LETTER TO THE EDITOR

Fluorescence Photobleaching Recovery: Control of Laser Intensities with an Acousto-Optic Modulator

Dear Sir:

Fluorescence photobleaching recovery (FPR; Axelrod et al., 1976) methods are widely used to measure rates of lateral movement of specific molecules in biological and artificial membranes. In this method, the molecules of interest are fluorescently labeled and examined with a fluorescence microscope equipped with a suitable laser. The laser beam is focused to a small spot on the membrane, and a brief but intense pulse of laser light irreversibly photobleaches the fluorescence of the spot. Subsequent recovery of fluorescence in the bleached spot occurs by lateral transport of unbleached molecules from the surrounding membrane, and is measured by excitation with the same laser beam but attenuated by a factor of 10^3–10^5. It is essential that the alignment of the laser beam at the membrane should not alter. A detailed analysis of the errors arising from misalignment has been given by Barisas (1980), who concluded that to measure diffusion coefficients accurate to within 10%, the displacement d of the measuring spot relative to the bleached spot should not exceed 0.3 ω, where ω is the $1/e^2$ radius of the laser beam focused at the membrane. Typically, ω is 1–2 μm.

Attenuation of the laser beam from photobleaching to measuring intensities in earlier FPR instruments was performed by insertion of neutral density filters (Koppel et al., 1976; Jacobson et al., 1976). Such filters usually have sufficient wedge to cause unacceptable spot displacement (Barisas, 1980). Alternative means of attenuation have been described by Leuther et al., (1979) and Koppel (1979), and involve splitting the laser beam into two beams which are subsequently recombined. One of the split beams is electromechanically shuttered to provide the photobleaching pulse, the other is permanently attenuated to provide the measuring beam.

I have developed an alternative and possibly more convenient method of controlling the bleaching and measuring beam intensities. The method uses a digital acousto-optic modulator (Model 304D, Coherent Associates, Danbury, Conn.) to attenuate the average intensity of the laser beam by variation of the on-off duty cycle. For example, to achieve an attenuation of 10^4 the measuring beam is delivered as 10-μs pulses at 10 Hz, whereas bleaching is achieved by an uninterrupted pulse of 0.1–10 s. The measuring and bleaching pulses are controlled with conventional digital electronics, as is the gate of the sample and hold amplifier that converts the pulsed photomultiplier signal into a d.c. signal for pen recorder display. Alternatively, the sample and hold amplifier can be replaced with a gated photon-counting system. There is no requirement for gating or shuttering the photomultiplier, because the pulses of bleaching and measuring laser light have the same intensity. Otherwise the optical geometry follows that shown in Fig. 2 of Koppel et al., (1976) except that the laser beam monitor is omitted and any neutral density filters are permanently in position. The acousto-optic modulator is attached directly to the laser (1.0 W Argon laser, Model 85-1, Lexel Corp, Palo Alto, Calif.) and adjusted for maximal first order diffraction at 488 or 514.5 nm. The diffracted beam is isolated with a 2-mm diam pinhole, and then passes through a spatial filter consisting of a 9-mm focal length input lens, a 10-μm diam pinhole, and an 80-mm focal length collimating lens (Model 1526 beam expander with spatial filter, Oriel Corp, Stamford, Conn.). The spatial filter performs two essential functions. First, it attenuates higher spatial frequencies superimposed on the otherwise Gaussian profile of the beam. (Profiles were measured with a scanning pinhole.) Second, it improves the contrast ratio (i.e., ratio of the peak to trough intensities) of the modulated beam from 10^4 to 2.5×10^5.

Because the bleaching and measuring beams differ only in their duty cycle times, they are expected to be perfectly aligned. Nevertheless, the alignment was checked by photobleaching experiments on rhodamine B immobilized in a thin film of collodion, using a $\times 100$ oil objective, $\omega \sim 1$ μm, and 514.5-nm

laser light. In these experiments advantage was taken of the fact that the fluorescence of the illuminated spot can be measured throughout the bleaching phase, because there is no need to shutter or to gate the photomultiplier. If the laser beam were to shift at the moment when it was switched from the continuous bleaching phase to the modulated measuring phase, then there could be an upwards jump in the observed fluorescence intensity as the beam moved from bleached towards unbleached sample. No such jump was observed. A quantitative analysis of the minimal displacement detectable by this method was based on Fig. 5 of Axelrod et al., (1976). This figure can readily be shown to be identical with a graph of the fractional fluorescence increase that occurs when the measuring spot is displaced relative to the bleached spot, plotted against d/ω. Under the conditions mentioned above, a displacement corresponding to 0.1 ω would have been easily detected; this value is well within the acceptable limit indicated by Barisas (1980).

The advantages of controlling the laser beam with an acousto-optic modulator are as follows: ease of alignment, convenient and rapid digital control, negligible spot displacement with the ability to check it, and direct display of the time course of bleaching. The optical rise and fall times of the acoustic-optic modulator depend on beam diameter, but 0.2 μs is typical. Thus the laser light pulses can be as short as 1–5 μs, which is sufficiently brief to be used in an entirely different application, namely, the measurement of rotational mobility of membrane proteins by depolarization of phosphorescence or delayed fluorescence (Austin et al., 1979; Grienert et al., 1979; Moore et al., 1979). In this context it may be noted that a 1-mW laser beam focused to a 10-μm^2 spot delivers 10 mJ cm^{-2} in 1 μs, and this is a suitable intensity (at 514.5 nm) for exciting delayed photoluminescence from triplet probes such as erythrosin or eosin (Garland and Moore, 1979). Consequently, a further advantage of the acousto-optic modulator is that it enables an FPR instrument (with polarization facilities) to be used for measuring rotational as well as lateral mobility of proteins in cell or model membranes.

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