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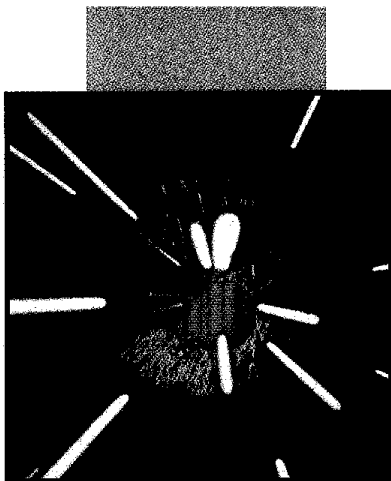
Detecting Membrane Lipid Microdomains by Two-Photon Fluorescence Microscopy

A Model for Estimating Domain Dimensions Using the LAURDAN Lipid Probe

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Two-photon excitation fluorescence microscopy is an emerging imaging tool for the study of biological samples [1]. The unique characteristics of two-photon excitation, such as the reduced sample photodamage and probe photobleaching, and a better background rejection compared to one-photon excitation, allow the prolonged observation and the study of samples that are difficult to measure using one-photon fluorescence microscopy [2, 3]. The spectroscopic properties of the emitting fluorophores can be characterized using two-photon microscopy [4].

This article discusses our ability to obtain, using two-photon excitation, microscopy images of the generalized polarization (GP) of the lipid probe 2-dimethylamino-6-lauroylnaphthalene (LAURDAN) in phospholipid vesicles and in natural membranes. The images show different distribution of the GP value depending on membrane composition. The use of linearly polarized excitation allowed the attribution of the GP heterogeneity to coexisting membrane domains of different dynamic properties. Based on the photoselection operated by the excitation polarization, we propose a model to explain our results and to estimate the domains' dimension.

Overview

Our interest in the study of the dynamic properties of lipids in membranes has been focused on establishing a methodology for the detection of lipid microdomains. In the bilayer aggregation form of lipids, microdomains are intended as locally separated areas with distinct dynamic properties. In biological systems, microdomains with specific properties may serve to separate compartments with specific functions and to modulate the activity and the delivery of information during the physiological life of the cell [5, 6]. Alteration of the topography and of the properties of such domains may also have a role in the development of cell malfunction and pathologies.

We have previously characterized the fluorescence response of the LAURDAN polarity lipid probe using "cuvette" spectroscopy, in both model and natural membranes [7, 8]. LAURDAN possesses a steady-state sensitivity to polarity changes in its lipid environment, showing a red spectral shift directly related to the increase in the polarity. The GP function has been used to quantify the spectral shift of LAURDAN:

$$GP = (I_B - I_R) / (I_B + I_R) \qquad (1)$$

where I_B and I_R are the steady-state intensities in the blue (440 nm) and the red (490 nm) part of the emission spectrum, respectively [7]. These blue and red spectral regions correspond to the maximum emission of LAURDAN in phospholipids in the gel and in the liquid-crystalline phase, respectively.

Calculation of the GP in the gel and in the liquid-crystalline phase following Eq. (1) gives high and low values, respectively. The polarity changes in the different phospholipid phases originate from variations of the water content around the probe fluorescent moiety, at the level of the glycerol backbone. In pure phospholipid bilayers of unknown composition, the dependence of the GP value on the excitation wavelength gives information on the coexistence of phase microdomains.

Similar to the fluorescence polarization, the GP possesses an additive property, so that the measured GP value

represents an average of the GP value of each emitting LAURDAN molecule. The general limits of the GP values for the gel and for the liquid crystalline phase have been determined, so that for samples of unknown composition, once the coexistence of domains has been ascertained, the fraction of the membrane domains can be quantitatively resolved. More difficult is the detection of coexisting domains of different dynamic properties when the bilayer is composed of unknown lipids, such as in the case of mammalian cell membranes. Moreover, the presence of cholesterol induces the formation of phase states with properties different from those of the pure phospholipid bilayer. Dynamic properties between those of the gel and of the liquid-crystalline phase have been measured when cholesterol is present. Phase diagrams have been constructed and phases such as the

solid-ordered, liquid-ordered, and liquid-disordered have been defined [9]. LAURDAN GP "cuvette" studies on mammalian cell membranes did not detect coexisting domains [10].

The next step for the investigation on coexisting domains in the membranes and, in particular, in cell membranes, was to use fluorescence microscopy. LAURDAN absorption occurs in the UV region, between 320 nm and 420 nm. Using one-photon excitation, the very fast fading of LAURDAN fluorescence due to photobleaching hindered any detailed spectroscopic study of its emission. However, by using two-photon excitation, prolonged observations on LAURDAN-labeled cell membranes were possible [11].

Images of LAURDAN GP values were obtained, revealing different GP values in the various cell membrane compartments [11]. More detailed fluorescence micros-

By using two-photon excitation, prolonged observations on LAURDAN-labeled cell membranes were possible.

copy studies on LAURDAN GP of multilamellar phospholipid vesicles and of natural membranes obtained by polarized two-photon excitation clarified the origin of the different GP values such as, for instance, the measurement noise, the bilayer orientation, and the coexistence of microdomains [12]. A model for the evaluation of the dimension of membrane phase domains was also developed.

Materials and Methods
Lipid Vesicle Preparation, Labeling, and Mounting

Multilamellar phospholipid vesicles were prepared by mixing appropriate amounts of solutions in chloroform of phospholipids [dioleoyl-, dilauroyl- and dipalmitoyl-phosphatidylcholine (DOPC, DLPC, and DPPC, respectively); Avanti Polar Lipids, Alabaster, AL] and LAURDAN (Molecular Probes, Eugene, OR) and evaporating the solvent by nitrogen flow. The dried samples were resuspended in a phosphate-buffered saline solution (Sigma Chem. Co., St. Louis, MO) (pH 7.4), heated above the phase transition temperature, and vortexed. The final total lipid and probe concentrations were 0.3 mM and 0.3 μM. A drop of the lipid vesicle suspension was evaporated on a #1.5 glass coverslip with a nitrogen stream. The coverslip was mounted on the microscope slide with a drop of deionized water.

Culture, Labeling, and Mounting of OK Cells

Opossum kidney renal tubular epithelial cells (OK cells) [13] were grown in
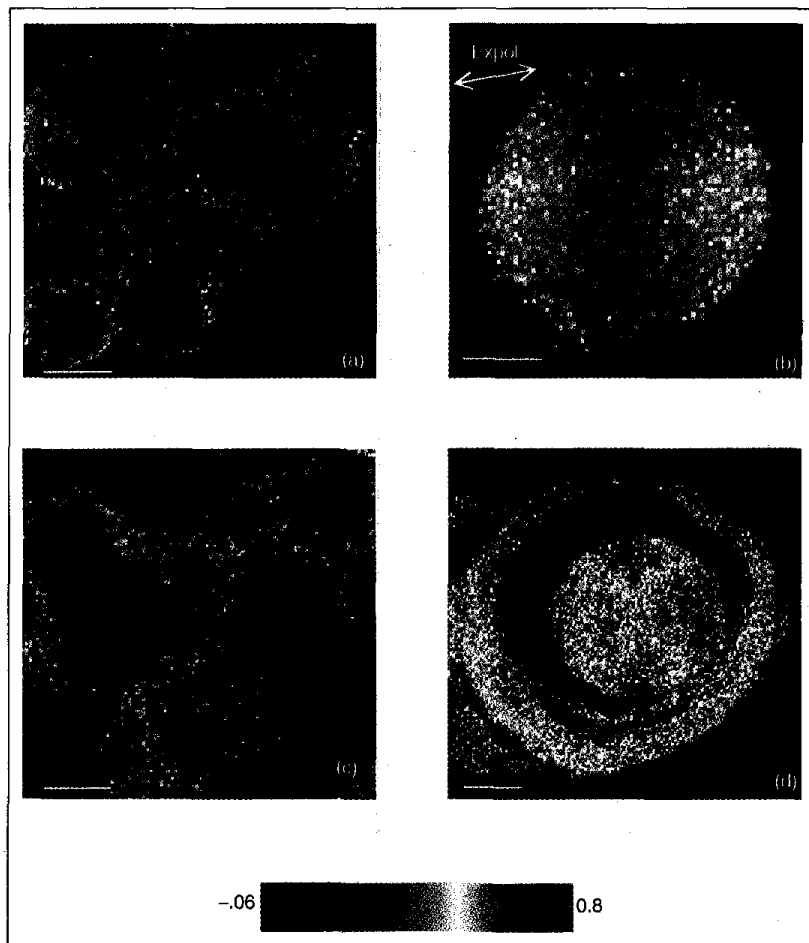
1. LAURDAN GP images of multilamellar phospholipid vesicles composed of DOPC (a), DLPC (b), DPPC (c), and of an equimolar mixture of DOPC and DPPC (d). Different colors indicate different GP values. The scale bar in all images is 5 μm. All images have been obtained using polarized excitation, indicated in panel B.

We could ascertain that the GP heterogeneity is due to real membrane heterogeneity, thus excluding possible artifacts due to measurements noise.

Dulbecco's modified Eagle's high-glucose medium (DMEM) containing 10% fetal calf serum, 100 IU/ml penicillin G, and 0.1 mg/ml streptomycin. For microscopy measurements, cells were seeded on dishes, containing microscope coverslips, for 2-4 hours. During this time, cells adhered to the coverslips, maintaining their round shape, which we preferred for the experiments with polarized excitation. LAURDAN labeling was performed by directly adding 1 μl of a 2 mM probe solution in DMSO per milliliter of the growth medium in the culture dishes. Cells were incubated for 30 min in the dark, then gently washed with fresh medium. The coverslips were mounted on the hanging drop microscope slides in fresh medium.

Brush Border and Basolateral Membrane Preparation, Labeling, and Mounting

Apical brush border (BBM) and basolateral (BLM) membranes from the rat renal cortex were simultaneously isolated by differential centrifugation, magnesium precipitation, and discontinuous sucrose gradient methods [14, 15]. Purified membranes were diluted to a concentration of 0.1 mg protein/ml. LAURDAN labeling was performed by adding 1 μl of the 2 mM probe solution in DMSO per milliliter of the membrane sample. The sample was vortexed for 30 s, then a drop was evaporated on a

coverslip with a nitrogen stream. The coverslip was mounted on the flat microscope slide with a drop of distilled water.

Two-Photon Microscopy Measurements

A titanium (Ti):sapphire laser (Mira 900, Coherent, Palo Alto, CA) pumped by an argon ion laser (Innova 310, Coherent) was used as the excitation light source. The laser wavelength was tuned at 770 nm. The laser light was guided by a galvanometer-driven x-y scanner (Cambridge Technology, Watertown, MA). The scanning rate was 26 s per frame (256 x 256 pixels). The incident laser power on the sample was about 2 mW. A quarter-wave plate (CVI Laser Corporation, Albuquerque, NM) was placed after the polarizer to change the polarization of the laser light from linear to circular for polarization-independent excitation. To change the laser polarization, a polarizer was placed after the quarter-wave plate. Two optical bandpass filters (Ealing Electro Optics, New Englander Industrial Park, Holliston, MA) were used to collect the fluorescence in the blue (440 nm) and in the red (490 nm) regions of the LAURDAN emission spectrum. The two filters were exchanged each time a full frame was scanned. To compensate for photobleaching, three successive pictures in a sequence of red-blue-red were collected, and the two

red frames were averaged. Then the GP was calculated following Eq. 1 [11]. A miniature photomultiplier (R5600-P, Hamamatsu, Bridgewater, NJ) amplified through a AD6 discriminator (Pacific, Concord, CA) was used for light detection in the photon-counting mode. The fluorescence intensity was collected using a custom-made data acquisition card located in a personal computer.

Results
Phospholipid Vesicles

GP images of vesicles composed of DOPC, DLPC, DPPC, and of an equimolar mixture of DOPC and DPPC, all at room temperature, are reported in Fig. 1. Increasing average GP values are observed in the order: DOPC < DLPC < DPPC. For the equimolar DOPC-DPPC mixture, the average GP values are between those of the individual components (Fig. 2). At room temperature, both DOPC and DLPC vesicles are in the liquid-crystalline phase and DPPC vesicles are in the gel phase, while vesicles of the equimolar mixture of DOPC and DPPC are composed of coexisting domains of the two phases [12, 16]. In DPPC vesicles, the GP values are high (Fig. 1c) with a relatively narrow histogram (Fig. 2c) and homogeneously distributed all over the vesicles. With the exception of DPPC vesicles, the polarized excitation resulted in higher GP values along the po-
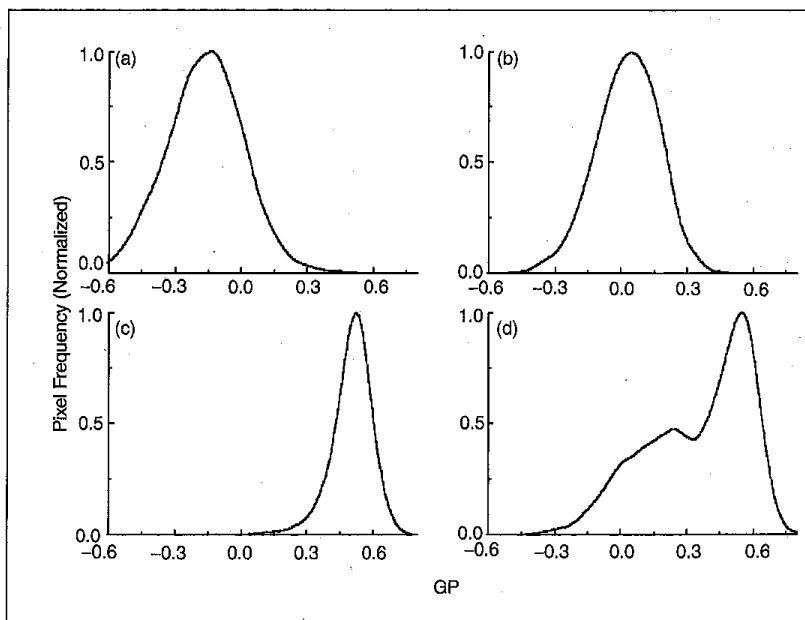
2. Distribution of the normalized pixel frequency of the GP values, from the images in Fig. 1, in vesicles composed of DOPC (a), DLPC (b), DPPC (c) and of an equimolar mixture of DOPC and DPPC (d).

larization axis. For DOPC and DLPC vesicles, higher GP values are observed along the excitation polarization [Figs. 1(a) and (b)], and lower GP values are observed perpendicularly to the excitation polarization [Figs. 3(a) and (b)]. A very clear separation between high and low GP values in the direction parallel and perpendicular to the laser polarization axis is obtained in the vesicles composed of equimolar DOPC-DPPC [Figs. 1(d) and 3(d)]. An interesting result of these GP images is the broad distribution of GP values, not only in the vesicles composed of the equimolar mixture, but also in the vesicles of pure liquid-crystalline phase. This heterogeneity can be well appreciated in the plots of the histograms of the GP value (Fig. 2).

Natural Membranes

The GP images obtained from OK cells show different GP values in the different cellular compartments (Fig. 4). In particular, the plasma membrane shows high GP values. For the round shape of the plasma membrane, an excitation
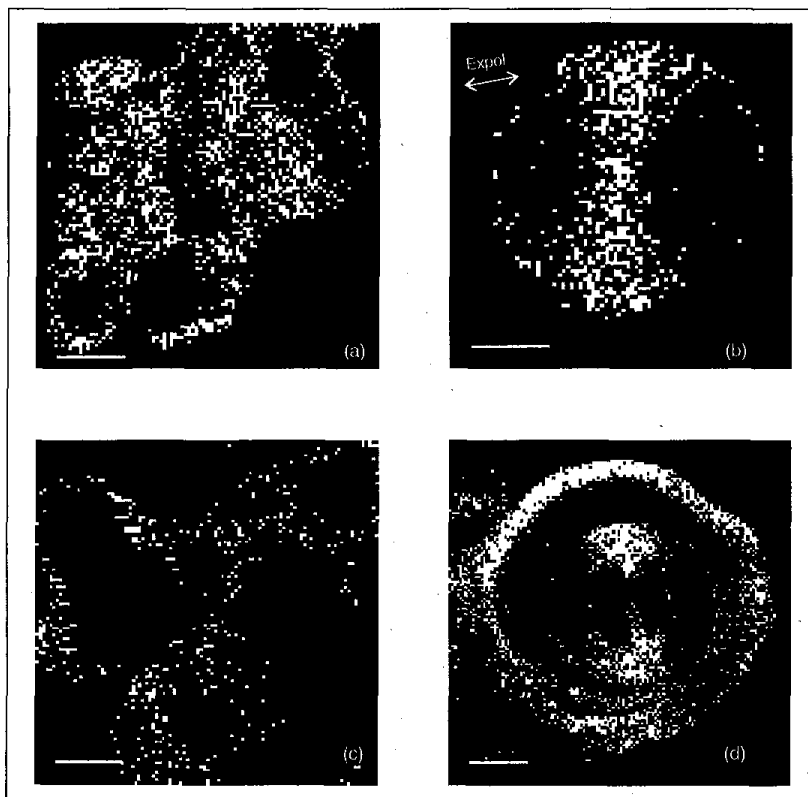
photoselection can be performed, and the high GP values are displayed along the direction parallel to the excitation polarization. By selectively plotting high and low GP values (Fig. 5), the low GP pixels mainly appeared in the direction perpendicular to the excitation polarization [Figs. 4 (a-d)].

The GP images obtained from the BBM and the BLM samples show relatively high values, with no photoselection effect (Fig. 4). Compared to the BLM, the BBM samples show higher GP values. A selective plotting of low GP values of both the BBM and the BLM images shows the localization of these low values at the border of the membranes (Fig. 5).

Discussion

We have obtained LAURDAN GP images in the phospholipid vesicles and in the natural membranes using two-photon excitation. These measurements have been made possible for the reduced overall sample photobleaching when using two-photon excitation. Im-

We found that the high GP values are associated with membrane regions where the fluorescent probe is aligned with the excitation polarization.

ages of LAURDAN GP previously reported in mouse fibroblasts [11], as well as the GP images of the OK cell presented in this work, showed a heterogeneous distribution of the GP values. Both in fibroblasts and in OK cells, higher GP values were observed in the plasma membranes and in inner membranes corresponding to the Golgi apparatus. Lower GP values were observed in the complex membrane cytoplasmic network, especially around the nucleus. These different GP values are consistent with the lipid composition of cell membrane compartments [17]. By the use of polarized excitation, we could ascertain that the GP heterogeneity is due to real membrane heterogeneity, thus excluding possible artifacts due to measurements noise. We also attempted an evaluation of the size of the domains in the membrane, based on the spatial separation of the different GP values.

Our rationale for using polarized excitation in imaging LAURDAN GP in membranes is based on our previous characterization of LAURDAN spectroscopy. We determined that the orientation of the transition dipole of the fluorescent moiety of LAURDAN was parallel to the phospholipid acyl chains [8]. Moreover, high values of both LAURDAN polarization and GP are observed in the gel phase, where the phospholipid molecular motion is hin-

3. Images of selected pixels with low GP values from Fig. 1. The scale bar in all images is 5 μm. The plotted GP values are less than –0.25 for DOPC (a); less than 0.10 for DLPC (b); less than 0.40 for DPPC (c); and less than 0.10 for the DOPC-DPPC mixture (d).

dered and the water content in the bilayer is low. In the liquid-crystalline phase, the molecular dynamics of the phospholipid is faster (i.e., more water penetrates the bilayer), and consequently, LAURDAN GP is also lower [7, 8]. By considering the round shape of the vesicles and the axial resolution of the two-photon excitation that corresponds to about 600 nm with our microscope, we associated the photoselection of the polarized excitation with the value of LAURDAN GP. The actual observation of this association allows the attribution of the GP heterogeneity to the heterogeneity of the membrane.

Excitation Photoselection is Associated With High GP Values

The possible orientations of the phospholipids in the round vesicle, with respect to the excitation polarization axis, are schematically represented in Fig. 6. The microscopic images correspond to a cross-section through the structure of the vesicles, with the phospholipids in a radial orientation. Using polarized excitation, we photoselect those LAURDAN molecules with their dipoles parallel to the excitation polarization [Panel (a) of Fig. 6]. LAURDAN molecules with a perpendicular orientation with respect to the excitation polarization will be weakly excited

only in the liquid-crystalline phase of the bilayer, where more rotational mobility is allowed (Panel (b) of Fig. 6) and a projection of LAURDAN dipole moment along the excitation polarization exists.

If the phospholipid environment is heterogeneous (i.e., when LAURDAN GP values are heterogeneous), the excitation polarization photoselects higher GP values in the regions where the phospholipids, and the probe, are oriented parallel to the excitation polarization. Examples of this case are represented by the images obtained with vesicles composed of DOPC, DLPC, equimolar mixture of DOPC and DPPC, and the plasma membrane of the OK cells. In regions of the vesicles where the bilayer is oriented perpendicularly to the excitation polarization, only the more fluid environments with lower GP values will be photoselected, since in these fluid environments the LAURDAN transition dipole possesses a higher rotational freedom. When the two-photon excitation occurs close to the bottom (or to the top) of the vesicles, the excitation photoselection results in GP images having a pattern as that sketched in Panel (c) of Fig. 6, with areas of higher GP values parallel to the excitation polarization, and areas of lower GP in the center and/or perpendicularly to the excitation polarization.

A clear example of this case is given by the image of the DLPC vesicle in Fig. 1(b). When the vesicles are very small or when we image a flat bilayer surface, we obtain medium and low GP values, respectively, and there is no apparent photoselection [Panels (d) and (e) of Fig. 6]. Examples of a flat bilayer surface with relatively homogeneous GP values are represented by the BBM and BLM samples and images. In these membranes, the cholesterol concentration is relatively high, and consequently, the average GP values are also high [18].

Evaluation of the Size of Membrane Domains

For this purpose, we considered the photoselection caused by the excitation polarization in relation with the spatial resolution of our images, which is about 300 nm in the radial direction. In the simplest case, the membrane domains are larger than the pixel size, so that pixels of similar GP values are present in large areas. This is the case of various cellular compartments in the OK cells, where we observed differences between the high GP values of the plasma and the Golgi mem-
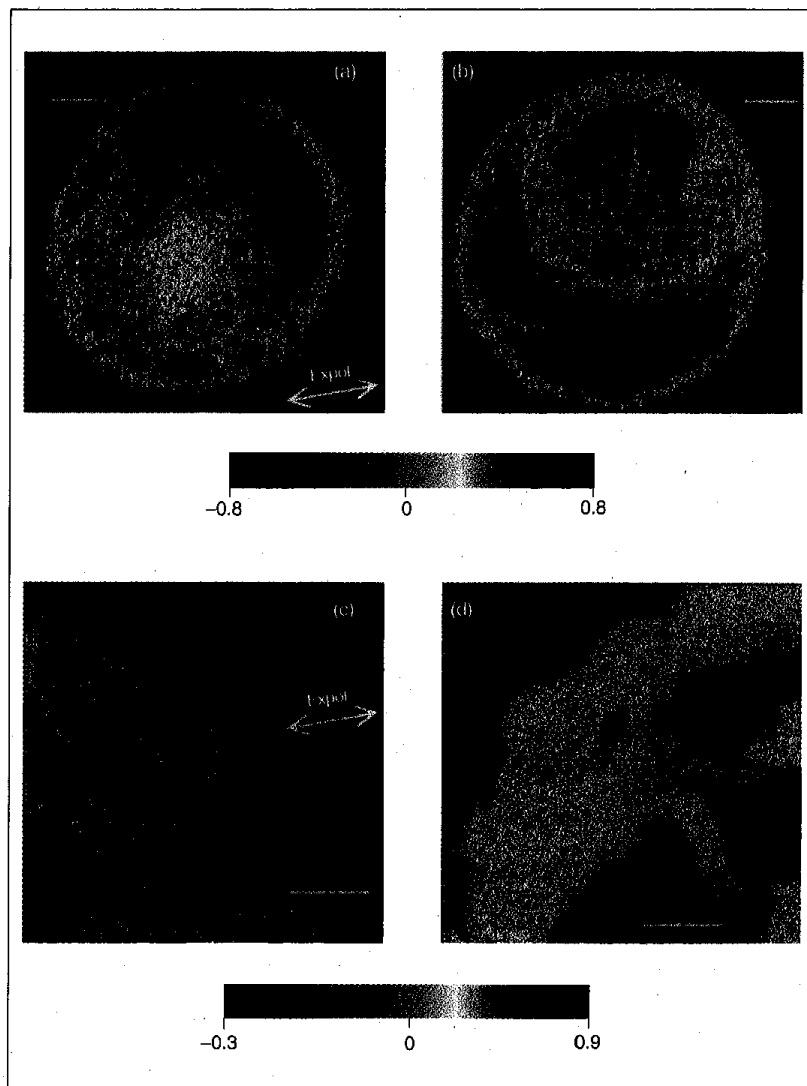
4. LAURDAN GP images of OK cells (A and B), BBM (C), and BLM (D) purified membranes. In panel B, two cells are imaged. The different colors indicate different GP values. The scale bars in panels A and B are 5 μm, and 10 μm in panels C and D. All images have been obtained using polarized excitation, in the direction indicated in panels A and C.

branes and lower GP values in the complex network of cytoplasmic membranes, including the membrane of the rough and smooth endoplasmic reticulum. Nevertheless, also in the OK cells, a more complex heterogeneity is present within each of these cellular compartments. As discussed above, the GP heterogeneity of the membranes is not due to measurement errors, but it represents a real heterogeneity due to the presence of spatially distinct domains. We now propose a model to evaluate the dimension of these membrane microdomains. We can separate the case of domains about the size of a single pixel and smaller. These two circumstances are schematically represented in Fig. 7. By using the excitation photoselection method, we can distinguish among them.

### Membrane Domains Comparable to the Pixel Size

In this case, each pixel contains only one of the various domains. Due to the rotational freedom of the LAURDAN molecule in the fluid liquid-crystalline phase, with polarized excitation the probe can be excited all around the vesicle circumference. Instead, in the rigid gel phase, the probe will only be excited when aligned along the excitation polarization axis. Consequently, a selective plotting of the high GP values will show pixels only in the direction parallel to the excitation polarization, while a selective plotting of low GP values will show pixels all around the circumference of the vesicle. This case was only observed in red blood cells [12]. We observed a homogeneous distribution of relatively low GP values also in the vesicles composed of DPPC. However, due to the overall homogeneity of the GP values in this sample, as judged by the narrow GP distribution [Fig. 2(C)], the separation of the high and the low GP values is not obvious. Moreover, in the DPPC sample we could not observe a clear photoselection effect of higher GP values along the direction of the excitation polarization [Fig. 1(c)], since the GP is always high.

Membrane Domains Smaller than the Pixel Size

In this case, each pixel contains both fluid and rigid domains. In a pixel with the phospholipids oriented along the excitation polarization, the rigid microdomains with higher GP values will be preferentially excited. In those pixels with the phospholipids oriented perpendicularly to the excitation polarization, fluid domains

will be weakly excited, while the rigid domains will not be excited. Consequently, a selective plotting of lower GP values will show pixels only in the perpendicular direction with respect to the laser polarization. A complete separation of regions with higher and lower GP values in the direction parallel and perpendicular to the excitation
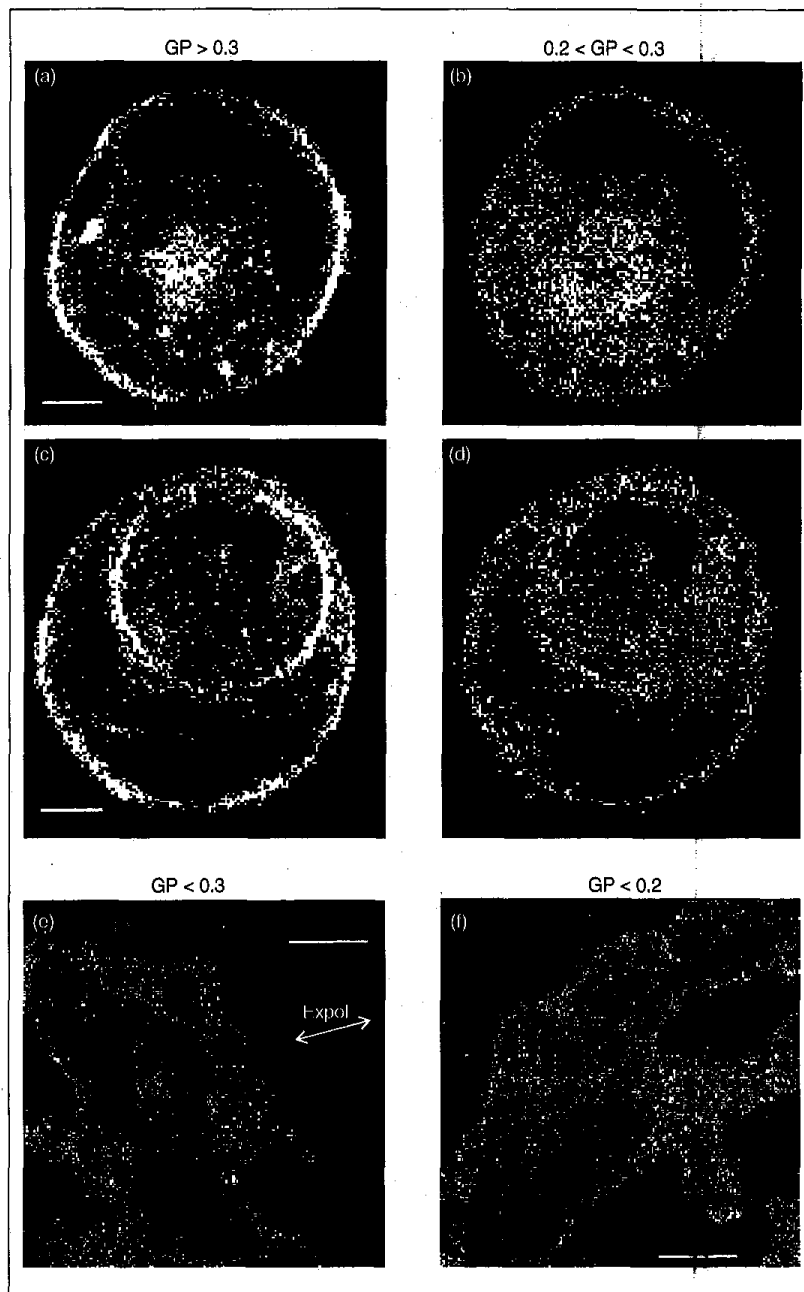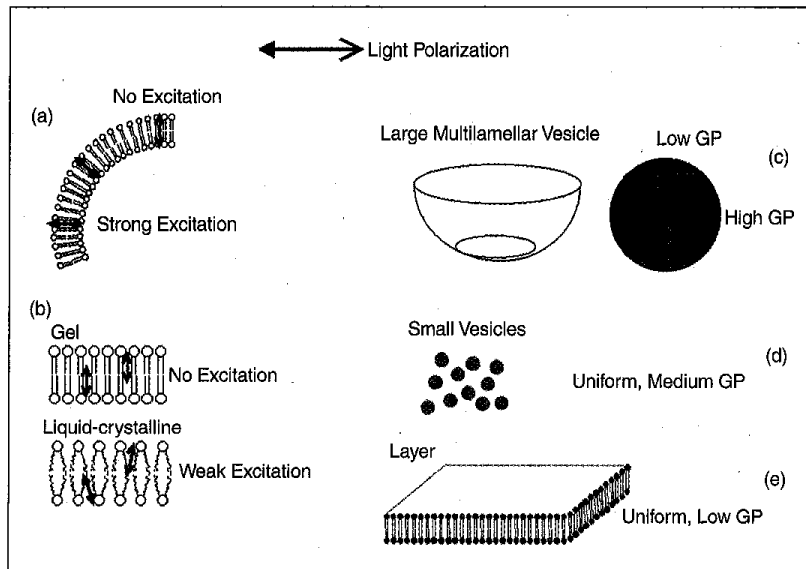

5. Images of selected GP values from the images in Fig. 4. To highlight the separation between the pixels of low and high GP in the OK cells' plasma membrane, GP values greater than 0.3 are plotted in panels (a) and (c), and intermediate values are plotted in panels (b) and (d), in the range between 0.2 and 0.3, so that the number of pixels of low GP values of the inner cytoplasmic membranes has been minimized. In panels (e) and (f), pixels with GP values lower than 0.3 and 0.2 of the BBM and BLM, respectively, are plotted. The scale bar in the images of the OK cells is 5 μm, in the BBM and BLM images is 10 μm. All images have been obtained using polarized excitation, in the direction reported in panel (e).

polarization, respectively, occurs only in this case of membrane microdomains smaller than the microscope image resolution. We observed this case in the vesicle samples (except for DPPC) and in the plasma membrane of the OK cells.

Using our model, we predict that the dimensions of the membrane domains in phospholipid vesicles and in OK cells are smaller than the pixel size. This result is in agreement with previous spectroscopic studies, from which the estimated domain

6. Schematic representation of the excitation photoselection relative to the bilayer orientation in the optical sectioning. Along the vesicle circumference (a), the direction of the LAURDAN transition dipole is perpendicular (no excitation), parallel (strong excitation), or has a projection parallel to the excitation polarization. This last case is also represented in (b), as the weak excitation in the liquid-crystalline phase. In (c), (d), and (e), the expected GP values and pattern are sketched in the case of membrane with fluid phases, following the photoselection and the vesicles' geometry.
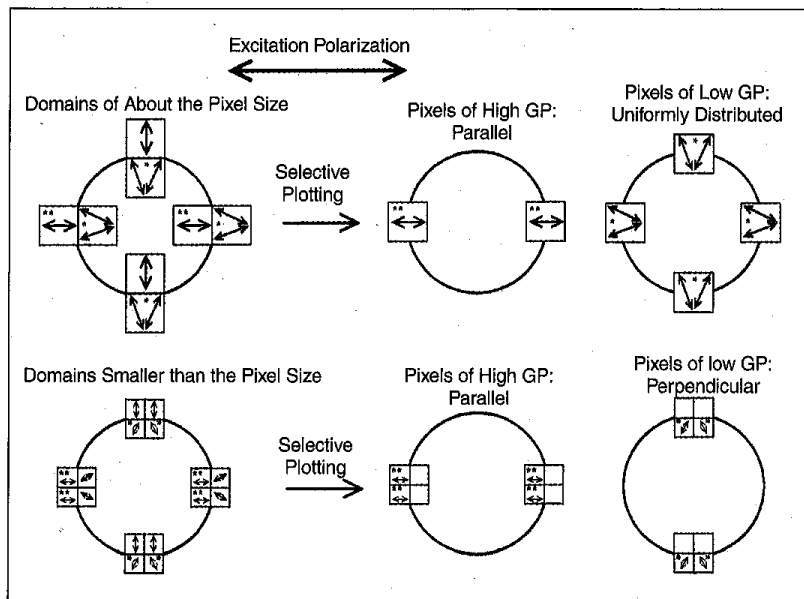
7. Schematic representation of the method for the evaluation of the size of membrane domains, comparable or smaller than the pixel size, in a selective plotting of low and high GP values and when using polarized excitation. The pixels are represented as squares along the circular vesicle.

dimensions were in the range between 2 nm and 5 nm [16]. In the case of BBM and BLM membranes, due to the random orientation of the probe molecules within these samples, the heterogeneity in terms of domain size is difficult to interpret using our model. Nevertheless, by selectively plotting the lower GP values, we can observe that the low GP values are confined at the border of the membranes [Figs. 5(e) and 5(f)], probably representing more fragile areas where membranes have been broken during their purification procedures.

Conclusions

Most fluorescent membrane probes are susceptible to photobleaching. The use of two-photon excitation opens new possibilities for microscopy studies of membranes. LAURDAN is a membrane probe with a steady-state sensitivity to the polarity of its environment, thus valuable for microscopy studies of membranes. When imaging LAURDAN GP with samples of natural membranes, we observed a large heterogeneity. By using the photoselection operated by a linearly polarized excitation, we found that the high GP values are associated with membrane regions where the fluorescent probe is aligned with the excitation polarization. Thus, we believe that the GP heterogeneity observed in natural membranes is due to coexisting domains. We have also discussed the possibilities of using polarized excitation for the evaluation of the dimension of the membrane domains.

Acknowledgments

Tiziana Parasassi received her doctorate degree in biological sciences in 1980 from the University of Rome. From 1980 to 1982, she was a postdoctoral fellow at the Serono Farmaceutici in Rome. She started working as a researcher at the Italian National Council of Research in 1982, at the Institute of Chemistry and Technology of Radioelements. Since 1988 she has been at the Institute of Experimental Medicine, where she is responsible for research in the fields of lipid molecular dynamics and of oxidative

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