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Scalable human neural stem cells produce mature neuronal model

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Introduction

Fully differentiated cultures of human neurons derived from stem cell sources provide the prospect of replacing methods typically performed in rodent derived primary cultures and tissue slices. A major hurdle to achieving this goal is to produce stem cell derived neurons and astrocytes and oligodendrocytes that are functionally mature enough to recapitulate normal physiology and are scalable for purposes of drug discovery and screening.

We have been able to reproducibly generate large scale cultures of functionally mature neural cultures derived from neural stem cells from specific brain region. These differentiated cultures contain neurons and astrocytes expressing mature neuronal markers such as beta-III-tubulin, MAP2 and Tau with a mature axonal/dendritic distribution and long branched processes. Neurites in these human neurons also show advanced synaptic maturation with extensive synaptophysin foci and a punctate distribution of VGAT and VGlut along axons, indicating the presence of both inhibitory and excitatory neurons. The brain region derived neural stem cells were able to produce these functional neurons due in part to the isolation and culture of the stem cells in the presence of a defined media formulations, which in addition to growth factors includes selective modulators of intracellular signaling pathways which maintains the stem cell phenotype and prevents phenotypic drift. The shift from a stem cell phenotype to differentiation is enhanced by a pre-differentiation step where manipulation of stem cell signaling pathways is removed. The cells are then differentiated into mature neuronal phenotypes in a defined basal neural media containing tissue specific growth factors and signaling molecules. The extensive use of High-Content imaging has allowed for the accurate characterization of phenotype during the differentiation process and the evaluation of novel factors for cell fate determination.

Materials and Methods

Human Neural Stem/Progenitor Cells Isolation, Expansion, Differentiation

- Giga-NSPCs were isolated from human fetal brain tissue from different brain regions.
- Cells were plated on laminin coated plates following isolation in Giga NSP-Gro™ growth medium (includes proprietary components, bFGF, EGF and laminin).
- Expansion carried out by plating the NSPCs into HyperFlasks or laminin coated plates in Giga NSP-Gro™ growth medium.
- Following expansion, NSPCs were cryopreserved in Giga NSP-Freez™ cryopreservation medium and stored in MVE LN2 vapor phase freezer.
- NSPCs were thawed into laminin coated plates in Giga NSP-Gro™ growth medium and expanded for differentiation potential experiment.
- Prior to differentiation NSPCs were split into 2 groups, one to be transitioned into Giga NSPC-Diff™ the other transitioned into Giga NSP-PreDiff™ medium for 3 days.
- For pan-neural *in vitro* differentiation, NSPCs were plated into 384-well poly-d-lysine coated plates in Giga NSPC-Diff™.
- Every 3 days half of the media was replaced with fresh Giga NSP-Diff for 28 days when the cells were fixed with 4% formaldehyde.
- Differentiation potential was determined by immunocytochemistry and image analysis.

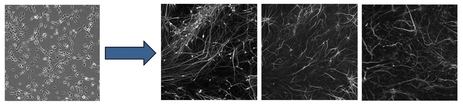
Immunocytochemistry of Differentiated Human NSPCs

- Antibody set 1: Nestin (green), GFAP (red), Tuj1 (far-red)
- Antibody set 2: Tuj1 (green), VGAT (red), VGlut1 (far-red)
- Antibody set 3: MAP2 (green), Synaptophysin (red), Tau (far-red)
  - Nestin: Intermediate filament; marker for neural stem cells.
  - Tuj1: Neuronal b-III-Tubulin; cytoskeletal neuronal marker.
  - GFAP: Intermediate filament; marker for astrocytes.
  - VGAT: Vesicular GABA transporter; found in vesicles at inhibitory synapses.
  - VGlut1: Vesicular Glutamate transporter; found in vesicles at excitatory synapses.
  - MAP2: Neuronal microtubule associated protein; associated with dendrites in mature neurons.
  - Tau: Neuronal microtubule associated protein; primarily associated with axons in healthy neurons.
  - Synaptophysin: Synaptic vesicle glycoprotein; abundant marker for synaptic vesicles at pre-synaptic termini.

Imaging & High Content Analysis (HCA)

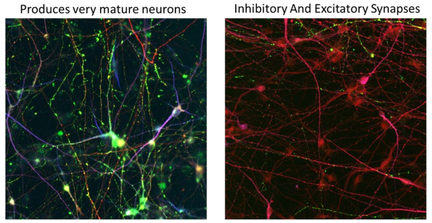
- Plates imaged and analyzed on the Cellomics VTI using the Target Activation and Neuronal Profiling 3.5 BioApplications.
- The settings were optimized for each antibody set and all instrument and software settings were the same across cell lines.
- Plates were imaged using a 20X 0.75NA objective at high-resolution, 15 fields were captured per well.
- Cellomics Target Activation algorithm was used on antibody set 1 to determine percentage positive for each marker.

Differentiation into Neurons, Astrocytes & Oligodendrocytes



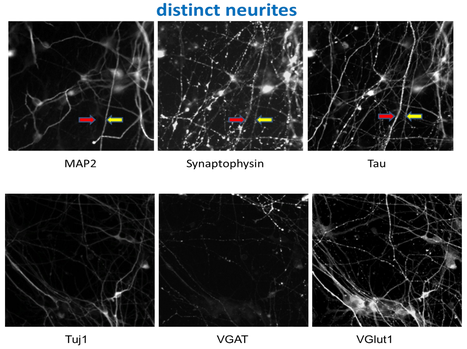
HIP009 neural stem cells differentiated for 28 days and immunostained for the presence of markers for neurons (Tuj1), Astrocytes (GFAP) and oligodendrocytes (04).

HIP-009 Hippocampal NSPC Derived Neurons



Differentiated HIP009 neural cultures were differentiated for 28 days and immunostained for the dendritic marker MAP2, the axonal marker Tau and the marker of pre-synaptic vesicles synaptophysin. Differentiated cultures where also immunostained for Tuj1 and markers for vesicular transporters for glutamate and GABA, VGlut1 and VGAT.

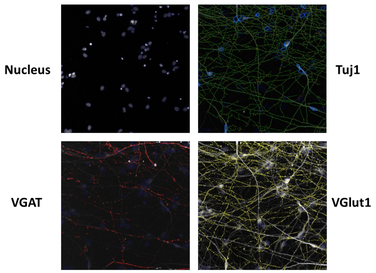
Mature axonal and dendritic markers in distinct neurites



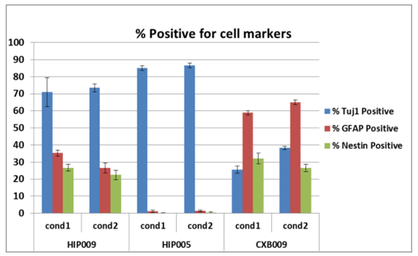
In HIP009 neurons immunostained for MAP2 and Tau there is a clear difference in the neurites staining for MAP2 and Tau indicating a functional maturation into axons and dendrites. Synaptophysin puncta are mainly found in Tau positive axons.

VGlut1 and VGAT puncta are found on distinct and separate neurites, with the VGlut1 staining being more abundant than that of VGAT.

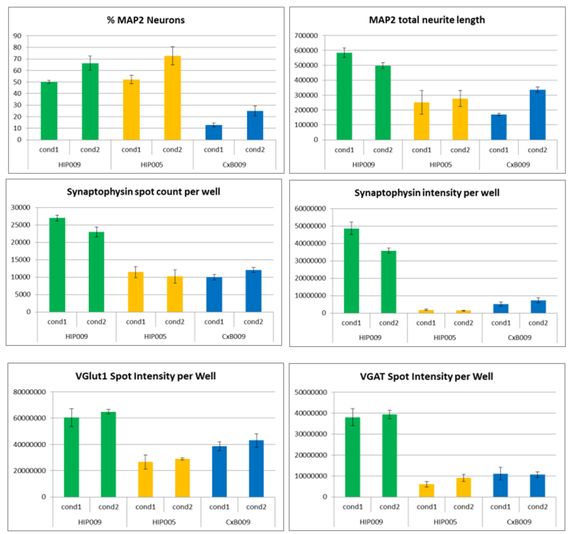
Multiple differentiated cell types analyzed using High-Content



Cellomics Neuronal Profiling 3.5 algorithm used to detect nuclei and neuronal cell bodies, then trace neuronal network. The mask for the neuronal network was then used to quantify VGAT and VGlut1 punta.

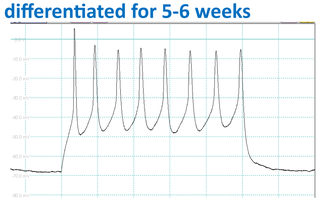


High-Content analysis for neuronal, astrocyte and neural stem cell markers following 28 days of differentiation with 3 GigaCyte neural stem cell lines, with (cond2) and without (cond1) a pre-differentiation step.



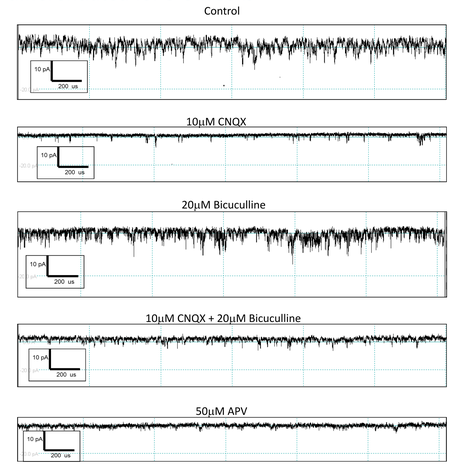
High-Content analysis for the dendritic marker MAP2, the pre-synaptic marker synaptophysin and the pre-synaptic markers for excitatory and inhibitory neurons, VGlut1 and VGAT. HIP009 has the most mature neuronal phenotype with long neurites and abundant synaptophysin. HIP009 has abundant markers for excitatory and inhibitory neurons and is consistent with patch clamp electrophysiology.

Action potentials in HIP009 Neurons differentiated for 5-6 weeks



Typical action potential from HIP009 neurons with –50pA injected current using patch clamp electrophysiology. Action potentials can be measured in cultures following 4 weeks of differentiation.

Spontaneous activity in HIP009 neurons differentiated for 5-6 weeks



Spontaneous network activity in HIP009 neurons can be measured following 5-6 weeks of differentiation using patch clamp electrophysiology. This activity can be blocked with either CNQX or APV and is potentiated in the presence of Bicuculline. Data is consistent with High-Content analysis which detected the presence of glutamatergic and GABAergic neurons.

Conclusions

- **GigaCyte neural stem cells can be expanded and differentiated in formats and scale appropriate for high-throughput screening.**
- **Mature neuronal cultures where analyzed by High-Content analysis and patch clamp electrophysiology.**
- **Data from both screening formats is consistent with active and mature excitatory and inhibitory neurons.**

Acknowledgements

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