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Zebrafish: an *in vivo* model for nano EHS studies

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Abstract

To assure a responsible and sustainable growth of nanotechnology, the environmental health and safety (EHS) aspect of engineered nanomaterials and nano-related products needs to be addressed at a rate commensurate with the expansion of nanotechnology. Zebrafish has been demonstrated as a correlative *in vivo* vertebrate model for such task, and the current advances of using zebrafish for nano EHS studies are summarized here. In addition to morphological and histopathological observations, the accessibility of gene manipulation would greatly empower such a model for detailed mechanistic studies of any nanoparticles of interest. The potential for establishing high-throughput screening platforms to facilitate the nano EHS studies is highlighted, and a discussion is presented on how toxicogenomics approaches represent a future direction to guide the identification of toxicity pathways.

Keywords

zebrafish; nano EHS; high throughput; transgenic; toxicogenomics

1. Introduction

Nanotechnology, an emerging multi-billion to trillion dollar business industry envisioned in early 21st century, holds a critical role in the growth of global economy.^[1–3] The benefits promised by the incorporation of engineered nanomaterials into commercial products have greatly stimulated the research and production efforts on creating novel nanoscale materials

in both academic and industry settings. As a result, engineered nanomaterials possessing diverse physiochemical properties based on various compositions, sizes, shapes, surface properties etc., are created on a daily basis. Collectively, there are over thousands of nano-related products in the market containing engineered nanomaterials as either the major components or the additives for better performances.^[4–5] However, the wide applications of nanotechnology also unavoidably lead to human and environment exposure that may cause potential hazardous effects.^[6–9] In order to ensure a sustainable growth of nanotechnology, it is important to understand the implications of engineered nanomaterials and nano-related products from the perspective of environment and human safety (EHS).^[10] The knowledge generated from nano EHS studies will in turn be instrumental in managing risk and providing guidance in the implementation of quality controls for minimizing exposure, protective protocols for reducing hazardous effect, and safe-by-design strategies for improving nanomaterials and nano-related products.^[11–14]

Much effort has been made in the past decades on the investigation and understanding of the potential hazardous effects of engineered nanomaterials on the environment and human health by bringing together multidisciplinary expertise on materials sciences, biological sciences, toxicology and environmental sciences.^[15–16] In fact, there is an increasing body of literature that documents the toxicity profiles of a wide range of engineered nanomaterials, including carbon based nanomaterials (fullerenes, carbon nanotubes and graphenes), metal and metal oxides, amorphous and crystalline materials, and nano-sized polymers (such as dendrimers and polystyrene nano-beads). The toxicity models used in these studies ranged from biomolecules (such as peptides and proteins), membranes, organelles and cells, to multicellular organisms (such as daphnia and sea urchin), and animal models (such as mice and rodents). While the biological complexity and relevance of these models increase from *in vitro* to *in vivo*, the throughput level and numbers of replicates that can be performed are inevitably reduced. Considering the continuously rising numbers of engineered nanomaterials and products generated each year, it is of critical need to develop models that would ideally maintain the biological representativeness as well as the screening throughput level. In this regard, zebrafish as an *in vivo* model organism has attracted much interest because of its unique features, including high fecundity, embryo transparency, fast and well-characterized development, low cost, gene manipulation accessibility, short reproduction time etc.^[17–19] These advantages potentiate this model to fit in between the traditional cell culture and mammalian models, providing validation of *in vitro* toxicity and prioritization of *in vivo* animal experiments (Figure 1).

This review has therefore focused on the current advances of using zebrafish for toxicity assessment of engineered nanomaterials, with the aim to better our understandings on the potentials as well as challenges present in this model for nano EHS studies.

2. The Advantages of Zebrafish Model

Zebrafish has long been recognized as a model organism for screening of environmental toxicants, man-made chemicals and drugs.^[20–25] It has also been considered as a “gold standard” for environmental toxicity assessment.^[26] Many fundamental cellular and molecular pathways involved in the response to toxicants or stress are highly conserved

between the zebrafish and mammals.^[27–28] The highly conserved genome of zebrafish compared to humans also makes it an analogous model to analyze developmental toxicity and disease pathogenesis.^[19, 29–30] More recently, the strengths of the zebrafish model have been realized from the perspective of nano EHS studies as well. The advantages of using zebrafish as a model organism for toxicity assessments of engineered nanomaterials can be summarized in four folds.

First, the multicellular organism zebrafish offers biological complexities that involve dynamic, interactive and multi-organ events that single cell lines fail to provide. It is well known that many biological processes cannot be reproduced in cultured cells and often the three-dimensional environment of cells determines their function. Furthermore, metabolism of chemicals may be profoundly different in whole organisms. Therefore, it is highly desirable to assess large numbers of nanomaterials for their potential effects on biological activity in whole organisms as early in the screening process as possible. Presently, this has become feasible using the zebrafish model.

Second, the high fecundity of the zebrafish makes it ideal for performing wide dose range of large numbers of nanomaterials screening in a single assay. The easiness in maintenance of adult zebrafish and the large numbers of embryos produced per mating enable medium to large numbers of replicates to be performed concurrently, which may provide statistical power comparable to *in vitro* cellular experiments. This feature is particularly attractive considering the ever-increasing numbers of nanomaterials and nano-related products generated each year and the need for developing high throughput platforms for toxicity screening.

Third, the well-characterized developmental stages of zebrafish allow screening strategies being designed and executed to target specific exposure scenarios or toxicity mechanism of nanomaterials (Figure 2). For example, the toxicity assessment in embryos provides rapid and early warnings of nanomaterials that induce abnormal morphology, low hatching and survival rates within 3~5 days post fertilization (dpf). Aqueous exposure to a later life stage of zebrafish resulted in skin, gastrointestinal tract, and gill exposure that may lead to sub-acute toxicity endpoints, including skin damage, GI tract malfunction, endocrine disruption, gill injury etc. Besides short-term acute toxicity assays, zebrafish is amenable for long-term exposure experiments that facilitates the investigation of chronic effect of nanomaterials.

Last but not least, the zebrafish is highly amenable to both molecular and genetic analysis and genetic manipulation, which allows in -depth investigation of specific toxicity mechanisms exerted by nanomaterials.^[26, 31–32] For instance, the transparency of zebrafish embryos makes examination of gene expression level in the whole embryo by RNA *in situ* hybridization (ISH) possible. Multiple zebrafish microarrays have also been developed to facilitate the rapid and simultaneous assessment of the expression profiles of thousands of genes.^[33–35] Furthermore, the availability of many zebrafish transgenic lines with fluorescent reporter genes expressed in specific cells/tissues/organs allows direct observation and real-time monitoring of biological changes in response to nanomaterial exposure in live animals. For example, the first stable transgenic zebrafish with tissue-specific expression using a fluorescent protein marker is the *gata1:eGFP* line in which

erythrocytes are labeled with green fluorescence recapitulating the expression pattern of the endogenous *gata1* gene.^[36] This line has been a valuable tool for examining erythrocyte status and circulation defects during development, and screening for chemicals that affect erythropoiesis and blood circulation. Up to date, hundreds of zebrafish transgenic lines have been established, each labeling specific type(s) of cells, tissues, organs or signaling molecules, and successfully utilized for genetic and molecular analyses (Table 1).

Like any model system, zebrafish has its limitations. As a relatively new model organism, a smaller body of knowledge is available on the zebrafish compared to rodents. In particular, limited data are currently available regarding metabolism and pharmacokinetics of most chemicals. Physiologic measurements and anatomical investigations could be rather challenging in such a small organism. Though a vertebrate, the zebrafish is a non-mammalian species and care must be taken when extrapolating findings from zebrafish studies to humans.

3. Current Advances in Toxicity Assessment of Nanomaterials using Zebrafish

In recent years remarkable progress has been made in using zebrafish for toxicity assessment of engineered nanomaterials, most of which depends upon the manifestation of abnormal morphological phenotypes of zebrafish under various exposure conditions. Although the toxicity screening process can be perceived as easy as adding “water” (nanomaterials in aqueous suspension) to the fish, the knowledge on the toxicity potential of the tested nanomaterials can only be obtained through careful screening designs based on zebrafish biology and rigorous physicochemical characterizations of the nanomaterials. As Bohnsack et al. also pointed out, the proper evolution of nanotoxicology demands a strong marriage between the physical and biological science.^[37] This section therefore categorizes the toxicity data available based on specific physicochemical properties of engineered nanomaterials, including particle size, shape, surface properties, and dissolution chemistry, with the aim to shed light on the property-toxicity relationship for nanomaterials in zebrafish models. The identification of nanomaterial properties that lead to hazard generation is pivotal to provide risk assessment as well as safe-by-design strategies for engineered nanomaterials and nano-related products.

3.1. Effect of Particle Size

Common consensus of nanomaterials is that at least one dimension of the materials falls in the scale of equal or less than 100 nm.^[38–39] With reducing particle size, the exponentially increased surface area per volume ratio may result in higher bioavailability or higher surface reactivity of materials that could lead to higher toxicity profile. So far, there are a few studies demonstrating the size-dependent toxicity of engineered nanomaterials in zebrafish.

Zhu et al. compared the toxicity of three metal oxide nanomaterials (ZnO, TiO₂ and Al₂O₃) with their bulk counterparts using zebrafish embryos.^[40] Through examining the morphology, hatching and survival rates of embryos exposed to suspensions of these materials, ZnO was found to be the most toxic one that exerted hatching delay, skin

ulceration, and high mortality, while TiO_2 and Al_2O_3 were non-toxic in both nano and bulk forms. For ZnO materials, there was no statistically significant difference in the LC_{50} values obtained from 96 hour post fertilization (hpf) zebrafish embryos exposed to nanosized ZnO and its bulk counterpart. The identical toxicity profiles could be explained based on the dissolution studies, in which comparable amount of shed Zn^{2+} ions were quantified in both nano and bulk sizes. Therefore, the metal ion shedding property, instead of the particle size, was the determining factor of toxicity in this case. Similar findings were reported by Ispas et al., in which they demonstrated that comparable LC_{50} values were obtained from three different sizes of Ni nanoparticles (30 nm, 60 nm and 100 nm).^[41] In the case of spherical-shaped Ag nanomaterials, the size-dependent toxicity of Ag particles with 3, 10, 50 and 200 nm in diameter was only observed in one time point of the developmental stage (24 hpf) of zebrafish embryos, while the mortality rate assessed at 96 hpf and later developmental stage did not resolve any size-dependent toxicity.^[42] It is important to note that, in these above-mentioned cases, zebrafish embryos were exposed to nanomaterials suspensions or their bulk counterparts with intact chorion. Although the pore sizes of the chorion are within the range of 300 nm to 1 μm , study using fluorescent-labeled SiO_2 (~200 nm) nanoparticles has shown that the nanoparticles failed to diffuse through the chorion pores, instead mostly adsorbed on the external surface.^[43–44] Only the stably mono-dispersed Ag nanoparticles at lower concentration (0.2 nM) were found to gain their way through the chorion pore canals into the perivitelline space.^[45]

While the studies conducted on zebrafish embryos did not show clear size-dependent toxicity of ZnO, Ni, and Ag nanomaterials, possibly due to the lack of nano-bio interactions between the nanoparticles and the embryos, the use of adult zebrafish allowed Xiong et al. to demonstrate that nano-sized TiO_2 nanoparticles are more toxic than its bulk counterpart.^[46] In this study, adult zebrafish was exposed to particle suspensions for 96 hours with particle suspensions replaced every 24 hours. This exposure scenario led to particle exposure to multiple zebrafish organs, including the skin, gill and intestine. As evidenced by the analysis of lipid peroxidation level and the histopathology of gill tissue, nano-sized TiO_2 nanoparticles were significantly more toxic than their bulk counterpart. The amount of Ti in the adult zebrafish exposed to nano-sized TiO_2 was significantly higher than their bulk counterpart, suggesting the higher bioavailability resulted from nano-size serves as the main reason for the differences in toxicity.

The studies discussed above highlight the importance of considering the physiological scenarios that the zebrafish model offers at different life stages for toxicity assessment. At embryonic stage, the acellular envelop, chorion, acts as a physical barrier that may prevent or minimize the nano-bio interactions of nanomaterials with embryos. Instead, the metal ions that shed from nanomaterials may serve as the dominating source of toxicity. Differently, in the case of hatched larvae and adult zebrafish, the exposure routes of nanomaterials extended to the skin, gills, intestine and other organs, which may lead to observable size-dependent toxicological responses.

3.2. Effect of Particle Shape

The shape of nanoparticles is emerging as one important materials design parameter to acquire unique properties and better performances.^[47] Beside the most common spherical shape, engineered nanomaterials are made in nanocubes, nanorods, nanotubes, nanowires and nanodendrites.^[48–50] The investigations of the shape effects on toxicity are also emerging and here we highlight some of recent demonstrations on shape-dependent toxicity in zebrafish. Dendritic-shaped Ni nanoparticles were compared with the spherical ones in zebrafish larvae.^[41] Under direct immersion exposure scenarios, the dendritic -shaped Ni nanoparticles resulted in significantly lower LC₁₀ than the spherical ones. And the LC₁₀ value of dendritic-shaped Ni nanoparticles was even lower than that of equivalent amount of Ni ions, demonstrating the additional hazardous effect from the nanoparticles. By comparing the histopathology of the intestine and skeletal muscle, the shape effects of Ni nanoparticles were further confirmed, with the dendritic ones exerted the most severe tissue damage. Interestingly, higher amount of dendritic-shaped Ni nanoparticles were found accumulated in the intestine of the surviving larvae. This result suggested that the dendritic shape might have prolonged the retention time of nanoparticles and hence increased the bioavailable Ni content, which could also be another reason for the relative higher toxicity compared to spherical Ni nanoparticles.

The shape-dependent toxicity was also demonstrated in the case of microinjecting nanoparticles into zebrafish embryos.^[51–52] Although such exposure scenario might not be environmental relevant, introduction of nanoparticles through microinjection assured the difference in the observed toxicity was due to the shape of the nanomaterials, rather than the variance in the bioavailable amount of nanomaterials or the difference in the developmental stage of embryos. Nelson et al. investigated the effects of SiO₂ nanowires compared with two different sizes (50 nm and 200 nm) of spherical nanoparticles.^[51] By microinjecting the nanoparticle suspensions into 1–2 cell stage zebrafish embryos, the researchers followed the development of embryos. While no effects were found in the embryos microinjected with spherical SiO₂ nanoparticles in both sizes, SiO₂ nanowires induced significant mortality starting at 8 hours after microinjection. Besides higher mortality rates, there were also large amount of embryos that developed abnormally at SiO₂ nanowire concentration of 3 pg/l and up, potentially due to the interference with neurulation and disruption of morphogen *sonic hedgehog* expression. In another study that focused on different lengths of multiwall carbon nanotubes (MWNT), Cheng et al. investigated the effects of microinjected MWNT with 0.2 μm and 0.8 μm in length.^[52] While the longer MWNT (high aspect ratio) showed no toxicity, the shorter ones exerted severe toxicity including failure of epiboly initiation and embryo malformation.

3.3. Effect of Surface Properties

The surface of nanomaterials represents one of the main components of the nano-bio interface.^[53] The interaction between nanomaterials and biological systems is mainly dictated by the surface properties of nanomaterials. The investigations on the surface properties dependent toxicity in zebrafish have been focused on surface charge, surface defects, and surface hydrophobicity/hydrophilicity of the engineered nanomaterials.

Through elegant design and synthesis, Harper et al. evaluated the toxicity profile of Au nanomaterials with positive, negative and neutral surface charges in zebrafish embryos.^[54] In this study, the chorions of fish embryos were chemically removed prior to the exposure of nanomaterials, since the chorions may act as a physical barrier for nanoparticles as shown in previous studies. The 5 day exposure assay resulted in clear surface charge dependent toxicity, i.e. high mortality in the embryos exposed to positively charged Au nanomaterials, more malformations occurred in the ones exposed to negatively charged ones, while no effects were found for the neutral ones. The surface charge dependent toxicity could be partially explained by the particle uptake and retention rate in the embryos. Although the embryos took up comparable amount of Au nanomaterials with different surface charges, positively charged ones elicited a much long retention time than the rest of the materials. The authors also postulated that the differences in alteration of cellular binding protein system or cellular redox changes could be two potential mechanisms that led to the surface charge dependent toxicity. Surface charge dependent toxicity was also evidenced in the study on poly amido amine dendrimers (PAMAM), a highly branched nano-sized polymer with tunable surface charges.^[55] In this study, high toxicity was found in positively charged PAMAM dendrimers, while the negatively charged ones were non-toxic. Interestingly, in this study, exposure of PAMAM to zebrafish embryos was conducted with the intact chorions and the removal of chorions resulted in a reduction of toxicity.

George et al. demonstrated the surface defect plays a role in determining the toxicity of Ag nanomaterials in both fish cell lines and zebrafish embryos.^[56] Despite the lower Ag⁺ ion dissolution rate of Ag nanoplate, the toxicity profile of this material was higher than other types of Ag nanomaterials (Ag nanospheres and Ag nanowires) in terms of both hatching interference and mortality of the embryos. In search for the cause of the higher toxicity profile of Ag nanoplate, the authors employed high-resolution transmission electron microscopy (HR-TEM) to exam the level of surface defects in Ag nanomaterials and showed that Ag nanoplates possess the highest degree of surface defects in the forms of point defect and stacking faults. Moreover, the authors confirmed their findings by demonstrating that the ligation of cysteine on the surface of Ag nanoplate can effectively mask the toxicity of Ag nanoplate.

Hydrophobicity is one of the typical characteristics of carbon based nanomaterials (such as fullerenes and carbon nanotubes). Because of their hydrophobic surface, carbon based nanomaterials tend to agglomerate to shield away from water molecules in aqueous environment. In order to investigate their potential hazardous effect on zebrafish, instead of using microinjection, several early studies had to use organic solvent (DMSO and DMF) or surfactants (sodium dodecyl sulphate) to render nanoparticle suspensions or chemically modify the nanoparticle surface with hydrophilic functional groups. In a study on C₆₀ fullerenes, Zhu et al. observed moderate developmental delay and reduced hatching success rate in zebrafish embryos exposed to C₆₀.^[57] Due to the presence of chorion and the significant aggregation of C₆₀ nanoparticles, the toxicity was likely due to the metal impurities instead of nanoparticle itself. In another study that focused on C₆₀, C₇₀ fullerenes and fullerene derivative C₆₀(OH)₂₄, Usenko et al. dechorionated the zebrafish embryos prior to nanoparticle exposures.^[58] In this case, high level of malformations, pericardial edema and mortality were observed in zebrafish embryos exposed to hydrophobic C₆₀ and C₇₀.

fullerenes. Significantly lower level of toxicity was found in embryos exposed to hydrophilic C₆₀(OH)₂₄, suggesting that high toxicity potential might be associated with the hydrophobicity of nanoparticle surface. Besides surface hydrophobicity, surface modification of nanoparticles also played a role in the toxicity. Henry et al. compared the toxicity profile of C₆₀ suspended in water with and without THF in larval zebrafish.^[59] By comparing the gene expression profiles, the toxicity induced by THF suspended C₆₀ was linked to a THF degradation product (γ -butyrolactone).

3.4. Effect of Dissolution and Metal Ion Shedding

Metals, metal oxides, semiconductor quantum dots (QD) and other metal-containing nanoparticles represent one major branch of engineered nanomaterials that may exert hazardous effects due to dissolution and metal ions shedding. ZnO is one of the most studied nanoparticles in zebrafish. The Zn²⁺ ions shed from ZnO nanoparticles, nano -aggregates and bulk ZnO have been shown to play a major role in determining the toxicological outcomes of each of these three types of materials in zebrafish embryos.^[60–62] Besides ZnO, other dissolvable transition metal oxides including CuO and NiO have also been shown to interfere with zebrafish embryo hatching.^[63] The shed metal ions effect was confirmed by the fact that the co-exposure of metal ion chelator DTPA was able to protect the embryos from hatching interference. The mechanism of hatching interference exerted by these metal ions was hypothesized to be the inhibition of zebrafish hatching enzyme 1 due to the ligation of shed metal ions with the active enzyme center. Based on the understanding of the toxicity mechanism of ZnO nanomaterials, Xia et al. has successfully demonstrated a safe-by-design strategy for ZnO nanomaterials by reducing the Zn²⁺ dissolution through Fe doping.^[61] Flame spray pyrolysis was used to dope increasing amount of Fe into the crystal structure of ZnO nanoparticles. The incorporation of Fe resulted in the increase of the lattice binding energy and therefore significantly reduced the dissolution rate of Zn²⁺. The recovery of hatching rates of zebrafish embryos exposed to Fe-doped ZnO demonstrated the successful implementation of such safe-by-design strategy.

Quantum dots, mostly constituted of trace metals such as Cd and Se, have raised concerns due to the high toxicity profile of their metal components. In order to ensure the biocompatibility of these materials, various synthesis strategies, including implementation of core/shell structure and surface modification with ligands, have been employed to prevent dissolution and metal ion shedding. Study of King-Heiden et al. provided systemic investigation on five different surface coating QDs using zebrafish embryos.^[64] Although the shedding of Cd ions in the exposure medium was insignificant, the toxicity as evidenced by embryo malformation and mortality did agree partially with the typical Cd toxicity profile. The results suggested that although not dissolvable *ex vivo*, degradation of QD might happen inside tissues after being taken up by zebrafish embryos.

Carbon based nanomaterials, as mentioned earlier, may also cause toxicological outcomes due to the release of residual heavy metal catalysts in their structures. In the studies of Cheng et al, hatching delay of embryos exposed to raw single wall carbon nanotubes (SWNT) and double wall carbon nanotubes (DWNT) was observed.^[65] The Ni and Co catalysts used in SWNT synthesis were postulated to be the cause for hatching delay. The

Lesser extent of hatching delay in the embryos exposed to DWNT was attributed to the partial encapsulation of Co catalysts that were not released during exposure.

It is important to note that the toxicity exerted by shed metal ions might mask the effects of nanoparticles if not being carefully examined. Although the toxicity profile of most metal-containing engineered nanomaterials share some common features with the corresponding metal ions, several studies have shown the toxicity was not always explained, or was only partially explained by the presence of free metal ions. For example, in the study of Cu nanoparticles in adult zebrafish, Griffith et al. observed moderate to high toxicity profiles of both Cu ions and Cu nanoparticles.^[66] However, based on the calculation of the amount of dissolved Cu ions from the nanoparticles, the majority of the observed mortality resulted from Cu nanoparticles exposure remained unexplained. Through detailed histopathological analysis on the gill tissue of the adult fish, higher level of gill injury was found in Cu nanoparticles exposed fish, demonstrating the contribution of nanoparticle effects to the high mortality.

4. High Throughput Screening, an Avenue to Speed up the Nano EHS Studies

The current literature has provided a solid foundation for the nano EHS studies using zebrafish. The existing screening assays based on zebrafish phenotypes have taken advantages of some of the unique features of this model such as their fast development, large fecundity that provides for biological replicates and statistical analysis, and embryo transparency for easy visual assessment. However, most of these assays involve repetitive processes that are labor-intensive and time-consuming, such as embryo selection, manipulation, quantifying abnormal and dead embryos under dissecting microscope, and microscopic imaging. One possible solution to this bottleneck is the use of high throughput screening (HTS), which has been one of the leading paradigms for drug discovery process.^[67–68] Such concept is particularly appealing considering the need to provide hazard assessment for the ever-increasing numbers and identities of engineered nanomaterials and nano-related products. Although zebrafish HTS is still in its infancy and validation of screening methods need to be developed in parallel, this review intends to inspire the research community by summarizing some of the promising examples of zebrafish HTS platform. The zebrafish HTS platform could be dissected into a collection of technologies that includes automated liquid handling and embryo manipulation, high content imaging that provides high resolution bright-field and/or fluorescence images, and computer-assisted image analysis or toxicity scoring systems (Figure 3). Here we highlight some of the promising progress being made recently.

4.1. Automation of Zebrafish Manipulation

Toxicity screening in zebrafish embryos starts with collecting embryos and placing them into multiwell plates, where they received exposure of engineered nanomaterials. In some cases, the embryos received nanoparticles exposure through microinjections.^[51–52] Both processes are tedious and laborious, which significantly hinder the throughput level of screening. Through streamlining of the routine process and introducing robotics technology,

a few proof-of-concept pilot studies have shown significant enhancement of the throughput level while keeping minimum disturbance in embryo development.

Zhang et al. established a batch transfer platform that allows parallel transfer of embryos into 96-well plates, with one embryo in each well.^[69] One of the main components of this platform is a vacuum-based cell holding device that traps embryos in a pattern that matches the layout of a standard 96-well plate. After the embryos immobilized by the low vacuum force on the holding device, a robotic mechanism was designed to flip the holding device, align on top of a 96-well plate, and release the embryos into each well. Such platform successfully eliminates large extent of human intervention and allows faster transfer of embryos for toxicity screenings, while maintaining 100% embryo survival rate.

A recent development of a microrobotic system showed how to automate the microinjection of foreign materials into individual embryos.^[70] This system overcomes several inherent inconsistencies in manual operation, such as human fatigue and large variation in success rate due to poor reproducibility. This system utilized vacuum force for immobilization of embryos, a real-time image processing system to recognize the orientation of embryos, and a microrobot carrying the injection needle accordingly. Such microrobotic system could be a reliable tool to facilitate large-scale screening of engineered nanomaterials.

The manipulation of zebrafish embryos or larvae can be more demanding when it has to be coupled with screening requirement that focuses on specific orientation or organ of the zebrafish. For example, retinal axons of zebrafish can only be directly observed from the hindbrain of zebrafish. This structure is obscured if observed at some other orientations. For a screening that focuses on the neuronal regeneration at this specific region, placing embryos in multiwell plates is simply not ideal for imaging. Instead, Yanik et al. demonstrated a fully automated system that employed a vision-guided robotic mechanism to deliver and orientate the zebrafish larvae under laser scanning confocal microscope.^[71] This system loads one zebrafish larva at a time from a multiwell plate or a reservoir, delivers it to a capillary assembly, positions and orientates the larva's brain region for automated image capture under the microscope, and dispenses the larva back to the plate or reservoir. Such a system can image a single larva in less than 20 seconds and an entire 96 -well plate in approximately 30 minutes.

4.2. High Content Imaging

Both wild type and transgenic zebrafish have been used for phenotype-based toxicity screening. While screening using wild type zebrafish focuses on the manifestation of morphological abnormalities, screening based on transgenic zebrafish lines provides unique opportunities to look at cell-type specific and/or pathway-specific toxicity. In both cases, the challenge for performing large-scale screening lies in the logistic limitation of providing a large number of high-resolution microscopic images for toxicity analysis. Such hurdle can be overcome by the advent of new technologies, high content imaging in particular, that automates such imaging process.

Lin et al. demonstrated for the first time the use of high content imaging to speed up the hazard ranking of transition metal oxide nanomaterials in zebrafish embryos.^[63] As

discussed previously, metal-containing nanomaterials could potentially affect the development of zebrafish embryos due to the shedding of metal ions. As a result, hatching interference of the embryos is one of the toxicological outcomes that can be evaluated based on bright-field imaging. However, the parallel comparison of a series of transition metal oxides (CuO, ZnO, NiO and Co₃O₄) at incremental dosages in one experiment was only made possible by the implementation of high content imaging. Zebrafish embryos that exposed to suspensions of transition metal oxides in 96-well plates were subjected to automated imaging using a high content imaging device (ImageXpress Micro). The device uses autofocus mechanism that allows acquisition of individual image of each well in multiwell plates within 2~3 seconds. The images acquired throughout the development of zebrafish embryos can be further analyzed for hazard identification, in this case the hatching success rate of embryos was evaluated at 3 dpf based on thousands of high resolution microscopic images.

While high content bright-field imaging provides rapid and accurate documentation of hatching interference caused by transition metal oxide nanomaterials, fluorescence-based high content imaging was subsequently developed to capture more subtle toxicological effects. Lin et al. used transgenic zebrafish larvae (*hsp70:eGFP*), in which enhanced green fluorescent proteins (eGFP) were expressed under the promoter of the stress response gene *hsp70*, to detect stress responses under the same exposure condition of transition metal oxides. The stress induction caused by the shedding of metal ions (Cu²⁺, Zn²⁺ and Ni²⁺) was successfully detected based on GFP fluorescence intensities that represent the expression level of stress response genes. Later, the transgene response was confirmed by expression of the endogenous *hsp70* gene, as determined by RT-PCR analysis. Thus, the combined use of fluorescence-based high content imaging and tissue/pathway specific transgenic zebrafish lines provides a unique opportunity to explore toxicological mechanisms exerted by nanomaterials in a timely manner.

So far, a number of transgenic zebrafish lines have been used for nanomaterials screening purposes. Bar-Ilan et al. used a transgenic zebrafish line (*ARE:eGFP*) for the detection of oxidative stress generation in the larvae exposed to TiO₂ nanoparticles.^[72] The oxidative stress was a result of the absorption and translocation of TiO₂ nanoparticles on the skin and the oxygen radicals generated by the nanoparticles under light activation. Vogt et al. has demonstrated another example of using a transgenic zebrafish strain *Tg(dusp6:d2eGFP)^{pt6}* that allows them to monitor the modulation of the FGF/RAS/MAPK pathway in zebrafish embryos.^[73] Given the fact that there are hundreds of well -established zebrafish transgenic lines labeling specific types of cells, tissues, organs or signaling molecules, we envisage that the zebrafish should soon become a valuable model in dissecting the molecular and genetic mechanisms underlying nanotoxicity, beyond morphology and survival assessment.

4.3. Computer-Assisted Image Analysis

The generation of tremendous amount of microscopy-based image data facilitated by high content imaging technology requires the development of image analysis tools to extract quantitative data from the images for toxicity analysis. Although bioimage informatics is in the infancy for application in toxicity assessment, there are a few studies that demonstrated

the utilities of computer-assisted analysis tools in providing quantitative analysis algorithms that can be applied on both bright-field and fluorescence images.^[74]

Tran et al. developed an automated, quantitative screening assay for monitoring the angiogenesis using transgenic zebrafish strain with GFP-labeled vasculature.^[75] High content fluorescence images were analyzed based on an automated algorithm developed to replace the traditional procedure of manually counting the numbers of blood vessels, such as the intersegmental vessels, the vertebral vessels, dorsal longitudinal anastomatic vessels etc. Vogt et al. have also demonstrated the use of the same transgenic zebrafish strain to identify other phenotypes, such as the size and shape of zebrafish at different developmental stages.^[76]

In contrast to fluorescence images, bright-field image analysis is more challenging due to the lack of contrast for feature extractions. Instead, Liu et al. were able to use a machine-learning based approach to build a phenotype recognition model that classifies three basic phenotypes of zebrafish embryos, i.e. hatched, unhatched and dead.^[77] In such an approach, a classification model was trained to recognize the three phenotypes initially identified based on an expert eye classification. The recognition model was built with a set of vectorial descriptors providing image color and texture information. The best performing model was attained with three image descriptors, i.e. color histogram, representative color, and color layout. The developed model was then used for automated phenotype recognition on unclassified images and the average recognition accuracy of the model was $97.40\% \pm 0.95\%$. The advantage of this model also lies in its flexibility in adapting to other phenotypes based on available high content images.

5. Summary and Future Perspectives

In summary, zebrafish has shown its great potential as an *in vivo* model for nano EHS studies. Their ability of rendering the physicochemical properties dependent toxicity profile is particularly promising for validations of *in vitro* screening and prioritization of detailed animal experiments. Moreover, another excitement this model brought to the nano EHS field is its potential in performing high throughput screening with the aim to speed up the hazard ranking of engineered nanomaterials and nano-related products. Last but not least, although most of the current nanotoxicity assays using the zebrafish rely heavily on morphological endpoints, the zebrafish model has exhibited great potential for studies of toxicological mechanisms. The amenability of the zebrafish system to various molecular techniques and the availability of multiple zebrafish microarrays, transgenic lines and vast genomic resources make it a highly versatile system for toxicogenomic studies in the near future.

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Figure 1.

Zebrafish, an *in vivo* model, possesses great potentials for facilitating nano EHS studies. With its high fecundity, embryo transparency, highly conserved cellular and metabolic activities etc., zebrafish offers higher biological relevance and complexities compared to *in vitro* cellular assays, while maintaining high throughput and high volume data generation capabilities.

Figure 2.

Well-characterized developmental stages of zebrafish offer versatile screening scenarios that target various toxicological responses, including hatching success rate, survivorship, developmental toxicity, skin damage, movement impairment, gill injury, neurotoxicity, reproduction toxicity etc.

Figure 3.

Example of a high throughput -screening platform that can speed up the hazard assessment of engineered nanomaterials and nano-related products using zebrafish embryos. The platform integrates a collection of technologies, including 1) automated liquid handling for nanoparticle suspension preparation; 2) automated embryo pick-and-plate for embryo selection and screening plates preparation; 3) high content imaging for automated microscopic image acquisition; and 4) machine-learning based image analysis for zebrafish embryo phenotyping.

Table 1

Selected list of available transgenic zebrafish strains useful for nano EHS studies.

Name of Transgenic Strains ^[a]	Labeling of specific cell/tissue/organ	References
ARE	antioxidant response pathway	[72, 78]
cmlc2	myocardium	[79–80]
flk-1/fli-1	blood vessels	[56, 75–76, 81–85]
gata-1	red blood cells	[36]
gut-GFP	esophagus, liver, pancreas, intestine	[86]
hsp70	heat shock response pathway	[63, 87–89]
insulin	endocrine pancreas	[90]
krt8	Skin epithelium	[91]
lysC	macrophages and possible neutrophils	[92–93]
mmp23b	liver	[94]
mpo	neutrophils	[95–96]
mylpfa	skeletal muscle	[97]
pdx1	pancreas	[90]
pomc	pituitary corticotrophs	[98]
vmat2	monoaminergic neurons	[99–100]

^[a] ARE: antioxidant response element; cmlc2:cardiac myosin light chain 2; flk-1:vascular endothelial growth factor receptor-2; fli-1: Friend leukemia integration site 1; gata-1: erythroid transcription factor; hsp70: heat shock protein 70; krt8: keratin 8; lysC: lysozyme C; mmp23b: matrix metalloproteinase 23b; mpo: myeloperoxidase; pdx1: pancreatic and duodenal homeobox 1; pomc: proopiomelanocortin; vmat2: vesicular monoamine transporter 2.