Neurotoxicity and Neurite Outgrowth Assay

For High Content Screening

For 5 x 96-well plates

Catalog No. HCS220

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

(i) High Content Screening and Drug Discovery

High Content Screening (HCS) technology offers a major opportunity to improve the drug discovery and development process [1]. HCS enables the evaluation of multiple biochemical and morphological parameters in cellular systems and facilitates characterization of the subcellular distribution of fluorescent signals with labeled reagents. By combining automated imaging of cells with validated detection reagents and powerful image analysis algorithms, scientists can now acquire deeper knowledge of multiple pathways at the single-cell level, usually in a single assay, at an early stage in the development of new drugs [2]. HCS platforms such as the IN Cell Analyzer (GE Healthcare), ArrayScan (ThermoFisher Scientific), or Opera (Perkin Elmer), can be used to deliver detailed profiles of cellular responses [3].

Successful HCS assays rely on high quality reagents [4]. With the commercial availability of thousands of immunoreagents and fluorescent probes, large numbers of fixed-endpoint HCS assays are possible. However, incompatibility of reagents when integrated into a single assay can lead to a significant drop-off in assay performance. Immunoreagents for HCS assays carry special requirements. Strong antigen affinity is required, minimal non-specific binding must be observed, interactions between multiple primary or secondary immunoreagents must be minimized, and the signal to background ratio must be sufficient to ensure an adequate screening window [4]. Additionally, to enable scale up of HCS assays, the sample preparation protocol must be highly reproducible, and the reagents must exhibit minimal assay-to-assay variability.

(ii) Neurite outgrowth and its significance in drug discovery

During development, neurons and neuronal-like cells extend numerous processes that differentiate into dendrites and axons. These processes, known as neurites, are critical for communication between neurons. The characterization of neurite formation, maturation and collapse/resorption is an area of intense interest, since these cellular processes are essential for interconnection of neuronal cell bodies. Neurite formation and neuronal regeneration hold particular promise for development of therapies for neurodegenerative conditions such as Alzheimer's and Parkinson's diseases, neuronal regeneration following spinal cord or brain injury, stroke, and diabetic neuropathy [5-8]. Therefore, major efforts in central nervous system drug discovery research are focused on the identification of compounds that affect neurite outgrowth.

The use of HCS for measurement of neurite outgrowth has become a valuable tool for neuroscience research and drug discovery [9, 10]. A large number of cell types have been successfully utilized for HCS neurite outgrowth analysis. These include immortalized/stable cell lines such as PC12, SY5Y and N2A; primary hippocampal, cortical and dorsal root ganglia (DRG) neurons; and neuronal embryonic stem cells. By labeling neuronal cells with an antibody recognizing a neuronal-specific marker, HCS provides the opportunity to perform high-throughput, non-subjective, quantitative neurite outgrowth assays, and enables morphological screening of multiple parameters in individual cells. Parameters which may be readily detected using HCS platforms include neuronal number, neurite count, neurite length, neurite area and branch point count. Accordingly, HCS has been demonstrated to be an effective method to screen for inducers of neurite outgrowth and neuronal regeneration. Compared to traditional approaches which are labor-intensive and subjective, HCS-based neurite outgrowth assays offer the opportunity for greatly improved productivity in neuroscience research and drug discovery.

(iii) HCS as a novel tool for neurotoxicity assays

Neurotoxicity assessment represents an important part of drug safety evaluation, as well as being a significant focus of environmental protection efforts [11, 12]. Additionally, neurotoxicity is also a well-accepted *in vitro* marker of the development of neuronal diseases, such as Alzheimer's disease [13].

In an attempt to develop more sensitive, higher-throughput neurotoxicity assays, researchers are increasingly adopting HCS technology [14-19]. Neuronal morphology has been demonstrated to provide an effective method for quantification of neuronal cell injury [14]. Since HCS represents the most effective method for quantitation of neuronal morphology, allowing accurate measurement of multiple parameters, HCS offers the opportunity to develop a new generation of neurotoxicity assays. Several groups have recently reported the application of HCS-based neurotoxicity screens in a variety of cellular models [14-19]. In each study, immunodetection was used successfully to quantitate neurotoxic effects. Thus, HCS represents a novel, highly-sensitive neurotoxicity screening tool.

Application

Millipore's HCS220 Neurotoxicity and Neurite Outgrowth Assay is a High Content Screening kit comprised of high-quality, validated, target-specific detection reagents for profiling neurotoxicity, neurite outgrowth and neuronal morphology in a wide variety of mammalian cell types. The highly-validated reagents provided with this kit allow the user to standardize their assays, minimize assay-to-assay variability, and to reproducibly generate images with a high signal-to-background ratio.

The kit is immunofluorescence-based, and uses a high quality βIII-tubulin primary antibody which specifically labels neurites and neuronal cell bodies from a wide variety of mammalian species, including human, mouse and rat (see Figures 1-3). The assay has been designed to enable quantification of multiple neuronal morphological parameters, and the classification of compounds that induce neurotoxicity or promote neuronal regeneration, even within a mixed cell population. The nuclear dye (Hoechst 33342) may be used for measurements of cell number, DNA content and nuclear size. Additionally, the assay can be multiplexed with other probes for correlating neuronal morphology with other biological markers, *e.g.*, for drug efficacy or toxicity.

The specificity of the primary antibody is demonstrated in Figure 1, where neuronal cell bodies and neurites can be clearly identified amongst a mixed population of cells. The ability of the kit to be used for screening both inducers of neurite outgrowth and neurotoxic agents is demonstrated in Figures 2-5. In addition, working solutions of the primary and secondary antibodies have been demonstrated to be stable for at least 24 hours at room temperature (Figure 6), a great benefit for large-scale screening applications.

The high quality Millipore reagents provided with this kit enable the user to reproducibly generate images with a high signal-to-background ratio, greatly facilitating subsequent High Content Analysis. The reagents and protocols contained within the kit provide a complete solution for specifically labeling neurites and neuronal cell bodies for High Content Imaging.

Sufficient reagents are provided for 5 x 96-well microplates – *i.e.*, sufficient to perform 480 separate experiments in 96-well microtiter plates. The kit includes a mouse βIII-tubulin primary antibody highly specific for neurites and neuronal cell bodies, a FITC-conjugated secondary antibody, Hoechst HCS Nuclear Stain, HCS Fixation Solution, Neurite HCS Immunofluorescence Buffer, HCS Wash Buffer, and Plate Sealers.

Kit Components

1. Mouse Anti-βIII-Tubulin HCS Primary Antibody, 200X: (Part No. 2007639) 1 vial containing 150 µL.
2. HCS Secondary Antibody (goat anti-mouse IgG, FITC conjugate), 200X: (Part No. 2007638) 1 vial containing 150 µL.
3. Hoechst HCS Nuclear Stain, 50X: (Part No. 2007640) 1 vial containing 600 µL.
4. HCS Fixation Solution, 2X: (Part No. 2007641) 1 bottle containing 100 mL.
5. Neurite HCS Immunofluorescence Buffer, 1X: (Part No. 2007642) 1 bottle containing 1000 mL.
6. HCS Wash Buffer, 1X: (Part No. 2007643) 1 bottle containing 500 mL.
7. Plate Sealers: (Part No. CS200443) 10 each.

Materials Not Supplied

1. Sterile, tissue culture-treated black/clear bottom microplates suitable for High-Content Imaging.
2. Cell-type for assay, *e.g.*, PC12 (rat pheochromocytoma, ATCC # CRL-1721).
3. Tissue culture instruments/supplies (including 37ºC incubator, growth media, flasks/plates, detachment buffer, etc.) for cell type of interest.
4. Differentiation media/growth factors for cell type of interest, *e.g.*, nerve growth factor for PC12 cells (Millipore # GF028).
5. Extracellular matrix microplate coating protein for cell type of interest, *e.g.*, bovine collagen I for PC12 cells.
6. 1X phosphate buffered saline (PBS) with Ca^{2+}/Mg^{2+} or alternate buffer for dilution of microplate coating protein.
7. High Content Screening imaging/analysis system, *e.g.*, GE Healthcare IN Cell Analyzer 1000 with Investigator software. Imaging system must be equipped with beam-splitters and filters capable of reading emission spectra in the blue and green ranges. Example filter ranges are shown in Table 2 below.

Storage

Store kit components under the conditions indicated on the labels. Hoechst HCS Nuclear Stain, HCS Fixation Solution, Neurite HCS Immunofluorescence Buffer and HCS Wash Buffer should be stored at 2-8ºC. Plate Sealers may be stored at room temperature. Primary and Secondary antibodies should be stored at -20ºC, avoiding repeated freeze/thaw cycles. Discard any remaining reagents after 6 months.

(Note: If kit is expected to be used for multiple experiments, rather than a single use, thaw Primary and Secondary Antibodies and dispense into appropriately sized aliquots. Store aliquots at -20ºC.

Warnings and Precautions

1. This product contains hazardous material. Refer to MSDS for further information.

Component	Hazardous Constituent	Warnings (See MSDS)
HCS Fixation Solution	Formaldehyde	Toxic, carcinogen, combustible, readily absorbed through skin
Hoechst HCS Nuclear Stain	Hoechst 33342	Harmful, potential mutagen

2. For Research Use Only. Not for use in diagnostic procedures.

Related High Content Screening Products from Millipore

HCS226	HCA Kit for Neurite Outgrowth and Synaptic Activity
HCS221	HCA Kit for Gliosis
HCS222	HCA Kit for Co-Culture of Neurons and Astrocytes
HCS214-215	High Content Screening Reagent Toolkits
HCS100	Hepatotoxicity Assay, Human HepG2 cells
HCS201–213, 216	HCS Kits for Cell Cycle and Proliferation
HCS223-225	DNA Damage HCS kits

Assay Instructions

Note: This protocol has been optimized for PC12 rat pheochromocytoma cells (ATCC #CRL-1721). However, the kit is suitable for HCS analysis of neuronal cells derived from a wide variety of mammalian species, including human, mouse and rat (see Figures 1-3).

Cell Preparation:

1. Prior to initiation of the assay, culture PC12 cells in growth media until ~70-80% confluent. ATCC recommends that PC12 growth media consist of Ham's F12K medium with L-glutamine, 82.5%; horse serum, 15%; fetal bovine serum, 2.5%. Penicillin/streptomycin (1%) may also be added to PC12 growth media.

2. If desired, protein-coat assay microplates to encourage neurite outgrowth. Prepare fresh matrix protein (*e.g.,* collagen I) at a desired working concentration in 1X phosphate buffered saline (PBS, with Ca^{2+}/Mg^{2+}) and coat each well. Remove protein coating solution prior to cell seeding.

3. Detach cells from culture flasks/plates via method appropriate for cell type of interest. Adjust cell density to 2-4 x 10^4 cells/mL in growth media. Add 100 µL of this cell suspension (2000-4000 cells) to each well (for a 96-well plate, 2000-4000 cells/well is approximately equivalent to 6,000-12,000 cells/cm^2 of well surface). After adding cells to plate, allow plate to sit on a level surface at room temperature for 15-30 min. This allows for even cell distribution. Well-dispersed, single-cell suspensions are optimal for later neurite segmentation and analysis. Following this period, incubate cells in growth media (37ºC/5% CO_2) for 24 hours.

4. For neuronal cell differentiation and neurite outgrowth to occur, undifferentiated cells must be exposed to specific external stimuli to induce cessation of proliferation and increased tubulin production. For example, for PC12 cells, growth media may be replaced with differentiation media consisting of Ham's F12K medium with L-glutamine + 1% horse serum + 100 ng/mL NGF. Under these conditions, PC12 cells should be cultured for 6 days, replacing medium with fresh differentiation media every 2-3 days. Cell treatments (test compounds, neurite outgrowth promoters, neurotoxins, etc.) can be introduced at any point during this culture period, as appropriate for time course of treatment of interest.

Cell Fixation and Immunofluorescent Staining:

Note: Staining time is ~2.5 hours post-fixation. Do not allow wells to dry out between staining steps. Aspiration and dispensation of reagents should be conducted at low flow rates to diminish any cell/neurite loss due to fluid shear. All recommended 'per well' volumes refer to a single well of a 96-well microplate. All recommended 'per 96-well plate' volumes include 25% excess for liquid handling volume loss. All staining steps are performed at room temperature (RT). All buffers and antibody solutions are stable for at least 24 hours at RT if long-duration automated handling is required (see Figure 6).

5. At end of neurite outgrowth culture period, pre-warm HCS Fixation Solution (2X) to 37ºC (12 mL/96-well plate). In a chemical fume hood, add 100 µL/well directly to culture media and allow to fix for 30 min at RT. Remove fixative and dispose of in compliance with regulations for hazardous waste (see MSDS). If proceeding immediately to staining, rinse twice with 200 µL of Neurite HCS Immunofluorescence Buffer. Alternatively, if plates are to be stained at a later time, rinse twice with 200 µL of HCS Wash Buffer, then leave second rinse volume in wells and store plates tightly sealed at 4ºC until staining.

6. If fixed samples have been stored at 4ºC prior to staining, rinse twice with 200 µL Immunofluorescence Buffer before proceeding with staining protocol.

7. Prepare working solution of Mouse Anti-βIII-Tubulin HCS Primary Antibody (6 mL/96-well plate) as follows: Add 30 µL of thawed Primary Antibody to 5.97 mL of Immunofluorescence Buffer (see Table 1). Mix well. Remove previous Immunofluorescence Buffer rinse. Add 50 µL of Primary Antibody solution to each well and incubate for 1 hour at RT.

8. Remove Primary Antibody solution. Rinse three times with 200 µL Immunofluorescence Buffer.

9. Prepare working solution of HCS Secondary Antibody/Hoechst HCS Nuclear Stain (6 mL/96-well plate) as follows: Add 30 µL of thawed HCS Secondary Antibody (goat anti-mouse IgG, FITC conjugate) and 120 µL of Hoechst HCS Nuclear Stain to 5.85 mL of Immunofluorescence Buffer (see Table 1). Mix well, protecting solution from light. Remove previous Immunofluorescence Buffer rinse. Add 50 µL of Secondary Antibody/Hoechst HCS Nuclear Stain solution and incubate for 1 hour at RT, protected from light.

10. Remove Secondary Antibody/Hoechst HCS Nuclear Stain solution. Rinse twice with 200 µL Immunofluorescence Buffer.

11. Remove previous Immunofluorescence Buffer rinse. Rinse twice with 200 µL of HCS Wash Buffer, leaving second rinse volume in wells.

12. Seal plate and image immediately, or store plate at 4ºC protected from light until ready for imaging.

HCS220 Detection Reagent Specifications			
Primary Antibody working solution			
Reagent	**Required dilution of initial reagent**	**Vol. required for 1 well (50 µL)**	**Vol. required for 1 plate (6 mL)**
Mouse Anti-βIII-Tubulin HCS Primary Antibody	1:200	0.25 µL	30 µL
Neurite HCS Immunofluorescence Buffer	None	49.75 µL	5.97 mL (5970 µL)
Secondary Antibody/Hoechst HCS Nuclear Stain working solution			
Reagent	**Required dilution of initial reagent**	**Vol. required for 1 well (50 µL)**	**Vol. required for 1 plate (6 mL)**
FITC-Goat Anti-Mouse HCS Secondary Antibody	1:200	0.25 µL	30 µL
Hoechst HCS Nuclear Stain	1:50	1 µL	120 µL
Neurite HCS Immunofluorescence Buffer	None	48.75 µL	5.85 mL (5850 µL)

Table 1. Detection Reagent Specifications

HCS 220 Image Acquisition Guidelines			
Detection Reagent	**Objective Lens**	**Excitation Filter Range [peak/bandwidth (nm)]**	**Emission Filter Range [peak/bandwidth (nm)]**
Hoechst HCS Nuclear Stain	10X/20X	360/40	460/40 or 535/50 (if necessary)
HCS Secondary Antibody, FITC-Goat Anti-Mouse IgG	10X/20X	480/40	535/50

HCS 220 Image Analysis Guidelines			
Cell Parameter	**Detection**	**Segmentation/ Measurement**	**Rationale**
Cell Number, Nuclear Characteristics	Hoechst HCS Nuclear Stain	Nuclear region (460 nm emission channel. Count number of nuclei. DNA content (nuclear intensity) or nuclear area analyses are also possible	Use cell number, nuclear characteristics to determine cell loss, toxicity phenotypes, etc.
βIII-Tubulin Expression, Neuronal Morphology, Neurite Outgrowth	HCS Secondary Antibody, FITC-conjugated	Cytoplasmic region (535 nm emission channel). FITC signal may be used to distinguish neuronal cell bodies from neurites (*e.g.*, via minimum/average cell body areas, minimum/ maximum neurite lengths and widths). Determine parameters such as total neurite length, neurite count/cell, etc.	Neurite outgrowth measurements may be modulated during neuronal differentiation or as a result of chemical injury, disease states, etc.

Table 2. Image Acquisition and Analysis Guidelines

Sample Results

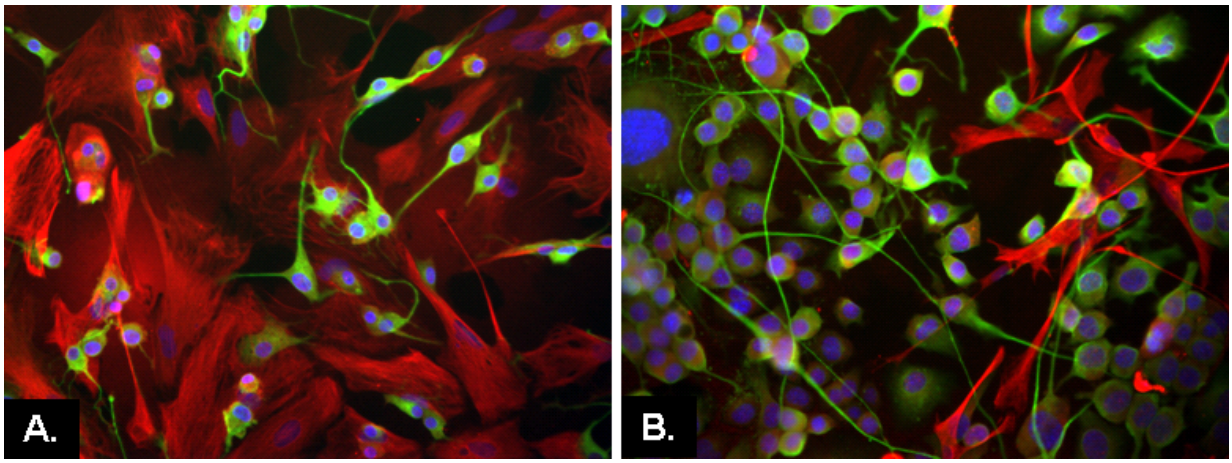

Figure 1. Specificity of βIII-Tubulin Primary Antibody

Rat PC12 pheochromocytoma (A) or mouse N1E-115 neuroblastoma (B) cells were co-cultured with rat hippocampal astrocytes on 96-well clear-bottom plates. Astrocytes were cultured in growth media (4 days), followed by 3 days in growth media + 20 ng/mL bFGF. PC12 or N1E-115 cells were seeded on top of this cell layer. PC12 cells were seeded in low serum differentiation media with 100 ng/mL NGF and cultured for 2 days. N1E-115 cells were cultured in growth media for 24 hours, then in differentiation media (no serum) for another 24 hours. Samples were fixed in Millipore's HCS Fixation Solution before immunofluorescent staining for neuronal cells (FITC (green) secondary antibody, under HCS220 assay conditions) and astrocytes (glial-cell specific GFAP with rhodamine (red) secondary antibody). Nuclei are counterstained with Millipore's Hoechst HCS Nuclear Stain (blue), under kit conditions. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) using a 20X objective.

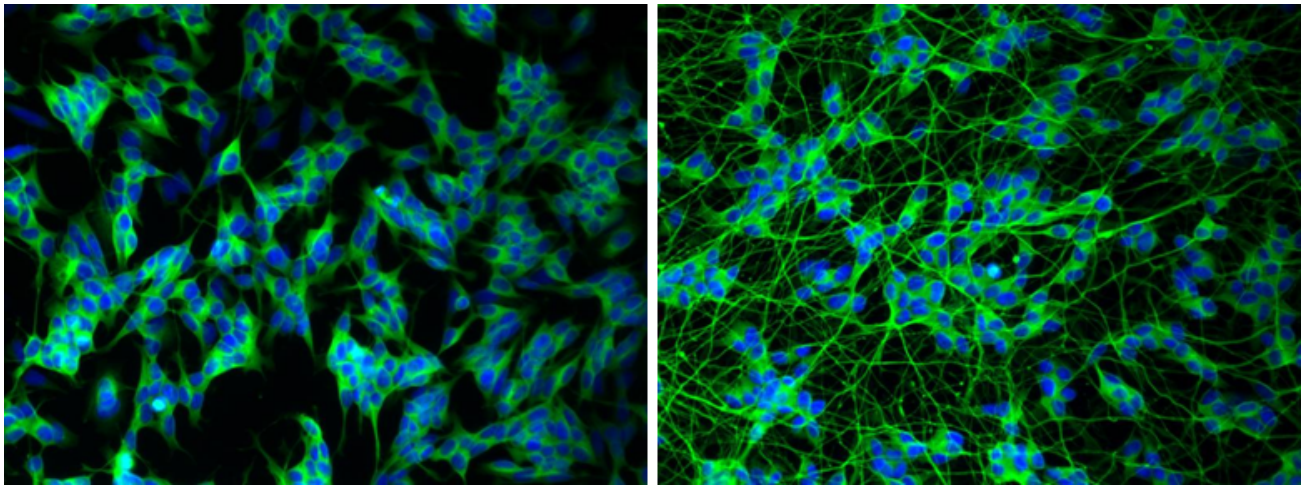

Figure 2. Immunofluorescence of differentiated and non-differentiated human SH-SY5Y cells

Merged images of staining with Hoechst HCS Nuclear Stain (blue) and HCS220 Primary/Secondary Antibodies (green). **Left Panel:** *Non-differentiated SH-SY5Y cells*. Cells were cultured in growth medium, replacing medium every 2-3 days. **Right Panel:** *Differentiated SH-SY5Y cells.* Cells were cultured in growth medium containing 10 µM retinoic acid for 5 days, replacing media/retinoic acid every 2-3 days, followed by 50 ng/mL BDNF in serum-free medium for 5 days. Cell fixation and immunostaining were performed as according to HCS220 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) using a 20X objective.

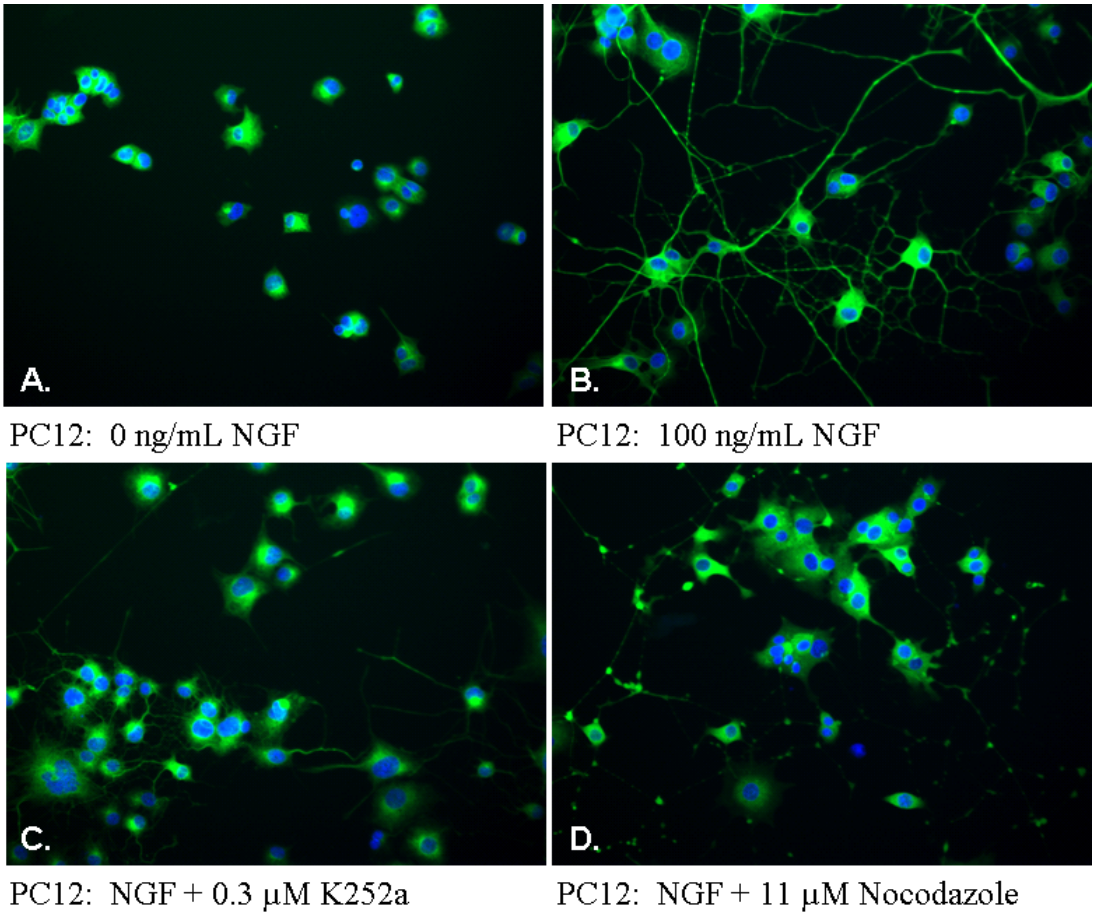

Figure 3. Immunofluorescence of treated and untreated PC12 cells

Merged images of staining with Hoechst HCS Nuclear Stain (blue) and HCS220 Primary/Secondary Antibodies (green). **A.** *Unstimulated PC12 cells*. Cells were cultured in low serum differentiation media containing no NGF for 6 days, replacing media every 3 days. **B.** *NGF-stimulated PC12 cells.* Cells were cultured in low serum media containing 100 ng/mL NGF for 6 days, replacing media/NGF every 3 days. **C & D**. *NGF-stimulated PC12 cells treated with neurotoxins.* Cells were cultured in low serum media containing 100 ng/mL NGF for 6 days, replacing media/NGF every 3 days. Cells received treatment with either the protein kinase inhibitor K252a (0.3 µM), for the final 3 days of culture (C), or the microtubule depolymerization agent nocodazole (11 µM), for the final 4 hours of culture (D). Cell handling, fixation and immunostaining were performed as according to HCS220 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) using a 20X objective.

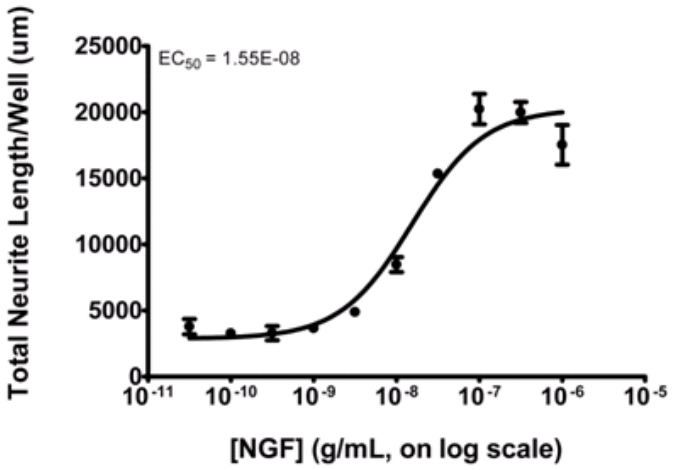

Figure 4. Dose response of NGF-induced neurite outgrowth stimulation

PC12 cells were cultured in low serum differentiation media containing serial dilutions of NGF (max. concentration = 1000 ng/mL) for 6 days, replacing media/NGF every 3 days, then fixed and stained according to HCS220 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) at 20X (5 fields/well) and analyzed using the GE IN Cell Analyzer 1000 Workstation (3.4) Neurite Outgrowth algorithm. (Mean ± SEM; *n* = 4)

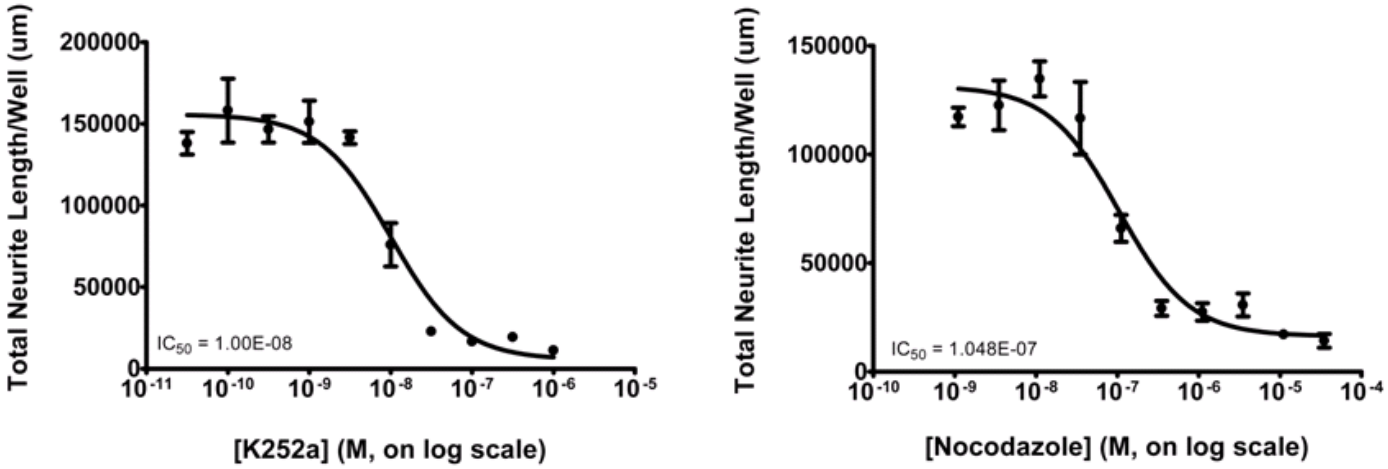

Figure 5. PC12 dose response to neurotoxic drugs

PC12 cells were cultured in low serum differentiation media, containing 100 ng/mL NGF for 6 days, replacing media/NGF every 3 days. Cells received treatment with either serial dilutions of the protein kinase inhibitor K252a, for the final 3 days of culture (max. concentration = 1000 nM), or the microtubule depolymerization agent nocodazole, for the final 4 hours of culture (max. concentration = 35097 nM). Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) at 10X (10 fields/well) and analyzed using the GE IN Cell Analyzer 1000 Workstation (3.4) Neurite Outgrowth algorithm. (Mean ± SEM, *n* = 4).

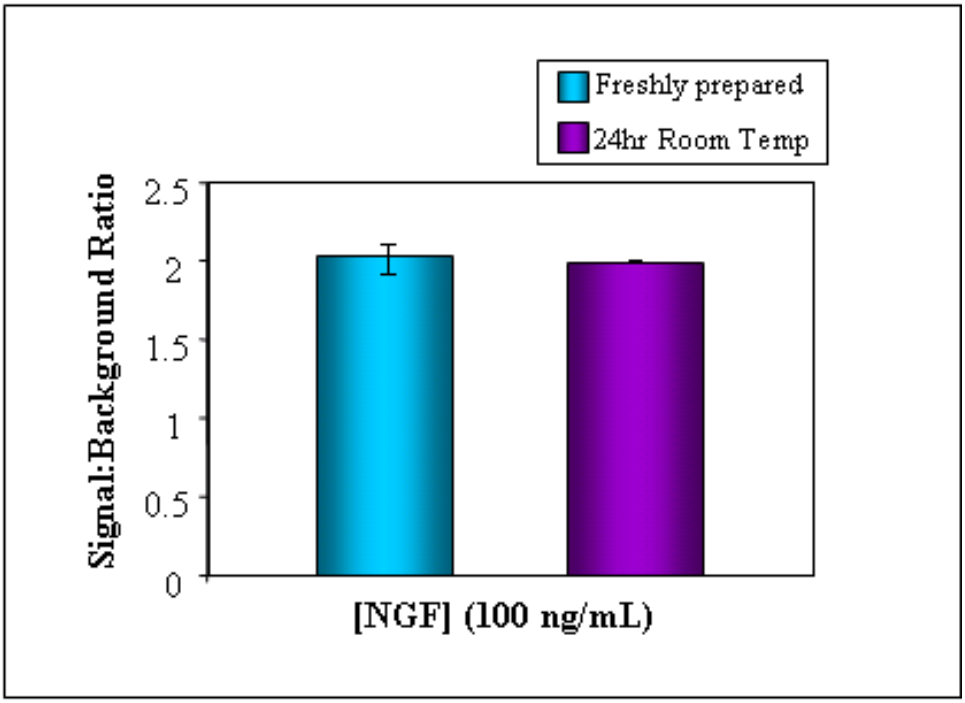

Figure 6. Reagent stability at room temperature

PC12 cells were seeded at 2000 cells/well on 96-well plates in growth media. After 24 hours of culture, growth media was replaced with low serum differentiation media containing 100 ng/mL NGF. Cells were cultured for an additional 6 days, replacing media/NGF every 3 days. Samples were fixed and stained under kit conditions, using either fresh buffers and antibody/Hoechst solutions, or buffers and antibody/Hoechst solutions that had been allowed to sit at room temperature (protected from light) for 24 hours prior to staining. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) at 20X magnification (7 fields/well) and analyzed for cell (FITC) signal:background ratios using the GE IN Cell Analyzer 1000 Workstation (3.4) Multi Target Analysis algorithm. Data presented are mean ± SEM; *n* = 2. No statistically significant difference was observed between freshly prepared and 24 hour samples.

Troubleshooting

Problem	**Potential Explanations/Solutions**
Weak FITC signal	Improper storage or preparation of Primary/Secondary antibody – retry stain with fresh antibody/solution. Inadequate primary or secondary antibody concentrations for cell type – titrate dilutions to optimize signal. Signal may diminish in extremely dense cultures – decrease cell seeding concentration or increase primary/secondary antibody. βIII-tubulin expression may change with cell type, passage number, seeding density or culture conditions – optimize cell model for parameters of interest. Optimize exposure times and/or fluorescence filters appropriate to fluorophore.
Weak Hoechst signal	Improper storage or preparation of Nuclear Stain – retry stain with fresh dye/solution. Inadequate Nuclear Stain concentration for cell type – titrate dilutions to optimize signal. Signal may diminish in extremely dense cultures – decrease cell seeding concentration or increase Hoechst concentration. Optimize exposure times and/or fluorescence filters appropriate to fluorophore.
Excessive background	Improper reagent storage or preparation – retry with fresh reagent (antibodies/dyes and/or buffers). Samples may have dried during staining – retry stain on fresh samples. Excessive primary or secondary antibody concentrations for cell type – titrate dilutions to optimize signal. Check for autofluorescence of microplate.
Excessive FITC/ Hoechst signal	Improper preparation of antibody/dye – retry stain with fresh antibody/dye solutions. Inappropriate antibody/dye concentrations for cell type – titrate dilutions to optimize signal. βIII-tubulin expression may change with cell type, passage number, seeding density or culture conditions – optimize cell model for parameters of interest. Optimize exposure times and/or fluorescence filters appropriate to fluorophore.

Problem	**Potential Explanations/Solutions**
Cell loss	Optimize liquid aspiration/dispensation rate to reduce shear. Optimize protein-coating conditions to improve cell adhesion to microplate. Optimize cell seeding concentrations for better cell adhesion and neurite extension. Cell loss during staining may actually increase for more dense neurite networks – decrease cell seeding concentration or duration of neurite outgrowth culture. Cell loss due to toxic treatments may hinder statistically relevant analysis; alter toxin dosages/treatment times to reduce cell loss levels.
Poor cell/ neurite segmentation during analysis	Effective segmentation parameters can be HCS system/software-dependent. Consider decreasing cell seeding concentrations or duration of neurite outgrowth culture, especially for difficulty in analysis of dense cultures. More vigorous resuspension of cells (*e.g.*, trituration through a 20G needle) prior to seeding may be necessary to enable more accurate segmentation.
Large measurement deviations; inconsistent dose-responses	Consistency of data may be improved by increasing the number of cells analyzed per condition. Consider increasing the number of imaged cells available for analysis (*e.g.*, use lower objective magnification, increase number of imaging fields per well, etc.).

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