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Surface analysis of membrane dynamics

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ABSTRACT

Cellular membranes change shape and composition in a controlled way in order for life to prosper. They are set apart from other cellular structures by their two-dimensional nature, which governs their properties and the processes happening in or on them. During the last decade, a boom of new techniques has allowed the study of the surface of biological membranes in unprecedented ways and has greatly advanced our understanding of its molecular organization. In this review, we first describe the principles of state-of-the-art microscopy methods and the suitable model membranes for the study of dynamic processes. Then, we explain how they can be used to investigate lipid dynamics, the lateral organization of membrane domains and the dynamic structure of cellular membranes.

1. Introduction

Membranes are essential components of cells and have both functional and structural roles. On one hand, cellular membranes form closed volumes that define the cell and its compartments. On the other, membranes act as impermeable barriers to polar molecules and isolate the cell and the organelles from their environment. Membranes control the exchange of matter, energy and information with the extra-cellular environment or with the cytosol in the case of organelles, which allows life to develop. As a result, biological membranes are highly dynamic structures with a very complex organization. The importance of membranes for life is reflected in the genome, where around one third of the coded information corresponds to membrane proteins [1]. Moreover, most current drugs target membrane components. However, our knowledge about the molecular basis of the organization of biological membranes lags behind other cellular components because of the difficulties to handle and study them.

Biological membranes are composed of a complex mixture of lipids and proteins, both of which can be modified with sugar moieties. The hydrophobic effect drives membrane lipids into two-dimensional, anisotropic bilayers, in which their hydrophobic tails are buried in the core, while their polar heads are exposed to the surrounding aqueous environment. Membrane proteins exhibit different ways of association with the lipid bilayer. Many of them are bound to the membrane surface via electrostatic interactions, some are anchored to the membrane through a lipid tail and others insert part of their polypeptide chain into the lipid bilayer. Due to the huge diversity of membrane proteins and the several hundreds of different lipid species that can be found in membranes, the composition of cellular membranes is extremely complex [2]. Indeed, heterogeneity is an important parameter in cellular membranes. Their protein and lipid composition varies not only between cell types, but also between different organelles inside a cell and even between the two leaflets that form the bilayer. During the last decade, it has become clear that lateral protein and lipid heterogeneities are important for a number of cellular processes [3]. The multitude of membrane-associated cell functions probably requires finely tuned changes in lipid and protein composition, which are spatially and temporally regulated.

The two-dimensional nature of biological membranes distinguishes them from three-dimensional environments in the cell. In this sense, the dynamic properties of such systems and the reactions happening at or within them have distinct features from physical and chemical points of view. During the last years, several techniques have been developed for the study of membranes with a surface approach. They allow the study of certain membrane processes that were not accessible with traditional bulk techniques. As a result, novel concepts have been developed to explain the

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organization of lipid membranes in terms of molecule and system dynamics, lipid/lipid and protein/lipid interactions, or phase behavior.

In this review, we will focus on microscope-based techniques that have become most popular for the investigation of dynamic processes in lipid membranes from a surface point of view. Such studies can be complemented with other surface techniques, such as atomic force microscopy (AFM), to provide more quantitative and complementary information. We will also describe the most common model systems used in such studies and the recent advances achieved with those approaches, mainly in terms of lipid dynamics, membrane fluidity, lateral organization and protein dynamics at membrane surfaces.

2. Microscopy-based methods to study lipid bilayers

The rapid development of fluorescent dyes during the last decade has boosted the use of fluorescence microscopy techniques for the study of biological systems. Dynamic processes in membranes can be followed by epifluorescence microscopy using membrane-targeted probes and CCD cameras. While this is sufficient for some membrane model systems, in many cases undesired out-of-focus fluorescence reduces the signal-to-noise ratio and, as a result, the resolution of the images. Microscopy strategies that improve this, such as confocal laser scanning microscopy (LSM) and total internal reflection fluorescence (TIRF) microscopy, are extremely useful to study dynamic processes in membranes.

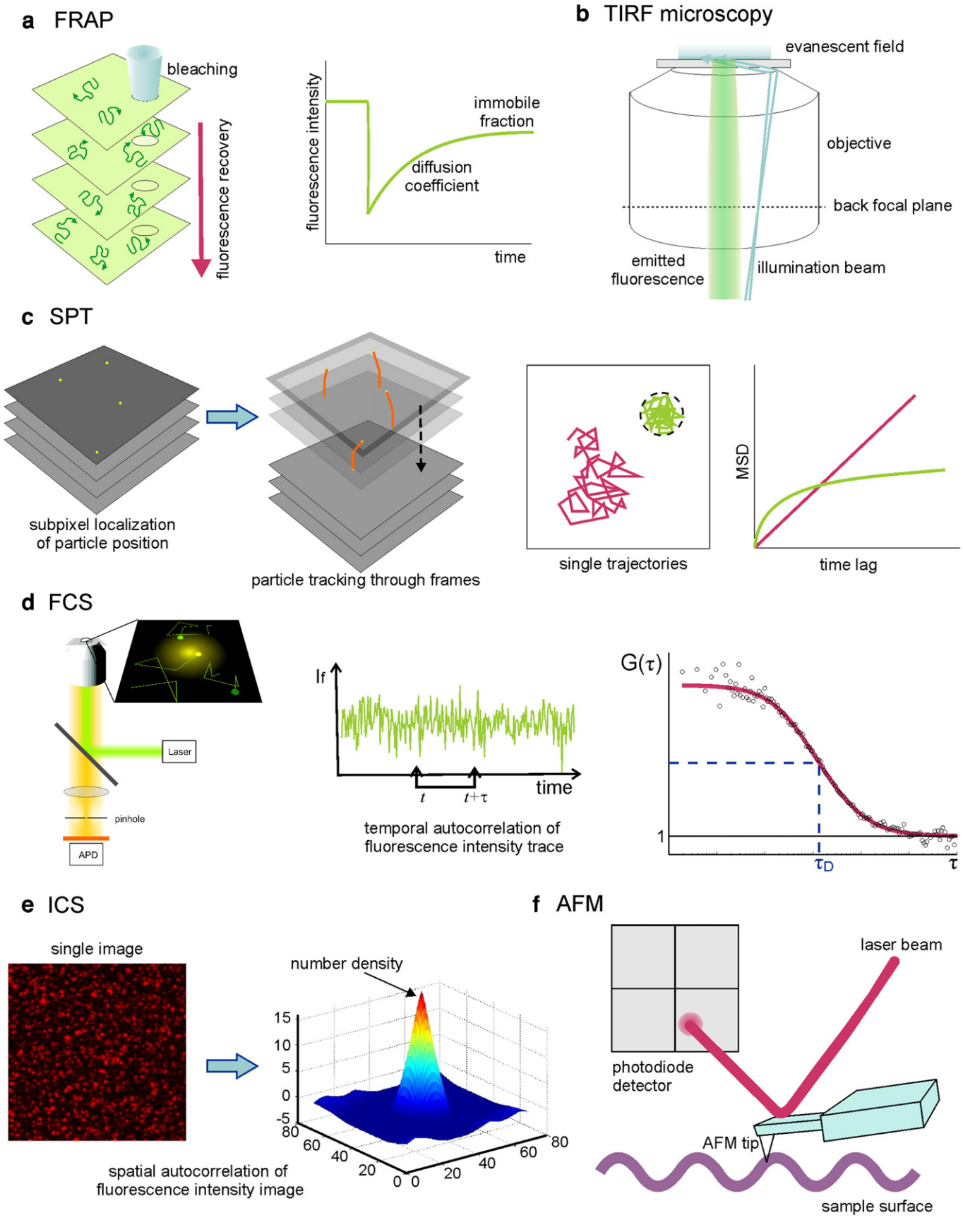
Confocal microscopy has become so widely available and used, that it is already a routine microscopy technique in many labs. Its spatial resolution power is increased by measuring only the fluorescence of thin sample sections [4]. This is achieved with a small pinhole in front of the detector that only allows the passage of the fluorescence emitted in the focus of the sample, while blocking the out-of-focus fluorescence. To obtain an image, the collimated light of a laser beam is focused onto the sample and scanned over it. Then, the fluorescence intensity of the focal volume at each position is used to process the image. Depending on the imaging conditions, the scan rate, and thus, the time resolution of the technique, can vary from several to less than one second. Confocal microscopy is very useful to follow membrane dynamics on that time scale because flat membranes can be easily included in the focused thin sections. In the case of three-dimensional membrane structures, their surface can also be accessed at the expense of time resolution by acquiring consecutive z-scans that can be later processed to obtain a 3D view of the sample.

Fluorescence recovery after photo-bleaching (FRAP) is a microscopy technique implemented in most commercial confocal microscopes that provides information about the dynamic properties of the fluorophores in the sample. Depending on the sophistication of the data analysis, the results can qualitatively describe changes in the sample dynamics or yield quantitative data about diffusion coefficients and membrane binding constants of the molecules under study. As schematically depicted in Fig. 1a, FRAP is based on the analysis of the increase in fluorescence intensity with time in a region of the sample that has been previously photo-bleached with high-power laser illumination. Once the region of interest has been depleted of fluorophores, the two-dimensional diffusion of fluorophores from the surrounding regions and/or the binding of new fluorophores from the solution immediately begin to repopulate the region of interest and, as a result, its average fluorescence intensity increases. The recovery curve of fluorescence intensity represents the dynamics of the system. It can be qualitatively compared under different experimental conditions, or fitted with adequate mathematical models that yield values for immobile fractions, diffusion coefficients and membrane binding constants, depending of the nature of the system under study. The main disadvantages of this technique are the high illumination power required, which can damage the sample or even affect the dynamics of the system, and the fact that it is a destructive technique based on the irreversible photo-bleaching of the fluorophores. Similar methods have been described in recent years, like fluorescence loss in photo-bleaching (FLIP), photo-activation and photo-conversion. For reviews on them, see [5,6].

TIRF microscopy exploits the physical phenomenon of total internal reflection, which occurs between an optically dense medium, such as glass, and a less dense medium, like an aqueous solution, to restrict illumination to around 100–200 nm above the glass/water interface. When light impinges on the interface at an angle higher than the so-called critical angle, it is completely reflected back into the dense medium. As a consequence, an evanescent wave is induced perpendicular to the reflecting surface, whose intensity decays exponentially with the distance. Only the fluorophores located within the evanescent field are excited. The presently most common configuration of TIRF microscopes is the lens illumination (Fig. 1b). In this method, an objective with high numerical aperture is used to make the illumination beam enter the sample above the critical angle. Light entering the objective must be confined at the back focal plane of the objective to the portion of the aperture cone that allows it to exit the optical surface above the critical angle. Otherwise, epi-illumination is produced and the signal-to-noise ratio decreases. One of the main advantages of TIRF microscopy is its high signal-to-noise ratio, which allows even the detection of single molecules. Its illumination strategy makes it very suitable for membrane applications [7,8]. Moreover, data are collected with cameras that provide a very high temporal resolution for monitoring dynamic processes at surfaces. Because of these reasons, TIRF microscopy has become a widely used method for single molecule applications (see below).

Single particle tracking (SPT) can be used to follow the motion of single proteins or lipids within membranes [9–11]. Single particle sensitivity is necessary for this technique. For that purpose, nanoparticles (i.e., latex beads or colloidal gold) coated with antibodies or other ligands are used to label the molecules of interest. Because these particles are smaller than the light wavelength, they scatter light and produce a diffraction pattern that is used to calculate the centroid position with around 10 nm precision. The molecules under study can also be fluorescently labeled with organic dyes, quantum dots or fluorescent proteins. Fluorescent probes are of smaller size than nanoparticles and probably interfere less with the biomolecule function and dynamics. However, they experience blinking and photo-bleaching, which hinder the particle tracking and reduce the achievable acquisition time. A number of different approaches including analysis software, simulations and biochemical strategies have been implemented to keep acquisition times in the ms regime [12–15]. The motion of particles in the two-dimensional plane of the membrane is captured with video microscopy. Usually, a contrast bright field method is used for nanoparticles, although during the last years the use of TIRF microscopy for the tracking of individual, fluorescently labeled particles has become very popular. The time resolution is very good, usually ranging between milliseconds and microseconds, and depends on the characteristics of the camera and the signal-to-noise ratio. The video images are recorded in real time and digitally analyzed a posteriori. For that, specialized software is used that allows tracking of single particles with high precision and without confusing them with neighboring particles (Fig. 1c). At the same time, the tracking software must be flexible to changes in the particle brightness due to out-of-focus movements. From the analysis of the individual trajectories, the mean square displacement can be calculated and associated with a diffusion coefficient. If the analysis of the trajectories is performed with an algorithm that can statistically separate various diffusion modes, SPT becomes a very powerful method to distinguish motion types, such as Brownian diffusion, confined diffusion, anomalous diffusion or directed flow.

Fluorescence correlation spectroscopy (FCS) is a powerful and well-established technique for the quantitative investigation of the dynamic properties of fluorescent samples [16]. In the case of


a FRAP
bleaching
fluorescence recovery
fluorescence intensity
immobile fraction
diffusion coefficient
time
b TIRF microscopy
evanescent field
objective
back focal plane
emitted fluorescence
illumination beam
c SPT
subpixel localization of particle position
particle tracking through frames
single trajectories
MSD
time lag
d FCS
Laser
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single image
number density
15
10
5
0
-5
80
60
40
20
0
spatial autocorrelation of fluorescence intensity image
f AFM
laser beam
photodiode detector
AFM tip
sample surface

membranes, it has proven very useful to characterize the fluidity and the phase of lipid bilayers of different complexity [9]. FCS is based on the statistical analysis of fluorescence fluctuations over time, usually due to the diffusion of single fluorophores in and out of the detection volume (Fig. 1d). To achieve this aim, the FCS setup requires very sensitive detectors that can detect single fluorophores, like avalanche photodiodes (APDs), and a tiny detection volume (in the order of fl), which normally is the focal volume of a confocal microscope. During the last years, several commercial setups have become available. Because FCS works in the single molecule regime, very dilute samples are required and protein overexpression is not necessary. Another advantage is the low laser power required for the measurements. Either with an on-line correlator or with off-line computing, the collected fluorescence intensity trace is subjected to autocorrelation analysis, a mathematical tool that yields the so-called autocorrelation curve. The amplitude of that curve is inversely proportional to the number of fluorophores in the sample, while the curve decay time is related to the average residence time of the fluorophores within the detection volume and, hence, to their diffusion properties. Fitting the autocorrelation curve to a mathematical model that correctly describes the diffusion mode of the fluorophores provides quantitative results about their concentration and diffusion coefficients. For that, calibration of the focal volume is necessary. Recently, new strategies, like two-focus, line-scan or z-scan FCS, overcame this limitation [17–20]. Another very powerful application is the two-color cross-correlation analysis, which quantifies dynamic interactions between biomolecules [21]. Though in principle FCS can also be used in membranes, it faces special challenges like the need of long measurement times due to slow diffusion and associated photobleaching problems, focal volume distortions due to refractive index mismatch and membrane movements or instabilities during the time of the measurement. To avoid these artifacts, z-scan FCS or several implementations of scanning FCS approaches have been successfully applied to membranes. For reviews on FCS in membranes, see [22] and [23].

Several image correlation techniques have been implemented during the last years. They are conceptually similar to FCS [24]. For example, image correlation spectroscopy (ICS) calculates the spatial correlation function of the fluctuations in fluorescence intensity of an image, which can be acquired by confocal or two-photon LSM or with TIRF microscopy (Fig. 1e) [25]. In this series of techniques, the homogeneity of the image in time and space is very important since strong artifacts are otherwise introduced into the analysis. As for FCS, the autocorrelation function is fitted to an analytical model, from which the number of independent fluorophore particles or the cluster density is calculated. Also, image correlation methods depend on external calibration of the point spread function (PSF) of the microscope and are insensitive to movements or interactions below the diffraction limit. The most important parameters affecting the quality of the results are statistical sampling (the number of spatial intensity fluctuations collected) and the background noise [26]. However, ICS cannot extract dynamic information from the sample because it only analyzes one image. The collection of a temporal stack of images allows temporal image correlation spectroscopy (TICS), from which the mode of mobility can be extracted [27]. Importantly, the imaging rate should match the time scale of the process under study. This is limited by the microscope setup, but it is usually within the adequate time range for the study of slow movements typical for membranes. Additional information, e.g., about the flow direction of fluorophores, can be obtained from spatio-temporal image correlation spectroscopy (STICS), which calculates the full spatial and temporal correlation function of a stack of images [28]. Slowly diffusing particles and/or the immobile fraction must be removed previous to the analysis. kICS calculates the two-dimensional Fourier transform of each image before time correlation, such that no previous knowledge of the system photophysics or PSF is needed [29]. Raster image correlation spectroscopy (RICS) is another alternative that allows the measurement of faster dynamic processes because it exploits the fact that imaging with the scanning microscope already introduces temporal sampling [30,31]: each pixel is acquired at a different, known time. The recently reported particle image correlation spectroscopy (PICS) is a hybrid method that combines ICS and SPT [32].

The microscopy strategies explained above can also be used to monitor FRET (Förster resonance energy transfer) [33–35]. The phenomenon of FRET is based on the non-radiative transfer of energy between donor and acceptor pairs. The most important parameters for FRET to occur are the spectral overlap, the quantum yield, and the distance and orientation between the donor and acceptor molecules. Since the efficiency of energy transfer decreases with the sixth potency of the distance, FRET can be used as a ruler to detect molecular interactions or conformational changes that occur below the optical resolution limit (less than 10 nm). The most common approaches to detect FRET with microscopy are sensitized emission (acceptor fluorescence intensity is detected upon donor excitation), acceptor photo-bleaching (the fluorescence intensity emitted by the donor increases after the acceptor has been bleached with high-power illumination) and fluorescence lifetime microscopy (FLIM) FRET (the fluorescence lifetime of the donor decreases due to FRET). Intensity-FRET measurements present bleed-through or spectral contamination between donor and acceptor fluorophores that needs to be corrected. This is avoided with the use of FLIM-FRET, because the fluorescence lifetime is independent of changes in dye concentration or excitation intensity. FLIM can be measured in the time domain or in the frequency domain and, in both cases, specialized instrumentation is required to detect the fluorescence lifetimes.

AFM can be combined with the above mentioned techniques for powerful approaches to the study of membrane dynamics. It is a label-free method that belongs to the scanning probe microscopy (SPM) techniques [36]. Initially developed for materials sciences, it has become very popular for biological applications because the surface scanning of the sample can be done in the presence of a physiological buffer. As shown in Fig. 1f, the principle of AFM relies on the force experienced by the AFM tip when it is brought close to or into contact with the sample surface. The sample is scanned underneath the tip and, as a result of the interactions between sample and tip, the flexible cantilever that holds the AFM tip is deflected. Structural information is obtained from the position at which a laser beam focused on the cantilever is reflected on a photodiode detector. As a result of the scanning, a topographical image of the sample surface can be calculated. This makes AFM very convenient for the study of membrane organization from a surface

Fig. 1. Schematic representation of surface-sensitive microscopy techniques. (a) FRAP. A small region of the fluorescent sample is photo-bleached with high-power laser illumination. The diffusion of fluorophores into the bleached region leads to an increase in fluorescence with time. From the quantitative analysis of fluorescence recovery, diffusion coefficients and immobile fractions can be estimated. (b) TIRF microscope. Critical illumination is achieved by positioning the laser focus far from the objective's optical axis. As a result, an evanescent wave is created that limits sample illumination to a thin section. (c) SPT. After acquisition of images with single molecule sensitivity, the position of particles are calculated with subpixel resolution. Then, particle tracking in consecutive frames allows calculation of single trajectories and of the mean square displacement with time. The curve shape depends on the motion type. (d) FCS. The diffusion of single fluorophores in and out of the focal volume induces fluorescence fluctuations with time. The autocorrelation curve is obtained from the fluorescence intensity trace. The number of particles and the diffusion coefficient can then be inferred by fitting the curve to the adequate diffusion model. (e) ICS. The spatial correlation of fluorescence intensity is performed on an image and provides the number density and the aggregation state of fluorescent particles (courtesy of S. Chiantia). (f) AFM. The interaction of the AFM tip with the sample surface causes a deflection of the cantilever that is detected by the position of the reflected laser on the photodiode detector. This information is used to build a topographical image of the sample surface.

point of view. An important advantage of AFM is that the spatial resolution is only limited by the size of the tip and can be as low as a few nanometers. In addition, it can be easily combined with optical microscopy. However, only the sample surface can be accessed. Moreover, it usually takes several seconds or even minutes to obtain a high quality image of the sample, which limits the range of dynamic processes that can be studied with this method. Recently, some fast AFM methods have been developed with enhanced time resolution. See [37–39] for recent reviews on the application of AFM to biological membranes.

There are other techniques to study membrane dynamics, though many of them lack the spatial approach of the methods described above and are out of the scope of this review. Recently, a mass spectrometry approach has been adapted to provide high resolution spatial information. Secondary ion mass spectroscopy has been used to provide a map of the chemical composition of supported bilayers with phase separation [40]. In addition, the last years have seen development of fluorescence-based super-resolution techniques, such as STED (stimulated emission depletion) microscopy and photoactivated localization microscopy (PALM), which improve the resolution to a few 10 of nm. Some efforts towards the measurement of dynamic processes in live cells have been reported [41,42]. Another promising approach is near-field scanning optical microscopy (NSOM), which is a SPM technique that measures the fluorescence intensity on the sample surface with a very sharp optical fiber [43,44]. It has similar advantages to AFM, but in this case the molecules of interest must be fluorescently labeled, which can be used to add an additional level of specificity to the measurement. On the down side, the setup is complicated and the measurement times are long, which has so far hindered the spreading of this technique.

3. Model membranes

The most common artificial membrane systems used in combination with microscopy-based techniques are supported lipid bilayers (SLBs) and giant unilamellar vesicles (GUVs). Among the methods to prepare SLBs, thermal fusion of small liposomes and the Langmuir–Blodgett film deposition are the most popular [45]. The solid supports can be glass or freshly cleaved mica, which offers an optically transparent and especially flat surface very convenient for AFM studies. In spite of a thin layer of water molecules separating the solid support and the lipid bilayer, several studies have pointed out that the proximity between them hinders the diffusion of membrane-embedded molecules, lipids included, when compared to free-standing bilayers. This is especially relevant for membrane proteins with large protruding regions. For this reason, there is a lot of interest in the development of polymer or gel cushions that can be used as a spacer between the solid support and the bilayer such that the diffusion of membrane molecules is not impeded. Long-chain tethers based on polyethyleneglycol can be used to keep a certain distance between the membrane and the support [46–48]. Despite this inconvenience, SLBs are generally very useful model membrane systems because of their stability and homogeneity, the possibility of patterning, simple preparation protocols, the ability of using many different lipid and protein compositions, and the relative ease to incorporate membrane proteins once they have been successfully reconstituted into small liposomes. An example of SLB is shown in Fig. 2a.

GUVs are lipid vesicles that vary from a few to hundreds of micrometers in diameter and that can be observed with optical microscopes. They have become useful model systems because they are free-standing bilayers with controlled composition and sizes in the same range as cells. Also is the same way as cells, their membrane area can be observed with optical microscopy (see Fig. 2b) and they have also been used to study dynamic processes on their surface. However, because they are deformable, they cannot be studied with "contact" techniques like AFM. Among the described methods for preparation of GUVs, electroformation is the most popular because of its simplicity and the quality of the vesicles obtained [49]. The main drawbacks are that it yields relatively heterogeneous vesicle sizes and compositions and that it works best in the absence of salts. In addition, the sample has to be dried, a harsh treatment which can prevent the successful reconstitution of membrane proteins. However, Montes et al. have recently described an electroformation protocol that can be applied under physiological conditions and with complex membrane compositions, like red blood cell membrane extracts [50].

More complex model systems like giant plasma membrane vesicles, cell membrane patches or cultured cells have also been used to investigate dynamic processes with surface-sensitive techniques (Fig. 2c and d). The main advantage is that their composition is closer to physiological conditions, but in turn, it becomes more difficult to control experimental parameters. There are a couple of reported methods to prepare giant vesicles derived from plasma membranes, but they consist on relatively harsh chemical treatments that could affect the membrane composition [51,52]. Cell membrane patches can be prepared by ripping off the upper membrane of cultured cells with a coverslip coated with poly-L-lysine [53]. In that way, the cytosolic side of the plasma membrane is on top and facing the buffer. If supports with holes are used to grow the cells on, then free-standing membrane patches are obtained that seal the holes and can be accessed from both sides [54].

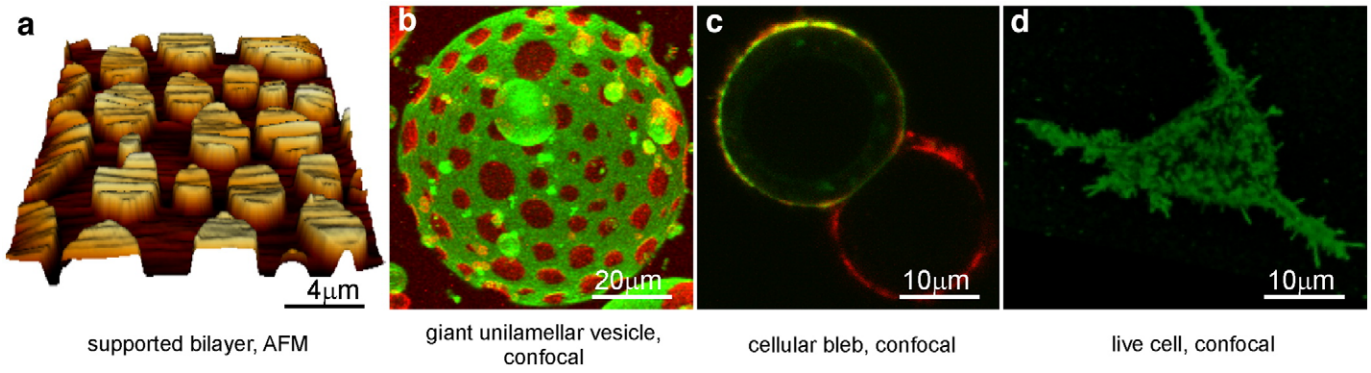

Fig. 2. Membrane model systems. (a) Supported lipid bilayer with phase separation. The height differences between the L_o (light) and L_d (dark) phases are detected with AFM. (b) Three dimensional reconstruction of a giant unilamellar vesicle with lipid domains, acquired with a confocal microscope. The L_o (green) and L_d (red) domains are labeled with specific, fluorescent probes. (c) Plasma membrane vesicle or bleb attached to the cell body imaged with confocal microscopy. GPI-GFP is shown in green and equinatoxin II labeled with Atto655 is shown in red. (d) Three dimensional reconstruction of the plasma membrane of a living cell stained with fluorescently labeled equinatoxin (green). Image obtained with confocal microscopy.

4. Membrane dynamics and phase behavior

The lateral organization of membranes is important for many of the specialized processes, such as trafficking or signaling, that take place in the bilayer. Research during the last years supports an important role of protein and lipid heterogeneities in the positioning of these processes at certain membrane spots. Indeed, the concentration of proteins in domains or clusters can modulate their collision probability and, thus, their activity. Some of these membrane domains, like the ones described by the raft theory [3], are characterized by distinct lipid/lipid interactions and packaging from the surrounding medium. As a result, the membrane in these microdomains has different physico-chemical properties, such as fluidity or thickness, which could be exploited by the cell in order to compartmentalize certain membrane functions [55]. Thus, the dynamics of lipids and membrane proteins provide useful information about the fluidity of the bilayer in which they are embedded. This can be directly associated with membrane organization and used to characterize the role and nature of lateral heterogeneities.

When model membranes, such as SLBs or GUVs, are prepared with a lipid composition that mimics that of lipid rafts, lipid domains are observable by fluorescence microscopy and AFM. These domains are organized into a more densely packed phase, enriched in sphingolipids and cholesterol, known as liquid ordered (L_o) phase, and a more fluid, liquid disordered (L_d) phase. FCS has been extensively used to investigate the organization of phase-separated membranes [22,56]. Early on, FCS was used to assign phases to lipid domains in GUVs based on fluidity differences [57,58]. The diffusion coefficients of lipids measured by FCS proved useful to evaluate membrane organization and were used to build FCS-based phase diagrams that show how the dynamic properties of lipids vary between the different phases [59]. With similar approaches, the effects of acyl chain saturation, cholesterol concentration and sterol structure in membrane organization and dynamics were evaluated [60–62]. Using SLBs, protein binding was shown to modulate lipid lateral mobility and the phase transition temperature of the membrane [63]. The combination of FCS with AFM offered the possibility of characterizing the dynamic and structural organization of phase-separated membranes simultaneously [64]. It was used to show that above a certain concentration, ceramide forms gel-like domains that can coexist with L_d and L_o phases [65], depending on the acyl chain length [66]. Recently, the partition coefficients of different lipophilic probes between L_o and L_d phases and the temperature dependence of the diffusion coefficient in membranes were evaluated with line-scan FCS [67].

Several studies have addressed the effect of the solid support on the lipid dynamics within SLBs. FRAP studies showed that the support material and the membrane preparation method affect the dynamic properties of SLBs [68]. However, when FCS measurements were performed specifically on the inner or outer leaflet of SLBs prepared under different conditions, the diffusion coefficients were basically the same, except for a small systematic tendency to be slower (<5%) in the inner leaflet [69]. Similar results were obtained in the case of asymmetric, phase-separated SLBs measured by SPT, where the diffusion of lipids in the L_o and L_d phases was not affected by transbilayer coupling [70]. Recently, the effect of a nanostructured solid support on the formation and dynamics of SLBs was investigated by a combination of FRAP and AFM. The quantitative analysis of the lipid dynamics showed that the nanopatterned support induced anisotropy in the macroscopic diffusion of fluorophores [71].

The quantitative analysis of the motion of membrane molecules in more complex model systems, such as plasma membranes, has prompted a new understanding in the organization of biological membranes. In such systems, not only lipophilic dyes but also fluorescently labeled proteins have been used as probes to monitor membrane fluidity and organization. Plasma membrane vesicles are interesting model systems because they partially retain the complexity of cellular membranes and have been shown to phase-separate under different conditions [51,52]. With the use of scanning FCS, the diffusion coefficients of probes in both phases were found to be reminiscent of the L_o and L_d phases found in GUVs [52].

FRAP was also used to test whether the mobility of membrane proteins in cells was in agreement with the raft hypothesis. The obtained results pointed to the membrane anchor as the main determinant of protein diffusion [72]. However, in a FRAP study on the apical membrane of MDCK epithelial cells, diffusion differences were found between raft and non-raft proteins that were attributed to a membrane state close to the raft percolating threshold [73].

In an elegant approach, Wawrezinieck and coworkers varied the size of the focal volume to measure FCS at different spatial scales [74]. From those data, they could calculate the so-called diffusion laws of typical raft and non-raft associated proteins in living cells, which followed lipid-dependent or cytoskeleton-dependent diffusion, respectively [11]. Interestingly, when they measured FCS diffusion laws including sub-diffraction apertures, they could access the nanostructural organization of cell membranes and estimate the size of lateral heterogeneities [75]. Recently, they have used this strategy to investigate the dynamic properties of E-cadherin in cell junctions and the nature of raft nanodomains involved in Akt/PKB signaling at the plasma membrane [76,77]. With a similar strategy of reducing the measured membrane area under the diffraction limit, Eggeling et al. combined FCS with STED microscopy to detect and characterize the size and dynamic properties of raft-like nanodomains [78].

The use of SPT to study the motion of membrane molecules has significantly improved our understanding of how the cytoskeleton affects membrane dynamics and organization. Kusumi and coworkers analyzed the single trajectories of lipid analogs and several types of membrane proteins, including raft and non-raft markers, to find that all these molecules experienced hop diffusion and transient confinement in nanometer-sized zones, in a cytoskeleton-dependent manner [79–83]. This led to the proposal of the “picket and fence” model for membrane organization, in which the membrane is compartmentalized by the actin-based membrane cytoskeleton that acts like “fences,” and anchored transmembrane proteins that would be the “pickets” [10,84]. This model is supported by a recent study in which simultaneous measurements of FcεRI motion and GFP-tagged actin dynamics showed that actin not only affects protein confined diffusion, but also long-range mobility, sequestration and ligand susceptibility [85].

SPT experiments have also been used to investigate the existence of microdomains in the cytoplasmic leaflet of the plasma membrane. Despite the existence of domains with two different diffusional populations of H-Ras that depend on the activation state, no evidence was found to support their association with lipid rafts [86,87]. However, similar activation-associated changes in diffusion were found for odorant receptors [88]. Moreover, experiments in T cells showed that the formation of protein complexes in the plasma membrane during signaling had a trapping effect and affected the diffusion of other membrane proteins [89]. Interestingly, modification of the overall plasma membrane fluidity affected not only the diffusion of oxidized fluorescent lipids, but also their uptake via endocytosis [90].

5. Dynamics of membrane organization

At a higher level of organization, the dynamic properties of lipid and protein domains have also been extensively investigated with surface-sensitive techniques. Here, the motions of lipid heterogeneities or protein clusters in the membrane, and not that of their constituents, are the objects of interest. The characterization of the dynamic behavior of such entities is crucial for our understanding of the principles of organization of biological membranes.

Model membranes that exhibit phase separation, like SLBs and GUVs, are excellent systems for the investigation of the dynamics of

domain formation and growth. While the translational motion of lipid domains is hindered by interactions with the solid support in SLBs, the slow diffusion of L_o domains within the membrane plane can be observed in GUVs. In recent studies, the 2D diffusion of such micrometer objects was found to be Brownian and to fall out of the application range of the Salman–Debrück approximation, though it could be described with an analytical model derived from the Hughes model [91,92]. An interesting issue here is the coupling of lipid domains across the two leaflets of asymmetric bilayers. Kiessling and coworkers showed that ordered domains can be induced in the inner leaflet of supported bilayers by domains in the outer leaflet [93,94]. Interestingly, a recent report shows that tuning the lipid composition can induce or suppress domain formation across leaflets of pure-lipid asymmetric bilayers [95]. In the case of gel phases, asymmetric membranes were shown to evolve differently, depending on the relative area fraction of gel phase between the two leaflets [96].

Several studies have addressed the kinetics of domain growth in model membranes [97–102]. The nucleation of lipid heterogeneities below the transition temperature leads to the formation of domains. In the case of raft-like membranes, the different thickness of the L_o and L_d phases introduces a height mismatch between the two phases at the interface. This is energetically unfavorable and a key parameter causing line tension, an energetic cost that depends on the perimeter of the domain boundaries. In a study combining confocal microscopy and AFM with SLBs, we showed that line tension strongly influences the lateral organization of raft-like membranes [102]. We found that the temperature of phase separation, the kinetics of domain growth and the domain size in equilibrium greatly depend on the phase height mismatch, and thus, on the line tension at the domain edges. However, the relationship between line tension and height mismatch is not clear. Our work and other theoretical considerations point to a quadratic relationship, while other studies evaluating the role of cholesterol on line tension suggest that it is linear [99]. Another important effect of line tension is domain budding, which has been observed in GUVs. A link between line tension at domain edges and membrane budding and vesicle formation has been suggested in a number of reports [103–105].

An appealing recent concept is the role of critical fluctuations on the lateral organization of biological membranes. Using NMR, Veatch and colleagues identified a line of critical points in the phase diagram of dioleoylphosphatidylcholine, chain perdeuterated dipalmitoylphosphatidylcholine and cholesterol [106]. They showed that composition fluctuations smaller than 50 nm appeared in the membrane plane near critical points. Careful analysis of GUVs with critical compositions revealed that as the critical point is approached from low temperature, the line tension decreases to zero. Miscibility transitions near the critical temperature are of the order of microns, while they are expected to become submicron at higher temperatures, in agreement with the two-dimensional Ising model for critical phase transitions [107]. Interestingly, a similar critical behavior was found in giant plasma membrane vesicles derived from cells [108]. Based on these observations, the authors suggested that the composition of the plasma membrane of mammalian cells is finely tuned to reside near a miscibility critical point that allows composition fluctuations smaller than 50 nm and that may be functionally related to lipid rafts.

The effect of proteins on the lateral organization of membranes has also been investigated with surface-sensitive techniques. With a combination of AFM and confocal microscopy, we found that a membrane active peptide derived from the apoptotic protein Bax induced the reorganization of phase-separated membranes into irregular domains (Fig. 3), which was associated to the reduction of the line tension at the domain interface [109]. Moreover, cross-linking of GM1 with cholera toxin subunit B was shown to induce phase segregation in GUVs and in giant plasma membrane vesicles [52,110]. Similarly, Shiga toxin-induced clustering of the glycolipid Gb(3) promoted membrane reorganization and domain formation in SLBs [111]. Another example of protein-induced alteration of the membrane organization is the formation of pores. Using AFM, Lam et al. investigated the dynamics of pore formation and membrane disruption by the pore forming peptide protegrin-1 [112].

On the other hand, the lateral organization of membranes can also affect the distribution of membrane proteins and lipids. For example, modulation of the local concentration of ceramide in raft-like membranes can induce the formation of a third, ceramide-rich, gel phase that coexists with L_o and L_d phases. The dynamics of membrane reorganization during enzymatic production of ceramide were followed by fluorescence microscopy and AFM [113]. Under those conditions, the GPI-anchored protein PLAP and the ganglioside GM1, which are usually enriched in the L_o phase, partitioned preferentially to the ceramide domains [114]. The role of protein coat formation on sorting was investigated with a biotin/streptavidin system on GUVs. Individual tethered proteins localized to the L_d phase, while ordered protein domains distributed to the L_o phase [115]. However, COPI coating assembly was found to occur specifically in the L_d phase and to induce membrane deformations depending on the membrane tension of the vesicle. Interestingly, the deformed regions seemed to have a different lipid composition because they were protected from phase transitions [116]. Another situation in which the membrane has been shown to contribute to the spatial organization of proteins is the case

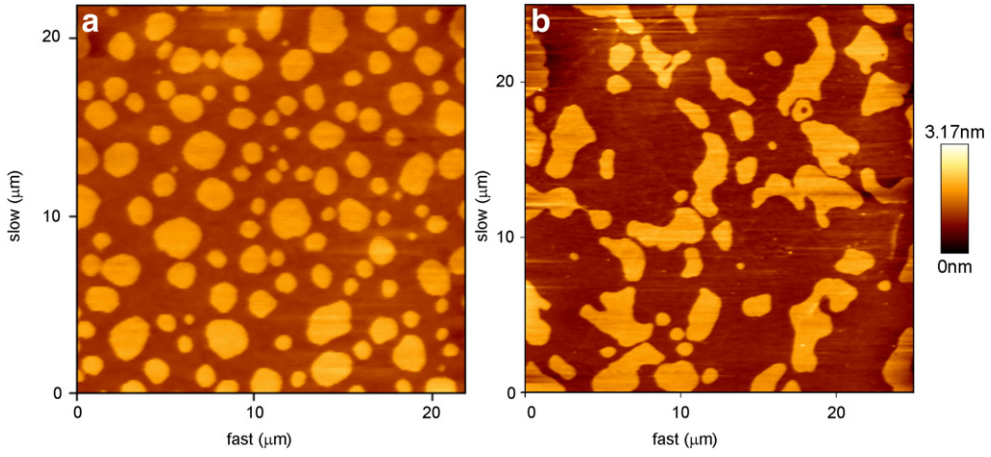

Fig. 3. Changes in domain shape induced by a membrane active peptide imaged with AFM. (a) Pure SLBs composed of DOPC:SM:Chol (1:1:0.67) exhibit phase separation. Circular L_o domains (light) are surrounded by a thinner L_d phase (dark). (b) L_o domains become irregular after addition of a peptide derived from helix 5 from Bax (L/P = 109).

of the Min proteins, which are involved in determining the center position for bacterial cell division. In this case, the membrane forms part of an oscillatory three-component system that combines 3D and 2D geometries. In a reconstituted system, the sequential binding/dissociation of the proteins to the membrane results in dynamic planar waves of proteins on the membrane that can be described with a reaction–diffusion model (Fig. 4) [117]. Recently, the membrane has been shown to enhance the affinity between the apoptotic proteins tBID and BCL_{XL}, which has implications for the regulation of apoptosis at mitochondria [118].

Several studies have addressed the role of curvature on the lateral organization of membranes. The lipid distribution in thin tubes pulled from GUVs with raft-like composition was found to differ from the rest of the GUV [119]. This curvature-induced lipid sorting was found to depend on the collective behavior of lipids and to be affected by lipid-clustering proteins, whose sorting is also curvature sensitive [120,121]. Parthasarathy and coworkers used micropatterned substrates to constrain SLBs to different curvatures. They found that beyond a certain threshold, the membrane curvature governs the spatial distribution of the L_o and L_d domains due to differences in the bending rigidity between the phases [122].

A similar strategy was used to study the activation of the immunological synapse in T cells. The spatial translocation of T-cell receptors was found to regulate signaling in a system with T cells on top of SLBs on a microstructured support that spatially limits the formation of the synapses [123]. Synapse activation induced flow driven by acting polymerization that sorted T-cell receptor clusters depending on their size. These observations led to a model in which frictional coupling to the cytoskeleton controls cluster sorting [124,125].

As in the example above, TIRF microscopy has been extensively used to study the motion, oligomerization and interactions between membrane proteins. Some examples include dynamics of receptors involved in gradient sensing [126] and PI3K signaling during chemotaxis [127], studies on EGFR activation by EGF binding [128–130], dynamics of PLCβ1 during GPCR stimulation [131], the role of FERM domain proteins in cell–substrate adhesion [132] or the distinct sorting of EGF and transferrin prior to endocytosis [133]. This strategy has also proved very useful to investigate membrane trafficking processes. It has been employed to follow the interaction of vesicles with the target plasma membrane and the subsequent fusion process. Kyoung and Sheets used a TIR-FCS to investigate the effect of salt concentration, pH and lipid composition on the diffusion of small vesicles on supported bilayers [134]. Moreover, viral infection was studied by tracking the fusion dynamics of individual virus particles with SLBs [135]. Using cellular systems, TIRF microscopy was used to investigate the exocytosis of synaptic vesicles [136]. Other exocytic processes, like the release of glutamate and ATP from astrocytes [137–140], the insulin-stimulated secretion of GLUT4 [141], the exocytosis of secretory granules in chromaffin cells [142–144] and of insulin granules [145–147], have also been studied with TIRF microscopy. Recently, this strategy has been applied to the exocytic pathway in polarized cells [148]. Such approach has also proved very useful to improve our understanding of clathrin-mediated endocytosis [149–151] and the regulatory role of PIPs [152–154].

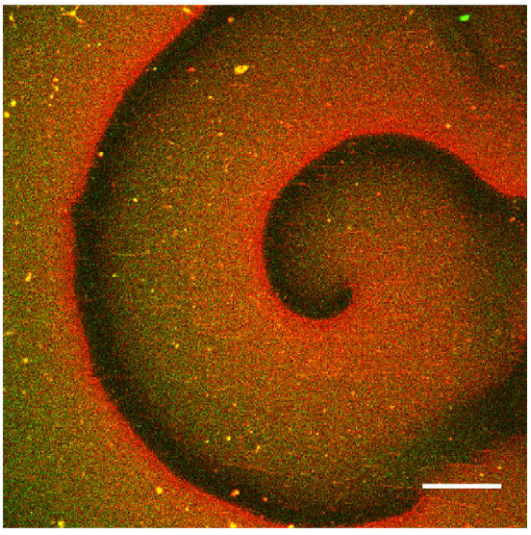

Fig. 4. Self-organized waves of Min proteins on a SLB. Surface organization of labeled Min D (green) and Min E (red) can be followed with confocal microscopy. Scale bar, 25 μm (courtesy of M. Loose).

FRET studies have also been used to investigate the dynamic structure of membranes. For example, FLIM-FRET was used to monitor changes in lipid order in cell membranes upon cholesterol depletion [155], the coalescence of rafts during EGF signaling [156] or dynamic protein/lipid interactions in living cells [157]. But mostly, FRET has been used to characterize protein organization in cell membranes, including the formation or protein microdomains [158–160], trafficking [161] and ligand/receptor dynamics [162]. In an elegant approach, Varma and Mayor used Förster resonance energy transfer between equal dyes, called homoFRET, to investigate the organization of the plasma membrane in living cells. They found that GPI-anchored proteins assemble in cholesterol-dependent nanoclusters, in contrast to other transmembrane proteins that showed a random distribution [163]. Cross-linking of GPI-anchored proteins also affected the distribution of protein domains and their endocytosis, thus revealing a functional, lipid-dependent clustering of these proteins [164]. A high temporal resolution analysis of the cluster dynamics showed that they are immobile and that the redistribution dynamics between monomers and clusters is heterogeneous. Interestingly, depletion of cortical actin affects the dynamics and organization of the nanoclusters [165].

6. Conclusions

In this review, we have explained the basic principles of the surface-sensitive microscopy techniques that have proven most useful for the study of dynamic processes in biological membranes. The selection of one or another depends mainly on the time scale of the process under study and on the spatial resolution required. Lately, the combination of several of these approaches is becoming quite popular, since it provides complementary information that gives a more comprehensive view of the system of interest. We have also commented on the most convenient membrane model systems used with such methods, including giant vesicles, supported bilayers and the plasma membrane of living cells. Artificial membranes are simple systems that allow easy control of the experimental conditions. They have shown to be extremely useful to investigate the principles that govern membrane organization. On the other hand, cell-derived model membranes are closer to the complexity found in organisms and the information obtained from them is expected to better resemble the natural situation. However, both are necessary to achieve a satisfactory understanding of how membranes organize in living systems. From lipid dynamics and composition fluctuations near critical points, to lateral heterogeneities and cytoskeleton effects on membrane organization, the combination of surface-sensitive techniques and membrane model systems has already aided significantly our knowledge of the dynamic organization of biological membranes. And surely, it will be exciting to follow the new discoveries in the field during the next years.

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