

Laurdan Fluorescence Lifetime Discriminates Cholesterol Content from Changes in Fluidity in Living Cell Membranes

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ABSTRACT Detection of the fluorescent properties of Laurdan has been proven to be an efficient tool to investigate membrane packing and ordered lipid phases in model membranes and living cells. Traditionally the spectral shift of Laurdan's emission from blue in the ordered lipid phase of the membrane (more rigid) toward green in the disordered lipid phase (more fluid) is quantified by the generalized polarization function. Here, we investigate the fluorescence lifetime of Laurdan at two different emission wavelengths and find that when the dipolar relaxation of Laurdan's emission is spectrally isolated, analysis of the fluorescence decay can distinguish changes in membrane fluidity from changes in cholesterol content. Using the phasor representation to analyze changes in Laurdan's fluorescence lifetime we obtain two different phasor trajectories for changes in polarity versus changes in cholesterol content. This gives us the ability to resolve *in vivo* membranes with different properties such as water content and cholesterol content and thus perform a more comprehensive analysis of cell membrane heterogeneity. We demonstrate this analysis in NIH3T3 cells using Laurdan as a biosensor to monitor changes in the membrane water content during cell migration.

INTRODUCTION

From the biological perspective, cell membranes influence many cell functions and are involved in most of the cellular communication processes (1–3). With respect to membrane dynamics, one biophysical parameter found to be especially critical is membrane fluidity. Membrane fluidity is an important parameter at a molecular level, as it determines the rate of motion of molecules in the membrane, with an inversely proportional relationship to membrane microviscosity. Changes occurring in membrane fluidity play a key role in regulation of membrane properties under physiological conditions and in the pathogenesis of disease (4–6). For example, changes in the plasma membrane fluidity of cancer cells may affect antigens and receptors (7,8), cancer cell motility (9), and the deformability potential of metastatic cells (10). Evaluating membrane fluidity is essential for understanding complex mechanisms regulated by membrane properties. Moreover, fluidity appears to be influenced, and to a certain extent spatially regulated, by membrane domains characterized by a different composition with respect to the rest of the membrane (11). It has been suggested that these domains, which are enriched with cholesterol and sphingolipids, function as platforms for partitioning of membrane proteins (12,13). In other words, cell membranes have a complex lipid composition where lipids can be continuously added to or removed from these microdomains (14). Thus, the total number of chemical species that compose the lipid bilayer of these membranes is very large, and the binary and ternary model systems studied so far could only be prototypical examples

that provide guidelines for understanding the complexity of cellular membranes.

Laurdan is a fluorescent membrane marker used to investigate membrane fluidity (15–21). A distinctive feature of Laurdan is its ability to sense the polarity of its environment (22). In the case of lipid bilayers, Laurdan senses the presence of water in the membrane, which gives us information about water penetration, a property related to membrane fluidity. When originally used in model membranes, it was shown that Laurdan distinguishes membrane lateral packing in model bilayers according to at least two separate classes: solid-ordered and liquid-disordered (16). For the first class, the bilayer is a highly ordered quasi-two-dimensional solid, while for the second class it is a disordered quasi-two-dimensional liquid (14). To distinguish and quantify these two phases, a normalized ratiometric method of analysis was developed called generalized polarization (GP) (16,23,24). However, this method is limited when more than two states need to be distinguished. For example, if we want to distinguish additional phases formed in lipid bilayers at different cholesterol concentrations we need to expand the ratiometric approach to a method that will allow us to quantify more than two states. It is well established that in the presence of a certain amount of cholesterol, membranes can display a liquid-ordered phase (25–27). The flat and rigid molecular structure of sterols does not allow them to fit in the solid-ordered (or gel) phase (14). Cholesterol at adequate molar fractions can convert liquid-disordered and solid-ordered lipid phases to liquid-ordered phases (25).

The compositional complexity of cell membranes is vast, and given the distribution of sizes and shapes of membrane microdomains that have been detected, a more comprehensive spectroscopic method is required that can detect and

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quantify this heterogeneity. In this work, we develop a, to our knowledge, new approach for the analysis of Laurdan fluorescence in biological membranes. We propose that detection of Laurdan lifetime at specific emission wavelengths allows us to discriminate between changes in membrane fluidity and specific changes in cholesterol content. Using the phasor approach to fluorescence-lifetime imaging microscopy (FLIM) we transform the fluorescence decay data into a two-dimensional coordinate system where we obtain different trajectories for changes in polarity versus changes in cholesterol content. This gives us the ability to resolve *in vivo* membranes with different properties such as water content and cholesterol content and thus perform a more comprehensive analysis of cell-membrane heterogeneity.

Laurdan is a molecule whose spectroscopic properties are influenced by both the composition and dynamics of its local surroundings. In other words, Laurdan's fluorescence is dependent on two major factors: the polarity of the environment (ground state of the fluorophore) and the rate of dipolar relaxation of molecules or molecular residues that can reorient around Laurdan's fluorescent moiety during its excited-state lifetime. The original work of G. Weber on Prodan (22,28) was aimed at separating these two effects. These effects are more evident in different parts of Laurdan's emission spectrum. In this work, we propose a detection scheme that isolates different wavelength bands of Laurdan's emission in such a way that changes in polarity (ground state) are detected independent of changes in dipolar relaxation (excited state). That is, unlike conventional GP analysis, which centers the blue and green filters at 440 nm and 490 nm, respectively, our proposed method involves shifting the green filter toward longer wavelengths (540/50 nm, called green channel in this work) to highlight dipolar relaxations that are more evident in the extreme red part of the emission spectrum. In this way, dipolar relaxations can be detected in the green channel and the signal collected in the blue channel (460/80 nm) will detect changes mainly related to Laurdan's ground state. Given that these two parameters are interlinked in a biological context, it is difficult to separate the two effects using intensity-based measurements (such as GP analysis). For this reason, instead of intensity-based detection, we used fluorescence-decay-based detection and applied the phasor approach to decay analysis. By using this graphical approach, we could take advantage of the additive properties of phasor analysis and thus make a straightforward identification of the existing components and mutual interactions. The phasor approach provides a straightforward separation of true decay times from those apparently lengthened due to the relaxation effect. In fact, the dipolar relaxation appears as a delay of excitation that in the phasor approach results in a simple rotation of the phasor plot. A remarkable finding of our study is that changing the amount of cholesterol in the membrane of NIH3T3 cells (as well as in solu-

tions that we use as a control) causes an overall rotation of the phasor plot, as seen in the green channel. Therefore, by using Laurdan's lifetime decay in these two detection channels (blue and green) we can disentangle the effects of polarity from dipolar relaxations due to addition/subtraction of cholesterol, giving us unprecedented details about cholesterol and the heterogeneous distribution of polarity/fluidity in cell membranes.

MATERIALS AND METHODS

Cell culture and treatments

NIH3T3 cells were grown at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 5 mL of Pen-Strep, and 2.5 mL of 1 M HEPES. Freshly split cells were plated onto 35-mm Mattek glass-bottom dishes coated with fibronectin. The membrane marker Laurdan (6-dodecanoyl-2-dimethylamino naphthalene; Invitrogen) was dissolved in dimethylsulfoxide (DMSO), and 1.8 mM stock solution was prepared and added to the cell dishes at a 1:1000 dilution. Cells were incubated with Laurdan for 40 min at 37°C before imaging.

Cholesterol depletion

For the cholesterol-depletion experiments, a 50 mM stock solution of methyl- β -cyclodextrin (M- β -CD; Sigma-Aldrich, St. Louis, MO) was prepared by dissolving in nanopure water. The solution was added to the cell dishes to a final concentration of 1.0 mM or 5 mM, and the dishes were incubated for 1 h at 37°C. Cells were then rinsed with phosphate-buffered saline (PBS), and new medium was added. For the stimulation experiments, the cells were serum-starved in unsupplemented high-glucose medium for 2–4 h. They were then stimulated with 50 ng mL⁻¹ of epidermal growth factor (EGF) (Sigma-Aldrich) and imaged immediately afterward.

Solution experiments

Laurdan fluorescence decay was measured in 1-butanol and glycerol solutions. To assess the effect of water, we measured Laurdan fluorescence decay in pure 1-butanol or glycerol (0% water), to which nanopure water was added in increasing amounts of (2%, 5%, 10%, 20%, 30% water). For the cholesterol experiments, water-soluble cholesterol (Sigma-Aldrich) was dissolved in nanopure water, and 30 mM stock solution was prepared. To assess the effect of cholesterol, we used solutions of Laurdan in glycerol, maintaining constant the percentage of glycerol and water, and we added different amounts of cholesterol. We used solutions of 90%, 80%, and 70% glycerol, to separate the effect of glycerol from that of cholesterol. We prepared solutions, varying the amount of cholesterol from 0.9 mM to 3.0 mM for each glycerol concentration. The membrane probe Laurdan (6-dodecanoyl-2-dimethylamino naphthalene; Invitrogen) was dissolved in DMSO to make a stock concentration of 1.8 mM. The Laurdan stock solution was added to these solutions at a dilution varying from 1:1000 to 5:1000.

POPC vesicles

POPC was obtained from Avanti Polar Lipids (Birmingham, AL). Multilamellar phospholipid vesicles were prepared according to the procedure described in Parasassi et al. (16). Briefly, the proper amounts of chloroform stock solutions of POPC were mixed with the probe and cholesterol. Solvent was evaporated by nitrogen flux. The dry samples were

resuspended in PBS that had been deoxygenated by vigorous nitrogen bubbling for 1 h. The samples were then heated at 65°C, vortexed, and used immediately after preparation. All preparations were carried out in red light and a nitrogen atmosphere. The final probe/lipid concentration was in the range 1:1000–1:400.

Microscopy

FLIM and ratiometric GP data for both cells and solutions were acquired with a Zeiss LSM710 META laser scanning microscope coupled to a 2-photon Ti:Sapphire laser (Mai Tai, Spectra Physics, Newport Beach, CA) producing 80-fs pulses at a repetition of 80 MHz. An ISS A320 FastFLIM box was used to collect the decay data. A 40× water-immersion objective, 1.2 NA (Zeiss), was used for all experiments. Laurdan fluorophore was excited at 780 nm. Excitation at 780 nm induced negligible autofluorescence, since two-photon excitation of intracellular metabolites is centered at 740 nm (29). An SP 760 nm dichroic filter was used to separate the fluorescence signal from the laser light. The fluorescence signal was directed through a 495 LP filter and the signal was split between two photomultiplier detectors (H7422P-40, Hamamatsu, Hamamatsu City, Japan), with a Semrock (Rochester, NY) BrightLine single-band bandpass filter in front of each detector: 460/80 nm for the blue channel and 540/50 nm for the green channel. For image acquisition, the pixel frame size was set to 256 × 256 and the pixel dwell time was 25.61 μs/pixel. The average laser power at the sample was maintained at the mW level.

Data analysis

FLIM and ratiometric GP data were acquired and processed by the SimFCS software developed at the Laboratory for Fluorescence Dynamics (www.lfd.uci.edu). Calibration of the system and phasor plot for FLIM data was performed by measuring fluorescein (pH 11), which has a known single-exponential lifetime of ~4.05 ns. Calibration of the GP data was performed with Laurdan in DMSO. In the phasor approach, we first acquire the fluorescence decay, $I_{ij}(t)$, at each pixel of an image and then apply the phasor transformation to the decay measured at each pixel. The transformation consists of calculating the sine and cosine transformations of the decay curve at every pixel. The transformations are then plotted on the phasor plot. The equations used to calculate the g and s coordinates for the phasor plot are shown below: i and j indicate the pixel within the image, and ω is the angular modulation frequency, equal to $2\pi f$, where f is the laser repetition frequency. In our experiments, f is 80 MHz. The coordinate g represents the x -coordinate and s represents the y axis in the phasor plot.

$$\begin{aligned} g_{ij}(\omega) &= \frac{\int_0^{\infty} I_{ij}(t) \cos(\omega t) dt}{\int_0^{\infty} I_{ij}(t) dt} \\ s_{ij}(\omega) &= \frac{\int_0^{\infty} I_{ij}(t) \sin(\omega t) dt}{\int_0^{\infty} I_{ij}(t) dt}. \end{aligned} \quad (1)$$

The phasor is like a vector and follows the addition rules of vectors, i.e., pixels that contain a linear combination of two phasors are in the line joining the two phasors.

RESULTS

GP analysis

To compare our proposed FLIM approach to the GP approach, we performed both GP and FLIM analysis of Laurdan fluorescence in live NIH3T3. First, we use intensity-based detection to acquire a z -stack of live NIH3T3 cells (Fig. 1 A) and we show the conventional GP analysis in Fig. 1 B. This analysis provides the location of the membranes with high and low GP, according to binary criteria based on the decomposition of the GP pixel distribution in two Gaussian components (Fig. 1, C and D). (Note that the emission filters used for the GP analysis are different from those employed in the conventional GP analysis used originally by Parasassi et al. (16).) Then we use the newly proposed phasor analysis to show that it can distinguish several environments for the Laurdan probe.

Phasor analysis of polarity and dipolar relaxation effects on Laurdan solutions and POPC vesicles

We performed a series of solution experiments that separately test the influence of water and cholesterol on Laurdan's decay (Fig. 2). We are following a procedure to establish whether the decay of Laurdan fluorescence can distinguish polarity changes from dipolar relaxation effects, similar to the Macgregor and Weber approach using spectral analysis (28). In Fig. 2 A, we assess the influence of water molecules on Laurdan decay as detected in the blue channel. Laurdan decay was measured in 1-butanol, a relatively low-polarity solvent, increasing the amount of nanopure water added (10%, 20%, and 30% water). If we increase the water amount beyond 30%, the signal becomes dimmer because of the quenching of Laurdan fluorescence by water. The diamonds in Fig. 2 A represent the position of each solution's phasor distribution. These data show that increasing the water content causes a quenching of Laurdan lifetime, moving the phasor (as measured in the blue channel) toward shorter lifetime values (the 1,0 point in the phasor plot). In Fig. 2 A, we show the effect of adding water to 1-butanol in the blue filter, but very similar effects are also found for glycerol-water mixtures (data not shown).

In Fig. 2 B, we assess the influence of cholesterol as detected in the blue channel. We note that cholesterol's influence on Laurdan GP has already been shown in artificial and biological membranes (30). We use solutions of Laurdan in glycerol (70% glycerol and 30% water), adding different amounts of water-soluble cholesterol. Data in Fig. 2 B were obtained using a cholesterol content that varied from 0.9 mM to 3 mM. We also tested solutions of 90% and 80% glycerol, and a similar phasor shift was observed (data not shown). In these experiments, we observe a shift of the phasor position from longer lifetime values (apolar region, or high cholesterol) to short lifetime values (polar

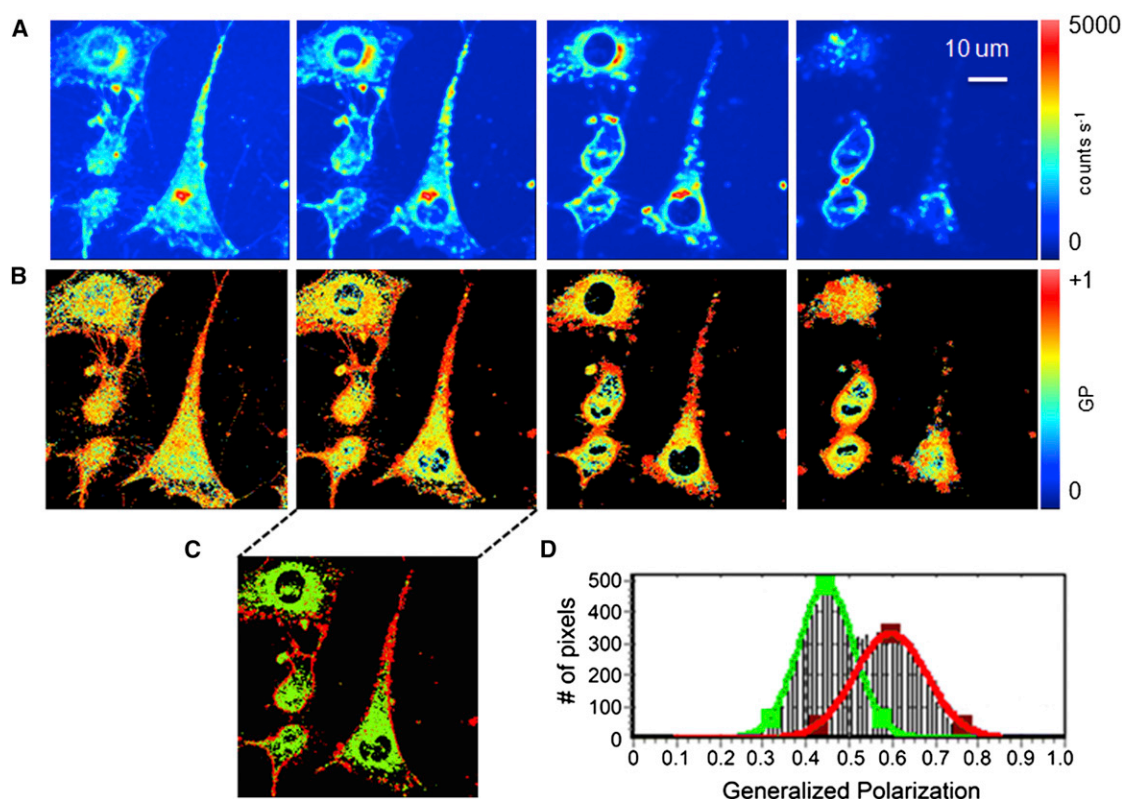


FIGURE 1 Laurdan GP analysis. (A) Fluorescence-intensity images of NIH3T3 cells at different z planes 1 μm apart (from the ventral region to the dorsal region) observed in the blue channel (460/80 nm). (B) GP images. The GP scale used to pseudocolor the intensity image is shown at right. (C) Binary GP image: the GP histogram from the corresponding image in B is decomposed into two Gaussian components. (D) Pixels belonging to the low-GP component (green Gaussian) are green; pixels belonging to the high-GP component (red Gaussian) are red.

region, or low cholesterol). Thus, analysis of the phasor position in the blue channel provides trajectories that align along lines of different lifetime values, which reflect the relative polarity (water content) of the medium. In contrast, changes due to dipolar relaxation cannot be observed at this emission wavelength.

To detect the characteristic signature of dipolar relaxations on Laurdan's emission we must move to the green channel. Fig. 2 C shows the phasor plot when the water content of the glycerol solution is changed. The change in polarity due to the increase in water content causes a shift of the phasor toward shorter lifetimes. Of more importance, the phasor distributions tend to be located outside the universal circle, despite the fact that every lifetime should be located inside the universal circle. This is due to dipolar relaxation processes (28,31). Due to dipolar relaxation, the red part of the emission spectrum appears at a later time with respect to the excitation. This causes an additional phase shift in the excited state of the probe (32), which in the phasor plot results in an overall rotation of the plot that shifts the phasor distribution outside of the universal circle (Fig. 2 E). In Fig. 2 D, we show that adding cholesterol causes a shift in the same direction as increasing dipolar relaxations, which is different from the direction of the phasor shift due to polarity. Therefore, the phasor

analysis in the green channel shows that the changes in phasor positions are due to different contributions, one related to polarity (the more polar, the shorter is the lifetime) and another that depends on the relaxation of water molecules in the proximity of the Laurdan fluorescence moiety (the larger the spectral shift due to the relaxation, the more the phasor distribution rotates and moves outside of the universal circle). In Fig. 2 F, we show the phasor distribution of POPC vesicles with different cholesterol content from 0 to 20% mole fraction. Also in this system, addition of cholesterol moves the phasor distribution toward the outside of the universal circle (Fig. 2 F). Cholesterol also displaces water in this system, causing the overall distribution to move toward longer lifetimes.

Phasor analysis of Laurdan as a biosensor of cholesterol in live cells

Next, we demonstrate that the overall rotation of the phasor plot is mainly caused by the presence of cholesterol. Once the basic rules for interpreting the directions of the phasor shifts in the blue and the green channels are established in solutions, we need to verify that these rules also apply to biological membranes. For these experiments, we used the same NIH3T3 cells used for the GP experiments in Fig. 1.

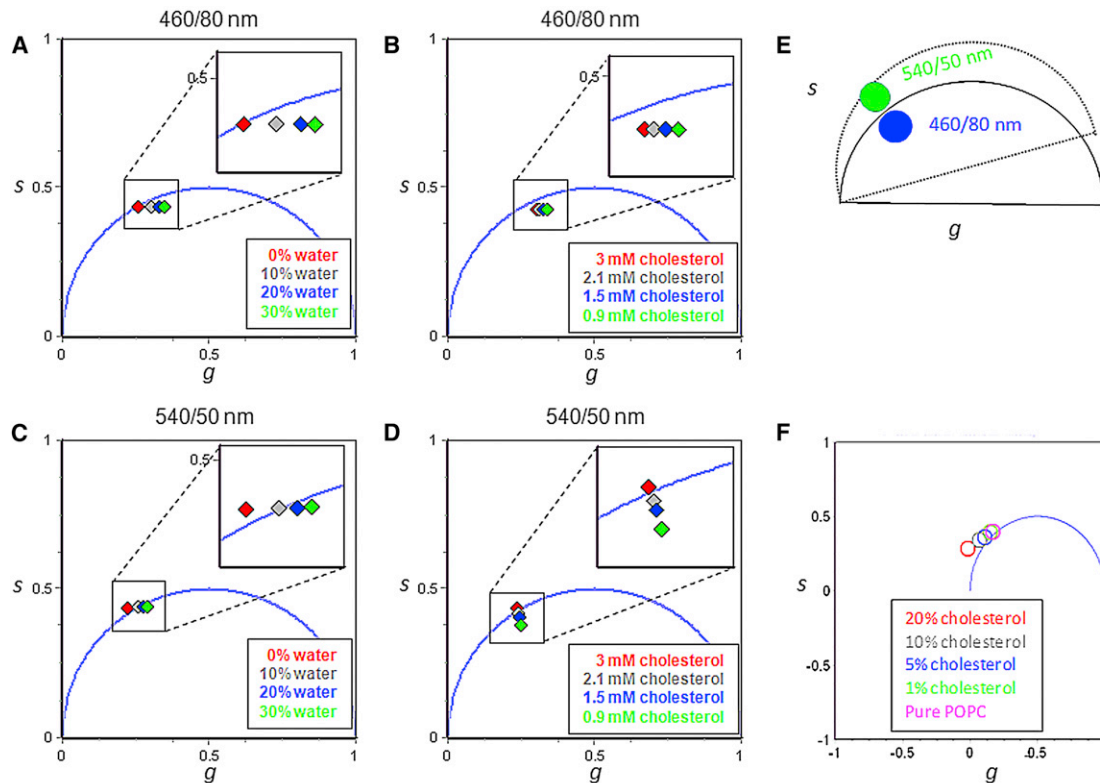


FIGURE 2 Laurdan decay transformed into a phasor plot for solution and POPC vesicle experiments detected at two wavelength ranges, 460/80 nm (A and B) and 540/50 nm (C, D, and F). In the blue channel (A and B), the phasor position is determined by polarity effects, whereas in the green channel (C, D, and F), dipolar relaxations determine the phasor position, which is outside the universal circle. Laurdan phasor is measured in pure 1-butanol (A) and glycerol (C) with increasing amounts of nanopure water. Water causes a shortening of Laurdan lifetime. In B and D, the effect of adding cholesterol to a solution of 30% water in glycerol shows that at 460 nm the phasor distribution moves toward a longer lifetime, whereas at 540 nm, the addition of cholesterol increases dipolar relaxations, as shown by the shift of the phasor distribution toward the outside of the universal circle. (E) In POPC vesicles, addition of cholesterol also moves the phasor outside of the universal circle but also toward longer lifetimes.

We have already established for these cells that the plasma membrane is less fluid than the internal membranes (Fig. 1, C and D). We treated NIH3T3 cells with 0 mM, 1 mM, and 5 mM M- β -CD for 2 h as a means of depleting the overall cholesterol content. We note here that there is a very small difference between treatment with 1 mM and treatment with 5 mM M- β -CD. Henceforth, we refer to samples simply as treated with M- β -CD or untreated. The phasor distribution integrated for $N = 4$ or 5 cells (in each category) is shown for the different detection wavelength ranges for the blue channel (Fig. 3) and the green channel (Fig. 4) before and after treatment with M- β -CD, so that the change in phasor position induced by cholesterol depletion could be directly compared in the figures. As can be seen in Fig. 3 A, the phasor distribution detected through the blue filter shows a distribution of phasors that remains inside the universal circle upon depletion of cholesterol. The color-coded cursors in Fig. 3 A are used to select pixels in the image that correspond to pixels in the selected region of the phasor plot. For the untreated sample, pixels in the plasma membrane of the cell tend to fall in the part of the phasor plot selected by the red cursor, although the separation is

marginal. This result implies that the membranes of the cell have similar polarity before cholesterol depletion. NIH3T3 cells treated with M- β -CD have a different distribution of phasors than untreated cells, as shown in Fig. 3 B. Fig. 3 B and the selection shown in Fig. 2 D show that cholesterol depletion makes the plasma membrane less polar, but that it has an opposite effect on internal membranes. After cholesterol depletion, we can clearly separate the plasma membrane from internal membranes using the same cursor positions that we placed for the analysis of the untreated cells (Fig. 3 D). In accordance with the common interpretation of the effect of water on Laurdan lifetime, we identify this trajectory with changes in water penetration in the membrane, which is also related to membrane fluidity and lipid packing. We obtain the well established result that the presence of cholesterol makes the plasma membrane more fluid and the internal membranes less fluid.

In contrast to the blue channel, the phasor distribution detected through the green filter (Fig. 4 A) is outside the universal circle. Treatments with M- β -CD move the phasor distribution toward the universal circle. Thus, we conclude

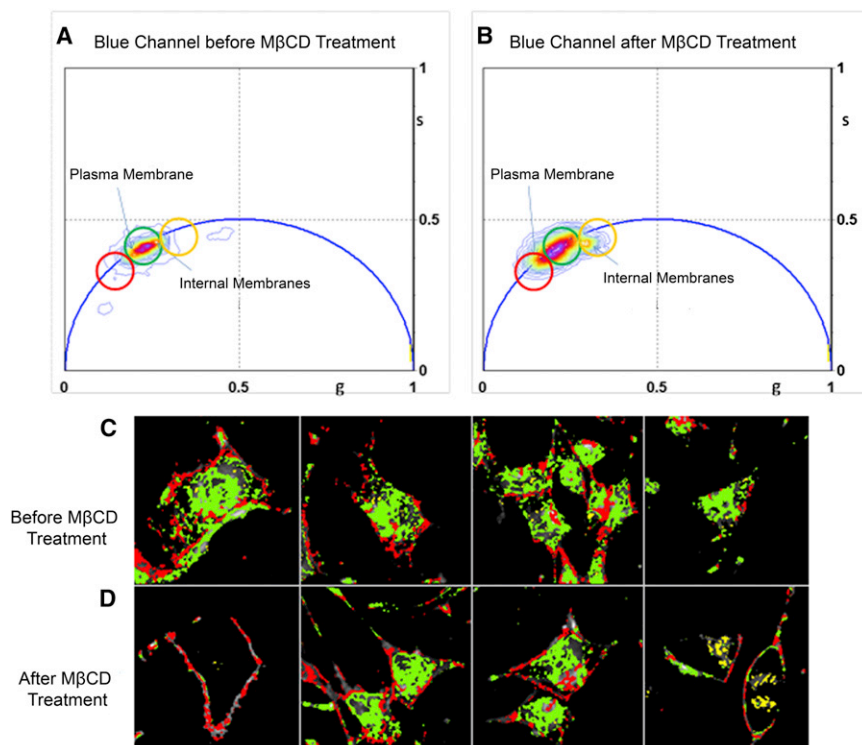


FIGURE 3 Influence of cholesterol on decay of Laurdan in the blue wavelength range. Phasor distribution of Laurdan in NIH3T3 cells detected through a 460/80-nm filter (blue filter) before (A) and after (B) treatment with 1 and 5 mM M- β -CD. (C) NIH3T3 cells pseudocolored according to the palette defined in A, where green pixels correspond to the average phasor position (*green cursor*), and yellow and red pixels correspond to movement of the phasor distribution toward the left or right, respectively, upon M- β -CD treatment. (D) The NIH3T3 cells of C after treatment with M- β -CD pseudocolored according to the same palette. Pixels within the plasma membrane move toward the lower-polarity region, whereas pixels corresponding to the internal membranes move in the direction of increased polarity.

that cholesterol has the effect of increasing dipolar relaxation in natural membranes. Using the principle that dipolar relaxations produce a rotation of the phasor plot as shown

in Fig. 2 E, we can generate two coordinates, one related to the movement along the polar trajectory and a second coordinate corresponding to the rotation of the phasor

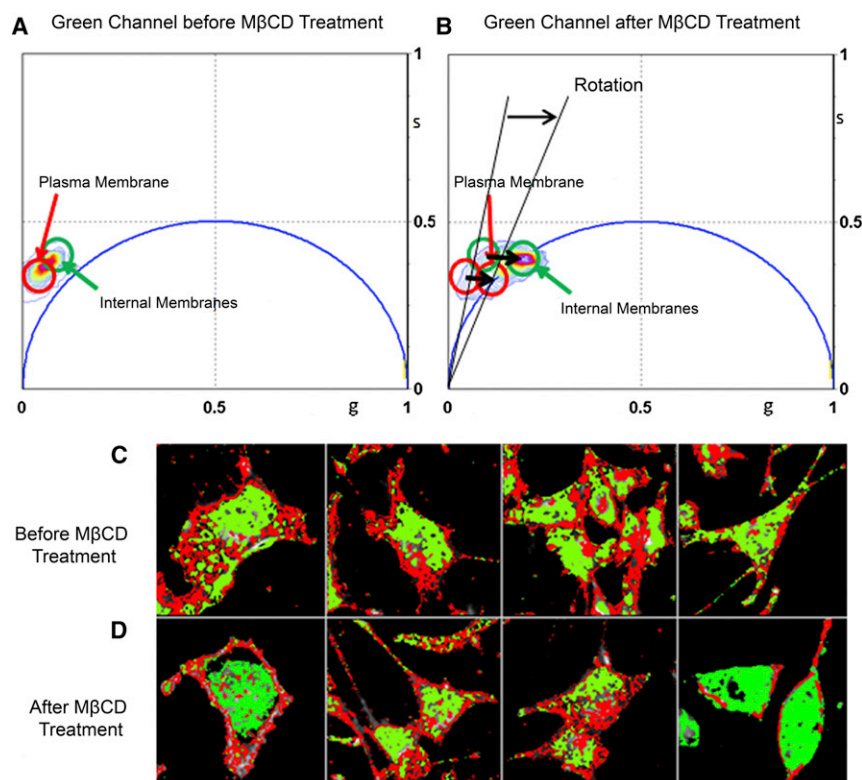


FIGURE 4 Influence of cholesterol on decay of Laurdan in the green wavelength range. Phasor distribution of Laurdan in NIH3T3 cells detected through a 540/40-nm filter (green filter) before (A) and after (B) treatment with 1 and 5 mM M- β -CD. Before treatment (A), the phasor distribution is completely outside of the universal circle, indicating that the emission decay is dominated by dipolar relaxations. After treatment with M- β -CD (B), the phasor distribution is moved inside the universal circle. (C) NIH3T3 cells pseudocolored according to the palette defined in A. (D) The NIH3T3 cells of C after treatment with M- β -CD pseudocolored according to the palette defined in B, where the two cursors in A are simply rotated to reflect the change in dipolar relaxation. After cholesterol removal, the two cursors select the plasma membrane (*red cursor*) and the internal membranes (*green cursor*).

plot, which is related to dipolar relaxations and correlates with cholesterol content. This correlation gives us the opportunity to separately estimate the amount of water (polarity) and cholesterol in the cell membrane. In Fig. 5, we show that the movement of the phasor toward the universal circle is gradual, as the cholesterol is depleted as a function of time after M- β -CD treatment. This observation opens up the possibility of producing a quantitative map of cholesterol concentration in the membrane. We also show that as we start cholesterol extraction by addition of M- β -CD, the cholesterol content is altered first in the plasma membrane, then in the internal membranes.

Changes in fluidity and cholesterol in response to EGF stimulation of cells

We performed experiments to determine whether the phasor distribution could detect biological changes in the membrane due to external stimulation. In particular, we monitored membrane lipid reorganization during cell migration in live NIH3T3 cells stained with Laurdan before and after epidermal growth factor (EGF) stimulation (Fig. 6). As can be seen from the intensity images in Fig. 6 A, the cell polarizes from the top left hand corner toward the bottom right hand corner upon stimulation. The leading edge and the retracting tail of the cell are indicated by arrows in Fig. 6. The

analysis of phasor distribution detected by the blue filter is shown in Fig. 6 B, and that detected by the green filter in Fig. 6, E and F. Using the blue filter, we color the pixels of the images with a palette that maps changes in membrane fluidity, as shown in the cursor selection in the phasor plot (Fig. 6 C). Pixels in the image characterized by low fluidity are red, and pixels characterized by high fluidity are green. The cell presents a decrease in fluidity (thus polarity) at both edges upon EGF stimulation. Using the blue filter, we cannot determine whether these changes occur independent of cholesterol content and/or cholesterol redistribution. For this purpose, we use the green channel. In Fig. 6 E, we select pixels with low cholesterol content by selecting the phasor distribution that is on (or inside) the universal circle. These pixels are found mostly at the retracting edge. Upon stimulation there is a large decrease in polarity in this part of the cell, since this part of the distribution becomes redder according to our color code. Then, as shown in Fig. 6 F, we select regions of high cholesterol content corresponding to pixels totally outside of the universal circle (Fig. 6 G). After stimulation, only the cytoplasmic membrane at the leading edge of the cell shows a definite decrease in polarity (it becomes redder according to our color code). We note that for this experiment, we started with cells that had been starved before stimulation. These starved cells present a different distribution of cholesterol than cells that have not

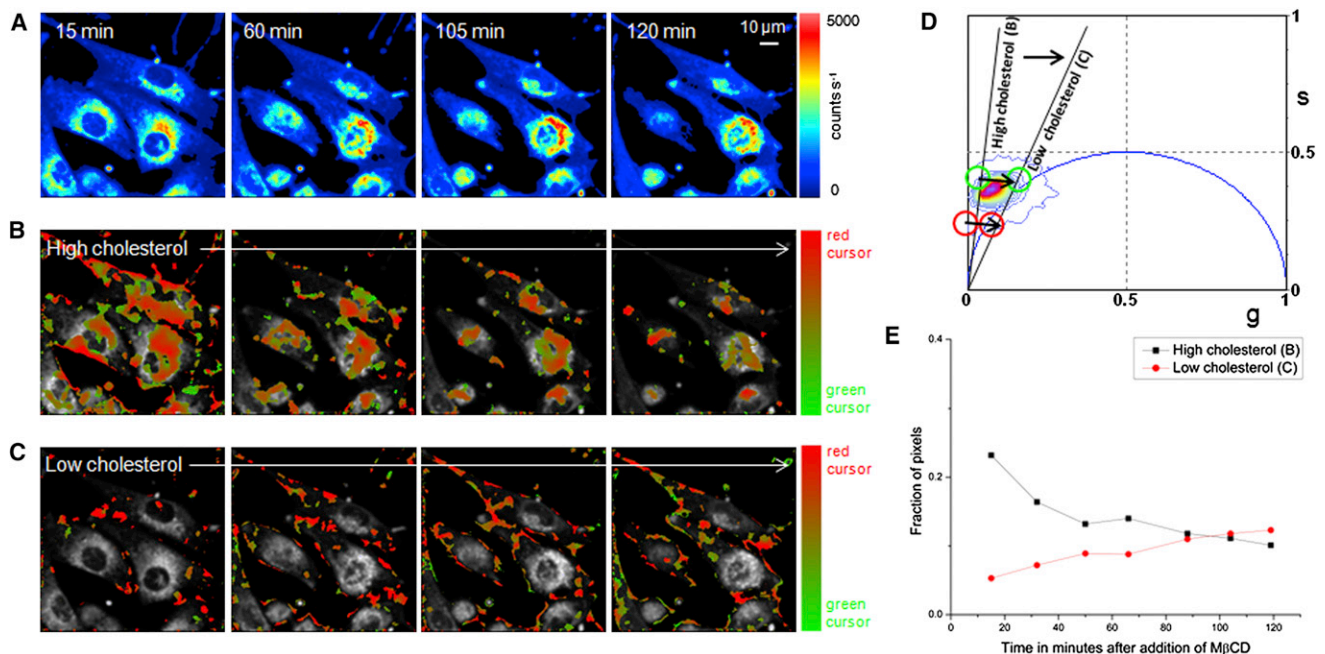


FIGURE 5 Gradual depletion of cholesterol from a single cell monitored by decay of Laurdan in the green wavelength range. (A) Time series of the fluorescence-intensity images of a group of NIH3T3 cells observed in the green channel (540/40 nm), upon treatment with 5 mM M- β -CD. (B and C) Lifetime images of the time series depicted in A, pseudocolored according to the two different palettes defined in D, which highlight the high-cholesterol environment (B) and the low-cholesterol environment (C). The difference between the two palettes is a small rotation in the placement of the red and green cursors, to reflect the change in dipolar relaxation. As can be seen with increasing time after addition of 5 mM M- β -CD, more pixels are highlighted in the low-cholesterol environment. (E) Quantification of this effect, where we plot the fractions of pixels in the high- and low-cholesterol environments as a function of time during M- β -CD treatment.

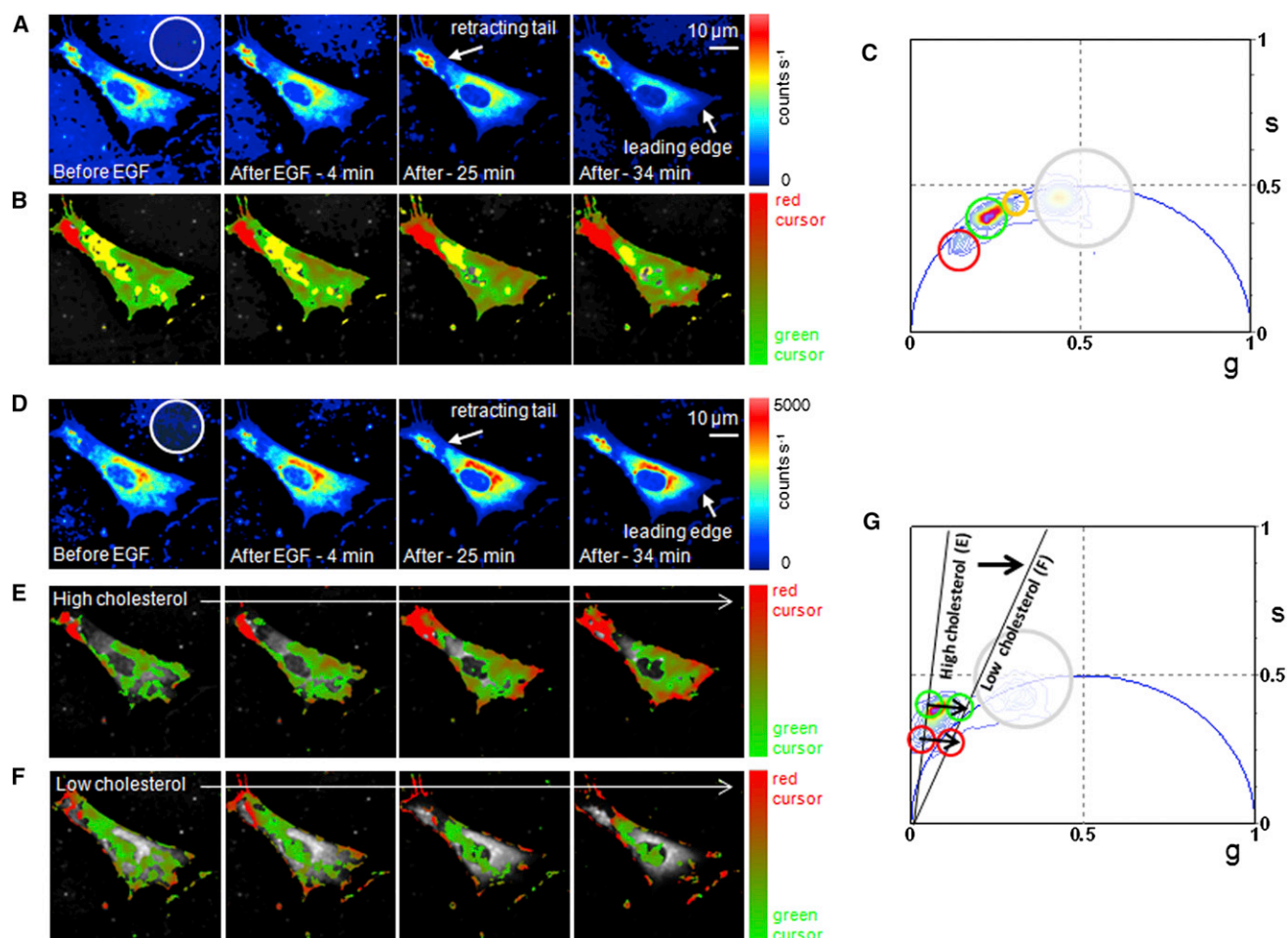


FIGURE 6 Monitoring fluidity changes in NIH3T3 cells after EGF stimulation by using Laurdan phasor distribution in the two detection wavelength ranges. (A) Intensity images collected in the blue channel before and after EGF stimulation; arrows point to the retracting tail and the leading edge of the cell, respectively. The fluorescence signal due to free Laurdan outside the cell (i.e., indicated by the white circle) is easily distinguishable and it can be ignored in our analysis. (B) FLIM images collected in the blue channel (460/80 nm): we highlight the fluidity/polarity changes using a color scale from red (rigid) to green (fluid). To obtain this color scale, in the phasor plot (C), the points lying on the line connecting the red and the green circle are used to color the corresponding pixels in FLIM images. (D) Intensity images collected in the green channel before and after EGF stimulation; arrows point to the retracting tail and the leading edge of the cell, respectively. (E–G) FLIM images collected in the green channel (540/50 nm). Pixels are first selected according to their position along the low- (E) or high-cholesterol content (F) and then colored according to a fluidity scale shown in the phasor plots (G). As the cell responds to stimulation, the retracting tail, which is characterized by low cholesterol content, becomes more rigid. The leading edge, which is characterized by higher cholesterol content, becomes more rigid.

been starved, as seen by the appearance of phasor clusters close to the universal circle before stimulation.

DISCUSSION

Here we present an analysis of fluorescence decay of Laurdan as a means to detect changes in membrane lipid packing in live cell membranes, based on polarity and dipolar relaxations. We show that the classical two-wavelength GP essentially discriminates two distributions of membrane lipid properties based on water penetration, which affects both polarity and dipolar relaxation of the membrane environment.

The phasor representation of the fluorescence decay has a simple graphical interpretation. The phasor plot is divided

into two regions by the universal circle. This semicircle that goes from the points (1,0) to (0,0) corresponds to all possible single exponential decays. Since a multiexponential decay is the sum of exponential decays, due to the rule of vector addition followed by the phasors, all pixels with a combination of multiple lifetimes must be inside of the universal circle. For a phasor to be outside of the universal circle, an additional process must be present, such as an additional delay of the emission. In the phasor plot representation, the delay caused by the excited-state reaction moves the phasor outside of the universal circle. If a phasor point is outside of the universal circle it can only come from a region of the membrane where we have dipolar relaxations. For Laurdan, this process is caused by dipolar relaxations that

involve a few molecules of water in the proximity of the Laurdan fluorescent moiety. We found that the presence of cholesterol brings the phasor distribution outside of the universal circle.

We first discuss the meaning of the phasor distribution shown in Fig. 3 in the blue channel. Each phasor plot in Fig. 3 is obtained as the sum of the phasor plots of four cells. In the blue channel, the phasor distribution is continuous and along a trajectory close to the universal circle. Selection of different parts of the distribution (low- and high-polarity parts represented by the red and green cursors, respectively, in Fig. 3 A) shows that both the plasma and internal membranes contribute to different parts of the phasor cluster (Fig. 3 C). This observation is still compatible with the theory that there are only two basic types of environments for the Laurdan probe. In the green channel, after cholesterol removal, the phasor distribution is broader and more complex (Fig. 4, B and D). This phasor distribution cannot be described by the linear combination in each pixel of only two environments. We need three or more environments. Our experiments using solutions of different polarity (with addition of cholesterol and water) show that there are at least two independent principles that determine the direction of changes of the phasor location. One is the polarity of the environment (which changes the true lifetime) and the other is the capability of water to give rise to the dipolar relaxation phenomenon (which moves the phasors outside the universal circle). Among our results was the remarkable finding that in the green channel, reducing the cholesterol content rotates the phasor plot toward the universal circle. This observation holds true in solutions as well as in cells. This behavior implies that in cells, cholesterol promotes dipolar relaxation. We cannot exclude the possibility that in natural membranes other factors can also affect dipolar relaxation and have the same effect as cholesterol, i.e., if this effect is specific to cholesterol. Whatever the reason, the phasor analysis in the green channel allows us to separate regions of the membrane with high or low dipolar relaxations. In this article, we ascribe the movement of the phasor toward the outside of the universal circle to the cholesterol content of the membrane. We note that an additional phasor cluster (Fig. 3 B, yellow cursor) becomes prominent after cholesterol removal. This cluster is only associated with internal membranes.

Cholesterol is an essential component in cell membranes, required for membrane lipid organization. It is recognized as playing an integral role in cell structure, function (e.g., by forming membrane domains), and regulation of cellular processes such as metabolism, compartmental homeostasis, and molecular interactions in extracellular and intracellular communication (33,34). Evidence of alterations in cholesterol distribution, transport, and function have been shown in neurodegenerative disease (33), tumor metastasis (4), transporters activity (34), and indeed formation and disruption of membrane domains. For all these reasons, a new tool

with which to investigate and visualize alterations in cholesterol content separately from general fluidity changes is highly advantageous.

We show here that phasor analysis is able to decouple changes in water content from changes due to cholesterol content in the membranes of live cells, whereas GP analysis cannot. By detecting Laurdan emission in the blue channel, we can identify the changes in polarity due to membrane composition; then, in the green channel, we can distinguish dipolar relaxation from polarity. In other words, we can draw a map of fluidity and cholesterol values based on Laurdan emission decay in cell membranes (Fig. 6), and we can read this map to assess changes in fluidity and cholesterol content during cellular processes of interest. We use this fluidity-cholesterol map to monitor membrane-packing changes during cell migration (Fig. 6). By using FLIM analysis in the green channel (Fig. 6, C and D), we found that when the cell is stimulated with EGF, the leading edge and the retracting tail undergo a polarity decrease (the lifetime phasor shifts toward the red region). The GP analysis cannot recognize similarities in polarity, because the emission spectrum of Laurdan measured at only two wavelengths cannot discriminate regions of high cholesterol content from regions of low polarity. FLIM-phasor analysis gives us information similar to that offered by GP analysis but with the additional ability to resolve intermediate membrane conditions that cannot be resolved by intensity-based detection.

Finally, the ability to discriminate changes occurring in the cell membranes while the cell is polarizing or migrating can give us further insight into the roles of membrane fluidity, cholesterol, and membrane-domain formation. In Fig. 6 D, we can observe a decrease in membrane fluidity in the moving front of the cell in a high-cholesterol environment. It has been shown that EGF-receptor activation can induce the formation of signaling domains by regulating the production of critical second-messenger lipids. This results in modification of the membrane lipid environment (35). Using the phasor approach to Laurdan decay we can directly and noninvasively monitor in vivo the local remodeling of the lipid bilayers (Fig. 6, C and D). This new approach allows us to visualize reorganization in membrane lipid packing of the cell after EGF stimulation. The cell response can be described by two distinct polarization processes in membrane fluidity. The first polarization is observed in the direction of cell migration (Fig. 6 D). In this high cholesterol environment we monitor a gradual increase in the rigidity of the leading front (represented in the color map by the gradual change from green to red). The second polarization process is observed in the opposite direction (Fig. 6 C): in this low-cholesterol environment, there is an almost symmetrical increase in the rigidity of the retracting tail. In conclusion, the membrane probe Laurdan used in conjunction with the phasor approach to FLIM can detect and distinguish membrane fluidity from

cholesterol-content changes. Laurdan can be used as a biosensor for cell membranes, since the phasor method allows us to observe the membrane lipid remodeling and reorganization that follows activation of EGF receptors in the cell membrane.

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