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# Significantly greater numbers of neurons, neurite outgrowth and neurite branch points in 21 day cultures of primary rat cortical neurons with NeuroCult™ Neuronal Media

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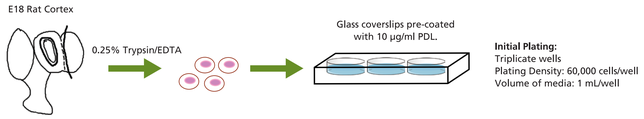
## Introduction

Primary neuronal cultures represent a useful system in which to study neuronal biology in a simplified and controlled environment. Fully mature neuronal cultures are complex in morphology, featuring highly branched dendrites and extensive networks of synaptic connections. The peak of dendrite growth and synaptogenesis does not occur until two to three weeks in culture, with some markers of synaptic maturity requiring three weeks or longer to reach their highest expression levels. As such, the ability to establish and maintain long-term primary neuronal cultures is essential for the comprehensive study of neuronal development and function. While short-term culture of neurons can be a straightforward process, long-term (>21 days) culture of relatively pure neuronal populations is technically challenging. The development of neuron-specific serum-free media formulations was pivotal to the evolution of modern primary neuronal culture techniques, enabling neurons to be cultured in the absence of undefined serum supplements or a supporting glial cell layer. However, variability in the performance of a commonly used serum-free supplement and discrepancies in the protocols for substrate coating and cell plating have resulted in inconsistency in the quality of neuronal cultures. Variability in protocols and culture media quality becomes more problematic when cells are plated at low density or cultured long-term, because these conditions place additional stress on the cultured cells. We have developed a neuronal culture kit comprising of a neuronal basal medium (NeuroCult™ Neuronal Basal Medium) and serum-free supplement (NeuroCult™ SM1) which, when used according to optimized protocols, consistently produces high yields of functional neurons from primary mouse and rat central nervous system (CNS) tissues. NeuroCult™ SM1, based on the published B27 formulation, is optimized to more reproducibly support the survival of mature neurons in long-term culture. Here we present data on the performance of NeuroCult™ SM1 for long-term culture of embryonic rat cortical neurons.

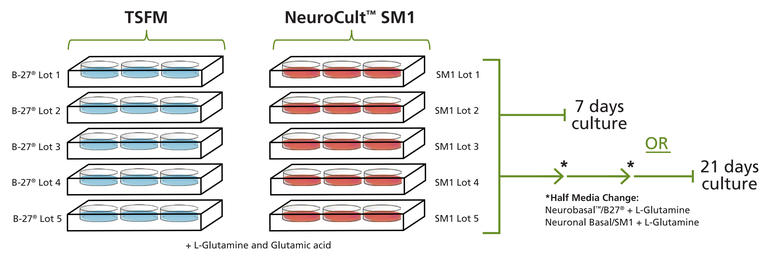
## Materials and Methods

### Primary Neuronal Culture

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



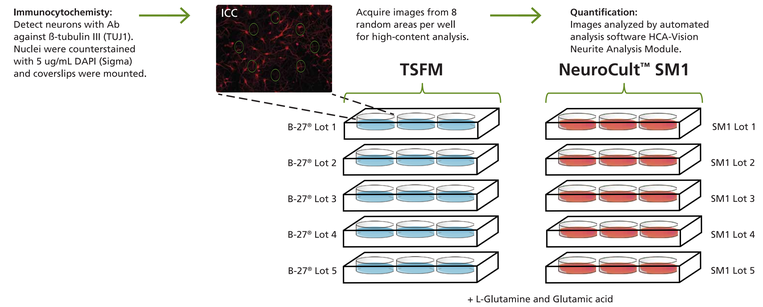

Glass coverslips were placed in individual wells of a 24-well plate and coated with 10 µg/mL poly-D-lysine. Cortices were dissected from E18 rat CNS (Brain Bits, Inc.) and enzymatically dissociated with 0.25% Trypsin/EDTA into a single-cell suspension. Cells were diluted in either NeuroCult™ SM1 plating medium (NeuroCult™ Neuronal Basal Medium, 2% NeuroCult™ SM1, 0.5 mM L-glutamine and 25 µM L-glutamic acid), or traditional serum-free plating medium [Neurobasal™ Medium, 2% B-27® (Life Technologies Inc.), 0.5 mM L-glutamine and 25 µM L-glutamic acid], and plated at a density of 60,000 cells/well or 320 cells/mm$^2$. Cells were plated in triplicate wells for each condition. Five lots of B-27® and SM1 were tested.




After 3 days, the medium was replaced with NeuroCult™ SM1 maintenance medium (NeuroCult™ Neuronal Basal Medium, 2% NeuroCult™ SM1 and 0.5 mM L-Glutamine), or traditional serum-free maintenance medium (TSFM; Neurobasal™ Medium, 2% B-27® and 0.5 mM L-Glutamine). Half-medium changes were performed weekly throughout the duration of the culture period. Cells were cultured for a total of either 7 or 21 days. Five experiments with the same experimental design were performed.

### Cell Counting, Neurite Outgrowth and Branching Analyses

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




Neurons were detected by immunolabeling for the neuronal marker class III β-tubulin and nuclei were counterstained with DAPI. Images of neuronal class III β-tubulin-labeled neurons were captured with a Thermo Scientific ArrayScan® Infinity High Content Reader [previously Cellomics High Content Analysis (HCA) and Screening]. Images were acquired from eight random areas per well in each of three replicate wells per condition for high-content analysis. The raw images from all samples were blinded and images were analyzed to determine which contained cell clumping as defined by more than three cells attached together. These images were not used for further quantification. The number of neurons (class III β-tubulin- and DAPI-positive), neurite outgrowth and neurite branch points were quantified using the HCA-Vision Neurite Analysis Module.

### Microscopy

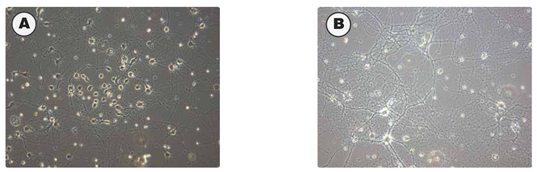
Phase contrast images were captured using an Olympus CKX41 inverted microscope. Confocal microscopy was performed using an Olympus Fluoview FV1000 laser scanning confocal microscope.

### Statistical Analyses

Data were analyzed by JMP® statistical software using ANOVA statistical analyses. Preliminary analysis revealed an effect of the different tissue samples (i.e. the cells obtained on each experimental date were significantly different). To remove this effect from the analyses and enable a comparison of the medium formulations, the experimental date was used as a blocking factor. Analyses were then performed by standard ANOVA to compare the effect of medium formulation on the dependent variables of 1) number of neurons, 2) neurite outgrowth and 3) neurite branching. In preliminary analyses, a strong inverse relationship between cell number (i.e. measured cell numbers per field of view in the captured images) and neurite outgrowth was observed. Measured cell number in the field of view was therefore included in the outgrowth analyses as an independent variable, to isolate the effects of medium formulation from this very significant cell density effect. Similarly, a strong linear relationship between the numbers of neurite branch points and neurite outgrowth was observed. Neurite outgrowth was therefore included in the branching analyses as an independent variable, to isolate the effects of medium formulation. Data are represented by mean ± 95% confidence intervals (CI).

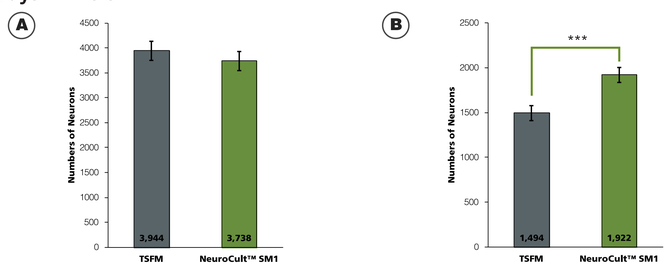
## Results

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



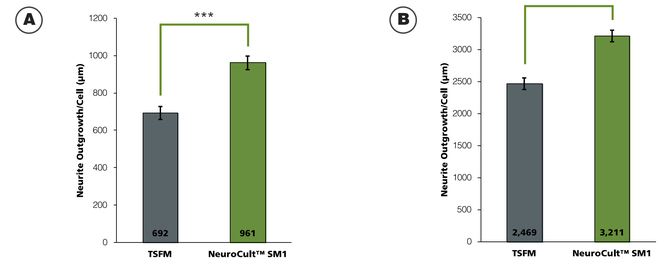

Primary rat E18 cortical neurons were cultured for 7 **(A)** and 21 **(B)** DIV in NeuroCult™ SM1 medium. **(A)** Phase contrast imaging at 7 DIV shows large numbers of viable neurons, with minimal cell clumping and extensive neurite outgrowth and branching. **(B)** After 21 DIV, large numbers of viable neurons with developed dendritic arbors remain in culture. Magnification 20x.

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



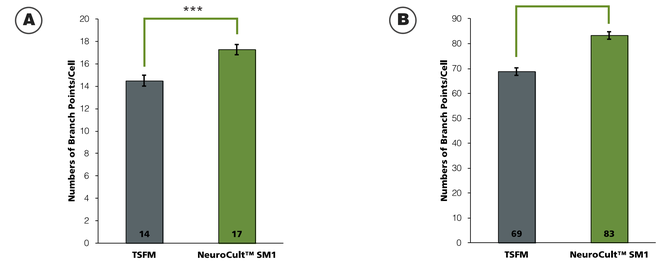

**(A)** Comparable numbers of neurons are obtained when cells are cultured for 7 days in NeuroCult™ SM1 compared to TSFM (n = 25; mean ± 95% CI; p > 0.05). **(B)** Significantly higher numbers of neurons are obtained when cells are cultured for 21 days in NeuroCult™ SM1 compared to TSFM (n = 25; mean ± 95% CI; ***p < 0.001; see Materials and Methods for details of statistical analyses).

**FIGURE 5. Neurite Outgrowth of Primary Neurons Cultured in NeuroCult™ SM1 and TSFM Cultures for 7 and 21 Days**



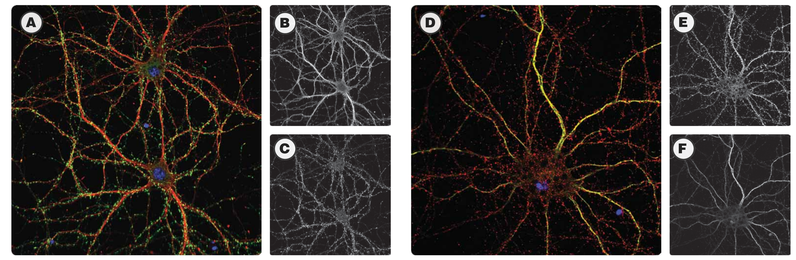

Significantly longer neurite outgrowth was observed for cells cultured for 7 **(A)** and 21 **(B)** days in NeuroCult™ SM1 compared to TSFM (n = 240 independent measures; mean ± 95% CI; ***p < 0.001).

**FIGURE 6. Neurite Branching of Primary Neurons Cultured in NeuroCult™ SM1 and TSFM for 7 and 21 Days**




Significantly more neurite branch points were observed for cells cultured for 7 **(A)** and 21 **(B)** days in NeuroCult™ SM1 compared to TSFM (n = 240 independent measures; mean ± 95% CI; ***p < 0.001).

**FIGURE 7. Expression of Pre- and Post-Synaptic Markers in Neurons Cultured for 21 Days in NeuroCult™ SM1**




Neurons cultured in NeuroCult™ SM1 for 21 days are phenotypically mature as indicated by the presence of an extensive dendritic arbor and appropriate expression and localization of pre- (Synapsin) and post-synaptic (PSD-95) markers. **(A - C)** Synapsin (green) staining is concentrated in discrete puncta distributed along the somata and dendritic processes, as defined by MAP2 (red) staining. **(D - F)** Dendritic staining observed for MAP2 and punctate staining for the postsynaptic marker PSD-95. Nuclei were counter-stained with DAPI. Scale bar = 10 µm.

## 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 when compared to primary neurons maintained in a traditional serum-free medium (TSFM)
- Visibly mature neurons, featuring extensive dendritic arborization and appropriate expression of synaptic markers, after 21 days in culture
- Minimal variability attributable to supplement lot