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Integrating 4-D light-sheet fluorescence microscopy and genetic zebrafish system to investigate ambient pollutants-mediated toxicity

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Abstract

Ambient air pollutants, including $PM_{2.5}$ (aerodynamic diameter d ~2.5 μm), PM_{10} (d ~10 μm), and ultrafine particles (UFP: d < 0.1 μm) impart both short- and long-term toxicity to various organs, including cardiopulmonary, central nervous, and gastrointestinal systems. While rodents have been the principal animal model to elucidate air pollution-mediated organ dysfunction, zebrafish (*Danio rerio*) is genetically tractable for its short husbandry and life cycle to study ambient pollutants. Its electrocardiogram (ECG) resembles that of humans, and the fluorescent reporter-labeled tissues in

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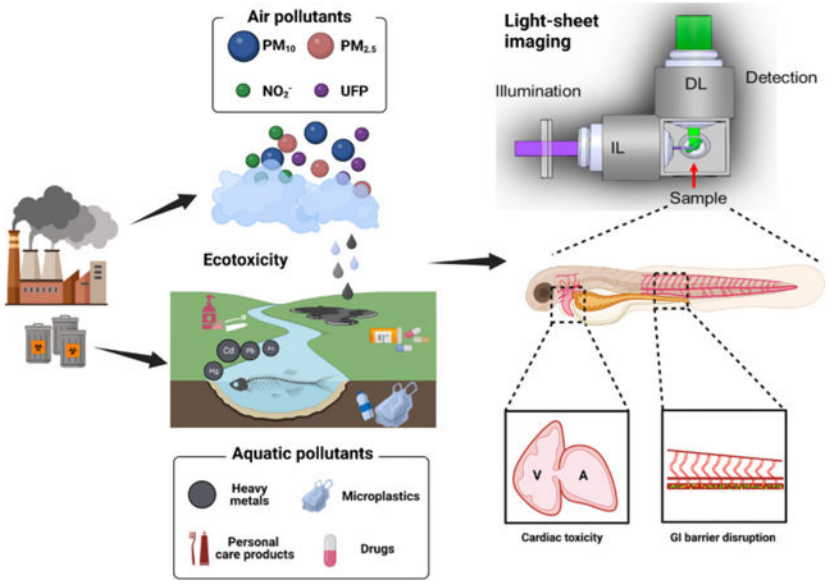
the zebrafish system allow for screening a host of ambient pollutants that impair cardiovascular development, organ regeneration, and gut-vascular barriers. In parallel, the high spatiotemporal resolution of light-sheet fluorescence microscopy (LSFM) enables investigators to take advantage of the transparent zebrafish embryos and genetically labeled fluorescent reporters for imaging the dynamic cardiac structure and function at a single-cell resolution. In this context, our review highlights the integrated strengths of the genetic zebrafish system and LSFM for high-resolution and high-throughput investigation of ambient pollutants-mediated cardiac and intestinal toxicity.

Graphical Abstract



Keywords

Ultrafine particles; Zebrafish; Cardiovascular health; Gastrointestinal health; Light-sheet fluorescence microscopy

1. Introduction

Pollution is a leading risk factor for death globally, responsible for around one in six premature deaths (Chen and Hoek, 2020; Committee on Assessing Causality from a Multidisciplinary Evidence Base for National Ambient Air Quality Standards et al., 2022; Fuller et al., 2022). Air quality standards established by the World Health Organization (WHO) were not met by 99 % of the world's population in 2019, leading to an estimated 4.2 million premature deaths worldwide (Vohra et al., 2021; Josey et al., 2023). Environmental pollutants have been identified as a major risk factor for multiple health conditions, including allergies, autism, colorectal adenomas, diabetes, inflammatory bowel disease, and obesity (Zanobetti and Schwartz, 2002; Wu et al., 2013; Guo et al., 2020; Celebi Sozener et al., 2022). Acute or chronic exposure to air pollutants further impairs the cardiovascular, gastrointestinal, and respiratory systems, increasing morbidities and mortalities (Vignal et al., 2021).

Particulate matter (PM) with a diameter of less than or equal to 0.1 μm, known as ultrafine particles (UFP), is an important health risk due to its high concentration, surface-volume ratio, and potential to translocate across the bronchial epithelium into the circulatory system (Nordenhäll et al., 2000; Donaldson et al., 2002). UFPs are predominantly composed of organic carbon that is redox active as they undergo photochemical reactions, particularly in the summer, leading to the formation of secondary organic aerosols to increase their biotoxicity (Sardar et al., 2005; Daher et al., 2013; Yang et al., 2020). Exposure to UFP increases plasma sCD40L levels, a soluble 18-kDa trimer released by the activated immune cells (T-lymphocytes) and blood coagulation components (platelets), promoting thrombus formation (Ruckerl et al., 2007). UFP has also been shown to reduce the production of endothelial nitric oxide (NO) via endothelial NO synthase (eNOS)-glutathionylation (Du et al., 2013). However, investigating the mechanism whereby $PM_{2.5}$ and UFP impair organ systems remains experimentally challenging *in vivo*.

To this end, light-sheet fluorescence microscopy (LSFM) enables to image the 3-D developing hearts, vascular network, and blood cells *in vivo* (Ding et al., 2018a, 2018b). LSFM illuminates the focal plane with a thin sheet of light, minimizing exposure time and photo-toxicity to the samples, thereby allowing for rapid imaging acquisition and time-lapse observation of the live zebrafish in response to ambient pollutant exposure. LSFM was demonstrated to quantify 2,4,6-Trinitrotoluene (TNT)-mediated developmental toxicity, revealing impaired heart tube looping and cardiovascular hypoplasia, which resulted in reduced cardiac contractility and vascular circulation (Eum et al., 2016). By virtue of its high spatial and temporal resolution, LSFM was also utilized to elucidate microvascular injury and regeneration after tail amputation using the green fluorescent protein (GFP)-labeled vascular network *Tg* (*fli:GFP*) transgenic line (Ding et al., 2018a; Roustaei et al., 2022). Furthermore, LSFM was instrumental in tracing the 3-D distribution of tissue-specific cardiac progenitor cells with the rainbow fluorescent reporters in the embryonic and neonatal mouse hearts (Ding et al., 2018a). Clinically, LSFM further demonstrated impaired myocardial contractility after chemotherapy-induced toxicity using the *Tg(cmlc2:GFP)* transgenic line to visualize the myocardium (Packard et al., 2017). In this context, integrating the high spatiotemporal resolution of light-sheet imaging with genetically labeled fluorescent reporters in the zebrafish lines can advance the field of ambient pollutant-mediated organ toxicity.

2. Comparison of small animal models to study environmental pollutants

Rodent exposure to air pollution has been used to demonstrate cardiovascular disorders. Ambient UFP exposure promotes oxidative stress, leading to a reduction in high-density lipoprotein (HDL) and an acceleration in atherosclerosis in the ApoE-knockout ($ApoE^{-/-}$) mice (Araujo et al., 2008; Bell et al., 2017; Holme et al., 2020). UFP exposure also initiates the pro-inflammatory signaling pathways to impair vascular endothelial homeostasis, priming the inflammatory responses and oxidative stress (Beck-Speier et al., 2005). UFP exposure studies in hyperlipidemic low-density lipoprotein (LDL) receptor knockout ($LDLR^{-/-}$) mice revealed that NF-κB signaling pathways promoted inflammatory responses and vascular calcification (Li et al., 2013; Dorans et al., 2016). Short-term exposure (1–7 days) to ambient fine particle concentrations also triggered elevated diastolic blood pressure

(Huang et al., 2018), acute coronary syndrome, myocardial infarction, cardiac arrhythmia, and heart failure, especially among susceptible populations living in urban areas (Lederer et al., 2021; Zhang et al., 2022). Furthermore, $PM_{2.5}$ exposure alters the intestinal flora of mice, causing gut microbiota dysbiosis, dysregulated liver metabolism, increased blood viscosity, vascular endothelial dysfunction, and risks of ischemic heart disease (Xu et al., 2019; Liang et al., 2020; Ran et al., 2021; Alexeeff et al., 2021).

While rodents have been the common model to assess the toxicity and biodistribution of nanomaterials, the zebrafish (*Danio rerio*) model has emerged as an efficient screening model for toxicity and counter-measures (Fako and Furgeson, 2009; Cassar et al., 2020). The zebrafish developmental genome is 80 % conserved with that of humans (Barbazuk et al., 2000). Despite having a two-chambered heart and a lack of pulmonary vascular system (Fig. 1a), the adult zebrafish electrocardiogram (ECG) is analogous to that of humans, exhibiting a typical P wave for atrial contraction, QRS for ventricular depolarization, and T wave for repolarization (Fig. 1b) (Vornanen and Hassinen, 2016). Due to their small size, short developmental stages, and transparent gut and circulatory systems, the zebrafish model is viable for investigating pollutants-mediated brain, cardiovascular, liver, and gut-vascular barrier impairments (Zhong et al., 2022) (Table 1). In addition, embryonic pigmentation can be suppressed by 1-phenyl 2-thiourea (PTU) (Karlsson et al., 2001).

Unlike rodents, zebrafish embryological development can be monitored *in vivo*, obviating the need for dissecting the uterus to harvest the embryos. Zebrafish embryos can survive the first five days of their development without active cardiac contraction, receiving oxygen by passive diffusion through the skin at the embryonic stage and obtaining nutrients from the yolk, which allows convenient embedding in gas-permeable agarose for stable long-term *in vivo* imaging (Kaufmann et al., 2012). These features render the zebrafish a viable model for genetic manipulation or experimental intervention to investigate various organ systems in real-time (Bakkers, 2011; Kamei et al., 2010). In contrast, avian and mammalian embryos die in the absence of a functional cardiovascular system during organ development (Table 2).

Numerous transgenic strains can serve as a genetically tractable system with fluorescently labeled proteins. They enable time-lapse *in vivo* imaging of the entire organ development, injury, and regeneration (Fig. 2). For example, we can observe neural crest cell migration to the cardiovascular systems at the early developmental stages with *Tg (−5sox10:nfsB-mCherry; myl7:nuc-EGFP)* transgenic line (Ding et al., 2018b; Gudapati et al., 2018). Whole-brain and cardiac functional imaging of calcium currents by GCaMP reporter under a pan-neuronal or cardiomyocyte promoter allows for the simultaneous recording of electrical activity with the use of high-speed LSFM (Ahrens et al., 2013). Quiescent and dividing cardiomyocytes can be detected *in vivo* with a fluorescent ubiquitylation-based cell cycle indicator (FUCCI) transgenic zebrafish line, and the ratio between proliferating and non-proliferating cardiomyocytes after an injury can be quantified to assess the cardiac regeneration (Chen et al., 2019). Furthermore, the zebrafish model provides a molecular toolbox for genetic manipulation, such as reverse genetics (i.e., gene knock-down) by morpholino injection or CRISPR/Cas9 editing (Kabashi et al., 2010; Li et al., 2021; Timme-Laragy et al., 2012; Yin et al., 2015). In the ensuing paragraph, we will demonstrate that this

gain- and loss-of-function manipulation of the tissue-specific phenotypes can be visualized via advanced imaging modalities.

3. Animal models to study environmental pollutants-mediated cardiovascular and gastrointestinal toxicity

3.1. Zebrafish as a model to study environmental toxicology

Ecotoxicology refers to the impact of contaminants on an organism, population, community, ecosystem, and biosphere level (Lynch et al., 2001), whereas environmental toxicology studies the exposure of chemicals and physical agents to living organisms (Shahid et al., 2020). Zebrafish have been used in toxicology studies long before their well-known applications in developmental biology. In 1952, almost two decades before the founding of the United States Environmental Protection Agency (EPA), Battle and Hisaoka examined the effect of ethyl carbamate (urethane) on cell differentiation and organization using zebrafish embryos (Battle and Hisaoka, 1952). Urethane was a carcinogenic compound widely used to treat cancer and as an analgesic before World War II, later found in many alcoholic beverages. The researchers documented the abnormalities in various embryonic structures after urethane exposure, including edema of the body cavities, malformation of the circulatory system, and impaired differentiation of the central nervous system. Subsequently, in 1957, Jones and Huffman published a method paper describing the protocol for testing chemicals that affected mitosis in zebrafish embryos (Jones and Huffman, 1957).

At the turn of the century, researchers from the University of Cincinnati first attempted to generate several transgenic zebrafish lines as aquatic sentinels (Carvan et al., 2006). The transgenic lines would carry the luciferase (*LUC*) reporter gene controlled by pollutant-inducible DNA response elements. They utilized the natural bioconcentration process to provide a more relevant measure of the toxicant level than directly testing the water. The designed system has been shown in zebrafish cell line ZEM2S to respond to aromatic hydrocarbons (e.g., dioxins), heavy metals (e.g., cadmium), and electrophiles (e.g., tBHQ) (Carvan et al., 2000). However, like many others at that time, the researchers had difficulty obtaining stable germline transmission by injecting plasmids into the embryo. Likely for this reason, the research with these sentinels was discontinued. Fortunately, the then-nascent *Tol2* transposon technology became the standard method to generate transgenic zebrafish for the next decade, developing a new generation of toxicant reporter lines (Bambino and Chu, 2017; Kawakami, 2005). The rapid growth of environmental health research using zebrafish also ensued, characterizing the toxicity of many pharmaceuticals and personal care products found in the modern-day aquatic environment, such as antibiotics, steroid hormones, and non-steroidal anti-inflammatory drugs (NSAIDs) (Porretti et al., 2022).

Moreover, air pollutants can affect aquatic organisms through atmospheric depositions and rain runoffs, contributing to the acidification of lakes and coastal waters (Lovett et al., 2009). The behavioral and physiological changes in zebrafish caused by these compounds served as a testament to the environmental impact of our ever-increasing urbanization. The following sections will focus on pollutant-mediated cardiovascular and gastrointestinal toxicity studies.

3.2. Environmental pollutants and cardiovascular health

Ambient air pollutants (PM_{10}, $PM_{2.5}$, UFP, and NO_2^-) from diesel exhaust, shipping and vehicle emissions, metal processing, and industrial combustion contribute to the increase in cardiovascular mortality (Franklin et al., 2015; Lin et al., 2018). Investigators have commonly employed the rodent model to elucidate the mechanisms supporting the epidemiological and clinical findings. Studies *in utero* and postnatal development using a mouse model have demonstrated that air pollution during this critical period increased the susceptibility to heart failure in adulthood (Weldy et al., 2014). Moreover, gestational exposure to air pollutants in mice revealed morphological and functional changes in the placenta, such as reduced volumes, calibers, and surface areas of maternal blood spaces, which compromised fetal weight (Veras et al., 2008). Despite being an excellent postnatal and fetal development model to study ambient air pollutant exposure, the mouse embryo requires uterus support and lacks optical clarity (Table 1).

On the other hand, chick embryos have been broadly used for angiogenesis studies due to their ability to survive *ex ovo* and translucent nature, allowing for direct visualization of blood vessels and angiogenic events using confocal microscopy and time-lapse imaging (Ribatti, 2012). However, the large size of the chick embryo renders high-throughput screening of toxins experimentally challenging. Alternatively, the zebrafish embryo has emerged as an experimentally conducive model to study cardiac development, toxicity, injury, and regeneration.

Zebrafish models have been selected to study aquatic ecotoxicity from natural and synthetic compounds (Bambino and Chu, 2017; Bhagat et al., 2020; Busquet et al., 2014). The marine ecosystem faces multiple sources of contamination (e.g., fungicides) that promote severe cardiovascular defects in zebrafish embryos (Chen and Li, 2021; Huang et al., 2020; Liu et al., 2017; Ma and Li, 2021). Medical drugs such as antibiotics are ubiquitous in the environment, and they are well-recognized for promoting cardiac defects in zebrafish embryos, including decreased calcium signaling and myocardial contraction (Gauthier and Vijayan, 2019; Ping et al., 2022; Shen et al., 2019; Zhang et al., 2020). Nanomaterials are widely used in biomedical and environmental fields, and researchers have shown that they induced developmental abnormalities in zebrafish, such as delayed hatching, vascular defects, and retardation of cardiac looping (Bai and Tang, 2020; Bangeppagari et al., 2019; Hu et al., 2017; Yang et al., 2021). Moreover, personal care products containing triclosan caused alteration in zebrafish heart looping and cardiac toxicity in a dose-dependent manner (Saley et al., 2016).

In mammals, the common route of air pollutant exposure to cardiovascular circulation is via the lungs (Kelly and Fussell, 2011). In zebrafish embryos, passive gas exchange primarily occurs via the skin during the first seven days post-fertilization (Kämmer et al., 2022; Lin et al., 2006). This embryonic or larval stage allows ambient ultrafine particles to enter the circulatory system when zebrafish are immersed in the pollutant-treated water. (McLeish et al., 2010). PM_{10} exposure promotes the production of reactive oxygen species (ROS) to impair cardiac development in zebrafish embryos, including increased sinus venous and bulbusarterious (SV-BA) distance, retarded vascular subintestinalis growth, accompanied

by functional defects such as decreased heart rate, obstructed blood flow, and reduced venous return (Cen et al., 2020). Similar to PM_{10}, extractable organic matter (EOM) from $PM_{2.5}$ affects cardiac development and function, while *N*-acetyl-L-cysteine (NAC), a ROS scavenger, rescues the impaired cardiac development (Ren et al., 2020). mRNA and miRNA microarray studies further revealed that zebrafish embryos exposed to silica nanoparticles and methylmercury (MeHg) developed NF-κB-mediated inflammation, which decreased myocardial contraction (Hu et al., 2017). Together, these studies support the use of zebrafish to advance cardiovascular toxicity research.

3.3. Environmental pollutants and gastrointestinal barriers

The gastrointestinal (GI) tract is among the primary organs that ambient air pollutants can access via direct consumption of contaminated food or indirect inhalation of ambient UFP or PMs (Li et al., 2015; Salim et al., 2014). In the latter, PMs can be indirectly deposited in the oropharynx via mucociliary clearance and be swallowed along with saliva and mucus (Mutlu et al., 2018). Once in the GI tract, PMs alter the gastric epithelium and gut microbiome and elicit a pro-atherogenic serum lipidomic profile (Li et al., 2017).

Murine models are commonly used in GI research due to mammals' highly conserved digestive systems, even though different species can have distinct digestive tract designs due to dietary habits (Nguyen et al., 2015). The composition of gut microbiota can also vary widely between animal species and individuals within a species. UFP exposure by $LDLR^{-/-}$ mice resulted in gut microbiota dysbiosis, leading to alterations in the microbiome diversity. The gut microbiota dysbiosis is associated with increased TNF-α and atherogenic metabolites such as lysophosphatidylcholine (LPC) 18:1 and lysophosphatidic acids (LPAs) in the intestines and blood circulation (Li et al., 2017). While mice models provide a host of gene knockouts, it is challenging to extrapolate the findings to humans when studying the composition of gut microbes (i.e., administering external microbes to germ-free mice) or conducting ecosystem interventions such as dietary adjustment. The ion transport via secondary messengers (cGMP and cAMP) differs between rodents and humans, making rodents a less favorable model to study ion transport in the gut (Foulke-Abel et al., 2020).

In zebrafish, the cellular composition and function of the intestine resemble those of higher vertebrate organisms, serving as both a digestive and immune organ (Wallace et al., 2005). The molecular pathways regulating injury and immune responses are also conserved (Zhao and Pack, 2017). The zebrafish model provides an optically transparent model to track nano-particles that transmigrate across the gut-vascular barrier (Dai et al., 2014; Zhong et al., 2022). The effects of air pollutants in an aquatic organism can be recapitulated by micro-gavage of pollutants into the intestinal bulb to assess gut-vascular permeability or by microinjection directly into the circulatory system for rapid biodistribution (Baek et al., 2020; Cocchiaro and Rawls, 2013). Zebrafish optical transparency further allows using laser-scanning confocal microscopy (LSCM) to assess the subcellular phenotypes. For instance, acute ingestion of UFP can be visualized to transmigrate across the embryonic intestinal barrier to the vascular circulation in the transgenic zebrafish line *Tg(flk1:mCherry)* (Fig. 3).

The gut-vascular barrier regulates paracellular trafficking and systemic dissemination of ingested microbes and toxins. UFP ingestion down-regulated Notch1-mediated tight-junction protein expressions such as zonula occludens1 (ZO-1) and Claudin-1, increasing endothelial permeability and impairing the gut-vascular barrier (Baek et al., 2020). In addition, UFP exposure inhibited FOXO1/Notch1 complex, hindering vascular regeneration after tail amputation (Baek et al., 2018b). These findings suggested that the downregulation of tight-junction proteins and the inhibition of FOXO1/Notch1 complex by UFP disrupted the gut-vascular development and regeneration.

Both mice and zebrafish are viable models for gastrointestinal and cardiovascular research. They provide complementary insights into disease development, injury, and repair. Mice have been widely used to study the underlying mechanisms of gastrointestinal diseases. At the same time, zebrafish models offer unique advantages such as rapid development, optical clarity, and high fecundity for genetically tractable studies. By combining the unique features of these two models, researchers can gain a comprehensive understanding of air pollutants-mediated organ dysfunction and develop therapeutic targets.

4. Imaging techniques to elucidate environmental pollutants affecting zebrafish vasculature and gastrointestinal barrier *in vivo*

To provide state-of-the-art imaging modalities, we will highlight the strengths of laser-scanning confocal microscopy (LSCM) and light-sheet fluorescence microscopy (LSFM), respectively, and to introduce high spatial and temporal resolution imaging of the entire zebrafish embryo *in vivo* (Fig. 4).

The fundamental principle of laser-scanning confocal microscopy (LSCM) is based on a pinhole fluorescence microscopy (Fig. 4d). In the illumination path, the light is focused onto the area of interest via a lens to reduce out-of-focus excitation. The fluorescent light will pass through a pinhole in the detection path before reaching the camera. This pinhole blocks the out-of-focus signal, providing confocal microscopes with a superior image contrast to a widefield system, effectively increasing the lateral and axial resolution. For example, Baek et al. demonstrated the UFP-mediated disruption of the gut-vascular barrier via the FITC-conjugated dextran (10 kDa) (Baek et al., 2020). LSCM was capable of localizing the dextran molecules leaking into the anterior venous capillary plexus (AVP) and caudal vein capillary plexus (CVP) from the intestine (Fig. 3e). The high spatial resolution of LSCM was necessary to resolve the embryonic capillaries (diameters <1 μm).

By virtue of its capability of large-volume and time-lapsed imaging with rapid acquisition and minimal photo-toxicity, LSFM has demonstrated an unparalleled strategy to image the entire zebrafish in real-time. Instead of a cone-shaped beam and point-by-point detection, LSFM illuminates the sample with a thin sheet of concentrated light and acquires the whole plane of fluorescence signal in one frame (Fig. 4e). In a classic design, the excitation beam path is orthogonal to the detection beam path. This orthogonal optical path grants LSFM superior resolving power for deep photon penetration into the thick sample because the incident path remains the same at each z-position. In other words, the scattering of excitation

light does not increase with depth. The result is that LSFM can image more than twice as deep as confocal (Pediredla et al., 2016).

In our custom-built LSFM (Fig. 5), beam splitters can be employed to align laser sources of varying wavelengths, facilitating multi-channel illumination. Rapid switching between distinct lasers is accomplished using an acousto-optic tunable filter (AOTF). Subsequently, a beam expander is deployed to broaden the beam, followed by a half mirror that bifurcates the beam. The divided beams are then illuminated from opposite directions to the sample chamber. The beam is subsequently modulated into a light sheet by combining a cylindrical lens and an illumination lens. The sample chamber is designed with multiple axes of freedom (5-axis in Fig. 5), allowing for adjustments of the illumination direction for the specimen. This custom design contributes to the versatility and efficacy of LSFM as a powerful imaging technique in biological research (Ding et al., 2018b; Wang et al., 2021a). With high-speed cameras, LSFM can take images at 200 frames per second, becoming the ideal tool for large-volume 3-D + time scanning with high spatial and temporal resolution. Another important application of LSFM is to allow 3-D imaging for prolonged periods. Unlike confocal microscopy, where most of the emitted light will be blocked by the pinhole, LSFM collects a larger portion of the signal and needs lower laser intensity for excitation, thereby preventing phototoxicity and photobleaching to the specimen.

The LSFM system can be custom-built for rapid data acquisition, followed by a post-image synchronization algorithm for 4-D registration and reconstruction (Wang et al., 2021a). This system provides high spatiotemporal resolution and minimal photobleaching and is suitable for *in vivo* visualization of developing zebrafish embryos' hearts (~0.4 × 0.5 × 0.6 mm^3 at ~30 s) (Ding et al., 2018b) and visualization of AV valve leaflet excursion at five days post-fertilization (dpf) in zebrafish (Baek et al., 2018a). This high spatiotemporal resolution can be experimentally conducive to studying cardiovascular function post-injury and during regeneration. For instance, our group has demonstrated the displacement analysis of myocardial mechanical deformation (DIAMOND) in a doxorubicin injury model using LSFM (Chen et al., 2019). LSFM allowed us to separate the 3-D-reconstructed heart into six radial segments and compare their contractile function after doxorubicin injury. The results showed spatial heterogeneity in myocardial damage and regeneration, revealing that the cardiac susceptibility to doxorubicin differed throughout the heart.

Compared to LSCM, LSFM provides higher imaging acquisition speed, lower phototoxicity, and deep tissue penetration and is uniquely suitable for 3-D imaging of larger tissues (Table 3). Although applications of LSFM in UFP studies have not yet been reported, Wiles et al. demonstrated the use of LSFM in tracing the interaction between two microbial species within the intestine of zebrafish embryos over 12–15 h (Wiles et al., 2016). The 4-D time-lapse imaging allowed for studying the change in size and spatial distribution of the native microbial community upon invasion by another species. In summary, 4-D imaging combined with computational fluid dynamics (CFD) simulation has transformed the study of zebrafish mechanobiology, enabling researchers to quantitatively characterize different components of the cardiovascular system, such as trabeculation (Lee et al., 2018), valves (Hsu et al., 2019), red blood cells (RBCs) (Wang et al., 2021a, 2021b), and vasculature

(Roustaei et al., 2022). Thus, both LSCM and LSFM hold great promises to empower the study of UFP in cardiovascular and gastrointestinal health.

In addition to LSCM and LSFM, several optical/non-optical methods have shown promise in advancing environmental toxicity research (Table 4). Two-photon fluorescence microscopy (2PFM) is another fluorescence imaging method to image living tissue up to about one millimeter in thickness (Helmchen and Denk, 2005). Unlike traditional fluorescent microscopes, where the excitation wavelength is shorter than the emission wavelength, two-photon fluorescence microscopy requires simultaneous excitation by two photons with longer wavelengths than the emitted light (Denk et al., 1990). The laser is focused onto a specific location in the tissue and scanned across the sample to sequentially produce the image. 2PFM is commonly used for intravital imaging of organs or time-lapse imaging of cellular dynamics (Al-Rekabi et al., 2023). For instance, it has been used to trace the real-time deposition of aerosol $PM_{2.5}$ in the murine lung and the clearance of instilled fluorescent beads by the tracheal mucosa (Li et al., 2019; Veres et al., 2017). Despite its low scanning speed and challenging focusing process, 2PFM is a powerful tool for research in the interaction between air pollutants and the pulmonary system at high-resolution.

Further advancements in the field include CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ-hybridization-compatible Tissue hYdrogel) and expansion microscopy (ExM), two groundbreaking strategies that augment imaging depth and resolution by transforming the opaque tissue into a porous transparent hydrogel (Du et al., 2018; Sim et al., 2021). Both CLARITY and ExM use acrylamide/bis-acrylamide to form the gel backbone, while ExM adds sodium acrylate to enable hydrogel swelling. The physical enlargement of a specimen enhances its resolution to the nanoscale, allowing the visualization of zebrafish intestinal microvilli and the bacterial colony residing on them (Sim et al., 2021). Such techniques are also compatible with fluorescent labeling and thus useful for anatomical characterization in toxicity research. For example, researchers have demonstrated the power of ExM to study dietary zinc-mediated toxicity on medaka retinal development (Wang and Wang, 2022). Similar method was used to examine silver nanoparticles-mediated toxicity in zebrafish skeletal muscle and notochord development (Wang and Wang, 2023).

Super-resolution imaging, such as stimulated emission depletion (STED) microscopy, has been extensively employed. STED ingeniously employs a mechanism that deactivates fluorescence in specific sample regions, yielding an extraordinarily concentrated, effectively super-resolved fluorescence spot (Blom and Widengren, 2017). Complementing optical innovations, machine learning-based algorithms, specifically focusing on deep learning, augment the resolution of digital images (Kaderuppan et al., 2020). Such computational techniques enable surpassing the diffraction limit of light, thereby facilitating super-resolution capabilities without necessitating specialized hardware. So far, ultra-high-resolution imaging mainly serves the observation of organelle dynamics and has limited applications in pollutant toxicity research. Nevertheless, these methods opened doors for studying the subcellular damage by pollutants with unprecedented details.

5. Future directions and conclusion

As we gain a deeper understanding of how ambient fine particle pollution by vehicle emissions and industrial combustion affects human health, we uncover another pressing issue: micro- and nano-plastic pollution. Over the last decades, microplastic (MP) abundance and distribution have increased in salt, fresh, drinking, and wastewater, posing an imminent threat to aquatic organisms and humans (Agathokleous et al., 2021; Xu et al., 2020). Plastics are non-biodegradable and become brittle upon ultraviolet (UV) exposure, breaking into smaller fragments that accumulate in marine environments, fresh water, and soil (Fackelmann and Sommer, 2019). Plastic pollution in marine ecosystems threatens marine biodiversity and contributes to the decline of coral reefs worldwide (Pinheiro et al., 2023). Recent studies have shown that nanoplastics (NPs) infiltration triggers cardiotoxicity in zebrafish embryos (Dai et al., 2023; Sun et al., 2021). Moreover, studies in both zebrafish and mouse models have demonstrated that MPs ingestion alters gut microbiota by disrupting the intestinal barrier and triggering metabolic disorders (Jin et al., 2019; Qiao et al., 2019).

However, few studies have shown the size- and shape-dependent accumulation of MPs/NPs in zebrafish and their toxicity (Lee et al., 2019). A study using nano-size polystyrene particles (20 nm) injected in the yolk of zebrafish embryos has unveiled that polystyrene particles can cross the blood-brain barrier and cause oxidative DNA damage in the brain (Sökmen et al., 2020). Another study showed that particles smaller than 50 nm appeared to travel away from the GI system toward the eyes compared to the larger particles that remained in the intestine (Van Pomeren et al., 2017).

Although the exact mechanism of MP/NP transport is still unclear, research has shown that RBCs carriage may be a potential route. A study by Geiser et al. reveals that ultrafine polystyrene spheres (< 1 μm) were up-taken by RBCs, and they are not membrane-bound (Geiser et al., 2005). In addition, treating macrophages with CytD (phagocytosis inhibitor) did not prevent particle movement into the cell. These findings suggest that endocytic pathways which involve vesicle formation are not likely to account for particle translocation. Instead, passive uptake due to particle adhesion caused by Van Der Waal electrostatic interactions proved to be a preferable dissemination course to multiple organs (Rimai et al., 2000).

Since MPs and NPs are ubiquitous in everyday life, they are likely also bound to organic matter. The zebrafish model could provide crucial information on mixture toxicity and the interaction of MPs/NPs with other environmental toxicants through fluorescent labeling, transgenic lines, advanced microscopy, and post-processing machine learning strategies.

In conclusion, due to their small size, short developmental stages, and optical clarity, the zebrafish model has emerged as a valuable animal model for studying environmental pollutants-mediated multi-organ toxicity. Developmentally, this model organism possesses some essential conserved physiology with humans as a promising strategy for forward and reverse genetic approaches. However, more efforts are needed to elucidate the effects of other pollutants, such as MPs and NPs, which threaten aquatic organisms' cardiac and gastrointestinal health. Thus, integrating the zebrafish model with light-sheet imaging

provides a reliable strategy to investigate environmental pollutants' impacts on human health and the underlying mechanism.

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Data availability

Data will be made available on request.

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HIGHLIGHTS

- Environmental pollutants threaten cardiovascular and gastrointestinal health.
- Zebrafish serves as a genetically tractable model to study environmental toxicity.
- 4-D imaging and transgenic reporters provide molecular insights into pollution-impaired organ development and function.

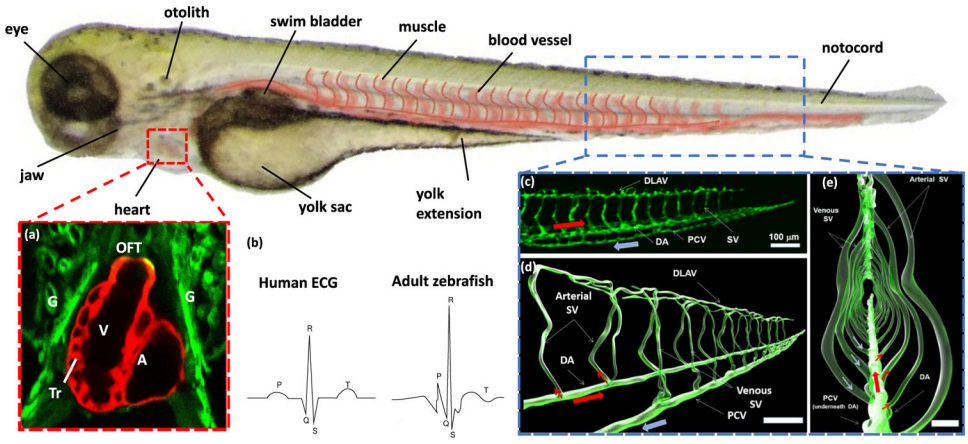

Fig. 1.

Zebrafish system. Anatomy of a zebrafish embryo. The heart is visible at ~1 mm below the skin. (a) Zebrafish have one atrium (A) and one ventricle (V). (b) Comparison between human and adult zebrafish ECG reveals resembling atrial activation and ventricular depolarization features. (c) Zebrafish embryonic cardiovascular circulatory system. Arterial circulation is denoted with arrows in red and venous in blue. (d) This the 3D view reveals that the DA originates from the ventricle, and the PCV returns to the atrium. Arterial SVs connect with the DLAV, which drains to the PCV via venous SVs. (e) Cross-*sec*tional view of 3D embryonic cardiovascular circulatory system; OFT, outflow tract; G, gills; V, ventricle; A, atrium; Tr, trabeculation; DA, dorsal aorta; SV, segmental vessel; DLAV, dorsal longitudinal anastomotic vessel; and PCV, posterior cardinal vein. Panel c, d, and e figures were adopted Mehrdad Roustaei Computational Simulations of the 4D Micro-Circulatory Network in Zebrafish Tail Amputation and Regeneration., doi:https://doi.org/10.1098/rsif.2021.0898.

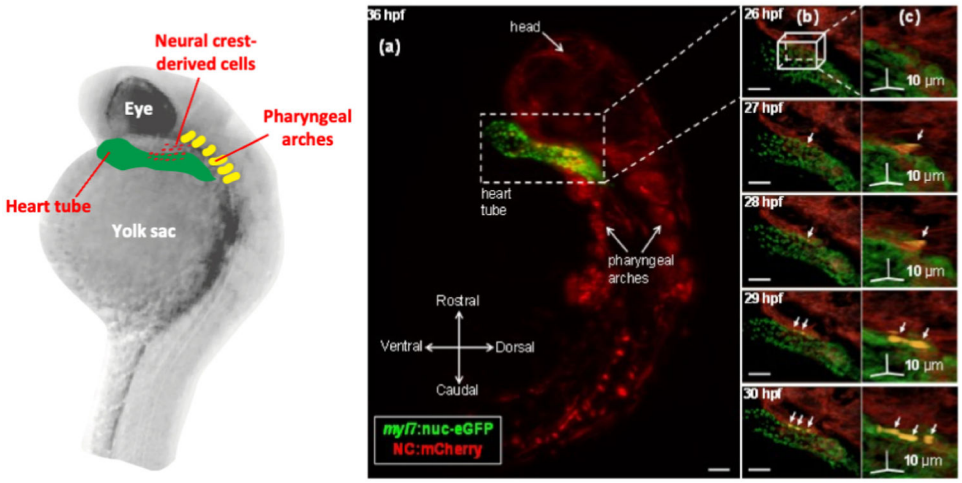

Fig. 2.

Light-sheet fluorescence microscopy imaging of neural crest-derived cells migrating to the developing heart tube. (a) Overview of 36 hpf transgenic zebrafish embryo expressing nuclear eGFP in cardiomyocytes (green) and mCherry in neural crest and neural crest-derived cells (red). By 36 hpf, colocalization of eGFP and mCherry indicates many neural crest-derived cells contribute to the heart tube (yellow signal). (b and c columns) Time-lapse image series of the developing embryonic heart from 26 hpf to 30 hpf shows the integration of neural crest cells into the heart. For *(a)* and *(b)*, scale bar: 50 μm. This figure is adopted from Varun Gudapati Visualization of Neural Crest Cell Migration to the Dorsal Surface of Developing Zebrafish Myocardium, doi:https://doi.org/10.1161/circ.138.suppl_1.17251.

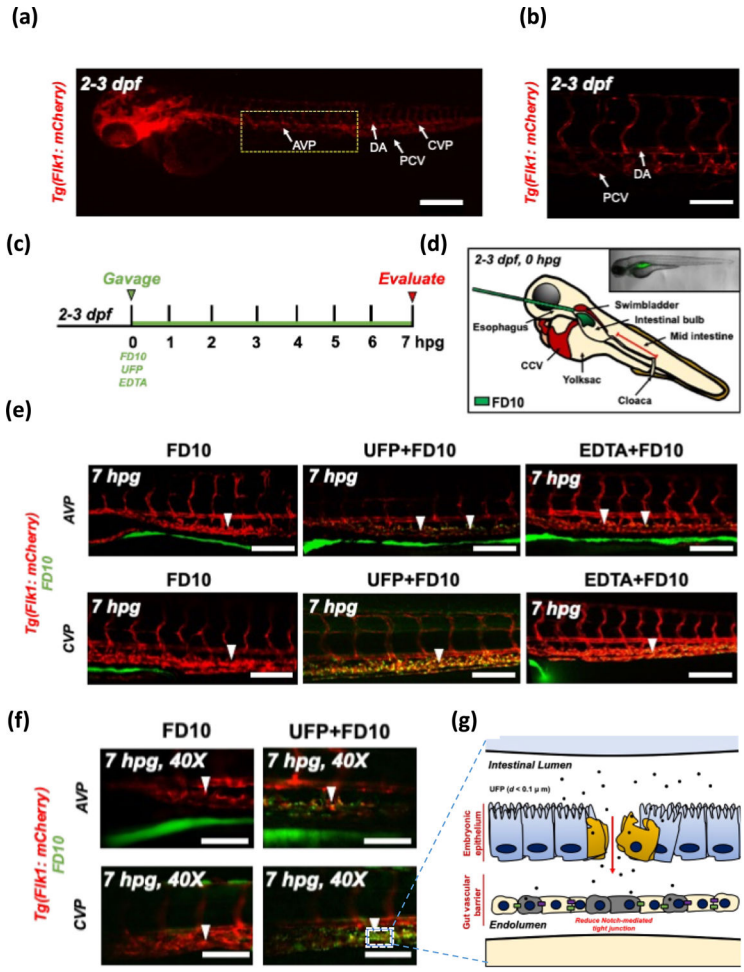

Fig. 3.

Acute UFP ingestion disrupts intestinal epithelial barrier integrity. Transgenic *Tg(flk1: mCherry)* zebrafish embryos at 2 dpf were micro gavaged with FITC-conjugated dextran (FD10, 10 kDa) and UFPs. (a-b) Anatomy of endothelial vasculature in the *Tg(flk1: mCherry)* zebrafish. (c) Experimental design: At 2 dpf, embryos were randomly chosen for micro-gavage with FD10 solution with or without UFP. In parallel, EDTA at 20 mM was gavaged as a positive control. Intestinal epithelial barrier integrity and translocation of FD10 to vascular endothelium (*flk1*$^{+}$) were evaluated at 7 h post gavage (hpg). (d) A schematic representation of micro gavage technique in an embryonic GI tract. FD10 solution was gavaged in the intestinal bulb without disrupting the esophagus, swimming bladder, and yolk sac. (e) Confocal images of the AVP and CVP at 7 hpg. In FD10 gavaged-controls, FD10 retained only in the intestinal bulb and mid-intestine. By contrast, co-gavaging FD10 with UFP or EDTA accumulated FD10 in the AVP and CVP (white arrowheads). Scale bar: 20 μm. (f) 40× confocal images of embryos exhibiting endoluminal FD10 fluorescence as an indicator of UFP infiltration. (g) Graphical representation of intestinal epithelium disruption upon UFP ingestion. DA: Dorsal aorta; PCV: Posterior caudal vein; AVP: Anterior venous capillary plexus; CVP: Caudal vein capillary plexus; Scale bar: 32 μm. This figure is adopted from Kyung In Baek An Embryonic Zebrafish Model to Screen Disruption of Gut-

Vascular Barrier upon Exposure to Ambient Ultrafine Particles., doi:https://doi.org/10.3390/toxics8040107.

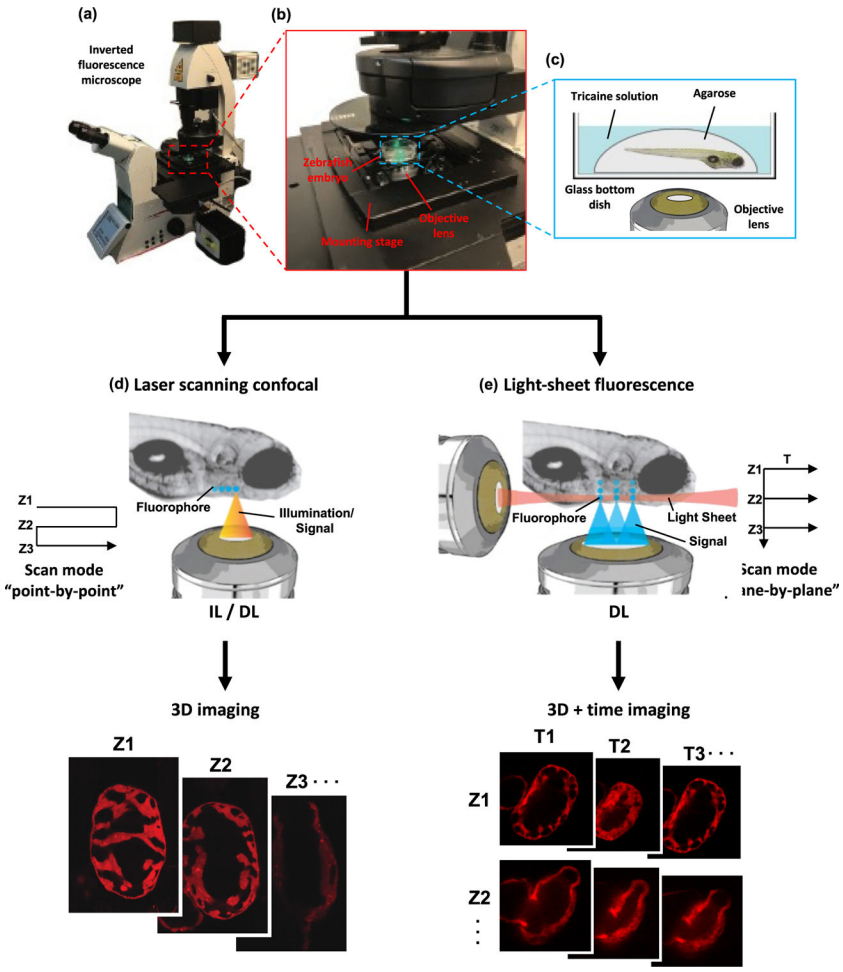

Fig. 4.

Imaging modalities and their working principles for zebrafish UFP study.

(a) Zebrafish embryos can be conveniently imaged on an inverted fluorescence microscope. (b) Enlarged view of the sample mounting region. (c) The embryos can be mounted inside a glass bottom dish with gas-permeable agarose. Submerging in tricaine solution allows for the immobilization of embryos. (d) Laser-scanning confocal microscopy (LSCM) allows high-resolution imaging of subcellular structures by eliminating the out-of-focus signal with a pinhole disk. Only the in-focus signal (orange beam) can pass through the pinhole and reach the camera. A single objective can be used for both the illumination lens (IL) and the detection lens (DL). Due to the "point-by-point" scanning of LSCM, imaging speed is limited, resulting in only 3D stacks. (e) Light-sheet fluorescence microscopy (LSFM) enables fast volumetric imaging by illuminating an entire cross-section of the sample with a thin sheet of light (red beam). The emitted fluorescence signal (blue beam) from each section reaches the camera within the same distance, drastically improving the contrast in the z-direction. Instead of point-by-point scanning, LSFM scans across the sample plane by plane and thus can produce 3D + time stacks.

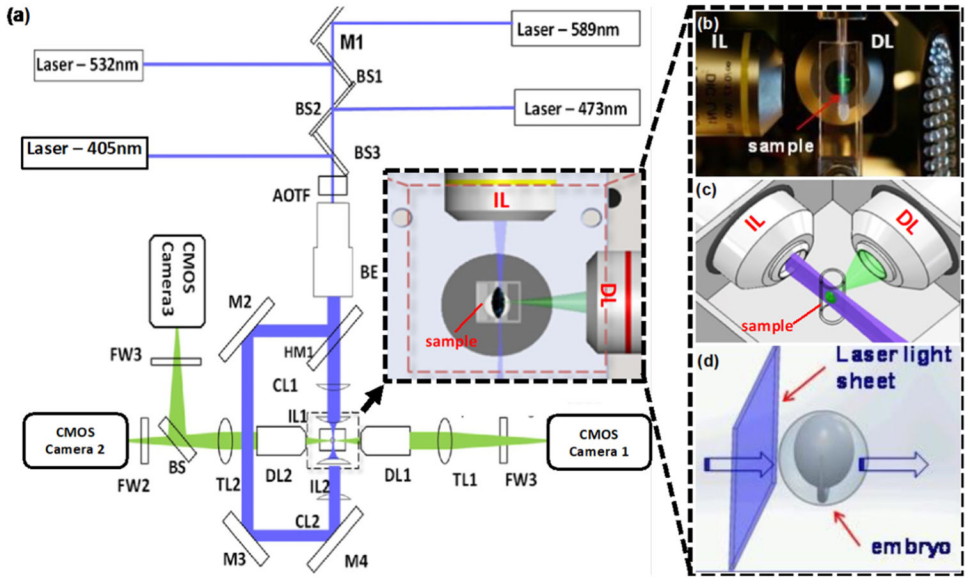

Fig. 5.

Key components and design of a custom multi-color light-sheet imaging system.

(a) The optical path layout of the system is shown, with two cylindrical lenses (CL1, CL2), each converting a laser beam into a sheet of light that illuminates a thin layer of the sample. The resulting fluorescent signal is captured by two detection lenses and passed to three high-speed sCMOS cameras. An acousto-optic tunable filter (AOTF) coupled with filter wheels (FWs) allows for multi-color acquisitions. (b) The sample is positioned at the orthogonal intersection of the illumination lens (IL) and detection lens (DL). (c) A laser light-sheet is applied to illuminate the sample rapidly. The illuminated planes are orthogonally detected by the detection lens (DL). (d) A sheet of light transverses the embryo to produce 3D + time scans. M: mirror; HM: half mirror; BS: beam splitter; AOTF: acousto-optic tunable filter; BE: beam expander; TL: tube lens.

Table 1

Multi-organ effects of air-pollutants on zebrafish system.

Organ	Effects		Publications
Brain	•	Neurotoxicity	(Arab-Bafirani et al., 2022; Barnhill et al., 2020; Ha et al., 2022; Hawkey et al., 2022; Jami et al., 2021)
	•	Neurobehavioral effects	
	•	Neurodegeneration	
Heart	•	Developmental defects	(Cen et al., 2020; Jia et al., 2022; Kompella et al., 2021; Park et al., 2023)
	•	Cardiotoxicity	
Liver	•	Structural changes	(Manjunatha et al., 2022, Manjunatha et al., 2021; Qamar et al., 2022)
	•	Inflammation	
	•	Developmental defects	
Intestines	•	Dysbiosis	(Baek et al., 2020; Chang et al., 2018; Rosenfeld, 2017; Sun et al., 2019)
	•	Gut-vascular-barrier disruption	

Table 2

Comparison of embryonic animal models and their biomedical applications.

Model organisms	Advantages	Limitations	Applications
 *Danio rerio*	• Fast, *ex utero* embryonic development. • Transparent embryos. • Easy genetic manipulation. • High regenerative capacity. • Low cost, fecundity.	• Absence of stomach, lungs, gallbladder, and adaptive immune system. • Primitive cardiovascular system (two-chambered hearts). • Embryos turn opaque after one week of development.	Models for congenital heart disease (Vicente et al., 2022; Xu et al., 2002). High-resolution *in vivo* imaging (Baek et al., 2018a). Fast phenotype identification (Lescouzères et al., 2022). Large-scale drug screening (Zon and Peterson, 2005). Pathway studies with interventions (Baek et al., 2018b; Dai et al., 2023; Schüttler et al., 2017).
 *Mus musculus*	• Developmental similarities with humans. • Innate and adaptive immune system. • Widely available transgenic and mutant lines. • Gold standard of biomedical models.	• The gut microbiota of mice and humans differ significantly. • Notable differences between mouse and human cardiac physiology, including heart rate. • Limited regenerative capabilities in the heart. • *In utero* embryonic development. • High cost, small litters.	Models for placenta and fetus development (Kulas et al., 2018; Weldy et al., 2014). Chronic toxicity study (Choi et al., 2022; Yoshizaki et al., 2015). Models for immune system (Shoenfelt et al., 2009).

Model organisms	Advantages	Limitations	Applications
 *Gallus gallus*	• Direct manipulation and monitoring of embryonic development *ex ovo*. • Closer developmental similarities with mammals compared to fish. • Fewer, smaller genes and fewer pseudogenes compared to fish and mammals. • Low cost, fecundity.	• Differences in cardiac physiology and digestive processes compared to mammals. • Limited regenerative capabilities in the heart. • Non-transparent embryos. • Limited availability of transgenic and mutant lines.	Study of fetus-specific effects in isolation (Sless et al., 2017). Cell fate studies using chimeras (Ballantyne et al., 2021). Testing gene function (Davey and Tickle, 2007; Sauka-Spengler and Barembaum, 2008).

Table 3

Comparison of laser-scanning confocal and light-sheet fluorescence microscopy.

40×, 0.8 NA water lens	Laser-scanning confocal	Light-sheet fluorescence
Lateral resolution (x-y)	0.25 μm	0.37 μm
Axial resolution (z)	1.33 μm	1.65 μm
Acquisition rate *	30 frames/sec **	200 frames/sec
Penetration depth	~100 μm	~250 μm
Photo-toxicity/–bleaching	High	Low

Theoretical values are adapted from Engelbrecht and Stelzer (Engelbrecht and Stelzer, 2006).

* 512 × 512 pixels per frame.

** Maximum speed using resonant scanner.

Table 4

Comparison of imaging methods and their applications.

Imaging methods	Advantages	Limitations	Applications
LSCM	• High resolution. • Easy to use. • Gold standard for biology.	• Slow imaging speed. • High photobleaching. • Short penetration depth.	• Study cellular details of thin samples. • *In vivo* imaging of slow/non-moving targets.
LSFM	• High lateral resolution. • Fast imaging speed. • Low photobleaching. • Long penetration depth. • Highly customizable.	• Lower axial resolution than LSCM. • May have stripe artifacts in thick samples. • Lower resolution than LSCM (long-wavelength laser).	• 3D imaging of large-volume samples. • *In vivo* 3D + time (4D) imaging of fast-moving targets.
2PFM	• Long penetration depth. • Low photobleaching.	• Slow imaging speed. • Difficult to find focus. • Expensive equipment.	• Intravital imaging of mouse organs. • *In vitro* time-lapse imaging of cells.
ExM	• Nanoscale resolution. • Tunable formula. • Does not require special equipment.	• Imaging speed and depth depend on equipment. • Only non-living samples. • Lengthy sample processing. • Distortion of organelle morphology.	• 3D imaging of tissue with complex cellular organization (e.g., brain). • 3D imaging of fine cellular structures.
STED	• Nanoscale resolution (higher than ExM). • Allows live-cell imaging.	• Slow imaging speed. • Short penetration depth. • Require special equipment and fluorescent probes. • High photobleaching and phototoxicity.	• 2D imaging of subcellular structure.