Introduction
Ligand gated ion channels (LGICs) are a large family of membrane-embedded proteins that enable the passage of ions across membranes, in response to the binding of ligands such as neurotransmitters. LGICs represent a class of highly attractive drug targets because of the pivotal role they play in many physiological functions, and their association with multiple human diseases. Among existing ion channel screening technologies, the fluorescent probes, especially the calcium sensing dye, have become indispensable tools for analyzing ion channel activities. The simplicity of the assay, the high throughput, as well as the low turning cost, makes this method especially attractive to large scale primary screening in the early phase of the drug discovery process. In an attempt to further improve such assay quality we evaluated the use of a novel calcium-sensing dye in LGIC assays. The unique molecular configuration of this dye, combined with a novel quenching technology, provides distinctive advantages over existing calcium probes by (1) providing a larger signal-to-noise ratio, and (2) removing the need of probenecid in the assay. Specifically, in this study we examined two calcium permeable ligand gated ion channels: acid sensing ion channel 1a (ASIC1a) and transient receptor potential vanilloid 1 (TRPV1).

Methods
Cells:
ChanTest™ ASIC1a-Glo cells (Cat # CT6012) are a stably transfected cell line with a human ASIC1a channel constitutively expressed in the CHO cell line.
ChanTest™ tTRPV1-HK293 (Cat # CT6105) are a stably transfected cell line with a human TRPV1 channel constitutively expressed in the HEK 293 cell line.
Both cell lines have been validated by ChanTest for use in manual patch-clamp recording, the PatchPlex® 7000A and IonWorks Barracuda Automated Patch Clamp Systems, and in conjunction with FLIPR Calcium Kits on the FLIPR® Tetra System.

Calcium assay on the FLIPR Tetra Instrument:
For the assay on the FLIPR Tetra Instrument, cells were washed with Hank’s Balanced Salt Solution, treated with trypsin and re-suspended in media without selection antibiotics. Cells were plated at a density of 30,000 cells/well into poly-L-lysine coated 384-well plates and incubated for 18-24 hours in a humidified 5% CO2, 37°C cell culture incubator.

Data reading: For TRPV1 Cells, culture medium was removed and replaced with 20 µL well FLIPR Calcium 6 dye (Molecular Devices) in HB-PS Buffer. Plates were incubated at 37°C for 120 minutes. ASIC1a cells were incubated in dye DPHB at 37°C for 120 minutes. Pre-incubation: For TRPV1 cells, a 5X volume of CaCl2 or ECGC ligand was pre-incubated in HB-PS buffer + 20 mM HEPES in 384-well polypropylene plates. Ligand was added during detection on the FLIPR Tetra Instrument at optimized parameters. Capsaicin was prepared at 5X concentration and added 15 minutes prior to addition of a 6X volume of EC50 concentration of challenge ligand. For ASIC1a Cells, buffer 1.5X Log pH 7 added to cells + dye. ASIC blockers were incubated for 15 min prior of addition of pH 5 buffer.

Data reduction: Relative Fluorescence Units (RFU) were calculated as the fluorescence minus the dye signal minus the baseline signal minus the noise signal. Data points were calculated using GraphPad Prism software.

Electrophysiology assay on the IonWorks Barracuda system:
The IonWorks Barracuda System is a fully automated patch clamp system with 384 parallel recording sites, for continuous recording of either voltage- or ligand-gated ion channels. External buffer was first added into the 384-well patch plate, before cells were re-suspended and dispensed into each well. Two seal tests were performed through the application of a 10 mV step, followed by the addition of amphotericin B (100 µg per mL internal solution) as perfusing agent to gain access into the cells. Once whole-cell access was achieved, a third seal test was performed before compound(s) were added to the wells, and the recording of the current started. The holding/recording potential was applied continuously at -70 mV for both channel types. All recordings were performed at room temperature.

Results
Ligand gated ion channels with calcium sensitive dye
ASIC1a Channel

![Figure 1. Fluorescent measurement of intracellular calcium triggered by activation of ASIC 1a channel at different pH levels.](image1)

![Figure 2. Fluorescent measurement of intracellular calcium triggered by activation of ASIC 1a at pH 5.0; in the presence of incremental concentrations of ASIC channel blockers.](image2)

![Figure 3. Concentration-dependent activation of TRPV1 channel by capsaicin. Data are Mean ± SD (n=6).](image3)

![Figure 4. Concentration-dependent inhibition of TRPV1 channel by capsaicine. Data are Mean ± SD (n=6).](image4)

![Figure 5. Pharmacological analysis of ASIC1a inhibitors using the IonWorks Barracuda system. A) concentration-dependent inhibition of ASIC currents by amiloride and benzamil. Data collected from representative experiments (n = 27-32 for each data point); B) representative ASIC1a currents recorded in the presence and absence of 50 µM amiloride; C) plate view of ASIC1a currents obtained on the IonWorks Barracuda system, in response to incremental concentrations of amiloride.](image5)

![Figure 6. Concentration-dependent activation of TRPV1 Channels by capsaicin. A representative TRPV1 current traces elicited with 2-second application of 1 mM capsaicin. The dose-response curve of TRPV1 activation with capsaicin. Data presented as Mean ± SD, N=4.](image6)

![Figure 7. Concentration-dependent inhibition of TRPV1 channel by capsaicine and Ruthenium Red. A representative TRPV1 current traces elicited with 2-second application of 1 mM capsaicine in the presence of capsaicine (0.01 – 30 mM). Capsaicine was added simultaneously with capsaicine (B), dose-response curve of TRPV1 inhibition with capsaicine, data presented as Mean ± SD, N=4, C, dose-response curve of TRPV1 inhibition with ruthenium red (ruthenium red was added simultaneously with capsaicine; data presented as Mean ± SD, N=4.](image7)

Discussion and conclusions
1. We have validated the utility of a novel calcium sensing dye for sensitive, high-throughput, fluorometric assays of two ligand-gated ion channels.
2. The internalization of this novel dye is selective for calcium ions. Under the experimental conditions described in this study, the dye itself does not alter the biophysical properties of the channels.
3. The fluorometric assays measure the change of global calcium intracellularly, in response to the activation of ion channels; whereas the electrophysiological assays measure primary signal of ionic movement through the channels. The molecular differences between these two methodology could explain the difference of pharmacology presented in this study.

References