Review

To see or not to see: Lateral organization of biological membranes and fluorescence microscopy ☆

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Abstract

In the last few years several experimental strategies based on epi-, confocal and two photon excitation fluorescence microscopy techniques have been employed to study the lateral structure of membranes using giant vesicles as model systems. This review article discusses the methodological aspects of the aforementioned experimental approaches, particularly stressing the information obtained by the use of UV excited fluorescent probes using two-photon excitation fluorescence microscopy. Additionally, the advantages of utilizing visual information, to correlate the lateral structure of compositionally simple membranes with complex situations, i.e., biological membranes, will be addressed.
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Keywords: LAURDAN; Cholesterol; Two photon excitation fluorescence microscopy; Giant vesicle; Lipid phase coexistence; Raft

Contents

1. Introduction

There are simple questions regarding lipids and membranes that still need to be answered. For example, why do cell membranes contain thousands of different molecular lipid species? Why do the molar fractions of these species vary among different membranes? Is there a coherent code still hidden and waiting to be discovered in order to predict the influence of lipids on membrane lateral structure? As Higelmann pointed out in his article "Getting ready for the decade of the lipids" [1] "...why not speculate that (phospho)lipids and their metabolites will soon be the subject of an information explosion, similar to that presently occurring for genes and proteins?".

For several years lipids were considered randomly organized building blocks of biological membranes. This interpretation was adopted from the Singer and Nicholson fluid mosaic model, proposed in 1972 [2]. Even though the Singer and Nicholson fluid mosaic model was extremely important in considering, for first time, the dynamical aspects of biological membranes, it is obvious that the lipid compositional heterogeneity was not considered as an important factor in this model. The idea that lipids are simply randomly organized building blocks of membranes has been changed since the coexistence of stable lipid domains in lipid bilayers was reported three decades ago [3,4]. However, the consequences of the non-random lateral organization of lipid membranes have not been acknowledged until recently when the raft hypothesis was postulated [5–8]. Although the role of lipid compositional complexity is acknowledged in the raft hypothesis, the physical basis behind membrane lateral organization (including potential links with membrane function) in biological systems still remains obscure. For example, based on the information obtained from model systems, lateral heterogeneity in biological membranes can be rationalized simply as local (transient) compositional fluctuations or phase separation phenomena. However, very little information is available about the occurrence of these two phenomena in biological membranes. For instance, the presence of liquid ordered phases is repeatedly claimed in the literature but not always strictly demonstrated in many systems invoking rafts. This last observation points to the necessity of developing new experimental approaches in order to clarify the abovementioned issues. In particular, experimental information that allows correlation between the lateral structure of compositionally simple and complex membranes (i.e., artificial lipid mixtures and biological membranes) may help to identify the basis of membrane lateral heterogeneity in biologically relevant situations.

A detailed understanding of temperature-dependent membrane phase equilibria in compositionally simple model systems (composed of one, two and three-component lipid mixtures) constitutes a very important step in order to understand the lateral organization of compositionally complex membranes. For instance, in the last 35 years there has been extensive research to elucidate the presence of particular lipid phases (including coexistence of different lipid phases) in particular membranous systems using an array of experimental techniques that provide structural and dynamical information (fluorescence spectroscopy, differential scanning calorimetry, infrared spectroscopy, FRAP, EPR, NMR, X-ray diffraction to mention a few, [3,9–26]), including theoretical treatments using computer simulations [27,28]. In several cases the data were utilized to construct phase diagrams for each particular lipid mixture. Phase diagrams provide important thermodynamic information regarding the different lateral organization of different lipid mixtures [29,30]. However, the construction of phase diagrams is limited to a few components (no more than 3 components, [30]) and extremely difficult to implement in the case of compositionally complex membranes. The interpretation of the experimental data obtained from the abovementioned experimental techniques becomes complicated in compositionally complex situations. It is particularly difficult to ascertain the influence of specific molecular species on the membrane's lateral

structure as the number of components in the lipid mixture increases. As a consequence, correlation of the lateral structure of well described, compositionally simple cases with those observed in the compositionally complex situations becomes difficult. It is important to note that the experimental techniques mentioned above provide mean parameters on the basis of data collected from bulk solution of many liposomes (or cells), and hence lack information about lipid lateral organization at the level of *single* vesicles (or cells). Such information can, however, be provided by microscopy techniques such as fluorescence microscopy (also atomic force microscopy, see below). The main advantage in using fluorescence microscopy techniques in membrane studies is clear, i.e., the sensitivity and flexibility of a microscope with the addition of fluorescence spectroscopy allows the collection of spatially resolved information. Ultimately this latter information bridges membrane morphology with the dynamical and structural information obtained at the molecular level using fluorescence spectroscopy (such as lipid mobility, hydration, etc.). The additional "visual" information obtained in these experiments has generated new information that complements very well the "bulk" information obtained with the aforementioned classical approaches. This "visual" information can be used as another alternative to link the lateral structure of compositionally complex membranes (more than three components) with that observed in the compositional simple cases.

2. Model membrane systems

Many papers have appeared in recent years which describe the use of giant vesicles (GUVs) as model systems to study different physical aspects of membranes (lateral structure, mechanical properties), particularly considering the effect of lipid–lipid, but also lipid–DNA, lipid–peptide and lipid–protein interactions [31–72]. One of the reasons why giant vesicles are suitable membrane model systems is their size, on the order of a few tens of micrometers, similar to the size of the plasma membrane of cells. Due to their size, single vesicles can be directly observed using microscopy related techniques (such as phase contrast and fluorescence microscopy). Additionally, because experiments are performed at the level of single vesicles, heterogeneity in shape and size and the presence of multilamellar vesicles are ruled out. One of the significant aspects in using giant vesicles as model systems is the ability to control the molecular composition of the membrane as well as the environmental conditions. For instance, studies of the lateral structure of membranes using giant vesicles as model systems were normally confined to giant vesicles composed of single lipids species or mixtures with no more than three to four components [33,35,41,44–46,48,52–54,58–60,72]. However, as recently reported in the literature, it is also possible to form giant vesicles from natural lipid extracts [39,50,53,55] and native membranes [37,39]. Additionally, incorporation of membrane proteins into GUVs composed of lipid mixtures can also be performed [34,38,40,42,51]. This last fact allows one to establish an interesting strategy, i.e., to perform comparative studies among artificial lipid mixtures, natural lipid mixtures (both with and without membrane proteins) and finally membranes containing the full composition under controlled environmental conditions

[39]. Regarding preparation of giant vesicles there is no general agreement about "unique" conditions required to obtain such vesicles mainly because the mechanism underlying giant vesicle formation is still obscure. A major consequence of this dearth of precise knowledge is that there are many different methods described in the literature to obtain GUVs [38,39,51,73–77]. However, almost all these methods are based on two main experimental protocols, the gentle hydration method, originally described by Reeves and Dowben [77], and the electroformation method introduced by Angelova and collaborators [74,75]. From these two experimental protocols, the electroformation method provides the most homogeneous population of GUVs, with sizes between 10 to 60 μm in diameter. Additionally, the electroformation protocol requires less time compared to the gentle hydration method (∼1–2 h vs. 12–24 h, respectively) and provides a high yield of giant unilamellar vesicles (∼95%) [78,79]. The reader can find additional information about the giant vesicle field in an excellent review by Menger and Keiper [62] and a book completely devoted to giant vesicles edited by P.L. Luisi and P. Walde [80].

3. Fluorescence microscopy and phase coexistence as seeing in GUVs

To begin this section, we should note the seminal contribution from Haverstick and Glaser who, in 1988, achieved the first visualization of lipid domains in GUVs using fluorescence microscopy with digital image processing [70]. These authors directly visualized Ca^{2+}-induced lipid domains in erythrocyte ghosts, GUVs formed of mixtures of phosphatidylcholine (PC) phospholipid and acidic phospholipids and GUVs formed from natural lipids from the erythrocyte membrane at constant temperature [69,70]. However, it was not until the end of the 1990s when several papers appeared applying fluorescence microscopy techniques (epifluorescence, confocal and two photon excitation fluorescence microscopy) to show for the first time images of the temperature dependent lateral structure of giant vesicles composed of different phospholipids, phospholipid binary mixtures, ternary lipid mixtures containing cholesterol, natural lipid extracts and native membranes [33,39,44,46,48,50,52–55,58–60,81]. These papers presented, for the first time, a correlation between micron-size (visual) domain structure and the local lipid dynamics under different environmental conditions (different temperatures for example). Additionally, the experimental data involving fluorescence microscopy and GUVs have offered a new alternative to construct lipid phase diagrams [30,44,46] for artificial lipid mixtures (i.e., phase diagrams that include visual information about membrane lateral structure).

It is important to mention that the visual information itself, based on the acquisition of fluorescence intensity images (particularly obtained using environmentally insensitive fluorescent probes), does not always provide information about the *local* physical properties of the different domains that coexist in the plane of the membrane. In other words, the fluorescence intensity images obtained will limit us to information about the lipid domain's shape and size and eventually the GUV morphology. Therefore, caution must be invoked in assigning lipid phases, particularly in compositionally complex mixtures (such as natural lipid extracts or native membranes) but also in simple lipid mixtures, using only the fluorescence probe's partition information obtained in representative model lipid mixtures. It is very important to keep in mind that the partition of the fluorescent probes to particular membrane domains *does not depend on the membrane phase state*. Instead, the partition of fluorescent probes generally depends on the local chemical environment of the lipid domains [82,83]. For instance, the fluorescent probe Rhodamine-DPPE shows partition to the fluid phase in DMPC/DSPC mixtures while in DLPC/DPPC it shows preferential partitioning to the gel phase [54,82]. In this last case, both lipid mixtures display gel/fluid phase coexistence (Fig. 1A). Another case is that observed in a "two probe experimental approach", based on the differential labeling of the coexisting membrane domains using different fluorescent probes (for example using DiC_{18} and Bodipy-PC probes). In POPC/sphingomyelin mixtures that display gel/fluid phase coexistence, the probe DiC_{18} shows a preferential partitioning to the gel phase while Bodipy-PC partitions to the fluid phase. Instead, in GUVS composed of a POPC/DPPC mixture (that also show gel/fluid phase coexistence), both probes shows preferential partitioning to the fluid phase (see Fig. 1B). Clearly, then, it is not wise to generalize the fluorescent molecule's affinity for the different lipid phases without careful probe characterization.

On the other hand, physical information pertaining to the membrane's local domain, such as lipid diffusion coefficient or lipid local order, will assist in answering important questions. For instance, have the lipid domains similar physical characteristics among artificial membranes exhibiting the same phase coexistence scenario (gel/fluid or fluid ordered/fluid disordered) but different molecular composition (binary, ternary lipid mixtures)? Are the local properties of the in-plane membrane domains comparable among compositionally complex and simple membranes displaying the similar coexistence scenario? Only a few fluorescence microscopy based experimental strategies were used to determine the physical characteristics of in plane membrane domains. Using the abovementioned "two probe experimental approach" and scanning confocal fluorescence microscopy Korlach et al. [60] performed fluorescent images of particular phospholipid mixtures (DLPC/DPPC) displaying gel/fluid phase coexistence. The fluorescent probes used in this experiments (DiC_{20} and Bodipy-PC) display different partition properties to the two different coexisting lipid phases. Using fluorescence correlation spectroscopy (FCS) the authors correlate the visual information with information about the diffusion coefficient of each fluorescent probe in the different lipid domains. Various lipid phases were assigned based on the diffusion coefficients obtained in different regions of the membrane [60]. This experimental strategy was applied to other lipid mixtures, including GUVs containing membrane proteins [34,35,40,43,46]. One of the main drawbacks of this experimental approach is the necessity of finding pairs of fluorescent probes that specifically label the coexisting lipid domains. For instance, in mixtures that display fluid ordered/fluid disordered phase coexistence (particularly DOPC/sphingomyelin/cholesterol or DOPC/DPPC/cholesterol) the vast majority of fluorescent membrane probes (particularly
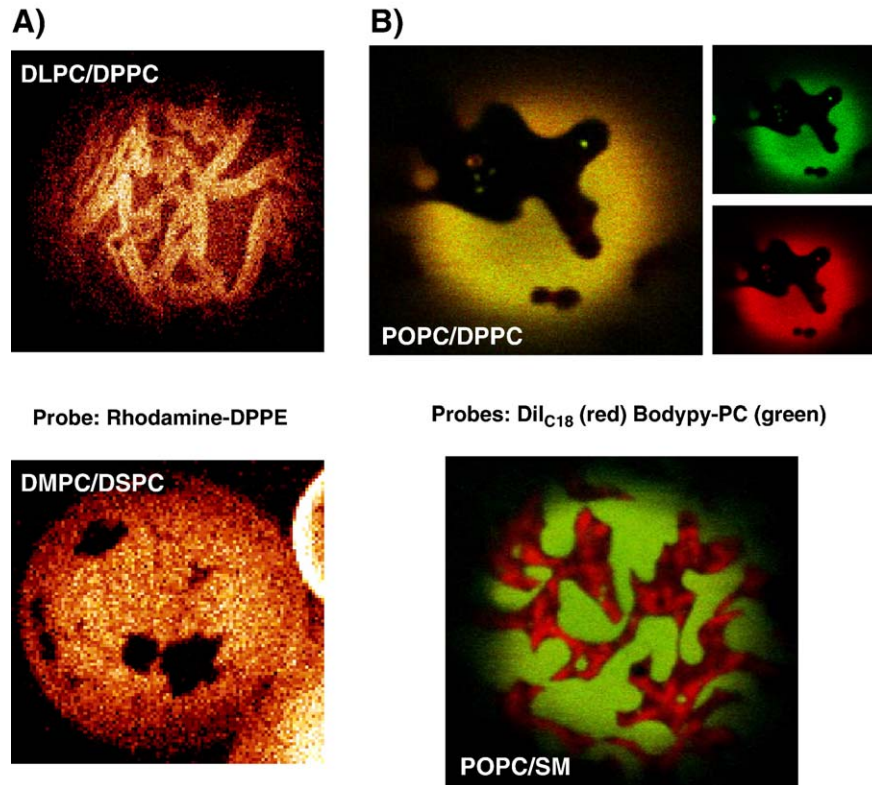
Fig. 1. (A) Two photon excitation fluorescence images of Rhodamine-DPPE labeled GUVs composed of two different phospholipids mixtures displaying gel/fluid phase coexistence. The fluorescent probe shows preferential partitioning to the gel phase in DLPC/DPPC mixture while in DMPC/DSPC mixture the probe is segregated to the fluid phase. (B) One photon excitation confocal images of GUVs composed of two different phospholipids mixtures displaying gel/fluid phase coexistence. Two fluorescent probes DiIC18 and Bodipy-PC are used in this case. Notice the different partition of the fluorescent probe DiI_{C18} in the different mixtures (see text). The GUVs diameter is approximately 25 μm.

those that are excited in the visible wavelength region and are normally used with epifluorescence or fluorescence confocal microscopes, such as the commercially available phospholipid-like derivates of Bodipy, NBD, carbocyanines and rhodamine) show preferential partitioning to the fluid disordered phase [45].

Nevertheless, the fluorescent probe perylene shows preferential partitioning to fluid ordered regions in DOPC/SM/cholesterol mixtures [45]; although, the partition of this fluorescent probe need to be further evaluated in other model systems displaying liquid immiscibility in order to corroborate if it can be used as a
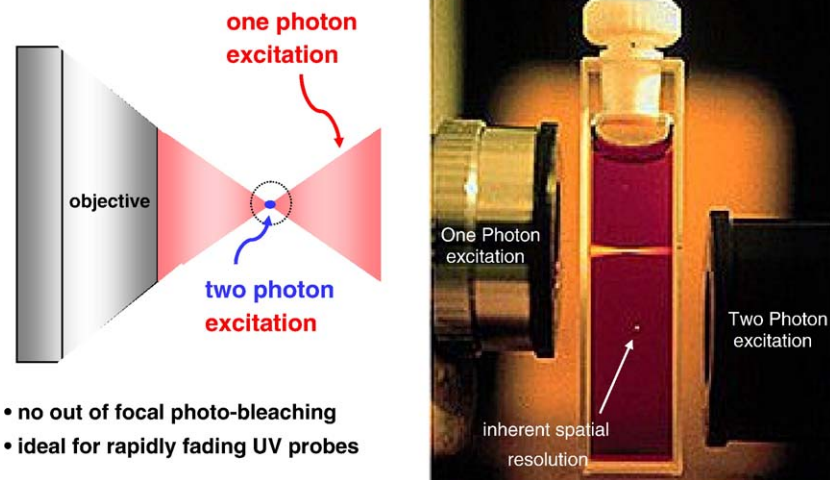

Fig. 2. One and two photon excitation effect. The high photon densities required for two-photon absorption are achieved by focusing a high peak power laser light source on a diffraction-limited spot through a high numerical aperture objective Therefore, in the areas above and below the focal plane, two-photon absorption does not occur, because of insufficient photon flux. This phenomenon allows for a sectioning effect (inherent spatial resolution) without using emission pinholes as in confocal microscopy.

fluorescent marker for fluid ordered phases. An alternative approach to label the liquid ordered phase is the incorporation of a small fraction of the ganglioside G_{M1} in the lipid mixtures [40,53]. The G_{M1}-enriched areas are assigned to be liquid ordered-like phase domains [40,53]. Using fluorescently labeled cholera toxin (that specifically binds G_{M1}), the G_{M1} enriched regions were visualized using fluorescence microscopy. In summary, visual information can be complemented with additional techniques such as FCS [35,46,60], FRAP or particle tracking to correlate visual spatial correlation with local membrane dynamics.

Another possible approach to gain correlated spatial and dynamical information in membranes displaying phase coexistence is to obtain images of other fluorescent parameters than simply fluorescent intensity. It is significant to mention that the vast majority of the studies about characterization of lipid membrane's lateral structure using fluorescence spectroscopy techniques (cuvette experiments) exploit other fluorescent parameters such as fluorescence lifetime, rotational correlation time (obtained by anisotropy measurements) and position of the emission spectral maximum. Several fluorescent probes, particularly those UV-excited fluorescent probes (such as Pyrene, DPH, TMA-DPH, trans-parinaric acid, LAURDAN, PRO-DAN), are used in these experiments [9,22,23,84–96]. Few papers concerned with the lateral structure of membranes (model system or cells) exploiting these UV-excited probes and fluorescent parameters were reported using fluorescence microscopy related techniques. The two main reasons for the dearth of such

studies are (i) the inaccessibility of these advanced microscopy techniques, i.e., lifetime microscopy or polarization fluorescence microscopy, (expensive and specialized equipment is required along with significant user expertise) and (ii) it is practically difficult to perform fluorescence microscopy experiments (one photon excitation, i.e., epifluorescence and confocal) with UV-fading fluorescent probes (such as LAURDAN or PRODAN), since the extent of photobleaching is high and is often technically difficult to obtain reliable fluorescence images.

4. Two photon excitation fluorescence microscopy

The application of two photon fluorescence microscopy in biology was introduced by Denk, Strickler and Webb in 1990 [97]. At present, two photon excitation (TPE) fluorescence microscopy constitutes one of the most promising and fastest developing areas in biological and medical imaging [98]. Two-photon excitation is a nonlinear process in which a fluorophore absorbs two photons simultaneously. Each photon provides half the energy required for excitation. The high photon densities required for two-photon absorption are achieved by focusing a high peak power laser light source on a diffraction-limited spot through a high numerical aperture objective [97,99,100]. Therefore, in the areas above and below the focal plane, two-photon absorption does not occur, because of insufficient photon flux. This phenomenon allows for a sectioning effect (inherent spatial resolution) without using emission pinholes as in confocal
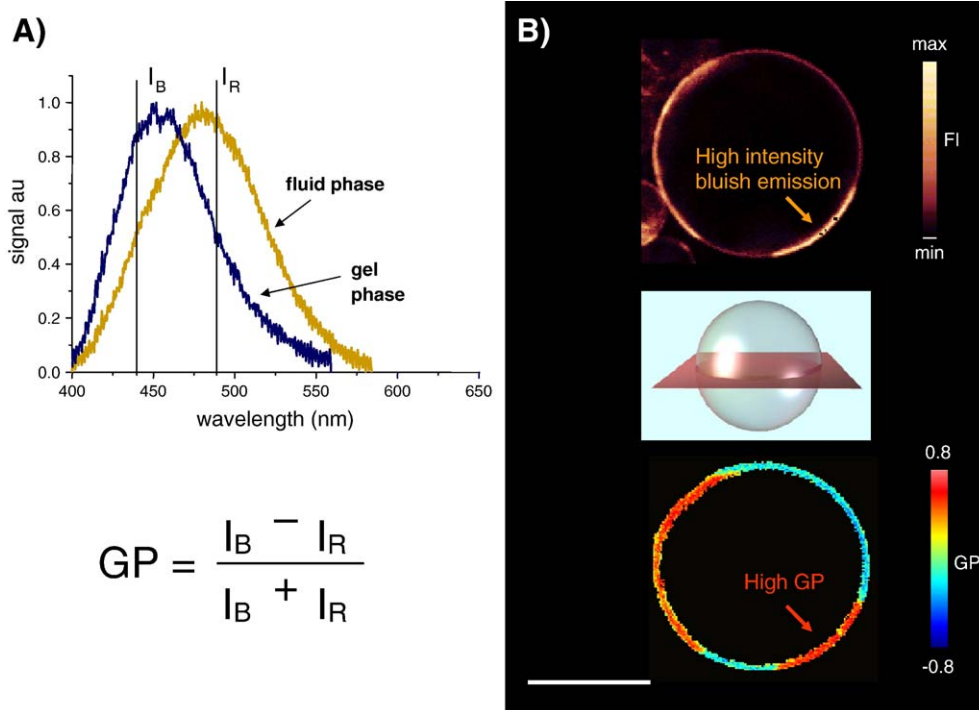

Fig. 3. (A) LAURDAN emission spectra in gel (blue) and fluid (green) phase membranes. The emission spectrum shift is around 50 nm. The Generalized Polarization parameter that depends on the position of the LAURDAN emission spectrum contains information about solvent dipolar relaxation processes which occur during the time that LAURDAN is in the excited state, and is related to water penetration in the lipid interfaces. (B) Two photon excitation fluorescence images obtained at the equatorial region of the GUV composed of DLPC/DAPC (at temperatures corresponding to gel/fluid coexistence). Intensity image obtained using a blue bandpass filter 446 ± 23 nm (Top image). The high intensity areas in this mixture are related to a gel-like phase. LAURDAN GP image (bottom). Notice the correlation between the high intensity areas (blue emission spectra) and the high GP values.

microscopy, see Fig. 2 [97,99,100]. The benefits of two photon excitation includes: improved background discrimination, reduced photobleaching of the fluorophores, minimal photodamage to living cell specimens and excitation of multiple fluorescent probes using a single excitation wavelength (including those probes that require more than one excitation wavelength in the one photon excitation mode) [97–101]. In the particular case of membranes, the characteristics of multiphoton microscopy allows the use of UV-excited fluorescent probes to fully combine and exploit two important pieces of information: i) the fluorescent parameters that are sensitive to membrane lateral structure, such as phase-dependent emission shift, fluorescent lifetimes or polarization as was done in the earlier studies in bulk, i.e., liposome solutions; (ii) visual information (morphological and topological data). Since their introduction, few TPE fluorescence microscopy experiments using UV- (but also visible-) excited fluorescent probes were reported to explore the lateral structure of lipid membranes [45,47,53–55,58,59,82,102–105]. This methodological approach offer a very consistent picture of relevant events such as lipid phase separation in lipid bilayers and cellular membranes (as will be discussed below) [45,47,53–55,58,59,82,102–105], enzymatic reactions in membranes [47,49] and insertion of peptides in lipid membranes [106,107]. Although many UV-excited fluorescent probes can be useful to

study membrane lateral heterogeneity (*vide supra*), the discussion in the coming sections will be limited primarily to results obtained with one of these probes, LAURDAN, in many different model systems and cell membranes and tissues. Despite the fact that photobleaching of LAURDAN using epifluorescence and confocal microscopy is severe, hampering the acquisition of reliable fluorescent images, TPE fluorescence microscopy is an alternative technique to collect images on LAURDAN-labeled specimens. Because this fluorescent probe has very interesting spectroscopic and partition properties in membranes it is possible to perform quantitative spectroscopic studies at each pixel and hence to obtain detailed information (e.g., correlation between morphology and dynamics) from a fluorescence-based image related to the sample at hand, be it a living cell, an extended surface polymer or a giant unilamellar vesicle.

4.1. LAURDAN: an ideal fluorescent probe to study lateral structure of membranes

LAURDAN belongs to the family of polarity-sensitive fluorescent probes, first designed and synthesized by Gregorio Weber for the study of the phenomenon of dipolar relaxation of fluorophores in solvents, bound to proteins and associated with lipids [92,108–111]. When inserted in lipid membranes,
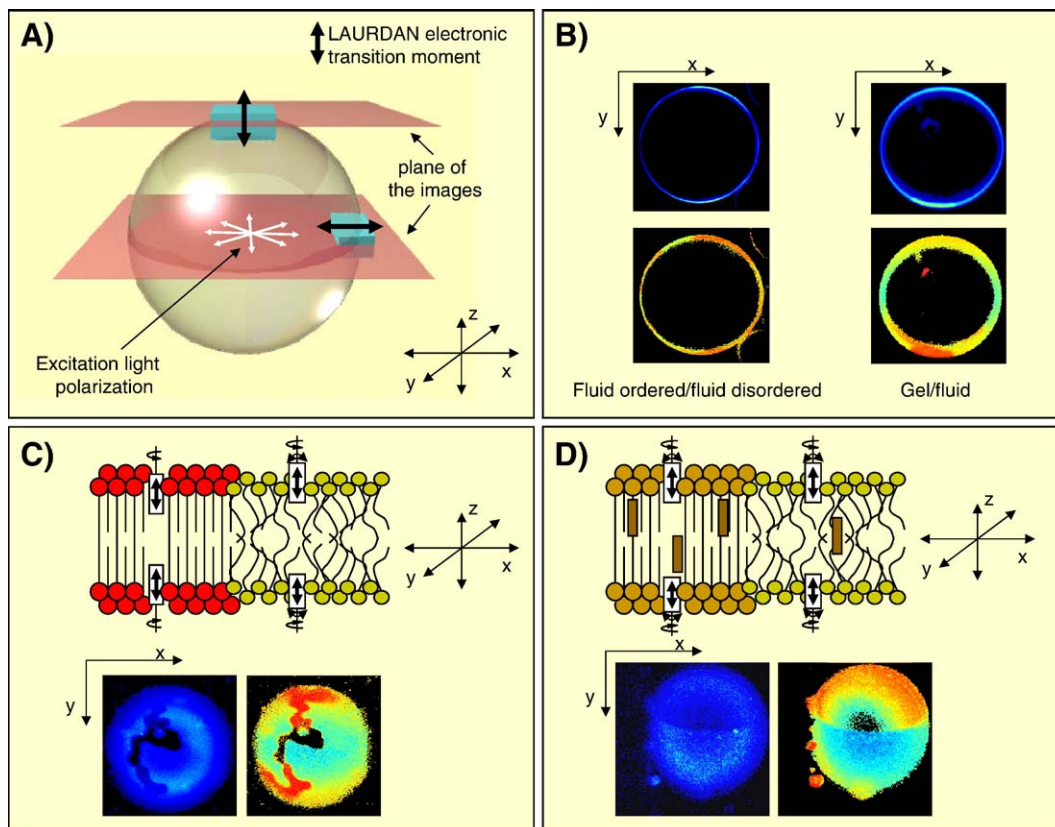

Fig. 4. The photoselection effect is dictated by the relative orientation of the LAURDAN electronic transition moment respect to the polarization plane of the excitation light. The last is also influenced by the lipid bilayer phase state (see text). (A) Sketch of the GUV and the position of the LAURDAN electronic transition moment; (B) the photoselection effect do not operate in the equatorial region of the GUV independent of the phase coexistence scenario; (C) gel/fluid phase coexistence observed at the polar region of the GUV composed of Ceramide/POPC 1:5 mol (fluorescence intensity and GP), notice the strong photoselection effect observed in the gel phase area respect to the fluid phase; (D) fluid ordered/fluid disordered phase coexistence observed at the polar region of the GUV (fluorescence intensity and GP) composed of DOPC/SM/cholesterol 1:1:1 mol. The photoselection effect in the fluid ordered phase is less pronounced to that observed in the gel phase (compare C and D).

LAURDAN displays unique characteristics compared with other fluorescent probes namely: (i) it shows a phase-dependent emission spectral shift, i.e., bluish in the ordered lipid phase and greenish in the disordered lipid phase (this effect is attributed to the reorientation of water molecules present at the lipid interface near LAURDAN's fluorescent moiety, i.e., water dipolar relaxation process); (ii) it distributes equally into the ordered and disordered-like lipid phases; (iii) the electronic transition moment of LAURDAN is aligned parallel to the hydrophobic lipid chains allowing use of the photoselection effect in the microscopy images to qualitatively discriminate between different lipid phases; (iv) LAURDAN is negligibly soluble in water; [82,83,112–114]. LAURDAN's homogeneous distribution in membranes (even when different lipid phases coexist) and its lipid phase-dependent emission spectral shift offer a great advantage over fluorescent probes (such as DPH, pyrene and parinaric acid) that show preferential partitioning to particular regions of the membrane and whose fluorescence intensities and spectral maximums are generally insensitive to the lipid phase state. For example, in the case of LAURDAN labeled membranes, *lateral packing information can be obtained directly from the fluorescent images* just by using the proper set of emission filters. A way to quantify the extent

of water dipolar relaxation, which in turn is related to the phase state of the lipid membrane, is based on a useful relationship between the emission intensities obtained on the blue and red side of LAURDAN's emission spectrum. This relationship, called Generalized Polarization (GP) was defined by analogy to the fluorescence polarization function [93,113,114]. The Generalized Polarization parameter contains information about solvent dipolar relaxation processes, which occur during the time that LAURDAN is in the excited state, and is related to water penetration in the lipid interfaces. In the GP function, the relative parallel and perpendicular polarizer orientations in the classical polarization function were substituted by the intensities at the blue and red edges of the emission spectrum (I_B and I_R respectively) using a given excitation wavelength (Fig. 3A). It is important to notice that polarizers are not required in the experimental set up even though the name of this function contains the word polarization [93,113,114]. GP images can be constructed from the fluorescence intensity images obtained with blue and green band-pass filters on the microscope, allowing further characterization of the phase state of the coexisting lipid domains [81–83,112]. Fig. 3 shows the particular emission spectra obtained in the ordered and disordered membrane regions (Fig. 3A). Discrimination of two different fluorescent intensity regions
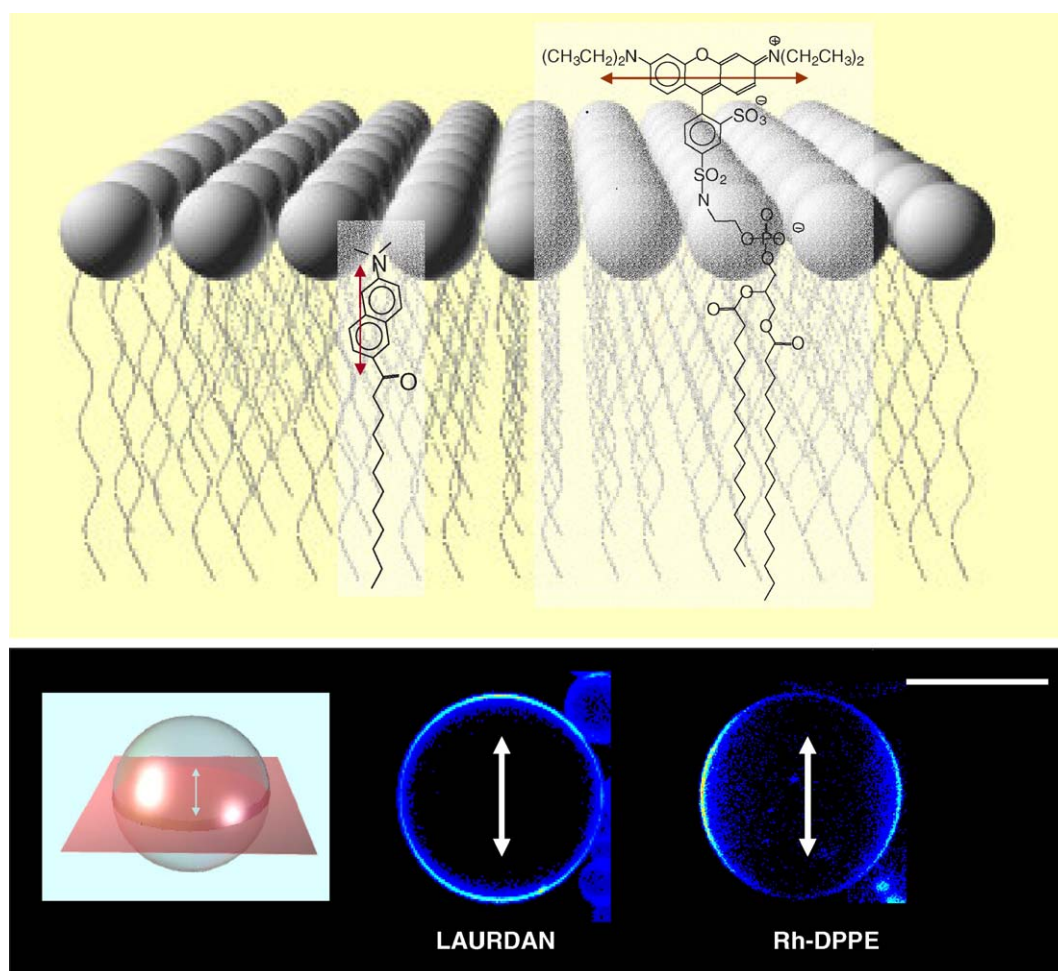

Fig. 5. Sketch of the probe's transition moment orientation (top panel) respect to the surface of the bilayer. Two photon excitation fluorescence intensity images of GUVs composed of DMPC obtained at the equatorial region of the vesicle (bottom panel). The white arrows indicate the linear polarization orientation of the excitation light. GUVs were labeled with LAURDAN and Rhodamine-DPPE. The bar corresponds to 20 μm.

can be observed by using appropriate emission filters (generally blue and green band pass filters, 446 ± 23 nm and 499 ± 23 nm respectively, Fig. 3B). Additionally, computing the GP function allows further characterization of the different lipid phases as shown in Fig. 3B [54,58,82,102]. The particular spherical shape of giant vesicles allows application of the photoselection effect to qualitatively distinguish between the different lipid phases. The photoselection effect arises from the fact that only those fluorophores which have electronic (absorption) transition moments aligned parallel or nearly so to the plane of polarization of the excitation light are excited, i.e., the excitation efficiency is proportional to the cosine squared of the angle between the electronic transition moment of the probe and the polarization plane of the excitation light. Considering a circularly polarized excitation light confined in the x–y plane, exploring different regions of a spherical vesicle (at a given vertical section) allows observation of different excitation efficiencies depending on the position of the probe's electronic transition moment relative to the plane of excitation light polarization (Fig. 4A). At the equatorial region of the vesicle, the circularly polarized excitation light allows excitation of all LAURDAN molecules present in the equatorial plane of the GUV with the same efficiency, independent of the lipid phases present in the vesicle (e.g., one phase or phase coexistence). In this particular case the probe's electronic transition moment is always parallel to the polarization plane (the photoselection effect does not operate). This last fact allows calculation of the GP images without the influence of the photoselection effect (as seen in Fig. 4B for coexistence of fluid/gel and fluid ordered/fluid disordered phases). Instead, at the polar region of the vesicle, and when the GUV

displays gel/fluid phase coexistence, only fluorescence coming from the fluid part of the bilayer will be observed (Fig. 4C). This selection occurs since a component of LAURDAN's electronic transition moment is always parallel to the excitation polarization plane (because of the relatively low lipid order, i.e., the wobbling movement of LAURDAN molecules is present in this phase). This last phenomenon does not take place in the gel phase (even though LAURDAN molecules are present in this region of the bilayer) because the high lipid lateral order precludes the wobbling movement of LAURDAN in the gel region, Fig. 4C. In the case of fluid ordered/fluid disordered phase coexistence scenario, components of LAURDAN's electronic transition moment will be present (parallel to the excitation light polarization plane) in both phases by observing the GUV in the polar region. This situation is mainly due to the fluid nature of the membrane's phases, i.e., because of the relatively low lipid order the wobbling movement of LAURDAN molecules is present in both lipid phases. This last fact will allow collection of fluorescence signals from both phases discriminating them by the position of the LAURDAN fluorescence spectrum. Particularly, the fluid ordered phase will present a lower impact of the photoselection effect compared to that observed for gel phase regions (compared Figs. 4C and D). The message here is that the photoselection effect allows extraction of qualitative information about lipid phases directly from the intensity images.

The photoselection effect can also be exploited to determine the orientation of the probe's transition moment relative to the membrane plane. Using *linear* polarized light as the excitation source the fluorescent images obtained in the equatorial region of the vesicle will present particular characteristics depending on the
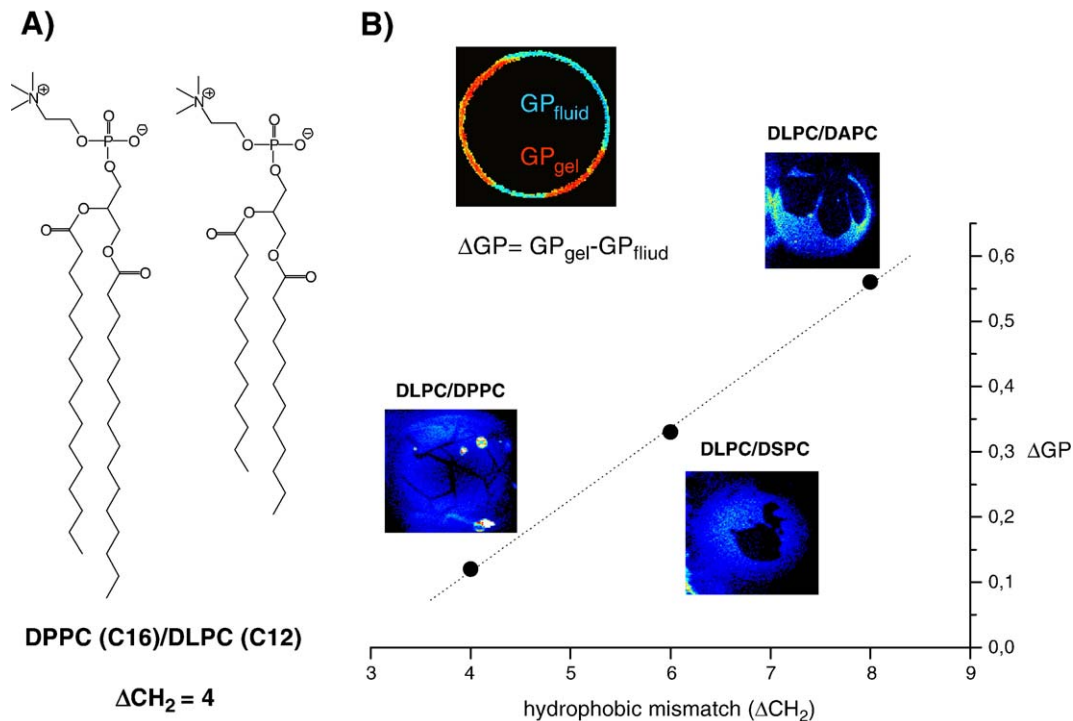

Fig. 6. (A) Molecular structure of DLPC and DPPC showing the phospholipid hydrophobic mismatch. (B) LAURDAN GP differences between the fluid and gel phase domains (ΔGP) plotted versus the hydrophobic mismatch between the components of the binary mixtures. (●) DLPC containing GUVs (DLPC/DPPC, DLPC/DSPC and DLPC/DAPC 1:1 mol, hydrophobic mismatch equal to 4, 6 and 8 respectively). Notice the different shape of the gel-like domains as the hydrophobic mismatch changes.

probe orientation in the membrane [50,55,58]. For example in Fig. 5 the high intensity areas in the fluorescence image are populated by fluorescent molecules with their transition moments oriented parallel to the direction of the polarized excitation light (because the photoselection rule). The immediate conclusion here is that the rhodamine-PE probe has its transition moment oriented 90° in the membrane with respect to that observed for LAURDAN [58]. A similar situation was reported for LAURDAN, where the probe shows different orientations depending of the lipid composition or the particular phase present in the membrane; i.e., the orientation of the probe inserted in GUVS composed of phospholipids with that observed in GUVs composed of bipolar archibacteria lipids [55], or pulmonary surfactant lipid extracts at low temperature [50]. This type of information, for instance, is quite relevant in energy transfer experiments, where the spatial orientation of the fluorophores is one of the parameters used to calculate the distance between the donor and the acceptor. LAURDAN microscopy experiments using linear polarization in the excitation light can be further explored to obtain relevant information about the presence of lipid phases. For example, LAURDAN GP images obtained at the GUV's equatorial region using *linear* polarized light can be exploited to obtain information about the coexistence of lipid domains with sizes below the resolution of the microscope (the resolution of the microscope is ~250 nm radial) [59,81,83,112]. When the lipid domain's size is smaller than the image pixel size, it is not possible to visualize these particular membrane regions. However, in this last case each

pixel of the image will contain this information (displaying an average GP value). Since the polarized light, which photoselects appropriately oriented LAURDAN molecules, also selects LAURDAN molecules associated with high GP values, pixels with high and low GP values can be discriminated. In other words, if the image contains separate domains (pixels) of different GP values because of lipid phase coexistence, the higher GP value domains appear parallel to the orientation of the polarized excitation light and not in the perpendicular direction [59,81,83,112]. This effect was used to ascertain lipid domain coexistence in GUVs composed of single phospholipid species (where the lipid domains have sizes below the microscope resolution) [59] and multilamellar vesicles composed of phospholipids mixtures (with and without cholesterol), including some intact cell membranes [81]. Particularly in this last study, domains of sizes below, in the same range and above the microscope resolution limit (0.3 μm radial) were observed in OK cells, red blood cells and brush border native membranes respectively. For a more detailed technical description on the use of LAURDAN GP images to ascertain lipid domains below the microscope resolution the reader is encouraged to consult the following references [59,81,83,112].

4.2. Membrane lateral structure information: from model system to biological membranes

Since its introduction in 1986, LAURDAN has demonstrated an exquisite sensitivity to detect temperature induced phase
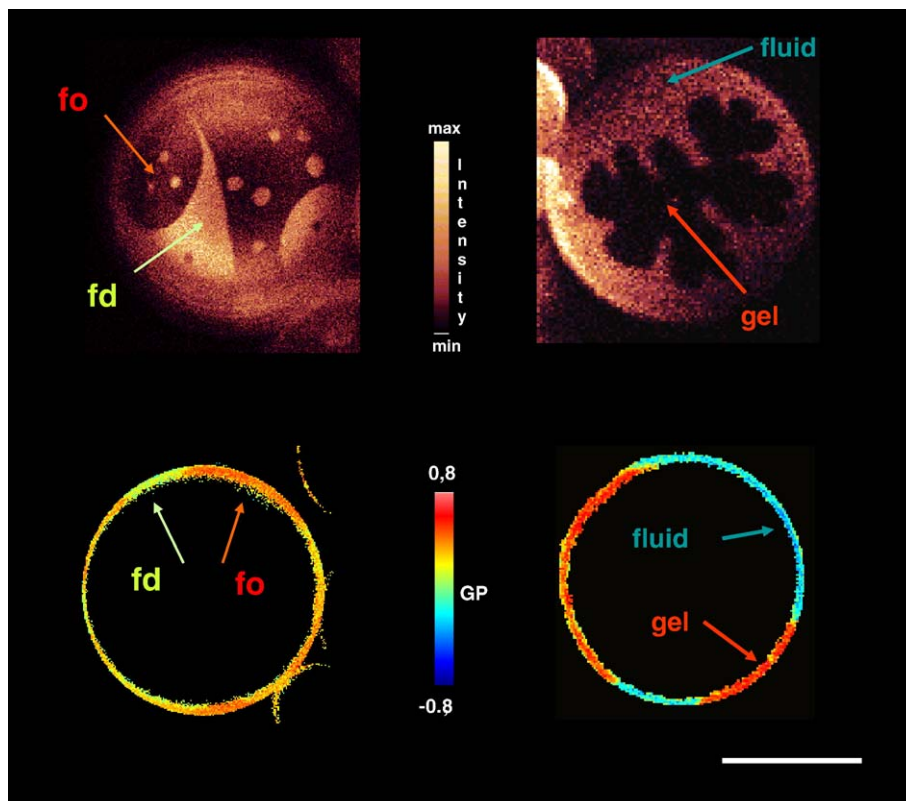

Fig. 7. Two photon excitation LAURDAN fluorescent images (taken at the polar region of the vesicle) and GP images (taken at the equatorial region of the vesicle) of DOPC/Cholesterol/Sphingomyelin 1:1:1 mol displaying fluid ordered/fluid disordered phase coexistence (left panel) and DPPC/DPPE 3:7 mol displaying gel/fluid phase coexistence (right panel). The differences between the two different phase coexistence scenarios are clearly showed by LAURDAN (see text). The bar corresponds to 20 μm.

transitions using liposome solutions [109,110]. The first two photon excitation fluorescence microscopy study using membrane model system (multilamellar vesicles) and LAURDAN was reported in 1997 by Parasassi et al. [81]. The non-random organization of single component phospholipids bilayers in the fluid phase as well as the coexistence of gel and fluid areas in DOPC/DPPC multilamellar vesicles at room temperature were reported in this pioneering work. Additionally, the lateral structure of red blood cell membranes, renal tubular cell line and renal brush border and basolateral membranes using LAURDAN GP was also characterized by using two photon excitation microscopy [81]. The use of giant *unilamellar* vesicles (GUVs) as model system (instead of multilamellar vesicles) further improved the characterization of the lateral phase coexistence scenario in membranes composed of several lipid mixtures using the LAURDAN-based fluorescence microscopy approach. The temperature effect on the lateral structure of membranes composed of pure phospholipids and their different mixtures at the level of single vesicles were first reported in 1999–2000 [54,58,59]. In these reports the gel/fluid phase coexistence was first visualized at different temperatures in free standing bilayers. Gel lipid domains of micrometer size with particular shapes depending on the composition of the lipid mixture and temperature were observed in these experiments [54,58,59]. This information was important to clarify aspects of the gel/fluid phase coexistence scenario, particularly questioning the general assumption about presence of nanometer size gel domains in

membranes (bilayers) displaying gel/fluid phase coexistence. These results led to a correlation between domain shape and lipid miscibility for the different phospholipids binary mixtures displaying gel/fluid phase coexistence [54]. For example, the difference in LAURDAN's GP between the gel and fluid phases ($\Delta GP = GP_{gel} - GP_{fluid}$) for each lipid mixture showed a linear dependence with the hydrophobic mismatch of the different phospholipids mixture (see Fig. 6). This result reflects the sensitivity of the ΔGP to monitor the compositional differences between the gel and fluid phases among the different mixtures, that in turn are related with the miscibility of the lipid components.

The visual information extracted with the experimental strategy discussed above was also implemented to explore the effect of cholesterol in lipid mixtures. Particularly, the lateral structure of canonical raft mixtures (DOPC/SM/cholesterol) was visualized for the first time in free standing bilayers using LAURDAN and TPE fluorescence microscopy [53]. This information was correlated with that obtained in planar membranes composed of the same lipid mixtures and was utilized to compare the lateral structure of compositionally complex membranes (as will be discussed below, [53]).

The information obtained using LAURDAN and TPE fluorescence microscopy in several lipid mixtures aids in the characterization of distinct phase coexistence scenarios, providing particular signatures of the lateral phase separation phenomena in membranes. For example, Fig. 7 compares the gel/fluid and fluid
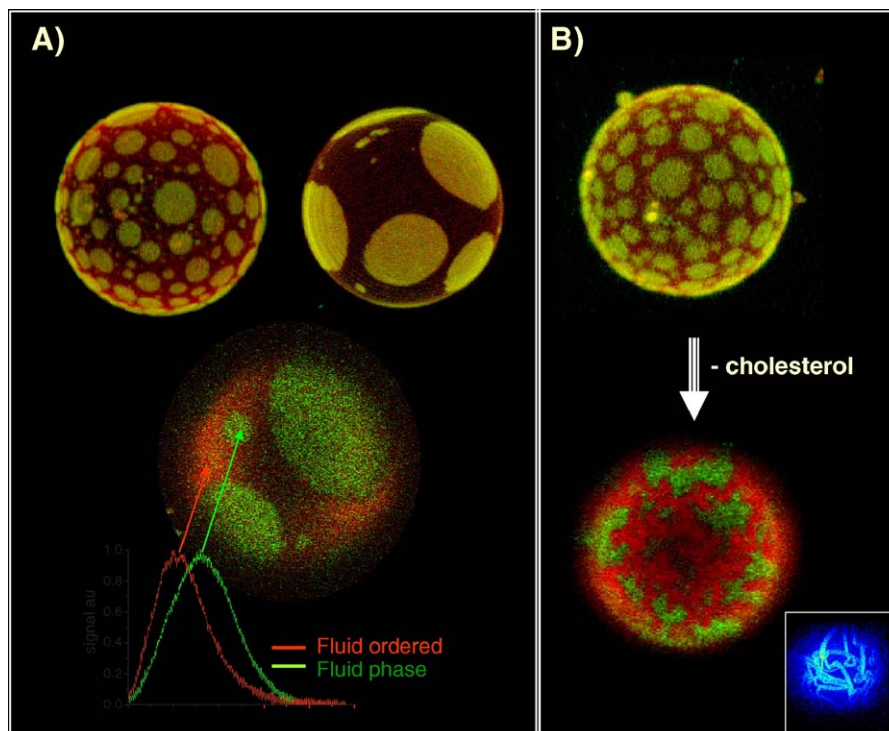

Fig. 8. (A) One photon confocal images of GUVs composed of native pulmonary surfactant membranes (top left) and DOPC/DPPC/chol (top right) obtained using the fluorescent probes DiI$_{C18}$ (red areas) and Bodipy-PC (yellow area). LAURDAN intensity image of a GUV composed of Native pulmonary surfactant membrane obtained with two bandpass filters (428±37 nm and 500±20 nm) which reflect the blue and red side contribution of the LAURDAN emission spectrum respectively (ordered and disordered fluid phases). The similar phase coexistence scenarios between the model system (DOPC/POPC/chol) and the native membrane are evident. (B) Cholesterol extraction by addition of cyclodextrine. The change of the phase coexistence scenario is evident in this experiment. The insert shows the DLPC/DPPC binary mixture displaying gel/fluid phase coexistence (see text).

ordered/fluid disordered phase coexistence scenarios as revealed by LAURDAN. As a first glance, the shapes of the domains are quite different between these two phase coexistence scenarios. Particularly the coexistence of two fluid phases (ordered/dis-

ordered) is characterized by the presence of perfectly round domains. When fluid domains are embedded in a fluid environment, circular domains will form because both phases are isotropic and the line energy (tension), associated with the rim of
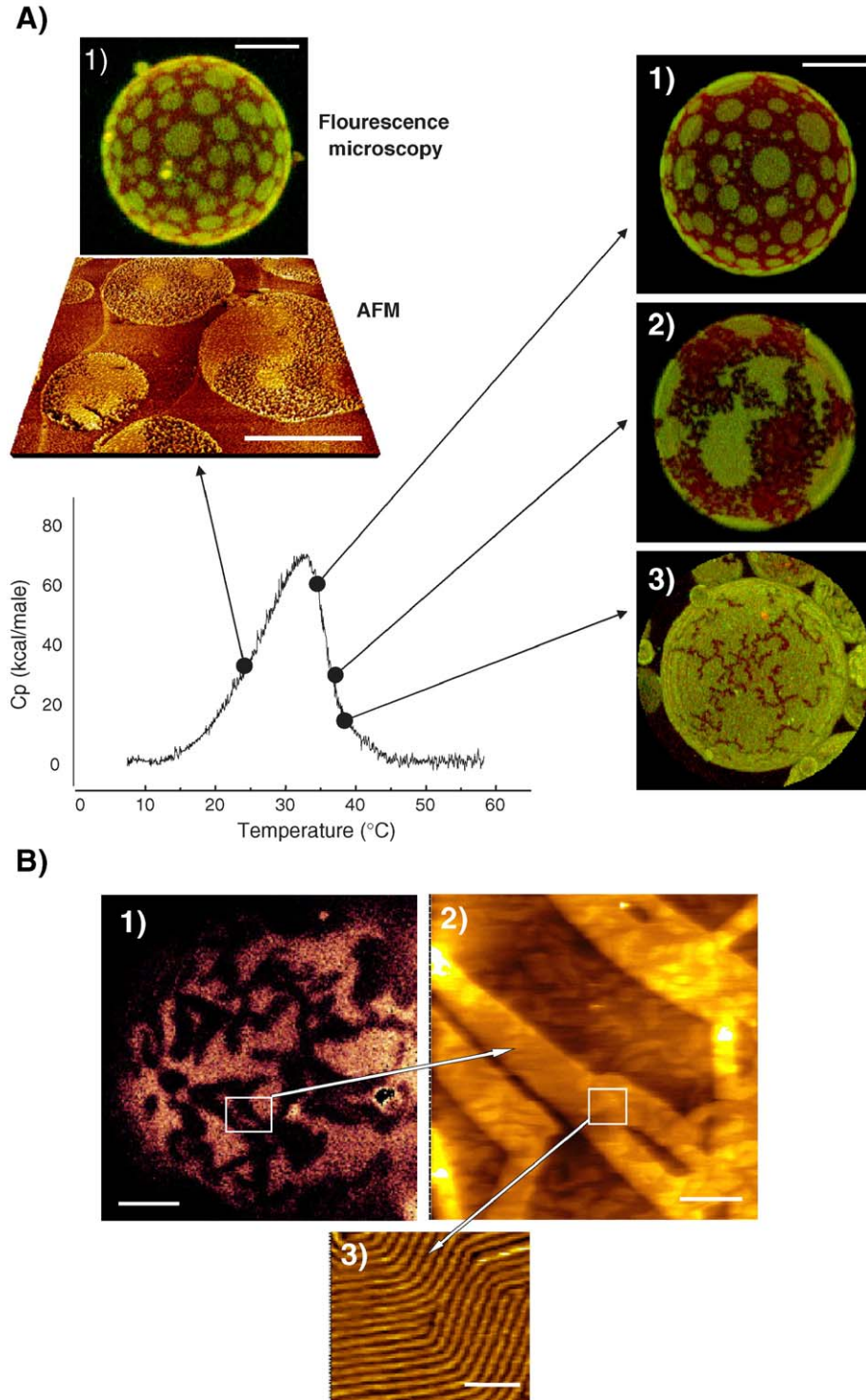

Fig. 9. (A) Thermogram of native pulmonary surfactant membranes obtained using differential scanning calorimetry. Confocal images of a DiIC18/Bodipy-PC labeled GUV composed of pulmonary surfactant membranes at 24, 36, 37.5 and 38.0 °C (images 1, 2 and 3). Image (1) includes an atomic force microscopy figure of planar pulmonary surfactant membranes. The bars correspond to 10 μm. (B) Two photon excitation fluorescent image of a single LAURDAN labeled GUV composed of DMPC/DSPC 1:1 mol mixture. The dark areas correspond to the gel phase (photoselection effect). (B and C) AFM images of double planar bilayers composed of DSC/DMPC 1:1 binary mixture. The brighter areas in B correspond to gel areas. The nanoscopic details inside these areas reveal the presence of metastable ripple phase [57,58]. The bars are (A) 4 μm, (B) 1 μm, (C) 0.1 μm.

two demixing phases, is minimized by optimizing the area-to-perimeter ratio [53]. This last fact is not observed when gel/fluid phase coexistence is present. Additionally, the coexistence of fluid ordered and fluid disordered phases is characterized by a strong reduction of the photoselection effect (particularly comparing the fluid disordered phase with that observed in gel phases) in the polar region of the GUV, and a reduction in the GP differences between the coexisting phases compared with that found in the gel/fluid phase coexistence [53,58].

All the information obtained in compositionally simple model systems, as described above, can aid in the characterization of membrane structure in complex cases such as those that occur in biological situations. As pointed out in Introduction, we must consider how we can correlate the information obtained in simple cases with the lateral structure of compositionally complex mixtures.

5. The importance of visual information to ascertain lateral structure in compositionally complex mixtures

One of the challenges in the membrane field is how to correlate lateral structure information between compositionally simple model systems (normally membranes composed of few lipid species) and compositionally complex mixtures (natural lipid extracts or native membranes) under the same environmental conditions. Because of the complex composition of the natural membranes this correlation is difficult to achieve using bulk solutions containing many liposomes (or cells). However, the visual information provide by fluorescence microscopy is key to perform this correlation. As we mentioned in the model system section, GUVs can be prepared using natural lipid mixtures and native membranes [39,50,53]. Such GUVs can be seen as an intermediate step between a simple lipid mixture and the biological membrane under study. For example, correlations among compositionally complex lipid mixtures displaying gel/fluid [50] and fluid ordered/fluid disordered phase coexistence [53] with binary phospholipids mixtures and cholesterol containing ternary mixtures were reported using LAURDAN. In particular, extraction of cholesterol from the brush border membranes lipid extracts showed a change in the phase coexistence scenario (from fluid ordered/fluid disordered phase coexistence to gel/fluid phase coexistence [53]). Similar experiments were recently reported for pulmonary surfactant membranes and DPPC/DOPC/cholesterol mixtures, where similar lateral structure (fluid ordered/fluid disordered phase coexistence) was observed between artificial mixtures composed of DOPC/DPPC/cholesterol and native pulmonary surfactant membranes (full composition in this case, i.e., lipids and proteins), see Fig. 8A [39]. Additionally, in this particular membrane system, extraction of cholesterol but not extraction of the membrane proteins affects the observed membrane lateral pattern. Specifically, extraction of cholesterol in this compositionally complex mixture generates a pattern that can be linked with the presence of gel/fluid like phase coexistence observed in model lipid mixtures (Fig. 8B insert). Additionally, the observed lateral structure observed in the native pulmonary surfactant at physiological temperatures is linked with functional aspects of

this material [39]. This observation suggests that pulmonary surfactant could be one of the first membrane systems reported where the coexistence of specialized membrane domains may exist as a structural basis for its function [39].

What is clear from the aforementioned studies is that the presence of key lipid species, which exists at particular concentrations in distinctive biological membranes, can trigger phenomena that can be linked with the presence of lateral phase coexistence. However, caution is invoked in generalizing information about the lateral structure of membranes. Biological membranes are adapted to different functions, and so also are their molecular compositions. The latter consideration will be crucial in order to predict the influence of particular membrane components to the lateral organization of the membrane.

6. Combination with other experimental techniques

Differential scanning calorimetry (DSC) experiments provide extremely useful thermodynamical parameters to characterize the temperature behavior of lipid mixtures. However, as the number of components in the lipid mixture is increased the data analysis becomes very difficult. For example, a differential scanning calorimetry profile of pulmonary surfactant membranes is shown in Fig. 9A. Although thermodynamic information from the DSC experiment can be extracted from the system, no detailed information about the physical characteristics of lipid lateral structure at different temperatures can be obtained using this technique. As observed in Fig. 9A, from the GUV data three different temperature regimes with particular membrane lateral structure (phase state) are observed and characterized using fluorescence microscopy. Results using this combination of experimental techniques was also reported for bovine pulmonary surfactant lipid extracts (BLES) using LAURDAN [50]. Other techniques can
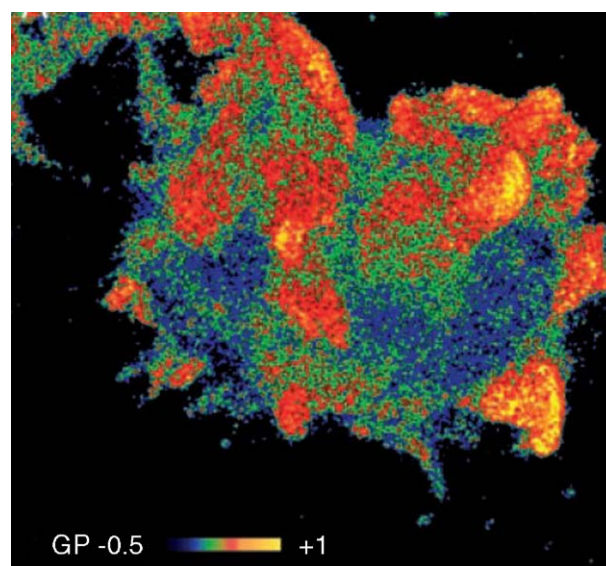

Fig. 10. GP image of a living macrophage, 3D-reconstructed pseudocolored GP images of RAW264.7 cell (image plane parallel to coverslip, viewed from above). Notice the different discrete GP regions on the cell membrane indicating membrane lateral heterogeneity (adapted from ref 105, "Copyright (2003) National Academy of Sciences, U.S.A.").

provide information that complements that obtained from GUV experiments. Atomic force microscopy (AFM), for instance, allows bridging the micrometer size scale with the nanometer size scale. As shown in Fig. 9 details about the protein distribution inside the fluid disordered domains in native pulmonary surfactant can be observed [39]. Additionally, as shown in Fig. 9B the nanoscopic details of a binary mixture (DMPC/DSPC) can be obtained using a probe free technique [115,116]. In this case the phase information obtained with the probe LAURDAN (that is related with water dipolar relaxation processes in the membrane) is expanded with the nanoscopic information (lateral packing structure and height differences) obtained by using AFM. In this case the highly lateral packed areas (gel phase) observed using LAURDAN fluorescence have a nanometer size structure corresponding to ripple phase as observed with AFM. The similarities in shape and size are remarkable in both model systems. Additionally, the combination of epi-fluorescence microscopy with AFM in the same specimen was recently reported offering another possibility to ascertain membrane lateral structure and correlate different size information among different membrane model systems. For example, using this approach a direct correlation between lipid domains in GUVs and planar membranes using the same fluorescently labeled lipid mixtures were reported [117–119]. In these particular cases the partition properties of the fluorescent probe for a particular lipid domain is utilized to compare the lipid domain's features in the free standing bilayer (GUVs) and planar membranes. Additionally, combination of fluorescence correlation spectroscopy (FCS) with AFM was also recently reported allowing linkage of dynamical information and structural features of the lipid domains [120]. It is noteworthy that the height difference between the coexisting lipid phases or presence of domains with sizes of nanometers (not detectable by fluorescence intensity microscopy because the diffraction limit of optical imaging) can be obtained using AFM. The information obtained from these AFM and TPE fluorescence microscopy is hence rather complementary and very useful to characterize the lateral structure of membranes.

6.1. LAURDAN in cell membranes and tissues

From the pioneering work of Parasassi et al., and Yu et al. [81,121], LAURDAN was proposed to be very promising in exploring cell membranes. In these studies, domains of sizes below, in the same range and above the microscope resolution limit (0.3 μm radial) were observed in OK cells, red blood cells and brush border native membranes respectively [81,121]. Recently, LAURDAN GP differences observed in compositionally complex mixtures and artificial lipid ternary mixtures containing phospholipids, sphingomyelin and cholesterol were exploited to interpret LAURDAN GP images in cell membranes [105]. In this report the LAURDAN GP function was used to directly observe transient micron size high GP regions surrounded by low GP areas in living macrophages (see Fig. 10). This paper demonstrated the presence of lateral phase separation in these particular cells in vivo supporting strongly the cholesterol effect observed in the model systems [53]. Interestingly, this last result is in line with the work reported by Gousset et al., where micron size domains were also observed in platelets upon

activation [122] and to that observed in native pulmonary surfactant membranes [39]. Although micron size domains are observed in the membranes mentioned above (macrophages, platelets, pulmonary surfactant) generalization of this phenomenon must be done cautiously. Following the literature in this respect, the presence of micron size domains in biological membranes seems to be the exception instead of a general case. This last fact can be simply related to the membrane composition and the particular functions of the membranes under favorable environmental conditions (such as temperature). Lastly, LAURDAN GP was applied also to tissues. Sun et al. [123] have demonstrated that both LAURDAN multiphoton polarization and generalized polarization (GP) can be combined using a two photon excitation fluorescence microscope to characterize the structural changes of intercellular lipids in skin tissue. This work demonstrated how treatment with oleic acid results in a skin surface with a more random packing of lipid molecules, which facilitates water penetration.

6.2. Concluding remarks

As demonstrated in this review, the combination of particular membrane model systems (such as GUVs) and fluorescence microscopy techniques can be exploited to learn about processes that occur in the complex framework of biological membranes. Some experimental strategies are available, as discussed in this review, to first understand the particular molecular interactions of the membrane components in compositionally simple model system and to finally understand the basis of either lipid–lipid or lipid–protein interactions in more complex situations. As discussed above, the visual and dynamical information obtained using multiphoton excitation microscopy and UV-excited probes provides a powerful tool to learn about similar phenomena in complex systems such as biological membranes and tissues.

Acknowledgements

References

[1] D.W. Hilgemann, Getting ready for the decade of the lipids, Annu. Rev. Physiol. 65 (2003) 697–700.

[2] S.J. Singer, G.L. Nicolson, The fluid mosaic model of the structure of cell membranes, Science 175 (1972) 720–731.

[3] E.J. Shimshick, H.M. McConnell, Lateral phase separation in phospholipid membranes, Biochemistry 12 (1973) 2351–2360.

[4] C. Gebhardt, H. Gruler, E. Sackmann, On domain structure and local curvature in lipid bilayers and biological membranes, Z. Naturforsch., C 32 (1977) 581–596.

[5] K. Simons, W.L. Vaz, Model systems, lipid rafts, and cell membranes, Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 269–295.

[6] M. Edidin, The state of lipid rafts: from model membranes to cells, Annu. Rev. Biophys. Biomol. Struct. 32 (2003) 257–283.

[7] D.A. Brown, E. London, Structure and origin of ordered lipid domains in biological membranes, J. Membr. Biol. 164 (1998) 103–114.

[8] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572.

[9] L.A. Bagatolli, B. Maggio, F. Aguilar, C.P. Sotomayor, G.D. Fidelio, Laurdan properties in glycosphingolipid–phospholipid mixtures: a comparative fluorescence and calorimetric study, Biochim. Biophys. Acta 1325 (1997) 80–90.

[10] V. Schram, H.N. Lin, T.E. Thompson, Topology of gel-phase domains and lipid mixing properties in phase-separated two-component phosphatidylcholine bilayers, Biophys. J. 71 (1996) 1811–1822.

[11] T. Parasassi, M. Loiero, M. Raimondi, G. Ravagnan, E. Gratton, Absence of lipid gel-phase domains in seven mammalian cell lines and in four primary cell types, Biochim. Biophys. Acta 1153 (1993) 143–154.

[12] K. Arnold, A. Losche, K. Gawrisch, 31p-NMR investigations of phase separation in phosphatidylcholine/phosphatidylethanolamine mixtures, Biochim. Biophys. Acta 645 (1981) 143–148.

[13] A. Blume, R.J. Wittebort, S.K. Das Gupta, R.G. Griffin, Phase equilibria, molecular conformation, and dynamics in phosphatidylcholine/phosphatidylethanolamine bilayers, Biochemistry 21 (1982) 6243–6253.

[14] M. Caffrey, F.S. Hing, A temperature gradient method for lipid phase diagram construction using time-resolved x-ray diffraction, Biophys. J. 51 (1987) 37–46.

[15] B. Maggio, G.D. Fidelio, F.A. Cumar, R.K. Yu, Molecular interactions and thermotropic behavior of glycosphingolipids in model membrane systems, Chem. Phys. Lipids 42 (1986) 49–63.

[16] W.L. Vaz, E.C. Melo, T.E. Thompson, Translational diffusion and fluid domain connectivity in a two-component, two-phase phospholipid bilayer, Biophys. J. 56 (1989) 869–876.

[17] W.L. Vaz, E.C. Melo, T.E. Thompson, Fluid phase connectivity in an isomorphous, two-component, two-phase phosphatidylcholine bilayer, Biophys. J. 58 (1990) 273–285.

[18] T. Bultmann, W.L. Vaz, E.C. Melo, R.B. Sisk, T.E. Thompson, Fluid-phase connectivity and translational diffusion in a eutectic, two-component, two-phase phosphatidylcholine bilayer, Biochemistry 30 (1991) 5573–5579.

[19] P.F. Almeida, W.L. Vaz, T.E. Thompson, Lateral diffusion and percolation in two-phase, two-component lipid bilayers. Topology of the solid-phase domains in-plane and across the lipid bilayer, Biochemistry 31 (1992) 7198–7210.

[20] A.G. Lee, Fluorescence studies of chlorophyll a incorporated into lipid mixtures, and the interpretation of "phase" diagrams, Biochim. Biophys. Acta 413 (1975) 11–23.

[21] S. Mabrey, J.M. Sturtevant, Investigation of phase transitions of lipids and lipid mixtures by sensitivity differential scanning calorimetry, Proc. Natl. Acad. Sci. U. S. A. 73 (1976) 3862–3866.

[22] B.R. Lentz, Y. Barenholz, T.E. Thompson, Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. 2 Two-component phosphatidylcholine liposomes, Biochemistry 15 (1976) 4529–4537.

[23] B.R. Lentz, Y. Barenholz, T.E. Thompson, Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. 1. Single component phosphatidylcholine liposomes, Biochemistry 15 (1976) 4521–4528.

[24] P.W. van Dijck, A.J. Kaper, H.A. Oonk, J. de Gier, Miscibility properties of binary phosphatidylcholine mixtures. A calorimetric study, Biochim. Biophys. Acta 470 (1977) 58–69.

[25] R.F. de Almeida, A. Fedorov, M. Prieto, Sphingomyelin/phosphatidylcholine/cholesterol phase diagram: boundaries and composition of lipid rafts, Biophys. J. 85 (2003) 2406–2416.

[26] R.F. de Almeida, L.M. Loura, A. Fedorov, M. Prieto, Lipid rafts have different sizes depending on membrane composition: a time-resolved fluorescence resonance energy transfer study, J. Mol. Biol. 346 (2005) 1109–1120.

[27] K. Jorgensen, O.G. Mouritsen, Phase separation dynamics and lateral organization of two-component lipid membranes, Biophys. J. 69 (1995) 942–954.

[28] J.H. Ipsen, O.G. Mouritsen, Modelling the phase equilibria in two-component membranes of phospholipids with different acyl-chain lengths, Biochim. Biophys. Acta 944 (1988) 121–134.

[29] J.R. Silvius, Partitioning of membrane molecules between raft and non-raft domains: insights from model-membrane studies, Biochim. Biophys. Acta 1746 (2005) 193–202.

[30] S.L. Veatch, S.L. Keller, Seeing spots: complex phase behavior in simple membranes, Biochim. Biophys. Acta 1746 (2005) 172–185.

[31] C. Nicolini, J. Baranski, S. Schlummer, J. Palomo, M. Lumbierres-Burgues, M. Kahms, J. Kuhlmann, S. Sanchez, E. Gratton, H. Waldmann, R. Winter, Visualizing association of N-Ras in lipid microdomains: influence of domain structure and interfacial adsorption, J. Am. Chem. Soc. 128 (2006) 192–201.

[32] E.E. Ambroggio, F. Separovic, J.H. Bowie, G.D. Fidelio, L.A. Bagatolli, Direct visualization of membrane leakage induced by the antibiotic peptides: maculatin, citropin, and aurein, Biophys. J. 89 (2005) 1874–1881.

[33] S.L. Veatch, S.L. Keller, Miscibility phase diagrams of giant vesicles containing sphingomyelin, Phys. Rev. Lett. 94 (2005) 148101.

[34] N. Kahya, D.A. Brown, P. Schwille, Raft partitioning and dynamic behavior of human placental alkaline phosphatase in giant unilamellar vesicles, Biochemistry 44 (2005) 7479–7489.

[35] N. Kahya, D. Scherfeld, P. Schwille, Differential lipid packing abilities and dynamics in giant unilamellar vesicles composed of short-chain saturated glycerol-phospholipids, sphingomyelin and cholesterol, Chem. Phys. Lipids 135 (2005) 169–180.

[36] E.E. Ambroggio, D.H. Kim, F. Separovic, C.J. Barrow, K.J. Barnham, L.A. Bagatolli, G.D. Fidelio, Surface behavior and lipid interaction of Alzheimer beta-amyloid peptide 1–42: a membrane-disrupting peptide, Biophys. J. 88 (2005) 2706–2713.

[37] Q. Ruan, M.A. Cheng, M. Levi, E. Gratton, W.W. Mantulin, Spatial-temporal studies of membrane dynamics: scanning fluorescence correlation spectroscopy (SFCS), Biophys. J. 87 (2004) 1260–1267.

[38] P. Girard, J. Pecreaux, G. Lenoir, P. Falson, J.L. Rigaud, P. Bassereau, A new method for the reconstitution of membrane proteins into giant unilamellar vesicles, Biophys. J. 87 (2004) 419–429.

[39] J. Bernardino de la Serna, J. Perez-Gil, A.C. Simonsen, L.A. Bagatolli, Cholesterol rules: direct observation of the coexistence of two fluid phases in native pulmonary surfactant membranes at physiological temperatures, J. Biol. Chem. 279 (2004) 40715–40722.

[40] K. Bacia, C.G. Schuette, N. Kahya, R. Jahn, P. Schwille, SNAREs prefer liquid-disordered over "raft" (liquid-ordered) domains when reconstituted into giant unilamellar vesicles, J. Biol. Chem. 279 (2004) 37951–37955.

[41] S.L. Veatch, I.V. Polozov, K. Gawrisch, S.L. Keller, Liquid domains in vesicles investigated by NMR and fluorescence microscopy, Biophys. J. 86 (2004) 2910–2922.

[42] G. Koster, M. VanDuijn, B. Hofs, M. Dogterom, Membrane tube formation from giant vesicles by dynamic association of motor proteins, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15583–15588.

[43] D. Scherfeld, N. Kahya, P. Schwille, Lipid dynamics and domain formation in model membranes composed of ternary mixtures of unsaturated and saturated phosphatidylcholines and cholesterol, Biophys. J. 85 (2003) 3758–3768.

[44] S.L. Veatch, S.L. Keller, Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol, Biophys. J. 85 (2003) 3074–3083.

[45] T. Baumgart, S.T. Hess, W.W. Webb, Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension, Nature 425 (2003) 821–824.

[46] N. Kahya, D. Scherfeld, K. Bacia, B. Poolman, P. Schwille, Probing lipid mobility of raft-exhibiting model membranes by fluorescence correlation spectroscopy, J. Biol. Chem. 278 (2003) 28109–28115.

[47] L.A. Bagatolli, D.D. Binns, D.M. Jameson, J.P. Albanesi, Activation of dynamin II by POPC in giant unilamellar vesicles: a two-photon fluorescence microscopy study, J. Protein Chem. 21 (2002) 383–391.

[48] S.L. Veatch, S.L. Keller, Organization in lipid membranes containing cholesterol, Phys. Rev. Lett. 89 (2002) 268101.

[49] S.A. Sanchez, L.A. Bagatolli, E. Gratton, T.L. Hazlett, A two-photon view of an enzyme at work: Crotalus atrox venom PLA2 interaction with single-lipid and mixed-lipid giant unilamellar vesicles, Biophys. J. 82 (2002) 2232–2243.

[50] K. Nag, J.S. Pao, R.R. Harbottle, F. Possmayer, N.O. Petersen, L.A. Bagatolli, Segregation of saturated chain lipids in pulmonary surfactant films and bilayers, Biophys. J. 82 (2002) 2041–2051.

[51] N. Kahya, E.I. Pecheur, W.P. de Boeij, D.A. Wiersma, D. Hoekstra, Reconstitution of membrane proteins into giant unilamellar vesicles via peptide-induced fusion, Biophys. J. 81 (2001) 1464–1474.

[52] G.W. Feigenson, J.T. Buboltz, Ternary phase diagram of dipalmitoyl-PC/dilauroyl-PC/cholesterol: nanoscopic domain formation driven by cholesterol, Biophys. J. 80 (2001) 2775–2788.

[53] C. Dietrich, L.A. Bagatolli, Z.N. Volovyk, N.L. Thompson, M. Levi, K. Jacobson, E. Gratton, Lipid rafts reconstituted in model membranes, Biophys. J. 80 (2001) 1417–1428.

[54] L.A. Bagatolli, E. Gratton, A correlation between lipid domain shape and binary phospholipid mixture composition in free standing bilayers: a two-photon fluorescence microscopy study, Biophys. J. 79 (2000) 434–447.

[55] L. Bagatolli, E. Gratton, T.K. Khan, P.L. Chong, Two-photon fluorescence microscopy studies of bipolar tetraether giant liposomes from thermo-acidophilic archaebacteria *Sulfolobus acidocaldarius*, Biophys. J. 79 (2000) 416–425.

[56] M.I. Angelova, I. Tsoneva, Interactions of DNA with giant liposomes, Chem. Phys. Lipids 101 (1999) 123–137.

[57] J.M. Holopainen, M.I. Angelova, P.K. Kinnunen, Vectorial budding of vesicles by asymmetrical enzymatic formation of ceramide in giant liposomes, Biophys. J. 78 (2000) 830–838.

[58] L.A. Bagatolli, E. Gratton, Two photon fluorescence microscopy of co-existing lipid domains in giant unilamellar vesicles of binary phospholipid mixtures, Biophys. J. 78 (2000) 290–305.

[59] L.A. Bagatolli, E. Gratton, Two-photon fluorescence microscopy observation of shape changes at the phase transition in phospholipid giant unilamellar vesicles, Biophys. J. 77 (1999) 2090–2101.

[60] J. Korlach, P. Schwille, W.W. Webb, G.W. Feigenson, Characterization of lipid bilayer phases by confocal microscopy and fluorescence correlation spectroscopy, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8461–8466.

[61] M.I. Angelova, N. Hristova, I. Tsoneva, DNA-induced endocytosis upon local microinjection to giant unilamellar cationic vesicles, Eur. Biophys. J. 28 (1999) 142–150.

[62] F.M. Menger, J.S. Keiper, Chemistry and physics of giant vesicles as biomembrane models, Curr. Opin. Chem. Biol. 2 (1998) 726–732.

[63] M.L. Longo, A.J. Waring, D.A. Hammer, Interaction of the influenza hemagglutinin fusion peptide with lipid bilayers: area expansion and permeation, Biophys. J. 73 (1997) 1430–1439.

[64] R. Wick, M.I. Angelova, P. Walde, P.L. Luisi, Microinjection into giant vesicles and light microscopy investigation of enzyme-mediated vesicle transformations, Chem. Biol. 3 (1996) 105–111.

[65] D. Needham, E. Evans, Structure and mechanical properties of giant lipid (DMPC) vesicle bilayers from 20 degrees C below to 10 degrees C above the liquid crystal-crystalline phase transition at 24 degrees C, Biochemistry 27 (1988) 8261–8269.

[66] D. Needham, T.J. McIntosh, E. Evans, Thermomechanical and transition properties of dimyristoylphosphatidylcholine/cholesterol bilayers, Biochemistry 27 (1988) 4668–4673.

[67] E. Sackmann, The seventh Datta Lecture. Membrane bending energy concept of vesicle- and cell-shapes and shape-transitions, FEBS Lett. 346 (1994) 3–16.

[68] D.M. Haverstick, M. Glaser, Influence of proteins on the reorganization of phospholipid bilayers into large domains, Biophys. J. 55 (1989) 677–682.

[69] D.M. Haverstick, M. Glaser, Visualization of domain formation in the inner and outer leaflets of a phospholipid bilayer, J. Cell Biol. 106 (1988) 1885–1892.

[70] D.M. Haverstick, M. Glaser, Visualization of Ca2+-induced phospholipid domains, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 4475–4479.

[71] L. Yang, M. Glaser, Membrane domains containing phosphatidylserine and substrate can be important for the activation of protein kinase C, Biochemistry 34 (1995) 1500–1506.

[72] J. Sot, L.A. Bagatolli, F.M. Goñi, A. Alonso, Detergent-resistant, ceramide-enriched domains in sphingomyelin/ceramide bilayers, Biophys. J. 90 (2006) 903–914.

[73] K. Akashi, H. Miyata, H. Itoh, K. Kinosita Jr., Preparation of giant liposomes in physiological conditions and their characterization under an optical microscope, Biophys. J. 71 (1996) 3242–3250.

[74] M.I. Angelova, D.S. Dimitrov, Liposome electroformation, Faraday Discuss, Chem. Soc. 81 (1986) 303–311.

[75] M.I. Angelova, S. Soléau, P. Meléard, J.F. Faucon, P. Bothorel, Preparation of giant vesicles by external AC fields. Kinetics and application, Prog. Colloid Polym. Sci. 89 (1992) 127–131.

[76] A. Moscho, O. Orwar, D.T. Chiu, B.P. Modi, R.N. Zare, Rapid preparation of giant unilamellar vesicles, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11443–11447.

[77] J.P. Reeves, R.M. Dowben, Formation and properties of thin-walled phospholipid vesicles, J. Cell Physiol. 73 (1969) 49–60.

[78] L.A. Bagatolli, T. Parasassi, E. Gratton, Giant phospholipid vesicles: comparison among the whole lipid sample characteristics using different preparation methods: a two photon fluorescence microscopy study, Chem. Phys. Lipids 105 (2000) 135–147.

[79] N. Düzgüneş, L.A. Bagatolli, P. Meers, Y.K. Oh, R.M. Straubinger, in: V. Weissig, V. Torchilin (Eds.), Liposomes: A Practical Approach, 2nd ed., Oxford Univ. Press, Oxford, 2003, pp. 105–147.

[80] P. Luisi, P. Walde, Giant Vesicles, John Wiley and Sons Ltd, England, 2000.

[81] T. Parasassi, E. Gratton, W.M. Yu, P. Wilson, M. Levi, Two-photon fluorescence microscopy of laurdan generalized polarization domains in model and natural membranes, Biophys. J. 72 (1997) 2413–2429.

[82] L.A. Bagatolli, E. Gratton, Direct observation of lipid domains in free standing bilayers using two-photon excitation fluorescence microscopy, J. Fluoresc. 11 (2001) 141–160.

[83] L.A. Bagatolli, S.A. Sanchez, T. Hazlett, E. Gratton, Giant vesicles, Laurdan, and two-photon fluorescence microscopy: evidence of lipid lateral separation in bilayers, Methods Enzymol. 360 (2003) 481–500.

[84] L.A. Bagatolli, T. Parasassi, G.D. Fidelio, E. Gratton, A model for the interaction of 6-lauroyl-2-(N,N-dimethylamino)naphthalene with lipid environments: implications for spectral properties, Photochem. Photobiol. 70 (1999) 557–564.

[85] H.A. Garda, A.M. Bernasconi, R.R. Brenner, Possible compensation of structural and viscotropic properties in hepatic microsomes and erythrocyte membranes of rats with essential fatty acid deficiency, J. Lipid Res. 35 (1994) 1367–1377.

[86] M.E. Jones, B.R. Lentz, Phospholipid lateral organization in synthetic membranes as monitored by pyrene-labeled phospholipids: effects of temperature and prothrombin fragment 1 binding, Biochemistry 25 (1986) 567–574.

[87] E.K. Krasnowska, L.A. Bagatolli, E. Gratton, T. Parasassi, Surface properties of cholesterol-containing membranes detected by Prodan fluorescence, Biochim. Biophys. Acta 1511 (2001) 330–340.

[88] E.K. Krasnowska, E. Gratton, T. Parasassi, Prodan as a membrane surface fluorescence probe: partitioning between water and phospholipid phases, Biophys. J. 74 (1998) 1984–1993.

[89] B.R. Lentz, Use of fluorescent probes to monitor molecular order and motions within liposome bilayers, Chem. Phys. Lipids 64 (1993) 99–116.

[90] C.R. Mateo, M.P. Lillo, J. Gonzalez-Rodriguez, A.U. Acuna, Lateral heterogeneity in human platelet plasma membrane and lipids from the time-resolved fluorescence of trans-parinaric acid, Eur. Biophys. J. 20 (1991) 53–59.

[91] T. Parasassi, F. Conti, M. Glaser, E. Gratton, Detection of phospholipid phase separation. A multifrequency phase fluorimetry study of 1,6-diphenyl-1,3,5-hexatriene fluorescence, J. Biol. Chem. 259 (1984) 14011–14017.

[92] T. Parasassi, F. Conti, E. Gratton, Time-resolved fluorescence emission spectra of Laurdan in phospholipid vesicles by multifrequency phase and modulation fluorometry, Cell Mol. Biol. 32 (1986) 103–108.

[93] T. Parasassi, G. De Stasio, G. Ravagnan, R.M. Rusch, E. Gratton, Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence, Biophys. J. 60 (1991) 179–189.

[94] T. Parasassi, M. Di Stefano, M. Loiero, G. Ravagnan, E. Gratton, Influence of cholesterol on phospholipid bilayers phase domains as detected by Laurdan fluorescence, Biophys. J. 66 (1994) 120–132.

[95] M. Velez, M.P. Lillo, A.U. Acuna, J. Gonzalez-Rodriguez, Cholesterol effect on the physical state of lipid multibilayers from the platelet plasma membrane by time-resolved fluorescence, Biochim. Biophys. Acta 1235 (1995) 343–350.

[96] P.K. Wolber, B.S. Hudson, Fluorescence lifetime and time-resolved polarization anisotropy studies of acyl chain order and dynamics in lipid bilayers, Biochemistry 20 (1981) 2800–2810.

[97] W. Denk, J.H. Strickler, W.W. Webb, Two-photon laser scanning fluorescence microscopy, Science 248 (1990) 73–76.

[98] A. Diaspro, Confocal and Two Photon Microscopy. Foundations, Applications and Advances, Wiley-Liss, New York, 2002.

[99] B.R. Master, P.T.C. So, E. Gratton, Fluorescent and Luminescent Probes, 2nd ed., Academic Press, New York, 1999, pp. 414–432.

[100] P.T.C. So, T. French, W.M. Yu, K.M. Berland, C.Y. Dong, E. Gratton, in: X.F. Wang, B. Herman (Eds.), Fluorescence Imaging Spectroscopy and Microscopy, John Wiley and Sons, New York, 1996, pp. 351–374.

[101] P.T.C. So, T. French, W.M. Yu, K.M. Berland, C.Y. Dong, E. Gratton, Time resolved fluorescence microscopy using two-photon excitation, Bioimaging 3 (1995) 49–63.

[102] L.A. Bagatolli, Direct observation of lipid domains in free standing bilayers: from simple to complex lipid mixtures, Chem. Phys. Lipids 122 (2003) 137–145.

[103] L. Kuerschner, C.S. Ejsing, K. Ekroos, A. Shevchenko, K.I. Anderson, C. Thiele, Polyene-lipids: a new tool to image lipids, Nat Methods 2 (2005) 39–45.

[104] E. Quesada, A.U. Acuna, F. Amat-Guerri, New transmembrane polyene bolaamphiphiles as fluorescent probes in lipid bilayers, Angew. Chem., Int. Ed. Engl. 40 (2001) 2095–2097.

[105] K. Gaus, E. Gratton, E.P. Kable, A.S. Jones, I. Gelissen, L. Kritharides, W. Jessup, Visualizing lipid structure and raft domains in living cells with two-photon microscopy, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15554–15559.

[106] S. Fahsel, E.M. Pospiech, M. Zein, T.L. Hazlet, E. Gratton, R. Winter, Modulation of concentration fluctuations in phase-separated lipid membranes by polypeptide insertion, Biophys. J. 83 (2002) 334–344.

[107] S. Janosch, C. Nicolini, B. Ludolph, C. Peters, M. Volkert, T.L. Hazlet, E. Gratton, H. Waldmann, R. Winter, Partitioning of dual-lipidated peptides into membrane microdomains: lipid sorting vs. peptide aggregation, J. Am. Chem. Soc. 126 (2004) 7496–7503.

[108] M. Lasagna, V. Vargas, D.M. Jameson, J.E. Brunet, Spectral properties of environmentally sensitive probes associated with horseradish peroxidase, Biochemistry 35 (1996) 973–979.

[109] R.B. Macgregor, G. Weber, Estimation of the polarity of the protein interior by optical spectroscopy, Nature 319 (1986) 70–73.

[110] G. Weber, F.J. Farris, Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-propionyl-2-(dimethylamino)naphthalene, Biochemistry 18 (1979) 3075–3078.

[111] E. Bismuto, D.M. Jameson, E. Gratton, Dipolar relaxations in glycerol: a dynamic fluorescence study of 2-dimethylamino-6-naphthoyl-4′ cyclohexane carboxylic acid (DANCA), J. Am. Chem. Soc. 109 (1987) 2354–2357.

[112] T. Parasassi, E. Gratton, H. Zajicek, M. Levi, W. Yu, Detecting membrane lipid microdomains by two-photon fluorescence microscopy, IEEE Eng. Med. Biol. Mag. 18 (1999) 92–99.

[113] T. Parasassi, E. Krasnowska, L.A. Bagatolli, E. Gratton, LAURDAN and PRODAN as polarity - sensitive fluorescent membrane probes, J. Fluoresc. 8 (1998) 365–373.

[114] T. Parasassi, E. Gratton, Membrane lipid domains and dynamics detected by LAURDAN, J. Fluoresc. 5 (1995) 59–70.

[115] T. Kaasgaard, C. Leidy, J.H. Crowe, O.G. Mouritsen, K. Jorgensen, Temperature-controlled structure and kinetics of ripple phases in one- and two-component supported lipid bilayers, Biophys. J. 85 (2003) 350–360.

[116] C. Leidy, T. Kaasgaard, J.H. Crowe, O.G. Mouritsen, K. Jorgensen, Ripples and the formation of anisotropic lipid domains: imaging two-component supported double bilayers by atomic force microscopy, Biophys. J. 83 (2002) 2625–2633.

[117] A.C. Simonsen, L.A. Bagatolli, Structure of spincoated lipid films and domain formation in supported membranes formed by hydration, Langmuir 20 (2004) 9720–9728.

[118] M. Fidorra, L. Duelund, C. Leidy, A. Simonsen, L.A. Bagatolli, Absence of fluid-ordered/fluid-disordered phase coexistence in ceramide/POPC mixtures containing cholesterol, Biophys. J. 90 (2006) 4437–4451.

[119] C.D. Blanchette, W. Lin, T.V. Ratto, M.L. Longo, Galactosylceramide domain microstructure: impact of cholesterol and nucleation/growth conditions, Biophys. J. 90 (2006) 4466–4478.

[120] S. Chiantia, N. Kahya, J. Ries, P. Schwille, Effects of ceramide on liquid-ordered domains investigated by simultaneous AFM and FCS, Biophys. J. 90 (2006) 4500–4508.

[121] W. Yu, P.T. So, T. French, E. Gratton, Fluorescence generalized polarization of cell membranes: a two-photon scanning microscopy approach, Biophys. J. 70 (1996) 626–636.

[122] K. Gousset, W.F. Wolkers, N.M. Tsvetkova, A.E. Oliver, C.L. Field, N.J. Walker, J.H. Crowe, F. Tablin, Evidence for a physiological role for membrane rafts in human platelets, J. Cell Physiol. 190 (2002) 117–128.

[123] Y. Sun, J.-W. Su, W. Lo, S.-J. Lin, S.-H. Jee, C.-Y. Dong, Multiphoton polarization imaging of the stratum corneum and the dermis in ex-vivo human skin, Opt. Express 11 (2003) 3377–3384.