HIGH-CONTENT IMAGING OF IPSC-DERIVED HUMAN NEURONS FOR TOXICITY SCREENING

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Introduction

There is a significant need for in vitro systems that more closely model the human nervous system and its response to environmental toxins. Such a platform would have greater predictive power to indicate which compounds pose a risk. Toward this goal, we have developed a platform centered on the use of iPSC-derived human neurons. First, iPSCs were gene-edited to ubiquitously express eGFP. We then patterned these iPSCs to a neuroepithelial fate and next to neuronal progenitors before finally differentiating them into neurons. Spinal motor neurons were generated in this manner and used for this proof-of-concept project. They were plated in 384-well format for high-content imaging. Optimizing imaging in this manner required attention to the source of cells, plate surface coating, medium composition, staining protocol, imaging parameters, and the timeline for neuron maturation and neurite outgrowth. Optimization of all parameters yielded a sensitive and robust system with a Z-prime value greater than 0.5.

Materials and Methods

Establishment of eGFP Reporter iPSC Line The human iPSCs line WC-30 (normal wildtype) was used in this study. iPSCs were cultured on irradiated mouse embryonic fibroblasts (MEFs) as described in the standard protocol (www.wicell.org). We applied CRISPR technology to integrate the CAG-eGFP reporter into the AAVS1 safe harbor site. CRISPR guide RNA pairs, Cas9-nickase, and the donor plasmid were introduced into iPSCs by electroporation. Neomycin was added in the culture medium to select the resistant cells. The neomycin-resistant iPSC colonies were picked and screened by imaging to detect the integration of the reporter. In the donor plasmid, we flanked the neomycin resistant cassette with two loxP sites to allow removal by Cre recombination.

Neuron Differentiation from Human iPSCs Motor neuron differentiation from human iPSCs was based on protocols described previously (Du et al. 2015. Nat Commun. 6:6626). Briefly, human iPSCs were treated with small molecules for one week to induce their fate to neuroepithelial progenitors (NEPs). The NEPs were split and treated with additional patterning molecules for another week to generate subtype-specific neuron progenitors. These progenitors were expanded with a combination of small molecules and frozen in cell freezing medium. To accelerate maturation after thawing and seeding, neurons were cultured in medium supplemented with BrainXell Seeding Supplement.

Culture Conditions Thawed motor neurons were seeded on 384-well plates coated with collagen, fibronectin, laminin, or PDL. Neurons were cultured for two to five days before exposure to the toxin rotenone, an insecticide. Rotenone was applied from 0.02-40 µM with serial dilutions of 2-fold. Exposure time before imaging was 24, 48, or 72 hours.

High-Content Imaging Motor neurons were imaged live using a PerkinElmer Operetta high-content imaging system. Images were collected using either a 10X or 20X air objective. They were subsequently processed using the Neurite Outgrowth module within the Harmony High-Content Imaging and Analysis software package.

Use of Human Motor Neurons in High-Content Imaging

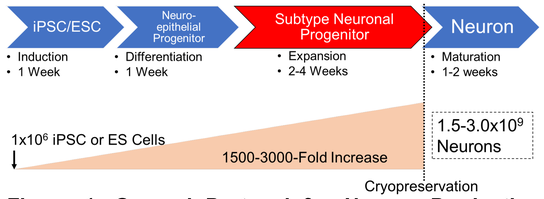


Figure 1. General Protocol for Neuron Production Scheme showing the general production protocol. The time from initiation of iPSC culture until cryopreservation is 4-6 weeks. Neurons mature 1-2 weeks after plating with BrainXell maturation supplements.

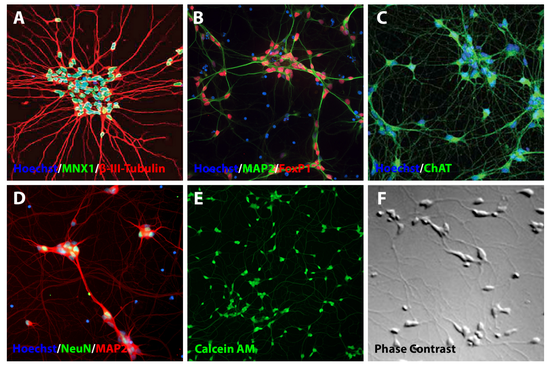


Figure 2. Expression of Motor Neuron Markers *(A-C)* Neurons express markers associated with spinal motor neuron identity, including MNX1, FoxP1, and ChAT. *(D)* Approximately 60-65% of motor neurons are positive for the mature neuronal marker NeuN. *(E-F)* Extensive neurite outgrowth, beginning within a day of plating, is shown by calcein AM staining and phase microscopy.

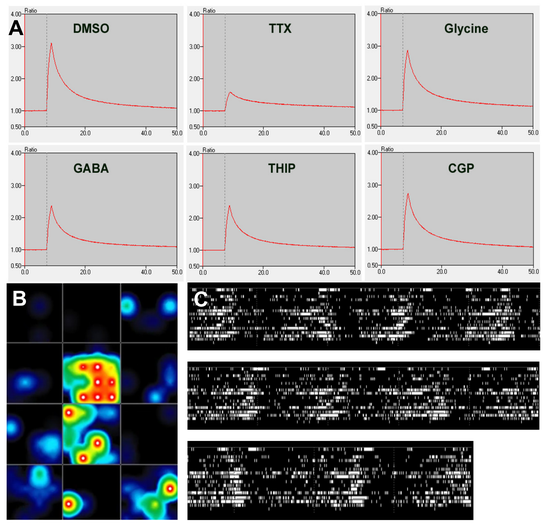


Figure 3. Functional Activity *(A)* Calcium changes after electrical stimulation at Day 10 in the presence of DMSO (no compound), TTX (sodium channel blocker), glycine, GABA, THIP (selective GABA-A receptor agonist), and CGP64626 (selective GABA-B antagonist). *(B-C)* As measured by multielectrode array (MEA), neurons display robust spontaneous activity, including spikes, bursts, and synchronous network activity on Day 12 and Day 15.

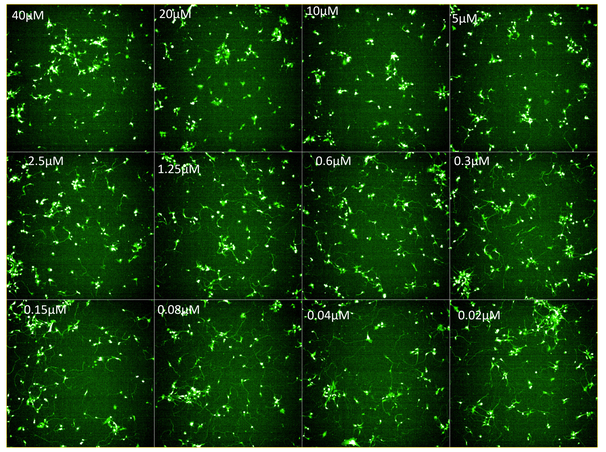


Figure 4. Rotenone Dose-Response at 24 Hours. Images of neurite deterioration on PDL-coated plates with exposure to increasing concentrations of rotenone.

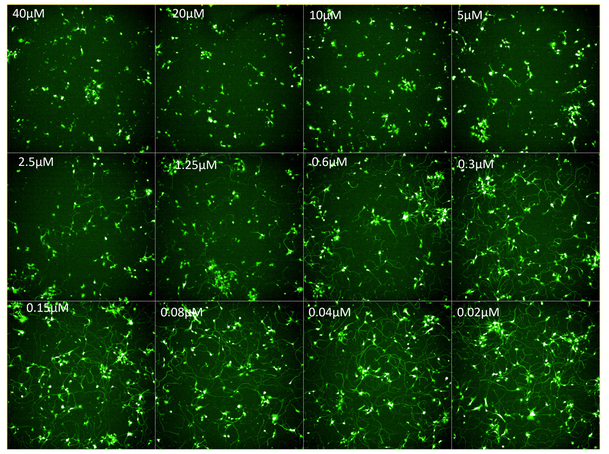
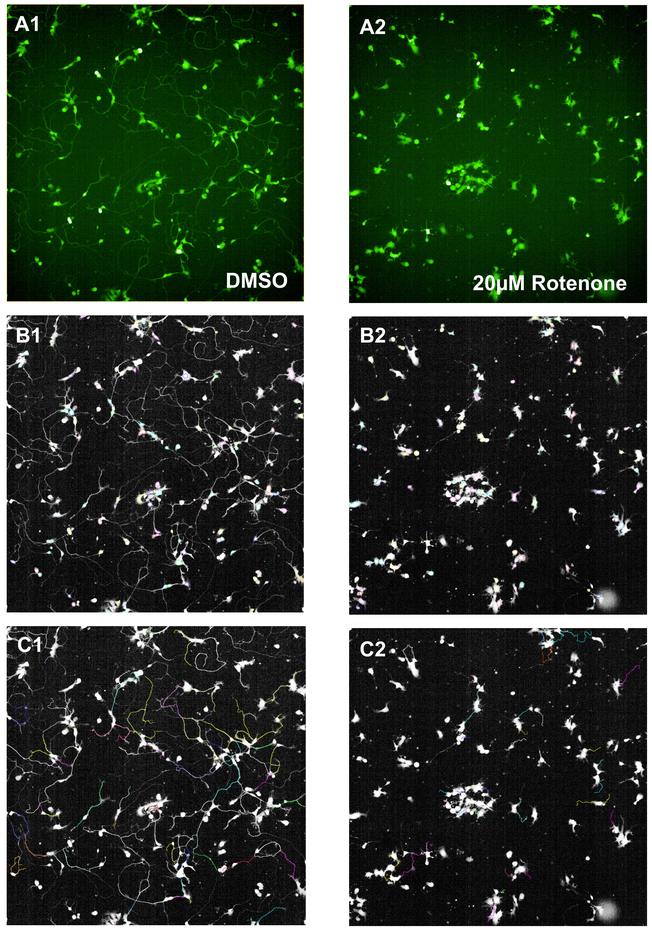


Figure 5. Rotenone Dose-Response at 48 Hours. Images of neurite deterioration on PDL-coated plates with exposure to increasing concentrations of rotenone.

Conclusions

- Assay performance was best with a coating of PDL versus collagen I, fibronectin, or laminin.
- Use of a 10X versus 20X air objective provided faster data collection with similar assay performance.
- Toxin exposure of 48 versus 24 or 72 hours led to a larger response window and higher Z-prime value.
- High-content imaging to screen potential neurotoxins is feasible using normal human neurons.



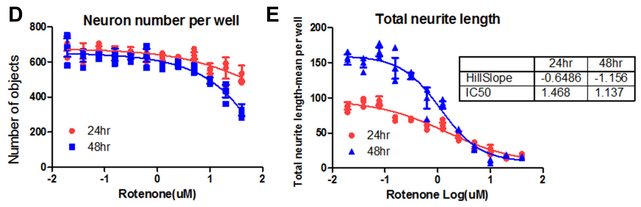


Figure 6. Detection of Neurons and Neurites. Original images (A) were processed to detect cell bodies (B) and neurites (C) for the control (DMSO) and 20 µM rotenone conditions. Dose-response curves for both neuron number (D) and total neurite length per well (E) were determined.

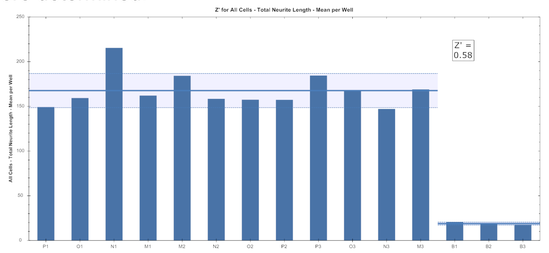


Figure 7. Z-Prime Determination. A Z-prime value of 0.58 was obtained for 48 hours of treatment with 20 µM rotenone. (Value at 24 hours was 0.28.)