

# Liquid Perfusion Without the Overhead: Fast, Serial Liquid Replacement for PAM Assay Development

## Introduction

Ion channels are a class of trans-membrane proteins prolific in nearly all cell types, with extensive biodiversity in controlling a vast array of physiological processes from cell-to-cell signaling all the way to homeostasis<sup>1,2</sup>. It is possible to separate ion channels into two separate classes: ligand gated and voltage gated. Ligand-gated ion channels (LGICs) represent the class that are activated, or “gated”, in the presence of a ligand, or “agonist”. Functional impairment of LGICs represent a variety of disorders and diseases, and as such they are attractive targets for drug research focusing on various levels of modulation.  $\gamma$ -Amino butyric acid (GABA) activated receptors (GABAA) are chloride conducting channels that function by inhibiting neuronal cell excitability and represent the most abundant inhibitory receptor in the brain<sup>3</sup>. This receptor has a heteropolymeric structure<sup>4</sup> with many identified subunits leading to a high degree of molecular diversity in mammalian species<sup>5</sup>. With GABA acting as an agonist, other compounds can modulate this evoked chloride current by binding to an allosteric site on the receptor<sup>6</sup>. These allosteric modulator compounds are used in various therapeutic treatments including insomnia, anxiety, and schizophrenia. Allosteric enhancers of channel activity, also known as positive allosteric modulators (PAMs), are major targets for drug discovery and can act either by binding to the extracellular domain or to the pore domain<sup>3</sup>. Regardless of their binding sites, PAM activity is only observed in the presence of an agonist and hence their study can be complicated, with multiple serial additions of various compounds needed to properly quantify their effect.

Automated patch clamp (APC) systems have become indispensable tools in the drug development pipeline by allowing high throughput recording of channel activity. With the ability to record currents from multiple cells in parallel, these systems can measure channel activity in the presence of an agonist, antagonist or a PAM in real time. Developing successful PAM assays can be a delicate process requiring extensive flexibility in extracellular liquid displacement. Often, specialized PAM assays are hampered by time and physical



**Figure 1:** The IonFlux plates are based on the 96 or 384 SBS-standard format. (Left) an IonFlux HT system capable of 64 parallel recording. (Right) the microfluidic network attached to the bottom of a 384 well plate. This plate is used with the IonFlux HT

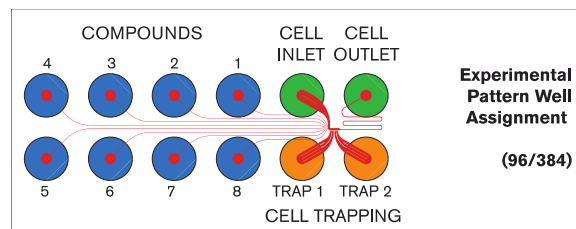
limitations imposed by the use of complex robotic liquid handlers. These systems can be either incapable of adequately changing the extracellular bathing solution rapidly or are forced to work in an asynchronous manner, decreasing their speed and reducing their throughput. The IonFlux system deploys specialized microfluidics plates where all liquid delivery and exchange occurs within the plate itself, removing the need for external liquid handling during system operation. The applied benefits of continuous solution flow, and increased control and speed in liquid management made possible by the IonFlux system provides the user with the high degree of flexibility required for PAM drug screening. This report looks into

various capabilities of the system that answer the need for implementation of complex PAM assays.

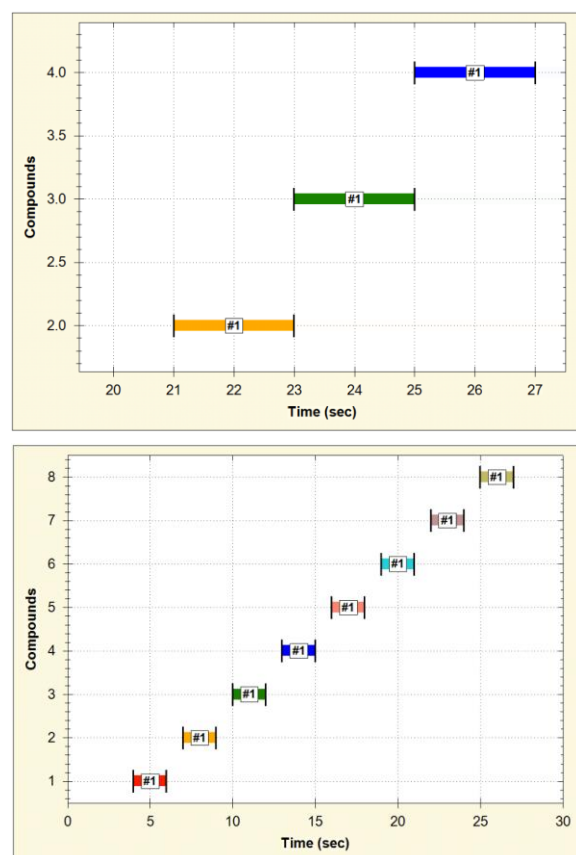
## Technology

The IonFlux system uses pneumatics to effectively move all solutions within a recording plate. The lack of requirement for a liquid handling robot allows for continuous flow of solution, while the unique experimental pattern layout in the recording plate allows compounds to be added using laminar flow.

The IonFlux plate is divided into patterns of 12 wells (Fig 2), where two are used to trap and record from the cells, one to introduce the cells, and one for waste collection. The remaining 8 wells are used for various combinations of serial additions. These can be different compounds, or varying concentrations of the same compound for EC50/IC50 determination. Each intracellular well is connected to a distinct recording electrode allowing two individual recordings per pattern. Once introduced, cells flow through the microfluidic channels and are “trapped” by gentle suction from the patch holes before stronger suction ruptures their membrane allowing for whole-cell patch clamp recordings. In ensemble plates, 20 cells are trapped per recording site, providing higher success rates. With single-hole plates, a single cell is trapped per recording site allowing for high seal resistance (Giga-seal) recording. After successful capture, the cells are continuously bathed by flowing extracellular buffer solution. Pressure can be applied to compound wells in any user-defined sequence allowing the flow of compound from their reservoirs to the captured cells. These compounds are delivered with laminar flow providing a complete fluidic displacement with compound of interest eliciting the intended pharmacological response. The definition of this process in the software provides great flexibility in design of compound application permitting the execution of complex experiments. Fig 3 shows a simple schematic of a dose response experiment where each dose is followed by a brief washing period. Fig 3 (bottom) shows the same experiment with no washing period and staircase effect of compound additions.



**Figure 2:** Each pattern in an IonFlux plate has 8 compound wells, 2 trap/recording wells, 1 inlet well for cells and an outlet well for waste. The cells and compounds are loaded at the same time, eliminating the need for liquid handling robotics. There are two distinct recordings per experimental pattern. IonFlux 16 records: 16 data recordings, 8 experimental patterns. IonFlux HT: 64 data recordings, 32 experimental patterns.

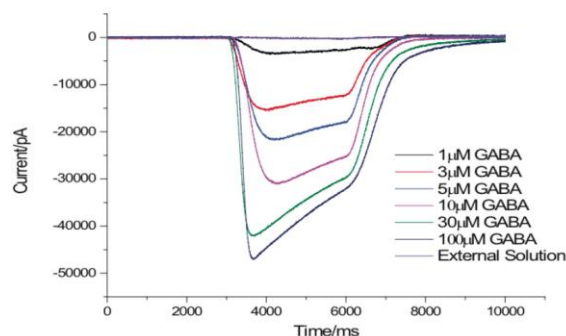


**Figure 4:** Assay development and application of compounds in the IonFlux software. Colored bars indicate when compound is added from the compound wells. Between compound applications, the trapped cells are continuously bathed in flowing extracellular bath solution.

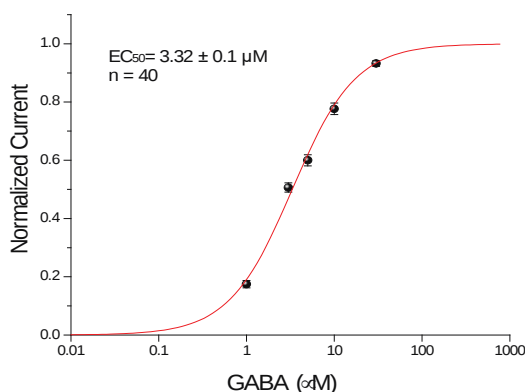
## Agonist Dose Response Assays

The Activation of GABAA receptors by application of agonist results in chloride ions flowing outward and the inward current shown in Fig 4. Recording buffers used

to obtain these results are as follows. Intracellular buffer (in mM): 60 KCl, 70 KF, 15 NaCl 5 HEPES, 5 EGTA pH 7.2 295 mOsm. Extracellular buffer (in mM): 138 NaCl, 4 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 5.6 Glucose, 10 HEPES, pH 7.4 295 mOsm. The receptor is sensitive to a number of agonists including GABA, muscimol, isoguvacin, and even ethanol.



**Figure 4:** Representative sweeps showing the response of the same cell ensemble exposed to increasing GABA concentrations. Experiments conducted HEK cells expressing  $\alpha 1\beta 3\gamma 2$  GABAA receptors.



**Figure 5:** GABA dose response curve (0.1  $\mu$ M to 100  $\mu$ M) in recombinant HEK cells expressing  $\alpha 1\beta 3\gamma 2$  GABAA receptors (n=40)

Current amplitude tends to vary directly with receptor affinity for the agonist of interest. Fig 4 shows activation of the receptor in response to an increasing concentration gradient of extracellular GABA. These experiments use HEK cells expressing GABAA (Eurofins PrecisiON™ hGABAA  $\alpha 1\beta 3\gamma 2$ -HEK recombinant cell line CYL3053) as an experimental model. When compiled together (Fig 5), a dose response curve can be plotted showing an EC<sub>50</sub> of 3.32  $\mu$ M, which is consistent with published values for GABA<sup>7</sup>.

The replacement of a liquid handling apparatus with pneumatic control for compound application provides

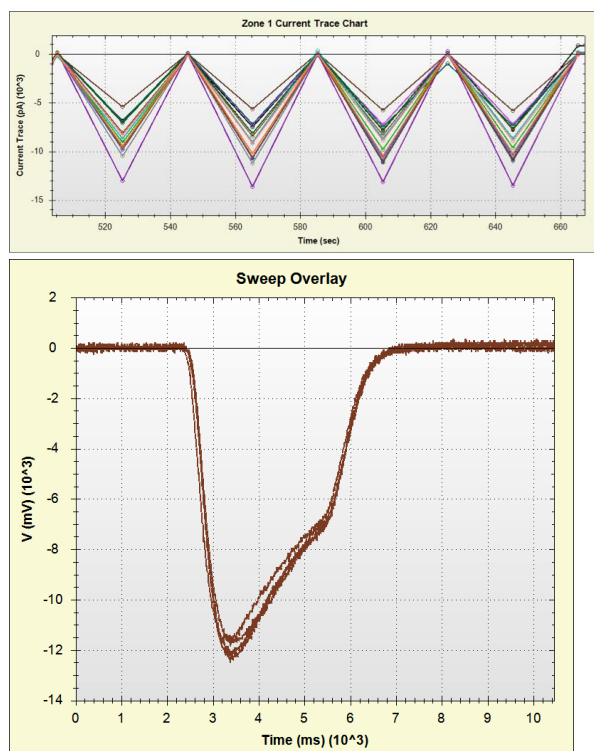
the IonFlux the perfect ability to add compound sequentially to all recording pattern in parallel. Wash steps are no longer required, but rather the cells are washed by simply separating compound addition steps in the application strategy (Fig 3 top). This parallel ability is demonstrated in Fig 6. Currents initiated by 10  $\mu$ M GABA additions are plotted vs. time for all wells. Recording cursors calculate a minimum current value during peak activation and a mean value of the current right after its peak for each recording well. The negative peak activation is then subtracted from the baseline value (mean) and the resultant values are plotted on a current vs. time plot. This experiment not only shows parallel addition of agonist on all recording wells, but also the effectiveness of agonist removal between compound applications. This is further demonstrated by the superimposable aspect of successive sweeps (Fig 6 bottom).

## Modulator Effect Assays

Positive allosteric modulators do not activate the receptor in question on their own. PAMs merely modulate the current in response to an agonist. Co-application of a PAM plus agonist potentiates current conducted by the ion channel in question. The study of receptor modulation benefits from consistent serial response to the effects of agonist. Therefore proper quantification of PAM effect requires minimal variability in the measured control currents.

Diazepam, marketed as Valium, is a very common PAM for the GABAA receptor. Fig 7 shows the effect of increasing concentrations of diazepam in the presence of 1  $\mu$ M GABA.

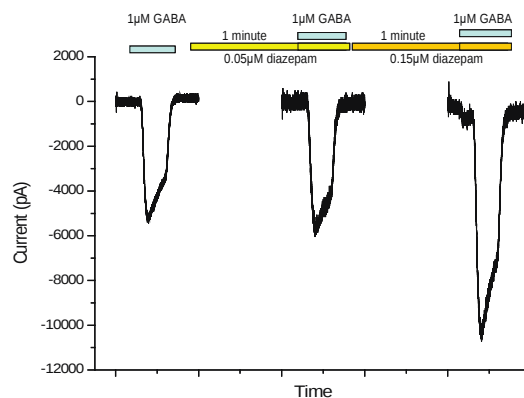
First, 1  $\mu$ M GABA is added establishing a baseline response to agonist alone. Next 0.05  $\mu$ M diazepam is added and incubated for 1 minute. As expected, no current is observed in the incubation period. However when diazepam and GABA are co-applied, a slight increase in the current is observed. Next another incubation phase is performed, but now with 0.15  $\mu$ M diazepam, which also shows no evoked current. Finally 1  $\mu$ M GABA is co-applied with 0.15  $\mu$ M diazepam, and



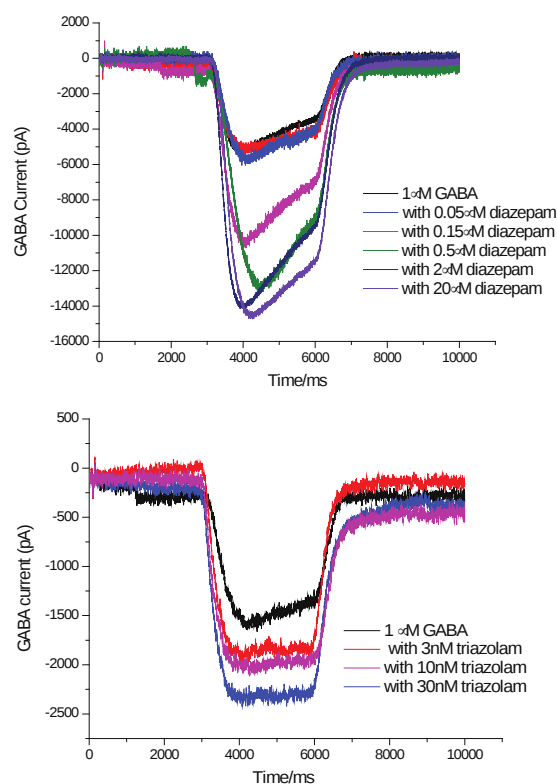
**Figure 6:** Consecutive addition of 10  $\mu\text{M}$  GABA on 16 cell ensembles within the same plate. (Top) IT plot showing points measured within the sweeps, with and without GABA additions: points at 0 indicate no presence of GABA, negative values occur when GABA activates the channel. (Bottom) superimposed serial sweeps from one recording well showing consistency of current.

the resulting current is substantially larger. Fig 8 (top) shows sequential additions of increasing concentrations of diazepam co-applied with 1  $\mu\text{M}$  GABA. The superimposed sweeps demonstrate the effect of current modulation.

Triazolam is another PAM belonging to the benzodiazepine compound family which is used to treat insomnia. Fig 6 (bottom) shows superimposed current traces evoked by co-application of 1  $\mu\text{M}$  of GABA with increasing concentrations of triazolam. Note the higher potency of triazolam as compared with diazepam, and the distinctive effects on receptor potentiation at much lower concentrations. Zolpidem, marketed as Ambien, is a potent hypnotic drug outside of the benzodiazepine family, and is also a positive modulator for GABAA receptors<sup>8</sup>. Zolpidem has higher potency than diazepam but lower than triazolam. Fig 9 is a comparative EC<sub>50</sub> plot of diazepam, zolpidem, and triazolam all in the presence of 1  $\mu\text{M}$  GABA. These EC<sub>50</sub> values are (in nM) 425, 84, and 12 respectively.



**Figure 7:** PAM serial protocol setup: pre-incubation with the modulator for 1 minute, followed by a co-application of the modulator with 1  $\mu\text{M}$  GABA. Diazepam was first added at 0.05  $\mu\text{M}$  then at 0.15  $\mu\text{M}$

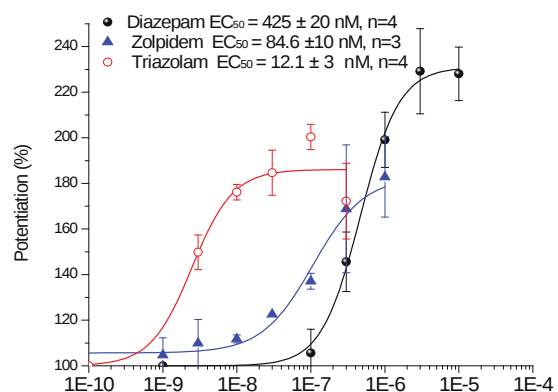


**Figure 8:** Two PAM effects on GABA receptors: (Top) superimposed sweeps from the same recording well showing effect of 0.05, 0.15, 0.5, 2 and 20  $\mu\text{M}$  diazepam. (Bottom) effect of 3, 10, 30 nM triazolam additions. All PAM additions co-applied with 1  $\mu\text{M}$  GABA.

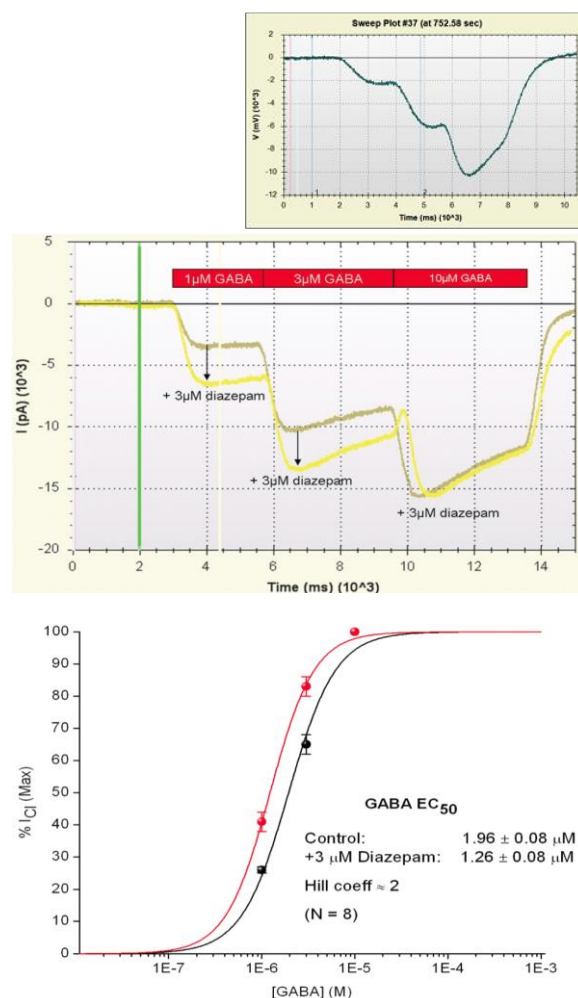
## Rapid Liquid Displacement Assays

Pneumatic control and a plate-based microfluidics design allow the IonFlux to sequentially change extracellular solution from one compound well to another with no wash step in between (Fig 3 bottom). This capability opens up the potential feasibility for automated assays that are challenging if not impossible to perform on systems dependent upon liquid handlers. This unique compound application strategy avoids problems of stagnant liquid stacking such as concentration mixing between stacked solutions layers in a pipette. Continuously flowing extracellular solution allows for rapid exchange of bath solution with near immediate effect on the target receptor. In response to agonist concentration challenge, activated GABAA receptor current shows a fast activation, followed by a slow deactivation. By sequentially increasing the agonist dose faster than the rate of deactivation, we can effectively increase activation of current in a staircase effect. An example of this staircase activation is shown in Fig 10 (top). The receptor was activated with 1 $\mu$ M GABA, followed by 3 $\mu$ M then 10 $\mu$ M. The compounds are applied for 2 seconds at each concentration.

To show the potentiating effect of PAMs, a similar experiment is conducted where a constant application of modulator is added and compared to control. Fig 10 bottom shows the same staircase GABA activation with and without co-application of 3 $\mu$ M diazepam, clearly showing the expected allosteric shift in current magnitude. It is important to note the above dose effect of GABA and the allosteric shift of diazepam were conducted in only a few minutes and within a single recording sweep, making this unique experiment a convenient and fast measurement of EC<sub>50</sub>s within each recording well. Fig 10 (right) shows the allosteric shift in the EC<sub>50</sub> seen in these experiments.



**Figure 9:** EC<sub>50</sub> of diazepam, zolpidem and triazolam. Values were 425 nM (n=4), 84.6 nM (n=3) and 12.1 nM (n=4) respectively.



**Figure 10:** (Top and Middle) Cumulative staircase GABA activation in response to sequential addition of 2 minutes 1, 3 and 10 $\mu$ M of GABA. (Middle) control addition and co-application with 3  $\mu$ M diazepam reproducing the known shift in GABA potency attributable to positive allosteric modulators (B) (ref 7). EC<sub>50</sub> of control vs diazepam co-application showing the shift (left)

## Conclusion

The advent of high-throughput and data-rich electrophysiological screening methods has allowed the development of many functional assays in the study of ligand gated channels and their modulation by pharmacological agents. While research for agonists, co-agonists, and antagonists of important neuronal receptors such as GABAA is ongoing, modulation of said receptors represents a target rich environment. Discovery and research of new effective positive allosteric modulators can be a challenge for most high throughput screening systems due to intermittent buffer exchange, unreliable serial additions, or non-parallel execution of assays. The IonFlux system, equipped with its unique liquid exchange and continuous flow system, provides an excellent platform for complex PAM assays. The ability to provide a consecutive and fast switch from one compound to another or to serially increase compound concentrations with no wash steps or liquid stacking problem, gives the platform the unique edge and speed a complex PAM qualification assay requires.

## References

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