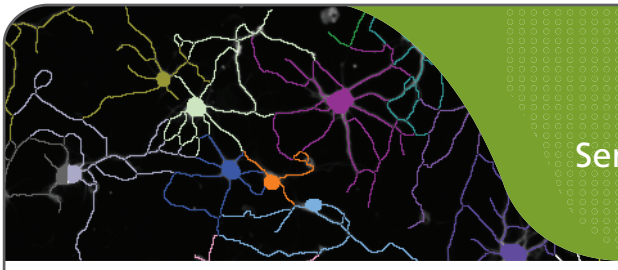

TECHNICAL BULLETIN

Semi-Automated Neurite Analysis Of Neurons Cultured With NeuroCult™ SM Neuronal Supplements

Background

The study of neuronal biology is highly dependent upon robust culture methods and sensitive analytical tools. Recent reports have shown that variability in a traditional serum-free supplement can lead to poor neuron survival or maturation.[1,2] To provide more standardized and consistent neuronal cultures, STEMCELL Technologies Inc. developed NeuroCult™ SM Neuronal Supplements. NeuroCult™ SM1 and substrate-independent NeuroCult™ SM2 were optimized from the published B27 formulation, to minimize variability from raw materials, manufacturing processes, or protocols.[3,4]

When primary neurons are isolated from CNS tissues and cultured under appropriate culture conditions, each developing neuron extends cellular projections, referred to as neurites, from the cell body.[5-7] Typically, as the neuron matures, one immature neurite grows substantially longer than the other neurites and develops into the axon, whereas the remainder of the neurites develop into dendrites. Distinguishing axons from dendrites is difficult in an in vitro culture system where the axons intersect the dendrites and become intertwined in the extensive dendritic arbors.[7] As a result, the term neurite is often used to refer to immature cellular projections that have not yet acquired the properties of mature dendrites or axons, as well as more mature projections that have begun to acquire the properties of axons or dendrites but were not identified as such due to difficulties of doing so in culture.

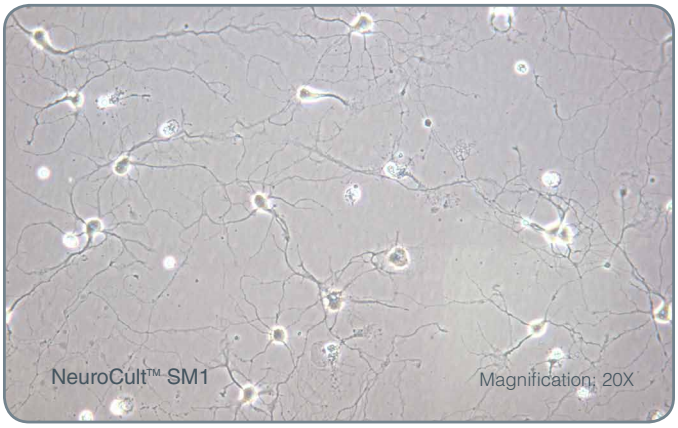

FIGURE 1. Representative image of primary neurons cultured in medium supplemented with serum-free NeuroCult™ SM1 Neuronal Supplement

Summary

- Neurite evaluation is key to analyses of neuronal culture quality.
- A convenient, reliable semi-automated protocol for neurite tracing and analysis is presented here.
- Using this method, neurons cultured with NeuroCult™ SM Neuronal Supplements were shown to have longer neurites with more branch points, as compared to those cultured with a traditional serum-free supplement.

The development of neurites and their subsequent maturation into dendrites and axons is fundamental to the biology of neurons. As a result, evaluating neurons according to neurite, axonal and dendritic properties can serve as a useful tool to analyze in vitro neuronal culture quality. Because the extent of process outgrowth and branching serves as an indicator of the level of arborization and maturation of a neuron, methods for in vitro neurite tracing or profiling have been used.[5-7] In order to ensure high performance of the NeuroCult™ SM Neuronal Supplements, a fast, reliable and objective assay for measuring key neurite properties to assess culture quality was established. A semi-automated neurite tracing protocol and analytical program was chosen as it avoids both the tedium and inconsistencies of manual analysis.[8,9]

In this technical bulletin, we describe the semi-automated method we used to measure and compare the properties of neurons cultured in medium supplemented with NeuroCult™ SM1 Neuronal Supplement (Catalog #05711) or a traditional serum-free supplement. Data presented here indicates that neurons cultured in NeuroCult™ SM1-supplemented medium have significantly greater average neurite length and average number of branch points, as compared to neurons cultured with a traditional serum-free supplement. Similar methods were used to qualify new, substrate-independent NeuroCult™ SM2 Neuronal Supplement (Catalog #05721).

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Neuronal Culture

Primary mouse and rat neurons were cultured using NeuroCult™ SM1 and a traditional serum-free supplement on poly-D-lysine coated coverslips. All test conditions and corresponding controls were cultured in duplicate.

Coverslip Preparation

- Glass coverslips (Catalog #633029, Carolina) were coated with 10 µg/mL poly-D-lysine (PDL; Catalog #P7280, Sigma-Aldrich) for 2 hours at 37°C or overnight at 2 - 8°C. Coated coverslips were washed 3 times with sterile PBS before use.

Note: It is recommended to prepare the PDL solution in sterile water (increased cell clumping in cells cultured with NeuroCult™ SM1-supplemented medium is observed when PDL is prepared in borate buffer).

Cortical Neuron Culture

- Cortical tissues from embryonic day 14 (E14) mice or E18 rats were dissociated with 0.25% Trypsin-EDTA for 15 - 30 minutes at 37°C. To neutralize the trypsin, cells were washed twice with DMEM containing 10% FBS.
- 30,000 cells were seeded into each well of a 24-well plate containing a PDL-coated coverslip in Complete Neuronal Medium [(Neurobasal™ (Life Technologies) containing 2% NeuroCult™ SM1 Neuronal Supplement (Catalog #05711) or 2% Traditional Serum-Free Supplement, 0.5 mM L-Glutamine (Catalog #07100), 25 µM L-Glutamic acid (Catalog #G8415, Sigma-Aldrich)].
- Cultures were incubated at 37°C for 7 days. Every 3 days, half of the culture medium was removed from each well and replaced with fresh Complete Neuronal Medium lacking L-Glutamic acid.

Neuronal Staining and Imaging

The neuronal cultures were stained for β-tubulin III expression to detect neurites, and counterstained with DAPI to detect the individual cell bodies. Neurites can also be detected using a variety of other markers such as MAP2 and tau.

Indirect Immunohistochemistry

- After 7 days, the primary cortical neuron cultures were fixed with 4% paraformaldehyde at room temperature for 30 minutes. Cultures were then washed three times with PBS and permeabilized with 0.3% Triton X-100 in PBS for 5 minutes.
- To detect neurites, the cultures were labeled overnight at 4°C with mouse anti-β-tubulin III antibody (1 µg/mL; Catalog #01409) diluted in PBS containing 10% normal goat serum. The next day, cells were washed three times with PBS and incubated with goat anti-mouse IgG Texas Red (diluted 1:100 in PBS containing 2% normal goat serum) for 30 minutes at 37°C. Cells were then washed again three times with PBS to remove residual antibodies.
- To detect the cell bodies, 4',6-diamidino-2-phenylindole (DAPI; Catalog #H1200, Vector Laboratories) diluted 1:100 in water was used to counterstain the neurons.

Microscope Imaging

- 8 random areas from each culture well were imaged using the fluorescent microscope system Cellomics® ArrayScan® VTI HCS Reader (Thermo Scientific).
- For each individual field of view, positive signals to DAPI were detected with the excitation wavelength 365 nm and emission wavelength 535 nm, while positive signals to β-tubulin III were detected using the excitation wavelength 575 nm and the emission wavelength 640 nm.

Neuronal Image Analysis

We chose to use the semi-automated image analysis software HCA-Vision to analyze the neurite properties of our cultured neurons (refer to Figure 2 for an overview of the analysis process).[6,7] At least three image pairs (i.e. DAPI image combined with β-tubulin III image in the same field of view) were randomly selected to set and double check that the parameter profiles for the automated Neuron Body Detection, Neurite Detection and Neurite Analysis Modules were accurate for analyzing all images. To verify that the established parameters were accurate, three additional randomly selected image pairs were analyzed using the automated analysis. If the automated analysis of the test images was deemed sufficiently accurate, the remaining images were analyzed using the batch processing feature. Batch processing the images minimizes labor and ensures consistency across a large sample size, which allows statistical analysis to be conducted on the experimental data.

Cell Body and Neurite Identification

A. To detect individual cell bodies, the raw DAPI-stained images were analyzed with the Neuron Body Detection Module. Thresholds for image contrast, intensity and size were manually adjusted to include at least 95% of the cell bodies while excluding debris (which appears brighter and smaller than the cell bodies) and neurite-like structures. The size and area thresholds were also adjusted to detect one cell body per neuron (i.e. distinguish between clumped cells).

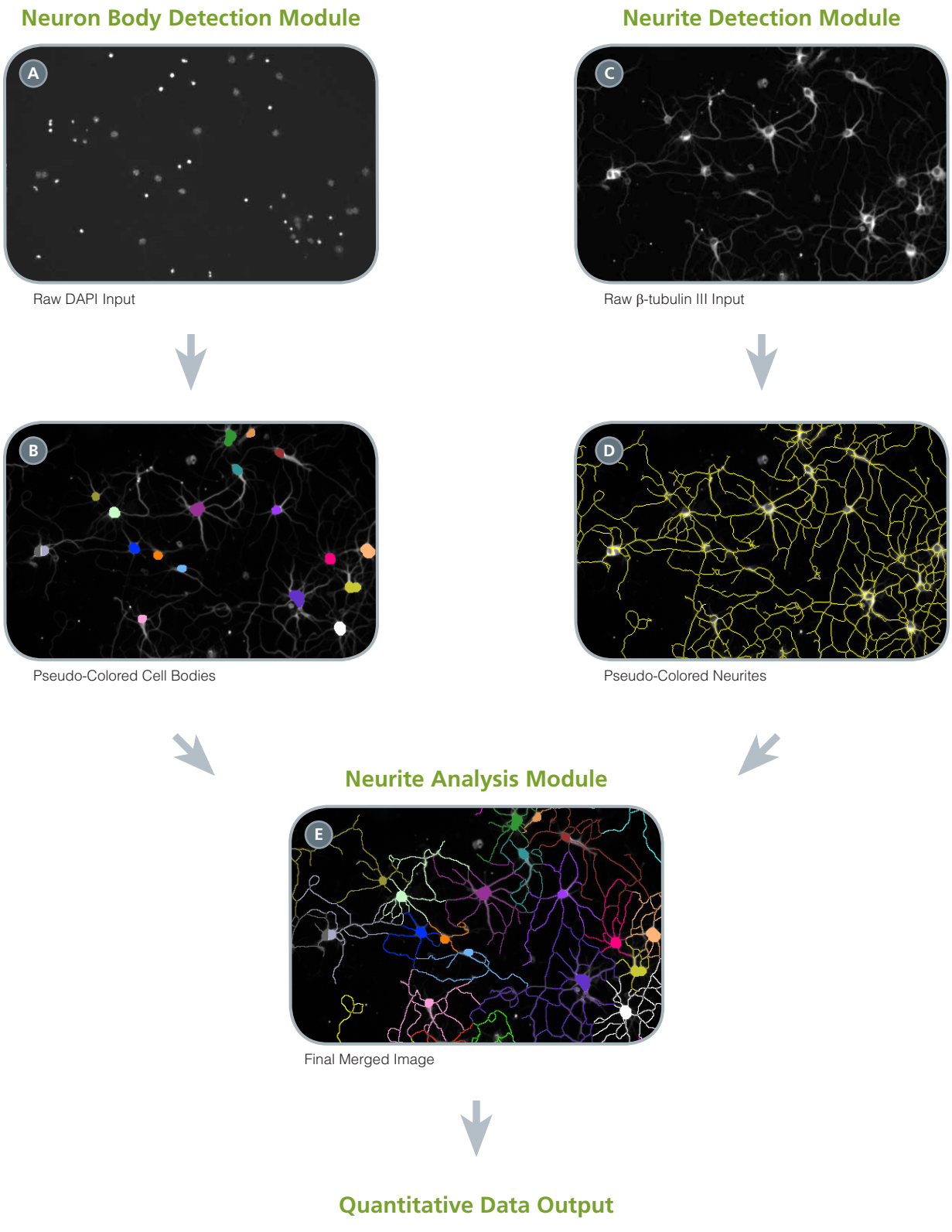
B. The output of the Neuron Body Detection Module is an image with pseudo-colored cell bodies. Note that small, bright structures (debris) apparent in the raw DAPI input image do not appear as cell bodies in the output image (i.e. a pseudo-colored representation of the cell body is not apparent at that location in the image).

C. To detect the neurites, the raw β-tubulin III-stained images were analyzed with the Neurite Detection Module. Thresholds for neurite diameter and image contrast were manually adjusted to balance the identification of and discrimination between neurite extensions.

D. The output of the Neurite Detection Module is an image with pseudo-colored neurite structures.

E. Finally, the Neurite Analysis Module merges the pseudo-colored cell body and neurite images together into a single image, where each cell body is associated with the neurites extending from it.

FIGURE 2. Overview of Neuronal Profiling Image Analysis Process



Semi-Automated Neurite Analysis

Of Neurons Cultured With Cultured With NeuroCult™ SM Neuronal Supplements

Quantitative Neurite Analysis

- Based on the merged cell body-neurite images, the Neurite Analysis Module in HCA-Vision was used to generate quantitative data on: the number of neurons, the length of neurite outgrowth, the number of root neurites, and the number of neurite branch points.
- The entire experiment (from Neuronal Culture to Image Analysis) was repeated four times. Data were then compiled and exported to Excel for statistical analysis (Table 1) and graphical display (Figure 3).
- Within Excel, data were expressed as the mean ± standard error of the mean (SEM) and then analyzed for statistical significance via Student's t-test. A p-value < 0.05 was considered significant.

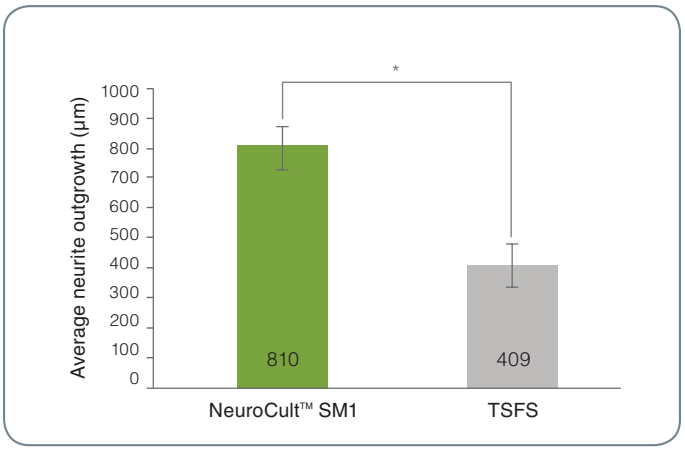
Note: Total number of neurons for each well in a 24-well plate = (Average number of neurons counted by analysis software)/ 1.098mm^2 x 190mm^2. Surface area of each image captured = 1.098mm^2. Growth area of a well in a 24-well plate = 190mm^2.

TABLE 1: A representative data set showing the average number of neurons, average length of neurite outgrowth and average number of neurite branch points for 4 experiments. Data are represented as the mean ± standard error of the mean (SEM), and p-value.

	NUMBER OF NEURONS		LENGTH OF NEURITES		NUMBER OF BRANCH POINTS	
	TSFS	**SM1**	**TSFS**	**SM1**	**TSFS**	**SM1**
Experiment 1	2671	2682	292	656	2.5	9.7
Experiment 2	3439	3126	460	930	5.0	16.8
Experiment 3	851	2307	289	701	2.3	11.1
Experiment 4	1128	1527	593	953	7.3	16.2
Mean ± SEM (n = 4)	2022 ± 619	2410 ± 339	409 ± 73	810 ± 77	4 ± 1	13 ± 2
p-value	0.4		0.001		0.001	

TSFS: Traditional Serum-Free Supplement. SM1: NeuroCult™ SM1 Neuronal Supplement.

FIGURE 3. Representative graph of the average neurite outgrowth for cortical neurons cultured in medium supplemented with NeuroCult™ SM1 or a traditional serum-free supplement (n = 4, Mean ± SEM; * p = 0.001)



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