Optical Materials: X 12 (2021) 100085

Contents lists available at ScienceDirect

Optical Materials: X

journal homepage: www.journals.elsevier.com/optical-materials-x

Invited Article

(INVITED) Fluorescent probes for optical investigation of the plasma membrane

Gaia Bondelli [a,b], Giuseppe Maria Paternò [b,*], Guglielmo Lanzani [a,b]

[a] Department of Physics, Politecnico di Milano, Milan, Italy
[b] Center for Nano Science and Technology @PoliMi, Istituto Italiano di Tecnologia, Milan, Italy

ARTICLE INFO

Keywords:
Membrane probes
Fluorescence
Optical spectroscopy
Membrane viscosity
Lipid order

ABSTRACT

The biological functions of the cell membrane are regulated by its physicochemical properties, such as the transmembrane potential, phase state and hydration. Therefore, the *in situ* investigation of these properties is attracting a rising interest in the field of membrane biophysics. In these regards, a wide variety of fluorescent membrane probes has been developed, providing powerful tools for fluorescence microscopy and/or fluorescence spectroscopy to study the microscopic analogs of viscosity, polarity, and hydration, as well as the molecular order and electrostatic potential at the sites of their location. Despite the success of this approach, further developments rely on the design of fluorescence probes with optimized photophysical properties. This review discusses the most successful fluorescent molecular probes and their application to the study of the plasma membrane viscosity, lipid order and potential.

1. Introduction

Fluorescent probes are powerful and widespread tools in biophysics that are widely employed for functional imaging *in vitro* and in vivo. They rely on both steady state and time-resolved detection where spectral properties or dynamic are the telltale signal, respectively. These techniques, originally employed in biochemistry and biophysics, are now becoming dominant also in the biotechnology research area, and in some practical techniques such as flow cytometry, DNA sequencing and forensics, to name a few [1–4]. Here, we are interested in their use for probing of the plasma membrane, not only because it delimits the cell surface, but also because its shape directly provides information regarding the cell status and its functions [5]. In neuronal networks, for instance, this ultimately enables the display of neuronal organization and membrane trafficking involved in the synaptic transmission [6]. In biological processes like cellular uptake, neural communication, muscle contraction, and cell trafficking and signaling [7] the plasma membrane is a key player. Furthermore, it has been suggested that nonlinear bioelectrical pulses, such as action potentials, can only exist if the membrane state lies in vicinity of a phase transition [8,9]. Indeed, many studies point towards the role of the plasma membrane on the excitability of living systems, and on the propagation of the related bioelectric signaling [8]. Therefore, it is crucial to understand which general properties a membrane must possess in order to support such pulses. This ultimately correlates with health of the cells in the organism. It is well known that cancer cells exhibit increased membrane fluidity and are characterized by a higher polarity compared to healthy cells [10–12]. Probing the state of the plasma membrane by optical means qualifies as a non-invasive method to screen healthy from malignant cells.

In these regards, recent years have witnessed the development of a number of fluorescent molecular probes for monitoring cellular events [7,13,14]. Emerged as an alternative to protein-based membrane markers, membrane probes include fluorescent analogs of natural lipids, as well as lipophilic organic dyes that have little structural resemblance to natural biomolecules. The small size of molecular probes, comparable to those of the membrane constituents, allows their precise location in the lipid bilayer, providing space resolved data on the lateral lipid organization of biomembranes (lipid rafts [15,16]), the Förster Resonance Energy Transfer (FRET), with membrane proteins [17] and super-resolution imaging [18,19]. Fluorescent dyes used in microscopy should exhibit simple decay kinetics, high stability, invariant fluorescence parameters, and highly selective localization. The requirements for probing membrane properties are however quite different [20]. For instance, the probe fluorescence properties should be highly responsive to changes in the physicochemical parameters (e.g. solvent polarity) of

* Corresponding author.
E-mail address: giuseppe.paterno@iit.it (G.M. Paternò).

https://doi.org/10.1016/j.omx.2021.100085
Received 21 July 2021; Received in revised form 5 August 2021; Accepted 11 August 2021
Available online 17 August 2021

their environment [21], in such a way that the interaction between the molecule and its environment shifts the spectrum or changes the deactivation rate. On top of this, fluorescence probes have to show high affinity towards the membrane without possibly distorting its native structure. Moreover, their response mechanism must be understood and their location and orientation must be known. The latter is particularly challenging to be assessed, as the fluorescence-related information here cannot be obtained and treated at the atomic level, making often hard to understand at which precise location the probe monitors a given parameter. Smart fluorophore design, location-selective quenching techniques and computer simulations [22] can be used to provide such information, but high precision still cannot be reached [20]. Besides few other limitations, such as low selectivity, heterogeneous staining, and fast internalization, the advantages of fluorescence probe methods are countless. These include extremely high sensitivity, at the limit of single molecule detection, ultrahigh resolution in interaction energies and sub-nanosecond resolution in time. Finally, the versatility of fluorescence spectroscopic techniques is enhanced due to the possibility of recording several parameters that can bring complementary information on the studied systems [20].

Within the plethora of existing fluorescent probes, the appropriate type of molecule should be selected in order to fulfill a number of experimental requirements. Among them usually stringent ones are the excitation and emission wavelengths, to allow the excitation and to avoid spectral overlap of the emission with any other existing signal, and the appropriate fluorescence lifetime that should be experimentally accessible and possibly well matched with the characteristic lifetime of the phenomenon under investigation. This review contains an overview of the various types of fluorescent membrane probes, their applications and spectral properties. First, we will introduce the class of probes assessing the membrane viscosity, to which both molecular rotors and fluorescence anisotropy probes belong; next, we will review the group of solvatochromic dyes, able to give information on the polarity, hydration and lipid order of their local microenvironment; finally, both fast- and slow-response membrane potential probes will be discussed.

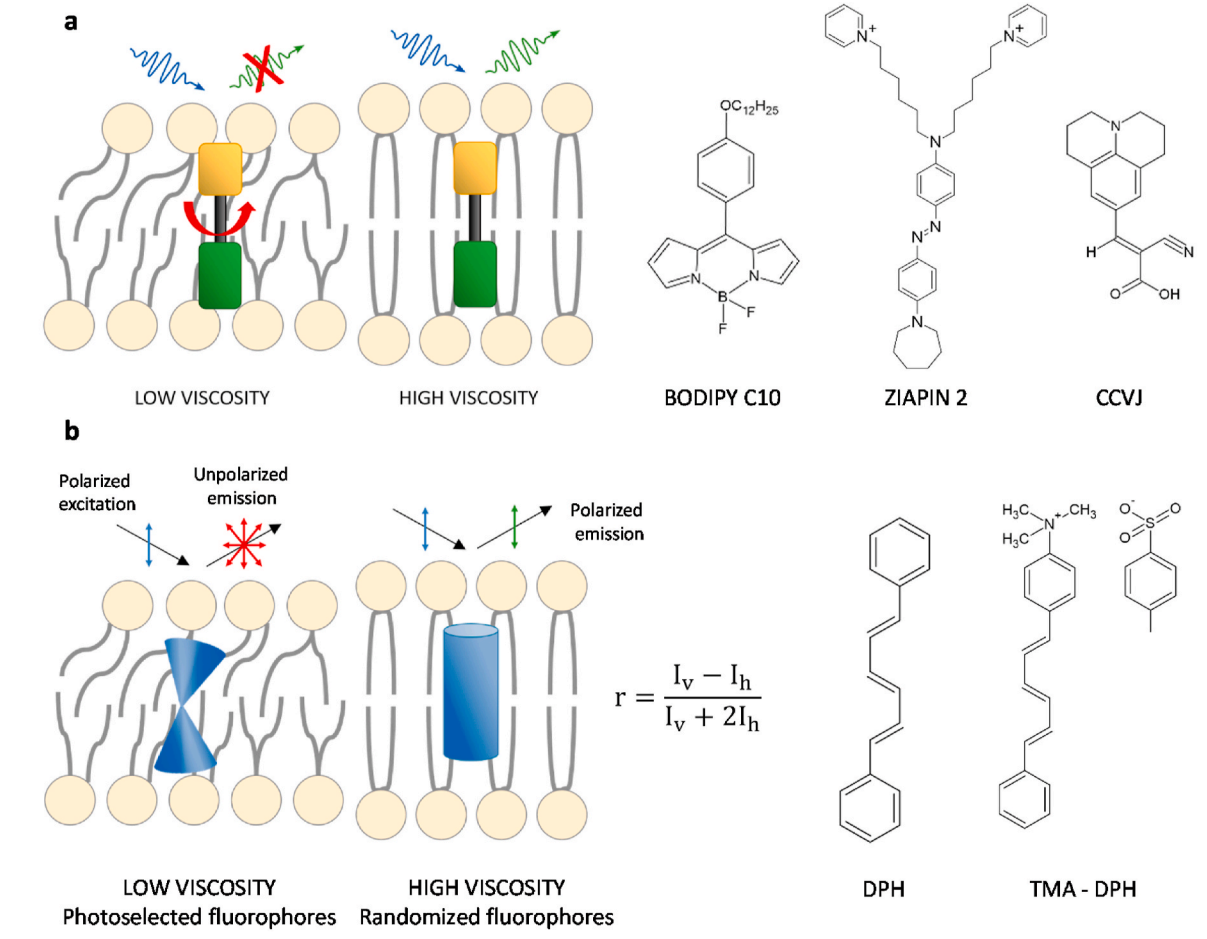
2. Membrane viscosity probes

Living organisms maintain their membranes in a fluid state, while the exact value of membrane viscosity can change depending on the culturing conditions and in turn, can influence crucial functions associated to the membrane. For example, *Escherichia coli* bacteria grown at lower temperatures are known to adjust the fatty acid composition of their plasma membranes to maintain membrane fluidity in a process termed ‘‘homeoviscous adaptation’’ [23]. Cell membrane viscosity affects the translational and rotational diffusion of proteins, lipids and other small bio-molecules within the membrane, hence controlling the rates of intracellular reactions and influencing their functions. Moreover, membrane viscosity is thought to play a key role in protein–protein interactions (e.g. via the formation of lipid rafts), the action of anesthetics and other drugs, disease development, resistance to shear stress, and more generally in any processes that require diffusion of reagents within and across the membrane bilayer [24,25]. For these reasons, there is significant interest in the direct and quantitative assessment of membrane viscosity.

A forefront category of probes for the investigation of membrane viscosity is that of molecular rotors, whose principle of mechanism is illustrated in Fig. 1a. These molecules are small synthetic fluorophores able to report on variations in viscosity by changes in their fluorescence lifetime, intensity or spectrum [24,25]. Upon photon absorption, fluorophores belonging to this class can undergo either radiative (emission of a photon) deactivation or non-radiative decay assisted by torsional motion. The non-radiative decay of the excited state, and thus the overall lifetime, display an extremely strong dependence on the viscosity of the microenvironment, which allows precise correlation between fluorescence parameters and viscosity and hence spatially resolved imaging of viscosity in living cells. It is worth highlighting that there is here a subtle shortcut in establishing a connection between non-radiative decay and viscosity, for the local interactions occurring between the probe and the solvent molecules are not necessarily the same responsible for the fluid internal friction giving rise to viscosity. Indeed such a correlation it is a practical statement validated by the experimental calibration. In the aftermath, a rational behind that is the observation that probe conformational adjustment drags along many solvent molecules whose motion is indeed ruled by viscosity.

While specially designed membrane-soluble molecular rotors have been successfully used for viscosity probing in model lipid bilayers [26], the imaging of the plasma membrane in living cells was hindered by the effective endocytosis of the dyes. Consequently, such molecular rotors have been reported as able to probe the viscosity of internal organelles only [25,27–29]. In this framework, BODIPYs (4,4-difluoro-4-bora-3a, 4a-diaza-*s*-indacenes) and their derivatives have attracted considerable attention because of their excellent chemical and physical properties, such as easy functionalization, high molar extinction coefficients, tunable fluorescence quantum yields and visible to red excitation wavelength, as well as excellent photostability [30–32]. Kuimova’s group reported the design and detailed characterization of a series of BODIPY-based molecular rotors unaffected by endocytosis owing to the double positive charge located on their hydrocarbon tail [25,33,34]. Specifically, they reported [33] on the use of fluorescence lifetime imaging microscopy (FLIM) of the molecular rotor BODIPY C10 in the plasma membranes of living *E. coli* bacteria to directly quantify the viscosity (Fig. 1c–d). Interestingly, this work reports an *E. coli* membrane viscosity of 950 cP, exceeding by almost two orders of magnitude the estimate of a very recent work [35] based on the use of an azobenzene-based molecule, ZIAPIN2 (Aze-2(C6Pyr)), whose molecular structure appears in Fig. 1a. The azobenzene isomerization mechanism was demonstrated to be hindered in highly viscous media, leading to an increase of the photoluminescence (PL) quantum yield and of the excited state lifetime in such condition. The discrepancy in the reported values of viscosity between ZIAPIN2 and the BODIPY-based molecular rotor might originate from the different probe size and PL lifetime timescale, indicating different mechanisms of conformational adjustment in the two molecules. Despite the wide use of azobenzene derivatives in material chemistry and in many different biological applications [36–41], and the existence of computational studies assessing the feasibility of the use of such molecules [22], to date the azobenzene photoswitching activity has been scarcely exploited for the investigation of viscosity and phase recognition. Another class of molecular rotors is CCVJ [9-(2-carboxy-2-cyanovinyl)julolidine], which displays internal rotation and charge transfer in the excited state at low viscosity. In contrast, a highly viscous environment hinders this path, as the molecule cannot distort, resulting in radiative decay [42,43]. As a result, the quantum yield of CCVJ, assessed as relative intensity changes, can be employed to assess membrane viscosity.

On the other hand, with DPH (1,6-diphenyl-1,3,5-hexatriene)-based probes that display a quantum yield only weakly dependent on the viscosity, the observable of choice is fluorescence anisotropy [42]. Fluorescence anisotropy measurements, schematically shown in Fig. 1b, are performed exciting the sample with polarized light. This photoinduces in the original isotropic sample an anisotropy pattern ($\sim cos^2\,\Theta$) in the excited population due to the preferential selection of those molecules whose absorption dipole moment has a component along the electric field of the excitation (according to the angle Θ). Since the emitted light is polarized along a fixed axis in the fluorophore, such anisotropy distribution results into a polarized emission. The time dependent change in polarization of the fluorescence light provides information about the rotational degree of freedom of the fluorophore, which depends on viscosity. An anisotropy parameter (r, see formula in Fig. 1b) is usually defined in such a way to depend only on the reorientation and not on the population dynamics. The anisotropy parameter r is equal to zero for fluorophores in non-viscous solutions, due to



(caption on next page)

Fig. 1. Membrane viscosity probes. Working principle of molecular rotors (**a**) and fluorescence anisotropy probes (**b**) together with the chemical structures of few examples discussed in the text. (**c**) Fluorescence spectra and decay traces recorded for BODIPY C10 in methanol/glycerol mixtures of different viscosity. Adapted from Ref. [34], copyright 2008, with permission from American Chemical Society. (**d**) Fluorescence lifetime imaging (FLIM) of BODIPY C10 in live *E. coli* cells, showing preferential localization to the membrane. On the left, cell grown at 37 °C and imaged at room temperature (23 °C); on the right, cell grown and imaged at 37 °C. False color scale represents lifetime in picoseconds. Scale bars are 1 mm in length. Adapted from Ref. [33] under the terms of the Creative Common CC BY license, copyright 1969 Elsevier. (**e**) Decay of fluorescence anisotropy of TMA-DPH in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposomes at (top) 25 °C, (middle) 50 °C and (bottom) DPPC with 30 mol% cholesterol at 50 °C. The lipid composition and temperatures were selected to represent gel (DPPC at 25 °C), liquid disordered (DPPC at 50 °C) and liquid ordered (DPPC-30 mol% cholesterol at 50 °C) phases. Dots represent the experimental data and solid lines represent the fitted curves. Adapted from Ref. [48], copyright 2003, with permission from EBSA.

the very fast loss of polarization memory. If not null, any change in the orientation of the transition dipole moment will be responsible for a partial or total depolarization of fluorescence that can be used to monitor membrane viscosity. Several DPH derivatives have been developed to selectively measure the fluidity of plasma membranes in living cells [44,45], including DPHpPC and TMA-DPH [46]. Such derivatives avoid ambiguities about the location of the DPH fluorophore in the membrane. Indeed, both molecules have hydrophilic headgroups and, as such, are anchored to the interface region of the bilayer, ensuring that the DPH moiety is aligned with the phospholipid acyl chains [47]. As an example, the decays of fluorescence anisotropy of TMA-DPH in artificial membranes at different temperatures and conditions are shown in Fig. 1e: the more viscous phase results in a considerably slower decay compared to that observed in the more fluid phases [48].

3. Membrane order probes

Membrane viscosity is closely connected to membrane order, as lipid packing can influence the fluidity of the membrane. Lipids and proteins are not homogenously distributed in the plasma membrane, and give raise to biochemically and biophysically distinct domains. Particularly relevant is the comparison between the so-called liquid-disordered (Ld) and liquid-ordered (Lo) phases, which differ in the degree of lipid packing [49], thought to influence numerous cellular processes including receptor signaling [50] and membrane trafficking [51]. An Ld phase can be assimilated to a fluid phase composed of unsaturated phospholipids and cholesterol, characterized by high mobility of lipids and exhibiting fast relaxation dynamics and high level of hydration. In contrast, a Lo phase consists of saturated lipids, either phosphatidylcholine or sphingomyelin, and cholesterol, comparable to a gel phase, characterized by slow lipid mobility and much lower hydration [20]. These highly ordered domains or "rafts" float in a pool of Ld domains

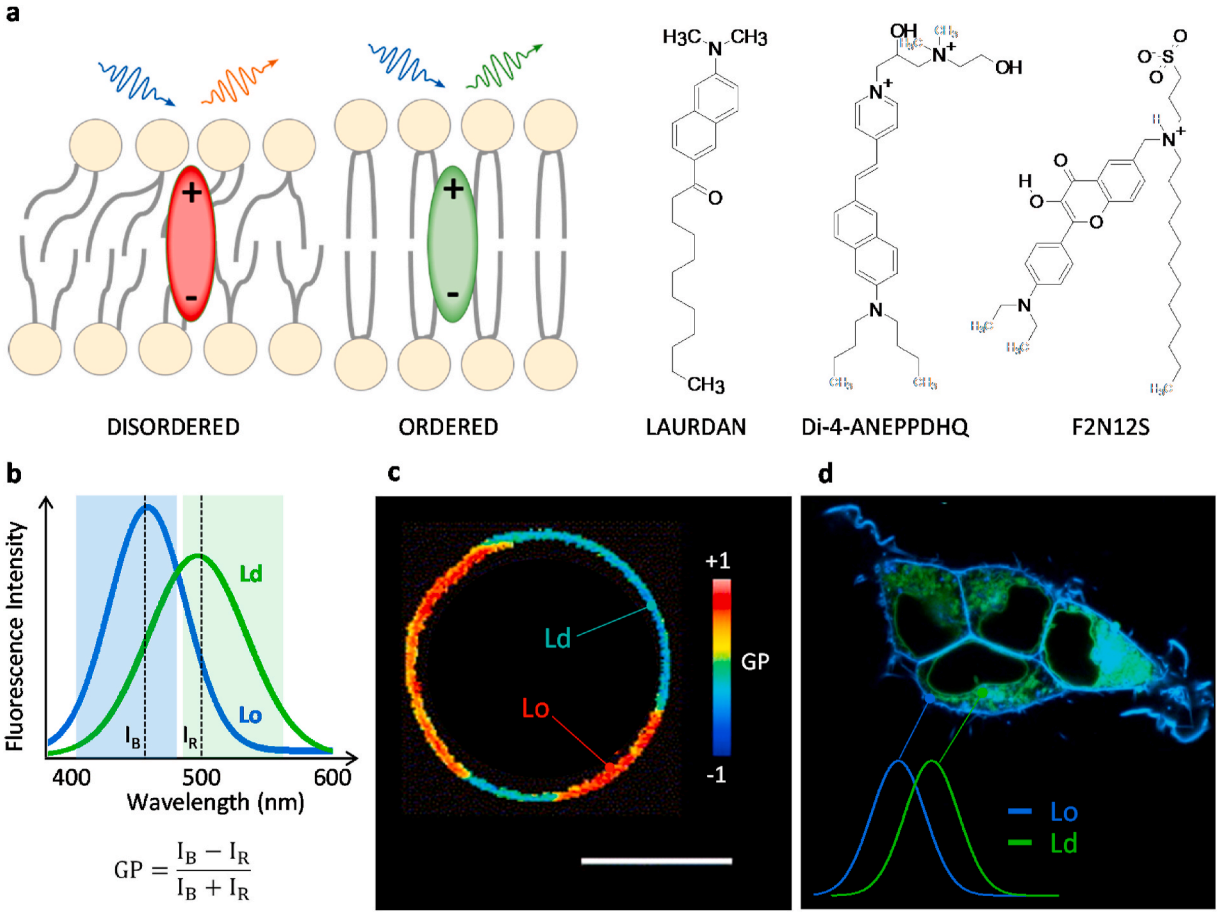


Fig. 2. Solvatochromic probes and examples of their application in model and cellular membranes. (**a**) Working principle of solvatochromic probes (left) and examples of their chemical structure (right). (**b-d**) Application of Laurdan for studying phase separation in membranes. Fluorescence spectra of Laurdan in model membranes of Lo (blue) and Ld (green) phases (**b**), their ratiometric (GP) image in giant unilamellar vesicles (GUVs) presenting both phases (SM/DOPC/Cholesterol mixture) (**c**), and in HEK293 cells membranes with inset showing the fluorescent spectra in separated phases (**d**). (**c**) Adapted from Ref. [62], copyright 2006, with permission from Elsevier.

[52,53] and are believed to play a crucial role in the regulation of a number of functions involving membrane proteins and membrane transport [20,54]. Moreover, rafts take part in a number of pathologies [55,56], such as Alzheimer's disease. In this case, changes in the structure and composition in neuronal membranes may trigger amyloid toxicity, due to the interaction of amyloid-beta peptides (Aβ) with the cellular membrane [57]. Indeed, previous studies on the brain membrane lipid composition of Alzheimer's disease patients have revealed changes in lipid composition that occur during disease progression, including lowering the content of several types of phospholipids found in the inner leaflet of the membrane [58] and a decrease in sphingomyelin content [59]. Visualization and quantification of lipid order is thus an important tool in membrane biophysics and cell biology, and the knowledge of membrane phase is essential to determine the health or illness of a cell [60]. A plethora of fluorescent probes is available for this purpose, even though the choice of the proper molecule for the requested analysis may be challenging.

Environment sensitive membrane dyes constitute a valid and direct approach for distinguishing Ld from Lo phases. Their working principle and some examples of their chemical structure are shown in Fig. 2a. This class of molecules, also referred to as solvatochromic probes, change their optical properties (HOMO-LUMO energy distance) depending on the polarity of their local environment, owing to the different polarities of the ground and excited state [61]. Accordingly, liquid-ordered and liquid-disordered domains can be distinguished by the differential penetration of polar water molecules into the otherwise non-polar bilayer interior. An archetypical example is represented by Prodan (6-propionyl-2-(dimethylamino)-naphthalene), which exhibits clear-cut differences in its spectra when internalized in these two phases [53,62]. Indeed, in the Lo phase, this probe reveals a less polar environment, with a blue-shifted emission compared to the Ld phase. Based on the success of this molecule that contains a 3-carbon acyl chain, Laurdan and Patman were introduced with longer tails to better anchor them to a specific location in the membrane bilayer [63]. Synthesized by Weber and Farris in 1979 [64], Laurdan (6-lauryl-2-dimethylamino-naphthalene) is nowadays one of the most used and studied polarity-sensitive dyes for the investigation of membrane organization. When intercalates into the membrane bilayer, the molecule is fluorescent with at least two excited states: an intrinsic locally excited (LE) state, and an internal charge transfer (ICT) state characterized by a large permanent dipole moment. The latter is stabilized by the reorientation of the surrounding water molecules with the Laurdan dipole moment, so that the frequency of the emitted photons is decreased [65]. Thus, the emission spectrum of the dye undergoes a shift to longer wavelengths in more polar environments (Fig. 2b) [61,66,67]. Thanks to its 50 nm red-shift between the ordered and disordered phases, Laurdan is largely used to quantify membrane order by 2-channel ratiometric imaging [68] and to visualize lipid rafts in living cells by the generalized polarization (GP) approach [69] (Fig. 2c–d). The latter consists in defining a parameter constituted by a ratio of the two emission intensities (at the blue and green edges of Laurdan emission spectrum) for a given excitation wavelength [70]. As such, GP measurements do not depend on local probe concentrations or surface area. Laurdan variants have been synthesized in order to improve its performances and overcome few drawbacks (e.g. phototoxic UV excitation). In 2006, Anthradan (2-propionyl-6- dimethylaminoanthracene) was developed, which absorbs in the red range, avoiding overlap with cell autofluorescence and making this dye more convenient for biological applications [71]. A more sensitive and photostable Laurdan derivative is C-Laurdan, that exhibits improved solubility in aqueous media [72,73].

Besides these Prodan derivatives, an alternative probe to quantify membrane order is di-4-ANEPPDHQ [49,74,75], despite being originally designed as a voltage-sensitive dye to monitor electrical activity in cells and tissues. Di-4-ANEPPDHQ shows red-shifted absorption and emission compared to Laurdan, and a deeper insertion into the membrane. Its 60 nm red shift between Lo and Ld phases makes it a perfect candidate for confocal microscopy using single-photon excitation as well as TIRF microscopy and flow cytometry [49]. However, its broad emission spectrum in the green-red region makes multi-labeling challenging. Designing new membrane probes is an area of ongoing research in order to overcome the limitations affecting each probe and to enlarge their field of application. For instance, Kwiatek et al. [49] presented a series of novel fluorescent dyes whose excitation and emission properties may enable unique combinations for multi-labeling and multi-modal imaging. Interestingly, besides determining quantitatively the degree of membrane order through ratiometric imaging, they show that the fluorescence lifetime of the dyes is also dependent on bilayer order.

The order and level of lipid packing within the cell membrane directly affects membrane tension. Membrane shape changes can cause bending, shearing and stretching, and membrane tension is defined as the derivative of the membrane free energy with area change [77]. Membrane tension plays an essential role in numerous cell processes, such as cell migration [78,79], cell spreading and phagocytosis [80,81] and cell division [82]. Moreover, membrane tension regulates the opening of mechanosensitive ion channels [83]. Because of its multiple roles, membrane tension is constantly regulated by the cell and despite its importance remains notoriously difficult to be measured in cells. Colom et al. [76] provided a non-invasive tool for the measurement of membrane tension in living cells, by a planarizable push–pull called FliptR (fluorescent lipid tension reporter, molecular structure shown in Fig. 3a). This fluorescent probe can monitor changes in membrane tension by changing its fluorescence lifetime as a function of the twist between its fluorescent groups. The fluorescence lifetime depends linearly on membrane tension, which relies on a membrane-tension-dependent lipid phase separation. As a consequence, the FliptR probe is sensitive to lipid composition by detecting the various packing of lipids in different phases with different order (Lo, Ld), as illustrated in Fig. 3b.

Another interesting feature of biomembranes is the asymmetric distribution of lipids between their two leaflets. Indeed, while the outer leaflet contains a large amount of sphingomyelin, its fraction in the inner leaflet is marginal [84]. Therefore, a correct evaluation of Lo phases requires probes presenting high specificity to this phase together with a high selectivity to the outer membrane leaflet and negligible flip-flop between the two leaflets. The challenge to develop an environmentally sensitive fluorescent probe staining quite exclusively the outer leaflet of cell membranes has been only recently solved through the use of 3-hydroxyflavone (3-HF) dyes, namely F2N12S [85]. This class of probes attracted big attention because of their ability to respond to small changes in their microenvironment via a dramatic alteration of the relative intensities of their two well-separated emission bands. These bands result from an excited state intramolecular proton transfer (ESIPT) reaction and belong to normal excited (N*) and photo-tautomer (T*) states [86]. The N*/T* ratio is a powerful indicator of polarity, hydration and lipid order in membranes, independently from the dye concentration and the instrument. The asymmetric incorporation at the outer membrane leaflet was also proven for the NR12S probe, that has recently been obtained by modifying the solvatochromic fluorescent dye Nile Red with the same amphiphilic anchor group as for F2N12S [87].

4. Membrane potential probes

Molecules sensitive to the electrical potential across the membrane constitute another class of membrane probes. The total electrostatic potential of a biomembrane (Ψ) follows a complex profile given by the contribution of three different electrostatic potentials [88]: the transmembrane potential (ΔΨ), associated to the difference in ionic concentrations between the interior and the exterior of the cell; the surface potential (ΨS), originating from charged residues at the membrane interfaces; and the dipole potential (ΨD), resulting from the alignment of phospholipid dipolar residues and from associated water molecules within the membrane. Transmembrane potential (ΔΨ) is the most

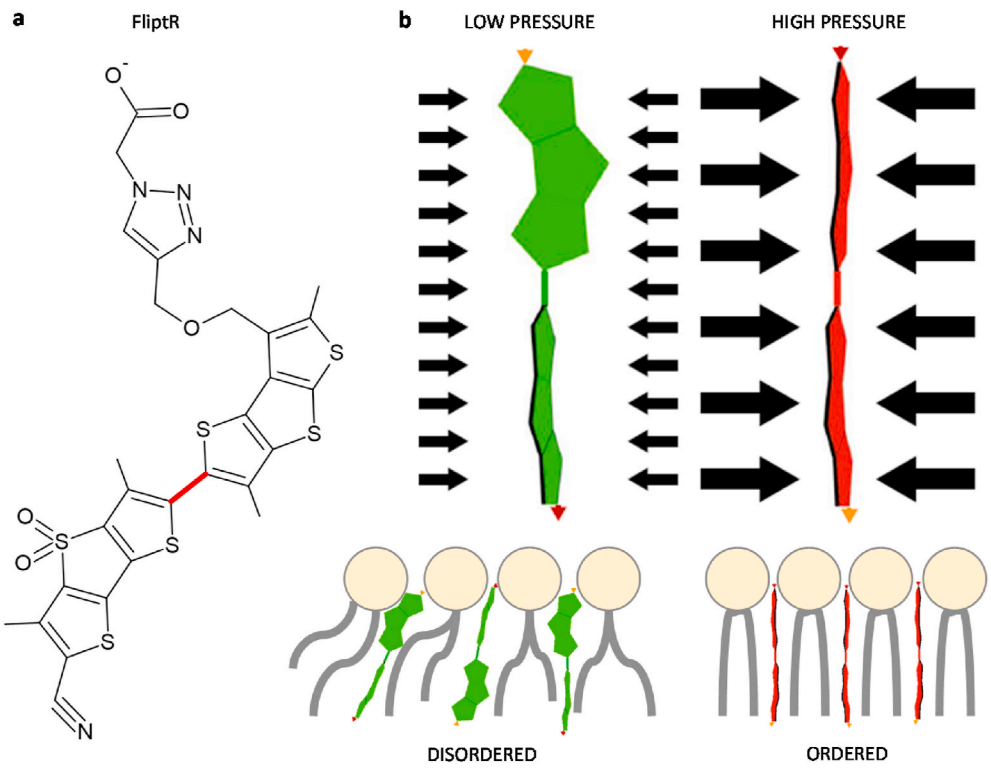


Fig. 3. The FliptR membrane tension probe. (**a**) Chemical structure of the molecule, the carbon bond around which the fluorescent groups can twist is shown in red. (**b**) Pressure along the axis of the FliptR probe can planarize the two fluorescent groups, leading to changes in excitation maxima and fluorescence lifetime. The probe is sensitive to lipid composition by detecting the various packing of lipids in different phases with different order (Lo, Ld). Adapted from Ref. [76], copyright 2018, with permission from Springer Nature.

dynamic of these components, increases and decreases in its value – referred to as membrane depolarization and hyperpolarization, respectively – play essential roles in a variety of cellular functions and physiological processes. Fast changes in $\Delta\Psi$, from milliseconds to seconds, control our heartbeat and trigger the electrical signals in brain cells. On the other hand, slower changes in transmembrane potential, from minutes to hours/days, play important roles linked to many cellular processes like mitosis, cell cycle progression, and differentiation [89,90]. $\Delta\Psi$ is negative (inside) with values ranging typically from −40 to −80 mV, depending on the cell type, and it is due to K^+, Na^+ and Cl^- concentration gradients that are maintained by active transport processes. Intracellular microelectrode (or patch-clamp) recording and optical microscopy are the two major methods to monitor $\Delta\Psi$ in cells. Microelectrodes allow the recording of $\Delta\Psi$ with extremely high temporal resolution and precision. However, the investigation of its spatial distribution within a single cell or cell assemblies with microelectrodes is not trivial and requires multi-electrode techniques. This crucial limitation can be partially overcome using optical methods and fluorescent probes allowing the recording of $\Delta\Psi$ in single cells and multicellular assemblies with both high temporal and spatial resolution. In addition, membrane potential probes enable to perform measurements in organelles and in bacterial cells that are too small for microelectrodes [91]. Together with imaging techniques, these probes can display variations in membrane potential across excitable cells, with extraordinarily high spatial resolution and sampling frequency. These potentiometric probes include the cationic or zwitterionic styryl dyes, the cationic carbocyanines and rhodamines, the anionic and hybrid oxonols and merocyanines. A number of mechanisms are thought to be responsible of their sensitivity to electrical potential, including partitioning of the dye from water to the membrane phase, reorientation and/or aggregation of the molecules in the membrane (*vide infra*), and the inherent sensitivity of the dyes to the electric field [42,92,93]. The styryl dyes seem to respond directly to the electric field, in contrast, the carbocyanines, upon application of a voltage tend to partition and/or aggregate in the membranes [94]. Merocyanines respond to membrane potential by both mechanisms [42]. Substantial variations in their optical responses, phototoxicity and interactions with other molecules can complicate the selection of the best potentiometric probe for a particular application. Membrane potential probes can be divided into two categories based on their temporal response: fast- and slow-response probes. Fig. 4 illustrates the response mechanisms of these two classes of probes, together with the molecular structures of some exemplificative probes. In fast-response probes (Fig. 4a), a change in the electric field induces a change in their electronic structure, and consequently in their fluorescence properties. Even though this process is fast enough to detect transitory (millisecond) polarization changes in excitable cells, the magnitude of their potential-dependent fluorescence change is often small, as fast-response probes typically show a 2–10% fluorescence change per 100 mV. On the other hand, slow-response probes (Fig. 4b) exhibit potential-dependent changes in their transmembrane distribution resulting into a much larger fluorescence change (~ 1% fluorescence change per mV). Slow-response probes, which include cationic carbocyanines and rhodamines and anionic oxonols, are suitable for detecting changes in average membrane potentials of non-excitable cells caused by respiratory activity, ion-channel permeability, drug binding and other factors [95].

Voltage-sensitive dyes of the RH series were among the first membrane probes developed for the detection of transmembrane potential, facilitating in vivo imaging and leading to the possibility of voltage sensitive dye imaging [96]. Originally synthesized by Rina Hildesheim, the RH dyes include an extensive series of dialkylaminophenylpolyenylpyridinium dyes that are principally used for functional imaging in neurons. The existence of numerous RH dye analogs (e.g. RH 237, RH 414, RH 421 and RH 795) reflects the observation that no single dye provides the optimal response under all experimental conditions [95]. These dyes bind to the external surface of cell membranes and act as molecular transducers transforming changes in $\Delta\Psi$ into changes in emitted fluorescence that occur on the microsecond time scale. However, some drawbacks are remaining such as responses in emission intensity, which are difficult to calibrate, being dependent on the probe concentration. Moreover, because of the small size of fluorophores it is often difficult to obtain a significant change in voltage

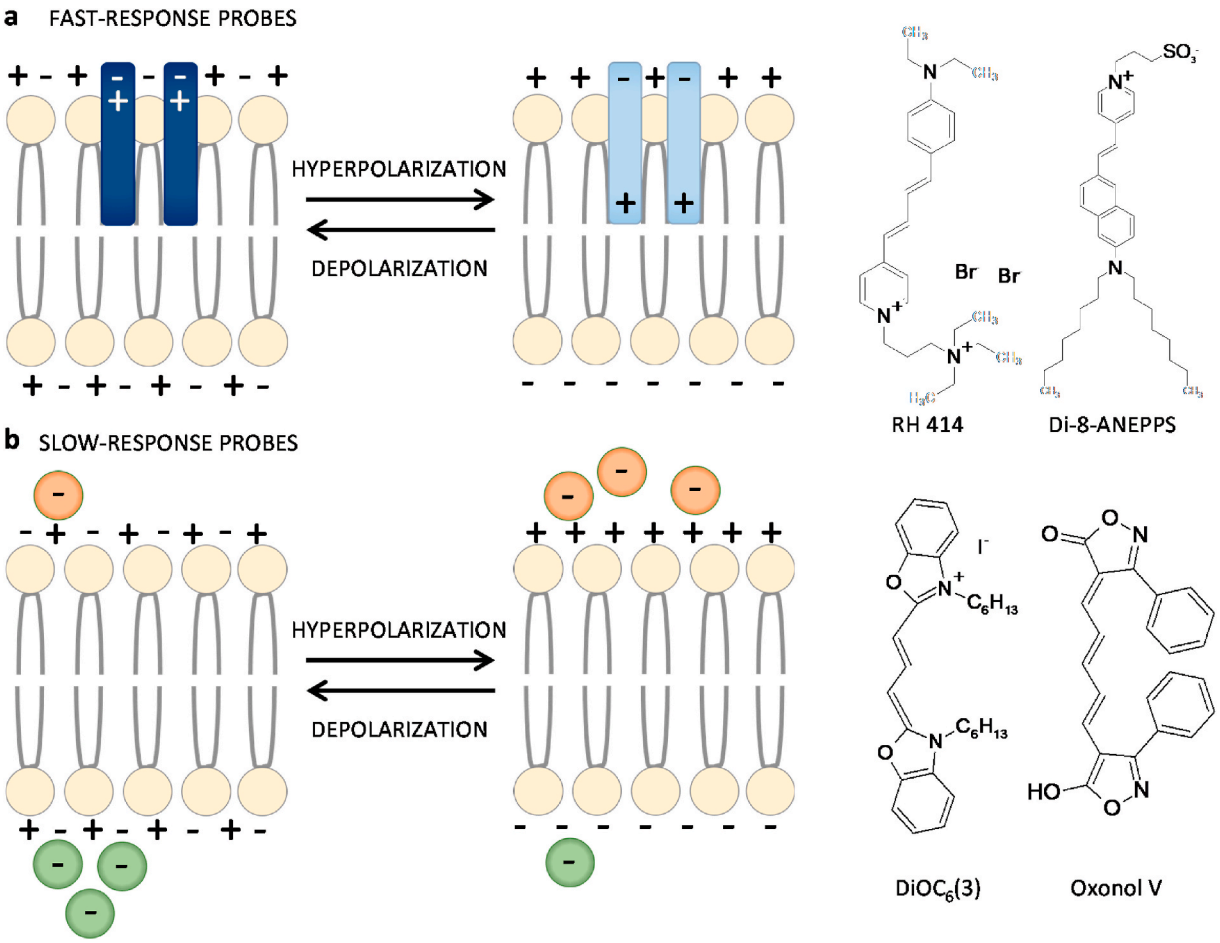


Fig. 4. Response mechanisms and chemical structures of fast- and slow-response membrane potential–sensitive probes. (**a**) In response to a change in electric field, fast-response probes undergo intramolecular charge distribution changes, generating corresponding changes in their fluorescence intensity or spectral profile. (**b**) Slow-response probes are lipophilic anions or cations translocating across the membrane by an electrophoretic mechanism. The probe sensitivity to intracellular and extracellular environments is reflected in its changes in fluorescence, associated with transmembrane redistribution. Accordingly, potentiometric response speeds directly reflect the time constants of the underlying processes - fast intramolecular redistribution of electrons versus relatively slow transmembrane movement of entire molecules [95].

across the fluorophore. The sensitivity to voltage can be improved by using fluorescence resonance energy transfer (FRET), employing the hydrophobic oxonol dye as a FRET partner [97,98]. This is accomplished by positioning a static donor fluorophore (e.g. carbocyanines, DiOC18 (3) or DiOC16(3)) on the membrane surface and allowing a second mobile dye (e.g. oxonol or dipicrylamine) to partition into the membrane interior, working as an acceptor for the first fluorophore. Alterations in voltage result in changes in the mobile dye concentration near the donor and hence change in intensity of the acceptor emission [42]. The modifications in light intensity give an estimate of the magnitude of the change in membrane potential, allowing the analysis of many cells without harming them. Besides being not very accurate, this method cannot report the absolute value of membrane potential nor quantitatively assess its changes. Moreover, difficulties arise from variations in dye environment, dye loading, illumination intensity and fluorophore bleaching, which make the calibration and determination of membrane potential variation not trivial or even impossible. A promising solution to these problems was suggested by the development of a fluorescence lifetime-based approach (VF-FLIM) to visualize and optically quantify the transmembrane potential with single-cell resolution [99]. Measuring the excited state lifetime of a population of fluorophores at specific voltages, rather than their intensity, Lazzari-Dean et al. [99] reported $\Delta\Psi$ distributions over thousands of cells, resulting in a 100-fold improvement relative to electrophysiological approaches, without artefacts. As illustrated in Fig. 5, the VF-FLIM approach is based on fluorescent voltage indicators that use photoinduced electron transfer as a voltage-sensing mechanism, namely, changes in the transmembrane potential alter the rate of photoinduced electron transfer from an electron-rich aniline donor to a fluorescent reporter, modulating the fluorescence properties of the dyes [99].

It is challenging to measure the dipole potential (ΨD) when compared to $\Delta\Psi$, and can only be theoretically calculated or inferred from indirect experimental methods. One of these indirect methods is the use of fluorescent voltage-sensitive probes with a location of their fluorophore in the region where ΨD is effective, namely between the apolar bilayer center and the membrane surface. The ANEP (Amino-NaphthylEthenylPyridinium) dyes were the first popular molecules for the determination of ΨD [20,86]. Given their millisecond-range temporal characteristics, zwitterionic di-4-ANEPPS and di-8-ANEPPS belong to the class of fast-response probes, displaying, however, a modest amplitude response in fluorescence intensity (10% per 100 mV changes). Both di-4-ANEPPS and di-8-ANEPPS allow a ratiometric recording of ΨD through the shift of their excitation spectra, as long as the sample is excited with two different wavelengths, which introduces experimental challenges. A more convenient solution is provided by dyes adapted for multicolor imaging microscopy, with a ratiometric response in emission rather than in excitation. 3-hydroxyflavone (3-HF) dyes fit well with these requirements. In particular, two probes (F4N1 and BPPZ) were developed in which the fluorescent moieties and consequently the dipole moments are oriented vertically in lipid bilayers

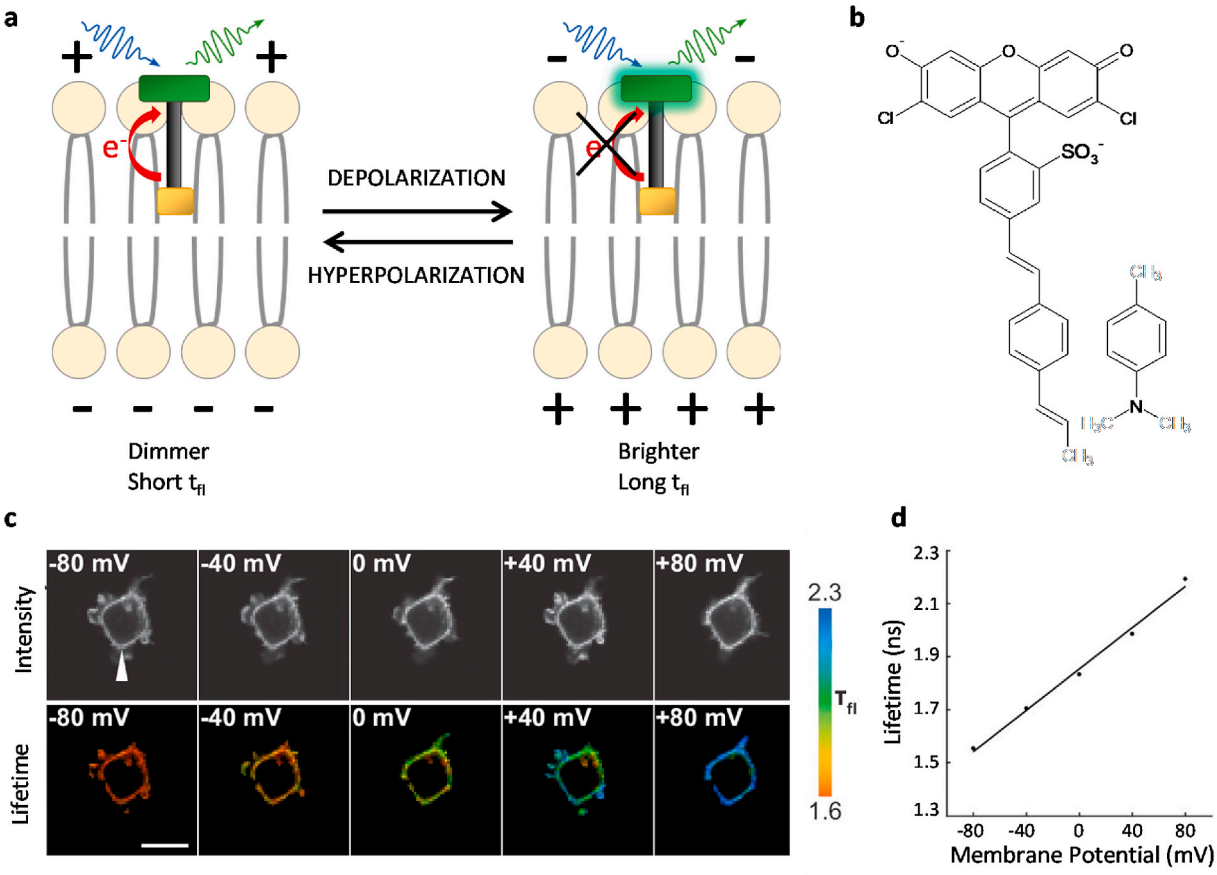


Fig. 5. Membrane potential probe VF-FLIM linearly reports the absolute membrane potential. (**a**) Mechanism of VF-FLIM dyes, in which depolarization of the membrane potential attenuates the rate of photoinduced electron transfer. (**b**) Structures of the molecule used in the study [99]. (**c**) Intensity (top) and lifetime (bottom) images of HEK293T cells voltage-clamped at the indicated membrane potential. Scale bar represent 20 mm. (**d**) Quantification of the single trial shown in (**c**), with a linear fit to the data. (**c-d**) Adapted from Ref. [99] under the terms of the Creative Common CC BY license.

but inversely with respect of each other [100]. The response to a ΨD variation was observed as a change in the intensity ratio of their two emission bands in an opposite way for the two probes, in accordance with their inverse orientation. However, since these probes were not applicable for cellular studies due to their fast internalization and/or poor staining of plasma membranes, a second generation of probes was introduced, namely F8N1S and PPZ8. This second generation of probes demonstrates a fair selectivity for cell plasma membranes and exhibits strong ratiometric variations of their two emission bands versus ΨD in living cells [101].

5. Conclusions

The investigation of the structure and properties of biological membranes is a major challenge in biology and medicine. Routine techniques are based on electrodes that are invasive, sometime destructive, hard to be applied in vivo and limited to single event detection. Being fast, noninvasive, and applicable *in situ* on live cells, optical techniques are a promising alternative. Indeed, these techniques have become powerful and widely used tools for studies of biochemical and biophysical processes occurring in biological membranes. Fluorescence is the indicator of choice, because it can be background free, highly sensitive and easy to collect. Various fluorescence methods have played and continue to play key roles in modern membrane science; in particular, fluorescent membrane probes constitute major tools for obtaining information about the structure and dynamics in biomembranes, allowing the study of essential cellular processes at the plasma membrane level. Choosing a suitable molecule to visualize and probe a specific membrane property can be daunting, given the countless molecules available either commercially or through *ex novo* design and synthesis. Moreover, the choice of the probe depends on the targeted question posed, the membrane system studied, and the instrumental technique used. In this review, we discussed the fluorescent membrane probes used to study three major cellular membrane properties, namely viscosity, lipid order and membrane potential, in order to provide the background information to identify a range of suitable fluorescence methods to successfully design and conduct experimental studies on model lipid bilayers and biological membranes. A few constrains still hamper a wide use of such probes and their replacement of standard electrophysiology set-ups. One is sensitivity and the uncertainty related to the experimental conditions, such as dose or distribution. Another is the response time, mostly not fast enough to allow dynamic sampling. One more is the requirement for stimulation, still largely delivered by electrodes. A careful photophysical exploration of the probes' properties could help defining their response characteristics. The resulting scenario could be an input for the tailoring of new compounds with better performances. Finally, a recently developed set of light actuators that enable cell stimulation could be associated to optical detection, paving the way to all optical physiology.

Funding

G.M.P. thanks Fondazione Cariplo (grant no 2018-0979) for financial support.

CRediT authorship contribution statement

Gaia Bondelli: wrote the, Writing – original draft, and created the figure. **Giuseppe Maria Paternò:** Conceptualization, the idea of the article and together with. **Guglielmo Lanzani:** Writing – review & editing, the manuscript, All authors contributed to manuscript drafting and revising, and figure creation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] T. Morikawa, T. Shibahara, T. Nomura, A. Katakura, M. Takano, Non-invasive early detection of oral cancers using fluorescence visualization with optical instruments, Cancers (Basel) 12 (2020) 1–16, https://doi.org/10.3390/cancers12102771.

[2] R.T. Ranasinghe, T. Brown, Ultrasensitive fluorescence-based methods for nucleic acid detection: towards amplification-free genetic analysis, Chem. Commun. 47 (2011) 3717–3735, https://doi.org/10.1039/c0cc04215c.

[3] S.E. Denny, S.S. Nazeer, T.T. Sivakumar, B.J. Nair, R.S. Jayasree, Forensic application of fluorescence spectroscopy: an efficient technique to predict the presence of human saliva, J. Lumin. 203 (2018) 696–701, https://doi.org/10.1016/j.jlumin.2018.07.022.

[4] A. Görgens, M. Bremer, R. Ferrer-Tur, F. Murke, T. Tertel, P.A. Horn, S. Thalmann, J.A. Welsh, C. Probst, C. Guerin, C.M. Boulanger, J.C. Jones, H. Hanenberg, U. Erdbrügger, J. Lannigan, F.L. Ricklefs, S. El-Andaloussi, B. Giebel, Optimisation of imaging flow cytometry for the analysis of single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material, J. Extracell. Vesicles 8 (2019), https://doi.org/10.1080/20013078.2019.1587567.

[5] S. Rello, J.C. Stockert, V. Moreno, A. G, Morphological Criteria to Distinguish Cell Death Induced by Apoptotic and Necrotic Treatments, 2005, pp. 201–208, https://doi.org/10.1007/s10495-005-6075-6.

[6] F. Hecht, B.K. McCaw, D. Peakman, A. Robinson, Structural basis of long-term potentiation in single dendritic spines, Nature 255 (1975) 243–244, https://doi.org/10.1038/255243a0.

[7] M. Collot, P. Ashokkumar, H. Anton, E. Boutant, O. Faklaris, T. Galli, Y. Mély, L. Danglot, A.S. Klymchenko, MemBright: a family of fluorescent membrane probes for advanced cellular imaging and neuroscience, Cell Chem. Biol. 26 (2019) 600–614, https://doi.org/10.1016/j.chembiol.2019.01.009, e7.

[8] C. Fillafer, A. Paeger, M.F. Schneider, The living state: how cellular excitability is controlled by the thermodynamic state of the membrane, Prog. Biophys. Mol. Biol. (2020), https://doi.org/10.1016/j.pbiomolbio.2020.10.003.

[9] M. Mussel, M.F. Schneider, Similarities between action potentials and acoustic pulses in a van der Waals fluid, Sci. Rep. 9 (2019) 1–10, https://doi.org/10.1038/s41598-019-38826-x.

[10] I. Nakazawa, M. Iwaizumi, A role of the cancer cell membrane fluidity in the cancer metastases: an ESR study, Tohoku J. Exp. Med. 157 (1989) 193–198, https://doi.org/10.1620/tjem.157.193.

[11] I. Nakazawa, M. Iwaizumi, A correlation between cancer metastases and the fluidity of cancer cell membrane, Tohoku J. Exp. Med. 137 (1982) 325–328, https://doi.org/10.1620/tjem.137.325.

[12] C. Händel, B.U.S. Schmidt, J. Schiller, U. Dietrich, T. Möhn, T.R. Kießling, S. Pawlizak, A.W. Fritsch, L.C. Horn, S. Briest, M. Höckel, M. Zink, J.A. Käs, Cell membrane softening in human breast and cervical cancer cells, New J. Phys. 17 (2015), https://doi.org/10.1088/1367-2630/17/8/083008.

[13] L.D. Lavis, R.T. Raines, Bright ideas for chemical biology, ACS Chem. Biol. 3 (2008) 142–155, https://doi.org/10.1021/cb700248m.

[14] M. Fernández-Suárez, A.Y. Ting, Fluorescent probes for super-resolution imaging in living cells, Nat. Rev. Mol. Cell Biol. 9 (2008) 929–943, https://doi.org/10.1038/nrm2531.

[15] A.S. Klymchenko, R. Kreder, Fluorescent probes for lipid rafts: from model membranes to living cells, Chem. Biol. 21 (2014) 97–113, https://doi.org/10.1016/j.chembiol.2013.11.009.

[16] D.M. Owen, D.J. Williamson, A. Magenau, K. Gaus, Sub-resolution lipid domains exist in the plasma membrane and regulate protein diffusion and distribution, Nat. Commun. 3 (2012), https://doi.org/10.1038/ncomms2273.

[17] I. Ziomkiewicz, A. Loman, R. Klement, C. Fritsch, A.S. Klymchenko, G. Bunt, T. M. Jovin, D.J. Arndt-Jovin, Dynamic conformational transitions of the EGF receptor in living mammalian cells determined by FRET and fluorescence lifetime imaging microscopy, Cytometry. A 83 (2013) 794–805, https://doi.org/10.1002/cyto.a.22311.

[18] L.M. Solanko, A. Honigmann, H.S. Midtiby, F.W. Lund, J.R. Brewer, V. Dekaris, R. Bittman, C. Eggeling, D. Wüstner, Membrane orientation and lateral diffusion of BODIPY-cholesterol as a function of probe structure, Biophys. J. 105 (2013) 2082–2092, https://doi.org/10.1016/j.bpj.2013.09.031.

[19] A. Honigmann, V. Mueller, H. Ta, A. Schoenle, E. Sezgin, S.W. Hell, C. Eggeling, Scanning STED-FcS reveals spatiotemporal heterogeneity of lipid interaction in the plasma membrane of living cells, Nat. Commun. 5 (2014), https://doi.org/10.1038/ncomms6412.

[20] A.P. Demchenko, G. Duportail, S. Oncul, A.S. Klymchenko, Y. Mély, Introduction to fluorescence probing of biological membranes, Methods Membr. Lipids (2015), https://doi.org/10.1385/1597455199.

[21] A.P. Demchenko, Y. Mély, G. Duportail, A.S. Klymchenko, Monitoring biophysical properties of lipid membranes by environment-sensitive fluorescent probes, Biophys. J. 96 (2009) 3461–3470, https://doi.org/10.1016/j.bpj.2009.02.012.

[22] S. Osella, S. Knippenberg, The influence of lipid membranes on fluorescent probes' optical properties, Biochim. Biophys. Acta Biomembr. 1863 (2021), 183494, https://doi.org/10.1016/j.bbamem.2020.183494.

[23] M. Sinensky, Homeoviscous adaptation - A homeostatic process that regulates the viscosity of membrane lipids in Escherichia coli 71 (1974) 522–525.

[24] M.A. Haidekker, E.A. Theodorakis, E.A. Theodorakis, Molecular Rotors — Fluorescent Biosensors for Viscosity and Flow, 2007, pp. 1669–1678, https://doi.org/10.1039/b618415d.

[25] I. López-Duarte, T.T. Vu, M.A. Izquierdo, J.A. Bull, M.K. Kuimova, A molecular rotor for measuring viscosity in plasma membranes of live cells, Chem. Commun. 50 (2014) 5282–5284, https://doi.org/10.1039/c3cc47530a.

[26] M.E. Nipper, M. Dakanali, E. Theodorakis, M.A. Haidekker, Biochimie Detection of liposome membrane viscosity perturbations with ratiometric molecular rotors, Biochimie 93 (2011) 988–994, https://doi.org/10.1016/j.biochi.2011.02.002.

[27] K. Luby-Phelps, S. Mujumdar, R.B. Mujumdar, L.A. Ernst, W. Galbraith, A. S. Waggoner, A novel fluorescence ratiometric method confirms the low solvent viscosity of the cytoplasm, Biophys. J. 65 (1993) 236–242, https://doi.org/10.1016/S0006-3495(93)81075-0.

[28] M.A. Haidekker, T. Ling, M. Anglo, H.Y. Stevens, J.A. Frangos, E.A. Theodorakis, New fluorescent probes for the measurement of cell membrane viscosity, Chem. Biol. 8 (2001) 123–131, https://doi.org/10.1016/s1074-5521(00)90061-9.

[29] A. Battisti, S. Panettieri, G. Abbandonato, F. Cardarelli, Imaging Intracellular Viscosity by a New Molecular Rotor Suitable for Phasor Analysis of Fluorescence Lifetime, 2013, https://doi.org/10.1007/s00216-013-7084-x.

[30] W. Miao, C. Yu, E. Hao, L. Jiao, Functionalized BODIPYs as Fluorescent Molecular Rotors for Viscosity Detection, 2019, pp. 1–6, https://doi.org/10.3389/fchem.2019.00825, 7.

[31] A. Loudet, K. Burgess, BODIPY Dyes and Their Derivatives : Syntheses and Spectroscopic Properties, 2007. https://doi.org/10.1021/cr078381n.

[32] H. Lu, J. Mack, Z. Shen, H. Lu, Structural Modification Strategies for the Rational Design of Red/NIR Region BODIPYs, 2014, pp. 4778–4823, https://doi.org/10.1039/c4cs00030g.

[33] J.T. Mika, A.J. Thompson, M.R. Dent, N.J. Brooks, J. Michiels, J. Hofkens, M. K. Kuimova, Measuring the viscosity of the Escherichia coli plasma membrane using molecular rotors, Biophys. J. 111 (2016) 1528–1540, https://doi.org/10.1016/j.bpj.2016.08.020.

[34] M.K. Kuimova, G. Yahioglu, J.A. Levitt, K. Suhling, Molecular rotor measures viscosity of live cells via fluorescence lifetime imaging, J. Am. Chem. Soc. 130 (2008) 6672–6673, https://doi.org/10.1021/ja800570d.

[35] A. Magni, G. Bondelli, G.M. Paternò, S. Sardar, V. Sesti, Azobenzene Photoisomerization Probes Cell Membrane Nanoviscosity, ArXiv:2107.03205 [Physics.Chem-Ph]. (n.d.).

[36] H.B. Cheng, S. Zhang, J. Qi, X.J. Liang, J. Yoon, Advances in application of azobenzene as a trigger in biomedicine: molecular design and spontaneous assembly, 2007290, Adv. Mater. (2021) 1–42, https://doi.org/10.1002/adma.202007290.

[37] D. Mulatihan, T. Guo, Y. Zhao, Azobenzene photoswitch for isomerization-dependent cancer therapy via azo-combretastatin A4 and phototrexate, Photochem. Photobiol. 96 (2020) 1163–1168, https://doi.org/10.1111/php.13292.

[38] V. Vurro, G. Bondelli, V. Sesti, F. Lodola, G.M. Paternò, G. Lanzani, C. Bertarelli, Molecular design of amphiphilic plasma membrane-targeted azobenzenes for nongenetic optical stimulation, Front. Mater. 7 (2021) 1–10, https://doi.org/10.3389/fmats.2020.631567.

[39] M.L. DiFrancesco, F. Lodola, E. Colombo, L. Maragliano, M. Bramini, G. M. Paternò, P. Baldelli, M.D. Serra, L. Lunelli, M. Marchioretto, G. Grasselli, S. Cimò, L. Colella, D. Fazzi, F. Ortica, V. Vurro, C.G. Eleftheriou, D. Shmal, J. F. Maya-Vetencourt, C. Bertarelli, G. Lanzani, F. Benfenati, Neuronal firing modulation by a membrane-targeted photoswitch, Nat. Nanotechnol. 15 (2020) 296–306, https://doi.org/10.1038/s41565-019-0632-6.

[40] G.M. Paternò, G. Bondelli, V.G. Sakai, V. Sesti, C. Bertarelli, G. Lanzani, The effect of an intramembrane light-actuator on the dynamics of phospholipids in model membranes and intact cells, Langmuir (2020), https://doi.org/10.1021/acs.langmuir.0c01846.

[41] G.M. Paternò, E. Colombo, V. Vurro, F. Lodola, S. Cimò, V. Sesti, E. Molotokaite, M. Bramini, L. Ganzer, D. Fazzi, C. D'Andrea, F. Benfenati, C. Bertarelli, G. Lanzani, Membrane environment enables ultrafast isomerization of amphiphilic azobenzene, Adv. Sci. 7 (2020), https://doi.org/10.1002/advs.201903241.

[42] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, 2006, https://doi.org/10.1007/978-0-387-46312-4.

[43] C. Rumble, K. Rich, G. He, M. Maroncelli, CCVJ Is Not a Simple Rotor Probe, 2012, https://doi.org/10.1021/jp309019g.

[44] A. Beck, D. Heissler, G. Duportail, Influence of the length of the spacer on the partitioning properties of amphiphilic fluorescent membrane probes, Chem. Phys. Lipids 66 (1993) 135–142, https://doi.org/10.1016/0009-3084(93)90038-5.

[45] B.R. Lentz, Membrane "fluidity" as detected by diphenylhexatriene probes, Chem. Phys. Lipids 50 (1989) 171–190. https://doi.org/10.1016/0009-3084(89)90049-2.

[46] J.G. Kuhry, P. Fonteneau, G. Duportail, C. Maechling, G. Laustriat, TMA-DPH, A suitable fluorescence polarization probe for specific plasma membrane fluidity studies in intact living cells, Cell Biophys. 5 (1983) 129–140, https://doi.org/10.1007/BF02796139.

[47] L.M. Loew, Spectroscopic Membrane Probes, vol. 1, 1988. https://books.google.co.uk/books/about/Spectroscopic_membrane_probes.html?id=fGTwAAAAMAAJ&pgis=1.

[48] M. Sinha, S. Mishra, P.G. Joshi, Liquid-ordered microdomains in lipid rafts and plasma membrane of U-87 MG cells: a time-resolved fluorescence study, Eur. Biophys. J. 32 (2003) 381–391, https://doi.org/10.1007/s00249-003-0281-3.

[49] J.M. Kwiatek, D.M. Owen, A. Abu-Siniyeh, P. Yan, L.M. Loew, K. Gaus, Characterization of a new series of fluorescent probes for imaging membrane order, PloS One 8 (2013) 1–7, https://doi.org/10.1371/journal.pone.0052960.

[50] D.M. Owen, S. Oddos, S. Kumar, D.M. Davis, M.A.A. Neil, P.M.W. French, M. L. Dustin, A.I. Magee, M. Cebecauer, High plasma membrane lipid order imaged at the immunological synapse periphery in live T cells, Mol. Membr. Biol. 27 (2010) 178–189, https://doi.org/10.3109/09687688.2010.495353.

[51] M.F. Hanzal-Bayer, J.F. Hancock, Lipid rafts and membrane traffic, FEBS Lett. 581 (2007) 2098–2104, https://doi.org/10.1016/j.febslet.2007.03.019.

[52] D. Scherfeld, N. Kahya, P. Schwille, Lipid dynamics and domain formation in model membranes composed of ternary mixtures of unsaturated and saturated phosphatidylcholines and cholesterol, Biophys. J. 85 (2003) 3758–3768, https://doi.org/10.1016/S0006-3495(03)74791-2.

[53] C. Dietrich, L.A. Bagatolli, Z.N. Volovyk, N.L. Thompson, M. Levi, K. Jacobson, E. Gratton, Lipid rafts reconstituted in model membranes, Biophys. J. 80 (2001) 1417–1428, https://doi.org/10.1016/S0006-3495(01)76114-0.

[54] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569–572, https://doi.org/10.1038/42408.

[55] P. Scheiffele, A. Rietveld, T. Wilk, K. Simons, Influenza viruses select ordered lipid domains during budding from the plasma membrane, J. Biol. Chem. 274 (1999) 2038–2044, https://doi.org/10.1074/jbc.274.4.2038.

[56] G.C. Carter, L. Bernstone, D. Sangani, J.W. Bee, T. Harder, W. James, HIV entry in macrophages is dependent on intact lipid rafts, Virology 386 (2009) 192–202, https://doi.org/10.1016/j.virol.2008.12.031.

[57] E. Drolle, S. Turnbull, N. Mei, C. Filice, B.Y. Lee, M. Robinson, E. Pavlov, E. Finot, Z. Leonenko, Changes in lipid membrane may trigger amyloid toxicity in Alzheimer's disease, Biophys. J. 116 (2019), https://doi.org/10.1016/j.bpj.2018.11.2298, 427a.

[58] J.W. Pettegrew, K. Panchalingam, R.L. Hamilton, R.J. McClure, Brain membrane phospholipid alterations in Alzheimer's Disease, Neurochem. Res. 26 (2001) 771–782, https://doi.org/10.1023/A:1011603916962.

[59] X. He, Y. Huang, B. Li, C. Gong, E.H. Schuchman, Deregulation of sphingolipid metabolism in Alzheimer ' s Disease. https://doi.org/10.1016/j.neurobiolaging.2008.05.010, 2010, 31, 398-408.

[60] M.F. Coughlin, D.R. Bielenberg, G. Lenormand, M. Marinkovic, C.G. Waghorne, B. R. Zetter, J.J. Fredberg, Cytoskeletal stiffness, friction, and fluidity of cancer cell lines with different metastatic potential, Clin. Exp. Metastasis 30 (2013) 237–250, https://doi.org/10.1007/s10585-012-9531-z.

[61] T. Parasassi, E.K. Krasnowska, L. Bagatolli, E. Gratton, Laurdan and prodan as polarity-sensitive fluorescent membrane Probes 8 (1998) 365–373. https://doi.org/10.1023/A:1020528716621.

[62] L.A. Bagatolli, To see or not to see: lateral organization of biological membranes and fluorescence microscopy, Biochim. Biophys. Acta Biomembr. 1758 (2006) 1541–1556, https://doi.org/10.1016/j.bbamem.2006.05.019.

[63] P. Jurkiewicz, A. Olzyńska, M. Langner, M. Hof, Headgroup hydration and mobility of DOTAP/DOPC bilayers: a fluorescence solvent relaxation study, Langmuir 22 (2006) 8741–8749, https://doi.org/10.1021/la061597k.

[64] G. Weber, F.J. Farris, Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-propionyl-2-(dimethylamino)naphthalene, Biochemistry 18 (1979) 3075–3078, https://doi.org/10.1021/bi00581a025.

[65] R.B. Macgregor Jr., G. Weber, Fluorophores in polar media: spectral effects of the Langevin distribution of electrostatic interactions, Ann. N. Y. Acad. Sci. 366 (1981) 140–154. https://doi.org/10.1111/j.1749-6632.1981.tb20751.x.

[66] M. Viard, J. Gallay, M. Vincent, O. Meyer, B. Robert, M. Paternostre, Laurdan solvatochromism: solvent dielectric relaxation and intramolecular excited-state reaction, Biophys. J. 73 (1997) 2221–2234, https://doi.org/10.1016/S0006-3495(97)78253-5.

[67] T. Parasassi, G. De Stasio, G. Ravagnan, R.M. Rusch, E. Gratton, Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence, Biophys. J. 60 (1991) 179–189, https://doi.org/10.1016/S0006-3495(91)82041-0.

[68] D.M. Owen, C. Rentero, A. Magenau, A. Abu-Siniyeh, K. Gaus, Quantitative imaging of membrane lipid order in cells and organisms, Nat. Protoc. 7 (2012) 24–35, https://doi.org/10.1038/nprot.2011.419.

[69] K. Gaus, T. Zech, T. Harder, Visualizing membrane microdomains by Laurdan 2-photon microscopy, Mol. Membr. Biol. 23 (2006) 41–48, https://doi.org/10.1080/09687860500466857.

[70] T. Parasassi, G. De Stasio, A. d'Ubaldo, E. Gratton, Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence, Biophys. J. 57 (1990) 1179–1186, https://doi.org/10.1016/S0006-3495(90)82637-0.

[71] Z. Lu, S.J. Lord, H. Wang, W.E. Moerner, R.J. Twieg, Long-wavelength analogue of PRODAN: synthesis and properties of Anthradan, a fluorophore with a 2,6-donor-acceptor anthracene structure, J. Org. Chem. 71 (2006) 9651–9657, https://doi.org/10.1021/jo0616660.

[72] H.M. Kim, H.-J. Choo, S.-Y. Jung, Y.-G. Ko, W.-H. Park, S.-J. Jeon, C.H. Kim, T. Joo, B.R. Cho, A two-photon fluorescent probe for lipid raft imaging: C-laurdan, Chembiochem 8 (2007) 553–559, https://doi.org/10.1002/cbic.200700003.

[73] J. Barucha-Kraszewska, S. Kraszewski, C. Ramseyer, Will C-Laurdan dethrone Laurdan in fluorescent solvent relaxation techniques for lipid membrane studies? Langmuir 29 (2013) 1174–1182, https://doi.org/10.1021/la304235r.

[74] L. Jin, A.C. Millard, J.P. Wuskell, X. Dong, D. Wu, H.A. Clark, L.M. Loew, Characterization and application of a new optical probe for membrane lipid domains, Biophys. J. 90 (2006) 2563–2575, https://doi.org/10.1529/biophysj.105.072884.

[75] L. Jin, A.C. Millard, J.P. Wuskell, H.A. Clark, L.M. Loew, Cholesterol-enriched lipid domains can be visualized by di-4-ANEPPDHQ with linear and nonlinear optics, Biophys. J. 89 (2005) L04–L06, https://doi.org/10.1529/biophysj.105.064816.

[76] A. Colom, E. Derivery, S. Soleimanpour, C. Tomba, M. Dal, N. Sakai, S. Matile, A fluorescent membrane tension probe, (n.d.) 1–22. https://doi.org/10.1038/s41557-018-0127-3.

[77] W. Helfrich, Elastic properties of lipid bilayers: theory and possible experiments, Z. Naturforsch. C Biosci. 28 (1973) 693–703, https://doi.org/10.1515/znc-1973-11-1209.

[78] B. Pontes, P. Monzo, L. Gole, A.-L. Le Roux, A.J. Kosmalska, Z.Y. Tam, W. Luo, S. Kan, V. Viasnoff, P. Roca-Cusachs, L. Tucker-Kellogg, N.C. Gauthier, Membrane tension controls adhesion positioning at the leading edge of cells, J. Cell Biol. 216 (2017) 2959–2977, https://doi.org/10.1083/jcb.201611117.

[79] A.D. Lieber, Y. Schweitzer, M.M. Kozlov, K. Keren, Front-to-rear membrane tension gradient in rapidly moving cells, Biophys. J. 108 (2015) 1599–1603, https://doi.org/10.1016/j.bpj.2015.02.007.

[80] N.C. Gauthier, M.A. Fardin, P. Roca-Cusachs, M.P. Sheetz, Temporary increase in plasma membrane tension coordinates the activation of exocytosis and contraction during cell spreading, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 14467–14472, https://doi.org/10.1073/pnas.1105845108.

[81] T.A. Masters, B. Pontes, V. Viasnoff, Y. Li, N.C. Gauthier, Plasma membrane tension orchestrates membrane trafficking, cytoskeletal remodeling, and biochemical signaling during phagocytosis, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 11875–11880, https://doi.org/10.1073/pnas.1301766110.

[82] J. Lafaurie-Janvore, P. Maiuri, I. Wang, M. Pinot, J.-B. Manneville, T. Betz, M. Balland, M. Piel, ESCRT-III assembly and cytokinetic abscission are induced by tension release in the intercellular bridge, Science 339 (2013) 1625–1629, https://doi.org/10.1126/science.1233866.

[83] Y. Wang, Y. Liu, H.A. Deberg, T. Nomura, M.T. Hoffman, P.R. Rohde, K. Schulten, B. Martinac, P.R. Selvin, Single molecule FRET reveals pore size and opening mechanism of a mechano-sensitive ion channel, Elife 3 (2014), e01834, https://doi.org/10.7554/eLife.01834.

[84] R.F.A. Zwaal, A.J. Schroit, Pathophysiologic implications of membrane phospholipid asymmetry in blood cells, Blood 89 (1997) 1121–1132, https://doi.org/10.1182/blood.V89.4.1121.

[85] V. V Shynkar, A.S. Klymchenko, C. Kunzelmann, G. Duportail, C.D. Muller, A. P. Demchenko, J.-M. Freyssinet, Y. Mely, Fluorescent biomembrane probe for ratiometric detection of apoptosis, J. Am. Chem. Soc. 129 (2007) 2187–2193, https://doi.org/10.1021/ja068008h.

[86] P.K. Sengupta, M. Kasha, Excited state proton-transfer spectroscopy of 3-hydroxyflavone and quercetin, Chem. Phys. Lett. 68 (1979) 382–385. https://doi.org/10.1016/0009-2614(79)87221-8.

[87] O.A. Kucherak, S. Oncul, Z. Darwich, D.A. Yushchenko, Y. Arntz, P. Didier, Y. Mély, A.S. Klymchenko, Switchable nile red-based probe for cholesterol and lipid order at the outer leaflet of biomembranes, J. Am. Chem. Soc. 132 (2010) 4907–4916, https://doi.org/10.1021/ja100351w.

[88] P. O'Shea, Intermolecular interactions with/within cell membranes and the trinity of membrane potentials: kinetics and imaging, Biochem. Soc. Trans. 31 (2003) 990–996, https://doi.org/10.1042/bst0310990.

[89] L.A. Kadir, M. Stacey, R. Barrett-Jolley, Emerging roles of the membrane potential: action beyond the action potential, Front. Physiol. 9 (2018) 1–10, https://doi.org/10.3389/fphys.2018.01661.

[90] X. Huang, L.Y. Jan, Targeting potassium channels in cancer, J. Cell Biol. 206 (2014) 151–162, https://doi.org/10.1083/jcb.201404136.

[91] G.M. Paternò, G. Bondelli, G. Lanzani, Bringing microbiology to light: toward all-optical electrophysiology in bacteria, Bioelectricity 3 (2021) 136–142, https://doi.org/10.1089/bioe.2021.0008.

[92] J. Plásek, K. Sigler, Slow fluorescent indicators of membrane potential: a survey of different approaches to probe response analysis, J. Photochem. Photobiol., B 33 (1996) 101–124, https://doi.org/10.1016/1011-1344(96)07283-1.

[93] P.R. Dragsten, W.W. Webb, Mechanism of the membrane potential sensitivity of the fluorescent membrane probe merocyanine 540, Biochemistry 17 (1978) 5228–5240, https://doi.org/10.1021/bi00617a024.

[94] P.J. Sims, A.S. Waggoner, C.H. Wang, J.F. Hoffman, Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles, Biochemistry 13 (1974) 3315–3330, https://doi.org/10.1021/bi00713a022.

[95] Thermo Fisher Scientific, Probes for membrane potential, Mol. Probes Handb. - A Guid. to Fluoresc. Probes Labeling Technol. (2010) 922–936, https://doi.org/10.7554/eLife.44522.

[96] A. Grinvald, R. Hildesheim, VSDI: a new era in functional imaging of cortical dynamics, Nat. Rev. Neurosci. 5 (2004) 874–885, https://doi.org/10.1038/nrn1536.

[97] A. Kuznetsov, V.P. Bindokas, J.D. Marks, L.H. Philipson, FRET-based voltage probes for confocal imaging: membrane potential oscillations throughout pancreatic islets, Am. J. Physiol. Cell Physiol. 289 (2005) C224–C229, https://doi.org/10.1152/ajpcell.00004.2005.

[98] T.W. Cacciatore, P.D. Brodfuehrer, J.E. Gonzalez, T. Jiang, S.R. Adams, R. Y. Tsien, W.B. Kristan, D. Kleinfeld, Identification of neural circuits by imaging coherent electrical activity with FRET-based dyes, Neuron 23 (1999) 449–459, https://doi.org/10.1016/S0896-6273(00)80799-0.

[99] J.R. Lazzari-dean, A.M.M. Gest, E.W. Miller, U. States, H. Wills, U. States, Optical Estimation of Absolute Membrane Potential Using Fluorescence Lifetime Imaging, 2019, pp. 1–29, https://doi.org/10.7554/eLife.44522.

[100] A.P. Demchenko, A.S. Klymchenko, G. Duportail, Y. Me, Ultrasensitive two-color fluorescence probes for dipole potential in phospholipid membranes. https://doi.org/10.1073/pnas.1934603100, 2003, 100, 11219-11224.

[101] V. V Shynkar, A.S. Klymchenko, G. Duportail, A.P. Demchenko, Y. Me, Two-color Fluorescent Probes for Imaging the Dipole Potential of Cell Plasma Membranes, 2005, pp. 128–136, https://doi.org/10.1016/j.bbamem.2005.03.015, 1712.