

# Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order

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## Abstract

Laurdan is a fluorescent probe that detects changes in membrane phase properties through its sensitivity to the polarity of its environment in the bilayer. Variations in membrane water content cause shifts in the laurdan emission spectrum, which are quantified by calculating the generalized polarization (GP). We tested whether laurdan fluorescence could be used to distinguish differences in phospholipid order from changes in membrane fluidity by examining the temperature dependence of laurdan GP and fluorescence anisotropy in dipalmitoylphosphatidylcholine (DPPC) vesicles. The phase transition from the solid ordered phase to the liquid disordered phase was observed as a decrease in laurdan GP values from 0.7 to  $-0.14$  and a reduction in anisotropy from 0.25 to 0.12. Inclusion of various amounts of cholesterol in the membranes to generate a liquid ordered phase caused an increase in the apparent melting temperature detected by laurdan GP. In contrast, cholesterol decreased the apparent melting temperature estimated from anisotropy measurements. Based on these results, it appeared that laurdan anisotropy detected changes in membrane fluidity while laurdan GP sensed changes in phospholipid order. Thus, the same fluorescent probe can be used to distinguish effects of perturbations on membrane order and fluidity by comparing the results of fluorescence emission and anisotropy measurements.

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## 1. Introduction

Laurdan is a useful fluorescent probe of membrane structure. It has been used successfully to study bilayer properties in both artificial and natural membranes because of its sensitivity to the solvent relaxation effect. For example, the maximum emission wavelength in phosphatidylcholine bilayers shifts approximately 45 nm from about 435 nm to about 480 nm when switched from a rigid membrane environment to one that is fluid [1]. Apparently, the change represents differences in the number and/or mobility of water molecules present at the level of the phospholipid glycerol backbones [2,3]. Accordingly, laurdan is sensitive to membrane phase transitions and other alterations to membrane fluidity [1,2,4–13].

Recently, we have used laurdan to explore physical properties of human erythrocyte membranes under various experimental conditions [12,13]. As part of those studies, membrane fluidity was examined using measurements of both laurdan emission spectral characteristics and anisotropy. Interestingly, conditions have been observed at which the two measurements vary independently [12]. Therefore, although both are sensitive to membrane fluidity, they apparently report different information.

We have hypothesized that the differences could reflect distinctions between phospholipid order and membrane fluidity, defined as the ability of molecules the size of laurdan to diffuse in the membrane. To test this hypothesis, we prepared liposomes composed of dipalmitoylphosphatidylcholine (DPPC) mixed with various concentrations of cholesterol. This strategy allowed us to manipulate both fluidity and phospholipid order. Pure DPPC has a main phase transition from a solid ordered phase to a liquid disordered phase at about 41.5 °C. Addition of appropriate concentrations of cholesterol results in a liquid ordered phase [14–17]. For each of the resulting phases, we

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compared laurdan anisotropy with the properties of its emission spectrum. The results suggest that joint measurement of laurdan emission spectra and anisotropy can distinguish between changes in membrane fluidity and phospholipid order. This observation is useful since both measurements can be obtained easily from the same sample using the same equipment. Importantly, they can be obtained with complex membranes and living cells in which other spectroscopic measurements may be difficult or prohibitive.

## 2. Materials and methods

DPPC was purchased from Avanti Polar Lipid (Birmingham, AL), laurdan from Molecular Probes (Eugene, OR), and cholesterol from Spectrum (Gardena, CA). All other reagents were from standard sources.

To form liposomes, cholesterol and DPPC were dissolved in chloroform and mixed at the mole percents indicated in the figures. The solvent was then removed by evaporation at room temperature under a nitrogen stream. The dried lipid was then hydrated at 50 °C for 1 h in 150 mM KCl, 20 mM sodium citrate/citric acid buffer at pH 7.0. The sample was mixed vigorously on a vortex mixer for several seconds every 10 min during the hydration step to form multilamellar vesicles (MLV). The total lipid concentration in the hydrated suspension was 10 mM. Samples were stored at room temperature.

Steady-state laurdan fluorescence emission was monitored with a photon-counting spectrofluorometer (Jobin Yvon, Edison, NJ). Temperature was controlled by a circulating water bath, and sample homogeneity was maintained by continuous magnetic stirring. Vesicles (50 µM lipid) were equilibrated in the fluorometer sample cell with 2.5 µM laurdan at 50 °C until fluorescence emission was stable (generally about 1 h). This relatively high concentration of laurdan was chosen to increase the sensitivity of anisotropy measurements and to minimize the relative contribution of scattered light from the MLV. Control experiments using the intensity of scattered light to assess the membrane phase transition in MLV devoid of laurdan indicated that the perturbation to the transition was minimal at the resolution used in this study. Emission intensity was then acquired for several hundred seconds at 435 and 500 nm (excitation = 350 nm, 4.25 nm bandpass) at 50 °C. Temperature was then lowered 5 °C, the sample reequilibrated, and fluorescence data again acquired. The procedure was repeated down to 25 °C. At the concentrations of vesicles and bandpass used, interference from scattered light was negligible. Generalized polarization (GP) was calculated from the emission intensities using the following equation adapted from the work of Parasassi et al. [2]:

$$GP = \frac{I_{435} - I_{500}}{I_{435} + I_{500}} \quad (1)$$

where  $I_{435}$  and  $I_{500}$  refer to the average emission intensities at those wavelengths.

Laurdan anisotropy measurements were obtained in the L-format using a PC1 fluorometer from ISS (Champaign, IL) equipped with Glan-Thompson polarizers and 16-nm bandpass on both monochromators. Samples were prepared as described above for steady-state emission intensity. Fluorescence intensity at 435 nm (350 nm excitation) was measured with excitation and emission polarizers parallel to each other (both at 0°,  $I_0$ ) and repeated with the polarizers perpendicular (excitation: 0°, emission: 90°,  $I_{90}$ ). The intensity of scattered light was measured at each permutation of polarizer angles using MLV without laurdan and subtracted from the data obtained above. The contribution of scattered light was minimal under the conditions used (low vesicle concentration, high laurdan). Anisotropy was then calculated using the traditional equation.

$$r = \frac{I_0 - GI_{90}}{I_0 + 2GI_{90}} \quad (2)$$

The correction factor ( $G$ ) was obtained from the ratio of emission intensity at 0 and 90° with the excitation polarizer oriented at 90° (after subtraction of scattered light).

To assess the amount and mobility of bilayer water, laurdan fluorescence lifetimes were determined from phase modulation data obtained with an ISS lifetime fluorometer located at the Laboratory for Fluorescence Dynamics (LFD) at the University of Illinois, Urbana. The light source was a 350 nm line obtained by doubling the frequency of the 700-nm emission from DCM dye pumped by a Nd:YAG laser (Coherent). The excitation light was also polarized at 35°. Lifetimes were measured at 10-nm wavelength increments from 410 to 530 nm with a bandpass of 16 nm using eight frequencies ranging from 7.62 to 198.12 MHz. 1,4-Bis(5-Phenyl-2-oxazole)benzene in ethanol was used as a standard (1.35 ns) for calculating lifetimes from the phase and

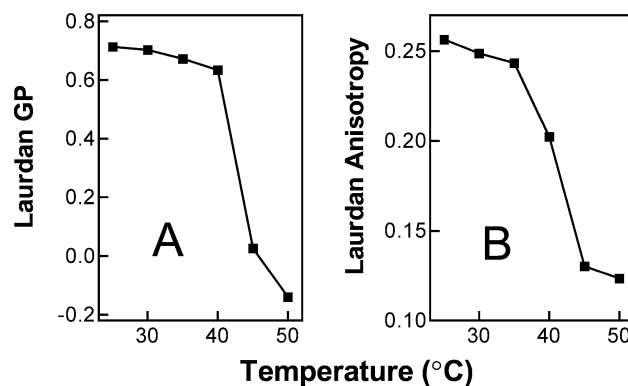


Fig. 1. Temperature dependence of laurdan GP (A) or anisotropy (B) values in DPPC MLV. Panel A: the value of GP was obtained at the indicated temperatures from laurdan emission at 435 and 500 nm as explained in Materials and methods. Panel B: anisotropy values were measured for the sample shown in (A) as explained in Materials and methods.

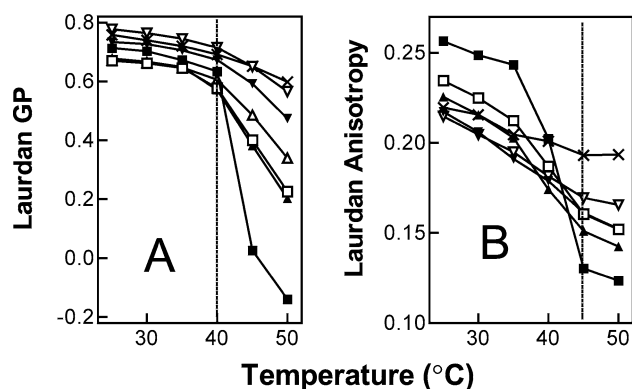


Fig. 2. Effect of cholesterol on the temperature dependence of laurdan GP (A) and anisotropy (B) values in DPPC MLV. The experiment of Fig. 1 was repeated with MLV containing the following concentrations of cholesterol: solid squares—0%, open squares—10%, solid triangles—15%, open triangles—20%, solid inverted triangles—25%, open inverted triangles—30%, crosses—40%.

modulation data. Lifetimes were estimated using the Globals Unlimited software available at the LFD. The time dependence of the emission spectrum was determined from the lifetime results and the corresponding steady state emission spectrum for each condition as described [10].

### 3. Results

Fig. 1A displays the temperature dependence of laurdan GP values for homogenous DPPC MLV. As expected, the value of GP decreased from about 0.7 at temperatures corresponding to gel phase lipid to about -0.14 for the liquid crystalline phase. The melting temperature appeared to lie between 40 and 45 °C. The typical value reported for calorimetric assessment of pure DPPC vesicles is about 41.5 °C (e.g. Ref. [18]; many examples given in Ref. [19]).

Fig. 1B represents repetition of the experiment of Fig. 1A using the same sample but with measurements of steady-state laurdan anisotropy instead of GP. Transition of the phospholipid from gel to liquid crystalline states was accompanied by a decrease in anisotropy values from about 0.25 to about 0.12. Again, the apparent melting temperature occurred between 40 and 45 °C.

The experiments of Fig. 1 were repeated with vesicles containing 10%, 15%, 20%, 25%, 30% and 40% cholesterol. Fig. 2A illustrates the effect of cholesterol content on laurdan GP values as a function of temperature. Four main effects were observed: first, the difference in GP values between high and low temperatures was progressively reduced. This observation reflected a large increase in GP values at the higher temperatures while values below the

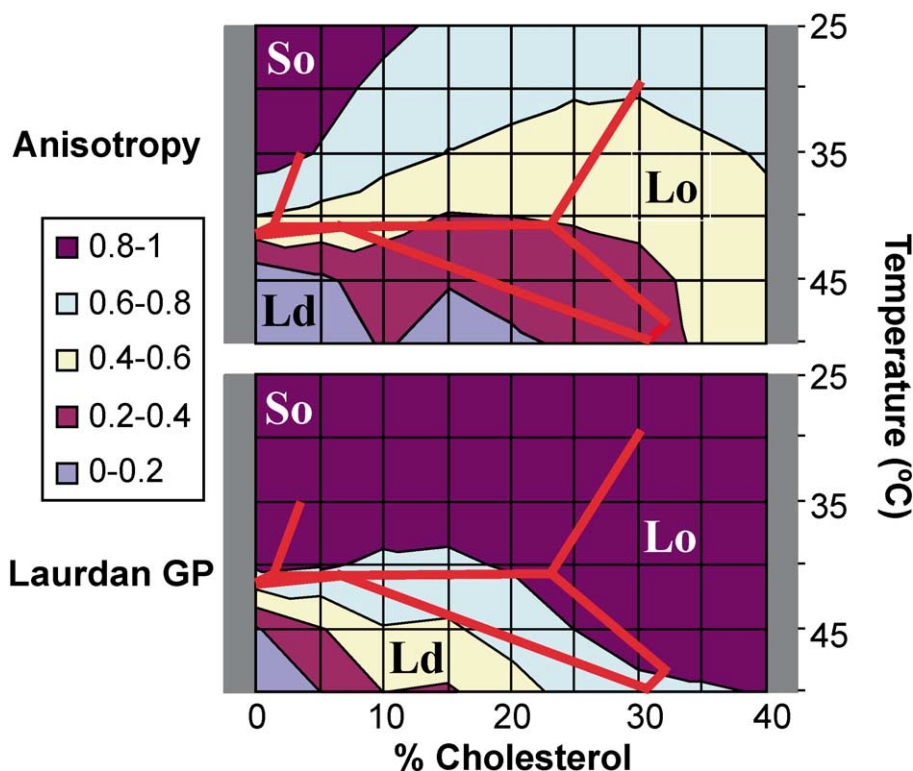


Fig. 3. Comparison of laurdan GP and laurdan anisotropy changes to a theoretical phase diagram of DPPC and cholesterol mixtures. The two panels represent contour plots of the data from Fig. 2A (lower panel) and B (upper panel). The data were normalized by subtracting the minimum value of the corresponding data set and dividing by the maximum change observed. The relative values of anisotropy (upper panel) or GP (lower panel) corresponding to the different colors are shown in the legend on the figure. The theoretical phase diagram (red lines) is based on Ref. [16]. “So:” solid ordered phase; “Lo:” liquid ordered phase; “Ld:” liquid disordered phase.

melting temperature remained similar at all cholesterol concentrations. Second, the range of temperatures over which the transition in GP values occurred was broadened. Third, the apparent lipid melting temperature increased. Fourth, the temperature of onset of the apparent transition was similar at all concentrations of cholesterol tested (dotted line in Fig. 2A).

Similar results were obtained using anisotropy measurement with three important differences. First, the progressive reduction in the difference between values at high and low temperature reflected both a reduction in anisotropy at low temperatures and an elevation of values at high temperatures (Fig. 2B). Second, the apparent melting temperatures decreased with cholesterol content. Third, instead of the onset, the completion of the apparent transition was similar at all cholesterol concentrations tested (dotted line in Fig. 2B).

These differences in anisotropy and GP measurements appeared related to complexities of membrane phase properties introduced by cholesterol. Fig. 3 displays in red an idealized phase diagram for DPPC and cholesterol patterned after that suggested by theoretical and experimental results [15,16]. Superimposed on this phase diagram is a contour plot of the data of Fig. 2A (lower panel) and B (upper panel). In these diagrams, both the anisotropy and GP data have been normalized to the maximum and minimum values for the data set for facilitated comparison.

To further explore the basis of changes in laurdan GP values caused by cholesterol, we estimated laurdan lifetime values for vesicles at each concentration of cholesterol at 45 °C. Lifetimes were assessed as a function of wavelengths corresponding to the emission spectrum in order to evaluate the time dependence of spectrum dynamics following photon absorption. As shown in Fig. 4, the spectrum center of mass increased from 449 to 484 nm with a

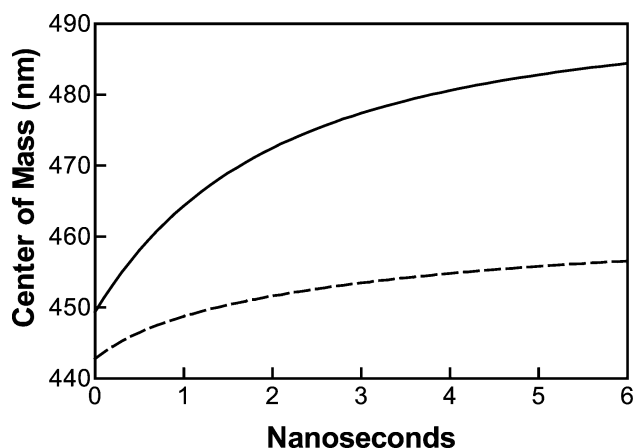


Fig. 4. Time dependence of the laurdan emission spectrum at 45 °C for DPPC MLV in the absence (solid curve) and presence (dashed curve) of 25% cholesterol. The emission spectrum center of mass was obtained from measurements of laurdan lifetimes as a function of wavelength and calculated as explained in Materials and methods.

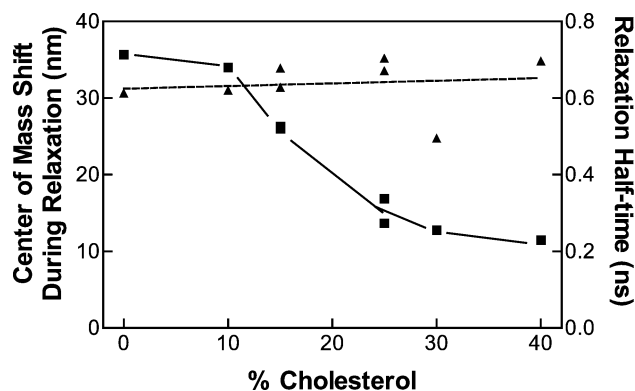


Fig. 5. Effects of cholesterol content on kinetics of the solvent relaxation effect on laurdan at 45 °C. Data such as that shown in Fig. 4 were gathered for MLV at the indicated concentrations of membrane cholesterol. Squares, solid lines: the extent of shift in the spectral center of mass during solvent relaxation. Triangles, dashed line: the half-time for completion of the spectral shift.

halftime of 1.4 ns, consistent with the strong solvent relaxation effect associated with this probe. Increasing amounts of cholesterol reduced the magnitude of the time-dependent shift in emission spectrum without altering the kinetics of the shift (example shown in Fig. 4, summary in Fig. 5).

#### 4. Discussion

Spectral and anisotropy measurements using fluorescent probes have been used commonly to assess changes in membrane phases [5–7,20–24]. Advantages of fluorescent probes include their sensitivity (experiments can be conducted with dilute samples), their ability to provide information from complex membranes including living cells, and the possibility of obtaining images of the vesicle or cell membrane being studied [11]. Laurdan is one probe that has proven useful for such studies because it is highly sensitive to the presence and mobility of solvent dipoles [2,6]. Changes in the emission spectrum associated with sensitivity to solvent are quantified by calculation of the GP value as demonstrated in Eq. (1) [2]. The value of GP varies between 1 (no solvent effects) and  $-1$  (complete exposure to bulk water). Since the presence and mobility of water molecules in the bilayer depends on the mobility of lipid molecules in the membrane, laurdan can be used to report changes in membrane phase (Fig. 1; [2,5,6,8,9,11,25]).

One disadvantage of fluorescence is that the amount of detailed information provided may be considerably less than from some techniques such as deuterium NMR. However, experiments with two-photon microscopy imaging of laurdan demonstrated that the amount of information obtained can be increased by using both the environment sensitivity of the probe as well the photoselection effect of excitation with polarized light [11,25]. Furthermore, recent evidence



from erythrocyte ghosts in bulk dispersion suggested that under some circumstances, measurements of laurdan GP and anisotropy do not report the same information [13]. In fact, comparison of the data of Fig. 2A and B verifies that such can be the case.

Superficially, it may seem that laurdan GP and anisotropy should report the same results. Changes in the anisotropy of a membrane probe can be interpreted as changes in the freedom of movement of that molecule in the membrane (if the probe lifetime does not change). Specifically, it refers to displacement of the emission dipole from that of absorption due to rotation of the probe during the lifetime of the excited state. Therefore, the anisotropy value depends on the viscosity of the local molecular environment, in this case, the membrane. GP, however, does not reflect laurdan motion; rather, it is influenced by local motion of polar molecules. It appears that for vesicles dispersed in aqueous solution, much of the effect of polar molecules in the vicinity of laurdan can be attributed to water penetrating the head groups of membrane phospholipids [2,6]. Although water penetration would be aided by lipid motion in the membrane, it is especially sensitive to phospholipid packing [6]. The degree of lipid packing, of course, depends on the degree of phospholipid chain order. When the chains are ordered, the head groups pack better, and the number of solvent molecules available to influence laurdan is reduced. These changes in phospholipid order must not be confused with the “order parameter” calculated from time-dependent anisotropy decay measurements that refers specifically to motion of the fluorescent probe.

Thus, it seems reasonable to argue that when membrane fluidity changes but phospholipid order remains constant, one would expect laurdan anisotropy, but not GP, to also change. To test this hypothesis, we created membranes with varied levels of cholesterol. Although pure DPPC shifts from a solid ordered to a liquid disordered phase, an intermediate liquid ordered phase forms at appropriate cholesterol concentrations [14]. If our hypothesis were correct, one would predict that laurdan anisotropy values should be low while GP values are still high when the membrane is in the liquid ordered phase. As shown in Fig. 3, this is exactly what was observed. Thus, we conclude that the combined measurement of laurdan anisotropy and GP allows one to distinguish changes in membrane fluidity and order with a single fluorescent probe.

That the change from disordered toward ordered phases represents an actual loss of water molecules from the bilayers is suggested by the data of Figs. 4 and 5. If the change in GP values upon addition of cholesterol were due to decreased mobility of water or some other dipole in the membrane (e.g. phosphoester moiety of the phospholipids), an increase in half-time for the solvent relaxation effect would have been observed. This result validates a previous interpretation of the effect of cholesterol on laurdan GP [6].

Interpretation of anisotropy values in terms of rotational rates requires consideration of the average lifetime of the fluorescent probe. Probe rotational rate ( $R$ ) is related to anisotropy ( $r$ ) by the following equation:

$$R = \frac{\left(\frac{r_0}{r} - 1\right)}{6\tau} \quad (3)$$

where  $\tau$  is the average lifetime of the laurdan excited state and  $r_0$  is the lower limiting anisotropy. The maximum value of anisotropy we have measured for gel phase vesicles is 0.26. The theoretical maximum value of  $r_0$  is 0.4. Therefore,  $r_0$  lays somewhere between 0.26 and 0.4 for laurdan.

Lifetime values of laurdan are, of course, sensitive to the solvent relaxation effect, and therefore change when the environment polarity changes. For example, we measured an average lifetime value of 5.9 ns for laurdan at 39 °C. This value decreased to 2.6 ns above the phase transition at 44 °C. If one tries to infer changes in laurdan rotation rate quantitatively from anisotropy data without considering lifetimes, the magnitude of the changes will be underestimated based on Eq. (3). In the case of 39 and 44 °C,  $R$  increases by 0.06 to 0.1 ns<sup>-1</sup> if one accounts for lifetimes (range relates to  $r_0 = 0.26$ –0.4). If one ignores lifetimes, the change will be underestimated as 0.05–0.7 ns<sup>-1</sup>. As shown in Fig. 6, cholesterol addition (to form a liquid ordered phase) alters anisotropy values (open squares) less than GP values (solid squares) in accordance with the observations displayed in Fig. 2. Calculation of  $R$  (triangles) using lifetimes produces changes that more closely resemble those

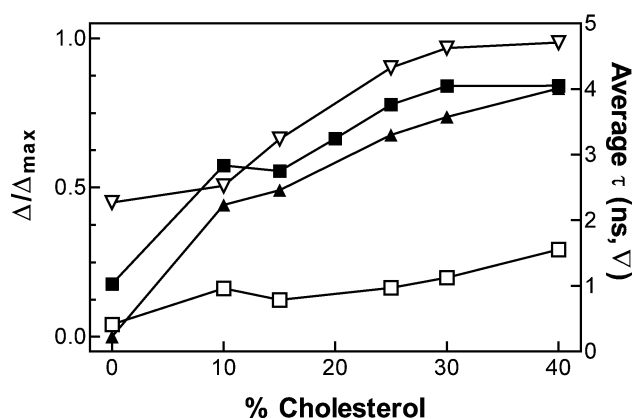


Fig. 6. Comparison of laurdan average fluorescence lifetimes ( $\tau$ , inverted triangles), anisotropy (open squares),  $R$  (triangles), and GP (solid squares) at 45 °C as a function of cholesterol concentration. GP and anisotropy values were obtained from the data of Fig. 2, lifetime values were calculated from the data of Figs. 4 and 5, and values of  $R$  were calculated from Eq. (3) using  $r_0 = 0.26$ . Repetition of the calculation at  $r_0 = 0.4$  gave a result that was qualitatively the same as that shown in the figure. In the case of GP, anisotropy, and  $R$ , data were normalized by subtracting the minimum value of the corresponding data set and dividing by the maximum change observed. In the case of  $R$ , the maximum value was that obtained with pure DPPC, and the minimum was set at 0.

obtained with GP measurements. Thus, the changes in laurdan lifetime that accompany changes in the emission spectrum tend to hide effects of membrane order on laurdan anisotropy. In contrast, anisotropy variations that occur under conditions at which GP does not change (i.e. at temperatures below 40 °C in Fig. 2) are not obscured by lifetime alterations. Hence, the anisotropy data are actually simplified by the effects of solvent on laurdan lifetimes as membrane order varies. Accordingly, laurdan anisotropy results selectively emphasize fluidity changes that are not accompanied by changes in lipid order. The net effect is the ability of the probe to distinguish membrane fluidity from lipid order as shown in Fig. 3.

In summary, the results of this study demonstrate that the combined use of GP and polarization techniques can increase the understanding of membrane phase properties. Specifically, anisotropy appears to report changes in membrane fluidity while laurdan GP is more sensitive to variations in membrane order. Other membrane perturbations such as interdigitation of certain lipids that could influence membrane water content without altering fluidity would also be distinguishable by comparing laurdan GP and anisotropy. Thus, the many advantages of using this fluorescent probe in artificial and biological membranes are enhanced by simple inclusion of a polarized excitation source. Furthermore, the results of this study facilitate interpretation of existing data in which laurdan emission spectra and anisotropy report contrasting results.

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