3-D Midbrain Floor Plate Model for Differentiation of PSC-derived Dopaminergic Neurons

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ABSTRACT

Accurate in vitro modeling of neurological diseases requires multiple cell types of the brain to interact and develop toward mature functionality. When human pluripotent stem cells (PSC) undergo neural differentiation in 3-D, self-organization of progeny cells results in organoids with brain-like structures and functions that are not observed in 2-D culture. However, the increased complexity of neural organoids often comes with the costs of low throughput and poor reproducibility. Disease models for drug discovery may therefore have to temper self-organized complexity with inductive specification of desired cell types.

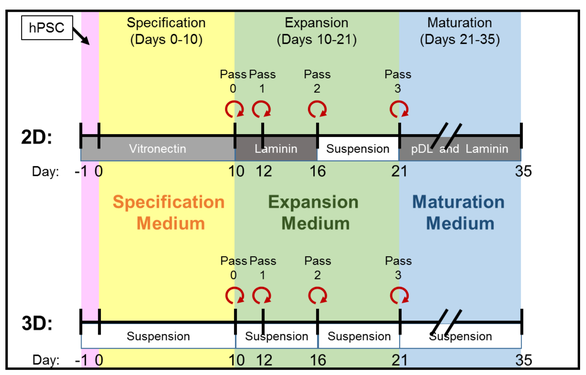
To model Parkinsons Disease (PD), we have developed a method for differentiation of human PSC to midbrain dopaminergic (DA) neurons that combines elements of 2-D dissociated culture and 3-D organoid culture. Cells are efficiently specified as midbrain floor plate (FP) in 2-D via an established protocol using a combination of small molecules and growth factors. FP cells are then seeded into suspension culture in defined numbers for spheroid formation and expansion, then maintained in suspension for two to five weeks of differentiation. Early differentiation of the 3-D cultures is marked by morphological change and the appearance of NurrI- and tyrosine hydroxylase (TH)-expressing DA neurons at the organoid surface. Single-cell analysis demonstrates that many neurons co-express Sox6+ TH+ and thus resemble midbrain DA neurons of the Substantia Nigra pars compacta (SNc). Replating of spheroids on extracellular matrix results in neurite outgrowth and outward migration of DA neurons. Multielectrode array (MEA) recording of replated spheroids shows spontaneous burst activity within a relatively short time, followed by gradual refinement toward coordinated rhythmic bursting.

In short, a hybrid 2-D/3-D culture system for iPSC-derived midbrain floor plate improves maturation of DA neurons and makes promising steps toward a reproducible in vitro disease model for Parkinsons.

INTRODUCTION

To be useful for drug screening, in vitro disease models must be reproducibly generated at large scale. As a first step toward this goal we tested whether spheroid models of the ventral midbrain could be produced using the simple and scalable Gibco™ PSC Dopaminergic Differentiation Kit. Differentiation in 2-D and 3-D were carried out in parallel, initially keeping the 3-D workflow as similar as possible to the optimized 2-D schedule of passages and medium changes.

Figure 1. Similar Workflows for 2-D and 3-D Floor Plate Differentiation



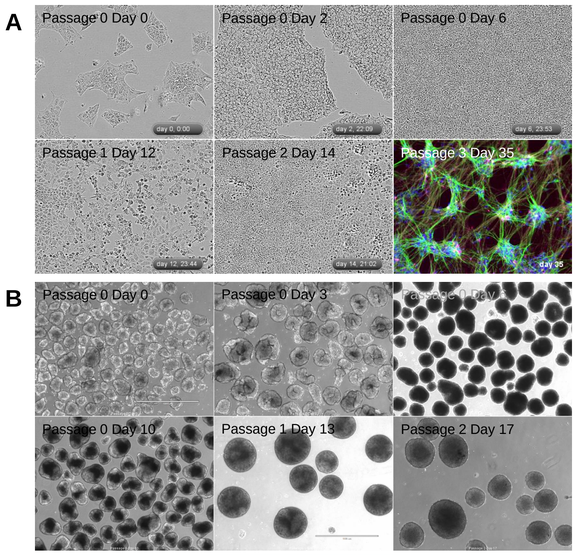
Schematic of the Floor Plate derivation process using the three media in the Gibco™ PSC Dopaminergic Differentiation Kit.

2-D specification occurs in attached culture on Vitronectin, followed by two passages onto Laminin. At Day 16, dissociated cells may be cryopreserved if desired. After Day 16, the Floor Plate cells are expanded in suspension culture for five days then passaged onto pDL/laminin for final maturation.

3-D Floor Plate differentiation follows the same schedule as 2-D, except that the hPSC are placed in suspension prior to specification and repassaged into suspension for the expansion steps at Days 10, 12, and 16.

RESULTS

Figure 2. Floor Plate Yield is Superior in 2-D Culture



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Specification in:	Lines	Starting Cell #	Passages	Average Bank Size (n=4)
2-D	2 x iPSC lines	0.75 - 1.0 e6	2	258.8 ± 12.2 e6
3-D	2 x iPSC lines	0.6 e6	2	9.5 ± 5.4 e6

A) Floor plate differentiation of hPSC in monolayer culture. Numbering of days and passages correspond to the schematic in Figure 1.

B) Floor plate differentiation of hPSC in suspension culture. Numbering of days and passages correspond to the schematic.

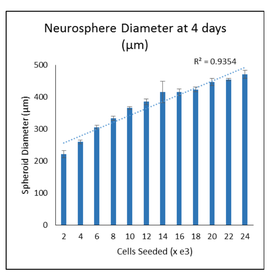
C) Yield of cryopreserved Floor Plate cells from monolayer (n=4) or suspension (n=4) cultures. Floor Plate cells from both methods are >80% double-positive for FoxA2 and Otx2 (data not shown).

- **2-D Specification of Midbrain Floor Plate is much more amenable to scale-up and banking than 3-D culture.**

Figure 3. Thawing Banked Cells into Nunclon™ Sphera™ Produces Consistent Spheroid Size

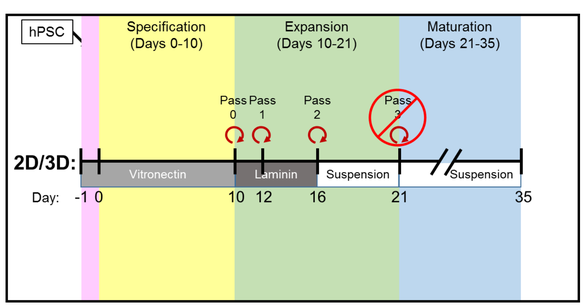
Pilot experiment in which hPSC-derived Neural Stem Cells were thawed into Nunclon™ Sphera™ 96U-well plates. 4 days later, a single NSC spheroid had formed per well with diameter varying linearly with the cell number input.

Bars indicate average diameter ± SD.



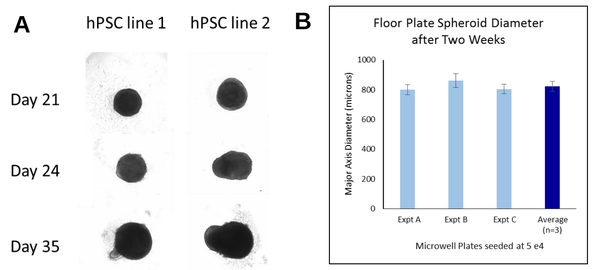
- **Nunclon™ Sphera™ 96U-well plates produce neural spheroids of predictable and uniform size.**

Figure 4. Hybrid 2-D and 3-D DA Neuron Workflow



Schematic of the hybrid 2-D/3-D Floor Plate derivation process using the three media in the Gibco™ PSC Dopaminergic Differentiation Kit. Floor Plate cells were derived in attached culture through Day 16, at which point they were cryopreserved and banked. Day 16 Floor Plate cells were thawed directly into suspension culture in Nunclon™ Sphera™ 96U-well plates and never re-passaged. These banks generated 3-D spheroid cultures for all the following data.

Figure 5. 3-D Spheroid Growth and Morphology is Consistent

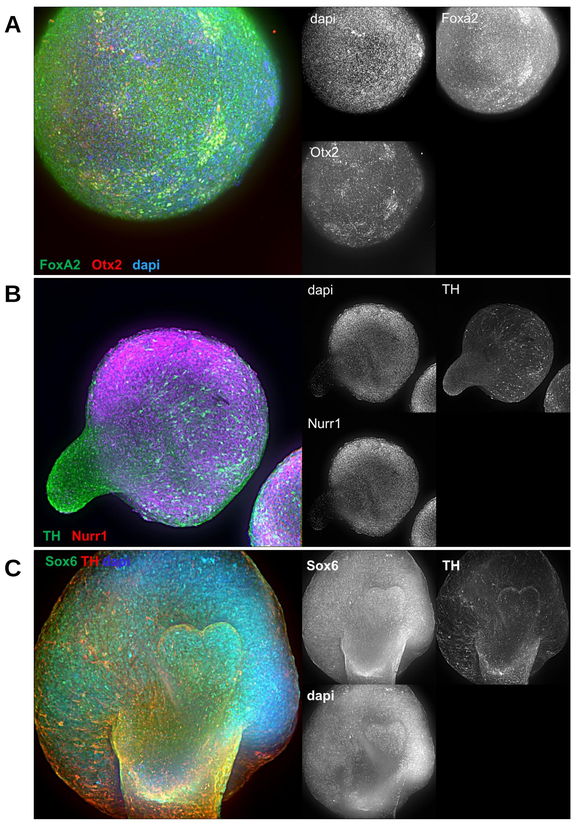


A) Examples of Floor Plate spheroids expanded in suspension in 96-well U-bottom plates. Greater than 75% of Floor Plate spheroids in unagitated plates transiently form a single large projection between Day 26 and Day 35.

B) hPSC-derived Floor Plate forms spheroids of highly regular size in Nunclon™ Sphera™ 96U-well plates. Bars indicate average diameter ± SD.

- **Nunclon™ Sphera™ 96U-well plates produce Floor Plate spheroids of predictable and uniform size and shape.**

Figure 6. Floor Plate Spheroids Produce Midbrain Dopaminergic Neurons



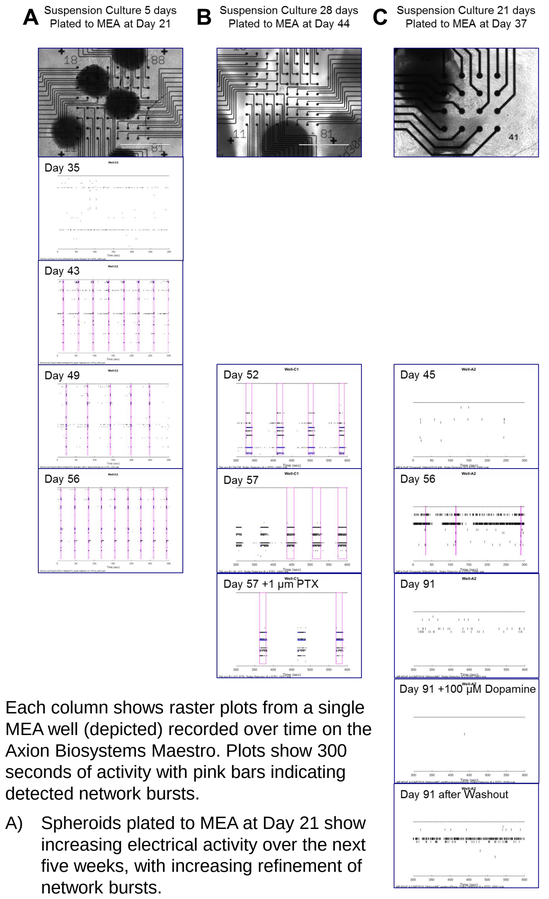
A) Maximal intensity projection of wholemount day 35 Floor Plate Spheroid stained for floor plate markers FoxA2 and Otx2. Imaged on the CellInsight™ CX7 LZR High Content Analysis Platform.

B) Maximal intensity projection of day 35 Floor Plate Spheroid stained for DA progenitor marker Nurr1 and DA neuron marker tyrosine hydroxylase (TH).

C) Maximal intensity projection of day 35 Floor Plate Spheroid stained for Sox6 (DA progenitors and SNc DA neurons) and DA neuron marker TH. Cleared with CytoVista™ 3-D Cell Culture Clearing Reagent and imaged on the CellInsight™ CX7.

- **Floor Plate spheroids at Day 35 are composed of both DA neuron progenitors and DA neurons similar to those of the Substantia Nigra.**

Figure 7. Floor Plate Spheroids Mature Functionally



Each column shows raster plots from a single MEA well (depicted) recorded over time on the Axion Biosystems Maestro. Plots show 300 seconds of activity with pink bars indicating detected network bursts.

A) Spheroids plated to MEA at Day 21 show increasing electrical activity over the next five weeks, with increasing refinement of network bursts.

B) Spheroids plated to MEA at Day 44 can show network activity by 8 days later, but still at more than 7 weeks in culture total. Plating of mature spheroids is much less consistent than immature ones. The observed activity is not sensitive to Picrotoxin.

C) Spheroids plated to MEA at Day 37 show network activity at about 7 weeks in culture total. Activity can be maintained in some cases as far as 13 weeks. Silencing in the presence of 100 µM Dopamine demonstrates that the recorded activity is due to DA neurons.

- **Electrophysiological activity of hPSC-derived DA neurons matures over time at similar rates in suspended or attached Floor Plate spheroids.**

CONCLUSIONS

- A Hybrid 2-D Specification with 3-D Maturation method is amenable to scale-up and banking of Midbrain Floor Plate progenitors.
- Banking of Floor Plate progenitors allows the reproducible formation of Midbrain-like spheroids.
- Dopaminergic neurons differentiate and reach functional maturity within Midbrain-like spheroids.
- Dopaminergic spheroids mature electrophysiologically at similar rates whether cultured in active suspension or attached to the MEA surface.

TRADEMARKS/LICENSING