An unbiased, quantitative plate reader method for monitoring neuronal cell health and neurite outgrowth

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ABSTRACT

Neurobiology researchers often need to monitor cell health during extended periods of neural cell culture and differentiation while also evaluating the effects of various treatments that affect cell morphology and function. While a number of cell viability assays exist that allow for assessing generic features of cell vitality, there are few options that are directly tailored to measure neural cell specific features like neurite outgrowth. Likewise, whereas monitoring changes in neurite outgrowth due to culture conditions and/or drug treatment is among the most widely performed phenotypic assays, facile methods for quantifying this feature are lacking in the field. Traditional approaches have largely relied upon subjective imaging approaches, e.g., calcein AM fluorescence and beta-III tubulin immunostaining that typically require tedious wash, fix, and permeabilization workflows. To overcome these limitations we report the development of a new staining method that features a simple, high throughput screening compatible workflow. Using both primary neurons and PC-12 derived Neuroscreen™-1 cells, we have demonstrated that this assay allows for both image-based visualization as well as unbiased, quantitative plate reader analysis of neuronal cell viability and neurite outgrowth.

INTRODUCTION

Neurite outgrowth is typically monitored via immunostaining, which is both time-consuming and tedious (Figure 1). To generate a quick and simple method for measuring neurite outgrowth, a select combination of fluorescent and background suppression dyes was optimized to allow for simultaneous detection of relative neurite outgrowth (via outer cell membrane staining) and cell viability (via intracellular esterase activity) in the same sample (Figures 2 and 3). The dyes used are suitable for image-based or microplate reader detection using standard FITC and TRITC fluorescence settings. While the Molecular Probes® Neurite Outgrowth Staining Kit dyes are not inherently neural-specific and will stain any animal cell type, their optimized combination provides a useful means to visualize and quantify important cell morphology features such as neurite outgrowth.

Figure 1. Traditional immunostaining vs. the Molecular Probes® Neurite Outgrowth Staining Kit (time to results & pipette steps)

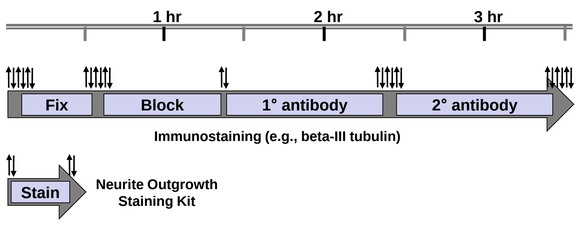

Figure 1. Traditional immunostaining requires 20 – 30 pipette steps (arrows) and >3 hours of time to obtain results. In contrast, the Molecular Probes® Neurite Outgrowth Staining Kit requires only 4 pipette steps and 15 – 30 minutes to complete.

Figure 2. Workflow using the Neurite Outgrowth Staining Kit

Neurite Outgrowth Staining Kit	Cat. No. A15001
Kit Component	Amount
Cell Membrane Stain (1000X)	100 µL
Cell Viability Indicator (1000X)	100 µL
Background Suppression Dye (100X)	2.5 mL

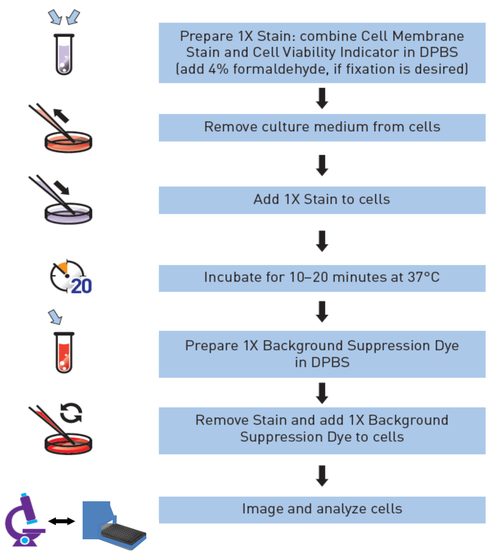

Figure 2. The Neurite Outgrowth Staining Kit was developed using a select combination of fluorescent and background suppression dyes, which work together to provide a simple, rapid workflow for simultaneously measuring relative neurite outgrowth and cell viability in the same sample.

MATERIALS AND METHODS

Neurite Outgrowth Staining Kit (Life Technologies A15001). Staining was performed as outlined in Figure 2.

Cell culture. Cryopreserved rat cortex neurons (Life Technologies A10840-02) were thawed and plated onto poly-D-lysine coated 96-well black-wall, clear-bottom microplates at a cell plating density of 5 – 10,000 cells/well. These cells were grown in Neurobasal® medium (Life Technologies 21103-049) supplemented with B-27® Serum Free Supplement (Life Technologies 17504-044) and 0.5 mM GlutaMAX™ (Life Technologies 35050). PC-12 derivative Neuroscreen™-1 cells were obtained from Thermo Fisher Scientific® and plated onto collagen I-treated 96-well plates (Life Technologies A11428-03) at 10, 000 cells/well. These cells were cultured in RPMI 1640 (Life Technologies 72400-047*) supplemented with 10% final FBS (Life Technologies 26140-111*) and 100 U/mL Penicillin/100 µg/mL Streptomycin (Life Technologies 15140-122). To stimulate neurite outgrowth, NGF (Life Technologies 13257-019) was added at the indicated concentrations to the Neuroscreen™-1 cells at the same time they were plated. *For In Vitro Diagnostic Use.

Compound treatment. 17-AAG and Bortezomib (LC Laboratories®), MG-132 (EMD Millipore®), Nocodazole (Thermo Fisher Scientific®), Paclitaxel (Life Technologies P3456), Staurosporine (Life Technologies PHZ1271), and U0126 (Life Technologies PHZ1283), SAHA (USBiological), CCCP, Cisplatin, Colchicine, Doxorubicin, Roscovitine, Rotenone and Verapamil (Sigma-Aldrich®). Compounds were prepared at 1000X in DMSO or water. Where DMSO was used as a solvent, the final amount added to the cells did not exceed 0.1%.

Imaging. Cells were imaged using a Zeiss® Axiovert® 25 inverted fluorescence microscope using standard FITC or TRITC filter sets. Images were taken using an integrated Pixera® Penguin 600CL camera and processed using ImageJ software (NIH, http://imagej.nih.gov/ij).

Fluorescence plate reader detection. Relative cell viability and neurite outgrowth was measured using a Tecan Safire^2 fluorescence plate reader to detect the fluorescence intensity from each well, expressed in relative fluorescence units (RFU). Bottom-read fluorescence detection was performed using monochromator excitation/emission settings of 483/525 nm (12 nm bandwidths) for the green-fluorescent Cell Viability Indicator and 554/567 nm (5 nm bandwidths) for the orange-fluorescent Cell Membrane Stain. Cell-free controls were included and used for background subtraction. Cell treatments were typically performed in triplicate, from which the mean +/- SEM was plotted. Curve fitting was performed with GraphPad Prism™ software using a nonlinear regression equation for variable slope sigmoidal dose-response.

RESULTS

Figure 3. Image examples using the Neurite Outgrowth Staining Kit

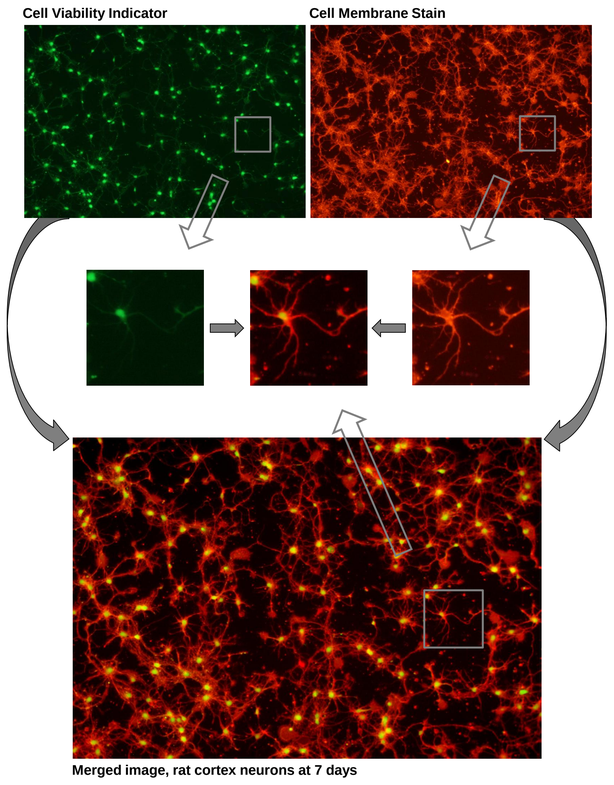

Figure 3. The Neurite Outgrowth Staining Kit allows for visualizing cell viability and neurite outgrowth in the same sample. To illustrate how this works, representative images are shown here for rat cortex neurons grown in Neurobasal® medium supplemented with B-27® Serum Free Supplement and 0.5 mM GlutaMAX™. Top, left: the cell-permeant green fluorescent Cell Viability Indicator brightly stains the cell bodies of live cells and only faintly stains the extending processes. Top, right: in contrast, the orange-red fluorescent Cell Membrane Stain brightly stains the outer surfaces of both the cell bodies as well the neurite extensions. Note that the Cell Membrane Stain will also bind nonspecifically to any animal cell type, dead or alive, and to debris. Bottom: merged images from neurons at 7 or 16 days in culture.

Figure 4. Measuring relative neurite outgrowth and cell viability

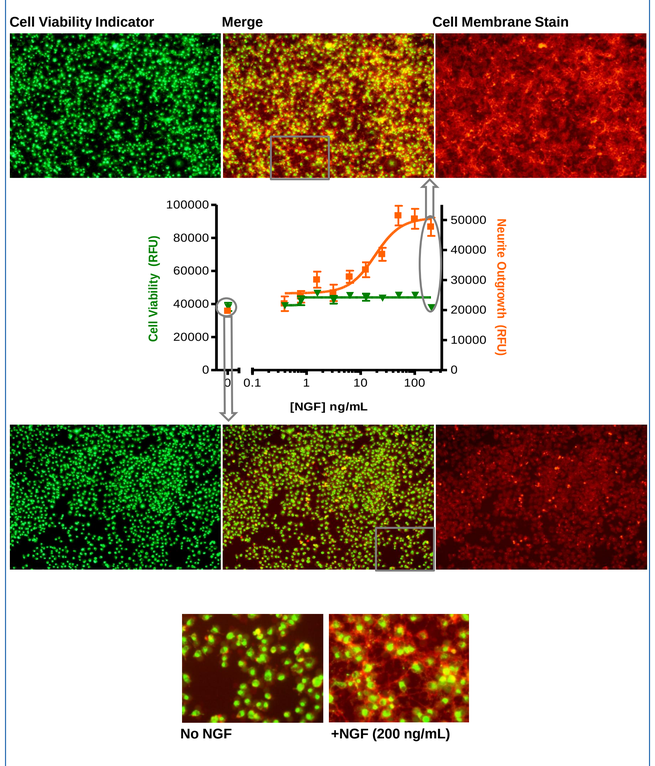

Figure 4. To demonstrate the ability of the Neurite Outgrowth Staining Kit to measure neurite outgrowth, PC12-derived Neuroscreen™-1 cells were plated in 96-well format and treated with a serial dilution of nerve growth factor (NGF) for 4 days to induce varying degrees of neurite outgrowth. Relative cell viability and neurite outgrowth was measured using a bottom-read fluorescence microplate reader to detect the fluorescence intensity from each well. For visual reference, representative images are also shown. The Neurite Outgrowth Staining Kit simultaneously measures relative cell viability (green fluorescence), which was unchanged in this experiment, and neurite outgrowth (orange-red fluorescence) which increased in an NGF dose-dependent manner.

Figure 5. Fluorescence microplate reader quantification of compound effects on relative neurite outgrowth and cell viability in the same sample

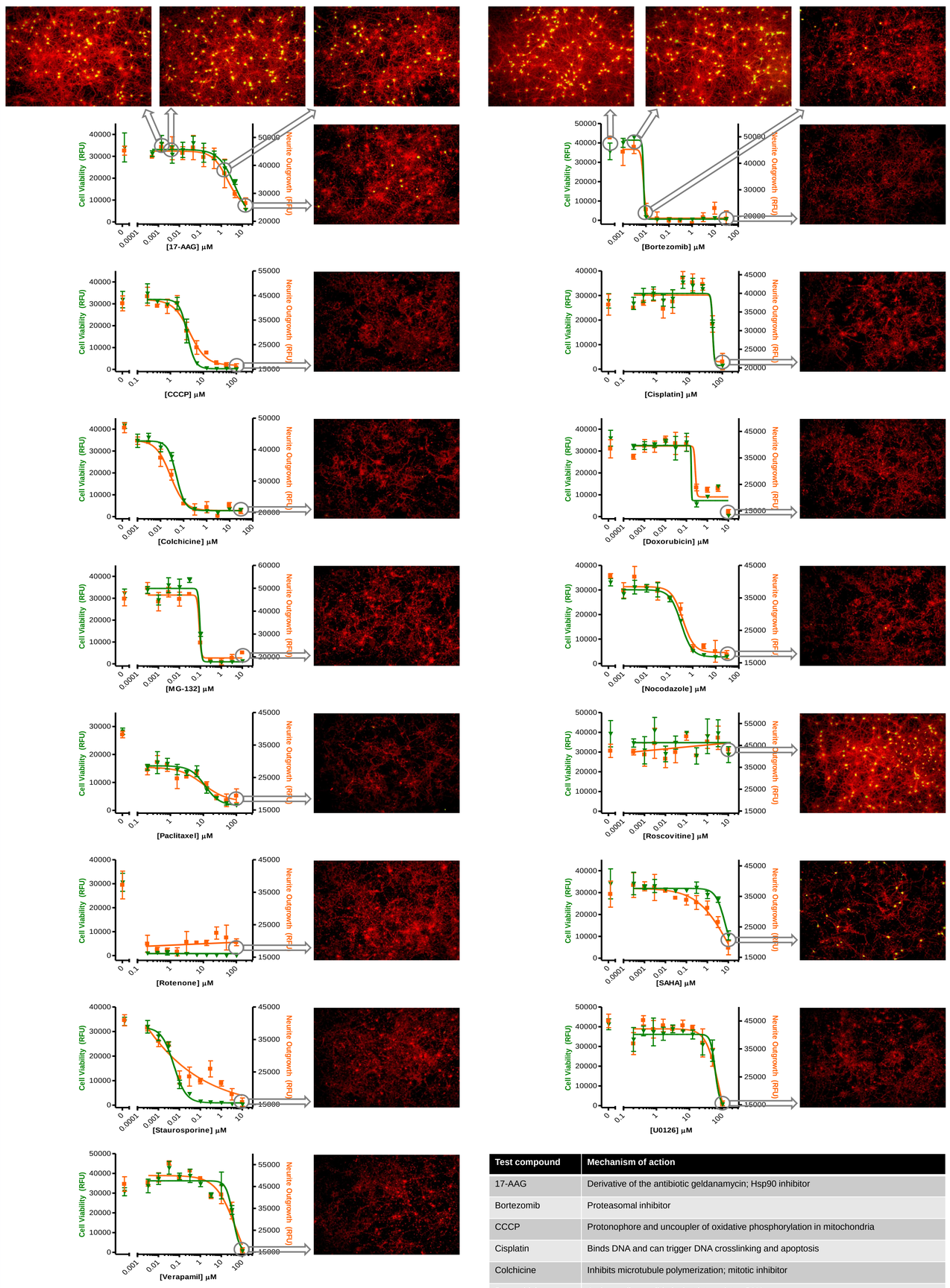

Figure 5. The Neurite Outgrowth Staining Kit can be used to measure the effects of compounds on relative neural cell viability and neurite outgrowth. Rat cortex neurons were plated in 96-well format and grown in Neurobasal® medium supplemented with B-27® Serum Free Supplement and 0.5 mM GlutaMAX™. After 7 days in culture, serial dilutions of the indicated compounds (15 total) were applied to the cells and the cells were incubated for an additional 4 days prior to staining them and measuring the average well fluorescence using a microplate reader. Also shown are merged images of the green fluorescent Cell Viability Indicator and the orange-red Cell Membrane Stain at select compound concentrations.

Test compound	Mechanism of action
17-AAG	Derivative of the antibiotic geldanamycin; Hsp90 inhibitor
Bortezomib	Proteasomal inhibitor
CCCP	Protonophore and uncoupler of oxidative phosphorylation in mitochondria
Cisplatin	Binds DNA and can trigger DNA crosslinking and apoptosis
Colchicine	Inhibits microtubule polymerization; mitotic inhibitor
Doxorubicin	Anthracycline antibiotic that intercalates DNA
MG-132	Proteasomal inhibitor
Nocodazole	Inhibits microtubule polymerization; mitotic inhibitor
Paclitaxel	Stabilizes microtubule polymers; mitotic inhibitor
Roscovitine	Cyclin-dependent kinase inhibitor
Rotenone	Inhibitor of mitochondrial electron transport
SAHA	HDAC inhibitor
Staurosporine	Pan-kinase inhibitor
U0126	MEK1/2 kinase inhibitor
Verapamil	Blocks voltage-dependent calcium channels

CONCLUSIONS

- ❑ The Neurite Outgrowth Staining Kit enables faster and easier time-to-results than traditional immunocytochemistry.
- ❑ The Neurite Outgrowth Staining Kit allows for simultaneously measuring relative cell viability and neurite outgrowth in the same sample.
- ❑ The Neurite Outgrowth Staining Kit can be used to visualize and quantify the effects of biological and chemical modulators of cell viability and neurite outgrowth.

TRADEMARKS/LICENSING