Video Article

A Neuronal and Astrocyte Co-Culture Assay for High Content Analysis of Neurotoxicity

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Abstract

High Content Analysis (HCA) assays combine cells and detection reagents with automated imaging and powerful image analysis algorithms, allowing measurement of multiple cellular phenotypes within a single assay. In this study, we utilized HCA to develop a novel assay for neurotoxicity. Neurotoxicity assessment represents an important part of drug safety evaluation, as well as being a significant focus of environmental protection efforts. Additionally, neurotoxicity is also a well-accepted *in vitro* marker of the development of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Recently, the application of HCA to neuronal screening has been reported. By labeling neuronal cells with βIII-tubulin, HCA assays can provide high-throughput, non-subjective, quantitative measurements of parameters such as neuronal number, neurite count and neurite length, all of which can indicate neurotoxic effects. However, the role of astrocytes remains unexplored in these models. Astrocytes have an integral role in the maintenance of central nervous system (CNS) homeostasis, and are associated with both neuroprotection and neurodegradation when they are activated in response to toxic substances or disease states. GFAP is an intermediate filament protein expressed predominantly in the astrocytes of the CNS. Astrocytic activation (gliosis) leads to the upregulation of GFAP, commonly accompanied by astrocyte proliferation and hypertrophy. This process of reactive gliosis has been proposed as an early marker of damage to the nervous system. The traditional method for GFAP quantitation is by immunoassay. This approach is limited by an inability to provide information on cellular localization, morphology and cell number. We determined that HCA could be used to overcome these limitations and to simultaneously measure multiple features associated with gliosis - changes in GFAP expression, astrocyte hypertrophy, and astrocyte proliferation - within a single assay. In co-culture studies, astrocytes have been shown to protect neurons against several types of toxic insult and to critically influence neuronal survival. Recent studies have suggested that the use of astrocytes in an *in vitro* neurotoxicity test system may prove more relevant to human CNS structure and function than neuronal cells alone. Accordingly, we have developed an HCA assay for co-culture of neurons and astrocytes, comprised of protocols and validated, target-specific detection reagents for profiling βIII-tubulin and glial fibrillary acidic protein (GFAP). This assay enables simultaneous analysis of neurotoxicity, neurite outgrowth, gliosis, neuronal and astrocytic morphology and neuronal and astrocytic development in a wide variety of cellular models, representing a novel, non-subjective, high-throughput assay for neurotoxicity assessment. The assay holds great potential for enhanced detection of neurotoxicity and improved productivity in neuroscience research and drug discovery.

Protocol

Cell Preparation:

1. Prior to cell seeding for assay, culture neurons/astrocytes in growth media until ~70-80% confluent (unless cell seeding directly from thaw or isolation).
2. Detach cells from culture flasks/plates via method appropriate for cell type of interest. If necessary, coat assay plate wells with poly-D-lysine or extracellular matrix protein to enhance cell adhesion. Co-cultures of astrocytes and neurons may be seeded simultaneously or sequentially. For sequential seedings, plating of astrocytes first as a "basal" layer is recommended, followed by neuronal plating and differentiation, if necessary. Adjust cell density as appropriate for cell type, subsequent culture time, parameters of interest, etc. Depending on culture age, cell source and seeding density, primary cultures may vary greatly in rate of proliferation, GFAP expression or neurite outgrowth - it is important to characterize and optimize your cell system to provide the most biological relevance for your experimental model, as well as to provide for effective imaging, segmentation and analysis using HCS (see Figure 1). After adding cells to plate (cells may be seeded in 90 µL media, to facilitate toxin treatment as described in Step 3 below), allow plate to sit on a level surface at room temperature for 15-30 min, enabling even cell distribution. Following this period, incubate cells in growth media (37°C/5% CO_2) for at least 24 hours, then switch to differentiation culture conditions (e.g., low serum/NGF for PC12 cells), as appropriate. Continue culture until cells reach desired level of confluence or differentiation, changing media at intervals appropriate for cell type.
3. Cell treatments (control compounds, test compounds, etc.) can be introduced at any point during this culture period, as appropriate for time-course of treatment of interest. Acrylamide, hydrogen peroxide and K-252a are provided as neurotoxic control compounds. Sufficient reagents are provided for duplicate 12-point dose response curves (including one dH_2O or DMSO-control set within the dose response) for all five 96-well plates. The compounds are provided at 250X concentration (for K-252a, assuming a maximum treatment of 1 µM) or 10X (for acrylamide and hydrogen peroxide, assuming maximum treatments of 100 mM and 10 mM, respectively). Recommended treatment preparation involves half-log (1:√10) serial dilution of the 250X compound in DMSO, followed by dilution in Compound Dilution Buffer to 10X (10X control compounds originating in dH_2O may be serially diluted directly in sterile dH_2O or Compound Dilution Buffer). 10 µL of each treatment may then be added to the 90 µL of culture media already present in each well, for a final 1X concentration (0.4% DMSO or 10% dH_2O). Sample data is provided for 24 or 96 hours of compound treatment at 37°C prior to fixation.

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 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.

4. At end of culture period, pre-warm HCS Fixation Solution (2X) to room temperature (RT) or 37ºC if desired (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/toxin-containing media and dispose of in compliance with regulations for hazardous waste (see MSDS). If proceeding immediately to staining, rinse each well twice with 200 µL of HCS Immunofluorescence Buffer. Alternatively, if plates are to be stained at a later time, rinse twice with 200 µL of Wash Buffer, then leave second rinse volume in wells and store plates tightly sealed at 4ºC until staining.
5. If fixed samples have been stored at 4ºC prior to staining, rinse twice with 200 µL HCS Immunofluorescence Buffer before proceeding with staining protocol.
6. Prepare working solution of Rabbit Anti-βIII Tubulin/Mouse Anti-GFAP HCS Primary Antibodies (6 mL/96-well plate) as follows: Add 60 µL of each thawed primary antibody to 5.88 mL of HCS Immunofluorescence Buffer. Mix well. Remove previous Immunofluorescence Buffer rinse. Add 50 µL of Primary Antibody solution to each well and incubate for 1 hour at RT.
7. Remove Primary Antibody solution. Rinse three times with 200 µL HCS Immunofluorescence Buffer.
8. Prepare working solution of HCS Secondary Antibody/Hoechst HCS Nuclear Stain (6 mL/96-well plate) as follows: Add 30 µL of each thawed secondary antibody and 30 µL of thawed Hoechst HCS Nuclear Stain to 5.91 mL of HCS Immunofluorescence Buffer. Mix well, protecting solution from light. Remove previous HCS Immunofluorescence Buffer rinse. Add 50 µL of HCS Secondary Antibody/Hoechst HCS Nuclear Stain solution and incubate for 1 hour at RT, protected from light.
9. Remove HCS Secondary Antibody/Hoechst HCS Nuclear Stain solution. Rinse twice with 200 µL HCS Immunofluorescence Buffer.
10. Remove previous HCS Immunofluorescence Buffer rinse. Rinse twice with 200 µL of HCS Wash Buffer, leaving second rinse volume in wells.
11. Seal plate and image immediately, or store plate at 4ºC protected from light until ready for imaging.

Image Acquisition and Analysis:

12. Imaging and analysis of stained plates may be performed upon a variety of available HCS platforms, including the IN Cell Analyzer (GE Healthcare), ArrayScan (ThermoFisher Scientific) or Opera (Perkin Elmer). Some guidelines for imaging and analysis are provided in Table 1:

HCS222 Image Acquisition Guidelines

Detection Reagent	Objective Lens	Excitation Filter Range [peak/bandwidth (nm)]	Emission Filter Range [peak/bandwidth (nm)]
Hoechst HCS Nuclear Stain	20X	360/40	460/40 (or 535/50 if necessary)
HCS Secondary Antibody, FITC-Donkey anti-Rabbit IgG	20X	480/40	535/50
HCS Secondary Antibody, Cy3-Donkey anti-Mouse IgG	20X	535/50	600/50

HCS222 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. Can "filter" nuclei for those associated with βIII-tubulin or GFAP expression to obtain separate neuronal and astrocytic cell counts/characterizations (strongly recommended).
βIII-Tubulin Expression, 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 counts/cell, etc.	Neurite outgrowth measurements may be modulated during neuronal differentiation or as a result of chemical injury, disease states, etc. Can "filter" cell bodies for those associated with βIII-tubulin expression to obtain distinct neuronal characterization (strongly recommended).
GFAP Expression, Astrocyte Area	HCS Secondary Antibody, Cy3-conjugated	Cytoplasmic region (600 nm emission channel). Cy3 signal may be used to define astrocytic cytoplasmic segmentation. Determine parameters such as average cytoplasmic signal intensity, cell area, etc.	GFAP expression and astrocyte cell area may be modulated during astrocyte development or as a result of chemical injury, disease states, etc. Can "filter" cell bodies for those associated with GFAP expression to obtain distinct astrocytic characterization (strongly recommended).

Table 1. Image Acquisition and Analysis Guidelines – HCS222 βIII-Tubulin/GFAP (Co-Culture) Assay

Representative Results:

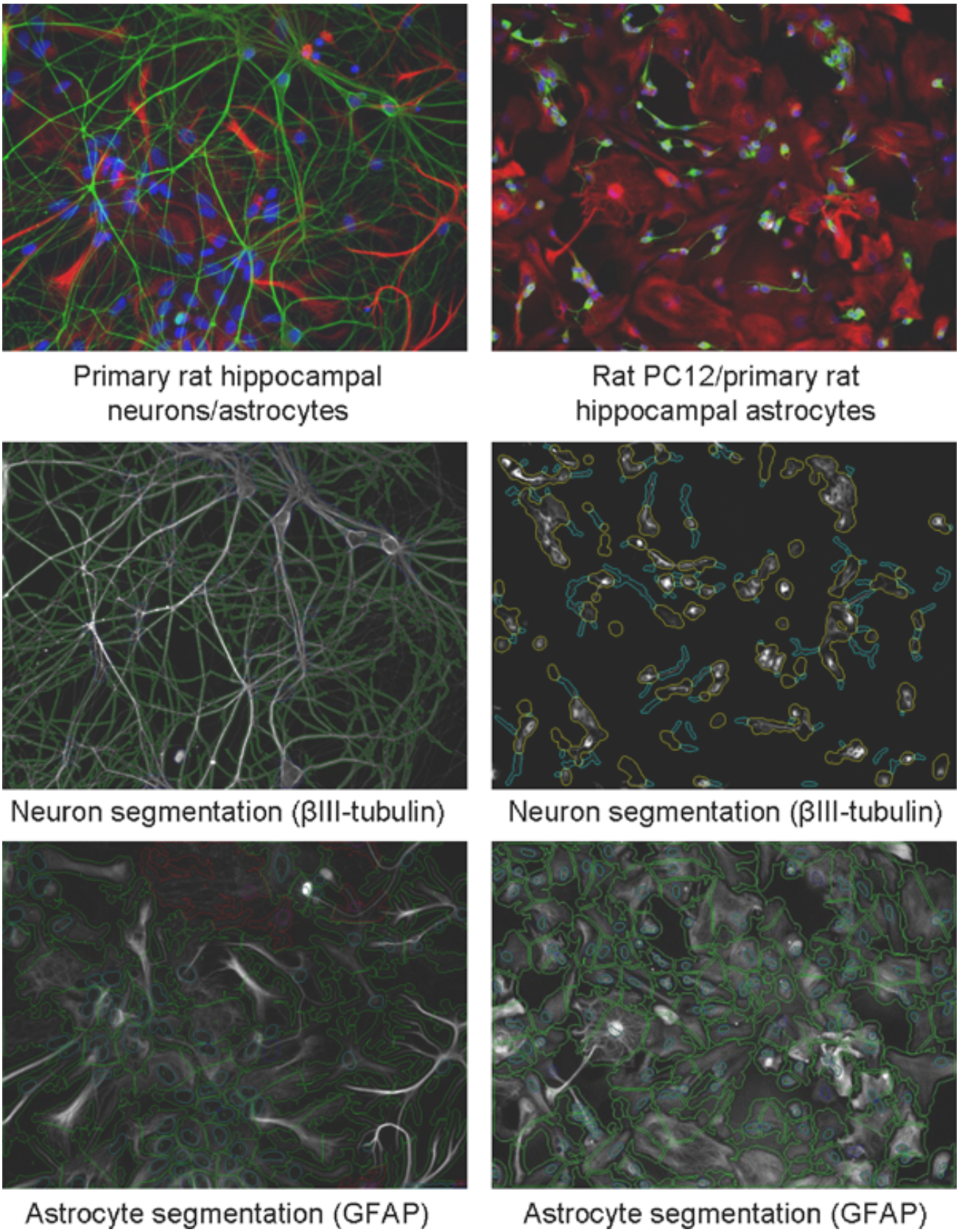

Figure 1. βIII-tubulin and GFAP immunofluorescence of mixed rat neural cell cultures.
Co-cultures of primary rat hippocampal astrocytes with either primary rat hippocampal neurons or rat PC12 cells were generated by pre-plating astrocytes for several days in culture, followed by neuronal seeding. Primary neurons were cultured for an additional 11 days, while PC12s were cultured for an additional 2 days under differentiation conditions (low serum/NGF). Cell handling, fixation and immunostaining were performed according to HCS222 assay protocols. Cells were imaged on the GE IN Cell Analyzer 1000 (3.3) at 20X (primary neurons) or 10X (PC12) objective magnification and analyzed using the GE IN Cell Analyzer 1000 Workstation (3.4) Neurite Outgrowth and Multi Target Analysis algorithms. *Top panel:* Fused images of Hoechst HCS Nuclear Stain (blue), βIII-tubulin (green) and GFAP (red) fluorescence. Separate analysis of the βIII-tubulin fluorescence channel allows for neurite outgrowth segmentation (*middle panel:* cell bodies outlined in blue (primary neurons) or yellow (PC12), neurites in green (primary neurons) or light blue (PC12)). Analysis of the GFAP channel allows for astrocyte segmentation (*bottom panel:* nuclei outlined in blue, cytoplasm in green). GFAP segmentation for the primary rat hippocampal neuron/astrocyte co-culture demonstrates

the ability to distinguish between nuclei/cell bodies that are GFAP (+) (green outlines) and those that are GFAP (-) (red), enabling separate cell counts for neurons and astrocytes in a mixed culture setting.

