High Content Analysis of Neural Stem Cell Expansion and Differentiation

Oksana Sirenko, Allan C. Powe*, Steven L. Stice*, Karen Cook, Nick Callamaras, Jayne Hesley, Xin Jiang, and Evan F. Cromwell

Molecular Devices, Inc., 1311 Orleans Drive, Sunnyvale, CA 94089
***ArunA Biomedical, Inc., 425 River Drive, Athens, GA 30602**

Introduction

Neurogenesis is an important process for nervous system development and maintenance. During neurogenesis, neural stem cells generate neural progenitors that mature into functional neurons. Pharmacological enhancement of neurogenesis represents a potential therapeutic approach for neuronal loss in neurodegenerative diseases, such as stroke, brain damage, Parkinson's, and Alzheimer's Disease. Accordingly, there is great interest in using neural stem cells as tools for screening neurogenic compounds during early drug development.

Here we describe automated assay methods for monitoring neural stem cell expansion and differentiation using stem cell derived neural cell lines and high content imaging systems. The assays greatly increase reproducibility and throughput and are suitable for screening of pharmacological agents.

hESC-derived Neural Cell Lines

STEMEZ™ hNP1™ neural progenitors

- Derived from approved WA09 (H9) hESC cell line
- Derived, maintained and propagated as adherent monolayers using serum- and feeder-free, defined medium
- Possess a stable karyotype for multiple (>10) passages with a doubling time of ~36 hours
- Robust and scalable for HTS format (96-, 384-well) assays
- Proneural: >90% Nestin+; <5% Oct4+; positive for Musashi1, CD133
- Capable of differentiation into multiple neuronal and glial phenotypes

STEMEZ™ hN2™ differentiated neurons

- Differentiated from early stage neural progenitor cells under defined (feeder & serum free) conditions
- Neuronal morphology; >90% β-III tubulin (Tuj1)+, >60% MAP2+, <5% Oct4+; mostly post-mitotic
- Large populations of glutamatergic and GABA-ergic – can be used as a general neuronal model
- Cultured in an adherent 96-well format with defined medium conditions
- Can be cryopreserved for thaw-and-use applications
- Can be maintained in culture for >2 weeks

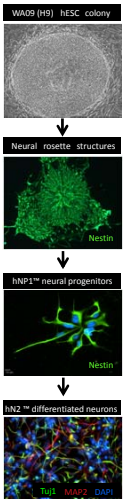


Fig 1. Steps involved in transformation from stem cells to neuronal phenotype.

High Content Imaging

High content imaging provides an efficient tool to monitor and evaluate neuronal expansion and differentiation. The ImageXpress® Micro System provides a fast, automated way to acquire high resolution images of appropriately stained cells throughout the assay processes. The MetaXpress® Software package then can analyze the images to characterize cell phenotype, quantify extent of differentiation based on marker expression, and monitor neurogenesis and neurotoxicity.

- Images acquired with ImageXpress Micro High Content Screening System using a 20X objective and two exposures per well
  - AlexaFluor 488 anti-β-tubulinIII: 488nm Ex, 520nm Em
  - Hoechst dye label for nuclei: 405nm Ex, 450nm Em
- Images were analyzed using standard algorithms from MetaXpress Software
  - Cell Scoring – identify number of Neurons
  - Neurite Outgrowth – identify total outgrowth, number of neurons, neuron length, branching, and number of processes

ImageXpress Micro System

Neural Progenitor Proliferation and Differentiation

Neural progenitors hNP1 were expanded in appropriate media supplemented with leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF) on Matrigel coated dishes. We have tested 2 assays using the hNP1 cell line:

1. Proliferation of neural progenitors: the effect of different agents on neural progenitor proliferation can be tested in a 3-5 day assay in 96-well format.

2. Differentiation of neural progenitors: hNP1 neural progenitors can be differentiated into cells with a neuronal phenotype. Effects of different growth factors can be evaluated in a 14 day assay in 96-well format.

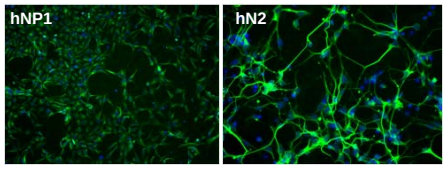


Fig. 2. Overlayed images from wells containing hNP1 and differentiated hN2 cells. Images were acquired with ImageXpress Micro System using a 20X objective.

Image analysis was done using the Cell Scoring and Neurite Outgrowth modules in MetaXpress Software, and data visualization and analysis was done using AcuityXpress™ Software. The Cell Scoring module determines a "positive" cell by presence of both nuclear and β-III tubulin stains. Statistics on number and phenotype of cells in each well are then calculated.

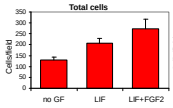


Fig. 3. Image analysis results of MetaXpress Software from Cell Scoring module

The Neurite Outgrowth module finds neural cells and then characterizes β-III tubulin labeled neurites extending from those cells. Output parameters include number of neurites, length, and branches per cell or per field.

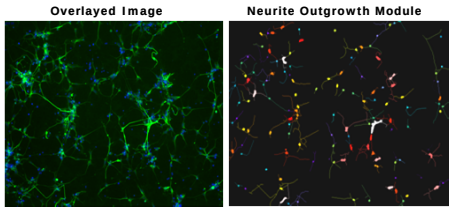


Fig. 4. Image analysis results of MetaXpress Software from Neurite Outgrowth module.

Principal Component Analysis

An advantage of high content imaging is the multi-parametric data output that provides cell-by-cell information about biology. However, it can be difficult to determine which parameter, or groups of parameters are most significant and provide the best assay result. The Principal Component Analysis (PCA) tool in AcuityXpress Software provides a way to statistically analyze various output parameters and determine the optimum way to assay a particular biology. We demonstrate the use of PCA for separating the phenotypes of undifferentiated (hNP1) and differentiated (hN2) cells.

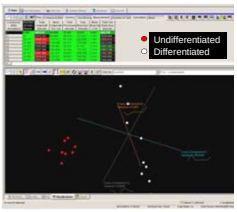


Fig. 5. Results of a PCA analysis of outputs from Cell Scoring and Neurite Outgrowth modules. The analysis optimized separation of the neuronal progenitors from differentiated cell populations.

LIF and EPO Promote Neuronal Differentiation

hNP1 neuronal progenitors were differentiated into cells with neuronal phenotype. Effects of different growth factors were evaluated in a 14 day assay in 96 well format. Cells were cultured in the presence of different growth factors and analyzed with the Cell Scoring and Neurite Outgrowth modules of MetaXpress Software. Presence of LIF or EPO increases neuronal differentiation. Shh and BDNF had marginal effects.

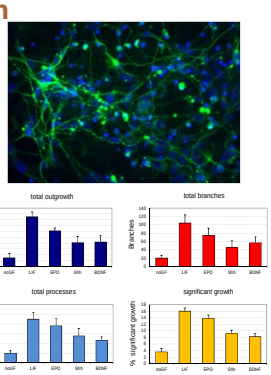
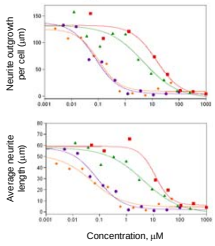


Fig. 6. Top: Image of cells differentiated in culture for 14 days (Hoechst and β-III tubulin). Lower panel: Output parameters from Neurite Outgrowth analysis: Total outgrowth per field, number of neuronal branches and processes, significant growth (>6μm). Error bars: SEM, N=4

Disintegration of Neuronal Networks by Cytotoxic Agents

The hN2 cells can be used for evaluation of potential toxic effects on neuronal development. Nitric Oxide (NO) was found to contribute to neuronal death and brain damage in neurological diseases. Inhibition of neuronal development by NO inducer (SNAP) and several other cytotoxic compounds was evaluated using various output parameters after visualizing cells with β-III tubulin.



Agent	IC50 (μM)
SNAP	15
Antimycin A	4
Staurosporine	0.1
Mitomycin C	0.07

Fig. 7. Dose response curves for the effect of toxic agents on neuronal development. Cells were cultured in the presence of cytotoxic compounds for 72h.

Summary

We have developed high content imaging methods that allow automatic evaluation of proliferation and differentiation of hESC derived neuronal progenitors

We have demonstrated the effect of positive and negative factors on neuronal development:
- EPO promotes differentiation of human neural progenitors toward neurons
- NO inducer SNAP, Antimycin A and staurosporine inhibit neuronal development

These methods can be used to automate assays for:
- Testing biologics or chemical compounds on neuronal development
- Screening and validation of drug candidates
- Evaluating potential neurotoxic effects of different agents

Stem cell derived neurons and high content imaging provide a powerful platform for neurobiology research and drug screening