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The Laurdan Spectral Phasor Method to Explore Membrane Micro-heterogeneity and Lipid Domains in Live Cells

Ottavia Golfetto, Elizabeth Hinde, and Enrico Gratton

Abstract

In this method paper we describe the spectral phasor analysis applied to Laurdan emission for the assessment of the fluidity of different membranes in live cells. We first introduce the general context and then we show how to obtain the spectral phasor from data acquired using a commercial microscope.

Key words Laurdan, Spectral phasor, Membrane fluidity, Lipid domains

1 Introduction

Lateral organization and lipid packing of cell membranes, both plasma and intracellular, allows for the regulation of numerous cellular processes essential to cell life and death. Membrane heterogeneity, which arises from variation in lipid composition, lipid order and diversified lipid-protein interaction, is essential for the correct functioning of biological membranes. It has been shown that lateral membrane heterogeneity influences and regulates cellular polarization, signaling events, transduction pathways, trafficking, and secretion of molecules [1–3]. Within this concept, two related aspects of membrane heterogeneity are particularly relevant: membrane fluidity (or order) and membrane domains.

Membrane fluidity plays a key role in the formation of membrane domains. A more rigid membrane environment influences the lateral diffusion and concentration of membrane proteins [4], which in turn regulates interactions among proteins and therefore the efficiency of signaling pathways through the formation of membrane domains. Some examples are viral entry/budding [5], cell polarization and adhesion [6], membrane trafficking [7, 8], and B cell signaling [9]. The membrane lipid composition defines the degree of lipid packing, the thickness of the bilayer, and the rotational freedom that the lipids have in the bilayer.

All these parameters are included in the general concept of membrane fluidity or order. In order to evaluate membrane fluidity a key role is played by water molecules in the membrane. The degree of lipid packing can be quantified because more ordered packing excludes polar water molecules from the nonpolar lipid bilayer. This results in a change in the local environment polarity, which can be sensed by polarity environment-sensitive fluorescent probes, such as Laurdan.

Membrane domains are (micro) regions of the membrane, which are enriched in cholesterol and sphingolipids, such as gangliosides. According to their composition their intrinsic fluidity is different from the rest of the membrane. In response to signaling, membrane domains may fuse into larger and more stable structures, which could become efficient signaling platforms. In other words, they are thought to modulate cellular signaling and to initiate cellular processes before/after sequestering specific proteins from the rest of the membrane. A large effort has been spent to investigate the existence and nature of membrane domains or rafts, via several single-molecule high resolution techniques such as [10–14]. These techniques support the existence of the postulated domains and the importance of cholesterol in their composition and existence. In these studies the use of probes that bind to various membrane constituents is very common. One of the most widely used examples is Cholera Toxin subunit B (CT-B), from the bacterium *Vibrio cholerae*. This protein binds to the ganglioside GM1, which, similarly to other glycosphingolipids, has been found to be concentrated in ordered and cholesterol-enriched membrane domains.

As mentioned previously, fluorescent probes are extensively used to study membrane heterogeneity. In order to address questions oriented toward measuring subtle changes of either membrane fluidity and/or order in membrane domains, two different classes of fluorescent probes can be distinguished: environment sensitive and binding probes. The first class of probes (environment sensitive) is particularly suited to explore membrane fluidity and order. These probes have equal and high affinity for every region of the membrane. Some examples of these fluorescent membrane markers are Laurdan (with its derivatives) [15–21], di-4-ANEPPDHQ [22, 23], and other solvatochrome derivatives [24–26]. In this work we focus on the Laurdan probe and its capability to sense the polarity of its environment [27]. In the case of lipid bilayers, Laurdan senses the presence of water in the membrane, thus providing information about water penetration, which is directly related to membrane fluidity. When originally used in model membranes, it was shown that Laurdan distinguishes lipid domains in model systems according to at least two separate classes: gel (alias solid-ordered) and fluid (alias liquid-disordered) phases [28]. To quantify the contribution of these two phases in membranes,

a normalized ratiometric method of analysis based on a two-channel detection was developed the Generalized Polarization (GP) function [29].

The second class of probe (binding) includes probes that have affinity for membrane ordered domains, and therefore are considered to stain selectively only the liquid-ordered phase in membranes. Some examples are probes that bind to most glycosphosphatidylinositol-anchored (GPI-anchored) proteins as well as palmitoylated proteins. As we mentioned before, the fluorescently labeled Cholera Toxin subunit B (CT-B), which binds to the ganglioside GM1, belongs to this family of probes [30–32]. It is usually assumed that CT-B binds to specific membrane domains but there are some issues. First, the ganglioside GM1 is not expressed by all cells, therefore in some cases CT-B is not useful as a membrane domain probe. Moreover, a significant fraction of GM1 is found outside ordered regions, at least when judged by detergent resistance [33, 34], and the toxin may also bind to other molecules such as glycosylated proteins [35]. Therefore, binding of CT-B alone should not be taken as evidence of a microdomain, but instead an indication, in the instance it is confirmed by additional approaches.

In this work we aim to explore membrane lateral heterogeneity using a new detection scheme based on a dual fluorescent labeling and novel approach to spectral analysis. More precisely, we combine the fluorescent labels Laurdan and CT-B, with the aim to detect the fluidity of the regions which CT-B binds. The reasoning behind this is that, despite CT-B being useful for imaging ordered domains due to its binding properties, it is not able to provide information about the actual micro-environment of these domains and is not very specific in live cells. Thus since Laurdan labels all the membranes in a cell and is sensitive to the domain micro-environment, a dual labeling could determine the specific fluidity of the regions where CT-B binds. Then for image analysis of the combined Laurdan and CT-B signal, we propose a novel detection scheme which exploits the additive property of the phasor to identify the specific “local fluidity” of the membrane regions where CT-B binds.

Laurdan fluorescence has been used to study lateral membrane heterogeneity both in model systems and in live cells due to the dependence of the Laurdan emission on the packing and water content of the membrane. This spectral sensitivity originates from the dipolar relaxation effect in which few water molecules in the proximity of the Laurdan molecules reorient after excitation to lower the energy of the excited state in times comparable to the Laurdan fluorescence lifetime producing a gradual shift of the spectrum. Traditionally, this shift is captured by the Generalized Polarization function or GP which is essentially a normalized ratio-metric measurement obtained by measuring the emission at two

wavelengths. Since the shift is gradual, the measurement at two wavelengths only reflects the projection of the spectral shift on the wavelength axis as captured at these two wavelengths. Therefore a limitation of the GP function is that all the spectra that give equal projections at these two wavelengths appear indistinguishable. This limitation is particular severe for the analysis of the GP of microscopy images of live cells where many different membranes are presents, each with different lipid composition and packing.

Here we describe the Laurdan spectral phasor approach, a detection scheme and image analysis of Laurdan emission in model systems and in live cells, that has the potential to capture in greater detail the process of dipolar relaxation as it occurs in time and at different pixels of microscope images. Since the possible spectral combinations in the pixels of an image arise from a continuum of Laurdan environments, it is difficult to establish a priori the number and shape of the different spectra in a given image. In this respect, the spectral phasor analysis offers two important advantages. First, due to the linear properties of the phasor transformation it could identify spectral species occurring in each and every pixel of an image. Second, using higher harmonics of the phasor transformation it could identify subtle changes in spectral width and spectrally overlapping components.

In this work, we first present the phasor analysis in the case of two well-established lipid phases, gel and fluid (liquid-disordered) in model membrane systems. Then, we perform a comprehensive analysis of Laurdan spectral phasor in NIH3T3 and HEK293 live cells. We found that the spectral phasor approach has the potential to identify different membranes in a live cell on the basis of the Laurdan spectral emission. We use these spectral signatures and their linear combinations to investigate micro-heterogeneities in live cells' membrane. Treatment with cholesterol reducing agents shows that we can quantify the abundance of regions with a given spectral signature. Finally, by coupling two different fluorescent membrane probes (Laurdan and fluorescently labeled Cholera Toxin subunit B) we are able to characterize the fluidity of the membrane around different GM1-enriched domains identified by Cholera Toxin binding.

Laurdan [27] is a fluorescent dye used to measure dipolar relaxation effects on a membrane, which are related to the extent of water penetration in the bilayer. Laurdan is therefore capable of determining the degree of membrane fluidity. When lipids are highly packed, it has an emission maximum at 440 nm. When lipids are in a disordered fluid phase its emission is centered at 490 nm. The GP function uses two emission channels centered at 440 nm and 490 nm, respectively, to determine membrane water content. In this work we use instead 32 separate channels over the entire emission wavelength range (416–728 nm). This results in a much higher spectral sensitivity, which gives us the chance to detect

subtle and smaller changes in membrane lateral packing when compared with the GP function.

As a means of enabling a simple and fit free analysis of spectral imaging data, in this work we applied the phasor transformation to Laurdan spectral images. In the case of Laurdan studies this feature of the phasor approach is extremely relevant. In order to fit the Laurdan fluorescence response many assumptions are needed and they can easily lead to artifacts and wrong interpretations. The phasor approach has been proved to be very valuable for frequency and time domain lifetime imaging data [36–39]. The phasor approach provides a global analysis of entire images and does not require a priori knowledge of the number or spectral shape of the components in the sample. Fereidouni et al. recently applied the phasor approach to spectral imaging data as a simple graphical method for spectral un-mixing [40]. Here we use the spectral phasor approach to distinguish Laurdan interactions with different environments in a pixel. We use spectral images acquired at 416–728 nm wavelength range with 32 separate channels (bandwidth of 9.7 nm). Each pixel in the spectral image contains the emission spectrum at that pixel. Therefore, an emission curve is associated with every pixel. The spectral phasor transformation calculates the Fourier sine and cosine transforms of a given spectrum. For each Fourier harmonic, two coordinates are calculated: g corresponds to the cosine transform (x coordinate in a polar plot) and s corresponds to the sine transform (y coordinate in a polar plot). We use the following equations:

$$g = \frac{\sum_{\lambda} I(\lambda) \cos(2\pi n \lambda / L)}{\sum_{\lambda} I(\lambda)} \quad (1)$$

$$s = \frac{\sum_{\lambda} I(\lambda) \sin(2\pi n \lambda / L)}{\sum_{\lambda} I(\lambda)} \quad (2)$$

A point at coordinates (g, s) is called a phasor and is represented in a polar plot. L in Eqs. 1 and 2 is the total wavelength range, $I(\lambda)$ represents the intensity at a given wavelength and given pixel, and n is the harmonic order.

Figure 1 illustrates the basic principles of the spectral phasor transformation. Gaussian spectra (Fig. 1a) are transformed into a spectral phasor (Fig. 1b). The angular position in the polar plot (the phasor angle) is proportional to the spectral center of mass. The distance from the origin (the phasor radius) is inversely proportional to the spectral width. Figure 1c, d show reference phasors obtained varying spectral center of mass and width of Gaussian spectra, using the first harmonic ($2\pi\lambda/L$) and the second harmonic ($4\pi\lambda/L$) in Eq. 1. Basically, it's easier to interpret phasor positions and phasor visual properties using the first harmonic when the spectral response extends over the entire wavelength range (416–728 nm) and the spectral components have emission wavelengths

Fig. 1 Basic principles of the spectral phasor approach. Gaussian spectra in (a) are transformed into phasors and represented in (b). Changes in the spectral center of mass are translated into different phasor angle (from blue spectrum/phasor to turquoise spectrum/phasor). Changes in the spectral bandwidth correspond to different phasor radius (red, turquoise and pink spectra/phasors). (c) Spectral phasor simulation using the first harmonic: blue pixels in the plot corresponds to Gaussian spectra with varying center of mass and bandwidth. (d) Analogous phasor simulation using the second harmonic

distant from each other. However, phasors can be deformed at the shortest wavelengths (Fig. 1a) due to the lack of points in the initial part of the spectrum. The second harmonic allows resolving spectral species that have close emission wavelengths, and it is less affected by distortion at the shortest wavelengths. However when spectral components are distant along the wavelength range, it can be more complex to visually interpret results due to the wrap around effect in the polar representation of Fig. 1. In other words,

different harmonics of the Fourier transform provide additional polar plots that reveal finer details of the spectral response. This capability is particularly advantageous when considering that each harmonic can have an optimal sensitivity for different spectral components. In this way we can specifically tune our analysis to discriminate spectrally separated as well as spectrally overlapping components using a different harmonic transform/analysis.

We performed a series of simulations in order to understand the level of sensitivity we can reach using the spectral phasor approach. Essentially, by coupling a low resolution instrument for spectral image detection (only 32 channels, therefore a 32-points-spectrum for each pixel in the image) with the spectral phasor image analysis, we obtain discrimination in the spectral domain due to subtle changes in the spectral position and width. Figure 2 shows Gaussian spectra detected using 32-channels acquisition and the corresponding calculated phasors. In Fig. 2a, ten spectra with increasing spectral width are represented. We are able to identify and correctly transform spectra with up to 2 nm difference in the bandwidth. This can be done using both first and second harmonic, and the results are represented in Fig. 2b, c. In Fig. 2d, ten spectra with a 2 nm shift in the central emission wavelength are represented. The spectral phasor transformation allows us to discriminate the spectra and to correctly transform each pixel (alias each spectrum) into a phasor without distortions. This can be done both by using first harmonic and second harmonic Fourier transform, as represented in Fig. 2e, f. In Fig. 2g very narrow spectra were reconstructed using 32 channels/points; The corresponding phasor plots for the first and the second harmonic analysis are shown in Fig. 2h, i.

In this work, we used the second harmonic when we analyze Laurdan labeled cells to better resolve the small spectral shifts induced by differences in the membrane micro-environment. Conversely, we used the first harmonic when we analyze cells labeled with both Laurdan and CT-B-Alexa 594 because the emission spectra of the two probes are distant and using the first harmonic we can better visualize a wider spectral range.

1.1 Laurdan Spectral Phasor Analysis in Solutions

Preparation of Giant Unilamellar Vesicles was performed using the electroformation method, originally developed by Angelova et al. [41]. Phospholipids were diluted with chloroform to a final concentration of 0.2 mg/ml. Two platinum wires attached to a Teflon chamber were coated with 2 μ l of the lipid mixture and dried under N_2 (g). The water jacketed chamber was sealed with a No. 1.5 cover slip and it was attached to a circulating water bath. Phospholipids were rehydrated with 1 mM Tris-HCl pH 7.4. The platinum wires were attached to a frequency generator with alternating current set to 10 Hz and 2 V. A thermocouple was used to monitor the temperature of the chamber. GUVs were grown for 30 min and then imaged.

Fig. 2 Phasor simulations to test spectral sensitivity. (a) Spectra with increasing bandwidth are reconstructed using 32 channels/points from 416 nm to 728 nm; (b, c) corresponding spectral phasor plot for the first harmonic (b) and second harmonic (c). Each spectrum is transformed into a pixel in the phasor plot, it's possible to resolve and discriminate spectra which are 2 nm apart. (d) Spectra with shifted spectral center of mass were reconstructed using 32 channels/points; (e, f) spectral phasor plot for the first harmonic (e) and second harmonic (f). Each spectrum has been transformed into a phasor. Using the phasor transformation, it's possible to obtain a spectral resolution of 2 nm. (g) Narrow spectra were reconstructed using 32 channels/points; (h, i) corresponding phasor plot for the first and the second harmonic analysis. Considering 32 channels/points, the phasor transformation is capable of correctly calculate the phasor position using only three points of the spectrum

For cell culture NIH3T3 and HEK293 cells were grown at 37 °C and in 5 % CO₂ in Dulbecco modified Eagle medium (DMEM) from Invitrogen supplemented with 10 % of 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 probe Laurdan (6-dodecanoyl-2-dimethylamino naphthalene) was purchased from Invitrogen, it was dissolved in DMSO (dimethyl sulfoxide), 1.8 mM stock solution

was prepared and added to the cell dishes at a dilution 1:1,000. Cells were incubated with Laurdan for 40 min at 37 °C before imaging.

For the chronic cholesterol depletion experiments, cells were incubated with lipoprotein-deficient serum (DMEM plus 5 % LPDS) for 2 days to deplete cholesterol. For the acute cholesterol depletion experiments, a 50 mM stock solution of methyl- β -cyclodextrin (Sigma-Aldrich) was prepared by dissolving in nano-pure water. The solution was added to the cell dishes with a final concentration of 2 mM and they were incubated for 1 h at 37 °C. Cells were then rinsed with PBS and new medium was added.

Spectral data were acquired with a Zeiss LSM710 META Laser scanning microscope, coupled to a 2-Photon Ti:Sapphire laser (Spectra-Physics Mai Tai, Newport Beach) producing 80 fs pulses at a repetition of 80 MHz. A 40 $\times$ water immersion objective 1.2 N.A. (Zeiss) was used for all experiments. The Laurdan fluorophore was excited at 780 nm. Excitation at 780 nm induced negligible autofluorescence since 2-photon excitation of intracellular metabolites is centered at 740 nm [42]. For Laurdan and CTB-A594 co-localization measurements the excitation was set at 800 nm. Spectral images were acquired in 32 channels, each with 9.7 nm bandwidth, the first channel is centered at 421 nm and the last one at 723 nm. For image acquisition, the pixel frame size was set to 256 $\times$ 256 and the pixel dwell time was 177 μ s/pixel. The average laser power at the sample was maintained at the mW level. Spectral data were processed by the SimFCS software developed at the Laboratory for Fluorescence Dynamics (www.lfd.uci.edu).

First, we performed measurements in different solvents to visualize Laurdan spectral phasor in solutions (Fig. 3a). We measured the Laurdan's spectral phasor in dimethyl-sulfoxide (DMSO), methanol (MeOH), glycerol, and glycerol with 10 % water. For the latter two samples, the shift caused by the change in polarity due to the addition of water is evident in the phasor plot. The images were taken using the Zeiss LSM710 microscope and 780 nm two-photon excitation is used in order to reduce photo-bleaching issues. The emission spectrum is detected from 416 nm to 728 nm in 32 wavelength channels, each having a 9.6 nm bandwidth. In Fig. 3a normalized fluorescence emission spectra of Laurdan in solution are shown. The emission spectrum at every pixel is Fourier transformed, the real and imaginary components of the Fourier transform are used to calculate the x and y coordinates in the spectral phasor plot, according to Eqs. 1 and 2. Here we use the second harmonic analysis, the corresponding phasor distributions are represented in Fig. 3b. The first major advantage of spectral phasor analysis is that for every pixel of the image we obtain a value identified by the two coordinate g and s , while conventional GP function calculates a single value. This means that instead of representing every pixel onto a graph with one dimension, we represent every pixel into a

Fig. 3 Laurdan spectral phasors in solutions and GUVs. **(a)** Normalized fluorescence spectra of Laurdan in different solvents. **(b)** Spectral phasors of Laurdan in different solvents using the second harmonic analysis. All the phasor distributions are very well resolved in the phasor plot. **(c)** Spectral images of DPPC, DMPC (at different temperatures) and DLPC single lipid GUVs. The diameter of the GUVs varies from 20 to 50 μm . **(d)** Spectral phasor analysis using the second harmonic, the phasor distributions of all images are represented in the same phasor plot. **(e)** The images in **(c)** were pseudo-colored using the color map represented in the phasor plot. Each pixel in the spectral images has been colored depending on the phasor position, the color cursors that have been used are represented in **(d)**

phasor plot in two dimensions. Indeed a two-dimensional space is more informative than a one-dimensional scale which results in an improved resolution and a better capability of discrimination.

Next, we show Laurdan spectral phasor analysis in single lipid Giant Unilamellar Vesicles (GUVs) (Fig. 3c–e). To prepare GUVs we used the electroformation method, originally designed by Angelova et al. [16, 41, 43, 44]. We used phospholipids with different saturation (different rigidity) and at different temperatures. We imaged GUVs of dipalmitoyl phosphatidyl choline (DPPC) at 20°, GUVs of dimyristoyl phosphatidyl choline (DMPC) in a temperature range varying from 14.7° to 30.0°, and GUVs made of dilauroyl phosphatidyl choline (DLPC) at 20°. Figure 3c shows spectral images taken at the equatorial center of GUVs. For these measurements we used *linear* polarized excitation light so that in the equatorial region, only those molecules with an absorption transition moment parallel or nearly parallel to the plane of excitation

light polarization are excited, and the efficiency is proportional to the cosine squared of the angle between the transition moment of the probe and the polarization plane of the excitation light [45]. The diameter of GUVs varies from 20 to 50 μm . In Fig. 3d the spectral phasor analysis using the second harmonic is shown, the phasor distributions of all the images are shown in the same polar plot. Using the second harmonic analysis, phasors of GUVs made of single lipids are distributed along two positions, corresponding to two Laurdan environments such as gel and liquid phases. GUVs made with different lipid composition and at different temperatures have different phasor positions. If we know the phasor position of these differently packed membrane environments, using the linear combination properties of phasors, we can determine the fractional contribution of liquid versus gel like phase in a given membrane. In Fig. 3d clusters of points in the phasor plot are selected by cursors of different colors. The points in the image that correspond to each specific cluster are colored with the same color of the cursor (Fig. 3e). Red cursor/color identifies the shortest wavelengths of emission, corresponding to a highly packed phospholipids phase (gel like). Pink cursor/color identifies the longest wavelengths, corresponding to a disordered lipid phase (liquid).

1.2 Laurdan Spectral Phasor in Live Cells

After determining Laurdan's response using spectral phasor image analysis in model systems, we performed Laurdan spectral imaging in live cells. In Fig. 4, we show images obtained at different z -values of a NIH3T3 and HEK293 cells, respectively. Live cells were labeled with Laurdan; spectral images were obtained at 1.2 μm and 1.4 μm distance, respectively, along the z -axis (Fig. 4a, d). First, for both cell types phasor plots show that the phasors fall on a different line with respect to the GUV's line and the total range of the phasor cluster is reduced in cells. This confirms that cellular membrane heterogeneity cannot be explained solely as a combination of gel and liquid phase. Moreover, the fluidity values obtained within a live cell membrane are not as extreme and separate as in model membranes. Using the four color cursors represented in the phasor plot (Fig. 4c, f) we can distinguish at least four groups of membranes, which are characterized by increasing fluidity values (or water content). When we color the pixels in the images according to their location in the phasor plot, we can identify small regions of relatively low fluidity along the plasma membrane (red pixels), plasma membrane (green pixels), and intracellular membranes (blue and pink pixels), including the nuclear envelope and other internal membranes, which could be associated to the Golgi, ER, and small organelles such as endosomes (Fig. 4b, e). Interestingly, the plasma membranes of both NIH3T3 and HEK293 cells display regions of different fluidity according to the Laurdan spectral phasor analysis. We cannot identify these small regions with membrane micro domains at the present stage of this research, since we

Fig. 4 Laurdan spectral phasor analysis in live NIH3T3 and HEK293 cells. **(a)** Spectral images of NIH3T3 live cells in different z-planes. The planes are 1.2 μm apart. **(b)** Phasor images: pixels of the images are colored using the color cursors for the phasor distribution in **(c)**; *red* pixels are characterized by high rigidity, they are the pixels in which Laurdan emits at very short wavelengths (non polar environment). **(c)** Spectral phasor distribution of the images in **(a)** using the second harmonic: we used four cursors (*red, green, blue* and *pink*) to map cell membranes in order of increasing fluidity (therefore decreasing rigidity). **(d)** Spectral images of HEK293 live cells in different z-planes. The planes are 1.4 μm apart. **(e)** Phasor images: pixels of the images are colored using the phasor distribution in **(f)**; *red* pixels are characterized by high rigidity according to the spectral phasor analysis. **(f)** Spectral phasor distribution of the images in **(d)** using the second harmonic: we used four cursors (*red, green, blue* and *pink*) to map cell membranes in order of increasing fluidity

cannot distinguish between membrane folds, small vesicles attached to the plasma membrane or proximity of membranes with different phasor values.

Since cholesterol plays a major role in influencing membrane fluidity/order and it is a major component of membrane domains, we monitored membrane fluidity changes during cholesterol removal using the Laurdan spectral phasor analysis. We induce chronic cholesterol depletion by cultivating the cells with lipoprotein-deficient serum (LPDS) for 44 h. During this time the cell should be able to partially compensate cholesterol depletion. We also induced acute cholesterol depletion by treating the cells with methyl- β -cyclodextrin (M β CD) for 1 h. Results of these two treatments are shown in Fig. 5. Intensity images of the control, and cells treated with LPDS and M β CD, respectively, are shown in Fig. 5a. A low intensity and high intensity threshold were applied

Fig. 5 Laurdan spectral phasor analysis show fluidity changes in cell membranes' fluidity upon chronic (LPDS) and acute (MβCD) cholesterol depletion. **(a)** Intensity images for control, LPDS, MβCD: pixels with very high or very low intensity were threshold, scale and thresholds are represented on the right. **(b)** Phasor images for control, LPDS, MβCD: pixels of each image are colored using the corresponding phasor distribution in **(c)**; *red* pixels are characterized by high rigidity according to the spectral phasor analysis. **(c)** Spectral phasor distribution of the images in **(a)** using the second harmonic: we used four cursors (*red*, *green*, *blue* and *pink*) to map cell membranes in order of increasing fluidity. **(d)** Scatter plot representing the fractional contribution of red pixels (pixels characterized by a very rigid environment) in control images ($N=32$), LPDS images ($N=28$) and MβCD images ($N=24$). For each image, we plot the percentage of pixels for which their phasor is located in the area represented by the red cursor in the phasor plot

to the images in order to highlight changes in the plasma membrane. Figure 5b shows pseudo-colored intensity images obtained using the second harmonic analysis and the cursor location in the phasor plots shown in Fig. 5c. The color cursors' positions are the same for all the three images. Examining the phasor plots in Fig. 5c, we observe that the control (before cholesterol depletion) shows two defined large phasor clusters, which can be associated to pixels belonging to plasma (red and green cursors) and intracellular membranes (blue and pink cursors). Upon cholesterol removal, the number of pixels in the red cursor (very rigid domains) decreases. This decrease is prominent in the plasma membranes while the internal membranes are less affected by the treatment. Also, we are able to monitor the difference between the two treatments. Particularly, the number of red pixels (representing the most rigid regions in the plasma membrane) decreases differently for the two treatments, as can be seen in Fig. 5d. We used $N=32$ cells for the control, 28 cells for LPDS treatment and 24 cells for MβCD treatment. For each image, we plot the percentage of pixels having phasors highlighted by the red cluster. The control shows large variations among cells of the number pixels corresponding to

the red cursor (rigid domains); the fractional contribution of red pixels has values between 0.12 and 0.01 (12–1 %). The LPDS population presents a fractional contribution of red pixels between 0.04 and 0 (4–0 %), while the M β CD population does not present heterogeneity anymore and the fractional contribution of red pixels is very close to 0 % for all cells. While for the control population the two groups of phasor (plasma and intracellular membranes) are quite separated and distant in the phasor plot, for LPDS treated cells it is difficult to separate the two groups (the phasors appear all along a line), and for M β CD treated cells the two phasor groups appear even closer.

1.3 Cholera-Toxin Co-labeling Experiments

To further illustrate the capability of the spectral phasor approach we co-labeled live cells with Laurdan and a putative marker of membrane domains, the Cholera Toxin subunit B (CT-B), fluorescently labeled with Alexa Fluor 594 which binds to ganglioside GM1. In performing these measurements we use a specific property of the phasor transformation, which is related to the linear combination of properties in the same pixel [38, 39]. Specifically, if in a pixel we have the linear combination of two fluorophores, the phasor distribution is found along a straight line joining the phasor distributions of the two independent fluorophores. The schematic of this concept is represented in Fig. 6b; as we mentioned in the introduction we use the first harmonic for this analysis. For Laurdan and CT-B-A594 this means we can visualize pixels having fluorescence emission due to a rigid Laurdan environment and the presence of CT-B-A594 in these pixels because they must lie along the line connecting the two separate phasor distributions (red line). Similarly, we can identify pixels characterized by the contributions of a fluid Laurdan environment and CT-B-A594 along the blue line. Spectral images are shown in Fig. 6a. Spectral phasor analysis is shown in Fig. 6c–h. Pixels of the spectral image have been colored according to cursors in the phasor plot. Yellow colored pixels correspond to pixels where CT-B-A594 is dominant; blue colored pixels show the co-localization of CT-B and Laurdan in fluid environment; red colored pixels show the co-localization of CT-B and Laurdan in rigid environment. In Fig. 6d, f, h we show spectral phasor distributions of the images in Fig. 6c, e, g, respectively. Using the phasor approach we can directly visualize the pixels where Laurdan and CTB-A594 are both emitting. Using the trajectory that represents the co-localization between CTB-A594 and the most rigid part of Laurdan emission, we can identify regions of the plasma membrane which are rigid and enriched in GM1 (red pixels).

Laurdan spectral phasor analysis allows us to investigate the heterogeneity of membrane fluidity in live cells. By coloring pixels according to the clusters in the phasor plots using four cursors to select these clusters, we identify four groups of membranes in the

Fig. 6 Spectral phasor analysis of live NIH3T3 cells labeled with Laurdan and CT-B-Alexa 594. **(a)** Spectral image of NIH3T3 cells co-labeled with Laurdan and CT-B-A594; zoomed-in image on the right. Colors in the image correspond to the dominant emission wavelength. **(b)** Schematic representation of where the spectral phasor locates depending on the contribution of Laurdan and CT-B-A594. The phasor plot was calculated using the first harmonic analysis. Phasor distributions of Laurdan and CT-B-A594 alone are represented. Also, we represent where the pixel phasor is expected to be located when the emission spectra of Laurdan and CT-B-A594 are both present in the same pixel. The phasor position also changes depending on the Laurdan environment (fluid versus rigid). **(c, e, g)** Phasor image (zoomed-in on the right): pixels are colored according to the color cursors in the phasor plot in **(d, f, h)** respectively. *Yellow* colored pixels correspond to pixels where CT-B-A594 is dominant; *blue* colored pixels show the co-localization of CT-B and Laurdan in fluid environment; *red* colored pixels show the co-localization of CT-B and Laurdan in rigid environment. **(d, f, h)** Spectral phasor distribution of images in **(c, e, g)** respectively using the first harmonic analysis

imaged cells (Fig. 4). We can use fractional contribution analysis of the pixels in the image to quantify the contribution and the percentage of each group over the rest of cellular membranes. In this work we applied the fractional contribution analysis to quantify the percentage of pixels of the plasma membrane characterized by a rigid environment of the Laurdan probe (Fig. 5) when treated with different agents. We pseudo-colored these pixels in red and we monitored changes in their number after chronic and acute cholesterol depletion, respectively LPDS and M β CD treatments. Control cells are characterized by a number of rigid regions along the plasma membrane (Fig. 5d), while this number is reduced in cholesterol depleted cells. Different control cells show a large heterogeneity of the relative number of pixels corresponding to the red cursor. LPDS treated cells still maintain some heterogeneities,

while rigid regions are basically absent after M β CD treatment. We anticipate that the level of detail revealed by the spectral phasor method will prove invaluable to measure cell membrane heterogeneity and for monitoring membrane compositional changes, upon drug treatment or pathogenesis.

Considering the intrinsic biological complexity of cell membranes, the spectral phasor analysis of Laurdan significantly improves the sensitivity to different spectral components with respect to GP analysis. The Laurdan spectral phasor is able to discriminate subtle changes in membrane fluidity, which shows significant and consistent differences between plasma and intracellular membranes. In particular, using higher harmonics, the phasor analysis can be tuned to obtain different degrees of spectral separation. In addition, it can easily identify the presence of different environments (such as for Laurdan itself) or different fluorophores (Laurdan and CTB-A-594) in the same image pixel. We took advantage of this phasor property when we co-labeled cells with Laurdan and CT-B-A594. The purpose of these experiments was to characterize the membrane fluidity around GM1 domains, labeled by CT-B; more specifically we identified domains that are enriched with GM1 and also characterized by high rigidity. Laurdan labels all membranes but has different spectral properties depending on the membrane fluidity. The combination of the two probes provides a way to characterize the environment of the CT-B in the different locations in the cell. The linear combination property of the phasor transformation gives a simple way to identify the fluidity of the CT-B environment. In Fig. 6 we show that only some of the domains stained with CT-B co-localize with rigid domains. In particular, by visualizing the pixels that correspond to the contribution of both Laurdan in a rigid environment and CT-B-A594, we are able to specifically select only some of the domains stained by CT-B. We identify these domains with rigid regions of the plasma membrane possibly enriched with GM1.

In conclusion, Laurdan spectral phasor analysis substantially improves sensitivity toward discrimination of different emission spectra as compared to conventional methods of membrane characterization such as GP. This increased sensitivity stems from the fact that we measure all wavelengths of a spectrum in parallel and the phasor transformation results in a two coordinate system (the *g* and *s* phasor value) for each pixel of the image. Using this analytical approach we can better discriminate changes in Laurdan's environment and interaction with other probes. In particular, we can tune our analysis to detect subtle shifts in Laurdan's emission due to micro-environment heterogeneity or significant differences in emission spectra when Laurdan is co-labeled with CT-B by using a higher or lower harmonic analysis, respectively. Considering the large number of questions that still need to be answered about membrane fluidity and conformation, lipid composition and

domains, Laurdan's spectral phasor analysis has proven to be a valuable method that can be applied to many other biological problems concerning cell membranes.

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