$Na_v1.5$ sodium channel assay using IonWorks™ HT

IONWORKS HT APPLICATION NOTE #3

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INTRODUCTION

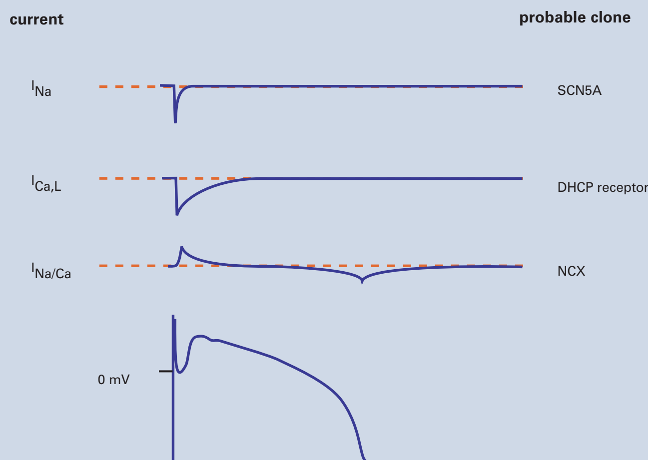
Voltage-gated sodium channel currents depolarize the membrane in excitable cells and are therefore critical for action potential initiation and propagation.[1] As a result, they have been implicated in many pathophysiological states[2–3] and thus represent important drug discovery targets. Until recently, robust, high-throughput assays that directly measure the function of voltage-gated ion channels were not available.[4] The IonWorks HT system can be used for screening and pharmacological studies of the cardiac voltage-gated sodium channel $Na_v1.5$.

CARDIAC SODIUM CHANNELS

$Na_v1.5$ sodium channels participate in the initial depolarization phase of action potentials in cardiac myocytes[5] (Figure 1) and are encoded by the *SCN5A* gene.[2] Mutations in the *SCN5A* gene have been identified for one type of familial Long QT Syndrome (LQT3).[2] The known mutations for LQT3 result in abnormalities in $Na_v1.5$ inactivation kinetics[5], resulting in sustained depolarizing currents that can lead to *Torsades de Pointes,* a potentially fatal cardiac arrhythmia.[2]

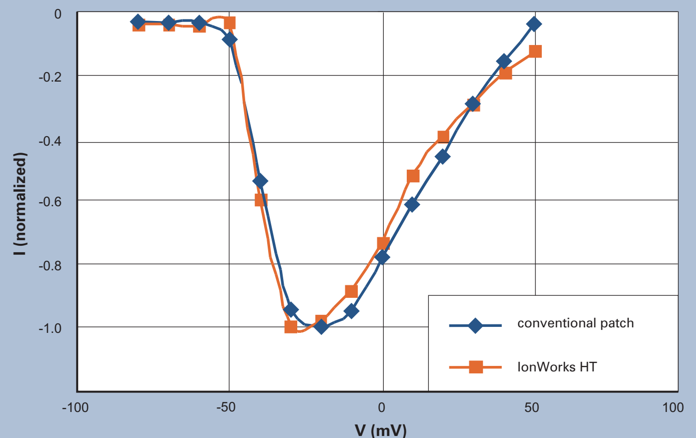
Experiments described in this application note used a cell line expressing the α subunit of $Na_v1.5$, which has among the fastest currents of voltage-gated ion channels. Here we report the utility, fidelity and performance of the IonWorks HT system for fast voltage-gated sodium channel studies.

cardiac action potential (figure 1)



Ionic currents underlying the cardiac action potential. Probable clone for each current type is indicated. $Na_v1.5$ channels underlie I_{Na} currents in cardiac myocytes. Potassium currents not shown. (Adapted from reference 3.)

$Na_v1.5$ I/V relation (figure 2)



Superimposed current-voltage (I/V) curves from cells measured with conventional patch clamp methods and with the IonWorks HT system. In both cases the peak currents were near 3 nA.

MATERIALS

- Cells: Chinese hamster lung (CHL) cells expressing the Na$_v$1.5 sodium channel
- Reagents and buffers: Amphotericin (Sigma Cat. # A-4888), DMSO (Sigma Cat. # D-2650); Internal buffer: high K^+, low Cl^- internal buffer containing (in mM): 100 K^+ Gluconate (Sigma Cat. # G-4500, 40 KCl (Sigma Cat. # P-9333), 3.2 $MgCl_2$ (Sigma Cat. # M-2670), 5.0 EGTA (Sigma Cat. # E-0396), 5.0 HEPES (Sigma Cat. # H-7523), pH 7.25 with KOH; External buffer: Phosphate Buffered Saline (PBS, Gibco Cat. # 14040)
- Tissue culture flasks: Cells grown in T-75 flasks (Corning Cat. # 430641)
- Cell culture media: Dulbecco's Modified Eagle Medium (Gibco, Cat. # 11965-092) supplemented with the following: 50 ml Fetal Bovine Serum (FBS, Irvine Cat. # 3000), 5 ml Penicillin-Streptomycin (Irvine Cat. # 9366) and 5 ml Geneticin (G418, Gibco Cat. # 10131) was used to grow cells; Versene™ (Gibco Cat. #15050) used to remove the cells from the flasks
- PatchPlate™ consumables (Molecular Devices Cat. # 9000-0688)
- Compound plates: Costar® 96-well plate (Cat. # 3355)

METHODS

Preparation of antibiotic solution

Aliquots of amphotericin (5.0 ±0.3 mg) were pre-weighed and stored at 4°C. Prior to cell preparation, 180 µl DMSO was added to an aliquot of amphotericin. Amphotericin/DMSO solution was vortexed until soluble (~30 seconds), added to a 50 ml conical tube of Low Chloride Internal Buffer and vortexed for ~1 minute. The solution was stored in the dark until ready for use.

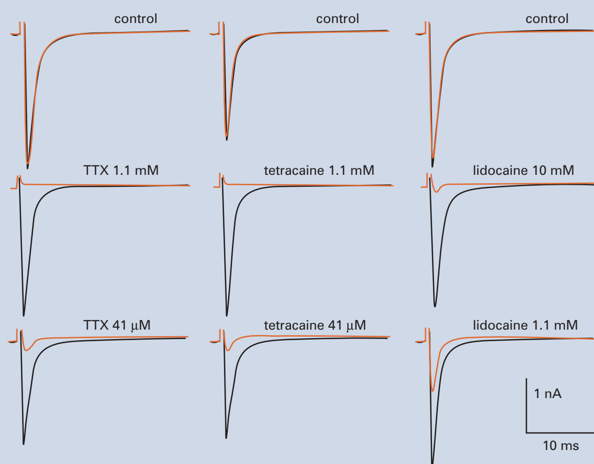
Preparation of cells

Step 1: Cells were grown to 70-90% confluence in a T-75 flask and removed from the incubator (37°C, 5% CO_2) 1-2 days after plating.

Step 2: Growth media was aspirated from the culture flasks using a 2 ml aspirating pipette attached to a vacuum pump. Cells were gently rinsed with 2.5 ml Versene solution for approximately 10 seconds before the solution was aspirated.

Step 3: The cells were again immersed in 2.5 ml Versene solution at 37°C. After 4 minutes, visibly rounded cells were easily dislodged from the bottom of the flask with a few brief taps on a solid surface. 20 ml of PBS was added to the flask and the resulting solution was used to wash the sides of the flask; the cell suspension was divided equally into two 15 ml conical tubes.

Na$_v$1.5 sodium currents recorded on the IonWorks HT system (figure 3)



Representative traces of control, full- and partial-antagonist doses are indicated for tetrodotoxin (TTX), tetracaine and lidocaine. Pre-compound traces are shown in black and post-compound traces are shown in orange. Control solution was phosphate-buffered saline (PBS).

compound plate setup (figure 4)

	1	2	3	4	5	6	7	8	9	10	11	12
A		0.5E-6			0.5E-6			4.6E-6				
B		1.5E-6			1.5E-6			1.4E-5				
C		4.5E-6			4.5E-6			4.1E-5				
D	TTX	1.3E-5		tetra	1.3E-5		lido	1.2E-4		PBS		
E		4.1E-5			4.1E-5			3.7E-4				
F		1.2E-4			1.2E-4			1.1E-3				
G		3.7E-4			3.7E-4			3.3E-3				
H		1.1E-3			1.1E-3			1.0E-2				

Concentration of drug in compound plate; each compound was replicated in three separate columns, indicated by color. TTX=tetrodotoxin (yellow); Tetra=tetracaine (green); Lido=lidocaine (purple); PBS=Phosphate buffered saline (white). Values are [drug] M.

Step 4: The two 15 ml tubes were centrifuged at 800 rpm for 4 minutes. The cell supernatant was decanted, 1.5 ml of PBS was added per tube, the cell suspensions were combined, and the cells were gently triturated for 1 minute using a p200 pipettor.

Step 5: A 3 ml volume of cell suspension was added to the cell boat on the IonWorks HT instrument just prior to beginning the experimental run.

Preparation of compound plate

Stock solutions of 3.3 mM tetrodotoxin (TTX), 3.3 mM tetracaine and 100 mM lidocaine were prepared in PBS. The tetracaine stock solution also included 0.3% DMSO. In the compound plate, the last row (H) contained the highest concentration of drug, and 1:3 serial dilutions were made from row H to G, G to F, etc. For TTX and tetracaine, 1.1 mM was used as the highest concentration bathing the cells, whereas for lidocaine 10 mM was used as the highest concentration. Between serial dilutions, the solution was mixed ten times with the pipettor and the pipette tips were replaced after each mixing step. Figure 4 shows the concentrations of TTX, tetracaine and lidocaine in the compound plate; each drug dilution series was replicated in three columns (as indicated by color in the figure). Each concentration was diluted 3-fold during the experiment by addition of 3.5 µl drug to 7 µl buffer/cell solution in the PatchPlate.

Electrophysiology

$Na_v1.5$ currents were elicited by a voltage step from the holding potential of -100 mV to -20 mV for 40 ms. A modified P/N subtraction[6] was used whereby a linear leak current is estimated from a voltage step from -100 to -110 mV. The values from this measurement were used to extrapolate a leak I/V curve; leak current values were estimated and subtracted digitally from all current recordings. There is no subtraction of the capacitive transients as in traditional P/N subtraction. Drugs were incubated for 118-229 seconds. Similar protocols were used for the conventional patch clamp recordings with leak subtraction disabled. Pipettes were pulled from TW-150 glass (WPI) with tip sizes about ~2 MΩ. Bath perfusion of compound (180-240 seconds) was used on the conventional patch clamp setup. A PC-505 amplifier (Warner) was used in all conventional experiments.

Data analysis

Concentration-response curves for TTX, tetracaine and lidocaine were fitted to a four parameter logistic equation:

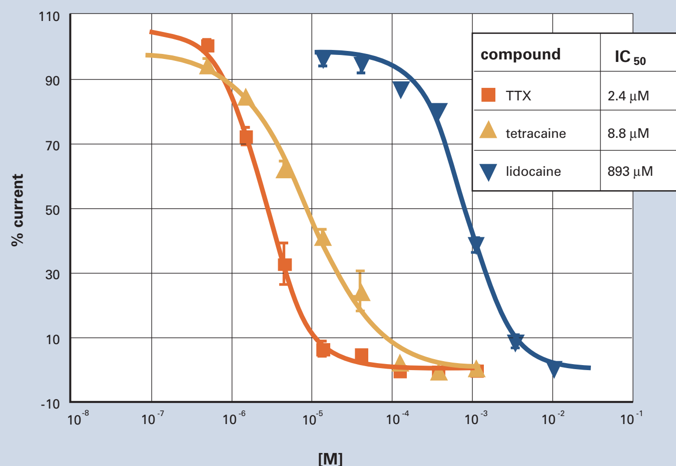
$$\% \text{ of control} = 100\,(1 + ([\text{drug}]/IC_{50})^{p})^{-1},$$

where IC_{50} is the concentration of drug required to inhibit current by 50% and p is the Hill slope.

Z-factor was calculated as:

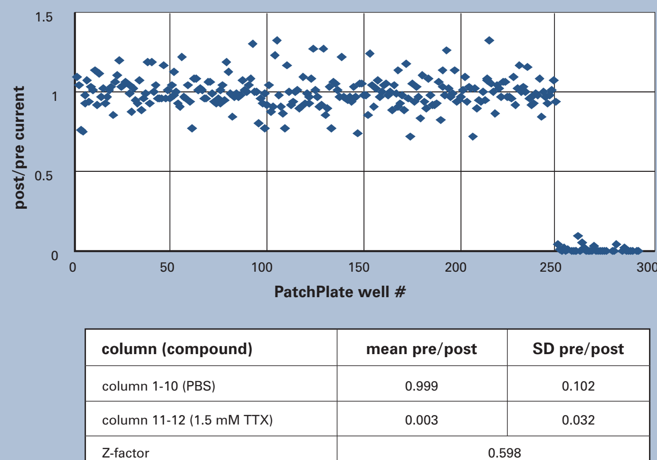
$$Z = 1 - \frac{[3(SD_{sample}) + 3(SD_{control})]}{|\text{mean of sample} - \text{mean of control}|}$$

dose-response curve for TTX, tetracaine and lidocaine (figure 5)



IC_{50} values for TTX, tetracaine and lidocaine obtained from a single 45-minute PatchPlate experiment. Mean ±SD shown; n=12 per data point. IC_{50} values are shown in table.

Z-factor statistical analysis of $Na_v1.5$ inhibition by TTX (figure 6)



column (compound)	mean pre/post	SD pre/post
column 1-10 (PBS)	0.999	0.102
column 11-12 (1.5 mM TTX)	0.003	0.032
Z-factor	0.598	

Mean (±SD) values for post-compound control and TTX addition, plotted as percent of pre-compound currents (n=4 per compound). Values used to calculate the Z-factor are shown in the table.

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RESULTS

Sodium current recording

Current-voltage (I/V) relation and representative current recordings from cells expressing $Na_v1.5$ channels are shown in Figures 2 and 3. Figure 2 demonstrates that sodium currents measured on IonWorks HT are similar to conventional patch clamp methods. Full and partial blockade by Na^+ channel inhibitors TTX, tetracaine and lidocaine are shown in Figure 3.

High-throughput IC_{50} curve generation

The 384-well PatchPlate allows the IonWorks HT system to rapidly perform high-throughput dose response experiments. Eight-point dilution series of three sodium channel antagonists were made in columns of a 96-well plate. Figure 4 shows the concentrations of TTX, tetracaine and lidocaine in the compound plate; each drug dilution series was replicated in three columns (as indicated by color). In one 40-minute PatchPlate experiment, data was collected for three dose-response curves at n=12 per data point; the resultant IC_{50} values for TTX, tetracaine and lidocaine are shown in Figure 5.

Z-factor analysis of TTX blockade

Evaluation of assay robustness was determined by performing Z-factor statistical analysis of TTX blockade of $Na_v1.5$ channels. Results from one PatchPlate experiment are summarized in Figure 6. The compound plate in this experiment had ten columns of control solution (PBS) and two columns of sample (1.5 mM TTX). Data was expressed as a percent of the pre-compound current at -20 mV test potential used to elicit sodium currents. Wells treated with TTX were readily identified (Z=0.598).

CONCLUSIONS

The IonWorks HT system can be used for high-throughput screening of modulators of voltage-gated sodium channels. Although clamping fast activating currents can be difficult, IonWorks HT still has sufficient fidelity for pharmacology studies with fast currents such as $Na_v1.5$. In addition, the IonWorks HT system can be used to generate eight-point dose-response curves at n=4 per data point, for up to 100 compounds per day.

REFERENCES

1. Hille, B. *Ionic Channels of Excitable Membranes,* 3rd edition. Sinauer Associates Inc, Sunderland, MA, 2001.
2. Ashcroft, F.M. *Ion Channels and Disease.* Academic Press, San Diego, CA, 2000.
3. Haverkamp, W., G. Breithardt, *et al.* (2000). The potential for QT prolongation and pro-arrhythmia by non-antiarrythmic drugs: Clinical and regulatory implications. Report on a policy conference of the European Society of Cardiology. *Eur Heart J*, 21:1216–1231.
4. Schroeder, K., B. Neagle, *et al.* (2003). Ionworks HT: a new high-throughput electrophysiology measurement platform. *J Biomol Screen* 8(1): 50–64.
5. Yu, F.H. and W.A. Catterall (2003). Overview of the voltage-gated sodium channel family. *Genome Biol* 4:207.
6. Bezanilla, F. and C.M. Armstrong (1977). Inactivation of the sodium channel. I. Sodium current experiments. *J Gen Physiol* 70(5): 549–66.

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