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COMPARATIVE ANALYSIS OF MEMBRANE LIPID ORDER IMAGING WITH TWO FLUORESCENT PROBES IN INTACT CELLS

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Abstract: Cell membranes include vast range of lipids which form segregated regions of structure and function. They assemble small, dynamic clusters of lipids in a more ordered state called "rafts". These microdomains mediate cellular processes such as protein sorting, signal transduction, membrane trafficking and cell adhesion. Large part of our knowledge about the behaviour of lipid mixtures in bilayers is shaped from studies on model membranes which are with well-defined composition. Our aim was to observe domains on the more complex membrane systems of intact cells.

We applied membrane probes which give information on the degree of lipid packing to make membrane domains visible. We compared two polarity sensitive fluorescent probes – namely Laurdan and di-4-ANEPPDHQ. Two cell lines with distinct differences in their plasma membrane composition and two fluorescent methods for analysis of rafts – microscopy and spectroscopy were chosen. We obtained reliable assessment of the state of the membrane order through both methods. Di-4-ANEPPDHQ provided better micrographs with the available microscope technique.

INTRODUCTION

Cell membranes have an important role in many cellular processes as well as in maintaining the cell integrity and communication with the environment. Their functions are directly dependent on their composition and structure. The plasma membrane is a dynamic, heterogeneous structure, composed of discrete domains. The fluid mosaic model has been modified by Simons and colleagues with the concept of lipid-lipid interactions and lipid raft hypothesis (Symons & Ikonen, 1997). This new hypothesis states that the cell membrane displays two

co-existing lipid phases. The liquid ordered (Lo) phase is sterol-dependent state, which consists mainly of saturated lipids. In this state lipid acyl chains are tightly packed and extended and lipid molecules form microdomains, where they still retain high degree of lateral diffusion. These specialized domains, enriched in cholesterol, sphingomyelin and certain proteins, called rafts (membrane lipid rafts, MLR), are characterized with unique physical and biological properties. Rafts are placed among milieu of unsaturated lipids in liquid disordered state (Ld). Diverse functions of biological membranes like membrane trafficking, signal transduction and activity of membrane proteins are considered to be related to the lateral partitioning of lipids into domains (Simons and Toomre, 2000). Scientists have gathered knowledge about membrane composition through proteomics and lipidomics. Cell membrane molecules involved in a particular cellular function participate in coordinated interactions with each other during the performance of this function. The complexity of plasma membrane's dynamics has become obvious, but despite the vast knowledge acquired about its composition and structure, our comprehension of its dynamic processes is still on basic level.

The clarification and visualization of the membrane structure and dynamics is of fundamental importance for cell biology. One traditional method of studying raft-associated proteins is isolation of Lo-phase detergent-resistant membranes (DRMs) from cells (Brown and Rose, 1992; Lingwood and Simons, 2007; London and Brown, 2000). This technique is reasonably criticized in several publications (Heerklotz, 2002; Lichtenberg *et al.,* 2005; Sot *et al.,* 2006). The main argument states that the detergent itself may induce phase separation and render influence on membrane proteins' partitioning.

One method that accounts for the actual diversity of lipids and proteins in cell membranes and demonstrates fluid/fluid phase separation in biological membranes is the induction of giant plasma membrane vesicles (GPMVs) in cultured mammalian cells (Baumgart *et al.,* 2007). Phase separation in these vesicles was observed in 2007 and canonical raft proteins and lipids were found in one of the phases (Baumgart *et al.,* 2007). This phase separation was proven to be dependent on membrane cholesterol (Levental *et al.,* 2009). It was shown that the coexisting domains have different diffusivity (Levental *et al.,* 2009) and order values (Kaiser *et al.,* 2009). This verified that biological membranes indeed have the ability to separate into coexisting fluid phases with particular constitution and physical properties.

Other methods like mass spectrometry (Kraft *et al.,* 2006), NMR spectroscopy (Guo *et al.,* 2002), atomic force microscopy (AFM) (Goksu *et al.,* 2009) are either destructive or unsuitable for investigation of membrane dynamics of intact cells. Currently, optical techniques prevail as the most adequate approach for investigation of rafts in intact cells. Fluorescence methods that permit visibility in the submicron scale are fluorescence correlation spectroscopy, hetero- and homo-

fluorescence energy transfer and single molecule techniques (Bacia *et al.,* 2004; Kenworthy *et al.,* 2000; de Almeida *et al.,* 2005; Varma and Mayor, 1998; Schütz *et al.,* 2000).

The selection of the fluorescent dye is crucial for the outcome of these studies. The choice of a fluorophore depends on the type of sample (model or cellular membrane), the available equipment and the scientific aim. Most of the lipid probes, available nowadays can be associated to one of three main classes:

Class I. Probes that specifically label lipid components of the membrane, such as gangliosides (GM1 for example) or cholesterol;

Class II. Probes that selectively partition to either Lo or Ld phase. Several studies are dedicated to lipid-like dyes that show high affinity and preference to liquid ordered or disordered phase (Baumgart *et al.,* 2007; Sengupta *et al.,* 2008; Sezgin *et al.,* 2012; Shaw *et al.,* 2006). It is found that the vast majority of these probes (especially lipid - like probes) associate preferentially to Ld phase, since they are excluded from the tightly packed ordered phase. There is a small number of fluorescent markers as LcTMA-DPH and diI-C20, which possess long alkyl chains and readily partition in Lo domains;

Class III. Environment-sensitive fluorophores are probably the ones most recently introduced to lipid raft research. Among them, molecular rotors and solvatochromic and electrochromic dyes are most commonly used for membrane investigations. The fluorescence dimensions of molecular rotors exhibit variations according to their intramolecular rotation, which depends on the viscosity of the environment. In more viscous vicinity the fluorescence intensity is increased, because the rotations are slowed down. These fluorophores report the microviscosity of their surroundings (Haidekker and Theodorakis, 2007). Representative examples of such probes are DCVJ, its carboxy analog and some newly developed analogs of Prodan. Some of them like BODIPY-FL-C12, BODIDY-FL-DHPE and DiOC18 are suitable for monitoring viscosity and discriminating between Lo and Ld phases, but are preferentially partitioned in the Ld phase (Kuimova *et al.,* 2008). Fluorescence features of solvatochromic dyes are environmentally-sensitive and dependent on the polarity of their local solvent (Parasassi *et al.,* 1998). Due to probe's intrinsic properties, its fluorescent emission spectra are determined by the presence of water molecules in their surroundings. They are distributed evenly between the two phases, but emit from two excited states – relaxed one with loose lipid packing and a non-relaxed state when the bilayer packing is tight.

The shift in emission spectra can be quantified by Generalized Polarization (GP) analysis (Gaus *et al.,* 2003; Parasassi *et al.,* 1997). This function (GP) is proposed by Parasassi et al. in 1990 and represents normalized fluorescence intensity ratio. The spectral channels that correspond to Lo and Ld phases are used for the GP equation. The main superiority of GP analysis is that data acquisition can be acquired either on spectrophotometer or confocal microscope.

Quantitative and qualitative analysis can be performed simultaneously on the same single micrograph.

In this work we compared two polarity-sensitive dyes – Laurdan and di-4-ANEPPDHQ. Most of the routinely used fluorescent dyes for detection of lipid phases are UV - excitable probes like Laurdan. This could generate difficulties for microscopic analysis because of the special technique parameters these probes require (lasers, lenses) and the fact that living cells have high autofluorescence in this spectral region. One approach to overcome these problems is to excite them with two-photon absorption process.

The fluorescent probe 6-dodecanyl-2-dimethylaminonaphthalene (Laurdan) is a classic example for polarity-sensitive dye. The fluorescent group of Laurdan is located ~10A ° from the center of the bilayer (Antollini and Barrantes, 1998). The probe's dipole molecules are aligned in the interface between the hydrophilic head groups and the hydrophobic acyl chains in the phospholipid bilayers (Bagatolli *et al.,* 1999). When the dye is in an apolar milieu, the shift in emission spectra is blue and in polar solvents is a red-shifted emission is observed due to its structure.

The styryl di-4-ANEPPDHQ dye is originally used to monitor electrical activity in cells and tissues (Obaid *et al.,* 2004) and designed as a voltage-sensitive dye. It is another environmentally-sensitive probe that can be used to discriminate lipid phases in model membranes and intact cells (Jin et al., 2006; Owen *et al.,* 2012). Di-4-ANEPPDHQ attaches to membrane differently and its fluorescence lifetime differs from Laurdan. The acyl heads of di-4-ANEPPDHQ are inserted deep into the membrane. The presence of two positive charges reduces flip-flop movement between the inner and outer layers compared to Laurdan. The lifetime of di-4-ANEPPDHQ (1.8 ns) is shorter in the Ld phase and longer in the Lo phase (3.5 ns), compared to Laurdan.

MATERIALS AND METHODS

We have carried out optimized protocols according to Owen *et al.,* 2011 and Wheeler et al., 2011 and obtained micrographs of two cell lines with distinct differences in their plasma membrane composition (namely MDCKII and A549). MDCKII cells originate from non-cancer tissue (kidney epithelium). They form well defined tight junctions and have raft-enriched apical plasma membrane, covered with microvilli. A549 cell line originate from human lung carcinoma. Their cytoplasm is rich in organelles, lamellar bodies and active cytosis is executed. Cells were grown in CO_2 incubator Sanyo MCO – 18 AC (standard conditions – humidified atmosphere with 5% CO_2, at 37°C) in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic solution (streptomycin 100 μg/ml, penicillin 100 U/ml and amphotericin B 0.25 μg/ml). Cells were plated in 24-well plates on sterile

cover slips. Prior to microscopy, cells were incubated in serum free medium for 40 min. +Laurdan (up to 30 μM final concentration) or di-4-ANEPPDHQ (5 μM final concentration) were added to the medium. At this point some of the samples, stained with Laurdan were fixed with 4% paraformaldehyde solution. Fixation was precluded when di-4-ANEPPDHQ was applied.

For spectroscopy studies cells were washed three times with PBS and Laurdan (0.05 μM final concentration) was added to the cell suspension. After 1h of incubation in dark and under mild stirring, cells were washed with PBS and measured on highly sensitive fluorescence spectrophotometer Hitachi F-7000.

RESULTS AND DISCUSSION

Our results suggest that both of the used probes visualize Lo and Ld phases and that membranes appear more ordered in the areas where cells adhere to surface and to one another. As expected, the cancer cell line (A549) showed more fluid plasma membranes while membranes of non-cancer cells (MDCK II) were more ordered. Advantages of Laurdan are that it can be applied both on live and fixed cells and that its spectra allows easy co-localization with another fluorescent dye. Considerable drawback of Laurdan is that it requires microscope with specific parameters. Laurdan is excited at ~ 400 nm, which makes it particularly suited for multiphoton microscopy (~800 nm) as this prevents from excessive photobleaching. Here we obtained images of Laurdan stained cell membranes with standard confocal microscope (**Fig. 1 and 2**). Images of A549 cells (**Fig. 1**) - primarily the signals from both filters (for Lo and Ld phase) co-localized and were the highest intensity of the signal was detected in the cytoplasm, which confirmed our expectations that this cell line has more fluid membranes.

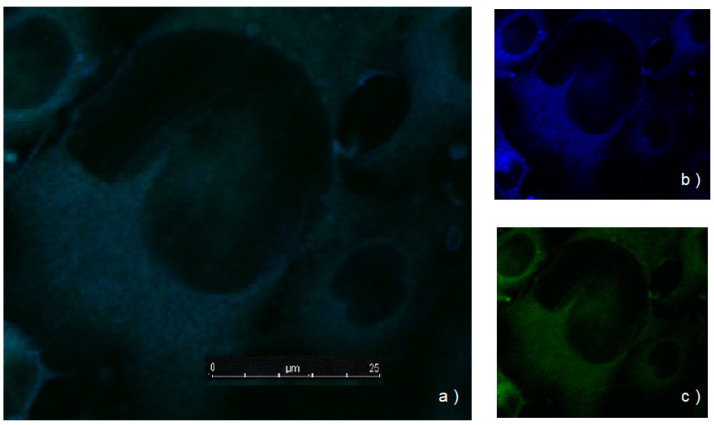

Fig. 1 A549 cells, labeled with Laurdan. a) Merged picture; b) Fluorescence from membrane areas in Lo phase; c) Fluorescence from membrane areas in Ld phase.

Images of MDCKII cells (**Fig. 2**) – shows that membranes are more ordered in the cell periphery and region of cell contacts, specific for these cells. Many raft-like structures can be found where the plasma membrane is visualized.

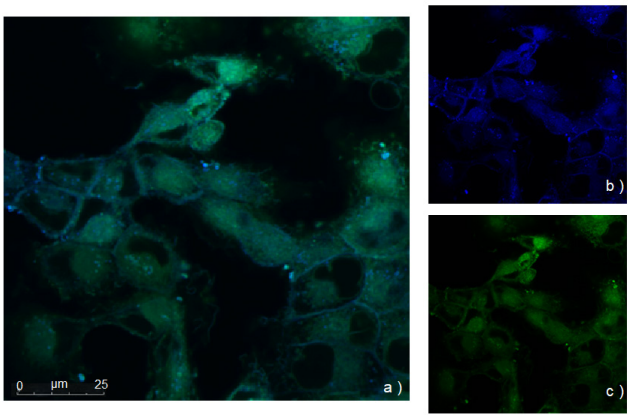

Fig. 2 MDCK II cells, labeled with Laurdan. a) Merged picture; b) Fluorescence from membrane areas in Lo phase; c) Fluorescence from membrane areas in Ld phase.

Di-4-ANEPPDHQ's advantages include more clear distinction between the signals from the two phases and compatibility with conventional confocal microscope (**Fig. 3**). Inconvenience is caused by its spectra, which makes co-localization with another fluorophore very difficult. We observed similar cell characteristics as with Laurdan, but the image is clearer.

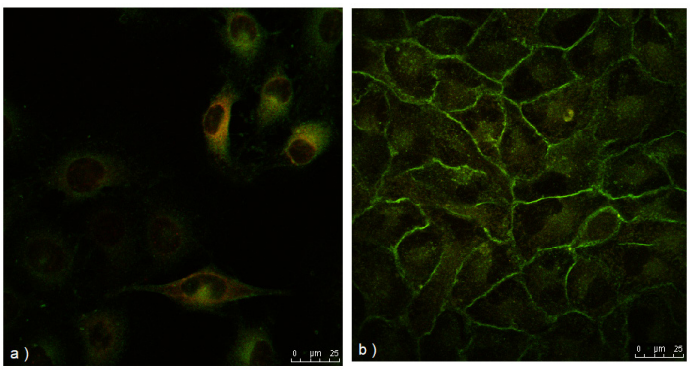

Fig. 3 Cells, labeled with di-4-ANEPPDHQ. a) A549 cells; b) MDCK II cells.

Fluorescence spectroscopy has a number of advantages for biological membrane studies. The main advantage is that this technique is highly sensitive, allowing low concentrations of reporter molecules and therefore minimal impact of the probe on the system. The speed of response is in the range of nanoseconds, which is the considered time-scale of many biochemical processes. The fluorescence signal can be characterized by several parameters. These parameters can be recorded in steady-state and time-resolved manner. The conventional detection regime is the steady-state method, when the fluorescence intensity is determined as a function of the emission wavelength. We implemented the same two cell lines (A549 and MDCKII) for the spectroscopy studies which we used for microscopy. The fluorescence spectroscopy experiments were carried out on fluorescence spectrophotometer Hitachi f7000 and were performed according to Parasassi *et. al.,* 1993. The calculated GP values also confirmed that membranes of MDCK II cells are more ordered than those of A549 cell line.

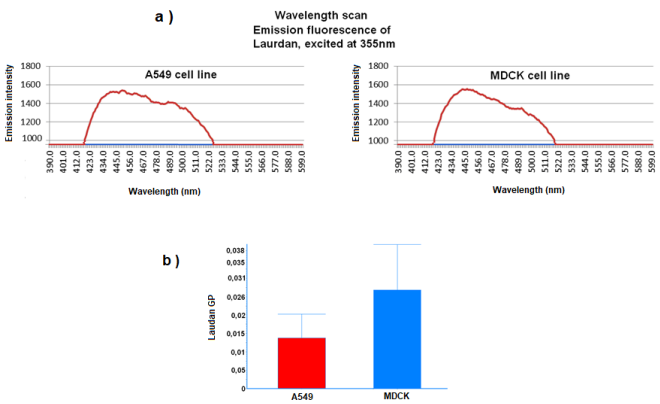

Fig. 4 Fluorescence spectroscopy. a) Wavelength scan; b) Calculated GP values.

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DECLARATION OF INTEREST STATEMENT

The authors declare no existing conflict of interest.

AUTHORS CONTRIBUTION STATEMENT

GS designed the experiments. RV performed the experiments and processed the data. TT wrote the manuscript.

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