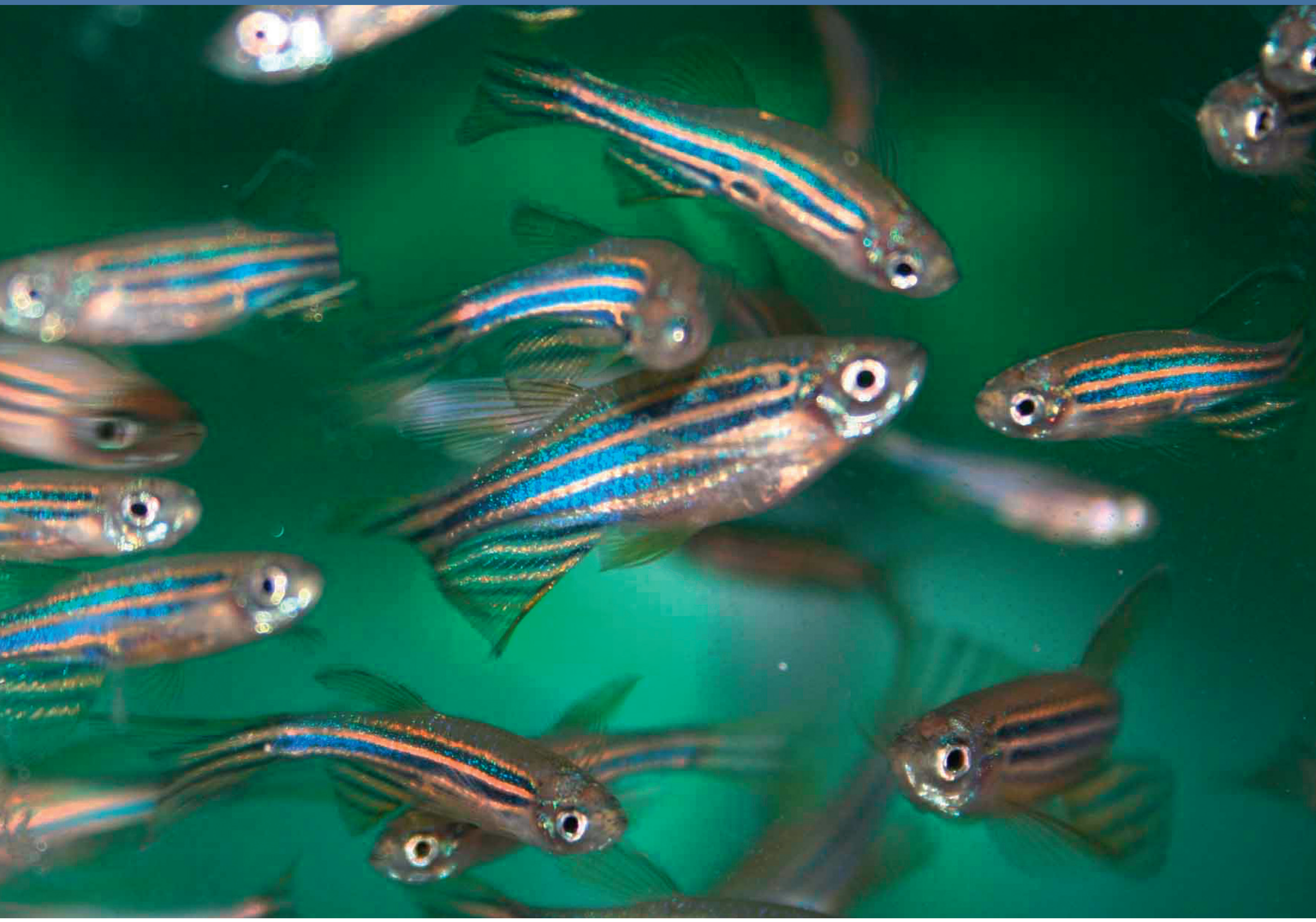
Basic behavioral neuroscience in zebrafish

A practical guide



A publication from Noldus Information Technology

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CONTENT & INTRODUCTION

CONTENT

INTRODUCTION

Welcome to the Noldus neuroscience zebrafish e-book! This e-book serves as a stepping stone for your behavioral research, providing background information and references per topic.

The e-book will delve into the significant transformation zebrafish undergo through their developmental stages: from the embryonic stage to larvae, and finally adult zebrafish. These stages directly impact their behavior and the methods used to measure it, thereby influencing the possible information extracted from each distinct stage.

Use this e-book to gain information on specific behavioral research topics, and how these are studied in the current scientific field. Noldus Information Technology strives to provide you with the best solutions and the most accurate information to advance your research.

Enjoy reading, and feel free to contact us if you have any questions on or suggestions for improving the contents of this e-book.

A BRIEF HISTORY OF ... ZEBRAFISH

George Streisinger pioneered the use of a then-popular tropical aquarium fish species, known today as the zebrafish (*Danio rerio*), in his research at the University of Oregon in 1972. Born in Hungary, Streisinger made a name for himself in the American academic landscape, eventually co-founding the Institute of Molecular Biology at the University of Oregon.

A geneticist by training, Streisinger initially conducted research on T-even bacterial viruses. In search of a suitable vertebrate model to take his research with phages to the next level, he soon discovered the ease of genetic modification in zebrafish to replicate specific diseases or their characteristics. Notably, he became the first person to clone a vertebrate, namely, the zebrafish.

From curiosity to cornerstone

Today, both the pharmaceutical industry and research scientists employ zebrafish to develop models for an array of diseases. These include leukemia, melanoma, immune and infectious diseases, developmental biology, (eco)neurotoxicity, neuronal defects, and neurodegeneration. The zebrafish's journey from an aquarium curiosity to a cornerstone of disease research represents its remarkable versatility and value in the scientific community.

The resilience of Streisinger in championing the zebrafish as a disease model has led to invaluable contributions to scientific progress in understanding human diseases. Since then, the zebrafish has evolved into more than just a unique model in basic research; it has become a preferred choice in biomedical research, leveraged for creating human disease models and investigating the impacts of drugs and toxins.

ADVISORY NOTE

In this e-book we describe a number of behavioral tests, their scientific background, and present protocols. This information is gathered from years of experience in the field of behavioral neuro-science and literature research. However, this information is not intended as a substitute for academic research. We advise you to always do your own research. We at Noldus are here to help you in providing solutions for your behavioral research.

WHY
DO WE MEASURE BEHAVIOR IN ZEBRAFISH

Zebrafish have steadily gained popularity in behavioral neuroscience and ecotoxicology. Their appeal lies in their rapid development, transparency, and, notably, their genetic similarity to humans. Moreover, as a vertebrate species with a well-characterized genome, zebrafish serve as valuable resources in understanding the genetic and neural basis of behavior. By evaluating zebrafish behavior at various developmental stages, we can acquire precious insights into many aspects of human health and disease. This chapter will explain why and how the zebrafish has been enriching this understanding, and where its future as a model organism lies.

GENETIC AND NEURAL SIMILARITIES WITH HUMANS

One of the key reasons zebrafish are utilized in behavioral research lies in the genetic and neural similarities they share with humans. Around 70% of human genes find a counterpart in zebrafish, with a significant portion of these being functionally conserved. This genetic resemblance provides researchers with the opportunity to investigate the influence of specific genes on various behavioral expressions, and in turn, draw connections to human conditions.

Moreover, the fundamental organization and function of the zebrafish nervous system mirror those of other vertebrates, including humans. This structural and functional conservation allows scientists to delve into essential aspects of neural circuits, neurotransmitter systems, and molecular pathways that shape behavior. Therefore, zebrafish serve as an indispensable tool in uncovering the complex dynamics of behavior and its underlying neural and genetic mechanisms.

The number of disease models

The zebrafish has initially made its name in research within the domain of developmental biology and gene function. But more and more human disease models are emerging and are proving to be of great importance in understanding underlying (genetic) processes of diseases like cancer[1], neurodegenerative diseases and other brain disorders[2,3], and even orthopedic research[4].

RAPID DEVELOPMENT AND HIGH FECUNDITY

Zebrafish are notable for their fast development and high fecundity, which make them highly suitable for behavioral research. Their entire nervous system develops within just a few days after fertilization. Additionally, with the capability to produce hundreds of eggs per week, zebrafish are an ideal model organism for large-scale behavioral studies. This combination of fast development and high fecundity allows researchers to quickly obtain a large sample size, enhancing the detection of minor behavioral differences and increasing the statistical power of their experiments.

The fact that zebrafish are externally fertilized and can be easily maintained in large laboratory populations contributes to their practical use in high-throughput screening and large-scale genetic studies. These traits enable researchers to conduct thorough behavioral analyses and accelerate the identification of genetic factors associated with various behavioral traits.

DRUG DISCOVERY AND SCREENING

The above-mentioned traits of zebrafish allows researchers to expose zebrafish embryos or larvae to a variety of compounds and on a relatively short time scale evaluate their impacts on behavior, neural development, and function. Common readouts are physical changes, behavioral alterations, or molecular responses.

These studies are not limited to any specific group of disorders. From neurological conditions to cardiovascular diseases and cancers, the use of zebrafish can assist in the early stages of drug development for a broad spectrum of human disorders. This approach is efficient and more cost-effective than traditional methods or using rodents.

With zebrafish, through genetic studies and, for example, the use of morpholino oligonucleotides (small molecules that can block gene function), researchers can systematically study the roles of individual genes in diseases [5]. This makes them a promising tool for finding and validating new drug targets. In these studies, researchers have found zebrafish with conditions that look like human diseases. The genes causing these conditions in zebrafish could lead to the discovery of new drug targets. Alternatively, these disease-like conditions in zebrafish could be used as a basis for more studies to find genes or drugs that can correct the condition [6].

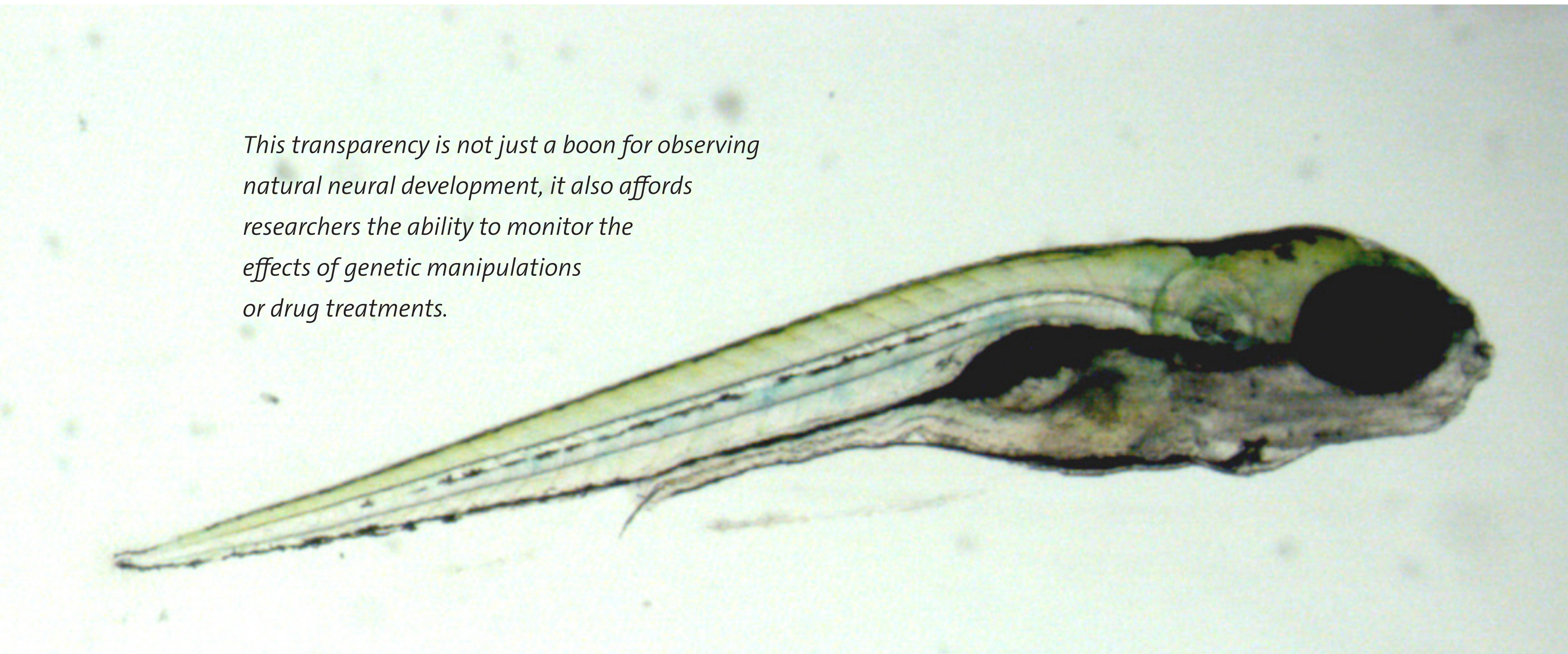
TRANSPARENT EMBRYOS AND LARVAE

The transparency of zebrafish embryos and larvae offers researchers a unique window into the real-time development and functioning of their nervous system. By deploying advanced imaging techniques like confocal microscopy or two-photon imaging, scientists can visualize the activity of individual neurons and track their influence on behavior throughout development.

This transparency is not just a boon for observing natural neural development, it also affords researchers the ability to monitor the effects of genetic manipulations or drug treatments. This allows them to see first-hand how these alterations can change neural function and development and how these changes, in turn, impact behavior. By enabling the direct observation of these processes, the transparency of zebrafish embryos and larvae contributes to a more holistic understanding of the complex interplay between genetics, drug effects, neural development, and behavior.

HIGHER THROUGHPUT, LOWER COSTS

Zebrafish, due to their relatively small size and the ability to be housed in large numbers, offer a cheap(er) option for behavioral research. Zebrafish can produce many eggs, which take a short time to develop: gastrulation begins as early as 6 hours post-fertilization and hatching occurs 2 days later, resulting in free-swimming larvae. It is thus possible to obtain large amounts of larvae/adult animals in a short amount of time, however, little space is needed for housing these large numbers of zebrafish as the embryos/adults are small of size [7]. Thus, compared to bigger animal models, like mice or rats, the cost of maintaining zebrafish is more manageable.



This transparency is not just a boon for observing natural neural development, it also affords researchers the ability to monitor the effects of genetic manipulations or drug treatments.

Zebrafish are also easy to care for. Well-established husbandry protocols exist for their maintenance and they require minimal space and resources compared to many other model organisms. Best practices in housing and husbandry will be further explored in the final chapter of this e-book.

ETHICAL CONSIDERATIONS AND THE 3 R'S

Incorporating zebrafish as a model organism in behavioral research presents an approach that addresses ethical concerns tied to animal experimentation. Positioned lower on the phylogenetic scale compared to mammals, the use of zebrafish may be considered a more ethically acceptable alternative under certain conditions.

The 3 R's—*Replacement, Reduction, and Refinement*—form a framework promoting the humane and responsible use of animals in research. Adherence to the principles of the 3 R's enables scientists to mitigate the impact on animal welfare while enhancing the quality, reliability, and reproducibility of their research.

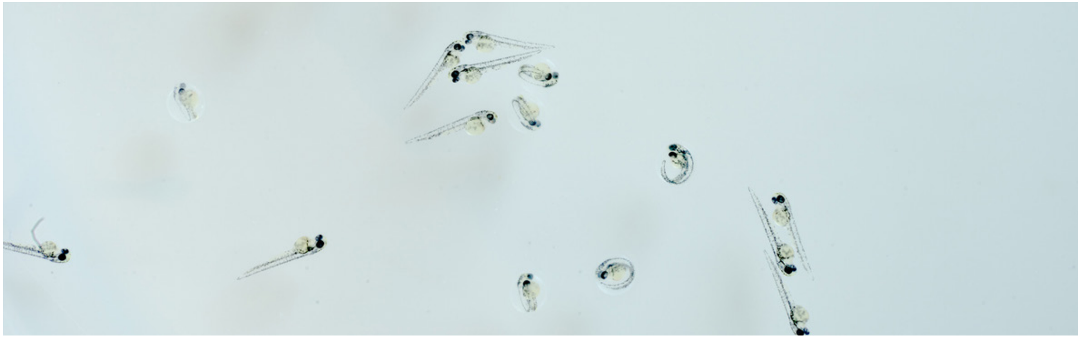
Replacement focuses on incorporating alternative methods that can avoid or replace the use of animals in research. Zebrafish allow researchers to replace the use of mammals, like rodents, in certain behavioral and genetic studies due to their significant genetic and neural similarities with humans. Moreover, the transparent embryos and larvae of zebrafish facilitate non-invasive imaging, reducing the need for invasive procedures and replacing less suitable animal models in some research settings.

Reduction is aimed at minimizing the number of animals used in experiments while still delivering reliable and valid scientific results. Given their high fecundity and fast development, zebrafish enable the study of large numbers of individuals within a short time frame, obtaining statistically significant data with fewer animals. Furthermore, advanced experimental designs and data analysis techniques can assist researchers in reducing the number of zebrafish needed for their studies, maximizing the information derived from each individual.

Refinement involves modifying experimental procedures and animal husbandry practices to minimize the pain, distress, and suffering of the animals. In zebrafish research, this includes optimizing housing conditions, handling practices, and experimental procedures to reduce stress and uphold high animal welfare standards. During zebrafish behavior measurement, researchers should mirror the natural environment as closely as possible to minimize stress and discomfort, resort to non-invasive or minimally invasive techniques, and apply appropriate anesthesia and analgesia when needed.

GOING FORWARD

Wrapping up, the unique characteristics and considerable benefits of zebrafish position them as an ideal model organism in behavioral studies. The use of zebrafish as a model enables researchers to delve into the genetic, neural, and environmental elements that influence behavior, thereby progressing our understanding of the complex realm of animal behavior, including human behavior.

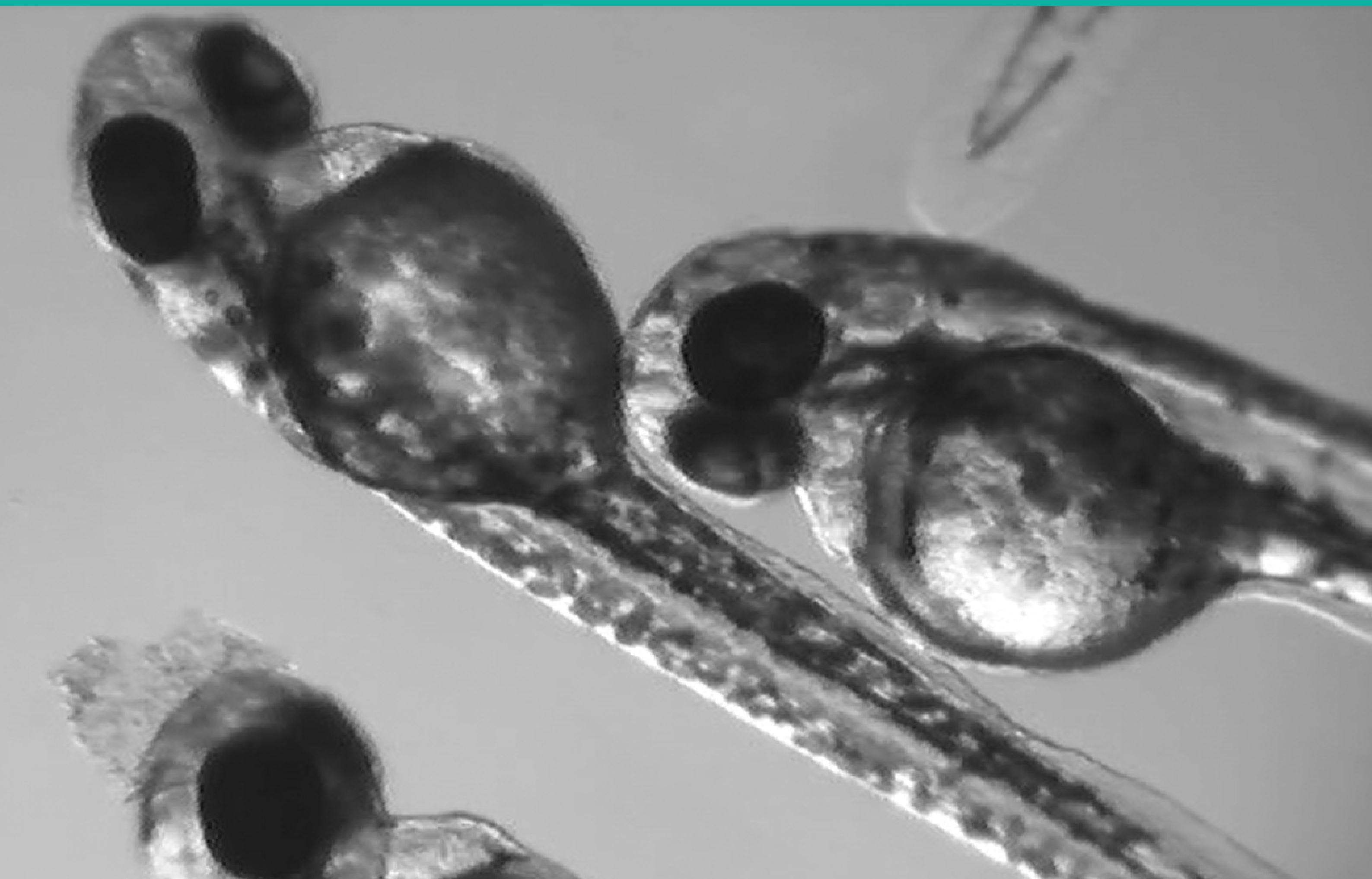


Many of the behavioral traits that zebrafish display are directly linked to survival and reproduction in their native environments. This includes *predator avoidance, foraging,* and *complex social interactions*. The diversity of this behavioral repertoire offers researchers a robust platform for studying the neural and genetic underpinnings of a variety of behaviors. However, the display of certain behavioral traits and their interpretation are very dependent on the age, or developmental stage, of zebrafish. In the following chapter each stage, and its (behavioral) characteristics will be explored further.

WHEN

DO WE MEASURE BEHAVIOR IN ZEBRAFISH

The age of a zebrafish significantly alters its body and behavior, and depending on the measurements and readouts you seek, and the formulation of your research question, selecting the appropriate age/stage for investigation is crucial. In this chapter, we'll go over the different growth stages of zebrafish and their associated behaviors, thereby facilitating the planning of your experiments and ensuring the acquisition of useful results by understanding what to measure (and what you can measure) at each specific stage.



When do we measure behavior in zebrafish

EMBRYONIC STAGE

During the embryonic stage (0-3 days post-fertilization - *dpf*), zebrafish develop rapidly, with many key developmental events occurring within the first few days post-fertilization. At this stage, zebrafish are transparent and display limited motor capabilities. The embryonic stage spans from fertilization to hatching, which typically occurs within 48 to 72 hours post-fertilization (*hpf*) at 28.5°C. During this stage, zebrafish embryos exhibit spontaneous muscle contractions, initially in response to touch, and later in a coordinated manner. Researchers can study the development of basic motor and sensory functions, as well as the formation of neural circuits during this stage.

Hpf & dpf

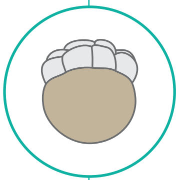
Hours post-fertilization (hpf) and days post-fertilization (dpf) are the common ways of denominating the age of zebrafish embryos, larvae, and even adult fish.

Key behaviors to measure:

- **Spontaneous movements** - Early in development, zebrafish embryos exhibit spontaneous muscle contractions, which can be used to assess the functionality of the neuromuscular system. The presence and frequency of spontaneous movements and coiling can be observed as early as 17 hpf.
- **Touch-evoked response** - By gently touching the embryos, researchers can evaluate their sensory and motor systems. Touch-evoked responses are a crucial measurement in zebrafish embryos. This response can be observed around 27 hpf, where the embryos exhibit motor responses to tactile stimuli, typically involving a swift, jerky movement or coiling. This allows researchers to study motor function, development, and the involvement of neural circuits in response to sensory input.
- **Photomotor responses** - Embryos exhibit changes in movement in response to light stimuli.

The development of a zebrafish can be studied throughout the different stages, from the zygote until the creation and growth of the organs, including the central nervous system due to the transparency of the embryo.

The embryonic stage starts with a newly fertilized egg, zygote, at 0 hpf and lasts until about 48-72 hpf (or 2-3 days dpf).

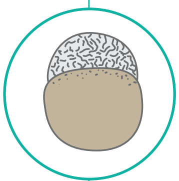


ZYGOTE AND CLEAVAGE PHASE

(0 – 3 hpf)

The *zygote*, the first cell to form after fertilization. An animal pole is formed inside the egg at this stage. This cell has multiplied and created a partial *cleavage* in just 45 minutes. These two cells will now divide in a series of cycles to create the blastomeres.

Although there are no overt behaviors to measure during this stage, researchers can observe the early cell divisions and the establishment of embryonic cell lineages.

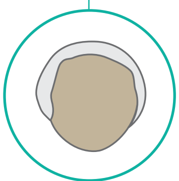


BLASTULA

(3 – 6 hpf)

The *blastula* stage occurs between 2 ¼ - 3 and 6 hpf. The phase begins with 128 cells, which multiply by one every fifteen minutes to reach roughly 1,000 cells in about three more hours. Basically, you want to be looking for the formation of a hollow ball of cells called the blastula. This occurs around 4 hours and at that point, a dome-shaped structure may be observed within.

Studies on acute toxicity can begin as soon as 2 hpf. There are still no observable behaviors at this stage. However, you can study the mechanisms that drive early embryonic patterning and the establishment of the body axes.



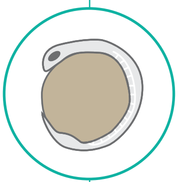
GASTRULATION

(6 – 10 hpf)

The term *epiboly* refers to the cell movements that take place during the gastrulation stage of embryonic development. This stage takes place between 6 and 10 hpf, and is characterized by extensive cell movements and the formation of the three primary germ layers: ectoderm, mesoderm, and endoderm. These germ layers give rise to different tissues and organs in the developing embryo.

During this stage, the neural plate forms, representing a primitive brain alongside cells destined to become the notochord, axial somite-derived muscles, and specific neurons in the hindbrain.

Although no specific behaviors can be measured during this stage, it is essential to understand the processes that drive tissue specification and organogenesis.



SEGMENTATION

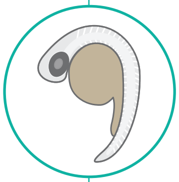
(10 – 24 hpf)

The *somites* (segmented blocks of tissue) form between 10 and 24 hpf during a phase known as segmentation. The fish's simple main organs start to show. At this point, the body begins to move as the cells morphologically differentiate, which basically shows the emergence of the basic body plan. After 15 hours, the embryo's body length and somite count can be utilized as a developmental index. The embryo's body length increases fast during this time. Along the somites, the muscle fibers and spinal cartilage are organized. Under the third somite pair, pronephric kidneys and ducts form. By the end of this stage, the major organ systems, including the nervous system and the heart, have started to form.

At this point, you can begin to measure touch-evoked responses and spontaneous muscle contractions, providing insights into the development of motor and sensory functions.

The development of the optic, olfactory, and ear tissues also begins at this stage. It is clear from this that the sensory neurons and the motor neurons also have a significant impact on the zebrafish's behavioral reactivity and motor reaction to light.

This reaction to light and dark stimuli will be crucial for behavior studies during the larval phase, which are typically conducted after 5 dpf, once the majority of the zebrafish's neurons are present.



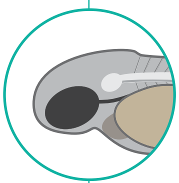
PHARYNGULA

(24 – 48 hpf)

The production of the *pharyngeal arches*, which will give birth to the mandibula and gill, is what gives the period its name. The pharyngula stage lasts from 24 to 48 hpf and during this stage, the embryo becomes more responsive to external stimuli.

The embryo's growth slows down at this stage, and the head condenses, the fins begin to form, the cells begin to pigment, and last but not least, the circulatory system forms and the heart starts to beat. One crucial aspect of this animal that will be used to demonstrate the cardiotoxicity of new chemical substances is its heart function.

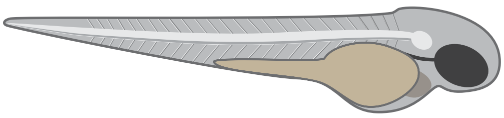
Researchers can start to observe more coordinated muscle contractions. Key behaviors to measure at this stage include touch-evoked responses and spontaneous, coordinated muscle contractions.



HATCHING

(48 – 72 hpf)

Hatching, the embryo's last stage, can take place between 48 and 72 hpf. Fish may hatch at different times, but regardless of whether they are within the chorion or not, developmental phases occur at the same times. Hatching occurs as the embryo secretes enzymes to break down the chorion, allowing it to escape.



The fish starts this phase with a length of about 3 mm, and by day three (3 dpf), it is 3.5 mm long. By this time, the majority of organs have finished their morphogenesis, and the fins, jaws, and gills grow swiftly. Early embryonic development before this point may be connected to pesticides' acute toxicity. They are no longer in the chorion and begin self-feeding after 5 days and are generally regarded as experimental animals under the Animal Welfare Act at this point.

This stage allows you to study the process of hatching, as well as the development of more complex sensory and motor functions.

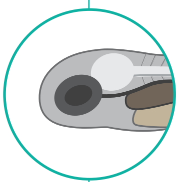
In summary, the embryonic stage of zebrafish development is a dynamic period marked by rapid changes in the embryo. By studying the different stages of embryonic development and the associated behaviors, researchers can gain valuable insights into the genetic, neural, and environmental factors that shape the formation of neural circuits and the emergence of early motor and sensory functions.

Check out the study by Kimmel et al. to read more details about the embryonic stages in zebrafish.[8]

When do we measure behavior in zebrafish

LARVAL STAGE

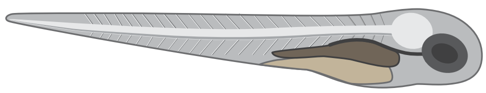
As zebrafish transition into the larval stage, their anatomy becomes more complex and their range of behaviors expands. The larval stage begins after hatching and lasts until approximately 30 dpf. During this period, zebrafish display a variety of behaviors that can be measured as they continue to develop and mature. Researchers can study locomotion, predator avoidance, social interactions, and the effects of environmental stimuli on behavior.



EARLY LARVAL STAGE

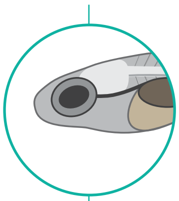
(3 – 7 dpf)

In the early larval stage, zebrafish start to swim freely and begin to eat external food as their digestive systems become functional. They still have a yolk sac, which is a source of nutrition that gradually gets absorbed. Key organs, like the heart and liver, become visible and the zebrafish develop pigmentation and more mature eyes. Morphological changes begin, such as the ossification of the jaw and skeletal system, and the swim bladder inflates, aiding buoyancy.



In terms of behavior, their motor activity increases significantly and they start to respond to visual stimuli. The initial behaviors include:

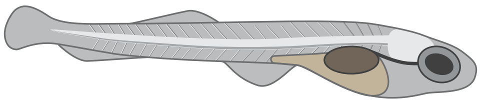
- **Free swimming -** The larvae start swimming freely. This behavior can be used to study their motor functions.
- **Feeding behavior -** As their digestive system develops, they begin to feed on microscopic organisms. This offers an opportunity to study feeding behavior.
- **Visual responses -** The maturation of their eyes allows them to respond to visual cues. Researchers can study behaviors such as phototaxis, where larvae move toward or away from light.



MID-LARVAL STAGE

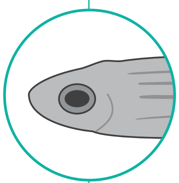
(7 – 14 dpf)

At the mid-larval stage, the zebrafish start to resemble adult zebrafish. Their pigmentation develops further, their fins start to take shape, and the jaw and other craniofacial bones become more developed, allowing them to feed on larger prey. The lateral line system, a series of sensory organs that detect water movement, also develops at this stage.



Behaviorally, this stage is characterized by:

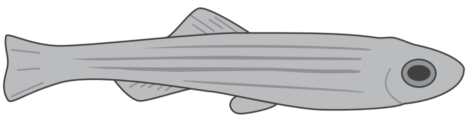
- **Improved locomotion** - The development of fins enables more advanced swimming patterns, which can be assessed using various behavioral assays.
- **Predator avoidance** - The maturation of their sensory systems, especially vision and the lateral line, enables larvae to better respond to potential threats. Researchers can study predator-prey interactions and defensive behaviors.
- **Social behavior** - Around this time, larvae start to display early signs of shoaling, a social behavior where they swim in groups. This can be used to study social interactions and the neural circuits involved.



LATE LARVAL STAGE

(14 – 30 dpf)

During the late larval stage, zebrafish experience a growth spurt, and their appearance starts to resemble adult zebrafish. They display adult-like pigmentation patterns and their fins fully develop. The first signs of sexual dimorphism (physical differences between males and females) may start to appear, but it's challenging to differentiate sexes at this stage.



Behaviorally, these larvae demonstrate:

- **Complex locomotion** - The larvae display more complex swimming patterns, similar to those of adults. Studying these patterns can provide insights into motor functions and neuromuscular coordination.

- **Enhanced social interactions** - As the larvae grow, their shoaling behavior becomes more pronounced. Studying these group behaviors can provide insights into social cognition and behavior.
- **Learning and memory** - Researchers can begin to study learning and memory at this stage, as larvae can be trained to associate specific stimuli with rewards or punishments. For example, a study by Widder et al.[9] demonstrates the capability of zebrafish larvae to learn and remember associations between environmental conditions and social rewards. Zebrafish larvae (6-8 dpf) were trained in an associative learning model to associate an adverse spatial location (the dark side of a chamber) with a social reward: a clear window to view conspecific fish. The larvae were initially transferred individually into chambers separated by opaque infrared-transparent dividers. After a 5-minute acclimation period, a video projector superimposed light or dark areas on alternative sides of the test chamber for 15 minutes, establishing the normal light/dark preference pattern for each fish; typically, the light side is preferred. The position and movement of each fish in the light and dark areas were automatically collected with a DanioVision system using Noldus' EthoVision XT software. Following the 30-minute pre-training assessment, the fish were trained for 3 hours, using 45-minute alternating light and dark projections, to associate a view of conspecific fish with the dark side of the chamber. After training, the fish were held overnight and placed back into the chamber with opaque infrared-transparent dividers, for 24 hours post-training. The results showed that socially paired fish exhibited a significant change in preference for the dark environment when compared to pre-training, indicating retention of memory associating a dark environment with prior social reward. This increased movement in the dark environment suggests that trained fish are searching for social cohorts in the dark environment even 24 hours after training.

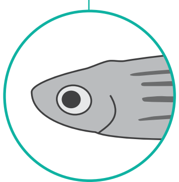
When do we measure behavior in zebrafish

JUVENILE & ADULT STAGES

As zebrafish transition from the larval stage and enter their juvenile and adult stages, we see further development and maturation. This period, beginning approximately 30 dpf and extending into the fish's life beyond 90 dpf, brings about significant physical, physiological, and behavioral changes. The distinction between the juvenile and adult stages is typically marked by sexual maturity, which happens around 90 dpf, although this can vary depending on factors such as diet and environmental conditions.

During these stages, the zebrafish's body shape finalizes, pigmentation finishes developing, and sexually dimorphic features become pronounced. Their behavior also matures, and more complex behavioral patterns emerge. These stages offer researchers the opportunity to study a range of behaviors, from complex social interactions to reproductive behavior and long-term memory.

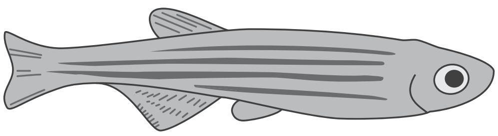
During these stages, the zebrafish becomes an even more powerful model for scientific study, not just for genetic and developmental research, but also for understanding complex behaviors and studying diseases.



JUVENILE STAGE

(30 – 90 dpf)

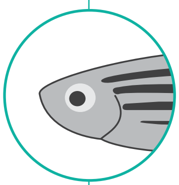
The juvenile stage marks the transition from larvae to adult zebrafish. During this period, zebrafish undergo significant morphological changes that signal maturation. This period sees a significant growth spurt where their body size more than doubles.



By approximately 30 dpf, the juveniles exhibit adult-like pigmentation patterns and their fins are fully developed. The sex of the zebrafish can now be determined based on morphological characteristics, such as the shape of their anal fin (pointed in males, fan-shaped in females) and the body shape (males are more streamlined while females have a more rounded belly). This is also the stage where the gonads are fully developed, paving the way for sexual maturation.

Behaviorally, juvenile zebrafish are capable of exhibiting a wide range of complex behaviors:

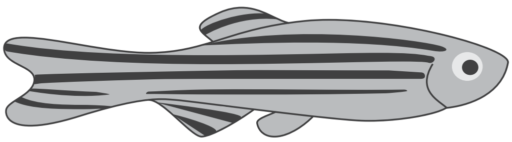
- **Complex locomotion** - The fully developed fins allow for intricate swimming patterns and increased exploration, revealing more about the zebrafish's motor functions and neuromuscular coordination.
- **Social interactions** - As juveniles mature, their social behaviors become more complex. Researchers can observe and measure shoaling and courtship behaviors, both of which provide valuable insights into social cognition and behavior.
- **Learning and memory** - The capacity for learning and memory continues to develop. By using training paradigms, researchers can study these cognitive functions in depth.



ADULT STAGE

(90+ dpf)

In the adult stage, zebrafish have completed their physical development. They reach sexual maturity around 90 dpf, at which point they are capable of reproduction. Adult zebrafish display sexually dimorphic features, including a rounded belly in females and a slim, torpedo-shaped body in males. This period of their life is marked by consistent growth, and their average lifespan can range from 3 to 5 years.



Behaviorally, adult zebrafish display a wide range of complex behaviors that can be studied:

- **Reproductive behaviors** - Adult zebrafish exhibit a range of sexual and reproductive behaviors including courtship rituals, mating, and parental care. These behaviors can be studied to understand reproductive strategies and the influence of various factors on reproductive success.
- **Social hierarchy** - Adult zebrafish establish social hierarchies, which can be observed and studied to understand social dynamics in animal groups.
- **Learning and memory** - At this stage, zebrafish have a fully developed capacity for learning and memory. This allows researchers to use a variety of training paradigms to study cognitive functions in depth.

- **Stress and anxiety-related behaviors** - Adult zebrafish are often used in studies of stress, anxiety, and related behaviors. This can be done by exposing them to various stressors and measuring their behavioral responses.

It's important to remember that the timing of these stages can vary depending on the temperature of the water, with development proceeding more quickly in warmer water. The ages given are approximate and apply to zebrafish raised at a typical laboratory temperature of 28.5°C.

HOW

DO WE MEASURE BEHAVIOR IN ZEBRAFISH

There are some great tools you can use to measure behavior in zebrafish. However, related to the previous chapter, the use of these tools can differ quite a bit depending on the developmental stage of the zebrafish. Powerful tools such as DanioScope (morphology in embryos and larvae), DanioVision (behavior in larvae) and EthoVision XT (behavior in larvae and adults) provide the means to accurately measure physiological and behavioral parameters at every stage. This chapter will guide you through the proper use of these tools, in what research fields they are used, and some common behavioral protocols in zebrafish (and what their outcome means!).

How do we measure behavior in zebrafish

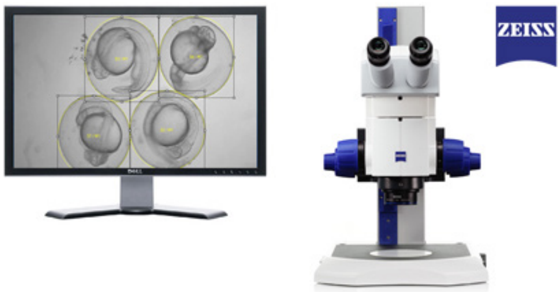
RESEARCH TOOLS

DANIOSCOPE

Automated detection of zebrafish embryos

During zebrafish's early development, body transparency and behaviors like spontaneous movements and touch-evoked responses are prominent. DanioScope is a specialized tool used by researchers to measure these behaviors and physiological aspects. This software solution is coupled with an imagery microscope, such as the ZEISS SteREO Discovery.V8, to acquire detailed images and video files that can be analyzed by DanioScope.

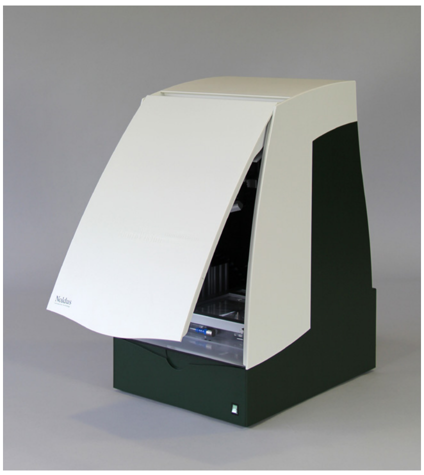
Research using DanioScope often examines zebrafish embryo models, focusing on morphological traits and metrics like body length, yolk size, and heart rate. These metrics are pivotal in developmental biology, neurology, toxicology, and cardiology, providing insights into development, mutations, or drug impacts.



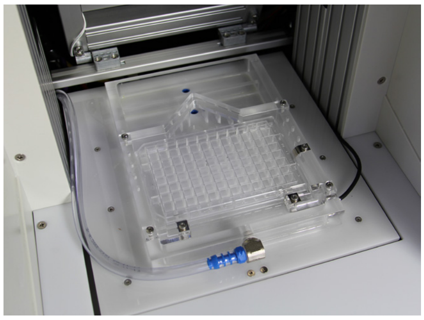
DANIOVISION

Complete and controlled observation chamber

DanioVision is a complete observation system designed for high-throughput tracking of zebrafish larvae and other small organisms. In larval zebrafish, DanioVision is employed for analyzing locomotion and social behaviors. This system houses an observation chamber for zebrafish behavior, offering controlled environments for experimentation. The observation chamber includes a high-quality camera and the system is delivered with its dedicated software, EthoVision XT, pre-installed on a computer. EthoVision XT can track and analyze individual or group zebrafish behaviors, like swimming speed and location preference.



DanioVision is often used in studies related to drug development, behavioral genetics, toxicology, and circadian rhythmicity. In these studies, larvae activity and movement patterns are basic measurements that can reveal information on stereotypic and epileptic behaviors, circadian rhythmicity, motor control, movement disorders, neural development, and more.



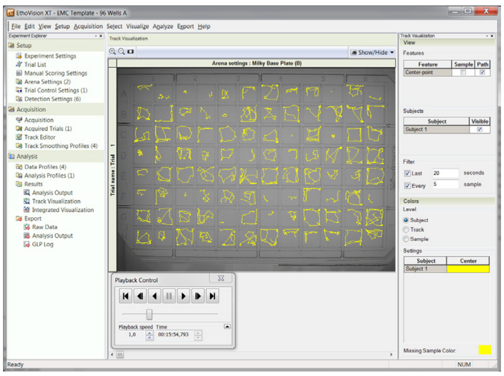
ETHOVISION XT

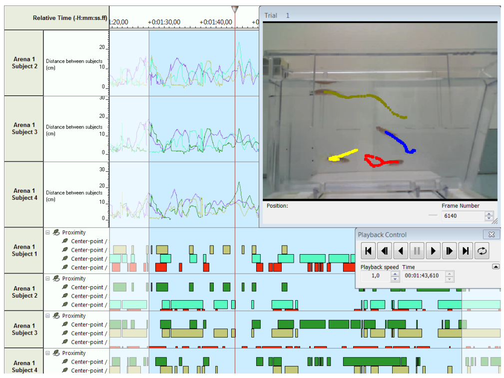
Powerful video tracking

EthoVision XT is the world's most used video tracking tool, as well as a software platform to build your set-up on. Video tracking essentially means that EthoVision XT recognizes your zebrafish (adult or larva) from a live video feed or video file and is able to track its movement, activity, velocity, rotations, etc.

As *'the engine of DanioVision'*, you can use EthoVision XT to set the intensity of white light in the DanioVision observation chamber, which lets you induce a startle reflex or mimic a day/night cycle. You can also control the frequency, timing, and duration of the light stimulus. All actions are logged in the software, which facilitates a nearly automated analysis. Add-ons for the DanioVision system, such as the *tapping device* or *optogenetics equipment*, are also programmed and controlled with EthoVision XT.

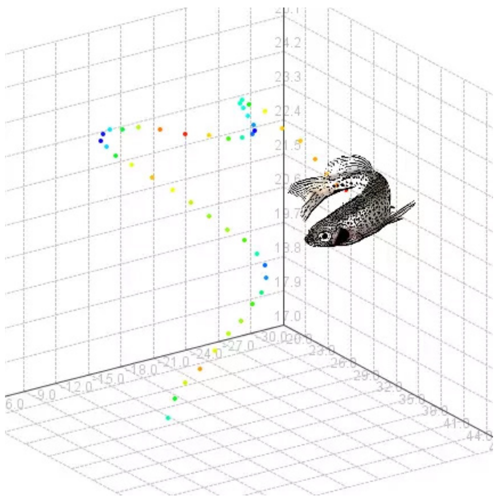
The EthoVision XT software that comes with the DanioVision system is not restricted to the DanioVision system, but can be used for other experiments as well. For example, you can track adult zebrafish in *shoaling tests*, *T-maze learning*, *shuttlebox tests, place preference*, and so on.





TRACK3D
Three dimensional video tracking

While EthoVision XT excels at two-dimensional tracking, zebrafish behaviors in 3D spaces, like diving or wall-hugging, require additional tools. Here, Track3D complements EthoVision XT by offering 3D tracking capabilities, allowing depth-based behavior analysis in zebrafish, making it invaluable for comprehensive behavioral studies.



For instance, in the novel tank test, fish may exhibit the behavior of thigmotaxis (swimming close to the walls) or diving. Tracking in three dimensions is very useful to distinguish these two behaviors. Likewise, although measurements of velocity and distance moved carried out in 2D will often give a good approximation, there are occasions in which information can be lost, simply because a fish is swimming straight towards the camera while no movement is recorded.

Make sure to also check out this short video about Track3D.

Find a full overview of behavioral research tools for (zebra)fish on our website.

How do we measure behavior in zebrafish

RESEARCH FIELDS

CARDIOLOGY AND VASCULAR RESEARCH

The heart and blood vessels develop early and rapidly in zebrafish embryos, bearing remarkable similarities to human cardiovascular development. This makes them a valuable model for cardiology and vascular research. Key metrics in these studies include heart rate, inter-beat intervals, and blood flow.

With the DanioScope system, you can designate the heart area in the video footage, allowing the system to automatically calculate beats per second (BPS) and beats per minute (BPM).

Beyond heart rate, DanioScope can also be used to assess blood flow, a critical measurement in studying heart function and vascular health. Using changes in pixel counts on a frame-by-frame basis, the system measures the flow in a designated blood vessel, providing an activity percentage.

Additionally, DanioScope is adaptable to measure gut flow, another significant metric that is particularly relevant in studies of digestive system development and health. Much like the measurement of blood flow, researchers select the part of the gut to measure from wall to wall in the video image, and the system uses frame-by-frame pixel changes to estimate flow.

A study by Isa Da'as et al. (2022) demonstrates the use of Noldus DanioScope to assess cardiac heart chamber measurements and cardiac contractility in a zebrafish (embryo) model of inherited hypertrophic cardiomyopathy.[10]

MORPHOLOGICAL STUDIES

Morphological changes can offer significant insights into the growth, development, and health of zebrafish embryos and larvae. DanioScope provides an invaluable tool for such morphological studies. Through its capability to process images or snapshots from videos, researchers can track changes over time and identify potential malformations. By defining distances or areas of interest and calibrating the system accordingly, it's possible to measure lengths and surfaces automatically, including tail length, eye size, pericardial area, or any custom measurements as required.

Varshney et al. (2022) use Noldus DanioScope to quantitatively measure the total body length of larvae (from head to tail), eye size, and swim bladder size. This was done to investigate the impact of a widely used antioxidant in automobile tyres and many rubber products on acute toxicity, morphology, swimming behavior, heart rate, and oxygen consumption in zebrafish larvae. [11]

ONCOLOGY

Oncology research is consistently evolving, and zebrafish embryos and larvae, due to their unique characteristics and rapid development, offer new insights into cancer biology. These embryos not only complement traditional research models, but also enhance our understanding of cancer development and treatment. Zebrafish tumors closely mimic human ones, as noted by Völkel et al. (2018), enabling a detailed exploration of cancer mechanisms and spread [12].

The transparency of zebrafish embryos is advantageous for xenotransplantation studies with human cancer cells, facilitating real-time tracking of cancer cell behavior, dissemination, and metastasis. Additionally, their suitability for high-throughput molecular screening accelerates the discovery of potential anti-cancer treatments. Recent research, like that by Virtanen et al. (2023), delves into specific cancer targets using zebrafish, by using a DanioVision system to look at the behavioral response in a phototaxis assay with 4 dpf old larvae [13].

In summary, zebrafish, due to their genetic adaptability, early-stage transparency, natural cancer development, and drug screening capability, are invaluable in oncology research, further supported by Noldus' behavioral tools.

These articles highlight the potential of the zebrafish model in oncology research:

- Barriuso et al. (2015): *Zebrafish: A new companion for translational research in oncology.* [14]
- Astone et al. (2019): *Fishing for cures: The alLURE of using zebrafish to develop precision oncology therapies.* [15]
- Kwiatkowska et al. (2022): *Zebrafish—An optimal model in experimental oncology.* [16]

NEUROSCIENCE AND BEHAVIOR

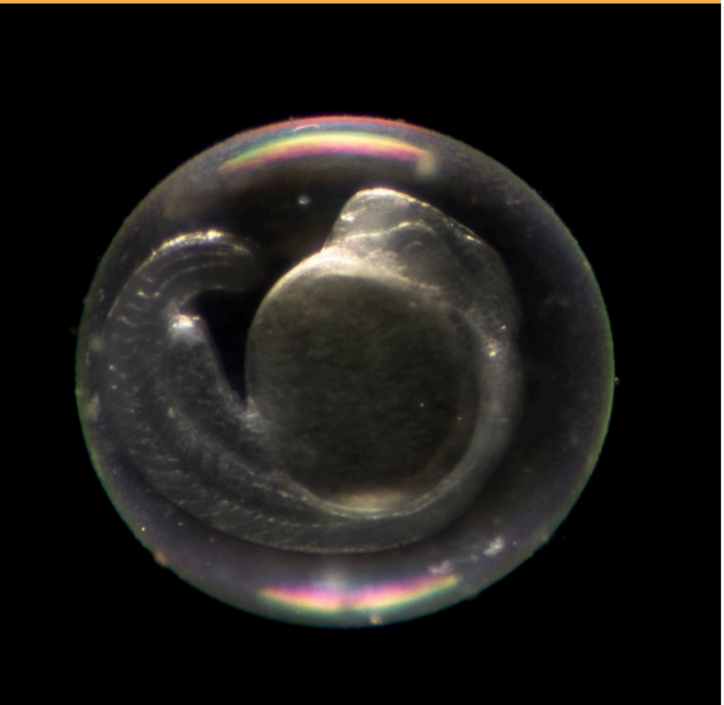
In zebrafish embryos, the visible developing nervous system offers a unique window into neurodevelopment, degeneration, and neurotoxicity. Researchers utilize techniques such as neural migration tracking, neuron connection observation, and neural activity analysis.

Zebrafish embryos are favored in neuroscience due to their genetic parallels with humans, ease of genetic alteration, and early-stage transparency. Using tools like DanioScope, researchers can evaluate the effects of drugs on zebrafish behavior, offering insights into neuroactive substances and neurodevelopmental disorders.

STUDYING AUTISM SPECTRUM DISORDERS WITH ZEBRAFISH EMBRYOS

The way zebrafish embryos are used in more specific areas of neuroscience, such as studying Autism spectrum disorders (ASDs), is thoroughly explained in a review paper by Rea et al. (2020). They state the complexity of ASDs, which are neurological disorders characterized by varied and often unique symptoms [17]. It also is the genetic diversity of ASDs that has posed significant challenges in the field, especially as different genetic mutations can result in similar phenotypes. Zebrafish models, due to their genetic tractability and high genetic homology with humans, can thus be used to investigate the functions of ASD risk genes. For instance, there are now 12 ASD risk genes with validated zebrafish mutant models. These models can reveal how specific genetic mutations alter neurodevelopment and contribute to ASD symptoms.

In addition to genetic models, zebrafish embryos are also used in non-genetic ASD models, including pharmacological and gnotobiotic models. These models allow for the exploration of environmental factors contributing to ASD, adding another dimension to our understanding of this complex disorder.



Studying zebrafish behavior helps us decipher the influence of environmental and genetic factors on behavior. This understanding can lead to treatments for human behavioral disorders. Zebrafish also serve as models for brain development studies, offering potential breakthroughs in understanding and treating neurological diseases.

Notably, zebrafish embryos display behaviors as early as 17 hpf, like tail flicks. These behaviors, measurable non-invasively, shed light on nervous system development. Changes in such behaviors can indicate neurodevelopmental issues and are essential for testing potential neuroactive drugs' impacts. Researchers can then gauge the effectiveness and safety (and toxicity) of experimental treatments.

Other commonly studied neurological disorders in zebrafish models

- *Epilepsy* - Zebrafish larvae have been used to model various forms of epilepsy, including both genetic and chemically-induced forms of the disorder. Researchers can use a variety of techniques to induce seizures in the larvae, such as administering convulsant drugs or exposing them to specific light stimuli, and then study the underlying neural mechanisms that drive the seizures.
- *Parkinson's disease* - Zebrafish have been used to model various aspects of Parkinson's disease, including motor deficits, neurodegeneration, and dopaminergic dysfunction. Researchers can use a variety of techniques to induce Parkinson's-like symptoms in the fish, such as exposing them to environmental toxins or manipulating specific genes associated with the disorder.

- *Alzheimer's disease* - Zebrafish have also been used to model various aspects of Alzheimer's disease, including beta-amyloid accumulation, neuroinflammation, and cognitive deficits. Researchers can use a variety of techniques to induce Alzheimer's-like pathology in the fish, such as exposing them to specific toxins or manipulating specific genes associated with the disorder.

ECO-NEUROTOXICOLOGY

Eco-neurotoxicology is an interdisciplinary field that bridges the gap between toxicology, ecology, and environmental science. It is aimed at unraveling the relationships between environmental contaminants, nervous system function, and the health and stability of ecosystems. By studying these interactions, researchers aim to inform policies and practices that safeguard both wildlife and the environments they inhabit.

Eco-neurotoxicology primarily focuses on pollutants and contaminants found in the environment. These may include heavy metals (e.g., mercury, lead), pesticides, industrial chemicals, pharmaceuticals, and other substances that can negatively affect the health of organisms and ecosystems.

Please also check out this white paper on eco-neurotoxicology writing by renowned professor Donald Wlodkowic.

READ

The central aspect of this field is the study of neurotoxic effects. Neurotoxicity refers to the harmful impact of substances on the nervous system. These effects can range from changes in neural development and function to disruptions in neural signaling, which may lead to behavioral abnormalities or neurological disorders. An example of behavioral endpoints commonly used in zebrafish (usually in larvae or juveniles) to assess these neurotoxic effects are:

- **Spontaneous locomotor activity** - Speed alteration tests, overall changes in motility (hyperactivity, hypoactivity), and specific swimming patterns such as thigmotaxis (*wall hugging*).
- **Sensory-motor biotests** - All aquatic animals are able to respond to external stimuli and alter their behaviors accordingly. The analysis of more complex behaviors can be very advantageous due to their sensitivity as well as high physiological and ecological relevance. The startle response is an example of sensory-motor testing. It is the animal's defensive reaction to a sudden and unexpected stimulus, such as a sound, vibration, light change, or a moving shadow simulating a predator. You usually see a pronounced increase in non-directional locomotion, often followed by more complex behaviors, such as avoidance or shelter seeking.

Check out this study by Xu et al., that investigates the (neuro)toxic effect of Cadnium. [18]

Unlike traditional neurotoxicology, which often focuses on human health, eco-neurotoxicology examines the effects of contaminants on wildlife species, including birds, fish, mammals, and invertebrates. It also considers the broader ecological implications of neurological disruptions within ecosystems. Researchers in eco-neurotoxicology investigate how exposure to environmental contaminants can alter an organism's behavior and physiology.

Furthermore, they also research how contaminants move through the food chains in an ecosystem. This field also informs conservation and environmental management efforts. Understanding how contaminants affect wildlife can help develop strategies to mitigate the impact of pollution and protect vulnerable species and ecosystems.

OTHER COMMON BEHAVIORAL TESTS IN ZEBRAFISH

- The *larval photomotor response (LPR) assay* is a sensory-motor biotest performed on freely swimming zebrafish larvae at the age of 5 to 7 dpf. At that developmental stage, fish show stereotypic non-directional light seeking behavior, which is a rapid increase in locomotion in response to turning off the light.
- *Phototaxis* is a directional response to light. It can be positive when the test animal moves toward the light, and negative when the animal moves away from it. Qian et al. (2021) found a significant inhibition of the phototactic response in zebrafish larvae following exposure to Boscalid, a neurotoxic fungicide.[21]
- *The light-dark preference test* is a simplified version of the phototaxis assay and has been a well-validated test in zebrafish larvae and adults. Upon acclimation, the animals are exposed to a binary light stimulus: one half of the chamber is illuminated while another half is kept in darkness.

Examples of the use of lpr assays

Holloway et al. (2021) studied the neurotoxicity of the polycyclic aromatic hydrocarbon Benzo(a) pyrene, which caused locomotor hypoactivity at dpf 6 in zebrafish larvae, as measured with the LPR test. Using this test, they measured changes in swimming patterns and overall distance moved.[19]

Bownik and Wlodkowic (2021) describe the LPR test as a tool to observe a reproducible startle response to repeated light-dark changes. Habituation and sensitization to these light/dark changes can also be used to study the impact of toxicants on non-associative learning.[20]

The light-dark preference test can, for example, be performed in a Light Dark Tank (credit: MazeEngineers/Conduct Science), which is an acrylic tank "(15 cm height x 10 cm width x 45 cm length) that is divided equally into one black half and one white half. Walls and bottom are either black or white.

How do we measure behavior in zebrafish

PROTOCOLS FOR EMBRYOS & LARVAE

The following protocols serve as foundational guidelines for when you take your first steps into zebrafish behavioral research. While these suggestions are designed to help you take these initial steps, it's essential to adapt and refine them based on specific research objectives and experimental conditions.

PHOTOMOTOR RESPONSE (PMR) IN ZEBRAFISH EMBRYOS

This is a crucial behavioral assay that provides insights into several aspects of zebrafish development and physiology, such as neural development, but also the functionality of the visual system. This test can be used to assess the effects of drugs or toxic agents on the nervous system. Compounds that affect neural function may alter the PMR. The most common developmental stage for assessing PMR is between 22 to 28 hpf. At this stage, zebrafish embryos have developed the necessary neural circuits to exhibit a photomotor response, but they have not yet started free swimming.

Objective
To assess the motor response of zebrafish embryos to light stimuli.

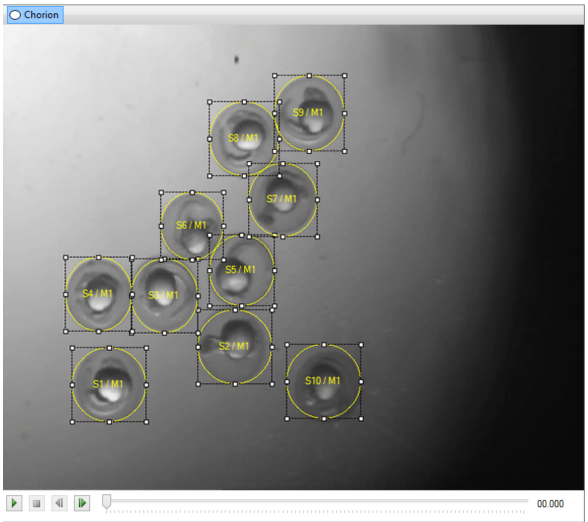
Materials
- Multi-well plate.
- Light source and video recording setup: for example, a WEISS SteREO Discovery.V8 microscope to acquire crisp and detailed images and video files.
- Computer with tracking software: DanioScope.

Procedure
1. Place individual embryos in the wells of a multi-well plate filled with embryo medium.
2. Allow embryos to acclimate for 5 minutes.
3. Expose embryos to a brief light flash.
4. Record the motor response for 1 minute.
5. Analyze the frequency and intensity of tail flicks or coiling.

Interpretation
A clear control group is crucial in determining the effects of the PMR. Compared to control, after (for example) toxicity exposure there can be either hypo- or hyperactivity of this response, indicating an aberration.

Parameters reported by DanioScope

- *Burst activity* – percentage of time (from total measurement duration) the embryo was moving
- *Inactivity* – percentage of time of inactivity (100% - burst activity)
- *Burst duration* – total time spent active (sum of all movement durations)
- *Inactivity duration* – total time spent inactive
- *Burst count* – number of times the embryo moved
- *Burst count / per minute* – number of movements per minute



Add Subject(s)... Delete Subject(s) Delete Measurement(s)

Subject	treatment	age	Measurement	Start	Stop	Duration	Status	Burst Activity (%)	Inactivity (%)	Burst Activity Duration (s)	Inactivity Duration (s)	Burst Count	Burst Count / Minute
S1	0.10	24 hpf	M1	00.000	01:01.040	01:01.080	Acquired	2,82	97,18	1,72	59,36	10	9,82
S2	0.10	24 hpf	M1	00.000	01:01.040	01:01.080	Acquired	4,32	95,68	2,64	58,44	11	10,81
S3	0.10	24 hpf	M1	00.000	01:01.040	01:01.080	Acquired	5,50	94,50	3,36	57,72	16	15,72
S4	0.10	24 hpf	M1	00.000	01:01.040	01:01.080	Acquired	3,93	96,07	2,40	58,68	17	16,70
S5	0.10	24 hpf	M1	00.000	01:01.040	01:01.080	Acquired	4,06	95,94	2,48	58,60	10	9,82
S6	0.10	24 hpf	M1	00.000	01:01.040	01:01.080	Acquired	3,21	96,79	1,96	59,12	13	12,77
S7	0.10	24 hpf	M1	00.000	01:01.040	01:01.080	Acquired	1,51	98,49	0,92	60,16	6	5,89
S8	0.10	24 hpf	M1	00.000	01:01.040	01:01.080	Acquired	5,11	94,89	3,12	57,96	11	10,81
S9	0.10	24 hpf	M1	00.000	01:01.040	01:01.080	Acquired	4,26	95,74	2,60	58,48	12	11,79
S10	0.10	24 hpf	M1	00.000	51.960	52.000	Acquired	1,00	99,00	0,52	51,48	4	4,62
			M2	52.440	01:01.040	08.640	Acquired	0,00	100,00	0,00	8,64	0	0,00

With DanioScope, you are able to accurately determine parameters, such as Burst activity, Inactivity, Burst duration, Inactivity duration, and Burst count when performing the PMR assay in zebrafish embryos.

TOUCH-EVOKED RESPONSE IN ZEBRAFISH LARVAE

The touch-evoked response is a fundamental reflex that indicates the proper functioning and connectivity of sensory neurons, interneurons, and motor neurons. This test also provides insights into the development and functionality of the sensory system, particularly the mechanosensory neurons. Any anomalies in the response can suggest defects in the development or function of these sensory cells. The touch-evoked response can be observed in zebrafish larvae as early as 2 dpf. At this stage, the larvae have developed sufficiently to exhibit a clear motor response to tactile stimuli. The response becomes more pronounced and consistent as the larvae continue to develop. Typically, researchers assess the touch-evoked response between 2 to 5 dpf, depending on the specific objectives of the study and the developmental milestones they are interested in examining.

Objective

To evaluate the motor response of zebrafish larvae to tactile stimuli.

Materials

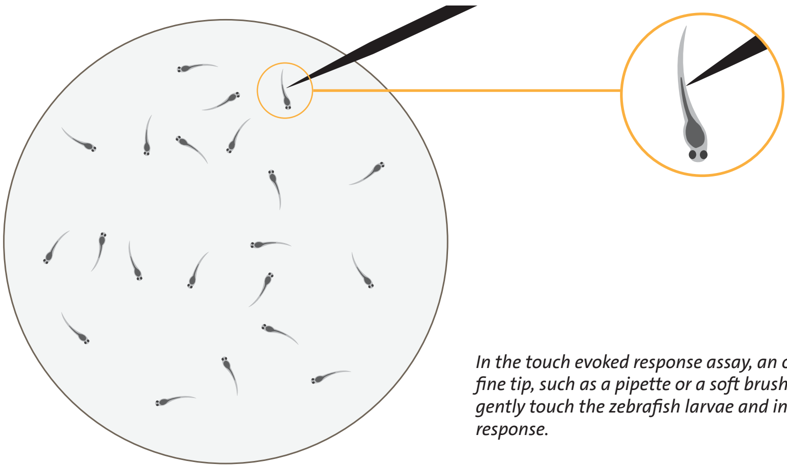
- Petri dish.
- Soft brush or hair or pipette or forceps.
- Video recording setup: a (stereo)microscope with diffuse light source and mounted camera.
- Computer with tracking software: EthoVision XT.

Procedure

1. Place larvae in a petri dish filled with embryo medium.
2. Gently touch the larvae using a soft brush, hair pipette, or forceps.
3. Record the escape response.
4. Analyze the speed, direction, and duration of the response.

Interpretation

Variations in the escape response can indicate motor deficits or altered sensory perception, whether higher or lower. In this case, a proper comparison to a control group is crucial.



In the touch evoked response assay, an object with a fine tip, such as a pipette or a soft brush can be used to gently touch the zebrafish larvae and initiate the 'escape' response.

STARTLE RESPONSE IN ZEBRAFISH LARVAE

The startle response in zebrafish larvae primarily evaluates the functionality of the auditory and vestibular systems. A pronounced startle response indicates that the inner ear structures, responsible for detecting sound and changes in position, are functioning correctly. This test involves a rapid and coordinated motor reaction, suggesting that the neural circuits responsible for processing auditory or vibrational stimuli and initiating motor responses are intact. Any anomalies in this response can hint at defects in neural development or connectivity. The startle response can be observed in zebrafish larvae starting from 5 dpf. By this stage, the inner ear structures and associated neural circuits have developed sufficiently to detect and process auditory or vibrational stimuli. The response becomes more robust and consistent as the larvae continue to develop.

Objective

To evaluate the motor response of zebrafish larvae to tactile stimuli.

Materials

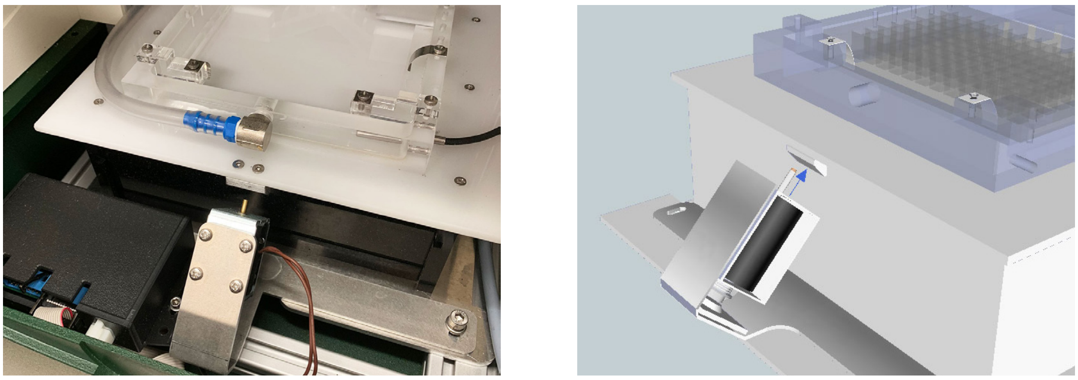
- Multi-well plate.
- Vibrational or sound stimulus source.
- Video recording setup: provided by the DanioVision Observation Chamber.
- Computer with tracking software: EthoVision XT.

Procedure

1. Place individual larvae in the wells of a multi-well plate.
2. Allow larvae to acclimate for 5 minutes.
3. Deliver a brief vibrational or sound stimulus. This can, for example, be achieved with a tapping device, which is a DanioVision add-on.
4. Record the startle response for 1 minute.
5. Analyze the latency, magnitude, and duration of the response.

Interpretation

Alterations in the startle response can indicate sensory or motor deficits. A longer latency can indicate a retardation of the neural circuitry responsible for the response. Similarly, a longer duration of the response can indicate a heightened stress response (or vulnerability).



The tapping device add-on for the DanioVision system evokes a startle response in the zebrafish larvae. This can all be controlled from the EthoVision XT software.

THIGMOTAXIS IN ZEBRAFISH LARVAE

Thigmotaxis, or the preference for staying close to the walls or edges of an enclosure, is considered an anxiety-like behavior in many animal models, including zebrafish. Larvae that spend more time near the edges than in the center of an arena are displaying thigmotactic behavior, which can be interpreted as a sign of heightened anxiety or stress. The behavior observed in thigmotaxis can provide insights into the neural circuits and neurotransmitter systems involved in anxiety and stress responses. Alterations in thigmotactic behavior can hint at imbalances in neurotransmitter systems or defects in specific neural pathways. Thigmotaxis can be observed in zebrafish larvae starting from 6 dpf. By this stage, the larvae have developed sufficiently to exhibit clear behavioral preferences in an open arena. The assay is typically conducted between 6 to 9 dpf, but it can also be extended to older larvae or even juvenile zebrafish, depending on the specific objectives of the study. It's essential to ensure that the arena size is appropriate for the age and size of the zebrafish being tested.

Objective

To evaluate the preference of zebrafish larvae for the edge or center of an arena, often used as an indicator of anxiety-like behavior.

Materials

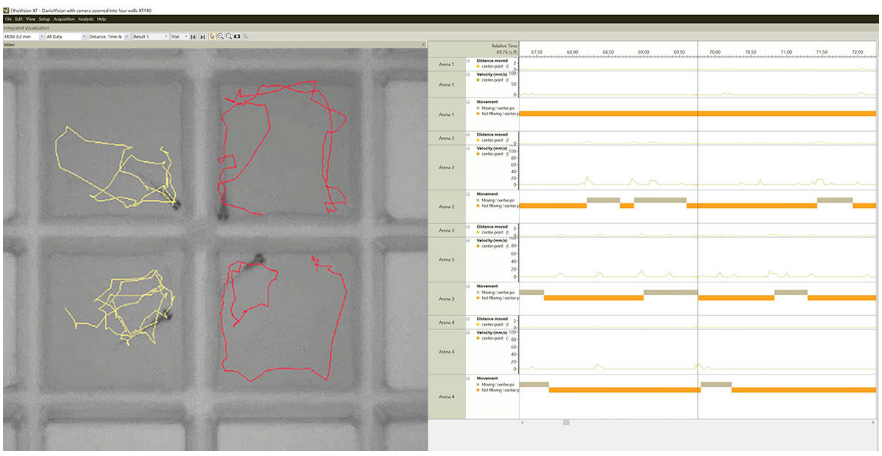
- Multi-well plate, circular arena or petri dish, or tank for fish older than 9 dpf.
- Video recording setup: provided by the DanioVision Observation Chamber.
- Computer with tracking software: EthoVision XT.

Procedure

1. Place larvae in the center of the arena filled with embryo medium.
2. Record the swimming behavior for 10 minutes.
3. Analyze the time spent near the edge versus the center of the arena.

Interpretation

A preference for the edge (thigmotaxis) is considered an anxiety-like behavior.



Thigmotaxis can be easily, and automatically, quantified using EthoVision XT. Zebrafish larvae that spend more time near the edge (red track) versus the center (yellow track) are thought to have a higher degree of anxiety-like behavior.

How do we measure behavior zebrafish

PROTOCOLS FOR JUVENILES & ADULTS

The following protocols serve as foundational guidelines for when you take your first steps into zebrafish behavioral research. While these suggestions are designed to help you take these initial steps, it's essential to adapt and refine them based on specific research objectives and experimental conditions.

OPEN FIELD TEST FOR ZEBRAFISH

The Open Field Test (OFT) is instrumental in assessing the general locomotor activity and exploratory tendencies of zebrafish. The total distance traveled, speed, and trajectory provide insights into the fish's activity levels and willingness to explore a new environment. The time spent in the center versus the periphery of the tank is indicative of anxiety-like behaviors. A preference for the periphery suggests heightened anxiety, while more time spent in the center indicates reduced anxiety levels. The Open Field Test is typically conducted on adult zebrafish, usually older than 3 weeks post-fertilization. At this stage, zebrafish have reached a level of development that allows for a comprehensive assessment of locomotor activity, anxiety-like behaviors, and exploratory tendencies. However, the exact age may vary depending on the specific objectives and design of the study. The test can also be adapted for juvenile zebrafish or older larvae, with modifications to the tank size and test duration to suit the developmental stage of the fish.

Objective

To assess general locomotor activity, anxiety-like behaviors, and exploratory tendencies.

Materials

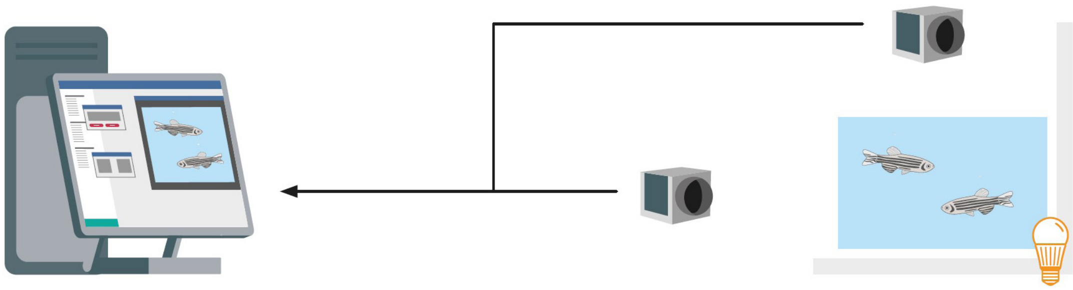
- Transparent tank (e.g., 30 x 30 x 30 cm).
- Video recording setup.
- Computer with tracking software: EthoVision XT.

Procedure

1. Fill the tank with dechlorinated water to a depth of 15 cm.
2. Allow the zebrafish to acclimate to the testing room for at least 30 minutes.
3. Place the zebrafish in the center of the tank.
4. Record the fish's movement for 10 minutes.
5. Analyze the video to measure parameters like total distance traveled, time spent in the center vs. periphery of the tank, and speed of movement.

Interpretation

Increased time spent in the center indicates reduced anxiety-like behavior. A higher total distance traveled suggests increased locomotor activity.



To measure horizontal and vertical movements in the same experiment, a setup with simultaneous two-camera tracking and/or recording can be installed and used with EthoVision XT. This creates the most accurate tracking setup to perform the OFT in zebrafish.

NOVEL TANK DIVING TEST

The Novel Tank Diving Test is a widely recognized behavioral assay to assess anxiety-like behaviors in zebrafish. Fish displaying anxiety-like behaviors will spend more time at the bottom of the tank, indicative of a heightened state of stress or fear. This test provides insights into the neurobiological mechanisms underlying anxiety and stress responses. It can help elucidate the role of specific brain regions, neurotransmitter systems, and neural circuits in modulating anxiety-like behaviors. The Novel Tank Diving Test is typically performed on adult zebrafish, usually older than 3 weeks post-fertilization, when they are developmentally mature and exhibit clear behavioral responses. However, depending on the research objectives, this test can also be adapted for juvenile zebrafish.

Objective

To evaluate anxiety-like behaviors.

Materials

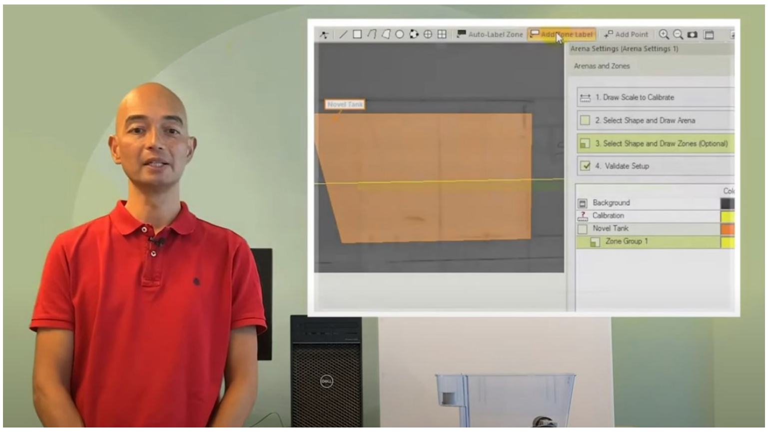
- Tall, transparent tank (e.g., 30 x 15 x 15 cm)
- Video recording setup.
- Computer with tracking software: EthoVision XT.

Procedure

1. Fill the tank with dechlorinated water up to 25 cm.
2. Allow the zebrafish to acclimate to the testing room for 30 minutes.
3. Introduce the zebrafish to the top of the tank.
4. Record for 5-10 minutes.
5. Analyze the time spent in the top vs. bottom of the tank.

Interpretation

Zebrafish displaying anxiety-like behaviors will spend more time at the bottom of the tank.



Check out this video, where our resident trainer and consultant Patrick explains exactly how to perform the Novel Tank Diving Test.

WATCH

LIGHT/DARK TEST

The Light/Dark Test is a prominent behavioral assay to evaluate anxiety-like behaviors in zebrafish, based on their innate preference for dark over light environments. Spending more time in the dark compartment is interpreted as a manifestation of anxiety-like behavior. This test offers valuable insights into the neurobiological substrates of anxiety and fear. It aids in understanding the neural circuits, neurotransmitter systems, and brain regions involved in the processing of anxiety and light preference and is typically conducted on adult zebrafish, generally older than 3 weeks post-fertilization, when they have attained developmental maturity and exhibit distinct behavioral preferences.

Objective

To assess anxiety-like behaviors based on the fish's preference for light or dark environments.

Materials

- Tank divided into a light and dark compartment.
- Video recording setup.
- Computer with tracking software: EthoVision XT.

Procedure

1. Introduce the zebrafish into the light compartment.
2. Record for 10 minutes.
3. Analyze the time spent in the light vs. dark compartment.

Interpretation

Spending more time in the dark compartment indicates anxiety-like behavior.

This test is also available for zebrafish larvae.

READ

T-MAZE TEST

The T-Maze Test is a pivotal assay to assess cognitive function and decision-making in zebrafish. It evaluates the ability of the fish to make choices and can provide insights into their preference, discrimination, and learning capabilities. By repeating the test and altering the conditions, researchers can assess both short-term and long-term memory of zebrafish. Consistency or changes in choices over repeated trials can indicate memory retention or deficits. The T-Maze Test is typically performed on adult zebrafish, usually older than 3 weeks post-fertilization, when they have reached a level of developmental maturity that allows for a comprehensive assessment of cognitive functions, memory, and decision-making.

Objective

To evaluate cognitive function, memory, and decision-making.

Materials

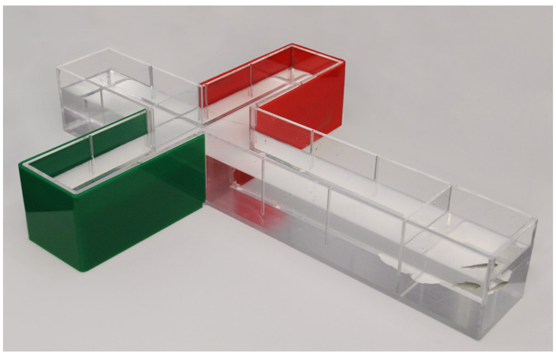
- T-shaped maze.
- Video recording setup.
- Computer with tracking software: EthoVision XT.

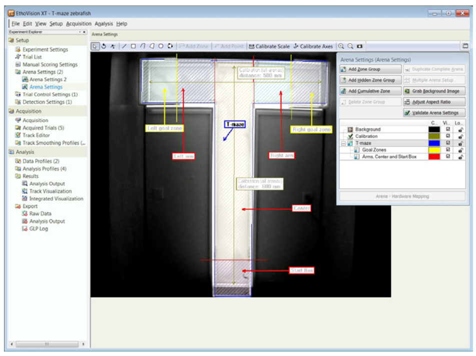
Procedure

1. Place the zebrafish at the base of the 'T'.
2. Allow the fish to choose between the left and right arm.
3. Record the choice and the time taken to make a decision.
   Reinforcing this choice can be done with, for example, a (food) reward or shock.
4. Repeat several times to assess consistency and memory.

Interpretation

A preference for one arm or changes in choices over repeated trials can indicate memory or cognitive deficits.





In the T-Maze, you teach your animal to choose the left arm, the red-colored one, or the deeper one. Memory plasticity can then be tested by reversal learning: the previously 'incorrect' arm is now the 'correct' arm. How long does it take the zebrafish to figure this out?

Often, colors or patterns discriminate the two goal arms. The arms have painted walls or are fitted with a colored or patterned sleeve. It is useful to alternate the colors left and right to make sure the zebrafish is recognizing the color rather than the side it is on. The same goes for visual cues from the testing environment.

A brief explanation can also be found in this video.

SOCIAL INTERACTION TEST

The Social Interaction Test is crucial for evaluating the social behaviors and preferences of zebrafish. It assesses the inclination of zebrafish to interact with conspecifics, providing insights into their social affinity or avoidance. This test can reveal the neurobiological underpinnings of social behaviors, helping to understand the role of specific brain regions, neurotransmitters, and neural circuits in modulating social interactions and preferences. This test is typically conducted on adult zebrafish, generally older than 3 weeks post-fertilization, when they are developmentally mature and exhibit clear and consistent social behaviors.

Objective

To assess social behaviors and preferences.

Materials

- Tank with a transparent divider.
- Video recording setup.
- Computer with tracking software: EthoVision XT.

Procedure

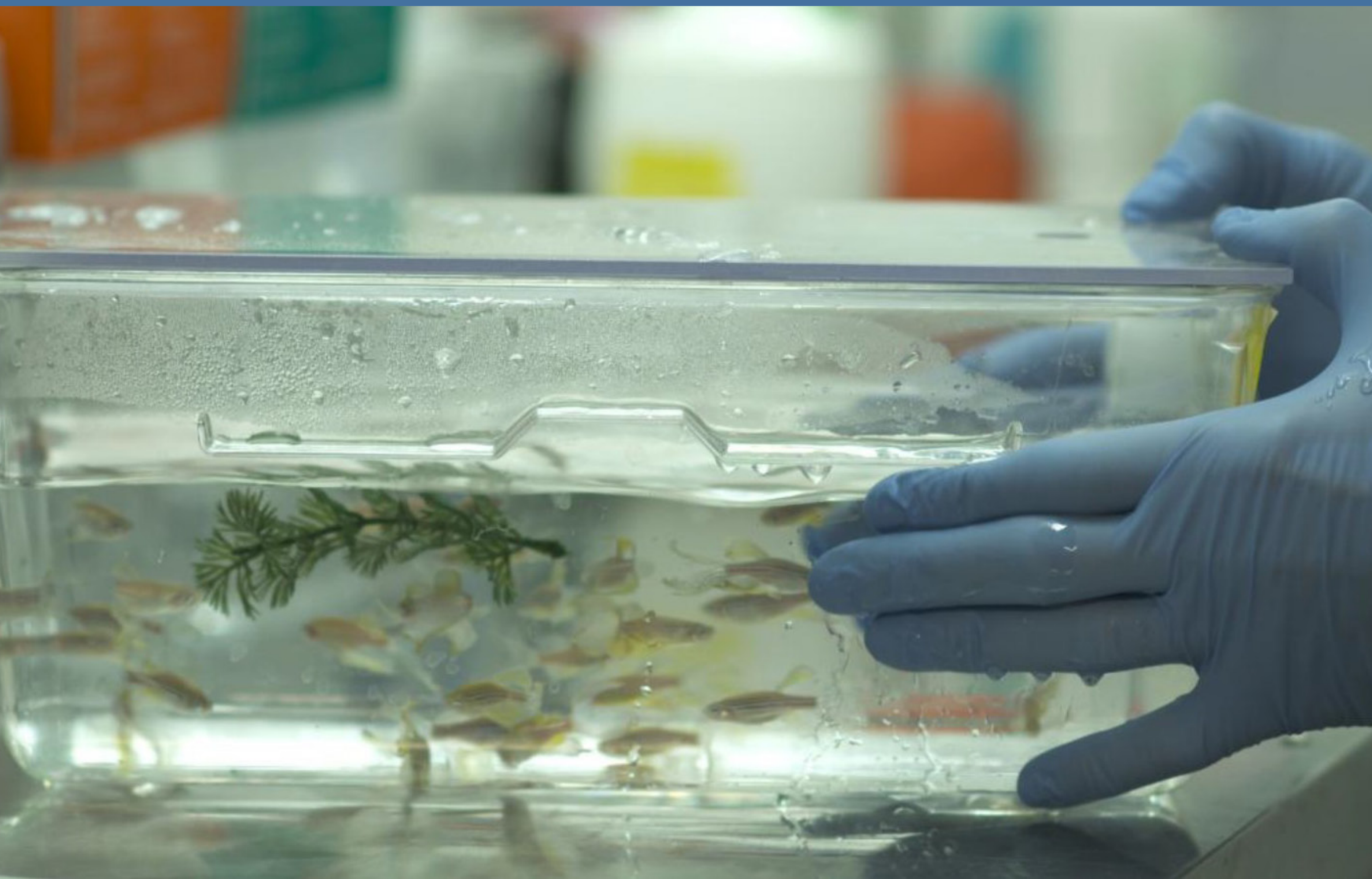
1. Place the test zebrafish on one side of the divided tank.
2. Introduce a group of zebrafish on the other side.
3. Record for 10 minutes.
4. Analyze the time the test fish spends near the divider vs. away from it.

Interpretation

Spending more time near the divider indicates a preference for social interaction.

BEST PRACTICES IN HOUSING & HUSBANDRY

Effective housing and husbandry practices are essential for maintaining healthy zebrafish colonies in laboratory settings. Proper care not only ensures the welfare of the zebrafish but also enhances the quality and reproducibility of research outcomes. In this section, we will discuss some of the best practices in zebrafish housing and husbandry, including the use of multi-well plates for various applications.



Housing & Husbandry

GENERAL PRACTICES

AQUATIC SYSTEMS FOR ZEBRAFISH HOUSING

- **Recirculating systems** - Many research facilities use recirculating aquatic systems that filter and maintain water quality. These systems provide a stable environment and minimize water consumption, making them efficient and cost-effective.
- **Flow-through systems** - Flow-through systems continuously supply fresh water while removing waste. They are ideal for breeding colonies and maintaining specific environmental conditions.

ENVIRONMENTAL PARAMETERS AND WATER QUALITY

- **Temperature** - Zebrafish are ectothermic, so maintaining a stable water temperature within the recommended range (usually 28-32°C or 82-89.6°F) is crucial. This temperature range is ideal for promoting normal growth, reproduction, and immune function. Temperatures outside this range, but primarily fluctuations, can lead to increased stress, reduced growth and reproductive success, and increased susceptibility to diseases. 28.5°C (83.3°F) is the most commonly used temperature in laboratory settings.
- **Light cycles** - Maintaining a consistent light-dark cycle is essential. A 14-hour light and 10-hour dark cycle is commonly used to mimic natural conditions and support normal behavior and reproduction.

Maintaining optimal water quality is crucial for the health and well-being of zebrafish. Zebrafish require clean, well-aerated water with stable parameters.

A proper filtration system is essential for maintaining water quality in zebrafish tanks. Filtration systems can include mechanical, biological, and chemical components to remove particulate matter, break down harmful compounds, and maintain stable water chemistry. Whilst adequate aeration is necessary to ensure sufficient oxygen levels in the water. Air stones or diffusers can be used to provide gentle aeration and water movement in zebrafish tanks.

IMPORTANT PARAMETERS TO MONITOR & MAINTAIN IN THE TANK(S)

- **pH and water hardness** - Regular monitoring and maintenance of pH levels and water hardness are vital to prevent adverse effects on fish physiology. Zebrafish prefer slightly alkaline water (pH 7-7.8). Maintaining a stable pH within this range is essential for the health of zebrafish, as sudden changes in pH can cause stress and harm the fish.

WHY IS TEMPERATURE SO IMPORTANT?

Development

Zebrafish embryos and larvae develop optimally within this temperature range. Deviations from this range can lead to developmental abnormalities or delays.

Metabolism

The metabolic rate of zebrafish is influenced by temperature. Maintaining the fish within the optimal temperature range ensures proper growth, digestion, and overall health.

Reproduction

Zebrafish reproduce most efficiently within their optimal temperature range. Temperatures outside this range can result in reduced fertility or abnormal reproductive behaviors.

Behavior

The behavior of zebrafish, including locomotion, social interactions, and learning, is influenced by temperature. Maintaining a stable, optimal temperature ensures that observed behaviors are representative of natural conditions.

- **Conductivity** - Conductivity measures the total dissolved salts in the water and should be maintained between 400 and 800 µS/cm for zebrafish. Proper conductivity ensures an optimal ionic balance for zebrafish health.
- **Nitrogenous waste** - Ammonia, nitrite, and nitrate levels should be monitored and kept as low as possible. High levels of these compounds can be toxic to zebrafish and may cause stress, disease, and even death.

FEEDING & NUTRITION

- **Nutrient-rich diet** - Providing zebrafish with a well-balanced, high-quality diet is essential for their health and reproductive success. Commercial zebrafish diets are available and can be supplemented with live or frozen food such as brine shrimp or *Daphnia*.
- **Feeding schedule** - Establish a consistent feeding schedule and avoid overfeeding, which can lead to water quality issues. Monitor fish health and adjust feeding amounts accordingly.

SPACE & DENSITY

- **Tank size** - Zebrafish tanks come in various sizes, but overcrowding should be avoided. Consider the size of the fish, their social behavior, and the research objectives when selecting tank sizes. Common zebrafish tanks (for juveniles and adults) range from 2-3 liters to up to 10 liters.

Read more on the effects of tank size on zebrafish behavior and physiology:
Abudusaimaiti et al. 2020. [22]

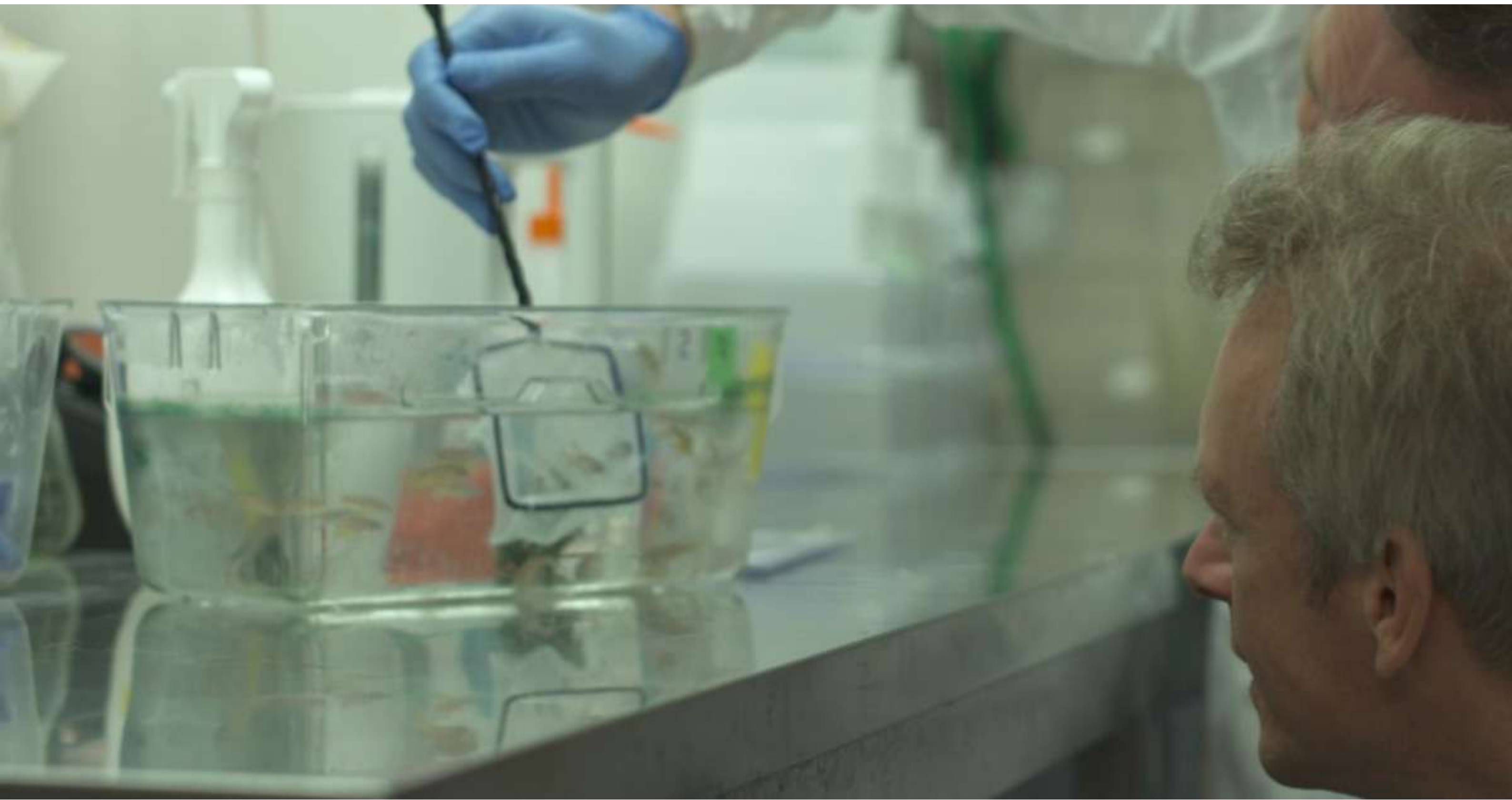
- **Group housing** - Zebrafish are social animals that thrive in groups, so they should be housed in groups to promote natural behaviors and reduce stress. However, be mindful of territorial behaviors and provide adequate hiding places.
- **Tank material** - Zebrafish tanks can be made of glass or clear plastic and should provide adequate space for the fish to swim and display natural behaviors. Tanks should be equipped with secure lids to prevent fish from jumping out.
- **Environmental enrichment** - Providing environmental enrichment, such as plants or artificial structures, can promote natural behaviors and reduce stress. However, avoid overcrowding the tank, as this can impede swimming and create territorial disputes.

BREEDING & REPRODUCTION

- **Breeding tanks** - Use specialized breeding tanks or chambers to isolate breeding pairs and collect embryos. These tanks often have dividers to separate males and females until breeding is desired.
- **Embryo collection** - Zebrafish embryos are typically collected in Petri dishes or multi-well plates. The choice between 24, 48, or 96 well plates depends on the research needs and the scale of the experiment, but should also be considered as a factor that can heavily impact the parameters of your study.

ZEBRAFISH FACILITIES - SEGREGATION

Whenever possible, separate zebrafish of different developmental stages in dedicated facilities or rooms to prevent cross-contamination and maintain specific environmental conditions.



Housing & Husbandry

EMBRYONIC & LARVAL ZEBRAFISH

SPECIAL CONSIDERATIONS

Zebrafish, as a model organism, undergo significant developmental changes throughout their life cycle. Therefore, it's crucial to adapt housing and husbandry practices to accommodate these developmental stages.

Embryos and larvae

- *Housing* - Zebrafish embryos and larvae are typically housed in multi-well plates or Petri dishes.
- *Feeding* - Embryos primarily rely on yolk reserves and do not require external feeding during the first few days. Larvae start feeding on live or powdered food (e.g., paramecia, powdered commercial diets) around 5-7 dpf.

Juvenile stages

- *Housing* - As zebrafish grow, they require more space. Transition juveniles to larger tanks, such as 1- or 2-liter tanks, to provide room for swimming and growth. Group housing is still common at this stage.
- *Feeding* - Juveniles can be fed a combination of live or powdered food, including *Artemia*, brine shrimp, and commercial diets. Monitor feeding behavior to avoid overfeeding.

HANDLING

Handling zebrafish, as with many laboratory animals, can introduce variables that might affect research outcomes, and it's essential to consider these aspects when planning and interpreting experiments.

Handling during different stages

- *Embryonic stage* - Zebrafish embryos are usually handled minimally. When required, they can be transferred using a pipette. At this stage, they are less susceptible to stress from handling, but physical damage is a potential risk.
- *Larval stage* - Larvae are more active than embryos and can be transferred using a pipette or a soft mesh net. Overhandling or rough handling can cause stress or physical damage.
- *Adult stage* - Adult zebrafish can be scooped using a net (or small beaker/tank). It's essential to transfer them swiftly to reduce stress and avoid keeping them out of water for extended periods.

Behavioral effects of handling

- Zebrafish can experience stress from handling, which can manifest as altered swimming patterns, changes in social behavior, increased cortisol levels, or reduced exploration in novel environments.
- The stress response in zebrafish might not be as pronounced or well-documented as the handler effect seen in rodent research, but it's still a factor to consider.

Proper housing and husbandry = reliable data!

Proper housing and husbandry practices are essential for maintaining the health and well-being of zebrafish. By providing appropriate care, including stable temperature conditions, researchers can ensure the welfare of their animals and obtain accurate, reliable data from behavioral studies.

Minimizing stress

- Using nets with a fine mesh can help reduce the chance of injuring the fish.
- Using a beaker or smaller tank can also be used to gently transfer the fish.
- When transferring zebrafish between tanks or other environments, it's beneficial to match water temperatures to minimize thermal shock.
- Using a light sedative, like tricaine methanesulfonate (MS-222), can be helpful for specific procedures, but researchers should be aware that any sedative might affect physiological parameters.

Acclimation

It's essential to give zebrafish time to acclimate after handling, especially if they've been moved to a new environment. This acclimation can help reduce the impact of any stress they might experience during handling.

TYPES OF WELL PLATES

Multi-well plates are indispensable in zebrafish research, particularly when dealing with high-throughput applications. The choice of well plate format depends on the specific needs and constraints of your experiment. Below are the pros and cons of using different well plate formats.

6-WELL PLATES

- (+) *Spacious wells* - The large well size facilitates detailed observation of developing embryos and larvae and allows for ample space for their growth while also having more room for behavioral observation.
- (+) *Ease of handling* - The size of the wells makes it convenient to perform manual manipulations and treatments on the embryos.
- (+) *Low throughput applications* - Ideal for experiments that do not require a high number of samples but need more space per sample.

- (−) *Space consumption* - Due to fewer wells, more plates may be needed to accommodate the same number of embryos as higher well count plates, consuming more lab space.
- (−) *Not suitable for high throughput* - The lower number of wells limits the throughput of experiments.

24-WELL PLATES

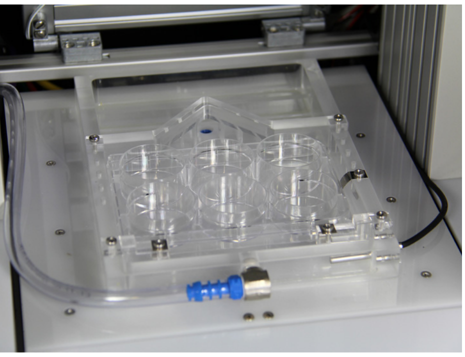
- (+) Relatively large wells allow for good observation of developing embryos and behavior.
- (+) You can keep embryos and larvae for a longer period of time (days as opposed to hours) in these well plates - striking a balance between more space to live in and less evaporation.
- (+) Suitable for experiments with moderate throughput.
- (+) Easier handling of embryos during manual manipulations.

- (−) Larger footprint requires more space. (You need more plates for the same amount of fish).
- (−) Limited to a moderate number of embryos per plate.

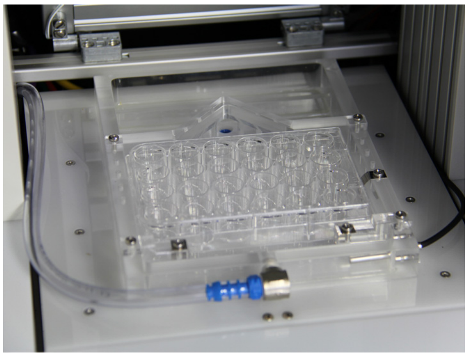
96-well plates

- High throughput with the smallest foot-print.
- Ideal for automated systems and large-scale screening.
- Suitable for experiments involving a large number of embryos.

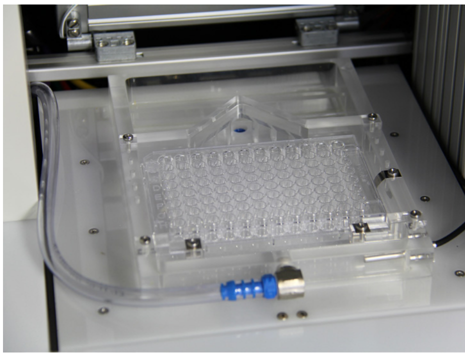
- Smaller individual wells may limit observation and manual manipulations and hamper natural behavioral traits.
- May require specialized equipment for efficient handling.



6-well plate.



24-well plate.



96-well plate.

Making a choice

The choice of well plate format depends on your specific research needs, available space, and equipment. Researchers conducting large-scale screenings may prefer 96-well plates, while those requiring very detailed observations might opt for 6-well plates. 24-well plates and 48-well plates offer a compromise between space efficiency and ease of handling.

Want to know more about the effects of well plate size on zebrafish (behavior)? Make sure to check out this blog: Does well plate size matter? A methodological study in zebrafish

CONCLUDING REMARKS & REFERENCES

CONCLUDING REMARKS

Wrapping up this guide, we've learned a lot about zebrafish! They are great for studying behavior because they share many genes with humans, develop quickly, and are easy to observe. We can watch how they grow, learn, and behave from the time they are embryos to when they become adults.

We've also seen why it's important to study zebrafish. They help us understand how genes work and how their bodies can develop as a result of their manipulation. They have proven their usefulness for finding out how different things in the environment, like toxins, can affect health. Zebrafish are proving to be a very cost-effective and ethical replacement for rodent research.

We've also gone over when and how to study zebrafish behavior. We've looked at different tests and what they can tell us about how zebrafish, and other animals, think and feel. We've discussed the importance of considering the age of the zebrafish and ensuring that we are conducting our studies ethically and responsibly.

In short, zebrafish are a powerful tool for learning more about the world around us. We hope this guide has been helpful and has given you a good starting point for your research, or or that it helps you to advance your research in zebrafish behavior. Keep exploring, keep learning, and keep discovering!

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