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The imaging FCS diffusion law in the presence of multiple diffusive modes

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ABSTRACT

The cellular plasma membrane is the barrier over which cells exchange materials and communicate with their surroundings, and thus plays the central role in cellular sensing and metabolism. Therefore, the investigation of plasma membrane organization and dynamics is required for understanding of cellular functions. The plasma membrane is a heterogeneous matrix. The presence of structures such as lipid and protein domains and the cytoskeleton meshwork poses a hindrance to the free diffusion of membrane associated biomolecules. However, these domains and the cytoskeleton meshwork barriers are below the optical diffraction limit with potentially short lifetimes and are not easily detected even in super-resolution microscopy. Therefore, dynamic measurements are often used to indirectly prove the existence of domains and barriers by analyzing the mode of diffusion of probe molecules. One of these tools is the Fluorescence Correlation Spectroscopy (FCS) diffusion law. The FCS diffusion law is a plot of diffusion time (τ_d) versus observation area. For at least three different diffusive modes – free, domain confined, and meshwork hindered hop diffusion – the expected plots have been characterized, typically by its y-intercept (τ_0) when fit with a linear model, and have been verified in many cases. However, a description of τ_0 has only been given for pure diffusive modes. But in many experimental cases it is not evident that a protein will undergo only one kind of diffusion, and thus the interpretation of the τ_0 value is problematic. Here, we therefore address the question about the absolute value of τ_0 in the case of complex diffusive modes, i.e. when either one molecule is domain confined and cytoskeleton hindered or when two molecules exhibit different diffusive behavior at the same position in a sample. In addition, we investigate how τ_0 changes when the diffusive mode of a probe alters upon disruption of domains or the cytoskeleton by drug treatments. By a combination of experimental studies and simulations, we show that τ_0 is not influenced equally by the different diffusive modes as typically found in cellular environments, and that it is the relative change of τ_0 rather than its absolute value that provides information on the mode of diffusion.

1. Introduction

The cell membrane is a highly dynamic, complex system with manifold functions. It provides stability as a mechanical barrier, acts as a semi-permeable boundary, and is the site for several key cellular processes including cell signaling and transduction. In order to understand the kinetics of protein interactions involved in cell signaling on the cell membrane, knowledge about their binding affinities and concentration alone is inadequate. It is also crucial to understand the dynamics on the membrane, and the factors that influence it [1]. Proteins cannot freely diffuse in a Brownian fashion on the cell membrane and are constrained by several barriers [2,3]. Various protein and lipid domains that can vary in size from nanometers to micrometers, and can be stable or transient, can influence protein and lipid dynamics [4]. Lipid rafts, for instance, contain a higher concentration of sphingolipids and cholesterol, and are dynamically and structurally different from the rest of the matrix [4–8]. The hydrolysis of sphingomyelin generates ceramide molecules which self-associates to form small caveolae of ceramide rich micro domains [9,10]. Certain proteins like DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin) form clusters on the membrane, leading to the formation of protein domains [11,12]. These domains are portals for regulating several vital cellular functions

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such as receptor signaling, membrane trafficking, or host-pathogen interactions among others [5,11–19]. These membrane domains act as transient trapping sites and thus hinder the free diffusion of proteins [20,21]. Apart from these rafts, the cytoskeleton meshwork also acts as compartmentalization barriers for transmembrane proteins. The 'picket-fence model' postulates that membrane proteins associated with the cytoskeleton display free diffusion within the compartment, but for long range diffusion across compartments of the meshwork, they must undergo hop-diffusion [22–26]. Several optical techniques such as Fluorescence Recovery after Photo bleaching (FRAP), Single Particle Tracking (SPT), and Fluorescence Correlation Spectroscopy (FCS) have been employed to investigate the molecular dynamics of these membrane proteins, and gain insight into the diffusion barriers influencing it [1,27–29]. FRAP can detect domains in bilayers [30] and in cell membranes [31,32] by measuring the apparent diffusion coefficient at varying illumination spot sizes. However, FRAP demands a high laser power for photo bleaching, and high concentration of fluorescent probes at the region of interest. Single particle tracking (SPT), a single molecule sensitive technique with sufficient spatiotemporal resolution, has also detected the presence of domains and compartmentalization barriers on the cell membrane. SPT plots the fluorescent probe's time averaged mean square displacement (MSD) versus time, and identifies the different diffusion states of an individual molecule using appropriate fitting models [19,22,33–35]. However, use of large fluorescent labels such as antibodies and gold particles to obtain good signal to noise ratio with a temporal resolution in the microseconds scale pose a challenge as they might change the diffusive behavior they are supposed to measure [36,37]. A method that uses smaller fluorescent tags such as organic dyes and fluorescent proteins is FCS. FCS measures the diffusion time of fluorescent tags in a small observation area or volume based on an autocorrelation analysis of the fluctuations in the fluorescence intensity at a given area or volume [38]. FCS is a single molecule sensitive technique, that generates a good S/N ratio using standard fluorescent dyes and proteins, hence a valuable technique to obtain information regarding membrane organization and dynamics [39–41]. Imaging FCS (ImFCS) is a multiplexed modality of FCS [42–45], where a fast sensitive camera is used to collect signal from the illuminated sample plane in a total internal reflection fluoresce microscope (TIR-FM) [46] or single plane illuminated microscope (SPIM) [47,48]. Each pixel is treated as an observation area, and an autocorrelation function (ACF) is calculated for each pixel, thus measuring across a larger area of the sample in a single measurement [49,50]. Conventional FCS is, however, diffraction limited, and one cannot measure the dynamics of the proteins in the aforementioned barriers such as lipid rafts and cytoskeleton meshwork which are below 200 nm [51–53]. Hence, the 'FCS diffusion law' was introduced which helped differentiate between free diffusion, domain confined diffusion, and hop-diffusion due to cytoskeleton meshwork compartmentalization. The FCS diffusion law is a plot of diffusion time versus observation area, and an analysis of these plots helps identify the mode of diffusion [54,55]. The FCS diffusion law was extended to ImFCS, where varying observation areas can be created by software post acquisition pixel binning, thus requiring only a single measurement [36,44,50,56]. A plot of diffusion time (τ_d) versus effective observation area (A_{eff}) was generated, and the intercept (τ_0) of the plot was calculated. The dependence of τ_d on A_{eff} is linear with a zero intercept for free diffusion ($\tau_0 = 0$), positive intercept for hindered diffusion due to domain confinement ($\tau_0 > 0$), and negative intercept for hop-diffusion due to meshwork compartmentalization ($\tau_0 < 0$). Stimulated emission depletion FCS (STED-FCS), which can achieve observation areas below the diffraction limit by changing the intensity of the STED beam, have also been applied to investigate the diffusion of membrane proteins in domains [21,51,57]. Using varying spot sizes in STED-FCS, the transition regions of the diffusion law plot can be observed, however multiple sequential measurements and sample damage due to high STED laser power are major drawbacks [36].

The FCS diffusion law has been successfully applied to a range of biological systems. However, there remain unsolved problems. If a protein is simultaneously raft associated and hindered by the cytoskeleton, as has been suggested for the epidermal growth factor receptor (EGFR) [58], then it is not clear what the absolute value of the diffusion law intercept will be, or how it will change when either the raft association or the cytoskeleton hindrance is relaxed, e.g. by drug treatments. Furthermore, as the observation areas in ImFCS are diffraction limited and thus cover a large area compared to the size of many domains and cytoskeleton compartments, is it possible that two different diffusion modes can co-exist in the same area, i.e. can one molecular species be entrapped by domains while another is diffusing freely or is hindered by the cytoskeleton? Lastly, can two different diffusion modes be distinguished in a single multi-component autocorrelation function? To address these questions (Fig. 1), we identified probes with non-overlapping spectra as standard diffusion markers, which only display a single mode of diffusion. We then selected a combination of two probes and first performed sequential ImFCS, and then simultaneous dual color ImFCS measurements. The τ_0 value obtained by simultaneous measurements of two different probes was compared with τ_0 value obtained when the two probes were measured independently in the same spot. We confirm our findings with simulations to observe a similar trend in the τ_0 values when two probes showing different diffusion modes are present in the same spot. Through simulations, we also show that two fluorescent probes of the same wavelength, which show different diffusion modes, can be distinguished in a single autocorrelation function, and the diffusion law can be reconstructed separately for the two components. The understanding of the complex behavior of the diffusion law in the case of mixed or convoluted diffusive modes is a pre-requisite for the correct interpretation of FCS diffusion law experiments.

2. Materials and methods

2.1. Cell culturing, cell labeling and cell transfection

Chinese Hamster Ovary (CHO-K1) cells were cultured at 37 °C, 5% (v/v) CO_2 in a 75 cm^2 flask containing Dulbecco's Modified Eagle Medium (DMEM; Invitrogen; Singapore), 10% Fetal Bovine Serum (FBS; Invitrogen, Singapore), and 1% Penicillin-Streptomycin (PS; PAA, Austria).

When the cells were ~90% confluent, the cells were seeded on glass covered dishes (35 mm dish, No. 1.0 cover glass 0.13–0.16 mm, MatTek Corporation, US) supplemented with DMEM + 10% FBS + 1% PS and incubated for ~24 h until confluent. For DiI labeling, a stock solution of DiI-C18 (Molecular Probes, Invitrogen, Singapore) in DMSO was prepared initially. A 100 nM working solution of DiI-C18 was prepared by diluting the DiI-C18 stock solution in Hank's Balanced Salt Solution (HBSS; Invitrogen, Singapore). To avoid aggregate formation, the working solution was thoroughly homogenized and vortexed for 3 min. The culture medium in the seeded cells was replaced with the DiI-C18 working solution and incubated at 37 °C for 20 min. After incubation, the cells were washed with HBSS twice and then imaged using imaging medium (Phenol red free DMEM + 10% FBS).

The GFP-GPI plasmid was a gift from Dr. John Dangerfield (Anovasia Pte Ltd, Singapore), and the construction of PMT-mRFP is described by Ping Liu and colleagues [59]. For transfection of cells with GFP-GPI and PMT-mRFP plasmids, electroporation by the