Fluorescence photobleaching recovery techniques for translational and slow rotational diffusion in solution and on cell surfaces

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Fluorescence photobleaching recovery techniques allow the accurate quantification of lateral and rotational motions of small numbers of specific molecules in complex systems. The technique was first applied to the study of translational diffusion in cell plasma membranes (Peters et al., 1974). This continues to be the principal area of application. More recently, it has been extended to the study of macromolecular diffusion, binding and polymerization in solution (e.g. Barisas & Leuther, 1979; Lanni et al., 1981; Icenogle & Elson, 1983), and in situ, in the cytoplasm of living cells (e.g. Wojcieszyn et al., 1981; Wang et al., 1982; Salmon et al., 1984). Fluorescence photobleaching analysis of slow rotational motion has generated considerable theoretical interest (e.g. Koppel, 1983; Wegener & Rigler, 1984; Wegener, 1984) but few experimental results to date (Johnson & Garland, 1981; Smith et al., 1981; Jovin, 1986). Features and capabilities of the technique, emphasizing the advantages of rotational diffusion over translational diffusion, will be illustrated in this report with recent data from mammalian sperm cells and erythrocyte membranes.

**Underlying principles**

As in nanosecond fluorescence depolarization (e.g. Beechem et al., 1986) or polarized phase fluorometry (e.g. Edgerton et al., 1986), the underlying basis of fluorescence photobleaching is photoselection. The probability of chromophore absorption (and possible subsequent 'bleaching' to a long-lived photochemically distinct state) is proportional to the product of the local incident intensity and the cosine-squared of the angle between the incident polarization and the absorption dipole moment. Photobleaching with non-uniform polarized illumination thus introduces non-equilibrium distributions of probe concentration and orientation.

The approach employed is generally that of a 'pump-and-probe' technique, using intense photobleaching pulses, short compared with the characteristic times of interest. Recovery kinetics are followed with time-resolved measurements of fluorescence depletion after bleaching, monitored with an attenuated constant light source.

Two technical variations have been reported in which the absolute distinction between bleaching and monitoring has been eliminated. In one (Peters et al., 1981), the incident intensity during bleaching is reduced to the point that the rate of diffusion of unbleached molecules into the illuminated region is comparable with the rate of bleaching. Under these conditions, diffusion coefficients can be deduced from the kinetics of fluorescence decay under continuous bleaching illumination. More recently, preliminary reports (Yoshida et al., 1986; Garland & Birmingham, 1986) describe the implementation of polarized fluorescence depletion methods in the frequency domain. Following the lead of recent technical advances in polarized phase fluorometry (Lakowicz et al., 1985), rotational correlation times can be deduced from the amplitudes and phases of fluorescence depletion signal frequencies measured by lock-in amplifiers phase-locked to the intensity and polarization modulation of the incident illumination.

Translational and rotational effects are usually well separated on the basis of time-scale, with even 'slow' (μs-ms) rotational recovery long over before translational recovery starts to become apparent. In exceptional cases, however, it may be necessary to take special measures to distinguish between the two, such as varying the angle between the polarization vectors of the bleaching and monitoring beams (Wegener & Rigler, 1984; Dale, 1985).

Although translational diffusion measurements and rotational diffusion measurements are similar in principle, the latter are technically more demanding, requiring improved time-resolution and rigidly bound fluorescent probes. The presence of significant segmental flexibility or local probe rotation will lead to marked reductions in the initial post-bleach anisotropy (eqn. 30 of Koppel, 1983), restricting the type of label that can be used. Packard et al. (1986) have recently reported a promising fluorescent probe with two sulphhydryl groups designed to rigidly label disulphide-containing peptides.

**Experimental methods and results**

Fig. 1 presents an updated schematic diagram of a fluorescence microscope-based optical system designed for measurements of translational diffusion (Koppel, 1979). For single-cell experiments, the imaging aspect of the microscope is essential for visual inspection, and selecting the appropriate fluorescence excitation and collection. While one can use a microscope system for solution studies, a well-designed reflecting collector can give improved collection efficiency. Moreover, an unbiased collection over a solid angle of 2π or 4π steradians greatly simplifies the theoretical analysis of rotational diffusion (Wegener & Rigler, 1984).

The combination of shutter Sr2 and beam-splitters BS1 and BS2 act to switch the laser intensity between bleaching levels (Sr2 open) and monitoring levels (Sr2 closed). Physically separating the bleaching and monitoring beams between the two beam-splitters also allows the possible selective modification of one or the other in other ways, as shown. For the faster relaxation rates typical of rotational processes, one can control the illumination intensity with an electro-optic or acousto-optic modulator. Furthermore, when necessary, one can automatically control the polarization of laser excitation with a Pockels cell without polarizers.

SM is a servo-activated galvanometric optical scanning mirror, which controls the precise orientation of the incident laser beam and hence the location on the sample of the beam along a scan axis. This additional degree of freedom has proved to be extremely useful in a variety of operational modes employing photomultiplier tube (PMT) detection. The low-light-level video-detector is used in conjunction with a digital image processor whenever a detailed two-dimensional analysis of a complex cellular phenomenon is needed.

Fig. 2 outlines four methods of analysis (I-IV) that are used in this laboratory to characterize lateral transport. The corresponding panels in Fig. 3 present recent data sets illustrating the methods.

(I) Photobleaching with a focused spot or line (e.g. Axelrod et al., 1976; Koppel, 1979) analyses local characteristics. The corresponding data (I) in Fig. 3 is from a guinea-pig sperm cell, surface-labelled with PH22 Mab, a monoclonal antibody specific for an integral membrane protein restricted to the surface of acrosome-intact cells to the posterior head region (Myles et al., 1981; Myles & Primakoff, 1984). The data show near-complete fluorescence recovery within this local domain. The width of the bleached profile, needed for absolute calibration of diffusion coefficient, can also be determined from the series of fluorescence scans after bleaching.

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Abbreviations used are: ND, neutral density filter; Sr, shutter; M, mirror; D, diaphragm; BS, beam-splitter; SM, scanner mirror; L, lens; DM, dichroic mirror; BF, barrier filter; \((L)^T\) TV, low-light-level television; PMT, photomultiplier tube.

(II) Periodic pattern bleaching (e.g. Smith & McConnell, 1978; Lanni & Ware, 1982; Koppel & Sheetz, 1983) analyses characteristics averaged over extended areas. Scanning the monitoring illumination pattern across the bleached pattern gives independent measures of the average fluorescence intensity within the bleached area, and the amplitude and phase of the fluorescence modulation. In this way, one can follow lateral diffusion over two distinctly different characteristic distances (the radius of the illuminated area and the pattern repeat distance), and detect possible systematic flow with high sensitivity (Koppel & Sheetz, 1983).

The corresponding data (II) in Fig. 3 are for a guinea pig sperm cell, surface labelled with PT1 Mab, a monoclonal antibody specific for an integral membrane protein restricted on the surface of uncapacitated cells to the posterior tail region (Myles et al., 1981, 1984; Myles & Primakoff, 1984). The data show near complete fluorescence recovery (> 90%), within this local domain, with a diffusion coefficient \((2.5 \times 10^{-9} \text{ cm}^2/\text{s})\) near the theoretical maximum for an integral membrane protein (Myles et al., 1984). This finding of localization without immobilization strongly suggests that the surface regionalization of these cells is maintained by inter-regional barriers.

(III) A normal mode analysis (Koppel et al., 1980;...
Koppel, 1985) has proved useful in the study of global lateral diffusion on small closed surfaces, such as erythrocyte membranes. One computes decay rates of the amplitudes of particular normal modes of the concentration distribution (and hence D) from the series of high-resolution fluorescence scans.

The corresponding data (III) in Fig. 3 are for a discoidal human erythrocyte labelled with a fluorescent lipid probe, N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (A. Bürkli, M. P. Sheetz & D. E. Koppel, unpublished work). The bleaching beam in this experiment was defocused to cover the central portion of the cell, producing an initial post-bleach distribution closely approximating the second-order normal mode for this geometry (Koppel, 1985). It is seen that redistribution after bleaching is essentially complete, with later scans approaching the initial pre-bleach distribution.

(IV) Bleaching and monitoring with a sharply defined scanning slit image allows one to introduce uniform fluorescence depletions over extended areas. This approach has been designed to study the resistance to inter-regional transport on cell surfaces (Koppel et al., 1986).

The corresponding data (IV) in Fig. 3 are for a guinea-pig sperm cell surface labelled with PT1 Mab, after in vitro capacitation has been initiated. A fraction of the PT1 antigen at this point has migrated from the posterior tail region, across the annulus, to the adjacent anterior tail region (Myles & Primakoff, 1984). In this experiment, the entire surface of the anterior tail region and a small adjacent part of the posterior tail region were bleached. After bleaching, there is clear evidence of inter-regional diffusion, with detailed data simulation indicating a marked restriction of permeability at the inter-regional boundary (Koppel et al., 1986).

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