Bright future of optical assays for ion channel drug discovery

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Ion channels are a key target class for drug discovery. The introduction of new and optimized optical probes, including fluorescent protein-based calcium sensors, luminescent photoproteins, voltage-sensitive probes and ion indicators, allows tackling a wide variety of ion channel targets. To make optical assays more physiologically relevant, tools to control the conformational states of ion channels via manipulation of the membrane potential have to be developed. There is no doubt that progress in optical methods will streamline the ion channel drug discovery process.

Ion channels are pore-forming transmembrane proteins that regulate the rapid movement of ions across the cell membranes [1]. Ion channels use a variety of 'gating' mechanisms to open and close their pores in response to biological stimuli such as the ligand binding or membrane potential changes. During gating, ion channels undergo conformational changes to assume several drastically different activation states, i.e. closed (non-conducting, deactivated), open (conducting, activated), inactivated (non-conducting). Different types of ion channels exist throughout the body, and besides setting the resting membrane potential and controlling cellular excitability, ion channels mediate critical physiological functions including heartbeat, signal transduction, cell secretion, and gene expression. Ion channel dysfunctions produce a diverse set of disorders, including arrhythmias, seizure disorders, neuropathic pain, glaucoma, hypertension, migraine, cystic fibrosis, and renal disease [2–4]. Among the 100 top-selling drugs, 15 are ion-channel modulators with a total market value of more than $15 billion, including anti-hypertensive amlodipine besylate (Norvasc; Pfizer) and anti-insomnia zolpidem tartrate (Ambien; Sanofi Aventis).

To realize the full potential of ion channels as a target class, the drug discovery community is actively searching for drugs targeting the specific states of ion channels, as it would allow the fine-tuning of drug effects as a function of the degree and frequency of channel activity [4,5]. This state-dependent or use-dependent mechanism enables drugs to specifically modulate pathological channel activity without affecting normal function. Drugs such as anticonvulsants (i.e. carbamazepine) and local anesthetics (i.e. lidocaine) targeting voltage-gated sodium channels in neurons work by this principle [6,7]. Novel state-dependent and use-dependent drugs are expected to display minimal side effects because of their selectivity for target channels versus similar ion channel subtypes with different gating behavior.

To discover such drugs, screening technologies must be able to repeatedly trigger ion channel conformational changes (physiological activation), detect small transient changes in ion channel gating in the presence of a drug (high information content), and collect 100,000 data points per day (high throughput).

The current gold standard technique for probing ion channel activity is the patch-clamp method. This high-content low-throughput method allows to measure small ion currents with sub millisecond temporal resolution while controlling the membrane potential [8]. Compared to manual patch clamp, automated patch clamp (APC) instruments have a 10–100-fold improved throughput [9,10]. However, the highest throughput using commercially available APC instruments is currently still only ~2000 data points per day (IonWorks® Quattro™, Molecular Devices), which falls short of the requirements for high throughput screening (HTS) campaigns. Moreover, these instruments are rather costly. Therefore, APC instruments are primarily used for lead optimization, hit confirmation, and safety screening. Currently, developments in APC technologies are focused on higher throughput, lower cost, and miniaturization.

Optical ion channel assays can achieve a throughput of 100,000+ data points per day. In addition, they are cheap, simple to perform, non-invasive, amendable to miniaturization and can

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GLOSSARY

Buffering capacity of Ca^{2+}-specific optical indicators these indicators contain a Ca^{2+}-binding motif to capture ions. As a result, they may act as buffers and affect the intracellular Ca^{2+} concentration by reducing the amplitude and prolong the decay of Ca^{2+} changes. The higher the affinity of a Ca^{2+}-specific indicator to Ca^{2+}, the higher its buffering capacity and consequent perturbation of Ca^{2+} concentration.
Caged compound a bioactive molecule linked via a covalent bond to light-sensitive chemical groups that render the bioactive molecule inactive. UV illumination of a caged compound irreversibly cleaves the bond, releasing the bioactive molecule in a spatially and temporally controlled manner.
Environmental insensitivity of fluorophores the spectral characteristics of fluorophores are not affected by environmental factors, such as temperature, pH and solution composition.
Ratiometric measurements parallel multiple wavelength measurements using dyes that exhibit shifts in excitation and/or emission spectra in response to external stimuli. An increase in intensity at one wavelength is accompanied by a decrease in intensity at second wavelength. In ratiometric experiments, the ratio of fluorescence outputs in two wavelengths is directly correlated to characteristics of external stimuli. Ratiometric measurements significantly reduce experimental artifacts caused by variations in fluorophore loading and retention, as well as by instrumental factors such as illumination stability and optical path length. Specialized instrumentation with dual excitation and/or dual emission hardware capabilities is required for ratiometric measurements.

test multiple cells at a time [11], which makes them preferable methods for primary screening campaigns. The introduction of new probes, such as fluorescent protein (FP)-based calcium sensors, luminescent photoproteins, improved voltage-sensitive probes and ion indicators, have resulted in optimized assays for a wide variety of ion channels [12,13]. However, the inability to control the membrane potential and interrogate ion channels in different conformational states remains a major problem for optical assays. In this review we will evaluate the existing optical assay technologies for ion channel drug discovery, discuss the current trends in their development, and highlight emerging novel approaches to address their crucial shortcomings.

Detection methods

Conformational changes of an ion channel in response to activation stimuli result in its transition to an ion-conducting state that allows the passing of ion currents through its open pore resulting in membrane potential changes. Ion channel assay platforms (Table 1) are based upon either detection of changes in membrane potential or ion flux using a variety of specialized optical indicators such as voltage sensors and ion indicators, respectively [11,14].

Voltage sensors

The membrane potential is a generic readout of any ion channel activity regardless of its ion selectivity. Since the membrane potential is determined by gating states of all ion channels in the cell, it is an indirect measurement of a specific ion channel activity [5,11,14]. Note that relationship between the membrane potential and ion channel activity is non-linear since the channel gating is voltage-dependent [1]. Because of this non-linearity, voltage-sensitive dyes are not appropriate for determining structure–activity relationships [5]. Moreover, drug potency determined by using voltage-sensitive dyes exhibits a compound-dependent shift from patch-clamp recordings of ion channel currents [4].

Fluorescent membrane-potential assays utilize voltage-sensitive dyes that are loaded in the cell membrane. These dyes are categorized as 'slow-response' or 'fast-response' dyes according to their temporal resolution [15].

'Fast-response' dyes (e.g. styryl dye di-8-ANEPPS) undergo electrical field-triggered changes of intramolecular charge distribution and, consequently, change their fluorescent properties [16]. Their rapid optical response enables measurements of transient potential changes on a millisecond timescale. However, since their small fluorescence changes are difficult to detect (~0.1% per 1 mV) [17], these dyes are used primarily in basic research. Other 'fast-response' dyes, the hemicyanines ANNINE-6 and ANNINE-6plus exhibit significantly higher fluorescence changes (~0.35% per 1 mV at 515 nm excitation) and have an improved temporal resolution [18,19]. The limiting factors for HTS adoption of ANNINE-6 dyes are their photostability, solubility and need for specialized light sources to achieve optimal performance.

'Slow-response' dyes, i.e. rhodamine, merocyanine, and oxonol probes, demonstrate voltage-dependent changes in their intramembrane distribution, which is accompanied by fluorescence change [16]. For example, negatively charged oxonol probes, such as DiBAC, migrate across the cell membrane from the outside to inside surface in response to depolarization while increasing their fluorescence signal and exhibiting a red spectral shift. The slow response of these dyes is determined by the fact that the transmembrane movement of entire molecules is significantly slower process than intramolecular redistribution of electrons in immobile molecules of 'fast-response' dyes. $DiBAC_{4(3)}$, currently the most popular reagent for membrane potential assays, has the voltage sensitivity of 1% per 1 mV with $t_{1/2}$ ~40 s [15]. Assays with oxonol probes excel at detecting large and slow responses, but have some technical issues such as response fluctuations because of temperature dependence, dilution of the dye, and complicated dye loading procedure. Additionally, the signal-to-noise may be compromised by interactions of charged dye molecules with changes in membrane potential and drug compounds [5,20].

Optical detection of membrane potential changes in cells could be further improved by using kits with supplementary components that can enhance the dye performance. The FLIPR Membrane Potential Assay Kits from Molecular Devices contain an oxonol-type voltage-sensitive dye and a quencher molecule [14,21,22], which selectively suppresses fluorescence from extracellular dyes (Figure 1a). These kits generate a large fluorescence response (~1.5% per 1 mV) and reasonable temporal resolution ($t_{1/2}$ ~10 s). Problems with unforeseen pharmacological activity of the quencher are mitigated by two kit formulations with different quenchers.

The voltage sensor probes (VSP) from Invitrogen is based on fluorescence resonance energy transfer (FRET) between a

TABLE 1

Summary of current and emerging technologies for ion channel drug discovery screening

		Name	Provider	Comments
Detection	Membrane Potential assay	FLIPR Membrane Potential Assay Kits	Molecular Devices	Contains a quencher
		Voltage Sensor Probe	Invitrogen	FRET-based ratiometric measurements No quencher
	Ion flux Ca^{2+} Assay Kits	FLIPR Calcium Assay Kits	Molecular Devices	Contains a quencher
		Fluo-4NW Calcium Assay Kit	Invitrogen	No quencher Contains probenecid
		BD™ Calcium Assay Kit	BD Biosciences	Contains a quencher Contains probenecid
		BD™ PBX Calcium Assay Kit	BD Biosciences	Contains a quencher No probenecid
		Aequorin		Genetically encoded luminescent photoprotein
		Photina®	Axxam (PerkinElmer)	Genetically encoded luminescent photoprotein with high quantum yield and improved kinetics
		Premo™ Cameleon	Invitrogen	FRET-based ratiometric measurements
	Ion flux K^+ Assay Kits	FLIPR Potassium Ion Channel Assay Kit	Molecular Devices	Contains a quencher Fluorescent-based detection of surrogate ion flux through potassium ion channels
Activation	Chemical	'High K^{+}' solution	Generic	Non-physiological stimulus Irreversible action User-friendly
		Pharmacological channel openers	Generic	Non-physiological stimuli Irreversible action Potential interference with drug compounds
	Electrical	Electric Field Stimulation	Vertex Pharmaceutical	Physiological stimulus 96-well format Enables screening for use-dependent modulators of voltage-gated ion channels
		Trans Cell Layer Electrical Field Stimulation	Merck	Physiological stimulus Higher screening format Enables screening for use-dependent modulation of voltage-gated ion channels
	Optical	'Caged' compounds	Invitrogen	Activation of ligand-gated ion channels in fast and irreversible manner
		Channelrhodopsin-2 Halorhodopsin		Natural light-sensitive ion channels Allows bidirectional control of membrane potential when these channels are co-expressed
		Synthetic Photoisomerizable Azobenzene-Regulated K channels: D-SPARK and H-SPARK		Chemically modified light-sensitive ion channels
		LiGluR		Chemically modified light-gated glutamate receptors
		Nanoparticle-based light-controlled activation platform		Physiological stimulus Compatibility with optical readout

coumarin-linked phospholipid attached to the outer surface of membrane and a mobile oxonol acceptor [either $DiSBAC_{2(3)}$ or $DiSBAC_{4(3)}$] [20,23] (Figure 1b). These FRET-based VSPs are currently the most advanced membrane potential sensors [24–26], because they combine the voltage sensitivity of oxonol dyes (~1% per 1 mV), with a fast response time (e.g. 20 ms for $DiSBAC_{4(3)}$) and exhibit reduced assay artifacts because of a RATIOMETRIC readout (see Glossary). Unfortunately, the temperature dependence of oxonol translocation and accumulation of oxonol molecules inside cells could limit the number of repetitive measurements [11,20].

Currently, membrane potential assays are used in ~40% of all ion channel primary screens [9], because they produce robust and homogeneous cell population measurements. Since their temporal resolution reflects properties of voltage-sensitive dyes rather than the gating kinetics of ion channels, the further improvement of these assays would require new sensors that can measure sub millisecond membrane potential changes.

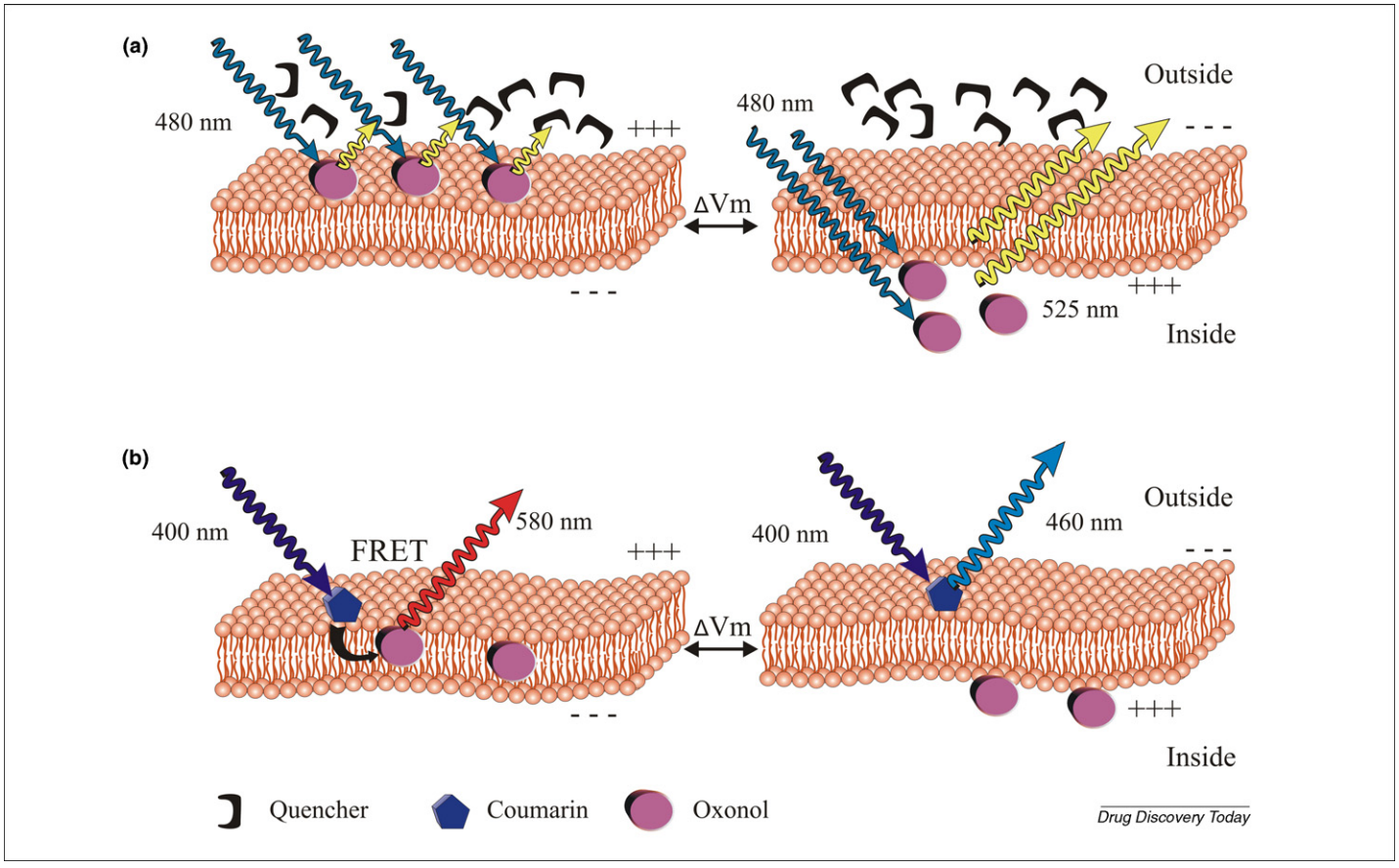

FIGURE 1

Mechanism of action of membrane potential assay kits. **(a)** The FLIPR Membrane Potential Kit (Molecular Devices). This kit combines an oxonol-type voltage-sensitive dye (purple circle) with a fluorescence quencher (black staple). In the resting cell, an oxonol molecule is located on the outside of the cell membrane, and its fluorescence is absorbed by the quencher. When an oxonol molecule migrates across the cell membrane in response to depolarization (ΔV_m), the fluorescent response is greatly enhanced because of two concurrent events: increase in signal amplitude from the voltage-sensitive dye and disappearance of quencher effect. **(b)** Voltage sensor probes (VSP) (Invitrogen) exploit a fluorescence resonance energy transfer (FRET)-based technology, where the FRET donor is a membrane-bound, coumarin-phospholipid (blue pentagon), and the FRET acceptor is a mobile, negatively charged, hydrophobic oxonol (purple circle). In resting cells, the two probes associate with the exterior of the cell membrane, resulting in efficient FRET. Exciting the coumarin donor (at 400 nm) generates a fluorescence signal (at 580 nm) from the oxonol acceptor. During the cell depolarization (ΔV_m), the oxonol translocates to the interior of the membrane. This translocation separates the FRET pair, resulting in a blue fluorescence signal (at 460 nm) from the coumarin donor only.

Ion indicators

Regardless of multitude of channel types present in cells, ion-specific indicators allow the detection of activity of a particular ion channel type based on its ion selectivity only. Therefore, ion flux assays provide a direct assessment of a specific ion channel function.

Indicators for calcium-permeable ion channels

Calcium flux assays are important screening techniques for Ca^{2+}-permeable ion channels because of the availability of excellent Ca^{2+}-specific indicators and a large dynamic range of intracellular Ca^{2+} concentrations [11,12]. There are two main approaches to measure intracellular Ca^{2+} concentration resulting from Ca^{2+} influx: using organic dyes or genetically encoded proteins with either natural or engineered Ca^{2+} sensitivity [27].

Organic fluorescent Ca^{2+} Indicators

These calcium indicators are organic fluorophores coupled to calcium chelators and designed to detect Ca^{2+} concentration changes over the physiological range (0.1–1 mM). Advantages of Ca^{2+}-specific fluorescent dyes are their high specificity, brightness, temporal resolution, dynamic range, and ENVIRONMENTAL INSENSITIVITY (see Glossary). Their limitations include dye compartmentalization in the cell, inability to differentiate between Ca^{2+} influx through ion channels and Ca^{2+} release from intracellular stores, light-stimulated release of reactive oxygen species, and BUFFERING CAPACITY (see Glossary) [14].

Ratiometric Ca^{2+} indicators exhibit a shift in excitation spectrum (i.e. fura-2) or a shift in emission spectrum (i.e. indo-1) upon Ca^{2+} binding. Ratio of the normal and shifted signals is directly correlated to Ca^{2+} concentration. These dyes have found limited use in HTS, partly because of dual-wavelength readout. Non-ratiometric indicators, such as fluo-3, fluo-4, rhod-2, and Calcium Green, exhibit Ca^{2+}-triggered changes in intensity of fluorescent response and do not require specialized instrumentation. Fluo-4 (K_d ~350 nM) is especially useful in HTS applications because of low autofluorescence in the absence of Ca^{2+} [12].

Recent efforts to improve the use of ion indicators for HTS were largely aimed at optimization of protocols for existing Ca^{2+} dyes rather than designing new indicators and have resulted in the

introduction of several Ca^{2+} assay kits. These kits utilize mix-and-read no-wash protocols leading to decreased data variability and allowing accurate detection of Ca^{2+} concentration.

The first on the market, the FLIPR Calcium Assay Kits from Molecular Devices include a quencher that improves the signal-to-noise ratio by reducing dye fluorescence outside cells [14]. Although the earlier FLIPR Calcium Assay Kits had some performance issues such as non-specific potential interference of a quencher with drug compounds and significant 'addition artifact', the FLIPR Calcium 4 Assay Kit partially addressed these problems using a novel quencher with the greater light extinction and more inert chemical nature.

The Fluo-4NW Calcium Assay Kit from Invitrogen is formulated without a quencher. Enhanced performance of this kit is achieved by increasing the fluo-4 dye loading efficiency with exclusive loading buffers and blocking the dye extrusion from cells with probenecid, an inhibitor of non-specific anion transporters.

Another option for detecting intracellular Ca^{2+} levels is offered by BD Biosciences. The kit for standard applications (BD™ Calcium Assay Kit) contains a calcium indicator, a chemical signal enhancer, and probenecid, which is excluded from the kit for probenecid-sensitive applications (BD™ PBX Calcium Assay Kit). A signal enhancer in all BD™ kits can significantly reduce the 'addition artifact', but at the same time it might have adverse pharmacological effects on cells.

Since all Ca^{2+} kits contain formulation additives, systematic evaluation of each kit is required to identify the one with the optimal performance for the specific cell type and target [28].

Genetically encoded Ca^{2+}-sensitive probes

Ca^{2+}-sensitive genetically encoded proteins can be targeted to specific cellular regions to measure localized Ca^{2+} concentrations, which is an advantage over the organic dyes. This technology includes probes based on the photoprotein aequorin and FPs [27].

Ca^{2+}-activated luminescent photoproteins

Aequorin is a Ca^{2+}-activated photoprotein from the jellyfish *Aequoria victoria*. Aequorin emits light as a result of irreversible luminescent reaction upon binding of Ca^{2+} ions (Figure 2a) [29,30]. Important advantages of aequorin-based assays are low background, large signal-to-noise ratio, heterologous expression of apoaequorin, measurements of Ca^{2+} concentrations at specific cellular sites, wide dynamic range (50 nM–50 μM), and low Ca^{2+} buffering effect because of low Ca^{2+}affinity (K_d = 10 μM). Shortcomings of aequorin-based assays are the irreversible nature of aequorin reaction with Ca^{2+}, requirement for addition of an exogenous cofactor, lengthy incubation period with a cofactor, and low quantum yield [12]. Recently Axxam developed a novel Ca^{2+}-activated chimeric photoprotein (Photina®) that shows an enhanced quantum yield, slower reaction kinetics, wide range of Ca^{2+} sensitivity, and a high signal-to-noise ratio [31]. This technology has been optimized for various Ca^{2+}-permeable ion channels, including TRP channels, 5-HT, P2Y and P2X receptors [32,33].

Fluorescent protein-based Ca^{2+} sensors

FPs are extensively used as tags in cellular imaging and can also serve as building blocks for novel ion and physiological sensors [34]. Ca^{2+}-sensitive probes are the most advanced FP-based biosensors. The majority of genetically encoded FP-based Ca^{2+} indicators incorporate the calcium-binding protein calmodulin (CaM) as a molecular switch with Ca^{2+} sensitivity. Among these indicators, there are two structurally different groups constructed with either single or double FP fluorophores [27].

The camgaroo and pericam probes are based on a single circularly permutated YFP that incorporates CaM in a strategic location within YFP structure [35,36]. Unfortunately, both probes have inherently high pH sensitivity, as their Ca^{2+} detection is based on the changes in protonation of YFP upon Ca^{2+} binding. Besides, these probes have misfolding problems because of the required rearrangements in the YFP structure to render it Ca^{2+}-sensitive.

The cameleons are dual-fluorophore sensors comprised of two GFP variants connected by CaM and a CaM-binding peptide. Cameleons exhibit the increase in FRET because of Ca^{2+}-dependent binding of CaM to its target peptide and consecutive decrease of the distance between the two FPs surrounding a Ca^{2+}-sensitive domain (Figure 2b) [37,38]. The ratiometric nature of these FRET-based sensors helps to reduce the assay variability. Additional advantages include bright fluorescence, no need for cofactors, and the ability to run iterative screens. The use of cameleons is limited by a relatively small signal change upon Ca^{2+} binding, large molecular size that might impair their targeting efficiency, photoisomerization of FPs, slow kinetics of Ca^{2+}-induced conformational change, and sensitivity to intracellular environmental factors, especially pH. For these reasons, adoption of FP-based Ca^{2+} sensors for HTS has been limited. Nonetheless, there is a significant promise for these approaches as probes and instrumentations continue to improve.

Recently, Invitrogen released the first commercially available cameleon, Premo™ Cameleon calcium sensor (K_d ~0.24 μM) [39] that exhibits the enhanced brightness compared to other cameleons. It is achieved by optimization of the relative orientation the two FPs that results in enhanced efficiency of energy transfer and five-fold increase in dynamic range. Additionally, Premo™ Cameleon has improved signal-to-noise ratio because of relatively small pH-dependent changes and a large Ca^{2+}-dependent response.

Indicators for potassium ion channels

Potassium ion channels attract huge attention, both as targets for therapeutic indications and for safety profiling [2]. In contrast to Ca^{2+}-permeable ion channels that greatly benefited from a variety of primary functional HTS methods, no equivalent resources are available for potassium channels. In order to facilitate the pharmaceutical development of potassium channel modulators, high throughput potassium-specific optical assays are critical. A major challenge in designing such assays is the shortage of K^+-specific fluorescent indicators capable of detecting narrow physiological variations of extracellular K^+ concentration.

K^+-conductive ion channels are also permeable to other ions such as rubidium and thallium [1]. Therefore, in the absence of K^+-specific fluorescent indicators, the flux of these surrogate ions is used as a measure for the activity of K^+ ion channels. Unfortunately, because of the differences in the mechanism of channel block in the presence of surrogate ions [40], the compound potency values are less accurate. Rubidium efflux is routinely detected with reasonable sensitivity using Atomic Adsorption

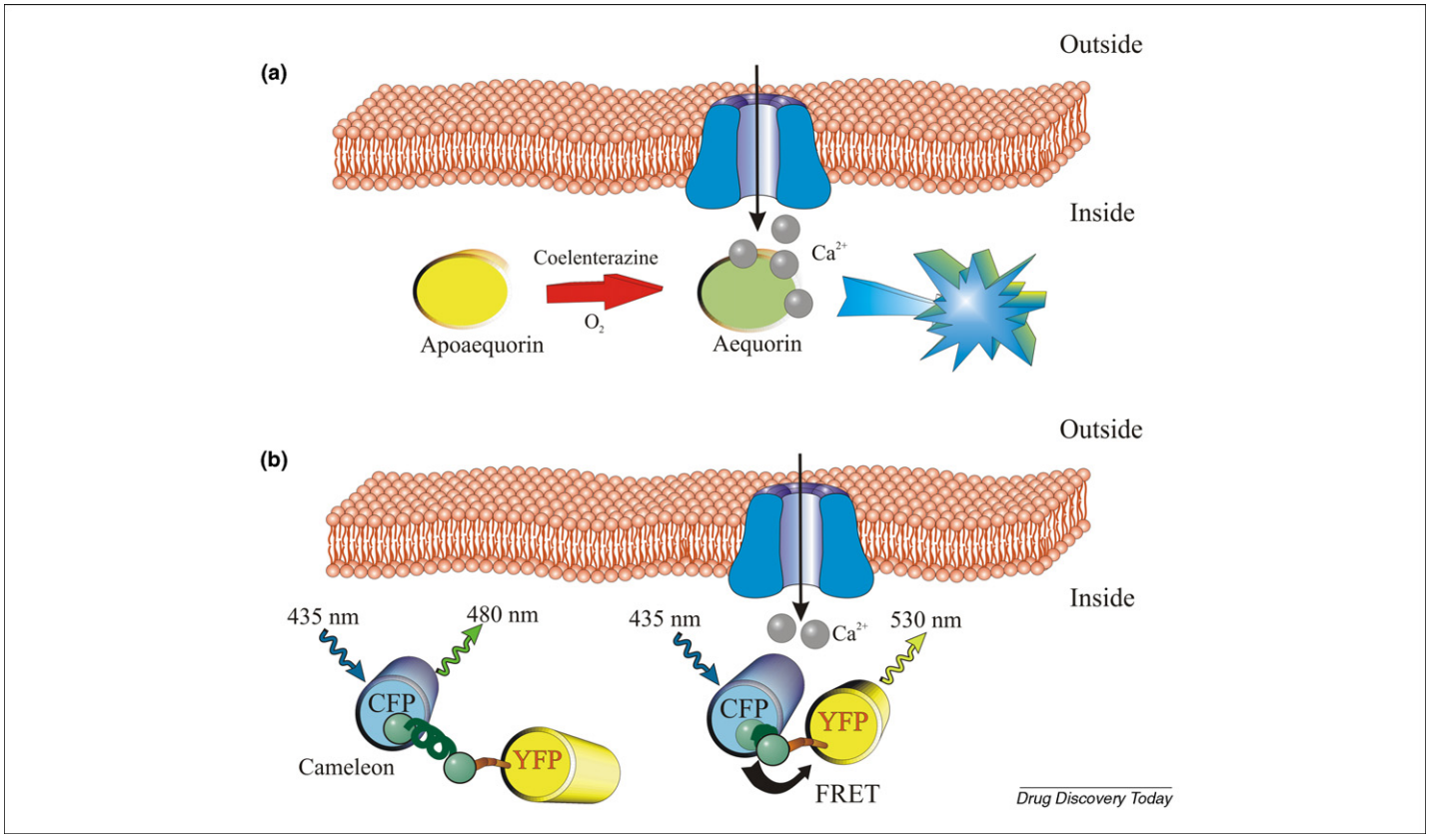

FIGURE 2

Genetically encoded Ca^{2+}-sensitive probes. **(a)** Aequorin, a Ca^{2+}-sensitive photoprotein, is assembled from the apoprotein apoaequorin, with three binding sites for Ca^{2+} ions, and the prosthetic group, coelenterazine. In the presence of molecular oxygen these parts reconstitute spontaneously to form a functional protein. Upon binding of Ca^{2+} ions, aequorin undergoes the conformational change leading to oxidation of coelenterazine and emission of a flash of blue light. **(b)** Cameleon is a fluorescent protein-based Ca^{2+} sensor that incorporates calmodulin as a Ca^{2+}-sensitive switch. The CFP and YFP parts of cameleon are connected by a Ca^{2+}-sensitive domain consisting of a calmodulin and a M13 protein. Upon binding of Ca^{2+} ions, the calmodulin-M13 moiety undergoes a conformational change bringing the fluorescent protein-domains into close proximity. The resulting fluorescence resonance energy transfer (FRET) signal allows ratiometric measurements of intracellular Ca^{2+} detected as an increase in YFP emission (535 nm, green–yellow emission) and a decrease in CFP emission (485 nm, blue emission).

Spectroscopy or radioactive methods [14,41]. The only fluorescence-based functional method to measure potassium channel activity is to monitor thallium influx using Tl^+-sensitive fluorescent probes [42]. Along with the efflux of K^+ ions from cells, activation of K^+ channels allows the influx of thallium ions from the extracellular solution through open channels into cells. Thus, Tl^+-sensitive fluorescent dyes inside cells can detect the activity of K^+ channels and its modulation by drugs.

Recently, Molecular Devices introduced a no-wash assay for potassium channels using ThalKal, a Tl^+-sensitive dye, along with a quencher. This assay offers an alternative way to perform safety profiling and primary screening of modulators of ligand- and voltage-gated potassium channels. This kit has several characteristics that could adversely affect its performance: (1) requirement for chloride-free buffer because of precipitation of thallium halides; (2) ability to elicit only small depolarization (~10 mV) with 10 mM K^+ solution to avoid competition between K^+ efflux and Tl^+ influx through a channel pore; (3) slow kinetics of Tl^+ influx; (4) potential interference of quencher molecules with drug compounds; (5) limited detection sensitivity because of non-specific background fluxes [23,43,44]. Improvements addressing these shortcomings could take this enabling fluorescence-based assay to the next level.

Activation protocols

Regardless of a detection modality, activation must be performed in such a way that drugs will have access to all conformational states of ion channels. Currently, pharmacological intervention ('high K^+' solution or pharmacological channel openers) is used to activate voltage-gated ion channels in optical assays [3,4]. These methods, however, are non-physiological, since they employ chemicals rather than electrical stimuli to activate ion channels. Other serious issues include the lack of control of the membrane potential, the irreversibility of elicited changes, the potential interference of the chemicals used for activation with compounds tested and low temporal resolution. Moreover, these methods are not suited for ion channels with complex and fast gating kinetics [4,11,14].

The 'wish-list' for optimal physiological activation of ion channels includes the ability to change the membrane potential quickly (milliseconds), reversibly, and repeatedly, as well as to control the amplitude, duration, and direction of these changes (depolarization or hyperpolarization). These tools should be minimally invasive and compatible with optical detection methods (Table 1).

Electrical stimulation

An alternative protocol for the activation of ion channels in optical assays is the electric field stimulation (EFS) method. In

this method, eight parallel electrode pairs enter the wells of one column of a 96-well plate, and cells in each well are subjected to the electrical field created by this electrode array. As a result, EFS triggers variable and repetitive depolarization of cells which allows ion channels to 'cycle' through various conformation states. To enable an optical readout for voltage-gated ion channel assay, the EFS stimulation protocol is combined with FRET-based voltage-sensitive probes [20,25]. This method offers a unique high-throughput optical platform for the sensitive detection of use-dependent modulation of voltage-gated ion channels. The limitations of this method include the transient nature of membrane potential changes, accelerated bleaching of voltage-sensitive dyes, risk of occasional electroporation of membrane, and difficulties in adaptation to a higher screening format.

Further improvement of the EFS method has resulted in the introduction of the Trans Cell Layer Electrical Field Stimulation system [45]. In this design, a wire electrode is placed above the cell layer of each well, and a whole-plate perimeter electrode resides beneath the cell-containing filter layer. Although this approach shares some limitations with the EFS method, it allows the electrodes to be placed away from cells to minimize effects on cell viability and dye bleaching. Because of the limited availability of the EFS-based ion channel-screening platform, other physiologically relevant methods to activate the voltage-gated ion channels are needed.

Light-controlled activation

Light is a perfect external trigger for manipulation of the membrane potential in temporally precise and reversible manner. Currently, there are several novel approaches for light-controlled activation of ion channels.

Light-triggered 'uncaging' of chemically modified signaling molecules

Light-triggered release of 'CAGED' molecules (see Glossary) allows the specific and localized activation of cells [46]. Several molecules that can activate various ligand-gated ion channels are available in the 'caged' form, including glutamate, dopamine, serotonin, ATP, cyclic nucleotides, etc. Although the 'uncaging' event is very fast (1–3 ms), it is irreversible and non-iterative. Additionally, 'caged' systems show some precursor instability, phototoxicity, and require UV illumination. Irreversibility can be addressed by employing photochromic molecules that can be switched between the active and inert configurations using light (i.e. nicotinic acetylcholine receptor agonists, glutamate agonists) [47,48]. Further improvements are required to overcome the difficulties in achieving the perfect on/off activity [49].

Genetically encoded light-sensitive proteins

The ability to control the membrane potential with millisecond resolution can be achieved using microbial light-sensitive proteins. The naturally occurring protein Channelrhodopsin-2 from the algae *Chlamydomonas reinhardtii* (ChR2), a rapidly gated light-sensitive cation channel, is maximally activated at 470 nM [50]. The kinetics and amplitude of the ChR2 currents are dependent on the duration and intensity of light. ChR2 is an 'on-switch', as its activation causes membrane depolarization (Figure 3a, left) [51]. The light-driven chloride pump halorhodopsin (NpHR), a protein from the archaebacterium *Natronobacterium pharaonis,* is activated at 580 nm [52]. NpHR works as an 'off-switch', since chloride influx results in membrane hyperpolarization (Figure 3a, right) [53,54].

The incorporation of a ChR2-based approach in an activation protocol of optical assays would require the development of engineered cell lines and could face several hurdles: (1) high ChR2 expression levels for substantial depolarization because of its small single channel conductance (50fS) [50]; (2) addition of endogenous co-factor all-trans-retinal; (3) potential impact of exogenous functionally active protein on cell function; (4) interference of drug compounds with light-sensitive proteins; (5) requirement of continuous light exposure to maintain depolarization; (6) permeability for Ca^{2+} ions; (7) compatibility of activation signals with optical readout. Thus, further improvements of light-sensitive proteins regarding their ion selectivity, ion conductance and spectral sensitivity are needed.

Another approach is to chemically modify ion channels to make them light-sensitive. Potassium ion channels were engineered to sense light via a synthetic photoisomerizable 'switch' molecule azobenzene attached to a channel pore blocker. These so-called 'synthetic photoisomerizable azobenzene-regulated K' (SPARK) channels now exist in two versions that differ in their effects on the membrane potential. Light illumination of potassium-selective H-SPARK channels results in hyperpolarization [54], whereas non-selective D-SPARK channels cause the depolarization of cells [55] (Figure 3b). Modified light-gated glutamate receptors (LiGluR) were developed using a similar paradigm involving a photoswitchable glutamate analogue irreversibly bound to the channel surface via an azobenzene linker [56]. This approach has several disadvantages, such as the lack of specificity of TEA-azobenzene compounds in respect to cystein-containing proteins, necessity to add photoisomerizable compounds, and inability to pair these channels to enable bidirectional control, whereas its advantages include rapid switching, more optimal conductance levels, and the ability to retain effects for many minutes after a brief light pulse.

Nanotechnology

Semiconductor nanoparticles (quantum dots) are inorganic materials governed by the laws of quantum physics. They are transforming life science imaging because of their extraordinary photostability, brightness, broad excitation, narrow emission, long fluorescence lifetimes, and multiplexing capability. Currently, quantum dots are mainly used for the detection of macromolecules [57,58]. The next step is to utilize quantum dots beyond the 'light bulb' applications by exploiting their *unique* physical and photochemical properties.

A nanostructured biocompatible interface is currently being developed as a light-controlled activation platform for cell-based assays [59,60]. Its mechanism of action is based on creation of free charge carriers (e.g. electrons) in nanoparticles upon light illumination. When these nanoparticles are placed in close proximity to a cell, the cumulative electromagnetic field generated by photo-excited nanoparticles will modulate the cell membrane potential. Thus, this nanoparticle-based light-controlled activation platform allows to stimulate cells physiologically (electrical field) and repeatedly [60]. Because of the broad absorption spectrum, semiconductor nanoparticles can be excited by any light shorter than

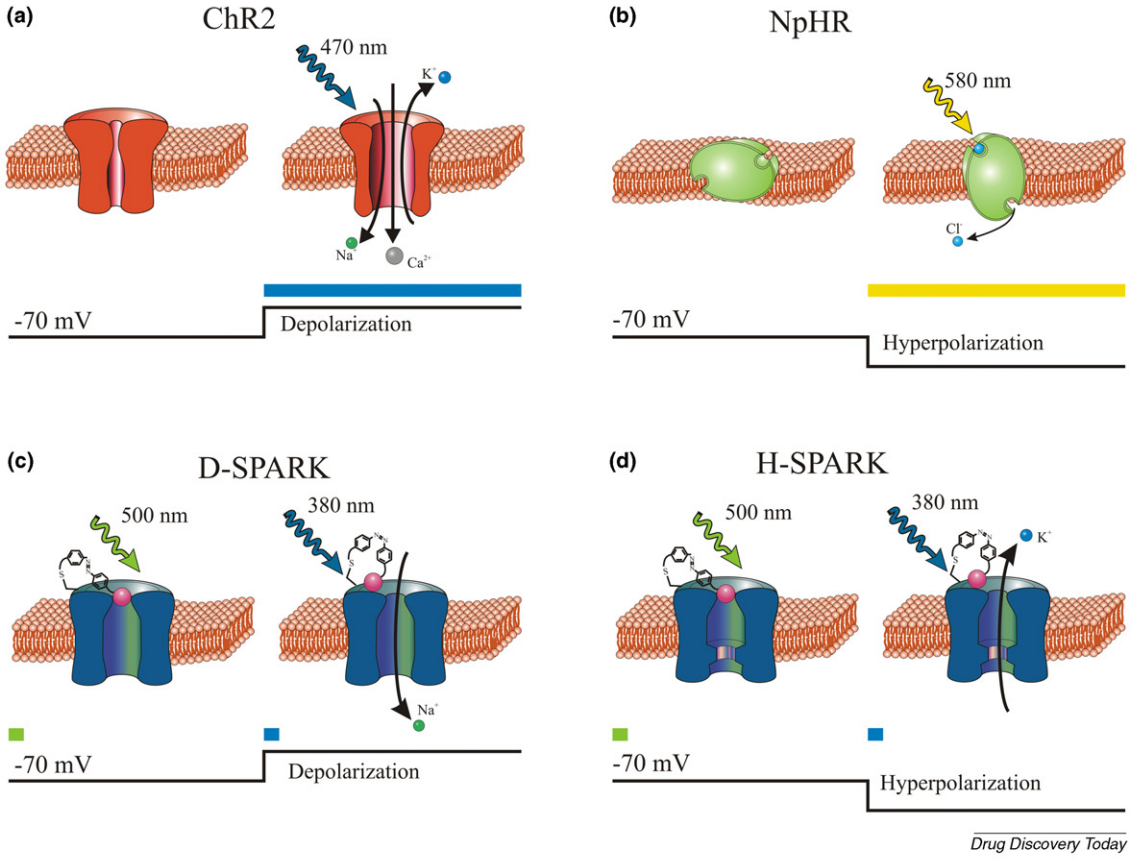

FIGURE 3

Genetically encoded tools for light-controlled activation of cells. **(a)** Naturally occurring light-sensitive proteins. (Left) Channelrhodopsin (ChR2) is a light-sensitive, non-selective ion channel. Light illumination (~470 nm) triggers the opening of ChR2 and consecutive cation influx across the cell membrane, leading to the membrane depolarization. (Right) Upon light illumination (~580 nM), halorhodopsin (NpHR), a light-sensitive chloride pump, allows the chloride anions entry into the cells which results in the membrane hyperpolarization. **(b)** Chemically modified light-sensitive ion channels. Synthetic photoisomerizable azobenzene-regulated K (SPARK) channels are ion channels with engineered light-sensitivity. At longer wavelengths (500 nm) the blocker molecule on a stretched *trans*-linker is positioned in the pore of ion channel, whereas at shorter wavelength (380 nm) it is withdrawn from the pore by shortened linker in the *cis*-configuration. (Left) Activation of D-SPARK channels leads to the membrane depolarization because of sodium influx into the cells. (Right) Activation of H-Spark channels leads to the membrane hyperpolarization because of potassium influx into the cells.

their emission wavelength, thus making them compatible with many fluorescent dyes.

There is a considerable potential for implementing light-controlled activation technologies for HTS optical assays in ion channel drug discovery. However, these methods for physiological stimulation of ion channels are not reality yet and remain to be enabled in HTS mode to be useful for the drug discovery community.

Conclusions

To accelerate development of new ion channel modulators, the drug discovery community needs screening technologies that offer both high throughput and high information content in a cost-effective way. There are two leading technologies in ion channel screening: patch clamp and optical detection methods, none of them satisfying both requirements. As a result, these methods are used at distinct stages of the drug discovery process: the information-rich patch clamp method is currently utilized mainly in secondary screening and safety profiling, whereas HTS-compatible optical assays domineer the primary screening.

In optical assay technologies more emphasis is now being placed on information content and physiological relevance of the screens. The next generation of predictive and reliable optical assays for functional ion channel screening would require a method to simulate the physiologically relevant conditions for ion channel activation in time- and voltage-dependent manner. Multidisciplinary approaches are aimed at finding a solution that could combine tools for manipulation of ion channel gating with fast optical detection of channel activity. Innovation efforts in detection protocols have resulted in more sensitive and selective methods tailored for specific ion channel targets and homogenous assays with fewer artifacts. However, the ability to manipulate the membrane potential is still lagging. Currently, several methods for light-controlled activation that would allow to reversibly and repeatedly activate ion channels in a physiological manner are emerging. Implementation of these technologies would have a major impact on ion channel drug discovery by allowing the primary screening of ion channels with high throughput without sacrificing the information content.

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