

Introduction

- In vitro neurotoxicity assays remain a key approach to address the impact of environmental, occupational and medicinal chemical exposures on neuron function.
- Single-step live-cell dyes provide a rapid solution for image-based analysis of neuron culture viability and morphology.
- Automated benchtop imaging and analysis is an accessible solution to improve the scale, efficiency and reproducibility of in vitro assays to advance neurotoxicity research.
- A panel of neurotoxicants across a range of mechanism-of-action classes and previously reported phenotypes were evaluated in a proof-of-principle study.
- Automated analysis confirmed effect classes and provided quantitative insights, validating this approach as a sensitive and robust solution to determine neurotoxic effects across a broad range of assay outcomes.

Results and Discussion

Image-based live-cell imaging and analysis platform



Figure 1. Agilent BioTek imaging systems compatible with live-cell neurotoxicity analysis.

Validation of dye-based assay approach

Calcein AM: single-dye viability and outgrowth analysis

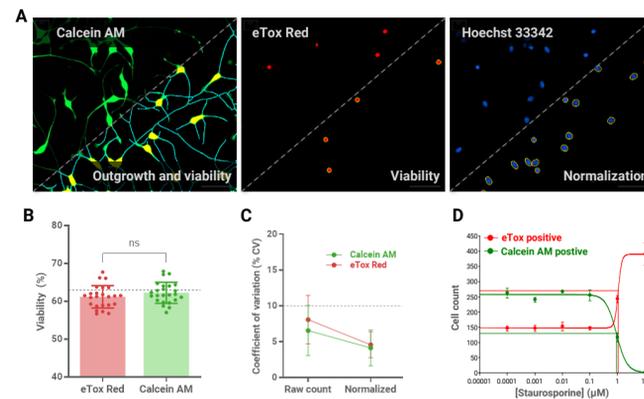


Figure 2. (A) Example images of calcein AM, eTox Red, and Hoechst 33342 live-cell staining of iPSC-derived neuron cultures. Calcein AM analysis identifies neurite outgrowth morphology and cell counts for viability. eTox Red labels non-viable cells as an orthogonal method for viability determinations. Hoechst 33342 labels all cells for normalization purposes. (B) Percent viable cell determinations for all control wells (n=24) by eTox Red or calcein AM staining analysis. Individual well data are displayed over bars, showing mean and standard deviation. The dashed line corresponds to the initial cell viability measured at thaw. (C) Comparison of percent coefficient of variation (CV) of cell viability measurements before and after normalization with total cell counts from the Hoechst 33342 analysis. Data points indicate the mean and standard deviation of controls (N=4 replicate sets, n=4 wells each set). Raw cell counts across both methods demonstrate less than 10% CV (dashed line). Normalization further reduces %CV by ~3%. (D) Comparison of staurosporine treatment toxicity evaluated by eTox Red and calcein AM analysis. Data points correspond to the mean and standard deviation of replicate wells (n=4) for each concentration, with 4-parameter fit overlay (solid lines) and corresponding EC₅₀/IC₅₀ interpolation (dashed lines).

Automated image analysis and z-score calculation

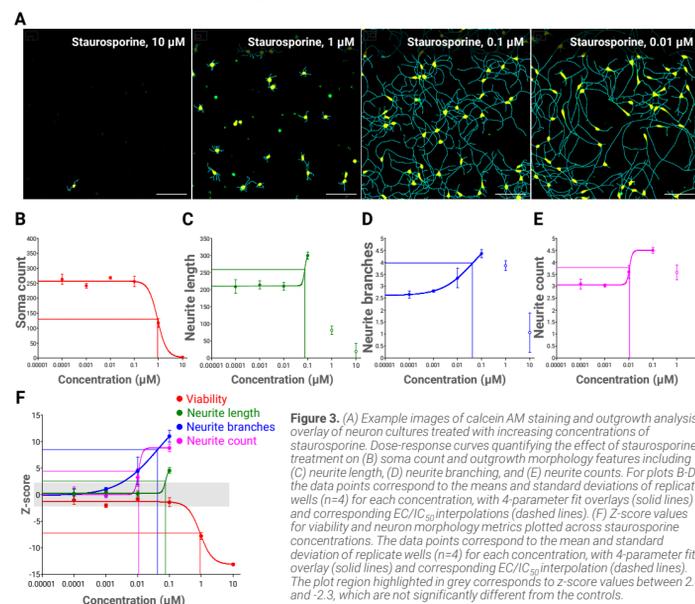


Figure 3. (A) Example images of calcein AM staining and outgrowth analysis overlay of neuron cultures treated with increasing concentrations of staurosporine. Dose-response curves quantifying the effect of staurosporine treatment on (B) soma count and outgrowth morphology features including (C) neurite length, (D) neurite branching, and (E) neurite counts. For plots B-E, the data points correspond to the means and standard deviations of replicate wells (n=4) for each concentration, with 4-parameter fit overlays (solid lines) and corresponding EC₅₀/IC₅₀ interpolations (dashed lines). (F) Z-score values for viability and neuron morphology metrics plotted across staurosporine concentrations. The data points correspond to the mean and standard deviation of replicate wells (n=4) for each concentration, with 4-parameter fit overlay (solid lines) and corresponding EC₅₀/IC₅₀ interpolation (dashed lines). The plot region highlighted in grey corresponds to z-score values between 2.3 and -2.3, which are not significantly different from the controls.

Results and Discussion

Establishing neurotoxicity across treatment panel

Evaluation across a range of expected neurotoxicity profiles

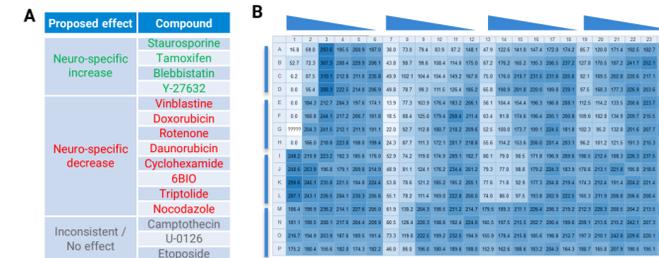


Figure 4. (A) List of potential neurotoxicants tested and their predicted effects based on previously published work across various model systems and techniques. (B) Example plate overview depicting the treatment layout with a heatmap corresponding to the results for average neurite length.

Dose-response analysis to establish effect specificity

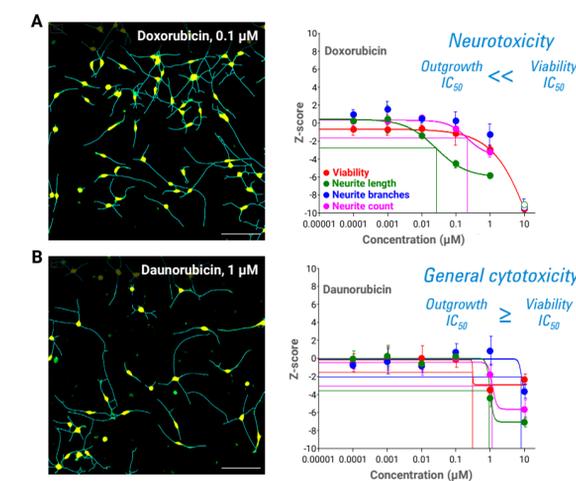


Figure 5. Dose-response analysis enables quantitative comparison of viability and neurite outgrowth effects. (A) Example image of doxorubicin treatment and dose-response analysis, demonstrating neurite morphology effects with significantly lower IC₅₀ values than viability effects, indicating an example of specific neurotoxic outcome. Data points correspond to the mean and standard deviation of replicate wells (n=4) for each concentration, with 4-parameter fit overlay (solid lines) and corresponding IC₅₀ interpolation (dashed lines). (B) Daunorubicin treatment example image and dose-response analysis that demonstrates similar IC₅₀ values for both viability and neuron morphology effects, indicating a general cytotoxicity effect. Data points correspond to the mean and standard deviation of replicate wells (n=4) for each concentration, with 4-parameter fit overlay (solid lines) and corresponding IC₅₀ interpolation (dashed lines).

Identification of neurotoxic response profiles

Neurotoxicants that reduced outgrowth

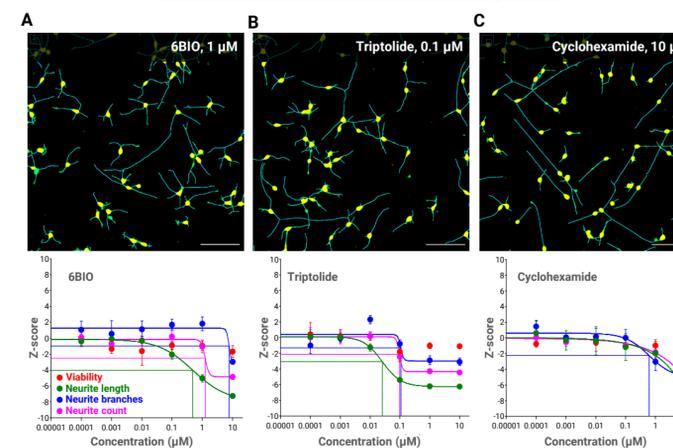


Figure 6. Dose-response profiles for neurotoxic treatments that generally reduced neurite outgrowth across morphology features. Example images and dose-response analysis for (A) 6BIO, (B) Triptolide, and (C) Cyclohexamide treatments, where concomitant decreases in both length and branching features of neuron morphology were observed. Six of the fifteen treatments demonstrated a similar effect profile, with or without significant viability effects observed in the concentration range tested. Data points correspond to the mean and standard deviation of replicate wells (n=4) for each concentration, with 4-parameter fit overlay (solid lines) and corresponding IC₅₀ interpolation (dashed lines).

Results and Discussion

Neurotoxicants that increased outgrowth

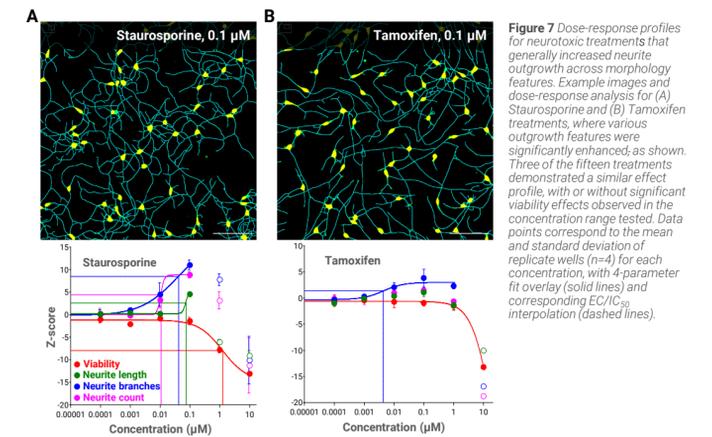


Figure 7. Dose-response profiles for neurotoxic treatments that generally increased neurite outgrowth across morphology features. Example images and dose-response analysis for (A) Staurosporine and (B) Tamoxifen treatments, where various outgrowth features were significantly enhanced, as shown. Three of the fifteen treatments demonstrated a similar effect profile, with or without significant viability effects observed in the concentration range tested. Data points correspond to the mean and standard deviation of replicate wells (n=4) for each concentration, with 4-parameter fit overlay (solid lines) and corresponding EC₅₀/IC₅₀ interpolation (dashed lines).

Neurotoxicants that reduced length and increased branching

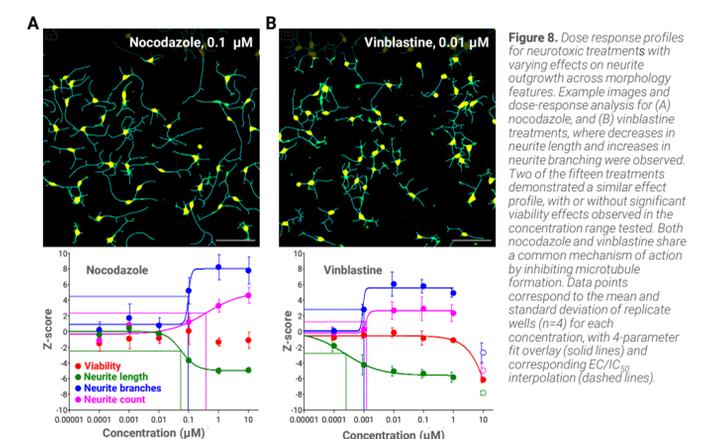


Figure 8. Dose response profiles for neurotoxic treatments with varying effects on neurite outgrowth across morphology features. Example images and dose-response analysis for (A) nocodazole, and (B) vinblastine treatments, where decreases in neurite length and increases in neurite branching were observed. Two of the fifteen treatments demonstrated a similar effect profile, with or without significant viability effects observed in the concentration range tested. Both nocodazole and vinblastine share a common mechanism of action by inhibiting microtubule formation. Data points correspond to the mean and standard deviation of replicate wells (n=4) for each concentration, with 4-parameter fit overlay (solid lines) and corresponding EC₅₀/IC₅₀ interpolation (dashed lines).

Summary of assay outcomes

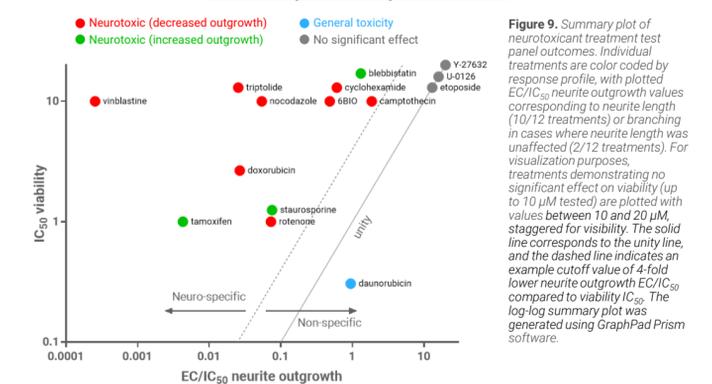
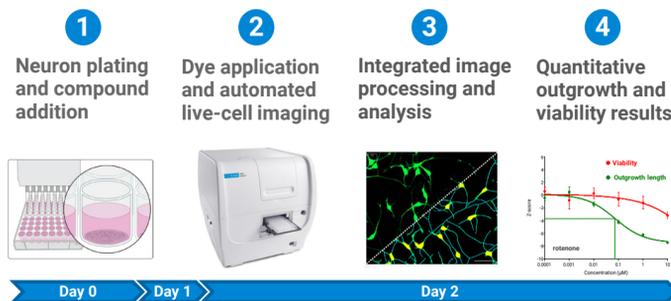


Figure 9. Summary plot of neurotoxicant treatment test panel outcomes. Individual treatments are color coded by response profile, with plotted EC₅₀/IC₅₀ neurite outgrowth values corresponding to neurite length (10/12 treatments) or branching in cases where neurite length was unaffected (2/12 treatments). For visualization purposes, treatments demonstrating no significant effect on viability (up to 10 μM tested) are plotted with values between the unity line, and the dashed line indicates an example cutoff value of 4-fold lower neurite outgrowth EC₅₀/IC₅₀ compared to viability IC₅₀. The log-log summary plot was generated using GraphPad Prism software.

Conclusion

- The Agilent BioTek automated imaging and analysis platform is a robust solution for neurotoxicity analysis
- Live-cell labeling with calcein AM dye captures essential viability and neurite outgrowth features, providing an effective alternative to antibody-based methods
- Agilent BioTek Gen5 software automated image analysis identifies salient neuron morphology features for dose-response analysis, providing a sensitive and efficient method to capture a broad range of neurotoxicity assay outcomes

Neurotoxicity assay overview



Experimental

Neuron culture

Reagents were sourced from Sigma, unless noted. iPSC-derived neurons (FUJIFILM iCell GlutaNeurons, p/n R1061) were cultured according to product recommendations for media composition, culturing protocol, and plate coating procedure. Neurons were plated at 2,000 cells/well on 384-well microplates (Agilent, p/n 204628-100). At three hours post-plating, a panel of fifteen treatments were added, resulting in a final concentration range of 0.001 to 10 μM.

Neuron labeling

After 48 hours of drug treatment, live-cell dye calcein AM (Invitrogen, C34852), eTox Red (Agilent, p/n 711009), and Hoechst 33342 (p/n H3570) were added to a final concentration of 0.3, 0.3, and 0.6 μM, respectively. Cells were incubated for ~1 hour at 37 °C before imaging began.

Imaging

The Agilent BioTek Lionheart LFX instrument automatically captured 20x magnification images for GFP, CY5, and DAPI widefield fluorescence channels in a 2 x 2 montage across each well. Laser autofocus was used for image capture focus. Similar performance should be expected when using other Agilent BioTek imaging instruments, including all Cytation models that support multichannel live-cell fluorescence widefield imaging and laser autofocus.

Analysis

Image acquisition and analysis were performed with Agilent BioTek Gen5 software with the neurite outgrowth analysis module. Dose-response visualization and analysis was also performed in Gen5 software. Additional statistical comparisons and plotting were performed in GraphPad Prism software.

Expanded materials and methods are available in the application note "Rapid, Image-Based Viability and Outgrowth Analysis for Neurotoxicity Assays."

