

Establishing and Functional Characterization of 3D Neural Spheroid Models from Monolayer Expanded NSCs

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INTRODUCTION

The expanding application of 3D culture methods to generate stem cell derived models of neuronal development, maturation and disease enables the creation of more complex cellular models which more faithfully recapitulate in vivo neural architectures and physiology than traditional 2D cultures.

Here we evaluated the differentiation and functional maturity of NSCs (neural stem cells) generated and expanded in monolayer, and then transferred to conditions which promote 3D spheroid formation. Using a range of analysis tools we demonstrated that our Sox2/Nestin positive NSCs are capable of forming 3D spheroids that could be matured to generate electrically active neurons with robust expression of genes related to functional synapses and neurotransmitter trafficking

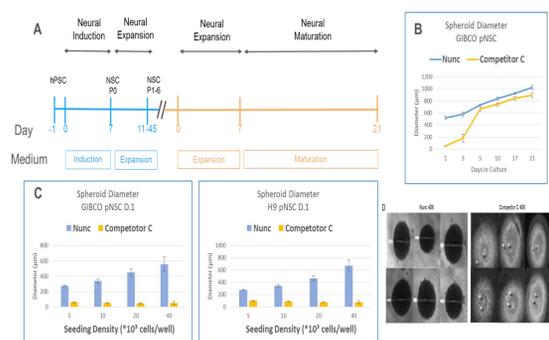
Currently, many approaches to create 3D models rely on reagents and tools designed for 2D monolayer systems. Incorporating multiple reagents from different vendors into the organoid workflow raises compatibility questions for many researchers. In our study, multiple specialty culture ware, media combinations, culture conditions and reagents were assessed for optimal differentiation of NSCs to neurons and the influence these many variables had on proliferation, gene expression and maturation.

Our results clearly demonstrate the feasibility of transitioning from a 2D NSC culture system to 3D and the importance of optimizing several key culture system parameters in order to reproducibly generate neural spheroids and influence the rate of maturation. Defining the relative benefits of modeling and analyzing neural biology in 2D versus 3D is an area of active investigation in which our study highlights several points of consideration.

MATERIAL AND METHODS

Figure 1. Establishing Neural Spheroid Models

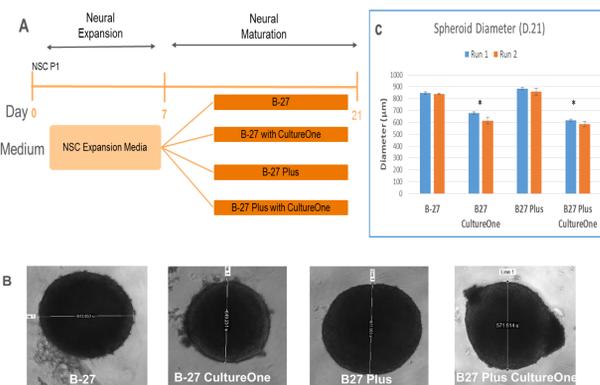
GIBCO Human Episomal iPSC Line (Cat No. A18945) or H9 hESC were differentiated to NSC using Gibco® PSC Neural Induction Medium (Cat No. A1647801). Cells at P6 were cryopreserved and upon thaw seeded into Neural Expansion media in Nunclon™ Sphera™ Microplates, 96U-Well Plate (Cat No. 174925). After 7 days in culture spheroids were transitioned to maturation media consisting of either Neurobasal™ Medium (Cat No. 21103049) with B-27™ Supplement (Cat No. 1750404) or the B-27™ Plus Neuronal Culture System (Cat No. A3653401) with or without the addition of CultureOne™ Supplement (Cat No. A3320201)



(A) Workflow for generating and maturing neural spheroid models. (B & D) Compared to competitor products, NSCs seeded in Nunclon™ Sphera™ plates rapidly establish uniformly sized spheroids. (C) NSC generated from two PSC lines (iPSC left panel, ESC right panel) seeded at a range of densities formed single spheroids within 24 hours. (D) Phase contrast images of neural spheroids at 1 day in culture captured on EVOS™ FL auto. Equivalent exposure settings used for both plates, differences in plate dimensions affects image brightness

RESULTS

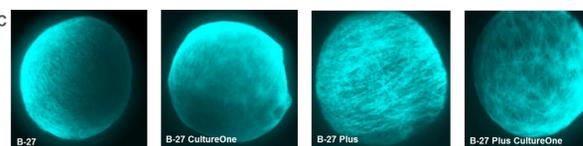
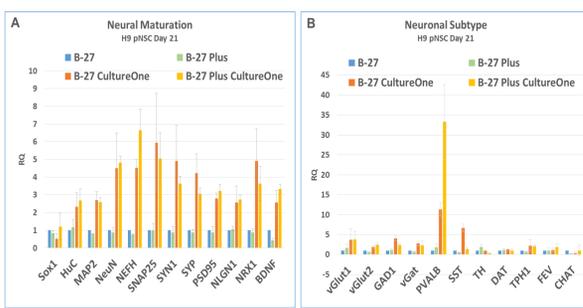
Figure 2. Maturation of Neural Spheroids



(A) Maturation workflow, after 7 days in culture spheroids were transitioned to 4 different maturation media (indicated). (B) Phase contrast images of neural spheroids at 21 days in culture captured on EVOS™ FL auto show morphological changes observable in spheroids cultured in the presence of CultureOne. (C) Spheroid diameter using EVOS™ FL Auto measurement tools from two independent experiments. Differences observed with addition of CultureOne™ is statistically significant (students t-Test, p-value <0.001)

- Maturation media containing either “classic” B-27™ or B-27™ Plus yielded spheroids of similar size and shape
- Addition of CultureOne™ results in smaller spheroids with a more compact morphology- likely due to reduced progenitor proliferation

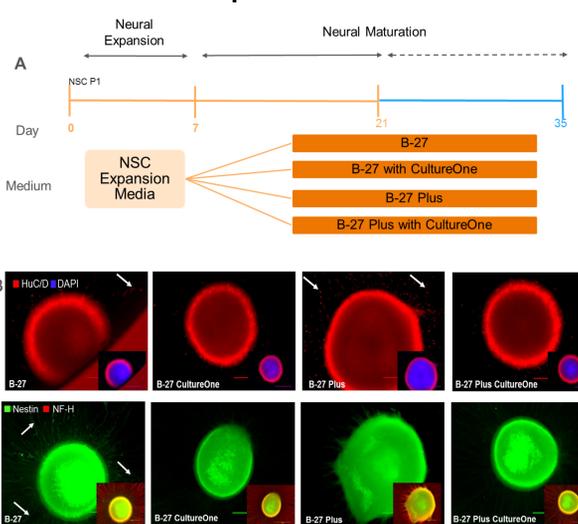
Figure 3. Characterization of Neural Spheroids



Gene expression analyses of neural maturation (A) and subtype markers (B) from spheroids at 21 days in culture assessed using TaqMan® assays. (C) Spheroids stained with Tubulin Tracker™ Deep Red (Cat No T34077) to label neuronal processes, images acquired using CellInsight™ CX7 LZR HCA Platform.

- Addition of CultureOne™ elevates expression level of genes related to functional synapses and neurotransmitter trafficking.
- Culture in B-27™ Plus and CultureOne™ resulted in high levels of PVALB expression, suggesting increased numbers of this subtype of GABAergic neurons
- Spheroids matured in the B-27™ Plus CultureOne™ media system appear to contain denser and more structured neurite networks.

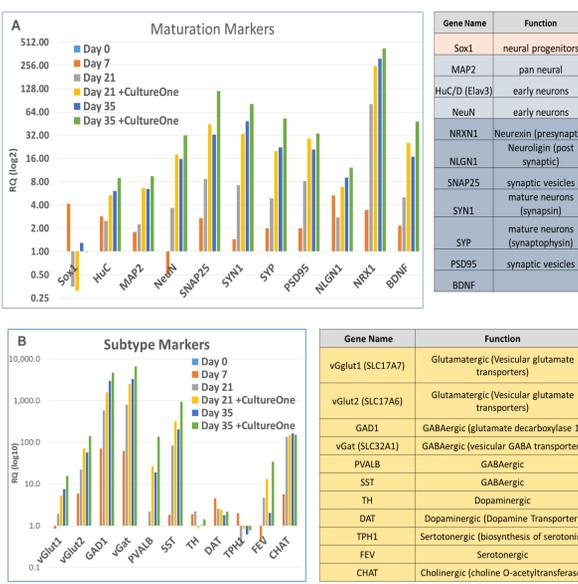
Figure 4. 2D Attachment and Outgrowth from Adherent Spheroids



(A) Alternative maturation workflow, after 14 days of maturation in suspension, spheroids were transferred to poly-D-Lysine and Laminin coated wells and allowed to attach. Spheroids were matured for a further 14 days then fixed for downstream imaging. (B) Immunostaining using HuC/D antibody (red; pan-neuronal maker), nuclei are counterstained with DAPI (blue); see white arrows; to assess migration of neurons away from spheroid, and (C) Nestin (green), Neurofilament Heavy (red, NF-H) inset image, marks mature neuronal cells

- Addition of CultureOne™ reduces migration of neurons away from the adherent spheroids.
- Spheroids matured in the B-27™ media system retain a greater amount of immature neuronal cells.

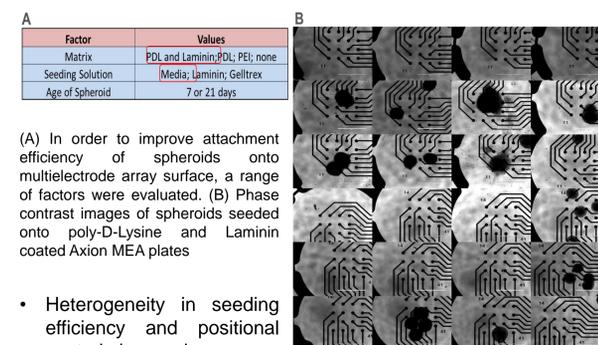
Figure 5. Tracking of Neural Spheroid Maturation



Time course analysis of neural maturation (A) and subtype markers (B) from spheroids at 0, 7, 21 and 35 days in culture. Gene expression levels assessed using TaqMan® assays. See Figure 4 A for workflow outline. Note- B-27™ and B-27™ Plus samples were pooled for RNA analysis.

- Spheroids mature over time with elevated levels of mature and functional synaptic markers
- Majority of neurons mature into Glutamatergic and GABAergic neurons.
- CultureOne™ treated spheroids maintain elevated expression level of genes related to functional synapses and neurotransmitter trafficking

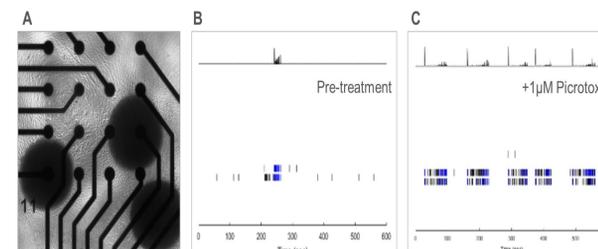
Figure 6. Seeding Neural Spheroids on Multi-Electrode Array plates



(A) In order to improve attachment efficiency of spheroids onto multielectrode array surface, a range of factors were evaluated. (B) Phase contrast images of spheroids seeded onto poly-D-Lysine and Laminin coated Axion MEA plates

- Heterogeneity in seeding efficiency and positional control observed

Figure 7. Functional Assessment of Neural Spheroids



Multi-electrode array (MEA) analysis using the Axion Biosystems Maestro platform (A) Phase contract image of neural spheroid attached to MEA surface. Raster plots of spiking activity (B) before and (C) after treatment with 1µM Picrotoxin (GABA antagonist)

- Neural spheroid are electrically active and contain a mixed population of excitatory and inhibitory neurons

CONCLUSIONS

- We established the compatibility of existing reagents to generate 3D neural spheroid cultures
- Addition of CultureOne™ Supplement modulates the rate of proliferation and maturation of Neural Spheroids
- Spheroids matured in the B-27™ Plus media system develop complex neurite networks and are electrically active
- Transitioning spheroids onto MEA wells remains a challenging step in the workflow

TRADEMARKS/LICENSING

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