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Ion-channel assay technologies: *quo vadis*?

Jia Xu, Xiaobo Wang, Brooks Ensign, Min Li, Lei Wu, Antonio Guia and Junquan Xu

Although many existing methods are used to study the functions of ion channels and to screen lead compounds for important ion-channel targets, new technologies are being developed for improved performance. It is important to identify the advantages and disadvantages of each technology. In this review, we segment the ion-channel assay market according to distinguishable applications, and compare the needs of each market segment with the capabilities of different technologies in terms of multiple assay attributes. We further discuss the future directions in the development of ion-channel assays.

***Jia Xu**
Xiaobo Wang
Brooks Ensign
Lei Wu
Antonio Guia
Junquan Xu
AVIVA Biosciences Corporation
11568 Sorrento Valley Road,
Suite 9
San Diego, CA 92121, USA
*tel: +1 858 259 8288
fax: +1 858 259 0965
e-mail: jxu@avivabio.com
Min Li
Department of Physiology
The Johns Hopkins University
School of Medicine
725 North Wolfe Street,
Baltimore, MD 21205 USA

▼ All methods that measure ionic conduction across a biological membrane require two ionic compartments separated by a lipid bilayer in which the ion channels reside. Voltage-clamping of membrane patches (referred to as patch-clamping) and radioactive efflux assays are direct methods to measure ionic flux. Indirect assays include fluorescent voltage-sensitive probes, which report membrane-potential change as a result of ionic flux and fluorescent ion-sensitive probes, such as calcium-sensitive dyes, which quantify the change of the concentration of the conducted ions. In addition to the above functional assays, binding assays are also available to study ion-channel targets. Further, new assay approaches to achieve better performance and lower cost are under development[1].

Previous reviews have described many of these ion-channel assay technologies[2–4]. But few have provided a systematic comparison with respect to market segments (applications) and assay attributes. Ion channel scientists, purchasing managers and investors are primarily concerned about the following questions:

- What are the suitable applications for a certain technology?
- What are the optimal technologies for a certain application?
- What is the trend of technology competition?
- Is it a worthy investment?

The objective of this article is to dissect through various assay attributes, applications and assay technologies to achieve a comprehensive understanding of the competitive position of each ion-channel assay technology, and to begin answering some of these strategic questions.

Assay attributes

It is important to know what attributes to look for when comparing technologies and their applications. Among many assay attributes, the most often considered are: sensitivity, specificity, throughput, information content, robustness, flexibility, cost and physiological relevance. Although these are the most important attributes for ion-channel assays, they apply to other bioassays as well.

'Sensitivity' describes the minimum change of signal that can be reliably detected. In the context of pharmaceutical screening, it describes the ability of an assay to detect weak stimuli. Sensitivity can be measured by the false-negative rate; a low false-negative rate corresponds to the high sensitivity of an assay.

'Specificity', also called selectivity, measures the ability of an assay to distinguish between different types of stimuli. The false-positive rate is a measurement of specificity. A low false-positive rate corresponds to a high-specificity assay.

'Throughput' measures the speed and volume of an assay. It is usually expressed by the number of data points performed in unitary

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time (e.g. per day), by unitary resources (e.g. per person, or per instrument).

'Information content' is a multi-dimensional attribute that describes the amount of data output from one test unit (e.g. a well of a microtiter plate) of an assay. It includes kinetic aspects (such as temporal resolution) as well as static or steady state aspects of the measurements (such as fluorescence measurement at multiple wavelengths, simultaneous detection of fluorescence intensity, polarization and lifetime, multiple morphological attributes, and so on, or the combinations of these measurements).

'Robustness' measures the ability of the assay to tolerate interference and changing experimental conditions. High reproducibility corresponds to the high robustness of an assay.

'Flexibility' relates directly to the ability of an assay to be adapted for different tests or different targets.

'Cost' measures the consumption of resources such as money, manpower and time by an assay. Cost is usually measured by the cost per data point.

Finally, 'physiological relevance' describes how relevant the result of an assay is to a physiological situation. A close approximation to physiological conditions correlates with a more meaningful or relevant assay.

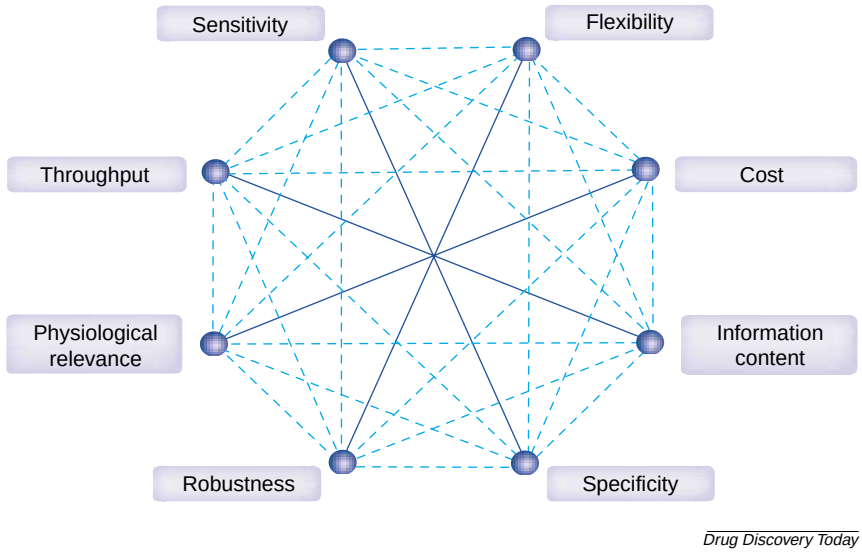

Figure 1. Interdependence of assay parameters. Solid lines indicate opposing relationships – the achievement of one attribute is usually at the cost of the other. Dashed lines indicate other relationships – a change of requirement of one attribute could have positive and/or negative impacts on other attributes.

The relationships among the above assay attributes are illustrated in Fig. 1. The attributes in the opposing positions somewhat antagonize each other, that is, the achievement of one attribute is usually at the sacrifice of the other. Although this is not always the case, this observation is true to many assay scenarios. Within a technology's developmental stage, information content often has to be sacrificed to achieve higher throughput. Fluorescence-based assays, for example, offer high throughput, but have relatively poor information content. However, patch-clamping is excellent for providing high information content, but suffers from low throughput. Another example is clinical trials, which, despite providing the most physiologically relevant data, are also the most expensive step in drug development. By contrast, binding assays are perhaps the least expensive method available to screen a compound library for a given ion-channel target, yet they provide less relevant information.

Overall, these attributes are inter-related, such that changing the requirement of one attribute could have positive and/or negative impacts on several other attributes. Because of this interdependency, a single attribute should not be used to determine the superiority of a technology. Strongly interdependent attributes, especially opposing attributes, are usually considered at the same time. One such example is sensitivity and specificity. In drug screening, a subjective-hit threshold is usually used to classify responses into 'hits' or 'non-hits'. Such classification affects both the false-positive and false-negative rates and, therefore, the sensitivity and specificity of an assay. Because of the subjective threshold setting, talking about sensitivity or specificity alone is not meaningful. However, given a certain specificity, assays that offer better sensitivity are superior in this aspect. In this review, we refer to 'sensitivity' under a fixed specificity within the same context. For detailed discussion, please refer to previous publications[5,6].

Market segmentation

Ion-channel assays are used widely in basic research for investigating ion-channel-related phenomena and in drug discovery for screening compounds directed to ion-channel related targets. We identify five market segments in the drug-discovery value chain, where ion-channel assays significantly add to the value-generation process. They are: basic research, primary screening, secondary screening, safety screening (cardiac safety screening is part of ADME/Tox) and animal testing. Market attributes and technical requirements vary with respect to each specific market segment.

Basic research

The goal of basic research is to acquire knowledge of how a biological system works in its normal physiological condition, how its physiology is perturbed by a pathological condition, and how new therapeutic intervention might be used to alter the pathological state. Target identification and target validation are two aspects of basic research to which ion-channel assay technologies can be applied. Basic research needs assay methods that offer high flexibility, but demand less in throughput. Traditionally, patch clamping, ion indicator dyes and radioactive flux assays are the most commonly used assays for this market segment.

Primary screening

During primary screening, a large collection of chemical entities is tested against an ion-channel target for desired modulation using a specific assay format. The screening data are used to construct structure–activity relationships (SARs), which result in the identification of lead compounds. Throughput and robustness are crucial for assays in this market segment. High information content, although desired, is not essential. Currently, most primary screenings are done using fluorescence and flux-based methods.

Secondary screening

The knowledge obtained from primary screening is usually used to construct focused libraries, which are then used in secondary screenings to find related compounds that have improved properties. In comparison to primary screening, fewer compounds are tested during secondary screening, but they are scrutinized in more detail for their physical, chemical and physiological properties as they become more advanced lead pharmaceutical candidates. Assay methods that offer great sensitivity, selectivity and temporal resolution such as patch clamping are preferred for secondary screening.

Safety screening

Since the late 1990s, several approved drugs have been found to prolong cardiac repolarization (long QT). This led to the withdrawal of several drugs from the market and the termination of the development of several compounds[7]. In certain cases, patients taking these compounds developed polymorphic ventricular dysrhythmia that is known to cause ventricular fibrillation and sudden death. Hence, the Committee for Proprietary Medicinal Products (CPMP) recommended cell-based methods to determine the potential of drug candidates to prolong cardiac repolarization[8]. HERG, one of the several cardiac potassium channels, was shown to regulate cardiac repolarization[9–11] and was identified as the target for many of the compounds that prolong QT. This creates a new market opportunity for ion-channel assays to be performed on all drug candidates, from the whole-cell to whole-tissue level, to obtain relevant toxicological data. Both patch clamping and fluorescence-based assays are currently used for HERG screening.

Animal testing

During animal testing, ion-channel assays can be performed using *in vivo* electrophysiological techniques (e.g. on anesthetized dogs). In addition, genetically encoded fluorescent polypeptides such as green fluorescent protein (GFP) and its mutants could be used as a fluorescent energy resonance transfer (FRET) partner of voltage sensors for *in vivo* detection of voltage changes; for example, in targeted cells of small animals such as *Caenorhabditis elegans*, where a mobile FRET partner such as oxonol can reach the cell membrane by diffusion[12].

The previous paragraphs indicate that the requirements with regard to the eight attributes are different for each of the market segments we have identified (Fig. 1). The following are the primary differences between the requirements in the different market segments, as summarized in Table 1. Because there is significantly less overlap between the techniques used in the animal testing phase and those used in other market segments, this segment has been omitted:

- The research market has significant requirements for information content, cost competitiveness, physiological relevance and flexibility, but has less demand for throughput and robustness. The primary screening market has great need for sensitivity, throughput, robustness and cost competitiveness, but has less demand for selectivity, information content, physiological relevance and flexibility. However, the secondary screening and safety screening markets require high sensitivity, selectivity, information content and physiological relevance, but have less demand for throughput, robustness, cost competitiveness and flexibility.
- The requirements for throughput, information content, flexibility, robustness and cost competitiveness vary substantially with different market segments, whereas there are consistent requirements for sensitivity, selectivity and physiological relevance across different market segments.
- Although it is not always the case, antagonizing attributes of an assay (Fig. 1) tend to have opposite requirements for each market segment. This suggests that it might be possible to apply technologies that maximize certain attributes to different market segments.

Table 1. Requirements for each market segment

Assay parameters	Market segments			
	Research	Primary screen	Secondary screen	Safety screen
Sensitivity	+++	+++	+++	+++
Selectivity	+++	++	+++	+++
Throughput	+	+++	++	++
Information content	+++	+	+++	+++
Robustness	+	+++	++	++
Flexibility	+++	+	+	+
Cost	+++	+++	+	+
Physiological relevance	+++	++	+++	+++

Symbol designation: +, moderate requirement; ++, high requirement; +++, highest requirement.

- Secondary and safety screening markets have similar requirements. This suggests that technologies could be simultaneously well-suited for both market segments.

Technologies

This section will describe the most popular ion-channel assays and introduce several types of emerging ion-channel assay technologies along with their characteristic assay attribute profiles.

Patch clamping

Patch clamping is the gold standard for determining ion-channel function[13,14]. The patch-clamp technique controls, or clamps, the electrical potential difference across a small patch of membrane or across the plasma membrane of an entire cell and directly assesses the current carried by ions crossing the membrane at that voltage through ionic channels. This technology provides high quality and physiologically relevant data of ion-channel function at the single cell or single channel (within a small patch of membrane) level. Patch clamping is unparalleled in its microsecond to sub-millisecond temporal resolution for measuring the intrinsic biophysical properties of ion channels such as the time constants of transitions between closed, open and inactivated states. For pharmacological testing of compounds, it provides a standard for measuring the potency of compound–channel interactions.

Although the patch-clamp technique is able to resolve ionic currents in the pico-amp to sub-pico-amp range, setting up patch-clamping experiments is a complicated process requiring highly trained personnel to make the system less vulnerable to interference from vibration and electrical noise. Throughput of a veteran patch-clamper is, at best, 10–30 data points per day. Such low throughput and high labor-cost is far from acceptable for HTS purposes. Currently, patch clamping is used extensively for basic research. It is also a major method for secondary and safety screening.

Patch clamping is a highly flexible technique that can be performed in either whole-cell configuration; cell-attached, inside-out, or outside-out configurations in macro-patch or single-channel mode[13]; or even with membrane vesicles and reconstructed lipid bilayer configurations[15,16]. Single-channel patch-clamp recording provides event-based measurements such as unitary conductance, sub-conductance states, open and closed statistics, bursting behavior, and so on. No other existing technology can provide such a direct, precise and detailed measurement of the activity of an ion channel down to the single-molecule level.

Xenopus oocyte has become a popular ion-channel recording and screening system for its faithful and high-level expression, as well as relatively low endogenous background current. Its large size, together with its tolerance for being impaled by multiple microelectrodes, makes it easy to inject mRNA for expression cloning and drug screening. Because of the large size of oocytes, two-electrode voltage clamp (TEVC) is used for whole-cell recordings of *Xenopus* oocytes to enable one of the electrodes to be entirely dedicated to injecting sufficient current to clamp the voltage across its entire membrane. It is also impossible to control the intracellular ionic composition of oocytes because of their size. Oocyte recordings suffer from lower sensitivity during compound screening and pharmacological testing, largely because of the absorption of the compounds by egg yolk. In addition, seasonal variation of expression makes it difficult to perform screenings during the summer months, whereas the requirement of low incubation temperature can reduce the physiological relevance of the data[14,17].

Fluorescence-based methods

Fluorescence-based methods do not directly measure ionic current but, rather, measure either membrane-potential-

dependent or ion-concentration-dependent changes of fluorescence signals (from fluorescent dyes loaded into the cytosol or cell membrane) as a result of ionic flux. Because fluorescence-based methods give robust and homogeneous cell population measurement, these assays are relatively easy to set up and achieve high throughput.

Fluorescent voltage-sensor dyes are used to measure voltage changes across the cellular membrane through either the potential-dependent accumulation and redistribution[18,19] or the FRET mechanism[20]. The lipophilic, negatively charged oxonol dyes, such as *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] are examples of redistribution dyes. DiBAC-based voltage-sensitive dyes have low fluorescence in an aqueous environment, but show increased quantum yield upon binding to hydrophobic intracellular molecules. Because the change of fluorescence occurs minutes after the change of membrane potential, these dyes are best suited to detecting steady state changes in membrane potential. Second and sub-second resolution of changes in membrane potential cannot be detected with these dyes. During HTS, compound fluorescence and compound–dye interactions are major sources of artifacts that result in a relatively high false-positive rate. Molecular Devices (Sunnyvale, CA, USA) recently introduced an improved membrane potential kit that gives faster response time (in tens of seconds) for use with its Fluorometric Imaging Plate Reader (FLIPR™) HTS platform. FLIPR uses a 384-well microplate format that gives a throughput of up to 10 plates per hour depending on the assay format. Dye cost is about 10–30 US cents per data point depending on the type of plates (96- or 384-well) being used[21,22].

In FRET-based voltage sensors, different negatively charged, membrane-soluble oxonol dyes are used as voltage-sensing FRET acceptors – *bis*-(1,3-dialkyl-2-thiobarbituric acid) trimethine oxonol [$DiSBAC_n(3)$]. The FRET donors are coumarin-tagged phospholipids that are integrated into the outer leaflet of the membrane when loaded into the cells. An increase or decrease of FRET in response to membrane hyperpolarization or depolarization produces fast, ratiometric changes. The ratiometric nature of the assay helps to eliminate many of the artifacts associated with DiBAC assays. Unlike DiBAC assays, the use of phospholipid-anchored FRET donor restricts the location of FRET in the plasma membrane, which ensures the measurement of potential changes that occur at the cell membrane, rather than in other subcellular compartments such as the mitochondria.

FRET-based voltage sensors also give sub-second temporal resolution, which allows for kinetic reading. Because the mobile oxonol molecules are charged, a dye-concentration-dependent dye-current might interfere with the change of membrane potential caused by the ionic current through the ion channels, especially when the current is less than a few hundred pico-amps. One way to reduce this is to use less dye, which might cause a decrease in the signal-to-noise ratio if the dye concentration is below a certain limit. Recently, Aurora Biosciences (San Diego, CA, USA) has developed the Voltage Ion Probe Reader (VIPR™) as a high-throughput instrument for screening ion-channel-related targets using these FRET-based voltage sensors[3]. The newer VIPR II, like FLIPR, also adopts the 384-well format, capable of screening several plates per hour. The dye cost is about 10–30 US cents per data point plus a technology access fee. Like the DiBAC assays, VIPR assays are approximately one-log value less-sensitive during the screening of certain compounds compared with results obtained using patch-clamp recordings[21–23].

Ion-specific fluorescent probes that measure intracellular ionic concentrations are also widely used in research and pharmaceutical screenings for ion channels. Among these dyes, calcium indicator dyes that alter their fluorescence emission upon calcium binding are the most popular[24]. Depending on the application, calcium dyes are available in a range of affinities to calcium ions (10^{-8}–10^{-5} M), excitation and emission spectra, and chemical forms (membrane permeable or not)[25]. They show different temporal resolution (from milliseconds, like Fluo-3, to tens of seconds, like Fura-2), and different degrees of accuracy for each range of calcium concentration. For example, Indo-1 is preferable to Fluo-3 for measuring the large, relatively slow intracellular calcium transients associated with cellular contraction, whereas Fluo-3 is preferred for measuring small, fast transients associated with calcium 'sparks'[26]).

These calcium dyes are usually used in conjunction with a FLIPR-type kinetic fluorescent reader to achieve high-throughput, low-noise detection of both absolute levels and changes of cytosolic calcium concentration. Dye cost for a typical high-throughput assay is less than one US cent per data point. Calcium dye-based ion-channel assays suffer from the interference from other cellular processes that produce changes in intracellular calcium concentration. Overall, ion-channel assays using ion indicator dyes are largely limited to the availability of high-performance ion-specific indicator dyes. To date, only calcium dyes give a robust performance that can be used for HTS of calcium channels or non-selective cation channels.

It must be emphasized that temporal resolution in cell-based fluorescence ion-channel assays (FLIPR, VIPR) is different from the ion-channel characteristics that can be measured in patch-clamp analysis. In particular, the slower kinetics recorded with voltage-sensor dyes do not mirror the behavior of the ion channels under investigation

(which open or close in the order of microseconds) but, rather, is related to the time of redistribution of the dye(s) upon membrane depolarization or hyperpolarization. What is measured is the convolution of the aggregated change in membrane potential over multiple cells as a result of ion-channel gating and dye-charge movement, and the dye response determined by the biophysical properties of the dye, the membrane fluidity, and so on. Therefore, fast dyes with response times in the micro- to milli-second range could provide more relevant information about the ion channels than slow dyes at the cell population and single cell, but not single channel, level. It should be noted, however, that temporal resolution is not necessarily a prerequisite to monitor the effects of compounds on ion-channel activity using voltage-sensitive dyes.

Flux assays

Radioactive isotopes have been used to trace the cellular influx or efflux of specific ions. A commonly used assay format is $^{86}Rb^+$ efflux for K^+ channels or non-selective cation channels[27]. In this format, cells expressing the ion channel of interest are incubated with buffer containing $^{86}Rb^+$ for several hours before they are washed and incubated with agonist to allow for $^{86}Rb^+$ efflux. The cells and supernatant are then collected for radioactive counting[2].

The flux assay is a format preferred by many screening laboratories because it measures ionic flux. It gives much higher throughput (several hundred data points per hour) than patch clamping and is robust against artifacts such as those seen in fluorescence-based assays. In addition, it gives a highly sensitive readout when measuring compound potency, comparable to that of patch clamping[28]. The typical overall cost is around 20 US cents per data point. However, these assays suffer from low temporal resolution (typically from seconds to minutes), less information content compared with voltage-clamping, and lower throughput compared with fluorescence-based assays.

These are among the reasons that radioactive-efflux methods are more suitable for secondary assays to confirm primary hits before patch clamping is used for in-depth characterization of compounds. However, radioactive-efflux assays also suffer from the inconvenience associated with handling radioactive material, and the requirement for different radioisotopes for channels selective for different ions. A high level of channel expression is also necessary to achieve an acceptable signal-to-noise ratio[29].

Binding assays

Generally, binding assays are not considered functional assays because they detect the binding of a compound to an ion channel rather than the compound's ability to alter channel function. A typical binding assay involves the labeling of a known ligand for the ion channel, such as a neurotoxin, possibly with a radioisotope. Activity of the test compound is indicated by the displacement of the labeled ligand. Such method is prone to false-negatives because ligand displacement can only detect compounds that bind to the same, or allosterically coupled, sites. However, it is also subject to frequent false-positives because compound–channel binding does not guarantee functional modulation. Secondary assays are always necessary to find out if the compound is either an agonist, an antagonist or neither.

The sensitivity of a binding assay is often determined by the affinity of the known ligand. A high affinity ligand might not allow the detection of weak binders. However, the selection of a low affinity ligand could lead to increased detection of non-specific binding. When the affinity of the ligand is within a certain range (e.g. from nano- to micro-molar concentration) the EC_{50} (IC_{50}) values obtained from binding assays have a reasonable correlation with those obtained from patch clamping[28]. Binding assays can be performed in 96-, 384-well or even higher density formats with reasonable reagent cost that varies with respect to the ligand used.

Emerging technologies

There has never been a better time to be in the ion-channel research field. Genomic and information technologies are helping to uncover a new level of ion-channel diversity and complexity, the crystallization of ion-channel proteins is just starting to unveil the related structure information[30], and new detection technologies are emerging to create new tools for studying ion-channel function[1]. The following introduces a few types of emerging ion-channel assay technologies.

Automated recording using conventional electrodes This includes automated patch-clamping and automated oocyte recording. The idea is to use a computer to completely control and automate the otherwise complicated, tedious and repetitive manual process, to achieve higher throughput and reproducibility. During the 1990s, in collaboration with Pfizer (New York, NY, USA), NeuroSearch (Ballerup, Denmark) developed NeuroPatch™ robots for automated patch-clamping on mammalian cells, which is now marketed under the name Apatchi-1™ by Sophion Bioscience (Ballerup, Denmark), a subsidiary of NeuroSearch (http://www.sophion.dk/). The system uses an automatic cell recognition and positioning system for the identification, selection and patching of up to eight independent cells in

separate recording chambers. The current throughput is ~30–100 datasets per day. Because the cells are cultured and patched on solid surfaces, it requires a precise cell selection and pipette positioning system to avoid breaking the glass recording electrodes.

Recently, CeNeS (Cambridge, UK) introduced a novel, automated patch-clamping system for mammalian cells (http://www.cenes.com/channelwork/autopatch.html). This system presents the cells to the patch electrode at an air–liquid interface in a glass capillary. The advantage of this 'interface patch' is that it avoids problems associated with vibration and does not require sophisticated positioning of the patch electrode. The current product, AutoPatch™ 1 (AP1), with a single recording chamber, has a throughput similar to manual patch-clamping with a >50% success rate for whole-cell recording[31]. The next generation products AP2 and AP3 will have 18 or more recording chambers for simultaneous recordings.

Robotic oocyte recording systems were invented in the 1990s independently in several pharmaceutical laboratories. In 1994, Bayer AG (Leverkusen, Germany) developed the prototype for Roboocyte™, a fully automated sequential oocyte recording system using a 96-well format, which is now manufactured by Multi Channel Systems (Reutlingen, Germany) and marketed by ALA Instruments (Westbury, NY, USA) (http://www.roboocyte.com/). Similarly, AstraZeneca (London, UK) developed RoboClamp™ for oocytes, which is now licensed to, and further developed, at Axon Instruments (Union City, CA, USA) (http://www.axon.com/notes/Axon_Shareholder_Update_2000_Oct.doc).

Recently, Abbott Laboratories (Abbott Park, IL, USA) also released its eight-channel version of the automated oocyte recording system. The current throughput of this type of technology is several hundred cells per day.

Neither automated patch-clamping nor automated oocyte recording is particularly amenable for a high degree of parallelism and miniaturization. Further improvement of throughput could become increasingly difficult because of the complexity of controlling and positioning multiple recording electrodes.

Chip-based ion-channel assays There are two ways of developing on-chip ion-channel assays: chip-based patch-clamping (patch-on-a-chip) and chip-based fluorescence measurement. Chip-based patch-clamping is currently being developed by several companies and academic laboratories worldwide[32–37]. The objective is to replace traditional patch electrodes with a planar array of recording interfaces miniaturized on the surface of either a silicon, polymer or glass substrate ('a hole on a substrate'). In this design, two perfusion chambers, the intracellular and extracellular chambers, are separated by a substrate. During the assay, cells are loaded into the extracellular chamber. A negative pressure is usually required to pull the cells into the holes to form high-resistance seals. The membrane below the seal is subsequently ruptured to obtain whole-cell configuration.

This simple design completely eliminates the need for an electrode control system and, therefore, has the potential to achieve high throughput without significantly losing temporal resolution. The companies competing in this area include Cytion (Lausanne, Switzerland; acquired by Molecular Devices in 2001), Axon Instruments, Sophion Bioscience, Nan]i[on Technologies (Munich, Germany), Genion (Hamburg, Germany; fully owned by Evotec, also Hamburg, Germany) and AVIVA Biosciences (San Diego, CA, USA). Recently, Essen Instruments (Ann Arbor, MI, USA; in which Molecular Devices has 20% equity investment and exclusive marketing rights for its patch-clamping product) has reported achieving 200 data points in 30 minutes in a planar system[34]. However, it is not known whether it is based on microfabricated chip(s). Most other competitors have also demonstrated reproducible whole-cell recordings and are progressing towards miniaturization. By the end of 2002, a few types of patch-on-a-chip products will be available in the ion-channel screening market.

The other direction for on-chip ion-channel assays is represented by Caliper Technologies (Mountain View, CA, USA), which is developing a fluorescence-based method for detecting single-cell voltage signals on microfluidic chips[38]. The measurement is based on the pseudo-ratiometric change in fluorescence from $DiBAC_4(3)$ and Syto 62 upon membrane depolarization or hyperpolarization. Pre-loaded cells are introduced to the detection site via microfluidic channels after they are mixed with compound solutions. The advantage of this flow-through system is that it could potentially achieve high throughput using a single chip with low compound consumption.

Novel fluorescence-based assays Aurora Biosciences is developing a technology capable of performing electrical stimulation to cell populations and recording millisecond voltage-responses using FRET-based voltage-sensor probes[39]. Traditional voltage-sensor assays using VIPR or FLIPR stimulate the cells through careful manipulation of ionic concentrations in the pre-incubation and addition buffers[3]. For example, cells expressing potassium channels have negative membrane-potentials in buffers with low potassium concentration. Addition of high-potassium solution to the cells would cause a depolarization detectable by voltage dyes. During the assays, the cells are usually exposed to non-physiological ionic concentrations. Electrical stimulation assays can be done in normal saline, which is a

more physiological experimental setting. In addition, it can be performed in the absence of liquid handling, which leads to the possibility of high-density miniaturization. However, the applications of electrical stimulation are currently limited to voltage-gated ion channels.

Recently, Abbott Laboratories introduced its Micro-Arrayed Compound Screening (μARCS™) technology[40]. With μARCS, the same set of compounds is spotted at high density (~10^4 spots per microtiter plate) in different arrangements on two filters. Each of the filters is pressed against an agarose gel precasted with dye-loaded cells and a second agarose gel precasted with agonist to allow for diffusion. Compound inhibition will appear on the filters as negative spots. The identity of the inhibitory compounds can then be obtained through deconvolution of the data from the two filters using a computer. μARCS is a simple and low-cost screening platform that offers enzyme or cell-based ultra-HTS (~100,000 compounds per day) for a variety of target classes. In particular, μARCS was shown to work in a FLIPR-type ion-channel assay. Fluorescent dye-related artifacts and poor temporal resolution are the disadvantages of this technology. μARCS is currently marketed through Discovery Partners International (San Diego, CA, USA).

Novel flux assays George Terstappen at Bayer took advantage of atomic absorption spectroscopy and developed a Rb^+ efflux assay for potassium channels and other non-selective cation channels without using radioisotopes[29]. This makes the traditional $^{86}Rb^+$ efflux assay more convenient and easier to integrate into HTS platforms. A fully automated system is being commercialized by Aurora Biomed (Vancouver, BC, Canada) with a throughput of 3–4 samples per minute at ~ US$1 per data point.

Competitive landscape

We compared the current and emerging ion-channel assay technologies discussed previously with the following six criteria: throughput, information content, overall cost, sensitivity, assay type and product development stage. The results are summarized in Fig. 2. Limited by the ability to quantify all different attributes in one graphic presentation, the following have not been covered in this figure: specificity, robustness, flexibility and physiological relevance. We chose information content and throughput as the x- and y-axes, respectively, because they are perhaps the most important attributes to consider when comparing ion-channel-assay technologies. From Fig. 2, we can make the following observations:

- Electrophysiology based technologies including patch-clamping, automated patch-clamping, robotic oocyte recording, interface patch and patch-on-a-chip stand out as a distinct group from other technologies in their ability to offer superior sensitivity and information content with single-cell or single-channel readouts. These technologies, however, suffer from the lowest throughput and highest overall cost. On the one hand, fluorescence-based technologies including FLIPR, VIPR, μARCS, electrical stimulation and chip-based fluorescence-detection technology can achieve high throughput and relatively low cost for measuring signals from cell populations (with the exception of Caliper Technology's chip-based fluorescence detection). On the other hand, they are unable to provide the level of information content that voltage clamp measurement offers.
- Currently, the best throughput is given by μARCS. The best information content is offered by patch-clamping-related methods done on mammalian cells. If a technology that offers some degree of both is needed, the VIPR type of technology stands out.
- Looking into the future, there is a market demand for technologies that offer both high throughput and high information content. High-throughput patch-clamping methods and electrical stimulation might be the likely candidate technologies competing for these markets. The future of μARCS will be secured by its continued leadership in cost and throughput. Flux assays are likely to be replaced by high-throughput electrophysiology in the future. Finally, binding assays might not see much improvement as these technologies become increasingly obsolete.

From these observations, it is clear that there are two mainstream technologies on which ion-channel assays are based: patch clamping and fluorescent probes. Currently, fluorescence-based assays dominate the primary screen market where throughput is more valued than information content, whereas patch-clamp-based methods are used more in the basic research market where the opposite is true. For secondary screening and safety screening markets, both types of technologies are being used, although the need for patch-clamp analysis increases as the development of a particular compound progresses. Which technology will win the future competition depends on the degree of improvement that each method can achieve over the next few years. In fact, most emerging technologies are aimed at improving these two detection methods to increase their respective market share.

To achieve higher throughput and lower cost, developers for patch-clamp-based technologies will miniaturize the recording units to enable large-scale parallel recording. However, temporal resolution could be compromised if multiplexing must be used to reduce the number of amplifiers (which represent a large consumption of space and

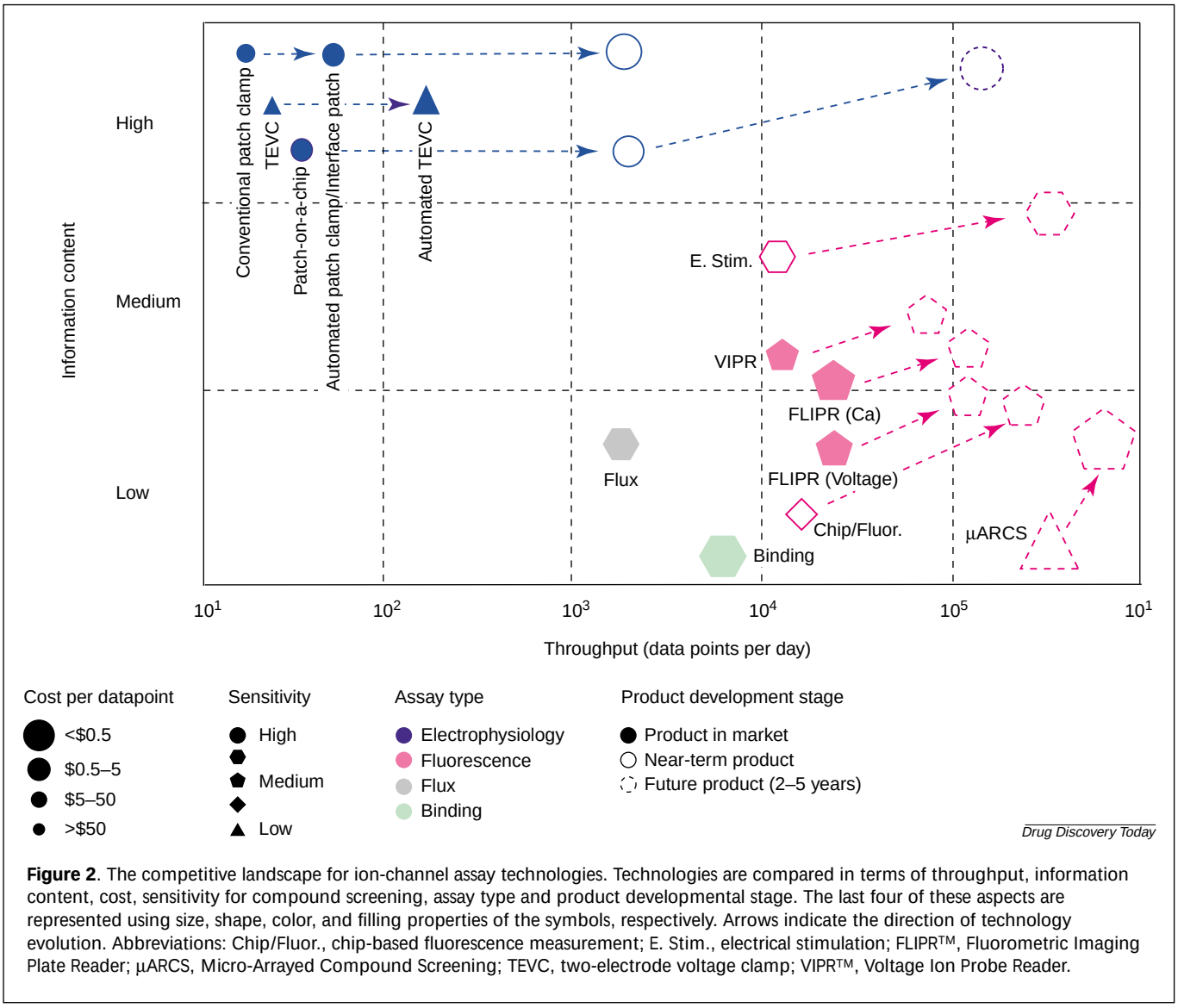

Figure 2. The competitive landscape for ion-channel assay technologies. Technologies are compared in terms of throughput, information content, cost, sensitivity for compound screening, assay type and product developmental stage. The last four of these aspects are represented using size, shape, color, and filling properties of the symbols, respectively. Arrows indicate the direction of technology evolution. Abbreviations: Chip/Fluor., chip-based fluorescence measurement; E. Stim., electrical stimulation; FLIPR™, Fluorometric Imaging Plate Reader; µARCS, Micro-Arrayed Compound Screening; TEVC, two-electrode voltage clamp; VIPR™, Voltage Ion Probe Reader.

overhead costs) needed for electrophysiological signals. The issue concerning developers of fluorescence-based technologies is how to achieve high information content, in particular, temporal resolution.

Traditional 'fast dyes' that give a sub-millisecond response include polymethines such as *p*-dialkylaminostyryl pyridinium compounds[19]. However, they typically give small changes of fluorescence levels per 100 mV. Improved oxonol dyes such as [$DiSBAC_6(5)$] were shown to achieve a response-time constant of ~400 µsec[20] when used as a FRET acceptor, with about 10–30% ratio changes per 100 mV. With this temporal resolution, the voltage dyes could be used to track the fast physiological voltage signals. Even so, the fact that fluorescence-based technologies cannot directly measure ionic current, to date, suggests that it is unlikely to replace patch clamping as technologies advance, but the reverse could be true.

Conclusion and prospective

In 2000, the average number of compounds tested per screen was ~350,000 for large pharmaceutical companies and 190,000 for smaller pharmaceutical and biotech companies[41]. The forecasted number in 2005 is approximately one million for both categories. From our analysis, ion-channel assays that offer both high temporal resolution (millisecond) and high throughput (>10,000 per day) will probably become available within the next two to five years. HTS will then no longer be the rate-limiting step for ion-channel drug development. So, what's next?

The ultimate goal of target identification, besides identifying targets, is to obtain as much knowledge about the targets as possible. The ultimate goal of screening, besides identifying lead compounds, is to obtain as much knowledge about the compounds as possible. The overall goal of discovery, besides filling up the pipeline with leads, is to

obtain high quality leads so that uncertainty (risk) is reduced to the lowest point for later stages of development (clinical trials).

Beyond the next generation, ion-channel assays are likely to be part of a cell-based screening system to provide high information content. In these screening systems, multiple detection methods including those for ion-channel functions are employed simultaneously for monitoring multiple physiological processes or multiple biochemical and signaling pathways. The enormous amount of data generated from the time and space integral of simultaneous detection will be fed into a multidimensional screening database, which can be cross-referenced to target, disease, patient and clinical databases. Highly sophisticated software systems will be used to analyze the database to extract quality lead information for multiple targets of a disease for patient populations with categorized genetic profiles.

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References

1 Lachnit, W. *et al.* (2001) Drug discovery technology for ion channels. *Drug Discov. Today* 6, S17–S18

2 Denyer, J. *et al.* (1998) HTS approaches to voltage-gated ion channel drug discovery. *Drug Discov. Today* 3, 323–332

3 Gonzalez, J. *et al.* (1999) Cell-based assays and instrumentation for screening ion-channel targets. *Drug Discov. Today* 4, 431–439

4 Clare, J. *et al.* (2000) Voltage-gated sodium channels as therapeutic targets. *Drug Discov. Today* 5, 506–520

5 Pagano, M. and Gauvreau, K. (1993) *Principles of Biostatistics* (Sugarman, M.J. and Kugushev, A., eds), pp. 124–128, Duxbury Press

6 Zhang, J. *et al.* (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screening* 4, 67–73

7 De Ponti, F. *et al.* (2000) QT interval prolongation by non-cardiac drugs: lessons to be learned from recent experiences. *Eur. J. Clin. Pharmacol.* S6, 1–18

8 Netzer, R. *et al.* (2001) Screening lead compounds for QT interval prolongation. *Drug Discov. Today* 6, 78–84

9 Curran, M.E. *et al.* (1995) A molecular basis for cardiac arrhythmia: HERG mutations cause Long QT Syndrome. *Cell* 80, 795–803

10 Sanguinetti, M.C. *et al.* (1995) A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the I_{kr} potassium channel. *Cell* 81, 299–307

11 Taglialatela, M. *et al.* (1998) Human ether-a-gogo related gene (HERG) K^+ channels as pharmacological targets. *Biochem. Pharmacol.* 55, 1741–1746

12 Oades, K. *et al.* (2000) Targetable voltage-sensitive FRET probes based on GFP and exogenous oxonols. *Society For Neuroscience 30th Annual Meeting*, 4–9 November 2000, New Orleans, LA, USA (Abstract No. 876)

13 Hamill, O. *et al.* (1981) Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391, 85–100

14 Sakmann, B. and Neher, E., eds (1995) *Single-Channel Recording* (2nd edn), Plenum Press

15 Miller, C., ed. (1986) *Ion Channel Reconstitution*, Plenum Press

16 Schlue, W. and Hanke, W., eds (1993) *Planar Lipid Bilayers*, Academic Press

17 Wagner, C.A. *et al.* (2000) The use of *Xenopus laevis* oocytes for the functional characterization of heterologously expressed membrane proteins. *Cell. Physiol. Biochem.* 10, 1–12

18 Waggoner, A. (1976) Optical probes of membrane potential. *J. Membrane Biol.* 27, 317–334

19 Loew, L.M. (1988) How to choose a potentiometric membrane probe. In *Spectroscopic Membrane Probes* (Loew, L.M., ed.), pp. 139–151, CRC Press

20 Gonzalez, J. and Tsien, R. (1997) Improved indicators of cell membrane potential that use fluorescence resonance energy transfer. *Chem. Biol.* 4, 269–277

21 Cronk, D. *et al.* (2001) Cell-based high-throughput screening of ion channels using FLIPR and VIPR membrane potential assay technology. *Society for Biomolecular Screening 7th Annual Conference*, 10–13 September 2001, Baltimore, MD, USA (Abstract No. 119)

22 Johnston, P. *et al.* (2001) Ionotropic glutamate receptor (iGluR's) HTS assays: comparison of FLIPR and VIPR formats. *Society for Biomolecular Screening 7th Annual Conference*, 10–13 September 2001, Baltimore, MD, USA (Abstract No. 123)

23 Gonzalez, J.E. (2001) Fluorescence technologies for ion transport targets. *Cambridge Healthtech Institute's Eighth Annual High Throughput Technologies*, 11–14 June 2001, Philadelphia, PA, USA

24 Bers, D.M. *et al.* (1994) A practical guide to the preparation of Ca^{2+} buffers. *Methods Cell Biol.* 40, 3–29

25 Haugland, R.P. and Spence, M.T.Z., eds (1996) *Handbook Of Fluorescent Probes And Research Chemicals* (6th edn), Molecular Probes

26 Rios, E. (1999) Calcium release flux underlying Ca^{2+} sparks of frog skeletal muscle. *J. Gen. Physiol.* 114, 31–48

27 Weir, S.W. and Weston, A.H. (1986) The effects of BRL 34915 and nicorandil on electrical and mechanical activity and on ^{86}Rb efflux in rat blood vessels. *Br. J. Pharmacol.* 88, 121–128

28 Hanson, D.C. (1999) UK-78,282, a novel piperidine compound that potently blocks the Kv1.3 voltage-gated potassium channel and inhibits human T cell activation. *Br. J. Pharmacol.* 126, 1707–1716

29 Terstappen, G. (1999) Functional analysis of native and recombinant ion channels using a high-capacity nonradioactive rubidium efflux assay. *Anal. Biochem.* 272, 149–155

30 Doyle, D.A. *et al.* (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* 280, 69–77

31 Trezise, D.J. *et al.* (2001) Rapid electrophysiology screening of ion channels using the CeNeS Autopatch system. *Society for Biomolecular Screening 7th Annual Conference*, Baltimore, MD, USA (Abstract No. 5016)

32 Schmidt, C. *et al.* (2000) A chip-based biosensor for the functional analysis of single ion channels. *Angew. Chem., Int. Ed. Engl.* 39, 3137–3140

33 Fertig, N. *et al.* (2000) Stable integration of isolated cell membrane patches in a nanomachined aperture. *Appl. Phys. Lett.* 77, 1218–1220

34 Schroeder, K. (2001) High-throughput electrophysiology – a reality. *Society for Biomolecular Screening 7th Annual Conference*, 10–13 September 2001, Baltimore, MD, USA (Abstract No. 146)

35 Mathes, C. (2001) Whole-cell recordings from planar patch clamp electrodes: a step toward high-throughput electrophysiology. *Society for Biomolecular Screening 7th Annual Conference*, 10–13 September 2001, Baltimore, MD, USA (Abstract No. 5039)

36 Klemic, K. *et al.* (2001) Patch clamping with a planar electrode array. *Biophysical Society 45th Annual Meeting*, 17–21 February 2001, Boston, MA, USA (Abstract No. 1412)

37 Farre, C. *et al.* (2001) Whole-cell patch clamp recordings performed on a chip. *Biophysical Society 45th Annual Meeting*, 17–21 February 2001, Boston, MA, USA (Abstract No. 1414)

38 Farinas, J. *et al.* (2001) A microfluidic device for measuring cellular membrane potential. *Anal. Biochem.* 295, 138–142

39 Maher, M.P. and Gonzalez, J.E. (2000) Ion channel functional analysis using electrical stimulation. *Society for Neuroscience 30th Annual Meeting*, 4–9 November 2000, New Orleans, LA, USA (Abstract No. 713)

40 Warrior, U. (2001) Arrayed compound screening – ARCS, a well-less screening platform with advantages of miniaturization. *Cambridge Healthtech Institute's Eighth Annual High Throughput Technologies*, 11–14 June 2001, Philadelphia, PA, USA

41 Fox, S. (2001) New trends and directions. *Cambridge Healthtech Institute's Eighth Annual High Throughput Technologies*, 11–14 June 2001, Philadelphia, PA, USA