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High-Content Screening Assay for Identification of Chemicals Impacting Spontaneous Activity in Zebrafish Embryos

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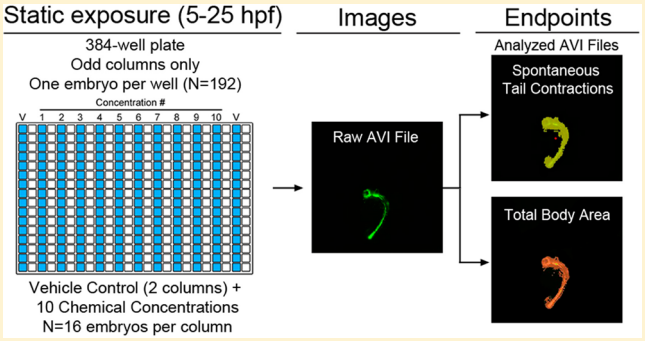
Supporting Information

ABSTRACT: Although cell-based assays exist, rapid and cost-efficient high-content screening (HCS) assays within intact organisms are needed to support prioritization for developmental neurotoxicity testing in rodents. During zebrafish embryogenesis, spontaneous tail contractions occur from late-segmentation (~19 h postfertilization, hpf) through early pharyngula (~29 hpf) and represent the first sign of locomotion. Using transgenic zebrafish (*fli1:egfp*) that stably express eGFP beginning at ~14 hpf, we have developed and optimized a 384-well-based HCS assay that quantifies spontaneous activity within single zebrafish embryos after exposure to test chemicals in a concentration−response format. Following static exposure of one embryo per well from 5 to 25 hpf, automated image acquisition procedures and custom analysis protocols were used to quantify total body area and spontaneous activity in live embryos. Survival and imaging success rates across control plates ranged from 87.5 to 100% and 93.3−100%, respectively. Using our optimized procedures, we screened 16 chemicals within the US EPA's ToxCast Phase-I library, and found that exposure to abamectin and emamectin benzoate—both potent avermectins—abolished spontaneous activity in the absence of gross malformations. Overall, compared to existing locomotion-based zebrafish assays conducted later in development, this method provides a simpler discovery platform for identifying potential developmental neurotoxicants.



INTRODUCTION

The developing nervous system is a sensitive target for chemical exposure in both humans and animal models,[1,2] and early life-stage exposures can lead to long-term effects on motor activity, sensory function, and cognition.[3−5] Developed and issued by the Organization for Economic Co-operation and Development (OECD), the developmental neurotoxicity (DNT) test guideline (http://www.oecd-ilibrary.org/environment/test-no-426-developmental-neurotoxicity-study_9789264067394-en) is used to assess the potential effects of pre- and postnatal chemical (mainly pesticide) exposure on the morphology and function of the developing nervous system within preweaning, adolescent, and young adult rodents. Commonly used strains of rats are preferred for this guideline, and a minimum of 20 litters per treatment group and 3−4 dose levels plus a vehicle control are needed for successful study completion. Therefore, assuming an average litter size of 8−12 pups,[6] each DNT study would require 640 to 1200 rats (excluding dams) per chemical.

Currently, there are minimal to no DNT data available for thousands of chemicals used in commerce.[1,7,8] In addition to animal use considerations, it is impractical to screen these chemicals using the existing DNT test guideline, as this test is costly, time-intensive, and low-throughput. Although significant advancements in development and application of high-throughput screening (HTS) and high-content screening (HCS) assays have occurred over the last 5−10 years, a large majority of assays relevant to DNT are focused on key molecular and cellular events in vitro and, as such, do not address the potential for chemically induced DNT within an intact organism.[9,10] Therefore, there is a recognized need to use alternative nonmammalian models to support screening and prioritization of chemicals for DNT testing.[9,10]

Zebrafish have long been used as a model for understanding vertebrate neurodevelopment[11,12] and, as a result, the nervous system circuitry within zebrafish is well characterized and understood.[13,14] Due to the small size, transparency, and ex utero development during embryogenesis, the potential for adverse effects on the developing nervous system can be readily evaluated at both the morphologic- and functional-level. For example, several zebrafish-based locomotion assays have been used to examine spontaneous tail contractions, touch-response, and/or swimming patterns in response to chemical and/or light stimuli in microplate format.[15−18] Although valuable for providing information on behavioral responses following

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developmental exposure, these assays can be complex, requiring treatment solution renewals (and, therefore, more technical material), excessive embryo/larval handling, and cost-prohibitive infrared cameras needed for imaging under dark conditions. Moreover, typical zebrafish-based locomotive assays are conducted using a larval stage (6–7 days postfertilization (dpf)) that is protected by animal use regulations around the world, likely resulting in minimal animal use reductions for DNT testing.

Despite significant advancements in zebrafish locomotion assays, rapid and streamlined HCS assays are needed to facilitate screening and prioritization of chemicals for DNT testing within rodents. Therefore, for this study, we focused on spontaneous tail contractions—the first sign of motor activity—within zebrafish embryos as a behavioral end point amenable for identification of potential developmental neurotoxicants. Although other assays have focused on this end point, the HCS assay reported here provides a streamlined discovery platform with (1) increased sample sizes; (2) broad concentration–response format; (3) short assay duration (~22 h total); and (4) minimal technical-grade test material needed for screening.

MATERIALS AND METHODS

Animals. For this assay, we relied on a robust line of transgenic zebrafish (*fli1:egfp*) that stably express enhanced green fluorescent protein (eGFP) within vascular endothelial cells.[19] This strain begins expressing eGFP at ~14 h postfertilization (hpf), allowing us to accurately track spontaneous tail contractions in the absence of shading effects observed under transmitted light. Adult *fli1:egfp* zebrafish were maintained on a 14-h/10-h light/dark cycle within a Mini Mass Embryo Production System (mini-MEPS) (Aquatic Habitats, Inc., Apopka, FL) containing a photoperiod light cycle dome and recirculating conditioned reverse osmosis (RO) water (~27–28 °C). Adult females and males were bred directly within the mini-MEPS, directly on-system using in-tank breeding traps suspended within 3 L tanks, or off-system within a light- and temperature-controlled incubator using breeding traps suspended within 1 L tanks. For all experiments described below, newly fertilized eggs were staged according to previously described methods.[20] All fish were handled and treated in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols at the University of South Carolina—Columbia.

Chemicals. Chemicals were purchased from ChemService, Inc. (West Chester, PA) and Sigma Aldrich (St. Louis, MO). Chemical names, chemical formulas, CAS registry numbers, vendor, and purities are provided within Supporting Information, SI, Table S1. Within 24 h of test initiation, stock solutions of each chemical were prepared by dissolving chemicals in high performance liquid chromatography (HPLC)-grade dimethyl sulfoxide (DMSO) (50 mM), and then performing 2-fold serial dilutions into DMSO to create stock solutions for each working solution. All stock solutions were stored at room temperature within 2-mL amber glass vials containing polytetrafluoroethylene (PTFE)-lined caps. For each individual plate, working solutions of all treatments were freshly prepared by spiking stock solutions into embryo media (EM) (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$), resulting in 0.1% DMSO within all vehicle control and treatment groups.

High-Content Screening (HCS) Assay. Exposure Setup. Black 384-well microplates containing 0.17-mm glass-bottom wells (Matrical Bioscience, Spokane, WA) were used for this assay. Newly fertilized eggs were collected immediately after spawning and placed in groups of approximately 50 per glass Petri dish within a light- and temperature-controlled incubator until 5 hpf. Although dechorionation may help decrease uncertainties regarding uptake across the chorion, previous attempts within our laboratory to dechorionate embryos prior to exposure resulted in unacceptable survival within control embryos (data not shown); therefore, chorionated embryos were used for all exposures. Vehicle control (0.1% DMSO) or treatment solution (50 μL per well) was added to odd columns of a 384-well plate at 5 hpf. Using clean forceps, 192 viable *fli1:egfp* embryos were then manually arrayed into the plate over a 20-min time period, resulting in one embryo per well and 16 initial embryos per column. Each plate was checked to ensure that all embryos were intact or undamaged following loading. Initial attempts to use 384 embryos were unsuccessful due to increased imaging duration, resulting in variation in spontaneous activity within vehicle control embryos across the plate (data not shown). For chemical screening assays, vehicle control groups occupied two columns flanking the left and right sides of the plate (columns 1 and 23) to account for potential control variance in spontaneous activity from initiation to termination of image acquisition. The plate was then incubated at 28 °C under a 14-h/10-h light/dark cycle and static conditions until 24 hpf.

Image Acquisition. At 24 hpf, the plate was removed from the incubator and allowed to acclimate in a second incubator at 25 °C in treatment solution for 1 h with the lid removed. This acclimation was done to ensure that changes in temperature from the incubator (28°C) to the imager (25°C) did not affect spontaneous activity, as previous experiments have demonstrated that temperature alterations can affect this behavior.[26] The plate was then centrifuged for 3 min at 200 rpm to ensure that the majority of embryos were near the bottom of each well. Using an automated image acquisition protocol (SI Figure S1) and parameters (SI Table S2) optimized for our ImageXpress Micro (IXM) Widefield High-Content Screening System (Molecular Devices, Sunnyvale, CA), each embryo was imaged in treatment solution over a 6-s time period to assess the presence or absence of spontaneous tail contractions. Image acquisition began within 5 to 10 min after centrifugation, commencing at well A01 and proceeding down each column across the plate from columns 1 to 23. Throughout this process, one 6-s stream was acquired at a time for each well. This time period was chosen to minimize variation in spontaneous activity across the plate due to increased imaging duration. Image acquisition procedures were identical for each independent plate. During the entire ~1.4-h image acquisition period, internal temperature within the IXM system was maintained between 25 and 27 °C by removing panels on both sides of the IXM system and blowing air from left to right through the IXM with a portable fan; internal temperature was monitored and recorded at initiation and termination of imaging using a digital thermometer. In accordance with National Institutes of Health (NIH) guidelines,[21] embryos were then euthanized by placing the plate at −20 °C.

Data Extraction. Custom journal scripts for video generation were developed using MetaXpress 4.0.0.24 software (Molecular Devices, Sunnyvale, CA). Prior to data extraction, stream acquisitions within each well were inspected within

MetaXpress to assess survival. As heart morphogenesis is not complete until ~48 hpf, presence or absence of heartbeat was not used as a criterion for survival. Rather, coagulated embryos and embryos with undeveloped or absent tails were considered dead and were not analyzed for total body area or spontaneous activity. Fully automated journal scripts were used to generate videos of each well by converting each stream acquisition to an .AVI file. Video file sizes were reduced within Format Factory (http://www.pcfreetime.com/) by conversion to MPEG4 (DivX). Converted videos were then imported into EthoVision XT 9.0 (Noldus Information Technology, Leesburg, VA) to determine the mobility state of each embryo. On the basis of user-defined detection settings that identify the embryo as the subject, embryos were considered highly mobile (exhibiting spontaneous tail contractions) if the change in subject pixel area from one frame to another was above a previously optimized user-defined threshold. Initial attempts to use EthoVision to quantitatively assess the number or duration of spontaneous tail contractions were unsuccessful due to variation in this behavioral phenotype within individual control embryos (data not shown). Rather, EthoVision output was used to assign spontaneous activity values to each embryo. Inactive embryos were defined as embryos that were immobile during the entire 6-s stream acquisition and were assigned an activity value of 0. Active embryos were defined as embryos that moved for at least 0.1 s and were assigned an activity value of 1. Within EthoVision, duration is assessed in 0.1-s increments; therefore, we used this value as a criterion for activity in order to include embryos exhibiting partial tail contractions as active embryos.

Automated custom journal scripts within MetaXpress were used to analyze total embryo body area in order to (1) reliably identify treatment-related effects and (2) focus our analyses on targeted impacts on spontaneous activity in the absence of significant effects on embryonic growth. Treatments resulting in a significant decrease in total body area were not analyzed for spontaneous activity. Embryos exhibiting notochord malformations that were not detected by analysis of total body area were also excluded from analysis of spontaneous activity. Additional information about parameters used in EthoVision XT 9.0 is provided in SI Table S3. Additional details about the data extraction and analysis process are provided in SI Figure S1.

Statistical Analysis. All statistical procedures were performed using SPSS Statistics 20.0 (Chicago, IL). Nonparametric tests were used for analyzing spontaneous activity data, as these data were categorical and did not meet normality assumptions. A Kruskal–Wallis test was used ($\alpha = 0.05$) to test for main effect of treatment, and Mann–Whitney pairwise comparisons were used to test for differences between vehicle control columns 1 and 23 and treatment columns relative to vehicle control columns ($\alpha = 0.05$). For chemical screening assays, treatments were only considered significant if different from both control columns. For total body area analysis, a general linear model (GLM) analysis of variance (ANOVA) ($\alpha = 0.05$) was used, as these data did not meet the equal variance assumption for non-GLM ANOVAs. Pair-wise Tukey-based multiple comparisons of least-squares means were performed to identify significant treatment-related effects. To estimate the median lethal concentration (LC_{50}) after static exposure to each chemical from 5 to 25 hpf, a four-parameter concentration–response curve was fit to percent mortality data using log-transformed chemical concentrations within Prism 6.0 (GraphPad Software Inc., La Jolla, CA).

RESULTS

HCS Assay Variability. To determine assay variability, we imaged three independent control plates containing embryos incubated in EM at 28 °C from 5 to 24 hpf, and then acclimated for 1 h at 25 °C prior to imaging. Parameters provided in SI Figure S1 and Tables S2 and S3 were used to analyze total body area and spontaneous activity for each control plate. Percent survival and image success rates for each plate were consistently above 95% and 99.5%, respectively (Table 1 and SI Figure S2A). When summarized by column, survival ranged from 87.5 to 100%, while image success rates ranged from 93.3 to 100% (SI Table S4). While total body area was consistent within and across control plates (SI Figure S2B), spontaneous activity within and across control plates was variable, with an average percent by column ranging from 36.3 to 63.4% across all three plates (Figures 1 and SI S2C).

Table 1. Image Success Rates for Analysis of Spontaneous Activity and Total Body Area within Control 25-hpf Zebrafish Embryos Across Three Independent Plates Containing an Initial Sample Size of 192 Embryos Per Plate[a]

	live embryos		spontaneous activity		total body area	
control plate	total (no.)	total (%)	analyzed (no.)	analyzed (%)	analyzed (no.)	analyzed (%)
1	192	100.0	192	100.0	192	100.0
2	185	96.4	185	100.0	184	99.5
3	192	100.0	191	99.5	191	99.5

[a]The percentage of live embryos was relative to an initial sample size of 192 embryos per plate, whereas the percentage of analyzed embryos was relative to the number of live embryos.

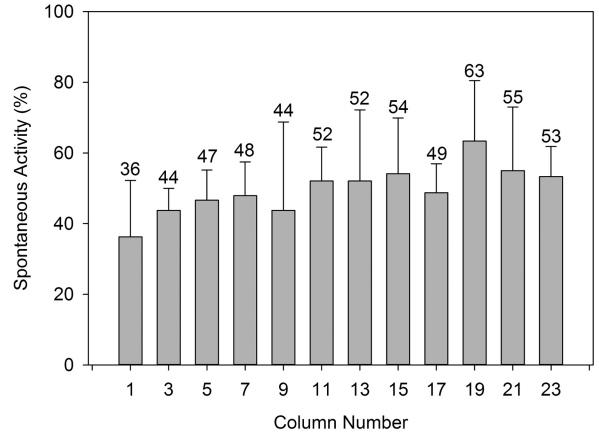

Figure 1. Spontaneous activity (%) following exposure to 50 μL embryo media from 5 to 25 hpf in a 384-well plate containing 192 initial embryos. Spontaneous activity (%) data are presented as mean ± SD across three independent control plates. Numbers above each bar denote percent spontaneous activity within that column.

On the basis of performance within control plates, we then developed decision criteria for assay success (SI Figure S3). The percent of embryos with spontaneous activity per column ranged from 18.75% to 75% across all three plates (36 columns total), with the majority of columns ranging from 25 to 74% (Figure S4). Therefore, based on these data, we concluded that spontaneous activity within each control group (columns 1 and 23 for chemical screening assays) must be >18% in order to be

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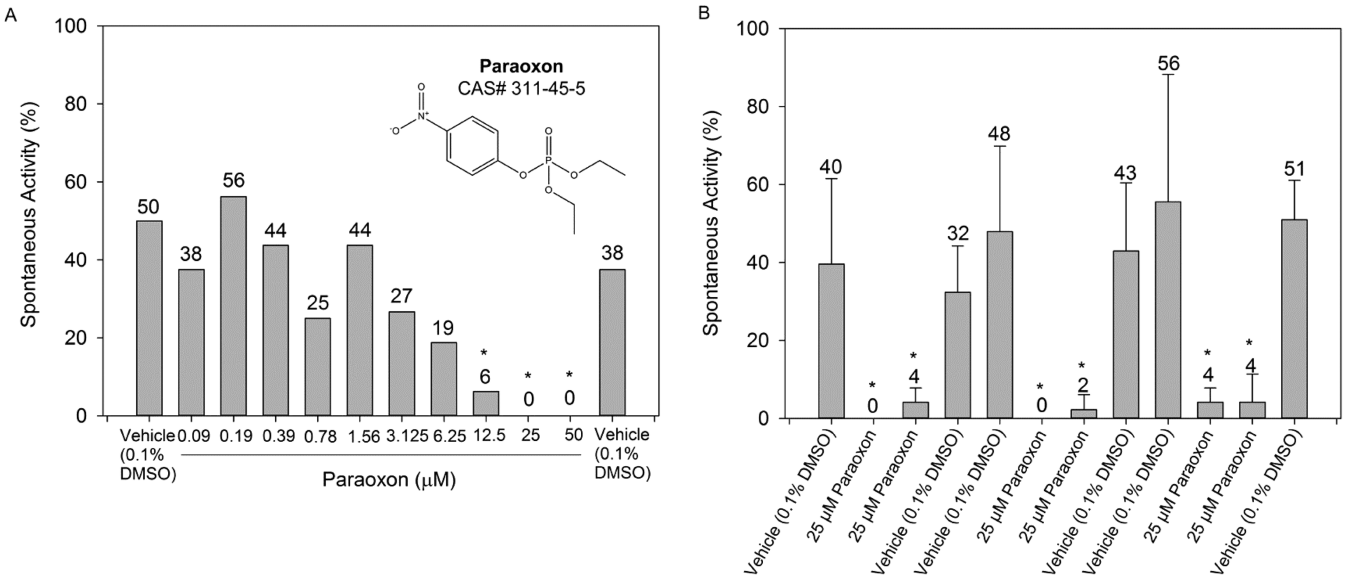

Figure 2. Spontaneous activity (%) following (A) exposure to paraoxon in a concentration response-format or (B) exposure to vehicle (0.1% DMSO) or 25 μM paraoxon (reference plates) from 5 to 25 hpf in a 384-well plate containing 192 initial embryos. Paraoxon significantly decreased spontaneous activity in the absence of effects on survival and total body area (SI Figures S6A, S6B, S7A, and S7B). Spontaneous activity (%) data in Panel B are presented as mean ± SD across three independent reference plates. Numbers above each bar denote percent spontaneous activity within that column. Asterisk denotes significant difference from both vehicle control columns 1 and 23 ($p < 0.05$).

Table 2. Image Success Rates for Analysis of Spontaneous Activity and Total Body Area within Reference Chemical Plates Consisting of 25-hpf Zebrafish Exposed to Vehicle or 25 μM Paraoxon[a]

		live embryos		spontaneous activity		total body area	
plate no.	treatment	total (no.)	total (%)	analyzed (no.)	analyzed (%)	analyzed (no.)	analyzed (%)
1	vehicle (0.1% DMSO)	93	96.9	93	100.0	92	98.9
	25 μM paraoxon	93	96.9	93	100.0	91	97.8
2	vehicle (0.1% DMSO)	95	99.0	95	100.0	95	100.0
	25 μM paraoxon	93	96.9	93	100.0	91	97.8
3	vehicle (0.1% DMSO)	95	99.0	95	100.0	92	96.8
	25 μM paraoxon	95	99.0	95	100.0	94	98.9

[a]Three independent plates contained an initial sample size of 96 embryos per treatment per plate. The percentage of live embryos was relative to an initial sample size of 96 embryos per treatment per plate, whereas the percentage of analyzed embryos was relative to the number of live embryos.

considered a successful plate (SI Figure S3). Additionally, spontaneous activity within columns 1 and 23 must not be significantly different ($p > 0.05$) from each other based on a Mann–Whitney test. If both criteria were met, then the entire plate was then analyzed for total body area and spontaneous activity (SI Figure S3).

HCS Assay Reproducibility. On the basis of previously published data, nicotine and paraoxon were selected as reference chemicals to evaluate assay reproducibility.[22,23] Nicotine exposure resulted in no effect on survival, total body area, or spontaneous activity (SI Figure S5). However, in the absence of an effect on survival and total body area at all concentrations tested (SI Figures S6A and S6B), paraoxon exposure resulted in a concentration-dependent decrease in the percent of embryos with spontaneous activity at 12.5, 25, and 50 μM (Figures 2A and SI S6C). On the basis of this concentration–response test, three reference plates containing 96 initial embryos per treatment were then exposed to vehicle or 25 μM paraoxon. Percent survival, total body area, and image success rates of paraoxon-treated embryos were similar to vehicle controls (SI Figures S7A and S7B and Table 2), and the hypoactive effect of paraoxon on spontaneous activity was reproducible within and across all three reference plates (Figures 2B and SI S7C). On the basis of results of control and reference chemical plates, we concluded that this assay is reproducible based on sample sizes of 16 initial embryos per column.

Chemical Screening. Following assay development and optimization, we tested a small chemical library to evaluate the potential utility of this HCS assay for screening and prioritizing chemicals for DNT testing. On the basis of data available from previous teratogenesis screens using zebrafish embryos,[24] acute toxicity benchmarks (AC_{50}) for chemicals within the U.S. Environmental Protection Agency's (EPA's) ToxCast phase-I chemical library were ranked from most to least potent based on survival and gross malformations present at 6 dpf following a 8-hpf to 5-dpf exposure. Using protocols described in Figure 1, the most potent 18 chemicals (SI Table S1, excluding nicotine and paraoxon) were selected to evaluate the ability of this assay to detect targeted effects on spontaneous tail contractions. Two chemicals (tefluthrin and milbemectin) were not commercially available (as of August 2013), and fentin was insoluble in DMSO at concentrations as low as 2.5 mM. Therefore, these three chemicals were not included in the final screen, resulting in a total of 15 chemicals tested in a concentration–response format.

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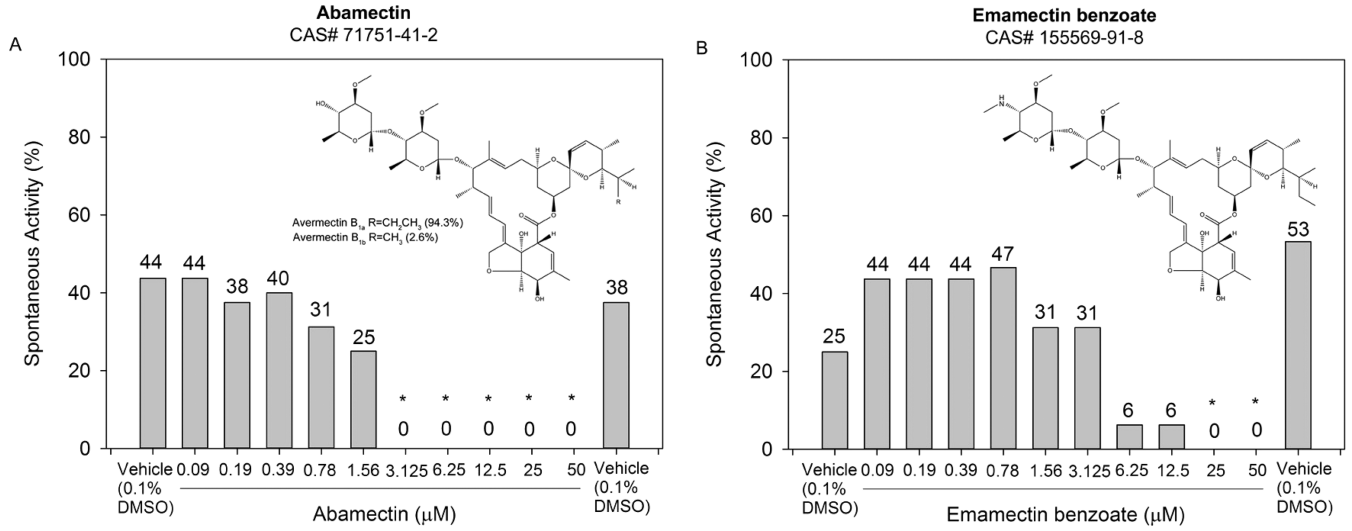

Figure 3. Spontaneous activity (%) following exposure to (A) abamectin or (B) emamectin benzoate from 5 to 25 hpf in a 384-well plate containing 192 initial embryos. Both chemicals significantly decreased spontaneous activity in the absence of effects on survival and total body area (SI Figures S15A, S15B, S24A, and S24B). Numbers above each bar denote percent spontaneous activity within that column. Asterisk denotes significant difference from both vehicle control columns 1 and 23 ($p < 0.05$).

A summary of statistical results and overall effects on spontaneous activity for all chemicals screened is provided within SI Table S5. Of the chemicals tested, rotenone was the most acutely toxic based on survival and total body area, as 0.09 μM was the highest concentration analyzed due to a significant decrease in total body area at 0.19 μM and 0% survival in all other concentrations (SI Figure S8). On the contrary, butafenacil, flumetralin, propargite, tribufos, and pyraflufen-ethyl showed no evidence of acute toxicity in this assay, with no effect on survival or total body area across all concentrations tested (0.09−50 μM) (SI Figures S11, S13, S16, S19, S22). Approximately one-third of the chemicals screened (thiram, fluthiacet-methyl, pyraclostrobin, (*Z*,*E*)-fenpyroximate, and trifloxystrobin) resulted in steep concentration−response curves based on survival, where only a 2-fold increase in concentration was required to decrease survival from >85% to 0% (SI Figures S9, S14, S17, S18, S23).

To determine the potential for DNT, spontaneous activity was assessed within treatment groups with >85% survival and no significant decrease in mean total body area relative to vehicle controls. On the basis of our chemical screen, exposure to abamectin—a widely used insecticide and chloride channel activator belonging to the avermectin class—resulted in a significant decrease in spontaneous activity at concentrations as low as 3.125 μM (Figures 3A and SI S15C). Out of all 15 chemicals screened, abamectin was the only chemical that affected spontaneous activity in the absence of effects on gross embryonic development (SI Figures S15A and S15B). On the basis of these data, the entire ToxCast phase-I chemical library was then searched for other commercially available avermectins to determine whether exposure to chemicals within the same class resulted in similar adverse effects on spontaneous activity. On the basis of this search, emamectin benzoate was screened and, similar to abamectin, significantly decreased the percent of embryos with spontaneous activity (at 25 and 50 μM) in the absence of effects on gross embryonic development, albeit with approximately 8-fold less potency than abamectin (Figures 3B and SI S24A-C).

DISCUSSION

Although assays quantifying zebrafish locomotion have been developed, the HCS assay reported here provides a simpler discovery platform for identifying potential developmental neurotoxicants. Using 384-well plates, we were able to (1) increase sample size to 16 individual embryos per treatment (192 embryos total), (2) examine a broad concentration−response (vehicle control and 10 chemical concentrations), (3) decrease assay duration and test material, and (4) identify chemicals that target the developing nervous system at nonteratogenic concentrations. After confirming assay variability and reproducibility using control plates and reference chemicals known to affect spontaneous activity, we then screened 16 chemicals within the US EPA's ToxCast phase-I chemical library and found that exposure to abamectin and emamectin benzoate—both potent avermectins—abolished spontaneous activity in the absence of effects on survival and total body area.

Contrary to other vertebrates, zebrafish neural tube formation and central nervous system development overlaps with the segmentation period (10−24 hpf).[20] By 18 hpf (18-somite stage), the anterior neural tube develops into ten distinct brain neuromeres while the posterior neural tube develops into the spinal cord.[20] Within each brain neuromere and spinal cord segment, primary interneurons differentiate and extend axons to form a simple scaffold along the anteroposterior and dorsoventral axis. Within the spinal cord, the axons of primary motoneurons innervate target axial muscles in a highly stereotyped, error-free manner,[13] resulting in initiation of weak, spontaneous muscle contractions within the developing embryo.[20] This form of locomotion is controlled by neural networks within the spinal cord, as brain lesions nor isolation of the spinal cord from descending brain inputs do not affect this behavior.[25] Although spontaneous activity has been suggested to occur in the absence of external cues such as touch,[26,27] there is evidence that spontaneous activity may be affected by high-intensity light stimuli.[15] Despite controlling for developmental stage, temperature, and light conditions, we observed a high degree of natural variability in the percent of

control embryos exhibiting spontaneous activity. We attempted to minimize this variation by loading two embryos per well and extending the imaging duration per embryo; however, these changes had no impact on minimizing variation of this behavioral phenotype (data not shown). Therefore, since parameters were tightly controlled within our assay, the environmental or biological factors responsible for variation observed in our assay are currently unclear.

Despite the natural variation in control spontaneous activity, our assay detected a significant decrease in spontaneous activity after exposure to paraoxon, and we demonstrated that this effect was reproducible across reference plates. However, contrary to previous studies in zebrafish,[23] nicotine did not affect spontaneous activity, a discrepancy that was likely due to differences in experimental design and exposure duration. In a study conducted by Thomas et al.,[23] chorionated zebrafish embryos were incubated from fertilization to 23 hpf in 50-mm Petri dishes and placed in groups of three embryos per dish. Embryos were then transferred to 30 μM nicotine for 5 min, and an increase in the number of spontaneous tail contractions was observed over this 5-min imaging period under transmitted light. In our assay, chorionated embryos were individually exposed to treatment solution from 5 to 25 hpf within single wells of a 384-well plate, and then imaged for 6 sec each under fluorescence. Therefore, the absence of a nicotine-induced effect on spontaneous activity within our assay may be a result of different rearing conditions, extended exposure duration, decreased image acquisition duration, and/or the use of fluorescence rather than transmitted light.

Out of 16 ToxCast phase-I chemicals screened, exposure to 14 of these chemicals resulted in no impact on spontaneous activity using this assay. Out of these 14 chemicals, five chemicals had no impact on survival, total body area, or spontaneous activity up to 50 μM (the highest concentration tested), suggesting that these chemicals exhibited limited embryonic uptake—due to high rates of chemical absorption to microplate wells or low rates of chemical transport across the chorion—and/or negligible toxicity during this stage of development. However, exposure to nine out of these 14 chemicals resulted in significant effects on survival or total body area at one or more concentrations yet did not affect spontaneous activity at nonteratogenic concentrations, suggesting that the primitive nervous system during this stage of development was not a target organ for these nine chemicals.

Using this HCS assay, we discovered that exposure to abamectin and emamectin benzoate—both potent avermectins—significantly decreased spontaneous activity in the absence of effects on survival and total body area. Avermectins are primarily used for control of insects and helminthes [28] and are thought to induce neurotoxicity by activation of either glutamate- or gamma aminobutyric acid (GABA)-gated chloride channels, interfering with signal transmission between nerve cells.[29,30] Although registered in the U.S. since the 1980s,[30] to our knowledge very few studies have examined the neurotoxic effects of avermectin exposure on mammalian cells and model organisms, particularly during early development. In mouse neuroblastoma N2a cells, exposure to abamectin and doramectin resulted in a significant concentration-dependent inhibition of neurite growth.[31] In zebrafish, semistatic exposure to abamectin resulted in a decrease in swimming ability at 96-hpf.[32] Most importantly, within a DNT study similar to OECD Test Guideline 426,[6] exposure of mated female Sprague–Dawley rats to emamectin benzoate from gestation day 6 through lactation day 20 resulted in a dose-dependent decrease in motor activity in male and female offspring on postnatal day (PND) 17 but not PND 21; this decrease was also observed on PND 59 in females only.[33] Therefore, on the basis of our results and limited data available within the published literature, exposure to avermectins appears to result in disruption of nervous system function during early development.

In summary, this HCS assay provides a streamlined platform with sufficient replication and exposure concentrations to screen and identify potential developmental neurotoxicants. Given that we relied on an embryonic stage (25 hpf) with a less developed nervous system compared to later larval stages (6–7 dpf), we recognize that our assay has the potential to be biased toward identifying chemicals with specific neurotoxic modes-of-action. However, compared to existing zebrafish-based locomotion assays conducted within 96-well plates,[34] this assay provides shorter exposure duration (1 day vs 6 days) and decreased treatment solution volumes needed for screening (50 μL vs 250 μL per well for static exposures). As a result, within a tiered testing framework, this assay has the potential for rapid, cost-effective prioritization of chemicals for DNT assays that rely on later stages of zebrafish and rodent development. Moreover, in the future, this HCS assay can be readily coupled with reverse genetics approaches to identify the potential role of aberrant neurotransmitter signaling in chemically induced effects on early neurodevelopment within zebrafish embryos. Over the long-term, we envision that this HCS assay will help uncover mechanisms of DNT for pesticides and understudied high-production volume chemicals in commerce.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental File 1: Chemical names, chemical formulas, CAS registry numbers, vendors, and purities (Table S1); imaging and analysis parameters (Tables S2–S3); control image success rates (Table S4); a summary of chemical screening results (Table S5); figures for assay optimization (Figures S1–S7); and figures for chemical screening (Figures S8–S24). Microsoft Excel spreadsheets containing raw data for all assays are provided within Supplemental File 2. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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