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Fluorescence correlation and lifetime correlation spectroscopy applied to the study of supported lipid bilayer models of the cell membrane

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ABSTRACT

Supported Lipid Bilayers (SLBs) are versatile models capable of mimicking some of the key properties of the cell membrane, including for example lipid fluidity, domain formation and protein support, without the challenging complexity of the real biological system. This is important both from the perspective of understanding the behaviour and role of the lipid membrane in cell structure and signalling, as well as in development of applications of lipid membranes across domains as diverse as sensing and drug delivery. Lipid and protein diffusion within the membrane is vital to its function and there are several key experimental methods used to study membrane dynamics. Amongst the optical methods are Fluorescence Recovery After Photobleaching (FRAP), single particle tracking and Fluorescence Correlation (and Fluorescence Lifetime Correlation) Spectroscopy (FCS/FLCS). Each of these methods can provide different and often complementary perspectives on the dynamics of the fluid membrane. Although FCS is well established, FLCS is a relatively new technique and both methods have undergone a number of extensions in recent years which improve their precision and accuracy in studying supported lipid bilayers, most notably z-scan methods. This short review focusses on FCS and FLCS and their recent applications, specifically to artificial lipid bilayer studies addressing key issues of cell membrane behaviour.

1. Introduction

The lipid membranes of the living cell are dynamic and complex structures whose fluidity is central to their function. Membrane fluidity permits lipid reorganisation, protein diffusion, conformational change and aggregation essential for transmembrane ion/molecular transport and the signalling processes that the membrane mediates.

Artificial models of biological membranes can provide important insights into the behaviour of lipids and membrane associated proteins by mimicking key facets of the cell membrane structure decoupled from the challenging complexity of the living cell. A key prerequisite for a membrane model is that the fluidity of the lipid matrix is maintained. Amongst the many approaches to mimicking the cell membrane are liposomes, black lipid membranes, supported lipid bilayer (SLB) models and their variations [1–3]. SLBs are generally superior in terms of reproducibility, stability and versatility. However, interactions of lipid and particularly incorporated proteins with the underlying substrate can lead to undesirable impediments to the diffusion of these moieties, or worse, degradation of the protein from direct surface adsorption. In recent years there have been several advances which have addressed these issues, including cushioned and tethered lipid bilayer models [4–7]. Study of the dynamics of such models, in particular lipid and/or protein diffusion rely commonly on optical measurements, most traditionally on fluorescence recovery after photobleaching, FRAP. In FRAP, a volume, typically on the order of several μm^3 is treated to an intense pulse of laser which serves to bleach or photodegrade the luminescent probe within this volume. The recovery of the fluorescence intensity to the bleached region, due to diffusion of unbleached probe into the bleached volume, is then measured over time. The resulting intensity-time curve is then modelled to obtain a diffusion rate for the probe. FRAP has the key advantage that it can be carried out on most conventional confocal microscopes. In addition, by measuring the extent of recovery of the initial fluorescence intensity, it can provide an estimate of the fraction of a given species that is mobile. This is a useful quantity in validating the absolute mobility of SLBs. In particular, in those containing transmembrane proteins, it is a means of assessing if there are non-physiological substrate–protein interactions occurring [8,9]. However, because of the large interrogation area in complex samples the diffusion recovery may be complex and difficult to resolve, although this can be of value in studies of hindered diffusion, for example on protein

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meshes. In contrast the FCS/FLCS methods are non-destructive and monitor diffusion through a volume which is diffraction limited (although in some applications it may be selected to be larger). Other advantages of FCS and FLCS over FRAP are that they can provide insights into the absolute concentration of the fluorophore and so are useful in determination of, for example, membrane binding and kinetics. Both FRAP and F(L)CS rely on confocal optics therefore signal can be optimised in the z-direction so that accurate selection of a region of interest in a given cell or membrane is possible. However, F(L)CS methods also require only very low levels of fluorophore, reducing over-labelling of background and limiting any influence such exogenous agents may impose on the dynamics of the bilayer or its components, as discussed below. Furthermore, multi-detector formats permit dual-colour experiments and dual-foci experiments which permit multiple positions or multiple fluorophores to be studied simultaneously.

This short review outlines, with selected examples from recent literature, some of the issues that can be addressed by FCS and FLCS in the study of SLBs and some associated model membrane structures. In particular, the study of lipid diffusion, protein diffusion and the effects of nanostructures on lipid or protein diffusion. We describe here the basic principles of the techniques of FCS and FLCS and direct readers as well to some of the excellent reviews of these techniques in recent years [10,11].

2. Methodology and theoretical principles of fluorescence correlation spectroscopy (FCS) and fluorescence lifetime correlation spectroscopy (FLCS)

2.1. Fluorescence correlation spectroscopy (FCS)

FCS is a technique based on the statistical analysis of fluorescence intensity fluctuations. These fluctuations typically originate from the translational diffusion of fluorescent particles through a small observation volume (≈1 fL). In confocal FCS the size of the observation volume is minimized by employing spatially-selective excitation, provided by a focused laser beam, and spatially-selective detection, provided by a pinhole (diameter ≈ 50 μm). Minimizing the observation volume is essential, as ideally the best quality data is obtained when the observation volume contains only one fluorescent particle [12,13]. Under these circumstances, the diffusion of the fluorescent particles into the observation volume yields fluorescent intensity fluctuations which can be autocorrelated according to Eq. (1) [14].

$$G(\tau) = \frac{\langle \partial F(t) \partial F(t+\tau) \rangle}{\langle F \rangle^2} \tag{1}$$

where $\langle\rangle$ denotes the time average, and $\partial F(t)$ and $\partial F(t+\tau)$ are the fluorescence intensity fluctuations around the mean value, $\langle F \rangle$, at time t and $t+\tau$, respectively. In the case of fluorescent particles diffusing freely in solution, a three-dimensional expression for the autocorrelation function should be utilized, whereas in the case of fluorescent particles whose diffusion is limited to two dimensions, such as fluorescently-labelled lipids (or proteins) immersed in a lipid bilayer, a two-dimensional expression for the autocorrelation function is more appropriate. Therefore, in this contribution, we will focus mainly on the two-dimensional expressions which are commonly used for the study of probes embedded in lipid bilayers. If the diffusion of the fluorescent particles is constrained to a detection profile that can be approximated by a two-dimensional Gaussian, the theoretical shape of the autocorrelation function (ACF) is given by Eq. (2):

$$G(\tau) = \frac{1}{N} \cdot \frac{1}{1 + (\tau/\tau_D)} \tag{2}$$

where N is the average number of fluorescent particles in the observation volume, and τ_D is the diffusion time [15]. The diffusion time is related to the diffusion coefficient, D, of the fluorescent particles by Eq. (3):

$$\tau_D = \frac{\omega^2}{4D} \tag{3}$$

In this expression ω is the e^{-2} radius of the laser focus [16]. Unlike D, τ_D is not a molecular property because its value depends on the size and shape of the observation volume. Although N does not depend on molecular brightness, defined as the number of photons emitted per second for a single fluorescent particle, a high molecular brightness improves the signal-to-noise ratio. High molecular brightness can be achieved by using fluorophores with high fluorescent quantum yields, large absorption cross-sections, and high photostability [16]. The molecular brightness also increases with increasing excitation intensity. However, high excitation intensities can lead to photobleaching (photochemical destruction of the dye) and can increase the fraction of fluorophores in the triplet state (see below). To avoid these undesirable phenomena, the excitation intensity in FCS is typically lower than 100 μW (<20 kW cm^{-2}) [17,18], although it is dye dependent and may need to be considerably lower than this value. In general, using a suitable fluorophore and a suitable excitation wavelength a molecular brightness of 1–5 × 10^3 counts per second per molecule should be obtained [19]. A low molecular brightness (i.e., 700 counts per second per molecule) can in principle be compensated for by using long acquisition times. As a rule of thumb, the acquisition time must be at least 10,000 times longer than the diffusion time of the fluorescent particles [11]. Therefore, when working with lipid bilayers the measurements must be recorded for 10 s or more, as the diffusion times of labelled lipids usually range from 3 to 10 ms. In practise, long acquisition times are challenging to perform because the laser focus tends to drift from the plane of the lipid bilayer over time. This drifting of the focus introduces artifacts in the autocorrelation curve, especially at long correlation times. Although complete elimination of instrumental drift is impossible, allowing the instrument and the sample to reach thermal equilibrium prior to commencement of experimentation can reduce mechanical instability.

Apart from translational diffusion, FCS permits the study of all processes that result in microsecond or millisecond fluorescence intensity fluctuations. One such process is intersystem crossing to the triplet state, which is facilitated by the use of high excitation intensities. Therefore, when the use of high laser powers is required an additional term can be added to Eq. (2), so as to yield Eq. (4) [20]:

$$G(\tau) = 1 + [1 - T + T\exp(-\tau/\tau_T)] \frac{1}{N(1-T)} \frac{1}{1 + (\tau/\tau_D)} \tag{4}$$

where T is the fraction of molecules in the triplet state and τ_T is the intersystem crossing relaxation time. It should be emphasized that in the case of dyes with a high triplet quantum yield the use of Eq. (4) may be required even at low excitation intensities.

FCS can provide information about the type of diffusion that is being observed. In the case of normal diffusion, also termed Fickian diffusion the mean square displacement of the particles, $\langle r^2(t) \rangle$, increases linearly with time: $\langle r^2(t) \rangle = 4Dt$ [21]. In the case of anomalous diffusion, however, the dependence becomes non- linear: $\langle r^2(t) \rangle = 4\Gamma t^\alpha$, where α is the anomalous coefficient and Γ is the transport coefficient, which has the dimensions of area per fractional time (μm^2 s$^{-\alpha}$). Anomalous diffusion occurs, for example, where the mobility of the probe is hampered, such as in the case of lipid–protein binding interactions, the presence of immobile randomly-distributed obstacles, the formation of lipid microdomains, or the existence of regular protein networks (i.e., the cyto-