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Zebrafish: An under-utilized tool for discovery in host-microbe interactions

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Abstract

Zebrafish are relatively new to the field of host-pathogen interactions, although they have been a valuable vertebrate model for decades in developmental biology and neuroscience. As transparent larvae, zebrafish have most components of the human innate immune system, while adult zebrafish also produce cells of the adaptive immune system. Recent discoveries using zebrafish include mechanisms of pathogen survival and host sensing of microbes. These discoveries were enabled by zebrafish technology, which is constantly evolving and providing new opportunities for immunobiology research. Some recent tools include CRISPR-Cas9 mutagenesis, *in vivo* biotinylation, and biosensors. Here, we argue that the zebrafish model -- which remains under-utilized in immunology -- provides fertile ground for a new understanding of host-microbe interactions in a transparent host.

Advantages of the Zebrafish Model

Zebrafish have been extensively used to model human development and disease, including genetic diseases such as cystic fibrosis, neurodegenerative disorders such as Alzheimer's disease, and several cancers (reviewed in [1], [2], [3]). The zebrafish genome contains orthologues of 71% of human genes [4]. In addition, zebrafish intrinsically possess many features that make them an ideal model host, such as rapid external development of embryos, a short sexual maturation time of 2-3 months, and large **clutch sizes** (see **Glossary**) [5]. Female zebrafish lay about 300 eggs/week, enabling high-throughput experiments comparing hundreds of siblings. High efficiency genome editing can be performed by injecting eggs [6]. Furthermore, zebrafish embryos and larvae are optically transparent, as are the adults of some transgenic lines [7]. This permits high-resolution *in vivo* imaging, as well as time lapse imaging experiments within intact animals. With a long working-distance objective and a confocal microscope, any site within the body of a larva can be imaged.

To model infectious diseases, zebrafish larvae or adults can be inoculated with human pathogens (Table 1) or natural pathogens of fish. Zebrafish tools, including gene editing or transgenic lines that express fluorescent proteins, can be combined with an infection model of interest (Figure 1). In this article, we examine the use of zebrafish in host-microbe research, with a focus on larval infection models. First, we compare the zebrafish and human

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immune systems. Then, we discuss host-pathogen studies using zebrafish. These include discoveries in cell sensing of pathogens, immune cell responses, and pathogen survival strategies. Finally, we assess recent zebrafish tools that could be modified for use in host-pathogen research. Together, these data argue that zebrafish technology can be leveraged to provide powerful insights into infection biology.

Similarities Between the Zebrafish and Human Immune Systems

The immune systems of zebrafish and humans are highly similar. Larvae (1-2 weeks post-fertilization) can be used to study the innate immune system in isolation, while the adaptive immune system develops by 4-6 weeks post fertilization [8]. Zebrafish possess innate immune cell types, such as macrophages and granulocytes, that functionally resemble their mammalian counterparts. For example, zebrafish macrophages engage in phagocytosis [9], produce pro- and anti-inflammatory cytokines [10], and form **granulomas** [11]. Two main types of granulocytes are characterized in zebrafish: neutrophils and eosinophils [12]. Zebrafish neutrophils are capable of phagocytosis [13], respiratory burst [14], and production of **neutrophil extracellular traps (NETs)** [15]. Eosinophils in zebrafish have been shown to respond to parasitic helminths [16], as in humans [17]. *In vitro,* zebrafish eosinophils degranulate upon exposure to the rodent pathogen *Heligmosomoides polygyrus*, and increase in number in the gut in response to infection by the zebrafish pathogen *Pseudocapillaria tomentosa* [16]. Mast cells have also been characterized in zebrafish and are similar in morphology to those in humans [18]. Zebrafish and human mast cells function similarly, including degranulation and participation in systemic anaphylaxis, as measured in adult zebrafish by plasma **tryptase** concentration [19]. Given these similarities, host-microbe findings in zebrafish infection can often be translated to the human disease, especially in the zebrafish-*M. marinum* model of tuberculosis (TB). For example, a zebrafish gene associated with susceptibility to TB in humans, *lta4h,* emerged as a susceptibility locus for *Mycobacterium marinum* in a zebrafish genetic screen [20]. Remarkably, the zebrafish-*M. marinum* model predicted improved survival of TB meningitis patients treated with dexamethasone, based on the patient's *LTA4H* genotype [21]. Therefore, the immunological similarities between human and zebrafish enable zebrafish tools to be applied to questions addressing infectious diseases in patients (see Clinician's Corner).

Zebrafish have conserved innate immune receptors, including **toll-like receptors (TLRs)** [28]. Most zebrafish TLRs, as well as their adaptors, function similarly to those in humans ([28], [29], [30], [31]). TLR adaptors identified in zebrafish include **myeloid differentiation factor 88 (Myd88)**, **Myd88-adaptor like (Mal2)**, **sterile α, and HEAT-Armadillo motifs (Sarm1)** ([28], [29], [30], [31]). A **morpholino,** used to inhibit Myd88 translation, impaired clearance of a normally-nonpathogenic strain of *Salmonella enterica* [31]. This showed a functional role for TLR signaling in zebrafish. Indeed, zebrafish and human TLRs respond to similar **pathogen-associated molecular patterns**, such as dsRNA and flagellin (reviewed in [32]). Downstream of **pattern recognition receptors**, zebrafish have conserved signaling pathways, including nuclear factor kappa B (NF-κB) [33] and type I and II interferons [34]. In addition, zebrafish have a conserved cytosolic DNA sensing pathway through cyclic GMP–AMP synthase (cGAS) [35]. In zebrafish, as in humans, cGAS activates stimulator of interferon genes (STING), which mediates type I interferon production [36]. However,

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zebrafish STING has a C-terminal tail with an additional motif that results in stronger NF-κB activation and weaker interferon regulatory 3 (IRF3) signaling than human STING [37]. Additionally, most human chemokine receptors have at least one zebrafish ortholog and are conserved in function (reviewed in [38]). However, some chemokines and their receptors, such as Cxcl12 [39] and Cxcr3 [40], have additional paralogues with functional differences in zebrafish. Despite some differences, the conservation of most immune cell types and signaling pathways lays the foundation for modeling human infections in zebrafish.

Using Zebrafish to Understand Cell Autonomous Sensing of Microbes

The role of Myd88 signaling downstream of TLRs in immune cell responses has been examined in zebrafish. TLR signaling through Myd88 is important in the immune response to *Mycobacterium tuberculosis* in mice [42]. Similarly, Myd88 signaling contributes to the host response to *M. marinum* in zebrafish [41], where it causes a tuberculosis-like disease (reviewed in [43]). Indeed, in *M. marinum*-infected zebrafish embryos lacking functional Myd88, bacterial burden increased [41]. To understand the role of Myd88 in fungal infection, *myd88* mutant larvae were infected with *Aspergillus fumigatus* and were more susceptible to infection compared to wild-type larvae [44]. Moreover, a study comparing **germ-free** and **conventionalized larvae** showed that intestinal colonization by microbes decreased expression of *myd88* and downstream signaling components, such as the **Activator protein 1** (**AP-1**) **transcription complex** [45]. The decreased *myd88* expression observed in wildtype was absent in *tlr2* mutant larvae, demonstrating that the effects on Myd88 were Tlr2-mediated [45]. Collectively, these studies indicate that zebrafish have been a useful tool to examine how cells sense microbes.

Signaling pathways of polarized innate immune cells, such as macrophages, have been explored in zebrafish. Differentiated macrophages have been visualized *in vivo* using double transgenic zebrafish expressing eGFP from the *tnfa* promoter and dsRed from the macrophage-specific promoter, *mpeg1* [10]. Confocal microscopy identified *tnfa*$^{+}$*mpeg1*$^{+}$ macrophages, which correspond to **pro-inflammatory macrophages**, responding to infection by *Escherichia coli* [10]. Additionally, to understand macrophage polarization in zebrafish, single-cell RNA-sequencing was used to identify subpopulations of macrophages undergoing **type 2 signaling** within granulomas [46]. Specifically, these signals were necessary for macrophage epithelialization (determined by E-cadherin immunostaining) and necrotic granuloma formation (visualized by l-plastin leukocyte immunostaining and fluorescently labelled bacteria) [46]. Also, in a zebrafish model of leprosy, caused by *Mycobacterium leprae,* infection increased the number of **inducible nitric oxide (iNOS)**-positive macrophages [47]. In this model, macrophage-depleted larvae had reduced myelin damage compared to wildtype controls, demonstrating that macrophages mediated early demyelination [47]. Another type of pro-inflammatory macrophage, **foamy macrophages,** were identified in larvae infected with the eukaryotic parasite *Trypanosoma carassii.* In infected larvae, foamy macrophages were identified by high lipid content and expression of pro-inflammatory genes, such as *tnfa* and *il1b* [48]. This study suggests a potential inflammatory role for foamy macrophages, often seen in inflammatory metabolic disorders such as hyperlipidemia, in extracellular trypanosome infection [48]. These examples

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demonstrate the contributions of zebrafish transgenics and transcriptomics in defining how cells respond to microbes *in vivo*.

Using Zebrafish to Understand Pathogen Survival Strategies

Zebrafish have been used to understand how pathogens disseminate and survive in a host. For example, studies in zebrafish have revealed the complex roles of macrophages within granulomas during *M. marinum* infection ([49], [50]). Although macrophages are important in controlling bacterial numbers, infected macrophages can spread *M. marinum* deeper into tissues [49]. Live confocal microscopy in zebrafish shows infected macrophages leaving a granuloma and seeding secondary granulomas [50]. A similar process is thought to occur in mice, where *M. tuberculosis* disseminates from the lung within alveolar macrophages [42]. Further, zebrafish fungal infection models show that infected macrophages protect pathogens *Talaromyces marneffei* and *A. fumigatus* from neutrophil-mediated killing ([44], [51]) as there was decreased fungal burden upon macrophage depletion ([51], [44]). These findings have implications for host-directed therapies in *A. fumigatus* and *T. marneffei* infections. Specifically, therapeutics that increase neutrophil recruitment or decrease the ability of macrophages to serve as a protective niche may be beneficial. Thus, zebrafish can be used illuminate some of the intracellular survival strategies of microbes.

Some pathogens can facilitate the spread of infection by transferring to a more favorable cell type. For example, *M. marinum* can escape from resident macrophages to more growth-permissive monocytes after a transfer event between the two cells [52]. Confocal microscopy enabled visualization of macrophage – monocyte convergence, then separation of the two cells, followed by appearance of the bacteria in the monocyte [52]. Transfer to monocytes was dependent on the surface glycolipid, phenolic glycolipid (PGL) [52]. Additionally, some fungal pathogens have been shown to exploit intercellular exchange to transfer to a new cell without being exposed to the environment. In zebrafish, a new process was discovered termed “shuttling” in *T. marneffei* and *A. fumigatus,* which was observed by confocal microscopy [53]. Shuttling is the transfer of fungal spores, or conidia, from live neutrophils to live macrophages [53]. This process is mediated by β-glucan, a polysaccharide component of the fungal cell wall, as β-glucan-coated beads were sufficient to induce shuttling [53]. Shuttling enables the pathogen to move from a fungicidal neutrophil to a growth-permissive macrophage [53]. Further, fluorescently-labeled zymosan was transferred from murine neutrophils to macrophages *in vitro*, demonstrating that shuttling is likely a conserved process among vertebrates [53]. Indeed, research on intercellular pathogen exchange has expanded our understanding of how pathogens navigate a complex, multicellular environment. These studies further demonstrate that pathogen-dependent factors can mediate phagocyte-phagocyte interactions.

Studying Host-Pathogen Interactions With Zebrafish Tools

Gene editing

Recent genetic, transcriptomic, and imaging technologies in zebrafish can be adapted to advance host-pathogen research. CRISPR-Cas9, a gene editing tool, can rapidly generate stable knockout or knock-in zebrafish lines ([23], [24]). CRISPR-Cas9 has been used in

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zebrafish infection models, as when a *stat6* mutant was used to show the role of type 2 signaling in granuloma formation [46]. Cell-type specific mutations can be made by expressing Cas9 from a cell-specific promoter [54]. CRISPR technology can be used for rapid **reverse genetic screens**, by injecting zebrafish eggs with Cas9 and pooled guide RNAs that target many genes simultaneously [25]. Rapid reverse genetic screens, while not feasible in mammals, are possible in zebrafish due to their large clutch sizes, and the high efficiency of CRISPR-Cas9 genome editing [6]. Reverse genetic screens offer an unbiased approach to discover host factors important in infection, while providing a streamlined way to connect a gene to its associated mutant phenotype. While CRISPR-based reverse genetic screens are relatively new in zebrafish, **forward genetic screens** have been a successful high-throughput approach for some time ([55], [56]). For example, in the zebrafish-*M. marinum* model, a forward genetic screen identified susceptibility loci shared by human TB, including *lta4h* [20]. These genetic tools combined with zebrafish infection models hold promise for the discovery of new host factors important in infection.

Fluorescent transgenic lines

Other approaches include fluorescent transgenic lines, which when combined with *in vivo* imaging, are powerful tools in zebrafish. The Zebrafish Tol2kit [57] uses the Tol2 transposase and **Gateway cloning** to simplify generation of cell-specific fluorescent reporter lines. Thus, transgenic fluorescent zebrafish lines that label specific cell types are commonly used to study immune cell behavior and migration ([58], [59]). Transgenics can be infected with fluorescently-labelled microbes to visualize encounters between pathogens and host cells. For example, fluorescent *M. marinum* can be observed in transgenic zebrafish with labelled blood vessels and macrophages (Figure 2). In another example, the ISG15:GFP transgenic zebrafish line expresses GFP under the interferon-stimulated gene 15 (*isg15*) promoter to enable live imaging of type I interferon signaling [60]. Photoconvertible fluorophores, such **Dendra2** [61], can enable tracking cells at specific locations with localized application of light from a confocal microscope. For example, Dendra2 was expressed in neutrophils for cell tracking in larvae lacking **myeloid-derived growth factor (MYDGF)** [62]. Neutrophils were photoconverted at the wound site to label the cells responding to injury [62]. Increased photoconverted neutrophils remained in the wound in the *mydgf* mutant, suggesting a deficiency in inflammation resolution [62]. Tracking the fate of specific cells is a unique feature of zebrafish that holds promise to advance our understanding of immune cell migration.

Optogenetics

Along with photoconversion, optogenetic tools have emerged as a strategy to obtain precise spatiotemporal control over gene expression or protein activity in animal models, including zebrafish. This technology uses transgenic expression of a light-activated protein, which can be expressed in a tissue-specific manner. Optogenetic tools have been used to control gene expression, regulate protein localization, or induce cell ablation *in vivo* ([63], [64], [65]). For example, the transcription factor **TA4-EL222 (TAEL)** is activated by blue light [63]. The TAEL system was used in zebrafish for light-induced expression of *sox32*, a transcription factor known to direct formation of endoderm [63]. *sox32* was induced ectopically at specific locations by using light to activate TAEL, causing conversion of ectoderm into

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endoderm [63]. In another example, cells were ablated by using light-activated **GAVPO** to induce expression of the cytotoxic **M2 protein** ([64],[65]). GAVPO is a light-activated form of the GAL4 transcription factor [65]. To achieve targeted cell ablation, a cytotoxic version of the M2 channel can be expressed from the GAVPO promoter. This system was expressed in zebrafish neurons for light-inducible neuron ablation [64]. Many of these emerging optogenetic tools have not yet been used in combination with zebrafish infection models, but could be applied to study host-pathogen interactions. For example, GAVPO could be expressed in macrophages for light-activated depletion of macrophages at particular locations or specific time points in embryonic development prior to infection. This could help identify roles of different populations of macrophages based on their location of origin.

Transcriptomics

From another angle, transcriptomic methods in zebrafish offer the ability to understand global gene expression changes during infection. RNA-sequencing (RNA-seq) can identify host gene signatures [66] and cell populations important for host defense [46]. RNA can be obtained from whole zebrafish larvae, adult organs, or FACS-sorted cells. Recently, *in vivo* biotinylation has emerged as a method to isolate cell type-specific RNA [67]. Constructs in the *in vivo* biotinylation ("biotagging") toolkit express the biotin ligase, BirA, from a cell-specific promoter so it can biotinylate an avidin tag attached to nuclei or ribosomes [67]. The RNA isolated in this way represents actively transcribed genes (nuclei), or actively translated proteins (ribosomes) [67]. Another application of RNA-seq is the simultaneous transcriptome analysis of pathogen and host (**dual RNA-seq**), which allows the identification of linked host and pathogen phenotypes [68]. Dual RNA-seq analysis was performed in zebrafish infected with a clinical isolate of *Pseudomonas aeruginosa* to analyze RNA expression of both the pathogen and the host [69]. This identified virulence genes upregulated in *P. aeruginosa*, such as proteases and **superoxide dismutases**, and the upregulation of host iron and phosphate acquisition genes, suggesting that these nutrients may be limited during *P. aeruginosa* infection [69]. Studies such as this in zebrafish may identify new therapies that disrupt key interactions between host and pathogen.

Biosensors

Another zebrafish tool is genetically-encoded **biosensors,** which typically use fluorescence to indicate the concentration of a biomolecule or a biophysical force. For example, biosensors can sense calcium concentrations [70], Annexin V (apoptosis) [71], or mechanical forces [72]. A calcium biosensor based on calmodulin, GCaMP3 can be expressed in zebrafish under a cell-specific promoter to enable *in vivo* imaging of calcium signaling [70]. This approach was used in *Tg(LysC:GCaMP3)* larvae, which express GCaMP3 under the neutrophil-specific *LysC* promoter [70]. In this study, GCaMP3 fluorescence was imaged by light-sheet microscopy and radiometric imaging [70]. When larvae were infected with *P. aeruginosa*, a whole-cell, pulsatile calcium increase in neutrophils was observed after phagocytosis of bacteria [70]. This response is similar to a pulsatile rise in calcium observed in cultured human neutrophils after phagocytosis of opsonized antigens [73]. Thus, this biosensor can be used to visualize calcium signaling *in vivo,* at different steps of the infection process. In summary, zebrafish genetic, imaging,

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and transcriptomic tools (Figure 3) provide unique opportunities to examine mechanisms underlying host-pathogen interactions.

Concluding Remarks

In this opinion article, we argue that the many experimental advantages of zebrafish make this model an ideal system to investigate host-pathogen interactions. We provided examples to illustrate the breadth of discoveries that come from using zebrafish for host-pathogen research. Use of imaging, genetic, biosensors, and transcriptomic tools in zebrafish can thus provide novel insights into infectious disease pathogenesis. As with all model systems used in biomedicine, findings must be confirmed in human cells or clinical settings. Differences between the zebrafish and human immune systems, such as instances of gene duplication and divergence of chemokine receptors ([39], [40]) must be considered for results to be meaningful. While these differences may complicate translatability of findings in some cases, they can also be leveraged to understand the evolutionary conservation of pathways in host-pathogen interactions. Another limitation of the zebrafish model is the limited availability of zebrafish-specific antibodies. This limits the use of immunofluorescence or Western blotting, although genetic approaches are often sufficient.

Future work in zebrafish will be important to expand our understanding of pathogens that cause disease in humans (see Outstanding Questions), particularly with infections that have limited therapies, poor prognosis, and damaging side effects. Further, zebrafish may also serve as a useful preclinical model to find new therapies for infectious diseases through screening of compounds. Essentially, zebrafish enable high-throughput experiments, facile genetics, and live imaging in a transparent vertebrate with a similar immune system to humans. As technologies continue to advance, zebrafish infection models can be used to understand emerging pathogens as well as infectious diseases that continue to become more resistant to antimicrobials.

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Glossary:

Activator protein 1 (AP-1) transcription complex
Transcription factor family that regulates gene expression in response to environmental stimuli, including cytokines and growth factors

Biosensors
Indicators of biomolecule concentration; typically use fluorescence

Conventionalized larvae
Previously germ-free larvae that are colonized by microbiota communities, which may be extracted from non-germ free fish intestines or the media of non-germ free fish

Clutch size

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Number of eggs laid during a spawning event

Dendra2

Green fluorescent protein that emits red fluorescence in response to visible blue or UV-violet lights

Dual-RNA Sequencing

Method to measure gene expression changes in pathogen and host simultaneously

Foamy macrophages

Large, granular macrophages with high cytoplasmic lipid content

Forward genetic screen

Assay that induces random mutagenesis, often through chemicals or irradiation, to generate mutant phenotypes

Gateway cloning

A method to assemble multiple DNA fragments using recombination strategies

GAVPO

Light-activated form of the GAL4 transcription factor

Germ-free larvae

Raised in absence of microorganisms from embryos sterilized by a treatment procedure often containing antibiotics and bleach

Granulomas

Organized aggregates of immune cells; predominantly macrophages

Inducible nitric oxide (iNOS)

Enzyme that produces nitric oxide as an immune defense mechanism

Morpholino

Antisense oligomers for gene knockdown

Myeloid-derived growth factor (MYDGF)

Bone-marrow derived secreted protein with functions in cardiac repair

Myeloid differentiation factor 88 (Myd88)

Adaptor protein that interacts with TLRs to initiate signaling, leading to the activation of downstream transcription factors, such as NF-κB

MyD88-adaptor like (Mal2)

Toll like receptor adaptor molecule that transduces signals from the membrane to the cytosol

M2 protein

Influenza A proton channel normally activated by low pH; constitutively active mutant form causes rapid call death

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Neutrophil extracellular traps (NETs)

Extracellular DNA-histone complexes and proteins that can trap pathogens

Pathogen-associated molecular patterns (PAMPs)

Conserved microbial molecules that can be recognized by pattern recognition receptors

Pattern recognition receptors (PRRs)

Proteins that recognize conserved microbial molecules; have a key role in innate immunity

Pro-inflammatory macrophages

Phagocytic cells that produce pro-inflammatory cytokines and microbicidal molecules such as reactive oxygen species

Reverse genetic screens

Assay that disrupts known genes to test for mutant phenotypes

Sterile α and HEAT-Armadillo motifs (Sarm1)

Toll like receptor adaptor molecule that transduces signals from the membrane to the cytosol

Superoxide dismutase

Breaks down a harmful reactive oxygen species into oxygen (O_2) and water

TA4-EL222 (TAEL)

Transcription factor that activates when illuminated with blue light

Tol2 transposase

Enzyme that catalyzes transposition of DNA from a transposon donor plasmid into a genome

Toll-like receptors (TLRs)

Pattern recognition; often membrane-bound, initiate the innate immune response through interactions with adaptor proteins

Tryptase

An enzyme released from activated mast cells

Type 2 Signaling

Immune polarization commonly observed in allergic inflammation or parasitic helminth infection

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Clinician's corner box:

- The zebrafish immune system is highly similar to that of humans. Researchers can study innate immune responses to infection in zebrafish larvae (1-2 weeks post fertilization), and adaptive responses in adults (after about 4-6 weeks) [8].
- Researchers can infect larvae or adults using immersion, injection, or foodborne routes [22]. The type of injection can be determined based on where the pathogen is studied (localized vs. systemic infection) and what tissue or cell type is being analyzed.
- Zebrafish can complement clinical data with rapid and efficient gene editing tools. For example, CRISPR-Cas9-mediated knockouts in zebrafish can provide functional data for a locus associated with susceptibility to infection ([23], [24], [20]). Furthermore, reverse genetic screens in zebrafish could be a useful tool to discover novel susceptibility loci to different pathogens [25].
- Patient alleles in zebrafish, or "avatars," can be functionally studied through CRISPR-mediated knock-ins of specific mutations (reviewed in [26]). Zebrafish patient avatars could be tested for susceptibility to infections or used for drug screens, for a personalized medicine approach to finding host-directed treatments.
- Xenotransplantation of patient samples or human cell lines is possible in zebrafish larvae [26]. Kaposi's sarcoma associated herpesvirus (KSHV)-infected human B cells and epithelial cells were able to engraft and proliferate into the yolk sac of larvae to understand viral activity *in vivo* [27].

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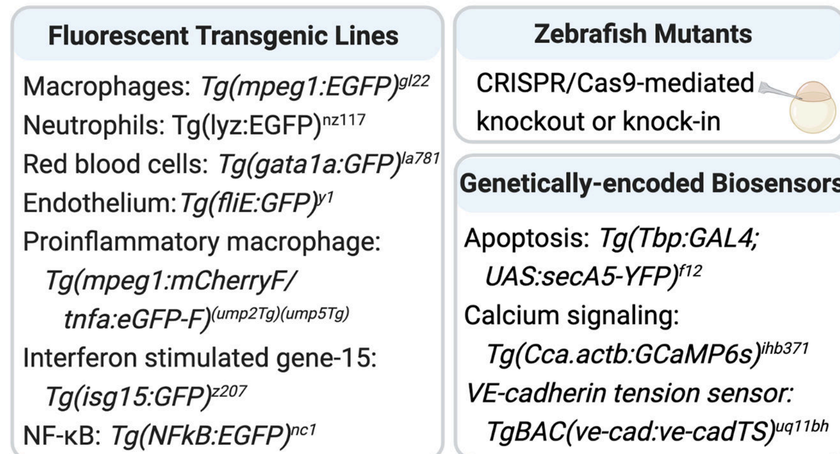
Outstanding Questions:

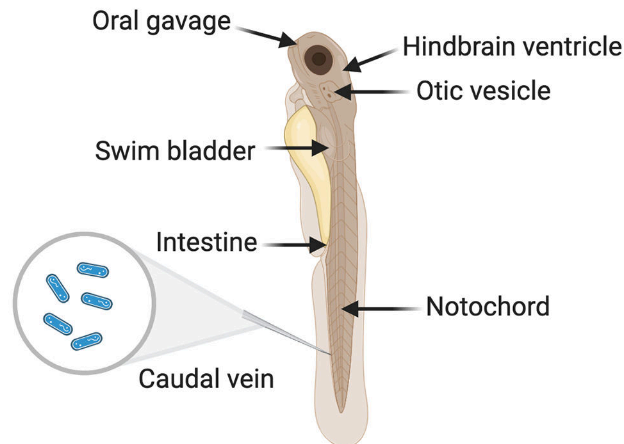
- How do host factors, such as genetics and the microbiome, mediate different immune responses across individuals responding to the same pathogen?
- Can reverse genetic screens using CRISPR/Cas9 in zebrafish be used to identify new targets for host therapy in infections that have acquired antibiotic resistance?
- How can researchers best utilize the increasing power of transcriptomics, proteomics, and lipidomics in zebrafish to uncover new mechanisms in host defense?
- How should discoveries in host-pathogen research in zebrafish be confirmed in humans?

How to Design a Zebrafish Infection Experiment

1 Select or create a zebrafish line



2 Infect fish



3 Collect and analyze data

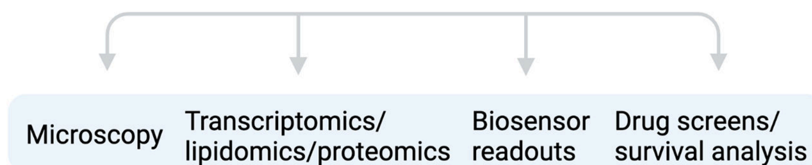


Figure 1.

How to Design a Zebrafish Experiment

The basic workflow for designing a zebrafish infection experiment, highlighting relevant technology for studying host-pathogen interactions. (1) Select an established transgenic line, generate a transgenic line using Tol2 transposase [57], or create a stable knockout or knock-in line using CRISPR/Cas9 gene editing [23], [24]. The fluorescent transgenic reporter lines listed may be particularly useful in host-pathogen studies. Names of these transgenic lines are as follows: Tg(*mpeg1:EGFP*)^{gl22} (macrophages) [58], Tg(*lyz:EGFP*)^{nz117}

(neutrophils) [74], Tg(*gata1a:GFP*)^{la781} (red blood cells) [75], Tg(*fliE:GFP*)^{y1} (endothelium) [76], Tg(*mpeg1:mCherryF/tnfa:eGFP-F*)$^{(ump2)(ump5)}$ (pro-inflammatory macrophages) [10], Tg(*isg15:GFP*)^{z207} (interferon stimulated gene-15) [60], and Tg(*NFkB:EGFP*)^{nc1} (NF-κB) [77]. Transgenic lines expressing genetically-encoded biosensors may also be adapted to infection experiments. For example, TgBAC(*ve-cad:ve-cadTS*)^{uq11bh} enables imaging of tension forces across endothelial cells (VE-cadherin tension sensor) [72]. Tg(*Tbp:GAL4; UAS:secA5-YFP*)^{f12} allows for detection of apoptosis by expressing YFP tagged to Annexin V under the control of a ubiquitous *Tbp* promoter [71], and Tg(*Cca.actb:GCaMP6s*)^{ihb371} enables imaging of calcium signaling [78]. (2) Possible methods of larvae infection include immersion [79] or injection of pathogens. Oral microgavage [80] may also be used to deliver pathogens directly into the intestinal lumen through the mouth. Caudal vein injections [81], [82], [83] have been useful for widely distributing pathogens through the body, while hindbrain ventricle [44], [84], [85], [86] otic vesicle [87], swimbladder [88], and intestinal injections [80] allow assessment of cell recruitment to specific tissues. (3) Examples of data collection/analysis include microscopy, "-omics" (transcriptomics, lipidomics, and proteomics), biosensor readouts (typically fluorescence). For screens, the analysis depends on the phenotype.

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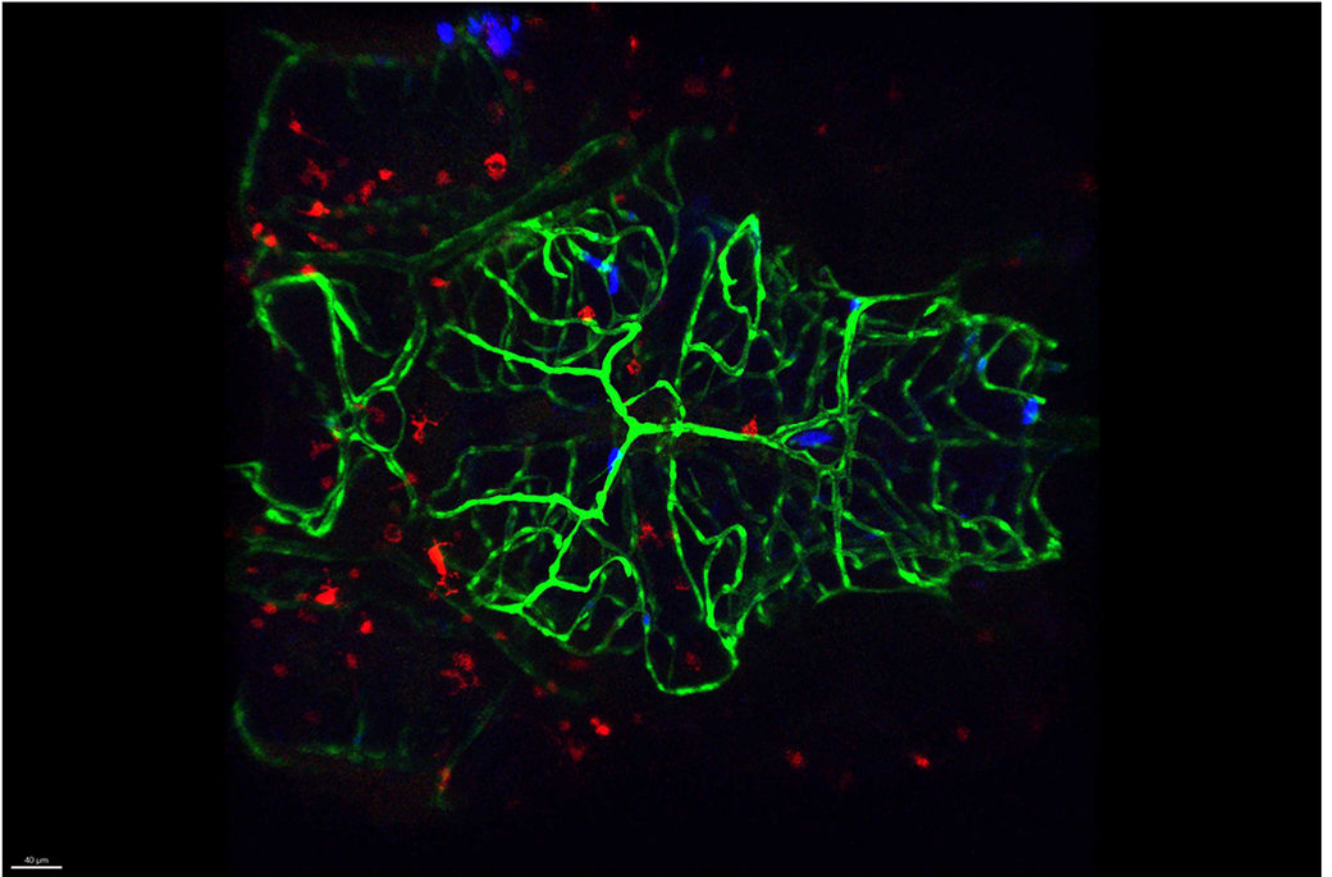

Figure 2.
Whole brain confocal image of *M. marinum*-infected larva

Whole brain confocal image of *mpeg1:dsRed;flk:GFP* larva at 5 days post infection (8 days post fertilization). Larvae were infected by caudal vein injection with blue fluorescent protein-labelled *M. marinum*. *flk:GFP* labels blood vessels, and *mpeg1:dsRed* labels macrophages. Bacteria (blue) can be observed in the vessels (green) near surrounding macrophages (red). Scale bar, 50 µm.

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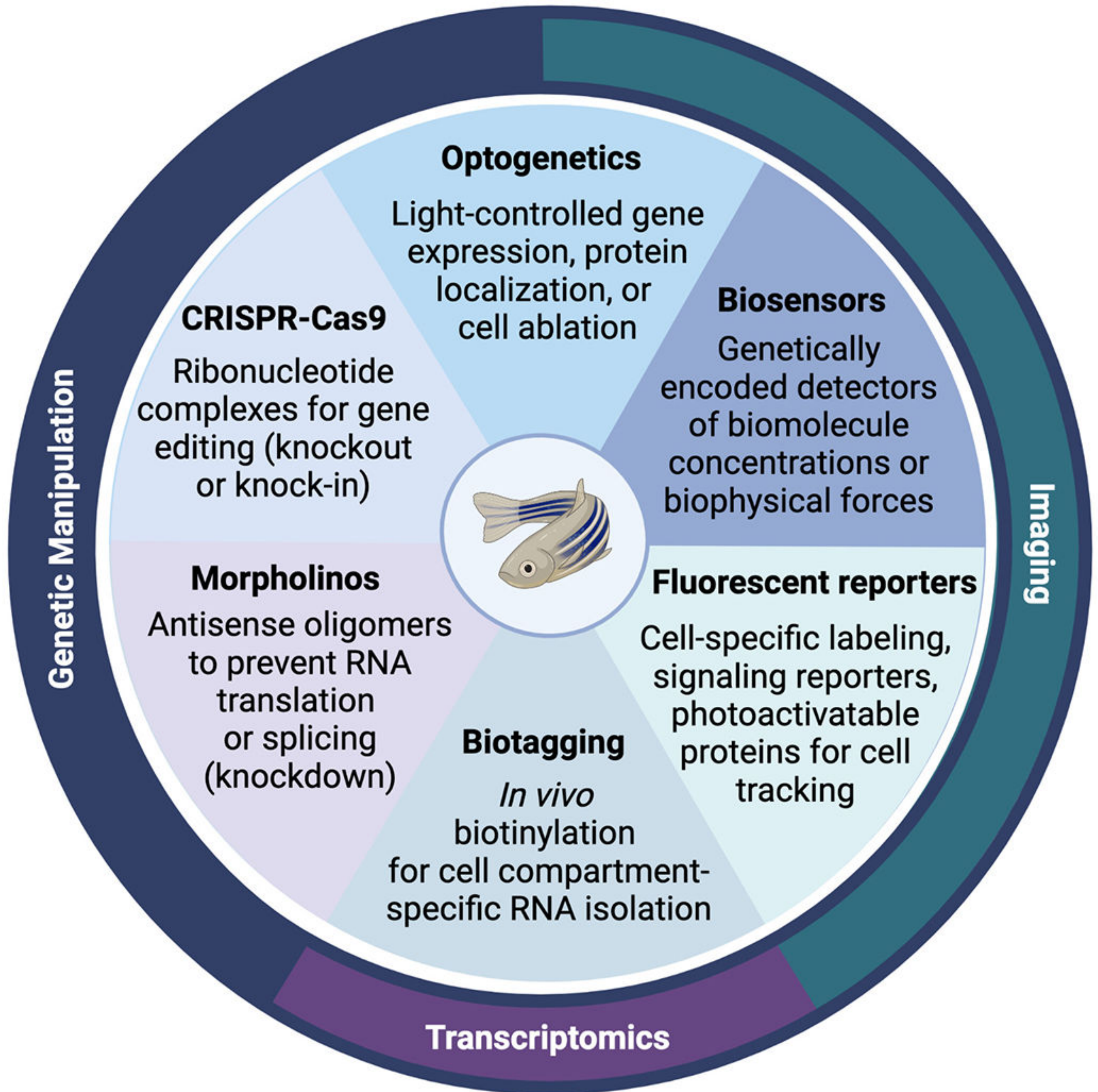

Figure 3.

Zebrafish Technology

Overview of zebrafish tools and technology discussed in this article. Zebrafish are highly amenable to genetic manipulation by CRISPR-Cas9 or morpholinos. CRISPR-Cas9 gene editing is achieved by injection of single guide RNA and Cas9 protein into single-cell eggs to for targeted mutations [23]. Knock-in mutations can be generated by addition of donor plasmid DNA [24]. Morpholinos are antisense oligomers delivered by injection into single-cell eggs [89]. Morpholinos prevent RNA translation or splicing, resulting in

knockdown of a target gene [89]. In addition, optogenetics tools use light to regulate of gene expression, protein localization, or cell ablation *vivo* ([63], [64], [65]). Optogenetic tools allow for *in vivo*, light-directed manipulation of a gene of interest, which is enabled by the transparency of zebrafish larvae. Furthermore, imaging tools include transgenic fluorescent reporters and biosensors. A fluorophore is placed under a specific promoter to label a cell type or signaling pathway. Biosensors are indictors of the concentration of a biomolecule or biophysical force, allowing localization and quantification through imaging. One example is the GCaMP3 calcium indicator, which can enable live imaging of intracellular calcium concentrations [84]. Lastly, transcriptomics in zebrafish offer ways to measure differential RNA expression. The biotagging toolkit [67] enables isolation of cell-specific RNA from actively transcribed genes or actively translated proteins.

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Table 1.

Examples of human infection models in zebrafish embryos and larvae.

	Developmental stage	Infection method	Citation
Bacteria			
Burkholderia cepacia	Embryos	Injection into blood island or axial vein	[80]
Clostridioides difficile	Larvae	Microgavage; injection into intestinal lumen	[81]
Escherichia coli	Embryos	Intravenous or hindbrain injection	[82]
Listeria monocytogenes	Larvae	Immersion; injection of yolk sac, hindbrain ventricle, or blood island	[83]
Mycobacterium abscessus	Embryos, larvae	Caudal vein injection	[84]
Mycobacterium leprae	Embryos	Injection into hindbrain ventricle, caudal vein, or spinal cord	[43]
Mycobacterium tuberculosis	Larvae	Caudal vein injection	[85]
Pseudomonas aeruginosa	Embryos	Yolk circulation valley injection	[86]
Salmonella enterica (serovar Typhimurium)	Embryos	Injection into yolk sac or otic vesicle	[87]
Shigella flexneri	Larvae	Injection into hindbrain ventricle or caudal vein	[88]
Staphylococcus aureus	Embryos	Injection into yolk or yolk sac circulation valley	[89]
Staphylococcus epidermidis	Larvae	Immersion	[90]
Streptococcus pneumoniae	Embryos	Blood circulation valley injection	[91]
Vibrio cholera	Embryos, larvae	Immersion	[92]
Viruses			
Chikungunya virus	Larvae	Injection into caudal vein or aorta	[93]
Herpes simplex virus 1	Larvae	Injection into hindbrain ventricle or caudal vein	[31]
Influenza A virus	Embryos, larvae	Injection into swim bladder or Duct of Cuvier	[94]
Kaposi's sarcoma-associated herpesvirus (KSHV)	Embryos	Xenograft – injection of KSHV-infected B cells or epithelial cells into yolk sac	[3]
Norovirus	Larvae	Yolk sac injection	[95]
Sindbis virus	Larvae	Injection into caudal vein, aorta, optic tectum, or retina (eye)	[96]
Fungi			
Aspergillus fumigatus	Embryos	Hindbrain ventricle injection	[40]
Candida albicans	Embryos	Hindbrain ventricle injection	[97]
Candida auris	Larvae	Hindbrain ventricle injection	[98]
Talaromyces marneffei	Embryos	Injection into muscle, hindbrain ventricle, or Duct of Cuvier	[47]
Cryptococcus neoformans	Embryos	Yolk sac circulation valley injection	[99]
Eukaryotic Parasites			
Schistosoma mansoni	Embryos	Incision and deposition of *Schistosoma* eggs into hindbrain ventricle using Capillary-Assisted Implantation Needles	[100]
Trypanosoma carassi	Larvae	Duct of Cuvier injection	[44]