Interpretation of fluorescence photobleaching recovery experiments on oriented cell membranes

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Both quantitative and qualitative aspects of the interpretation of fluorescence photobleaching recovery experiments as typically practised to obtain information on lateral diffusion processes in cell membranes are called into question in view of the polarized nature of the laser light sources routinely employed. Protocols which will eliminate the effects elicited under these conditions by any concurrent slow rotational diffusion are delineated.

FPR *FRAP* *Fluorescence polarization* *Lateral diffusion* *Rotational diffusion* *Mobile fraction*

1. INTRODUCTION

The technique of fluorescence photobleaching recovery (FPR, also widely known as fluorescence recovery after photobleaching: FRAP; see e.g. [1]) has been utilised extensively in the past 8 years or so in attempts to obtain quantitative information on the rates of lateral diffusion of proteins in a variety of model membranes (see e.g. [2]) and in the plasma membranes of living eukaryotic cells (see e.g. [3–5]). These rates may be important to the efficacy of transport or regulatory processes mediated at the plasma membrane which require association between membrane components [6] and therefore have potential significance also in abnormal, pathological and malignant cell states. Using this technique it has been adduced that the lateral diffusion of small molecules and unaggregated proteins in model membranes above their gel-liquid crystalline phase transition temperature is of the absolute and relative magnitudes expected, with diffusion coefficients D of the order of 5×10^{-8} and 5×10^{-9} $cm^2 \cdot s^{-1}$, respectively. While in real cell membranes the small molecules show similar behaviour, the majority of proteins appear to exhibit a $10–10^4$-fold reduction in D [4]. These proteins are thus either very extensively aggregated or, more probably, attached to relatively immobile submembranous, e.g. cytoskeletal, elements which may themselves be aggregated.

In its conceptually simplest form, the FPR experiment consists of irreversible bleaching of fluorophoric labels introduced into the membrane, e.g. covalently bound to membrane protein, with a powerful pulse of laser light of short duration delivered via a microscope, to an area ('spot') of the order of $1–10$ μm^2, followed by monitoring of the observed recovery of the fluorescence excited in this area by the same laser beam attenuated $10^2–10^4$-fold. This recovery is supposed to be due entirely to lateral diffusional exchange of mobile labelled material between bleached and unbleached regions, and for this [7] and other technical variants of the method such as 'pattern' photobleaching [8], the kinetics of such a recovery have been worked out in detail. The recovery is routinely fitted to one, or sometimes two independent, diffusion coefficients, but is commonly observed to be incomplete, i.e., the pre-bleach intensity monitored is not re-attained. The difference in pre-bleach and long-time limiting post-bleach values is interpreted to represent a fraction of the labelled material which is immobile on the time scale considered (D less than about 3×10^{-12} $cm^2 \cdot s^{-1}$).

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A number of artifacts that might affect these measurements (non-coincidence of bleaching and monitoring beams, local heating, photochemical damage to the membrane) have previously been considered and eliminated, and the theory and measurement in membranes of rotational diffusion in the absence of appreciable lateral diffusion using polarized FPR techniques have also been described [9,10] (see also [1]). However, the effects on FPR lateral diffusion determinations in membranes of the fact that the laser beam is highly polarized per se, and their implications not only for the quantitative, but also for the qualitative, interpretation of these data, do not seem to have been considered explicitly. Actually, these questions have recently been addressed in part for the case of lateral and rotational diffusion of molecules in solution by Wegener and Rigler [11,12]. In particular, using symmetry arguments, they showed that, for unbiased detection of all emitted photons, the electric vectors of linearly polarized bleaching and monitoring excitation beams were required to be oriented to each other at the 'magic angle', $\cos^{-1}(\sqrt{1/3}) \simeq 54.7°$, if translational diffusion in a heterogeneous system was to be cleanly separated from possible rotational diffusion occurring on the same time scale.

2. THEORY AND EXPERIMENTAL CONSIDERATIONS

The conditions considered by Wegener and Rigler do not correspond to those in a typical oriented membrane FPR experiment. There the bleaching and monitoring laser beam, of uncontrolled but high polarization [10], falls perpendicularly onto the, at least locally, essentially planar membrane sample in which the fluorescent probe is embedded. Fluorescence emitted along the same line is detected essentially without bias as to its state of polarization. For heuristic purposes, it will be assumed in the following discussion that:
(i) the bleaching and monitoring excitation beams are perfectly linearly polarized at the sample;
(ii) there is zero bias in collection of emitted photons of different polarization;
(iii) the relevant absorption and emission vectors of the label are perfectly parallel within the frame of the rotating unit to which they are rigidly fixed;
(iv) the only rotational motion is uniaxial about the normal to the perfectly planar membrane along which all beams considered propagate; and
(v) there is complete absence of lateral diffusion of the labelled material in the plane of the membrane.

These conditions maximise the extent of the effects noted. Except for the last they are not usually very widely departed from in typical membrane FPR experiments, in which the additional condition that the electric vectors of bleaching and monitoring excitation light be parallel obtains. They imply no loss in generality of the conclusions. The effects of partial relaxation of these conditions will be considered elsewhere [13].

Only the projections of the absorption and emission vectors onto, and components of emission polarized parallel to, the membrane plane are important. Using the relevant symmetry argument (cf. [11]), or developing the expressions from first principles, as presented more generally and in detail elsewhere [13], it can readily be shown that, relative to unit pre-bleach intensity, the evolution of observed post-bleach fluorescence intensity $F_\psi(t)$, where ψ is the angle subtended between the electric vectors of bleaching and monitoring excitation light, is given by:

$$F_\psi(t) = F(\infty) - (2\cos^2\psi - 1)\{1 - F(\infty)\}p(t) \qquad (1)$$

In this expression, $F(\infty)$ is the final recovery level (independent of ψ, but explicitly dependent on the form and extent of bleaching) and $p(t)$ is the relevant emission anisotropy decay function which here equates with a classically defined polarization decay function:

$$p(t) = \{F_\parallel'(t) - F_\perp'(t)\}/\{F_\parallel'(t) + F_\perp'(t)\} \qquad (2)$$

where the primes signify the difference between pre- and post-bleach signals ($F' = 1 - F$), and the subscripts $\parallel$ and $\perp$ represent the normal designations in fluorescence depolarization spectroscopy for $\psi = 0$ and $\pi/2$ respectively. The anisotropy decay function for a single rotating species is of particularly simple form:

$$p(t) = p_0\exp\{-t/\phi\} \qquad (3)$$

with $0.5 \geq p_0 \geq 0$ in the limiting case considered, depending on the form of illumination and the extent of bleaching, while ϕ is the rotational correla-

tion time, related to the uniaxial rotational diffusion coefficient D_r by $\phi = (4D_r)^{-1}$.

3. DISCUSSION

Firstly, it is noted from eqn 1 that all the time dependence of $F_\psi(t)$ is contained in the term scaled by the factor $2\cos^2\psi - 1$, the analogue in 2-D of the Legendre polynomial $P_2(\cos\psi) = (3/2)\cos^2\psi - (1/2)$ for the 3-D solution case described by Wegener and Rigler [11]. It disappears here for the 2-D magic angle analogue $\psi = \cos^{-1}(\sqrt{1/2}) = \pi/4$ rad. Thus, FPR measurements of this kind require an angle of 45° between the electric vectors of bleaching and monitoring excitation beams to free translational recovery from any possible rotational artifact. Alternatively, if the bleaching and/or monitoring beams exhibit no linear polarization bias, an equivalent condition is achieved. The magic angle alternative may be attained by inserting an appropriately rotatable polarizer or half-wave plate in the excitation beam path, and this will also allow rotational information in the signal to be extracted via $p(t)$. It should be noted, however, that $p(t)$ will in turn be affected by the occurrence of lateral diffusion which will introduce randomly oriented label contributing zero polarization (i.e., dichroism), and thus also contribute to the observed depolarization kinetics. The second alternative involves abrogation of polarized photoselection in the bleaching and/or monitoring beam. This may most simply be achieved by insertion into the excitation path of a quarter-wave plate or suitable scrambling wedge, which may be oriented to depolarize effectively either or both beams.

Secondly, if ψ in eqn 1 is set to zero, the usual experimental FPR condition is approximated and, in the complete absence of lateral diffusion, an FPR curve of qualitatively the form expected for this process will be observed. It will correspond roughly to a putative laterally mobile fraction f_M given by:

$$0 \leq f_M = \{F(\infty) - F(0)\}/\{1 - F(0)\} = p_0/(1 + p_0) \leq 1/3 \quad (4)$$

displayed as a function of bleaching efficiency $1 - F(\infty)$ in fig.1. As can be seen, it constitutes a significant artifact even at bleaching efficiencies as high as 80–90%, and will have an arbitrarily high apparent lateral diffusion rate whose relation to the actual uniaxial rotational diffusion coefficient depends on the form and extent of the bleaching. If, for example, it is calculated at the $t_{1/2}$ point in a 'spot' FPR experiment, the apparent lateral diffusion coefficient will be given by:

$$D = \gamma_D \omega^2 D_r / \log_e(2) \quad (5)$$

where ω characterizes the spot size and the 'constant' γ_D depends on the form and extent of bleaching [7]. Of course, the shape of the recovery curve in the simple case quoted, being monoexponential, is quite unlike that due to lateral diffusion [7]. On the other hand, if 2 or more species of fairly widely separated rotational correlation times are contributing, or in the case of very slow recovery, the curves may be difficult or impossible to differentiate. Rotational correlation times of the order of 0.1 s and longer, typically several tens to several hundreds of seconds, would give rise in this way to the magnitudes of lateral diffusion coefficients reported. For fluid membranes these would correspond to massive aggregates – larger than the spot size in many cases. Unaggregated membrane proteins exhibit rotational correlation times in the micro- to millisecond time range if they are not at-

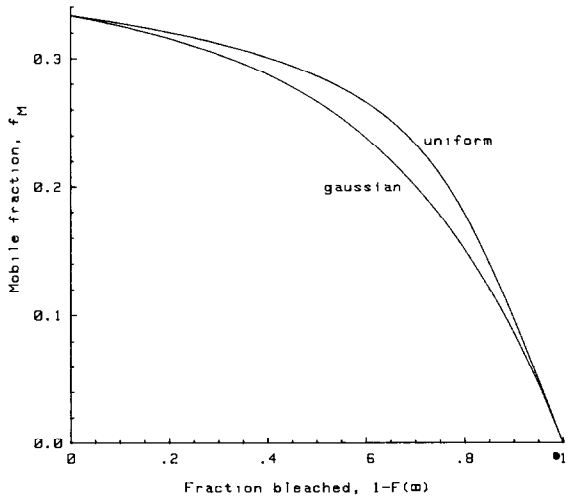

Fig.1. Apparent laterally mobile fraction f_M arising from rotational relaxation in the absence of lateral diffusion as a function of the bleaching efficiency $\{1 - F(\infty)\}$ for linearly polarized laser excitation with uniform disc and Gaussian intensity profiles [7,13].

tached to submembranous cytoskeletal components (e.g. [14]), and for such cases the effects would be randomized out before they could be detected. On the other hand, even small molecules have been shown to exhibit rotational correlation times of about 1–100 s in gel-phase model membrane systems [10].

In conclusion, it seems reasonable to suppose that, in a complex, multi-domain system such as the cell membrane, there may be ample opportunity, in both the presence and absence of lateral diffusion, for rotational diffusion effects of the above kind to influence considerably the observed course of the recovery, and hence at least the quantitative, and sometimes even the qualitative, interpretation of FPR data as routinely obtained heretofore. It is simple to eliminate such possible effects by the insertion of an appropriate polarizing or depolarizing element into the laser beam path.

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