FLIPR^{TETRA}™ Membrane Potential kit assay development in 384-well and 1536-well format

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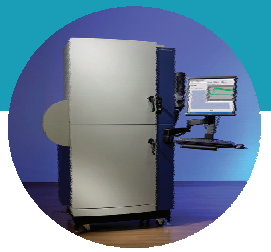


Abstract

Ion channels are involved in a variety of cellular processes. Dysfunction of ion channels is associated with cardiovascular disease, CNS disorders, and diabetes making them important targets for drug discovery. The gold standard for measuring ion channel activity has been patch-clamp recording. However, this technique requires highly skilled operators, and its very low throughput is a limitation for high-throughput screening (HTS). While automated patch clamping technology such as Molecular Devices IonWorks™HT has set a standard in medium throughput screening, fluorescence-based membrane potential assays provide a excellent option for primary ion channel HTS.

The FLIPR^{TETRA} system delivers an entirely new screening platform to address real-time kinetic cell-based assays. Excitation wavelengths have been expanded beyond standard calcium fluorescence, a separate excitation source for membrane potential fluorescence assays. In addition, FLIPR^{TETRA} broadens the FLIPR® system's capabilities to include simultaneous 536 liquid transfer using Molecular Devices' novel elastomeric technology. 1536 contact liquid transfer occurs with the same height, speed and volume flexibility offered in traditional FLIPR 96 and 384 pipettors. Here, we developed 384- and 1536-well protocols using the Membrane Potential Blue and Red assay Kits on FLIPR^{TETRA}. Results are compared to assess potential performance differences.

Introduction

Fluorescence based membrane potential assay is favored for primary screens for its sensitivity, versatility and high throughput capability. FLIPR Membrane Potential Assay Kit (Molecular Devices Corp.) has been shown to outperform the conventional DiBAC$_4$(3) assay that uses bis-oxonol fluorescent dye .[1,2] The FLIPR reagent kit offers fast kinetics with an easy mix-and-read assay format; eliminating the cumbersome preparation and temperature sensitivity issues associated with DiBAC. However, as has been previously shown, interaction between a test compound and membrane potential dyes may cause significant change in fluorescence response and lead to altered estimation of compound activity.[3] To minimize potential compound-dye interferences, Molecular Devices offers two Membrane Potential Kits with different formulations. This, in conjunction with FLIPR^{TETRA} technology broadens the range of targets that can be successfully screened using membrane potential as a primary method.

FLIPR^{TETRA} System Features

FLIPR^{TETRA} utilizes proprietary LED modules with distinct ranges to excite cellular fluorescence. Filtered light is captured using a CCD camera and displayed in real time in the software. LEDs assisted in reducing instrument footprint and facility requirements. In addition, 1536 simultaneous liquid transfer has been added to increase throughput while reducing screening costs. These features are controlled by the drag-and-drop user software which displays data in real-time kinetics.

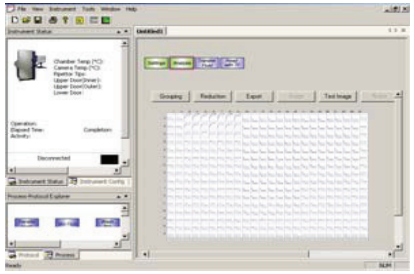


Figure 1. FLIPR^{TETRA} data screen

Materials and Methods

Cells used were WT3 CHO-M1 Cell line, Cat# CRL-1985, American Type Culture Collection, Manassas, VA A CHO-K1 cell line transfected with the rat muscarinic M1 acetylcholine receptor.

Cells were seeded 1:30 2x per week in Ham's F12 Media (Cat# 9058, Irvine Scientific, Santa Ana, CA), 10% Fetal Bovine Serum (Cat# SH30071.03, Hyclone, Logan, UT), 1% Pen/Strep/Glutamine (Cat# 10378-016, Invitrogen, Carlsbad, CA), 50ug/ml Geneticin (Cat#10131-035, Invitrogen). Cells were washed with PBS w/o Calcium and Magnesium (Cat#14190-144, Invitrogen) and incubated 5 minutes at 37°C with Versene 1:5000 (Cat# 9314, Invitrogen).

Assay plate preparation:

To create 384 well assay plates, cells were re-suspended in culture media and plated at 12,500 cells/well in Costar black-wall clear-bottom 384-well plates (Cat# 3702, Costar, Corning, NY). Plates were incubated overnight at 37°C in 5%CO_2.

For 1536-well assay plates, 2000 cells /well were plated in Greiner low base black-wall clear-bottom 1536-well plates (Cat# EK-16092, E&K Scientific, Campbell, CA). Plates were incubated overnight at 37°C in 5%CO_2.

Wash Buffer:

Wash buffer was prepared fresh daily. 10X HBSS (Cat#1406-5056, Invitrogen) was diluted in sterile water for injection (Cat# 9309, Irvine Scientific), and 20mM HEPES (Cat# 15630-080, Invitrogen) was added. pH was adjusted to 7.4.

384-well FLIPR Membrane Potential red and blue kit protocol:

Dye loading buffer for 10 plates was prepared by completely dissolving contents of one bulk-kit reagent vial with 100ml wash buffer. Cell plates were removed from the incubator, then 25ul dye loading buffer added directly to each well without washing. Dye-loaded plates were incubated for 30 minutes at room temperature.

1536-well FLIPR Membrane Potential red and blue kit protocol:

Dye loading buffer for 10 plates was prepared by completely dissolving contents of one bulk-kit reagent vial with 67ml wash buffer. Cell plates were removed from the incubator, then 2ul dye loading buffer added directly to each well without washing using an AquaMax® DW4. Dye-loaded plates were incubated for 30 minutes at room temperature.

Membrane potential assay:

For 384-well assays, a 5x dose response and for 1536-well assays, a 7x dose response of Carbachol and KCl agonists were prepared in 384 well polypropylene plates. Instrument set up parameters for both plate configurations are shown in Figure 2 below. Plates were read 30 minutes after incubation at room temperature.

FLIPR^{TETRA} Setup Parameters for Membrane Potential

Parameter	384 well	1536 well
Excitation	510-545 LED Module	510-545 LED Module
LED Intensity	50-80%	50-80%
Filter	565-625nm	565-625nm
Camera gain	30-50	30-50
Camera F stop	N/A	N/A
Exposure	0.4 Sec	0.4 Sec
Interval	1 Sec	1 Sec
Pipetting volume	12.5 ul	1 ul
Height	40 ul	2ul
Speed	25ul/sec	5ul/sec
Tip up speed	5mm/sec	2mm/sec
Tips in well	Yes	Yes

Figure 2. Instrument Setup parameters

FLIPR^{TETRA} and Membrane Potential Blue and Red Kits: CHO-M1 Membrane Depolarization by KCl in 384 and 1536 Well Format

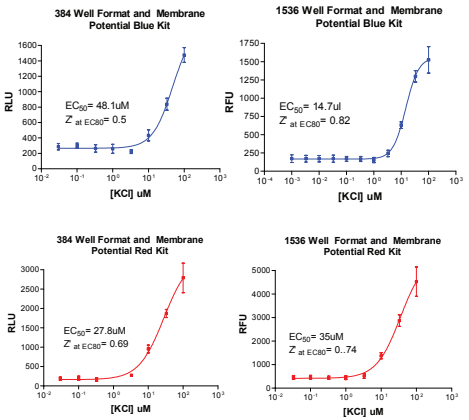


Figure 3. Membrane Depolarization by KCl

FLIPR^{TETRA} and Membrane Potential Blue and Red Kits: CHO-M1 Membrane Hyperpolarization by Carbachol in 384 and 1536 Well Format

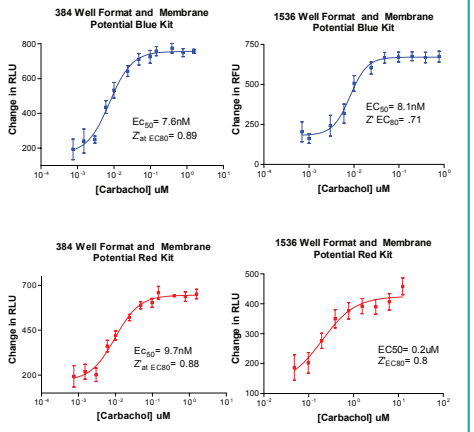
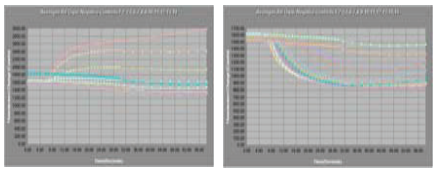


Figure 4. Membrane Hyperpolarization by Carbachol

Discussion



a. **b.**

Figure 5. (a.) FLIPR^{TETRA} trace of MP Blue 384-well depolarization of CHO-M1 cell membrane with KCl. (b.) FLIPR^{TETRA} trace of MP Blue 1536-well hyperpolarization of CHO-M1 cell membrane with carbachol

Data analysis: Graphs were generated from peak fluorescence values minus the minimum fluorescence. EC_{50} values were determined by sigmoidal dose response curve calculation performed in Graph Pad Prism (San Diego, CA). Z' values were determined at EC_{80} concentration[4].

Molecular Devices' Membrane Potential Red and Blue Assay Kits were used with a membrane potential depolarizing compound and a membrane potential hyperpolarizing compound to compare results between 384-well and 1536-well assays on FLIPR^{TETRA}. Depolarization with KCl gave an increase in signal (Figure 5a). Comparison of graphs between 384-well and 1536-well assays with Red and Blue kits shows values between 14uM and 47uM. Z' factors at EC_{80} were all above 0.5 (Figure 3). Hyperpolarization with carbachol showed a decrease in signal (Figure 5b). Comparison graphs between 384-well and 1536-well assays with Red and Blue kits shows values between 7nM and 200nM. Again Z' factors at EC_{80} were all above 0.71 (Figure 4). In this case, the both the Red and Blue performance was equivalent.

Conclusions

Availability of two Membrane Potential kits of different formulations broadens applications and increases data quality in HTS primary screening efforts for ion channel targets. Membrane potential assays read on FLIPR^{TETRA} using both kit formulations deliver equivalent results in both 384-well and 1536-well format.

References

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