

NEURITE OUTGROWTH

Measurement of neuronal morphological changes is an important tool in the search for drugs that may impact neuronal degenerative diseases, such as Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis (ALS), and others. Conversely, compounds that exhibit neuronal toxicity need to be avoided. The ability to assess neuronal toxicity in early stage drug development would aid in preventing unexpected adverse effects during clinical studies. One important endpoint for assessing neurotoxicity is neurite outgrowth, such as neurite number and length. Scoring of neurite morphological features has traditionally been done manually. These methods are usually labor intensive, time consuming and subject to bias. Automated detection and analysis of neurite outgrowth is an advantageous tool for screening compounds in early drug discovery.

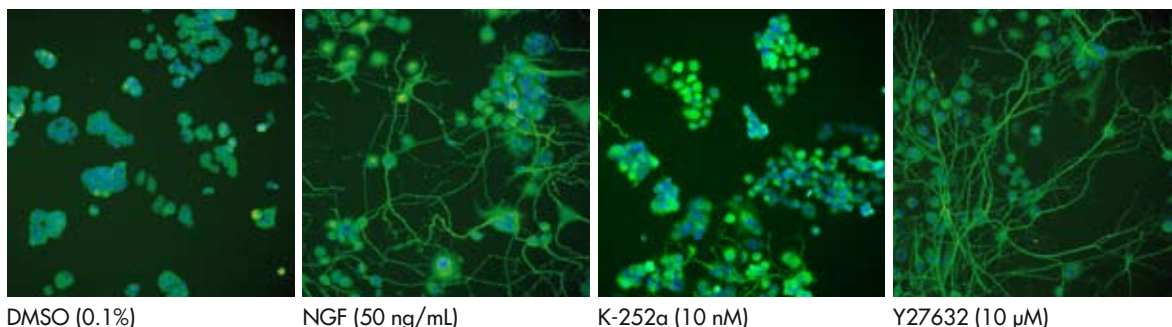
Cerep has developed a high content analysis assay using the Cellomics ArrayScan (ThermoFisher) for assessing neurite outgrowth in nerve growth factor (NGF)-stimulated rat pheochromocytoma (PC12) cells, a widely used *in vitro* neuronal model. This cell-based, automated, screening assay allows a rapid turnaround time and un-biased scoring for assessing potential neuronal toxicity and can also serve as a valuable tool in drug discovery for neuronal degenerative disease.

NEURITE OUTGROWTH ASSAY

The neurite outgrowth assay assesses four endpoints which were measured simultaneously in individual cells. The endpoints, definitions and principles are described in the following table.

End-Point	Staining	Definition	Principle
Cell number	Hoechst	Cell number in each well.	Decrease in cell number is a strong indicator of cytotoxicity
Neurite number	β III-tubulin	Average number of neurites in each cell.	Chemicals that disrupt molecular signaling pathways during neuronal differentiation may interrupt neurite initiation, resulting in changes of neurite numbers.
Neurite length	β III-tubulin	Average length of neurites in each cell.	Neurite elaboration or axonal degeneration caused by chemical treatment may result in changes in neurite length.
% cells with neurites	β III-tubulin	Cell population that are neurite bearing.	Changes in % of cells with neurites is an indication of effect on neurite outgrowth.

Representative images of NGF-induced neurite outgrowth in PC12 cells. All images were taken using a ThermoFisher Cellomics ArrayScan 4.5 with a 10X objective. All compound-treated cells were co-treated with 50 ng/mL NGF for 6 days. Final concentration of DMSO was 0.1% (v/v) ▼

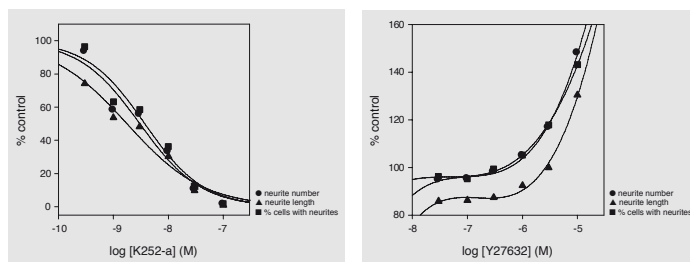


Effects of K-252a and Y27632 on NGF-induced neurite outgrowth ►

PROTOCOL

PC12 cells (from ATCC) are seeded in 96-well collagen IV-coated plates (~3,000 cells/well). Cells are allowed to attach overnight and then treated with a test compound and 50 ng/mL NGF, in triplicate, in assay medium containing 1% HS and 0.5% FBS. The final DMSO concentration is 0.1% (which is at the highest DMSO tolerance level for this assay). After incubation at 37°C/5% CO₂ for 6 days, the cells are fixed and then stained with Hoechst and β III-tubulin antibody.

After the staining process, the plates are scanned with an automated fluorescent microscope-conjugated camera (ThermoFisher Cellomics ArrayScan 4.5). Cellomics image-analysis software is used to detect nuclei, cell bodies, and neurites in PC12 cells.



NEURITE OUTGROWTH ► APPLICATION NOTE

Negative controls are wells that are treated with only vehicle (0.1% DMSO, no NGF). Positive controls are wells that are co-treated with vehicle (0.1% DMSO) and 50 ng/mL NGF.

Compounds are tested at multiple concentrations and the default test concentrations are 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μ M. Raw data are normalized to the positive controls and expressed as % of effect relative to NGF. IC₅₀ values, if calculable are reported for neurite outgrowth inhibitors. K-252a is used as a reference for neurite outgrowth inhibition, and Y27632 is used as a reference for neurite outgrowth enhancement.

■ ADVANTAGES OF THE NEURITE OUTGROWTH ASSAY

► SMALL AMOUNT OF COMPOUND REQUIRED

The neurite outgrowth assay requires only about 1 mg of compound to test at the top concentration of 100 μ M (assuming a MW of approximately 500).

► RAPID TURNAROUND TIME

The results are delivered within 2 weeks upon receipt of the compounds at the Cerep testing site. Data are available online as soon as they are validated.

► OBJECTIVE AND CONSISTENT SCORING

Automated scoring bypasses the subjectivity and inconsistency of manual scoring.

■ NEURITE OUTGROWTH ASSAY COMPOUND TRAINING SET

A set of training compounds were tested in the neurite outgrowth assay in order to validate this assay at Cerep. IC₅₀ values were calculated for the compounds that inhibited neurite outgrowth. For the compounds that enhance neurite outgrowth or have no effect on neurite outgrowth, the % effect relative to NGF treated cells were listed at the highest non-cytotoxic doses or the highest tested doses.

► COMPOUNDS THAT INHIBIT NEURITE OUTGROWTH

Compound	IC ₅₀ Cell number	Neurite length	Neurite number	% cells with neurites
K-252a	28.2±4.4 μ M (n=10)	2.7±0.6 nM (n=16)	3.2±0.6 nM (n=16)	3.5±0.6 nM (n=16)
SU6656	> 1 μ M	29.4±5.1 nM (n=8)	37.9±8.7 nM (n=8)	42.6±6.6 nM (n=8)
U0126	> 25 μ M	9.9±2.2 μ M (n=4)	16.7±3.0 μ M (n=4)	21.5±6.0 μ M (n=2)
retinoic acid	> 100 nM	1.9±0.8 nM (n=9)	5.3±2.2 nM (n=10)	3.2±2.8 nM (n=10)
sulpiride	> 100 μ M	77.0±16.0 μ M (n=2)	>100 μ M	>100 μ M
flvoxamine	41.0±10.3 μ M (n=3)	36.5±11.2 μ M (n=4)	53.5±6.1 μ M (n=4)	56.0±5.3 μ M (n=4)

Note: Data are expressed as the mean±S.E., when applicable, from separate experiments. - n = number of different replicates

► COMPOUNDS THAT ENHANCE NEURITE OUTGROWTH

Compound	Highest non-cytotoxic dose ¹	Cell number	Neurite length	Neurite number	% cells with neurites
Y27632	20.6±2.7 μ M (n=13)	89.7±2.7% (n=13)	170.7±12.6% (n=13)	162.2±5.8% (n=13)	153.9±7.1% (n=13)
fasudil	12.9±4.1 μ M (n=4)	82.2±2.0% (n=4)	190.6±21.2% (n=4)	177.1±10.4% (n=4)	147.0±17.2% (n=4)
dopamine	4.2±2.2 μ M (n=4)	87.4±3.8% (n=4)	148.6±23.4% (n=4)	130.1±14.7% (n=4)	124.0±12.5% (n=4)

¹ A non-cytotoxic dose is defined as the dose that caused less than 25% cell loss based on cell nuclei scoring.

Note: Data are expressed as the mean±S.E., when applicable, from separate experiments. - n = number of different replicates

► COMPOUNDS THAT HAVE NO EFFECT ON NEURITE OUTGROWTH

Compound	Highest tested dose	Cell number	Neurite length	Neurite count	% cells with neurites
tetradotoxin	10 μ M	101.8 ± 3.3% (n=15)	87.7 ± 2.3%(n=15)	90.1 ± 1.1%(n=15)	88.9 ± 0.9%(n=15)
galathamine	100 μ M	119.3 ± 11.4% (n=9)	88.8 ± 9.7%(n=9)	100.5 ± 7.6%(n=9)	100.3 ± 5.8%(n=9)
picrotoxin	100 μ M	97.8 ± 3.8% (n=15)	92.3 ± 7.3% (n=15)	94.3 ± 6.0% (n=15)	92.3 ± 5.1% (n=15)

Note: Data are expressed as the mean±S.E., when applicable, from separate experiments. - n = number of different replicates

Our assay showed a high sensitivity by correctly predicting the reported inhibitory effects of compounds k-252a, retinoic acid, SU6656 and U0126; as well as the known enhancement effects of compounds Y27632, fasudil and dopamine on neurite outgrowth (1-3).

The observation that tetradotoxin has no effect on neurite outgrowth is consistent with a previous study (4). PC12 cells are also known for not having a functional target for picrotoxin, a potent neurotoxin (5). Indeed, the neurite outgrowth in PC12 cells is not affected by picrotoxin treatment in our assay. These results demonstrate the specificity of our assay.

Overall, our assay is a rapid and reliable tool for assessing neurite outgrowth in PC12 cells. This assay can be useful in both drug discovery programs for identifying neuronal protective compounds and in early drug development for potential neurotoxicity screening.



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