

Cell-based assays and instrumentation for screening ion-channel targets

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Ion channels are an important class of drug targets. They comprise the molecular basis for essential physiological functions including fluid secretion, electrolyte balance, bioenergetics and membrane excitability. High-throughput screening for ion-channel function requires sensitive, simple assays and instrumentation that will report ion channel activity in living cells. This article will review relevant assay technologies for ion channels and describe voltage-sensitive probes and instruments based on fluorescence resonance energy transfer (FRET) that enable ion-channel drug discovery.

Ion channels make good drug targets because they are physiologically essential, are pharmacologically accessible, are encoded by a variety of genes and usually operate as multimeric protein assemblies, resulting in a high degree of functional and anatomical specificity^{1,2}. Through molecular cloning, heterologous expression and electrophysiological characterization by patch-clamping, it is clear that the complexity of ion-channel biology also offers multiple opportunities for small-molecule drugs to achieve a specific, desired functional effect. For example, small molecules might influence a variety of biophysical properties of ion channels, such as voltage-dependence,

permeability, use-dependence, activation and inactivation. In contrast to simple blockers or openers, the discovery of modulatory compounds could allow the development of drugs that specifically act on cells or tissues exhibiting aberrant levels of ion-channel activity.

To screen compounds against functional ion channels or assemblies, it is necessary to reconstitute and measure channel function in a relevant biological context, such as in a cell. It is also preferable for the assay to be compatible with automated high-throughput screening (HTS) of large compound libraries. These assays would ideally be sensitive and fast, allow probing of various functional states and be amenable to miniaturization to 96-well plates and beyond. The importance of, and requirements for, assay miniaturization in drug discovery have been previously discussed^{3,4}. Current formats for ion-channel assays have been recently summarized in an excellent review⁵ and will only be discussed briefly here.

The purpose of this article is to discuss the implementation of ion-channel assay technologies that combine high-screening throughput with high-information content. Although there are a variety of approaches being explored, including automated patch-clamping, ion-channel measurements in lipid bilayers and viability assays based on the toxicity of constitutively opened channels⁶, it is not currently possible to use these methods for ion-channel HTS. Therefore, the article will focus on platform technologies that have a broad application to HTS, and in particular, will discuss the efforts of Aurora Biosciences Corporation (San Diego, CA, USA) to apply FRET-based voltage sensing to ion-channel HTS.

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Established assay methods

Ion-channel assay methods can be classified as either high-throughput, low-information assays based on ligand displacement or radiotracer flux, or low-throughput, high-information content electrophysiological assays. Assay and screening methods for ion channels are summarized in Table 1. Ligand-displacement assays require the synthesis of a labeled compound (usually a peptide or small molecule) that has a known pharmacological activity. Because the probe occupies a known binding site, the technique is most effective when detecting molecules that bind to the same site. Although other sites are often allosterically coupled to the ligand site, these assays are not ideal for probing weakly coupled allosteric sites that might go undetected. For compounds that are detected, the functional effect (e.g. agonist or antagonist) is unknown and must be followed up with low-throughput secondary assays.

Radioactive flux assays are also commonly used for HTS screening of ion channels. An important example is $^{86}\text{Rb}^+$, which permeates through K^+ channels with similar properties to K^+ . Protocols generally require long incubation

with the radioactive tracer to load the cells, followed by removal of the excess tracer, and then the channels are kept in an open conformation for long periods (minutes to hours), often with pharmacological modifiers. Non-specific background fluxes are often present and must be subtracted from the data in a similar way as the non-specific binding being taken into account for binding assays. Flux assays also require different radioactive ions for channels with different ion permeabilities, and present the inconvenience associated with radioactive waste. Neither ligand binding nor flux assays are particularly well suited for miniaturization.

Patch-clamp recording represents the gold standard for biophysical evaluation of compound action. The ability to clamp either voltage or current across a cell membrane, in addition to manipulating the ionic composition on either side of the membrane, enables a detailed characterization of ion-channel gating, permeability, and drug interactions. In addition, the technique is so sensitive that single ion channels can be studied. Although patch-clamping is unparalleled for detailed investigation of ion channel function,

Table 1. Comparison of ion-channel assay methods

Method	Information content	Throughput	Typical channels assayed	Sensitivity	Temporal response	Comments
Electrophysiology (patch-clamp)	Very high	Low	All	High	Sub-millisecond	Dialyses cell contents
Binding assays	Low	High	All	Medium	N/A	Requires synthesis of radiolabeled probe that binds to target
Radioactive flux assays	Medium	Medium to high	K^+ , Na^+ , Cl^-	Medium	Seconds to minutes	Radioactive assay Often requires high-channel expression
Redistribution dyes	Medium	Medium	K^+	Medium	Slow response time	Prone to dye artifacts
Membrane-potential dyes	Medium	Medium to high	Ca^{2+} , Na^+	Medium to high	Several seconds to minutes	Na^+ -channel assays require co-expression of Ca^{2+} voltage-dependent ion channels
Ca^{2+} dyes	Medium	Medium to high	Ca^{2+} , Na^+	Medium to high	Several seconds to minutes	Limited for rapidly inactivating channels
FRET-based voltage sensors	High	High	All	High	Sub-second responses to voltage changes	Generic high-throughput method Potential for modifier-free assays Can measure hyperpolarizations Ratiometric

Throughput (compounds per day): low ≤ 100 , medium = 100–2000, high ≥ 2000 ; sensitivity means the relative number of channels per cell needed for good signal-to-noise ratio.

it is a cell-by-cell assay technology that is difficult to automate for HTS. Without the development of automated methodology, its role in ion-channel drug discovery will continue as a secondary or tertiary method to provide unequivocal biophysical data regarding the activity and action of hits from primary screens.

Optical readouts of channel function

Recently, optical methods for assaying ion-channel activity have begun to bridge the gap between the high-capacity, low-information assays and low-throughput, high-information assays. These assays utilize fluorescent probes to measure ion-channel-dependent changes to either intracellular ion concentration or membrane potential and are desirable because they are sensitive, can report channel activity in real-time, do not involve radioactivity, and are amenable to miniaturization and automation. Most importantly, these assays can report the activities of all pharmacologically active functional sites on the target.

Intracellular Ca^{2+} measurements

Because the Ca^{2+} concentration inside cells can increase dramatically when Ca^{2+} channels are opened, one particularly successful and sensitive approach has been the measurement of intracellular Ca^{2+} with fluorescent indicators. A breakthrough for the application of fluorescent dyes to HTS occurred with the introduction of sophisticated plate readers, such as the Fluorescence Imaging Plate Reader (FLIPR), (Molecular Devices, Sunnyvale, CA, USA), which have integrated liquid handling (used to elicit a response) and kinetic detection (used to record the response)⁷. FLIPR uses an argon laser to rapidly scan a microtiter plate containing dye-loaded cells and a semi-confocal detection method, resulting in excellent sensitivity and a high signal-to-noise ratio. Ca^{2+} levels are measured using indicators such as fluo-3 or Calcium Green, which are efficiently excited at the 488 nm wavelength of the argon ion laser. The system can scan and read a plate every few seconds using a charge-coupled device (CCD) camera, quickly enough to detect most Ca^{2+} responses. The success of this approach has led to the design of assays that couple the activity of a target channel, such as a Na^+ channel, to activation of a voltage-gated Ca^{2+} channel (i.e. conversion to a Ca^{2+} readout)⁸. Although this approach does work, the reporter cell needs to contain relevant endogenous or engineered Ca^{2+} channels. Furthermore, the assay can only detect activity that the Ca^{2+} channel can detect (e.g. a specific voltage range, and in the case of voltage-activated Ca^{2+} channels, only depolarizations) and compounds that interact with the Ca^{2+} chan-

nel can interfere with target detection to produce false positive results.

Membrane potential measurements

An indicator of membrane potential is an attractive alternative to Ca^{2+} for ion-channel assays because it is sensitive and versatile. This sensitivity is because of the high electrical resistance of biological membranes, which allows small ionic currents across the plasma membrane to cause large changes in membrane potential. This allows voltage assays to be at least as sensitive as isotopic flux assays while being more convenient. By contrast to Ca^{2+} measurements, voltage assays are generic because changes in membrane potential can be generated by any ionic flux across the membrane. One caution with membrane-potential assays is that, because many cells express endogenous ion channels, care must be taken in the choice of cell lines and assay formats to maximize the voltage response from the target channel.

Membrane potential indicator dyes have historically been divided into slow dyes (response time in minutes) with high sensitivity, and fast dyes (sub-millisecond response times) that have poor voltage sensitivities. The fast dyes generally operate via an electrochromic mechanism in which the transmembrane electric field directly interacts with the excited state of the dye. These 'styryl' dyes have sensitivities of 1–10% per 100 mV and their use has been restricted to basic research using highly optimized, low-noise equipment specially built for neurobiological problems requiring a high temporal resolution^{9,10}. The slow dyes are comprised of lipophilic, negatively-charged oxonol dyes, such as bis-(1,2-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)], which have very low fluorescence in aqueous extracellular solution and greatly increase their quantum yield upon binding to hydrophobic cellular sites^{11–13}. The basis of the assay is that depolarized cells accumulate the negatively charged dyes and increase their fluorescence to a greater extent than hyperpolarized cells (Fig. 1a,b). Only the slow dyes, with their relatively good sensitivity, have been applied to HTS. Indeed, the earliest application of a FLIPR-type system was for membrane potential assays using DiBAC₄(3). Redistribution dyes can work well for assays configured to detect steady-state changes in membrane potential. However, many ion channels, including Na^+ , K^+ and Ca^{2+} channels, rapidly inactivate or desensitize after opening, and hence, these channels must be pharmacologically modified to hold them open for the duration of these slow assays^{14,15}. From an HTS perspective, slow redistribution assays also limit throughput. In addition, the dyes are prone to experimental

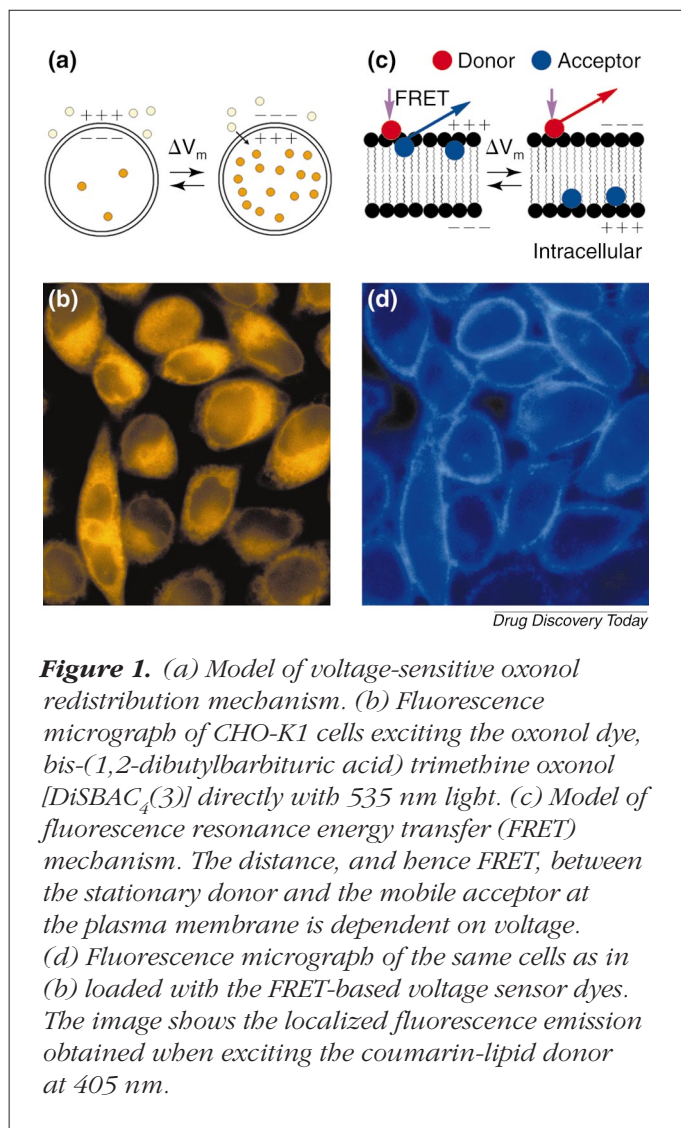


Figure 1. (a) Model of voltage-sensitive oxonol redistribution mechanism. (b) Fluorescence micrograph of CHO-K1 cells exciting the oxonol dye, bis-(1,2-dibutylbarbituric acid) trimethine oxonol [DiSBAC₄(3)] directly with 535 nm light. (c) Model of fluorescence resonance energy transfer (FRET) mechanism. The distance, and hence FRET, between the stationary donor and the mobile acceptor at the plasma membrane is dependent on voltage. (d) Fluorescence micrograph of the same cells as in (b) loaded with the FRET-based voltage sensor dyes. The image shows the localized fluorescence emission obtained when exciting the coumarin-lipid donor at 405 nm.

artifacts including temperature fluctuations and fluorescence artifacts from test compounds.

FRET-based voltage sensor dyes

Improved membrane-potential sensors based on FRET between voltage-sensing oxonol dyes and voltage-insensitive donor fluorophores associated with cell membranes have been demonstrated to retain the voltage sensitivity of the oxonol probes while reporting real-time kinetics of the membrane potential^{16–18}. The mechanism of voltage-sensitive FRET is shown in Fig. 1c. In the configuration currently used at Aurora Biosciences, two dye molecules, a coumarin-linked phospholipid (CC2-DMPE) and an oxonol dye, are loaded into the plasma membrane of cells. CC2-DMPE partitions into the outer leaflet of the plasma membrane where it acts as a fixed FRET donor to the mobile,

voltage-sensitive oxonol acceptor. Cells with relatively negative potentials inside will push the negatively charged oxonol to the outer leaflet of the plasma membrane, resulting in efficient FRET (i.e. quenching of the coumarin donor and excitation of the oxonol acceptor). Depolarization results in rapid translocation of the oxonol to the inner surface of the plasma membrane, decreasing FRET. Because FRET can only occur over distances of less than 100 nm, excitation of the coumarin results in specific monitoring of oxonol movements within the plasma membrane. This is shown in Fig. 1d by the appearance of a ring of fluorescence when the coumarin donor is excited with violet light and this contrasts with the non-specific intracellular oxonol distribution revealed when the oxonol is excited directly (Fig. 1b).

Figure 2 uses simultaneous patch-clamping and rapid optical recording to demonstrate the speed, sensitivity and ratiometric nature of the FRET assay in a rat basophilic leukemia (RBL) cell. The responses of the both donor and acceptor fluorophores to depolarizing pulses can be observed in Fig. 2a. By dividing the signals from the coumarin donor and oxonol acceptor, a ratio is obtained (Fig. 2b) that is independent of the excitation intensity, the number of cells being detected and the optical path length. Analysis of these data (Fig. 2c) and similar experiments in other cell types, indicates a sensitivity of about 1% ratio per mV for DiSBAC₂(3) over the relevant physiological range. These dyes load quickly and effectively (in approximately 20 min.), appear to be non-toxic, and cells can be maintained with the dyes for hours without degrading the cell response. The dye loading and the assays are usually conducted at room temperature, whilst the FRET signal is minimally affected by normal ambient temperature fluctuations, therefore not requiring special care to control the temperature.

With a response time of a few seconds, the FRET assay using DiSBAC₂(3) as an acceptor is 100-times faster than oxonol redistribution assays, and is appropriate for liquid addition protocols which are often used to trigger membrane-potential changes in HTS assays (e.g. Fig 3). Even faster FRET assays are possible because the time constant of the response can be shortened by increasing the oxonol hydrophobicity. For example, Fig. 3a compares the response times of the FRET probes DiSBAC₂(3)-CC2-DMPE, DiSBAC₄(3)-CC2-DMPE and the slow redistribution dye DiBAC₄(3), in patch-clamped cells. The more hydrophobic butyl oxonol, DiSBAC₄(3), is clearly the quickest of the three compounds, with a time constant of approximately 10 ms. FRET dyes using DiSBAC₄(3) or other more hydrophobic oxonols are therefore quick

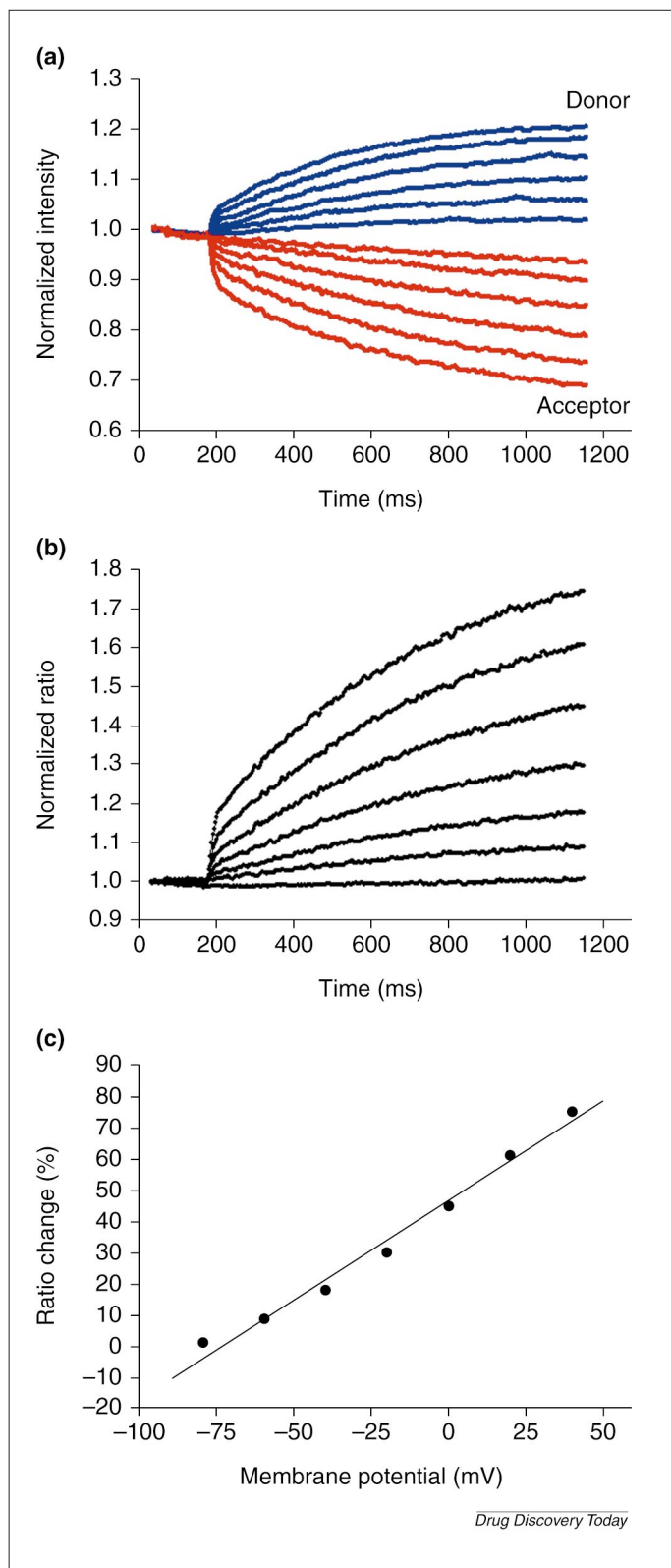


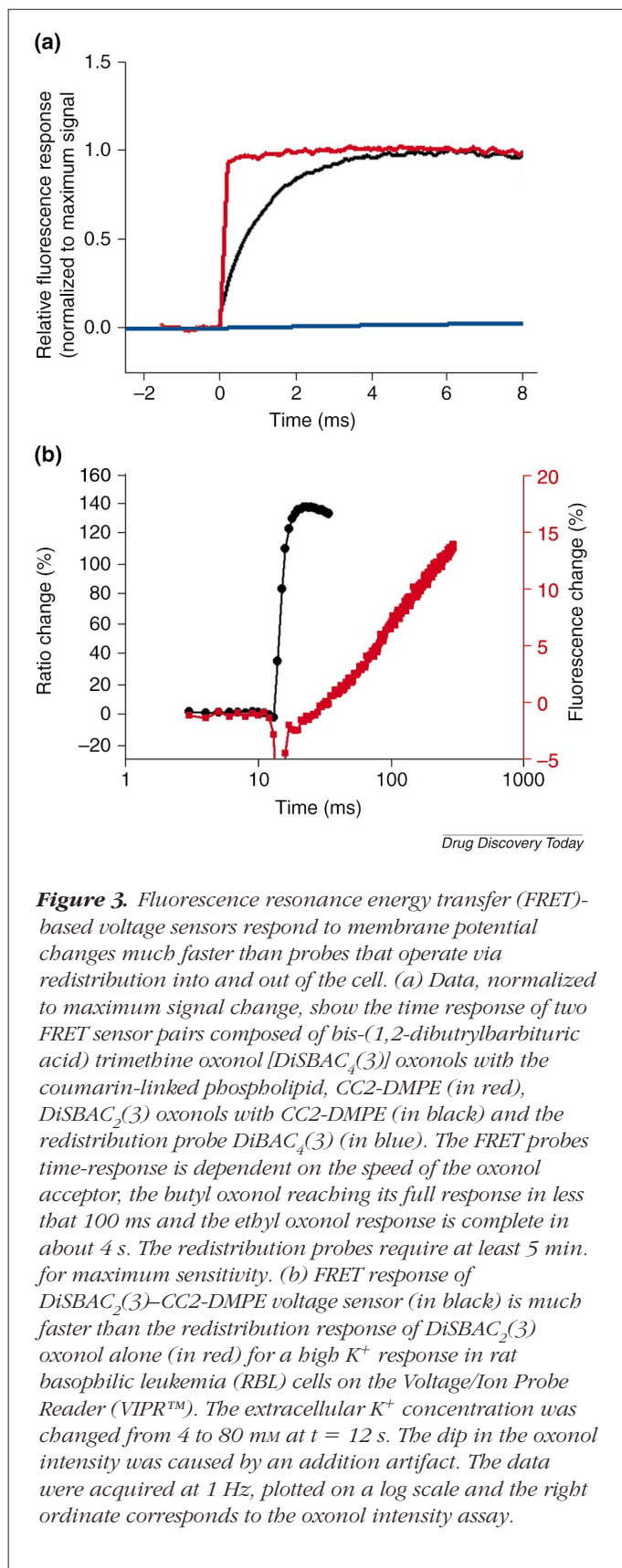
Figure 2. Voltage-sensitive fluorescence resonance energy transfer (FRET) probes provide a sensitive, ratiometric readout of membrane potential in mammalian cells. (a) Single-sweep fluorescence changes in a single voltage-clamped rat basophilic leukemia (RBL) cell stained with the coumarin-linked phospholipid, 15 μM CC2-DMPE (donor) and the oxonol dye, 6 μM bis-(1,2-dibutylbarbituric acid) trimethine oxonol [DiSBAC₄(3)] (acceptor) on the microscope. The individual FRET donor and acceptor intensities synchronously move in opposite directions in response to the voltage changes. For depolarizations, the donor intensities (blue traces) increase and the acceptor oxonol intensities (red traces) decrease. The cell was held at -80 mV and the voltage was stepped up to $+40$ mV in 20 mV steps. The data were collected at 300 Hz and normalized to a starting ratio of 1. (b) The normalized ratio is sensitive to voltage. (c) The percentage ratio change in cells as a function of membrane potential is approximately linear in the physiological relevant range from -80 mV to $+40$ mV.

development of more rapid stimulation methods for HTS. Figure 3b compares DiSBAC₂(3)-CC2-DMPE and DiSBAC₂(3) responses to a depolarizing stimulus in a kinetic plate reader.

Instrumentation for measuring ion-channel activity

A kinetic plate reader has been developed by Aurora that is compatible with the speed, sensitivity and ratiometric output of the FRET-based voltage sensors (and rapid kinetic assays in general). The first-generation instrument (the Voltage/Ion Probe Reader or VIPR™) is an integrated liquid handler and kinetic fluorescence reader for 96-well microtiter plates. The VIPR reader integrates an eight-channel liquid handler, a microplate positioning stage and a fiber-optic illumination and detection system (see Fig. 4). The system was designed to simultaneously measure fluorescence from a column of eight wells before, during and after the introduction of a liquid sample obtained from another microplate or trough. Liquid addition can be used to trigger a response from cells and the protocols used typically work by changing ionic conditions or adding a ligand. A xenon arc lamp provides excitation light, which allows the reader to be used with most fluorescent dyes. The VIPR reader excites and detects emission signals from the bottom of a microtiter plate by a novel design employing eight trifurcated optical fiber bundles (one bundle for each well). One leg of the trifurcated fiber is used as an

enough to measure action potentials. The high sensitivity and rapid temporal response, in conjunction with cell lines with rapid membrane-potential responses will drive the



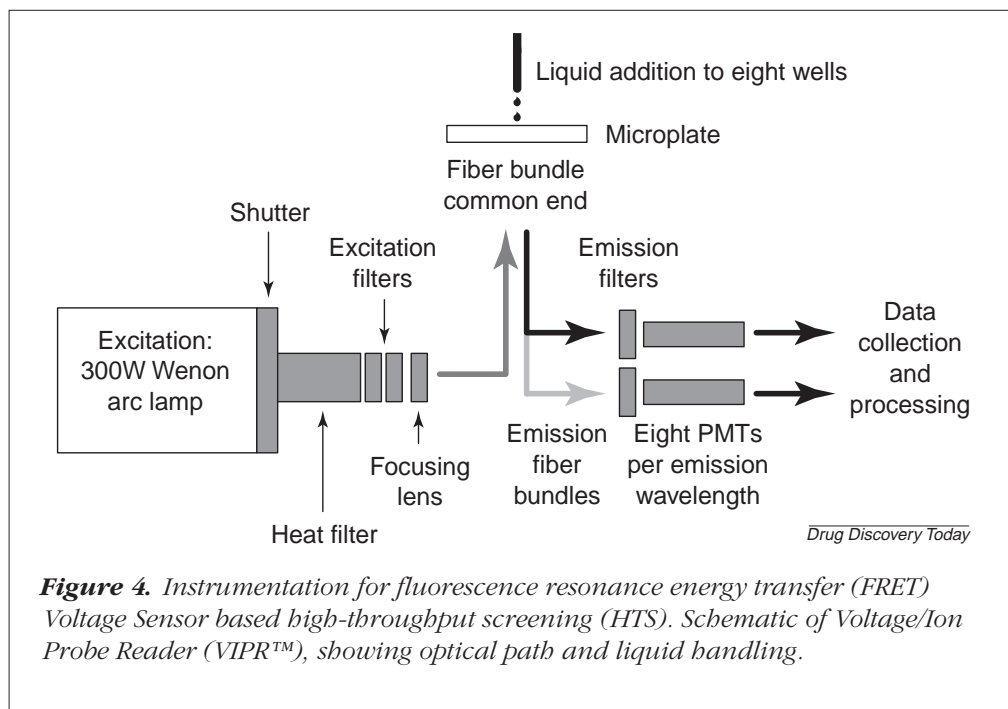
excitation source, the other two legs of the trifurcated fiber being used to detect fluorescence emission. A lens on the end of the fiber increases the efficiency of light excitation and collection. The bifurcated emission fibers allow the reader to detect two emission signals simultaneously and are compatible with rapid signals generated by the FRET-based voltage dyes. Photomultiplier tubes then detect emission fluorescence, enabling sub-second emission ratio detection for advanced applications.

The VIPR reader can be operated in either manual or automated formats. A Windows-based graphical user interface allows the user to customize assay routines, such as how long the data are acquired, how compound additions are performed and when the test compound is added. This allows rapid and flexible assay development. Manual operation is extremely useful for research, pharmacology and screen development purposes. Examples of typical reader data and assays are shown in Fig. 5, which demonstrates assay protocols and pharmacology for a voltage-dependent Na⁺ channel and a Ba²⁺-sensitive inward-rectifier K⁺ channel, respectively. The Na⁺ channel was heterologously expressed in CHO-K1 cells while the K⁺ channel was endogenously expressed in RBL cells. As seen from the examples above, these assays typically take 20 to 30 seconds per columns of eight wells, which includes the time for the liquid handler to supply each of the columns. By reading one column at a time, an entire 96-well plate is assayed in approximately 5 min.

The VIPR reader was specifically designed to be compatible with automated HTS and uses washable tips, has a robust robotic interface and a low-profile plate tray to allow robotic access. The reader has been successfully integrated into several automated systems and can typically perform 5000–7000 assays per day. For assays generating 15 mV changes or greater, compounds can be screened in single wells. An example of screening data is shown in Fig. 6, where 560 pharmacophores were assayed at a concentration of 10 μM in an assay for inhibitors of the RBL inward-rectifier ion channel. Figure 6a shows a scatter plot of the data, including both positive and negative controls used to calculate statistical windows, and the data are then shown as a histogram in Fig. 6b. Aurora and its partners have screened over a million data points against at least 12 targets in all major ion-channel classes and the results shown here are fairly typical.

Future enhancements

The development of sensitive, real-time, automation-compatible assays for Ca²⁺ levels and membrane potential has made ion-channel targets more accessible for cell-based



HTS. Further enhancements will allow more rapid and precise stimulation of cells, more sensitive signal detection and more sophistication in the application of molecular and cell biology to assay development.

Techniques for rapid stimulation of cellular membrane potential in microtiter plates would further bridge the gap between patch clamping results and optical assays while maintaining HTS compatibility. Approaches towards achieving this goal include rapid photolysis of chemically caged ligands^{19,20} that can rapidly activate channels that in turn induce membrane-potential changes. In this way, light is used to both elicit and measure changes in membrane potential. Alternatively, electrical stimulation can be used for rapid and repetitive stimulation in microtiter plates. For example, electrode arrays have been successfully used for large excitable cells in native tissue or primary cultures^{21,22}. Repetitive and rapid stimulation in combination with fast FRET probes and VIPR would potentially enable HTS assays in formats previously restricted to electrophysiology assays. For example, state-dependent blockers of Na⁺ or K⁺ channels could be screened as therapies for epilepsy, pain or cardiac arrhythmia.

Hypothetically, the maximum response possible using a distributed charge method such as the membrane-bound singly-charged oxonol is predicted by the Nernst potential to be 10-fold per 60 mV. Thus the dye sensitivity could still be improved. Probe performance might be improved by shifting the fluorescence to the red wavelength (to reduce

cell and system autofluorescence), using different classes of fluorophores and increasing the efficiency of voltage-sensitive energy transfer.

The use of genetically encoded fluorescent molecules such as green fluorescent protein (GFP), raises the prospect of engineering all or part of the FRET voltage sensor as a molecular construct. Recently, genetically targeted probes of Ca²⁺ and pH have been described^{23–25} and similar approaches could be applied to voltage probes. Such sensors could be expressed in specific subpopulations of cells or even targeted to specific intracellular organelles. Interest in mitochondrial function and its role

in disease and metabolism has revealed the importance of intracellular ion channels²⁶. However, because intracellular membranes are not easily accessible to patch electrodes and because isolated mitochondria lack cell-based regulatory factors, optical interrogation might be the only prospect for exploring the roles of ion channels inside cells.

Conclusions and perspectives

The rapid evolution of sensitive, function-based assays and related instrumentation is transforming the way the pharmaceutical industry is approaching both research and drug discovery. Assays using cloned molecular targets heterologously expressed in host cells are now standard for drug discovery. However, as cell-based screens begin to provide more biological information per well and screening-based research enables systematic biological exploration, it is likely that compound screening in native cell systems will be used to identify ion channel targets involved in more complex cellular processing, particularly in excitable cells. Responses of primary cultures or differentiated cells displaying complex behavior (e.g. action potentials, repetitive firing, synaptic transmission) are amenable to a miniaturizable, ensemble read-out such as membrane potential. In this way, the discovery tools described here will be used to both screen established targets in heterologous systems and to discover new targets in native cell lines.

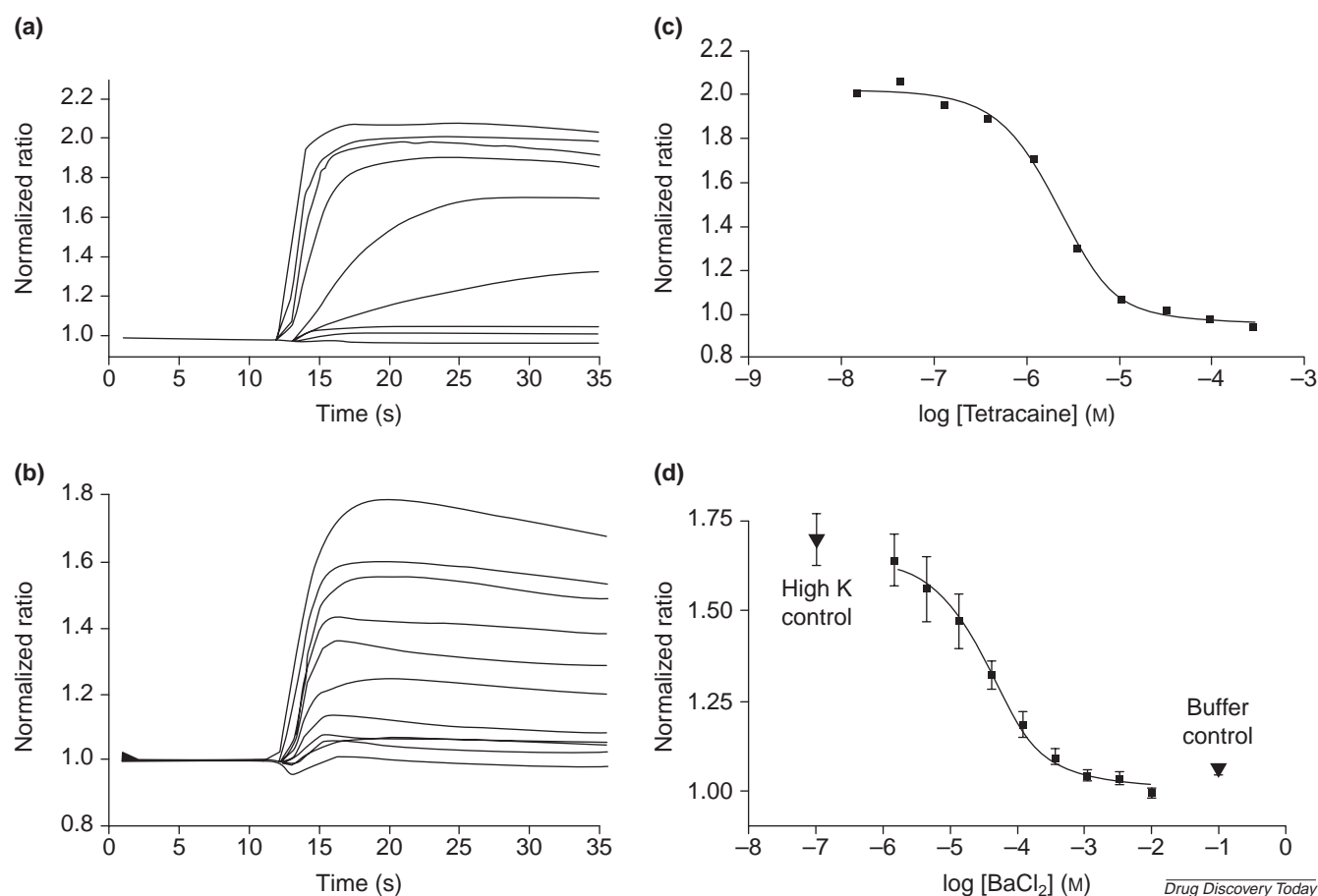


Figure 5. Examples of ion-channel pharmacology detected on a Voltage/Ion Probe Reader (VIPR™). (a) Na⁺-channel assay in CHO-K1 cells is shown. The cells are kept in a solution in which Na⁺ has been substituted with tetramethyl ammonium, a monovalent cation that does not permeate through Na⁺ channels. The assay uses veratridine to keep the channel partially open and a large depolarization is initiated with the addition of a Na⁺-containing solution. Single-well ratio traces with increasing concentrations of the antagonist tetracaine are shown (a). Single-well dose-response curve of the antagonist tetracaine is shown (c). The IC₅₀ is 2 μM, which is similar to the value of 0.7 μM measured electrophysiologically. (b) Assay for endogenous inward-rectifier K⁺ channel in rat basophilic leukemia (RBL) cells. The inward-rectifier sets the membrane potential to approximately the K⁺-equilibrium potential and addition of a high-K⁺ solution causes a channel-dependent depolarization. Single-well ratio traces with increasing concentrations of the antagonist Ba²⁺ are shown (b). Dose-response of the Ba²⁺ blockade of the inward-rectifier and K⁺-channel-dependent depolarization is shown. High K⁺ and control data are shown on the left and right of the dose-response curve respectively (d). Normalized ratio refers to the final ratio divided by the pre-stimulus ratio, error bars are ±SD of five wells.

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REFERENCES

- 1 Ackerman, M. and Clapham, D. (1997) *New Engl. J. Med.* 336, 1575–1586
- 2 Curran, M.E. (1998) *Curr. Opin. Biotechnol.* 9, 565–572

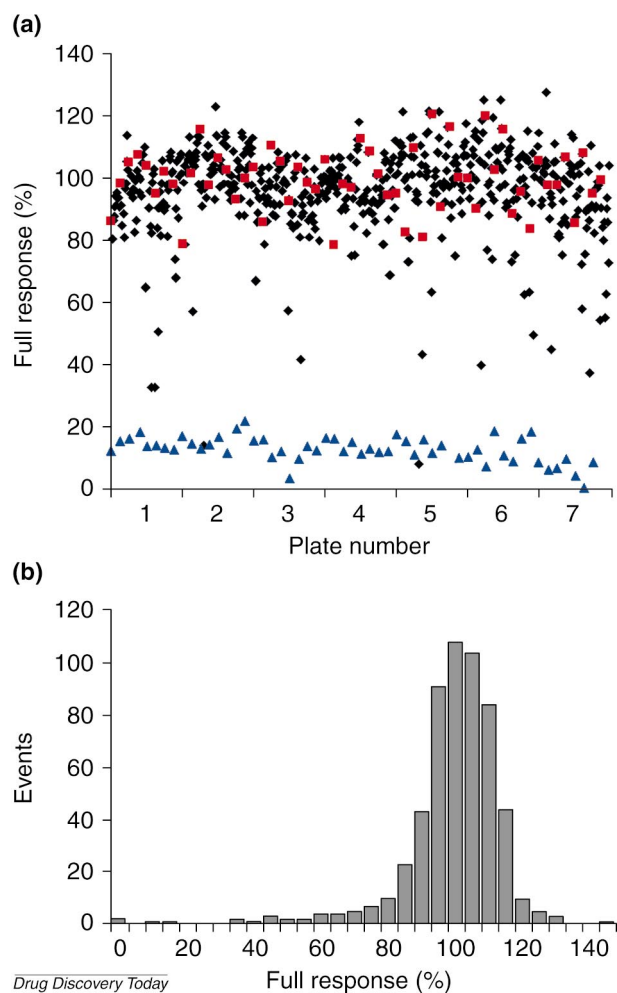


Figure 6. Sample screening data from a membrane potential assay using fluorescence resonance energy transfer (FRET) voltage probes. (a) Scatter plot of data from seven 96-well plates. Test compounds were screened at 10 μM (in black diamonds), negative controls (in red squares) and positive Ba^{2+} controls (in blue triangles). 10 μM of each of the compounds were obtained from Microsource Discovery Systems Inc. (Gaylordsville, CT, USA) and LOPAC library at the RBI (Natick, MA, USA). (b) A histogram of the data shows a desirable distribution with the majority of the compounds tightly clustered around 100% full response and a 2% bit rate (defined as more than 50% block). The screening window was $X = 3.5$ calculated according to the following equation: $X = [(mean_{(max)} - 3SD_{(max)}) - (mean_{(min)} + 3SD_{(min)})] / SD_{(max)}$. A window >1 means confidence for single-well screening²⁷.

- 3 Burbaum, J.J. (1998) *Drug Discovery Today* 3, 313–322
- 4 Gonzalez, J.E. and Negulescu, P.A. (1998) *Curr. Opin. Biotechnol.* 9, 624–631
- 5 Denyer, J. *et al.* (1998) *Drug Discovery Today* 3, 323–332
- 6 Manger, R.L. *et al.* (1993) *Anal. Biochem.* 214, 190–194
- 7 Shroeder, K.S. and Neagle, B.D. (1996) *J. Biomol. Screen.* 1, 75–80
- 8 Velicelebi, G. *et al.* (1999) *Methods Enzymol.* 294, 20–47
- 9 Loew, L.M. (1993) in *Fluorescent and Luminescent Probes for Biological Activity* (Mason, W.T., ed.), pp. 150–160, Academic Press
- 10 Grinvald, A. *et al.* (1998) *Physiol. Rev.* 68, 1285–1366
- 11 Epps, D.E., Wolfe, M.L. and Groppi, V. (1994) *Chem. Phys. Lipids* 69, 137–150
- 12 Rink, T.J. *et al.* (1980) *Biochim. Biophys. Acta* 595, 15–30
- 13 Holevinsky, K.O. *et al.* (1994) *J. Membr. Biol.* 137, 59–70
- 14 Narahashi, T. and Herman, M.D. (1992) *Methods Enzymol.* 207, 620–643
- 15 Strichartz, G., Rando, T. and Wang, G.K. (1987) *Annu. Rev. Neurosci.* 10, 237–267
- 16 Gonzalez, J.E. and Tsien, R.Y. (1997) *Chem. Biol.* 4, 269–277
- 17 Gonzalez, J.E. and Tsien, R.Y. (1995) *Biophys. J.* 69, 1272–1280
- 18 Tsien, R.Y. and Gonzalez, J.E. (1997) US Patent 5661035
- 19 Nerbonne, J.M. (1996) *Curr. Opin. Neurobiol.* 6, 379–386
- 20 Adams, S.R. and Tsien, R.Y. (1993) *Annu. Rev. Physiol.* 55, 755–784
- 21 Breckenridge, L.J. *et al.* (1995) *J. Neurosci. Res.* 42, 266–276
- 22 Wilson, R.J. *et al.* (1994) *J. Neurosci. Methods* 53, 101–110
- 23 Miyawaki, A. *et al.* (1997) *Nature* 388, 882–887
- 24 Llopis, J. *et al.* (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 6803–6808
- 25 Miesenbock, G., De Angelis, D.A. and Rothman, J.E. (1998) *Nature* 394, 192–195
- 26 Szewczyk, A. and Marban, E. (1999) *Trends Pharmacol. Sci.* 20, 157–161
- 27 Sittampalam, G.S. *et al.* (1997) *J. Biomol. Screen.* 2, 159–169

In short...

A two-year agreement has been finalized between **Scotia Holdings plc** (Stirling, UK) and **Bristol-Myers Squibb** (BMS; Princeton, NJ, USA) in which Scotia are to develop prototype formulations, utilizing their Galactosome technology for a number of molecules produced by BMS. BMS are to fund the initial feasibility studies, after which time they can develop any of the prototype formulations for registration and marketing. Under the terms of the agreement, Scotia will be entitled to milestone payments and royalties based on worldwide sales of the formulations.