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Characterization of Compound Effects on Neuronal Morphology and Neurite Outgrowth Using an Automated Image Analysis System

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Abstract

The search for therapeutic drugs for neurodegenerative diseases relies on suitable models. In vitro neuronal assays offer ease of manipulation, scalability, and ability to understand compound effects at the cellular level. We have validated an in vitro assay that provides these benefits as well as the ability to automatically and objectively analyze the results for more robust assays.

The assay was first validated against a sub-clonal cell line of the standard model system PC-12 cells to determine neurite functionality. Neurotrophic factor nerve growth factor (NGF) was used as a positive control (72 hr exposure), stimulating cells to differentiate into neuronal-like cells. After treatment and fixation, nuclei, neurons and neurites were fluorescently labeled and analyzed on an automated imaging system. Multiple neurite characteristics such as neurite count, maximum neurite length, and degree of branching, as well as changes in neuronal morphology (e.g. cell body area, intensity, and shape) were evident in wells treated with NGF.

We also validated the assay using primary rat cortical neurons. Culture conditions in 96-well plates were optimized for quantifiable neurite formation. Multiple compounds were tested in the assay to determine what effects these compounds had on various measures of neuronal morphology. Cells were treated, fixed, labeled, and imaged to analyze changes in morphological features. Decreases in neuronal cell number and inhibition of neurite length, with the maintenance of neuronal morphology (shape), was found in one compound (SU6656). Since primary cultures are not pure populations of neurons, validation of this assay included ensuring that non-neuronal cells did not interfere with the measurement, as well as accurately reporting the percentage of neuronal- and non-neuronal cells in the cultures.

Our data confirm that the algorithm is able to accurately characterize these cultures with respect to the population and cellular features mentioned above, and thus is a validated assay for conducting research into compounds and conditions that prevent neuronal degeneration and death.

Introduction

Abnormalities of neuronal cells are implicated in a variety of pathophysiological conditions such as Alzheimer's and Parkinson's disease. These disorders affect changes in neuronal cell morphology and/or changes in neurotransmitter expression. Some disease conditions affect outgrowth and elongation of neurites, branching of the neurites, changes in neuronal cell survival, cell body area, and expression of certain genes. Axonal outgrowth and functional recovery after nerve injury and the altering of various signaling cascades (including Raf/MEK/MAP kinase pathways) may change the amount of proliferation and neurite growth within a cell (Das et al., 2004). Being able to multiplex these and other changes can help understand the pathways leading to abnormalities and how to treat them.

High content screening (HCS) involves automatic imaging and analysis of cells using quantitative fluorescence microscopy. Cells are labeled with fluorescent probes whose emissions reflect the cell's phenotype, physiological state, or cellular distributions of targets. An HCS imaging instrument, such as a Cellomics ArrayScan® HCS Reader, can automatically acquire and analyze these cells on-the-fly by proprietary image processing algorithms, known as BioApplications. Informatics software allows data management of all images and results, automatically and seamlessly archiving the data and enabling the user to interact with it and assist in decision-making.

In order to evaluate changes in morphology, the Neuronal Profiling (NP) BioApplication, along with the Neurite Outgrowth HitKit® HCS Reagent Kit, were verified using Neuroscreen™-1 (NS-1) cells, a sub-clonal cell line of PC-12 cells (Cellomics, Pittsburgh PA). PC-12 cells are established as a standard model system for the study of neuronal cells (Greene et al., 1998; Tsuji et al., 2001; Wu and Bradshaw 1996) and develop biological characteristics similar to sympathetic neurons (Das et al., 2004). Neuroscreen-1 cells display several significant advantages over the parental PC-12 cell line, including a shorter doubling time, easier growth and culture, a lower tendency to aggregate, and visible neurite outgrowth in 48 hours, compared to 6-7 days in PC-12 cells. Neurotrophic factors such as nerve growth factor cause the NS-1 cells to differentiate into neuronal-like cells with neurites within 3-4 days.

Primary neuronal cultures were used in this study to validate the assay and compare compound results obtained with cultured cells. Primary cultures will grow neurites within 5 - 7 days (without NGF treatment). Morphological features of untreated cells were compared to see if changes occurred when treated with possible inhibitors (SU6656, Bisindolylmaleimide-I and U0126) as well as the agonist NGF.

Materials and Methods

Materials

Cells and Plates:

- NS-1 cells were passaged at 70% confluency. Cells were grown in collagen-I flasks in RPMI-1640 media with 10% HS, 5% FBS, 1% P/S, and 1% L-Glutamine (Hyclone, Logan UT). Cells were then plated onto Collagen-I coated 96-well microplates (Becton Dickinson, Franklin Lakes NJ) to ensure attachment.
- Primary striatal and cortical neurons (Cambrex, Walkersville MD) were cultured in Primary Basal Neurite Media (PBNM) containing Single Quots (Cambrex) and immediately plated onto Poly-L lysine plates (Becton Dickinson).

Compounds and Stains:

- Neurite Outgrowth HitKit HCS Reagent Kit (Cellomics) was used for initial analysis. An additional TRITC secondary (DyLight 549 GAM, Pierce, Rockford, IL) was also used.
- Compounds (EMD BioSciences, San Diego, CA) selected were the following:
 - SU6656 - inhibitor of the Src-Family of Kinases; can inhibit axon outgrowth once induced by Netrin-1 and GDNF
 - U0126 - inhibitor of MEK1 and MEK2
 - Bisindolylmaleimide-I (Bis-I) - cell permeable inhibitor of PKC
 - 7S-NGF (NGF) - promotes neuron survival and neurite outgrowth
- Fixation was by using Formaldehyde (Fisher Scientific, Fair Lawn NJ) diluted to 3.7%

Methods

NS-1 testing of agonist vs antagonist

NS-1 cells were plated at 3000/well onto collagen-I coated plates and incubated overnight. Media was removed and cells were treated with combinations of NGF (final concentrations 0 - 800 ng/mL) and potential inhibitors SU6656 (0 - 20 µM), Bis-I (0-8 µM), and U0126 (0 - 50 µM). Plates were incubated for 72hr. After incubation, solution was removed and cells were fixed with 3.7% formaldehyde for 20 min. Plates were washed twice with Neurite Outgrowth Buffer (NOGB) and incubated at RT for 1 hr with kit primary. After washing plates twice with NOGB, cells were incubated at RT for 1 hr with Dyomics secondary Ab (549). Plates were again washed twice with NOGB, once with Wash Buffer, and sealed in Wash Buffer. Plates were run with the NP BioApplication at 10x, analyzing 300 cells per well.

Primary neuronal cultures – antagonistic studies

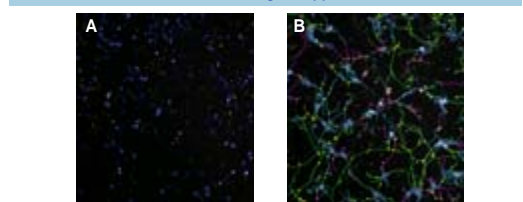
Primary striatal and cortical cells were purchased from Cambrex and plated according to their protocol. Total cell volume per well was decreased to 120 µl (~48,000 cells/well) allowing more wells for testing. After incubation for 4 hr, media was removed and replaced with 1:2 dilutions of Bis-I (0 - 8 µM), SU6656 (0 - 20 µM), U0126 (0 - 50 µM), or NGF (0 - 400 ng/mL). Plates were incubated for 7 days, with a media change (same compounds and concentrations) on day 4. Solution was then removed and plates were stained using the kit protocol. Plates were run with the NP BioApplication at 5x, analyzing 9 fields per well.

Cellomics Neuronal Profiling BioApplication

All testing was conducted on a Cellomics ArrayScan HCS Reader. The NP BioApplication quantifies morphological changes in neurons. It allows the user more control over selecting neurites based on differences in morphology and intensity, as well as selecting neurons based on parameters in the nuclear and neuronal channels. One can also identify subpopulations of cells, due to biology, by combining their cell features into events.

All nuclei (neuronal and non-neuronal) are identified in Channel 1 where nuclear features (size, shape, intensity, etc.) are measured. Neurons and neuronal-like cells are identified in Channel 2 with a primary antibody against a protein specific to neurons present in both neuronal cell bodies and neurites. Cell body (size, shape, and intensity) and neurite features (count, size, intensity, branch and cross point counts, etc.) are measured in Channel 2. Figure 1 is an example of overlays displayed in Channel 1 and 2, using primary cortical neurons stained with the Neurite Outgrowth Reagent Kit.

Figure 1. Identification of Channels and Overlays using the Cellomics Neuronal Profiling BioApplication



Images representing channels and overlays with the Neuronal Profiling BioApplication. (A) Channel 1: ValidNuclei (blue) and RejectedNuclei (orange). (B) Channel 2: ValidCellBody (blue), RejectedCellBody (red), NeuritePoint (yellow), and Neurites (green, teal, and pink). Differences in neurite colors is to help distinguish possession of the neurites to specific cell bodies.

The NP BioApplication allows the user to define and screen for occurrence of specific Events. The event feature used in these experiments was:
Event = [(Neurite Count) > threshold] OR [(Neurite Total Length) > threshold]

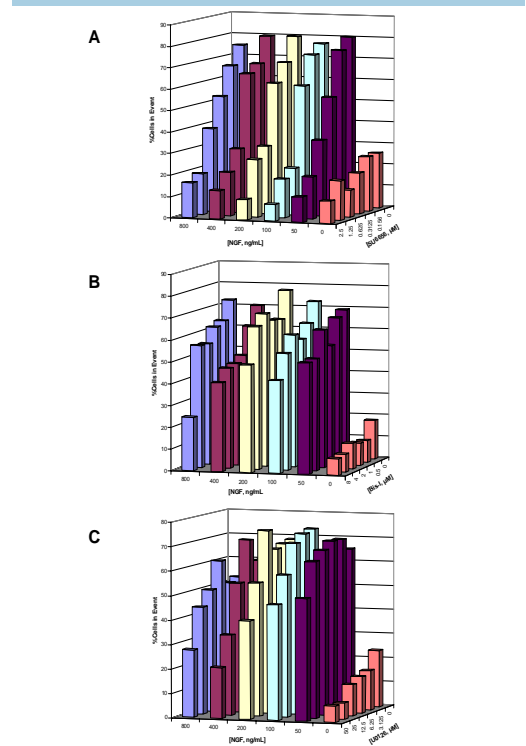
Results

NS-1 testing of agonist vs antagonist

Various morphological features were observed to see if Bis-I, SU6656, and/or U0126 inhibited the effects of NGF on NS-1 cells. Comparison of no NGF to any concentration of only NGF showed a mean increase in neurite count, length, area, and branch point; however, a true dose response when looking at the tested concentrations was not found.

To observe the effect of inhibitors against NGF, an Event was set in the BioApplication to look at the percent of cells that had neurite count or neurite total length above basal levels (no NGF, no inhibitor). Decreases in the frequency of this Event was evident upon increasing doses of SU6656, Bis-I, and U0126 (Figure 2A, 2B, 2C). Increasing inhibitor concentration also resulted in decreasing neurite area and branch point count. SU6656 caused the most inhibition, followed by Bis-I, and then U0126. Images (with overlays) of NS-1 cells treated with NGF (A) and NGF + inhibitor (B) can be seen in Figure 3.

Figure 2. Concentration Gradients of Inhibitor vs. NGF in NS-1 cells



Bar graphs representing dose responses of NGF plotted against dose responses of (A) SU6656, (B) Bis-I, and (C) U0126 with respect to the selected Event, defined as the percent of cells expressing a Neurite Count OR Neurite Total Length above the reference well-determined levels.

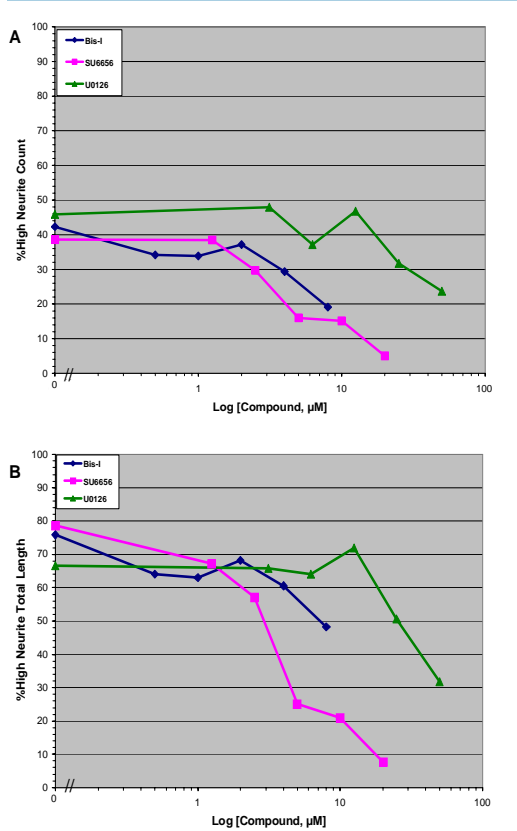
Figure 3. NS-1 Cells



Images (10x) exhibiting overlays of NS-1 cells treated with (A) 800 ng/mL NGF and (B) 800 ng/mL NGF + 8 µM Bis-I after running on the ArrayScan with the NP BioApplication. Note the difference in neurite length, number, and branches in cells treated with inhibitor.

Reference wells (without inhibitor) were analyzed to obtain population threshold values for specific output features. The percent of cells with feature values above threshold, as well as the average feature value of all cells in a well were evaluated. Figure 4 exemplifies the separation of this Event into %High Neurite Count and %High Neurite Total Length of these inhibitors against 200 ng/mL NGF. A dose-dependent decrease in the percent of cells with a neurite count and length above the calculated threshold was found for each.

Figure 4. Neurite Outgrowth inhibition in NS-1 Cells



Graph representing (A) %High Neurite Count and (B) %High Neurite Total Length for varying doses of SU6656, Bis-I, and U0126 with 200 ng/mL NGF. In all three inhibitors, increasing doses caused decreases in the percentages of cells exhibiting each feature.

Inhibition of Primary Striatal Neurons

Primary striatal neurons were plated and incubated over 7 days to evaluate possible changes in neurite and morphological characteristics when treated with the above inhibitors or NGF. Cells treated with inhibitor exhibited decreases in overall neurite length, as well as the number of neuronal cells. Figures 5A and 5B are a set of graphs representing % Event Cells (as mentioned in Figure 2) and % High Branch Point Count (% of cells with value above a set threshold). As inhibitor concentration increased, feature values of each decreased. Increasing the concentration of NGF caused an increase in these feature values. The average Cell Body area and average intensity of neurites did not change between inhibitors or NGF over untreated values (data not shown).

Figure 5. Inhibitory Effects in Primary Striatal Neurons

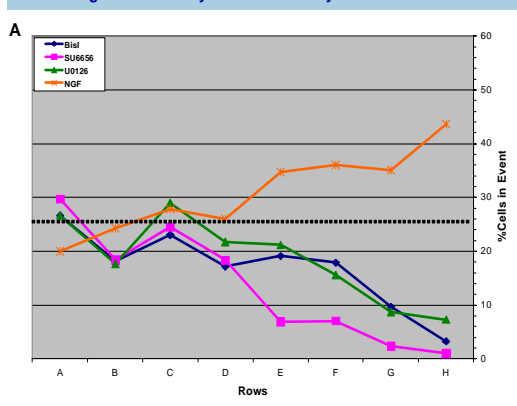


Figure 6. Inhibitory Effects in Primary Cortical Neurons

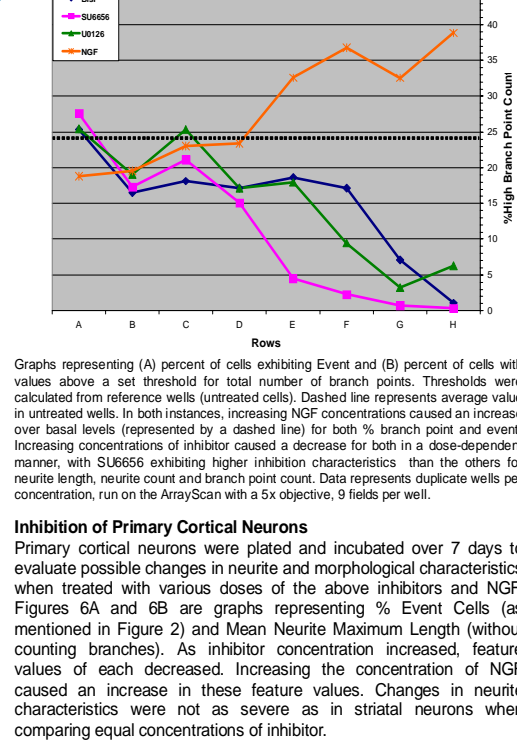


Figure 7. Evaluation of Non-Neuronal Cells

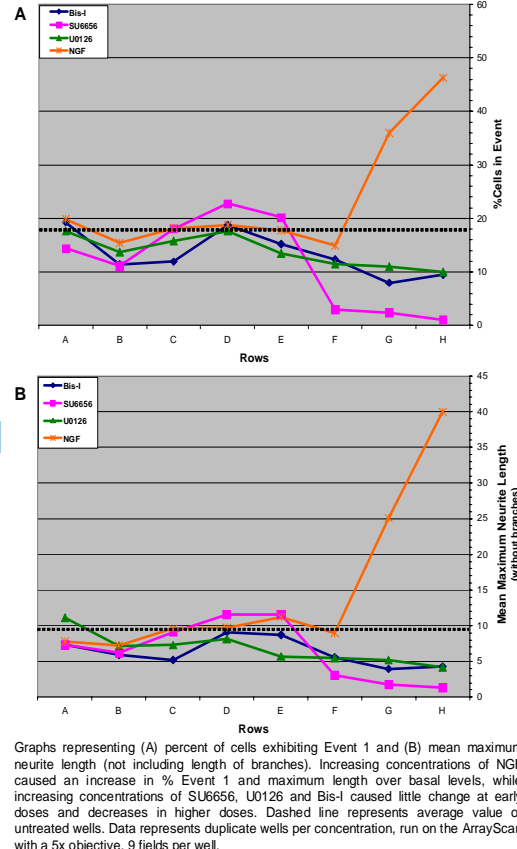
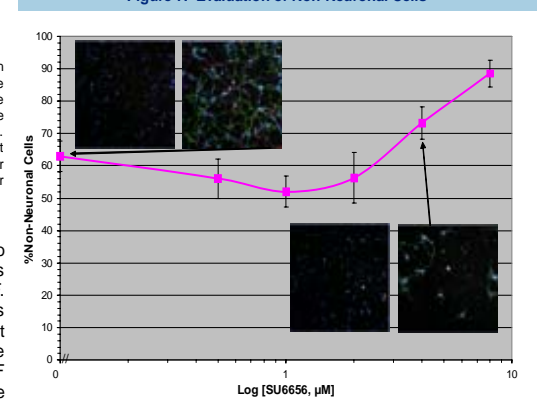


Figure 7 represents the percent of non-neuronal cells with increasing doses of SU6656 in primary cortical neurons. Hoechst dye stains the nuclei of both neuronal and non-neuronal cells and can be seen in the first channel of the BioApplication. The secondary antibody only stains neuronal cells and their neurites; thus the non-neuronal cells will not be seen in the second channel. As the concentration of SU6656 increased, the relative number of non-neuronal cells also increased. Inset images represent both channels for nuclei and cell bodies/neurites when untreated (top) and when treated with 4 µM SU6656 (bottom). In the untreated images, one can see a number of non-neuronal cells mixed with the neuronal cells. In the treated images, a large number of nuclei remain; however, the total number of cell bodies has diminished. Decreases in overall neurite length, as well as the total number of branch points, are also visible in the treated images.

Figure 7. Evaluation of Non-Neuronal Cells



Graph representing percent of non-neuronal cells over increasing concentrations of SU6656 with representative images. Data shown represents primary cortical neurons in triplicate wells per concentration, run on the ArrayScan with a 10x objective with a minimum of 300 cells/well.

Conclusions

The Neuronal Profiling BioApplication (in conjunction with the Cellomics HCS Readers and Neurite Outgrowth Reagent Kit) can easily detect and discriminate morphological changes in both NS-1 cells and primary neuronal cultures:

NS-1 cells

- In NGF-only treated cells, increasing doses of this agent does not elicit a dose-responsive increase in neurite characteristics. Instead, an immediate increase occurs in the lowest concentration of NGF over untreated levels. Differences in neurite length, branch point count, and cell body area are evident between treated and untreated wells.
- Increasing doses of inhibitor with NGF causes a dose-dependent decrease in the above characteristics
- Relative potency of compounds that inhibit neurite outgrowth of NGF are: SU6656 >> Bis-I >> U0126

Primary neuronal cultures

- Dose-dependent inhibition of neurite characteristics using the above compounds (without NGF) are similar as in NS-1 cells, with SU6656 exhibiting higher inhibition of neurite characteristics; however, some morphological differences (e.g., cell body area) are not different from basal levels (as was found in NS-1 cells)
- Relative potency of Bis-I to inhibit neurite morphology in primary cortical neurons is not as elevated as in primary striatal neurons
- Increased concentration of inhibitor resulted in a relative decrease in the number of neuronal cells, overall neurite length, and number of branch points

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