**Cell Counting, Neurite Outgrowth and Branching Analyses**

**FIGURE 2. Data Acquisition and Analyses for Each Experiment**

Neurons were detected by immunoblotting for the neuronal marker class III β-tubulin and were counted with DAPI. Images of neuronal class III β-tubulin-labeled neurons were captured with a Thermo Scientific ×1000 objective high-content reader (Thermo Scientific Cy3 High-Content Reader [HCR] and ArrayScan). Images were acquired at the right random areas per well in each of three replicates per well or condition for high-content analysis. The raw images from all samples were divided and images were analyzed to determine which captured cell type was as defined by more than three cells attached together. These images were used for further quantitation. The number of neurons (class III β-tubulin and DAPI-labeled), neurite outgrowth and neurite branch points were quantified using the ImageJ software.

**Microscopy**

Phase contrast images were captured using a Nikon Eclipse Ti-U inverted microscope. Confocal microscopy was performed using a Leica TCS SP5 laser-scanning confocal microscope.

**Statistical Analyses**

Data were analyzed by ANOVA statistical analysis using ANOVA statistical analysis. Prevalence analysis revealed an effect of the different culture conditions (i.e., the cells obtained on each experimental trials were significantly different). To remove this effect from the analyses and obtain a comparison of the medium formulas, the experimental data was used as a blocking factor. Analyses were then performed by standard ANOVA to compare the effect of medium formulations on the dependent variables of neuron numbers, neurite outgrowth, and neurite branching. In preliminary analyses, a strong reverse relationship between cell number (i.e., measured cell number per field of view in the captured images) and neurite outgrowth was observed. Measured cell number (in the 50×) of neurons was therefore included in the downstream analysis as an independent variable. To make the effects of medium formulation more visible, a very significant cell density effect, therefore, a strong linear relationship between the number of neuron branch points and neurite outgrowth was observed. Neurite outgrowth was therefore included in the downstream analyses as an independent variable. To make the effects of medium formulation visible, the data are represented by mean ± 95% confidence interval (CI).

**Materials and Methods**

**Primary Neuronal Culture**

**FIGURE 1. Experimental Design of Each Experiment**

Glass coverslips were placed in individual wells of a 24-well plate and coated with 10 µg/ml poly-L-lysine. Cortices were dissected from E18 rat CNS (brain, Inc. and extra-meningeal) dissected with 82% Trypsin/EDTA (1: 10,000 dilution) and enzymatically digested with 82% Trypsin/EDTA (1: 10,000 dilution). Cells were plated in either NeuroCult SM1 (StemCell Technologies Inc.) or NeuroCult SMi (StemCell Technologies Inc.) and 1% horse serum. Cells were plated in duplicate wells for each condition. Five wells of DMSO and SMi were treated. After 3 days, the medium was replaced with NeuroCult SM1. After 7 days, cultures were counted with DAPI. Images of neuronal class III β-tubulin-labeled neurons were captured with a Thermo Scientific ×1000 objective high-content reader (Thermo Scientific Cy3 High-Content Reader [HCR] and ArrayScan). Images were acquired at the right random areas per well in each of three replicates per well or condition for high-content analysis. The raw images from all samples were divided and images were analyzed to determine which captured cell type was as defined by more than three cells attached together. These images were used for further quantitation. The number of neurons (class III β-tubulin and DAPI-labeled), neurite outgrowth and neurite branch points were quantified using the ImageJ software.

**Results**

**FIGURE 3. Morphology of Neurons in Representative NeuroCult SM1 Cultures at 7 and 21 Days in Vitro**

Primary cortical neurons were cultured for 7 (A) and 21 (B) days in NeuroCult SM1 medium. Phase contrast imaging at 40× shows large numbers of viable neurons, with minimal cell lysis and extensive neurite outgrowth and branching. After 21 days, large numbers of viable neurons with developed dendritic arbors remain in culture. Magnification: 40×,

**FIGURE 4. Number of Neurons in NeuroCult SM1 and TSFM Cultures After 7 and 21 Days in Vitro**

(A) Comparable numbers of neurons are obtained when cell are cultured for 7 days in NeuroCult SMi compared to TSFM (N = 3; mean ± 95% CI ± 2.23). (B) Significantly higher numbers of neurons are obtained when cells are cultured for 21 days in NeuroCult SMi compared to TSFM (N = 3; mean ± 95% CI ± 3.85). See Materials and Methods for details of statistical analyses.

**Summary**

**NeuroCult SM1 Cultures Show:**

- Significantly greater cell survival after 21 days in culture.
- Significantly greater neurite outgrowth and branching at 7 and 21 days in culture compared to primary neurons maintained in a traditional serum-free medium (TSFM).
- Viable mature neurons, featuring extensive dendritic arborization and appropriate expression of synaptic markers, after 21 days in culture.
- Minimal variability attributable to supplement lot.