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

Hilary Sherman[1], Brad Larson[2], Hannah J. Gitschier, M.S.[1], and David H. Randle, Ph.D.[1]
[1]Corning Incorporated, Life Sciences, Kennebunk, ME USA; [2]BioTek Instruments, Inc., Winooski, VT USA

Abstract

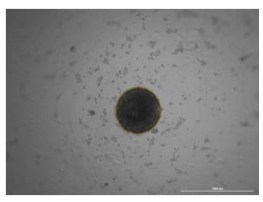
Neurosphere formation of neural stem cells (NSCs) is a widely used *in vitro* culture system and valuable model to study neurogenesis and neural development. This system allows for three dimensional expansion of NSCs within a more physiologically-relevant microenvironment. Having an easy to use, reproducible neurosphere culture and analysis method for studying NSC proliferation, migration, and neurotoxicity greatly enables their use for drug discovery and cell therapy applications. In this study, the Corning® spheroid microplate was used for neurosphere formation, NSC proliferation, and migration in an easy-to-use format that is amenable to high throughput screening. In this study, the spheroid microplates allow for NSC culture of uniform and directed neurosphere size over the course of 96 hours, throughout which multipotency is maintained, as assessed through Nestin and SOX2 marker expression. Analysis of spheroid size and quantification of migration was accomplished with the BioTek® Cytation™ 5 Cell Imaging Multi-Mode Reader. Further, differentiation of NSCs into neuronal, astrocytic, and oligodendrocytic lineages was achieved via harvesting neurospheres and plating in high content imaging microplates with growth factors removed.

Methods

- Human neural stem cells (hNSCs) and medium (Life Technologies Cat. No. N7800-200) were used to seed Corning 96-well spheroid microplates (Corning Cat. No. 4520) at various concentrations for a 96-hour culture. Media changes were performed every other day.
- Spheroid size was assessed daily using the Cytation 5 and cells were harvested with Accutase® (Corning Cat. No. 25-058-CI) and analyzed on the MACSQuant® flow cytometer (Miltenyi Biotech) to achieve total cell counts.
- Proliferation capacity was quantified by staining harvested cells for Ki-67 protein (BioLegend Cat. No. 652410 and 400506) using the MACSQuant flow cytometer.
- Multipotency was assessed via flow cytometry for expression of Nestin (R & D Systems Cat. Nos. IC1259F and IC002F) and SOX2 (R & D Systems Cat. Nos. IC2018A and IC003A) using hNSCs that were seeded at 16,000 cells/well and cultured as neurospheres for 4 days.
- Neurosphere differentiation to astrocytes, oligodendrocytes, and neurons were achieved by seeding harvested cells onto Corning 384-well film bottom microplates for high content imaging (Corning Cat. No. 4681) coated with Poly-L-Ornithine/Laminin (PLO/Laminin), then fixing and staining with appropriate fluorescently labeled antibodies.
- To asses neurosphere migration, spheroids seeded at 4,000 cells/well were grown for 4 days before being transferred to PLO/Laminin-coated Corning 96-well film bottom microplates for high content imaging (Corning Cat. No. 4680) using Axygen® wide bore 200 µL tips (Corning Cat. No. TF-205-WB-R-S). Growth factors were removed and the Cytation 5 was used to quantify migration over the course of 24 hours using a 40X objective and image stitching. After 24 hours, cells were fixed and stained to assess differentiation.

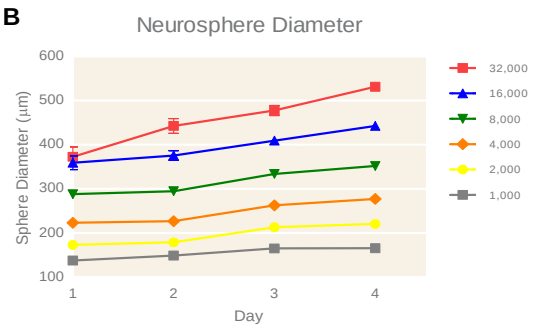
hNSC Proliferation, Neurosphere Size, and Multipotency

A

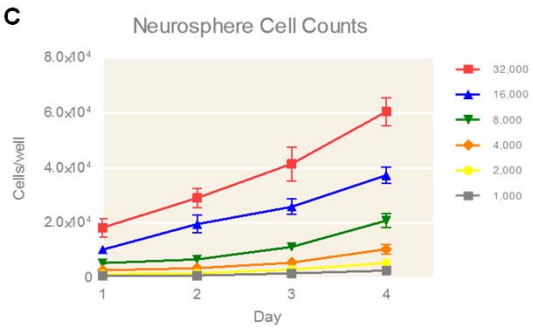


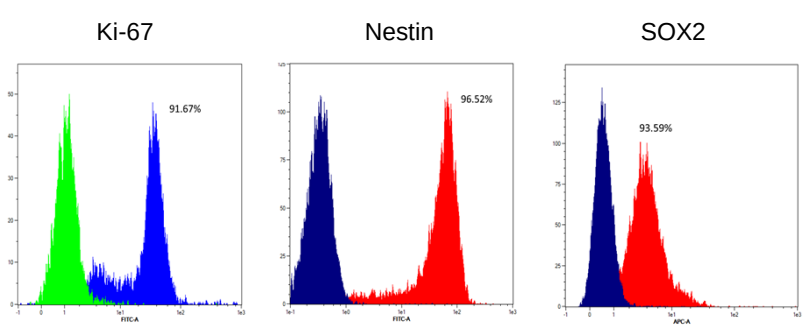
hNSC yields and neurosphere diameter increase with culture duration as calculated by Cytation 5. A) 4X photomicrograph of a neurosphere formed in a Corning 96-well spheroid microplate showing a cellular analysis object mask created using Gen5™ data analysis software (BioTek). Neurosphere diameter (B) and cell count yields (C) over time. Data is average from 2 independent studies.

B



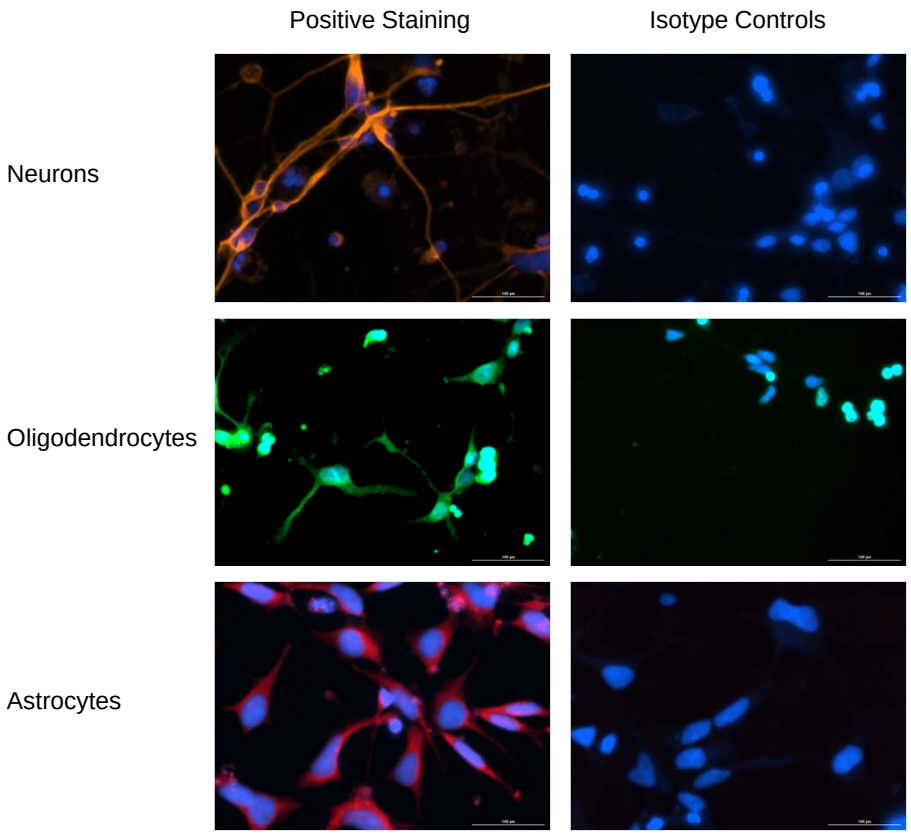
C





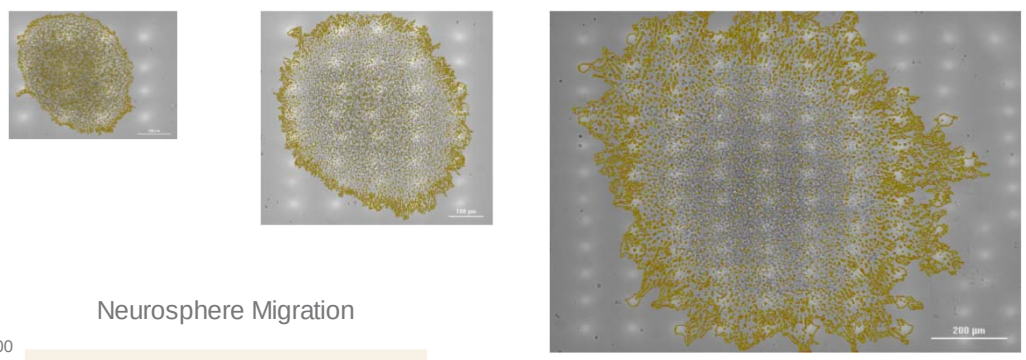
hNSCs maintain high proliferation and multipotency marker expression in neurosphere culture. Representative histograms of Ki-67, Nestin, and SOX2 expression from neurospheres harvested after 4 days of growth. Ki-67 proliferation maker expression was 91.67%, Nestin hNSC multipotency marker expression was 96.52%, and SOX2 marker expression was 93.59%.

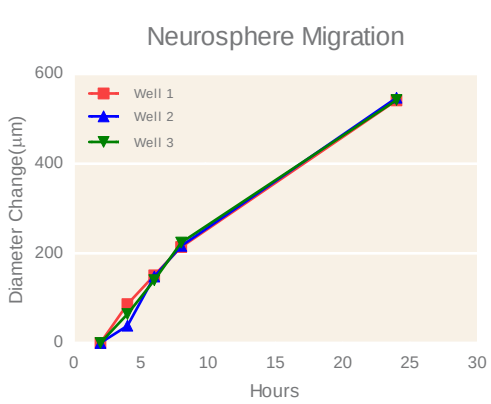
Differentiation of Neurosphere Derived Cells



Differentiation of hNSCs harvested from neurospheres. 40X images captured using the Cytation 5. Neurons were stained with neuron specific ß-III Tubulin or isotype control (R & D Systems Cat. Nos. 964673 and NL007). Oligodendrocytes were stained with A2B5 or isotype control (R & D Systems Cat. Nos. MAB1416 and NL020). Astrocytes were stained with human glial fibrillary acidic protein (GFAP) or isotype control (R & D Systems Cat. Nos. 965225 and NL011). Nuclei counterstained with Hoechst 34580.

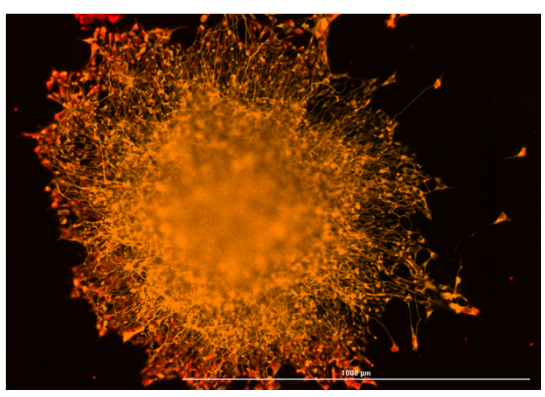
Neurosphere Migration





Migration of cells away from a neurosphere over the course of 24 hours. Above: Stitched images of migration at 3 separate time points captured using a 40X objective. Gold object mask of cell migration generated using primary and advanced cellular analysis. Left: Quantified cell migration distance away from neurospheres over time from 3 wells measured with the Cytation 5 cell imager.

Neuron and astrocyte migration away from a neurosphere after 24 hours. Stitched images of migration taken with a 40X objective showing astrocytes stained with GFAP (red) and neurons stained with neuron specific ß-III Tubulin (orange) using the Cytation 5 cell imager 24 hours after neurosphere transfer to a PLO/Laminin-coated Corning 96-well film bottom microplate for high content imaging.



Conclusions

- Corning 96-well spheroid microplates are an ideal tool for generating consistent, single hNSC spheroid per well cultures.
- Neurospheres produced in the Corning 96-well spheroid microplate maintain a consistent growth rate and proliferation capacity, as well as continued expression of multipotency markers Nestin and SOX2.
- hNSCs cultured in the Corning 96-well Spheroid Microplates maintain their ability to differentiate into astrocytes, oligodendrocytes, and neurons.
- Corning 96-well spheroid microplate, Axygen wide bore tips, Corning 96-well film bottom microplates for high content imaging, and the BioTek Cytation cell imaging multi-mode reader together make a valuable system for studying neurosphere proliferation, migration and differentiation.

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