Published in final edited form as:

Methods Mol Biol. 2015 ; 1232: 161–179. doi:10.1007/978-1-4939-1752-5_14.

Fluorescence Linear Dichroism Imaging for Quantifying Membrane Order

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Abstract

The plasma membrane of a cell is an ordered environment, giving rise to anisotropic orientations and restricted motion of constituent lipids and proteins. The membrane environment is also dynamic and heterogeneous, which is important for the regulation of membrane-localized signaling. A number of fluorescent microscopy approaches enable the membrane order to be quantified with high spatial and temporal resolution. A polarization-resolved fluorescence method, termed fluorescent linear dichroism (fLD) imaging, can quantify the orientation of membrane bound fluorophores which allows spatially resolved measurement of membrane order and sub-resolution membrane topology (ruffling). Here we describe the detailed methods for performing fLD imaging in biological membrane environments such as the plasma membrane of living cells. This includes the preparation of the sample with appropriate fluorescent dyes, the requirements of the microscope system, the data collection protocol, and post-acquisition image processing, analysis, and interpretation.

Keywords

Fluorescence; Microscopy; Polarization anisotropy; Plasma membrane; Orientation; Order; Topology

1 Introduction

The plasma membrane of a cell is an ordered environment of lipid molecules, proteins and other signaling molecules. The membrane environment is also heterogeneous; consisting of different classes of lipid molecules which determine properties of the membrane environment such as order, fluidity, polarity and mobility, and other factors. In addition to providing a barrier between the cytosol and extracellular space of a cell, the membrane can also act as a signaling platform for a diverse range of cellular signaling processes. These include exo/endocytosis, ion channel function and excitability, the function of GPCRs and other receptors or transporters, as well as intercellular communication. The regulation of many signaling molecules underlying these processes is recognized to be highly dependent on the membrane environment, such as the presence of ordered lipid phases or domains. Therefore the membrane environment is an important parameter that affects cellular function.

Several optical measurements have been developed to allow non-invasive measurement and quantification of the membrane environment. These include measurements of membrane polarity with dyes such as Laurdan and di-4-ANEPPDHQ [1] which can reflect changes in the environment of the membrane interior. Alternatively, measuring the fluidity of the membrane can be achieved by measuring the mobility of membrane bound fluorophores such as with FRAP [2] or fluorescence correlation spectroscopy [3].

We have previously developed a complimentary method for measuring membrane order which involves measuring the orientation of lipophilic fluorophores, where the chromophore resides on the fatty acid tail within in the plasma membrane interior. Due to the ordering of the lipids within the membrane, the chromophore is also ordered with anisotropic orientation (Fig. 1), and this orientation can be measured using polarized fluorescence microscopy [4]. A reduction in the order of the membrane interior, such as through reduced cholesterol content, leads to a greater range of orientations (tilt angles) of the fatty acid tails; therefore changing the tilt angle the chromophore makes relative to the membrane surface. Thus by measuring the average orientation of a fluorophore within the membrane, we can measure the membrane order.

The approach to measure the fluorophore orientation requires polarized excitation and polarization resolved fluorescence detection. Fluorophores have an absorption dipole aligned along some axis (Fig. 1a) and the more aligned the absorption dipole is with respect to the excitation polarization, the stronger the absorption and therefore the stronger the fluorescence emission. Furthermore, the more aligned the fluorophore emission dipole is with respect to the excitation polarization, the more polarized the fluorescence emission. A quantity called the fluorescence polarization anisotropy (r) represents the dependence of the detected fluorescence on emission polarization, which reflects both the fluorophore orientation and motion of the fluorophore between the absorption and emission process [5]. However fluorescence polarization anisotropy is generally used in samples with isotropic orientations to examine fluorophore mobility. A quantity called the reduced linear dichroism (LD^r) represents the dependence of fluorophore absorption on excitation polarization, which reflects just the fluorophore orientation [4]. These quantities can be compared to theoretical calculations based on geometrical considerations of fluorophore orientation distributions and dipole positions, which includes the relative orientation of the plasma membrane with respect to the excitation and detection polarization (Fig. 1b). For example, if a fluorophore dipole is perfectly orientated to be collinear with an experimental axis, it will have a LD^r of 1, whereas if it shows isotropic orientation it will have an LD^r of 0.

One advantage of this approach is that the position of the chromophore within the fluorescent lipid can be used to measure different orientation properties of the membrane environment. Within the membrane environment, the orientation of the probe will not be solely determined by the position of the plasma membrane (Fig. 1b). As highlighted above, a chromophore positioned on the fatty acid tail will tilt according to the order of the membrane interior (Fig. 1c); which can become disordered upon cholesterol disruption [4], leading to greater tilt of the fatty acid tail. Changes in head group can localize the fluorophore to different lipid phases in the membrane. However, the chromophore can also be positioned in the head group and the orientation at this position is largely independent of

the order in membrane interior. Instead, the chromophore positioned in the head group will tilt according to the local membrane topology (Fig. 1d). For example if the membrane shows a convoluted, “ruffled” topology, there will be a greater range of orientations (tilt angles) that the fluorophore can take, thus leading to a change in the polarization-dependence of absorption and fluorescence emission [6]. A powerful approach therefore is to measure the relative orientation of multiple fluorophores with different chromophore positions, to reflect changes in different properties of the membrane environment.

It should be noted that the flexibility of polarization resolved fluorescence imaging for measuring fluorophore orientation has applications beyond measuring properties of membrane environment. Other applications include membrane-bound biosensor measurements of calcium activity and GPCR activation [7]; the rigidity of membrane-bound proteins [8] and their interaction [5], and include protein complexes bound to other cellular membrane environments such as the nuclear pore complex [9].

In this protocol we will describe the measurement of membrane order and membrane topology in a cell line using lipophilic fluorophores with a chromophore that differentially localizes to the head group or tail group. We will describe the required microscope system, the preparation of the sample, the acquisition of time-lapse images, and the post-acquisition image processing, analysis, and interpretation.

2 Materials

All reagents for use with live cells should be sterile at the time of use. Appropriate institutional safety approval should be sought if primary human or animals cells are used.

2.1 Equipment for Imaging

1. A confocal microscope or other optical sectioning microscope (*see* Note 1) with appropriate excitation source (*see* Note 2) and emission filters and detector (*see* Note 3), (Fig. 2).
2. Linear excitation polarizer and control (*see* Note 4).
3. Linear emission polarizer and control (*see* Note 5).

¹The microscope does not necessarily have to be a confocal microscope, but does need to have optical sectioning capability to reject out of focus light such that fluorescence from a specific location in the membrane is detected. This could be in the form of structured illumination, two-photon excitation, Yokogawa spinning disk, or other fluorescent optical-sectioning method. If two-photon microscopy or other non-linear microscopy is used, the theory becomes more complex as the absorption is the square of the excitation intensity [4].

²This will depend on the microscope used, but must be suitable to excite the chosen fluorophore. BODIPY-PC and DiO have excitation maxima at 480–500 nm, such that a 488 nm Ar + laser line or diode laser will be suitable.

³This will also be dependent on the microscope, but must be suitable to detect fluorescence from the chosen fluorophore. BODIPY-PC and DiO have emission maxima at ~510 nm, such that a 500–550 nm band-pass filter will efficiently collect most fluorescence.

⁴The control must be able to rotate the linear polarizer between horizontal and vertical alignments. In cases where the excitation source is already polarized (such as the laser source in confocal microscope), a half-wave plate orientated at 45° to the excitation polarization should be used instead to flip the polarization between two orthogonal positions.

⁵The controller must be able to rotate the linear polarizer(s) to positions parallel and perpendicular to the excitation polarization direction (Fig. 2). In some microscope systems, such as a confocal or two-photon microscope, two detectors allow simultaneous detection. In this case a polarizing beam splitter and linear polarizers can be used. In a system utilizing a CCD camera, a “dual-view” system (Photometrics, Tucson AZ) containing a polarizing beam splitter and linear polarizers can be used to split an image into two adjacent images made up of orthogonal polarizations. In these of these two cases the polarizing beam splitter again must be aligned relative to the excitation polarization direction (Fig. 2).

4. Heated chamber for maintaining cell viability and function (*see* Note 6).
5. Power meter.
6. High-quality linear polarizer (e.g., from Thorlabs).

2.2 Reagents for Staining

1. DiO fluorophore (*see* Note 7), from Molecular Probes division of LifeTechnology.
2. β -BODIPY FL C5-HPC (BODIPY-PC) fluorophore (*see* Note 8), from Molecular Probes division of LifeTechnology.
3. Phosphate-buffered saline (PBS).
4. Scattering solution of milk diluted in buffer 1:100.
5. 10 μ M solution of fluorescein in buffer.
6. Chambered cover glass, for example Labtex wells (Nunc).

2.3 Other

1. Software for image analysis such as Matlab (as will be detailed here), ImageJ or Metamorph, etc. which can handle image arithmetic such as image addition, subtraction, multiplication, and division.

3 Methods

3.1 Calibration

1. First the excitation polarization is orientated. Place a linear polarizer just above the microscope objective parallel with the microscope stage, and orientate it such that its polarization is vertical within the image plane. The alignment of this polarizer will dictate the axes of the fLD analysis. Place a power meter just above the polarizer such that it detects the excitation illumination. Rotate the excitation polarization (or waveplate) until the detected signal is maximized—this is the “vertical” excitation polarization setting (Fig. 2). Now rotate the excitation polarization (or waveplate) until the detected signal is minimized—this is the “horizontal” excitation polarization setting (Fig. 2). These two polarization settings should be 90° with respect to each other.
2. Place 500 μ l of scattering solution in one well of the chambered coverglass and place on microscope stage. Bring the wall of one chamber into the field of view, where focusing on the interface between the wall and coverslip will indicate the z

⁶Membrane environment (order, polarity, etc.) is highly dependent on temperature. Therefore maintaining the sample under a physiological temperature is necessary during the imaging process. Depending on the medium or buffer used, appropriate CO₂ levels may also be required.

⁷DiO has the chromophore localized in the head group of the lipid. In principle other head group lipids such as DiI or DiD could also be used, albeit requiring different excitation and detection wavelengths.

⁸BODIPY-PC is a PC lipid molecule with BODIPY tagged to one of the tail groups. This can preferentially localize to more disordered membrane phases. If the orientation in more ordered phases is to be measured, a chromophore-tagged lipid with an alternative head group, such as GM can be used. Other fluorophore-tagged lipids can also be used where the chromophore is lipid-tail localized.

position (axial position) of the bottom surface of the well. Refocus to a z position above this, 25 μm into the well solution.

3. Set the microscope acquisition in free running mode, and select the vertical excitation polarization. Adjust the detection polarization settings (*see* Note 9) such that the signal in one channel is maximized, and the signal in the other channel is minimized. These channels will be “vertical” and “horizontal” detection channels respectively (Fig. 2), (*see* Note 10).
4. Next, to calculate the G and E factor, place 500 μl of fluorescein solution in a separate well of the chambered cover glass. Focus to a position as in **step 2**.
5. Collect images of the uniform fluorescein sample under all combinations (vertical, horizontal) of excitation and detection polarization. These are correction images C_{VV} , C_{VH} , C_{HV} , C_{HH} (Fig. 3), where the first subscript refers to excitation polarization, the second subscript the detection polarization, “H” represents horizontal and “V” represents vertical.
6. Under same acquisition settings, collect background images upon no excitation C_{OV} , C_{OH} (Fig. 3) (*see* Note 11).

3.2 Acquisition

1. Prepare a staining solution of 0.5 μM of DiO in 1 ml of 1 $\times$ PBS. Prepare a staining solution of 0.5 μM of BODIPY-PC in 1 ml imaging buffer. Take two dishes or wells of plated cells (*see* Note 12) and wash twice with PBS at room temperature. Incubate cells with the staining solution at 4 $^{\circ}\text{C}$ for 30 min. After incubation, again wash cells twice with 1 $\times$ PBS and store on ice until ready for imaging (*see* Note 13).

⁹This of course will depend exactly what form the detection channel will be in (Fig. 2). In the case of a polarization wheel, it must be rotated to maximize and minimize signal. In the case of a polarizing beam splitter, such as an optical insight system the beam splitter must be rotated.

¹⁰Depending on the form of detection polarization used it may be important to account for the presence of polarization bleedthrough. Linear polarizers are generally highly efficient, however polarizing beamsplitters are not. In a polarizing beamsplitter, the p polarization is transmitted, and the s polarization reflected. Generally the transmitted light has very high purity (>99 %), but the reflected light less purity (~90 %). Therefore additional linear polarizers are important, particularly for the reflected light (s polarization) channel. In the absence of additional linear polarizers, the reflected s-polarization channel can be corrected:

$$I_s^{\text{corr}} = \frac{1}{9}(10I_s - I_p)$$

In this case whether s is horizontal or vertically polarized depends on the orientation of the detection polarizing beam splitter or polarizers. But this can be checked using the scattering solution and vertical excitation: vertical detection will be higher for vertical excitation and horizontal detection will be higher for horizontal excitation.

¹¹For best results in collecting correction images, acquire using a long integration time and average multiple image frames (for a laser scanning microscope, use a long pixel dwell time, and average multiple lines).

¹²If cells do not require plating (for example, immune cell lines), these can be prepared through centrifugation and adding to chambered coverglass as a final step before imaging.

¹³The protocol for staining cells with BODIPY-PC and DiO may be different for different types of cells—this should be optimized so as not to disrupt the function of the cell but achieve good fluorescence signal.

2. Place the dish of cells stained with DiO on the microscope stage and focus on a healthy cell or set of cells. Switch to free-running fluorescence imaging and focus at the mid-way axial position of a cell.
3. Retaining the z position, set an acquisition to collect images of the sample under all combinations (vertical, horizontal) of excitation and detection polarization. These are sample images I_{VV} , I_{VH} , I_{HV} , and I_{HH} (Fig. 3), where the first subscript refers to excitation polarization, the second subscript the detection polarization, “H” represents horizontal and “V” represents vertical.
4. Under same acquisition settings, collect sample background images upon no excitation I_{OV} , and I_{OH} (Fig. 3).
5. To examine changes over time, set up a time-lapse acquisition to acquire each set of polarized images every 10 s for the desired time duration (*see* Note 14).
6. Acquire images, noting whether the object of interest is maintained within the field of view.
7. Repeat **steps 2–6** for cells stained with BODIPY-PC.
8. Check that the appropriate images were collected with appropriate polarizations. For BODIPY-PC staining, under vertical polarized excitation and vertical polarized detection the cell membrane should be brighter where they are aligned perpendicular to the vertical axis, at the top and bottom of the cell (the pole) where the chromophore dipole is parallel with the vertical axis (*see* Fig. 4). For DiO staining the areas with increased intensity should be rotated 90° with respect to the pattern observed under BODIPY-PC staining, for a given excitation polarization.

3.3 Analysis

1. Open Matlab, or other image analysis software, and load the set of acquired correction images and sample images for DiO-stained cells.
2. Background subtract all correction images: $C_{VV}' = C_{VV} - C_{OV}$; $C_{VH}' = C_{VH} - C_{OH}$; $C_{HV}' = C_{HV} - C_{OV}$; $C_{HH}' = C_{HH} - C_{OH}$ (Fig. 3) (*see* Note 15).
3. The G -factor corrects for differences in detection efficiency for the different detection polarization channels, to avoid fluorescence polarization-independent artifacts. The G -factor image represents this correction for the relative detection efficiency across the field of view for vertical and horizontal polarization (which are, respectively, parallel and perpendicular to vertical excitation). This is calculated by combining the correction images in **step 2** according to the following formula (Fig. 3) (*see* Note 16):

¹⁴For additional 3D resolution, a z stack of LD^F images can be collected at each time point, by acquiring the set of polarization resolved images at multiple z positions. Set the upper and lower bounds of the z position by refocusing under free running mode to image the top of the cell and the bottom of the cell. Acquire a single stack before setting up the time lapse to check the appropriate range of z values is sampled for the object. If the system is too slow to achieve this in the time required to resolve changes in LD^F or motion of the cell, discount collection of z stacks.

¹⁵Rather than subtracting the entire background image from the entire sample image on a pixel by pixel basis, an alternative is to calculate the average intensity for each background image and subtract that single value from all pixels of the equivalent polarized sample images.

$$G = \sqrt{\frac{C_{VV'}C_{HV'}}{C_{VH'}C_{HH'}}}$$

4. The *E*-factor corrects for differences in the excitation power/efficiency at the sample for the different excitation polarizations, to avoid absorption-polarization independent artifacts. The *E*-factor image represents this correction for the relative excitation efficiency across the field of view for horizontal and vertical detection polarizations. This is calculated by combining the correction images according to the following formula (Fig. 3) (*see* Note 16):

$$E = \frac{C_{VV'} + 2GC_{VH'}}{C_{HH'} + 2GC_{HV'}}$$

5. If the *G* factor and *E*-factor do not vary substantially across the field of view, the average *G* factor in the center of the image can be taken.
6. Background subtract all sample images: $I_{VV'} = I_{VV} - I_{OV}$; $I_{VH'} = I_{VH} - I_{OH}$; $I_{HV'} = I_{HV} - I_{OV}$; $I_{HH'} = I_{HH} - I_{OH}$ (*see* Note 15).
7. If the microscope objective has a high numerical aperture (NA), calculate the depolarizing effects on the images (*see* Note 17).
8. The linear dichroism (LD) is the difference in absorption following orthogonally polarized excitation, where absorption is calculated from the total fluorescence

¹⁶An example Matlab code for forming the *G* and *E* factor can be found below, which includes an optional correction for the s-polarization bleedthrough, where in the example s-polarization corresponds to horizontal:

```
%Control images: CVV, CVH, CHH, CHV.
%Control background images: C0V, C0H.
%First subscript excitation pol. second subscript emission pol.
CVVcorr = CVV-C0V; %background correction
CVHcorr = CVH-C0H; %background correction
CHHcorr = CHH-C0H; %background correction
CHVcorr = CHV-C0V; %background correction

G = sqrt((CVVcorr.*CHVcorr)./(CVHcorr.*CHHcorr)); %G factor
G = mean(mean(G)); %Optional spatial average
E = (CVVcorr + 2*G*CVHcorr)./(CHHcorr + 2*G*CHVcorr); %E factor
E = mean(mean(E)); %Optional spatial average

%Sample images: IVV, IVH, IHH, IHV.
%Control background images: I0V, I0H.
%First subscript excitation pol. second subscript emission pol.
IVVcorr = IVV-I0V; %background correction
IVHcorr = IVH-I0H; %background correction
IHHcorr = IHH-I0H; %background correction
IHVcorr = IHV-I0V; %background correction

%Optional polarization correction for polarizing beam splitter (PBS).
%If reflected light from PBS is 'Vertical' polarization:
IVVcorrcorr = (1/9)*(10*IVVcorr-IVHcorr);
IHVcorrcorr = (1/9)*(10*IHVcorr-IHHcorr);
```


(fLD). The reduced linear dichroism (LD^r) scales the LD to the isotropic absorption. The LD^r image is formed by combining the sample images as follows (Fig. 5) (*see* Note 18):

$$LD^r = \frac{(I_{VV'} + 2GI_{VH'}) - E(I_{HH'} + 2G^{-1}I_{HV'})}{(I_{VV'} + 2GI_{VH'}) + 2E(I_{HH'} + 2G^{-1}I_{HV'})}$$

9. For multiple points in the cell, calculate the LD^r value over a small region of interest approximately equivalent to the $1 \mu m \times 1 \mu m$ area, i.e., a few pixels (Fig. 6).
10. For each point calculate the angle the local membrane makes with the vertical orientation, corresponding to the vertical excitation polarization. This can be achieved by drawing a line that makes contact and is parallel with the membrane at which the region of interest is taken (Fig. 6). Measure the vertical and horizontal distance of this line. The angle with respect to the vertical is calculated as

$$\vartheta = \tan^{-1} \frac{\text{horizontal}}{\text{vertical}}$$

¹⁷A high numerical aperture can induce mixing of the polarizations. For emission polarizations, the effect of this mixing is calculated as

$$I_{\parallel} = K_a I_x + K_b I_y + K_c I_z \quad I_{\perp} = K_a I_x + K_c I_y + K_b I_z$$

where I_x is aligned parallel to the optical axis and perpendicular to the excitation polarization, I_y is aligned perpendicular to the optical axis and perpendicular to the excitation polarization, and I_z is aligned perpendicular to the optical axis and parallel to excitation polarization. In the limit of low NA $I_{\parallel} = I_z$ and $I_{\perp} = I_y$. Under high NA K_a dominates as >95 % of the depolarization, therefore the relevant polarizations are corrected as

$$I'_{\parallel} = I_{\parallel} - \frac{K_a}{K_a + K_c} I_{\perp} \quad I'_{\perp} = \frac{K_c}{K_a + K_c} I_{\perp}$$

In our notation, for vertical excitation (V) $I_{\parallel} = I_{VV}$ and $I_{\perp} = I_{VH}$. For horizontal excitation (H) $I_{\parallel} = I_{HH}$ and $I_{\perp} = I_{HV}$. In each case I_x is approximated as $I_{\perp}$. K_a , K_c are calculated as follows, where $\vartheta = \sin^{-1} NA/n$:

$$K_a = \frac{1}{3} (2 - 3\cos\vartheta + \cos^3\vartheta), \quad K_c = \frac{1}{4} (5 - 3\cos\vartheta - \cos^2\vartheta - \cos^3\vartheta)$$

¹⁸An example Matlab code for forming the reduced linear dichroism (LD^r) image:

```
%Corrected polarized images: IVV, IVH, IHH, IHV
%First subscript excitation pol. second subscript emission pol.
%Correction factors G, E

LDr_top = (IVV + 2*G.*IVH) - E.*(IHH + 2*(1./G). *IHV);
LDr_bottom = (IVV + 2*G.*IVH) + 2*E.*(IHH + 2*(1./G). *IHV);
LDr = LDr_top. / LDr_bottom;
```


11. Repeat sampling the LD^r and the corresponding membrane orientation angle for multiple points in the cell (*see* Note 19).
12. Plot the LD^r value as a functions of its corresponding angle α (Figs. 6 and 7) (*see* Note 20).
13. Repeat this process for data acquired from BODIPY-PC-stained cells (Fig. 5).

3.4 Interpretation

1. The LD^r vs. rotation angle is first fit to a model of the fluorophore orientation to calculate the mean tilt angle ϑ (*see* Note 21). First import the data set of BODIPY-PC LD^r values and DiO LD^r values as a function of the corresponding membrane orientation angle α .
2. For BODIPY-PC, the LD^r vs. alpha data is fit to the following function (*see* Note 22):

$$LD^r(\alpha) = \frac{(2\cos^2\alpha - 1)X}{1 + (1 - \cos^2\alpha)X}$$

where the order parameter is represented by $X = \frac{3(\cos^2\vartheta) - 1}{2}$

The maximum and minimum LD^r values are at $\alpha = 0^\circ$ and $\alpha = 90^\circ$, respectively, which are given as

$$LD^r(0^\circ) = X \quad LD^r(90^\circ) = \frac{-X}{1+X}$$

3. For DiO, the LD^r vs. alpha data is fit to the following function (*see* Note 22):

$$LD^r(\alpha) = \frac{\frac{1}{2}(1 - 2\cos^2\alpha)X}{1 + \frac{1}{2}(\cos^2\alpha - 1)X}$$

where again the order parameter is represented by $X = \frac{3(\cos^2\vartheta) - 1}{2}$

¹⁹If the cell image is close to a circular shape, an annular region of interest can be taken (*see* Fig. 7). Some image analysis software can unwrap the annulus to a linear array of pixels, which represents the LD as a function of angle around the cell.

²⁰Qualitatively, the greater the modulation of the LD^r with angle α , then the greater the level of order. For BODIPY-PC this corresponds to a more ordered membrane interior. For DiO this corresponds to a more ordered (less ruffled) membrane topology.

²¹Here, we present the data fitting for the mean tilt angle $\langle\vartheta\rangle$ assuming that the membrane is orientated at an angle α . However alternate LD^r models for fitting parameters describing the presence of vesicles, membrane nanotubes, and for quantifying the membrane ruffling can be generated. This is beyond the scope of this chapter but examples are described in [4, 6].

²²If the order parameters and mean title angles are very close between samples, then one option is to use a more accurate model which accounts for assumptions made in the main model where the formation of the isotropic fluorescence represents polarized light absorption. As a result of this assumption, high NA-induced depolarization and hindered motion of the fluorophore in the membrane can have a small (<5 %) effect on the calculated LD^r . The numerically solved model is beyond the scope of this protocol but is described in detail in [4].

Here, the maximum and minimum LD^r values are at $\alpha = 90^\circ$ and $\alpha = 0^\circ$, respectively, which are given as

$$LD^r(0^\circ) = -X/2 \quad LD^r(90^\circ) = \frac{X/2}{1 - X/2}$$

4. In each case determine the order parameter X which best fits the experimental data, from which the value of $\langle \cos^2 \vartheta \rangle$, and thus $\langle \vartheta \rangle$ can be obtained (Figs. 6 and 7) (*see* Note 23).
5. The greater the tilt angle (corresponding to a smaller modulation in LD^r vs. α), the more disordered the membrane in which the DiO or BODIPY-PC is orientated in. However, it should be noted that the measured tilt angle $\langle \vartheta \rangle$ represents the average tilt over a distribution. A fully disordered sample will show an isotropic distribution of orientations, from which $\langle \vartheta \rangle = 54.7^\circ$, which is the magic angle. An angle lower than this indicates anisotropic orientation, with a minimum orientation of $\langle \vartheta \rangle = 0^\circ$ indicating perfect orientation of the fluorophore within the membrane. However if $\langle \vartheta \rangle > 54.7^\circ$, this corresponds to an ordered fluorophore orientated perpendicular to its preferred orientation, such as parallel with the surface of the membrane. Thus we should note, if the fluorophore happens to be perfectly orientated at the magic angle, this will not be distinguishable from an isotropic orientation.
6. The order of the BODIPY-PC fluorophore reflects the order of the internal portion of the plasma membrane, such as ordered and disordered phases. For example if cholesterol is extracted from the plasma membrane, the interior of the membrane becomes more disordered (corresponding to a smaller modulation in LD^r vs. α) and $\langle \vartheta \rangle$ approaches 54.7° (Fig. 8).
7. The order of the DiO fluorophore reflects the order of the membrane surface topology. For example if membrane is highly ruffled at the edge of the cell-cell contact region in the immune synapse, the membrane surface is highly disordered (corresponding to a smaller modulation in LD^r vs. α) and $\langle \vartheta \rangle$ again approaches 54.7° (Fig. 9).

Acknowledgements

We would like to thank Bjorn nfelt (Karolinska Institutet) and Tim Lei (University of Colorado) for critical reading of the manuscript. This work was supported by NIH grant DK085145 (RKPB).

²³The mean tilt angle $\langle \vartheta \rangle$ will be averaged over the distribution of tilt angles. There may be a single fixed angle or a distribution of angles. $\langle \cos^2 \vartheta \rangle$ is calculated from an arbitrary tilt distribution as

$$\cos^2 \vartheta = \frac{\int_0^\pi \cos^2 \vartheta N(\vartheta) \sin \vartheta d\vartheta}{\int_0^\pi N(\vartheta) \sin \vartheta d\vartheta}$$

As a result, if $\langle \vartheta \rangle = 57^\circ$ as a single tilt, this will be equivalent to, and indistinguishable from, isotropic orientation.

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Fig. 1.

(a) Chemical structures of the two lipophilic fluorophores utilized in fLD imaging. Highlighted in *red* are the absorption dipoles of the chromophores, localized to the fatty acid tail for BODIPY-PC and to the head group for DiO. Note the opposite orientation of the dipole. (b) Orientation of the plasma membrane, where α represents the angle of the normal to the plasma membrane surface with respect to the vertical axis. (c) Schematic of BODIPY-PC in the plasma membrane of a cell with anisotropic orientation due to the ordered lipid environment. The orientation of the chromophore located in the tail with respect to the membrane normal reflects order of the membrane interior, which can tilt by an average angle $\langle \vartheta \rangle$. (d) Schematic of DiO in the plasma membrane of a cell. The orientation of the chromophore located in the head group with respect to the membrane normal reflects local topology of the membrane, where the local normal can tilt by an average angle $\langle \vartheta \rangle$ (Color figure online)

Fig. 2. Schematic of two possible microscope systems used for polarized fluorescence imaging. **(a)** In a representative wide field configuration, the excitation polarization is controlled by a rotating linear polarizer. The emission polarization is controlled by a polarizing beamsplitter imaging system, simultaneously imaging each emission polarization on a CCD camera. **(b)** In a laser scanning microscope, the excitation polarization is controlled by a half wave plate ($\lambda/2$). The emission polarization is controlled by a rotating linear polarizer. In each case underlined components are required to be sequentially rotated/adjusted

Control (fluorescein)

$$\begin{array}{ccc} C_{VV} & C_{HV} & C_{0V} \\ C_{VH} & C_{HH} & C_{0H} \end{array}$$

$$\begin{array}{ccc} C_{VV}' & C_{HV}' & \\ C_{VH}' & C_{HH}' & \end{array}$$

$$G \rightarrow E$$

Sample (stained cell)

$$\begin{array}{ccc} I_{VV} & I_{HV} & I_{0V} \\ I_{VH} & I_{HH} & I_{0H} \end{array}$$

$$\begin{array}{ccc} I_{VV}' & I_{HV}' & \\ I_{VH}' & I_{HH}' & \end{array}$$

$$(I_s^{corr})$$

$$(I_{||}' \quad I_{\perp}')$$

$$LD^r$$

Fig. 3.

Work flow for the images required for calculating the reduced linear dichroism. “C” refers to control (uniform sample) images. “I” refer to sample images. *First subscript* refers to excitation polarization direction, *second subscript* to emission polarization direction. “V” is vertical and “H” is horizontal polarization with respect to an axis in the sample. “0” refers to no excitation. *G* is the *G*-factor and “*E*” is the excitation factor

Fig. 4. Example images of BODIPY-PC stained cell acquired under different excitation and emission polarizations. For vertical excitation and detection there is increased intensity at the *top* and *bottom* of the cell image where the chromophore is aligned vertically. For horizontal excitation and detection there is increased intensity at the sides of the cell image where the chromophore is aligned horizontally

Fig. 5.

(a) Schematic of BODIPY-PC chromophore aligned in different parts of the plasma membrane of a cell, together with a false color scale image of LD^f in a BODIPY-PC stained cell, where increased LD^f at the *top* of the cell is associated with the chromophore dipole being more parallel with the vertical excitation axis (indicated, V). (b) Schematic of DiO chromophore aligned in different parts of the plasma membrane of a cell, together with a false color scale image of LD^f in a DiO stained cell, where increased LD^f at the *side* of the cell is associated with the chromophore dipole being more parallel with the vertical excitation axis (indicated, V)

Fig. 6.

Schematic showing how LD^r (from a DiO stained cell) can be calculated as a function of membrane orientation angle. **(a)** For a localized region-of-interest (*grey circle*), the average LD^r is taken. A *straight line* intersecting the membrane at the region-of-interest is taken, and the angle of this line with respect to the vertical is calculated (α'), and converted to the angle of the membrane normal ($\alpha = 90^\circ - \alpha'$). **(b)** A plot of LD^r vs. angle (α) can then be fitted to the model to obtain the order parameter (X) and the average chromophore tilt ($\langle \vartheta \rangle$)

Fig. 7. Schematic showing an alternative method how LD^r (from a DiO stained cell) can be calculated as a function of membrane orientation angle. **(a)** If the cell shows a regular circular structure, the LD^r verses α can be extracted directly, by unwrapping an annular mask (*dotted line*). **(b)** This can also be fit to obtain the order parameter (X) and the average chromophore tilt (ϑ)

Fig. 8. Example of how the LD^r for BODIPY-PC (fatty acid tail chromophore), can be used to measure changes in order of the membrane interior. In this case, measuring the effect of altered cholesterol levels over time. **(a)** False color scale LD^r images of DiO stained cell at various time points (indicated) following cholesterol extraction at time 0 showing no change in LD^r. **(b)** False color scale LD^r image of BODIPY-PC stained cell as in **a** showing loss of LD^r modulation around the cell. **(c)** Profile of LD^r around DiO stained cell before, during and after cholesterol extraction showing no change. **(d)** Profile of LD^r around BODIPY-PC stained cell before, during and after cholesterol extraction showing loss of modulation. **(e)** Change in order parameter over time after cholesterol extraction. Despite an already reduced order, the order further reduces close to 0 (isotropic orientation) for BODIPY-PC stained cell; but remains unchanged for DiO stained cells indicating no change in membrane topology

Fig. 9. Example of how the LD^r for DiO (head group chromophore) can be used to measure changes in membrane topology, in this case, measuring a spatially dependent increase in membrane ruffling across the cell-cell contact region during immune synapse formation. **(a)** Bright field and LD^r image of DiO-stained NK cell line interacting with a target cell. **(b)** Change in intensity and LD^r across the cell contact region indicating an increase in DiO fluorescence but reduction in LD^r at the edges of the interaction. **(c)** LD^r values vs. membrane normal orientation together with fits for plasma membrane outside the interaction zone (“plasma membrane,” black), plus the center (“synapse center,” red) and edge (“synapse periphery,” blue) of the interaction zone. **(d)** Additional micro-scale membrane topology of center and periphery of interaction zone required to change the LD^r and chromophore tilt $\langle\vartheta\rangle$, compared to the plasma membrane outside the interaction zone; assuming no ruffling outside the interaction zone (*see* Note 21)