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Zebrafishology, study design guidelines for rigorous and reproducible data using zebrafish

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The zebrafish (*Danio rerio*) is one of the most widely used research model organisms funded by the United States' National Institutes of Health, second only to the mouse. Here, we discuss the advantages and unique qualities of this model organism. Additionally, we discuss key aspects of experimental design and statistical approaches that apply to studies using the zebrafish model organism. Finally, we list critical details that should be considered in the design of zebrafish experiments to enhance rigor and data reproducibility. These guidelines are designed to aid new researchers, journal editors, and manuscript reviewers in supporting the publication of the highest-quality zebrafish research.

Science is an ever-evolving process, and our ability to formulate and study scientific questions continuously improves in both experimental design and technological implementation. In the biological sciences, this evolution is associated with increased interdisciplinary and collaborative approaches, encouraging scientists to cross into new fields to take advantage of novel tools. During this transition, scientists may bring assumptions and practices accepted in their previous field and apply them to the new one. However, many disciplines have independent assumptions and experimental design expectations that are taught and passed down through training, mentorship, and expertise. These rules or design guidelines are often unwritten, as is the case within the zebrafish community. Therefore, this review aims to outline critical resources available to the zebrafish researcher, describe major biological characteristics to consider when using the zebrafish as compared to other vertebrates, and propose guidelines for taking advantage of these attributes. We will review statistical perspectives that assist experimental designs and data analysis when using zebrafish. Finally, we will detail the critical reporting elements expected within a manuscript to judge methodological rigor and potential for data reproducibility.

Guidelines for rigorous and reproducible experimental results in the zebrafish

Overview of the zebrafish model and resources available

As a relative newcomer in the biological model field, the use of the zebrafish has expanded dramatically, with the number of publications rising steeply in the 2000s (Supplemental Fig. 1). This rapid expansion of zebrafish studies is due to the many advantages and relative accessibility of this model. Briefly, the genome of this vertebrate organism is fully sequenced and largely annotated[1]. Within the Online Mendelian Inheritance in Man website, 82% of disease-relevant genes contain a zebrafish ortholog[1]. Further, the zebrafish shares most organ systems[2–4] with other vertebrates. Like the invertebrate research organisms[5–8], the zebrafish genome is highly amenable to mutagenesis and transgenesis[9,10]. Zebrafish embryogenesis is rapid, with its developmental timeline more comparable to *Drosophila* than that of mammals. Notably, the embryos are optically translucent during development, facilitating imaging studies. As the zebrafish develops past embryogenesis, which is completed by "hatching" at 2–3 days post fertilization (dpf), the animal continues to grow and form pigment. However, it is possible to prevent pigment formation using phenyl-thio-urea (PTU)

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Fig. 1 | Examples of pigmentation patterns in zebrafish larvae due to genetic or chemical perturbations. Zebrafish are, by convention, oriented with their head to the left and tail to the right. The two common orientations are a lateral/side view (**a**, **c**, **e**) or a top/dorsal view (**b**, **d**, **f**). Labeled in the WT 5 pdf larvae (**a**) is the eye and swim bladder. **a** A side view of WT pigmentation pattern of the TLF line. **b** From the top view, the WT pigment obscures the zebrafish brain and upper digestive tract. **c**–**d** The zebrafish *casper* mutant line prevents most pigmentation from forming except for the eyes and around the swim bladder. E-F) WT embryos were exposed to PTU after gastrulation (at the start of somitogenesis) but before pigment started forming at 24 hpf. PTU can be added after the start of pigment formation, but this will cause an incomplete loss of pigment. Adding PTU prior to somitogenesis will inhibit gastrulation, and the embryos will not develop correctly. When comparing the WT and the WT + PTU zebrafish, despite these larvae coming from the same clutch, the PTU has slowed down development (compare swim bladder size and yolk). This is one example of when careful timing is important for direct comparison work.

Table 1 | A list of advantages of the zebrafish model

Zebrafish advantages	
Vertebrate model	70% of zebrafish genes have at least 1 obvious ortholog
Shares most organ systems with humans	Including immune system, eyes, mouth, brain, spinal cord, intestine, pancreas, liver, bile duct, spleen, kidney, esophagus, heart, ears, nose, muscle, blood, bone, cartilage, and teeth
Large number of offspring	50–300 embryos per mating pair
Fast development	Hatched from the chorion and swimming by 3 days old with most major organ systems patterned and functional by day 7.
Externally develops	Allows for visualization of development from the single cell stage (whereas mammalian development is in utero)
Translucent during development	No pigment formation before ~ 22 hpf. Addition of PTU after gastrulation inhibits pigment formation or translucent mutants are available
Genome highly accessible to manipulation	Knockdown, knockout and overexpression systems available for genetic manipulation
Large number of mutant and transgenic lines available	Many individual labs and societies have created mutant lines available to the zebrafish community (for examples see Table 2).
Genetic variability	Like humans, the zebrafish thrives on genetic variability.
Complex behaviors	Behavioral patterns have been defined throughout development, including embryonic (2 dpf), larval (5–7 dpf) and adult behaviors.
Relatively inexpensive housing cost	Zebrafish are smaller than mammalian models and prefer to live in large groups (shoals)

(Fig. 1c) until around 7 dpf[11–13]. Past 7 dpf, the translucency decreases, though there are genetic mutants, such as the *casper* (Fig. 1b), *absolute*, and *crystal* lines[14,15], that allow for imaging of both larval and adult tissues. Depending upon fish husbandry practices, it requires 2–4 months for the zebrafish to mature enough to breed[13]. Another advantage is the large sample sizes available for experimentation due to the ease of husbandry and number of embryos per mating pair, which is not easily accomplished in other vertebrate organisms[16,17]. These features make the zebrafish one of the only vertebrates wherein large-scale forward genetic studies are relatively inexpensive and can be performed by a single lab[9,18–22]. All these factors make the zebrafish an ideal model for bridging the gap between invertebrates and mammals (Table 1).

The early, large (1 mm) single-cell zebrafish embryo is accessible to rapid and scaled experimental manipulation, which has led to a diverse array of creative technologies in the field. Microinjection is perhaps the most notable, with gain of function from synthetic mRNA[23,24], loss of function using morpholinos[25–27], rapid transgenics via artificial transposons[28], and a rich array of gene editing tools[29] that can be directly delivered via this method. Each of these has important scientific constraints to consider for implementation and are discussed here (see below).

Additionally, the zebrafish community has embraced the ever-growing capability to collect and store large amounts of data by creating many online databases summarizing the knowledge and making these tools freely available. One critical resource is The Zebrafish Information Network (ZFIN, https://zfin.org/)[1,30]. This curated database has information or links to genetic sequences, mutations, targeted antisense reagents (morpholinos[26,27]), antibodies, publications, and more. Additionally, this website grants access to The Zebrafish Book[31], which contains practical standard operating procedures for general zebrafish husbandry and other experimental protocols. This text is an excellent resource when starting to work with the zebrafish. Directly linked to the ZFIN website is the Zebrafish International Resource Center (ZIRC, http://zebrafish.org/), which maintains many zebrafish lines available for purchase. This includes wild type (WT), transgenic and mutant lines. Laboratories worldwide send their mutant and transgenic lines to this facility for disbursement to other community members. In addition to these core resources, other free

databases, both internationally and locally, are available[32]. Finally, the zebrafish community is highly collaborative and has many national and international societies that foster strong connections (Table 2).

Genetic variability within the zebrafish

Next, we will discuss key characteristics that will help leverage the advantages of the zebrafish. When exploring differences between research organisms, one critical distinction between zebrafish and other vertebrates is its extensive genetic variability. While it is commonplace to use isogenic and highly inbred mammalian models to reduce variability, the laboratory-based "WT" strains of zebrafish show significant genetic heterogeneity. For example, one study looking at single nucleotide polymorphisms (SNPs) between different zebrafish lines demonstrated a 7% interstrain genetic variation in inbred research animals, with WT lines having as high as 37% genetic variation[33,34]. Despite multiple attempts, isogenic lines with stable fecundity are rare[35].

Due to this consideration, there are multiple laboratory-based "WT" lines, which include Tubingen (TU; strain used to sequence the zebrafish genome), AB, Tupfel long fin (TL), Sanger AB Tubingen (SAT), etc. Each of these lines behaves somewhat differently and has unique genetic and physical traits[36,37]. Additionally, when maintaining the WT lines within a facility, it is critical to maintain genetic diversity and prevent bottlenecks where the next generation offspring comes from a limited number of breeding pairs. The best solution is to obtain each new generation from a stock center or create new lines by combining clutches of at least 15–25 crosses.

In addition to genetic variability, around 340 million years ago, the zebrafish ancestor underwent a genome duplication event[38,39]. It has been found that, of the 70% of zebrafish orthologs to human genes, 47% have a single ortholog, whereas the remainder have more than 1 orthologue[1]. This has both advantages and disadvantages. With more than one gene performing the same function, it is possible to create a hypomorph by mutating one of the orthologs. Furthermore, many duplicate genes have subfunctionalized, leading to the actions of a given gene being split into two or more paralogs performing a distinct subset of the original gene's functions[40]. This can be advantageous, as a subset of the original gene functions can be more easily studied when subfunctionalized to one paralog. However, to create a null mutant comparable to a human genotype, multiple genes may need to be targeted.

This heterogeneity and genome duplication could hinder the adoption of an organism for experimentation if there is a limited number of offspring. For example, the average mouse litter size varies from 2–12 pups, limiting the sample number[41]. However, a single zebrafish mating pair will lay clutches of 70–300 eggs[17], allowing for a higher sample size and helping overcome genetic variability. In fact, when genetic variability is combined with an increased sample number, this becomes a distinct advantage of the zebrafish model, particularly when modeling human disease, where genetic heterogeneity is an important variable.

How genetic variability impacts experimental outcomes depends upon the hypothesis being tested, the experimental design, and the methods used. The power of the model is the ability to collect data from a single cell, an individual animal, or a subpopulation of fish, all of which can be defined as a single sample. Background variation provides noise that can make it challenging to link a genetic perturbation or a treatment condition to observed phenotypes. However, the background genetic diversity makes the zebrafish an excellent model for human disease, as humans are similarly diverse. Increasingly within mouse literature, it has been noted that isogenic lines limit the reproducibility and application of the data collected[42]. Combined with the large number of offspring available, the genetic diversity grants the zebrafish model further insight into the genotype-phenotype relationship. This is particularly critical when studying drugs. The zebrafish is an excellent model to study the variability of drug activity within a genetically heterogeneous population. Thus, using this model for drug testing will more truly emulate a human population as compared to an inbred mouse model. However, it is critical that the increase in variability, and thus larger error bars, be considered in the experimental design and carefully balanced with the number of animals needed to achieve statistical significance.

Maternal contribution to early development in zebrafish

Another benefit of the zebrafish is the ability to study maternal gene contribution to early development within a vertebrate animal. The onset of zygotic genome activation is preceded by the embryo's exclusive reliance on maternal gene products for development. These maternal RNAs and proteins in zebrafish progressively decrease as the zygotic genome takes over at ~3 h post fertilization (hpf)[43]. Many zygotically expressed genes are also expressed maternally. Embryos containing homozygous mutations in proteins required for development may develop for several days due to their heterozygous female parent providing the normal transcript[44,45]. Hence, even a complete loss-of-function phenotype may not be reflected as embryonic lethality. Maternal gene function must also be perturbed to examine the complete loss of both maternal and zygotic function.

Gene editing within the zebrafish

The zebrafish has a rich array of tools available for genetic manipulation. There are two broad categories of technology to decrease gene function. First, knockdown technologies decrease gene function without altering the genome. Morpholinos (MOs) were classically used and are still deployed today to knockdown genes[25]. There are two targets for MOs. First, MOs can target the start codon, which prevents translation. Second, the MO targets a splice site which prevents correct splicing and, in turn, likely causes protein truncation[46]. Using MOs enables the rapid screening for loss of function phenotypes and can be an excellent way to study a gene's function. This technology is most commonly used to study function during the first 2–3 days post fertilization (dpf). MOs have been shown to increase p53 signaling in the zebrafish embryo and larvae, with neurons the most sensitive cell type[27,47]. Therefore, conclusions on the effect of a MO on any developmental process, particularly within the neural tissue, should be carefully assessed for p53 signaling. In addition to MOs, CRISPR-based RNA targeting technology has recently become available. This enables a different mechanism than MOs for RNA targeting to knockdown gene expression or alter splice sites. As both these technologies improve, they offer options for development-specific timing of gene knockdown[48,49]. This is particularly important for genes critical for early development but whose function in later development or adulthood is still unknown due to early lethality[50].

The second category is gene knockout through DNA manipulation. A variety of techniques have been used to mutate the zebrafish genome, each with advantages and disadvantages. Classically, N-ethyl-N-nitrosourea (ENU) was used for chemical mutagenesis for large-scale genetic screens[20,51]. While this method provides many single base-pair genomic changes, phenotype screening and subsequent gene cloning can be laborious[51]. An alternative approach is insertional mutagenesis, which utilizes retroviruses or transposons to insert DNA, although the mutation frequency is considerably lower. Due to the size of the inserted sequences, these technologies allow for faster and more precise localization of the mutated target gene[52].

Lastly, when the gene of interest is known, using zinc fingers[53], transcription activator-like effector nucleases (TALENS)[54] or clustered regularly interspaced short palindromic repeats together with CRISPR-associated protein (CRISPR/Cas9)[55] to create double-strand breaks, which in turn is repaired either by non-homologous end joining or homology-directed repair[56,57], can be used to create mutant lines[58]. While the zinc finger technology[53] is mostly obsolete within the zebrafish, TALEN[56] and CRISPR[55] are still cost-effective and practical options. The CRISPR system is unique due to its effectiveness in multiplex genome editing as well as its versatility for both single gene of interest mutagenesis and large-scale targeted mutagenesis screens[59–61]. A shortcoming of using CRISPR technology is off-target effects, with a recent estimate of approximately 26% of founder offspring carrying an off-target mutation[62]. Therefore, careful design of CRISPR targets, Cas9 protein choice[63–65], and screening of multiple first-generation offspring are needed to decrease unwanted mutations. More recently, F0 crispants have emerged as a rapid screening tool. This technology allows for mutant phenotype screening in injected embryos at a similar efficiency to siRNA and MO[21,66]. As opposed to MO, the F0 crispant's

Table 2 | Examples of zebrafish databases and societies

Name	Content	Website
General databases		
• ZFIN	Aggregation of known publications, mutants, morpholinos and antibodies for a given gene	https://zfin.org/
• ZIRC	Mutants available in their inventory for a given gene	http://zebrafish.org/
	The zebrafish book	https://zfin.org/zf_info/zfbook/zfbk.html
• LAZEN	Latin American zebrafish resource center	http://lazen.fcien.edu.uy/
• EZRC	Europe's international zebrafish resource center	https://www.ezrc.kit.edu/
• CZRC	China's national zebrafish resource center	http://en.zfish.cn/
• TZCAS	Taiwan's national zebrafish resource center	http://icob.sinica.edu.tw/tzcas/fishlines_zeth.html
• Norwegian zebrafish platform	Norway's national consortium	http://zebrafish.no/#myAnchor
• Zebrafish–National BioResource Project	Japan's organization that collects and distributes zebrafish lines	https://nbrp.jp/report/reportProject.jsp?project=zebrafish
• ZeBase	Database for zebrafish inventory and streamline facility operations and organization	https://zebase.bio.purdue.edu/
Genomic databases		
• Ensembl	Ensemble zebrafish genome assembly	https://useast.ensembl.org/Danio_rerio/Info/Index
• BLAST	Zebrafish sequence analysis and searchable reference sequence	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=OGP__7955__9557
• zfishbook	Gene break transposon mediated mutants available	http://www.zfishbook.org/
• Genome browser gateway	Mapping genes within the zebrafish genome and between other sequenced species	http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=85282730&clade=vertebrate&org=Zebrafish&db=0
• Genomic resources for zebrafish ZInC	A Wikipedia page specifically for zebrafish genomic databases	https://wiki.zfin.org/display/general/Genomic+Resources+for+Zebrafish
	Insertional mutants in the zebrafish	https://research.nhgri.nih.gov/ZInC/
• ztrap	Expression patterns images of gene and enhancer trap lines	https://ztrap.nig.ac.jp/ztrap/
• M6A-Atlas	A database for many animals, including zebrafish, of the N6-methyladenosine epitranscriptome	http://180.208.58.19/m6A-Atlas/
• ZenoFishDb	A curated database of the xenograft studies performed in the zebrafish	https://konulab.shinyapps.io/zenofishdb/
Zebrafish single cell databases		
• Zebrahub	Zebrafish embryonic development that combines single cell RNA sequencing with live light-sheet imaging	https://zebrahub.ds.czbiohub.org/
• Daniocell	Single cell expression data of the TL/AB zebrafish throughout development	https://daniocell.nichd.nih.gov/
• ZSCAPE	Single cell RNA-sequencing comparing WT, genetic mutants and temperature perturbations	https://cole-trapnell-lab.github.io/zscape/
Anatomy or Atlas databases		
• Bio-Atlas	Zebrafish Anatomy with histological slides during development starting at 48 hpf through 12 months old	http://bio-atlas.psu.edu/zf/index.php
• ZFAP	The zebrafish anatomy portal contains annotated and 3D anatomy throughout development	http://www.rdvd.fr/
• Vascular Anatomy atlas	Zebrafish vascular anatomy throughout development, both 2D and 3D images.	https://zfish.nichd.nih.gov/Intro%20Page/intro1.html
• Z Brain Atlas	Zebrafish neuroanatomy reference at 6 dpf with regional definitions	https://engertlab.fas.harvard.edu/Z-Brain/home/
• mapzebrain	Zebrafish brain regions, neural connections and transgenic lines	https://fishatlas.neuro.mpg.de/
• Brain Browser	Transgene patterns at 6 dpf	http://vis.arc.vt.edu/projects/zbb/
• Zebrafishbrain	A high-resolution atlas of the developing zebrafish brain	https://zebrafishucl.org/zebrafishbrain#about-1
• AZBA	A 3D atlas of the adult zebrafish brain	https://azba.wayne.edu/
• FishFace	A database within FaceBase with zebrafish craniofacial development	https://www.facebase.org/resources/zebrafish/fishface/home/
Zebrafish societies		
• International Zebrafish Society	Fosters the collaboration within the international zebrafish community	https://www.izfs.org/
• European Zebrafish Society	Central contact point for the European zebrafish community	https://www.ezsociety.org/

Table 2 (continued) | Examples of zebrafish databases and societies

Name	Content	Website
• Polish Zebrafish Society	Zebrafish research society in Poland	https://zebrafish.org.pl/
• Nordic Zebrafish Network	Bring together zebrafish enthusiasts to improve quality of husbandry and science	https://ki.se/en/research/research-infrastructure-and-environments/core-facilities-for-research/zebrafish-core-facility/the-nordic-zebrafish-network
• Zebrafish Disease Society	Focuses on the use of zebrafish in clinical research	https://www.zdmsociety.org/home
• Zebrafish Foundation India	The society works towards creating a strong zebrafish research community	https://zebfin.res.in/
• InSDB	Zebrafish meetings and interactions in and around India are coordinated through the developmental biology society.	https://insdb.in/about-insdb/about-mission/

Due to the dynamic and expanding nature of the field, this table is an initial but incomplete list of the types of online databases and societies available to the zebrafish community. Many zebrafish societies are available regionally and in the area's native language.

DNA is mutated, and thus, its effect does not fade. Another benefit of the technology is a decrease in screening time because the mutated animals do not need to be raised to generate a stable line prior to phenotypic analysis[67,68].

Despite these technologies making genome manipulation straightforward, there are a few caveats to consider when creating a mutant animal. As stated previously, the zebrafish has high sequence diversity. Therefore, when using these specific double-strand DNA break technologies, it may be necessary to assess your gene of interest either through a SNP database or sequencing your zebrafish before TALEN/CRISPR design[69,70]. This is particularly true for fish that are not known laboratory lines, as these fish tend to have more sequence variability when compared to previously described zebrafish lines.

Next, as stated above, the zebrafish underwent a partial genome duplication[71]. Therefore, it is crucial to consider the paralogs and orthologs within the genome that can partially or completely compensate for the gene loss[39]. It may be that a single mutant within the zebrafish can act as a hypomorph, and observing a phenotype may require more than a single gene mutation. Alternatively, many duplicate genes have subfunctionalized thus displaying only a subset of phenotypes seen in other vertebrates[40], so again, multiple mutations may be required to completely phenocopy a human disease.

Additionally, maternal contribution to early development is critical. There are two strategies to mutate the maternal contribution to the embryo. First, germ-line replacement, where germ cells from homozygous zygotic mutant embryos are transplanted into WT embryos depleted of their germ cells and raised to adults[72], is used to create embryos with both maternal and zygotic homozygous mutations. Second, gene editing provides a feasible approach to investigating the maternal contribution during early development in zebrafish. Researchers can employ the CRISPR/Cas9 system directed to the germ line to study such maternal-effect or maternal-zygotic phenotypes[73,74]. Alternatively, rescue of the zygotic lethal mutation can be attempted by injecting WT mRNA of the gene into mutant embryos and raising them to adulthood to generate homozygous mutant mothers. The offspring could then be analyzed to determine maternal function[75,76].

The zebrafish is a strong developmental model

While there is a growing body of work in adult fish, the primary historical strength of the model has been within developmental biology[19]. Zebrafish embryos develop outside the body. Therefore, development is visible from the one-cell stage[77]. This enables a non-invasive, in-depth analysis of development in a vertebrate model. The zebrafish embryo also develops quickly. Within 24 h of fertilization (hpf), a complete body plan can be observed, and within 7 days, the embryo grows into a fully functional larva[11]. During this time, the zebrafish is largely translucent, especially in the first 24 h before pigment cells appear, making imaging straightforward. Additionally, during these first 7 days, the animals are simple to care for. Reliant on a yolk sac, zebrafish embryos, and larvae do not need to be fed and can live in simple aqueous media in a 28 °C incubator[31]. This allows zebrafish to be collected in large numbers at very specific developmental time points. A summary of many key zebrafish life cycle milestones can be seen in Fig. 2 [78–83].

Such rapid development and ease of observation come with some precautions. Because the zebrafish develop so quickly, it is critical to accurately stage zebrafish embryos and larvae. For example, mRNA expression within various organ systems can wax and wane within a relatively short period of time, as seen in the *short stature homeobox 1 (shox 1)* gene expression (Fig. 3a). To address the challenge, zebrafish development has been extensively studied and the stereotypical developmental milestones are carefully delineated. These developmental stages are defined by both morphological features and chronological time passed since fertilization ((hpf or dpf); https://zfin.org/zf_info/zfbook/stages/index.html)[11,12]. It is through carefully defined and standardized stages that rapidly developing organ systems such as the vasculature (Fig. 3b) have been contextualized in development. This enables data from different labs as well as different genetic strains and mutants to be compared within that developmental context.

When staging adult zebrafish, age, body length and morphology must be considered. Zebrafish are adults at ~90 dpf at 28.5 °C[13]. Because various factors can influence growth, age is not the most reliable measure of maturation. It is recommended to use standard length, a measure of the length from the snout to the tail (not including the tail fin), to assess maturation[12]. Fin morphology, specifically pattern, has also been used to determine growth[12,84]. Body weight is not typically used to stage zebrafish, but it can be used to evaluate the health status of the zebrafish[85,86].

Using both morphological markers and time post fertilization to stage development is critical because of the rapid development and the many exogenous factors that affect embryonic and larval development. Many factors can change the speed of zebrafish development[31]. For example, raising zebrafish at a warmer temperature accelerates development. At the standard 28 °C, zebrafish embryos reach the 20-somite stage at 19 hpf. At 33 °C, they reach the same stage by 14.5 hpf, and at 25 °C in 23.5 h—an almost 10-h difference (https://zfin.org/zf_info/zfbook/stages/figs/fig2.html)[11].

Another key example is that the density of zebrafish within a Petri plate can influence the embryo's development. Generally, it is considered best practice to raise around 50 embryos per 100 mm Petri plate. As the density increases, there is an increased likelihood of asynchronous growth rates, causing a large variation in the stage of the embryos[87]. This density effect continues into adulthood[88]. Additionally, the increased density causes an increased likelihood of water contamination with amoeba, bacteria, or mold, which also affects the health of the embryos. When considering juvenile and adult fish, the feeding regimen is another important example that can alter animal growth[89,90].

Finally, it is particularly critical to be aware of the developmental stage when comparing WT to an animal that has been manipulated. For example, when using knockdown technology such as morpholinos, their injection generally alters the embryo's development causing them to develop slower than their uninjected counterparts. It is critical to look at the embryo's

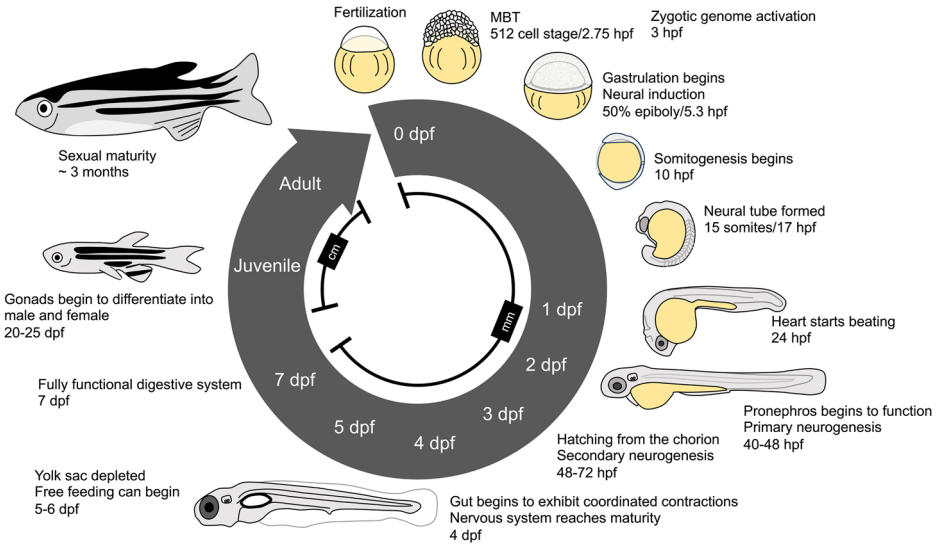

Fig. 2 | Important milestones in zebrafish development. Shown are critical milestones in zebrafish development (note images are not shown to scale). However, the embryos and larvae are mm in size, with the single cell at fertilization being 1 mm, whereas the juveniles and adults are cm in size. The mid-blastula transition (MBT) is when the time between cell cycles increases, and cell synchrony is lost[77]. Zygotic genome activation quickly follows, and maternal transcripts begin to be depleted. Gastrulation begins by just over 5 hpf, and somitogenesis begins 5 h later. The neural tube is formed by ~17 h, and primary neurogenesis begins at around 2 dpf, with secondary neurogenesis beginning at 3 dpf. The zebrafish have a mature nervous system by 4 dpf[78]. The heart starts beating by 24 h, and the larval kidney (the pronephros) begins to function at around 2 dpf[79]. The yolk sac is depleted by ~5–6 dpf. The mature digestive system is fully functional by 7 dpf, but the larvae can begin free feeding as early as 5 dpf[80,81]. The gonads begin to differentiate in the juvenile fish by around 20–25 dpf[82]. At ~3 months, the fish is fully mature and capable of mating. All times are developmental stages when the fish is developing at the standard 28.5 °C.

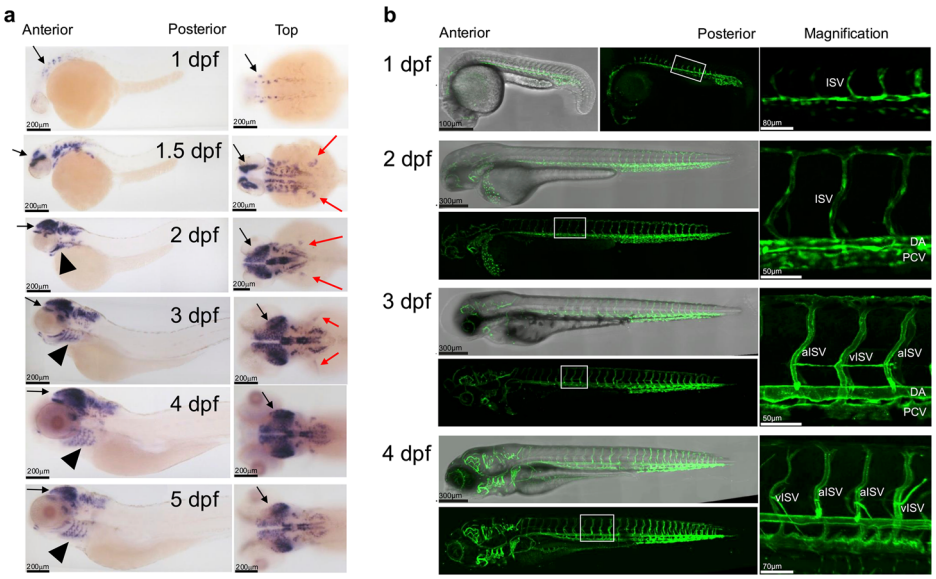

Fig. 3 | The zebrafish as a developing organism. **a** Whole mount in situ hybridization mRNA stain of *Short stature homeobox 1* (*shox1*) throughout the first 5 days of development exemplifies how a gene can change intensity (black arrows point to the brain) and location of expression (red arrows point to the fin bud, which is only expressed in 1.5–3 dpf; black arrowhead points to the pharyngeal arches which begin expression at 2 dpf) in a relatively short period of time. **b** The VEGF receptor (*kdr-like*) florescent line (Tg(*kdrl*:Hsa.HRAS-mCherry)s916) demonstrates how quickly an organ system, the vasculature, changes and develops in the first 4 days of zebrafish development. ISV—intersegmental vessels (a—arterial, v—venous); DA—dorsal aorta; PCV—posterior cardinal vein.

morphology to determine its stage and/or inject a control morpholino at the same concentration, which often causes a similar developmental delay. Within mutant lines, inbreeding of heterozygous mutants produces age-matched WT, heterozygous, and homozygous mutant animals within the same clutch. For experiments requiring exact timing, individual clutches should be collected soon after birth and kept separate because fish can produce embryos throughout the morning and, therefore, the embryos can be laid multiple hours apart. Even in homozygous mutants and maternal-effect mutants, stage matching should be done. Therefore, when comparing experimental and control groups, stage matching using both time and morphology should be considered while designing experiments and analyzing data[12].

A summary of common environmental factors that can alter the development and health of both the larval[91,92] and adult[84,93,94] zebrafish is summarized in Table 3. Many of these are listed above and have been formally studied. However, some examples in the table are from observations and have not been well quantified. For example, most of those who work with zebrafish embryos have observed that, if the density of embryos is too high, the embryos develop at a different rate, resulting in a plate with fish ranging from the 3-somite to 25-somite stages within that same dish. Additionally, most have observed that, if the water is not consistently changed, fungus or amoebas will grow and overrun the embryos, causing a slowing of development and generally unhealthy animals. With adult animals, we have observed changes in mating rates, particularly during daylight savings time, when the light/dark cycle is altered. While these are common observations, they have not been formally quantified.

There are many considerations when deciding the developmental windows suitable for particular experiments. For example, cell fate within organ morphogenesis, cell movement, and migration patterns tend to be studied within the first 24 h; however, they can be studied later in

Table 3 | Environmental exposures

Environmental exposure	Growth alteration
Embryo/Larva (0–7 days)	
• Temperature below 28 °C	Slower development[11]
• Temperature above 28 °C	Faster development[11]
• High population density	Slower development; increased heterogeneity of development[75]
• Water quality (fungal growth or amoeba overgrowth)	Slower development; unhealthy embryos
• Genetic mutations	Variable but can cause slower development
• Embryo manipulation (injections)	Slower development (active molecules more than control)
• Changes in salinity	Premature hatching[87]
Juvenile/Adult (8 days-2 years)	
• High population density	Slower growth[76,83]
• Low population density	Faster growth[76,83]
• Water quality (accumulation of waste products)	Slower growth, unhealthy fish[83]
• Low food quality	Slower growth[71]
• Insufficient food quantity	Slower growth, inadequate food for fish density[84]
• Genetic mutation	Variable, possible poor health
• Changes in light/dark cycle	Decreased mating rate, alters normal behavior patterns
• Noise exposure	Decreased mating rate, increased stress
• Excess inbreeding	Decreased mating rate, unhealthy fish[35]

Common Exposures that alter the growth and health of the embryonic/larval (those living in Petri plates) and juvenile/adult (those living in tanks) animals. While many of these exposures have been studied, some are observations made by many experts within the field but have not been formally quantified.

development using a subset of cells[95–97]. One specific example of this is the study of the connection between vascular and neural development, which occurs within the first 24–48 h. Choosing the correct developmental stage is critical, especially in neurological and behavioral experiments, because the behavioral complexity and neural connections may not be as developed in certain larval stages when compared to adult zebrafish or other mammalian models.

In addition, working with extremely young embryos, such as in the first hour of development, can be challenging. During this critical time of development, the embryo changes radically over a very short time span due to cell divisions occurring approximately every 15 min for the first 10 rounds, so even relatively subtle differences within a single clutch of embryos can have substantive consequences in experimental outcomes. For example, those embryos spawned first may be at a different stage than those laid last[11]. Therefore, it is critical to closely time the embryos using previously defined developmental markers[11]. This not only ensures consistency within your replications but also improves reproducibility. Moreover, for the first few divisions, the cells are very fragile, and manipulating these embryos is technically challenging. Even after fixation, the embryos are easily damaged during the RNA or protein staining protocols. Therefore, when first beginning to work with these young embryos, plan on additional time and embryos to replace the ones that are ruined during the process.

In contrast, when using older zebrafish larvae, other considerations arise. For this article, we focus on zebrafish dpf and beyond due to key developmental milestones. We also recognize that international regulatory bodies may use different criteria for specifying regulatory practices. Thus, local animal welfare oversight rules may be an important consideration in the study design for any zebrafish experimentation. First, the yolk sac, which supplies nutrients to the growing animal, is exhausted by 6–dpf, and the growing larva needs to start eating[98]. Experiments performed on larvae that have exhausted their yolk should have a feeding regimen. Some of the larvae may be starving and sick without feeding, making data more challenging to interpret. In addition, humanely handling and caring for experimental fish are an important issue after dpf, at which point neuroendocrine response to exogenous stress has developed[99–101]. While established protocols (IACUC or institutional animal care and use committee) differ by individual institutions, the beginning of 4 dpf usually marks when the experimental animal should be cared for with a full measure (e.g. analgesics, anesthesia) in the zebrafish field. Second, later-stage larvae are larger, and their epithelial tissue is no longer as permeable. Therefore, many imaging and staining techniques are more difficult at this stage. After 6 to 7 dpf, most RNA or protein staining protocols may need to be conducted on sectioned tissue or after manual skin removal due to increased tissue density depending on the target mRNA/protein depth within the animal.

Organ system size and scale

Another difference in the zebrafish model lies within the scale of the cell types and tissues seen within the animal. This is exemplified by comparing the zebrafish brain to the mouse or human brain. The human brain is large, on average 8.6×10^9 neurons[102], anatomically difficult to access through the skull, ethically complex to justify invasive studies, and biophysically challenging to investigate with non-invasive methods. The mouse brain, at approximately 7×10^7 neurons, is still too large to visualize in its entirety without removing the brain and examining sections or using disruptive clarity approaches[103]. The brain is encased within the skull, and looking at small live tissue sections is still difficult. On the other hand, the larval zebrafish brain contains approximately 100,000 neurons with the forebrain, midbrain, and hindbrain aligned along the anterior-posterior axis, which improves imaging capabilities. Still, the 100,000 neurons within the zebrafish larval brain provide analogous structures to the human and mouse brain[104,105]. The small size, translucency, and structural analogs enable the visualization of the entire brain and comparative studies. Other examples can be seen in the kidney, which contains a single glomerulus in the embryo, and the gastrointestinal system, which is a single straight lumen within the embryo. The zebrafish may be the only vertebrate model system where the cell biology of live intestinal cells can be assayed in the presence of bile mucus and symbiotic organisms[106,107]. These "scaled-down" analogs of human tissues allow for the rapid and single-cell-level resolution of cell-cell interactions and developmental processes within a live animal.

Experimental design and the zebrafish model

Sound experimental design and statistical analysis are foundational to collecting unbiased data and accurately interpreting experimental outcomes. To ensure scientific rigor, many scientific journals are starting to expect manuscripts to justify the statistical analysis[108,109]. This is also become an expectation within the zebrafish community. Here, we review unique considerations when creating sound experimental designs when using zebrafish.

Definition of replicates when using the zebrafish

In statistics, the "n" typically refers to the number of sampled organisms versus the population size "N". Within many areas of experimental science, the number of experimental replicates equals the "n". However, when using an in vivo research organism, there are additional requirements that must be accounted for to ensure the correct sampling of the population. According to the United States' National Institutes of Health (NIH) there are two distinct subsets of variation that must be accounted for when considering biological sampling (https://www.nigms.nih.gov/training/documents/module3-biological-and-technical-replicates.pdf). First, the samples must have independent technical replicates to account for the random noise of the methodology and equipment (reviewed in ref. 110). Second, biologically distinct samples that capture the variation of the animal or biological model system must be used to account for genetic and environmental noise (Fig. 4a, b).

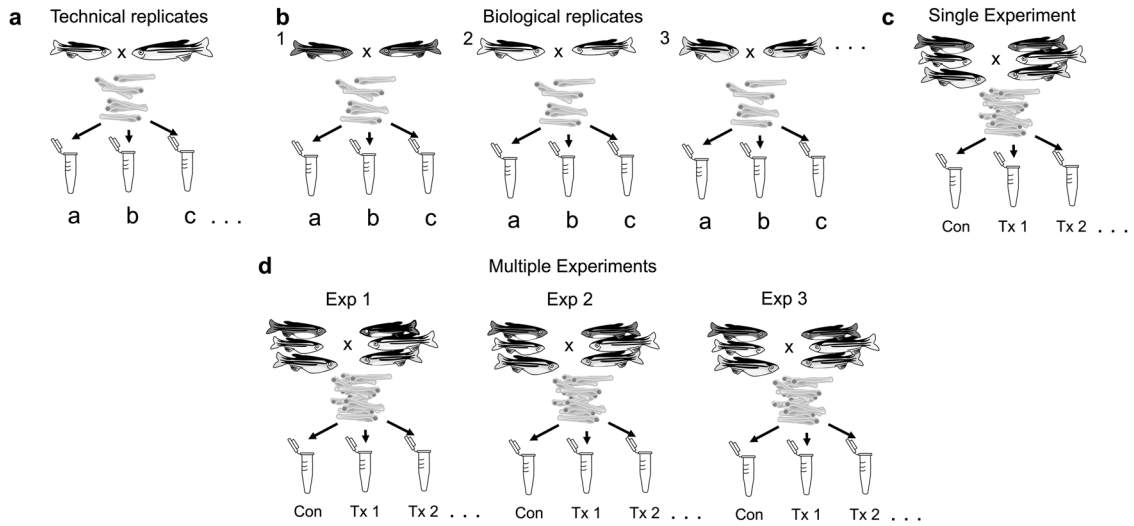

Fig. 4 | Defining replicates in the zebrafish model. a Using a single mating pair's progeny, technical replicates can be conducted (the Eppendorf tube stands in for any data output with **a**–**c** representing three technical replicates). This data will only give information on the variation due to the experimental method. **b** Biological replicates are also necessary to sample the population appropriately. Using the progeny from different mating pairs ensures genetic diversity, which is the basis of biological variability. **c** A single experiment typically uses many different mating pairs to create biological diversity within the pool of progeny tested. The experiment shown here has a single technical replicate with multiple conditions (control, con; treatment 1, Tx 1; treatment 2, Tx 2). However, technical replicates are achieved using these progenies, but these are not adequate biological replicates. **d** Multiple experiments using different tanks of outcrossed adult fish allow for both technical and biological replicates.

It is within the second type, the biological replicates, where field-specific expectations and standards come into play. As stated above, each zebrafish line is a genetically diverse population. Therefore, it is critical to account for both technical and biological variability within the experimental protocol. Additionally, it is customary that a minimum of three experiments be performed to obtain a confidence interval and account for both biological and technical replicates.

For example, imagine an experiment assessing the efficacy of two drugs at a single dose. There will be three treatment conditions (e.g. "Control", "Tx 1", and "Tx 2"). Now, depending upon the methodology downstream, this single experiment can have either one or multiple zebrafish within each sample. When using multiple zebrafish, if they are from a mix of multiple mating pairs, this helps account for biological/genetic diversity. Typically, with multiple animals within an experiment, the data are averaged to produce a single output toward the 'n'. Within this scenario, the number of zebrafish does not equal the number of experiments performed (Fig. 4). Next, this single experimental set should be repeated multiple times, ideally on different days. This way, the "Control", "Tx 1", and "Tx 2" groups will have multiple data points collected over the different experimental times (Fig. 4d). This helps avoid unforeseen and unnoticed factors that might affect the experimental outcome. For example, a malfunction within an incubator's thermostat, pH level in the aquarium widely fluctuates, room temperature changes due to weather, microbiomes in Petri dishes where larvae are kept vary, and many others. Performing experiments on different days ensures a data point's validity and the measurements' precision over a temporal scale. Additionally, performing experiments on separate days requires clutches derived from multiple different mating pairs, which is critical for genetic and biological variation. If a single animal is used for each experiment, it is important to ensure genetic diversity in the samples chosen (Fig. 4b). In the end, the expectation is that the experiment was performed, at a minimum, in triplicate to ensure adequate replicates regardless of the actual number of zebrafish tested.

Determine the number of zebrafish required per replicate

The number of zebrafish required per experiment depends largely upon two key elements, the type of data being collected and the variability of the data. Two distinct types of data that can be collected within the zebrafish, observational (or non-quantifiable) and quantifiable data. Observational data is considered data that can be described but not necessarily changed into a quantifiable number. For example, gene and protein expression patterns during development and how they are altered when a manipulation is performed are considered observational data. Which organ systems the gene is expressed in and how that changes over time cannot be readily converted into a number. It is critical, however, that this data be replicated. For example, when performing an in-situ hybridization experiment, approximately 10 or more embryos or larvae from at least three separate clutches at a given stage should be assessed to ensure that the pattern seen is consistent at that time point. Quantifiable data, alternatively, can be expressed as a number or percentage. So, while the examples above are observational, some aspects can also be quantitative by measuring the number of mRNA transcripts with quantitative PCR or the immuno-fluorescence intensity levels. Therefore, when considering the number of zebrafish per replicate, it is critical to decide the type of data output first.

Prior to experimentation, it is critical to define what you will consider a single replicate. Due to the large number of progeny available, the number of individual zebrafish within one biological replicate can range from 3–15 animals. In general, the more animals included in a single replicate, the more accurate the output tends to be. When deciding on the actual number of animals, it is important to consider how much variability is expected. Some data tend to have greater variability and require a larger number of both biological and technical replicates. Examples of such data are behavioral outcomes. Behavior fluctuates significantly between animals, and identifying the true mean takes many replicates. In such cases, the number of fish is often several tens (adults) to hundreds (larvae) of individual animals to produce a single data point. In comparison, some data sets have less variability or contain categorical data. Within the zebrafish community, such data can be those defining cell types or developmental processes.

Although a large number of zebrafish per replicate is desirable, it is understood that cost and technical limitations significantly influence the number of animals used. "The Guide for Care and Use of Laboratory Animals"[111] requests minimizing the number of vertebrate animals used. Therefore, to be good stewards of the animals within our care and the monetary resources awarded, it is important to consider using the minimum number of animals required while performing valid and reproducible experiments. Nevertheless, we cannot sacrifice experimental rigor, as a wrong or incomplete answer to a question wastes all of the animals.

As long as these ethical considerations are accounted for, measuring multiple data points for an experimental group and taking the average value

from the collective data points as a single data point is one of the unique strengths the zebrafish model has, whether the data type is qualitative or quantitative. Such a single data point represents a sample mean. By the central limit theorem, these data points are likely to be normally distributed regardless of the distribution of the population from which the samples are drawn[112,113]. Whereas making a composite data point from multiple animals or by averaging multiple measurements and combining multiple such experiments are a common practice in invertebrate model systems, zebrafish are among the few vertebrate models that permit this type of rigorous experimental design.

Randomizing samples

Due to the genetic heterogeneity in the zebrafish, randomization starts with obtaining embryos from multiple parents. Whether an experiment uses larval or adult fish, the subject fish of an experiment should be obtained from multiple mating pairs of different lineages, which ideally means different fish tanks, to avoid lineage-specific biases. The embryos obtained from multiple parental pairs should be combined and then placed into Petri dishes, ensuring that each dish will contain embryos from all lineages. Otherwise, some dishes will contain embryos from a mating of one pair of fish while other dishes contain embryos from a mating of another pair. This, in turn, produces data that may be confounded by family-specific responses. When embryos can be collected from only one mating pair, the experiment can still be performed. However, ideally repeat experiments should be done with a different set of embryos from different parental pairs and on different days to ensure both technical and biological replicates are performed.

How the collected fish are distributed into experimental groups is also important. This is particularly true after the animals are out of the chorion and freely swimming. At this point, collecting poorly swimming larvae first is a common error that inadvertently occurs because it is easier to catch such larvae. This type of error, in turn, introduces variations in the collected groups that are not factored in the original experimental design. Such variabilities may represent differing baseline activity levels, nervousness, hormonal levels, and developmental delay. Alternatively, if there is no obvious visual phenotype to differentiate mutant from WT siblings, mutant animals may be collected into a particular experimental condition, either earlier or later, depending upon whether the mutation leads to hyper- or hypo-activity. Similarly, where the larvae are located in the plate matter, the larvae collected from the central area of a dish and those collected by the wall of a dish may display different behaviors or biochemical profiles. It is essential to randomize those collections into experimental groups. The same criteria apply to adult fish. When collecting adult fish from a housing tank, earlier and later caught fish should be distributed randomly to diverse experimental groups.

Improving reproducibility

As the scientific community strives to achieve experimental reproducibility, this review is intended to create guidelines for rigorous and reproducible experiments using the zebrafish model. The key to reproducibility has two primary facets. First, as discussed above, the experiment should be designed with key details in place from the beginning, including technical and biological replicates and analytical methods determined based on the best understanding of the specific scientific context. Second, all pertinent information for the experiment replicates should be provided to the scientific community. Here, we review key topics to increase reproducibility in zebrafish research and list critical factors that should be included in a manuscript (Table 4).

Information about the zebrafish used during experimentation

As with other animal model systems, following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines is now strongly recommended[114,115]. When considering zebrafish specifically, the methods section of a manuscript should provide details on animal husbandry. These practices for zebrafish care are well defined and part of basic operating procedures found in sources such as Westerfield's Zebrafish book[31], Zebrafish: a practical approach[17], or Zebrafish in Biomedical Research[116]. If practices deviate from the standards found within those sources, those should be detailed within the manuscript. This includes but is not limited to the light/dark cycle, feeding regimen, and water conditions.

The zebrafish are genetically diverse, and it is important to maintain that diversity. Therefore, the outbreeding frequency and the lines used should be included within a manuscript. For those who do not have access to the laboratory lines or decide to use local fish from a pet store, fish farm, or the wild, it is critical to note this in any publications. Should a mutant be

Table 4 | Author and reviewer checklist

Zebrafish information	
1)	Zebrafish is spelled correctly (do not use zebra fish, which is the autocorrect default)
2)	IACUC number provided
3)	Animal husbandry
	a) Light/Dark cycle of the zebrafish
	• Specify if embryos are or are not raised in light/dark cycle
	• Specify timing of experiments within the light/dark cycle
	b) Feeding regimen
	c) Water conditions: Temperature, salinity, pH
	d) Breeding
	• General breeding practice (e.g. frequency of outbreeding)
	• Husbandry of carrier lines (e.g. frameshift or transgenic alleles) used in experiments (inbreeding or outbreeding) if different from general husbandry practice
	e) Embryos are typically raised with a 28 °C incubator. Specify alterations to this norm
4)	Zebrafish strains used
	a) "Wild type" Strains (Tübingen, AB, etc.)
	b) Carrier lines (e.g. frameshift or transgenic) with details on either derivation processes of the line or where it was obtained
	c) All zebrafish lines and strains named according to their ZFIN nomenclature with ZFIN link
5)	State sex of the zebrafish if adults are used in experiments
Experimental information	
1)	Nucleotide sequences
	a) Genomic coordinates, genome assembly version
	b) Provide an ENSEMBL ID number or gene ID for all genes discussed
	c) If pertinent, human orthologs or paralogs to the gene of interest
	d) Sequence of all genome perturbations
	e) Plasmids with sequence and annotation
	f) Morpholino sequences
	g) In situ hybridization probe sequences and experimental conditions
2)	Protocol Details
	c) Microscope settings
	d) Details of behavioral assays
Data analysis information	
1)	Define the "*n*"used in the statistical analysis (ex. Cells, mitochondria, whole zebrafish) and define the number of zebrafish used per experiment
2)	Number of independent replicates
3)	Specify blindedness of experiments. If not blinded, state the reasons.
4)	Informatics tool details and scripts available
5)	Statistical analysis used
6)	Share published raw data within a depository

This table lists the information that should be available in zebrafish manuscripts to ensure reproducibility.

created within that background and the sequence varies from the published genome, this should also be noted in the publication. Additionally, it is ideal that any new laboratory WT line established be sent to the local zebrafish repository.

How the zebrafish embryos and larvae are raised is another critical piece of information. In most institutions, young fish (until 6-7 dpf) are raised in a Petri dish in an incubator at approximately 28.5 °C. The light/dark cycle of the embryos is also standardized in The Zebrafish Book. However, when the lights are turned on and off can be adjusted for optimum experimental timing depending upon the data being collected. For instance, if experiments require the embryos to be raised without any light in an incubator or in a light/dark cycle, the protocol details should be reported. For many studies, reporting the times for in vivo experimental steps during the light/dark cycle may be important, as results may depend upon hormonal levels (e.g. the circadian rhythm) or time since feeding (for those animals 7 dpf and older). Experimentation on adult animals requires specific details along with the ones described for larvae. This includes any changes to the standard husbandry, such as changes to diet, temperature, pH of the water, the number of fish per tank above or below the standard practice, and the light/dark cycle. The adult zebrafish require the sex of the fish used in the experiment to be stated. If only a single sex is used, a reason should be given. If a mix of the sexes is used, a percentage of male to female should be stated.

Since the zebrafish has many strains of "WT" that are generally available through zebrafish vendors or propagated specifically in a laboratory, the WT strains used should be detailed within the methods and made available upon request. It is particularly important to report the background strain of any transgenic lines. Further, if multiple transgenic insertions are bred together, the background strain of each line crossed together should be noted, as well as the WT line used to outcross for propagation. The use of pet store zebrafish poses a particular challenge in scientific reproducibility due to the maintenance of genetic variation alone. Such stocks can become problematic for the researcher due to differences in sequences from the published zebrafish genome. Additionally, providing new WT lines to the corresponding stock center for distribution would also be expected. Consequently, any lower initial cost of using such fish is greatly balanced by the substantive overall costs of using such animals in publications, and the use of established WT lines is almost always the most practical choice for effective zebrafish research. In fact, a method whereby sperm can be stored for as much as 7 days at room temperature[117] has been described, thereby making it even easier for labs in remote locations to establish WT strains. Accordingly, any mutant or transgenic lines should have the information on the background lines from which they are generated. All lines should be named using ZFIN nomenclature and given a ZFIN link to connect the paper to previous data.

Critical methodological detail is required for experimental repetition

The "Experimental Information" in Table 4 highlights vital information for those wishing to replicate the experiment and validate the findings reported within the paper. DNA sequences required for identifying the genes are critical to report and include, but are not limited to, plasmids, genomic sequence changes, morpholino sequences, primer sequences, etc. Additionally, the genome coordinates and the genomic assembly version should be stated since the sequences and annotation can differ depending upon the assembly version used. To ensure the specific gene sequence is associated with other information known about the gene, the ENSEMBL identification number or the gene ID should be given. Protocol details should also be reported. It has become a trend to see a paper cited for the full protocol, when in fact that paper does not include the entire protocol. For example, when staining for mRNA with an in situ protocol, it is simple to cite (Thisse et al.[118]). However, most laboratories do not follow this protocol step by step. Any changes to the cited protocol should be added to the methods section of a paper.

Similarly, behavioral experiments using the zebrafish can be difficult to replicate without adequate details. It is essential to clearly state the details of the behavioral assay system used, whether it is commercially available or developed within the institution, the software used for data analysis, and any possible confounding environmental details (temperature, noise level, possible vibrations, etc.).

Details on data analysis used

The number of zebrafish used, and the replicates done should be reported. Additionally, the statistical method should be justified for the type of data collected. Within the data analysis discussion, each experiment should specify whether the data were scored blind (single or double). Any informatics tools used for data analysis or scripts created to run the analysis should be made available to aid in reproducing the data. Finally, it is becoming common practice to share raw data in an open data repository. While not zebrafish specific, there are many examples of excellent data deposit sites available: https://www.nature.com/sdata/policies/repositories#general, http://oad.simmons.edu/oadwiki/Data_repositories, https://datadryad.org/stash.

Conclusions

In conclusion, the zebrafish is a robust and valuable vertebrate model organism. The large number of animals available for experimentation compensates for and takes advantage of the high genomic diversity within this animal system. However, effective deployment requires rigorous planning for both the number of animals and replicates required for experimentation, as well as the thoughtful use of statistical methods. It is also critical to include methodological details not commonly listed for experiments using isogenic animals to enable experimental repetition and direct comparisons. The guidelines detailed above lay a foundation for understanding the expectations of the zebrafish field to ensure rigor and repeatability of data to fully unlock the potential of this amazing model system.

Methods

Zebrafish husbandry

All zebrafish experiments were ethically carried out and with the approval of the Mayo Clinic and the University of Pennsylvania IACUC boards. We have complied with all relevant ethical regulations for animal use. The adult animals were maintained using standard husbandry conditions[31]. The zebrafish embryos were raised and maintained at 28 °C before experimentation. The WT larvae were outcrossed TLF line mated with the *casper* background. Larvae from the WT line (Figs. 1e,f, and 3) were exposed to approximately 0.003% PTU before 24 hpf to prevent pigment formation. The VEGF receptor (*kdr-like*) florescent line (Tg(*kdrl*:Hsa.HRAS-mCherry)s916) was used to image the vasculature. All experimental data was obtained from embryos or larval zebrafish prior to any sexual differentiation.

Zebrafish imaging

In situ hybridization was used to assess the mRNA expression pattern of the *shox1* gene as previously described[118]. The mRNA probe was created from the predicted Danio rerio shox homeobox transcript (GeneID: 66448; ZfinGene-051030-21). PCR primers (Forward: 5′-CAGCGCCTCGGCGTGTTTTC-3′; Reverse: 5′-AACTCACGGCGTTCGTGTCG-3′) were used to clone into a pCR4 TOPO vector (ThermoFisher: K457501). The dig-labeled probe was made using the reverse strand by digesting using the speI restriction enzyme. The larval images were taken using SCORE imaging[119] within a capillary tube. The final image is a z-stack of multiple in-focus images manually stacked.

The Tg(*kdrl*:Hsa.HRAS-mCherry)s916 was imaged as previously described. Briefly, the embryos were embedded in 1% low-melt agarose in E3 with tricaine to prevent movement. They were imaged using an inverted SP5 Leica Microscope system[120].

Zebrafish research analysis

To approximate the number of papers that utilize the zebrafish, we searched PubMed using the keyword zebrafish. The number of papers published was binned into the year they were published and graphed using GraphPad

Prism software. Data is available on Dryad: https://doi.org/10.5061/dryad.nvx0k6f22.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The original data is available on dryad data under: Zebrafishology, study design guidelines for rigorous and reproducible data using zebrafish in situ images dataset (https://doi.org/10.5061/dryad.nvx0k6f22).

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V.M.B.: wrote and assembled the manuscript, made the figures, responded to the reviewers, and coordinated the authors. P.D.: helped write and research the manuscript. H.B.L.: wrote the statistics section of the manuscript, provided statistical expertise. D.S.B.: helped write the section on maternal genome manipulation. J.L.A.: helped write the initial manuscript specifically on the maternal genome. A.J.L.: helped write the initial manuscript. R.X.: helped write the statistical section and provided statistical expertise. E.V.L.: created the zebrafish vasculature figure. M.J.F.: edited the manuscript and provided zebrafish expertise to ensure quality guidelines. M.A.P.: edited the manuscript and provided zebrafish expertise to ensure quality guidelines. M.M.: edited the manuscript and provided zebrafish expertise to ensure quality guidelines. S.A.F.: edited the manuscript and provided zebrafish expertise to ensure quality guidelines. R.G.E.: edited the manuscript from a zebrafish novice point of view for clarity. S.C.E.: edited the manuscript and provided zebrafish expertise to ensure quality guidelines.

Competing interests

The Authors declare the following conflict of interests: SCE and Mayo Clinic have a financial interest in the technology used in this research and may gain financially from its successful outcome.

Additional information

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