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Validation of a cortical tri-culture axoModel™ for *in vitro* compound screening: a blinded compound study

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Abstract

Neurodegenerative diseases (NDDs) such as Alzheimer's Disease and Parkinson's Disease are a leading cause of physical and cognitive disability, affecting around 15% of the global population [1]. With rising prevalence due to an aging population, there is a growing need for better, safer therapies and curative treatments. The cortex is a key area affected by NDD and is also a common site of drug-induced neurotoxicity (a leading cause of drug attrition), hence it is a major target for drug discovery [2]. However, the lack of physiologically relevant cortical models is a major challenge, with traditional animal models failing to translate from "bench to clinic" and low complexity afforded by simple cell culture and cell line models.

In partnership with Sumitomo Pharma America, Inc., Axol Bioscience performed a blinded study testing eight reference compounds on an isogenic cortical tri-culture axoModel, using an Axion multi-electrode array (MEA) system to measure electrophysiological response. Here we demonstrate robust identification of reference compound mode of actions, validating this axoModel for use in human-relevant drug discovery and neurotoxicity screening.

Methods

axoCells™ isogenic human iPSC-derived cortical excitatory neurons [CENs, ax0015], cortical inhibitory interneurons [CINs, ax0667] and astrocytes [ax0665] were co-cultured on 48-well Axion Maestro MEA plates according to Axol's user guide. Cells were cultured from thaw for 26 days before compound treatment to allow full neuronal maturation and network formation.

On Day 26, recordings were taken at 10 minutes pre-compound addition (baseline data, R1), 10 minutes after compound addition (R2) and 60 minutes after compound addition (longer-term effects, R3). This process was then repeated two more times, with a second and third dose added in an accumulative manner. In total three concentrations of each compound were tested, the precise concentrations were specific to each compound. All compounds had been blinded prior to shipment and were only unblinded after the final data analysis and written report. For the drug additions, 30 µL for the cell culture medium was removed and replaced with 30 µL medium containing 10x concentrated compound, resulting in the desired final compound concentration. All compounds and concentrations were tested at n=3-14 wells per compound.

The Axion Maestro MEA system was used to record eighteen electrophysiological parameters, including individual electrode spike parameters, electrode burst parameters, well activity parameters and overall network parameters. To add comparison all parameters for each well were normalised to their R1 values.This enabled classification of each blinded compound into one of six groups and sub-groups, based on their observed actions:

Group 1 ("***Little Effect***") showed minimal significantly different effects from the vehicle control.

Group 2 ("***Activators***") induced an overall increase in activity. This was further sub-divided into Group 2A ("Hyperpolarizing": increased spike rate, decreased bursting), Group 2B –("Excitatory": increased spike rate, unchanged bursting) and Group 2C ("Pro-convulsant": increased spike rate and bursting, typical of pro-convulsant compounds).

Group 3 ("***De-Activators***") produced an overall decrease in activity. This was subdivided into Group 3A ("***Blockers***": reduced neuronal activity and spike amplitude) and Group 3B ("***Inhibitors***": decreased activity with unchanged spike amplitude).

After the compounds were put into categories and the final report submitted, they were unblinded and the results compared with their known pharmacologies.

axoCells iPSC-derived cells demonstrate key marker expression on immunocytochemistry (ICC)

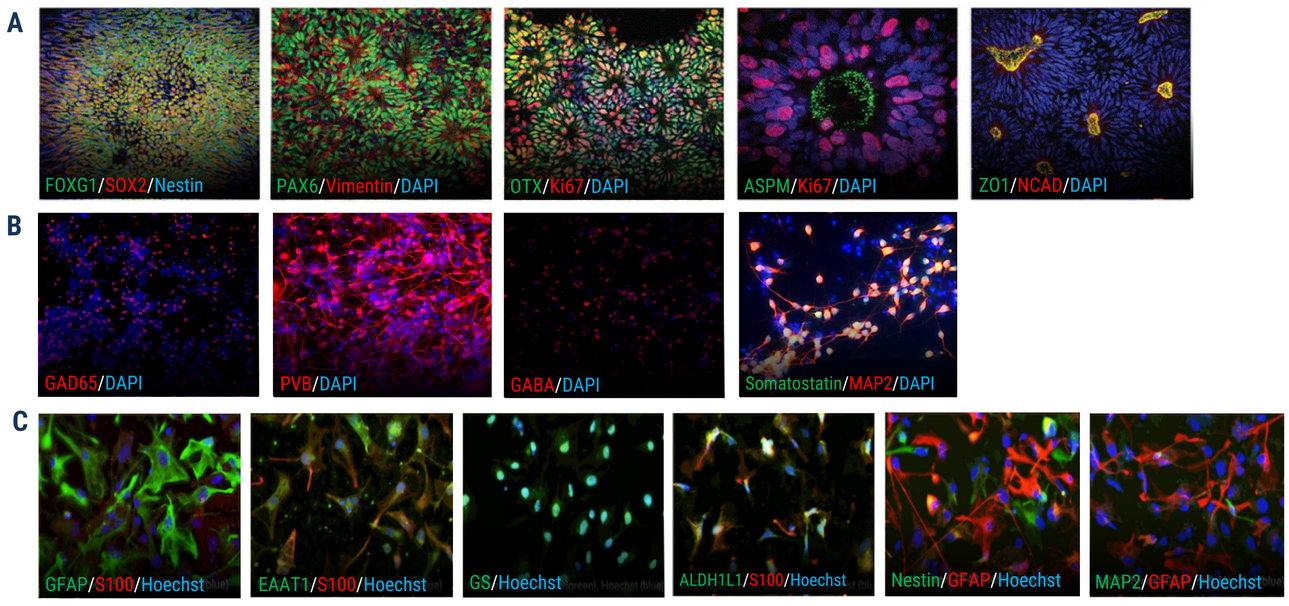


Figure 1. ICC shows expression of key markers for axoCells cortical excitatory neurons (CENs), cortical inhibitory interneurons (CINs) and astrocytes
(**A**) axoCells iPSC-derived neural stem cells (NSCs, ax0015) show expression of all standard NSC markers: FOXG1, SOX2, PAX6, Vimentin, OTX, Ki67, ZO1 and NCAD. (**B**) In contrast, axoCells CINs (DIV20, ax0667) express the key markers GAD65, Parvalbumin (PVB), Beta, Somatastatin and MAP2. (**C**) axoCells iPSC-derived astrocytes (ax0665) express key astrocyte-specific markers (GFAP and S100B) and astrocyte-associated markers, EAAT1, Glutamine Synthetase (GS) and ALDH1L1, while showing very low levels of neuronal progenitor markers Nestin and MAP2.

Modulation of cortical neuron firing in co- and tri-culture

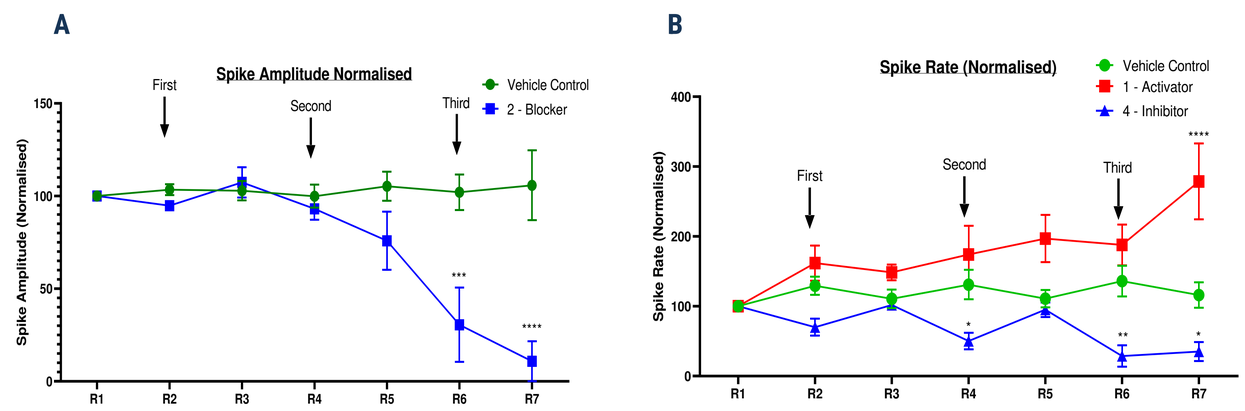
Monoculture: axoCells cortical excitatory neurons

Co-culture: axoCells cortical neurons & cortical inhibitory interneurons

Tri-culture: axoCells cortical neurons, cortical inhibitory interneurons & astrocytes

Figure 2. Axion MEA traces of axoCells CENs in monoculture, in isogenic co-culture with axoCells CINs and in isogenic tri-culture with axoCells CINs & astrocytes. The Axion MEA traces above show typical activities on DIV35. In monoculture (**Top**) the CENs fire frequently and spontaneously but with limited synchronicity. The addition of CINs (**Middle**) markedly reduces firing rate but switches activity to a more regular synchronized burst firing pattern. The addition of astrocytes (**Bottom**) increases both firing rate and spike amplitude while maintaining the regular synchronized burst firing seen in the co-culture model. This shows the expected functional modulation with increasing model complexity, demonstrating functional validation of the model.

Blinded *in vitro* compound screening using electrophysiological endpoints



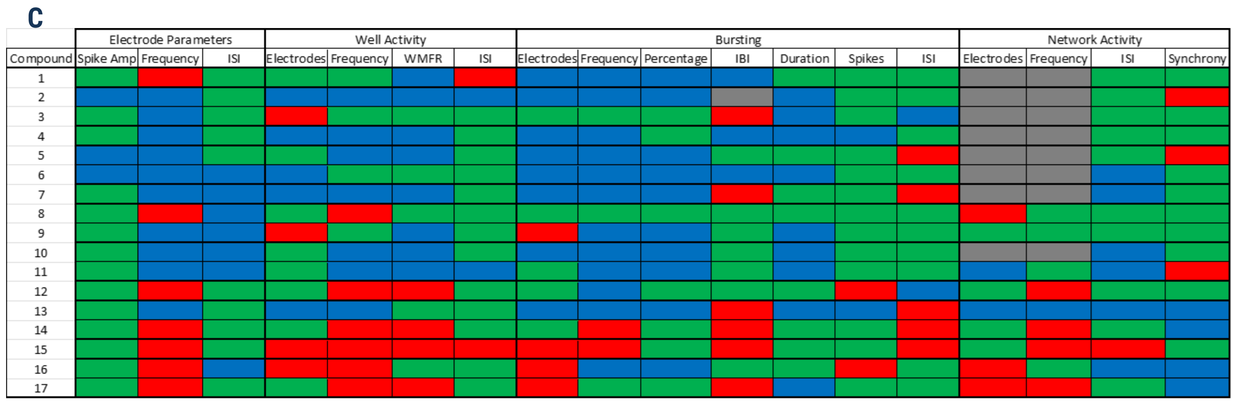


Figure 3. Blinded compound screening on a cortical tri-culture axoModel, with electrophysiological endpoints measured on the Axion Maestro MEA system.
For more details on Axol Bioscience's tri-culture protocol timeline, please see the QR code at the bottom. (**A**) Example spike amplitude data for vehicle control and Compound 2 (a "Blocker") (**B**) Example spike rate data for vehicle control, Compound 1 (a "Hyperpolarizing Activator") and Compound 4 (an "Inhibitor"). Statistical significance was assessed by two-way ANOVA. (**C**) Heatmap of the monitored parameters on the Axion MEA for the blinded compound list. Seventeen compounds were tested in total, with nine novel compounds of undefined action, two anonymized proprietary compounds, designated Compound A and Compound B, and six compounds of known action. Each parameter was color-coded GREEN for Unchanged, RED for Increased and BLUE for Decreased compared to the vehicle control.

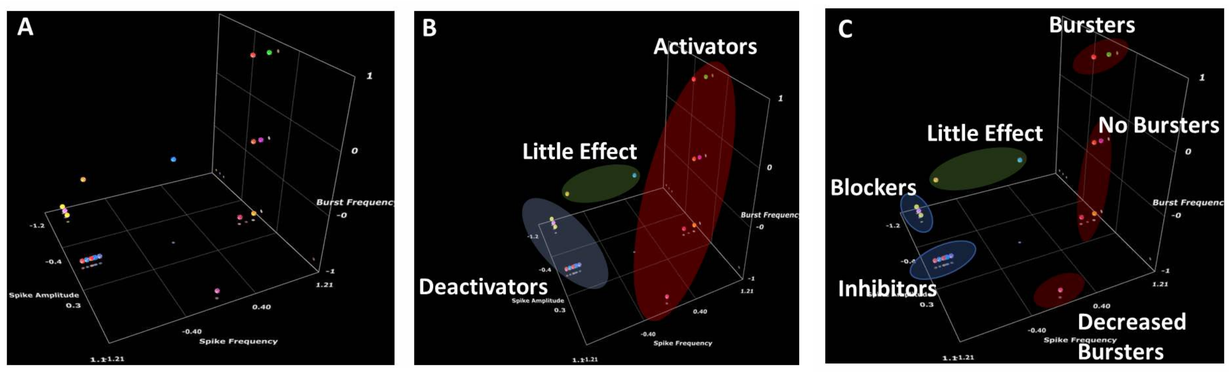


Figure 4. 3D scatter plots of measured MEA parameters for blinded compounds and vehicle control.
(**A**) Unclustered data (**B**) Data clustered by highest level groupings (**Little Effect**, **Deactivators** and **Activators**) (**C**) Data clustered by sub-groupings (**Little Effect**, **Blockers**, **Inhibitors**, **Increased Bursters**, **Unchanged Bursters** and **Decreased Bursters**). **Increased Bursters** were identified as potentially pro-convulsant, **Unchanged Bursters** are potentially non pro-convulsant and **Decreased Bursters** are hyperpolarizing. Created using the software available at https://miabellaai.net/.

Unblinding of compounds

Compound		Group		Subgroup	
Number	Name	Model classification	Unblinded/literature classification	Model classification	Unblinded/literature classification
1	**NS 309**	Activator	Activator	Activator – Decreased Bursting	Activator – Decreased Bursting
2	**Compound A**	De-activator	De-activator	De-activator- Blocker	De-activator - Inhibitor
3	**Nifedipine**	Little Effect	Little Effect	Little Effect	Little Effect
4	**Compound B**	De-activator	De-activator	De-activator- Inhibitor	De-activator- Inhibitor
5	**Lamotrigine**	De-activator	De-activator	De-activator- Blocker	De-activator- Blocker
14	**4-AP**	Activator	Activator	Activator – Increased Bursting	Activator – Increased Bursting
16	**Levetiracetam**	Activator	Activator	Activator – Unchanged Bursting	Activator – Unchanged Bursting
17	**Lithium Carbonate**	Activator	Activator	Activator – Unchanged Bursting	Activator – Unchanged Bursting

Figure 5. Table of unblinded compounds showing almost perfect fidelity between model-based predicted action and actual action.
After unblinding, the actual reported action of the compound was compared to the predicted effect using the Axion Maestro MEA system. Results demonstrated was alignment of the predicted and actual Group in 8 out of 8 compounds, with correct prediction of the Subgroup in 7 out of 8 compounds. These results were deemed by the client to be a valuable neurological compound screening test system, with future applications in drug discovery and neurotoxicity screening.

Conclusion

In this blinded study, a human iPSC-derived cortical tri-culture axoModel was used to correctly identify the effect of 8 compounds, with alignment of 8 out of 8 Groups and 7 out of 8 Subgroups. By incorporating human iPSC-derived cells into advanced *in vitro* platforms, it is possible to produce human-relevant test models that could better recapitulate the complex *in vivo* interactions of the human cortex compared to the equivalent heterologous cell lines and animal models.

Although these experiments were carried out using cells derived from a single healthy control donor, it is possible to incorporate lines derived from multiple donors and from patients with NDDs such as Alzheimer's Disease, to add further complexity and build disease-specific *in vitro* models for drug development.

References

[1] 1. Feigin V.L., Vos T., Nichols E., O Owolabi M., Carroll W.M., Dichgans M., Deuschl G., Parmar P., Brainin M., Murray C. The global burden of neurological disorders: Translating evidence into policy. *Lancet Neurol.* 2019;19:255–265. doi: 10.1016/S1474-4422(19)30411-9.
[2] Richard J Weaver, Jean-Pierre Valentin, Today's Challenges to De-Risk and Predict Drug Safety in Human "Mind-the-Gap", *Toxicological Sciences*, Volume 167, Issue 2, February 2019, Pages 307–321, https://doi.org/10.1093/toxsci/kfy270

Axol Tri-culture - Cerebral Cortical Neurons, Interneurons and Astrocytes User Guide

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