

Neurosphere Formation, Differentiation, and Migration of Human Neural Stem Cells Cultured in Corning® Spheroid Microplates

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Application Note

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Introduction

Neurosphere formation of human neural stem cells (hNSCs) is a widely used *in vitro* culture system and valuable model to study neurogenesis and neural development. This system allows for three-dimensional (3D) expansion of hNSCs within a more physiologically relevant microenvironment. Having an easy-to-use, reproducible neurosphere culture and analysis method for studying hNSC proliferation, migration, and neurotoxicity greatly enables their use for drug discovery and cell therapy applications. In this study, Corning spheroid microplates were used for neurosphere formation, proliferation, and migration of hNSCs in an easy-to-use format that is amenable to high throughput screening. The spheroid microplates were used for neurosphere culture of hNSCs over the course of 96 hours, throughout which multipotency was maintained as assessed through Nestin and SOX2 marker expression, followed by subsequent harvesting of neu-

rospheres for differentiation into neuronal, astrocytic, and oligodendrocytic lineages. Imaging of two-dimensional (2D) and 3D cultured hNSCs, in addition to analysis of spheroid size and quantification of migration, was accomplished with the Cytation™ 5 Cell Imaging Multi-Mode Reader and Gen5™ Data Analysis Software (BioTek Instruments, Inc.).

Materials and Methods

Neurosphere Formation and Proliferation

Cryopreserved human neural stem cells (Life Technologies Cat. No. N7800-100) were thawed, plated, and expanded per the vendor's recommended protocol on poly-L-ornithine (Sigma Cat. No. P3655) with a laminin (Sigma Cat. No. L2020) overlay. Cells were cultured in KnockOut™ DMEM/F-12 Basal Medium with StemPro® Neural Supplement, FGF Basic, and EGF (Life Technologies Cat. No. A10509-01). After at least 1 passage, hNSCs were harvested using Accutase® cell detachment solution (Corning Cat. No. 25-058-CI) and seeded into four 96-well Corning spheroid microplates (Corning Cat. No. 4520) at concentrations ranging from 1,000 to 32,000 cells/well in 200 µL of media per well. Neurospheres were maintained for 96 hours in a humidified 37°C 5% CO₂ incubator

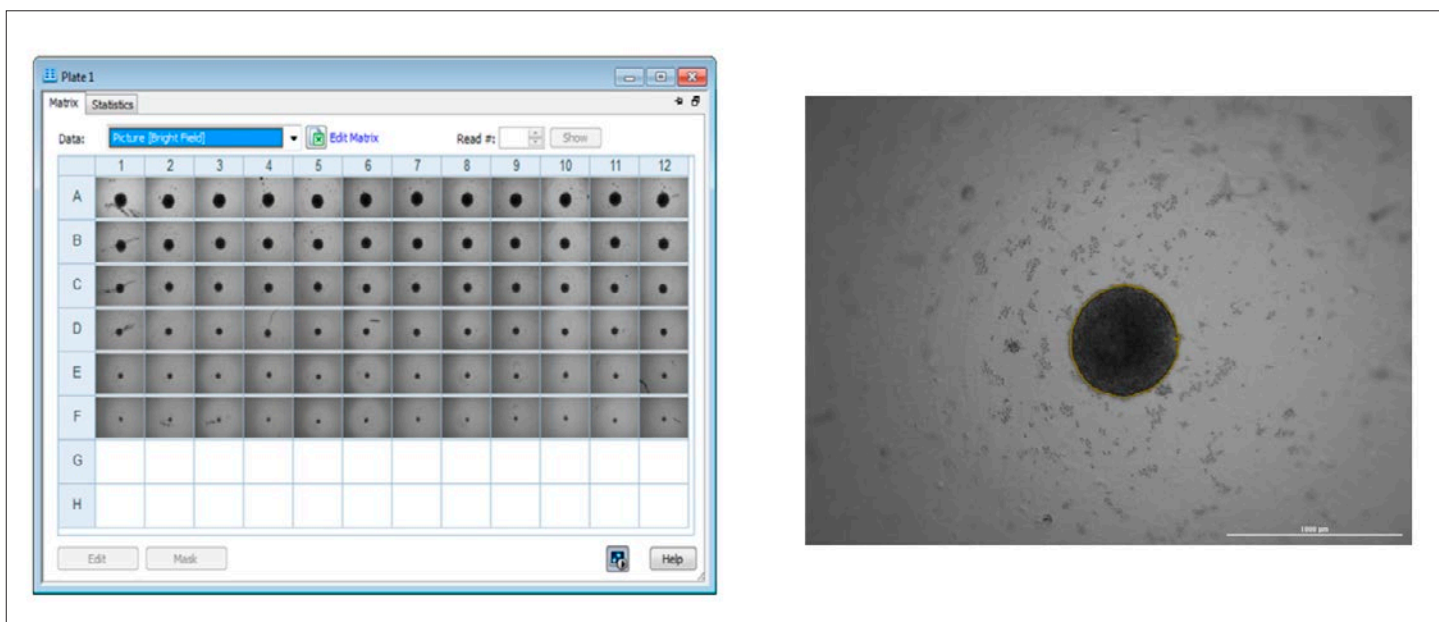


Figure 1. Screenshot of single spheroids of various sizes in each well of Corning spheroid microplate as imaged by Cytation 5 (left). Gold highlighted, spheroid cellular analysis object mask used to determine spheroid size (right).

with complete media changes every other day. Each day, one plate was imaged for neurosphere diameter using the Cytation 5 by creating a cellular analysis object mask via the Gen5 data analysis software (Figure 1). After imaging, spheroids were dissociated by incubating with 100 μ L of Accutase per well, for 1 hour at 37°C before being collected. Three wells per cell concentration were pooled for cell enumeration analysis using the MACSQuant[®] flow cytometer (Miltenyi Biotech). To confirm neurosphere proliferation, Ki-67 expression was assessed. Neurospheres cultured for 96 hours were harvested as described above and fixed with 70% cold ethanol. After 1 hour at 4°C, the cells were washed and stained following the vendor's recommended protocol with either Fluorescein isothiocyanate (FITC), anti-mouse Ki-67 (BioLegend Cat. No. 652410), or FITC rat IgG2a, κ isotype control (BioLegend Cat. No. 400506). Assessment then followed via flow cytometry.

Multipotency and Differentiation

To confirm maintenance of neurosphere multipotency, Nestin, and SOX2 expression were assessed via flow cytometry. Neurospheres were harvested as described previously, and fixed in 4% paraformaldehyde (Boston BioProducts Cat. No. BM-698) prior to staining with Nestin, SOX2, or appropriate isotype control (Table 1). Further, the harvested neurosphere cells were also plated onto 384-well high content imaging microplates (Corning Cat. No. 4681) coated with poly-L-ornithine with a laminin overlay. Cells for neuronal differentiation were seeded at 55,000 cells/ cm^2 , while cells for astrocytic and oligodendrocytic differentiation

were seeded at 40,000 cells/ cm^2 in standard growth medium. After 24 hours, the medium was changed to differentiation medium specific for the desired lineages, and the medium was changed every other day for the duration of culture time (Table 2). Cells were fixed with 4% paraformaldehyde and stained with the appropriate markers, secondary labeled antibodies, or isotype controls (Table 3) following the vendor's recommended protocols. All cells were counter-stained with Hoechst 34580 (Sigma Cat. No. 63493). Stained cells were imaged using the 40x objective of the Cytation[™] 5.

Migration

To assess neurosphere migration, the medium of 96-hour neurosphere cultures was exchanged with medium lacking growth factors. The neurospheres were then transferred to poly-L-ornithine/laminin-coated 96-well high content imaging microplates using Axygen[®] 200 μ L wide bore tips (Corning Cat. No. TF-205-WB-R-S). Distance of cell migration away from the spheroid post-transfer was quantified at various time points using the Cytation 5 and Gen 5[™] software. Specifically, images were stitched together and then an object mask was automatically applied using cellular analysis parameters to measure the sphere diameter. The delta in sphere diameter between time points of 3 spheres was averaged to determine migration distance. After 24 hours, neurospheres were fixed and stained for neurons and astrocytes, as described previously.

Table 1. Multipotency Markers

Antibody/Isotype Control (Dilution Ratio)	R&D Systems Vendor Cat. No.
Nestin fluorescein mouse IgG1 (1:20)	IC1259F
Fluorescein isotype control mouse IgG1 (1:20)	IC002F
SOX2 allophycocyanin mouse IgG2A (1:20)	IC2018A
Allophycocyanin isotype control mouse IgG2A (1:20)	IC003A

Table 2. Differentiation Media

Cell Type	Differentiation Media	Culture Time
Astrocyte	Corning glutagro [™] DMEM (Corning Cat. No.10-101-CV) supplemented with 1% N-2 supplement (Life Technologies Cat. No. 17502-048) and 1% fetal bovine serum (Corning Cat. No. 35-010-CV)	4 - 5 days
Oligodendrocyte	Neurobasal media (Life Technologies Cat. No. 21103-049) supplemented with 2% B-27 supplement (Life Technologies Cat. No. 17504-044), 1x GlutaMAX [™] (Life Technologies Cat. No. 35050-061), and 30 ng/mL of T3 supplement (Sigma Cat. No. T5516).	5 - 6 days
Neuron	Neurobasal with 2% B-27 supplement and 1x GlutaMAX. 1 mM dcAMP (Sigma Cat. No. D0627) was added after 2 days.	7 days

Table 3. Differentiation Markers

Cell Type	Antibody/Isotype Control (Dilution Ratio)	R&D Systems Vendor Cat. No.
Astrocyte	Sheep anti-human GFAP (1 μ g/100 μ L)	965225
	Donkey anti-sheep IgG NorthernLights [™] NL637 (1:200)	NL011
Oligodendrocyte	Anti-A2B5 mouse IgM (1.2 μ g/100 μ L)	MAB1416
	Goat anti-mouse IgM fluorescein, goat IgG (1:20)	F0118
Neuron	Mouse anti-neuron-specific β -III tubulin (0.5 μ g/100 μ L)	964673
	Donkey anti-mouse IgG NorthernLights NL557 (1:200)	NL007

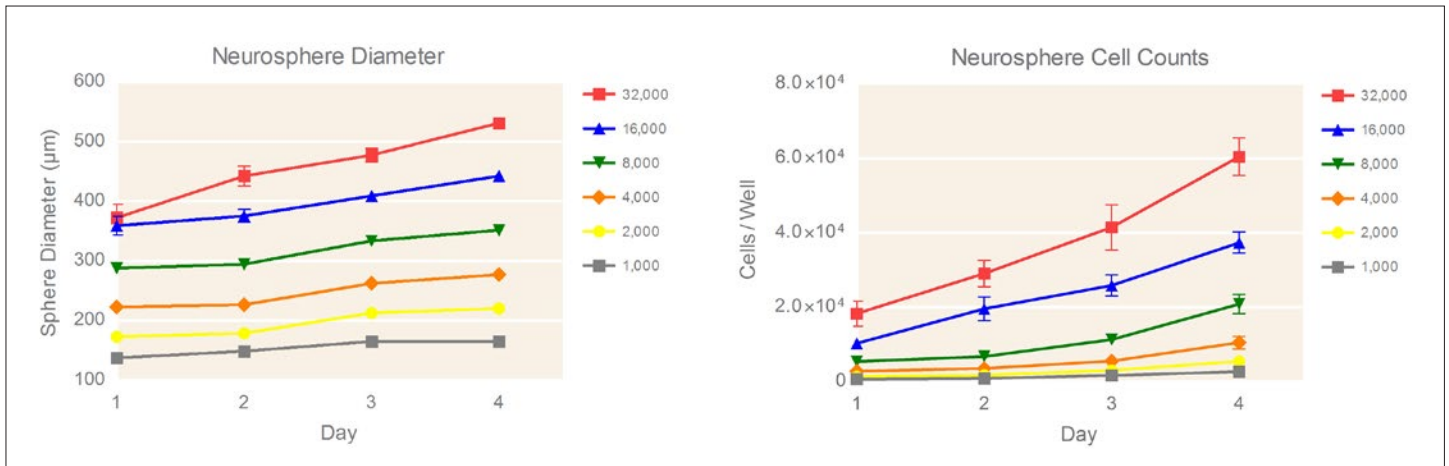


Figure 2. Neurosphere diameter and cell counts from hNSCs seeded at various concentrations (1,000 to 32,000 cells/well) and cultured for 96 hours. n = 24 per concentration for diameter quantification, and n = 6 pooled counts from cell harvest. All data is from 2 independent studies.

Results and Discussion

To assess the ability of Corning® spheroid microplates to form a single, uniform neurosphere in each well, spheroid diameters, as well as cell counts post-harvest were calculated from hNSCs seeded at various concentrations. Over the course of 96 hours, both the spheroid diameter and cell number increased over time with increasing seeding concentrations (Figure 2). To further confirm hNSC proliferation, Ki-67 expression was assessed after 96 hours of neurosphere culture. Ki-67 is a commonly used proliferation marker that is detected at high levels within the nucleus of actively dividing cells¹. Greater than 90% expression of Ki-67 was observed, as assessed by flow cytometry, from cells harvested 96 hours after seeding hNSCs at 16,000 cells/well (Figure 3). To confirm the proliferating hNSCs maintained multipotency, expression of two widely accepted markers for hNSC stemness, Nestin and SOX2, were assessed. Nestin is an intermediate filament protein that is required for hNSC self-renewal, and SOX2 is a transcription factor expressed in multipotent hNSCs^{2,3}. High expression levels of both Nestin and SOX2 were observed in neurospheres after 96

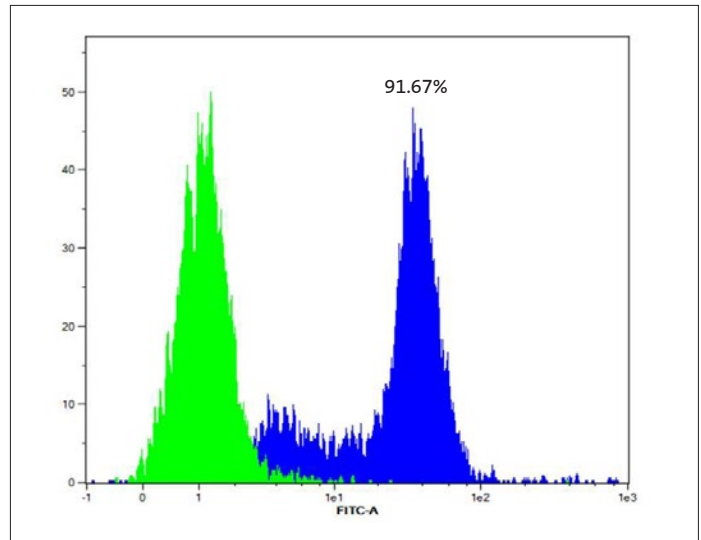


Figure 3. Representative flow cytometry histogram of Ki-67 expression compared to isotype control from neurospheres harvested after 96 hours of growth in Corning spheroid microplates.

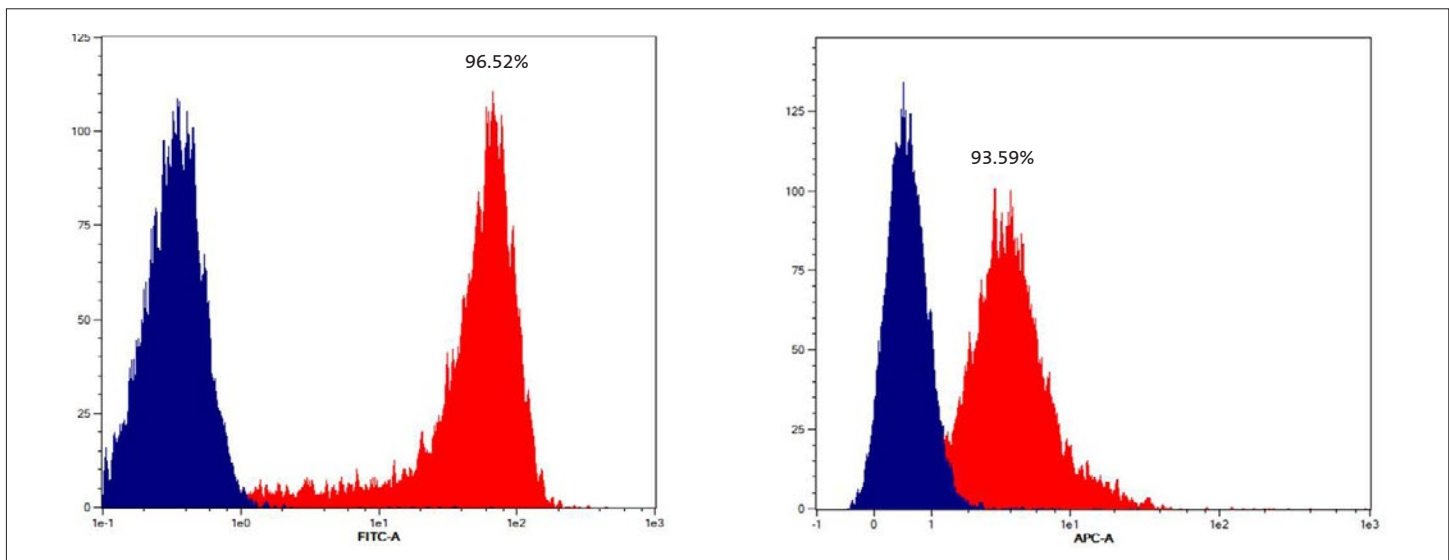


Figure 4. Representative flow cytometry histograms of Nestin (left) and SOX2 (right) expression from neurospheres harvested after 96 hours of growth in Corning spheroid microplates.

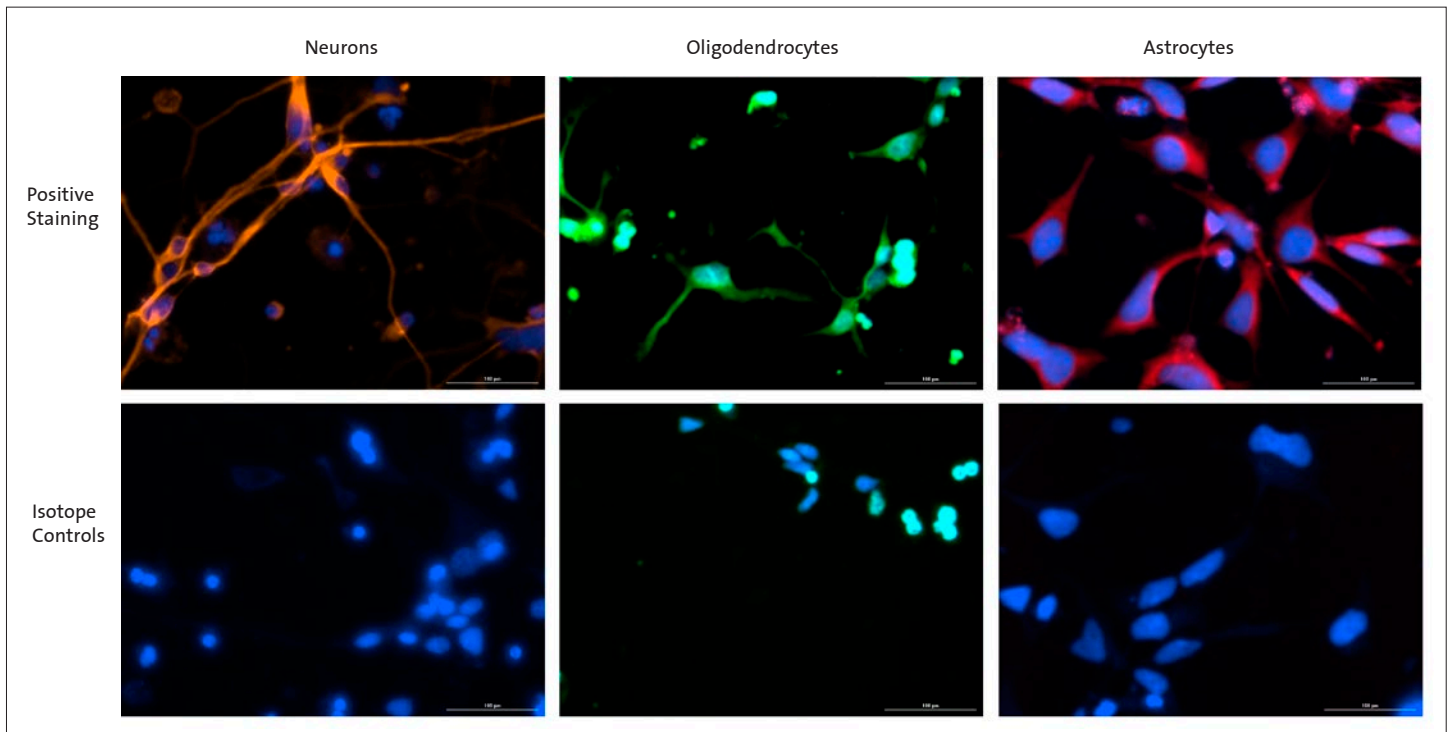


Figure 5. Representative images captured with the Cytation 5 using a 40X objective. Neurons were stained with β -III tubulin or isotype control, oligodendrocytes with A2B5 or isotype control, and astrocytes with human glial fibrillary acidic protein (GFAP) or isotype control. All cellular nuclei were counterstained with Hoechst 34580.

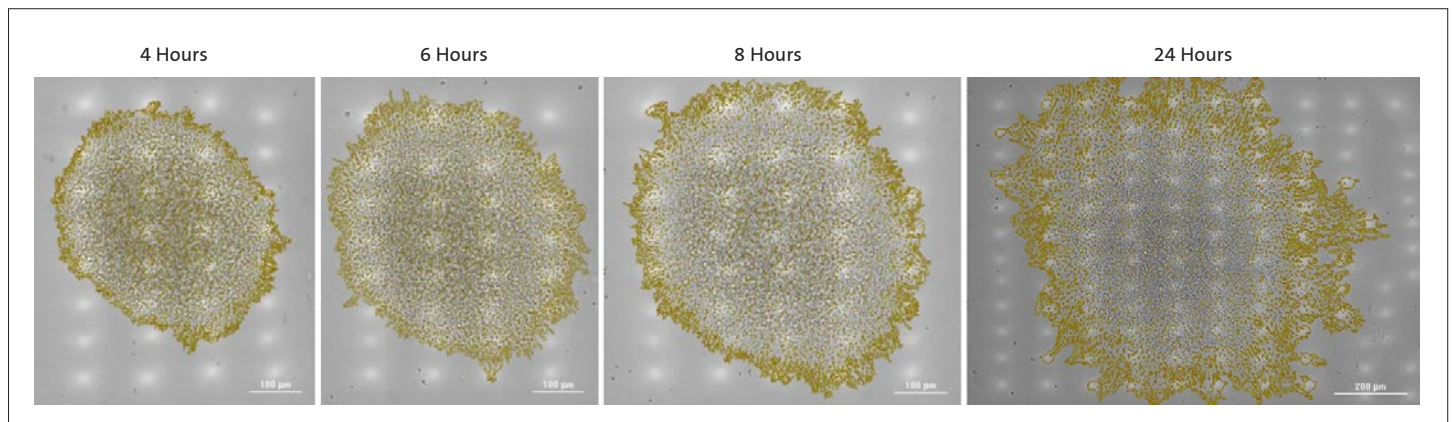


Figure 6. Final Cytation 5 stitched images of hNSC migration away from 96-hour neurospheres, assessed at 3 separate time points, and captured using a 40X objective and image montage. Gold object mask of cell migration was generated using primary and advanced cellular analysis parameters of Gen 5 data analysis software.

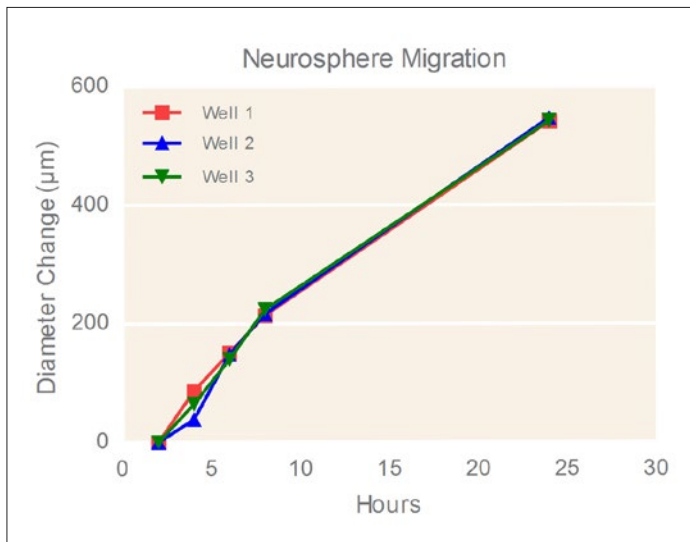


Figure 7. Quantified cell migration distance away from neurospheres over time from 3 representative wells as measured with (the Cytation 5 Cell Imager) Gen 5.

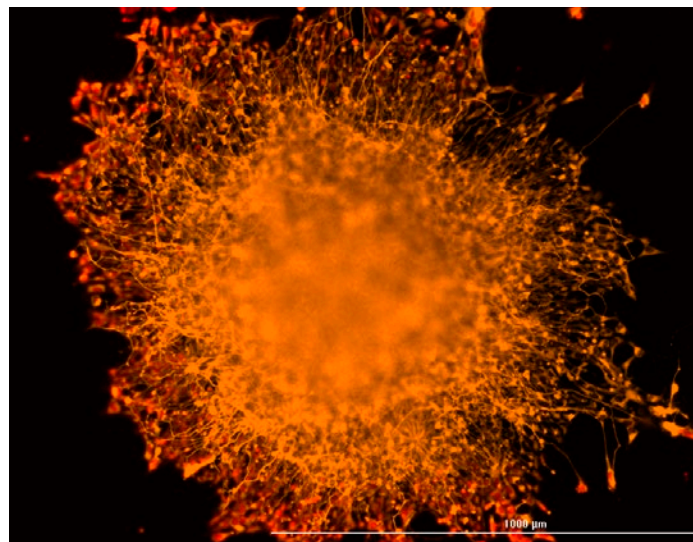


Figure 8. Final stitched image of representative fixed and stained migrated cells 24 hours after neurosphere transfer to poly-L-ornithine/laminin-coated Corning 96-well film bottom microplates for high content imaging. Image montage taken with a 40X objective using the Cytation 5. Astrocytes stained with GFAP (red) and neurons stained with β -III tubulin (orange).

hours in culture (Figure 4). To further confirm multipotency, neurospheres generated in the Corning spheroid microplate were differentiated into appropriate lineages: astrocytes, neurons, and oligodendrocytes. Positive staining for all 3 lineages was observed relative to isotype controls (Figure 5). For migration studies, Axygen® wide bore tips were used to transfer single neurospheres from the spheroid microplates to high content imaging microplates. The Cytation 5 was used to capture images of cells migrating away from the neurosphere over a 24-hour period (Figure 6). Additionally, by utilizing the Gen 5 software, acquired images were analyzed to quantify the cell migration over time (Figure 7). After 24 hours of migration, neurospheres stained positively for neuronal and astrocytic populations (Figure 8).

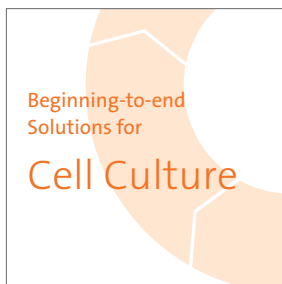
Conclusions

- ▶ Corning 96-well spheroid microplates are an easy to use and ideal tool for generating consistent and reproducibly sized, single neurospheres per well with hNSCs.
- ▶ Neurospheres produced in the Corning® 96-well spheroid microplate maintain a consistent growth rate and proliferation capacity, as well as continual expression of multipotency markers Nestin and SOX2.

- ▶ hNSCs cultured in the Corning 96-well spheroid microplate maintain the ability to differentiate into astrocytes, oligodendrocytes, and neurons
- ▶ The Cytation 5 and Gen5 Data Analysis Software can easily image neurospheres in spheroid or high content imaging microplates using high magnification, as well as accurately quantify changes to the 3D structures.
- ▶ Together, Corning 96-well spheroid microplates, Axygen wide bore tips, Corning 96-well film bottom microplates for high content imaging and the BioTek Cytation 5 Cell Imaging Multi-Mode Reader comprise a valuable system for studying neurosphere proliferation, migration, and differentiation.

References

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