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MECHANISMS OF TOXICITY (CJ MATTINGLY, SECTION EDITOR)

Zebrafish: A Marvel of High-Throughput Biology for 21st Century Toxicology

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Abstract The evolutionary conservation of genomic, biochemical, and developmental features between zebrafish and humans is gradually coming into focus, with the end result that the zebrafish embryo model has emerged as a powerful tool for uncovering the effects of environmental exposures on a multitude of biological processes with direct relevance to human health. In this review, we highlight advances in automation, high-throughput screening, and analysis that leverage the power of the zebrafish embryo model for unparalleled advances in our understanding of how chemicals in our environment affect our health and wellbeing.

Keywords Zebrafish embryo model · High-throughput screening · Environmental exposure · Human health

Introduction

The last 20 years witnessed the rate of studies using the zebrafish to elucidate mechanisms of vertebrate gene function increase exponentially, from approximately five publications in 1990 to over 2,500 in 2013. During this interval, a more modest increase in the publication of zebrafish-based toxicology studies was underway. However, with the new millennium came a new trend and we are in the midst of a shift away from using zebrafish exclusively for developmental studies and towards leveraging their many advantages to understand processes by which environmental exposures influence development and disease (Fig. 1). The reasons for this shift are complex and somewhat predicated on the need to move from in vitro to in vivo studies while adhering to principles that reduce, replace, or refine the use of animals in research [1]. As a result of this revolution, leveraging zebrafish to investigate the most pressing biological problems of human development, disease, and the role environmental exposures play in adverse biological outcomes is made possible, often at modest costs. In the past couple of years, reviews describing the potential use of zebrafish in toxicology research have been published [2–7]; therefore, this review, while not seeking to be comprehensive, aims to summarize the last few years of toxicology research in which zebrafish made significant contributions. We focus on results from a number of screening experiments, which are gradually informing our understanding of the complex interplay between environmental perturbation and human health and wellbeing.

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Zebrafish and Human Embryos: Living in a Fishbowl

Zebrafish experience a dramatically different environment than humans except during the embryonic period, during which human embryos develop in an aquatic environment—the amniotic fluid. The origins of amniotic fluid can be subdivided into pre-placentation and post-placentation [8]. Pre-placentation amniotic fluid derives from maternal plasma and enters the extracoelomic cavity by passive and active mechanisms, whereas post-placentation amniotic fluid originates in the embryo, from which it is exuded through pre-keratinized skin, urinated or (to a lesser extent) defecated. However, all amniotic fluid has its origins in maternal plasma, which transports nutrients, electrolytes, and water to the embryo, as well as any toxicants and xenobiotics that may be present in maternal circulation and are able to diffuse across the placenta. Not surprisingly, 'waterborne' exposures to

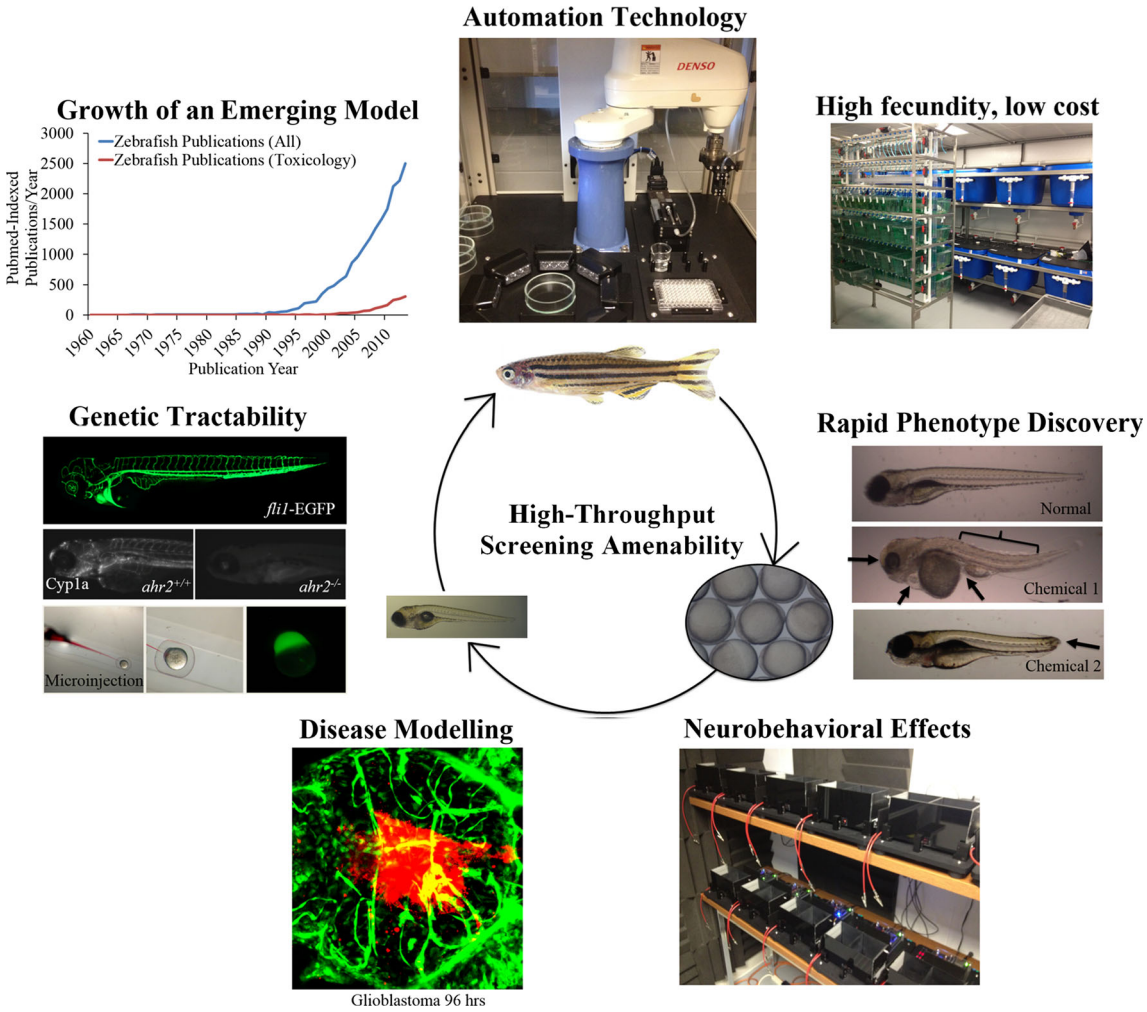

Fig. 1 Zebrafish are an emerging vertebrate model for HT toxicity screening, disease modeling, phenotype discovery, and chemical mechanisms of action. Their high fecundity, low cost, and rapid development make nearly all in vivo biological assays amenable to HT studies. As assays and endpoints become more standardized with the use of modern technologies, the zebrafish has rapidly become one of the premier vertebrate models for biological discovery. *HT* high-throughput

environmental factors occur; the presence of xenobiotics [9, 10], industrial pollutants [11, 12], medications [13], chemicals in household items [14, 15], and chemicals derived from lifestyle habits [16, 17] have been isolated from human amniotic fluid, which is swallowed, breathed in, and recycled by the developing fetus beginning at about week 10 and continuing throughout gestation [8]. Consequently, the absorption routes during embryogenesis in humans are probably similar to those in zebrafish, including dermal, gastrointestinal, and respiratory, although this has not been rigorously tested.

Performing waterborne exposures in the zebrafish embryo model is advantageous for several reasons:

1. Large numbers of zebrafish embryos can be exposed simultaneously in relatively small volumes (e.g. >10 embryos per ml), generating a robust sample for downstream applications, including transcriptomics, proteomics, and metabolomics.
2. The zebrafish chorion, an acellular envelope surrounding the embryo and riddled with pores between 0.5 and 0.7 μm in diameter [18], is highly permeable to a wide range of small molecules and xenobiotics. Instances in which the chorion is an effective barrier can be overcome by automated enzymatic dechorionating processes.
3. Short-duration exposures from 1 h to a few days will intersect with multiple developmental processes due to the accelerated growth rate of zebrafish embryos relative to humans (from fertilized egg to free-swimming hatchling in 3–5 days), possibly mimicking chronic exposures of weeks to months in humans.
4. Exposures timed to coincide with very specific developmental endpoints/events are possible.

For these reasons, and others discussed in the following sections, the zebrafish is imbued with the ability to enhance our understanding of potential risks of exposure to diverse

environmental challenges under a variety of experimental conditions.

Zebrafish: A Molecular 'Swiss Army' Knife

There are many advantages to using zebrafish for reductionist and systems biology, and for low-throughput (LT) and high-throughput (HT) toxicology research. Genetically, zebrafish are very similar to humans—approximately 70 % of human protein-coding genes have orthologs in the zebrafish, and over 80 % of human disease-associated genes have a zebrafish counterpart [19]. Furthermore, an ancestral genome duplication in teleosts led to the formation of ohnologs (paralogs that arise from whole genome duplication [20]) that in many cases underwent subfunctionalization [21], i.e. the ancestral function 'split' among ohnologs; therefore, the resolution of effects of environmental exposures on the mechanics of gene and/or protein function is often higher in zebrafish. An excellent example of this is found in the *SOX9* locus, encoded by one gene in humans but two—*sox9a* and *sox9b*—in the zebrafish [22]. In addition to its role in vertebrate male sex determination [23], SOX9 is required for chondrogenesis [24]. Proliferation, differentiation, and condensation of chondrocytes are regulated by SOX9 in humans, whereas in zebrafish, proliferation and differentiation are regulated by *sox9b* and condensation by *sox9a* [22]. It was subsequently discovered that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) impairs vertebrate chondrogenesis by interfering with the expression of *sox9b* but not *sox9a* [25]; thus, proliferation, but not condensation, is affected, which is consistent with the TCDD-induced craniofacial phenotype. Other examples of differential response by ohnologs to environmental factors have been reported [26].

Zebrafish have the capacity to spawn hundreds of developmentally synchronized embryos in a single spawning event. Embryos are small (diameter ≤1 mm), optically transparent, develop in an open environment, and are easily manipulated—properties that can be exploited in robotics-driven arraying schemes without resorting to complicated culturing protocols and sterile environments. Chemical screen assays can be implemented using in vitro-equivalent protocols, and screening large numbers of embryos simultaneously ensures sufficient replicates and robust statistical power. Existing and future integrated technologies will permit measuring multiple parameters during the course of an experiment, including growth rate, morphogenesis, behavior, morbidity and mortality, and gene and protein expression profiles. Multiple transgenic lines expressing fluorescent markers under cell- and tissue-specific promoters are being leveraged to study specific molecular pathways, often in a single-cell lineage or tissue, and to generate rapid mechanistic insights into the role of chemical exposures on biological function [27].

The transparency of zebrafish embryos opens opportunities previously unattainable in vertebrates. For example, light-sheet microscopy coupled with genetically engineered fluorescent zebrafish permit high-resolution imaging of cell movements during embryonic development while environmental conditions are altered in a controlled manner [28]. Therefore, the molecular and cellular basis of environmentally-induced developmental defects rooted in cell migration, for example, can be assayed in the context of a whole embryo in real time.

Forward [29, 30] and reverse [31, 32, 33••] genetic screens are now routine laboratory techniques in the zebrafish, and multiple consortia have ongoing projects to identify novel nonsense mutations across the entire zebrafish exome by TILLING (Targeting Induced Local Lesions in Genomes) [34], which are made available to investigators as heterozygous carriers for a nominal fee. The greatest advantage of using the zebrafish for these applications is cost; at a fraction of the cost of making a similar mutation in a mouse, the zebrafish is the most economical model organism in which to examine vertebrate gene function.

Zebrafish: Spawning a Revolution in In Vivo High-Throughput (HT) Screening

Over 80,000 chemicals are manufactured and used worldwide [35] in countless consumer products, including natural and processed foods; infant products, toys and clothing; furniture and housewares; building materials, and the workplace. In the US, enactment of the Toxic Substances Control Act of 1976 began an effort to regulate chemicals but exempted thousands of existing chemicals, and, consequently, it has generally been regarded as weak [36, 37]. Unlike the EU's REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) regulation [38], which mandates that manufacturer's provide adequate scientific evidence of a chemical's safety in human affairs if its manufacture or importation exceeds 1 metric ton, no formal regulatory mechanism exists in the US to ensure that chemicals conform to rigorous biological safety standards. Thus, scientific and lay communities are largely ignorant of the effect chemical exposures have on our health and well-being, with a few notable exceptions, such as polychlorinated biphenyls [39] and thalidomide [40]. Considering that most often we come into contact simultaneously with groups of chemicals, the number of possible combinations is astronomical (e.g. over 3 billion pairwise combinations of chemicals in an arsenal of 80,000), illustrating the daunting road ahead if we are to embark on understanding how environmental exposures of anthropogenic origin affect human development and health and the health of our planet.

Understanding how individual chemicals and their metabolites alter complex biological processes requires large-scale,

HT analytical studies, which are often performed in vitro using well-characterized cell lines of different origins—derived from different organs, ethnicities, sexes, and populations. In addition, HT chemical screens require simplicity, speed, assay validity, robustness, and statistical power in order to succeed, and these have been the driving forces behind in vitro cell-based systems. However, it is often very difficult to establish concordance between a chemical's effect in an in vitro cell-based two-dimensional assay and its role in human disease because of the complexity inherent in an animal: dimensionality; multiple organ systems; broad spectrum of cell types and physiology, including sessile versus motile cells, transport mechanisms, metabolic routes, and complexity of extracellular matrices; and differences in gene expression, including epigenetic variation and allelic composition. Advances in three-dimensional cell-based systems [41, 42] and three-dimensional prototype organs [43] have the potential to overcome some of these barriers to translation from in vitro results to in vivo predictions but we would argue that most are not amenable to modeling with current technology, neither in real nor virtual realms, because they lack the complexity, interorgan interactions, and structural organization characteristic of whole animals.

Under current paradigms, in vitro HT assays are the first line of inquiry to identify high-priority chemicals from the >80,000 in production because of the speed with which the assays can be performed. These assays are then followed by in vivo studies on a significantly reduced chemical space using model organisms, principally rodents, to identify the most acute and chronically pernicious chemicals that merit further investigation and possibly regulatory oversight. Unfortunately, this latter approach is very inefficient since the vast majority of chemicals cannot be tested in a timely manner and experiments with rodents are expensive. However, for reasons we enumerated earlier, a transition to the zebrafish as the first line of HT chemical screening could resolve many of these issues. This approach has gained traction at the US Environmental Protection Agency through its ToxCast program, in which the zebrafish embryo model played a significant role during the phase I analysis [44]; a subsequent study in the zebrafish with all 1,060 unique ToxCast phase I and II compounds has also been reported by one of the authors (RLT [45•]), in which 487 (46 %) compounds were seen to elicit significant adverse outcomes on development at environmentally relevant concentrations.

In the following sections, we describe some of the recent results of HT and scalable LT chemical screens using the zebrafish embryo as an alternative to in vitro cell-based HT assays with the aim of demonstrating that the zebrafish embryo rivals the best in vitro systems and contextualizes, in a complex organism, the effects of chemical and environmental exposures on development and disease.

Developmental Toxicity

Taking advantage of the zebrafish's external and transparent development, the zebrafish embryo model has been exploited in a wide array of developmental toxicology studies, including skeletal development [46, 47], immune development [48], neurodevelopment [49, 50], cardiovascular development [51–53], and regeneration [54–57]. A recent spate of screens have used the zebrafish embryo model to analyze potential developmental toxicity of small molecule libraries previously uncharacterized in in vivo models [44]: unknown contaminants in landfill soil [58]; nanomaterials (reviewed by Fako and Ferguson [59]); and ototoxic drugs [60]. Recent studies illustrate that the zebrafish embryo model can be used to screen and prioritize compounds suspected as human developmental toxicants [61]. In the recent past, limitations of chemical screening with zebrafish were largely due to laborious experimental setup and animal/chemical handling; data acquisition; and processing. However, in recent years, major strides have been made in automation technology and high-content image analysis. Therefore, developing pipelines and standardized methods for developmental toxicity screening is now possible for large chemical libraries [62]. While many specialized endpoints for toxicity exist (reviewed in other sections), an array of standardized phenotypes for general developmental toxicity assessments in zebrafish, including teratogenic effects, has been developed. These standardized phenotype screens enable measures of teratogenicity and malformation observed in a variety of embryonic structures, including notochord, yolk-sac, heart, body axis, eyes, snout, jaw, otic vesicle, brain, somites, trunk, pectoral and caudal fins, pigmentation, circulatory system, and swim bladder. Other measurable endpoints include mortality, spontaneous motion, developmental stage progression, and touch response. Observable phenotypes occurring as a result of chemically perturbed development are highly diverse and may be pleiotropic or polygenic depending on a chemical's mode of toxicity. Characterizing a chemical's developmental toxicity phenotype(s) is a necessary first step in the design of mechanistic studies, which can utilize reverse and forward genetics to determine a specific receptor or molecule's role in the phenotype. For example, developmental toxicity of TCDD has been studied extensively; TCDD toxicity generally includes pericardial and yolk-sac edemas [63, 64]. Mechanistic studies anchored to this phenotype in zebrafish have allowed for the dissection of the AHR pathway and its function in developmental toxicity [65, 66]. Due to advances in automation, high-content imaging, and forward/reverse genetics, phenotype-based chemical and mechanistic screens have placed zebrafish at the forefront of in vivo chemical screening. The utility of zebrafish in HT developmental toxicity screening will undoubtedly be invaluable for predictive toxicology for many years to come.

Cardiotoxicity

Studies of cardiotoxic effects from chemical exposures on the developing vertebrate heart are challenging because most vertebrates die in the absence of normal heart development. Zebrafish, on the other hand, survive up to 5 days post-fertilization (dpf) in the complete absence of a functional cardiovascular system, even though a functional heart normally develops within the first 24 h of life; therefore, exposure-driven cardiotoxic effects can be studied across the spectrum of heart development, including initial stages of cardiac mesoderm specification, patterning of the heart, and establishment of cardiac electrical conduction (see Staudt and Stainier for a review of zebrafish heart development [67]). Although the zebrafish heart is a two-chambered organ, many developmental and molecular landmarks are evolutionarily conserved in zebrafish and humans, including sarcomere formation [68], atrioventricular septum development [69], and apex-to-base ventricular activation patterns [70]. In addition, numerous mutations associated with human congenital heart defects and cardiomyopathies have been uncovered as a direct result of cardiovascular research performed in zebrafish (for review, see Bakkers [71]).

The zebrafish embryo model has anchored numerous cardiotoxicity studies. For example, studies of complex mixtures such as those found in oil spills (Alaska North Slope crude oil from the Exxon Valdez, and Mississippi Canyon 252 oil from the Deep Water Horizon) [72] identified polycyclic aromatic cardiotoxicants in zebrafish that induced cardiac edema, defective heart looping, hemorrhage, and reduction in arteriovenous circulation, which were consistent with observations by others that activation of the aryl hydrocarbon pathway by dioxins and dioxin-like substances leads to gross morphological and functional abnormalities in the zebrafish heart [73–76]. In other studies, retinoic acid and TCDD, both well-characterized cardiotoxicants, activated a common transcriptional response via distinct, non-overlapping sets of genes associated with heart failure [77].

The zebrafish embryo model has been successfully deployed to screen for cardiotoxic effects elicited by carbaryl and valproic acid [78], atypical antipsychotic drugs [79], kinase inhibitors [80], human cardiotoxic drugs [81], and chemotherapeutics [82], thus illustrating the value of zebrafish as a preclinical testing paradigm to predict cardiotoxic effects elicited by drugs under development. Recently, in vivo analysis of proliferating cardiomyocytes (the functional unit of cardiac muscle) in zebrafish embryos was leveraged to identify chemicals that could promote cardiomyocyte proliferation, thus opening a window into the possible therapeutic replacement of infarct-damaged cardiac tissue in humans [83].

Transgenic lines in which specific cardiac cell types are fluorescently labeled, including *cmlc2:EGFP* (differentiated cardiomyocytes [84]), *gata4:EGFP* (regenerative cardiomyocytes [85]), *pard3:EGFP* (endocardium [86]), and *tcf21:DsRed* (epicardium [87]), are available. These and other fluorescent lines are valuable resources with which to study the effects of chemical exposure on heart development with unprecedented granularity, including understanding the effects of cardiotoxicants in a cell-specific context. Coupled with HT automated visualization and sorting analysis, there is no better model for cardiotoxic research than the zebrafish embryo.

Hepatotoxicity

Liver toxicity is often a top concern during drug and pharmaceutical development and for chemical risk assessment. To evaluate hepatotoxicity, rodent liver assays are typically used but generally tend to be LT because studies are relatively expensive and slow, and as a result they suffer from low sampling sizes. In contrast, developing zebrafish can be utilized for hepatotoxicity screening, which is amenable to HT applications because of their transparency, speed of development, large sample sizes, and low cost. Hepatotoxicity studies in the zebrafish can therefore be conducted earlier in drug development, which may aid the decision-making process during research and development. There are a number of early indicators of liver toxicity in zebrafish [88].

Embryonic zebrafish develop a rudimentary liver by 24 h post-fertilization (hpf), which undergoes rapid growth and differentiation to become fully functional by 72 hpf (reviewed by Chu and Sadler [89]). Specialized endpoints can be screened in an HT manner by taking advantage of automated technologies, high-content imaging, transgenic lines, or assays for liver-specific enzymes. Similar to mammals, zebrafish respond to xenobiotics and oxidative stress with induction of phase I and II drug metabolism genes. Furthermore, there is a high degree of similarity between zebrafish and human cytochrome P450 (CYP) gene sequences and protein function [90] and the majority are expressed throughout development, which allows developing zebrafish to be used for toxicological and pharmacological studies. Expression of genes involved in drug metabolism can be visualized in the transparent embryonic and larval zebrafish, using transgenic strains or whole-mount in situ hybridization (WISH). For example, transgenic zebrafish expressing green fluorescent protein (GFP) regulated by the CYP1A promoter can be used as a rapid, non-destructive in vivo screen for induction of phase I metabolism [91, 92]. Other rapid in vivo non-destructive techniques that are amenable to HT screening include the ethoxyresorufin-O-deethylase (EROD) assay, which can be visualized and quantified by fluorescence microscopy [93]. Using this technique, many chemicals can be screened for CYP-specific activity. Additional assays, including high-resolution microscopy, liver morphometrics, apoptosis and necrosis, and other histopathological endpoints,

are readily applied using non-destructive methods. Advances in automation and software analysis have made screening these endpoints achievable in HT applications.

Endocrine Disruption and Reproductive Toxicity

No large-scale studies of endocrine disruption and reproductive toxicity have yet to utilize zebrafish, although endocrine disruption assays and reproductive toxicity screens in the zebrafish are amenable to HT applications. All major hormone receptors in humans have functionally conserved zebrafish orthologs that are active in developing zebrafish (e.g. thyroid [94], androgen [95], estrogen [96], and gonadotropin [97] receptors). Although understanding of sexual differentiation in zebrafish remains nebulous [98], gametogenesis is similar between humans and zebrafish [99, 100]. We anticipate that in the near future new and current standardized methods will come online for HT endocrine and reproductive toxicity screening in zebrafish; we predict that once these assays are available, zebrafish will be invaluable for identifying reproductive toxicants and novel endocrine disruptors. Using the zebrafish model as an in vivo whole animal endocrine screening tool offers advantages over in vitro cell-based screens and in vivo rodent studies, which suffer from low complexity and LT/high cost, respectively.

Endocrine-disrupting chemicals can be screened in vivo in a cost-efficient manner using rapid assays; when coupled to gene analysis studies, pathway-specific effects of exposure are readily measurable. There are many endocrine biomarkers useful for screening pathway-specific effects in developing zebrafish. For example, *VTG* (vitellogenin) and *CYP19a1b* (brain aromatase) genes are sensitive estrogen receptor-regulated biomarkers that are inducible in embryonic-larval zebrafish by xenoestrogens [101]. These biomarkers, and others, can be evaluated qualitatively by in situ hybridization, or quantitatively by real-time polymerase chain reaction (PCR). For example, in a rapid 4-day zebrafish embryo-larval bioassay, *VTG* messenger RNA (Mrna) expression was highly induced by estrogen, and completely inhibited (>95 % inhibition) by co-exposure to several dioxins, including TCDD [102], thereby demonstrating that the embryo-larval bioassay can be used to screen for potential endocrine system agonists and/or antagonists. The use of gene-specific mRNAs as biomarkers is largely a matter of arraying embryos, performing exposures, lysing embryos, extracting RNA, and coupling complementary DNA (cDNA) synthesis with PCR amplification; these steps are readily amenable to automation, which could speed the discovery of novel biomarkers when coupled with libraries of gene-specific primers. Other methodologies exploit the ability to make transgenic zebrafish that express GFP controlled by any promoter one chooses. One example—a transgenic in which the *CYP19a1b* promoter drives expression of GFP—was recently developed for rapid screening of estrogen mimics by measuring changes in fluorescence following exposure [103]. Currently, HT cell-based in vitro assays are commonly used to explore pathway-specific endocrine activities for large chemical libraries by deploying assays such as the CALUX assay [104]. We predict that, in the near future, the zebrafish embryo model will become the benchmark test for uncovering endocrine-disrupting compounds in in vivo screens at costs that rival traditional cell-based assays.

Although there are minor differences, zebrafish are an excellent substitute for mammalian reproductive toxicity studies because their reproductive processes are comparable to those observed in mammals. Developmentally, zebrafish and rodents require approximately the same time from conception to reproductive maturity (~3 months). Zebrafish primordial germ cells are migratory and detectable prior to 24 hpf. Their gonads, like all vertebrate gonads, are initially bipotential and develop into ovaries or testes between 21 and 45 dpf. This critical window of sexual differentiation is sensitive to reproductive toxicants that may disrupt gonadogenesis and result in reproductive deficits later in life. For example, exposure to TCDD during sexual differentiation in zebrafish results in reproductive toxicity later in life in males and females, which is also transgenerationally heritable [105, 106, 107•]. A large number of endpoints to evaluate effects on reproduction, whether exposures take place during sexual differentiation or in the adult, can be used, including fecundity, gonad histopathology (follicular development and progression, atresia, apoptosis, presence of ovotestis), organ morphometrics, reproductive biomarker expression (e.g. vitellogenin), embryo viability, and developmental toxicity in offspring of exposed adults.

The goal of screening chemicals for endocrine activity is to uncover those that may pose a risk to development and reproduction; moving towards an HT whole animal model is the best way to comprehensively evaluate these endpoints. HT screening in zebrafish far exceeds what can be done in rodent models at a fraction of the cost.

Neurotoxicity

The zebrafish is an excellent model for neurobiology studies, especially during the initial phases of nervous system development. Many examples of transgenic zebrafish expressing fluorescent reporters under the control of neural-specific promoters have allowed researchers unparalleled access to real-time investigations of neural architecture and function, thus paving the way towards comprehensive assessments of the effects of environmental exposures on diverse neurological endpoints, including molecular, cellular, anatomical, and functional phenotypes. Over the last 2 years, neural-specific

transgenic zebrafish have been at the forefront of chemical screens for compounds targeting the circadian clock [108], dopaminergic pathway [109], neurogenesis [110], spontaneous activity [111], pleotropic neurotoxicity [112], and environmental influences on axon growth and connectivity [113].

Advances in adapting zebrafish for use in Parkinson's research [114], amyotrophic lateral sclerosis and frontotemporal lobar degeneration [115–119], mitophagy and neurodegeneration (reviewed by Wager and Russell [120]), and understanding how drugs affect locomotor function [121] open promising avenues of research exploring the link between occupational–environmental exposures and neurological disorders, in which some degree of risk may be attributed to early life exposures. To this end, behavioral screens are underway in several laboratories (including the authors'), in which chemical exposures coupled with transgenics and neurophysiological measurements aim to elucidate the molecular and cellular basis of chemically-induced behavioral abnormalities at sublethal doses. These studies are also designed to address the long-term consequences of early-life exposures on nervous system function.

Nanotoxicity

Nanotechnology is a rapidly evolving field, and nanomaterials are increasingly used commercially for a number of products and applications. A nanomaterial is an object in which one of the three dimensions is between 1 and 100 nanometers (nm). Nanomaterials often exhibit complex and unusual physical, chemical, and biological properties compared with larger-scale materials, including differences in conduction, chemical reactivity, strength, and affinity to biological structures. Development of nanoparticle libraries (objects that scale between 1 and 100 nm on all three axes) have been reported [122] but have not been fully characterized for toxicity or evaluated for risk assessment in vivo. While no standard methods for evaluating nanotoxicity have been established, the zebrafish larval bioassay has been used to rapidly evaluate how physical properties such as size, shape, charge, and surface chemistry affect toxicity [123]. However, nanoparticle-specific exposure studies in zebrafish, including cadmium [124, 125], gold [126, 127], silver [127–132], silica [133], and titanium dioxide [134, 135], have been conducted to characterize physicochemical properties and correlate these with toxicity. The Nanomaterial-Biological Interactions Knowledgebase (NBI; http://nbi.oregonstate.edu/) is a repository of annotated and integrated data of nanomaterial characterization for which zebrafish have made substantial contributions. The sheer diversity of nanomaterials, combined with the pace at which they are being manufactured and utilized worldwide, requires using a model such as the zebrafish embryo for uncovering nanomaterial–biological interactions and potential mechanisms of toxicity.

Role of Automation in HT Screening

Advances in genetics and developmental biology have made the zebrafish the ideal small animal model to use for HT phenotype screening. However, one major bottleneck for chemical screening with zebrafish is embryo sorting and handling, and phenotype scoring. To overcome these major issues, advancement in two technologies will support the application of zebrafish in HT screens. The first is automation of embryo handling and sorting. The second is automation of phenotype-based endpoints using high-powered image analysis.

There are several handling steps required for chemical screening in zebrafish embryos that immediately need to be addressed for large-scale studies. Production of large clutches of embryos is relatively simple using mass embryo production systems, which can generate tens of thousands of embryos per day. However, manually sorting, dechorionating, and arraying clutches of this size for HT chemical screening is burdensome and time-consuming. Automation of these steps can free-up personnel time for other pressing tasks, which leads to increased throughput and productivity and improved consistency of animal handling [136]. Rapid dechorionation of large embryo clutches using enzymatic digestion and automated shaking/rinsing is possible. Using advanced image-based robotics, dechorionated embryos can be arrayed into single wells of a 96-well plate, allowing thousands of animals to be plated per day. These advancements in robotic automation have increased the throughput of chemical screening several-fold. Using these techniques, a single laboratory can conduct concentration-response studies for developmental toxicity with dozens of chemicals per day. This level of in vivo throughput is unrivaled by any other vertebrate model. The robotics for dechorionating and arraying are not currently commercially available, although several laboratories have implemented these technologies using commercially available robotic arms and machine-vision cameras to build custom instruments in-house. The automation of dechorionating and arraying lends itself to other downstream robotic applications, such as liquid handlers for automation of chemical exposures. Another emergent area of automation expected in the near future is large-scale microinjection of single-cell embryos. Currently, manual sorting and microinjection makes this technique impractical for HT applications. However, using automated microinjection, researchers can control dosing of chemicals, mRNA, or morpholinos to test specific hypotheses in an HT manner. The technology using modern robots to automate handling, arraying, and microinjecting is available but has not yet deployed in an HT context.

With advancements in automation of animal handling, a concomitant advance in phenotype screening is also necessary to ensure HT capability. Automated image-based screens are emerging for phenotype discovery. To accomplish this, high-resolution imaging to acquire photomicrographs of entire 96-well plates is being developed. Software-based algorithms can scan high-content images and detect phenotypes (e.g. pericardial edema, yolk-sac edema, axial defects, etc.) [137, 138, 139•]. However, to date, most large-scale chemical screens have relied on manual phenotype observation and analysis. While manual screening does have advantages, such as discovery of novel phenotypes, it is impractical for HT screening. Powerful customizable and trainable software algorithms will be required to automatically assess morphology parameters, including processing and analyzing large datasets. Overall, automation of handling and phenotype-based screening has vastly improved in recent years. Incremental improvements will increase the complexity of phenotypes that can be detected during automated screening and ultimately increase HT screening capabilities.

Pharma Adoption of the Zebrafish Embryo Model: Swimming in Savings

In 2010, the cost of bringing a drug to market in the US was estimated at $1.8 billion [140], requiring approximately 9 years of research and development before transitioning to the bedside. Drug discovery requires multiple rounds of in vitro assays, animal testing, and clinical trials before receiving regulatory approval if deemed safe and efficacious. A significant cost of bringing drugs to market is associated with animal testing, which includes safety assessments, toxicity testing, and pharmacokinetic studies to determine absorption rates, distribution, metabolism and excretion (ADME), which are performed in expensive mammalian animal models. To reduce the use of mammals in drug research for ethical and economic reasons, especially during the initial phase of safety assessment and in vivo toxicity testing, the use of the zebrafish embryo model has garnered early success (reviewed by Zon and Peterson [141]).Significantly, screening zebrafish embryos with panels of biologically active compounds uncovered 16,16-dimethyl prostaglandin E_2 as an important modulator of vertebrate hematopoietic stem cell homeostasis [142]. As a result, a novel compound is now in phase II clinical trials for the treatment of hematologic malignancies in humans (http://clinicaltrials.gov). It stands to reason that economies of scale can be realized using the zebrafish in drug discovery. Consequently, a consortium of multinational pharmaceutical companies initiated the process of validating the zebrafish for use in their drug discovery pipeline [143].

Conclusions

Deciphering the effects of chemical exposures on human health and the environment is a daunting task that lies ahead for those interested. It will require new technologies, new assays, and more efficient ways of analyzing and imaging complex and large data sets. The zebrafish is, by all measures, a powerful model that is facilitating rapid advances in these three areas, all of which will require prolonged efforts akin to swimming upstream for a long time. This is not a bad thing; after all, "A dead fish can float downstream but it takes a live one to swim upstream" (W.C. Fields).

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Compliance with Ethics Guidelines

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Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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