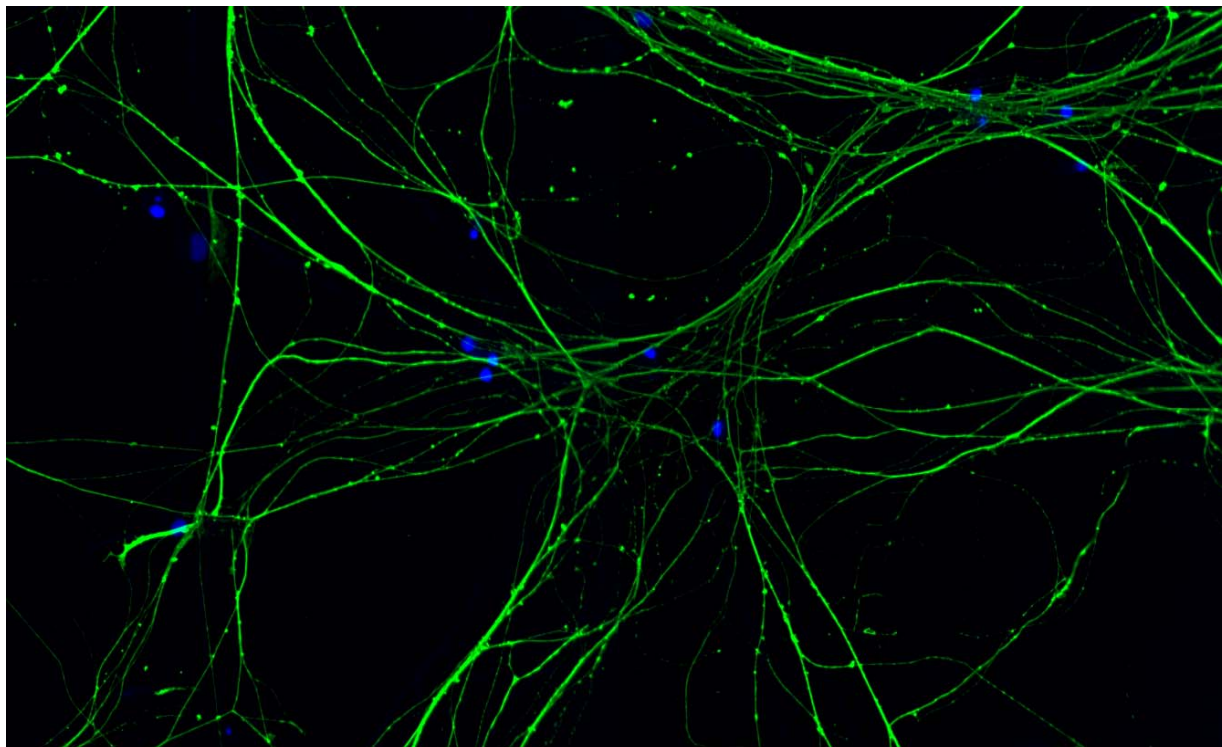


BD Biosciences

Quantitative High Content Analysis of Neurite Outgrowth



Introduction

The development of therapies for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis represents a major challenge to researchers in academia, biotechnology, and the pharmaceutical industry. In addition, therapeutics are also being sought for trauma-induced neuronal injuries such as cerebral ischemia and spinal cord injury¹. Consequently, many neuroscience projects are focused on identifying new drugs that affect the growth of neurites which is a critical event in neuronal development, formation and remodeling of synapses, response to injury, and regeneration². Changes in the pattern of neurite outgrowth have been implicated in neurodegenerative disorders as well as traumatic injuries. The discovery of new compounds that can positively affect neuritogenesis would be of immense importance for developing new therapeutics against both neurodegenerative diseases and injury. Measurement of neurite outgrowth using an automated image-based assay can be of use in the research, screening and validation phases of the drug discovery process. Moreover, a neurite outgrowth assay can also be used for testing toxic neuropathies.

High content screening (HCS) is an emerging technology that measures and analyzes cellular responses in individual

cells in a multi-well plate format. Cell line model systems that respond appropriately to external stimuli have enabled robust HCS assays to be developed for primary and secondary screening in the drug discovery process³. The BD Pathway™ bioimaging platforms are highly versatile systems that are ideally suited for HCS applications. The BD Pathway software enables a broad array of fluorescence-based biological assays to be performed, such as protein translocation, angiogenesis, apoptosis, cell cycle, and 3-dimensional cellular exploration, among others. Here we introduce a specialized algorithm for quantification of neurite outgrowth that can be used for analyzing both neuritogenesis and neurotoxicity.

We have tested the neurite outgrowth software using the rat clonal pheochromocytoma cell line PC12 which can be induced to display neurite outgrowth upon induction with nerve growth factor⁴. In this multi-parameter assay, the software segments the neuronal cell body and analyzes fluorescently labeled neurites with respect to their total length, maximum length, root count, extremities count, node points and segment count. In addition, we have used primary cells (rat dorsal root ganglion neurons) to demonstrate the ability of the BD Pathway platforms and the software to image and analyze very fine and complex primary neuronal structures.

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Methods

PC12 cell line (ATCC, CRL-1721) was maintained in DMEM with 10% FBS, 5% horse serum and 1% penicillin/streptomycin. For neurite generation, ~15,000 cells/well were plated in BD Falcon™ 96-well Imaging Plates (Cat # 353219) that were coated with BD type 1 rat tail collagen (Cat # 354236) using 1.8 µg collagen/well. After 24 h, the medium was replaced with differentiation medium (DMEM with 0.1% FBS, 0.05% horse serum) containing various concentrations of NGF (Calbiochem, 420298). The medium was replenished every 3rd day for 10 days. For inhibition of NGF-dependent neurite generation, cells were pretreated with various concentrations of a protein kinase inhibitor, K252a (Calbiochem, 480354) in differentiation medium for 1 hour and then treated with 100 ng/ml NGF in the continued presence of K252a for 10 days.

Primary rat dorsal root ganglion neurons (Cambrex, R-DRG-505) were plated at a density of ~5000 cells/well in BD Falcon™ 96-well Imaging Plates (Cat # 353219) coated with poly-D-lysine (Sigma, P6407) in primary neuron basal medium (Cambrex, CC-4461) supplemented with NSF-1, L-glutamine, penicillin/streptomycin and mitotic inhibitors as per suppliers' instructions. Neurites were allowed to extend for 7-8 days.

For imaging, cells were fixed with 3.7% paraformaldehyde (Electron Microscopy Sciences, 15714) for 20 minutes and permeabilized with 0.1% Triton-X-100 (Sigma, T-9284) for 5 min. Neurites were stained with a primary mouse anti-β-tubulin antibody (Cat # 556321) using 0.125 µg antibody/well followed by Alexa Fluor® 488 goat anti-mouse IgM (Invitrogen, A21042) at a concentration of 0.25 µg/well. Hoechst 33342 (Invitrogen, H3570) was used at 0.1 µg/well to stain the

nuclei. To prevent the dissociation and fracture of fragile neuronal networks, the number of washes in the fixation and processing steps were minimized and extra care was taken in aspirating and dispensing liquids in wells. In some experiments, anti-β-tubulin antibodies directly conjugated to Alexa Fluor® 488 (Cat # 558605), Alexa Fluor® 555 (Cat # 558608) and Alexa Fluor® 647 (Cat # 558606) were used at a pre-determined optimized concentration in a single staining step along with 0.1 µg/well Hoechst 33342.

Images were acquired on a BD Pathway™ 855 (Cat # 341036) or BD Pathway 435 (Cat # 641250) in non-confocal and confocal mode using a 20x objective (0.75 NA) in the form of 2x2 or 3x3 montage. For confocal mode, seven Z-sections separated by 2 µm steps were acquired which were collapsed into a single stack for analysis. The images were analyzed using BD's neurite outgrowth algorithm (Cat # 346203). Numerical data was graphed and dose response curves were generated. EC/IC₅₀ values and Z' scores were calculated using the BD Pathway software.

Results and Discussion

The neurite outgrowth software requires fluorescent images of the nucleus and neurites of a neuronal cell. The algorithm uses Hoechst images to generate segmentation masks of the nuclei and to create cell body boundaries around them. The software then employs the second fluorescence channel to define the neuronal network. The default algorithm settings work well for many cell types, however, settings can be customized to address issues such as cell clumping, missing or broken neurites, and debris or non-specific fluorescence. After analysis, image masks are generated (**Figure 1**) which allow

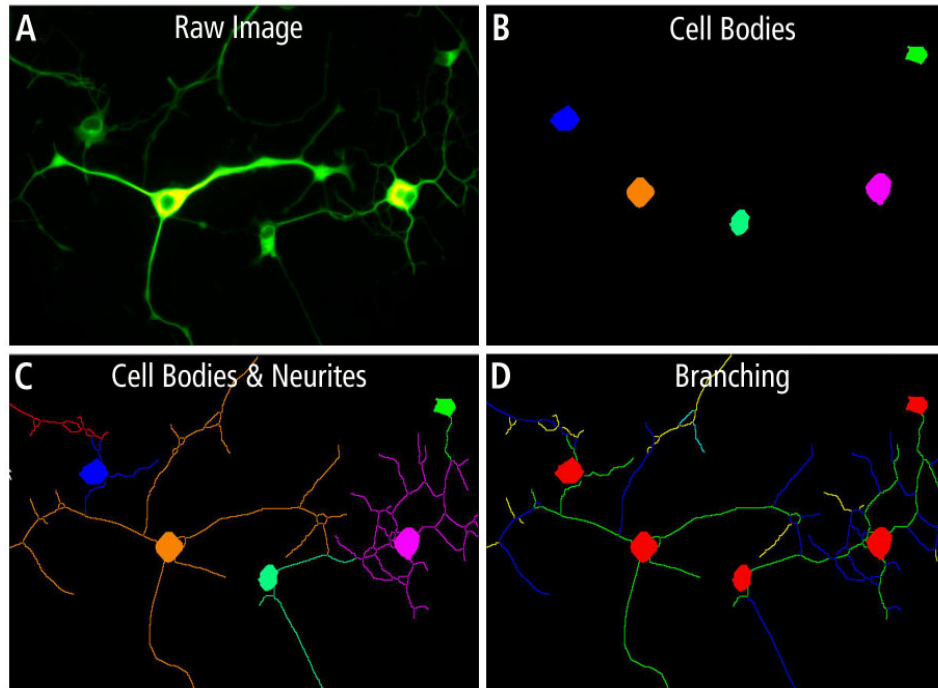


Figure 1. Image and segmentation masks. Panel A, a pseudo-colored raw Alexa Fluor® 488 image; Panel B, cell body image after segmentation of Hoechst image (not shown) to identify cells and Alexa Fluor® 488 image to establish boundaries of the individual cell bodies (colors are randomly assigned by the software); Panel C, cell body and segmentation mask displaying neurites associated with the cell bodies (each neuronal network is in a separate color); Panel D, branch level image of neurites showing the PC12 cell bodies (red) and associated neurite extremities identified as primary (green), secondary (blue), tertiary (gold) and quaternary (cyan).

the user to interpret the outcome of the software parameters that have been chosen for analysis. Modifications can be made to default settings and data can be reanalyzed as needed. Data output from the software analysis measures total, maximum and average length of neurites in addition to total number of roots, neurite extremities and segments along with the node points. The data can be analyzed using the BD Pathway™ software to generate graphs, EC/IC₅₀ curves, Z' score, Signal/Noise measurements and data tables, or can be exported as text files for analysis in other 3rd party software.

Measurement of Neurite Outgrowth

PC12 cells are the most widely accepted model for studying neurite growth as they respond to NGF in a dose and time dependent manner⁵. We validated the use of the neurite outgrowth algorithm on dose dependent neurite outgrowth in PC12 cells induced with NGF. **Figure 2** shows representative merged pseudocolored images from a dose response experiment of NGF and EC₅₀ curves for selected measurement parameters in the neurite outgrowth software. The data was generated using a contiguous 3x3 montage (9 image fields/well) which allows greater visualization of the neurite network. The EC₅₀ values of NGF using different neurite outgrowth parameters were found to be between 22-39 ng/ml.

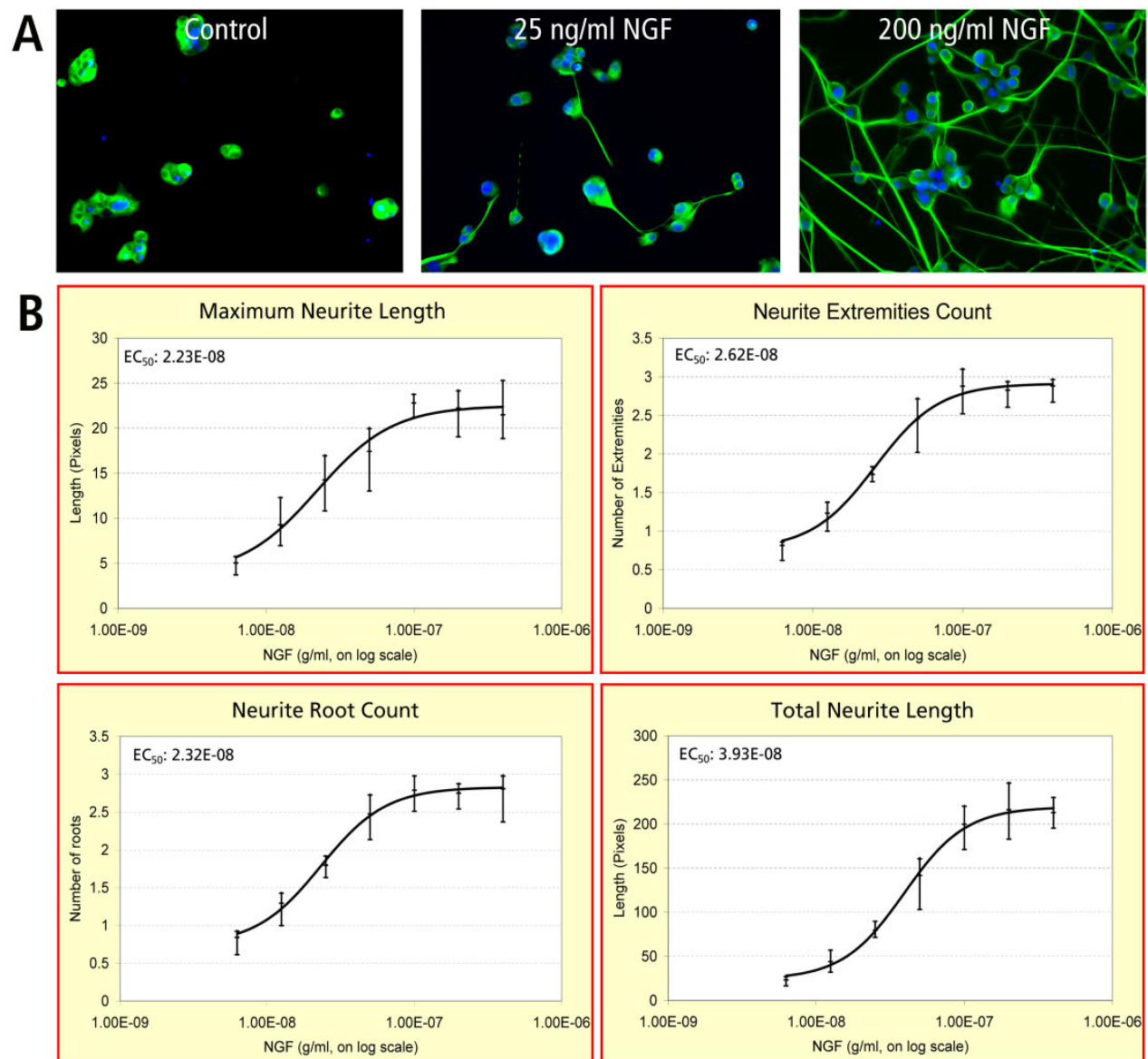


Figure 2. NGF induces neurite outgrowth in PC12 cells in a dose-dependent manner. Panel A, Pseudo-colored merged images of Alexa Fluor® 488 (cell bodies and neurites: green) and Hoechst (nuclei: blue) channels of control and NGF treated cells (single representative image per field); Panel B, EC₅₀ curves plotted in the BD Pathway software for maximum neurite length, extremity count, root count and total neurite length. Non-confocal 3x3 montage images were acquired using a 20x objective (0.75 NA). Data from n=10 wells is shown as mean ± SEM for all four parameters.

Inhibition of Neurite Outgrowth

The mechanism of NGF-induced cell differentiation and neurite outgrowth has been well investigated and involves binding of NGF to TrkA, a receptor tyrosine kinase. Upon binding, it stimulates dimerization and autophosphorylation of TrkA and initiates intracellular signaling cascades that propagate to the nucleus leading to expression of various genes involved in neuronal differentiation of PC12 cells⁶. K252a, a potent protein

kinase inhibitor, blocks NGF induced neurite outgrowth by inhibiting protein phosphorylation of TrkA elicited by NGF⁷. **Figure 3** shows the representative images and IC₅₀ curves for dose dependent inhibition of neurite outgrowth by K252a. The IC₅₀ values for the different neurite outgrowth parameters for K252a ranged from 14 to 18 nM which is consistent with published reports⁸.

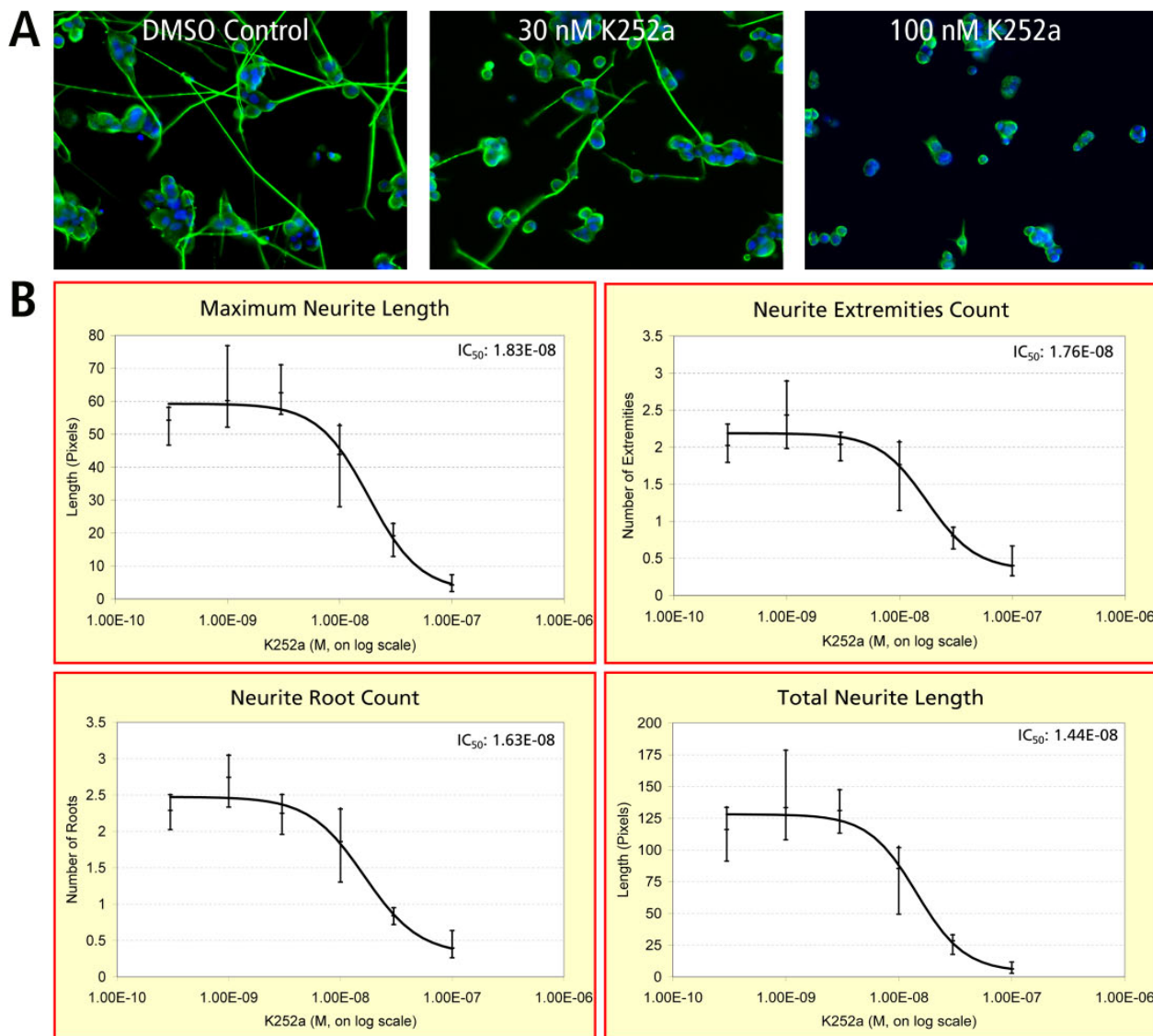


Figure 3. K252a inhibits NGF-induced neurite outgrowth in a dose-dependent manner. Panel A, Pseudo-colored merged images of Alexa Fluor® 488 (cell bodies and neurites: green) and Hoechst (nuclei: blue) channels of PC12 cells pretreated with DMSO, 30 nM and 100 nM of K252a followed by NGF treatment (single representative image per field); Data was analyzed in similar manner as in Figure 2. Panel B, IC₅₀ curves generated in BD Pathway™ software for maximum neurite length, extremity count, root count and total neurite length. Non-confocal 2x2 images were acquired using a 20x objective (0.75 NA). Data from n=4 wells is shown as mean ± SEM for all four parameters.

Advantages of Confocal Imaging

Clonal cell lines, such as PC12, are a relatively easy to use homogenous source of cells which can be grown in unlimited quantities. Nevertheless, primary cells are often desired for drug discovery and neurotoxicity applications because they better model their *in vivo* counterparts. We tested the neurite outgrowth application on primary rat dorsal root ganglia cells (neonatal) which form a neurite network after 7 days in culture. Since the neurite network formed by these primary cells was very complex (Figure 4) and included neurites that grew over one another in multiple focal planes, we compared the data quality from non-confocal and confocal images. One of the features of confocal imaging on the BD Pathway™ bioimagers is the ability to automatically collapse the Z-sections of a confocal stack into a single image for analysis. Figure 4 shows

the same 2x2 pseudocolored merged image field taken in confocal (collapsed stack) versus a single non-confocal plane. The improved clarity of the complex neuronal structures is evident in the enlarged region of the confocal collapsed stack. Furthermore, the data quantified from the two images using the neurite outgrowth algorithm confirmed the advantage of confocal over the non-confocal imaging (Table 1). An 11% to 28% increase in sensitivity of detection of neurite outgrowth measurement parameters was observed for the confocal data. A similar trend was observed with complex and dense network of neurites from PC12 cells (data not shown). The ability of the BD neurite outgrowth algorithm to segment and measure the complexities of neurites originating from dorsal root ganglion neurons highlights its flexibility and robustness in applications involving primary cells.

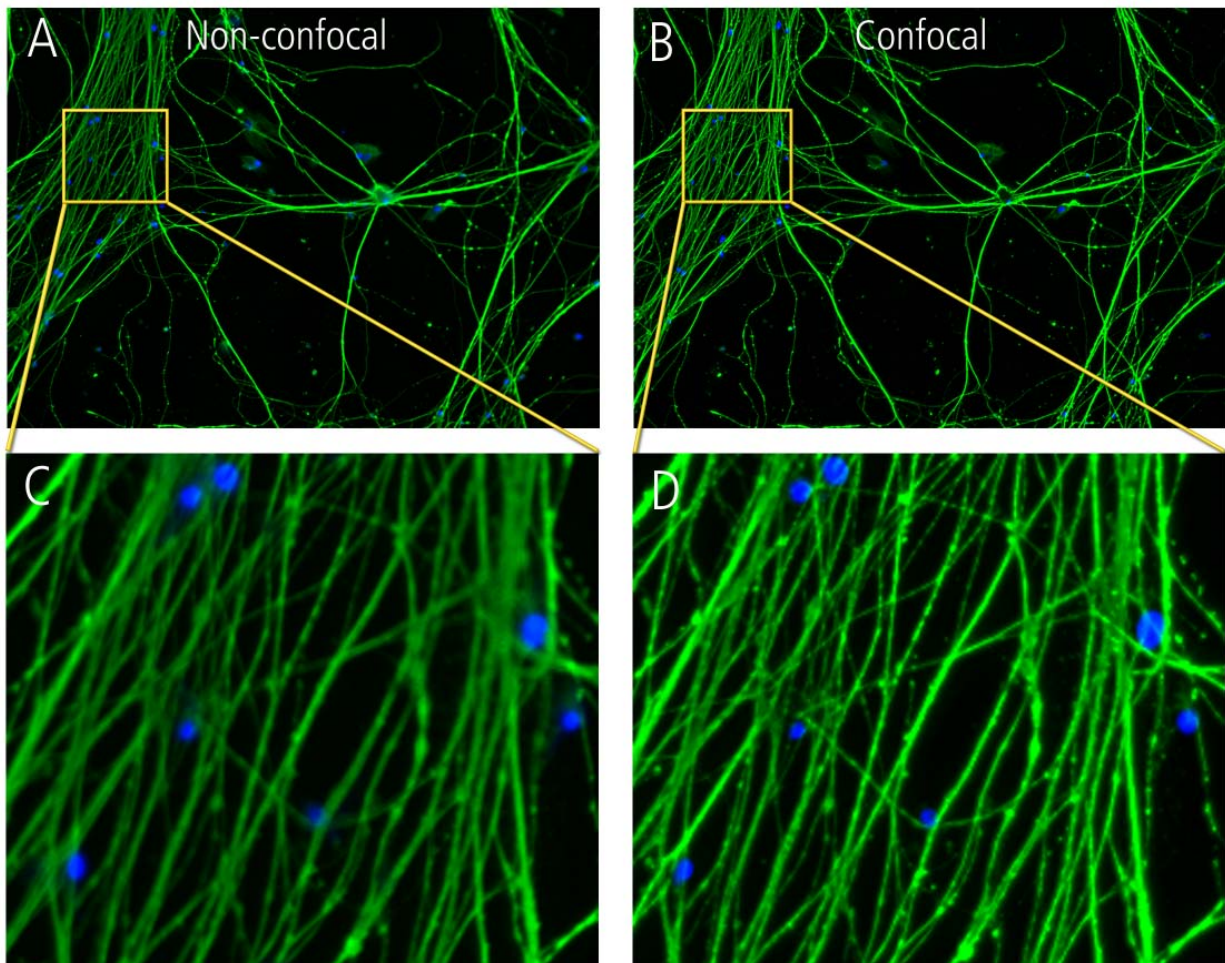


Figure 4. Representative images of rat dorsal root ganglion neurons. Panels A and B show pseudo-colored merged image of Alexa Fluor® 488 (cell bodies and neurites: green) and Hoechst (nuclei: blue) taken in non-confocal and confocal mode respectively. Images were acquired as a 2x2 montage, using a 20x objective (0.75NA). Panels C and D are enlargements of the areas marked by boxes in panels A and B, respectively.

Neurite Outgrowth Parameter (Average)	Non-Confocal	Confocal	% Increase
Total Neurite Length (Pixels)	607.8	696.8	12.8
Root Count	3.6	4.5	20.0
Segments	32.7	44.9	27.2
Extremities Count	3.1	3.5	11.4
Node Points	29.6	41.4	28.5

Table 1. Comparison of non-confocal and confocal neurite outgrowth data. Rat dorsal root ganglion neurons images (**Figure 4**) were analyzed using the neurite outgrowth algorithm and percent increase of each parameter was determined.

Improving Assay Quality (Z' score) using Montage Capture

Typical neurite outgrowth assays require cells to be plated relatively sparsely so as to leave adequate room for neurite extension. However, this sparse plating means that fewer cells are captured per image field which may lead to higher standard deviation. To address this concern and have statistically

relevant numbers of cells, BD Pathway™ systems are capable of assembling a montage of contiguous image fields (tiles) within a well that produces a single montaged image (**Figure 5**). The exact alignment of the montaged images and thus, neuronal networks is due to the precision and accuracy of the linear motors moving the objective. This hardware based approach to capture a montage is more precise than software based methods

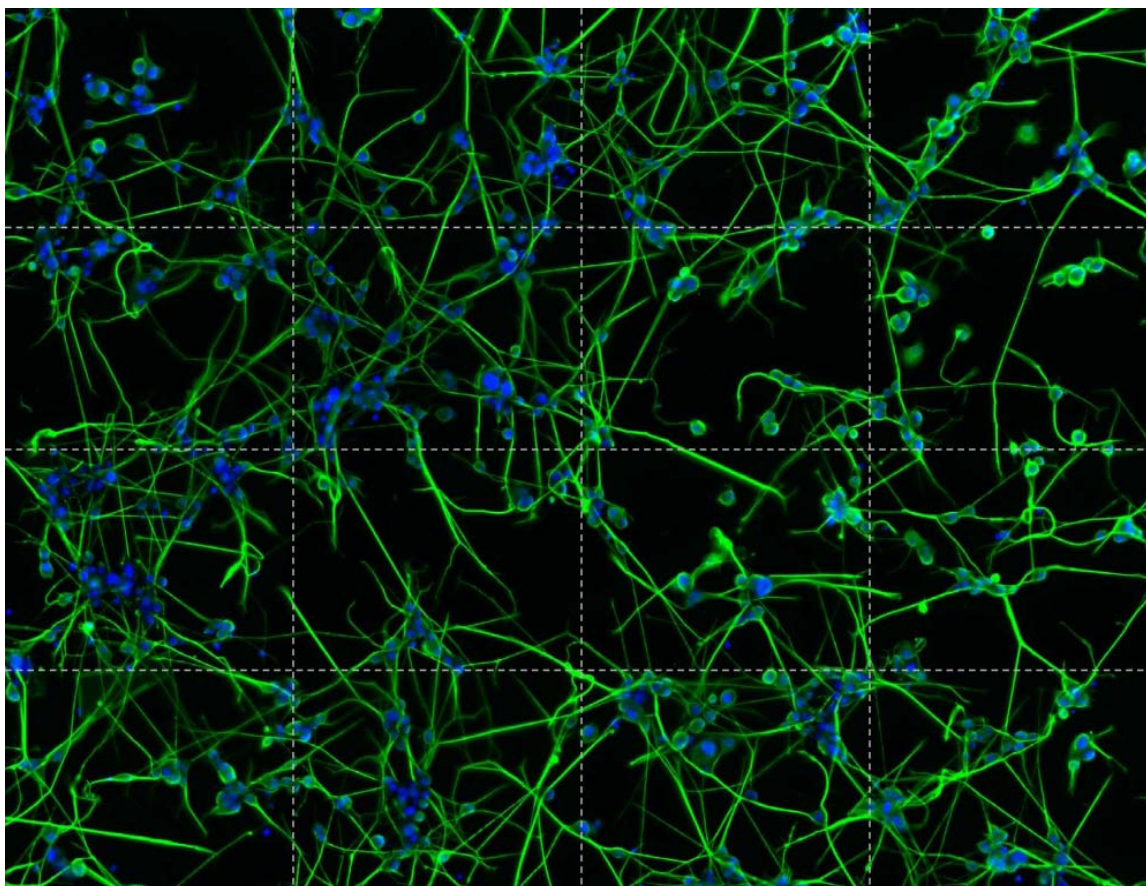


Figure 5. Precision and accuracy of montage. Pseudo-colored merged image of Alexa Fluor® 488 (cell bodies and neurites: green) and Hoechst (nuclei: blue) channels of PC12 cells differentiated with 100 ng/ml NGF. Images were acquired as a 4x4 montage using a 20x objective (0.75 NA). Grey dotted lines indicate the boundaries of each of the 16 image fields in the 4x4 montage.

and requires no additional image processing time. The montage feature ensures the continuity of neurites and provides improved image analysis over data from multiple random images within a well where neurites would be discontinuous. We evaluated the effect of montage size on the Z' value, which is a measure of assay quality and robustness⁹. The Z' value is based on the difference between the signal of the positive control and that of the negative control and also factors in the standard deviation of each signal. A min/max plate was analyzed in which half of the plate was treated with 200 ng/ml NGF (max) to induce neurite outgrowth and the other untreated half was used as a negative control (min). For all parameters tested, an increase in Z' value and a decrease in standard deviation (data not shown) were seen with larger montages containing higher numbers of cells (**Figure 6**). This result is typical for cell based assays that exhibit a fairly heterogeneous response to stimuli. Overall, contiguous montage and increased cell count in a neurite outgrowth assay ensures data quality and improves statistical parameters.

Improving Assay Performance using Primary Conjugated Antibodies

It is important to note that neurites are long, fragile structures that grow in three dimensional networks and thus, are very prone to shear stress during the fixing and staining procedure. A typical protocol includes fixation, permeabilization, primary and secondary antibody incubation with 2 washes after each step for a total of 12 dispensing and aspirating steps. One approach to safeguard the neurites from shear stress is to minimize these steps, a feature which is also highly desirable in high-throughput applications. However, reducing washes between steps can lead to non-specific artifacts and higher background. An approach which greatly reduces the number of required steps, but does not risk higher background or artifacts, is the use of primary antibodies directly coupled to a fluorophore. These reagents eliminate the need for secondary antibody incubation and the associated washes, thereby reducing the number of aspirating/dispensing steps by 25%. BD Bioimaging Certified™ primary anti- β -tubulin antibodies

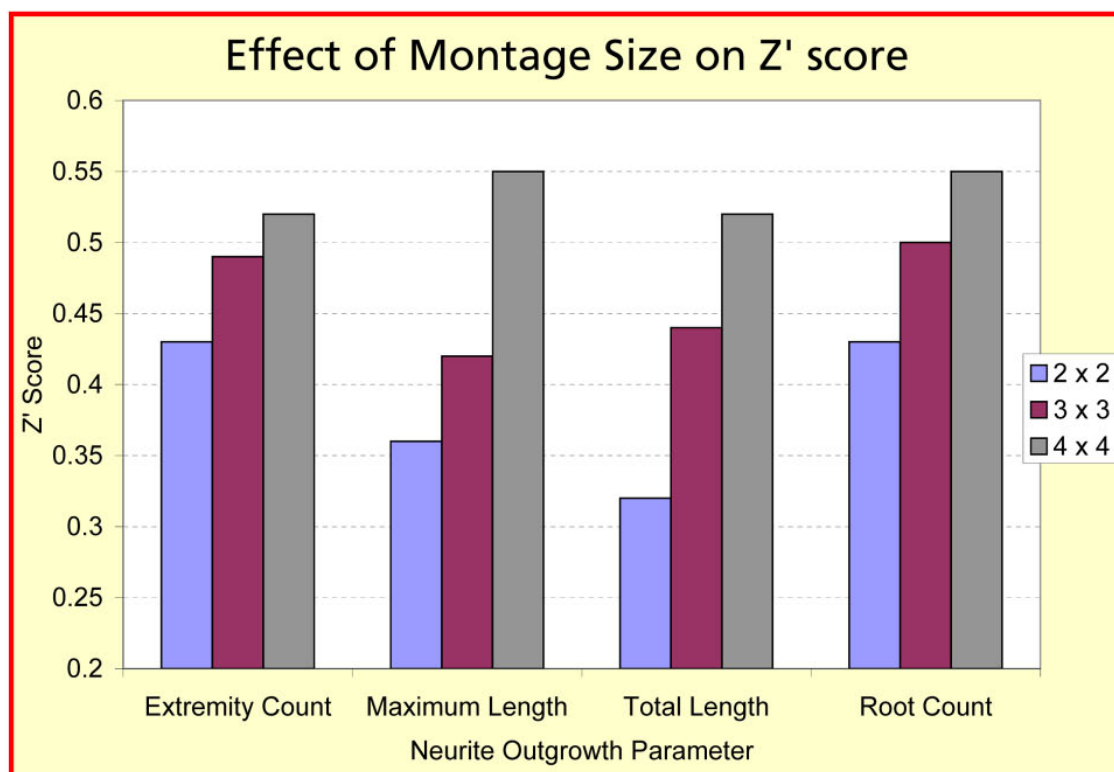


Figure 6. Effect of montage size on assay quality. A min/max experiment using 48 untreated and 48 NGF-treated wells of PC12 cells was imaged in non-confocal mode using different montage sizes with a 20x objective (0.75NA). The data from the different montage sized images was analyzed using the neurite outgrowth algorithm and Z' scores were calculated.

Application Note – Neurite Outgrowth

conjugated with Alexa Fluor® 488, 555 or 647 were used for labeling neurites and the results were compared with the conventional primary and labeled secondary antibody protocol. **Figure 7** shows representative images of PC12 neuronal networks labeled with the primary conjugates and conventional primary and labeled secondary reagents. In this experiment, the extent to which neurites were detected was unaffected by the reagents used for labeling. All three conjugated primary antibodies effectively labeled neurites and equivalent signal to noise was observed compared to primary antibody followed by labeled secondary antibody (data not shown). Camera exposure

times for primary conjugated antibodies varied depending upon the fluorophore used, but in general were in the same range as that used for primary antibody and labeled secondary antibody staining.

Primary conjugated antibodies showed a distinct advantage over primary/secondary reagents when using PC12 cells that formed very long and extensive neurite networks at higher NGF concentration. These longer neurites were especially prone to shear stress factors and were often severely disrupted when using the standard fixation, permeabilization and staining

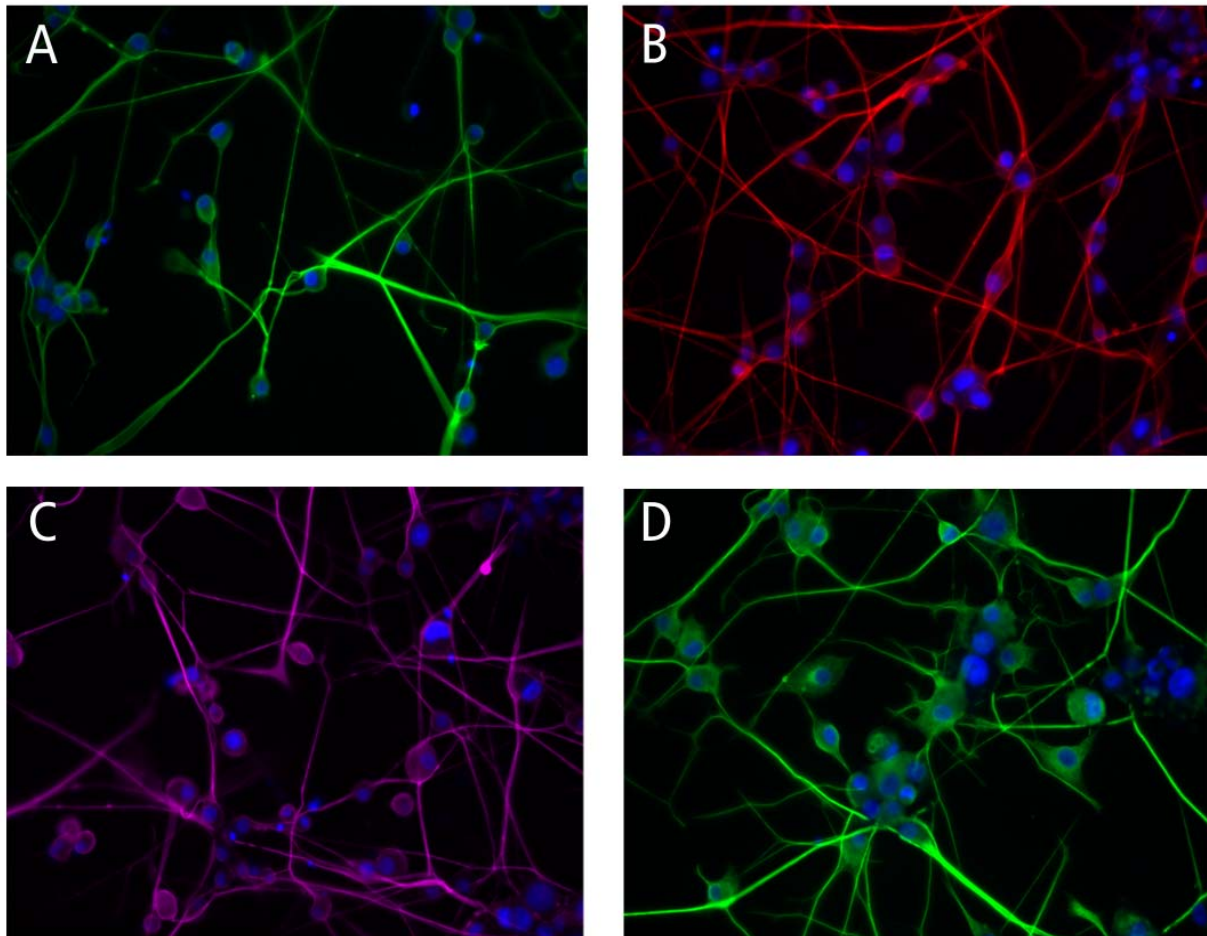


Figure 7. Representative images using primary conjugated antibodies. Pseudo-colored merged images of NGF treated PC12 cells. Nuclei are pseudo-colored blue in all images. Neurites were stained with: Panel A, Alexa Fluor® 488 conjugated anti β-tubulin antibody (green), Panel B, Alexa Fluor® 555 conjugate (red); Panel C, Alexa Fluor® 647 conjugate (magenta) and Panel D, unlabeled anti β-tubulin antibody and labeled Alexa Fluor® 488 conjugated secondary antibody (green). Images were acquired as non-confocal single tiles using a 20x objective (0.75 NA).

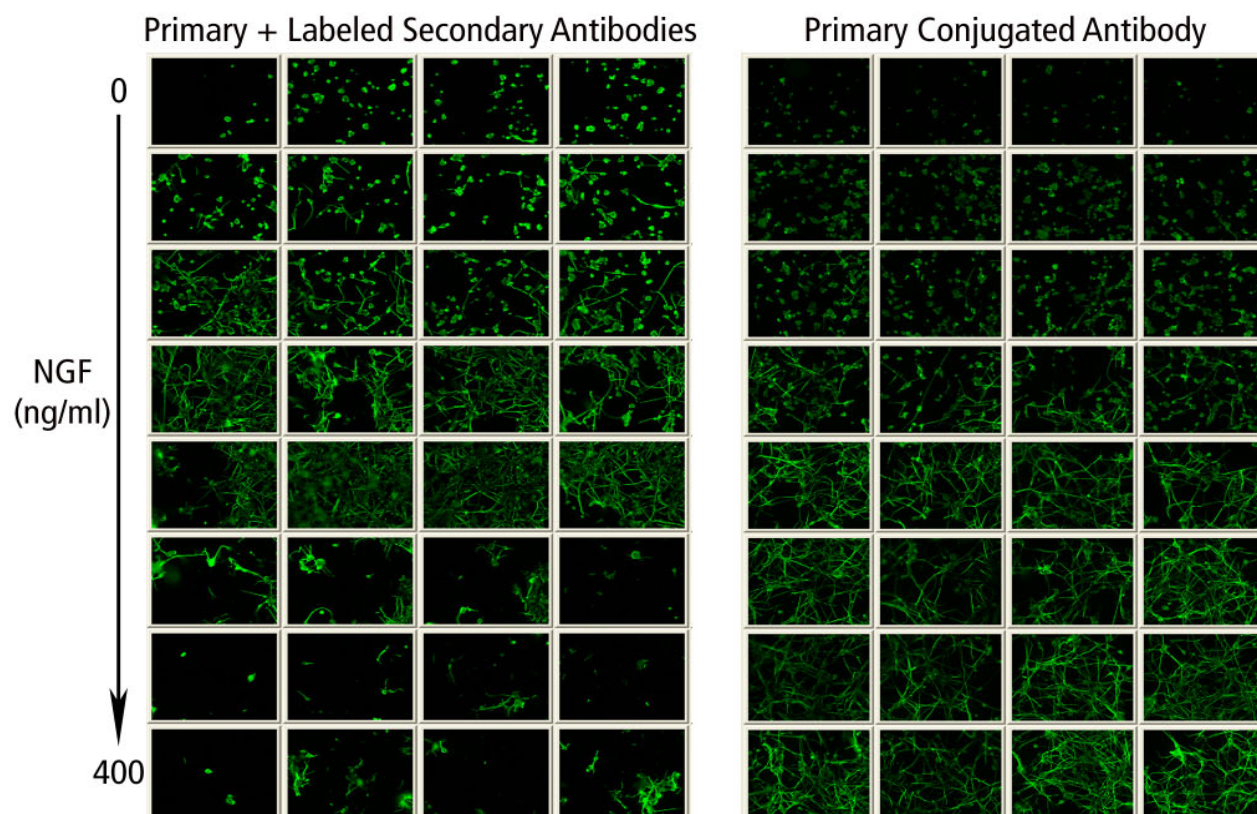


Figure 8. Comparison of conventional two step staining protocol to a one step protocol using primary conjugated antibody. A dose response of NGF (two fold dilutions, from 400 to 6.25 ng/ml, plus control) displayed as pseudo-colored thumbnail images of neurites (green) from 4 replicates of a 96 well plate. Left panel, images of wells processed using unlabeled anti- β -tubulin antibody and Alexa Fluor® 488 labeled secondary antibody; Right panel, images of wells processed using Alexa Fluor® 488 labeled primary conjugated anti- β -tubulin antibody.

protocols (**Figure 8**). The images substantiate that primary conjugated antibodies can save delicate neurites by reducing the number of wash steps. An additional benefit was reduced plate processing time.

Summary and Conclusions

The neurite outgrowth assay is an important tool for developing therapeutics for neurodegenerative disorders and injuries. Robust automated methods for integrating the neurite outgrowth assay into the drug discovery process are needed to ensure success. The neurite outgrowth application used in conjunction with BD Pathway™ bioimaging platforms is a robust and flexible algorithm which can be used for analysis of neuronal cell lines or primary cells. The algorithm can precisely count the total and average neurite length per cell, number of roots/neurites emerging per cell, neurite extremities (primary, secondary and tertiary branching) and neurite node points (total number of points where secondary and tertiary branches exist). We have successfully demonstrated dose response curves of neurite outgrowth with NGF as well as inhibition of neurite outgrowth using a protein kinase inhibitor.

The BD Pathway bioimagers' ability to acquire confocal collapsed stack images further improves the accurate analysis of neurites as demonstrated by use of primary dorsal root ganglion neurons which form complex neurite networks. Building precise montages that allow larger sections of the neuronal network to be analyzed also improves accuracy and reduces standard deviation. The use of primary conjugated antibodies not only reduces the number of steps in the assay protocol, but also dramatically improves the likelihood of intact neuronal networks post-processing. Furthermore, additional primary conjugates can be multiplexed along with anti- β -tubulin so that other cellular processes such as apoptosis, cell cycle or signal transduction events can be monitored in a true high content manner.

Overall, the simplicity, accuracy, flexibility and robustness of this neurite outgrowth assay makes it a useful tool for neuroscience research and in drug discovery for screening new compounds to prevent and treat neurodegenerative diseases and injuries, as well as testing existing drugs and chemicals for neuropathies.

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