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Review

Hierarchical organization of the plasma membrane: Investigations by single-molecule tracking vs. fluorescence correlation spectroscopy

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ABSTRACT

Single-molecule tracking and fluorescence correlation spectroscopy (FCS) applied to the plasma membrane in living cells have allowed a number of unprecedented observations, thus fostering a new basic understanding of molecular diffusion, interaction, and signal transduction in the plasma membrane. It is becoming clear that the plasma membrane is a heterogeneous entity, containing diverse structures on nano-meso-scales (2–200 nm) with a variety of lifetimes, where certain membrane molecules stay together for limited durations. Molecular interactions occur in the time-dependent inhomogeneous two-dimensional liquid of the plasma membrane, which might be a key for plasma membrane functions.
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1. Introduction

Just how proteins and lipids jostle around in the plasma membrane has been a source of debate for decades. Diffusion in the homogeneous membrane, a quasi two-dimensional structure immersed in water, is already a subject matter that has attracted great interest [1,2]. However, recent observations of the thermal movements of proteins and lipids in the plasma membrane, using single-molecule tracking and fluorescence correlation spectroscopy (FCS) at sufficient temporal and spatial resolutions, have revealed the unforeseen complexity of the motion. Essentially all of the molecules in the plasma membrane observed by high-speed single-molecule tracking exhibited non-Brownian diffusion. The majority of the molecules undergo suppressed diffusion: either their macroscopic (long-term) diffusion coefficients are smaller than their microscopic (short-term) diffusion coefficients or the molecules undergo temporary immobilization, making the long-term diffusion coefficients smaller than the short-term diffusion coefficients exhibited by the molecules during the mobile periods [3]. Therefore, one of the three important subject matters of the present review is to describe the technique of high-speed single-molecule tracking and its detection of the non-homogeneity of the plasma membrane.

Meanwhile, non-Brownian diffusion of membrane-incorporated molecules in non-homogeneous membranes often fails to be observed by low-speed single-molecule tracking [4,5] and FCS [6]. Therefore, the second of the three important themes of this review is to clarify why the results obtained by these techniques are different from those generated by high-speed single-molecule tracking.

Third, more concretely, we focus on two distinct membrane domains on the meso-scale (defined here as the scale between 2 and 200 nm). (1) The raft membrane domain, which is defined as dynamic, nano-sized, sterol–sphingolipid-enriched assemblies of molecules [7]. The plasma membrane consists of a non-ideal mixture of diverse molecules with differing mutual miscibilities in the fluid state, i.e., it contains dynamic meso-scale molecular complexes and domains, forming and dispersing continually within the plasma membrane on various time scales. These molecular complexes and domains range from small protein clusters with short lifetimes, such as transient dimers of rhodopsin [8], to stabilized raft domains, such as those induced by the receptors engaged in signaling, due to ligand binding and subsequent receptor clustering, including the signaling complexes/domains of T- and B-cell

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receptors [9–16], FcεR [17–19], and CD59 [3,20]. The raft domains in non-stimulated cells are likely to have properties between these two extremes.

(2) The other interesting feature of the plasma membrane, which distinguishes it from a simple two-dimensional ideal liquid, is our proposal that the plasma membrane is parceled up into apposed domains, for both proteins and lipids. Namely, the plasma membrane may be partitioned or compartmentalized for the translational diffusion of both proteins and lipids: all of the molecules incorporated in the plasma membrane undergo short-term confined diffusion within a compartment and long-term hop diffusion between the compartments. This is likely due to the filamentous actin meshwork associated with the cytoplasmic surface of the plasma membrane ("membrane skeleton" for short), and to various transmembrane proteins anchored to and aligned along the actin filaments.

In the present review, we propose how these two meso-scale structures are organized in the plasma membrane.

The research field of plasma membrane heterogeneity at various levels is presently very active, and many fine reviews have been published. Readers are directed to the following interesting reviews that generally cover this active field (limited to those published in 2007 and later), from a different viewpoint [21–29].

2. Single-molecule tracking of membrane molecules

New experimental techniques that allow researchers to track single molecules or small groups of molecules in the cell membranes of living cells are becoming important tools for investigating the dynamics, structures, and functions of the cell membrane. These techniques have given researchers the unprecedented ability to directly observe the movement, assembly, and even activation of individual single molecules in the plasma membrane of living cells in culture [30–33].

These methods are largely classified into two groups, based on the probes employed to track individual molecules. One of the methods, termed single fluorescent-molecule tracking (SFMT), employs fluorescent probes, which are usually fluorescent organic or protein molecules; each individual fluorescent molecule bound to a target molecule is visualized using fluorescence microscopy [32,34–38].

The other method, termed single particle tracking (SPT) by Sheetz et al. [39], employs colloidal gold particles of 20 or 40 nm in diameter; each individual particle bound to a single molecule (or small groups of molecules) in/on the plasma membrane is observed using Nomarski (differential interference contrast) or bright-field microscopy [30,31,39–44].

In successful SFMT, one always has to balance the spatial precision for determining the position of each single molecule, the time resolution (frame rate, e.g., how often a single molecule can be observed), and the duration of viewing a single molecule. If this balance fails, or the observation conditions are too limited by the capability of the instrument, then the information required for solving the problem will not be obtained. At typical position determination accuracies of 30–40 nm, several 10–100s of frames could be observed (at video rate, this corresponds to 1–10 s; at a 1 ms resolution, this corresponds to ~30–300 ms).

The prominent advantages of SPT are that it yields a much better signal-to-noise ratio than that of SFMT (because the contrast originates from the incident light scattered by the particle) and photobleaching or blinking is not a concern. Using Nomarski or bright-field microscopy, colloidal gold particles could be localized with position determination precisions of ~2 nm at video rate [42,44], and ~17 nm even at 40,000 frames/s (25-μs resolution [45]). However, the large size of the colloidal-gold particles (typically 20 or 40 nm) can cause crosslinking of the target molecules or steric hindrance effects, and prevents their application to intracellular molecules.

3. Observation of dynamics of membrane molecules and membrane heterogeneity using FCS and STED-FCS

FCS also has single-molecule sensitivity, and can detect single molecules in the focal area [46]. However, FCS will not allow the tracking of single molecules, and its raw data generally become useful only after many molecules are observed. Therefore, such observations are usually made under the conditions where several 10–100s of molecules simultaneously stay within the focal area, for better signal-to-noise ratios. The signal intensity in the focal area is observed as a function of time and is auto-correlated, and from the auto-correlation function (spectral density), the average number density and the residency time of the observed molecule in the focal area are determined. For further reviews of the general method, see Chen et al. [47] and Chiantia et al. [28].

The spatial resolution of FCS is basically the same as the focal area size of the excitation laser beam, which is usually given by the optical diffraction limit of ~240 nm. Recent efforts to limit the focal area size, to improve the spatial resolution, have generated important successes, yielding illumination area sizes of ~150 nmϕ [48] and ~30 nm [49–51]. The best spatial resolution of FCS accomplished thus far has been the 30-nm focal area, attained by stimulation-induced emission depletion (STED)-FCS. However, even at this spatial resolution, membrane heterogeneity on scales smaller than 30 nm will not be directly observed.

How does FCS or STED-FCS provide information on dynamics and structures occurring on scales less than ~30 nm (or ~240 nm for normal FCS)? The information must be obtained indirectly: educated guesses must be made of the models for molecular dynamics and substructures that might be occurring in the focal area, and then they must be translated to the information obtained in FCS measurements. For quantitative analysis, a Monte-Carlo simulation of molecular movements based on the model is performed, which gives a prediction of the FCS data, and then from the FCS data, based on the model, one obtains the parameters describing the model, such as the molecular residency time within the meso-domain, the time fraction of residency within a meso-domain vs. the bulk domain, the diffusion coefficient in the bulk domain, and the size of the meso-domain [6,52]. The accuracy of the assumed model can be tested, for example, by performing FCS experiments at various focal area sizes [6,51,53]. Therefore, creating suitable models and then translating them to analysis software for FCS data are critically important for using FCS to study the membrane structure and molecular dynamics occurring in the space scales smaller than the focal area.

4. High-speed single-molecule tracking revealed hop diffusion of membrane proteins and lipids in the partitioned plasma membrane

Fig. 1a and b shows representative trajectories of an unsaturated phospholipid (a typical non-raft phospholipid probe), L-α-dioleoylphosphatidylethanolamine (DOPE) in the plasma membrane of NRK cells, recorded at time resolutions of 33 ms and 25 μs (frame rates of 30 and 40,000 Hz), respectively, using SPT (due to the probe tagging in the headgroup, it no longer exhibits the characteristics of PE). A general statistical method for analyzing these trajectories has been developed, to classify them into simple-Brownian, suppressed, and directed diffusion modes ([44]; also see Fig. 4 and its related text in Suzuki et al. [54] for the statistical analysis of trajectories and hop-confined diffusion). Note that this classification is independent of the diffusion model (many scientists

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