Development and Characterization of Human iPSC-derived Neurons for Drug Discovery Applications

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Target Identification → Target Validation → **Compound Screening** → Lead Optimization → Preclinical Trials → Clinical Trials

Abstract

The human brain represents a complex organ that has consistently been proven difficult to model *in vitro*. Current models including primary rodent tissue and immortalized cell lines have served as mainstays in both academic research and the pharmaceutical industry. These models, while providing a means for numerous landmark discoveries, have suffered from various issues including biological relevance, reproducibility and scalability. Considerable efforts have been made specifically within the pharmaceutical industry to reduce late-stage drug attrition through the development of more relevant *in vitro* human model systems. One area given significant attention has been the development of platforms than can enable the modeling of human degenerative (e.g. Alzheimer's and Parkinson's disease) and genetic (i.e. Huntington's disease and muscular dystrophy) diseases as well as neurotoxicity. The recent discovery of induced pluripotent stem cells (iPSCs) not only overcomes the ethical and logistical issues associated with human embryonic stem cells, but also provides a flexible platform for generating various differentiated cell types from diseased individuals. Using this platform, we have developed a highly consistent and scalable protocol to differentiate and cryopreserve purified human iPSC-derived neurons, called iCell® Neurons. Phenotypically, these cells are >90% pure as measured using flow cytometry for presence of the neuronal marker class III beta tubulin (TuJ1) and absence of the progenitor marker nestin. Within 24hrs of thawing, these neurons display a typical neuronal morphology including a dense network of neurites. Detailed phenotypic analyses reveal that these neurons are comprised of a mix of predominantly GABAergic and Glutamatergic subtypes as measured at both the gene expression and protein levels and form characteristic synaptic connections. Functionally, these cells reveal typical electrophysiological characteristics as measured using single-cell patch clamp to detect both spontaneous and evoked action potentials as well as functional ion channels. Finally, when applied to high throughput applications, including cytotoxicity assays, iCell Neurons reveal characteristic pharmacological responses to known toxic compounds. The results demonstrate not only a novel cell model for use in various academic and pharmaceutical applications, but they also support the use of the iPSC technology as a platform capable of generating neurons against diverse genetic backgrounds.

Post-Thaw Morphology

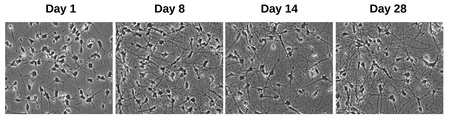


Figure 1. Post-thaw iCell Neurons exhibit a typical neuronal morphology. Cryopreserved iCell Neurons were thawed and plated in iCell Neurons Maintenance Medium with iCell Neurons Supplement on poly-L-ornithine/laminin-coated tissue culture plates. Reanimated neurons develop branched networks within 24 hours and remain viable and adherent for an extended period in culture (≥14 days).

Phenotype Characterization

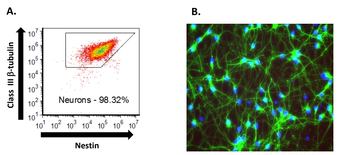


Figure 2. A highly pure neuronal population. iCell Neurons represent a highly pure population as demonstrated by (A) flow cytometry (Day 1 post-thaw) and (B) immunocytochemistry (Day 7 post-thaw) for class III β–tubulin (positive; neuronal marker) and nestin (negative, neural stem/progenitor cell marker).

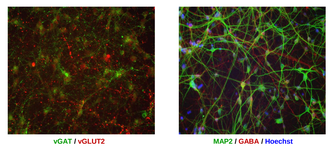


Figure 3. iCell Neurons display characteristic subtype protein expression. Post-thaw iCell Neurons were plated in iCell Neurons Maintenance Medium with iCell Neurons Supplement on poly-L-ornithine/laminin-coated tissue culture plates and immunostained on day 14 for (A) the synaptic markers vGAT (vesicular GABA transporter) and vGLUT2 (vesicular glutamate transporter 2), markers of GABAergic and Glutamatergic neurons, respectively; and (B) MAP2 (microtubule-associated protein 2) and GABA (gamma-aminobutyric acid). Magnification = 20x objective.

Figure 4. Gene expression analysis of iCell Neurons. Day 15 post-thaw iCell Neurons were analyzed against a focused panel of TaqMan Gene Expression Assays. The data demonstrate that iCell Neurons represent a population with largely a forebrain identity (top), are predominantly glutamatergic and GABAergic neuronal subtypes (middle), and express a number of characteristic receptors (bottom).

Electrophysiology

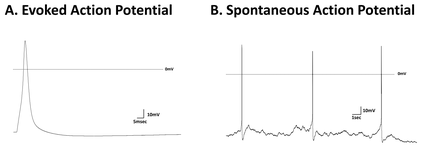


Figure 5. iCell Neurons exhibit neuron-like action potentials. Action potential tracings recorded from a single iCell Neuron using a whole-cell patch clamp methodology. (A) A representative evoked action potential from post-thaw day 11 neurons. Evoked action potentials from these cells display an average resting membrane potential of -46mV as early as 9 days post-thaw. (B) Representative spontaneous action potentials from a post-thaw day 14 neuron. All action potentials demonstrate an overshoot of the depolarization phase above 0mV and an undershoot of the repolarization phase below baseline before correction to steady-state.

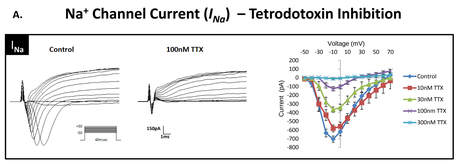


Figure 6. iCell Neurons respond to ion channel blockers. Addition of classical neuron ion channel antagonists tetrodotoxin (TTX,100nM), tetraethylammonium (TEA, 30mM) and nifedipine (10μM) blocks inward sodium outward potassium, and inward calcium currents, respectively, as measured using a single-cell patch clamp. (A) Sodium channel antagonist TTX blocks the inward current of post-thaw day 13 neuron from a holding potential of -70mV. (B) Potassium channel antagonist TEA blocks outward current of post-thaw day 12 neuron from a holding potential of -80mV. (C) Calcium channel antagonist nifedipine partially blocks inward calcium current of post-thaw day 19 neuron from a holding potential of -90mV. Calcium current was isolated by addition of TEA (50mM) and TTX (300nM) to block potassium and sodium currents, respectively.

High Content Image-based Assays

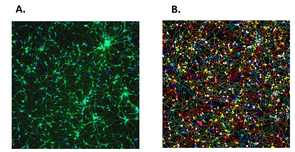
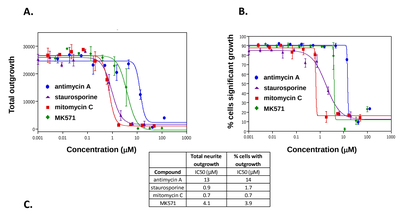


Figure 7. High content image analysis. (A) An example overlay image of post-thaw iCell Neurons stained with class III β-tubulin (green) and DAPI stain (blue) using the Molecular Devices ImageXpress® Micro system and MetaXpress® analysis software. Magnification = 10x objective. (B) Neurite outgrowth analysis using the Neurite Outgrowth module of MetaXpress Software.



Compound	Total neurite outgrowth IC50 (μM)	% cells with outgrowth IC50 (μM)
antimycin A	13	14
staurosporine	0.9	1.7
mitomycin C	0.7	0.7
MK571	4.1	3.9

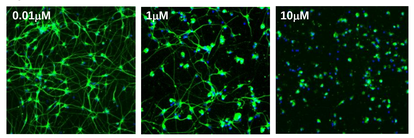
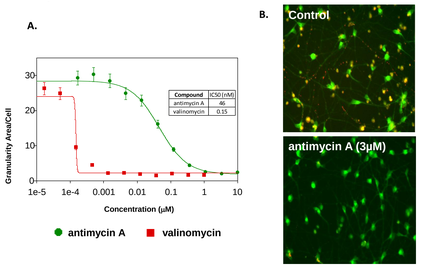


Figure 8. High content image-based assay for neurite outgrowth. (A and B) iCell Neurons treated with increasing concentrations of antimycin A, staurosporine, mitomycin C and MK571 were stained for nuclei (DAPI) and class III β-tubulin and analyzed using the Molecular Devices ImageXpress Micro system and MetaXpress software. The resultant dose response curves to cytotoxic compounds for 2 parameters: (A) total outgrowth and (B) % cells with significant growth, were generated. (C) Images of cells treated with increasing concentrations of mitomycin C and stained with class III β-tubulin. Magnification = 20x objective.



Compound	IC50 (nM)
antimycin A	46
valinomycin	0.15

Figure 9. High content image-based assay for mitochondrial integrity. (A) iCell Neurons treated with increasing concentrations of antimycin A and valinomycin and were stained for class III β-tubulin and JC-10. Images were analyzed using the Molecular Devices ImageXpress Micro system and MetaXpress software for JC-10 aggregates. Resultant data was used to generate dose-response curves. (B) Images of control and 30-minute antimycin A-treated iCell Neurons, causing a block of oxidative respiration.

Cell-based Assays

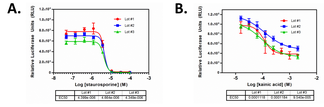


Figure 10. iCell Neurons display an expected sensitivity to known compounds. iCell Neurons were cultured for 7-14 days post-thaw on PLO/Laminin pre-coated 96-well plates and exposed to a dilution series of (A) staurosporine and (B) kainic acid. Viability (as measured using cellular ATP content) was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

BoNT Toxigenicity Testing

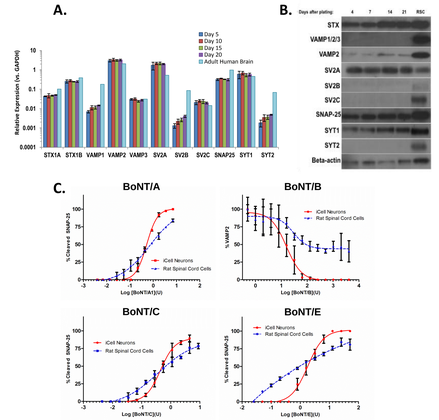


Figure 11. BoNT target characterization and toxigenicity testing. (A) TaqMan gene expression assays were used to detect Botulinum neurotoxin (BoNT) receptor and target protein expression in iCell Neurons cultured for 5-20 days post-thaw. Adult human brain was used as a positive control. (B) Protein expression of BoNT receptors and target proteins was analyzed via Western blot for iCell Neurons cultured for 4-21 days post-thaw. Rat spinal cord cells (RSC) were used as a positive control. (C) iCell Neurons (4 or 7 days post-thaw) and rat spinal cord cells were exposed to serial dilutions of BoNT/A, /B, /C, or /E for 48 hrs. Cell lysates were analyzed for respective SNARE target protein cleavage by Western blot. Data from three Western blots were quantified by densitometry and dose-response curves were plotted using GraphPad Prism 5. Protein expression of BoNT receptors and target proteins and BoNT toxigenicity assay data was generated by Regina Whitemarsh and Dr. Sabine Pellett, University of Wisconsin-Madison (Dr. Eric Johnson Lab).

Summary

iCell® Neurons represent a robust, consistent and commercially available population of human neurons for basic biological and drug discovery applications. This highly pure population of cells (Figure 2) displays a robust and stable neuronal morphology (Figure 1) and is comprised of largely Glutamatergic and GABAergic neuronal subtypes (Figures 3 and 4). iCell Neurons display evoked and spontaneous neuron-like action potentials (Figure 5) and possess functional sodium, potassium and calcium channels (Figure 6). In addition, these cells are amenable to various assay systems including high content image-based assays (Figures 7-9), standard cell-based assays (Figure 10) and toxigenicity testing (Figure 11). The results demonstrate a convenient, novel human cell model for neuroscience research which supports the use of iPSC technology as a platform capable of generating neurons from disease relevant genetic backgrounds.