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Cell membrane state, permeability and elasticity assessment for single cells and cell ensembles

Running title: Correlation of membrane order, permeability and elasticity

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Abstract

The phase state and especially phase transitions of synthetic lipid membranes are known to drastically modulate mechanical membrane properties like permeability and bending modulus. While the main transition of lipid membranes is typically detected employing differential scanning calorimetry (DSC), this technique is not suitable for many biological membranes. Moreover, often single cell data on the membrane state or order is of interest. We here first describe how to use a membrane polarity sensitive dye, Laurdan, to optically determine the order of cell ensembles over a wide temperature range from $T = -40^{\circ}\text{C}$ to $+95^{\circ}\text{C}$. This allows to quantify the position and width of biological membrane order-disorder-transitions. Second, we show that the distribution of membrane order within a cell ensemble allows for correlation analysis of membrane order and permeability. Third, combining the technique with conventional atomic force spectroscopy allows for the quantitative correlation of an overall effective Young's modulus of living cells with the membrane order.

Key Words

Cell membrane, lipid order, Laurdan, fluorescence spectroscopy, permeability, elasticity

1. Introduction

From synthetic lipid membranes it is known that the membrane state modulates non-linearly membrane characteristics like bending energy, membrane tension, permeability etc. As biological membranes are composed to a large extent of lipid molecules, the question arises, whether the physical membrane properties of living cells and, in consequence, cellular functions depend as well on the lipid phase state. So far, it was shown that the cell membrane phase state measured by the determination of the lipid order influences, e.g., the membrane bending rigidity, the immunological response in T cell membranes and focal adhesion domains[1–3]. To which extent other cellular functions are regulated by the membrane phase state needs to be investigated.

In this context, we here describe the technique to determine the cell membrane lipid order as measure of the phase state and provide a protocol to simultaneously measure the membrane properties ‘permeability’ and ‘elasticity’. For this, we use conventional epi-fluorescence microscopy / spectroscopy, the dye Laurdan for lipid order determination and rhodamine B and Hoechst 33342 as permeability probe. However, the cargo of interest simply can be exchanged. The elastic properties of the cell membrane are measured by atomic force microscopy / spectroscopy.

Exchanging the dye with a solvatochromic dye specific for the plasma membrane, like e.g., NR4A, could be an interesting modification of our protocol especially regarding the correlation of permeability and membrane order[4].

2. Materials

2.1. Solutions and reagents

1. Laurdan solution: 2.8 mM Laurdan in dimethyl sulfoxide (DMSO). Dissolve 1 mg of Laurdan powder in 1mL dimethyl sulfoxide (DMSO) in an amber glass vial. To speed up this procedure and to ensure full dissolution use an ultrasonic bath at T=50 °C for about 30 min. Each time after storage in the fridge the ultrasonic treatment should be repeated to dissolve all Laurdan crystals.
2. Rhodamine B solution: 2 mM rhodamine B in PBS buffer
3. Hoechst 33342 solution: 16 µM Hoechst 33342 in PBS buffer
4. Trypsin-EDTA solution: 0.05 %
5. Standard PBS buffer

2.2. Cell culture

1. Any cell line of interest
2. Standard cell culture equipment (e.g. clean bench, incubators, sterile equipment, etc.)
6. Petri dish
7. Coverslip
8. Cell culture medium (DMEM with 10% FBS and 1% Penicillin-Streptomycin)
9. 4 µL Poly-L-lysine (PLL, 0.1% in water with 0.01% thiomersal) with 996 µL PBS mixture

2.3. Other equipment

1. 440 nm filter cube: 360 nm excitation, 440 nm emission
2. 490 nm filter cube: 360 nm excitation, 490 nm emission
3. 645 nm filter cube: 585 nm excitation, 645 nm emission
4. 360 nm, 3W, LED
5. Spectrometer: minimum range 400 – 700 nm
6. Cantilevers of the desired type
7. Plasma cleaner

8. Atomic force microscope (AFM)

3. Methods

3.1 Laurdan staining and incubation

1. For cell membrane staining, the Laurdan solution is added to the culture medium in a volume ratio of 1:100. For example, for staining of adherent cells in a 25 cm² culture flask, 50 µL of the staining solution are added to 5 mL of culture medium (for further information about influences of the staining procedure see note 1). To avoid cell damage by locally high DMSO concentrations, premix the 50 µL staining solution with 1 mL of the culture medium in a separate vial and add this solution to the culture flask. Do not leave the amber staining solution vial open for too long. DMSO is hygroscopic and therefore, it will absorb water from the air. Because of this, after long usage of the same staining solution, it will not freeze any more in the freezer. At this point at latest, you should prepare another solution.
2. After incubation for 2h in the dark at T=37°C, remove all of the medium and rinse three times with 5mL PBS buffer to reduce the background signal (for further information about the staining time see note 2). You must be very careful during this washing procedure as the cells easily detach due to the high DMSO concentration during staining. For analysis of adherent cells, add 5 mL of PBS as imaging medium. Do not use media containing phenol red as it interferes with the Laurdan fluorescence emission signal.
3. For analysis of the cells in suspension, trypsinize with 1 mL of 0.05% trypsin-EDTA solution and incubate for 5 min. Again, care should be taken that no phenol red or other absorbing/fluorescing agents are added before spectral analysis.

3.2 permeability assessment

Membrane permeability is assessed by the analysis of rhodamine B or Hoechst 33342 diffusion into the cytosol.

1. Add the rhodamine B solution 1:1 to the culture medium already containing Laurdan 10 min prior to the end of the Laurdan staining procedure.
2. Remove the medium containing Laurdan and rhodamine B after 10 min and rinse the cells carefully three times with PBS buffer as described above.

While the use of rhodamine B allows for simultaneous phase state and permeability analysis, in contrast, the uptake of Hoechst 33342 must be analyzed after phase state determination due to channel cross talk. For the permeability measurement using Hoechst 33342, add the solution 1:1 to the imaging buffer (PBS). The uptake of Hoechst 33342 can be imaged without rinsing procedure as it only fluoresces after uptake and intercalation in the DNA.

3.3 Spectral analysis of Laurdan with two filter cubes and permeability measurement

1. Mount your sample into a tempered microscope setup. See note 3 or options to control the sample temperature.
2. Record two images per region of interest using the 440 nm and the 490 nm filter cubes. For more detailed information about how Laurdan measures lipid order see note 4.
3. Keep the ultraviolet exposure during image acquisition as low as possible to avoid artefacts as explained in note 5.
4. Record a third image for each region of interest for example using the 645 nm filter cube to measure membrane permeability by uptake of rhodamine B simultaneously to lipid order. Be careful about channel cross talk as discussed in note 6 or measure membrane permeability and lipid order sequentially as explained in note 7.

3.4 Spectral analysis of Laurdan with a spectrometer

1. Put your sample into a transparent sample container
2. Place the sample container in a tempered sample holder with optical fiber mount

3. Attach an optical fiber and a spectrometer
4. Excite the sample with an ultraviolet LED and record the spectrum with a spectrometer. The difference to the data obtained using a fluorescence microscope is discussed in note 8.

3.5 Acquisition of Elasticity data

1. Use a new cantilever or clean a used one with a short plasma procedure followed by a buffer bath.
2. Leave the PLL-PBS mixture to act on a coverslip in a Petri dish for one hour.
3. Rinse the incubated cover slip with PBS and cell medium.
4. Add the cell suspension and wait a few moments, e.g., 5 min.
5. Carefully drain off the remaining liquid and transfer the cover slip to a new Petri dish.
6. Fill the Petri dish with cell medium or PBS and start the AFM measurement with the desired parameters (see notes 9-14).

3.6 Microscopy Data Analysis

1. Correct the images for background intensity (see note 15):
2. Proceed with the image segmentation (see note 16)
3. Calculate GP for each pixel (see note 17).
4. Calculate GP for each cell (see note 18).
5. Calculate the cell membrane permeability (see note 19).

3.7 Spectrometer Data Analysis

1. Approximate the spectral data by two lognormal functions to calculate GP (see note 20).
2. Plot GP as function of temperature.

3. Calculate the derivative of GP with respect to temperature to obtain thermodynamic data (see note 21).

3.8 Mechanical Data Analysis

1. Subtract baseline and contact point of the AFM force curve.
2. Correct the cantilever bending.
3. Use the fit function appropriate for the tip geometry (see notes 22-23).
4. Apply descriptive statistics (see note 24).

4. Notes

1. Influence of environmental conditions on lipid order: The cell membrane phase state is a quantity depending on many variables such as temperature, pH, solvent content, salt concentration, lipid composition, cholesterol content and more. Therefore, care must be taken when you prepare your own culture media to ensure that these variables do not vary significantly between the single experiments. In addition, the local environment of each cell influences the membrane phase state as well: With increasing confluency in the culture flask, the lipid order increases as well [1, 10]. In consequence you must always ensure to work with cells of equal confluency state. Moreover, depending on the cell type, lipid order can increase or decrease with the number of the cell passages [11]. Care should also be taken when comparing cells in the adherent and suspended state. During trypsination-induced cell detachment, lipid order decreases [10](SI).
2. Influence of Laurdan incubation time and concentration: As this protocol is designed for lipid order analysis using conventional epifluorescence microscopes, where the fluorescence intensity should be high to compensate for losses due to by photobleaching, the dye concentration is comparably high and in consequence, the DMSO content as well. Both quantities again influence the lipid order: During staining, the lipid order decreases. For the above mentioned Laurdan/DMSO concentration, the staining-induced lipid order changes saturate after 2h [10](SI). To avoid that small variations in the staining process cause variability of the membrane phase state, the incubation time is set to 2h as well. If other concentrations are used, this time can be shortened or might have to be enlarged. The same holds true for other incubation temperatures: The staining-induced lipid changes might saturate later at lower temperatures.
3. Temperature control and temperature dependent lipid order: A key factor in the context of lipid order analysis is the temperature control. On the one hand, the precise control of temperature guarantees high comparability and reproducibility among data sets. On the

other hand, the analysis of the temperature dependence of the cell membrane lipid order allows for the determination of thermodynamic quantities such as cooperativity, enthalpy and melting temperature. At the microscope, temperature can be regulated by commercially available microscope incubators which are commonly limited to a temperature interval from room temperature to 20 K above. For wider ranges even below room temperature, custom made setups containing heat baths and Peltier elements (for example, available at Conrad Electronic SE or Reichelt Elektronik GmbH & Co. KG) are necessary. Independent of the setup, an *in-situ* temperature probe is strongly recommended as especially stage incubators exhibit often long temperature equilibration times.

4. The emission spectrum of Laurdan depends on the polarity and the relaxation dynamics of its environment [5, 6]. Therefore, the emission maximum shifts from 440 nm, meaning that the lipid chain order is high and the polarity in the membrane is low due to the lack of water molecules to 490 nm. This indicates that the lipid order is low and energy is lost due to solvent relaxation. This change can be monitored by the use of two fluorescence microscope filter cubes with the same excitation filters (360 nm) but different emission filters: 440 nm and 490 nm. To analyze the cell membrane lipid order, two micrographs have to be taken at the same position successively, one for each filter cube.
5. Possible artifacts: During image acquisition, photobleaching is not only an issue of intensity loss but the ultraviolet irradiation impacts the phase state of cell membranes such as it increases lipid order. Therefore, it is necessary to record reference images if cells are imaged multiple times to distinguish between UV induced artifacts and other phase state changes. Of course, the UV-effect is strongly setup dependent but as a rough estimate, we here give exemplary empirical values for our setup. We observed at a Zeiss Axiovert 200M epifluorescence microscope with a mercury-vapor lamp, a 10x objective and 1s exposure time, a change of the generalized polarization (GP) as measure of lipid order of roughly $\Delta GP = 0.005$ per recorded image at the same position.

6. If another quantity, such as permeability, is analyzed by fluorescence microscopy, care must be taken to avoid channel cross talk which could influence the lipid order measurements. In case of rhodamine B, the emission spectrum is far enough in the red compared to Laurdan so that permeability can be measured simultaneously to the lipid order analysis by the use of a third channel (645 nm filter cube). The TexasRed intensity is proportional to the uptake of rhodamine B and acts as a measure for permeability.
7. The use of Hoechst 33342 facilitates the experimental procedure as no rinsing steps are required because of the lack of background intensity. Therefore, it also allows for recording a time series of Hoechst 33342 uptake where increasing intensity again indicates a high membrane permeability. But in contrast to rhodamine B, there is spectral overlap of the Laurdan and Hoechst 33342 spectra. Therefore, the lipid order determination with the 440/490 nm filter cubes has to be done prior to the Hoechst 33342 addition. Afterwards, the fluorescence intensity of Hoechst 33342 should be measured with the 440 nm emission filter cube. By this, the permeability measurement can later be corrected for the Laurdan fluorescence intensity.
8. The use of a microscope with the mentioned filter sets for Laurdan analysis allows for spatially resolved lipid order analysis. But, as only a fraction of the spectrum is analyzed, namely the regions around 440 nm and 490 nm, the sensitivity to phase state changes is reduced as compared to the analysis of the whole spectrum with a spectrometer. The higher sensitivity is accompanied by the loss of spatial information. If the spectrometer is attached with an optical fiber to the microscope, the obtained spectrum is a mean value of the cell ensemble in the focal plane.
9. Atomic force microscopy: We recommend calibration before measurement on a cover glass without PLL.
10. To increase the validity of the results, it must be ensured that cells are not unintentionally measured twice and that the positioning of the cantilevers on the cell is always comparable.

11. Influence of tip geometry: Depending on the desired measurement intention, the use of different cantilevers should be considered. Measuring probes with a sharp tip could damage the cell [12], but at the same time provides a high spatial resolution. Cantilevers without a tip, on the other hand, can be used very well for determining the global cell properties without putting too much strain on the cell locally.
12. Influence of repeated measurements: When recording a larger number of force curves on a particular cell, it becomes apparent that, on the one hand, there is a significant scatter between the individual force curves and, on the other hand, a medium-term change in the Young's modulus can be observed. Therefore, it is recommended to record a comparatively small number of force curves (approx. 20) from a single cell.
13. Influence of variance: The measurement curves vary considerably between individual cells. It is, therefore, advisable to run several parameters on one cell to allow for the analysis of relative changes of the Young's modulus.
14. Spatial measurements: While classical imaging initially only reveals information about the topography, using a measuring grid in Force Spectroscopy mode allows both forces to be measured and topography data to be collected via the set-point height. Thus, even with a conventional atomic force microscope, Young's modulus maps of cells can be created without special software. Be aware of the overall geometry.
15. Prior to the calculation of the generalized polarization (GP) (see 3.6.3) values, the 440 nm and 490 nm images need to be corrected for background intensity resulting from thermal detector noise, stray light or not membrane-embedded Laurdan. If this is not done, the GP values are shifted towards zero with decreasing signal to background ratio.
16. For lipid order and permeability analysis on a single cell level, each pixel has to be assigned to the background or a cell (see figure 1). Neural networks are predestined for this task and platforms such as the python package "cellpose" are freeware, easy to use and do not have to be trained [7].

17. After assigning each pixel to the corresponding cell, the lipid order at each pixel can be quantified using the “generalized polarization” (GP) which is defined as the difference between the 440 nm and 490 nm intensities normalized to their intensity sum: $\frac{I_{440} - I_{490}}{I_{440} + I_{490}}$ [8] (see figure 1).
18. Cell-GP-calculation: Either different regions of each single cell can be analyzed separately, or a mean GP value can be calculated as sum of all pixel GP values divided by the number of pixels. Another method for mean GP value calculation would be to calculate the mean 440 nm/490 nm intensity of all cell pixels and use these mean values for GP calculation. The two approaches yield different results as in case of the second method, regions with lower fluorescence intensity are less weighted than regions of high fluorescence intensity. If microscopy data are compared to spectrometer data, the second method of mean GP calculation turns out to be more comparable.
19. In case of rhodamine B uptake measurements, the mean pixel intensity of the rhodamine B channel can be used after background correction as measure for membrane permeability (see figure 1). The mean intensity data of Hoechst 33342 recorded using the 490 nm filter set has to be corrected for the Laurdan intensity determined prior to the permeability assay. As a result of the microscopy image analysis, it is possible to quantify a cell membrane property, such as permeability, as function of the lipid order and test for correlation of both quantities.
20. Spectrometric data can be analyzed by the use of the two wavelength regions around 440 nm and 490 nm. Another more precise method that uses all of the spectral data is shown in figure 2: The data set is approximated by two lognormal functions centered around 440/490 nm [9]. The according fit function areas correspond to the number of ordered/disordered lipids and can be used for GP calculation.
21. Deduction of quantities of interest: As mentioned before, the temperature dependence of membrane lipid order provides insight into thermodynamic properties of the lipid system.

This is illustrated in figure 3: The temperature dependent GP values of different phospholipid vesicles indicate phase transitions as a steep decrease, meaning that lipid order is strongly reduced at the melting temperature. The derivative of GP with respect to temperature shows a peak at the melting transition as it is known from differential calorimetry measurements. The good agreement of optical and calorimetric data suggests that the Laurdan lipid order analysis can provide useful thermodynamic data such as melting temperature (peak position) and cooperativity (peak height and width) of lipid systems [10]. It is also possible to record such melting profiles in cellular membranes as shown in figure 4. But as the melting regime of biological membranes is markedly larger, the temperature range for GP determination has to be enlarged from -40°C to 90°C .

22. Fit limitations: Please note the assumptions and limitations associated with the fit [13].

23. Analysis of viscosity: In addition to the elasticity of the cells, their viscous behavior can also be analyzed. For example, after the set-point force has been reached, a pause at constant force can be built into the procedure, and the viscous properties of the cell can be inferred from the flow behavior [14].

24. Statistics: To maximize the informative value of the data obtained, the use of appropriate statistics is recommended. For example, intra-class correlation can be used, which deals with the variance between measurements and within measurements [15].

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Figure Captions

1. Raw and processed image data of a HeLa cell lipid order and membrane permeability assay. While the two Laurdan images captured with different filter sets allow for quantitation of lipid order measured by GP, the rhodamine B intensities provide data about the membrane permeability. Both quantities can be assigned to single cells using an image segmentation algorithm.
2. Spectral data of Laurdan embedded in the membrane of HeLa cells (black circles). The spectrum is approximated by the red line which is the sum of two lognormal functions represented in red and blue. The blue/green areas are used for GP calculation.
3. Phase state analysis of phospholipid vesicles. **a** GP as function of temperature of different phospholipid vesicle suspensions recorded with a spectrometer in a tempered cuvette. **b** Derivative of GP data in a) with respect to temperature. The peaks indicate the phase transition of the lipid vesicles. **c** calorimetric data of the vesicles analyzed in a) and b). The peak position and the peak height of optical and calorimetric data are in good agreement.
4. GP and the derivative of GP with respect to temperature as a function of temperature. The melting profile of HeLa cell membranes extends over a much larger temperature regime as compared to phospholipid vesicles.