. This facilitates describing the displacement d of the interrogation spot relative to the bleaching spot. Second, by considering only small extents of bleaching ($K < 0.2$), the initial bleaching-induced concentration fluctuation may be taken to have the Gaussian profile of the bleaching spot. Third, both the displaced interrogation spot intensity $I(x, y, d) = I_0 \exp\{-2[(x - d)^2 + y^2]/w^2\}$ and the post-bleach fluorophore concentration $C(x, y, t)$ are expressed as two-dimensional Fourier integrals with coefficients g_I *and* g_C, respectively. The time-evolution of C follows immediately from the Fourier coefficients $g_C(h, k, O)$ of the initial post-bleach concentration by observing that $g_C(h, k, t) = g_C(h, k, O) \exp[-(h^2+k^2)Dt]$.

The fluorescence $F(t, d)$ observed at time t is then given by

$$F(t, d) = 4\pi^2(q/A) \int \int q_I^*(h, k, d) g_C(h, k, t)\, \mathrm{d}h\mathrm{d}k$$

$$= F(\infty, d) - (c_0 \alpha T I_o^2 \pi w^2 q/4A) \frac{1}{1 + t/\tau_D} \exp\left[\frac{(d/w)^2}{(1 + t/\tau_D)}\right]. \quad (1)$$

Fluorescence recovery curves are conveniently displayed using the fractional recovery $f(t)$ defined by

$$f(t) \equiv [F(t, d) - F(o, d)]/[F(\infty, d) - F(o, d)]. \quad (2)$$

This quantity varies from 0 to 1 as recovery proceeds. Combining Eqs. 1 and 2 yields:

$$f(t) \equiv 1 - \frac{1}{1 + t/\tau_D} \exp(d/w)^2 \exp[-(d/w)^2/(1 + t/\tau_D)]. \quad (3)$$

The exponentials contain the effect of beam misalignment on $f(t)$; and, when d equals 0, the equation reduces to Axelrod's limiting case for small K. The important point is how the observed half-time $t_{1/2}$ for recovery varies with d. Eq. 3 shows that, with perfect alignment, $t_{1/2}$ equals the characteristic time τ_d

which is $w^2/4D$. Since K is small and γ thus approximates unity, the diffusion constant may correctly be calculated as $w^2/4t_{1/2}$. Any misalignment increases $t_{1/2}$ which, if incorrectly equated with τ_D, leads to calculation of artifactually small diffusion constants. For instance, if the degree of misalignment is such that d equals w, a situation very difficult to detect in a microscope photometer, the measured $t_{1/2}$ will be 3.3 times τ_d and thus diffusion constants 3.3 times too small will result. To measure diffusion constants accurate to within 10%, d cannot exceed 0.3 w.

In the usual FPR apparatus the bleaching beam is attenuated 10^3–10^4-fold to a suitable level for monitoring recovery kinetics by mechanically inserting neutral density filters into the unattenuated beam. Filter optical imperfections, primarily wedge, and errors in filter positioning adversely affect the superimposition of bleaching and interrogation spots on the sample. The nature and magnitude of these effects depend strongly on where the filter is placed in the optical system. Two possibilities for filter placement are either in the collimated laser beam before it enters the microscope or within the lens system of the instrument. These cases must be treated separately; and, because the first configuration has been the more frequently used, we will discuss it in greater detail.

A typical FPR microscope optical system is shown in Fig. 2 of Koppel et al. (1976) and all our designations of optical components refer to this figure. The collimated laser beam is focused by an auxiliary lens $L1$ to a spot in the intermediate image plane of a fluorescence epi-illuminator. This plane lies immediately to the left of $L1$. The microscope objective, and perhaps additional lenses such as that shown between $L1$ and the dichroic mirror, projects this image at reduced size onto the sample under study. This reduction factor is the product of the objective magnification M and a magnification r due to any additional lenses in the illuminator between the intermediate image and the objective. In the Zeiss III RS illuminator (Carl Zeiss, Inc., New York) r is 0.50. If a wedged filter (ND1 or ND2) is placed in the collimated laser beam and deviates it at an angle ϵ, the spot in the intermediate image plane is displaced an amount $f\epsilon$ where f is the focal length of the auxiliary lens $L1$. In this configuration only angular displacement of the beam matters and filter location, orientation, and thickness are without effect. The actual displacement at the sample is then $f\epsilon/rM$ and d/w becomes $\pi f\epsilon W/\lambda rl$ where l is the microscope tube length, typically 160 mm, and W is the $1/e^2$ radius of the collimated beam. Given that $W = 0.65$ mm, $\lambda = 514.5$ nm, and $f = 80$ mm, the above equation shows that ϵ cannot exceed 16 arc s if d/w is to remain < 0.3.

High quality filter substrates routinely have a maximum wedge specification of 5 min of arc. This implies beam deviation as large as 2.5 min of arc. We measured our own collection of Baird, Balzers, and other neutral density filters and found beam deviations ranging from a minimum of 48 arc s up to values of over 80 arc s. The best of our filters would thus yield apparent recovery time three times too long if inserted in the collimated laser beam to achieve attenuation after bleaching. Moreover, attempting to measure in the same apparatus the same diffusion constant using others of our neutral density filters would yield results spanning a five-fold range, i.e., D measured 3–15 times too small.

The troublesome aspect of this problem is that its presence may not be all obvious. As long as a laboratory uses a particular neutral density filter combination and a constant diameter laser beam, consistent results should always be obtained. Changing microscope objective magnification is inherently without effect and all filters from a given manufacturer may have comparable wedge. Thus, routine changes in experimental conditions within one laboratory may also not reveal the problem. On the other hand, this effect could be manifest in grossly inconsistent results for diffusion in the same or similar systems being obtained by different research groups. In any case, spot misalignment certainly causes errors in absolute diffusion constants larger than those attributable to the usual ±15% uncertainty in spot radius.

Active efforts to avoid spot misalignment seem preferable to correcting for its effects by such methods as determining diffusion constants relative to a standard dye in solution. Eq. 1 shows that spot misalignment strongly decreases the apparent fluorescence recovery signal achieved under given conditions; and, for low measurable fluorescence, this decreased signal may be unacceptably small. Where neutral density filters are inserted in the collimated laser beam, selection or custom manufacture of filters with extremely low wedge, whilte quite costly, is one way of meeting the problem. An

alternative is the use of a filter substrate coated only over part of its surface and left permanently in the optical path. If wedge is relatively constant over the surface, satisfactory attenuation should be obtained by shifting the filter so the laser beam, originally passing through an uncoated region, now strikes the coating. Our experimental solution has been to use an interferometer light pulse generator (Barisas and Leuther, 1979). The laser beam is separated into two components, one normally blocked by an electronic shutter and the other attenuated by neutral density filters. These beams are recombined by a beam splitter and bleaching pulses of 5 ms to 8 s are produced by opening the electronic shutter. No optical elements move during the operation of the device. The best theoretical alignment of the attenuated and unattenuated beams is ±3 arc s, as limited by mirror mount adjustment resolution. We can in practice achieve, for any particular filter, beam parallelism of ±15 arc s by superimposing the beams at and several meters from the interferometer. This translates into a d/w value of 0.3 and diffusion constants accurate to $+0\%$, -10%.

Instead of locating attenuating filters in the collimated laser beam, such filters may be placed within the lens system, i.e., to the left of $L1$. This permits effects due to filter wedge to be reduced greatly, although some other possible difficulties arise. Since the filter is now in a nonparallel beam, the spot displacement at the sample becomes a function of filter geometry, orientation, and, in particular, location. For minimizing wedge effects the optimal filter location is at the intermediate image plane of the fluorescence epi-illuminator. In such a case, if α is the filter wedge angle, n the index of refraction, t the filter thickness, and β the tilt of the filter away from perpendicularity with the optical axis, then d becomes $(t/rM)(\alpha/2 \pm \beta)(1 - 1/n)$. Calculation shows wedge effects indeed to be negligible. On the other hand the filter must now be kept rigorously perpendicular ($\beta < 34'$ for a 2-mm filter and a $\times 50$ objective) to the optical axis to maintain $d/w < 0.3$. This precise an orientation may be difficult to achieve. The possibility of laser damage to filters must be considered in this optical configuration. If all system parameters have the values used previously in this note, a 100-mW laser focused onto the neutral density filter will expose the filter coating to a damaging power density $>10^4$ W cm^{-2}. Using a longer focal length focusing lens or lens system to produce a larger spot at the filter solves the problem, but additional lenses between the filter and the objective become necessary to compensate for the increased size of the intermediate image. A final difficulty arises from the increased optical path length of a glass filter relative to the air it displaces. Suppose the unattenuated bleaching beam is focused on the sample, say a cell membrane. Then inserting a filter between the objective and $L1$ will raise the focal plane of the attenuated interrogation beam above the cell surface by $(n - 1)\, t/(rM)^2$ or 1.6 μm for a 2-mm filter and a $\times 50$ objective. The interrogation spot profile at the cell membrane will remain Gaussian, but the $1/e^2$ radius will be 8% larger than the bleaching spot. This problem seems best avoided by the use of filters coated only over part of their surfaces and left permanently in the optical path.

We hope these comments will help researchers using FPR to assess what relevance this problem might have to accuracy in their own results and to comparisons of results from different laboratories.

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REFERENCES

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.* **6**:1055–1069.

BARISAS, B. G., and M. D. LEUTHER. 1979. Fluorescence photobleaching recovery measurement of protein absolute diffusion constants. *Biophys. Chem.* **10**:221–229.

KOPPEL, D. E., D. ALEXROD, J. SCHLESSINGER, E. L. ELSON, and W. W. WEBB. 1976. Dynamics of fluorescence marker concentration as a probe of mobility, *Biophys. J.* **16**:1315–1329.

LEUTHER, M. D., B. G. BARISAS, J. S. PEACOCK, and H. KRAKAUER. 1979. Photobleaching recovery studies of membrane events accompanying lectin stimulation of rabbit lymphocytes. *Biochem. Biophys. Res. Commun.* **89**:85–90.

B. GEORGE BARISAS
E. A. Doisy Department of Biochemistry
St. Louis University School of Medicine
St. Louis, Missouri 63104