Lateral Diffusion in Heterogeneous Cell Membranes

Didier Marguet and Laurence Salomé

Abstract The plasma membrane is organized at numerous levels as a result of its large variety of molecular constituents and of selective interactions between them. Lateral diffusion, a direct physical consequence of the Brownian agitation, plays a key organizational role by constantly redistributing the membrane constituents among the possible molecular associations. In this context, we will first review the physical mechanisms contributing to the creation of inhomogeneity. We will then describe the current methodological approaches allowing us to measure diffusion in living cells. The different levels of membrane organization will be discussed before illustrating the impact of the dynamic organization of the membrane on cellular functions.

Keywords Cell membrane · Lateral diffusion · Fluorescence microscopy · Nanodomains

1 Introduction

Over the past four decades, extensive experimental work has been dedicated to the exploration of membrane organization and dynamics. Taking benefit from continuous and impressive methodological and technological advances, many facets of the cell membrane's complexity and exquisite subtleties have been revealed, providing new information that has enriched our concepts in membrane biology [1–4]. Still, the present consensual, but not yet definitive, view of biological

D. Marguet (✉)
Centre d'Immunologie de Marseille-Luminy, Aix Marseille Université, Inserm, CNRS, Marseille, France
e-mail: marguet@ciml.univ-mrs.fr

L. Salomé
Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, Toulouse, France
e-mail: laurence.salome@ipbs.fr

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membranes amazingly recalls the ideas proposed by Singer and Nicolson in 1972 [5, 6]. While textbooks, reviews, and papers recognize the influential step forward taken with the Fluid Mosaic model, they quite generally retain only the notion of the membrane as "a sea of lipids in which proteins are randomly distributed." However, Singer and Nicolson's seminal article claiming that "*valid generalization may exist about the way proteins and lipids are organized in membranes"* already predicted the existence of nanometer-scale domains which is now a commonly accepted idea. What visionaries were these scientists who proposed that "*the absence of long-range order (over distances of the order of tenths of a micrometer and greater) should not be taken to imply the absence of short-range order in the membrane*" and, even more, added "*It is more likely that such short-range order exists*." The concept of nanometer-scale domains was thus introduced, although their importance and functional role remain under scrutiny. Diffusion and heterogeneity, which represent the focus of this chapter, were the major membrane features that guided Singer and Nicolson in the elaboration of their original model grounded in thermodynamic principles.

Prior to the development of the arsenal of techniques dedicated to the measurement of diffusion by W.W. Webb [7–9], diffusion of the membrane components had been demonstrated by the now-famous cell fusion experiments by Frye and Edidin [10]. Diffusion results from the noncovalent nature of the interactions governing the self-assembly of membranes but leads to a dynamic organization of cellular membranes due to its interplay with selective molecular interactions. Both effects are at work in biological functions; they keep plastic and efficient for instance the cellular response following the stimulation by a specific signal.

The diversity of membrane components, lipids and proteins, is tremendous, as has been recognized for a long time. As a consequence of this diversity, "*the formation of a supra-molecular aggregate like a biological membrane is expected to be "heterogeneously" organized as a result of cooperative phenomena among a large number of different molecular species*" [11].

In this chapter, we first recall the general physical mechanisms governing the formation of lateral heterogeneities and the dynamics of membrane organization. We then compare the methodological and experimental approaches available to probe this dynamic membrane organization. Subsequently, we describe the different levels of organization observed in biological membranes, as well as their impact on cellular functions. Finally, we outline the questions that should be answered by future research in this field.

2 Physical Mechanisms Governing the Formation of Lateral Heterogeneity and the Dynamics of the Membrane Organization

As recalled in the introduction, the framework initially established by Singer and Nicolson to describe the structure of the cell membrane relies on proteins being wholly or partly embedded within lipid bilayers, in which it is assumed that

particular molecular associations take place at short range. The reason why biomembranes still are fascinating study objects from a physical and biological point of view has to do with these supramolecular aggregates being built upon weak intermolecular interactions between a broad variety of lipids and proteins. Therefore, this characteristic combined with the thermal agitation occurring between molecules at physiological temperature would ultimately generate local inhomogeneity. On first thought, these two features—i.e., weak intermolecular interactions and thermal agitation—might appear to be antagonistic: the former creates selective interactions, i.e., "order," whereas the latter introduces a tendency toward mixing, i.e., "noise," within the system. But, in a counterintuitive manner, and because of the large number of different molecular species, the Brownian agitation enables through molecular random motion the existence of a number of selective interactions which contribute to create the lateral heterogeneity [12]. Therefore, creating order as a result of noisy agitation should be considered to lie at the core of the mechanism behind the organization of cellular membranes by generating the heterogeneity and plasticity required for life processes [13, 14].

Indeed, the nonrandom distribution of the membrane components directly results from the balance between the energy involved in a molecular interaction and the thermal energy within the system. In cell membranes, the energy related to interactions among the membrane components is of comparable magnitude to the thermal energy occurring at physiological temperature. As a consequence:

- The characteristic length scales of the membrane organization strongly depend on temperature.
- The lifetime of an interaction is shortened if the thermal energy is higher than the characteristic energy required for a molecular association and vice versa.

Although long considered as only providing a passive fluid matrix to the proteins, lipids are now recognized to play effective roles in cellular membranes. Their physicochemical properties provide the fundamental principles from which the lateral heterogeneity and dynamics of membrane organization arise. Indeed, the primary physical mechanism organizing the cell membrane relies on the amphiphilic nature of lipids which are divided into a hydrophobic part, the hydrocarbon chains, and a hydrophilic part, the head group. When mixed with water, the biological solvent, lipids self-assemble by an entropic effect resulting from the incapability of the hydrocarbon chains to form hydrogen bonds and, concomitantly, from the capability of the polar head groups to collectively decrease the interfacial tension between water and the lipids. Altogether, the thermodynamic laws and intermolecular forces determine the complex dynamical organization of membrane components. However, favoring a state of aggregation by minimizing the free energy does not provide a complete understanding of the classical lipid bilayer organization observed by biologists. For instance, in artificial membranes, changes in the composition of lipid mixtures or in temperature can induce a transition from a lamellar organization to a micellar one or vice versa.

One should also consider the huge lipid diversity. Although all lipids share a very similar chemical structure with a polar head and hydrophobic hydrocarbon chains,

up to a thousand different molecular species can be found within a single cell. This has direct impacts on the cell membrane organization and more specifically on:

- The membrane thickness. The trans-bilayer structure is characterized by its thickness and relies on the lateral pressure profile of the bilayer. This profile is the consequence of the balance of forces occurring between (1) the interaction between lipid head groups, (2) the interfacial tension, and (3) the flexibility of the hydrocarbon chains;
- The lateral organization. The molecular organization within the plane of the bilayer is a consequence of a cooperative phenomenon generated by a number of selective intermolecular interactions among different lipid species, which arise through thermal agitation. As a consequence, phase separations which have been described in detail for lipid mixtures of different complexity contribute to the creation of lipid domains; the size, lifetime, or shape of such domains depends on thermodynamic conditions.
- The spontaneous curvature of the membrane. This parameter is influenced by the packing parameter calculated for individual lipid species. It takes into account the surface area of the hydrophilic group, and the volume, length, and degree of unsaturation of the hydrophobic chains [11]. The so-called shape of a lipid determines its capability to fit within a given lipid aggregate. In other words, the overall structure of a bilayer will tend to minimize the curvature elastic stress energy through an asymmetric distribution of lipid species between the two membrane leaflets and within each leaflet by developing selective lipid associations or exclusions.

Membrane proteins add another level of complexity to this basic membrane organization and ultimately contribute to generate lateral heterogeneity within cellular membranes. Indeed, cellular membranes also contain a large number of different proteins, either directly embedded within the lipid bilayer or bound directly or indirectly to it. Significant efforts have been made to integrate the following features in the current description of membrane dynamics:

- The diversity of the proteins with respect to their nature, function, and size, as well as their inherent asymmetric orientation within membranes
- The quaternary structure of proteins and the interactions between such supramolecular complexes both within the membranes and at their vicinity
- The interactions between lipids and proteins, the way they pack together (i.e., favorably accommodate each other), and the way that protein conformational changes occur during biological processes

All of these features are of particular significance and govern the organization and dynamics of cellular membranes.

As a consequence of the thermal agitation of molecules at physiological temperatures, lipids and proteins are driven by Brownian motion, but their diffusion is restricted to the membrane plane. If unhindered, such lateral diffusion allows the molecules to explore the cellular membrane in a short amount of time—for instance, a membrane component diffuses through the plasma membrane of a standard cell

size in a few tens of seconds—and should create a homogeneous distribution of the membrane components in the absence of selective interactions. However, the diversity of the membrane composition generates differential molecular interactions of various strengths which ultimately nucleate local heterogeneity. Thus, this leads to a switch of the behavior of the components from a strictly free diffusion regime to a constrained one, for example confined diffusion within subdomains or oriented diffusion by direct or indirect interactions with motors linked to the cytoskeleton.

Therefore, there is no doubt that collectively lipids and membrane-associated proteins contribute to create local heterogeneity. Experimentally, such heterogeneities in membranes have to be revealed by the description of the molecular distribution of their constituents with the appropriate spatial resolution. This has been achieved by electron and fluorescence microscopies (see for example [15, 16]) although experimental limitations due to fixation and labeling procedures remain [17, 18].

Ideally, a dynamic map of the molecular distribution of the membrane components should be established. The most recently developed techniques for measuring the lateral diffusion, providing the adequate spatiotemporal resolution, indeed tend toward this objective. This should allow the identification of the mechanisms prevailing in the membrane organization.

3 Methodological Approaches to Probe the Dynamics of the Membrane Organization

After the initial observation by Frye and Edidin [10] revealing the diffusion of membrane components by rapid intermixing of membrane proteins after cell fusion, a large panel of techniques, spanning several orders of magnitude in time and length scales, has been invented to investigate the lateral diffusion of membrane lipids and proteins (Fig. 1). These methods are mainly based on fluorescence microscopy. They owe their emergence to the impressive creativity of W.W. Webb who focused his research on the observation of the dynamics of the biomolecular processes of life. Nowadays renowned for the experimental demonstration of the two-photon absorbance phenomenon and its application to multi-photon microscopy, this scientist has also invented fluorescence recovery after photobleaching (FRAP) [7] and fluorescence correlation spectroscopy (FCS) [9, 19] and performed the first single-particle tracking (SPT) experiments [8] (see Fig. 2 for the basic principles of the techniques). Since then, improvements and variants of these three major techniques have been further developed. As an example related to FCS, fluorescence cross-correlation spectroscopy (FCCS) enables the observation of co-diffusion of molecules [20]. A variety of alternative image correlation spectroscopy (ICS) methods have emerged as well [21]. During the last decade, the use of single-molecule methods has literally exploded, first thanks to the increase of the sensitivity of the detectors making possible the imaging of single fluorophores with relevant

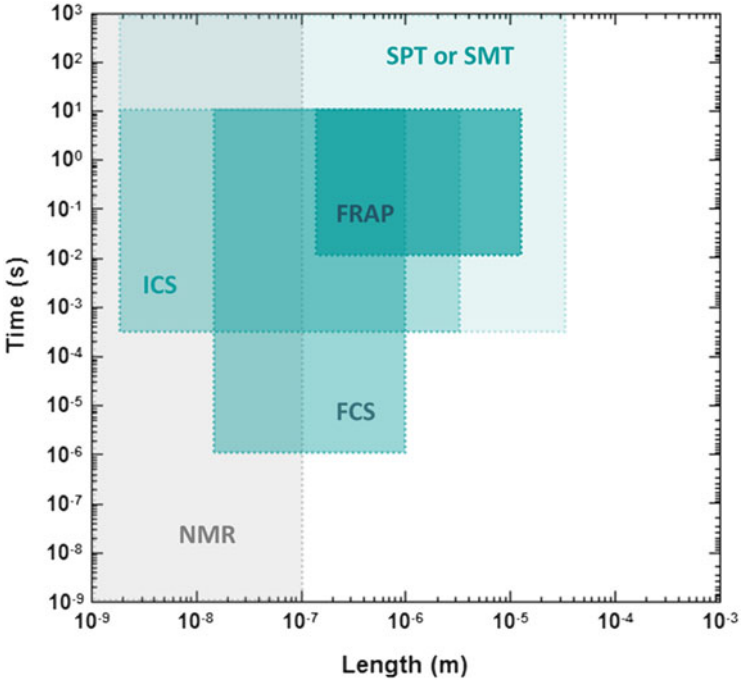

Fig. 1 Time and length scale ranges covered by the techniques dedicated to the investigation of membrane dynamics. Abbreviations: *NMR* Nuclear magnetic resonance, *FRAP* Fluorescence recovery after photobleaching, *FCS* Fluorescence correlation spectroscopy, *ICS* image correlation spectroscopy, *SMT* Single-molecule tracking, *SPT* Single-particle tracking

time resolution and more recently due to the capacity to control the fluorescence state or the illumination geometry of the probes, leading to the development of super-resolution microscopies such as PALM, STORM, etc. [22].

Our purpose in this chapter is not to discuss the general features of each of the techniques dedicated to membrane diffusion measurements. For this we invite the reader to consult recent reviews for useful information on the principles of these techniques, the proper choice of probe and instrumentation, the existing labeling strategies, and the basic analytic tools to compute the diffusion parameters from the experimental output of FRAP [23], FCS [24, 25] and SPT [21, 26]. Here, we will rather present a critical overview of the capability of these techniques to characterize heterogeneities and/or domains in membranes (Fig. 2).

First we propose to carefully delineate on which length and time scales these techniques yield information. This is of primary importance because obviously *one can find only what one is able to see* and none of the techniques covers the whole spatiotemporal range of lipid and protein diffusion. FRAP is usually considered to be a large-scale mobility assay inappropriate to go beyond a simple measurement of the diffusion coefficient of the mobile fraction of the tracer population. If a

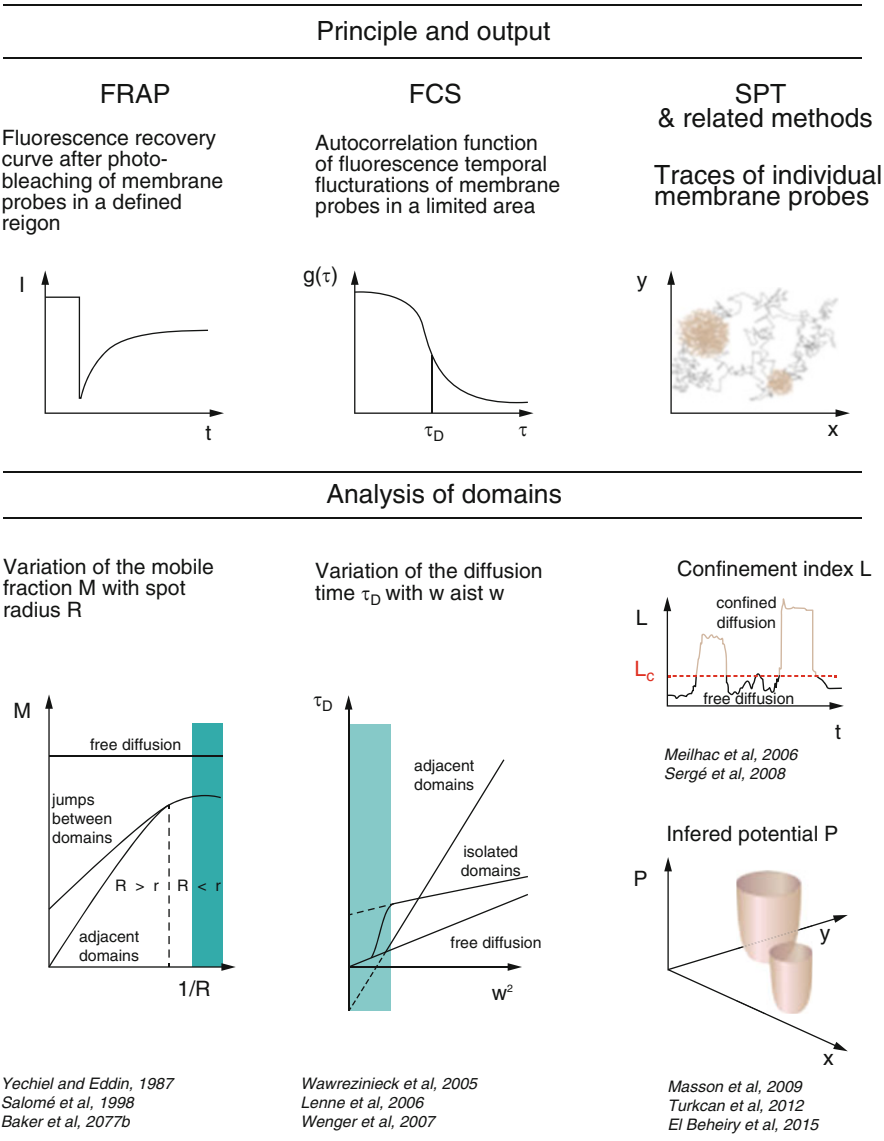

Fig. 2 Current methods for the analysis of membrane domains (of size *r*) based on diffusion measurements by FRAP, FCS, and SPT (see text for a more detailed discussion). In blue, the ranges of length scales inaccessible by conventional microscopy. In light brown, the periods of confinement and their corresponding analytical signatures

membrane is structured, this gives rise to incomplete fluorescence recovery (or an immobile fraction). The measured diffusion coefficients, which are estimated from the fit of the recovery curve assuming free diffusion within an area equal to the

bleached area, are then apparent ones. While this makes the comparison between data obtained on different cell lines problematic, it can turn out to be useful for the identification and characterization of submicrometer-sized domains by repeating measurements at variable spot size (see Fig. 2) as first demonstrated by Yechiel and Edidin [27]. Provided the microscope used can bleach areas down to about 1 μm in diameter and that the fluorescence recovery signal can be monitored without delay (these conditions are unfortunately not accessible with commercial confocal microscopes which are most often used), domain sizes as small as 200 nm can be measured together with diffusion coefficients inside these domains. A refined analysis enables to reveal whether the domains are joint and permeable or not [28–31]. The typical duration of a fluorescence recovery is of the order of a few tenths of seconds, precluding the identification of small short-lived domains by FRAP. In a similar way, FCS performed at variable beam waist gives information on spatiotemporal heterogeneities. This technique was first developed by Marguet and coworkers [32, 33] who, using nanoapertures, pushed the limit of the accessible length range down to 50 nm, well below the diffraction limit [34]. Yet another step has been taken by Eggeling and coworkers who implemented FCS on a STED microscope delivering directly a spatial resolution below 50 nm [35]. In addition to this advantage, FCS offers access to very short timescales (down to μs). Like FRAP, FCS measurements require careful and rigorous analysis to extract reliable information on the diffusion behavior based on the dependence of D (or τ) on spot size (see Fig. 2).

One would intuitively expect that it would be more straightforward to determine and characterize the deviations from free diffusion through the direct observation of the movements of individual molecules by single-particle tracking. In fact, due to intrinsic statistical fluctuations, identification of the diffusion mode from a single-molecule trajectory requires sophisticated tools from statistical physics. The identification of confined diffusion in domains usually proceeds by the search for confinement periods along the trajectories, taking care not to interpret as confinement a temporary reduction of the diffusion coefficient due to statistical fluctuations [36, 37]. Interestingly, the confinement index can also be used to detect jumps between adjacent domains in single trajectories [36], thus providing an unambiguous way to scrutinize hop diffusion [38]. An alternative method for the analysis of confined motion is Bayesian inference, particularly useful to infer diffusion coefficients and confinement potentials [39, 40] (see Fig. 2). This powerful technique has remained rather confidential but should gain notoriety in the near future with the availability of free software enabling the treatment of high-density SPT data such as those collected by, e.g., PALM [41]. Giving direct access to maps of the dynamic parameters of the molecules, this tool allows determining the physical origin of the observed motion without the intense modelization efforts needed to solve a complex inverse problem.

Nevertheless, the advent of such powerful analytical tools should not distract the experimentalist from a critical analysis of the experimental output. Although noninvasive, the techniques are not devoid of bias.

Especially for SPT techniques, the effects of time averaging of the particle's position by the detector and the influence of the probe's functionalization should be carefully considered. The former has been rigorously evaluated in the case of confined diffusion. Interestingly, corrections can be made to the apparent diffusion coefficients and domain size to recover the real values in an experimentally relevant range [42]. The latter concerns experiments making use of quantum dot (QD) nanoparticles coupled to antibodies. Monovalency, i.e., functionalizing the QD with (on average) a single antibody molecule, reduces the risk of crosslinking the targeted receptors. However, priority should be given to minimizing the friction of the particle with the membrane; hence the optimal antibody-to-particle ratio should be chosen as the one leading to the largest short-term diffusion coefficient [43].

A final and important bias, shared by all methods measuring lateral diffusion in membranes, is the topography of the cell surface which is not taken into account despite its influence. Very soon after the development of the FRAP technique, the question was raised whether invaginations or microvilli would affect the measurement of diffusion coefficients. After measuring the diffusion coefficients of lipophilic membrane probes in cells or cell regions devoid of or with a high density of microvilli, two research groups [44, 45] concluded that the presence of microvilli had no effect. Surprisingly, these authors did not question the procedure they used to analyze the FRAP recovery curves. Indeed, they computed the diffusion coefficient from the half-time of recovery without taking into account the roughness of the cell membranes and assuming that the diffusion area was equal to the bleached area. Three decades of extensive investigations of membrane dynamics neglecting the effect of membrane topology ensued, before observations by scanning ion conductance microscopy showed that the quite generally non-flat topography of the cell surface at the sub-micrometer scale compromises the interpretation of lateral diffusion measurements [46]. Curved surfaces can not only impact the diffusive timescales [47] but also lead to erroneous conclusions regarding the heterogeneities exhibited by membranes, such as apparent trapping [46]. Thus, it should be mandatory to accompany diffusion measurement by a characterization of the surface roughness [48]. As suggested by Jalink and van Rheenen [49], who even earlier pointed out the implications of membrane wrinkling in cell biology [50], one possible approach "*to control for local membrane content is by normalizing to the fluorescence of a homogeneously distributed membrane marker*." Alternatively, the identification of the existence of surface roughness and the evaluation of its extent can be obtained using an autocorrelation function approach [51]. Obviously, high-resolution 3D particle tracking provides in this respect an a priori ideal method [52, 53].

As a last advice, we recommend that experimentalists try as much as possible to confront results obtained by different techniques in parallel for a rigorous validation of their observations.

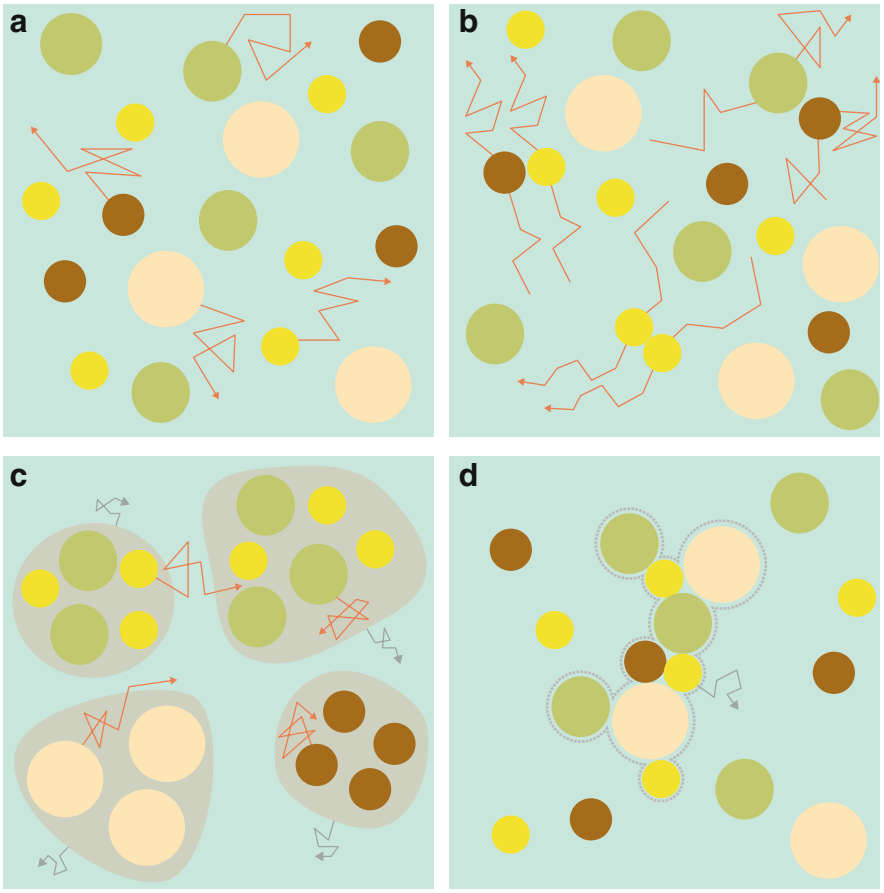

Fig. 3 Schematic representation of the different molecular organizations and their diffusional signatures. (**a**) Isolated molecules; (**b**) transient (top right and bottom) or permanent (left) oligomers; (**c**) dynamic clusters; (**d**) rigid aggregates. Orange arrows: individual movements; gray arrows: collective movements

4 The Different Levels of Molecular Organization

Nowadays, it is well accepted that different levels of molecular organization exist in living cells, leading to inhomogeneities such as submicrometer-sized entities named subdomains. Thus, the notion of *subdomain* is ill defined as it encompasses a broad range of spatiotemporal characteristics (Fig. 3). Both lipids and proteins are organized in supramolecular assemblies held together by noncovalent bonds—from the annular lipid shell to the clathrin-coated pits or caveolae, morphologically identifiable structures, and, on an intermediate scale, quaternary protein structure, lipid rafts, clusters, protein lattices, and aggregates.

Assembly of monomers into homo- or hetero-multimerized entities constitutes the first brick contributing to the organization of the membrane. FRET-related techniques are of particular interest to probe this level of organization. Note that lateral diffusion measurements can hardly inform on the formation of molecular complexes on the basis of their intrinsic mobility alone, because the diffusion coefficient of a membrane inclusion only moderately depends on its size (for a detailed discussion, see Chapter "Membrane domains under cellular recycling" by V. Démery and D. Lacoste). However, the interactions of supramolecular complexes with their environment will be different from those experienced by the monomers (Fig. 3a), and this influences the diffusion parameters beyond the diffusion coefficient. Single-molecule methods provide the possibility to count the proteins within an aggregate [54] and to evaluate the level of heterogeneity by a scrutiny of individual trajectories (Fig. 3b) [55]; FCS-related techniques provide the possibility to characterize such variations in the molecular organization by looking at the distribution of molecular brightness (Number & Brightness techniques) [56].

In the following paragraphs, we examine observations made on a well-characterized membrane receptor, the epidermal growth factor receptor (EGFR), to illustrate these different levels of membrane organization. This receptor is involved in the regulation of cellular growth and proliferation following its oligomerization through the binding of the EGF. Studies of the EGFR oligomeric state under resting and activated conditions have provided evidence that, in the resting state, the EGFR is mainly monomeric but also forms homo- and heterodimers, depending on its membrane density but not on the binding of EGF [57–60]. Further EGFR multimerization might be required for efficient signaling [61]. It is also interesting to notice that pre-assembled EGFR dimers, which have a finite lifetime, are prominent in lamellipodia but without the slow diffusion characteristic observed for ligand-bound EGFR dimers [57]. This effect is presumably due to the interactions with the signaling machinery observed in the presence of the ligand.

A higher level of membrane organization corresponds to the possible formation of subdomains from the bricks of multimeric complexes. At this point, we would like to distinguish between cluster, lattice, and aggregate. A molecular cluster relates to a dynamic assembly of membrane components, each of them individually maintaining a certain level of freedom within a cluster [62, 63]. Proteins, together with lipids, determine the diffusional properties of a cluster (Fig. 3c). Molecular aggregation corresponds more to the notion of molecules clumping together into weakly structured entities containing mainly proteins. In that case, each protein within the aggregate has the diffusional characteristics of the aggregate itself (Fig. 3d). Multimeric ligands can interact with a variety of membrane proteins, promoting their reticulation into supramolecular aggregates of undetermined size. For instance, multivalent lectins promote the formation of lattices by interacting with different membrane glycoproteins. As a consequence, the subdomains created by lectin-dependent lattices contribute to stabilizing the interactions among diverse membrane components. For instance, it has been shown that the inhibition of a specific N-glycosylation of EGFR essential for its functions results in a reduction of EGFR binding to a lattice of galectin. Moreover, the association of EGFR

with this lattice significantly reduces its diffusion in the plasma membrane and favors its association with the actin-based cytoskeleton, as demonstrated by FRAP measurements [64].

Let us now focus on specific subdomains in which lipids are critical (see also chapter "Lipid Rafts: A Personal Account" by K. Simons). Embedding a protein in a complex lipid mixture favors selective interactions that minimize hydrophobic mismatch between the length of protein's transmembrane domains and the thickness of the lipid bilayer. This is illustrated by the so-called lipid shell formed by lipids surrounding the transmembrane segment of a protein. It is assumed that such lipid shells behave like individual thermodynamically stable structures (Niemela et al. [65] and for review Anderson and Jacobson [66]).

It has also been hypothesized that lipid shells might have a certain affinity for the so-called lipid rafts. This concept had previously emerged from biochemical studies on principles governing sorting mechanisms in polarized cells through intracellular trafficking. It was initially defined as the capability of cholesterol and sphingolipids to mediate phase separation at the plasma membrane [4]. It has been postulated that the lipids within rafts are in a liquid-ordered phase. In fact, the direct translation of the thermodynamic phase observed in model membranes to cellular membranes denotes an oversimplification that neglects both the membrane's chemical heterogeneity and the nonequilibrium conditions of a biological system. Although the definition of lipid raft has evolved over time, the concept itself is still under debate, mainly due to semantic issues which make it difficult to group under a single denomination a huge diversity of molecular ensembles studied on different experimental models by methodologies that differ in terms of spatiotemporal resolution. This concept has been discussed in [67–69] and in chapter "Lipid Rafts: A Personal Account" by K. Simons.

The possible implication of lipid rafts in the organization of EGFR at the plasma membrane has been investigated by electron microscopy, demonstrating the localization of the receptor in subdomains enriched in cholesterol and sphingolipids [70]. Additional experimental evidence has been provided by depleting cholesterol from the cellular membrane; such conditions altered both the oligomeric state equilibrium of EGFR and its diffusional behavior [60, 71]. In fact, the activation of the receptor seems capable by itself of remodeling its lipid environment, allowing the formation of nanoclusters [72]. Conversely, it has been reported that EGFR can be activated in the absence of a ligand solely by disrupting the lipid-raft organization [73]. In that case, it is possible that the depletion of cholesterol by methyl-beta-cyclodextrin leads to receptor aggregation and consequently to its spontaneous activation.

So far, we have not considered the shape of the cell membrane except as a possible bias in the analysis of diffusion measurements. Recently, the potential influence of the membrane shape on cellular signaling via a modulation of the distribution of membrane components was hypothesized and the idea put forward that regions of high curvature would favor the recruitment of effectors during the activation of

receptors [74, 75]. This notion is supported by different experimental observations showing that interdependent mechanisms would be at play: (1) curvature favors the selective recruitment of specific peripheral proteins having curvature-sensitive domains [76, 77]; (2) curvature can induce a partitioning of lipids with consequences on the localization of lipid-anchored proteins [78]; (3) curvature can finely tune the activity of enzymes working at the membrane–water interface, especially the lipases [79]; and (4) curvature can result from the specific binding of a protein on planar membrane [80]. As recently exemplified for the coupling of BAR proteins with the membrane shape by Bassereau and coworkers [81], a refined understanding of the mechanisms driving these complex phenomena can be attained by a combination of numerical simulations and in vitro experiments on model membranes.

Finally, it is also important to connect the plasma membrane with its immediate molecular vicinity, both in the outer and inner cellular spaces in which selective interactions take place during biological processes. For instance, the membrane-associated actin-based cytoskeleton and the transmembrane proteins directly or indirectly associated with this cytoskeleton act as membrane organizers by corralling membrane constituents in this meshwork (see for review [3]).

Together, these different levels of organization contribute with their own dynamics to the compartmentalization of the plasma membrane in a diversity of subdomains. Presently, defining a hierarchy among these different organizing principles is still challenging and requires to refine our understanding or to create an alternative view of the cell membrane.

5 The Impact of the Dynamic Organization of the Membrane on Cellular Functions

It is commonly argued that the multiscale organization of lipids and proteins into clusters, nanodomains, or larger mesoscale domains plays a role in cellular processes [5]. Along this line, the idea is often invoked of specialized domains acting as operational platforms that concentrate specific proteins or lipids involved in a particular function. To counterbalance this ordering propensity, thermally driven motion, i.e., lateral diffusion, introduces the fluctuations "*fundamental to the function of biological systems*" and "*ubiquitous in life science*" [14]. Diffusion is obviously essential for membrane homeostasis. Diffusion promotes encounters between partners, increases the number of accessible distribution configurations, and extends the range of a perturbation, thereby boosting the reaction capability of the membrane. Confining diffusing molecules into domains in turn creates additional interesting properties. When signaling partners are sequestered in domains, this not only dramatically increases their frequency of encounters, hence improving the signaling efficiency, but it also avoids undesired interferences that could result from interactions with other proteins by keeping them spatially separated. With respect to the existence of receptor cross talk, this last feature might be the most

relevant one; see for example [82]. Combining order and disorder, i.e., clustering and diffusion, would thus facilitate the orchestration of the cellular activity at the cell surface by permitting a refined spatiotemporal regulation of the complex and manifold biological functions.

These concepts are extremely attractive due to their simplicity. They are systematically brought up in the discussion of experimental or theoretical results while clear demonstration that they are effectively at work is rarely provided. The existence of a relationship between the dynamic organization of a membrane component and the function it accomplishes is challenging to demonstrate, and understanding the underlying mechanisms of this coupling is even more difficult.

As already noted, most if not all membrane components—lipids and proteins—analyzed to date have been found to exhibit nonrandom diffusion for at least a fraction of their time and/or a fraction of their population. With the emergence of super-resolution microscopies and the improved tools for analysis, the description of membrane domains has become more precise. Thus, the confinement in nanoclusters of membrane proteins involved in extremely diverse functions, like GPI-anchored proteins [83], Ras proteins [84], and SNARE proteins [82, 85], is now firmly established. These nanoclusters are dynamic, with proteins diffusing in and out of them. Some clusters are found to be long-lived (up to minutes) [86]. Larger-scale organization of membrane proteins that tune signaling functions was also revealed. The TGF receptors, TβRI and TβRII, separate in distinct regions at focal adhesions but collapse to form a signaling complex upon release of cellular tension [87].

T cell receptors (TCR) represent another biological model on which extensive investigations have been performed to determine how the dynamics of membrane subdomains shapes the mechanism underlying the process of TCR transmembrane signaling, inducing CD3 phosphorylation, commonly called TCR triggering [88, 89]. The different mechanisms for TCR triggering proposed thus far are hotly debated, presumably because each one deals with one facet of the process [90]. In contrast, a consensus has emerged for the B cell receptor (BCR), another immune receptor. Indeed, recent work supports the notion that BCRs are organized into nanoclusters in resting B cells, whereas they dissociate during B cell activation by a mechanism whereby the Syk kinase induces an inside-out signaling [91]. In this dissociation-activation model, quiescent nanoclusters are not functional but switch to an activated state by a disassembly process.

Among membrane receptors, the superfamily of the G-protein-coupled receptors (GPCRs) deserves special interest as they constitute the largest and most diverse group. The Rhodopsin-like (or class A) GPCRs transduce extracellular signals through complex cascades of interactions with various partners starting with the heterotrimeric G-protein. To account for the rapidity and specificity of signaling, and based on indications that the receptors and G-proteins had a nonrandom diffusion and distribution, it was proposed in the 1990s that the receptors and their partners were localized in membrane compartments. The first experimental proof of the confinement of a GPCR was obtained 10 years later by the SPT analysis of hMOR, the main receptor of morphine [92]. These results were validated by different research groups [93]. Subsequent studies of other GPCRs using ad

hoc techniques found that at least an important fraction of them exhibit either a permanent dynamical confinement or transient confined diffusion [94]. So far, the integrated description and understanding of the relationship between the dynamical organization of the receptors during the signaling events that follow a ligand binding to a receptor are not yet established. In particular, the studies exploring the behavior of active receptors all report a correlation between the functional state of the receptors and their diffusion parameters. The emerging general feature is an increase of the confined population together with a constriction of the domains and a decrease of the receptor mobility [31, 95]. One exception is the serotonin receptor [96]. Interestingly, heterologous regulation by activation of other GPCRs can also change the dynamic organization of a receptor but in a different way from that induced upon homologous stimulation [97].

Constituting a specific subfamily of GPCRs, the metabotropic glutamate receptors have been the subject of intensive work, in particular using nanoscale microscopy, to unveil their trafficking pathway at neuronal synapses [98]. This study resulted in the most complete example of a tight link between function and dynamic organization with a new model of synapse organization and novel clues to potential pharmacological targets. Contrary to a historical paradigm, instead of being stably localized at the synapse, receptors are in a dynamical equilibrium between synaptic, extrasynaptic, and intracellular compartments "*governed by a tight interplay between surface diffusion and membrane recycling*" [99]. The synapse itself should be viewed as highly heterogeneous with neurotransmitter receptors distributed between stable but dynamic nanodomains and zones outside those nanodomains. Experiments have convincingly supported the idea that the rapidity of receptor exchanges between the vicinity and the interior of the postsynaptic density is a main factor of synaptic plasticity [100].

6 Concluding Remarks

Thermally driven diffusion is an essential phenomenon at the molecular scale, extremely important in membranes because of the absence of covalent links between their constituents. Manifold specific but also nonspecific interactions are nevertheless present, leading to multiscale heterogeneities in the distribution of the membrane components. Indeed, as asserted by Bigay and Antonny [76] "*collective effects arising from multiple low energy interactions have at least the same importance as biomolecular stereospecific interactions*." In this context, the lateral diffusion of proteins and lipids at the cell surface takes very complex forms. Characterization of this diffusion can ultimately reveal the underlying dynamic maps of the distribution of the various constituents and the forces between them. Such information is undoubtedly of great help to provide a mechanistic explanation of biological phenomena taking place at the membrane.

During the past decades, our understanding of the plasma membrane organization has benefited from massive technological advances, reaching unprecedented levels

of sensitivity and resolution allowing the measurement of relevant observables. The organization of the cell membrane into submicron domains has emerged as the major feature. We have presented the main techniques available to date for measuring diffusion and the associated analytic procedures dedicated to the identification and characterization of such organization. Those techniques are predominantly based on fluorescence microscopy. Particular attention has been given to the often neglected experimental and analytical pitfalls that should be considered before embarking upon such studies. Among the possible diffusion modes, we voluntarily did not discuss anomalous diffusion. The main reason is that no convincing evidence has yet been produced that such reported behavior is indeed due to an underlying self-similar structuration, as expected for genuine anomalous diffusion [101]. Most often, traces attributed to anomalous diffusion can be interpreted as a combination of a short-term confined diffusion with a longer-term and slower free diffusion [102]. As is customary in science, Occam's razor "*Pluralitas non est ponenda sine necessitate*" should also be privileged in this field. Combined with a panel of biochemical, biological, or physical techniques, diffusion measurements have succeeded in unveiling a variety of molecular organizations and influential parameters. However, we would like to point out a major difference between membrane proteins, which can be directly analyzed (ultimately native proteins can be labeled in situ), and lipids which are mainly studied through fluorescent analogs inserted in the membrane. Due to renewed interest in lipids, originating in part from the lipid-raft hypothesis, future progress can be expected in the design of novel probes or labeling schemes that more faithfully report on the lipid behavior in all its complexity.

Finally, even though the highly important notion of dynamic structure was clearly emphasized by Singer and Nicolson in their classic fluid mosaic model, it took several decades before the nonrandom distribution of membrane components at short, i.e., nanometer, length scales could be thoroughly documented through experimental investigations of the diffusion of membrane components, and accepted by the community.

In the future, efforts should continue to concentrate on a comprehensive description of membrane organization and dynamics. Progress is still needed to establish the physical laws governing specific features observed in membranes. In this respect, experiments on biomimetic systems permit to determine the minimal conditions necessary to reproduce a given behavior. In conjunction with theoretical modeling, in particular through numerical simulations which offer the possibility to bridge the gap between necessarily simplified models and highly complex cell membranes, biomimetic models constitute a promising approach to finally arrive at a functional model for the diffusion in the cell membrane.

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