Application Note

Toxicology Research

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# Rapid, Image-Based Viability and Outgrowth Analysis for Neurotoxicity Assays

## Single-step labeling and high-throughput, automated live-cell analysis to capture complex and specific neuron responses across a panel of neurotoxic treatments


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## Abstract

Neurotoxicity assays evaluate the potential of agents to produce unwanted effects on the nervous system through environmental, occupational, and medicinal exposures. Understanding the impact of chemical exposure on neurite outgrowth and cell viability is a key in vitro assay for neurotoxicity assessment. In this application note, we present a simple, rapid, and robust approach to evaluate neurotoxic effects on viability and outgrowth in a high-throughput neuronal cell model system. Readily available live-cell dyes, paired with an automated imaging and analysis solution, rapidly and efficiently quantified viability and outgrowth in an iPSC-derived neuronal model. Here, we identified significant effects after 48 hours of drug exposure for 15 potential neurotoxicants across disparate mechanisms of action. Dose-dependent $EC/IC_{50}$ evaluations quantified specific effects on both viability and neuron outgrowth morphology, including neurite length, branching, and number. This robust, image-based neurotoxicity assay presents a streamlined solution for the characterization of subcellular neuronal morphology and quantitative viability analysis, with the scale and reproducibility afforded by high-throughput, automated microscopy.

## Introduction

International efforts to enhance neurotoxicity and developmental neurotoxicity testing aim to reduce the risk of harm and address the growing global burden of brain disease.[1] Neurotoxicity evaluations consist of multifaceted approaches that rely on both in vitro and in vivo techniques. Image-based analysis of neurite outgrowth forms a critical assay for in vitro investigations of neurotoxicity.[2] Advanced automated microscopy and image analysis techniques are powerful tools leveraged for high-content imaging across disciplines, generating large datasets for phenotypic screening and analysis. Advancements in stem cell-derived neuronal models, including iPSC-derived models, open the door for scalable human neuron culture models for neurotoxicity studies, providing unparalleled access to genetic manipulations and patient-derived disease modeling for context-dependent neurotoxicity investigations.[3]

Image-based analysis of in vitro neurotoxicity often relies on immunohistochemistry-based techniques that are sensitive and specific, but also costly and time consuming. Live-cell dyes provide a convenient and inexpensive alternative for rapid labeling of neuron cultures, including stem cell-derived neurons. Calcein AM dye crosses cell membranes and is converted to a fluorescent molecule in live cells, making it useful as an in vitro marker of viable cells.[4,5] Calcein AM also labels neuron soma and neurites in culture[6,7], and was therefore assayed as an alternative method for image-based outgrowth and viability analysis. Viability measurements with calcein AM analysis were in excellent agreement with independent image-based viability analysis using eTox Red as a viability indicator. Additionally, we demonstrate an optional live-cell nuclear staining analysis with Hoechst 33342 that can readily be incorporated in this workflow for normalization, minimizing variability from cell density across plates or batches.

In this application note, we demonstrate that pairing calcein AM labeling of iPSC-derived neuronal cultures with automated live-cell imaging and analysis presents an efficient solution for neurotoxicity studies. We validate that this approach using a panel of 15 treatments with previously demonstrated effects across a range of neurotoxicity profiles and neuronal models.

## Experimental

### Materials

#### Chemicals

Unless otherwise stated, reagents were sourced from Sigma-Aldrich (Burlington, MA, USA). Neurotoxicity panel compounds were sourced from the following suppliers: Tocris Bioscience, Bristol, UK (staurosporine, p/n 1285; tamoxifen, p/n 0999; Y-27632, p/n 1254; vinblastine, p/n 1256; doxorubicin, p/n 2252; 6BIO, p/n 3194; triptolide, p/n 3253; U-0126, p/n 1144; camptothecin, p/n 1100; nocodazole, p/n 1228; and etoposide, p/n 1226), Sigma-Aldrich (blebbistatin, p/n B0560), Calbiochem, San Diego, CA, USA (rotenone, p/n 557368), Enzo Biochem Inc, Farmingdale, NY, USA (daunorubicin, p/n ALX-380-043-M010), Abcam, Waltham, MA, USA (cycloheximide, p/n ab120093).

#### Cell culture

iPSC-derived neurons (iCell GlutaNeurons Kit, FUJIFILM CDI, pn R1034) were cultured in BrainPhys Neuronal Medium (STEMCELL Technologies, p/n 05790) supplemented with iCell Neural Supplement B (kit component, FUJIFILM CDI, p/n R1034), iCell Nervous System Supplement (kit component FUJIFILM CDI, p/n R1034), N-2 Supplement (Gibco, p/n 17502048), and laminin (Sigma-Aldrich, p/n L2020). Neurons were plated on Agilent 384-well microplates (p/n 204628-100) coated with poly-L-ornithine solution (Sigma-Aldrich, p/n A-004-M), followed by complete media supplemented with laminin (Sigma-Aldrich, p/n L2020). Live-cell dyes were calcein AM (Invitrogen, p/n C34852), Agilent eTox Red (p/n 711009), and Hoechst 33342 (Sigma-Aldrich, p/n H3570).

#### Imaging instrumentation

The calcein AM neurotoxicity assay was imaged on the Agilent BioTek Lionheart FX automated microscope with wide-field-of-view camera. The Lionheart FX was configured with the following Agilent accessories: a 20x magnification objective (p/n 1220517), GFP imaging cube (p/n 1225101), laser autofocus cube (p/n 1225010), and $CO_2$ controller (p/n 1210012-S). Other Agilent optional accessories—imaging cubes DAPI (p/n 1225100, for Hoechst 33342) and CY5 (p/n 1225105, for eTox Red)—were included for normalization and assay validation purposes.

Note on imager compatibility: Similar performance should be expected using other Agilent BioTek imaging instruments, including all Cytation cell imaging multimode readers models supporting multichannel fluorescence widefield imaging.

## Methods

### Cell culture

iPSC-derived neurons (iCell GlutaNeurons Kit, FUJIFILM CDI, p/n R1034)) were thawed and plated according to the manufacturer's suggestions. The 384-well microplates were prepared for culture 24 hours before plating. Plates were coated overnight at room temperature with poly-L-ornithine. The following day, coated plates were rinsed five times with sterile phosphate-buffered saline (PBS) and incubated in a humidified tissue culture incubator at 37 °C and 5% $CO_2$ for at least 1 hour before plating cells with complete media. Live-cell estimations were performed with trypan blue exclusion method.[8] Cells were plated at a density of ~ 2,000 live cells per well in 50 µL complete media, and incubated for ~ 3 hours to adhere and initialize outgrowth. Treatments were added at three times the final concentration in 25 µL media, resulting in 75 µL final well volume. After 48 hours incubation in drugs, 25 µL of media was removed, and 25 µL of complete media containing live-cell dyes was added, resulting in a final concentration of, 0.3 µM calcein AM, 0.3 µM eTox Red, and 0.6 µM Hoechst 33342. Cells were incubated at 37 °C for 1 hour before imaging.

### Imaging setup

The Lionheart FX automated microscope with WFOV camera captured 20x magnification images in GFP, CY5, and DAPI channels with a 2 × 2 montage collected at the well center across the 384-well plate. Laser autofocus was used for image capture focus. Live-cell environmental $CO_2$ and temperature were managed by the Lionheart FX and gas controller.

### Imaging analysis

The default settings of the Agilent BioTek Gen5 neurite outgrowth module provided a starting point for soma and neurite detection, and were further optimized as shown in Table 1. Optimal values for the parameters indicated in Table 1 will vary based on experimental conditions, and users are expected to evaluate and adjust these parameters as needed.

**Table 1.** Agilent BioTek Gen5 neurite outgrowth analysis module.

| | |
|---|---|
| **Soma Detection** | **GFP (Calcein AM)** |
| Threshold Slider Level | 0 (Neutral) |
| Minimum/Maximum Size | 10/100 |
| Soma Closing Size | 0 |
| Rolling Ball Diameter | 30 |
| Image Smoothing Strength | 3 |
| **Neurites** | |
| Detection Mode | Intensity |
| Threshold Slider Level | 20 |
| Neurite Mask Closing Size | 0 |
| Rolling Ball Diameter | 20 |
| Image Smoothing Strength | 3 |
| Only Keep Neurites Connected to a Soma | Enabled |
| Discard Short Neurites | 5 |
| Discard Short Ending Branches | 10 |

### Data analysis and fitting

Agilent BioTek Gen5 software for imaging and microscopy was used for all data plotting and fitting. Dose-response relationships were fit with four-parameter logistic curves, and Z-scores were calculated in Gen5 software using the difference between the treatment mean and control mean expressed in multiples of the standard deviation of control. In Gen5 software syntax, the following formula was applied in a transformation step to calculate Z-score for outgrowth metrics: (X-MEAN(CTL1))/SD(CTL1). Additional statistical analysis was performed with GraphPad Prism software (GraphPad Software, Boston, MA, USA).

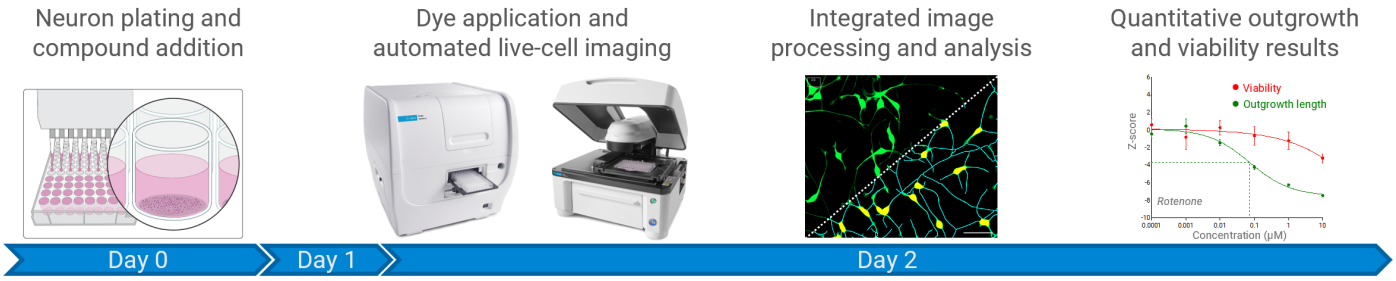


**Figure 1.** Assay workflow and timing. Neurons plated onto high-throughput 384-well imaging microplate, and treatments added ~ 3 hours after plating. Live-cell dyes added ~ 48 hours after treatment, and cultures imaged on the Agilent BioTek Lionheart FX automated microscope. Agilent BioTek Gen5 software with neurite outgrowth module integrates image analysis for both viability and neuron outgrowth. Dose-response analysis automatically quantifies neuron viability and outgrowth measurements across treatments and concentrations.

## Results and discussion

### Live-cell neurite outgrowth and viability assay workflow

This outgrowth and viability assay leverages rapid, one-step calcein AM dye loading in neuron culture as an indicator of both viability and neurite outgrowth for downstream image-based analysis. Figure 1 shows the example workflow for a neurotoxicity assay leveraging live-cell dyes. iPSC-derived neurons were plated on day 0 in a 384-well microplate, and treated with 15 drugs across a 6-decades concentration range. At 48 hours after treatment, cells were incubated with live-cell dyes for ~ 1 hour, and imaging commenced.

### Validation of single-dye assay for viability and neurite outgrowth

Here, we validated a live-cell analysis approach using calcein AM to measure viability and neurite outgrowth in response to a panel of known neurotoxicants. We validated this approach by including an independent viability marker for neurotoxicity using the dead-cell nuclear marker eTox Red. eTox Red is excluded from healthy cells, and only enters and labels DNA in cells with compromised membranes. Furthermore, we included Hoechst 33342 as marker for total cell number to normalize cell counts and determine the percentage of viable cells identified by each method. Figure 2 details example images from live neurons stained with each dye and captured in each fluorescence channel, and presented with corresponding Gen5 software analysis.

Calcein AM cell counts were reported using the Gen5 neurite outgrowth module "Soma Count" metric. eTox Red positive cells were independently identified by Gen5 cell analysis. Cell counts determined by each method were normalized to the total cell counts determined by Hoechst 33342 staining, yielding percentage of cells that were calcein AM or eTox Red positive.

For control wells ($n$ = 24), normalized cell counts indicated 62.2% ± 2.7 and 61.2% ± 3.0 viability, as measured by calcein AM and eTox Red, respectively. These values were not significantly different ($P$ = 0.21, unpaired t-test, GraphPad Prism software), and were also consistent with the viability measured at thaw (~ 65% viable cells, trypan blue exclusion method).[8] The coefficient of variance (CV) of raw counts was similar across calcein AM and eTox Red methods (7.6 and 8.1%, respectively), with relatively small reductions

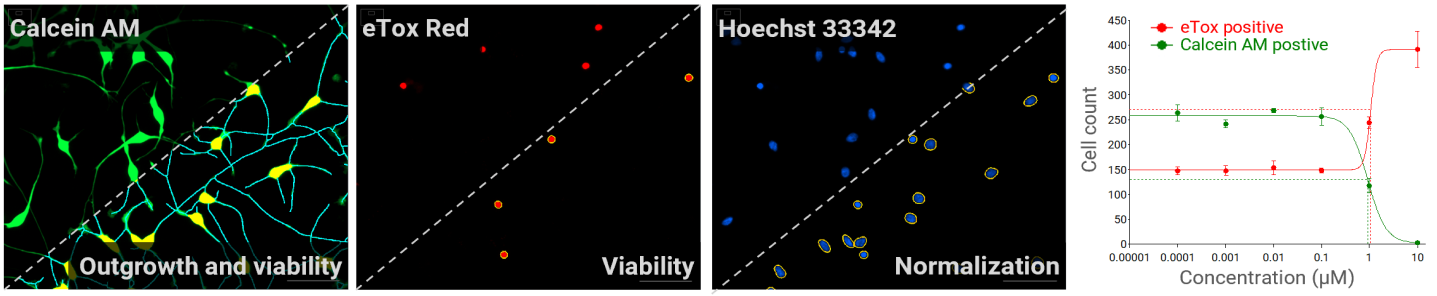


**Figure 2.** Validation of calcein AM approach for viability and neurite outgrowth. (A) Calcein AM imaging used for outgrowth and viability evaluation. (B) eTox Red is a dead-cell indicator, permitting independent evaluation of viability. (C) Hoechst 33342 is a cell-permeant DNA-binding dye, labeling all cells for normalization and calculation of viability as a percentage of total cells. (D) Dose-response analysis of staurosporine viability effects for both eTox Red (red) and calcein AM (green) by cell counts. Data points indicate mean and standard deviation of replicate wells ($n$ = 4), solid lines indicate four-parameter fits, and dashed lines indicate interpolated $EC/IC_{50}$ values.

in CV observed after normalization (4.5 and 4.9%, respectively). Together, these results support the use of calcein AM as a single-dye reporter of neurite outgrowth and viability. However, including a second dye to normalize for total cell counts, such as Hoechst 33342, may be more important to achieve consistent results when high variability in cell density is observed across wells, or in samples that exhibit neuron clustering.

In addition to control condition viability, treatment dose-response curves for viability were also compared across both calcein AM and eTox Red methods. Figure 2D compares viability analysis results for an example treatment, staurosporine, for which cytotoxicity is observed at higher concentrations. Dose-response determinations of $EC/IC_{50}$ values indicated excellent agreement across calcein AM and eTox Red methods.

### Analysis across viability and neurite outgrowth metrics

Fifteen potential neurotoxicants were applied across six decades of concentration for dose-dependent analysis on the Agilent BioTek automated imaging and analysis platform. Example images for a single treatment, staurosporine, are shown across the applied concentration range in Figure 3A. Gen5 neurite outgrowth module automatic image analysis results are overlayed on the images in Figure 3A, demonstrating detection of both neuron soma (yellow) and neurite compartments (cyan).

From these images, the automated analysis extracted four parameters that provide a relevant effect profile for viability and neuronal morphology: viable cell counts, and the per-cell average for neurite length, neurite branches, and number of neurites. The concentration-dependent results for each of these four measurements are shown for staurosporine in Figure 3B to E. Applied at 10 to 100 nM, staurosporine did not impact viability (Figure 3B) yet increased multiple neurite outgrowth parameters, including length (Figure 3C), branches (Figure 3D), and count (Figure 3E), consistent with previously reported effects as a neurotoxicant.[6] Starting at ~ 1 µM, however, reduced viability and concomitant reductions in outgrowth metrics were observed, suggesting nonspecific effects on neuronal morphology due to general cytotoxicity at higher concentrations.

For comparisons across viability and neuron morphology parameters, metrics were converted to Z-scores in Gen5 software, and dose-response analysis was performed (Figure 3F). Z-scores serve to normalize measurements while also indicating significant differences between test and control conditions and preserving directionality of effect. Individual treatment concentrations ($n$ = 4 replicates) were considered to be not significantly different from the control ($n$ = 24 replicates) for average Z-score values between 2.3 and –2.3.

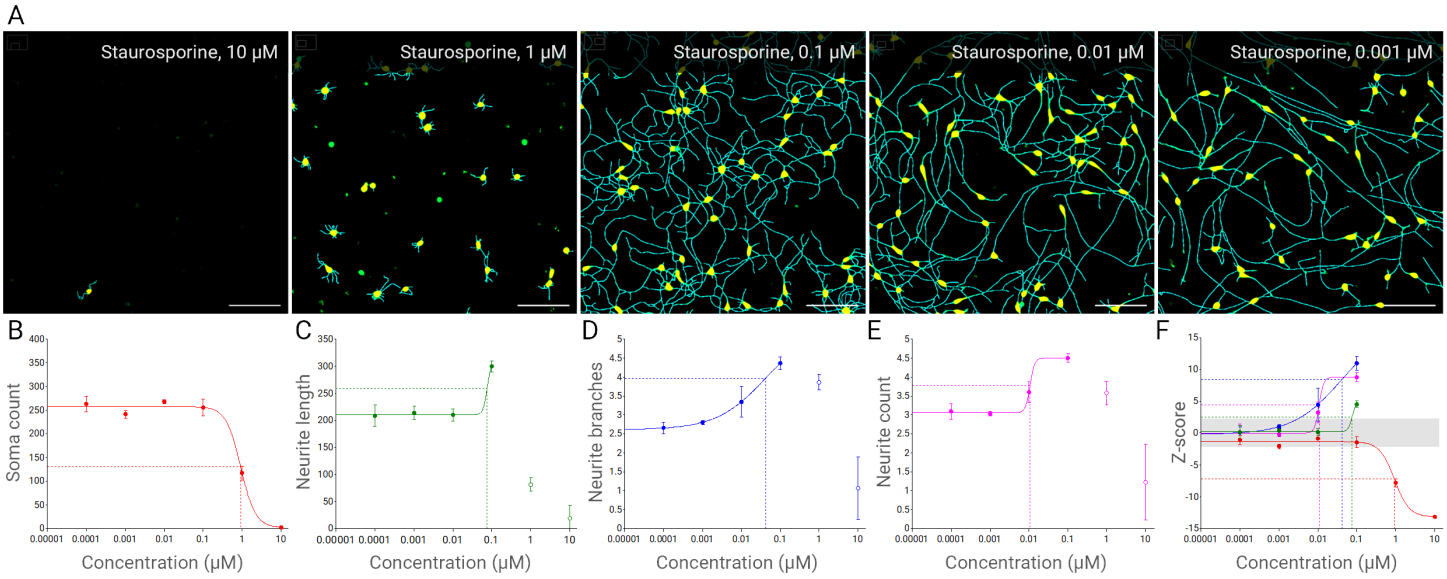


**Figure 3.** Dose-dependent outgrowth and viability analysis and Z-score. (A) Example images of iPSC-derived neuron cultures labeled with calcein AM and treated with increasing concentrations of staurosporine, as indicated. Neuron soma indicated with yellow overlay and neurites with cyan. Dose-response curves quantifying the effect of staurosporine treatment on (B) soma count and outgrowth morphology features including (C) neurite length (D) neurite branching, and (E) neurite counts. For plots B to D, data points indicate mean and standard deviation of replicate wells ($n$ = 4) for each concentration, with four-parameter fit overlay (solid lines) and corresponding $EC/IC_{50}$ interpolation (dashed lines). (F) Z-score values for viability and neuron morphology metrics plotted across staurosporine concentrations. Data points correspond to mean and standard deviation of replicate wells ($n$ = 4) for each concentration, with four-parameter fit overlay (solid lines) and corresponding $IC_{50}$ interpolation (dashed lines). Treatment Z-scores between 2.3 and –2.3 (gray highlight) were not considered significantly different from control.

### Neurotoxic treatments that reduce neurite outgrowth length

A range of treatments identified as neurotoxic across various neuronal culture models and analysis methods[6,9,10,11] were assayed to evaluate and validate the calcein AM assay on the live-cell imaging and analysis system. The neurotoxicants explored here included reported positive (increased) and negative (decreased) effectors of neurite outgrowth, with variable associated levels of cytotoxicity.

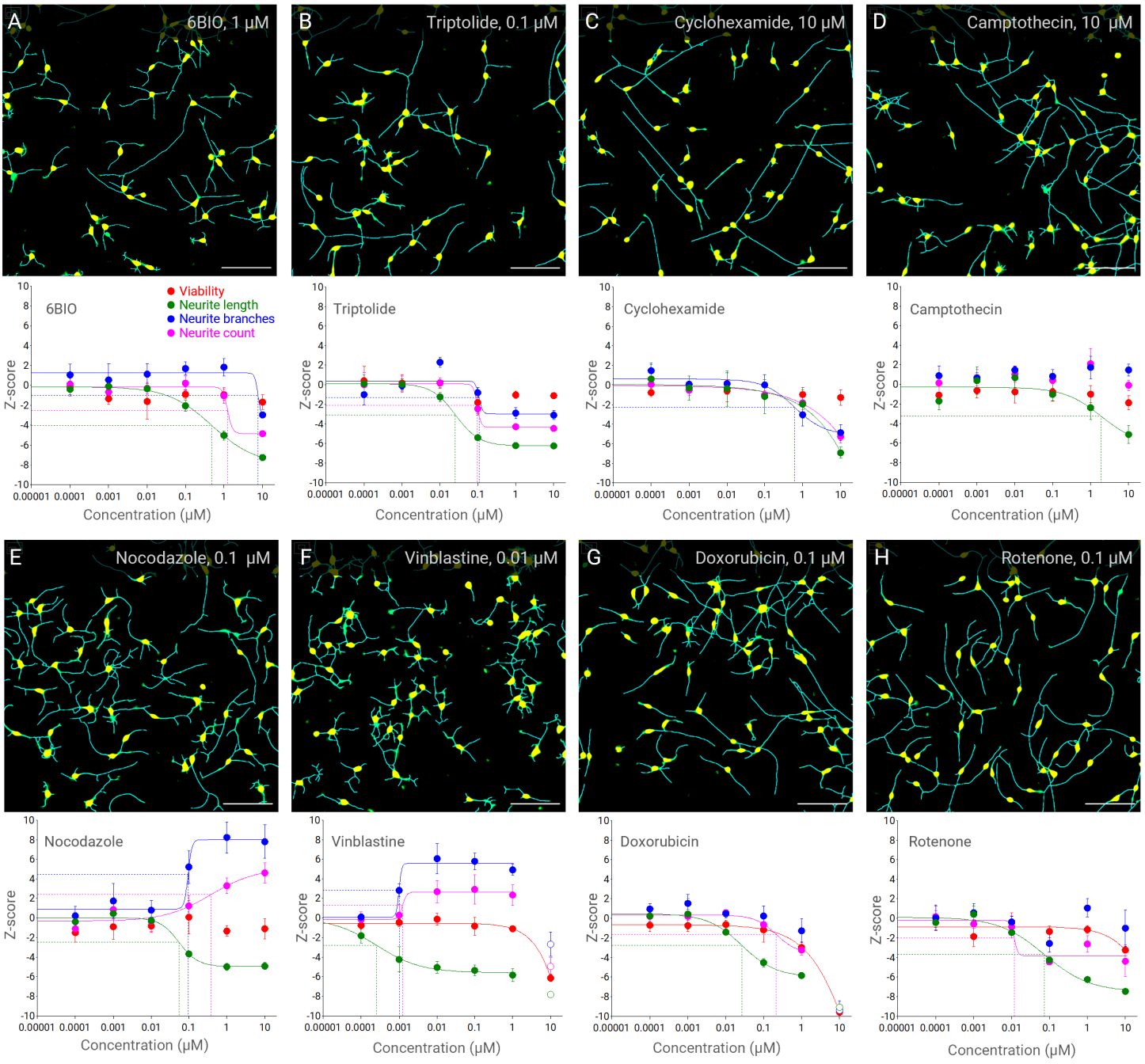


**Figure 4.** Neurotoxic treatments resulting in reduction of neurite outgrowth length. (A to H, top) Example images with analysis overlay indicating neuron soma (yellow) and neurite skeleton (cyan). (A to H, bottom) Dose-response analysis of viability and neuron morphology parameters for each treatment, including soma count (viability, red), neurite length (green), neurite branches (blue), and number of neurites per cell (neurite count, pink). Data points indicate mean and standard deviation of replicate well Z-score values ($n$ = 4), solid lines indicate four-parameter fits, and dashed lines indicate interpolated $EC/IC_{50}$ values. Open circles indicate values omitted from fit.

The most frequent outcome among the treatments tested was a reduction in neurite length, with or without cytotoxicity. Figure 4 depicts results for the eight of the fifteen tested neurotoxicants that demonstrated significant reductions in neurite outgrowth length. Five compounds significantly reduced neurite outgrowth length but did not affect viability up to the highest concentration (10 µM) tested here, including 6BIO (Figure 4A), triptolide (Figure 4B), cyclohexamide (Figure 4C), camptothecin (Figure 4D), and cycloheximide (Figure 4E). For two of these five treatments, dose-reponse analysis of neurite length was previously investigated. The $IC_{50}$ values reported here for 6BIO and triptolide effects on neurite length are in excellent agreement (< 2 fold) with previously reported values.[6]

Additional compounds that reduced neurite outgrowth length but also decreased viability at the highest concentrations included rotenone (Figure 4F), vinblastine (Figure 4G), and doxorubicin (Figure 4H). For rotenone and vinblastine, viability effects were only significant at the single highest concentration (10 µM), and consequently the $IC_{50}$ values are poorly defined from the fit, and instead here estimated as > 1 µM. Even using the conservative $IC_{50}$ estimate of 1 µM for rotenone and vinblastine, the $IC_{50}$ values for viability were > 10 times the $IC_{50}$ estimates for neurite outgrowth length effects, suggesting that all three of these treatments were specific neurotoxicants. Testing additional higher concentrations of rotenone and vinblastine would improve the accuracy of the viability $IC_{50}$ value, but would not alter the conclusion that they are specific neurotoxicants at lower concentrations.

Figure 4 demonstrates that decreases in neurite outgrowth length are often also associated with decreases in neurite branching or number, with two notable exceptions. Nocodazole (Figure 4E) and vinblastine (Figure 4F) result in significant reduction in neurite length, with concomitant increases in both branching and neurite count. Both nocodazole and vinblastine are microtubule inhibitors, acting with the same mechanism of action (MOA). Vincristine, a chemical analog of vinblastine with similar MOA, is a widely used chemotherapeutic with notable neurotoxic side effects and often investigated across in vitro neuron models.[6,7,10,11,12] Similar to the results identified here for vinblastine, vincristine has demonstrated significant inhibitory effects on neurite outgrowth when measured by neurite length[6,7,12] or area.[10,11] However, vincristine effects on branching and neurite number are comparably less explored.

### Neurotoxic treatments that increase neurite outgrowth

As shown in Figure 5, three neurotoxicants increased neurite outgrowth, with or without cytotoxicity at higher concentrations. For both staurosporine (Figure 5A) and

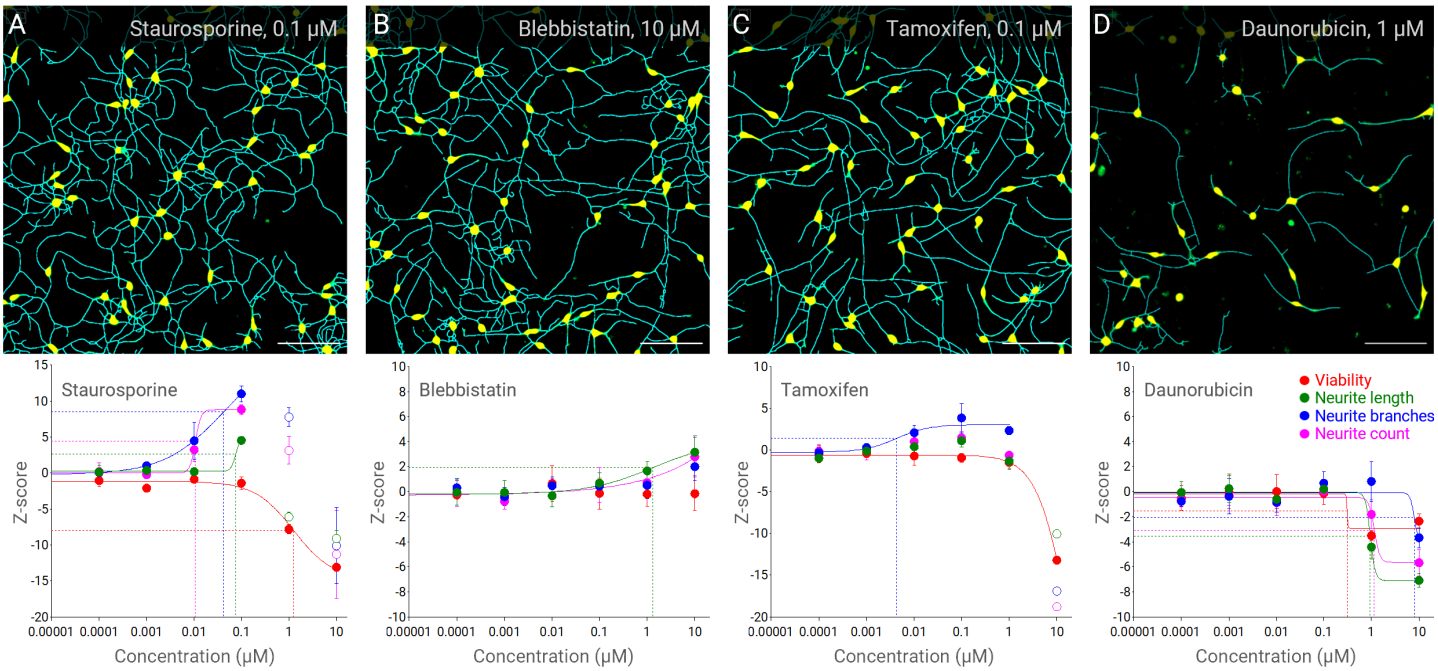


**Figure 5.** Neurotoxic treatments that do not reduce neurite outgrowth. (A to D, top) Example images with analysis overlay indicating neuron soma (yellow) and neurite skeleton (cyan). (A to D, bottom) Dose-response analysis of viability and neuron morphology parameters for each treatment, including soma count, neurite length, neurite branching, and number of neurites per cell (neurite count). Data points indicate mean and standard deviation of replicate wells Z-score values ($n$ = 4), solid lines indicate four-parameter fits, and dashed lines indicate interpolated EC/$IC_{50}$ values. Open circles indicate values omitted from fit.

blebbistatin (Figure 5B), neurite length was significantly increased, with neurite branching and count also significantly increased for the former. Furthermore, the $EC_{50}$ estimates for the staurosporine and blebbistatin enhancement of neurite length determined in this study were extremely similar (< 2 fold) to published $EC_{50}$ values for neurite length effects.[6] For tamoxifen (Figure 5C), a small but significant increase in branching was measured. While staurosporine and blebbistatin have also been identified as neurite outgrowth enhancers in multiple studies[6,10], tamoxifen treatment has demonstrated model- and cell-dependent effects on outgrowth.[6,13]

### Treatments exhibiting nonspecific cytotoxicity

Daunorubicin demonstrated significant cytotoxicity at lower concentrations than effects on neurite outgrowth (Figure 4D). Although daunorubicin had previously been identified in other systems as a specific neurotoxicant[6], suggesting daunorubicin effects may be cell-type or experimentally specific.

### Treatments exhibiting no significant effects

Three treatments (U-0126, Y-27632, and etoposide) did not show significant changes for any metric tested across the concentration range, including for both viability and outgrowth parameters (data not shown). Etoposide is a topoisomerase II inhibitor that demonstrates strong antiproliferative effects, but has not demonstrated significant neurite outgrowth effects[10,11], consistent with the results presented here. U-0126 testing has shown variable results, from no significant effect[11] to inhibition of outgrowth at relatively high concentrations across different models.[9,10] Further testing of U-0126 applied at higher concentrations in this neuron model may provide additional insight. Rho-kinase (ROCK) inhibitors, such as Y27632 tested here, have increased neurite outgrowth in PC12 models[14] and other stem cell-derived models.[6] However, ROCK inhibitors have demonstrated transient effects on neurite outgrowth in iPSC-derived neuron models[15], even at higher concentrations than tested here. Further investigation through kinetic neurite outgrowth analysis may provide important insights into ROCK inhibitor effects.

## Conclusion

The Agilent BioTek imager and software platform for neuron culture analysis of calcein AM labeled-neurons provided a rapid and robust solution for in vitro neurotoxicity analysis. This approach reduces sample preparation to a simple media addition to label cells before imaging, yielding both time and cost savings compared to antibody-based assay approaches. Automatic image collection and analysis streamlines the assay workflow, facilitating viability and outgrowth analysis in a single software interface, and reducing time to answer.

This approach was evaluated across 15 treatments that captured a broad range of assay outcomes, including identification of specific neurotoxic effects, general cytotoxic effects, and no significant effects. The results presented here are largely consistent with previously identified treatment results, including across a range of inhibitory and enhanced neuronal morphology effects, validating this platform and approach for in vitro neurotoxicity investigations.

The presented viability and neurite outgrowth results were determined solely through analysis of the calcein AM signal. Minimal reduction in variability was observed for this dataset when calcein AM signals were normalized to the additional all-cell marker, Hoechst 33342. However, it is important to note that normalization can be a key experimental element to improve reproducibility when cell culture density is variable, or when sample characteristics present challenging conditions for cell analysis, such as cell clustering. Normalization with an additional live-cell stain, such as a nuclear marker, is readily accommodated on the Agilent BioTek image and analysis platform across a spectrum of fluorescence channels.

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