Membrane bound protein diffusion viewed by fluorescence recovery after bleaching experiments: models analysis.

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2002, july 24th

Abstract

Diffusion processes in biological membranes are of interest to understand the macromolecular organisation and function of several molecules. Fluorescence Recovery After Photobleaching (FRAP) has been widely used as a method to analyse this processes using classical Brownian diffusion model. In the first part of this work, the analytical expression of the fluorescence recovery as a function of time has been established for anomalous diffusion due to long waiting times. Then, experimental fluorescence recoveries recorded in living cells on a membrane-bound protein have been analysed using three different models: normal Brownian diffusion, Brownian diffusion with an immobile fraction and anomalous diffusion due to long waiting times.

1 Introduction

Early models of the plasma membrane, notably the fluid mosaic model [1] postulated that transmembrane proteins were freely diffusing in a sea of lipids. During this last decade, it has become apparent that cell surface membranes are far from being homogeneous mixture of their lipid and protein components. They are compartmented into domains whose composition, physical properties and function are different. Numerous studies on transmembrane proteins by means of single particle tracking (SPT) or fluorescence recovery after photobleaching (FRAP) has shown the existence of micrometer and submicrometer size domains on both model membrane and living cells [2,3,4,5]. Kusumi et al. [4] have used FRAP to study the lateral diffusion of membrane proteins in human erythrocyte ghosts and have observed that the recovery of fluorescence intensity is slower than that expected for normal Brownian diffusion.

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al. recently formulated the "matrix" or "skeleton fence" model for hindered protein movements in which transmembrane proteins are corralled by a fence of cytoskeleton just beneath the membrane [4].

FRAP experiments have been used for determination of long-range molecular diffusion of proteins and lipids on both model system and cells for more than 30 years [6, 7]. Briefly, fluorescently labelled molecules localized within a predefined area are irreversibly photo-destructed by a short and intense laser pulse. The recovery of the fluorescence in this area is then measured against time. Since no reversible photoreaction occurs, recovery of the fluorescence in the photobleached area is due to diffusion. FRAP data are generally interpreted by assuming classical Brownian diffusion. Two parameters can then be obtained: D, the lateral diffusion coefficient and M, the mobile fraction of the diffusing molecule. When the radius of the photobleached area is small compared to the diffusion area, M must be equal to 1 for freely diffusing species. In fact, most of the data reported so far in biological membranes for transmembrane proteins shows a value of $M < 1$. This lack in total fluorescence recovery can be interpreted as a restriction to free-diffusion behaviour. Parameters obtained have then to be re-evaluated to recognize the effect of time-dependent interactions in a field of random energy barriers.

Membrane bound proteins should also be submitted to several interactions with their surrounding that should account for an anomalous subdiffusion behaviour. Sources of restriction to free diffusion may include lipid domains trapping, binding to immobile proteins and/or obstruction by cytoskeletal elements. Therefore, in this letter, diffusion of an intracellular membrane-bound protein domain (pleckstrin homology domain) has been analysed inside living cells by FRAP experiments. Previous structural studies have shown that these proteins are linked to the polar head of specific lipids by means of electrostatic interactions [8]. Furthermore, the protein used in this study (PH domain of Exchange Factor for ARF6) appears to have a functional requirement to be associated to the plasma membrane within cells [9]. After an analytical determination of the fluorescence recovery function based on an anomalous subdiffusion model, experimental recoveries obtained in living cells were analysed using random diffusion with or without an immobile fraction and compared to the analyse using time-dependent anomalous subdiffusion.

2 Anomalous sub-diffusion Modeling

A way to describe sub-diffusion is to start from a two dimensional random diffusion process. A particle walks from trap to trap and spend a certain (random) time in each trap. It is characterized by the following operation:

$$r \rightarrow r + \Delta; t \rightarrow t + \tau$$

(1)

$r$ and $t$ are respectively the two dimensional position and the age of the particle, where $\Delta$ is a two dimensional random (Gaussian) variable with variance $v = 2D$, and $\tau$ is the (random) time the particle spend in the trap.
In our model, the particle is supposed to diffuse very rapidly between two traps. This travel time is therefore neglected. The time $\tau$ the particle stays in a trap is supposed to have very strong fluctuations, this give rise to anomalous diffusion pattern.

As an example a generic distribution is used which leads, after a while, to a standard Levy law in time:

$$P_0(\tau) = \frac{\alpha}{(1 + \tau)^{\alpha+1}}$$

This distribution have been used in the same type of context by Naggle [10].

The Levy exponent $\alpha$ is the characteristic exponent of subdiffusive behaviour. For long times we have:

$$< r^2(t) > \propto t^\alpha$$

When $\alpha < 1$ a spatio-temporal Fourier (Laplace) analysis leads to the following asymptotic ($\omega, k \to 0$) Green function:

$$\tilde{g}(k, \omega) = \frac{1}{\omega(D_\alpha k^2 \omega^{-\alpha} + 1)} ; \quad D_\alpha = D/\Gamma(1-\alpha)$$

where $\omega$ and $k$ are respectively the conjugate variables of position $r$ and time $t$, where $k = |k|$. Notice that the solution of the inverse Laplace transform is a function of the variable $k^2 t^\alpha$. It follows that the Green function is a function of the variable $x = r^2/t^\alpha$. When $x$ is high enough one can perform an approximate inverse transformation via a saddle point method:

$$g(r, t) \propto \exp(-cst \, x^\nu) ; \quad \nu = \frac{1}{2-\alpha} , \quad cst : \text{a known constant}$$

Notice that the exponent $\nu$ interpolate nicely between the gaussian case ($\alpha = 1$) and the exponential case. The general solution of this type of anomalous diffusion process is then:

$$\rho(r, t) = \int \rho_0(r' - r)g(x(r', t)) \, d^2 r'$$

where $\rho$ is the probability density to find the particle at the point $r$ at instant $t$ and $\rho_0$ is the initial state.

As the green function is a bell-shaped fast decreasing function, one approximate it by a gaussian shape with the exact dispersion, $\tilde{D}_\alpha = D \sin(\pi \alpha)/(\pi \alpha)$, which can be calculated from eq.4. This permits to construct an analytical expression of the fluorescence recovery using standard properties of Gaussian functions.

Starting from Axelrod [1] initial density as it is immediately after a Gaussian laser beam profile extinction indeed:

$$\rho_0(r) = \exp(-K \exp(-2\frac{r^2}{R^2}))$$

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(K=photobleaching constant, depending on experimental conditions and using the standard properties of the Gaussian shape in the convolution operation, one can obtain the time evolved result as a series.

Once integrated upon a disk of radius $R$, and, after normalization to the surface of the disk, one obtain the FRAP signal:

$$I_R(t) = 1 + \sum_{n=1}^{\infty} \frac{(-K)^n}{n!} \frac{1}{2n} \left(1 - \exp\left(-\frac{2nR^2}{R^2 + 4nD_\alpha t^\alpha}\right)\right)$$

(8)

This function will be used to fit experimental data.

Systematic corrections of this procedure are determined using numerical Monte-Carlo simulations of the fluorescence recoveries, using known $\alpha$ and $D_\alpha$.

A more precise study of the Green function will be published later.

3 Experiments

Experiments were conducted on Baby hamster kidney cells (BHK) grown on a coverslip in cell culture medium for 2 days. Cells were transfected 24 hours before the FRAP experiment with a pC1EGFPPLHEF6 plasmid. This plasmid contains the sequence for both PH-EF6 domain and EGFP as a fluorescent label, linked to the N-terminus of the PH-EF6 domain in order to avoid any perturbation to the membrane linkage. FRAP measurements were made with a commercially available confocal microscope. Prebleached images were firstly acquired to ensure for the lack of photo-destruction during the observation. A brief laser pulse was then delivered to the cell (500ms). Images were thereafter recorded at given intervals (440ms). The intensity ratio between the extinction laser beam and the monitoring laser beam was fixed to 10\(^6\). Each fluorescence recovery was recorded for 80 s at 25 C, containing 150 experimental values. Focus of the laser by a 63x objective produced a Gaussian intensity distribution of the beam in the object plane.

4 Results

In order to validate our model, Monte-Carlo simulation of the fluorescence recovery have been made using different values of $\alpha$ and different value of $D$ (see fig. 1) with $K=4$ in every case since this value of $K$ was the one found in FRAP experiments. These simulations have then been fitted using a 10\(^{th}\) order limited development of the fluorescence recovery equation established for anomalous diffusion (eq. 8). Input $\alpha$ of respectively 0.6, 0.7, 0.8 led to fitted value of $0.5 \pm 0.02$, $0.65 \pm 0.03$ and $0.75 \pm 0.03$. Absolute error measured on $\alpha$ is found between $9 \pm 3\%$ and $6 \pm 3\%$, decreasing with increasing $\alpha$. Values found for $D$ are further from those input in our simulations (ranging from 0.25 to 10). For this reason, results were calibrated using appropriate factors for the
Figure 1: Monte-Carlo simulation of normalized fluorescence recoveries in the case of anomalous subdiffusion. Different values of $D$ and $\alpha$ have been tested in the simulations. Here, values of $D = 0.5; 1; 1.5$ are represented from bottom to top with different $\alpha$ in each case: 0.6 (dots); 0.7 (thin line); 0.8 (thick line). The Monte Carlo has been constructed with $10^7$ individual trajectories.

| $\alpha = 0.6$ | $\alpha = 0.7$ | $\alpha = 0.8$ |
|---------------|----------------|---------------|
| Input $D$     | Fitted $D$     | Correction    | Fitted $D$ | Correction | Fitted $D$ | Correction |
| 0.5           | 0.305          | 1.639         | 0.322      | 1.551      | 0.378      | 1.322      |
| 1             | 0.460          | 2.172         | 0.534      | 1.871      | 0.627      | 1.596      |
| 1.5           | 0.666          | 2.253         | 0.726      | 2.066      | 0.872      | 1.721      |

Table 1: Correction factors for $D$. Correction factors have been obtained by fitting Monte-Carlo simulations of normalized fluorescence recoveries with a $10^7$ order limited development of the analytical expression established here (eq. for D).
three values of \( \alpha \). A comparison of input and found values of \( D \) as well as some correction factors are given in Table 2.

Experimental fluorescence recoveries of the protein obtained in living cells (\( n=17 \)) were fitted by three different models (Fig. 2):

- classical diffusion without restriction (derived from eq.12 of Axelrod [1]),
- classical diffusion introducing an immobile fraction \( M \). For this model eq.12 from Axelrod [1] has been derived, leading to the following expression of \( I_R(t) \):

\[
I_R(t) = \frac{1-\exp(-K)}{K}(1-M) + M \sum_{n=1}^{\infty} \frac{(-K)^n}{n!(1+n+8nDt/R^2)}
\] (9)

- time dependent anomalous diffusion (for which the analytical fluorescence recovery has been established in the modeling section (eq.8)).

The quality of the fit was estimated using a \( \chi^2 \) statistical test.

Table 2 shows that fitting the experimental curves with the classical model of Axelrod led to very bad results, whereas using a limited development to the

Figure 2: Fit of the experimental normalized fluorescence recoveries:

Upper part: Experimental curve fitted using the three different models (see text for explanation). Lower part: Difference between experimental values and the three models: \( Fc-Fo \). Thin line: Normal diffusion without immobile fraction, Dashed line: Normal diffusion with immobile fraction, Thick line: Anomalous subdiffusion.
α  M  D(μm².s⁻¹)  $\tilde{D}_\alpha$(μm².s⁻α)  $\chi^2$
-  1  0.119 ± 0.061  -  5.7 ± 0.5
-  0.917 ± 0.004  0.217 ± 0.010  -  3.8 ± 1.6
0.63 ± 0.02  -  1.48 ± 0.05  2.9 ± 1.6

Table 2: Experimental values obtained with the different models. Values obtained after fit of the experimental recovery using the three different models described in the text.

10th order of eq.(8) and eq.(9) led to slightly different quality of the fit.

Once corrected by the previously determined factor, the calculated value of \( D \) for anomalous sub-diffusion using the expression \( D = \tilde{D}_\alpha(\pi\alpha)/\sin(\pi\alpha) \) gives \( D = 10.4 \pm 0.7 \mu m^2.s^{-\alpha} \).

It has to be noted that value of \( D \) found using the two models are (intrinsically) different. However they can be compared according the relation : \( D(t) = Dt^{\alpha-1} \). Therefore, if one estimate \( D(t) \) at time of half recovery of fluorescence \( (t = 14, 4s) \), as it is usually done for the estimation of \( D \) using classical diffusion model \[6, 7, 12\] a value of \( D(14, 4) = 3.9 \pm 0.4 \mu m^2.s^{-1} \) can be found which is more than ten time higher than the value found using classical diffusion with an immobile fraction, \( D = 0.217 \pm 0.010 \mu m^2.s^{-1} \).

5 Discussion

In this paper, anomalous subdiffusion in fluorescence recoveries experiments have been reexamined from the beginning. An analytical formulation of the recovery curve as a function of time have been calculated using a Gaussian extinction profile. This equation has been tested by fitting Monte-Carlo simulated fluorescence recoveries. As already observed by Feder et al. \[11\], \( \alpha \) was underestimated in the fit. This could be explain by asymptotic effects occuring because of the time-scale of the experiment \[11\]. More surprisingly, \( \tilde{D}_\alpha \) was also itself underestimated. This has not been underlined by Feder et al. \[11\], but seems to be crucial for correct estimation of \( D \). Combination of these two parameters led us to established correction factors, depending both on \( \alpha \) and \( \tilde{D}_\alpha \) to achieved measurement of \( D \).

The experimental recoveries obtained on PH-EFA6 showed that Brownian diffusion without immobile fraction did not fit the data. This strongly suggest that this membrane bound protein is also submitted to restricted motion at the surface of the membrane. Therefore, it was interesting to analyze the recoveries using the two limit models of restricted motion. Whereas this work show that it is not possible to firmly distinguish between free-diffusion with an immobile fraction and anomalous diffusion of the entire set of proteins only by statistical considerations on the quality of the fit, estimation of \( D \) at half time recovery obtained using anomalous diffusion \( (D \simeq 4 \mu m^2.s^{-1}) \) led to a value close to
that obtained for lipids in fluid-phase model membranes (lipid bilayers without proteins). This value would mean that diffusion of this protein from trap to trap is mainly due to its lipid links. This hypothesis is acceptable regarding biochemical data obtained on interaction of this protein domain with lipid membrane.

While analysis of SPT measurements using anomalous diffusion is now becoming usual it is still difficult to elucidate its relevance in FRAP experiments. Indeed, as a limit of the technique, FRAP inherently averages over a large number of particles. This could explain why anomalous diffusion model (in which complete but restricted diffusion is allowed) and classical Brownian motion with an immobile fraction (in which free diffusion occurs for one subpopulation and no diffusion for the other subpopulation) lead to the same statistical quality for fitting experimental data. Therefore there is still a challenge in trying to agreement SPT data with FRAP data on biological molecules in situ.

6 Acknowledgments

Authors would like to acknowledge M. Franco for kindly giving us the plasmid used for PH-EFA6 expression in cell lines and M. Partisani for cell culture and transfection. They are also indebted to A. Lopez for critical reading of the manuscript and fruitfull discussions.

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