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Application Note

Ion Channel Assay Development on the Synergy™HT Multi-Detection Microplate Reader from BioTek

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Introduction

Voltage Sensor Probes (VSP), a voltage-sensing Fluorescence Resonance Energy Transfer (FRET) assay technology from Invitrogen, provides a higher throughput assay format for ion channel drug discovery. VSPs can be used with any ion channel target that changes membrane potential, and is therefore well suited for sodium, potassium, calcium, chloride and ligand-gated ion channel research. The FRET-based detection method provides ratiometric results, which 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 areas may also utilize VSP technology on lower throughput instrumentation. In order to take full advantage of VSPs, a reader should be able to switch quickly between two emission filters in well kinetic mode while simultaneously injecting depolarizing stimuli. This Application Note demonstrates the use of BioTek's Synergy HT instrument as a suitable platform for development of VSP ion channel assays in both the pharmaceutical and academic environments. Further, we also show preliminary data demonstrating utility of the Synergy HT for ion channel assays using Fluo-4, the high-affinity calcium indicator, which is a more fluorescent version of Fluo-3.

Mechanism of the FRET-based VSPs

Voltage Sensor Probes (VSPs) are 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 $DiSBAC_2(3)$ or $DiSBAC_4(3)$], which will bind to either side of the plasma membrane in response to changes in membrane potential.

Fluorescence Resonance Energy Transfer (FRET) is the transfer of the excited-state energy from the initially excited donor to the acceptor. Typically the donor molecule emits light that overlaps the absorption or excitation spectrum of the acceptor molecule. The energy transfer takes place without the generation of a photon, with the rate of energy transfer being dependent on, among other things, the degree of overlap in spectrums and the proximity of the donor and acceptor. It is the distance dependence that is utilized with VSPs. Only when the acceptor $DISBAC_2(3)$ is located on the exterior of the cell membrane is FRET capable of taking place.

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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 ~590 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 (Figure 1). Thus, each oxonol probe "senses" and responds to voltage changes in the cell. This translocation separates the FRET pair, so exciting the CC2-DMPE donor probe now generates a strong blue fluorescence signal (at ~460 nm) from the CC2-DMPE probe. Depolarization of the cell, which causes $DISBAC_2(3)$ to relocate to the inner side of the membrane, would be expected to result in a decrease in FRET activity.

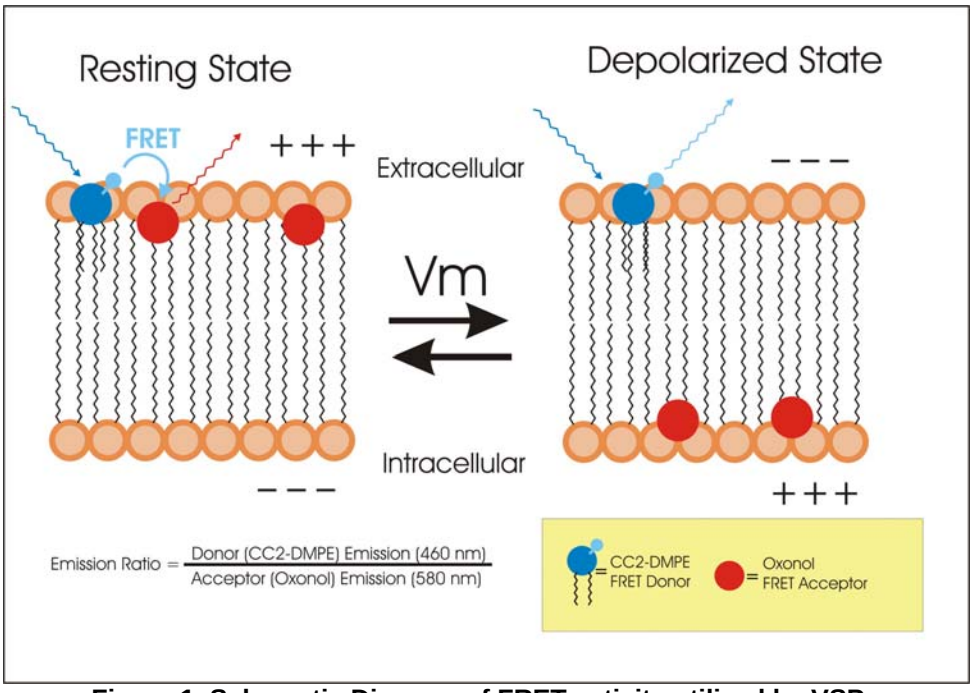

Figure 1. Schematic Diagram of FRET activity utilized by VSPs

The Synergy HT

The Synergy™ HT Multi Detection Reader (BioTek Instruments, Winooski, VT) is a robotic-compatible microplate reader that can measure absorbance, fluorescence, and luminescence in all plate formats up to 384-well plates (Figure 2). The Synergy HT utilizes a unique dual optics design that has both a monochromator/xenon flash system with a silicone diode detector for absorbance and a tungsten halogen lamp with blocking interference filters and a photomultiplier tube (PMT) detector for optimized fluorescence. The Xenon flash/monochromatic system can also be used for fluorescence measurements. Reagent addition is accomplished by an optional external injector module, which controls two independent injector syringes. Each syringe is connected to a separate injector tip that can be located adjacent to the top detector probe or above the bottom probe.

The absorbance optics are a single-channel system that has the capability of measuring absorbance from the UV to the near IR range. A xenon-flash lamp is used to illuminate a high-precision, diffraction-grating monochromator. The monochromator-based absorbance optics have a wavelength range of 200-999 nm, require no absorbance filters and can perform spectral scans of substances in increments as small as 1 nm.

Figure 2. The Synergy HT Multi-detection Microplate Reader

The fluorescence optics are capable of measuring conventional or time-resolved fluorescence. The reader is configured with both top and bottom probes in a variety of different sizes. The top probe adjusts up and down automatically via software, accommodating different plate heights. When conventional fluorescence mode is selected, the Synergy HT uses a tungsten-halogen lamp as a light source and band-pass filters in filter wheel cartridges to provide wavelength specificity for both excitation and emission wavelengths. When the reader is used in time-resolved mode, it automatically switches to a xenon-flash lamp light source with a monochromator to select wavelength. Control of reading parameters and data reduction is performed by KC4™ software running on an attached PC. Reading parameters include items such as top or bottom reading, PMT sensitivity, number of reads per well, collection time, and delay time.

Luminescence measurements are performed using the fluorescence optics path with the excitation lamp turned off and an excitation filter replaced with an opaque plug to ensure a dark reading chamber. Emitted luminescence is captured using either the top or bottom probes and the light measurements obtained using the Synergy HT's low-noise PMT operated in photon integration mode.

Materials and Methods

Cell Loading

RBL -2H3 (Rat Basophilic Leukemia, ATCC #CRL2256) cells were used for all assays. Cells were plated at 50,000 cells/well in Corning Costar 3603 96-well plates one day (18-24 hours) prior to experimental analysis. In all experiments, Column 1 of the microplate was the no-cells control.

Preparation of VSP Loading Buffers

Loading buffers were prepared from concentrated stock solutions. CC2-DMPE Loading Buffer (2.5 – 20 µM) was prepared by mixing the appropriate amount of 5 mM CC2-DMPE with an equal volume of 100 mg/mL Pluronic F-127. Next, 10 ml of VSP-1 solution (160 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) was then added and vortexed vigorously to mix.

$DiSBAC_2(3)$ Loading Buffer (2.5 – 10 µM) was prepared by mixing the appropriate amount of $DiSBAC_2(3)$ with 10 ml of VSP-1 solution and vigorously vortexed to mix. Both solutions were protected from light prior to use.

Loading Cells

Cells were sequentially loaded with the VSP Loading Buffers. Briefly, cell media were removed from all wells of the 96-well plates and replaced with 100µl VSP-1 The VSP-1 was immediately removed and replaced with 100 µl CC2-DMPE Loading Buffer and incubated at room temperature for 30 minutes, covered and protected from light. 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 $DiSBAC_2(3)$ Loading Buffer and incubated at room temperature for 30 minutes, covered and protected from light. The procedure is outlined in Figure 3.

Reading Parameters for VSP Assays

For VSP experiments, the Synergy HT reader was equipped with a 400/30 nm excitation filter and two emission filters, a 460/40 nm and a 590/35 nm. Using two filter sets, the Synergy was set up to rapidly switch between the two emission filters (Figure 4). The time to switch between filters was 140 milliseconds (ms). Measurements were made from the bottom with a PMT sensitivity setting of 65. The reader was programmed to read in kinetic well mode and to read each well starting at time 0 and ending at time 10090 ms. At 4000 ms, 100 µl of a High K^+ solution was injected to initiate depolarization (Figure 5).

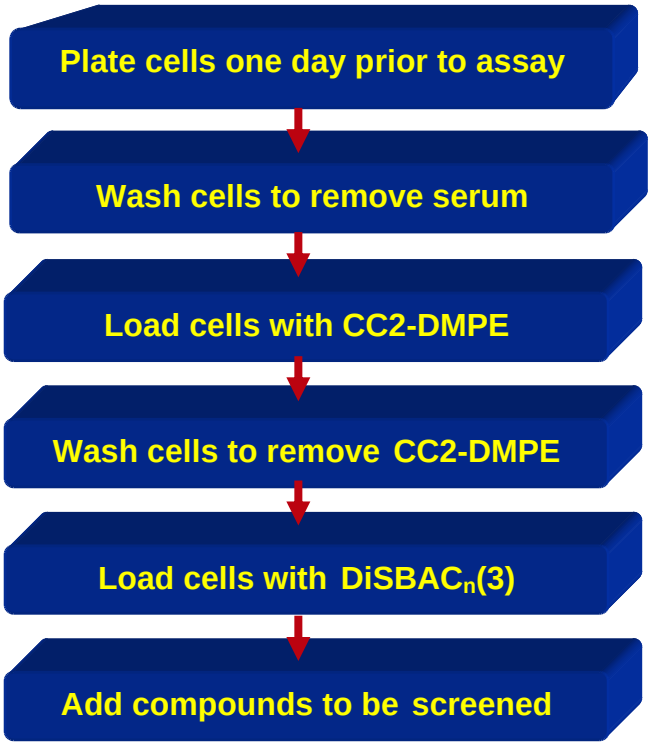

Figure 3. Outline of Cell Loading Procedure.

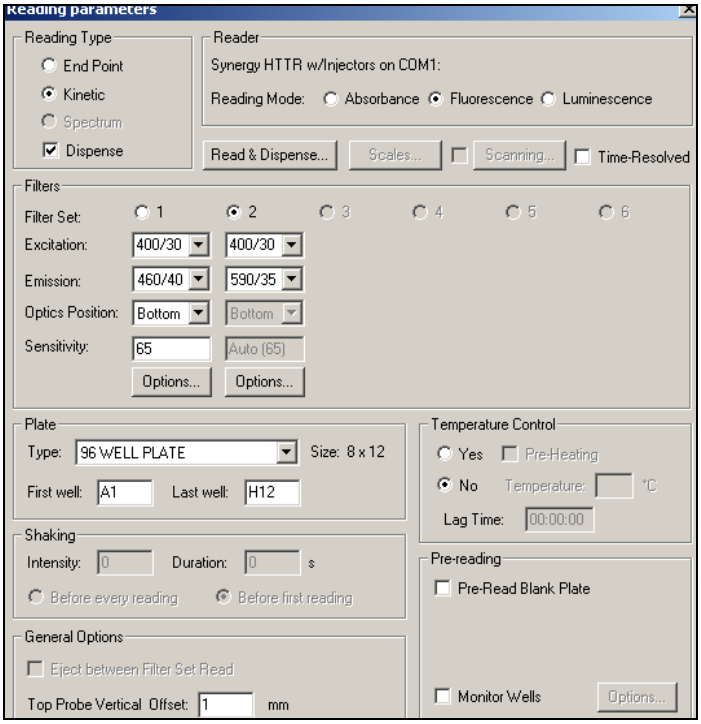

Figure 4. Screenshot from KC4 data reduction software depicting typical reading parameters for VSP assays. Parameters such as read type, filter pairs, probe selection, PMT sensitivity setting, plate type and wells to be read are selected using KC4 Data Reduction Software (BioTek Instruments, Winooski, VT). For VSP assays, plates were read kinetically from the bottom using a 400/30-excitation filter with both a 460/40- and a 590/35-emission filter.

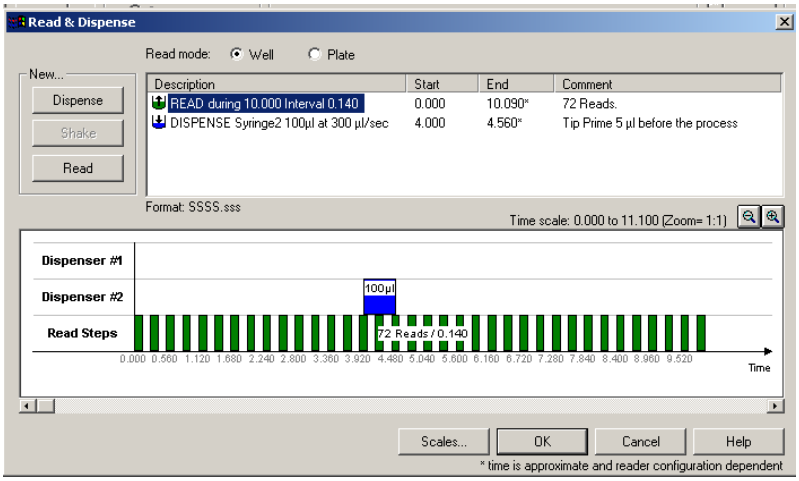

Figure 5. Screenshot from KC4 Depicting the kinetic reading and dispensing parameters used for a typical VSP assay. Several read and dispense parameters are available under the Dispense & Read Button. Read Mode, as well as reading parameters including time of initiation, duration and interval, can be selected or modified. Dispense parameters such as injection volume, rate and timing, are programmed as well

VSP Data Analysis

One of the advantages of the VSP technology is the ratiometric data analysis. Because the technology relies on FRET, a direct comparison can be made between 590-nm and 460-nm fluorescence. The data are collected in kinetic well mode, so a comparison can also be made between polarized and depolarized states. Figure 6 shows typical data obtained from the

Synergy HT. A template was set up on the Synergy HT to simplify the calculations explained below.

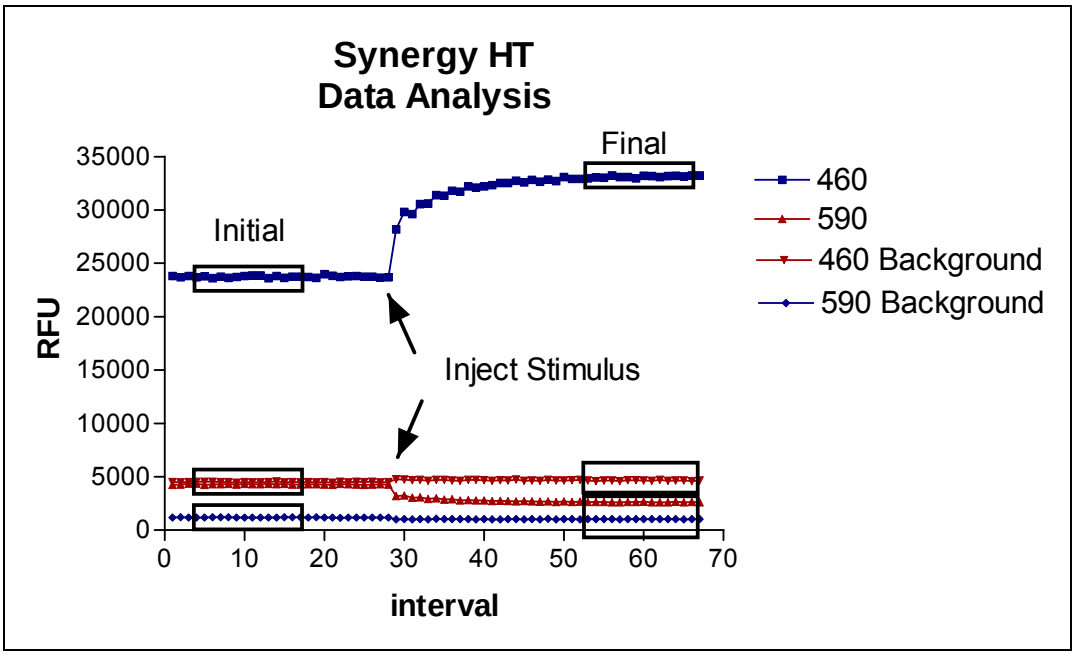

Figure 6. Typical VSP measurement with Data time windows indicated. Boxed regions depict reading time windows utilized for measurement calculations.

Using well-mode kinetics, fluorescence intensity measurements were obtained from cells in the polarized state using both sets of filter-pairs prior to the addition of depolarizing stimulant. The wells were monitored continuously until changes in fluorescence measurement intensity, as a result of the addition of depolarizing agent (depolarized state), reached a plateau. Identical data time-windows were then selected for both the initial and final 460 nm and 590 nm fluorescence intensity readings, and the average of the data points within each data window was determined to obtain: $460_{initial}$, $590_{initial}$, 460_{final} and 590_{final}. Background values were obtained by making identical measurements on control wells that did not contain cells. Background corrected (BLC) values were then calculated by subtracting the average background value from the corresponding average data window for $460_{initial}$, and $590_{initial}$, as well as, 460_{final} and 590_{final} emissions. Using the Baseline corrected data, the Emission $Ratio_{Polarized}$ is calculated from the ratio of the BLC $460_{initial}$ to the BLC $590_{initial}$ signals and the Emission $Ratio_{Depolarized}$ is the ratio of the BLC 460_{final} to the BLC 590_{final}. signals. The Determined Response Ratio is then expressed as the Emission $Ratio_{Depolarized}$ divided by the Emission $Ratio_{Polarized}$. (See Table 1.)

Emission Ratios:	
Eq. 1	Emission $Ratio_{Polarized}$ = BLC $460_{initial}$ / BLC $590_{initial}$
Eq. 2	Emission $Ratio_{Depolarized}$ = BLC 460_{final} / BLC 590_{final}
Response Ratio:	
Eq 3.	RR= Emission $Ratio_{Depolarized}$ / Emission $Ratio_{Polarized}$

Table 1. Basic Calculations Necessary to Determine Response Ratio.

Results

VSP Optimization

For each new assay developed, it is recommended to determine the optimal VSP concentrations to use. Most assays will require between 5 and 20 µM CC2-DMPE and 1.0 and 16 µM $DiSBAC_2(3)$. For these experiments, a VSP matrix was initially set up using the culture and loading conditions described above. CC2-DMPE concentrations varied across plate columns and $DiSBAC_2(3)$ down rows as shown in Figure 7.

		20 µm CC2-DMPE			10 µm CC2-DMPE			5.0 µm CC2-DMPE			2.5 µm CC2-DMPE		
		1	2	3	4	5	6	7	8	9	10	11	12
10 µM $DiSBAC_2(3)$	A												
	B												
	C												
5.0 µM $DiSBAC_2(3)$	D												
	E												
	F												
2.5 µM $DiSBAC_2(3)$	G												
	H												

Figure 7. Typical plate map of a VSP optimization experiment. Optimal results are indicated by shaded areas.

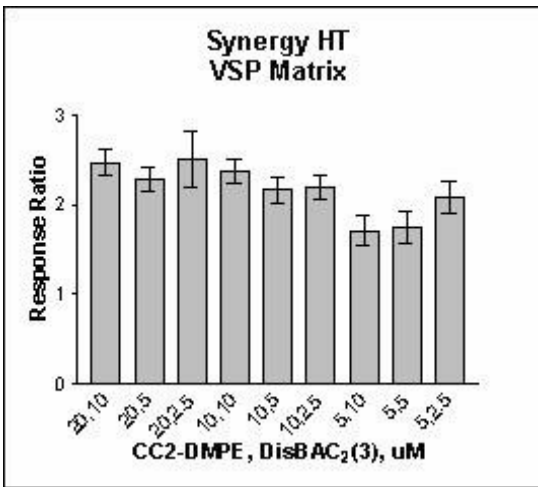

Figure 8. Graphical representation of Response Ratio from VSP matrix.

Only data for 20 µM and 10 µM CC2-DMPE are utilized in further experiments since standard deviations became higher at lower concentrations of CC2-DMPE. Note that lower concentrations of DiSBAC2(3) do not affect the Response Ratio as greatly. However, it is best to avoid the lower end of DiSBAC2(3) concentrations since assay sensitivity may become an issue. The data depicted in Figure 8 suggest that there are a few conditions that may work equally well. However, the two conditions that appear to be most consistent are 20 µM CC2-DMPE, 5 µM DiSBAC2(3) and 10 µM CC2-DMPE, 5 µM DiSBAC2(3). For subsequent experiments, the latter was chosen so as not to be near the saturation point for CC2-DMPE loading.

Barium Chloride Dose Response

Barium Chloride (BaCl2) is known to block inward rectifying potassium (Kir) channels, which are endogenously expressed at high levels in RBL cells. As demonstrated in Figure 9, 100 mM BaCl2 consistently blocks the depolarization response to KCl injection. Untreated (no BaCl2) cells consistently have a response ratio between 2 to 3, while treated (100 mM BaCl2) cells have a response ratio very near 1.0, which indicates little or no change.

Z' Factor Determination

Z' Factor is a statistical function used commonly to judge high-throughput screening assay robustness (Zhang, J. et al. (1999) *J. of Biomol. Screen.* 4(2): 67-73). Z' factors greater than 0.50 are indicative of an excellent assay. For cell-based assays, it is common to use a Z' factor greater than 0.4 as this indicator. The formula for Z' factor takes into consideration standard deviation as well as the difference in the means of the high and low values of an assay. It is represented by the following formula:

Eq. 4

$$1-\left(\left(3*\sigma_{exp}+3*\sigma_{blank}\right)/\left(\bar{X}_{exp}-\bar{X}_{blank}\right)\right)$$

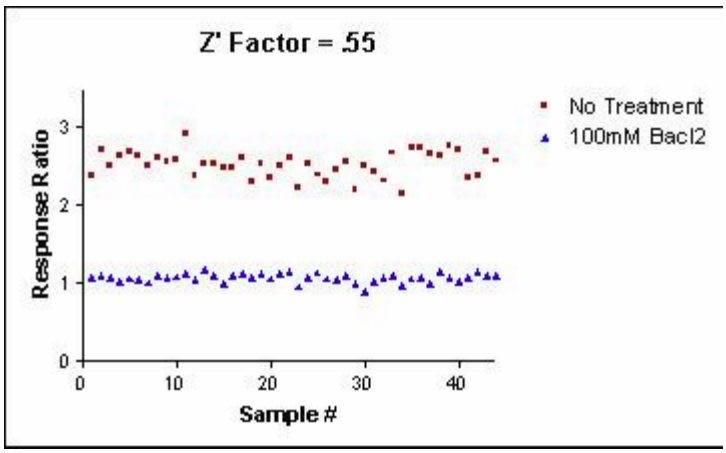

Figure 9. Response Ratio of 44 Different samples to 100 mM Barium Chloride Treatment. The response ratio was calculated for 44 different samples with or without 100 mM BaCl2 treatment. The Z' factor was then calculated for the data.

To demonstrate functionality of VSPs in this model, RBL cells were loaded with VSPs and treated with different concentrations of BaCl2, in replicates of 4 per concentration. Figure 10 demonstrates the dose response of increasing concentrations of barium chloride in regard to inhibiting depolarization. In addition, day-to-day dose responses were found to be consistent using VSP conditions of 10μM CC2-DMPE and 5μM DiSBAC2(3).

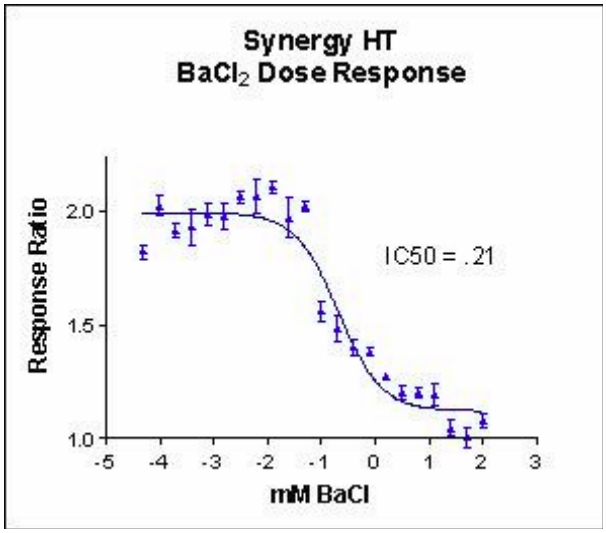

Figure 10. Barium Chloride dose response measured on the Synergy HT. Dose responses were measured using VSP conditions of 10 µM CC2-DMPE and 5 µM DiSBAC2(3) and the IC50 calculated. Graphing and IC50 calculations were accomplished with GraphPad Prism.

Fluo 4 Assay Development To assess the versatility of the Synergy HT, preliminary studies were performed to see if Fluo 4 (Molecular Probes # F23917) calcium assays could be quickly developed. Fluo-4 is an analog of fluo-3 with the two chlorine substituents replaced by fluorine. This structural modification results in increased fluorescence excitation at 488 nm and consequently higher signal levels for confocal microscopy, flow cytometry and microplate screening applications. RBL cells (50,000 cells/well, 96-well plates) were plated overnight. The following day, media were removed and replaced with 100 µl 3.0 µM Fluo 4 in Hanks Balanced Salt Solution (HBSS) + 25 mM HEPES. Cells were allowed to load for 60 minutes at room temperature. The Synergy HT was configured with a 485/25 nm excitation filter and a 516/20 nm emission filter. Well kinetic mode was used to take readings for 40 seconds with an injection of either 100 µl 2 µM Ionomycin (Sigma # I-0634) in HBSS or HBSS alone at +/- 4 seconds. Ionomycin induces the release of calcium from the endoplasmic reticulum (ER) that can be detected by Fluo 4 present in the loaded cells. KC4 Data Reduction Software was used to control reader function.

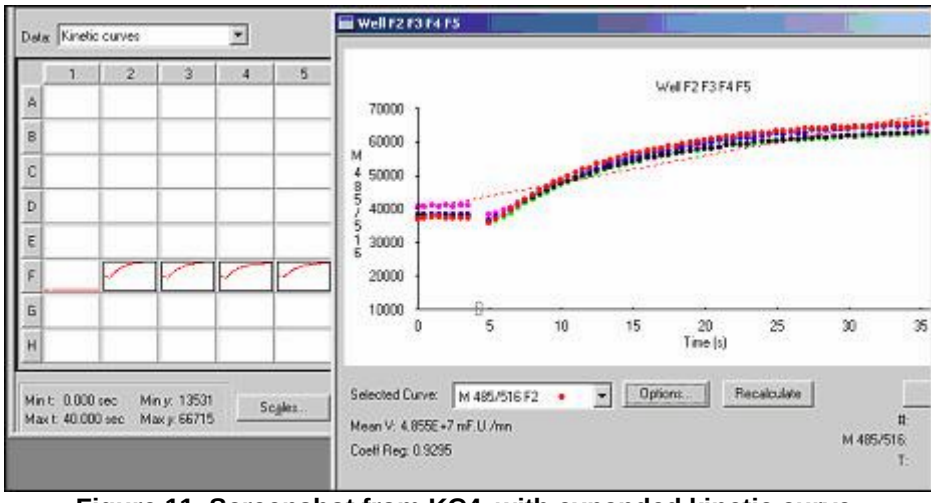

Figure 11. Screenshot from KC4, with expanded kinetic curve.

KC4 visually records kinetic data in real time. In addition, up to four wells can be viewed simultaneously in an expanded window. As demonstrated in Figure 11, when ionomycin is added to cells, a dramatic increase in fluorescence is seen in these wells. As demonstrated in Figure 12, while the fluorescence in cells that received only HBSS increased, cells that received Ionomycin returned a much more rapid and greater increase than the HBSS-only wells.

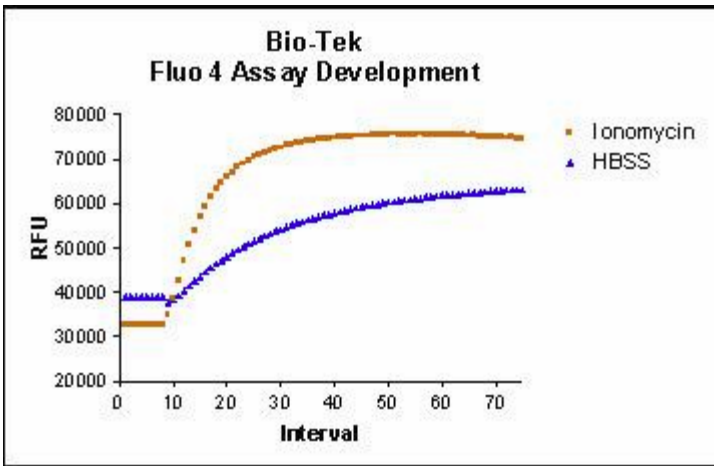

Figure 12. Representative data from a Fluo 4 development assay. The Synergy HT collected 80 data points, once every 0.5 seconds, during a 40-second kinetic read.

Discussion

These data indicate that the Synergy HT reader has the capability to perform VSP assays. The Synergy HT can inject depolarizing stimuli, as well as quickly switch filters, allowing accurate ratiometric analysis to be performed. The ability to assess cell depolarization response using ratiometric analysis eliminates many of the problems normally associated with other fluorescent ion channel technologies. Because the Synergy HT is capable of using 96-well microplates, variable condition matrices can be performed in one experiment. Besides saving time and reagents, the ability to assess all drug concentrations at one time eliminates the experiment-to-experiment variation associated with multiple experiments. The Synergy HT platform has also been demonstrated to be stable on a day-to-day basis, as well as consistent to other HTS technologies, such as the Voltage/Ion Probe reader (VIPR®) from Aurora Discovery (San Diego, CA). The data presented using the calcium indicator Fluo-4 demonstrate that the Synergy HT with Injector can be used for calcium flux assays. In these assays, the actual increase in fluorescence of one wavelength's emission was monitored rather than a change in the ratio of two different wavelengths.

Summary

- The Synergy HT's ability to quickly switch emission filters (140 milliseconds) and inject depolarizing stimuli allow it to use one of Invitrogen's Voltage Sensor Probes unique advantages over other fluorescent ion channel technologies.
- One 96-well VSP matrix assay allows determination of optimal loading conditions.
- Barium Chloride IC50s are consistent from day to day on the Synergy HT and compare to values obtained from HTS instrumentation such as the VIPR® from Aurora Discovery. (VIPR® data not shown).
- Voltage Sensor Probe assays can be developed on the Synergy HT and transferred to a high-throughput instrument like the VIPR®
- The Synergy HT's versatility allows it to be used to develop other cell-based assays such as Fluo 4 calcium assays.