FLUXION

APPLICATION NOTE

Transiently Transfected Cell Lines for GABA Receptor Screening using the IonFlux System

Introduction

$GABA_A$ receptors are ligand-gated ion channels found in the synapses of neurons that conduct chloride ions across the neuronal cell membrane. The receptor contains two binding sites for γ-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the central nervous system. Upon activation, the $GABA_A$ receptor selectively conducts Cl- through its pore. This results in a hyperpolarization of the neuron which has an inhibitory effect on neurotransmission by diminishing the chance of a successful action potential occurring and thereby decreasing the excitability of the neuron. There are at least nineteen different individual $GABA_A$ receptor subunits that assemble the pentameric structure in different individual combinations to form the native receptor (α1-6, β1-3, γ1-3, δ, ρ1-3, and minor subunits) [1]. Of these potential combinations, the receptors containing two of the α1-6 subunits, two of any β subunits and one of the γ2 subunit are the most prevalent in the brain. These receptors subtypes mediate the diverse effects of benzodiazepine modulation.

Here we present the establishment of an automated patch-clamp assay using transiently transfected mammalian cells (HEK293T) with the IonFlux instrument (Fluxion Biosciences). The assay was validated by measuring the electrophysiological response of benzodiazepine-based compound HZ166. The use of transiently transfected cells allows for maximum flexibility for the assembly of different $GABA_A$ receptor types for screening purposes and the robustness of the developed assay makes it well suited for the high-throughput screening using the IonFlux HT system. Transient transfection approaches will also enable the mutant screening for other ion channel proteins.

Figure 1. The IonFlux system utilizes a "plate reader" format to simplify workflow and increase throughput. The instrument operates on microfluidic channels with fluidic exchange rate on 100ms timescale and simultaneous patch clamp recordings. Twenty cells per ensemble under voltage clamp are exposed to a compound within a short time scale in parallel across a 96 or 384 well plate. Throughputs of 10,000 data points per day can be achieved. It integrates a temperature control module which enables users to perform temperature dependent electrophysiology assay screening.

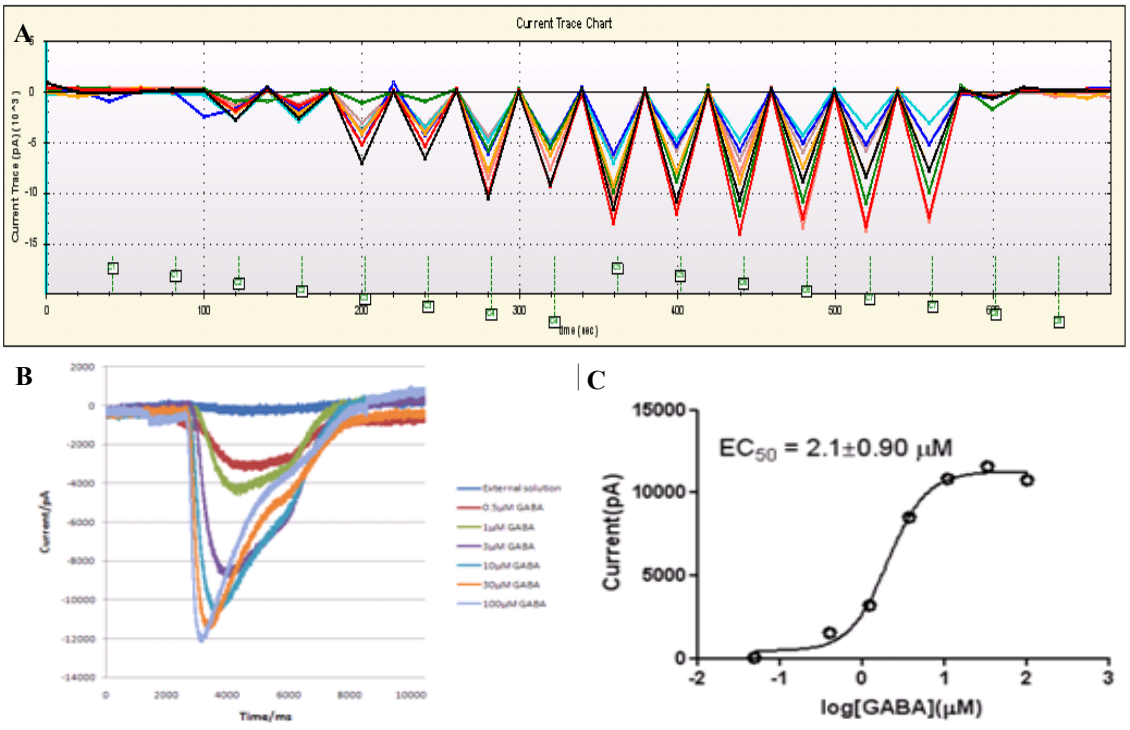

Figure 2. A. A screenshot from the IonFlux software showing the Cl^- peak current from cells transfected with the α1-β3-γ2 receptor subtype as they are exposed to two doses of a 0μM, 0.5μM, 1μM, 3μM, 10μM, 30μM, 100μM, and 0μM concentrations of GABA applied for a 3s interval. **B.** Current sweeps show the inward Cl- current from one ensemble of cells within one of the IonFlux traps. Cells expressed the α1-β3-γ2 receptor subtype and were exposed to GABA with increasing concentrations (0.5μM to 100μM). **C.** Dose-response curve for γ-aminobutyric acid in α1β3γ2 transfected HEK293T cells. .

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Materials and Methods

Cells: Human embryonic kidney 293T cells (ATCC) were cultured in 75 cm^2 flasks using MEM/EBSS without Phenol Red but with L-glutamine (2 mM), glucose (1 mM), non-essential amino acids, sodium pyruvate (1 mM), penicillin and streptomycin, and 10% dialyzed and heat inactivated FBS (Invitrogen). All flasks were treated for 10 minutes at 37°C with a 1% Matrigel (BD) solution in MEM/EBSS. At 50-70% confluency, 1.5 ml of serum free media was added containing 5 µg of each of the $GABA_A$ receptor subunit DNA2, LipofectamineTM LTX (75 µl), and PLUS™ reagent (25µl). Cell were washed with 5 ml of Ca- and Mg-free PBS, followed by 3 ml of Detachin solution, after which cells were incubated for two to five minutes. The cell solution was then spun down for two minutes at 1000 rpm and resuspended in DMEM media (5 ml). This was repeated two more times. Cells were then placed into serum free media (5 ml) and placed on a shaker for 30 minutes. The cells were centrifuged again and then resuspended in extracellular solution (5ml). They were spun down and resuspended in extracellular solution two more times resulting in cell suspension of 7M cells/ml.
The extracellular solution (ECS) contained (mM): 138 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 5.6 glucose, pH 7.4 with NaOH. The intracellular solution contained (mM): 60 KCl, 70 KF, 15 NaCl, 5 EGTA, 5 HEPES, pH 7.2 with KOH. Cell suspension in extracellular solution was dispensed into an IonFlux plate.

Experimental Procedures: The IonFlux plate layout consists of patterns of twelve wells; two wells contain intracellular solution (cytosolic compartment), one contains extracellular solution (ECS) plus cells, eight contain ECS plus varying concentrations of GABA and modulators, and one well is for waste collection. Cells are captured from suspension by applying suction to microscopic channels in ensemble recording arrays. Once the array is fully occupied, the applied suction breaks the cell membranes of captured cells, establishing whole cell voltage clamp. For compound applications, pressure is applied to the appropriate compound wells, introducing the compound into the extracellular solution rapidly flowing over the cells. For recording currents, cell arrays were voltage clamped at holding potential of -80mV.

Compounds: GABA was dissolved in deionized water to make a 10mM stock solution. This solution was serially diluted into ECS buffer. The highest concentration of GABA exposed to the cells was 100µM. Benzodiazepine analog Hz166 was dissolved as a 10mM solution in DMSO and serial diluted in ECS. The highest DMSO concentration was <1%. Vehicle control measured at the same DMSO concentration showed no significant response.

Data analysis and graphical presentation was performed using a combination of IonFlux software, Microsoft Excel, and GraphPrism. Data is shown with EC50 values.

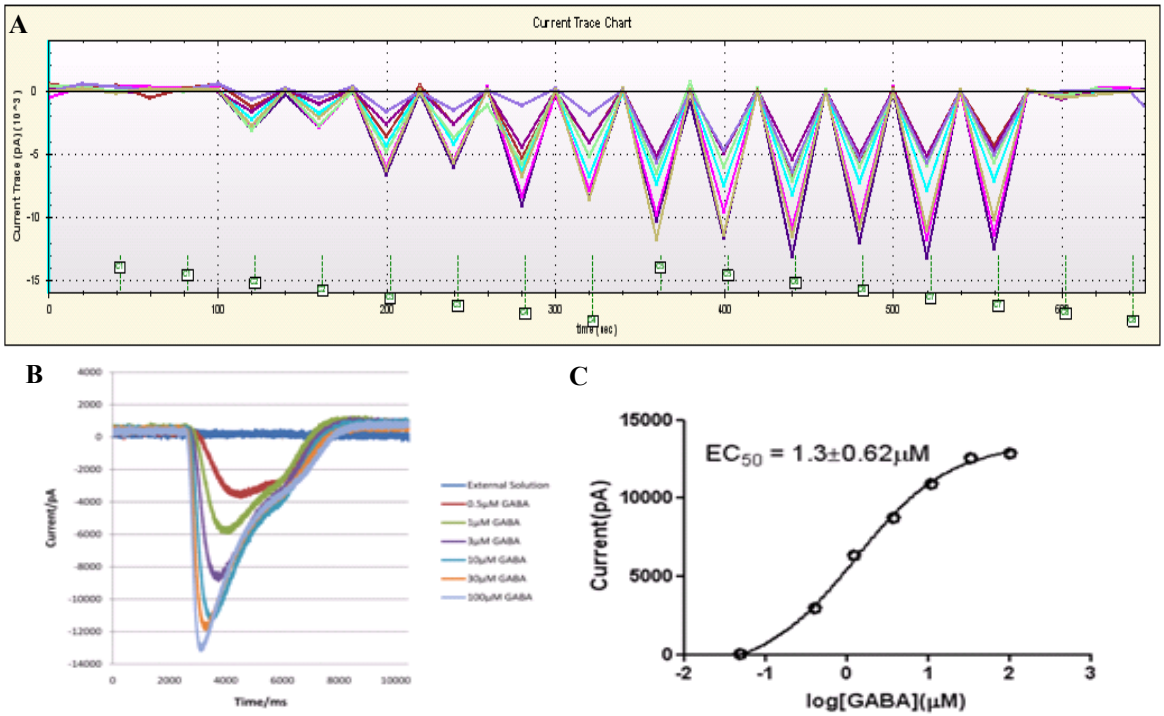

Figure 3. **A.** Screenshot from the IonFlux software showing the Cl^- peak current from cells transfected with the α2-β3-γ2 receptor subtype as they are exposed to two doses of a 0µM, 0.5µM, 1µM, 3µM, 10µM, 30µM, 100µM, and 0µM concentrations of GABA applied for a 3s interval. **B.** Current sweeps show the inward Cl- current from one ensemble of cells within one of the IonFlux traps. Cells expressed the α2-β3-γ2 receptor subtype and were exposed to GABA with increasing concentrations (0.5µM to 100µM). **C.** Dose-response curve for γ-aminobutyric acid in α2β3γ2 transfected HEK293T cells.

Transiently Transfected Cell Lines for GABA Receptor Screening

Results

A half plate experiment with α1-β3-γ2 $GABA_A$ receptor subtype transiently transfected cells showed a significant response when treated with GABA concentrations between 0.5-100 μM in comparison with cells only exposed to extracellular solution (Figure 2). The current sweeps of one experiment are depicted in Figure 2B showing saturation at GABA concentrations higher than 30 μM, while a concentration as low as 0.5 μM GABA already induced an inward Cl- current of 3000 pA. The determined GABA EC50 value for this cell ensemble was 2.10 μM (Figure 3B). This value corresponds with the reported EC50 value of 3.29 μM for transiently transfected HEK293 cells using a manual patch-clamp assay (2). All traps yielded an excellent seal resistance that averaged ~3.75MΩper ensemble (Figure 4) with comparable signals across a half plate. The success rate of the assay defined as detectable current (>1000pA) was 100% and subsequent assays yielded similar results. Peak currents in response to GABA ranged from 3 - 14nA.

Similarly, we determined the response towards GABA using a α2-β3-γ2 $GABA_A$ receptor subtype assembly (Figure 3). A set of sweeps is shown in Figure 3B. In comparison with the α1 subtype we observed similar results with currents of upwards to 13,000 pA and an EC50 value of 1.3μM, with faster desensitization after ligand is applied (Figure 3B). The seal resistance averaged ~ 6.52MΩ per ensemble (an average of 130MΩper cell) with a 100% success rate (Figure 4). Peak currents in response to GABA ranged from 4 - 15nA.

The seal resistance and success rates of transfected cells were similar to those of stably transfected cells expressing the GABA receptor, as reported in other GABA assay application notes using the IonFlux system. Therefore, the use of transient transfection did not affect either the average seal or success rates significantly.

Finally, we determined the change of current for the positive $GABA_A$ receptor modulator HZ166 (3). Modulators of the GABA response are often the target of screening campaigns in search of therapeutic compounds, and HZ166 has been reported to be selective for the α2 and α3-$GABA_A$ receptors and showed a dose-dependent antihyperalgesic effect in mouse models of inflammatory and neuropathic pain (4). We determined the dose-dependent effect of HZ166 using α1-β3-γ2 transiently transfected HEK293T cells in the presence of 2 μM GABA (EC20 modulation). The results are depicted in Figure 8. The EC50 value determined was 0.74 μM and the efficacy at higher concentrations of HZ166 was 294% of the control current. These values are similar to the reported values determined using transiently transfected oocytes and manual patch-clamp (3).

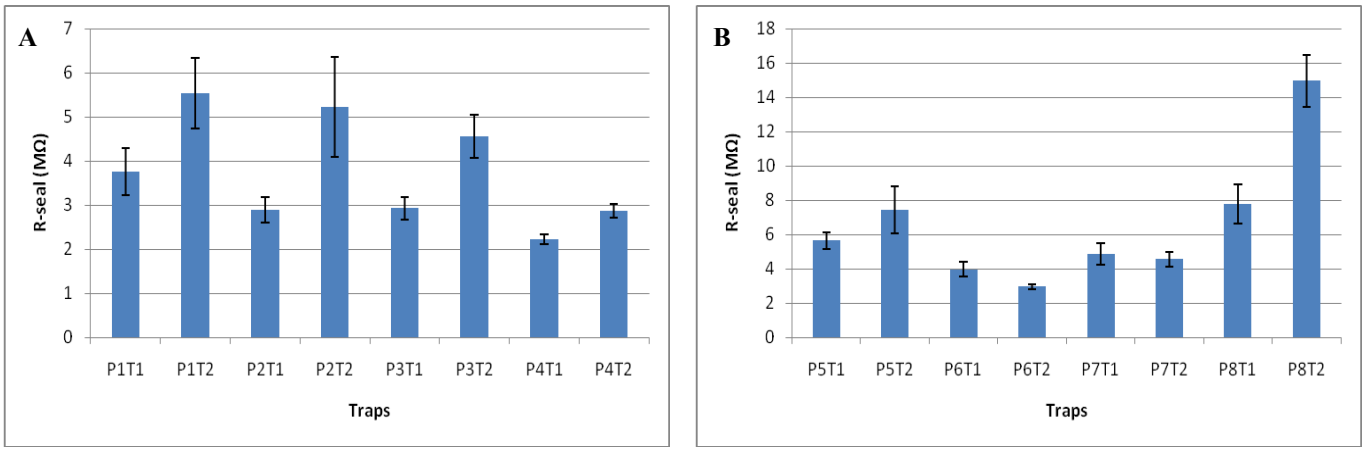

Figure 4. The average seal resistances for each trap are shown for half plate experiments using both the α1-β3-γ2 (**A**) and the α2-β3-γ2 (**B**) $GABA_A$ receptor subtypes. The seal ranged from 3MΩ to 17MΩ per 20-cell ensemble. Because the resistance is measured in parallel across all 20 cells, the average resistance per cell is 20x the ensemble value, or from 60MΩ to 340MΩ as measured after the establishment of the whole cell configuration. These seal levels yielded a 100% success rate (not shown) for both these receptor types in terms of being able to record currents of >1nA from all patterns at a good signal to noise ratios throughout the experiment (i.e. being able to complete a full dose-response response).

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Discussion

The IonFlux automated electrophysiology platform was compatible with the application of cells transiently transfected with GABAA receptor subtype DNAs. Ensemble recording post transfection had a success rate of 100% (40 completed experiments, I > 1nA for the duration of the experiment). These cells exhibited excellent electrophysiological response in the presence of GABA with an EC_{50} value of 2.1 μM for the α1-β3-γ2 assembly. Cells transfected with different $GABA_A$ receptor subtypes showed similar responses towards GABA. In addition, $GABA_A$ receptor modulators such as HZ166 were successfully evaluated in the presence of 2 μM GABA giving an efficacy increase of 294% in the α1-β3-γ2 assembly.

Overall, we showed that transiently transfected cells can be used with the IonFlux giving results similar to those determined by manual patch-clamp, with a very high success rate (100%) of experiments completed (full EC_{50} response curves at current peak values above 1nA).

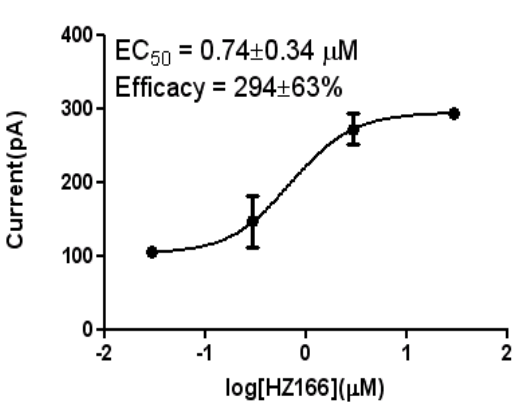

Figure 5 Dose-response curve for the modulator HZ166 in the presence of 2 μM GABA (EC20) in α1β3γ2 transfected HEK293T cells.

References

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