

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**New possibilities for investigating dipole potential in
the plasma membrane and intracellular membrane
systems of living, individual cells**

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2026

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
at 13:00, on 9th October 2026

I. Preface

In recent years, the dogma of the cell membrane being a passive, separating barrier has been challenged as more emphasis is being put on the role of membrane lipids in actively modulating the structure and function of transmembrane proteins. This effect arises both from direct lipid-protein interactions and their indirect influence on the structural organization of the cell membrane. Membrane fluidity and hydration – two parameters describing bilayer structure – are widely known and extensively studied, however a third component, the dipole potential, and its biological relevance remain mostly unexplored. As a result of this paradigm shift, the dipole potential and elucidation of its biological significance became the focus of our research. The magnitude of the dipole potential is determined mainly by the lipid composition of membranes, which exhibits considerable heterogeneity both laterally within a membrane system, and intracellularly, among different organelles. Consequently, the magnitude of dipole potential is expected to vary across these domains. Furthermore, the dipole potential complements the theory of direct lipid-protein interactions by introducing an electric field-modulated, indirect dimension.

The dipole potential was originally investigated in model membranes. Later, the examination of living cells became possible with the development of special fluorophores sensitive to local microenvironmental parameters, such as the magnitude of the local electric field. Despite these advances, even the *in vitro* effects of certain membrane components (e.g. cholesterol) on the dipole potential or the characteristics of intracellular organelles have remained largely unexplored.

Experimental modification of the dipole potential poses another challenge, since the limited number of chemicals capable of altering it are normally not present in human under physiological conditions. One such exception is cholesterol, which has been shown to increase the dipole potential in model membranes, however, an alternative to decrease its magnitude remains to be described.

Quantifying the magnitude of the dipole potential in living cells also remains an unresolved issue. Individual cells cannot be examined with the “gold standard” spectrofluorimetric approach, while confocal microscopic methods are usually slow and labor-intensive. Therefore, there is a need for a high-throughput method. A flow cytometric approach would be suitable, however, to date, neither an applicable probe nor an appropriate method has been reported.

In my dissertation, through presenting the results obtained during my PhD research, I address the problems outlined above while also highlighting the importance of dipole potential as a factor fundamentally influencing cellular functions.

II. Background and Introduction

II/1. From transmembrane approach to intramembrane regions

It is indispensable to understand the organizational characteristics – such as membrane potentials – of the eukaryotic cell membrane in order to explain the processes and underlying biological mechanisms that fundamentally influence cellular functions involving membrane systems. With the continuous advancement of investigative methods, a third component, the dipole potential, has been identified in addition to the transmembrane and surface potentials.

Among the “trinity” of membrane potentials, the dipole potential is the least characterized. It arises from the non-random orientation of molecular dipoles of sterols, phospholipids and interfacial, membrane-associated water molecules. Based on measurements in model membranes and *in silico* simulations, its magnitude ranges between 150 – 450 mV in a way that the inner, hydrophobic part of the bilayer is positive relative to the aqueous phase. The gradient of the dipole potential extends over only approximately 1 nm. Consequently, the electric field it generates exceeds those produced by the transmembrane and surface potentials by 2-3 orders of magnitude.

Dipole potential – besides hydration and fluidity – may also be regarded as a property describing the structural organization of bilayers, as it is fundamentally determined by the molecular composition of the lipid bilayer and the presence and organization of water molecules. Based on theoretical considerations these three parameters describe structural order at different depths within the membrane: while hydration and fluidity report on the deeper, hydrophobic regions, dipole potential may provide information about the more superficial, interfacial part. A deeper comprehension of the dipole potential may help us better understand the organization of membranes and lipid-protein interactions.

II/2. Factors determining the magnitude of dipole potential and possibilities for its experimental increase

A key factor determining – and offering the greatest opportunity for experimental modification of – the dipole potential is the composition of the lipid membrane: particularly the types of phospho- and sphingolipids and the concentration of cholesterol and its derivatives.

Incorporating sterols in lipid membranes is the most widely used experimental method to increase the magnitude of the dipole potential. The most effective molecule used in both model membranes and *in vitro* cellular experiments is 6-ketocholestanol (6KC), a cholesterol derivative not present under physiological conditions. Treatment of cells with these

hydrophobic molecules requires special carriers – the most effective of which for this purpose are the cyclic oligosaccharide cyclodextrins. The use of sterols precomplexed with the randomly methylated derivative (methyl- β -cyclodextrin, M β CD) has proven to be the most effective method in both *in vitro* and *in vivo* experiments.

II/3. Possibilities for the experimental reduction of dipole potential

Based on previous experimental results, the effective reduction of dipole potential could be achieved primarily using the flavone derivative phloretin. Results obtained with statins blocking cholesterol synthesis were contradictory and cyclodextrins tend to induce cytotoxicity. Polyunsaturated fatty acids (PUFAs) may represent physiological alternatives. Promising candidates include ω -3 and ω -6 PUFAs, such as α -linolenic acid (ALA) and γ -linolenic acid (GLA), which generally exert effects on membrane biophysical parameters opposite to those of cholesterol. However, their effects in living cells have not yet been investigated.

II/4. Examination of the magnitude of dipole potential in living cells

Ever since its theoretical definition, determination of the dipole potential – being an intramembrane potential – has posed difficulties. Techniques applied for its quantitative characterization, such as cryo-electron microscopy, atomic force microscopy, vibrational Stark-effect spectroscopy, or molecular dynamics simulations, are either not applicable or only of limited applicability in living cells.

Voltage-sensitive fluorophores, particularly the so-called fast-response sensors whose electronic structure changes depending on the magnitude of the electric field provided a solution to this problem. The earliest described group, the 4-p-aminostyryl-1-pyridinium derivatives functions on the basis of the electrochromic – also known as Stark – effect, which is associated with alterations in electronic structure, resulting in changes in both the excitation and emission spectra of the molecule. The most prominent member of this family, di-8-ANEPPS, a fluorophore widely used for measuring dipole potential, can be applied in an excitation-ratiometric procedure: by exciting the molecule at two different wavelengths and detecting emitted photons within the same wavelength range, the ratio of fluorescence intensities measured under these two conditions shows strong correlation with the magnitude of the dipole potential.

Recently characterized 3-hydroxyflavone (3-HF) derivatives offer another approach for determining dipole potential. Their biophysical basis lies in the so-called Excited State Intramolecular Proton Transfer (ESIPT) reaction occurring in the excited state of the

fluorophore. As a result of the ESIPT reaction, two excited states are formed: the normal (N^*) and tautomeric (T^*) forms, whose relative populations are fundamentally determined by the local electric field surrounding the molecule and thus by the magnitude of dipole potential. Accordingly, two well-separated emission bands can be observed in the emission spectrum, allowing the fluorophore to be used for determining the dipole potential via emission-ratiometric measurements. Specifically, the magnitude of dipole potential correlates negatively with the N^*/T^* ratio and positively with the T^*/N^* ratio.

F66 may be suitable for flow-cytometric measurement techniques, although this possibility has not previously been investigated. Furthermore, owing to its favorable spectral properties, the molecule may be combined with other conventional fluorophores.

II/5. Differences in the lipid composition and the magnitude of dipole potential of cellular membranes

Research concerning dipole potential—and membrane biophysical parameters in general—in living cells has so far focused primarily on the plasma membrane, whereas considerably less is known about the lipid bilayers of intracellular organelles and their structural organization. Numerous studies have suggested that membrane lipid compositions of organelles involved in secretory and endocytic pathways, as well as those of mitochondrial membranes, may differ markedly from one another. However, very little is known about whether these differences manifest as functionally significant variations in the biophysical properties of these membranes in living, intact cells. Moreover, comparative analyses of these features among different organelles have not yet been performed in living cells.

II/6. The biological relevance of dipole potential and the cell-penetrating peptides

In order to understand the precise pathomechanisms and identify potential therapeutic targets in disorders associated with altered membrane lipid composition – such as various forms of hypercholesterolemia, Smith-Lemli-Opitz syndrome characterized by reduced cholesterol but elevated 7DHC levels, neurodegenerative diseases, certain tumors, or Gaucher disease – it is first necessary to identify the interactions of disease-relevant molecules. Increasing evidence supports the pathophysiological significance of altered membrane biophysical properties arising from changes in membrane composition, primarily through influencing the structure and function of transmembrane proteins and thereby modulating various cellular functions.

The dipole potential, localized within the intramembrane region, may influence protein function mainly through its effect on transmembrane domains, particularly on charged and polar amino

acids, thereby modulating the stability of different conformational states and ultimately the functional activity of proteins.

Since its first description nearly 50 years ago, numerous studies have emphasized the biological relevance of dipole potential in proteins possessing transmembrane domains. Early studies described the effect of dipole potential on the association and conductance of various bacterial ionophores. Molecular dynamics simulations demonstrated its influence on the gating, activation, and deactivation processes of the hERG potassium channel, while investigations of small-molecule channel modulators revealed that the efficacy of the compounds is determined by dipole moment and thus – presumably – by the magnitude of their effect on dipole potential. Consistent with these findings, our research group recently demonstrated that modulation of dipole potential may contribute to the inhibitory effects of cholesterol and 7DHC on the gating of the voltage-gated potassium channels K_V1.3 and K_V10.1. Furthermore, distinct alterations in dipole potential may explain why ceramide and glucosylceramide inhibit K_V1.3 gating and voltage sensitivity through different mechanisms and at distinct intramolecular target sites.

The dipole potential may also modulate the function of active transporters. In the case of one of the most extensively studied members of this protein family, the Na⁺/K⁺ ATPase, a positive correlation was found between the magnitude of dipole potential and protein activity. Several studies examining P-glycoprotein (Pgp), an active transporter responsible for chemotherapy resistance in tumors, also demonstrated a positive correlation between transport activity and the magnitude of dipole potential.

The significance of dipole potential has also been demonstrated for various transmembrane receptors that are central to signal transduction processes. For example, increasing dipole potential enhances dimerization and clustering of ErbB proteins and consequently activates signaling pathways important in tumor proliferation, whereas decreasing dipole potential exerts distinct effects.

The magnitude of dipole potential has also previously been demonstrated to modify the membrane binding and bilayer permeation efficiency of drug molecules, β -amyloid, and other peptides. Among the latter, particular attention has been devoted to cell-penetrating peptides (CPPs), which may be promising alternatives for the non-toxic intracellular transport of otherwise membrane-impermeable compounds, including various drugs, peptides, DNA, and RNA. Both the direct permeation and endocytic mechanisms of CPP-mediated delivery involve interactions with cellular membranes. Consequently, the magnitude of dipole potential and the associated electric field may influence the efficiency of these processes. Our research group recently demonstrated that the decrease in dipole potential induced by phloretin or the

cholesterol-lowering agent atorvastatin results in increased cellular uptake of CPPs and particularly through their enhanced escape from the endolysosomal system.

Based on these observations, it can reasonably be assumed that a more detailed characterization of the relationship between CPP uptake and dipole potential, as well as the identification of novel physiological compounds capable of reducing dipole potential, may contribute to the development and optimization of therapeutic approaches and protocols that enhance CPP entry and ultimately improve the efficacy of the therapeutic molecules they deliver.

III. Objectives

As discussed in the Introduction, the investigation of dipole potential – particularly in living cells – is greatly hindered by the limited range of available methodologies.

In order to facilitate basic research, and, hopefully, to extend the analysis of clinical samples as well, we aimed to develop and optimize a novel flow-cytometric measurement method based on the voltage-sensitive fluorophore F66 that possesses favorable spectral properties. This method would enable the examination of large numbers of living, individual cells, as well as the discrimination of potential subpopulations within samples. To support the general applicability of our method, we additionally aimed to validate it in several different cell lines and to demonstrate that it indeed primarily quantifies the magnitude of dipole potential.

To understand the role of dipole potential in various pathological conditions, it is essential first to identify molecules that can change its magnitude. To our knowledge, no physiological compound capable of reducing dipole potential has yet been described; therefore, another objective of our work was to identify a suitable treatment modality for this purpose.

Cell-penetrating peptides (CPPs), although still relatively poorly characterized, represent highly promising tools for molecular therapy, since they enable the delivery of various molecules – otherwise impermeable to the plasma membrane – into the cytosol or different intracellular organelles. To the best of our knowledge, only a few studies have investigated the effect of dipole potential on the uptake of these peptides; however, previous findings have already suggested that its experimental reduction may facilitate cellular CPP uptake. The effects of fatty acids in this context remain completely unknown, and thus our goal was to explore the possible beneficial effects of these lipids.

Differences in the membrane composition of various cellular organelles are well characterized in many cases; however, in living cells the effects of these differences on the molecular organization of bilayers remain largely unexplored. One reason for this is the lack of techniques suitable for the comparative investigation of membrane properties among intracellular membranes. Therefore, another objective of our work was to optimize a novel and easily applicable method that would enable comparative analysis of the bilayers of the plasma membrane and various intracellular organelles in intact, living cells. Such a method would make it possible to reveal differences between individual compartments and, in the future, their alterations arising under various pathological conditions.

IV. Materials and methods

IV/1. Cell lines

All cell lines used in our work, including the JY Epstein-Barr virus-transformed human lymphoblastoid cells, THP-1 human monocytic cells, HEK-293T adenovirus-immortalized human embryonic kidney cells transfected with the gene encoding the SV40 T antigen, SKBR-3 human breast adenocarcinoma cells, HeLa human cervical adenocarcinoma cells, and SH-SY5Y human neuroblastoma-derived cells, were cultured according to the manufacturer's instructions.

For flow-cytometric and spectrofluorimetric experiments, the initial cell numbers were selected such that at least 150,000 cells remained in each sample after the expected losses during the washing procedures. For confocal microscopic measurements, cells were grown in 8-well chambered coverglass until approximately 80% confluence was reached.

IV/2. Compounds and reagents

To modify the dipole potential of cells, 6-ketocholestanol and phloretin were used, together with the non-ionic detergent Pluronic F-127 to facilitate their rapid incorporation into the plasma membrane. To alter the sterol content of the plasma membrane, complexes of 6KC, cholesterol, and 7DHC with M β CD were applied, with the relative sterol content being approximately 5% in each complex. To study the effects of fatty acids, saturated stearic acid (SA), polyunsaturated ω -3 α -linolenic acid (ALA), and polyunsaturated ω -6 γ -linolenic acid (GLA) were used.

The magnitude of dipole potential was measured using the fluorophores di-8-ANEPPS and F66. Plasma membrane fluidity was investigated with TMA-DPH, while membrane hydration was determined using the PY3174 fluorophore. For viability assays, cells were labeled with Sytox Green and Alexa Fluor 647-conjugated annexin V markers. Intracellular organelles were visualized using CellLight™ ER-GFP, BacMam 2.0, CellLight™ Golgi-RFP, BacMam 2.0, LysoTracker™ Deep Red, and MitoTracker™ Deep Red FM. Calibration pH solutions were prepared using valinomycin and nigericin.

IV/3. Modification of plasma membrane composition and dipole potential

During our experiments, in order to validate our novel measurement method and ensure comparability with previously published results, the dipole potential of THP-1 and JY cell membranes was increased using 6KC, whereas phloretin was used for its reduction at various

concentrations. To accelerate the incorporation of these compounds into the plasma membrane and thereby shorten incubation times, treatments were performed in the presence of the detergent Pluronic F-127. Based on our previous measurements, the compounds at the applied concentrations did not substantially affect either cell viability or the resting transmembrane potential.

To modify the sterol content of plasma membranes, cells were incubated for 60 minutes at room temperature in normal Ringer solution with M β CD complexes containing 6KC, cholesterol (CHOL), or 7-dehydrocholesterol (7DHC) at various concentrations. As controls, cells were treated with equivalent concentrations of “empty,” i.e., sterol-free, M β CD.

To investigate the effects of different fatty acids, THP-1 and JY cells were incubated for 48 hours in media containing saturated SA, polyunsaturated ω -3 ALA, or polyunsaturated ω -6 GLA at various concentrations.

IV/4. Determination of plasma membrane dipole potential using di-8-ANEPPS and spectrofluorometry

Untreated and treated THP-1 and JY cells were labeled with di-8-ANEPPS on ice following washing procedures, and after additional washing the samples were warmed to 37°C prior to measurements. Measurements were performed using a Fluorolog-3 spectrofluorometer while sample cuvettes were maintained at a constant temperature using a 37°C water bath. To detect changes in the dipole potential, excitation spectra of the probe were recorded. During measurements, samples were excited within a 5 nm bandwidth while the excitation wavelength was continuously varied between 380 and 550 nm in 1 nm increments, whereas emission was detected at a constant wavelength of 660 nm. According to literature data, the magnitude of dipole potential positively correlates with the ratio of the integrated fluorescence intensities detected following excitation between 410–440 nm and 490–520 nm.

IV/5. Determination of plasma membrane dipole potential using F66 and flow cytometry

Untreated and treated THP-1 and JY cells were labeled with F66 on ice following washing procedures, and after washing the samples were warmed to 37°C immediately before measurements. Fluorescence intensities of individual cells were analyzed at 37°C using a FACS Aria III flow cytometer. Samples were excited using a 405 nm laser, while the fluorescence emitted by F66 was detected using 480/20 nm and 585/42 nm bandpass filters corresponding to the fluorophore’s normal (N*) and tautomeric (T*) excited states, respectively. Data analysis was performed using FCS Express software. To determine dipole potential, cells exhibiting

normal morphology and considered viable were selected by gating on FSC-SSC density plots. Subsequently, the N*/T* emission ratio was calculated for individual cells, and the mean ratio value was determined for each sample. Based on previous data, the N*/T* emission ratio negatively correlates with the magnitude of dipole potential.

IV/6. Examination of cell viability

To investigate the possible cytotoxicity of the treatments used to modify dipole potential and plasma membrane composition, treated and untreated THP-1 and JY cells were labeled at room temperature in annexin-binding buffer using Sytox Green and Alexa Fluor 647-conjugated annexin V markers. Individual cells were then analyzed using a NovoCyte 3000RYB flow cytometer. For excitation and fluorescence detection, a 488 nm laser and a 530/30 nm bandpass filter were used for Sytox Green, whereas a 640 nm laser and a 660/20 nm bandpass filter were applied for Alexa Fluor 647-annexin V. Data analysis was also performed using FCS Express. In each sample, the proportion of viable cells negative for both Sytox Green and annexin V staining was calculated relative to the total cell population.

IV/7. Determination of plasma membrane fluidity using TMA-DPH and spectrofluorometry

We utilized the most widely accepted method in the literature to determine plasma membrane fluidity. Untreated and treated THP-1 and JY cells were suspended in Hank's buffer after washing, labeled with TMA-DPH at room temperature, and then, without further washing, warmed to 37°C immediately prior to measurement. Measurements were performed using a Fluorolog-3 spectrofluorometer, with sample cuvettes maintained at a constant temperature using a 37°C water bath.

The fluorescence anisotropy (r) of TMA-DPH was calculated using the following equation after excitation at 352 nm and detection at 430 nm:

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}$$

where I_{vv} represents the vertical component and I_{vh} the horizontal component of fluorescence following excitation with vertically polarized light. G is an instrument-specific correction factor used to compensate for differences in detector sensitivity toward horizontally and vertically polarized photons and is calculated according to the following formula:

$$G = \frac{I_{hv}}{I_{hh}}$$

where I_{hv} and I_{hh} represent the vertical and horizontal fluorescence intensities, respectively, following excitation with horizontally polarized light.

IV/8. Determination of plasma membrane hydration using PY3174 and confocal microscopy

To determine plasma membrane hydration, untreated and treated THP-1 and JY cells were labeled with the PY3174 fluorophore at room temperature following washing procedures. After washing, confocal images corresponding to the midplane of the cells were acquired using an LSM880 confocal laser-scanning microscope.

Excitation was performed using a 488 nm laser, while emission was detected in two wavelength ranges: between 500 and 540 nm (I_{blue}) and between 630 and 735 nm (I_{red}). Quantitative image analysis was performed using Matlab software.

During image processing, plasma membrane regions were identified using a custom watershed algorithm previously developed by our research group. Using exclusively the data from these pixels, the generalized polarization (GP) value, which is inversely proportional to membrane hydration, was calculated for individual pixels using the following equation:

$$GP = \frac{I_{blue} - I_{red}}{I_{blue} + I_{red}}$$

where I_{blue} and I_{red} are the background-corrected fluorescence intensities measured in plasma membrane pixels within the wavelength ranges defined above. Subsequently, the mean GP value was calculated from the plasma membrane pixel data for each image.

IV/9. Synthesis and fluorophore labeling of penetratin

Penetratin was manually synthesized by our collaborating partner, Dr. István Mándity (Semmelweis University, Budapest, Hungary), on TentaGel R RAM polystyrene PEG copolymer resin using standard solid-phase Merrifield Fmoc technique.

Following conjugation of the final arginine residue, the synthesized penetratin was conjugated with either AFDye532 N-hydroxysuccinimide ester or 5(6)-carboxynaphthofluorescein (NF) N-succinimidyl ester. Completion of the reaction was verified using Kaiser test, after which the compound was cleaved from the resin.

Following filtration and precipitation with cold diethyl ether, the crude product was purified by preparative reverse-phase HPLC using a C18 column and subsequently lyophilized. The purity of the resulting final product was assessed by reverse-phase HPLC on an analytical C18 column and was found to exceed 95%. The presence of labeled peptides was confirmed by Bruker electrospray ionization mass spectrometry.

IV/10. Investigation of penetratin uptake and endolysosomal release using flow cytometry

To investigate penetratin uptake and endolysosomal release, THP-1 and JY cells were incubated in media containing SA, ALA, or GLA and subsequently analyzed using a FACS Aria III flow cytometer. Immediately before measurement, 5 μ M AFDye532-penetratin and 5 μ M NF-penetratin were added to the cells, and fluorescence intensity measurements of individual cells were initiated immediately.

Excitation wavelengths and detection ranges were 488 nm excitation and 530/30 nm detection for AFDye532-penetratin, and 561 nm excitation and 670/14 nm detection for NF-penetratin. For all samples, measurements began immediately after addition of penetratins and continued for 15 minutes at a constant temperature of 37°C, maintained by a temperature-controlled sample holder.

Data analysis was initially performed using FCS Express software. Time-correlated fluorescence intensity data from viable cells showing normal morphology were exported, and moving averages using a 10-second time window, as well as their ratios were calculated using a custom Matlab algorithm.

AFDye532-penetratin provides information regarding the total amount of penetratin internalized by the cells, whereas NF-penetratin indicates the amount of penetratin released from the endolysosomal system into a non-acidic environment. Consequently, the NF/AFDye532 ratio reflects the extent of endolysosomal escape.

During analysis, fluorescence intensities were normalized to the values measured in the first time window of the control samples, after which fluorescence intensity ratios were calculated from the normalized values.

IV/11. Labeling of cells with intracellular organelle markers and the dipole potential-sensitive F66 fluorophore

For labeling of the endoplasmic reticulum and Golgi apparatus, SKBR-3, HeLa, and SH-SY5Y cells grown on 8-well chambered coverglass were transduced with CellLight™ ER-GFP, BacMam 2.0 and CellLight™ Golgi-RFP, BacMam 2.0 reagents, respectively. Lysosomes were visualized using LysoTracker™ Deep Red, while mitochondria were labeled with MitoTracker™ Deep Red FM.

To determine the dipole potential in membranes of intracellular organelles a dual-labeling protocol was employed. Cells were incubated for 24 hours with 10 nM F66 added to the culture medium to ensure appropriate accumulation of the fluorophore within intracellular membranes.

Subsequently, to enable unequivocal identification of the plasma membrane, an additional 10 nM F66 was added during the final 10 minutes of incubation.

For the analysis of the pH dependence of F66 spectral properties, the latter labeling procedure and confocal imaging were performed in calibration buffer solutions with pH values between 5.5 and 7.5 containing 10 μ M valinomycin and 10 μ M nigericin. These two compounds were used to equilibrate extracellular and intracellular pH.

IV/12. Determination of dipole potential in cellular membranes using F66 and confocal microscopy

Confocal images corresponding to the midplane of treated and labeled SKBR-3, HeLa, and SH-SY5Y cells were acquired using an LSM880 confocal laser-scanning microscope. For dipole potential determination, the F66 fluorophore was excited at 405 nm, and emission was detected within the ranges of 463–527 nm and 543–589 nm, corresponding to the normal (N*) and tautomeric (T*) excited states of the dye, respectively. In the same cells, the GFP, RFP, and Deep Red fluorophores used for organelle identification were sequentially excited at 488 nm, 543 nm, and 633 nm, respectively, while emission was detected within the ranges of 495–550 nm, 570–640 nm, and 650–735 nm, respectively.

Quantitative analysis of the acquired images was performed using Matlab software. Plasma membrane pixels and an intracellular region mask were identified using the previously mentioned manually guided watershed algorithm developed by our research group for each cell. For organelle identification, fluorescence intensities of the corresponding markers within intracellular pixels were analyzed. Using a so-called “maximum entropy” algorithm followed by manual verification, a threshold value was determined above which pixels were assigned to the investigated organelle. After subtraction of background intensities, the F66 T*/N* ratio – positively correlating with the magnitude of dipole potential – was calculated for each pixel. The median ratio value was then determined separately for each individual cell using the pixels corresponding to the plasma membrane and the membrane of the respective organelle.

To analyze relative differences between intracellular regions, we calculated the normalized value of the median F66 T*/N* emission ratio in organelle membranes relative to the median ratio determined for the plasma membrane of the same cell.

IV/13. Statistical analysis

Data obtained from at least five independent experiments (n) were analyzed in spectrofluorimetric and flow-cytometric measurements. In flow-cytometric experiments, at

least 20,000 cells per sample were analyzed in each independent experiment, while spectrofluorimetric samples contained an average of 150,000 cells. In experiments using sterol-M β CD complexes, values were normalized to samples treated with equivalent amounts of native M β CD, whereas in experiments involving fatty acids, normalization was performed relative to ethanol-treated controls. For confocal microscopic analysis of PY3174 generalized polarization, mean generalized polarization values from n=20 individual images obtained in five independent experiments were analyzed for each treatment. Each image contained data from 10–15 cells with normal morphology, corresponding to a total of 200–300 cells per treatment group. For the investigation of dipole potential in cellular membranes, median F66 T*/N* emission ratio values from n=107–154 individual cells with normal morphology obtained from five independent experiments were analyzed.

During statistical analysis, p values were generally calculated using Tukey's HSD test when preliminary ANOVA analysis revealed significant differences among groups. For comparisons of F66 ratios between the plasma membrane and the bilayers of intracellular organelles, p values were calculated using Welch's t-test. Differences were considered statistically significant when p<0.05. Statistical analyses were performed using GraphPad Prism software.

V. Results and discussion

V/1. Effects of phloretin and 6-ketocholestanol on the dipole potential

To develop and validate our novel flow-cytometric method for measuring dipole potential, we used the di-8-ANEPPS fluorophore as a reference, as it is the most commonly applied probe for this purpose in the literature. We utilized a widely accepted spectrofluorimetric method together with the compounds most frequently used to modify dipole potential, phloretin and 6KC. Incorporation of these molecules into the membrane was facilitated by incubation in the presence of Pluronic F-127, a hydrophilic, non-ionic detergent.

In accordance with previous literature data, our measurements showed that 6KC dose-dependently increased the ratio of integrated fluorescence intensities measured in the lower- and higher-wavelength regions of the excitation spectrum, namely 410–440 nm and 490–520 nm, respectively ($R_{\text{exc, di-8-ANEPPS}}$), indicating an increase in dipole potential in both examined cell lines, THP-1 and JY. Phloretin exerted the opposite effect by dose-dependently decreasing the excitation ratio; however, although the differences proved statistically significant, they were considerably smaller than those induced by 6KC.

To support the applicability of the F66 fluorophore, THP-1 and JY cells were treated with different concentrations of 6KC and phloretin as described above. Following labeling, the ratio of fluorescence intensities measured by flow cytometry in the ranges corresponding to the normal N^* and tautomeric T^* excited states of the fluorophore, 470–490 nm and 564–606 nm, respectively, was determined ($R_{\text{em, F66 } N^*/T^*}$). This ratio is inversely proportional to the magnitude of dipole potential.

In accordance with our previous results obtained using di-8-ANEPPS, we found that 6KC dose-dependently and significantly decreased $R_{\text{em, F66 } N^*/T^*}$ in both cell lines, indicating an increase in dipole potential. Although phloretin increased $R_{\text{em, F66 } N^*/T^*}$ in both cases, corresponding to a decrease in dipole potential, the change was not statistically significant in JY cells.

V/2. Effects of different sterol derivatives on the dipole potential

The magnitude of dipole potential is fundamentally influenced by the concentration of sterols, primarily cholesterol, present in the plasma membrane. Therefore, it was logical to validate our method using M β CD complexes of different sterols: 7DHC, CHOL, and 6KC. Based on data obtained mainly from model membrane studies, all three sterols are capable of increasing dipole potential.

In agreement with these literature data, our spectrofluorometric measurements showed that all three sterols dose-dependently increased the $R_{\text{exc, di-8-ANEPPS}}$ value, i.e., the magnitude of dipole potential. The most effective derivative was 6KC, followed by CHOL and then 7DHC. Identical results were obtained in both THP-1 and JY cells.

Following the same treatments, we also performed measurements by flow cytometry following labeling with F66. In both cell lines, fully consistent with the results obtained using di-8-ANEPPS, all sterol derivatives dose-dependently increased dipole potential, as reflected by the decrease in $R_{\text{em, F66 N}^*/\text{T}^*}$. The order of efficacy of the sterols was also identical.

V/3. Effects of different fatty acids on the dipole potential

In THP-1 and JY cells, we investigated the changes induced by the polyunsaturated ω -3 fatty acid ALA, the ω -6 fatty acid GLA, and their saturated analogue, SA. Cells were incubated for 48 hours in media containing the respective fatty acids at different concentrations, after which dipole potential was determined using both the reference spectrofluorometric method and the novel flow-cytometric procedure.

We found that ALA dose-dependently decreased dipole potential, as indicated by a reduction in $R_{\text{exc, di-8-ANEPPS}}$, in both THP-1 and JY cell lines. In contrast, slightly increased $R_{\text{exc, di-8-ANEPPS}}$ values were observed in the case of SA, which may indicate an increase in dipole potential; however, the observed changes were not statistically significant in either cell line. Contrary to our expectations, GLA significantly increased $R_{\text{exc, di-8-ANEPPS}}$, and thus dipole potential, in both THP-1 and JY cells.

We also examined the effects of the above fatty acid treatments using our newly developed flow-cytometric method. The results were consistent with those obtained using the reference method in both cell lines. In both THP-1 and JY cells, ALA dose-dependently and significantly increased $R_{\text{em, F66 N}^*/\text{T}^*}$, indicating a decrease in dipole potential, whereas GLA and SA exerted the opposite effect.

V/4. Effects of sterols and fatty acids on cell viability, plasma membrane fluidity, and hydration

To exclude the possibility that the observed changes in the F66 N^*/T^* emission ratio were due to cellular damage, we tested the effects of the abovementioned treatments on cell viability using Sytox Green as a necrosis marker and Alexa Fluor 647-conjugated annexin V as an apoptosis marker. Based on our flow-cytometric results, the treatments used to modify plasma

membrane composition did not affect the proportion of viable, double-negative cells, even at the highest concentrations, in either THP-1 or JY cells.

We also investigated whether treatment-induced changes in membrane fluidity could influence the alterations observed in the F66 N*/T* emission ratio. To examine this, control and treated THP-1 and JY cells were labeled with TMA-DPH, and the fluorescence anisotropy of the dye was determined by spectrofluorometry. This parameter is inversely proportional to membrane fluidity. In both cell lines, we found that at the maximum applied doses CHOL and 7DHC significantly increased anisotropy, indicating decreased fluidity, whereas the difference observed in response to 6KC was not significant. Thus, the relative magnitude of these changes differed substantially from that observed when examining the effects of the compounds on dipole potential. Among the fatty acids, ALA significantly decreased anisotropy, whereas neither SA nor GLA altered it significantly.

We also examined the effects of the treatments on membrane hydration. For this purpose, cells were labeled with the PY3174 fluorophore, which, based on literature data, is sensitive to the degree of local hydrophobicity and, owing to its favorable spectral properties, is reliably suitable for measurements using conventional imaging techniques. Using confocal microscopy and quantitative image analysis, we quantified the generalized polarization of the dye, which is proportional to the hydrophobicity of the local microenvironment and thus negatively correlates with the degree of hydration. The measurements yielded consistent results in both cell lines. Sterol derivatives increased generalized polarization, most strongly in the case of 6KC, to a lesser extent with CHOL, and least with 7DHC. Among the fatty acids, SA caused a slight positive shift, whereas both GLA and ALA exerted the opposite effect, increasing membrane hydration. All differences described above were statistically significant, except for the effects induced by 7DHC and SA in JY cells.

V/5. Correlation between changes induced by plasma membrane composition-modifying treatments in the spectral parameters of the environment-sensitive fluorophores

To support the applicability of our newly described flow-cytometric procedure for measuring dipole potential, we compared the results obtained from the analysis of the F66 N*/T* emission ratio after different treatments with those obtained using the reference spectrofluorometric method quantifying the excitation ratio of the di-8-ANEPPS fluorophore. The R^2 and p values obtained by linear regression analysis indicated a very strong, positive, statistically significant correlation between the two methods in both examined cell lines. This confirmed the applicability of our newly developed method for determining the magnitude of dipole potential.

Our findings were further supported by analysis of the relationship between the F66 N*/T* emission ratio and TMA-DPH fluorescence anisotropy, as no substantial correlation was found between these two parameters. This clearly argues against a major role for changes in membrane fluidity in the alterations of the F66 emission spectrum induced by treatments that modify plasma membrane composition.

When investigating the relationship between dipole potential and membrane hydration, a strong and statistically significant positive correlation was found between the F66 N*/T* emission ratio and PY3174 generalized polarization. This supports the close relationship between these two membrane biophysical properties, as also suggested by previous studies.

V/6. Effects of fatty acid pretreatments on the cholesterol-induced increase in dipole potential

We examined the combined effects of cholesterol and fatty acids on the magnitude of dipole potential using our flow-cytometric method. As a pretreatment, cells were incubated for 48 hours in media enriched with ALA, GLA, or SA, followed by treatment with CHOL.

Based on our results, pretreatment with ALA significantly attenuated the dipole potential-increasing effect of cholesterol in both examined cell types. In contrast, both SA and GLA enhanced the dipole potential-increasing effect of CHOL treatment, although in these cases the changes did not always reach statistical significance.

V/7. Effects of fatty acids on the cellular uptake and endolysosomal release of penetratin

Cell-penetrating peptides (CPPs) represent a promising therapeutic approach for delivering otherwise membrane-impermeable molecules into the cytosol and intracellular compartments. Both mechanisms of CPP entry into the cytosol and intracellular compartments – direct translocation across the plasma membrane, as well as endocytosis followed by endolysosomal escape – involve electrostatic interactions with membranes. Consequently, the efficiency of both pathways may be influenced by the dipole potential and changes in its magnitude.

We investigated the effects of ALA, GLA, and SA on the uptake and endolysosomal release of penetratin, a cationic CPP. Flow-cytometric measurements revealed that none of the investigated fatty acids significantly affected the normalized fluorescence intensity of AFDye532-conjugated penetratin, i.e., the total cellular penetratin uptake, compared to control cells. In contrast, treatment with the ω -3 polyunsaturated fatty acid ALA – and the resulting decrease in dipole potential – significantly increased the fluorescence intensity of the pH-sensitive NF-conjugated penetratin in the cells, indicating enhanced penetratin entry into the

cytoplasm. Conversely, the fully saturated SA and the ω -6 polyunsaturated GLA, both of which increase dipole potential, did not substantially alter this parameter.

In parallel, the NF/AFDye532 ratio, reflecting penetratin endolysosomal escape, was significantly increased by ALA, whereas neither SA nor GLA exerted a significant effect on this parameter. These findings confirm that the magnitude of the dipole potential influences the efficiency of CPP uptake primarily through enhancement of endolysosomal escape, and they emphasize both the dipole potential-reducing effect of ω -3 polyunsaturated fatty acids and its significance in cellular processes.

V/8. Heterogeneity of the dipole potential in intracellular membrane systems

The heterogeneity in the lipid composition of membrane systems in living cells would be expected to cause differences in the dipole potential as well. To support this assumption, we first examined cellular membranes in SKBR-3 cells using confocal laser-scanning microscopy and quantitative image analysis, employing the F66 fluorophore – which had proven to be a reliable method for dipole potential determination in the previous experiments – together with a dual-labeling protocol.

Our results demonstrated that the F66 T*/N* emission ratio, and thus the magnitude of dipole potential, exhibited substantial variability among cellular membranes. Specifically, significantly higher values were observed in the plasma membrane and in membranes located in the peripheral regions of the cell compared to more internal membranes.

Furthermore, analysis of the distribution of dipole potential values revealed markedly greater heterogeneity within intracellular membranes than in the plasma membrane, suggesting the presence of substantial differences among the bilayers of distinct organelles.

V/9. The dipole potential in lysosomal membranes

In the first part of our investigation of organelle-specific differences, we determined the dipole potential of lysosomal membranes relative to that of the plasma membrane. Simultaneously with dual labeling using F66, cells were incubated with LysoTracker™ Deep Red for visualization of lysosomes.

During analysis of confocal images, lysosomal membrane pixels were defined as those located within the intracellular mask and exceeding a fluorescence intensity threshold determined in the organelle-marker image. We then calculated the median F66 T*/N* emission ratio, which positively correlates with the magnitude of the dipole potential, separately for plasma membrane pixels and lysosomal membrane pixels within the same cell.

In SKBR-3 human breast tumor cells, the F66 T*/N* ratio calculated for lysosomes was significantly lower than that of the plasma membrane, indicating lower dipole potential in lysosomal membranes. Although it seemed plausible that this difference between plasma and lysosomal membranes could represent a general feature across different cell types, we performed similar measurements in two substantially different cell lines to support this assumption. Fully analogous results were obtained in both HeLa human cervical carcinoma cells and SH-SY5Y human neuroblastoma cells.

V/10. The dipole potential in the Golgi membrane system

We continued the investigation of intracellular membrane systems by examining the membranes of the Golgi apparatus. For this purpose, cells were transduced with CellLight™ Golgi-RFP, BacMam 2.0 in parallel with dual labeling using F66.

Confocal laser-scanning microscopy images were acquired in the emission ranges corresponding to the N* and T* states of the F66 fluorophore, as well as using settings appropriate for the RFP tag labeling the Golgi membranes. Plasma membrane and Golgi membrane regions were identified as described in detail for lysosomal analysis.

Measurements were performed in the same three cell lines used for lysosomal studies (SKBR-3, HeLa, and SH-SY5Y), and in all three cases we found that the dipole potential was substantially lower in Golgi membranes than in the plasma membrane.

V/11. The dipole potential in endoplasmic reticulum membranes

Next, we continued our experiments by examining the membrane of the endoplasmic reticulum, another component of the secretory pathway. In parallel with dual labeling using F66, cells were transduced with CellLight™ ER-GFP, BacMam 2.0.

As in the investigation of Golgi membranes, confocal laser-scanning microscopy images were acquired in the emission ranges corresponding to the N* and T* states of the F66 fluorophore, together with settings appropriate for the GFP tag labeling the endoplasmic reticulum membrane. Plasma membrane and endoplasmic reticulum membrane regions were identified as previously described.

Our measurements demonstrated that the F66 T*/N* ratio calculated for endoplasmic reticulum membranes was significantly lower than that of the plasma membrane, indicating lower dipole potential in all three cell lines examined: SKBR-3, HeLa, and SH-SY5Y cells.

V/12. The dipole potential in mitochondrial membranes

As the final step of our experimental series, mitochondrial membranes were also investigated. In parallel with dual labeling using F66, cells were incubated with MitoTracker™ Deep Red in a manner analogous to that used for lysosomes. Confocal laser-scanning microscopy images were acquired at the midplane of the cells using settings corresponding to the N* and T* states of F66 as well as to the Deep Red fluorophore labeling mitochondrial membranes.

Regions corresponding to plasma membranes and mitochondrial membranes were identified as described above. Similar to our previous observations, these measurements also revealed significantly lower F66 T*/N* ratios in mitochondrial membranes than in plasma membranes in all investigated cell lines, including SKBR-3, HeLa, and SH-SY5Y cells, indicating lower dipole potential in mitochondrial membranes.

V/13. Comparison of the magnitudes of dipole potential among intracellular organelle membranes

To compare dipole potential magnitudes among the membranes of the investigated organelles, median F66 T*/N* ratios calculated for organelle membranes were normalized to the median values calculated for the plasma membrane of the same cell. This normalization eliminated differences between measurements and cells, thereby enabling determination of relative differences among compartments.

Using this analysis method, we found that dipole potential exhibited an inward-decreasing gradient along the secretory/endocytic pathway from the plasma membrane toward the endoplasmic reticulum membrane. The highest F66 T*/N* emission ratio, and thus the highest dipole potential, was observed in the plasma membrane. This value decreased along the lysosome > Golgi > endoplasmic reticulum pathway in all investigated cell types. Differences among organelles proved statistically significant in all three cell lines, with one exception: the difference between endoplasmic reticulum and Golgi membranes in HeLa cells.

The magnitude of the F66 T*/N* ratio measured in mitochondrial membranes was similar to that of lysosomal membranes in SKBR-3 and HeLa cells, whereas in SH-SY5Y cells it was significantly higher.

V/14. pH dependence of the F66 T*/N* emission ratio

Considering the operating principle of F66 (excited-state intramolecular proton transfer reaction), it was possible that the differences described above in the T*/N* emission ratio of the fluorophore – particularly in acidic lysosomes – might be attributable to pH sensitivity of

the fluorophore. To test this possibility, SKBR-3 cells were treated with calibration buffers of different pH values in the presence of valinomycin and nigericin, ensuring equilibration of pH between the extracellular calibration solutions and intracellular compartments. Cells were simultaneously labeled with F66, after which confocal microscopic images were acquired at the midplane of the cells using settings corresponding to the tautomeric T* and normal N* excited states of the fluorophore. During quantitative image analysis, membrane pixels were identified as described previously, and the F66 T*/N* emission ratio, which positively correlates with the magnitude of the dipole potential, was calculated. Within the investigated pH range between 7.5 and 5.5, no significant differences were observed in this parameter.

VI. Novel findings and results

1. The F66-based flow-cytometric method developed during our work proved suitable for the reliable and highly reproducible determination of dipole potential in several different cell lines and under various dipole potential- and membrane composition-modifying treatments, based on comparison with the reference method.
2. In the case of the sterol derivatives investigated in our experiments – namely cholesterol, 6-ketocholestanol, and 7-dehydrocholesterol – we confirmed their dipole potential-increasing effects in living cells and also revealed the relative differences in efficacy among these sterols.
3. During the investigation of the effects of various fatty acids on dipole potential, we found that saturated stearic acid and polyunsaturated ω -6 γ -linolenic acid increased dipole potential, whereas ω -3 α -linolenic acid decreased it. The latter compound was also capable of counteracting the effect of cholesterol during combined treatments. Based on these findings, ω -3 α -linolenic acid may represent a promising physiological alternative for the experimental reduction of dipole potential.
4. ω -3 α -linolenic acid, and the dipole potential decrease induced by it, enhanced the cellular uptake of a cell-penetrating peptide, penetratin, primarily by promoting its endolysosomal escape, thereby emphasizing the biological relevance of dipole potential reduction.
5. During our work, we successfully adapted a dual-labeling protocol based on the F66 fluorophore, which, together with confocal microscopic imaging, demonstrated that the dipole potential of intracellular membrane systems exhibits a high degree of heterogeneity.
6. Using F66 together with specific organelle markers, we determined and compared the dipole potentials of individual intracellular membrane systems with that of the plasma membrane. The dipole potentials of all investigated cellular structures – including lysosomes, Golgi apparatus, endoplasmic reticulum, and mitochondrial membranes – were lower than that of the plasma membrane, a phenomenon that had not previously been demonstrated in living cells.
7. Our confocal microscopic method also enabled comparative analysis, allowing us to compare the magnitudes of dipole potential among individual cellular compartments. Based on our results, dipole potential decreases progressively along the secretory/endocytic pathway toward the cell interior in the order lysosome > Golgi apparatus > endoplasmic reticulum. The magnitude of dipole potential measured in mitochondrial membranes was similar to that observed in lysosomes. These findings were consistent across several markedly different human cell lines, suggesting a general biological principle.

VII. Summary

Characterization of the dipole potential arising from the ordered orientation of molecular dipoles of membrane phospholipids, sterols, and membrane-associated interfacial water molecules has remained difficult due to the lack of measurement methods suitable for investigating living individual cells, as well as the absence of physiologically relevant compounds capable of experimentally lowering its magnitude. Therefore, in the first part of our work we developed and optimized a high-throughput flow cytometric method based on quantifying the shift in the emission spectrum of the dipole-potential-sensitive F66 fluorophore. This method enables the acquisition of quantitative information on the magnitude of the dipole potential from large numbers of individual living cells. The applicability of our method was validated using several cell lines and treatments, as well as through comparison with the most widely accepted method for studying dipole potential. In our experiments, we also demonstrated the dipole-potential-increasing effects of various sterol derivatives, including 6KC, 7DHC, and cholesterol in living cells. Furthermore, by examining different saturated and polyunsaturated fatty acids, we showed that α -linolenic acid, an ω -3 polyunsaturated fatty acid naturally present in human cell membranes under physiological conditions, was capable of reducing the magnitude of the dipole potential, whereas saturated stearic acid and – interestingly – the ω -6 polyunsaturated γ -linolenic acid exerted the opposite effect. The beneficial action of α -linolenic acid was also observable in the presence of cholesterol, counteracting the latter's effect. The biological relevance of these changes in dipole potential was demonstrated through their impact on the uptake and particularly the endolysosomal escape of the cell-penetrating peptide penetratin. Due to the favorable spectral properties of F66, it can be excellently combined with other fluorophores, including fluorescent intracellular organelle markers. Based on this, in the work presented in the second part of this dissertation, we optimized a method based on confocal microscopy, quantitative image analysis, and emission ratio measurements of the F66 fluorophore, which enables the comparative investigation of the dipole potential of the plasma membrane and the membranes of various intracellular organelles in living individual cells. Using our novel technique, we demonstrated in several different human cell lines that the magnitude of the dipole potential is highest in the plasma membrane, whereas within intracellular membrane systems it decreases progressively “inward” along the secretory/endocytic pathway in the order lysosome > Golgi apparatus > endoplasmic reticulum. Somewhat unexpectedly, the magnitude of the dipole potential in mitochondrial membranes proved to be similar to that of lysosomes. Our method may serve as a useful tool for identifying

organelles in which pathophysiologically significant membrane biophysical changes occur during diseases associated with altered lipid levels.

VIII. Publications



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Registry number: DEENK/252/2026.PL
Subject: PhD Publication List

Candidate: Máté Szabó
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Szabó, M.**, Cs. Szabó, B., Kurtán, K., Varga, Z., Panyi, G., Nagy, P., Zákány, F., Kovács, T.: Look Beyond Plasma Membrane Biophysics: revealing Considerable Variability of the Dipole Potential Between Plasma and Organelle Membranes of Living Cells.
Int. J. Mol. Sci. 26 (3), 1-20, 2025.
DOI: <http://dx.doi.org/10.3390/ijms26030889>
IF: 4.9 (2024)
2. Zákány, F., **Szabó, M.**, Batta, G., Kárpáti, L., Mándity, I. M., Fülöp, P., Varga, Z., Panyi, G., Nagy, P., Kovács, T.: An [omega]-3, but Not an [omega]-6 Polyunsaturated Fatty Acid Decreases Membrane Dipole Potential and Stimulates Endo-Lysosomal Escape of Penetratin.
Front. Cell. Dev. Biol. 9, 1-17, 2021.
DOI: <http://dx.doi.org/10.3389/fcell.2021.647300>
IF: 6.081

List of other publications

3. Cs. Szabó, B., **Szabó, M.**, Nagy, P., Varga, Z., Panyi, G., Kovács, T., Zákány, F.: Novel insights into the modulation of the voltage-gated potassium channel KV1.3 activation gating by membrane ceramides.
J. Lipid Res. 65 (8), 1-17, 2024.
DOI: <http://dx.doi.org/10.1016/j.jlr.2024.100596>
IF: 4.1

Total IF of journals (all publications): 15,081

Total IF of journals (publications related to the dissertation): 10,981

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

04 May, 2026

