**Attention NTP Interagency Center for the Evaluation of Alternative Toxicological Methods Federal Register Notice: (81FR42718-42719)**

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**Comments:** The Tanguay laboratory has created an automated high-throughput zebrafish laboratory to investigate and compare chemical activities. Through automation, rigorous standard operating procedures, implemented innovative data analysis tools, quality control assurances, and other unique infrastructure attributes that allows for rapid reproducible data generation. Zebrafish are an ideal model for developmental toxicity studies due to their small size, short generation times, voluntary spawning, rapid embryonic development independent of the mother and transparent embryos that allow for visualization of every stage of early development. We routinely utilize specific pathogen free (SPF) Tropical 5D (5D) wild-type strain. Our zebrafish facility is the only one in the world that is SPF for *pseudoloma neurophilla* (Microsporidia), which is critical when it comes to obtaining unbiased results as microsporidia is known to impact survival, growth and reproduction (Ramsay et al. 2009). With the rise of the zebrafish as a model of human neurobehavioral, it is especially necessary to have utilize SPF-microsporidia fish as it has been demonstrated to cause aberrant behavioral phenotypes in adults (Spagnoli et al. 2015). The strain, Tropical 5D, was selected for its inherent genetic diversity. This helps to guard against strain specific susceptibility differences. On the practical side, this strain is very hardy and is highly prolific. In collaboration with the David Reif's lab, the Tanguay lab has fully sequenced the 5D strain and will be submitting this genome sequence for public use in the coming 2017.

In the Tanguay zebrafish toxicity testing workflow, the early life stage embryos are routinely staged and the chorions are enzymatically removed using pronase (63.6 mg/ml, ≥ 3.5 U/mg) at 4 hours post fertilization (hpf) with a custom automated dechorionator (Mandrell et al. 2012). Certainly keeping the chorion intact in experimentally easier, but numerous studies have revealed that the chorion can be a barrier for some chemicals and nanomaterials. This barrier could confound data interpretation. For example, recent studies have been conducted using carbon nanotubes (CNTs) in the Tanguay lab and revealed that in the absence of the chorion, CNTs are acutely toxic, but with chorions intact, the larvae develop normally; suggesting that the chorion is an effective barrier. This observation is not rare. To enable automated and reproducible dechorionation of the embryos, an automated dechorionator was developed (Mandrell et al. 2012).

Broad concentrations ranges need to be tested. Chemicals are tested in a concentration-response manner with 6-8 concentrations (including a control) in half-log with a highest concentration tested at 64 µM and a maximum DMSO concentration of 1% in all wells. All chemicals are typically tested in a 96-well format and run in in duplicate or triplicate to obtain the proposed 32 or 36 animal replication/treatment This large sample size allows for the statistical power needed to determine if the hit call is robust and with the proposed dose spacing, it allows for a more precise extrapolation of point of departure. A large N also allows helps to capitalize on the genetic diversity of the 5D strain.

Zebrafish embryos without the chorion are loaded 1 per well at 6 hpf into 100 µl of embryo medium in 96-well plates by an automated embryo placement system (AEPS) (Mandrell et al. 2012) A Hewlett-Packard D300 Digital Dispenser will be used to dispense the compounds immediately after embryo placement. This instrument dispenses picoliter-sized droplets of compounds carried in 100% DMSO vehicle, using inkjet technology. Full validation of this instrument for chemical dispensation for toxicology screening was completed in the Tanguay laboratory in October 2014. Volume delivery using the D300 is extraordinarily precise from a 10 mM stock and is direct to the experimental chamber. No prior dilution of samples into larger, human-pipettable volumes is required. We note that Hewlett-Packard does not recommend the use of stocks >10 mM because of solubility and viscosity concerns. The Tanguay laboratory has demonstrated the use of the D300 substantially improves control of chemical delivery that is not achievable with manual pipetteing and significantly reduces chemical losses due to sorption to plastic through multiple transfer steps (Truong et al. 2016a).

All plates are tightly sealed and blocked from light exposure to minimize evaporation and to reduce chemical degradation. Frequently chemical identity is not known (i.e. blinded studies), so the volatility or lability of the chemical is unknown. To improve reproducibility and rigor, the exposure plates are sealed and covered throughout the 5-day assay. The Tanguay lab routinely uses a static exposure to minimize handling of the developing embryo. A drawback of this approach is that over time, as a consequence of metabolism, the parent test compounds may be reduced or eliminated over the course of the exposure. For static renewal, there are two methodologies that can be used: 1) remove the embryos and transfer them into a new exposure chamber, which is time intensive, 2) remove and replace exposure solutions with the larvae in place, and dose the well again. In either methods, the bioavailability is different and is difficult to model. It is noteworthy that repeated dosing could lead to extremely high body burdens for chemicals that are not metabolized as the mass of test chemical added is additive in a renewal protocol. This could lead to a high false positive rate. We have conducted studies comparing static renewal with static exposure, and have found there are often shifts in the point of departure and is focusing efforts on better understanding dosimetry in this system.

Each plate undergoes embryonic and larval behavioral assessments and comprehensive phenotypic screening, as detailed in numerous manuscripts (Knecht et al. 2016; Reif et al. 2016; Truong et al. 2016a; Truong et al. 2016b; Truong et al. 2011; Truong et al. 2014; Truong et al. 2016c). Briefly, plates are evaluated at 24 and 120 hpf for 22 morphological endpoints, and 2 photomotor response assays. The Tanguay lab is in the process of reducing the morphological endpoints that are highly correlated (Zhang et al. 2016a) and developing so called ‘super endpoints’ to represent capture bioactivity scores. Additional efforts are being made to compute point of departures for the morphological and larval behavioral data (Zhang et al. 2016b)

Thus, our methodology is affordable, fast, and reproducible. Taking advantage of the technology we have developed to automate this process, we can screen large libraries such as ToxCast phase 1 and 2, in a few weeks and with the continued efforts to reduce variability, we are in position to assess larger libraries to fuel predictive model efforts to help move the field towards non-animal test methods.

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