

Screening Technologies for Ion Channel Targets in Drug Discovery

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Ion channels are important targets for therapeutic intervention. Increasing demand in screening throughput as well as the quality of ion channel screening assays has greatly driven innovation of assay technologies and screening instrumentation for ion channel targets in last 5 to 10 years. This review article will evaluate currently available screening technologies for ion channels including binding assays, ion flux assays, fluorescence-based assays, and automated patch-clamp instrumentation.

Introduction

Ion channels are membrane spanning proteins which facilitates the rapid movement of inorganic ions across the cell membrane down their electrochemical gradient. Ion channels play a vital physiological role in neuronal signal transduction, neurotransmitter release, muscle contraction, cell secretion, enzyme activation, signal transduction and gene transcription. To date, upwards of 300 different human ion channel genes have been identified. Mutations in genes encoding ion channels can lead to channelopathies which result from a dysfunction of the altered ion channel protein. Thus, ion channels represent an important class of molecular targets for drug development.

A recent analysis of all FDA approved drugs available in 2002 has revealed that only 273 human genes have been used as the therapeutic targets [1]. Ion channels correspond to 7% of the total recognized drug targets. It has been estimated that approximately 10% of the predicted 30,000 genes in human genome are druggable [2]. Ion channel subunits and splice variants may represent 10 to 20% of the 3,000 predicted druggable gene targets.

The role of ion channels in drug safety has emerged as a critical issue in last several years. Since 1985, five drugs have been withdrawn from market due to their adverse effect on prolongation of the cardiac QT interval and in server cases, “torsade de pointes.” The mechanisms underlying this toxic effect involve inhibition of one or more of the cardiac ion channels: (1) human ether-go-go related gene (hERG), a potassium channel (I_{Kr}); (2) KCNQ1/CNE1 potassium channel (I_{Ks}); and SCN5A sodium channel.

Ion channels can be grouped into two classes: voltage-gated and ligand-gated ion channels (Table 1). Ion channels can exist in multiple states such as the closed, open and inactivated states. Voltage gated ion channels transition (gate) between these states in response to

changes in membrane potential. Ligand-gated channels transition in between these states in response to the binding and unbinding of a ligand. In the open state, ions can flow through a single ion channel pore at prodigious rates of over 10⁷ ions per second. Cell-based functional assays are an essential requirement for the screening of ion channels at both the primary and secondary levels. Traditional methods developed for high throughput screening of ion channels, such as binding, ion flux and fluorescent probes, measure ion channel activity indirectly. Patch clamp electrophysiology is regarded as the gold standard for measuring ion channel activity and pharmacology. Patch-clamp allows for the direct, real time measurement of ion channel activity but in its traditional format is low throughput and requires a high degree of operator skill. Hence, drug screening assays for ion channels, in comparison with those for enzyme and receptor targets, have compromised data quality for throughput. However, a number of new screening technologies have been developed and improved for ion channel assays that are poised to change this. A summary of the currently available screening technologies is listed in Table 2.

Demands on Ion Channel Screening Assays

The current demands for ion channel screening in the drug discovery process can be grouped into in three main areas [3].

1. Primary Screening Assays; High Throughput Screening (HTS)

HTS has seen a tremendous advance during the last 10 years and remains a critical step in the discovery of lead chemical structures for novel drug targets including ion channels. To increase the probability of finding new leads from HTS, many companies have invested heavily in expanding both the diversity and quality of their compound libraries. For most mid and large sized companies, the library collection has grown to 400,000 to 1 million or more compounds. The standard paradigms used to screen these libraries have evolved to automated 384-well or higher density single compound test formats. Minimal throughput of 30,000 (ideally > 100,000) compounds per day is required. When considering materials needed for assay development, dead volume during the robotic screening, and positive and negative controls, the total reagent and consumable requirements for completion of a single target screen are typically 2 to 3 times what is required for the actual screen itself.

Table 1. Classification of Ion Channels

Channel type	Activator	Ion permeability	Transmembrane (TM) domains
Ligand-Gated Ion Channels:			
IP ₃ R	IP ₃	Ca ²⁺	6-TM
CNG	cAMP	Na ⁺ , K ⁺ , Ca ²⁺	
6-TM			
nAChR	Ach, nicotine	Na ⁺ , K ⁺ , Ca ²⁺	4-TM
5-HT ₃	5-HT	Na ⁺ , K ⁺ , Ca ²⁺	4-TM
GABA	GABA	Cl ⁻	4-TM
Glycine	GABA	Cl ⁻	
4-TM			
NMDA	Glutamate, NMDA	Na ⁺ , K ⁺ , Ca ²⁺	3-TM
AMPA	Glutamate, AMPA	Na ⁺ , K ⁺ , Ca ²⁺	3-TM
Kainate	Glutamate	Na ⁺ , K ⁺ , Ca ²⁺	3-TM
P2X	ATP	K ⁺	2-TM
P2Z	ATP	K ⁺	2-TM
Voltage-Gated Ion Channels:			
K ⁺ channels	*	K ⁺	6-TM
Na ⁺ channels	*	Na ⁺	24-TM
Ca ²⁺ channels	*	Ca ²⁺	24-TM
Cl ⁻ channels	*	Cl ⁻	12-TM

* Voltage-gated ion channels are activated and inactivated by depolarization of repolarization.

2. *Secondary Screening Assays; HTS Hit Conformation and Lead Optimization* The throughput requirement for these types of assays is much lower than HTS, but the demands on data quality are higher. Unlike primary screening, compound titration with 8 to 10 different concentrations in duplicate is usually needed to determine the IC₅₀ value for each compound tested in a secondary screen. Often one or more different types of assays are performed to confirm the activity of a compound. The required screening throughput for secondary assays is on the order of tens to hundreds of compounds per day.

3. *Ion Channel Safety Assessment* Cardiac ion channel safety has received a lot of attention in the past 5 to 7 years. Eliminating compounds which have the potential to produce QT prolongation early in the development process is of industry wide interest. Inhibition of cardiac hERG channels has been identified as the mechanism underlying the cardiac toxicity of several therapeutic agents. Considerable efforts have been devoted to developing a reliable high throughput assay for these channels. The FDA already requires that the activity of novel agents on hERG be evaluated. hERG assays must be of high quality, repeatability and reliability. Practically, the throughput requirement for these assays must be similar to those discussed in section 2.

Current Ion Channel Screening Technologies

I. Radioligand Binding Assay

The radiolabel ligand binding assay was developed in the 1960s. It has been extensively used for

Table 2. Screening Technologies for Ion Channel Targets

Assay type	Throughput	Format	†Cost per well	Comments
• Membrane binding	medium/high	*96well 384well (SPA)	median-high median	limited by ligand availability and structure. Not functional.
• Electrophysiology				
- Voltage-clamp /Patch-clamp	very low	*single cell		classic and gold standard
- Ion-Work HT	low/medium	*384well	high	small seal resistance (< GΩ)
- PatchXpress 7000	low	*16-well	very high	GΩ seal but low throughput
• Ion flux assay				
- Radioisotopes (15Ca ⁺⁺ , 22Na ⁺ , 86Rb ⁺)	low	*96well	median	low S/N ratio, not for IITS
- Rb flux assay (atomic absorbance)	medium	*96/384well	low	for K ⁺ channels
• Fluorescence dye				
(1) Ca ²⁺ dye	high	*96/384well	low-median	limited for Ca ⁺⁺ channels
(2) membrane potential				
-Membrane Potential Kit	high	96/384well	median-high	C ₅₀ shifted to right in certain types of channels
-FRET-based	high	*96/384well	low-median	

*Heterogenous assay (cell wash is required)

†Cost per well is calculated only for the special consumable reagent (eg. SPA beads, dye or special plate/chip) based on the highest plate density available.

drug screening of many targets including ion channels. Binding assays were utilized most extensively in the 1980s and 1990s before cell-based functional assays were made available for HTS. Binding assays incorporate the use of a ligand which is labeled with a radioactive tracer, such as ^3H or ^{125}I . Binding of the labeled ligand to a specific site on a channel protein can be displaced by an unlabeled compound if it binds to the same site on the protein. The activity of the unlabeled compound can be quantified by its ability (IC_{50}) to compete with the labeled ligand. Filtration binding assays utilize a glass fiber filter mounted 96-well plate to separate free ligands with the ligand-channel protein complex. This assay requires a plate wash step which limits the screening throughput. The scintillation proximity assay (SPA) uses solid scintillant containing beads to capture cell membranes. The labeled ligands bind to these membrane-coated beads which enables homogenous detection due to the transfer of energy from labeled ligands to SPA beads in proximity. This SPA binding assay can be miniaturized into 384 and 1536-well formats with a throughput of 50,000 -100,000 compounds per day at moderate cost.

Binding assays provide no information about the effect of novel agents on channel function. For example, an agonist cannot be distinguished from an antagonist in a binding assay. Additionally, if a compound interacts with the channel protein at a site distinct from the labeled ligand, it will not be detected in a binding assay. An example of this is the hERG ^3H -MK-499 binding assay. It has been reported that the potency of certain compounds in the ^3H -MK-499 binding assay can differ by more than a hundred fold when compared to potency of the same compounds measured in a functional voltage-clamp hERG assay. Voltage-gated ion channels do not have endogenous ligands and hence exogenous toxins or compounds are used as the labeled ligands. The structure diversity of hits identified by binding assay based primary screens for ion channel targets are often limited.

II. Fluorescent Dye Probes

Calcium-sensing dye

Fluo-3 and Fluo-4 are the most commonly used fluorescent dyes for the measurement of changes in intracellular calcium. Cells are loaded with the dye and an exogenous stimulus is applied to elicit the influx of calcium ions. The dyes emit a strong signal at 525 nm wavelength when excited at 480 nm. The fluorescence intensity of these dyes increases proportionally with the elevation of intracellular free calcium concentration. In a non-stimulated cell, the intracellular free calcium concentration ($\sim 0.1\text{-}0.2 \mu\text{M}$) is four orders of magnitude less than the extracellular calcium concentration of 2 mM. In comparison, the intracellular and extracellular concentration difference between K^+ , Na^+ , and Cl^- ions is much smaller (<20 fold) and fluorescent dyes which are sensitive to these ions are not widely utilized. Upon stimulation, influx of Ca^{2+} through open channels in the cell membrane can produce large changes in intracellular Ca^{2+} concentrations. These changes in Ca^{2+} concentration can be detected with these dyes. CCD-camera based detection instruments, first developed in the early 1990s, are used to detect and capture the fluorescent signal emitted by the dyes. This screening method is simple, and throughput has been maximized with the addition of a fluorescence quenching substance to the assay buffer to suppress extracellular dye fluorescence and to eliminate the need for a cell wash step. Throughput up to 80,000 data points per day in 384-well format with relatively low reagent cost can be achieved. Calcium-sensing dyes have been used extensively for voltage-gated channels and ligand-gated receptors which conduct Ca^{2+} ions. Since the membrane potential is not controlled in fluorescence based assays, the potency of compounds which display “state-dependent” or “voltage-dependent” antagonism may be significantly weaker when compared to potencies obtained in the voltage-clamp assay.

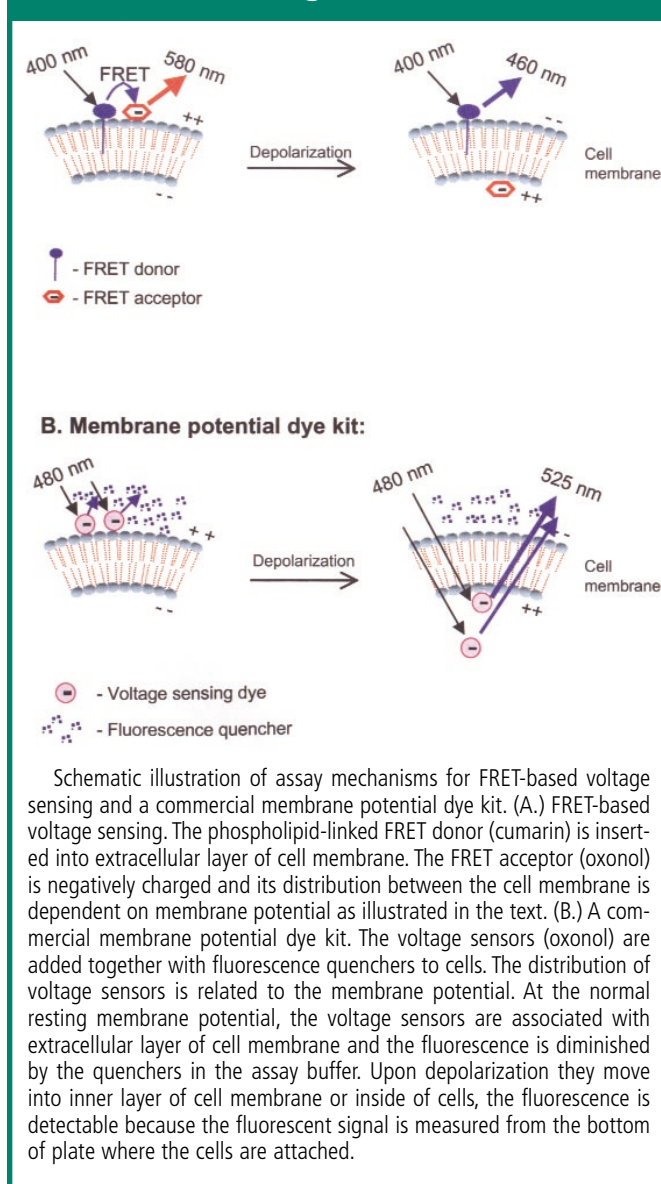
Voltage-sensing dyes

Voltage-sensing dyes are used to track changes in membrane potential in response to modulation of ion channels expressed in the cell membrane. Oxonol derivative voltage-sensing dyes are negatively charged and associate with the outside layer of the cell membrane. When the cell is depolarized, and the inner layer of the cell membrane becomes more positively charged, the dye moves into the inner layer of cell membrane. Voltage-sensing dyes were discovered in the 1960s but were not commercialized for use in large scale screening of ion channels until the mid 1990s [4].

FRET-based voltage-sensing dye

Fluorescent energy resonance transfer (FRET) incorporates the use of a pair of dyes to monitor changes in membrane potential. The FRET donor is a cumarin dye linked to a phospholipid that inserts into the outer leaflet of the cell membrane and the FRET acceptor is an oxonol derivative. In a hyperpolarized cell, 400 nm excitation of the cumarin donor produces FRET and excitation of the oxonol acceptor which then emits a fluorescent signal at 580 nm. When the cell is depolarized, the oxonol moves into the inner leaflet of the cell

Figure 1.



membrane, away from the cumarin dye. FRET is disrupted due to the physical distance (>100 nm) between the two dyes. Under these conditions, the emission from FRET donor cumarin (460 nm) is enhanced while the emission from the oxanol is reduced (Figure 1.A). The events are quantified as a ratio of emission detected from the FRET donor and FRET acceptor. FRET-based voltage dyes can provide a relatively rapid temporal resolution (~ seconds) in comparison to calcium sensing dyes. The ratiometric measurement of change in membrane potential helps to reduce assay artifacts. The throughput of FRET-based assays is 35,000-50,000 compounds per day in 384-well format. Drawbacks to this approach include: (1) the requirement of special and costly instruments, (2) only a select group of ion channels are compatible with this format and (3) two cell wash steps, which limit throughput, are required.

A special membrane-potential dye kit

This kit has been used for the homogenous measurement of changes in membrane potential with several potassium channels [5]. It utilizes a voltage-sensing dye mixed with unknown fluorescent quenchers. The temporal resolution of this dye is in the range of minutes, slower than the FRET-based voltage-sensing dye combination. Throughput is enhanced with the use of a quencher which enables homogenous assay format. The quencher's function is to absorb the emission of the voltage sensitive dye when it is positioned in the outer leaflet of the cell membrane. Upon membrane depolarization, the dye moves to the inner layer of cell membrane and upon excitation emits a detectable signal (Figure 1.B). The throughput of this assay in 384-well format is upwards of 60,000 to 80,000 compounds per day but the screening cost is relatively high due to the price of dye kit.

The homogenous nature of this assay combined with the use of a CCD-based imaging instrument which measures the entire plate at once helps to reduce well-to-well variation. This compensates the low signal-to-noise ratio of 1.5 to 2. By using this dye kit, we have recently performed an HTS on a chloride channel target in which the signal-to-noise ratio was only 1.4 fold. Despite the low signal-to-noise window, the hit confirmation rate was 56%.

A direct comparison of this assay with a FRET based voltage-sensing dye assay revealed that the activities of small molecule compounds are weaker in the no-wash assay (Zheng et al., unpublished data). We have also observed that in two distinct ion channel assays the activity of peptides and peptide/protein-based toxins were either greatly reduced or not detected in this assay but can be identified in the FRET-based dye assay. The noted reduction in activity may be a result of interference from the quencher/quenchers or other unidentified components in this no-wash dye kit. The weaker activity of compounds might also be due to the fact that the dyes used in this assay associate not only with the inner layer of cell membrane but also the membranes of subcellular organelles.

III. Ion Flux Assay

Radioactive ion flux assay

Radiotracers are used to measure the flux of ions moving in or out of a cell through ion channels expressed in the cell membrane. Radiotracers are available for every class of ion channels; $^{86}\text{Rb}^+$ for potassium channels, $^{22}\text{Na}^+$ for sodium channels, $^{45}\text{Ca}^{2+}$ for calcium channels and $^{36}\text{Cl}^-$ for chloride channels. Although these radiotracers have been used for over 20 years in ion channel assays, their application for HTS drug screening has been limited. Tracer assays are heterogeneous and slow, requiring both a tracer loading and wash steps. Only the steady state function of ion channels can be measured with radiotracers. Signal-to-noise ratio is low due to incomplete removal of extracellular tracer after loading or the continuous leak of tracer out of cells before stimulation. The concerns over excessive radioactive waste and safety for screeners also limit radioactive tracer based assay use in HTS.

Atomic absorbance spectroscopy (AAS)

Ion flux assay using non-radioactive ion tracers analyzed in AAS have been around since the 1950's but instrumentation for high-throughput screening has only emerged in the last few years. AAS assays have been developed for a variety of voltage-gated potassium channels, such as hERG. Briefly, non-radioactive rubidium (Rb^+) is loaded into cells expressing a channel of interest for 3 to 4 hours. Cells are then washed to remove extracellular Rb^+ from the assay buffer. A 50 to 80 mM K^+ addition is used to depolarize the cell membrane and open the channel. The concentration of Rb^+ in both supernatant and cells are measured and the percentage of Rb^+ efflux is calculated. The signal-to-noise ratio of this assay is 6 to 10 fold and reagent cost for screening is very low. Both single channel and multi-channel instruments are currently available for 96-well and 384-well screens with moderate throughput.

IV. Electrophysiology

Voltage-clamp and Patch-clamp

These methods have been regarded as the gold standard for measurement of compound activity on ion channels in vitro. Through a single electrode attached to the cell membrane, the current generated by the ions flowing through ion channels expressed in the cell membrane can be measured while the membrane potential is voltage-clamped. The activity of ion channels can be measured directly and in real time. Despite the high quality data generated by this method, in its traditional format, electrophysiology has limited use in drug screening for ion channel targets due to the extreme low throughput [6]. In last a few years, several automated patch-clamp instruments have been developed and are now commercially available.

Automated patch-clamp electrophysiology

Traditional patch-clamp electrophysiology incorporates the use of a glass micropipette electrode, micro-fabricated from glass capillary tubes, for controlling the membrane potential whilst measuring ionic current flow. Automating this process for truly higher throughput became a reality with the development of the planar patch-clamp technology. The breakthrough with this technology came when the traditional patch electrode was replaced with a planar substrate with an array of micro-apertures. The first commercially available instrument uses a planar 384-well disposable plastic plate. In order to perform patch-clamp recordings, cells (in suspension) are first added to electrically isolated wells on the plate. Each well contains a single aperture for the patch-clamping of cells. A slight negative pressure is used to pull the cell membrane into the aperture and achieve a 50-500 M Ω seal. Electrical access is achieved via a perforating agent. Recent reports show that the pharmacology on several voltage gated ion channels accurately reflects traditional patch-clamp data. The success rate of patch is between 60 to 90% and the assay speed for processing each 384-well plate is about one hour. 2000-3000 cells can be patch-clamped in a day and 50-100 dose responses can be acquired with this system representing a 100 fold increase in throughput over the traditional patch-clamp technique [6]. The low seal resistance limits the use of this technology to cell lines with robust and homogeneous expression of voltage-gated channels.

Another automated planar patch-clamp instrument which recently became commercially available uses a 16-well disposable glass chip. It has been reported that, like in the traditional patch-clamp, a gigaohm seal resistance is achieved on these chips with a success rate of 20 to 70%. In this system, electrical access is achieved by rupturing the membrane underneath the aperture. Detailed pharmacological studies have yet to be demonstrated with this instrument. The 2-10 fold increase in throughput this system offers remains attractive for detailed studies of ion channel pharmacology as well as directed screening of small sets of compounds. Ideally, this system fits well alongside the previously discussed 384-well instrument, where the 384-well instrument is utilized to filter through 1000 compounds and

the 16-well system is used to perform detailed studies of key leads. Cost of consumables will greatly impact the use of these instruments. The evolution of automated patch-clamp electrophysiology is just beginning. Several other automated planar patch-clamp systems are in late stage development and next few years promise to be an exciting time for this technology.

Perspectives for Ion Channel Screening Technologies

Automated patch-clamp electrophysiology

The quality and throughput of automated patch-clamp instrument will be further improved in the next 3 to 5 years with the advance of microfabrication and micro-machining technologies. The cost of consumables should see some reduction when this platform is widely used in ion channel screenings. The most immediate impact of this technology will be the secondary screening and ion channel safety assessment.

Non-invasive detection of ion channel activity

The current electrophysiological methods require the use of a microelectrode which is inserted into a cell, changing the physiological environment of a living cell. Microelectrode array technology is a new approach for non-invasive extracellular recording of ion channel activity [7]. Currently, this technology has been used to record ion channel activity in tissue slices and primary cardiac primary cells in single well/chamber format. It currently has low throughput but can generate high content information. With the miniaturization of microelectrode arrays and the development of multi-well detection, its application in ion channel screening will be further explored. Other label-free detection technologies, such as Resonant Acoustic Profiling, Microplate Differential Calorimetry, Atomic Force Microscopy and Microwave Spectroscopy, may also be developed for non-invasive and high throughput detection of ion channel functions in the future.

Next generation true high throughput instrumentation

The current version of automated patch-clamp technology cannot meet the demands of HTS for large size compound collections. The fluorescent dye based and ion flux assays only measure steady-state ion channel activity, which may not reflect the physiological condition of ion channels. Additionally, agonists or toxins are usually required to activate channels in these screens, whereas, under physiological conditions, the activity of these channels is controlled by the changes in membrane potential. The next generation of electrophysiology based screening technologies will have to incorporate even higher throughput and enhanced data quality.

Ion channel biologics

Unlike G-protein coupled receptors, ion channels are a complex protein with multiple subunits. The strategy for cell line generation of ion channels is complicated by the variety of auxiliary channel subunits. Voltage-gated calcium channels, for example, consist of α_1 , α_2 -d, b and g subunits and each of these subunits has multiple isoform and splice variants. It is difficult not only to stably transfect all the subunits into a cell line but also to make the "right" combination of these subunits. Validation of biological relevant ion channel targets including auxiliary subunit components will continue to be an important area for ion channel drug discovery. In addition, the use of transfected cell lines or native cell lines for ion channel screening warrants further investigation.

Table 3. Recommendation of Screening Methods for Ion Channel Targets

Primary screening (HTS):

Ca ²⁺ channels	- Fluorescent calcium dye (Fluo-3 and Fluo-4)
Na ⁺ channels	- FRET-based voltage-sensing dye
K ⁺ channels	- Membrane potential dye kit /FRET-based voltage-sensing dye
Cl ⁻ channels	- Membrane potential dye kit

Secondary screening or medicinal chemistry support:

(two or more methods are recommended for key compounds)

Certain cell lines (384-well device)	- Automated patch-clamp station
Most cells (including native ion channels)	- Automated patch-clamp station (16-well device)
K ⁺ channels	- Atomic absorbance spectroscopy (Rb ⁺ flux assay)
All channels	- Voltage-clamp (for few key compounds)

Ion channel safety assessment (HERG channel and others):

(two or more methods are recommended)

HERG channels	- ³ H-MK499 binding assay
	- Atomic absorbance spectroscopy
	- Automated patch-clamp station (384-well device and 16-well device)
	- Voltage-clamp

Conclusion

Currently, the fluorescence based assays remain the most frequently used method for the primary screening of large compound collections in ion channel drug discovery. Ion flux and automated patch-clamp assays are the choice for secondary screening and lead optimization (Table 3). Although the screening throughput and quality have been greatly improved compared to those ten years ago, ion channel screening technologies need further innovation, refinement and optimization to be ideal. Ion channel assays for future HTS will have to be miniaturized into 1536-well or a higher density to accommodate the increasing capacity for the screening of multimillion compound libraries. New screening technologies are needed for many ion channel targets, especially those with low expression in native cells, which cannot be screened by current assay technologies due to the lack of real high throughput electrophysiological instrumentation. In addition, more reliable and cost effective methods are needed for ion channel safety assessment.

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Prior to joining Merck & Co., Dr. Zheng was a Research Scientist in the HTS group at Amgen in Thousand Oaks, California (1996 – December 1997) and a Scientist in the screening group at Berlex Biosciences (1993 -1996).

In last 10 years, Dr. Zheng has worked in the area of high throughput screening for the assay development, robotic automation and screening. He has extensive experience in a variety of screening assays especially the cell-based assays including ion channel and receptor targets.