Chapter

Pore-Forming Proteins: Fluorescent Dyes to Study the Channel Functionality and Biophysical Properties

Guilherme Teixeira and Robson Faria

Abstract

Large conductance channels, when activated transiently, alter the plasma membrane permeability permitting the passage of molecules with the size of until 1000 Da. These pore-forming proteins are found in the plasma membrane of mammals and invertebrate cells such as the P2X7 receptor, transient receptor potential vanilloid 1 (TRPV1), transient potential melastin receptor 8 (TRPM8), pannexin-1, and hemichannel proteins. Other proteins may be secreted for organisms creating nonselective pathways to large molecules and proteins. Some examples are α-hemolysin and gramicidin. In both cases, an efficient manner to evaluate the functionality is using fluorescent dyes. Thus, we discuss the uses of the fluorescent dyes as tools to study the functionality and some biophysical properties of the proteins described above, among others.

Keywords: fluorescent dyes, ion channels, pore-forming proteins

1. Introduction

Techniques involving fluorescence have been used and improved in studies involving cells, the visualization, and research of their diverse biochemical structures and functions. Detection of proteins, recognition of organelles, nucleic acids, membrane permeabilization, and ions present in cells are widely explored through the feasibility of fluorescent dyes according to their physical-chemical characteristics such as excitation and emission lengths, electrical charges, chemical structures, and molecular weights [1, 2].

Ion channel-forming ionotropic receptors are extremely important proteins in maintaining the potential of cell membranes. In some cases, they are able to form pores (high conductance channels) in the plasma membrane, allowing the passage of certain ions of varying charges and different physical characteristics according to the concentration and electrochemical gradient. Thus, there are a large number of high conductance ion channels capable of opening pores, changing the permeability of the plasma membrane. As examples, we can mention the P2X7 purinergic receptor, transient receptor potential vanilloid (TRPV), voltage-dependent anion channel (VDAC), and others. When stimulated, they can allow ions to pass through

the formed pores and molecules with up to 1000 Da to the intracellular medium [3–7]. Thus, certain fluorescent dyes can be used to observe these pores of high ionic conductance, thus being able to further explore about the biophysical and chemical characteristics of these membrane proteins.

2. Pore-forming ionic channels in mammals

The formation of pores in the plasma membrane is a phenomenon often caused by defense proteins in mammalian cells as an immune response to infections by pathogens, tumors, or danger signs. As a response, several proteins are activated, forming pores such as perforin, gasdermin, complement system, mixed lineage kinase domain-like pseudokinase (MLKL), and granulysin, thus mediating cytotoxic and antimicrobial immune activities aimed at preventing and protecting the body from harmful agents [8, 9].

In addition to cellular defense proteins, ion channels are another class of proteins involved in the formation of pores in the plasma membrane, being responsible for the transit of ions such as selective to Ca^{2+}, N^{+}, K^{+}, and Cl^{-} and acting in a physiological and pathophysiological way. There are a large number of ion channels addressed in the literature related to the pore opening. Among them, the P2X7 receptor has been highlighted. The P2X7 receptor, which is the most studied among P2X, is a cationic channel activated by adenosine-5′-triphosphate (ATP) [10, 11]. This channel is responsible for several intracellular processes, such as apoptosis, NLRP3 inflammasome (one of the IL-1β study pathways), inflammation, and pain. When activated, P2X7 leads to an increase in ion flow that can intensify with a decrease in the influx of cations from divalent charges such as Ca^{2+} [12]. Electrophysiology (EP) studies addressed in 2005 by Faria et al. [4] about the high pore conductance of the P2X7 receptor tested on 2BH4 and peritoneal macrophage cells observed unitary conductance values corresponding to 400 pS. In addition to these data, experiments using high-molecular-weight cationic and anionic fluorescent dyes also confirm the high pore conductance. Regarding the permeabilization through the pore, fluorophores tested were able to penetrate the cell regardless of its load, ethidium bromide (1^{+}), propidium iodide (2^{+}), and Lucifer Yellow (2^{-}).

However, other channels in the literature also correlate with the pore of the P2X7 receptor showing high conductance and permeability to fluorophores through the membrane. As highlights are the TRP receptors as TRPV 1–4, with notoriety for TRPV1, which is the most studied subtype of TRPs. This subtype is expressed in several cells of the organism such as the liver, lung, and nociceptive neurons. TRPV1 is a nonselective cationic channel with high permeability for Ca^{2+}, with a unitary conductance of 35–77 pS [13, 14]; however, the unitary conductance of the pore formed was not record until now. Although the ionic channel may be activated for distinct stimuli (capsaicin, low pH, and heat), this receptor only permits the fluorescent dye uptake after capsaicin activation and low pH conditions. Other TRP subtypes related to high conductance channel formation are the transient potential ankhirin type-1 (TRPA1) cationic channels with a conductance of 87–100 pS and transient potential melastin receptor 8 (TRPM8) with a conductance of 40–83 pS, according to [14, 15]. Pannexin-1 [16, 17], connexin hemichannel [18], calcium homeostasis modulator 1 (CALHM1) [19], voltage-dependent anion channel (VDAC1 highlighted) [6, 20], maxi anion [21], P2X (P2X2 and P2X4) [11], and maitotoxin-induced pore [22] are some other different types of channels with unitary conductance values greater than 200 pS and permeable to molecules of until 1000 Da [4, 10, 23]. More details about these pore-forming proteins and some techniques used to record their functionality are shown in **Table 1**.

Pore-forming proteins	EF	PLP	FC	FPR	CA	CPA
P2X7R	[4]	[24]	[25]	[26]	[26]	[4]
TRPA1	[27]	[28]	N/A	[27]	[27]	[27]
Maxi anion	[17]	N/A	N/A	[17]	[17]	[17]
TRPV1	[15]	[59]	N/A	[71]	[24]	[15]
Plasma VDAC	[29]	[30]	N/A	N/A	[29]	[29]
Connexin	[31]	[32]	[33]	[31]	[31]	[31]
Pannexin	[34]	[16]	[35]	[17]	[17]	[17]
Maitotoxin	[22]	[36]	N/A	[22]	[37]	[37]
CALHM1	[31]	N/A	[38]	[31]	[31]	[31]
TRPM8	[39]	[40]	[41]	N/A	[40]	[42]

Table taken from Ref. [49] and adapted.

Table 1.
Pore-forming proteins and some assessment techniques as electrophysiology (EP), planar lipid bilayer (PLP), flow cytometry (FC), fluorescent plate reader (FPR), colorimetric assay (CA), and cell permeabilization assay (CPA) for functional detection of the high conductance.

Although they have similarities to conductance, these different channels have particularities related to biophysical properties, having different functionalities and mechanisms. Some channels exhibit a distinct selectivity for certain ions, some having preference for anions such as Cl^{-}, PO_4^{3-}, and glutamate in the case of VDAC1 and maxi anion or cations like Ca^{2+} for CALHM1 [6, 19, 43].

These channels are the subject of studies for their involvement in several cellular processes, such as intracellular signaling response, release of cytokines, inflammation, cell death, pain, tumors, and metabolism regulation. Interestingly, the same functions are performed for these large conductance channels with distinct cell types or tissues. We can highlight the role of TRPs (TRPV1 and TRPM8), P2X7 receptor, connexin 43, and pannexin-1 [11, 44–48].

Based on the study of these channels, several molecular and cellular biology techniques are employed to obtain the best understanding of the functions that these receptors perform. Among these assays, the cell death assay, flow cytometry, intracellular calcium quantification, cytokine assay, and cell permeabilization stand out. However, for the best performance of these techniques, fluorescent dyes are used according to their physical-chemical characteristics and molecular mass, serving as a strategy to obtain better results and performance of experimental tests [49]. For additional information about some of the techniques mentioned above, more details involving their respective methodologies can be found in the reviews published below [50–52].

3. Fluorescent dyes and their use in the study of pores by ion channels

Fluorescence is a kind of luminescence in which the electron can capture energy by entering an excited state for a short period and returning to its normal state by emitting light. The first discoveries involving this form of light were in the middle of the sixteenth century by physician and botanist Nicolás Monardes through a wood (*Lignum nephriticum*) from Mexico used to treat kidney diseases [53, 54]. Over the years, the term "fluorescence" was coined by physicist George Gabriel Stokes, in addition to presenting a list of fluorescent substances, confirming new

advances in the area. With the advancement of science and the years, the first fluorescent dye was synthesized, fluorescein, an anionic dye produced by German chemist Adolf von Baeyer, a molecule that would be used in several laboratory methods in the future [49, 55].

Fluorescent dyes are widely used in various specialties within science, among them; the staining of different structures in tissues and cells stands out. A vast number of laboratory techniques of molecular and cellular biology in broad fields of research use these molecules, based on their chemical and physical properties according to the method worked, thus being an excellent resource in the investigation and visualization of the topic addressed [56, 57]. Among many techniques, the opening function of the high conductance pores mediated through ion channels can be evaluated in experimental tests for the capture of intracellular dye [4, 49]. Many dyes are impermeable to the membrane due to their molecular weight and physical characteristics such as positive charge (cationic dyes) and negative charge (anionic dyes). However, due to the opening of the pores with a subsequent increase in permeability, it is possible to investigate these dyes through the capture by fluorescence reading. The interaction of fluorescent dyes in the intracellular medium occurs according to the electrical charge of the target structure to be marked. Several structures that make up the intracellular environment, such as ions, nucleic acids, and organelles, each are having a specific electrical character, thus enabling the connection with fluorophores after permeating through the high conductance pores [1, 58].

In 1987, one of the first works was carried out with the use of fluorescent dyes to determine pore permeability, in which there were significant results about the subject. Steinberg et al. [58] in a study using macrophages of the J774 strain found that the presence of extracellular ATP was able to induce increased cation flow through an unidentified membrane receptor. In order to better analyze the P2X7 receptor permeability, five fluorescent anionic dyes impermeable to the membrane with varying were tested. The authors tested Lucifer Yellow (457 Da, 2^-) being the Ca^{2+} pentavalent Fura-2 indicator (832 Da, 5^-), 6-carboxyfluorescein (376 Da, 2^-), trypan blue, and Evans blue with similar charge and molecular weight (960.81 Da, 4^-). The first three dyes showed permeability through the influence of ATP. However, trypan blue and Evans blue were not permeable to the cell and may suggest a specific conductance by the receptor through the different molecular weights of the fluorophores used by the authors. This data led to the exploration and better understanding of P2X pores, mainly from the P2X7 receptor [11, 12]. However, the data about the P2X7 receptor pore permeability to cationic fluorescent dyes with charge higher 2^+ (propidium iodide) was not investigated.

In another study, Faria et al. [59] evaluated the dye uptake involving proteins P2X7, TRPV1, TRPA1, and maxi anion in astrocytes. The selectivity of these pores for cationic dyes (ethidium bromide and propidium iodide) and anionics (fluorescein and calcein) was measured. The TRPV1 and TRPA1 receptors were selective for cationic ones allowing their permeability and maxi anion for anionic ones, suggesting a possible preference of these pores concerning the electric charge of the fluorophore. This aspect did not occur with the P2X7 receptor, already reported, and another study about the subject [4]. However, the data about the maximal size of cationic dyes able to permeate for TRPV1 and TRPA1 channels or anionic for maxi anion channels was not investigated. Additionally, the permeability to compounds with the higher charge was not discussed.

One of the points to be noted regarding the capture of dyes in the investigation of pore-forming proteins is the low diversity of fluorophores used in these tests. In

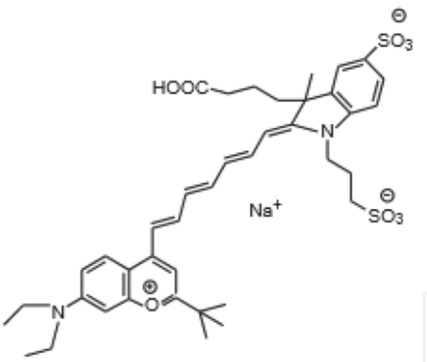
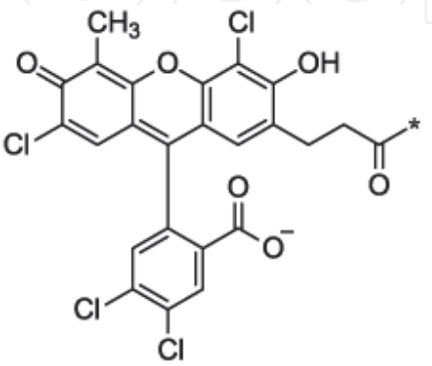
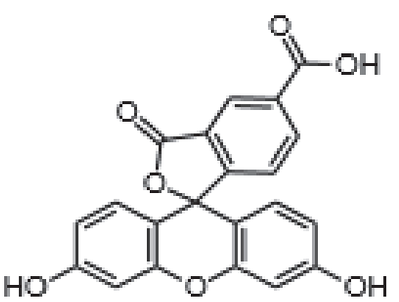
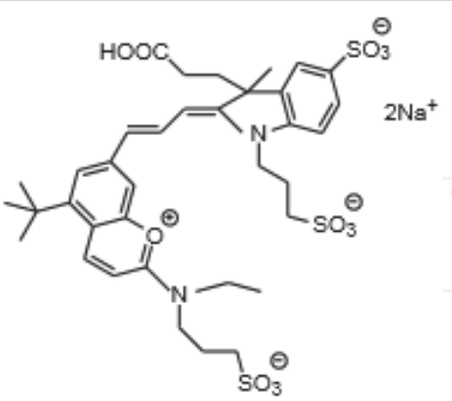
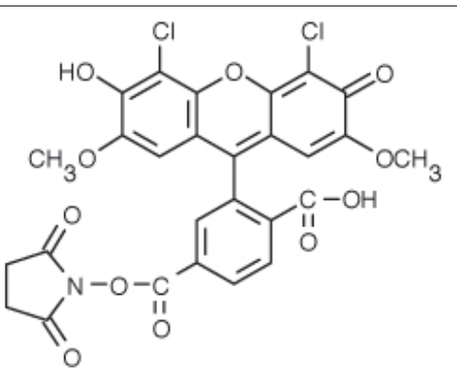
most experiments, "standard" dyes are used, among which are the cationic ethidium bromide (394 Da, 1^+), propidium iodide (668.39 Da, 2^+), YO-PRO-1 (629 Da, 2^+), and DAPI (350.25 Da, 2^+) which mostly interact with nucleic acids. Anionics such as Lucifer Yellow (457 Da, 2^-) Fluo-4 (736.63 Da, 4^-), Fura-2 (832 Da, 5^-), and fluorescein (332.31 Da, 1^-) are usually ion markers or intracellular structures [27, 59, 60]. A study by McCoy et al. [42] on the permeabilization of large molecules mediated by the TRPM8 receptor, the cationic dye PO-PRO-3 (605.30 Da, 2^+), was used to measure the dilation of the pore. However, it is not common to use fluorophores other than those mentioned above, having a great scenario to be explored in ionic conductance tests. Another impacting factor regarding dye testing is related to the electrical charge in which the molecule stands out. As seen previously, mainly in cationic dyes, the charges vary between the charge of 1^+ and 2^+, a fact little investigated, and the search for tests in dyes with more tri or tetravalent charges is relevant in the evaluation of these pores. However, pores similar to dyes also have charges, suggesting varied possibilities for molecular interaction of pores with the physicochemical properties of fluorophores, a fact that is not thoroughly investigated in scientific researches [49].

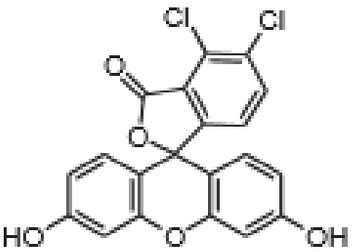
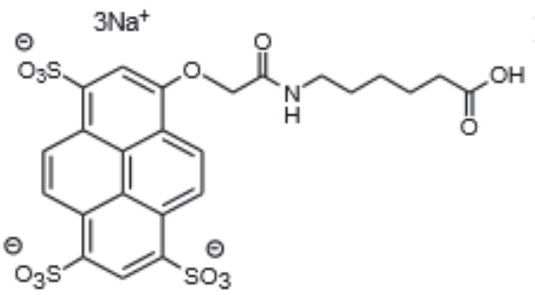
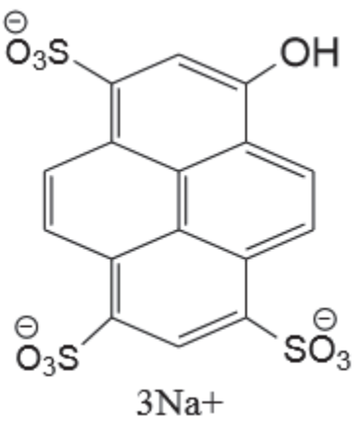
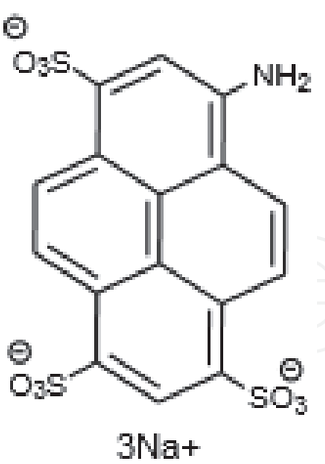
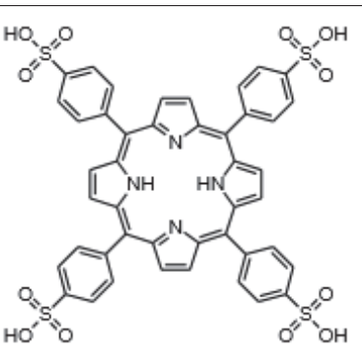
We consider it relevant to emphasize that some of the pore-forming proteins mentioned have no reports of the use of fluorescent dyes such as pl-VDAC. Other proteins possess basically the same ones studied at the P2X7 receptor, such as pannexin-1, maitotoxin, and maxi anion [22, 34, 59].

The only exception would be connexins, such as connexin 43, which is due to its prominent regulatory function in several vital processes, such as cardiac synchronism. However, these proteins have too much variability of homotypic and heterotypic channels with unitary conductance and permeability by size and charge of the variable molecule. Thus, the biophysical characterization of these channels can be models to generate protocols for the study of the dyes above in the other high unit conductance channels [61].

There is a plurality of fluorophores described in the literature still used in the evaluation of pore formation functionality [62], given a large field to be explored and researched regarding the use of these dyes in order to understand more clearly some specific characteristics of these pores. The following tables (**Tables 2** and **3**) contain a total of 24 different examples of fluorescent dyes with varied molecular weights, structures, and different charges (mono-, di-, tri-, or tetravalent), cutoff, within their physical-chemical characteristics, in which can be tested in future experiments to deepen the understanding about the biophysical properties of these high ion conductance channels. We hope that the data contained in these tables can contribute to the purpose of studies of the pore-forming channels, which may serve as a tactic and add discoveries to the scientific field, following more knowledge of cell physiology.

A detail to note is that the table corresponding to cationic lacks more examples of tetravalent dyes. The proteins addressed in the previous topic are reported to allow the permeabilization of molecules of up to 1000 Da. However, there are other types of tetravalent fluorophores found in the literature that exceed the permeable molecular weight limit for these pores [2]. BoBo 1 (1202.66 Da), BoBo 3 (1254.73 Da), ToTo-1 (1302.78 Da), and ToTo 3 (1354.85 Da) are some other examples of charged dyes (4^+) [62]. Thus, the lack of other viable tetravalent dyes to the corresponding permeability values of the described proteins prevents the exploration and evaluation of the pores with these molecules. Therefore, we consider it necessary to investigate other fluorescent dyes with this type of charge, aiming at possible future experiments on the molecular pore interaction with this level of net charge [56, 63].

Anionic dyes	MW	Charge	Molecular structure
PromoFluor 840	**788.96**	**1⁻**	
Yakima Yellow	**654.30**	**1⁻**	
5-Carboxyfluorescein	**376.32**	**1⁻**	
PromoFluor 680	**852.96**	**2⁻**	
JOE	**602.34**	**2⁻**	

Anionic dyes	MW	Charge	Molecular structure
Dichlorofluorescein	**401.2**	2^-	
PromoFluor 405	**695.59**	3^-	
Methoxypyrenetrisulfonic acid trisulfonate (MPTS)	**538.4**	3^-	
8-Aminopyrene-1,3,6-trisulfonic acid (APTS)	**523.38**	3^-	
Tetrakis(4sulfophenyl) porphine (TSPP)	**935**	4^-	

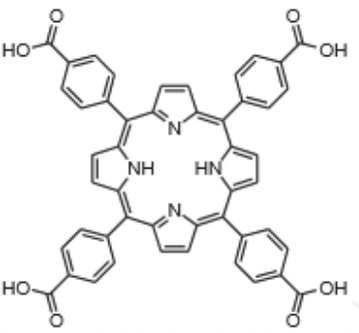
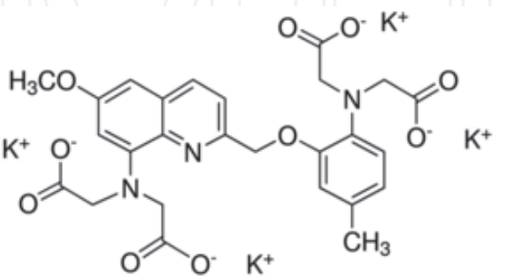
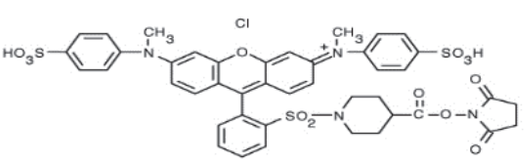
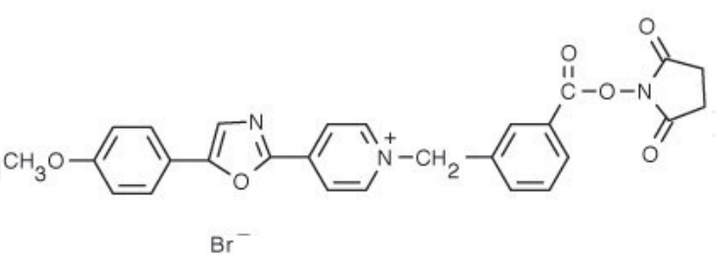
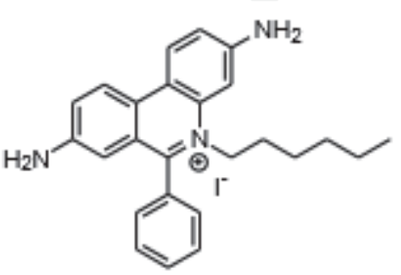
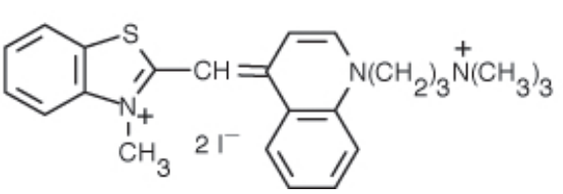
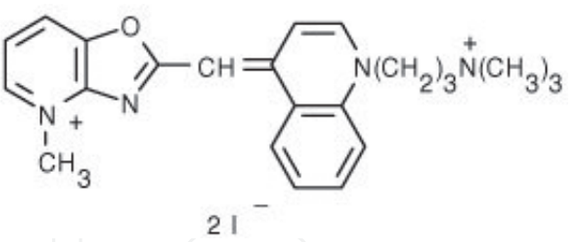
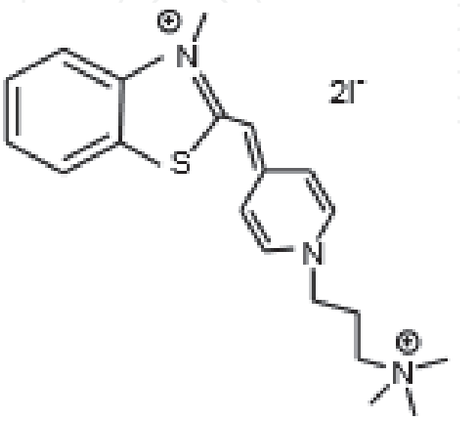
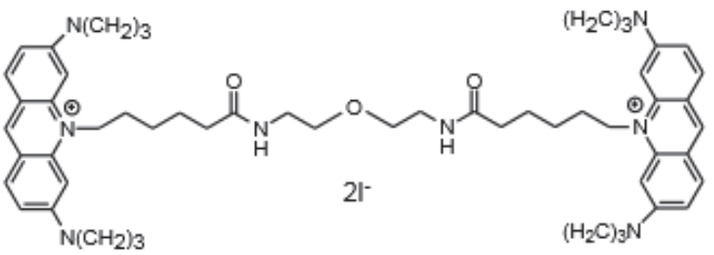
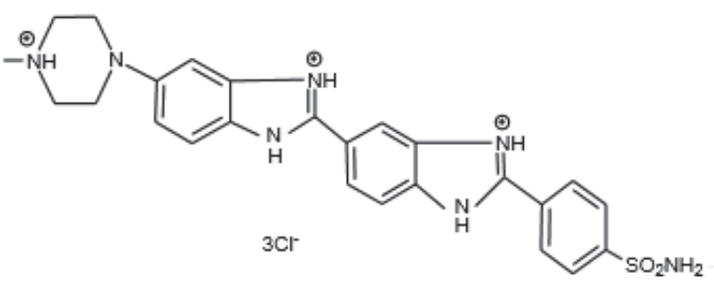
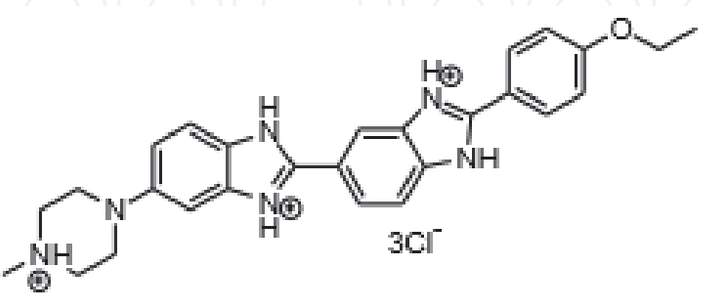
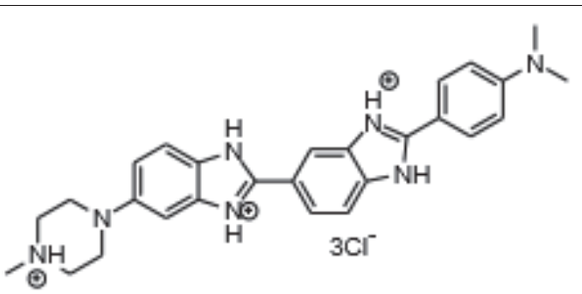
Anionic dyes	MW	Charge	Molecular structure
Tetrakis(4carboxyphenyl) porphine (TCPP)	**790.8**	**4⁻**	
Quin-2	**693.87**	**4⁻**	

Table 2.
Fluorescent anionic dyes.

Cationic dyes	MW	Charge	Molecular structure
QSY9 carboxylic acid succinimidyl ester	**951.43**	**1⁺**	
PyMPO	**564.4**	**1⁺**	
Hexidium iodide	**497.42**	**1⁺**	
TO-PRO-1	**645.38**	**2⁺**	

Cationic dyes	MW	Charge	Molecular structure
JO-PRO-1	**630.31**	2^+	
BO-PRO-1	**595.32**	2^+	
EvaGreen	**509.73**	2^+	
Hoechst 769121	**596.96**	3^+	
Hoechst 33342	**561.93**	3^+	
Hoechst 34580	**560.96**	3^+	

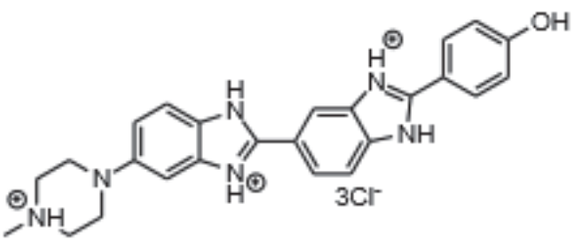
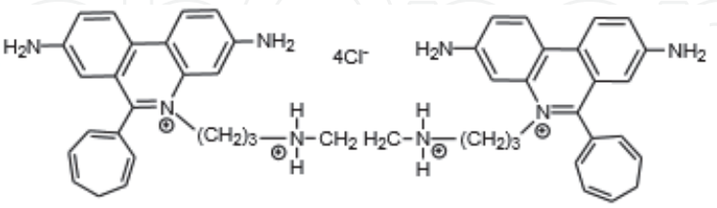
Cationic dyes	MW	Charge	Molecular structure
Hoechst 33258	533.88	3^+	
EthD-1	856.77	4^+	

Table 3.
Fluorescent cationic dyes.

4. Conclusion

Scientific advances with methods involving fluorescence, such as fluorimetry and colorimetry, have led to the significant discoveries in different horizons of biological knowledge, with the application of fluorophores in these methodologies having an immense importance in the purpose of evaluating different topics involving the study of cells. Studies on the permeabilization of the plasma membrane through high conductance pore-forming proteins are a topic widely addressed in laboratory research, with the use of fluorescent dyes being a useful device in the evaluation and investigation of the functionality of ionotropic receptors. Through the tables presented above, several anionic and cationic dyes with different charge values (mono-, di-, tri-, and tetravalent) can be used by researchers in numerous experiments, aiming to check these pores through their physical-chemical properties and conductance. We hope that this review will motivate further research on the applicability, mechanisms, and biophysical properties of these high ion conductance channels, aiming at new knowledge and discoveries for the cellular biological perspective.

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Conflict of interest

The authors declare no conflict of interest.

Notes/thanks/other declarations

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Author details

Guilherme Teixeira and Robson Faria*
Laboratory of Toxoplasmosis and Other Protozoans, Oswaldo Cruz Institute (IOC), Fiocruz, Rio de Janeiro, Brazil

*Address all correspondence to: robson.xavier@gmail.com

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