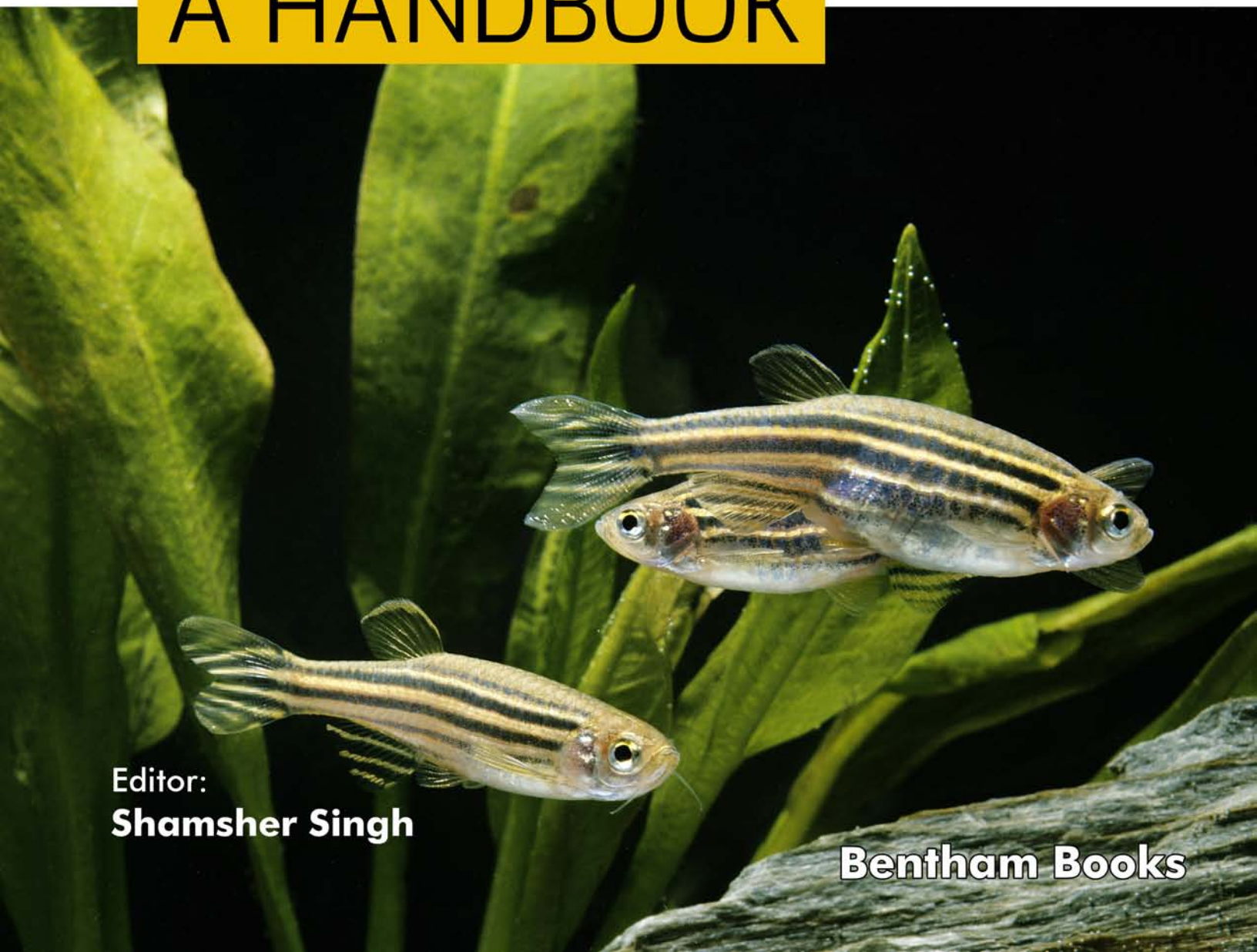
ZEBRAFISH MODELS FOR EXPERIMENTAL PHARMACOLOGY: A HANDBOOK



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Zebrafish Models for Experimental Pharmacology: A Handbook

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FOREWORD

The Zebrafish has emerged as an indispensable model organism for advancing experimental pharmacology, offering unique insights into human disease and drug discovery. This handbook, *Zebrafish Models for Experimental Pharmacology*, aims to provide researchers, scientists, and pharmacologists with a comprehensive guide to utilizing Zebrafish in their experimental studies.

Covering the latest methodologies, experimental setups, and translational applications, the book delves into the versatility and biological relevance of Zebrafish for evaluating pharmacological agents and studying disease mechanisms. By compiling cutting-edge techniques and best practices, this handbook aspires to bridge the gap between foundational research and clinical application, empowering readers to harness the full potential of Zebrafish in innovative pharmacological research.

I have thoroughly reviewed the content and am pleased to forward this book, which I believe will be highly valuable to the scientific research community.

Y.K. Gupta
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PREFACE

The goal of this book is to give readers a basic understanding of the subject's practical components, from handling Zebrafish and mounting tissue to the practical effects of several significant and intricate experimental techniques.

These days, experimental pharmacology has greatly strayed from the traditional method to focus on molecular and biochemical issues. Experimental pharmacology has been enhanced and expanded by advances in the fields of electrophysiology, biochemistry, molecular biology, electronic or digital recording methods, and software. Owing to the government's strict rules (CPCSEA), obtaining animals for research is a difficult undertaking.

Zebrafish have gained popularity as a model organism in biomedical research. Recently, researchers have been trying to better understand and cure conditions including cancer and various genetic and neurological abnormalities. One of Zebrafish's many benefits is that its embryo is transparent, making it possible to see how its tissues and organs grow and perform morphogenesis. This is crucial to comprehending the many systems that underlie health issues.

Some helpful elements of this book are available to the reader, such as the numerous worked-out examples that aid in putting theory into practice. A genuine effort was made to provide this book with as much pertinent material as possible, supporting it with appropriate examples and illustrated arguments. The subjects included in this book are carefully chosen in accordance with the majority of the contrived difficulties and the pharmacology curriculum. I believe this book will be useful to all recent graduates and postgraduates in the field of pharmacology, as well as to trainees and research personnel in their daily duties in allied health fields and toscientists in CROs with industrial drug development setups.

Several contemporary and straightforward experimental designs have been included to assist the students in their future drug discovery endeavors. Aside from this, the book covers a number of significant topics, including the ethics of using animals in experiments, how to handle and care for experimental animals, how to prepare solutions, and how to mount tissue for in vitro research.

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Introduction

Zebrafish (*Danio rerio*) have emerged as a cornerstone of recent biomedical research, bridging the gap between genetic studies and developmental biology. Their unique attributes—transparent embryos, rapid development, capability for regeneration, and genetic manipulability—make them an ideal model organism for exploring complex biological processes and diseases. This handbook serves as a comprehensive resource, providing foundational knowledge and practical methodologies to enhance the understanding and application of Zebrafish across various scientific disciplines. The Zebrafish model offers several advantages over mammalian models, such as cost-effectiveness, high fecundity, and external fertilization, which allow real-time observation of development. Its genetic similarity to humans (approximately 70%) and rapid life cycle make it ideal for large-scale studies. Compared to mammalian models, Zebrafish also enable efficient genetic manipulations and drug screenings, significantly accelerating biomedical research.

The first chapter of the handbook offers a detailed overview of Zebrafish anatomy, lifecycle, and care requirements, crucial for creating optimal conditions for their growth and experimentation. It highlights the rapid development of embryos, which undergo significant transformations within the first 72 hours post-fertilization, leading to the formation of major organs. Additionally, the chapter emphasizes the importance of maintaining suitable environmental conditions, such as water quality and temperature, to ensure the fish's health. The second chapter examines various drug administration routes, including oral, intraperitoneal, and water-based methods, focusing on critical factors like dosage, timing, and absorption rates that affect experimental outcomes. Following this, blood collection techniques essential for biochemical and genetic analyses are discussed, detailing methods such as caudal vein and cardiac puncture, while underscoring ethical considerations to minimize discomfort and ensure humane treatment.

Anaesthesia is essential for procedures that may induce pain or distress in Zebrafish. The fourth chapter details commonly used anesthetic agents, their dosages, and their physiological effects, enabling researchers to anesthetize fish safely while prioritizing their welfare. Additionally, the book emphasizes the importance of Zebrafish as models for studying human diseases, supporting drug discovery and insights into disease mechanisms through genetic manipulation.

A substantial portion of the book is dedicated to various behavioural assays that enable researchers to explore cognitive and emotional responses in Zebrafish including shoaling behavior that reveals social dynamics and environmental influences, while the Novel Diving Tank Test (NDTT) assesses anxiety responses in unfamiliar settings. The Social Preference Test evaluates social interactions, and the Mirror Chamber Test investigates self-recognition and social cognition. The Three Chamber Test measures sociability, whereas the Light-Dark Chamber Test and Plus-Maze Test gauge anxiety levels by examining preferences for safe versus open environments. Spatial learning is assessed through the Y-Maze Test, Square-Maze Test, and T-Maze Test, which explore navigation and memory. The Novel Object Recognition Test (NORT) provides insights into cognitive function, while the Open Field Test measures general locomotion and exploration. The Learning Test evaluates task

acquisition and retention, the Native Area Recognition Test assesses spatial memory, and the Zebrafish Rotation Test investigates motor coordination. Whether you are a seasoned researcher or a novice in the field, you will find valuable information that can enhance your research outcomes and inspire innovative applications.

As we navigate the intricate landscape of Zebrafish research, this handbook serves as your companion, equipping you with essential knowledge and practical techniques. By exploring the diverse applications of Zebrafish in experimental biology, we aim to inspire innovative research that advances our understanding of fundamental biological processes. Whether you are embarking on your first experiments or refining advanced methodologies, the insights and protocols contained within these pages will empower you to unlock the potential of this remarkable model organism. Together, let's dive into the fascinating world of Zebrafish and its contributions to science.

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CHAPTER 1

Introduction and Basics to Zebrafish

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INTRODUCTION

Zebrafish: Zebrafish (*Danio rerio)*, a freshwater teleost formerly used as an experimental model from the past few decades, is found in lakes and rivers [1]. They belong to the Minnow family Cyprindiae of Cypriniformes, native to South Asia [2]. Zebrafish derived its name from the five prominent, uniformly pigmented horizontal stripes that adorn its body, evoking the pattern of zebra-like stripes that extend longitudinally from the caudal fin and are distinctive characteristics of the species [3]. Zebrafish exhibit a fusiform (spindle-shaped) body plane, with lateral compression and a superiorly directed mouth, adapted for surface feeding and optimal maneuverability in its aquatic environment. Often referred to as tropical fish, this well-known aquarium fish is regularly offered for sale under the trade name Zebra Danio, also located in ponds. Female Zebrafish have silver stripes instead of gold and a wider white belly than males, a blue stripe separated by gold stripes in a torpedo design [4]. It takes three months to reach sexual maturity at 28.5°C and are considered adults in an ideal environment, Zebrafish commonly survive for 3-5 years [5, 6].

Nowadays, Zebrafish have evolved as a novel research model in recent decades as the species has many benefits such as being inexpensive to purchase, easy to maintain, handle, and breeding in the common laboratory. They are incredibly robust creatures. A single Zebrafish often yields between 20 and 200 offspring per breeding event. Other key advantages include: they are transparent during early developmental stages and have rapid development ability. Specifically, Zebrafish embryos develop from a single cell to a larva with a formed head, tail, and beating heart within 24 hours. In between 72 hours, the central nervous system is fully developed and becomes functional, and the fins become motile. By 5 days post-fertilization (dpf), they achieve full viability and are capable of independent swimming and foraging [7]. This rapid development and high reproductive output

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make Zebrafish an ideal model organism for both genetic and developmental biology research, particularly in high-throughput genetic studies. The basic organ system of Zebrafish is represented in Fig. (**1.1**).

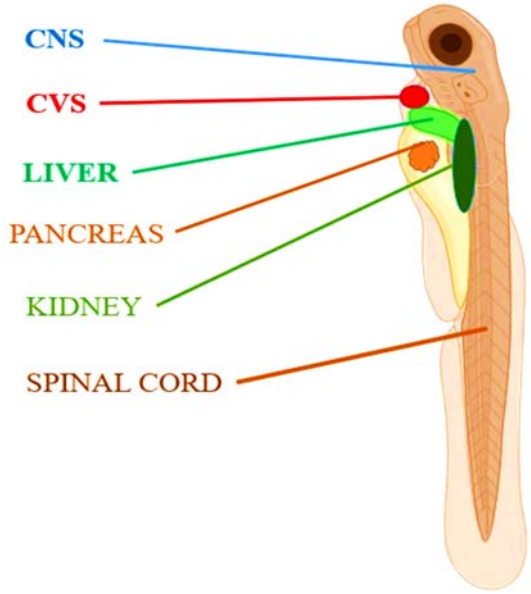

Fig. (1.1). Vital organs of Zebrafish.

ZEBRAFISH MAINTENANCE

As they are considered aquatic animals, they require good maintenance and care as per CPCSEA guidelines [8]. Various types of rotating systems constantly filter and oxygenate the water in which Zebrafish are kept to provide a marine environment that is favourable for their well-being. Fish waste products and extra foods are also filtered by the drainage system. Typically, the water and room temperature are kept between 26-28.5 °C and 12 hr light: 12 hr dark cycle. Un-purified water is passed through another tank after passing through a UV disinfection filter followed by a biological and active carbon absorption filter, a 50-micron canister filter, and finally 120-micron filter pad. To make dechlorinated water, it should be kept for at least 48 hours in a chamber and water should be circulated by a pump to keep it warm and speed up the dechlorination process [9].

The water pH-controller system should be monitored every day and pH should be kept around 6.8 to 7.5. $NaHCO_3$ can be used to raise pH levels while required. It is important to clean fish tanks regularly. While cleaning the tank, stop the water supply and tilt the tank back to release the remaining water, and then carefully

remove the tank from the system. Dirt accumulation and the growth of algae/fungus may be observed at the side walls and bottom of the tank. After filling the tank with dechlorinated water, place the baffle inside a clean tank. Using a fish net, carefully transfer fish into the tank and then shut the cover. Slide the cleaned tank into the system gently, then turn on the water supply. Before reusing the net, spray it with seventy percent ethanol, again rinse it with water, and allow it to dry. Take off the baffle from the unclean tank then spray with the same on both sides. Before reusing, give the tank and baffles a thorough rinse under tap water and let them completely dry. The filters need to be routinely checked and replaced with the circulation system to operate properly. Ensure that the water supply in each fish tank is neat and clean and that the filters are replaced regularly. Before replacing the 120-micron filter pad, make sure to reposition the filter toward the direction of water flow and utilize it completely before replacing it with a new one. Every week, the canister filter needs to be replaced. Use a wrench or your hands to spin the filter unit anticlockwise to remove it and then replace the canister filter and place a towel underneath to avoid or absorb water spills. Following the replacement of the old canister filter with a new one, carefully reinstall the filter into the aquatic system, manually adjusting its position as necessary. Twice every week or once every two weeks, the carbon filter needs to be replaced [10].

Use a wrench to carefully remove the carbon filter unit to replace the carbon filter. Replace the used activated carbon with fresh carbon by discarding it. Place the carbon holder back into the filter unit after re-fitting it. Reinstall the filtration unit inside the system. Before putting the filter into the system, flush some water through the pipes to clear all extra particles. Every six months, the biological filter needs to be washed. In a circulating system, a biological filter is often positioned between the carbon filter and the canister. Press the button for pressure release to reduce the filter pressure. For this phase, two people are usually required. After removing the filter's lid, extract the spore from the water and empty the contents of the filter into a container with a sieve. Nitrification and de-nitrification are two processes where a fine-pore bio-filter medium is used to separate spores [10].

Restore the spores in a fresh tank if the previous one is not clear. Replace the filter unit's lid, add de-chlorinated water, put it back into the system, and then turn on the water supply. Several nitrifying bacteria will reside in the spore in an acclimated aquarium. Because the nitrogen cycle in the aquarium depends on these microorganisms, remove the dirty spore which could cause a significant rise in ammonia (NH_3) and nitrite ($N{\equiv}C^-$) as compared to the new biological filter, which contains the clean spores. If it is not handled properly, either of these nitrogen cycle intermediate stages may be hazardous to aquatic life and even fatal to Zebrafish. Consequently, the Zebrafish housing system needs another

CHAPTER 2

Routes of Drug Administration in Zebrafish

Kousik Maparu[1] and **Shamsher Singh**[1,*]

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INTRODUCTION

Aim: To study various methods of drug administration in Zebrafish.

Scope and Outcomes: This chapter covers a thorough about various routes of drug administration techniques for Zebrafish with the goal of clarifying the steps involved and the results that are necessary to progress biomedical research. This chapter will cover a variety of techniques, including intramuscular (*i.m.*), intraperitoneally (*i.p.*), and intrathoracic injections, and will offer thorough guidance on performing these procedures successfully. By following these detailed protocols, researchers will obtain more reproducible results in their studies involving Zebrafish.

Theory: Drug delivery involves introducing medicinal substances into the body to target specific cells *in vivo* for the treatment of various disorders. It encompasses two key components: dosage form and delivery route. Effective drug administration ensures optimal drug action by regulating drug release, cellular absorption, and proper distribution within the body. Common methods of drug administration include enteral (*via* the digestive system), parenteral (injections), inhalation (*via* the nasal passages), transdermal (through the skin), topical (applied to the skin), and oral (*via* the mouth) [1]. Choosing the appropriate delivery method is critical to maximizing therapeutic effects while minimizing toxicity, particularly when targeting specific organs such as the brain or utilizing advanced drug delivery systems. In Zebrafish, drug administration presents unique challenges due to their small size and aquatic habitat. Various techniques have been developed to deliver drug substances effectively for research purposes.

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Below are some key considerations for drug administration in Zebrafish [2]:

1. **Water-soluble substances:** It can be difficult to keep the medicine concentrations that the fish ingest constant when water-soluble substances are added straight to the water in the aquarium [3].
2. **Food-based medicine pellets:** Medicated food pellets are an effective method for administering medication to adult Zebrafish, particularly for long-term therapies. This technique ensures precise, consistent, and non-invasive dosing, making it both practical and less stressful for the fish [4].
3. **Injections and oral gavage:** Although these techniques provide accurate dosage control, they are intrusive, potentially hazardous, and necessitate anesthesia, particularly when administered repeatedly.
4. **Inserts for spawning:** Zebrafish can be exposed to medicine by being submerged in a solution through inserts for spawning, which allows for accurate dosage for multiple fish at once. Different ways of drug administration with examples of models are described in Table **2.1**.

Table 2.1. Various routes of drug administration along with examples.

S. No.	Different Techniques	Examples of Drugs that Generally Administered with Different Techniques
01	**Bath immersion**	Antiepileptic drugs, such as Zebrafish larvae exhibit seizure-like behaviors that mimic human epilepsy. Useful when administering hydrophilic drugs targeting the GI tract.
02	**Microinjection**	Delivery of neurotoxins or chemotherapeutic agents to evaluate their effects on behavior and physiology. Allows conduct of toxicity assessments for compounds.
03	**Oral delivery**	Micropipette and needle-based method. Metabolic and obesity studies.
04	**Target drug delivery (Nanoparticles, Hydrogels, *etc.*)**	Target-specific drug delivery. Real-time measurement of accumulation and distribution.

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Syringe, micro-needles, micropipette, micro-injection.

PROCEDURES

Intra-muscular (*i.m.*)

Drugs deliver into dorsal muscle in adult Zebrafish (Fig. **2.1**).

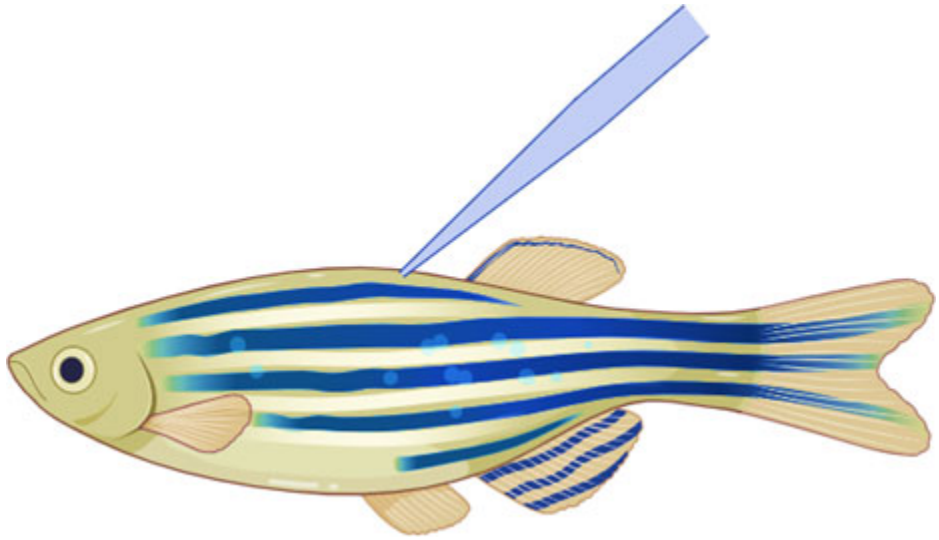

Fig. (2.1). Intra muscular (*i.m.*) in Zebrafish.

Preparation: Anesthetize the Zebrafish using tacrine, then position its dorsal side up on a moist sponge. Place the fish under a microscope and place a 45-degree angle micro-injection needle close to the dorsal muscle.

Injection: To put the needle, locate a scale-free location in front of the dorsal fin. Slowly inject phenol red and the desired ingredient (such as a DNA vaccination) into the muscle tissue after carefully inserting the needle.

Electroporation: If the chemical contains genetic material, use electrodes on both sides of the injection site to electroporate the region. Using an electroporator, deliver six pulses of 40 volts for 50 milliseconds each to help muscle cells absorb genetic material.

Recovery: After the procedure, move the fish right away to a recovery tank.

Monitoring: Use UV light to detect enhanced green fluorescent protein (EGFP) expression at the injection site as an indicator of successful uptake and expression of the genetic material.

Intra-peritoneal (*i.p.*)

Drugs are delivered into the abdominal cavity by *i.p.* route [5] (Fig. **2.2**).

CHAPTER 3

Blood Collection Methods in Zebrafish

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INTRODUCTION

Aim: To perform the blood collection methods in Zebrafish.

Scope and Outcomes: This chapter covers a thorough examination of Zebrafish blood collection techniques with the goal of clarifying the steps involved and the results that are necessary to progress biomedical research. It will discuss a variety of techniques, including the tail transection method, cardiac puncture, and micro-capillary tube from the dorsal aorta, and will offer thorough guidance on performing these procedures successfully. By following these detailed protocols, researchers will obtain more reproducible results in their Zebrafish studies.

Theory: A crucial area of study in both clinical medicine and biomedical research is hematology, or the study of blood and its problems. *Danio rerio* commonly known as a Zebra fish is endemic to South Asia and crops up as a treasure model for studying the hematological process, due to their genetic tractability and physiological similarities to humans [1, 2]. They are perfect for hematological studies due to their number of benefits. These benefits include that they are transparent in their early stages of growth, which allows the researcher to directly visualize the blood and blood vessels under the microscope without the need for intrusive operations [3]. This transparency makes it easier to image blood cell formation and circulation in real time, providing valuable information about the earliest phases of hematopoiesis. The maximum amount of blood collected in Zebrafish is described in Table **3.1**.

Table 3.1. Blood collection volume in different age groups of Zebrafish.

S. No.	Type of Zebrafish	Maximum Blood Collection Volume
1.	Juvenile Zebrafish	Collect up to 1% of body weight, not exceeding 10µl.
2.	Adult Zebrafish	Collect up to 2% of body weight, not exceeding 100µl.

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Blood collection from the Zebrafish is necessary for research on hematopoiesis, evaluating drug toxicity and efficacy, and genetic and environmental effects on the blood parameters [4, 5]. It contains a blood volume in the range of 8-10% of its total body weight, which is about 0.6-0.7 ml of blood per gram of body weight [6]. A single Zebrafish yields blood volume between 1-10 microliters. There are numerous techniques for collecting the blood from Zebrafish.

These techniques are:

1. Tail transection method
2. Cardiac Puncture
3. Micro capillary tube from dorsal aorta

REQUIREMENTS

Animal: Zebrafish

Chemicals: MS-222, Sterile Water, micro-capillary tube, eugenol.

PROCEDURE

Tail Transection Method

First, all the necessary materials were gathered and the work area was prepared under sterile conditions to avoid any contamination. Zebrafish were carefully selected for the procedure, ensuring that all were in optimal health and condition for sampling.

Anesthetized the Zebrafish using an appropriate anesthetic agent at the recommended concentration. Commonly used anesthetic agents are MS-222 and Eugenol. The depth of anaesthesia needed to immobilize the fish should be sufficient, but not excessive.

Method of Anesthesia

Prepare the stock solution of MS-222 (0.4 grams in one lit.) and dilute it to necessary conditions.

Place the zebrafish in a container filled with the anesthetic solution.

Monitor the fish closely for signs of anesthesia, such as loss of equilibrium, reduced opercula movement, and lack of response to external stimuli, like touch.

Now, transfer the anesthetized fish to a suitable container or petri dish filled with the anesthetic solution. Ensure that the fish is properly oriented and positioned so that the tail fin is easily accessible.

At the distal end of the tail fin, quickly make a transverse incision using sterilized surgical scissors or micro scissors as shown in Fig. (**3.1**).

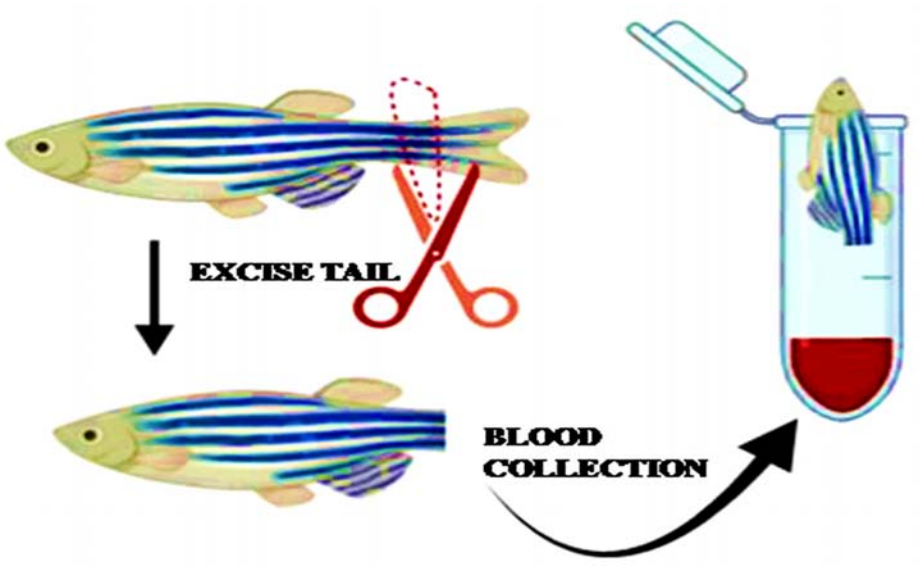

Fig. (3.1). Tail transection method.

To prevent excessive bleeding and stress to the fish, the incision should be made precisely and shallowly. Due to capillary action, blood will naturally pool at the incision time.

To collect the blood, gently position the micro-capillary tube close to the incision site as soon as the cut is made. Allow capillary action to fill the micro-capillary tube with blood. During this process, be careful not to disturb the fish too much.

After collecting enough blood (usually a small amount compared to the size of the fish, taking ethical considerations into account) remove the micro-capillary tube.

Transfer the drawn blood from the micro-capillary tube into a labeled micro-centrifuge tube or another appropriate container with sterile saline solution for analysis or storage.

To stop any bleeding, gently massage the tail fin incision with sterile gauze or a cotton swab. Observe the fish closely for signs of recovery from anesthesia. Move

CHAPTER 4

Anesthesia Techniques in Zebrafish

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INTRODUCTION

Aim: To study anesthesia techniques in adult Zebrafish.

Scope and outcomes: Anesthesia is necessary for various scientific and veterinary treatments involving Zebrafish(*Danio rerio*). Effective anesthetic techniques guarantee minimal stress and discomfort to the fish while enabling experimental manipulations, surgeries, and other treatments.

Theory: Anesthesia is commonly used in fish experiments, but using an insufficient anesthetic technique may harm the reliability of the data as well as the well-being of the animals. In recent years, Zebrafish (*Danio rerio*) have been used more frequently in biomedical research, emphasizing the significance of using appropriate anesthetic regimens for these species. Although the primary anesthetic medications along with a few analgesic techniques have been identified, further study is required before they can be applied to this species. A systematized observation of indicators is also recommended to assess the welfare of adult Zebrafish and minimize their pain [1].

General anesthesia in fish can be induced by diluting anesthetics in water, where they are absorbed primarily through the intestines. However, in some species, it can be absorbed through the skin [2]. Factors such as the method of administration, salinity, pH, temperature, nitrogenous substances, oxygenation, and other environmental parameters can influence how anesthesia affects fish [3]. Additionally, variables including length of anesthesia, concentration of anesthetics, body weight, metabolism of the fish, gill surface, health of the fish, age, strain, and the unique characteristics of each species of fish all affect the depth and recovery of anesthesia. As a result, before protocols are established, anesthesia trials with a limited number of fish or pilot studies must be carried out to ascertain the ideal dosage and exposure duration.

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Stages of Anesthesia in Zebrafish

A total of 8 phases were observed in Zebrafish after giving anesthesia [4] (Fig. **4.1**).

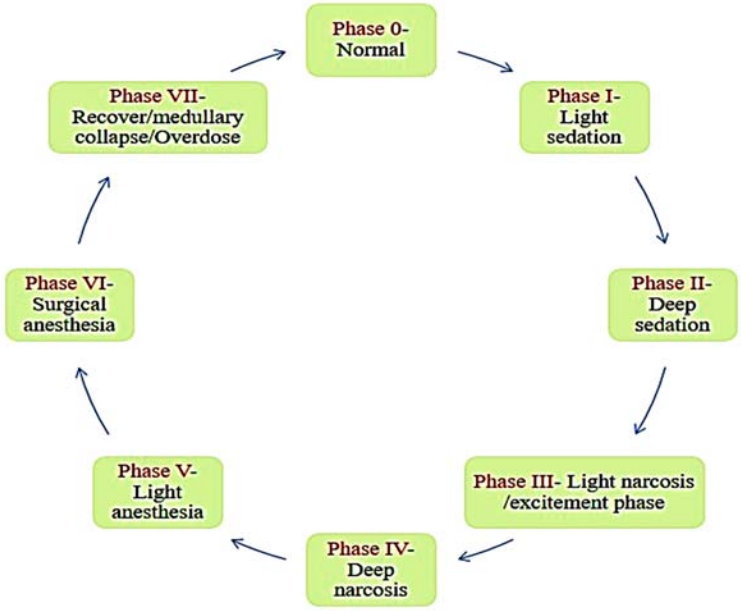

Fig. (4.1). Stages of anesthesia in Zebrafish.

1. **Phase 0-Normal:** Normal muscle tone, normal respiratory rate, and normal reaction to visual and tactile stimuli.
2. **Phase I-Light sedation:** Little reduction in response to touch and visual cues.
3. **Phase II-Deep sedation:** There is a minor decrease in respiration rate and muscle tone, with no response to light touch or visual stimulation.
4. **Phase III- Light Narcosis/Excitement Phase:** This phase is characterized by a partial loss of stability, poor reactions to postural changes, decreased muscle tone, and heightened sensitivity to touch and visual stimuli. Furthermore, there is either erratic breathing or an increase in the respiratory rate.
5. **Phase IV-Deep Narcosis:** Complete loss of equilibrium is present, along with no reactivity to small touch or visual cues or to changes in posture. The rate of breathing was close to normal.
6. **Phase V-Light Anesthesia:** Complete loss of muscular tone is present, along with a drop in heart and breathing rates and no response to unpleasant stimuli.
7. **Phase VI-Surgical Anesthesia:** Extremely low respiratory and slow cardiac rates are present, along with no response to intense stimulus.
8. **Phase VII-Recover/Medullary Collapse/Overdose:** After some time, fish will

recover or apnea is accompanied by a limping, non-respiratory tone that can cause cardiac arrest in a matter of minutes if the level of anesthesia is not rapidly reduced. In the end, this illness may be fatal.

Commonly Used Anesthetic Agents

Table 4.1. Commonly used anesthetic agents used for anesthesia in Zebrafish [4, 5].

S. No.	Anesthetic agents	Concentration	Preparation	Dosage for Different Phases	Induction Time	Recovery	Usage	Advantages	Disadvantages
01	Tricaine methanesuifonate (MS-222)	Typically used at 100-200 mg/L for anesthesia and 300-500 mg/L for euthanasia.	MS-222 is acidic and should be buffered to a neutral pH (7.0-7.5) using sodium bicarbonate.	**Stage V** - 100, 120, 140, 160, 180 and 200 mg/L (Immersion) **Stage III-IV** -100 and 120 mg/L (Immersion) **Stage V** - 140, 160, 180 and 200 mg/L (Immersion) **Stage VI** 150 mg/L (Immersion)	Anesthesia is usually achieved within 2-3 minutes	Fish typically recover within 5-10 minutes when placed in fresh water.	The most commonly used anesthetic for Zebrafish	Effective, readily available, and well-documented.	Requires careful pH adjustment and monitoring.
02	Eugenol (Clove Oil)	Typically used at 20-100 mg/L	Must be mixed with ethanol to dissolve in water for inhalation.	**Stage V** - 60, 80, 100, 120 and 140 mg/L (Immersion)	Anesthesia is generally achieved within 2-3 minutes	Fish recover within 5-10 minutes in fresh water.	An economical and effective alternative for Zebrafish anesthesia	Inexpensive and readily available.	Potential for variability in concentration and effectiveness; can be irritating at high concentrations.
03	Isoflurane	Typically used at 3-5% solution.	Fish are exposed to a saturated solution of Isoflurane in water or bubbled into the water.	**Stage I** 300 mg/L (Immersion) **Stage VI** 325 mg/L (Immersion) **Stage VI/ VII** 350 mg/L (Immersion)	Rapid, usually within a minute.	Quick recovery upon transfer to fresh water.	An alternative to MS-222, used primarily for short procedures.	Non-irritating and easy to administer.	Limited solubility in water, requiring specialized equipment for administration.
04	Metomi-date hydrochloride	Typically, 2 - 4 mg/L is used.	Used as inhalant anaesth-etic in a water bath in fish.	**Stage I** 2 and 4 mg/L (Immersion) **Stage IV** 6, 8 and 10 mg/L (Immersion)	Light anesthesia occurs between 2.3 and 4.3 min.	Recover in 2.8 min to 5.2 min.	Used for minor procedures.	No distressful behaviour observed during anesthesia. No mortality was seen during or after anesthesia.	Does not induce a surgical anaesthetic stage or analgesia, alter fish physiology by suppressing cortisol production.

CHAPTER 5

Shoaling Behaviour in Zebrafish

Pratyush Porel[1] and **Shamsher Singh**[1,*]

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INTRODUCTION

Aim: To study the shoaling behaviour in adult Zebrafish.

Scope and outcomes: Shoaling behaviour is a social interaction behavior where animals make social perceptions with other individuals followed by forming a group or schools to move together. A shoaling behaviour test is performed to evaluate the neurobehavioral parameters of Zebrafish.

Theory; Zebrafish as a Model to Study Behavioral Neuroscience:

Zebrafish is a tropical teleost, small fish, mainly found in freshwater. After 1970, Zebrafish became a widely accepted novel animal model for biomedical research purposes, including behavioral, genetic and ecotoxicological studies. The brain of Zebrafish shows a greater extent of similarities with the human brain in psychopharmacological and neurobehavioral aspects, so different neurological diseases can be studied by inducing that particular disease in Zebrafish. Over the commonly used animal models, including rodents, drosophila and *C. elegans*, Zebrafish makes itself feasible from some natural perspectives like its small size, availability, maintenance, acquisition and ease of taking care. Additionally, Zebrafish are highly similar in anatomical, and physiological prospects to mammals, which gives them more preference to establish themselves as animal model for biomedical research [1]. The average life span of Zebrafish is around 4-5 years whereas at the age of 10-12 weeks, the fish become reproductively mature and capable of laying 200-300 eggs per week. Generally, female fish are capable of reproducing every 2-3 days, and each clutch laid by them may contain several hundred eggs which give birth to hundreds of caviars. Within 3 months, fish become mature enough to be used in various experiments as an animal model [2]. Nowadays, Zebrafish are a prime model for studying genetics, mutagenetic

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screening and toxicology. In recent decades, an exact explanation of the pathophysiology of multifaceted behavioural disorders has been a main goal for researchers and Zebrafish provides an immense opportunity to study the basic functions of the brain through behavioural studies. Undoubtedly, rodents possess a closer anatomical structure to the human brain, but their complexity and expensive maintenance, provide more advantages to Zebrafish in establishing themself successfully as an alternative animal model. Zebrafish is also very much feasible for high throughput screening (HTS) methods for drug development in neuroscience, whereas rodent models may not be suitable for the same due to high maintenance [3].

According to taxonomical classifications, there are uncountable species around the world that express more intraspecies interaction. This is called preference for conspecifics. Previously, there were some attempts to use shoaling behaviour as a behavioural measure, but no standardized protocol or model has been established to study this. Adult Zebrafish and larvae are now widely used in the study of neurobehavioral parameters and shoaling nature. In simple words, "shoal" means, a group or aggregation of individuals and "school" means, shoals exhibiting polarized and synchronized motion. Shoaling behaviour is complex and dynamic behaviour, which is a characteristic behaviour that is predominantly observed in Zebrafish. Some behaviors are characterized by aggression, fear, alarm reaction, sleep and reward by studying the shoaling behaviour of Zebrafish. Due to some advantages like ease of breeding, handling and practical simplicity, its application is increasing as a translational tool in the drug discovery process [4].

REQUIREMENTS

Animal: Adult Zebrafish

Apparatus: Normal glass tank, Any-maze software, Graph-pad-prism, Camera

SHOALING BEHAVIOUR IN ZEBRAFISH

Behaviour is a complex phenomenon that possesses a core relationship with neurobiological mechanisms. Interaction with others and the expression of social perception are essential for all species to survive in the animal kingdom. Social behaviour can be expressed as foraging, aggression, competition and mating. Currently, several researches are ongoing to check animal behaviour and their mechanisms, and studies are designed in such a manner that correlate the nature of animal behaviour with human beings. Zebrafish spend most of their life in small, firm gatherings, called shoals and exhibit shoaling behaviour by synchronizing swimming in one direction, which is illustrated in Fig. (**5.1**). Zebrafish's shoaling nature develops slowly from about 7 days to adulthood.

Depending on the situation, circumstances and physiological conditions, shoal may tighten or loosen. Anxiety or fear causes the shoal to tighten and potentially form a school. Shoal becomes looser and its organization becomes less oriented if stimulation comes in the form of hunger or habituation. This behaviour can be observed manually and using automated video-recording software. The focal coordinates, which are used for tracking fish's movements by video recording software are diagrammatically represented in Fig. (**5.2**). Several endpoints are accessed as a goal of study including the inter-fish distance shown in Fig. (**5.3**), shoal area, nearest and farthest neighbor distances shown in Fig. (**5.4**), total time spent in the shoal and away from the shoal, and the number of animals leaving the shoal and polarization. Some neurological diseases like anxiety, depression, epilepsy and traumatic brain injury (TBI) significantly affect the shoaling behaviour of Zebrafish [5].

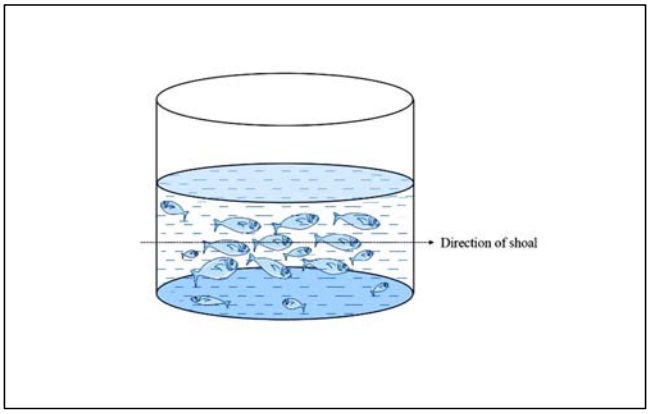

Fig. (5.1). Shoaling behavior and its direction.

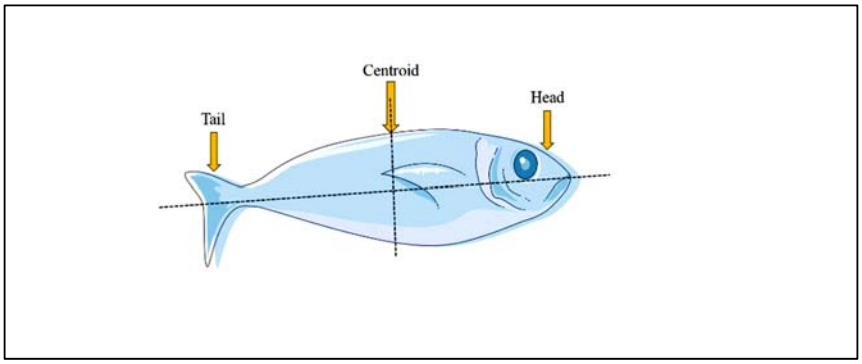

Fig. (5.2). Coordinates (head, centroid and tail) for the focal fish tracking system.

CHAPTER 6

Animal Models of Zebrafish

Romanpreet Kaur[1] and **Shamsher Singh**[1,*]

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INTRODUCTION

Zebrafish, scientifically known as *Danio rerio*, are small-bodied tropical, freshwater fish species originally from the rivers and streams of South Asia. They serve as a critical model organism in scientific research, particularly in the domains of genetics, developmental biology, toxicology, pharmacology, and neuroscience. Due to their extensive genetic homology to humans, Zebrafish has emerged as a widely utilized organism in biomedical and environmental studies, offering valuable insights into gene function, developmental processes, and disease mechanisms.

WHY ZEBRAFISH IS PREFERRED AS AN ANIMAL MODEL?

Zebrafish are popular species in biological research due to their diverse applications across various scientific disciplines. Despite their phylogenetic divergence from humans, Zebrafish exhibit several behavioral similarities to humans. These include complex social behaviors such as shoaling and courtship displays, as well as responses to environmental stimuli, including light and temperature variations. Additionally, Zebrafish demonstrate cognitive functions such as learning and memory formation. Notably, Zebrafish also shares analogous stress responses, reward system mechanisms, and patterns of social interaction, making it a valuable model for studying human behavior and neurobiology.

Additionally, Zebrafish and humans share several conserved molecular pathways and signaling networks that regulate essential physiological processes, including cell division, growth, and apoptosis (programmed cell death). Genomic analyses reveal that approximately 70% of human genes possess at least one identifiable homolog in Zebrafish, underscoring the significant genetic similarities between humans and Zebrafish.

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Zebrafish are the perfect model organism for genetic and developmental research due to their prolific reproduction and transparency of embryonic stages. Key advantages include:

1. Rapid embryonic development allows for efficient and accelerated research.
2. Low cost, high reproductive output, ease of genetic manipulation, and a simplified genome, facilitate experimental studies.
3. As a vertebrate, Zebrafish exhibits significant anatomical, genetic, and physiological similarities to humans, enhancing its relevance in biomedical research.
4. Tolerance to a broad range of environmental conditions, reflecting the species' natural habitat and enabling diverse experimental conditions.
5. Their small size and rapid developmental timeline make Zebrafish ideal for high-throughput screening of pharmacological agents and chemical compounds.

APPLICATIONS

1. **Disease modelling:** Zebrafish are used as a model organism for studying a range of human diseases, including cancer, mental, and neurological disorders, as well as developmental, and metabolic abnormalities. The specific areas of study in Zebrafish are described in Table **6.1**.
2. **Therapeutic compound screening:** Zebrafish are utilized in drug discovery and therapeutic compound screening processes to find possible treatments for human diseases.
3. **Environmental toxicology:** The impact of environmental pollutants on development and health can be studied using Zebrafish as a model to assess toxicological effects.
4. **Behavioural neuroscience:** Zebrafish are used in behavioural neuroscience research to explore the neuronal mechanisms underlying behaviour and cognition [1].

SCOPE OF ZEBRAFISH

Zebrafish research covers a wide range of scopes. The scope of Zebrafish in research is described in Fig. (**6.1**).

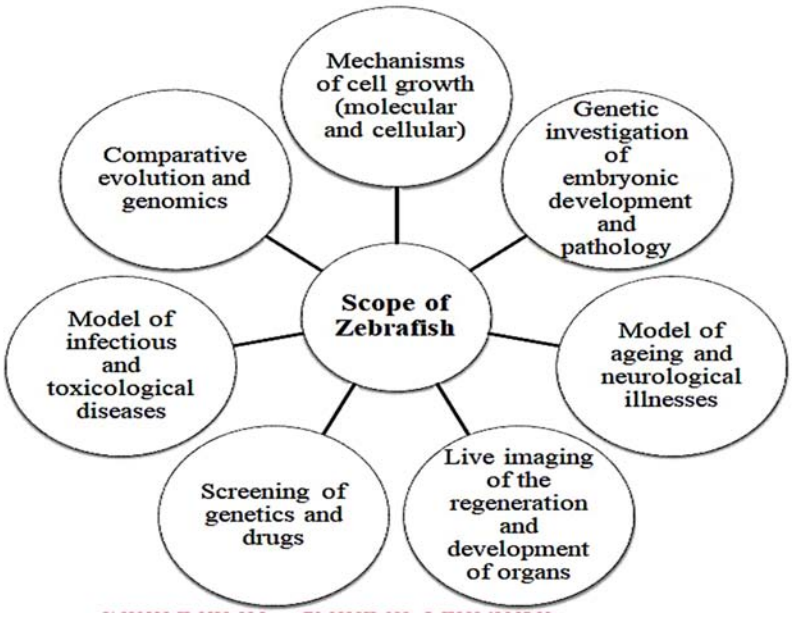

Fig. (6.1). Scope of Zebrafish.

Table 6.1. Specific areas of study in Zebrafish.

S. No.	System	Target Area
1.	Nervous System	Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS), Huntington's Disease, Autism Spectrum Disorders
2.	Cardiovascular	Atrial Fibrillation, Heart Failure, Atherosclerosis, Hypertension
3.	Endocrine System	Thyroid disease, Diabetes, Cushing's disease, Obesity, Adrenal insufficiency, Nonalcoholic fatty liver disease
4.	Excretory System	Polycystic kidney disease, Ciliopathy- associated human cystic kidney diseases, Acute kidney injury
5.	Regeneration Study	Kidney, Heart, Spinal cord, Retina, Telencephalon regeneration
6.	Toxicology	Digestive system, Liver, Brain, Heart, Kidney

As a vertebrate model organism, Zebrafish are generally appealing and hold considerable potential for biomedical research and the study of human diseases.

Classification of Diseases in which Zebrafish are used as an Animal Model

Zebrafish are utilized in laboratory settings to model a wide variety of human diseases, including neurological, cardiovascular, renal, and metabolic disorders such as diabetes, digestive system diseases, cancer, and musculoskeletal conditions. These Zebrafish models provide valuable insights into the

CHAPTER 7

Novel Tank Diving Test in Zebrafish

Falguni Goel[1], Romanpreet Kaur[1], Vaishali[1] and **Shamsher Singh[1,*]**

[1] Neuropharmacology Division, Department of Pharmacology, ISF College of Pharmacy, Moga, Punjab 142001, India

INTRODUCTION

Aim: To check for anxiety in adult Zebrafish by using a novel diving test.

Scope and outcomes: We would be able to know about the anxiety-like behavior in Zebrafish by this practical. A novel diving test is an apparatus that is wide from top and narrow from bottom. The top zone shows that the fish is active, and the bottom zone shows that the fish is anxious. Researchers observe and record their behavior, including diving depth, duration underwater, and swimming patterns.

Theory: A novel diving test in Zebrafish could be designed to assess their behavioral responses (anxiety) and physiological adaptations to underwater conditions [1]. A novel diving test is an innovative method to test anxiety by moving the fish (by net) to a unique tank for behavioral observation as well as phenotyping, Zebrafish are subjected to research testing in an initial treatment beaker. Using a beaker used for pre-treatment, groups of controls go through identical steps without interruption. The measurement of the tank is 60 × 30 × 46 cm (length × width × height). The novel diving test apparatus is shown in Fig. (**7.1**).

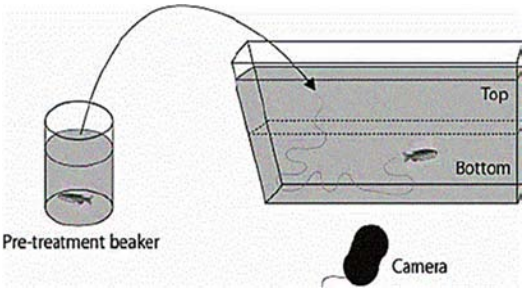

Fig. (7.1). Diagram illustrating the mechanism of the novel diving test.

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REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Novel Tank Diving apparatus, Any-maze software, Graph pad prism, Camera.

PROCEDURE

1. Firstly, take one healthy Zebrafish for the procedure.
2. Move it to the Novel diving apparatus with the help of a net.
3. Now make sure that there is no noise while following the procedure.
4. Leave the fish for 10 minutes in the tank for habituation.
5. Now place the camera to record the video and analyse it for 10 minutes.
6. If the fish spends more time in the bottom zone then it is showing anxiety-like symptoms.

Parameters Measured

1. **Diving depth:** The maximum depth reached by each Zebrafish during the test period could be measured using depth sensors or video tracking systems.
2. **Duration underwater:** The amount of time spent by each Zebrafish submerged could be recorded to assess their ability to stay underwater.
3. **Swimming patterns:** Researchers could analyse the swimming patterns of Zebrafish while diving, including speed, directionality, and any deviations from typical behaviour [1].

Physiological measurements: In addition to behavioral observations, physiological parameters such as oxygen consumption, heart rate, and metabolic rate could be measured to assess the physiological adaptations of Zebrafish to underwater diving.

Experimental conditions: The diving test could be conducted under various experimental conditions to study the effects of factors such as water temperature, oxygen levels, and environmental stressors on Zebrafish diving behavior and physiology.

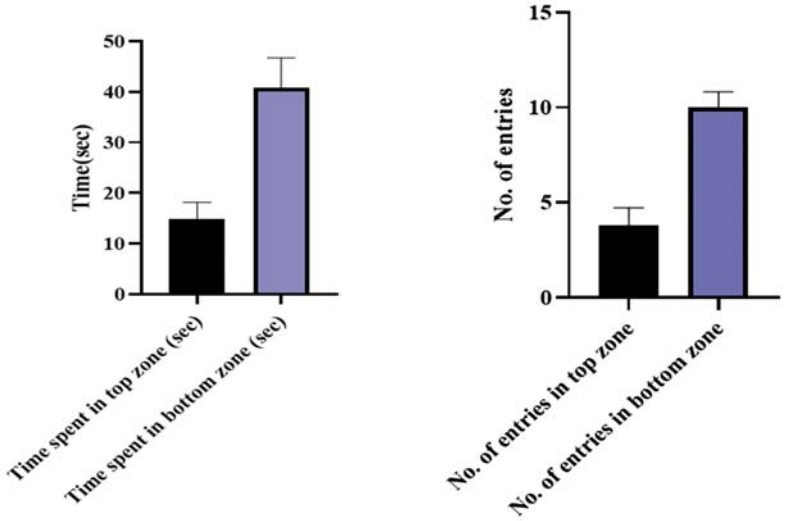
Data analysis: Data collected from the diving test could be analyzed using statistical methods to identify patterns and correlations between behavioral responses, physiological parameters, and experimental conditions.

OBSERVATION

A dummy analysis is given in Table **7.1**.

Table 7.1. Observation table of number of entry and time spent in top and bottom zones on the novel tank diving test apparatus of Zebrafish.

S. No.	Time Spent in the Top Zone (sec)	Time Spent in the Bottom Zone (sec)	No. of Entries in the Top Zone	No. of Entries in the Bottom Zone
1.	15	38	3	10
2.	10	49	3	11
3.	16	35	5	9
4.	18	41	4	10
Mean	14.75±3.4	40.75±6.0	3.75±0.9	10±0.8



Statistical analysis: Statistical analysis indicates that the time spent and the number of entries in the top zone have decreased, while the time spent and the number of entries in the bottom zone have increased.

RESULT

The novel diving test was performed, and the time was recorded successfully. The results have shown that the time spent and the number of entries in the top zone have decreased, while the time spent and the number of entries in the bottom zone have increased.

APPLICATIONS

1. **Anxiety assessment:** The test provides a valuable tool for assessing anxiety levels in Zebrafish by monitoring behavioral responses in unfamiliar

CHAPTER 8

Social Preference Test in Zebrafish

Falguni Goel[1], Mayank Attri[1], Khadga Raj[1] and **Shamsher Singh[1,*]**

[1] Neuropharmacology Division, Department of Pharmacology, ISF College of Pharmacy, Moga, Punjab 142001, India

INTRODUCTION

Aim: To study the social behavior preference test in Zebrafish (*Danio rerio*).

Scope and outcomes: The social preference test holds significant value for examining the potential translational effects of hormones, pharmaceuticals, medical interventions, and other pharmacological agents. Additionally, it serves as a critical tool for studying factors that may influence zebrafish social behavior in laboratory settings. This test offers promising utility in the development of a reliable zebrafish model for investigating various neurological disorders characterized by social deficits, as well as psychological conditions. A rectangular tank split into two compartments with a transparent barrier enabling direct vision among the experimental fishes and the shoal can be utilized to examine social interactions toward a single stimulus. The usage of a rectangular tank featuring three different areas is highly regarded for evaluating preference for two distinct social cues simultaneously.

Theory: The tendency of individuals to associate and reside near others who share similar characteristics is referred to as social preference. In Zebrafish, this social behavior is assessed by observing and analyzing the subject's responses to or interactions with a given social stimulus [1]. Similar to behavioral tests conducted on rodents, social preference tests have been developed for Zebrafish. These tests typically consist of two distinct phases to evaluate social behavior effectively [2]. The Zebrafish under scrutiny is preserved alone in a compartment of the test tank throughout the first phase, acknowledged as the habituation phase, to get accustomed to the unfamiliar surroundings. The initial introduction of the social stimulus, which usually involves the introduction of a couple of small groups of similar living species, commences the second phase, which is the interaction phase [3]. As an alternative, digitally animated (moving) visuals of

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Zebrafish, the augmented and virtual systems, or a pre-recorded film of live fish displayed on a computer screen could be used as the social stimulus [4] (Fig. **8.1**).

The social preference test remains a straightforward, accessible, and flexible method that holds the potential to offer novel insights into the mechanisms underlying neuropsychiatric and neurodevelopmental disorders characterized by impairments in social functioning. Two major benefits that may crop up by executing this test in neurobehavioral research are that contrary to the shoaling and schooling assays, the social preference test has the benefit of inspecting a single subject instead of a group of subjects [5].

Along with that, the social stimulus in the social preference test is less intimidating to those in the mirror biting and predator exposure tests, it could possibly be interpreted as a low-stress assay [6].

Fig. (8.1). Diagrammatic illustration for social preference test.

EXPERIMENTAL SETUP

The social preference test typically involves a tank divided into three compartments: a central chamber and two side chambers. The side chambers may either be identical or contain distinct visual cues to differentiate them.

Habituation phase: Zebrafish are first habituated to the experimental setup by allowing them to freely explore the entire tank without any social stimuli present. This phase helps acclimate the fish to the test environment.

Social preference phase: In this phase, a stimulus fish (a conspecific) is introduced into one of the side chambers, while the other side chamber remains empty or contains a non-social stimulus (*e.g.*, an object). The focal Zebrafish is then placed in the central chamber and allowed to freely interact with both the conspecific and non-social stimulus.

Behavioral observations: During the social preference phase, researchers observe and record the behavior of the focal Zebrafish. Key behavioral parameters, including the time spent in each chamber, the frequency and duration of interactions with the conspecific compared to the non-social stimulus, and specific social behaviors (*e.g.*, shoaling and courtship displays), are systematically measured.

Analysis of social preference: The preference of the focal Zebrafish for the conspecific *versus* the non-social stimulus is assessed based on the amount of time spent interacting with each stimulus and other relevant behavioral measures. An increased preference for the conspecific is indicative of social preference or sociability.

Experimental conditions: The social preference test can be conducted under different experimental conditions to investigate the effects of genetic mutations, drug treatments, environmental manipulations, or social experiences on Zebrafish social behavior. Additionally, the test can be modified to study specific aspects of social behavior, such as aggression, dominance, or social learning.

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Social preference test apparatus, ANY-maze, Camera, Graph-pad-prism.

PROCEDURE

The social performance test requires only three sessions: the habituation phase, training session, testing session

1. The optimal approach involves selecting healthy Zebrafish for the experiment, ensuring that each procedural step is conducted according to the guidelines established by the Committee for Control and Supervision of Experiments on Animals (CPCSEA).
2. Secondly, a net should be utilized for transferring the chosen Zebrafish directly into the test tank.

CHAPTER 9

Mirror Chamber Test in Zebrafish

Falguni Goel[1], Omkar Kumar Kuwar[1], Sania Grover[1] and **Shamsher Singh[1,*]**

[1] Neuropharmacology Division, Department of Pharmacology, ISF College of Pharmacy, Moga, Punjab 142001, India

INTRODUCTION

Aim: To check the anxiety-like behavior in adult Zebrafish using the mirror chamber test apparatus.

Scope and outcomes: This experiment allows us to assess anxiety-like behavior in zebrafish, particularly in response to social stimuli and novel situations. Zebrafish exhibiting anxiety-like behavior may find social interactions challenging or uncomfortable, displaying signs of anticipatory anxiety and situation avoidance. These behaviors may manifest as a reluctance to approach the mirror reduced exploration, or withdrawal from reflective areas. Such nervousness can be specific to certain social contexts or extend to encompass a broader range of interactions, providing valuable insights into the zebrafish's emotions and behavioral states under experimental conditions.

Theory: The mirror chamber test is also known as the mirror biting test. It is a well-established method for evaluating behavioral assays, such as boldness or aggressiveness, employed in the study of social behavior, self-recognition, and various neuropsychiatric and neurodevelopmental investigations in animals, including Zebrafish [1].

This apparatus typically comprises a tank with mirrored walls on one or more sides, enabling the Zebrafish to observe its reflection. The mirror chamber facilitates assessments of anxiogenic and anxiolytic effects and allows for the examination of how genetic and strain variations influence anxious behavior [2]. The mirror chamber test apparatus is shown in Fig. (**9.1**).

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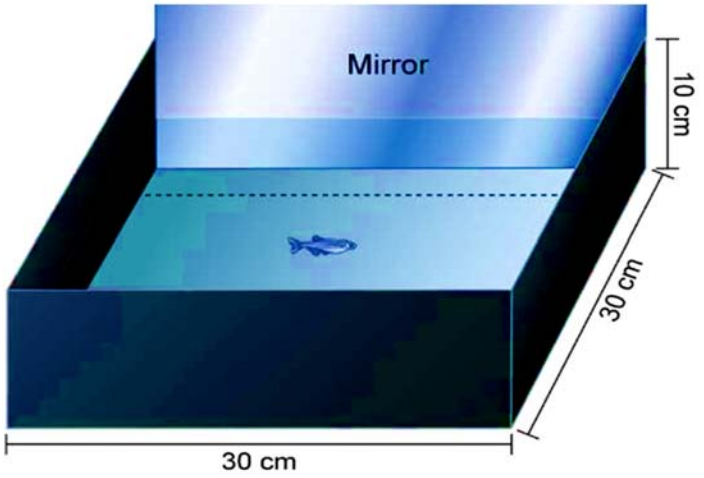

Fig. (9.1). Diagrammatic illustration of the mirror chamber.

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Mirror chamber test apparatus, ANY-maze, Camera, Graph-pad-prism.

PROCEDURE

1. Prepare the mirror chamber by rinsing it thoroughly with tap water.
2. Fill the mirror chamber with fresh water up to the designated level, ensuring that it does not exceed the capacity that will obstruct side viewing if the Zebrafish swims above the mirrored section.
3. Carefully transfer a healthy Zebrafish from the main aquarium to the mirror chamber filled with fresh water using a Fish net.
4. Ensure minimal disturbance and maintain a quiet environment throughout the experimental procedure.
5. Allow the Zebrafish to acclimate in the apparatus for 5 to 10 minutes.
6. Position the camera to record and analyze the fish's behaviour over 10 minutes.
7. Record the time spent near to the mirror or far from the mirror.
8. Behavioural indicators of anxiety and aggression include the fish approaching the mirror and attempting to attack its reflection.

Experimental setup: The Zebrafish is introduced into the mirror chamber and allowed to freely explore throughout the chamber. The fish can observe its reflection in the mirrors and interact with it as if it were another conspecific [3].

Behavioral observations: This is used to observe and record the behavior of the Zebrafish during its interaction with the mirror. Behavioral parameters such as time spent near the mirror, frequency, and duration of interactions with the reflection, and specific behaviors (*e.g*., aggression, courtship displays) are measured. Dummy observation data are given in Table **9.1**.

Assessment of self-recognition: Self-recognition in Zebrafish is inferred based on their responses to the mirror image. If the fish demonstrates behaviors indicative of recognizing themselves, such as repeated inspection of body parts not normally visible (*e.g*., gill covers), displaying social behaviors towards their reflection, or engaging in aggressive or courtship displays, it suggests self-awareness.

Control conditions: Control experiments may involve placing a non-reflective object (*e.g*., a plain wall) instead of a mirror to assess whether the observed behaviors are specific to the presence of the reflective surface [3].

Experimental conditions: The mirror chamber experiment can be conducted under different experimental conditions to investigate the effects of factors such as genetic mutations, drug treatments, environmental manipulations, or social experiences on Zebrafish self-recognition and social behavior [4].

Data analysis: Data collected from the mirror chamber experiment are analyzed statistically to determine significant differences in behavior between experimental groups or conditions. This analysis can provide insights into the cognitive abilities, neural circuits, and genetic factors underlying self-awareness and social behavior in Zebrafish.

Table 9.1. Observation table of time spent near or far from the mirror on mirror chamber apparatus of Zebrafish.

S. No.	Time Spent Near the Mirror in Seconds	Time Spent Far From the Mirror in Seconds
1.	260	340
2.	230	370
3.	265	335
4.	306	294
5.	320	280
6.	220	380
7.	340	260
Mean	277.3±45.73	322.7±45.61

CHAPTER 10

Three-Chamber Test in Zebrafish

Dhrita Chatterjee[1] and **Shamsher Singh**[1,*]

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INTRODUCTION

Aim: To check the memory function and other behavioral activities by using a three-chambered apparatus.

Scope and outcomes: This apparatus is widely used to study memory function, spatial learning activities, depression, and anxiety-like behavior.

Theory: The three-chamber paradigm is a standard method used to assess spatial and non-spatial learning and memory in Zebrafish. This test is particularly relevant for identifying cognitive disabilities in various neurodevelopmental disorders.

It is a modified version of the T-maze apparatus, consisting of three interlinked chambers. Two side chambers are green and red from the inside with a transparent chamber present between both these 2 chambers. The apparatus has two vertical sliding windows for entering into both the left and right chambers. The size of these two slides is approximately 12× 10 cm (height × width) (Fig. **10.1**) [1].

Eddins *et al.* (2009) constructed the most updated version of the three-chambered test to examine the impact of nicotine on the cognitive abilities of the fish. It is a Plexiglas maze having a length of 44cm, height of 40 cm, width of 22 cm and it is installed inside a fish tank [2].

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Three-chambered apparatus, Any maze software, Graph pad prism, and a Camera.

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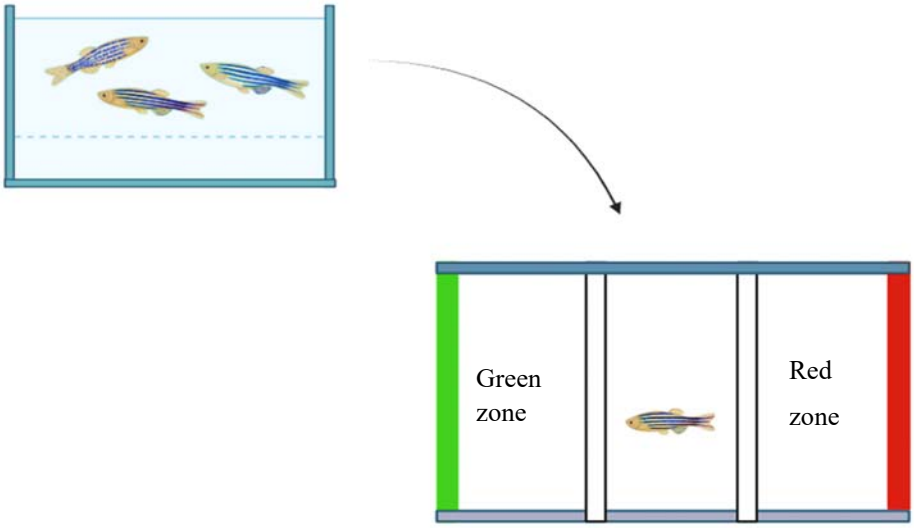


Fig. (10.1). Three-chamber test apparatus.

PROCEDURE

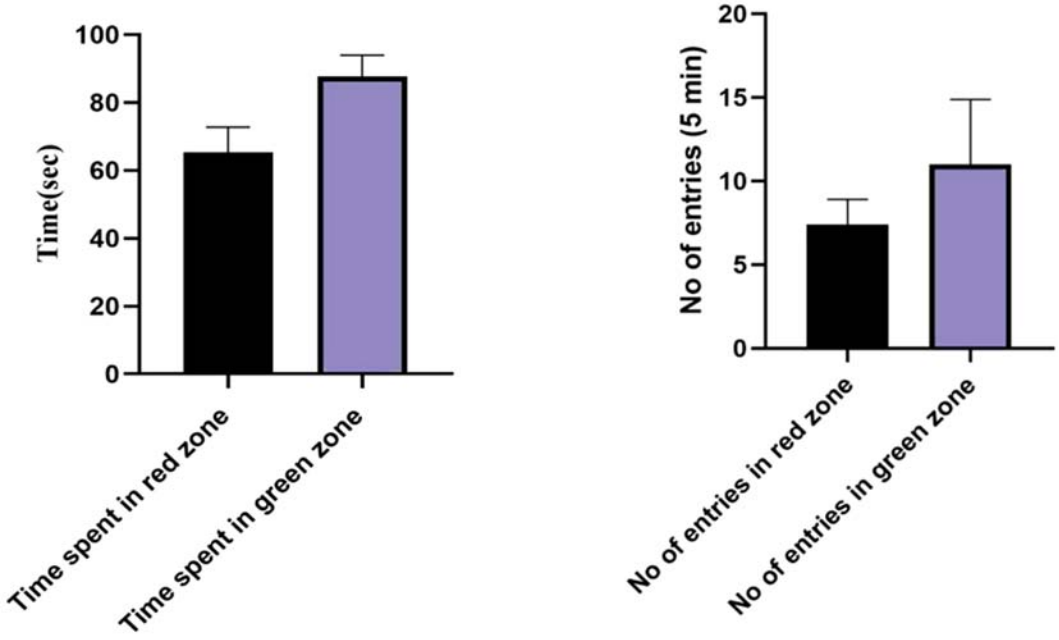
1. The Zebrafish is placed in the 'start zone' and allowed to freely explore the transparent chamber for 2 minutes before the test begins.
2. This phase is repeated 2-3 times to reduce experimental stress.
3. After completion of the habituation phase, Zebrafish is pushed towards the red chamber by opening the sliding door and it is allowed to remain there for at least 5 minutes.
4. In the red chamber, the fish is irritated with the help of a glass rod.
5. After 5 minutes, allow the fish to move towards the hybrid chamber without disturbances, where they remain stable in a calm and non-disturbing environment.
6. After 5 minutes, the fish is pushed to the green chamber for 5 minutes, and during this phase, it is rewarded with feed.
7. After the training phase, the fish is left to rest in the tank water for about 5 minutes.
8. In the testing phase, the fish is placed in the start zone again, and its chamber preference is recorded for 5 minutes [3].
9. Put the data on the observation table (Table **10.1**) and draw a graph by putting these data.

OBSERVATION

The observation parameter (Dummy analysis) is described in Table **10.1**.

Table 10.1. Observation table of number of entries and time of exploration on the three chamber test of Zebrafish.

Fish. No	No of Entries in the Red Zone	Time Spent in the Red Zone (Sec)	No. of Entries in the Green Zone	Time Spent in the Green Zone (Sec)
1.	5	56	7	78
2.	8	65	10	89
3.	7	62	8	92
4.	9	76	14	94
5.	8	68	16	85
Mean	7.4±1.5	65.6±7.4	11±3.8	87.6±6.3



Statistical analysis: Statistical analysis indicates that both the number of entries and the time spent in the green zone have risen, while the number of entries and time spent in the red zone have decreased.

RESULT

The memory of Zebrafish using three-chamber test was performed successfully and studied carefully. The result shows that both the number of entries and the time spent in the green zone have risen, while the number of entries and time spent in the red zone have decreased.

CHAPTER 11

Light-Dark Chamber Test in Zebrafish

Falguni Goel[1], Nileshwar Kalia[1], Lav Goyal[1] and **Shamsher Singh[1,*]**

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INTRODUCTION

Aim: The aim of this chapter is to check the anxiety in adult Zebrafish using a light-dark chamber.

Scope and outcomes: This chapter covers the entire investigation of anxiety evaluation in adult Zebrafish through the application of the light-dark chamber test. The behavioral assay uses the Zebrafish's innate preference for dark, protected areas to assess anxiety-like behaviors under carefully monitored conditions. We will examine the basic ideas that underpin the light-dark chamber test, including its construction and methods for assessing important behavioral traits that are suggestive of anxiety levels. The test protocols will be explained, with a focus on standard operating procedures to guarantee experimental rigor and study repeatability. The chapter will also cover the assay's uses in biomedical research, including how it might be used to investigate anxiety disorders and clarify neurological processes.

Theory: The light-dark chamber test is a widely used behavioral assay to evaluate anxiety-like behaviors in Zebrafish. This test leverages the Zebrafish's innate behavioral responses to light and dark stimuli, which are indicative of their anxiety levels and exploratory behavior. The principle behind this assay is the Zebrafish's preference for dark, safe areas and their tendency to explore brighter, potentially risky environments [1].

This apparatus consists of two compartments: a bright area and a dark area, connected by an opening that allows Zebrafish to move freely between them. Anxious fishes typically spend less time in bright areas and show reduced exploratory behavior characterized by fewer entries in the light zone [2]. Its non-invasive nature and high throughput capability make it an essential tool in behavioral neuroscience and psychopharmacology research, providing insights

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into neurobiological mechanisms underlying anxiety disorders and aiding in the development of new therapeutic strategies [3].

EXPERIMENTAL SETUP

Zebrafish are placed individually or in groups in the chamber and allowed to explore freely. The behavior of the fish is recorded by using video tracking systems or observed directly (Fig. **11.1**).

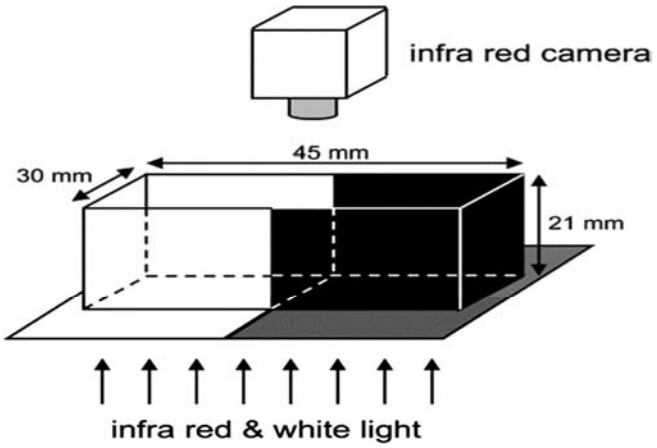

Fig. (11.1). Diagram illustrating the light-dark chamber.

Assessment of light preference: Researchers analyze the amount of time Zebrafish spend in the light compartment compared to the dark compartment. An increased preference for one compartment over the other indicates a preference for that light condition.

Water quality: To prevent any confusing stimuli, make sure the water is consistently clean.

Water Temperature: Fill the maze with either tank water or a combination of tank water and filtered tap water that has been treated with conditioner up to a height of around 8 to 10 cm. The ideal water temperature range is between 25.5 and 28.5 ºC, and a 25-watt heater is placed on the maze's floor to maintain temperature. It is advisable to time trials using a stopwatch. Food should be placed into the proper goal zone using stainless steel tweezers if the study involves rewards.

Behavioral parameters: Various behavioral parameters can be measured during the experiment, including:

1. Time spent in each compartment.
2. Number of transitions between compartments.
3. Swimming activity (speed, distance traveled) in each compartment.
4. Social interactions between fish in the chamber.

Experimental conditions: The light-dark chamber experiment can be conducted under different lighting conditions (*e.g.*, different light intensity and wavelength) to study the effects of light on Zebrafish behavior. Additionally, the chamber can be used to assess the influence of factors such as genetic mutations, drug treatments, or environmental manipulations on light preference.

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Light-dark chamber apparatus, Any-maze software, Graph-pad-prism, Camera.

PROCEDURE

1. Maintain consistent environmental conditions in the experimental room, including temperature (28 – 30 ^{0}C), light cycle, and water quality parameters for the Zebrafish.
2. Acclimate adult Zebrafish to the experimental room for at least 30 minutes before testing to minimize stress.
3. Position the light-dark chamber in a quiet area with minimal disturbances to ensure an accurate behavioral response.
4. Carefully transfer a single adult Zebrafish from its home tank to the light-dark chamber using a net.
5. Start the timer and allow the Zebrafish to explore freely for a pre-determined duration (5 minutes). Record the session with video equipment to capture detailed behavioral responses.
6. Observe and record the behaviors, such as latency to the dark compartment and time spent in each compartment.
7. Use appropriate statistical tests to compare results between experimental groups if different test conditions are applied.

Data analysis: Data collected from the experiment can be analyzed statistically to determine significant differences in behavior between the light and dark compartments, as well as the effects of experimental variables on Zebrafish behavior.

CHAPTER 12

Plus-Maze Test in Zebrafish

Vaishali[1], Kousik Maparu[1] and **Shamsher Singh[1,*]**

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INTRODUCTION

Aim: The aim of this chapter is to check the anxiety-like behavior in adult Zebrafish using a plus-maze apparatus.

Scope and outcomes: This experiment provides insights into anxiety-like behavior in Zebrafish. Individuals may find extremely uncomfortable social interactions with anticipatory anxiety and a tendency to avoid certain situations. The nervousness may be restricted to certain social contexts or broadened to include nearly all social interactions.

Theory: The "+" shaped device is made up of arms with varying depths, each corresponding to varying degrees of aversiveness, similar to the elevated plus-maze [1]. The plus maze is an enclosed environment created to observe Zebrafish as they make decisions and exhibit anxiety-like behaviors [2].

This experimental configuration consists of a cross-shaped labyrinth with two enclosed and two open arms (Fig. **12.1**), allowing researchers to evaluate how Zebrafish explore and navigate the place [3].

CONDITIONS OF THE ENVIRONMENT

Lighting: To simulate open *vs*. protected environments, the closed arms should be either entirely dark or somewhat dimmed, while the open arms should be often brilliantly illuminated.

Water quality: To prevent any confusing stimuli, make sure the water remains consistently clean.

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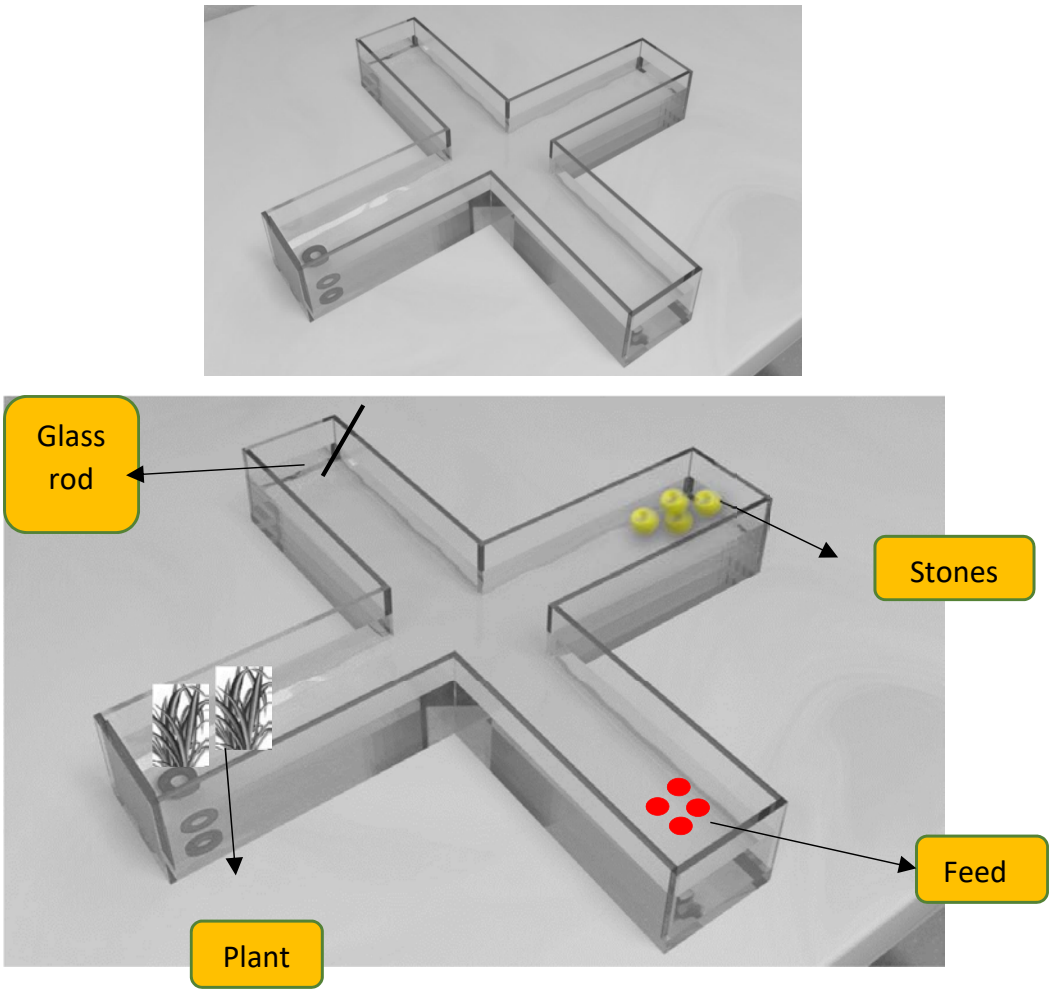

Fig. (12.1). Diagram illustrating the elevated plus maze.

Evaluation Criteria

1. **Anxiety level:** Compare the amount of time Zebrafish spend in the closed and open arms. Spending more time in the open arms indicates lesser anxiety, whereas spending more time in the closed arms is usually regarded as more anxiety level.
2. **Number of entries for every arm:** Count how many times Zebrafish enter each arm type. Increased entries in the open arms may suggest lower anxiety levels.
3. **Time to first entry:** After placing the fish in the maze, record how long it took the fish to reach the first open arm. A longer delay in entering the open arm may indicate an increased level of anxiousness.

4. **The total distance covered:** Track the total traveling distance of Zebrafish throughout the test. This may reveal anxiety-related behaviors and provide insights into overall activity levels.
5. **Investigative Behaviour:** Observe any exploratory actions, such as a Zebrafish dipping its head slightly into the open arms. These kinds of actions may provide more details regarding anxiety levels.
6. **Thigmotaxis or hugging the wall:** Record the Zebrafish's propensity to remain near the arm walls, especially while the arms are open. Elevated thigmotaxis levels are often linked to heightened anxiety.
7. **Changes in behavior:** Examine the frequency and pattern of behavioral changes between the arms. This helps in determining how exploratory behavior and anxiety-driven avoidance coexist.
8. **Freezing action:** Extended periods for freezing or immobility, particularly with arms extended, may be a sign of elevated stress or worry.
9. **Speed:** Calculate how quickly the Zebrafish go through the labyrinth. A slower gait with the fins outstretched may indicate nervousness.
10. **Risk assessment behavior:** Actions like stopping at the entrance of the open arm and looking around before going inside are examples of this behavior. Cautious behavior may indicate anxiety.

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: T-maze, ANY-maze, Camera, Graph-pad-prism.

PROCEDURE

The plus maze test apparatus has three sessions: The habituation phase, the training phase, and the testing phase [4].

1. Firstly, separate one healthy Zebrafish for the procedure and remove it from the water tank.
2. Transfer the fish to the plus-maze with the help of a net.
3. Ensure that the procedure is carried out in noise noise-free environment to reduce experimental stress.
4. Allow the individual fish to habituate in the apparatus for 10 mins.
5. Now, set up the camera to record the video and analyze it for 10 minutes.
6. To create mild irritation, put a glass rod in one arm and 5-6 stones in the second arm.
7. Place food as a reward in the third arm, and in the fourth arm, insert some plants.
8. A greater number of entries of fish toward the fourth arm indicates the healthy

CHAPTER 13

Y-Maze Test in Zebrafish

Dhrita Chatterjee[1], Kousik Maparu[1] and **Shamsher Singh[1,*]**

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INTRODUCTION

Aim: The aim of this chapter is to investigate the memory and cognition of adult Zebrafish using the Y maze apparatus.

Scope and outcome: This practical technique allows us to assess spatial memory by examining cognitive and behavioral parameters, such as the tendency of a fish to explore novel places. By performing the test, researchers can investigate the behavior of fish, such as environmental stimuli, exploration tendencies, spatial memory, and decision-making capabilities. The outcomes of the Y maze test can improve our knowledge in the fields of biology, neurology, and drug discovery.

Theory: The Zebrafish 'Y' maze apparatus is considered an aquatic adaptation of the traditional Y maze used for rodents. It is in the shape of capital 'Y' and used for evaluating the spatial working memory by analyzing the behavior of the fish during the procedure. This setup was first confirmed to be genuine in research conducted by Cognato *et al.* (2012).

The aquatic Y-shaped tank is made up of glass or acrylic materials with three arms of equal length. The length of each arm is 25 cm, width 8 cm, height 15 cm, and having a 120º angle between them (Fig. **13.1**). Compared to the T maze (where the arms have a 90º angle), it provides more area for fluid movement and exploration, which reduces the learning time. To accomplish the goal, food is used as a reward, and animals are forced to follow a particular search pattern or reduce the time or error while seeking the reward.

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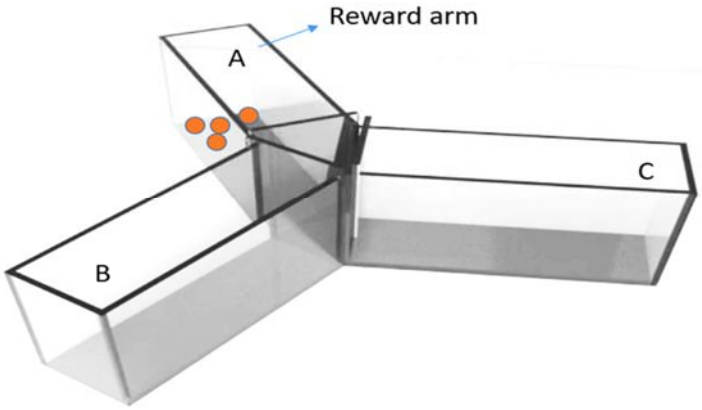

Fig. (13.1). Y-Maze test apparatus.

Each fish is placed at the starting point of the maze and allowed to explore. The three arms are equipped with doors at the entrance of each arm, which prevents the fish from entering more than one arm. If the fish reaches the reward zone in the first trial, then it is expected to explore another zone in the next trial. The capability of choosing the alternate arm determines the decision-making capabilities of the fish and the location-specific memory. The number of arm entries and the time the Zebrafish stays in each arm are recorded [1].

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Y-maze, ANY-maze, Camera, Graph-pad-prism.

PROCEDURE

1. Before beginning the test, set up the Y maze apparatus in a noise-free environment and fill the maze with system water from their home tank.
2. Before testing, habituate the fish to the maze by allowing it to freely explore throughout the maze to minimize experimental stress.
3. Observe the behavior of the animal and record which arm it mainly prefers.
4. Begin the pre-training phase, where the animal is introduced into the apparatus with a food reward in one of the arms.
5. The animal chooses the reward arm in the first trial. Then, in the second trial, the previous reward arm is closed, and then the animal is introduced to the apparatus and allowed to explore the end of the arm.

6. Food is placed in the alternative arm during the second trial. Repeat this process for an equal amount of time. This pre-training phase continues for about 3 minutes.
7. Then, in the testing phase, food is placed in both arms, and all three doors are open.
8. Observe the choice of the fish. If the fish chooses arm A, which means the correct arm choice, then it is rewarded with food.
9. And if it chooses arm B, then it will receive no reward. Thus, the decision-making capability of fish is determined.
10. If the fish does not choose any arm between A and B and stays in arm C, it means no decision-making capability of the fish is determined.
11. All the procedure is recorded to observe the behaviour of the animal. Collect data on different parameters like the number of entries to each arm and the time spent in arm A as well as arm B.
12. Ensure that all the procedures follow ethical guidelines to minimize the experimental stress of the animal [2].

Data Analysis

1. Review the previously recorded video and determine the type of behavioral nature shown by the fish during the experiment.
2. All study videos are recorded and analyzed in a spreadsheet.
3. Perform statistical evaluation to compare the obtained data about spending time in the A-arm (reward zone) and the B-arm (not reward zone).
4. Data should be checked by two independent study coordinators to keep the study from bias and produce more scientific as well as relevant data from the experiment.

CONDITIONS OF THE ENVIRONMENT

Water Quality: Ensure the water is kept clean to avoid any potential confounding stimuli.

Water Temperature: Fill the maze with either tank water or a combination of tank water and filtered tap water that has been treated with conditioner up to a height of around 8 to 10 cm. The ideal water temperature range is between 25.5 and 28.5 ºC, and a 25-watt heater is placed on the maze's floor to maintain temperature. It is advisable to time trials using a stopwatch. Food should be placed into the proper goal zone using stainless steel tweezers if the study involves rewards [3].

CHAPTER 14

Square-Maze Test in Zebrafish

Falguni Goel[1], Lovekesh Singh[1] and **Shamsher Singh[1,*]**

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INTRODUCTION

Aim: The aim of this chapter is to check the anxiety-like behavior in adult Zebrafish by using a square maze test apparatus.

Scope and outcomes: This practical will allow us to investigate anxiety-like behavior in Zebrafish. The square maze is a widely used behavioral assay for *Danio rerio*, employed to study various aspects of behavior, including anxiety, exploration, learning, and memory.

Theory: The Square Cognitive Maze (SCM) is a tool used to assess cognitive abilities in animals, including Zebrafish [1]. It consists of a square tank divided into four equal-sized sections, each containing 12 centimeters of water maintained at a temperature of 37°C (Fig. **14.1**). Food incentives are placed in the fourth section to motivate the fish to learn the location of the reward [2].

EXPERIMENTAL CONSIDERATIONS

1. **Consistent lighting and environment:** Ensure consistent environmental conditions to minimize variability in behavior due to external factors.
2. **Acclimation period:** Allow Zebrafish to acclimate to the maze environment before starting measures to reduce stress-induced behavior changes.
3. **Control groups:** Use control groups to compare against experimental groups, ensuring that observed behaviors are due to the experimental manipulation and no other variables.

CONDITIONS OF THE ENVIRONMENT

Water Quality: To prevent any confusing stimuli, make sure the water is consistently clean.

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Water Temperature: Fill the maze with either tank water or a combination of tank water and filtered tap water that has been treated with conditioner up to a height of around 8 to 10 cm. The ideal water temperature range is between 25.5 and 28.5 ºC, and a 25-watt heater is placed on the maze's floor to maintain temperature. It is advisable to time trials using a stopwatch. Food should be placed into the proper goal zone using stainless steel tweezers if the study involves rewards.

Parameters Measured

1. **Latency to enter specific zones:** The time taken for a Zebrafish to enter a predefined zone of the maze after being introduced is used to assess exploratory behavior and the initial response to the environment. Shorter latencies may indicate higher exploratory behavior or lower anxiety.
2. **Time spent in specific zones:** The total amount of time a Zebrafish spends in different zones of the maze helps to determine zone preferences, which can be indicative of anxiety levels or exploratory tendencies. For example, more time spent in the periphery might suggest anxiety-like behavior.
3. **Distance traveled:** The total distance covered by the Zebrafish while exploring the maze reflects general locomotor activity and exploratory behavior. Increased distance can indicate higher activity levels or reduced anxiety.
4. **Number of entries into specific zones:** The number of times a Zebrafish enters different zones of the maze indicates the frequency of zone transitions, which can be related to exploratory behavior and willingness to explore new areas.
5. **Path taken (Trajectory)**: It is the specific route taken by the Zebrafish through the maze. Analyzing the trajectory helps in understanding movement patterns, spatial learning, and decision-making processes.
6. **Thigmotaxis (Wall-hugging):** It is the tendency of the Zebrafish to stay close to the walls of the maze. Increased thigmotaxis is often interpreted as anxiety-like behavior. It reflects a preference for safer, enclosed areas over open spaces.
7. **Rearing and other vertical movements:** Vertical movements such as rearing (raising the body towards the surface) provide additional insights into exploratory behavior and anxiety. More vertical activity may indicate lower anxiety levels.
8. **Freezing behavior:** Periods of immobility, where the Zebrafish remains still, are referred to as freezing. Freezing is typically considered an indicator of fear or anxiety. Frequent or prolonged freezing suggests elevated levels of anxiety [3].

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Square-maze, ANY-maze, Camera, Graph-pad-prism.

PROCEDURE

1. The experiment begins by inducing a condition in the fish that may lead to cognitive impairments.
2. After the disease is introduced, the fish are placed in the first section of the SCM, and the time it takes them to reach the fourth section is recorded.
3. This process is repeated multiple times, and the average time is calculated. If the fish's cognitive abilities are intact, they will quickly learn the location of the reward and navigate to the fourth section of the maze in a short amount of time.
4. However, if their cognition has been altered due to the sickness, it will take them longer to reach their destination.
5. In addition to recording the time it takes for the fish to reach the reward, the time they spend in the fourth section is also recorded.
6. This is because cognitive impairments may cause the fish to have difficulty remembering the location of the reward or recognizing it as a desirable outcome.
7. Moreover, the SCM provides a reliable and efficient method for investigating potential cognitive flaws in the Zebrafish model.
8. By measuring the time it takes for the fish to navigate the maze and reach the reward, researchers can gain insights into the fish's cognitive abilities and potential cognitive impairments.

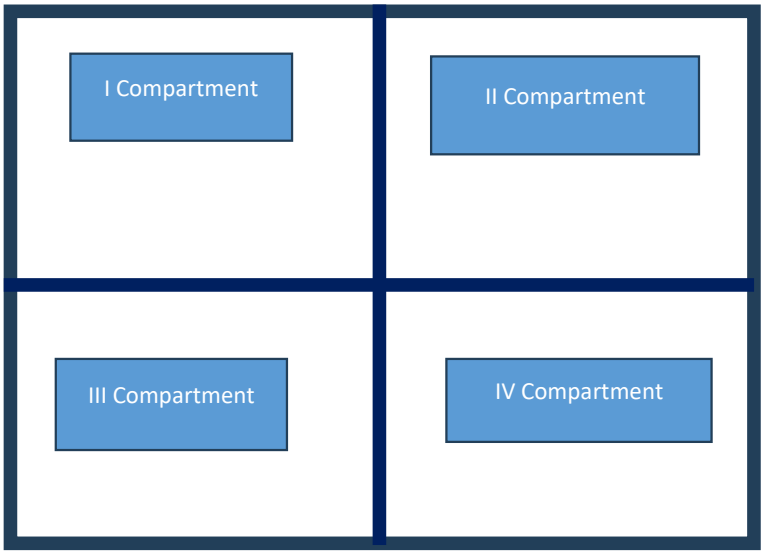

Fig. (14.1). Diagrammatic illustrations of square maze.

CHAPTER 15

Novel Object Recognition Task in Zebrafish

Dhrita Chatterjee[1] and **Shamsher Singh**[1,*]

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INTRODUCTION

Aim: The aim of this chapter is to investigate the memory and cognition of Zebrafish, a freshwater teleost, using a novel object recognition test (NORT).

Scope and outcomes: Through this practical technique, we can analyze the memory recognition and cognitive abilities of a fish by observing their preference for choosing and retaining a new object over a familiar object. This study provides insights into the fish's ability to recognize and remember new objects as well as to study their learning capabilities. By performing the test, researchers can better understand how various environmental and genetic factors influence the cognition of the fish.

Theory: The Novel Object Recognition (NOR) test has become an increasingly important method for evaluating learning and memory in Zebrafish, similar to rodents. In 1988, Ennaceur and Delacour first described the NOR test, primarily using rats. This test examines the ability of a fish to explore more frequently toward a new object than a familiar one. Consequently, a stronger preference towards unfamiliar objects indicates memory recognition and consolidation.

The NOR test is a quick and attractive method that requires no reward or punishment; instead, only minimal training or habituation is necessary. This test is very useful for assessing long-term, intermediate, and short-term memory by adjusting the retention interval. Basically, maze and open field apparatus are used for performing the NOR test in both fish and rodents. Ennaceur and Delacour (1988) used a wooden open box measuring 65 × 45 × 65 cm (Fig. **15.1**) [1].

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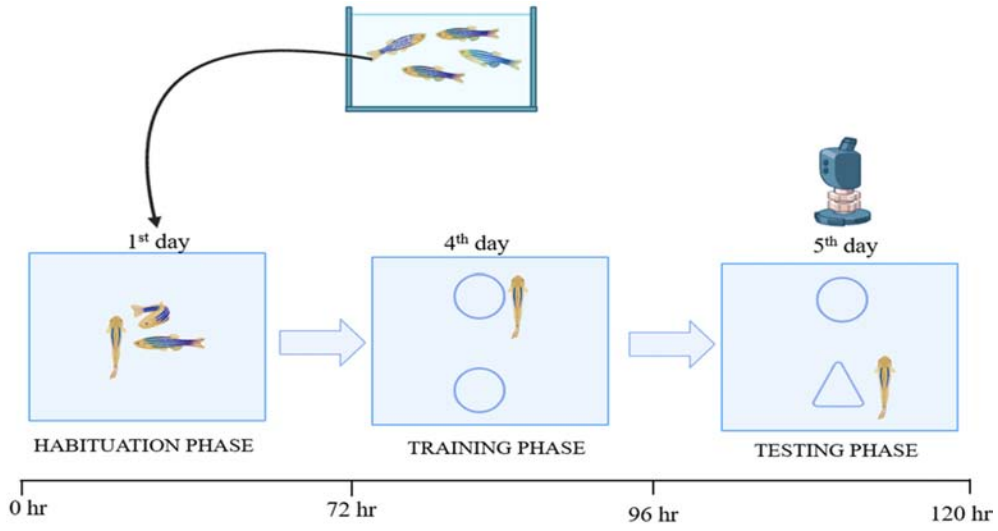

Fig. (15.1). In this picture, three sessions of novel object recognition test (NORT) are shown.

In contrast, Zebrafish were tested in 15L tanks measuring 29cm × 14cm × 18cm. Fishes were kept in the holding tanks in between trials and exposed to the experimental tanks containing the objects. Both tanks have similar dimensions. The novelty of performing the NOR test is to determine the recognition memory of fish. It is a highly validated test used to assess the effectiveness of drugs that improve memory, the (negative) impact of other substances on memory, or the impact of aging or heredity on memory [2].

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Novel Object Recognition test apparatus, Any maze software, Graph pad prism, Camera.

PROCEDURE

The NOR test requires only three sessions: (1) Habituation session, (2) Training session, (3) Testing session

1. During the habituation phase, remove the fish from the holding tank and expose it in the central portion of the experimental tank.
2. Make sure that the procedure is carried out in a quiet environment, and the temperature of the water should be maintained.
3. In the absence of objects, allow the fish to move freely throughout the

experimental tank for at least 5 minutes to reduce novelty stress.
4. Then, return the fish to the holding tank. The habituation session continues for 3 days.
5. On the 4th day, place two identical objects inside the test tank, and the training session starts after exposing individual fish to the tank for 10 minutes.
6. Objects are either cube (side = 1.5 cm), round (5 mm), or sphere (diameter 3 cm). After 10 minutes of training, return the fish to the holding tank.
7. In the testing session, place one familiar object from the training session and replace the other with a new object, and then expose the fish in the experimental tank.
8. Allow the fish to move between these familiar and unfamiliar objects for around and check which object it prefers mostly.

During these 10 mins of the novel object recognition test, all behaviors of the fish are captured using a webcam that is connected to a laptop for further evaluation *via* the Any-Maze video-tracking system [3].

$$\%\text{Preference} = \frac{\text{Time of exploration to the novel object} \times 100}{\text{Time of exploration to the familial object} + \text{Time of exploration to the novel object}}$$

Conditions of the Environment

Water quality: To prevent any confusing stimuli, make sure the water remains consistently clean.

Water temperature: The optimal water temperature range is between 25.5 and 28.5 ºC, and a 25-watt heater is placed at the bottom of the maze to maintain temperature. It is advisable to time trials using a stopwatch [4].

Data analysis: The data collected from the diving test can be analyzed using statistical methods to identify patterns and correlations between behavioral responses, physiological parameters, and experimental conditions.

OBSERVATION

The observation parameter (Dummy analysis) is described in Table **15.1**.

Table 15.1. Observation table of no. of entries and time of exploration on NORT of Zebrafish.

Fish No.	No. of Entries to Familiar Objects	Time of Exploration of the Familial Object (Sec)	No. of Entries to the New Object	Time of Exploration of the New Object (Sec)	% Preference
1.	8	65	18	62	48%
2.	12	75	9	79	51%

CHAPTER 16

Open Field Test in Zebrafish

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INTRODUCTION

Aim: The aim of this study is to study the locomotor activities of Zebrafish (*Danio rerio*) using open-field apparatus.

Scope and outcomes: The open field apparatus allows for the assessment of Zebrafish exploration (locomotion) and instances of freezing behavior. Both adult rodents and Zebrafish exhibit strong scototaxis (preference for dark environments over light ones) and thigmotaxis (preference for staying close to the walls). Zebrafish exhibit habituation responses like those of rodents, altering their exploratory behavior as they go across new habitats. Researchers would likely observe and record the number of times the lines are crossed by Zebrafish in 5 minutes, time spent on corners, mirror biting, and freezing behavior, which is recorded by using various tracking software such as ANY-maze, Zebra Lab, Bonze, *etc*. In addition, researchers may conduct histopathological studies to validate the preliminary findings from the open field apparatus (OFA) and provide definitive evidence of any abnormal alterations in various regions of the brain.

Theory: A standard method for evaluating Zebrafish behavior and motility is the open field apparatus test (with ideal dimensions of 30×30×30 cm), typically made of glass or plastic (Fig. **16.1**). This test is used to assess locomotion, aversion, and anxiety-like responses by observing changes in the Zebrafish's preferred locations. Formerly, various rodent-based models were utilized to find novel neuroactive medications, especially in the field of neurology. These approaches are productive, yet they have significant drawbacks (such as being time-consuming, expensive, and low output), which substantially decrease the chances of finding novel drugs.

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In contrast to previous models, the modern Zebrafish-based model offers several advantages for improved drug discovery and preclinical screening. Recently, the OFT has been used to analyze the swimming and locomotion patterns of Zebrafish, demonstrating a few similarities in the ways that rodents and Zebrafish explore unfamiliar environments [1]. The OFT is an animal anxiety/locomotion analysis model based on how animals naturally respond to unexpected circumstances. It can reveal a range of behaviors, from increased anxiety, which manifests as avoidance behavior (*e.g.*, decreased exploration, increased freezing, and/or disorganized, erratic locomotion), to heightened activity associated with exploration, demonstrating the animal's ability to explore new areas [2, 3].

A shift in behavior that could be an indication of adaptability to the new environment comes after this initial phase. For instance, as they get used to their new environment, the study subjects often decrease their mobility and develop an assortment of comfort behaviors. Also, the researchers might immensely benefit from the usage of OFA in hopes of accomplishing a spectrum of ground-breaking outcomes, such as executing novel research on links between numerous degenerative neurological conditions with specific newly discovered molecules, medicines, toxins, and chemicals.

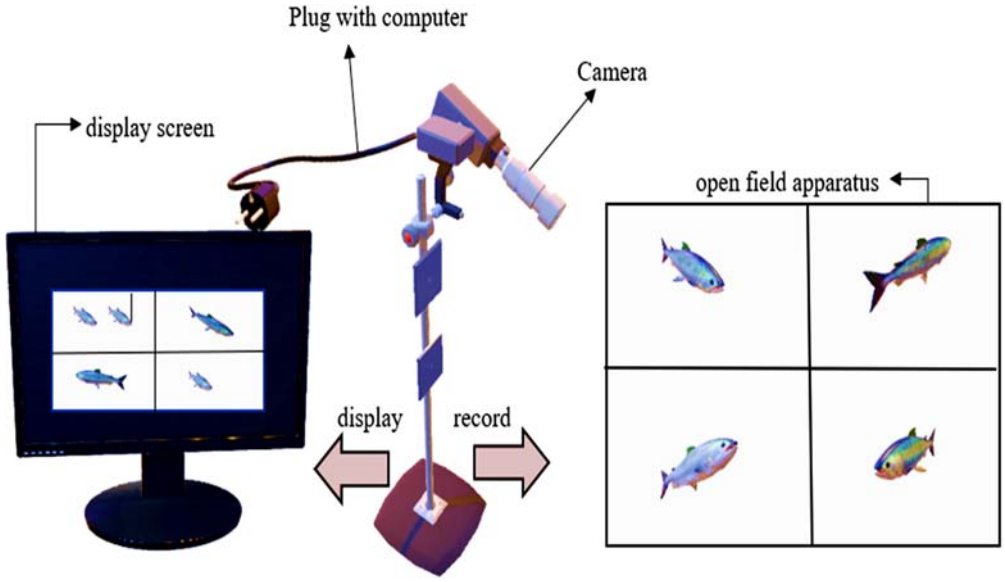

Fig. (16.1). Illustration of open-field apparatus.

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Open field apparatus, Any maze software, Graph pad prism, Camera.

PROCEDURE

1. Healthy Zebrafish should be selected for the conduction of the experimentation. All the procedures should be conducted with ethical guidelines provided by CPCSEA (The Committee for the Purpose of Control and Supervision of Experiments on Animals).
2. Usually, for the conductance of the study, the groups are divided into Control *vs*. Disease *vs*. Treatment with equal ratio of Zebrafish in each group.
3. The afflicted (diseased) group receives toxin, whereas the control group of Zebrafish receives no toxin, and the intervention group receives the desired drug dose for treatment as per the requirement of the researcher (the control group is used as a reference to compare the results of diseased *vs*. treatment).
4. Training is provided for four to five days for five minutes each morning and evening before the experiment day so that the Zebrafish could get habitual to the apparatus.
5. The study groups must keep on fasting for about 30 to 40 minutes before the start of trials on the sixth day, that being the day of the experiment.
6. The following behavior characteristics are recorded after Zebrafish are moved to the arena for the open field test, where they have five minutes (cut-off time) to explore.
7. Several behavioral outputs that are measured include time spent in the arena's center, freezing behavior, thigmotaxis, no. of lines crossed, and mirror biting behavior, and then control *vs*. diseased *vs*. treatment groups are recorded and compared.
8. In the mirror biting test, Zebrafish interact with their mirror images, and the number of times a fish attacks its reflection during a five-minute period is used to specifically measure aggression.

OBSERVATION

The observation parameter (Dummy analysis) is described in Table **16.1**.

Table 16.1. Different parameters of open field test apparatus of Zebrafish.

S. No.	No. of Times Lines were Crossed in 5 Mins	Time Spent in Corners (Sec)	Freezing Behavior (Sec)	Mirror Biting
1.	8	120 sec	40 sec	12
2.	12	280sec	60 sec	10
3.	16	180sec	95sec	8

CHAPTER 17

T-Maze Test in Zebrafish

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INTRODUCTION

Aim: The aim of this study is to check the memory function of an adult Zebrafish by using a T-maze apparatus.

Scope and Outcomes: T-maze is behavioral activity testing equipment widely used to study exploratory behavior in animals such as rodents and Zebrafish, particularly in the context of the central nervous system (CNS). It is employed to assess memory function, spatial learning activities, depression, and anxiety-related behavior. By conducting this experiment, researchers can gain valuable insights into how genetic and environmental factors influence cognitive functions in animals.

Theory: The T-maze apparatus consists of three arms: a main arm that brunches into two secondary arms, typically differentiated by colored markers. The arms are covered with colored paper: red to indicate a "stressed" arm and green to signify a "relaxed" arm [1]. The apparatus is usually constructed from Plexiglas and is available in various sizes and configurations to suit experimental models.

A range of experimental protocols can be applied with T-maze. For instance, researchers can evaluate visual discrimination by designating specific target zones or enrichment chambers within the maze's arm using colored or patterned sleeves. Environmental enrichment or food rewards may be placed in the goal zone to provide positive reinforcement during the test.

Although the proportions can be changed, we advise selecting a maze design that is either a symmetrical-shaped configuration (50 cm x 50 cm x 10 cm) or a cross-

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shaped configuration (70 cm x 50 cm x 10 cm) (Fig. **17.1**). It is best to utilize detachable opaque Plexiglas doors to keep the maze's arms separated from the goal and start zones. Make sure the arms of the maze are closed off from the goal and start zones with Plexiglas doors [2].

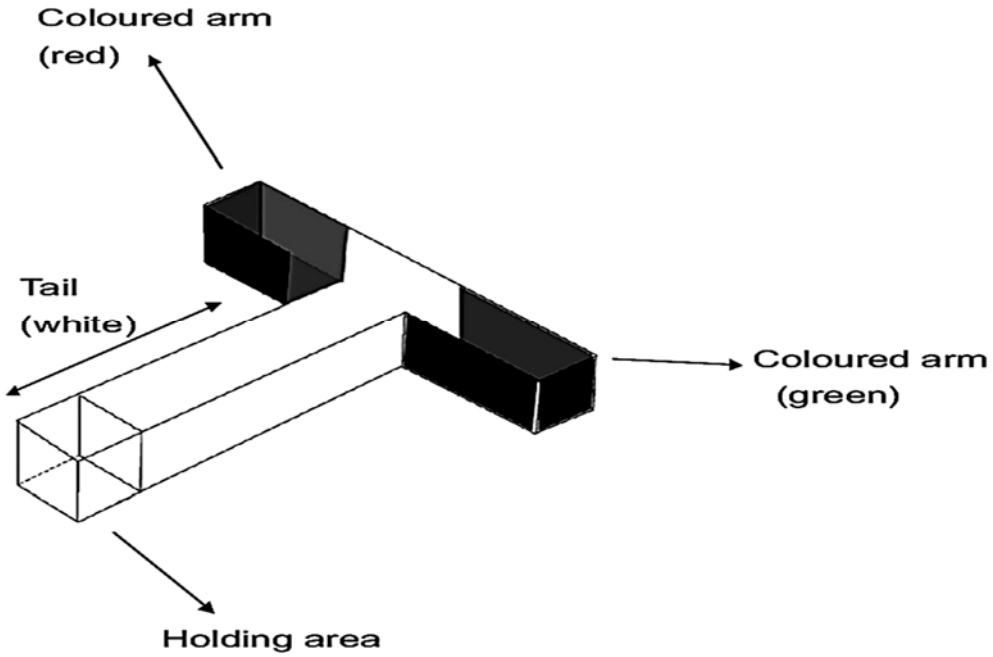

Fig. (17.1). Diagram illustrating the T-maze apparatus.

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: T-maze, ANY-maze, Camera, Graph-pad-prism.

PROCEDURE

The T-maze apparatus has three phases, including the habituation phase, the training phase, and the testing phase.

1. The Zebrafish is allowed to freely explore the maze for 5 minutes during the initial habituation phase.
2. The Zebrafish is then introduced into the main arm of the maze for 2 minutes.
3. After 2 minutes, the Zebrafish is gently guided toward the red chamber, which is then closed with a glass lid.

4. In the red chamber, the Zebrafish is exposed for 5 minutes, during which time it is gently stimulated using a glass rod to introduce irritation in the Zebrafish.
5. After 5 minutes, Zebrafish is pushed toward the green-colored chamber for 5 minutes, where it is rewarded with feed.
6. This activity is performed three or more times for habituation and training to reduce handling and novelty stress.
7. In the training phase, follow the same procedure as in the habituation phase and conduct training trials 2 times at an interval of 24 hours.
8. In the testing phase, follow the same procedure as in the trial phase, and after the testing session, return the fish to its home tank [3].
9. In the testing phase, observe the effect of the drug on the learning and memory behavior in Zebrafish and count the number of entries and time spent on each arm.

Conditions of the Environment

Water Quality: To prevent any confusing stimuli, make sure the water is consistently clean.

Water Temperature: Fill the maze with either tank water or a combination of tank water and filtered tap water that has been treated with conditioner up to a height of around 8 to 10 cm. The ideal water temperature range is between 25.5 and 28.5 ºC, and a 25-watt heater is placed on the maze's floor to maintain temperature. It is advisable to time trials using a stopwatch. Food should be placed into the proper goal zone using stainless steel tweezers if the study involves rewards [4].

OBSERVATION

A dummy analysis is given in Table **17.1**.

Table 17.1. No. of entry and time spent in different zones on the T-maze apparatus of Zebrafish.

S. No.	No. of Entry in the Red Zone	Time spent in the Red Zone (Sec.)	No. of entry in the Green Zone	Time spent in the Green Zone (Sec.)
01	2	30	3	112
02	3	26	5	98
03	2	25	3	147
04	3	37	5	182
05	4	29	4	128
Mean	2.8±0.8	29.40±2.11	4.0±1.0	133.4±14.64

CHAPTER 18

Learning Test in Zebrafish

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INTRODUCTION

Aim: The aim of this chapter is to perform the learning test in Zebrafish.

Scope and outcomes: The learning test is a behavioral activity test for analyzing exploratory behavior regarding central nervous system (CNS) disorders across various species like rodents and Zebrafish. It is used to study memory function and spatial learning activities, depression, and anxiety behavior.

Theory: To evaluate Zebrafish's cognitive abilities, specifically their capacity for learning and memory, a learning test is used. The goal of these tests is to assess the fish's capacity for learning and memory related to tasks, like rewarding a stimulus or avoiding a certain location after a bad experience. Measurements of the fish's behavior and performance over time are made in controlled environments, including tanks with characteristics or mazes.

Novelty in learning tests with Zebrafish often involves assessing their responses to new environments or stimuli. Apart from assessing anxiety and stress responses, these tests can also evaluate traumatic brain injury (TBI) in Zebrafish.

TYPES OF LEARNING

Zebrafish demonstrate various forms of learning, such as:

Non-associative learning

1. **Habituation:** A decrease in the response to a repeated stimulus, such as a reduced startle response to a sound that is presented repeatedly.
2. **Sensitization:** An increased sensitivity and heightened locomotor activity following repeated exposure to a stimulus, such as nicotine, leading to a more pronounced response with subsequent exposures.

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Associative Learning

Classical (Pavlovian) Conditioning

1. **Appetitive conditioning:** An enhancement or attenuation of a reflexive response after the association of a cue with a favorable stimulus.
2. **Aversive conditioning:** An enhancement or attenuation of a reflexive response after the association of a cue with an unfavorable stimulus.

Operant (Instrumental) Conditioning

1. **Positive reinforcement:** An increased response to obtain an appetitive stimulus, such as approaching a sensor that dispenses food.
2. **Negative reinforcement:** An enhancement in response to removing an unpleasant stimulus, such as entering a section without shock upon encountering a light previously associated with shock.
3. **Positive punishment:** A reduction in response after the association of behavior with an unpleasant stimulus.
4. **Negative punishment:** A reduction in response after the attribution of behaviors to the absence of an appetitive stimulus.

Other Types of Learning

1. **Social (shoaling) learning:** Groups of Zebrafish exhibit faster learning rates compared to individuals.
2. **Motor learning:** Zebrafish adjust their locomotor commands using sensory feedback to perform accurate movements, thereby enhancing accuracy and coordination.

Behavioral Learning in Zebrafish

Like many other animals, Zebrafish acquire new behaviors through experience and contact with their surroundings. This process is known as behavioral learning. Because of their transparent larvae, which allow researchers to see their brain activity in real time, and relatively basic neural circuitry, Zebrafish are particularly fascinating subjects for studying behavioral learning. These are some significant aspects of Zebrafish behavioral learning, such as associative learning, spatial learning, social learning, habituation, sensitization, operant conditioning, and neural mechanisms.

Research on behavioral learning in Zebrafish offers valuable insights into the fundamental principles of learning and memory across a variety of species, including humans. It also enhances our understanding of the cognitive abilities of Zebrafish, providing a comparative framework for studying learning processes in

more complex organisms [1].

Behavioral Learning in Various Diseases

The study of behavioral learning in Zebrafish has been examined for several illnesses, especially those that impact behavior and brain function. Several notable examples consist of:

1. **Autism spectrum disorders (ASD):** Zebrafish's social nature and capacity to pick up on socially significant cues have made them useful models for several aspects of ASD. Scientists have investigated the effects of ASD-related gene mutations on Zebrafish's social interaction, communication, and learning.
2. **Epilepsy:** To investigate seizure behavior and its underlying causes, Zebrafish models of epilepsy have been created. In addition to testing possible antiepileptic medications, researchers utilize these models to study how seizures impact Zebrafish learning and memory functions.
3. **Alzheimer's disease:** Alzheimer's disease-related cognitive abnormalities, such as learning and memory problems, have been studied using Zebrafish. Models of transgenic Zebrafish that exhibit mutations in the tau or amyloid-beta proteins have been used to study prospective treatment approaches and disease processes.
4. **Parkinson's disease:** Parkinson's disease-affected Zebrafish models show motor deficits like human symptoms. Additionally, studies have examined how learning and cognitive processes are affected in these models by mutations linked to Parkinson's disease.
5. **Fragile X syndrome:** Fragile X syndrome is a genetic condition linked to intellectual incapacity and behavioral difficulties. It has been studied using Zebrafish. Researchers investigated altered behavior and learning deficits in Zebrafish models with mutations in genes associated with fragile X.
6. **Drug addiction:** The behavioral impact of substances of abuse and behaviors connected to addiction has been studied using Zebrafish. Zebrafish models of drug-seeking behavior, reward processing, and behavioral sensitization can be evaluated by researchers, offering valuable insights into the brain circuits implicated in addiction.
7. **Anxiety and depression:** Models of Zebrafish have been used to investigate behaviors like depression and anxiety. Researchers investigate how stress responses, fear conditioning, and other behaviors associated with anxiety and depression are affected by genetic mutations or pharmacological interventions [2].

Researchers can investigate the underlying mechanisms of neurological and psychiatric diseases, particularly how these conditions affect behavior-learning

CHAPTER 19

Native Area Recognition Test in Zebrafish

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INTRODUCTION

Aim: The aim of this chapter is to check the social interaction or social activity of an adult Zebrafish.

Scope and outcomes: The native area recognition test is a neurobehavioral test that may help evaluate those factors that may affect the social behavior and memory of Zebrafish in the lab as well as look at the potential translational effects of hormones, medicines, and other medical interventions. In the pursuit of a reliable Zebrafish model for psychiatric as well as neurodevelopmental disorders in humans with social deficits, this social preference test can prove to be beneficial.

Theory: The term "memory" is defined as the ability to recall or recognize a past after receiving stimuli in any one of the forms of visual, taste, smell, sound, touch, *etc* [1]. The native area recognition test is nothing but a test of cognition where the stimuli are present in the form of vision. Diagrammatically, the concept of study and native areas is illustrated in Fig. (**19.1**). To check the ability of the subject to distinguish the presence or absence of visual stimuli and the movement followed by them is the prime goal of the study. During the experiment, any other external clues should be avoided to provide a uniform and neutral experimental condition [2].

NATIVE AREA RECOGNITION TEST APPARATUS

The apparatus is made up of rectangular-shaped plexiglass with a length of 30 cm, a width of 22 cm, and a height of 11 cm. The reasons behind selecting plexiglass as a constructive material are its inertness to chemicals, non-toxic nature, good quality, and high level of transparency. The original chamber is divided into two equal chambers by

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a divider with a diameter of 4 cm, which is also made of plexiglass. The left-hand chamber, or floral chamber, was recently made, like the larger tank, where the fish belongs, and another part is left vacant and termed an empty chamber. There is sufficient water level for the fish to swim, and they can migrate to any of the two zones according to their interests. A camera is placed to record the video during the experiment with a holding stand, which is tightly attached to the tank wall through a clamp. The upper side tank is open, and it is used to introduce the experimental animal inside the apparatus. Now, the entire setup, as diagrammatically represented in Fig. (**19.2**), is ready to experiment [3].

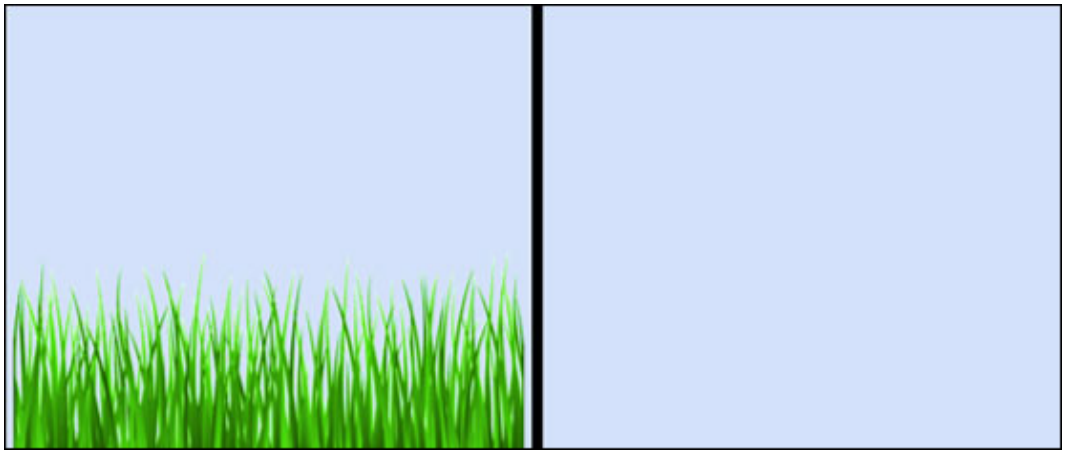

Fig. (19.1). Diagram illustrating the native area recognition.

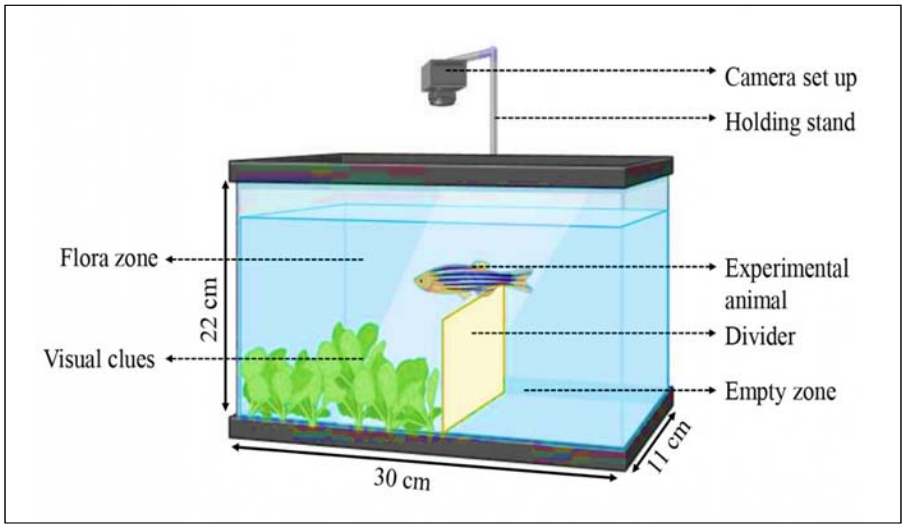

Fig. (19.2). Experimental setup for native area recognition test.

Features of the Apparatus

1. Shape: Rectangular
2. Length: 30 cm
3. Width: 11 cm
4. Height: 22 cm
5. Material: Plexiglass

ANY-maze Software

To make the study more scientific and reproducible, nowadays, researchers are highly aware of man-made mistakes. In the era of computer science and developmental technology, along with artificial intelligence, numerous applications are available on networking sites to make research more precise.

ANY-maze is a type of computational application with multidirectional features like video tracking, data analysis, solving mathematical equations, data transfer, and so many more. This application can be used to perform almost all behavioral tests, including Morris water maze, open field, Y-maze, T-maze, plus-maze, radial arm maze, forced swim test, *etc*. Due to its lower price, ease of operation, and, most importantly, multipurpose use, it is undoubtedly a promising software to be used in experimental pharmacology and enhances its acceptance among researchers throughout the world.

REQUIREMENTS

Animal: Adult Zebrafish

Equipment: Native area recognition apparatus, Any-maze software, Graph-pad-prism, Camera

PROCEDURE

Preparation of the Setup

1. Select one healthy fish from the larger tank and transfer it carefully into a smaller tank with the help of a net.
2. Keep the fish inside the small tank for 10 minutes to habituate itself to the environment.
3. Keep silence in the experimental room and avoid any other external stimuli such as an extra source of light, vibration, *etc*.
4. Maintain the temperature and humidity of the room throughout the experiment and provide a uniform experimental condition.
5. Properly clean the glass tank and apparatus with plenty of water and keep it

CHAPTER 20

Visual Impairment Testing in Zebrafish Using Rotation Test Apparatus

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INTRODUCTION

Aim: The aim of this chapter is to investigate visual impairments of adult mutant Zebrafish using a rotation test apparatus.

Scope and outcomes: This experimental technique allows us to examine abnormalities and levels of visual sensitivity. Adult mutant Zebrafish are often identified using the Zebra Rotation Test, which features two containers: a transparent inner container and an outer drum wrapped in white paper with black segments. With this configuration, adult mutant screening is made easier by being able to measure the rod and cone visual thresholds. Modifications to the test include adjustments to light intensity and changes to the dimensions of the equipment.

Theory: The Zebrafish Rotation Test, also known as the Adult Escape Response Test, is a critical behavioral assay for identifying vision abnormalities in adult mutant Zebrafish [1]. This test quantitatively evaluates visual sensitivity by measuring the absolute thresholds of the Zebrafish's rod and cone photoreceptors [2]. During this experiment, Zebrafish are placed in a stationary glass container encircled by a revolving drum coated with white paper and a black stripe that represents a predatory threat. As the drum rotates, the fish automatically gravitate towards an opaque post in the middle of the container to protect themselves from the black stripe. Researchers can evaluate their visual performance and gain insights regarding their visual acuity through this activity. The test further allows for the measurement of rod and cone threshold after light exposure and enables the study of dark adaptation by varying the intensity of the white light source.

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Features of the Apparatus

- Rotation range: 0-20 rpm
- Centre post diameter: 3 cm
- Main Rotating Chamber: 10 cm diameter, transparent
- Rotating drum: 15 cm diameter

Instrument Specifications

- This instrumental setup includes a circular, transparent container approximately 10 cm in diameter, surrounded by a rotating acrylic drum wrapped in white paper.
- The drum features a 5 x 5 cm black stripe designed to mimic a threatening stimulus. A central opaque post, about 3 cm in diameter, keeps fish from swimming across the midline inside the container.
- Illumination is provided from above by a white light source with adjustable intensity tailored to the equipment's requirements. The drum is rotated at 10 rpm by a motor connected *via* a belt mechanism.
- Zebrafish behavior is tracked and captured with video tracking software like Noldus EthoVision XT. With this configuration, scientists may examine the behavioral responses of Zebrafish to visual stimuli, gaining knowledge about their sensitivity to light and how they react to it in controlled environments. The Zebrafish rotation test apparatus is shown in Fig. (**20.1**).

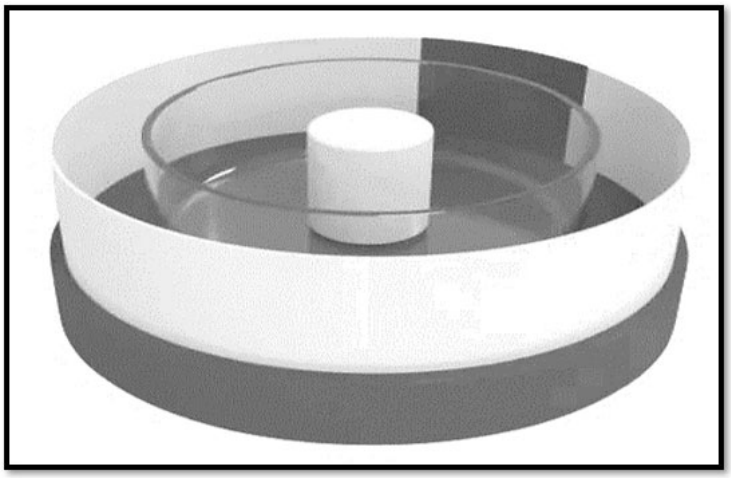

Fig. (20.1). Zebrafish rotation behaviour test apparatus.

REQUIREMENTS

Animal: Adult mutant Zebrafish

Instruments: Rotation test apparatus, Noldus EthoVision XT software.

PROCEDURE AND EVALUATION

Evaluation Method for Control Zebrafish

- **Housing:** Adult Zebrafish are individually housed in transparent containers before the experiment.
- **Light Adaptation:** Subjects undergo light adaptation by exposure to a bright light source emitting approximately 3.25×10^3 $\mu W/cm^2$ for about 20 minutes.
- **Dark Adaptation:** After light adaptation, the fish experience complete darkness (dark adaptation) for approximately 2 minutes.
- **Experiment Setup:** The fish are then introduced into the apparatus designed to assess their threshold light intensity for an escape response.
- **Light Intensity Adjustment:** Initially, the light intensity is set at log I = -3.0 and can be adjusted upwards in increments of 0.5 log units using neutral density filters on the drum [2].
- **Response Measurement:** If the fish do not respond to the initial intensity, the light intensity is increased by half a log unit until an escape response is observed.
- **Threshold Determination:** The lowest light intensity at which the fish exhibit an escape response is recorded as their absolute threshold.

Evaluation of Mutant Zebrafish

- **Mutant Screening Setup:** Light intensity is set to log I = -5.0 for screening mutants, which is approximately 1 log unit higher than the absolute rod threshold observed in wild-type Zebrafish. Individuals that do not exhibit the escape response at this intensity are segregated for further screening in subsequent days [2].
- **Generation of Mutant Individuals:** To generate mutant individuals, subjects undergo chemical mutagenesis using ENU (N-Ethyl-N-nitrosourea). Mutagenized individuals are then crossed with wild-type Zebrafish to produce the F1 generation.
- **Evaluation in F1 Generation:** In the F1 generation, individuals showing traits of night blindness are identified based on their behavior in the escape response test. These mutant individuals from the F1 generation are subsequently crossed with wild-type fish to produce the F2 generation.
- **Assessment in F2 Generation:** Both the F1 and F2 generations are examined to assess visual thresholds for both rods and cones using the escape response paradigm. This assessment helps in identifying and characterizing mutant Zebrafish with visual impairments across successive generations.

Conclusion

In conclusion, this book has explored the multifaceted role of Zebrafish (*Danio rerio*) as a vital model organism in scientific research. From their unique biological characteristics and rapid developmental processes to their genetic similarities with humans, Zebrafish offer unparalleled opportunities for studying various aspects of biology and medicine. We examined key methodologies such as drug administration routes, blood collection techniques, and anaesthesia methods, emphasizing the importance of ethical considerations and welfare in experimental design. Behavioural assessments, including the Novel Diving Tank Test, Mirror Chamber Test, and various maze tests, have demonstrated Zebrafish's capacity to reveal insights into anxiety, memory, and social behaviour. These tests not only highlight the complexities of Zebrafish behaviour but also their utility in understanding neurological and psychological phenomena. This book serves as a foundation for exploring Zebrafish in diverse research contexts, encouraging the use of advanced imaging and genetic modifications to deepen our understanding of behaviour. By incorporating ecological factors and fostering interdisciplinary collaborations, researchers can further enhance the applications of Zebrafish in drug discovery and developmental biology.

By integrating these diverse topics, this book underscores the significance of Zebrafish in advancing our knowledge of developmental biology, pharmacology, and disease mechanisms. As research continues to evolve, Zebrafish will remain an invaluable asset in scientific inquiry, paving the way for breakthroughs in health and disease understanding. Their contributions to both basic and applied sciences ensure that they will be at the forefront of research for years to come.

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Shamsher Singh

Prof. Shamsher Singh is working as a professor and head of the Department of Pharmacology, ISF College of Pharmacy, Moga, affiliated to IK Gujral Punjab Technical University Jalandhar, Punjab, India. He did Ph.D. from ISFCP, Moga. He has contributed dedicatedly towards pharmaceutical education and research for many years. He is a prolific writer with more than 150 publications in various reputed peer-reviewed journals to his credit. Besides this, he has contributed 27 International book chapters related to experimental animal models in Springer Nature, 10 books, and 11 granted design patents from the Government of India (The Patent Office). He has guided more than 22 students in UG projects, 4 pharmacy doctoral (Pharm.D) students, 53 M. Pharm. (pharmacology), 09 Ph.D. students in neurological disorders. Recently, he received 06 research projects and 05 travel grants from different government bodies (ICMR, DST, IBRO, MDS) in neurological areas. He received 07 research awards from Malaysia and India and was awarded 2% scientist in 2024 as per Elsevier data by Stanford University, USA.