

Ion Channel Assay Development using Voltage Sensor Probes (VSP) Technology on BMG LABTECH POLARstar OPTIMA and NOVOstar Instruments

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Introduction

Ion channels are important drug targets because of their critical role in nerve, cardiac, endocrine and skeletal muscle tissues. The lack of sufficiently sensitive screening systems has hampered research in this area. This application note focuses on Voltage Sensor Probes (VSP), a Fluorescence Resonance Energy Transfer (FRET)-based voltage-sensing assay technology from Invitrogen. This technology enables detection and measurement of rapid changes in membrane voltage and quickly reports them as fluorescence signals from living cells. This technology can be used with any target that changes membrane potential, and is therefore well suited for sodium, potassium, calcium, chloride and ligand-gated ion channel research. The ratiometric method used to detect and quantify changes in cellular membrane potential significantly reduces errors arising from well-to-well variations in cell number, dye loading and signal intensities, plate inconsistencies, and temperature fluctuations. These combined features make VSP technology highly amenable for high-throughput screening (HTS) applications.

Assay development and therapeutic groups develop and validate ion channel assays prior to HTS and may have lower throughput instrumentation that may or may not be amenable to VSP technology. This application note demonstrates the use of BMG LABTECH's POLARstar OPTIMA and NOVOstar instruments as suitable platforms for development of ion channel assays using VSP technology.

Assay Principle

Voltage Sensor Probes (VSP) is a Fluorescence Resonance Energy Transfer (FRET)-based assay technology used for high-throughput ion channel drug discovery. The FRET donor is a membrane-bound, coumarin-phospholipid (CC2-DMPE), which binds only to the exterior of the cell membrane. The FRET acceptor is a mobile, negatively charged, hydrophobic oxonol [either DiSBAC₂(3) or DiSBAC₄(3)], which binds to either side of the plasma membrane in response to changes in membrane potential (Figure 1)

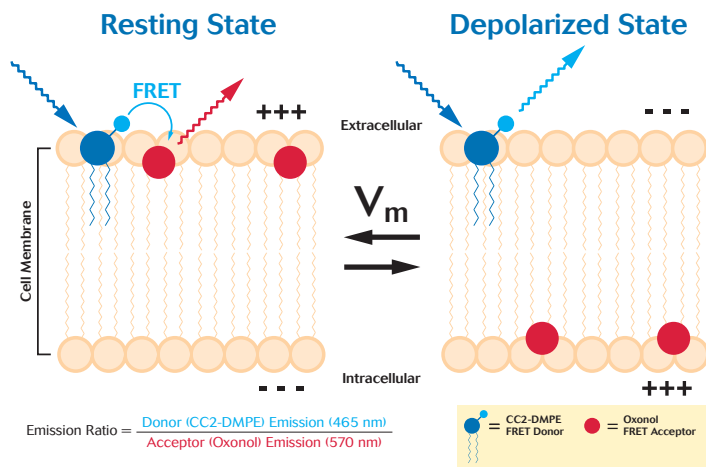


Figure 1. Schematic illustration of the mechanism of voltage-dependent, FRET-based VSP technology. Resting cells have a relatively negative potential, so the two probes associate with the exterior of the cell membrane, resulting in efficient FRET. Exciting the CC2-DMPE donor probe (at 400 nm) generates a strong red fluorescence signal (at 580 nm) from the oxonol acceptor probe. When the membrane potential becomes more positive, as occurs with cell depolarization, the oxonol probe rapidly translocates (on a subsecond time scale) to the other face of the membrane. Thus, each oxonol probe "senses" and responds to voltage changes in the cell. This translocation separates the FRET pair; therefore excitation at 400 nm now generates a strong blue fluorescence signal at 460 nm from the CC2-DMPE probe.

In this application note, we demonstrate the compatibility of two BMG LABTECH instruments for studying or screening ion channels using VSP technology. The ion channel model we have used is endogenously expressed inward rectifying potassium (Kir) channels present in rat basophilic leukemia cells. High potassium concentration in the culture medium causes a change in the membrane potential due to action of the Kir channels. This membrane potential change can be modulated by the addition of barium chloride (BaCl₂). This Kir channel model demonstrates the utility of VSPs on BMG LABTECH instruments.

Materials and Methods

Cell Culture RBL-2H3 (Rat Basophilic Leukemia, ATCC #CRL2256) cells were plated at 50,000 cells/well in Corning® 3603 96-well plates 18-24 hours prior to the experimental procedure.

Preparation of VSP Loading Buffers

5 μM CC2-DMPE Loading Buffer: 10 μl of 5 mM CC2-DMPE (Invitrogen Cat. no. K1016) and 10 μl of 100 mg/ml Pluronic® F-127 (Sigma) were premixed in a 15 ml tube. 10 ml of VSP Solution 1 (160 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4) was added and vortexed vigorously to mix. The solution was protected from light until use.

10 μM DiSBAC₂(3) Loading Buffer: 8.3 μl of 12 mM DiSBAC₂(3) (Invitrogen Cat. no. K1016) and 12.5 μl of 200 mM VABSC-1 (Invitrogen Cat. no. K1019) were premixed in a 15 ml tube. 10 ml of VSP Solution 1 was added and vigorously vortexed to mix. The solution was protected from light until use.

Loading Cells Media was removed from all wells and washed with 100 μl VSP Solution 1 (VSP-1). 100 μl CC2-DMPE Loading Buffer was added and incubated at room temperature for 30 minutes, covered and protected from light.

After 30 minutes, the CC2-DMPE Loading Buffer was removed and the plates washed once with 100 μl VSP-1. The VSP-1 was immediately replaced with 100 μl DiSBAC₂(3) Loading Buffer.

Experiments using the POLARstar OPTIMA instrument: BaCl₂ (Sigma) dilutions were added and incubated with the DiSBAC₂(3) Loading Buffer at room temperature for 30 minutes, covered and protected from light.

Experiments using the NOVostar instrument: BaCl₂ dilutions were added after the 30 minute DiSBAC₂(3) incubation and immediately prior to the depolarizing stimulant, High K⁺ buffer (164.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4).

Following the 30 minute incubation with the DiSBAC₂(3) Loading Buffer, the plates were analyzed with either the POLARstar OPTIMA or NOVostar instrument. Both instruments follow the same protocol of reading the plate with two "simultaneous" emissions at 460 nm and 580 nm wavelengths, before K⁺ addition, and continuing after K⁺ addition.

Data was analyzed by comparing the baseline subtracted ratio of donor (460 nm) to acceptor (580 nm) before (Emission Ratio_{Polarized}) and after (Emission Ratio_{Depolarized}) K⁺ addition. The final normalized assay ratio equals Emission Ratio_{Depolarized} / Emission Ratio_{Polarized}.

Results

POLARstar OPTIMA Instrument The POLARstar OPTIMA instrument is enabled to inject a solution (in our case, High K⁺) and collect data, once per second, at two wavelengths. The software allows real-time visual readout in both plate and well views, which is automatically exported to the BMG LABTECH evaluation software package for analysis. Experiments using the POLARstar OPTIMA demonstrate the compatibility of this instrument with VSP technology. Figure 2 shows typical data acquired using the Kir channel model. A useful feature of the instrument is the ability to view the data in real time using the current state window view. Also, because the POLARstar OPTIMA can rapidly switch filters, it is amenable to assay development for many ion channel targets.

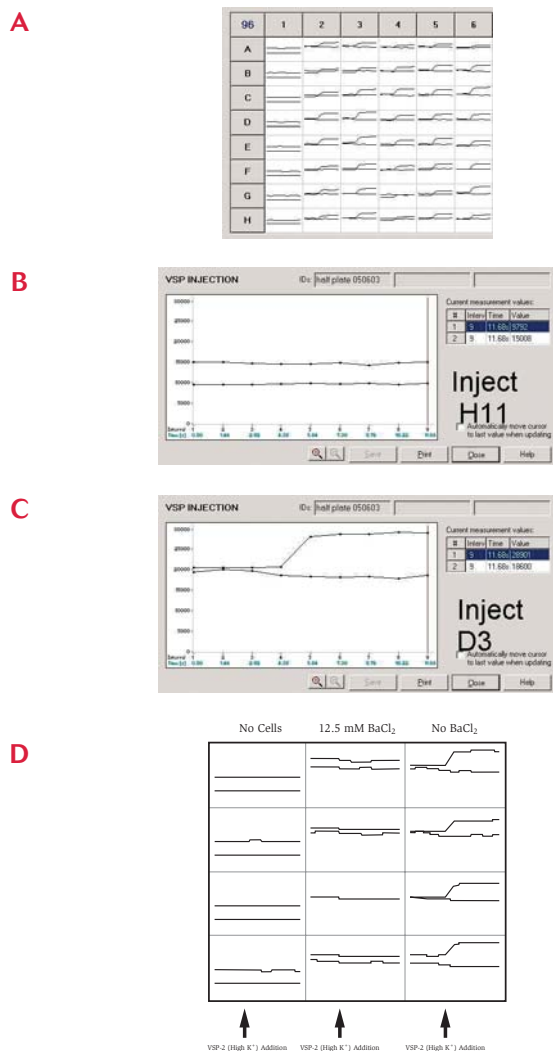
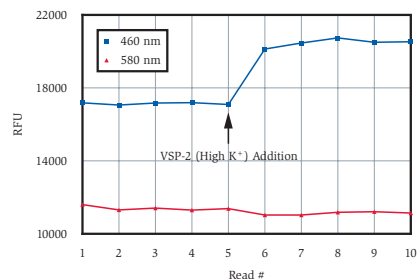


Figure 2. VSP Data on the BMG LABTECH POLARstar OPTIMA. Real-time readout of the VSPs on the POLARstar OPTIMA. A) Columns 2-6 depict a series of wells showing the increase in 460 nm emission following K⁺ addition. Column 1 is the no cells control. B) The current state window view of the no cells control shows no change in 460 nm upon high K⁺ addition. C) The current state window view of a well responding to K⁺ stimulation shows active depolarization (seen as an increase in 460 nm emission) upon injection of VSP-2. D) Enlarged views of the current state window from the BaCl₂ dose response experiments. Note the lack of increase of the 460 nm emission reading in both the no cell control well and the 12.5 mM BaCl₂ wells (compared to the untreated wells).

NOVOstar Instrument In addition to having all the POLARstar OPTIMA features, the NOVOstar instrument can be used to perform plate-to-plate transfers, important for addition of compounds for screening or titration. This instrument also has the ability to incubate cells at the various temperature and CO₂ conditions. In our experiments, the NOVOstar was programmed to use the autopipettor to add BaCl₂ dilutions directly from one 96-well plate to the assay plate containing the RBL cells. Next, the instrument injected the High K⁺ solution using one of its two additional injectors. Data was collected at two wavelengths once per second (Figure 3).

A—NOVOstar no barium chloride control



B—NOVOstar 12.5 mM barium chloride

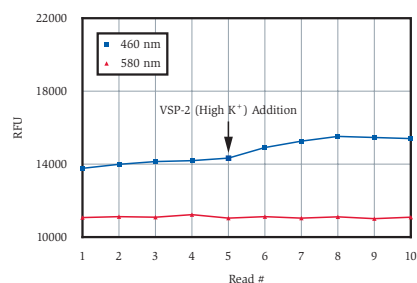


Figure 3. VSP Data on the BMG LABTECH NOVOstar. Effects of barium chloride on Kir channels using the VSPs on the NOVOstar. A) Results from a control assay well that did not receive BaCl₂. The 460 nm emission increased as expected upon K⁺ addition. B) Results from an assay well that received 12.5 mM BaCl₂. Only a very slight increase was seen in 460 nm emission due to the effect of BaCl₂ on the Kir channel.

Discussion

This application note demonstrates the compatibility of Voltage Sensor Probes (VSP) technology with the BMG LABTECH POLARstar OPTIMA and NOVOstar instruments. To utilize the full potential of VSP technology, any instrument must be able to measure two wavelengths in rapid succession. This specification takes advantage of the ratiometric FRET readout, which greatly reduces well-to-well variation common to many cell based assays.

Our model for ion channel assay development is endogenously expressed inward rectifying Kir channels present in rat basophilic leukemia cells. With both instruments we showed real-time readouts of the ion channel following K⁺ addition and blockage of the Kir channel with BaCl₂. The NOVOstar injectors were able to inject both the BaCl₂ inhibitor and the K⁺ stimulant.

The ability of both BMG LABTECH POLARstar OPTIMA and NOVOstar instruments to inject ion channel stimulant, switch filters at approximately 1 Hz, and continue to take readings during the kinetic event make these suitable instruments for therapeutic groups in developing ion channel assays for lower throughput applications and screening.



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Voltage Sensor Probes are covered by one or more of the following U.S. patents, as well as corresponding pending or issued foreign patents: 5,661,035; 6,107,066; 6,342,379; other U.S. patents pending.

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