Zebrafish*

A Versatile Learning Tool

Padmshree Mudgal, Chitra Bhasin, Adita Joshi and Radhika Gupta

Zebrafish, a freshwater teleost fish, is a popular vertebrate model to study embryo development, organogenesis, gene mutations, and adult physiology. Owing to its biological and genetic advantages, zebrafish is a preferred system for toxicological studies, modeling human diseases, and studying innate immunity. Its usage in studying psychosomatic behavioral disorders has been appreciated recently. Zebrafish is an easy-to-culture, non-invasive vertebrate system with potential multidisciplinary utility in undergraduate teaching and research.

Zebrafish (*Danio rerio*) is a small tropical freshwater fish endemic to India and is found in the Indo-Gangetic Plains (*Figure* 1). It can be procured from aquarium pet shops and can be easily maintained at a minimum cost in mini aquariums or tanks in laboratories. Zebrafish is a vertebrate and shares 70% genetic homology with the human genome and approximately 84% homology with disease-causing genes in humans [1]. Most organ systems and bioprocesses in zebrafish are similar to human systems.

It has become challenging to attract students' interest in biological sciences. Strict guidelines and restrictions on animal experimentation at the undergraduate level have taken away all the excitement of hands-on and inquiry-based learning in biological sciences. Zebrafish model system offers many advantages to fill up this void which include:

1. Zebrafish embryos are easy to collect due to external fertilization and are available in large numbers owing to high fecundity. A steady supply of embryos can be maintained as female fish breed

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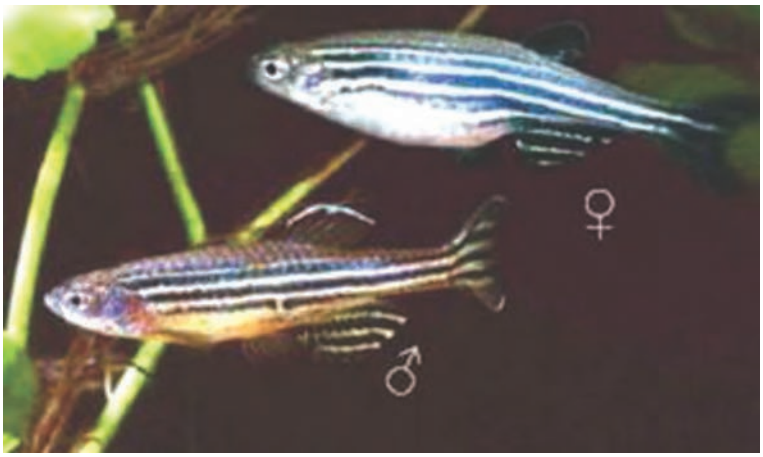

Figure 1. Male and female zebrafish. Male fish have a thin streamlined body and are yellowish golden in colour. Female fish have a white protruding belly and prominent silver stripes in between blue stripes. (Picture: Erik Leist)

Keywords
Zebrafish, learning tool, development, toxicology, regeneration, angiogenesis, behavior.

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every 7—10 days, laying up to 200 eggs/fish.
2. Zebrafish embryos are transparent in nature, which allows real-time observation of early embryonic stages and organ development under a simple stereo microscope in a non-invasive manner.
3. Experiments with zebrafish embryos up to 5 dpf (days post-fertilization) do not require animal ethics considerations as they are not free-feeding [2].
4. Live visualization of bioprocesses in a vertebrate system adds an enabling dimension to student learning and exploring.

1. Learning Tool

Developmental Model

Zebrafish is a valuable model system for studying vertebrate developmental biology. The transparency of the embryo and the chorion[1] allows visualization of developmental processes under a simple stereo microscope. Different developmental periods, including the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching, are very well defined in zebrafish [3]. This allows students to study and follow the development stages in real-time from the first cleavage of the single-cell zygote to multicellular zygote, to morphogenetic movements of involution during gastrulation (shield stage), somite formation, the appear-

[1] A chorion is the outer envelope that surrounds the Zebrafish egg. It is acellular in nature.

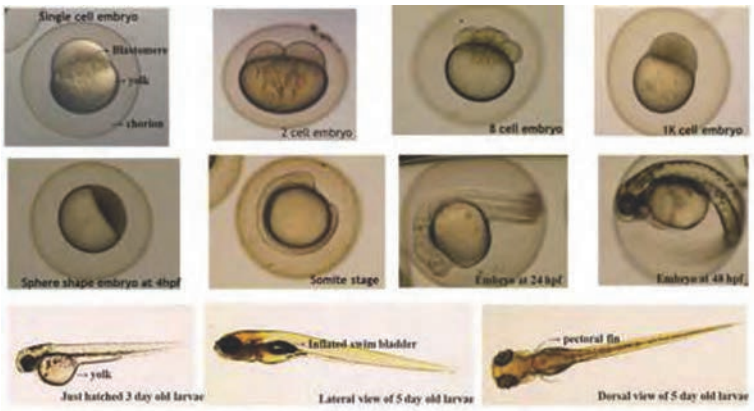

Figure 2. Zebrafish: Developmental stages. (Pictures: Zebrafish lab facility, Daulat Ram College)

ance of the tail, a beating heart, fin development, hatching, inflation of swim bladder and finally a free-swimming, actively feeding larva. Zebrafish development is rapid—the first cleavage occurs within 45 minutes after fertilization, gastrulation starts by 5 hpf (hours post-fertilization), blood circulation and heartbeat can be observed by 24 hpf. All major organ systems develop, and the embryo hatches to become a juvenile larva by 72 hpf. By 5 dpf, the swim bladder inflates, and the larvae are ready to swim and feed (*Figure* 2).

Toxicological Model

Embryonic and larval zebrafish have become a popular toxicological model to conduct rapid *in vivo* tests and toxicity assays. Zebrafish toxicological model provides many advantages such as

1. High fecundity and external fertilization, resulting in a large number of embryos available for toxicological assays for robust reproducible data.
2. *In vivo* toxicology evaluation can be done in a much shorter time frame (7 days) as compared to other vertebrates or mammalian assays.
3. Optical transparency allows for easy visualization of developmental stages and identification of toxicity-induced morphological and cellular defects.
4. High genetic homology and organ similarity to humans allows

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for extrapolation of developmental phenotypic/morphological deformities observed in zebrafish to similar effect in humans.

Zebrafish embryo toxicity assays can be performed in a high throughput manner. Morphological and physiological deformities such as yolk sac edema, pericardial edema, body curvature defects, blood pooling, swim bladder growth retardation, hepatotoxicity, brain toxicity, etc., can be easily observed under low magnification (*Figure* 3). Early life stage toxicity indicators such as chemical or drug-induced changes in survival rate and hatching rate can also be measured easily. Acute toxicity studies to measure 'lethal concentration' 50% (LC50) and chronic toxicity studies to measure 'no observed effect concentration' (NOEC) and maximum nonlethal concentration of chemicals/drugs can be determined. Zebrafish embryo toxicity assays have been performed to test the toxicity and assess the risk of environmental pollutants, industrial chemicals, and drugs [4–6]. Transgenic zebrafish expressing fluorescent proteins such as green fluorescent protein are useful tools with applications in toxicology [7]. Scientists have constructed transgenic zebrafish that express fluorescent reporter proteins for biomonitoring and studying environmental pollutants such as heavy metals [8]. The transgenic zebrafish is designed such that it selectively allows the expression of the fluorescent protein only in the presence of a pollutant, toxin, or heavy metal, thus detecting toxic substances. Students can be encouraged to perform zebrafish embryo toxicology assays for drugs, food additives, water, and other chemicals that humans are exposed to in everyday life.

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Human Disease Model

The use of animal models for human diseases has been helpful in understanding the molecular mechanisms, the pathological manifestation of diseases and syndromes, and determining drug efficacy for disease prevention and cure. Zebrafish was first used as a genetics and biomedical model by George Streisinger and his colleagues at the University of Oregon in the 1970s, who found it a much simpler model to work with as compared to

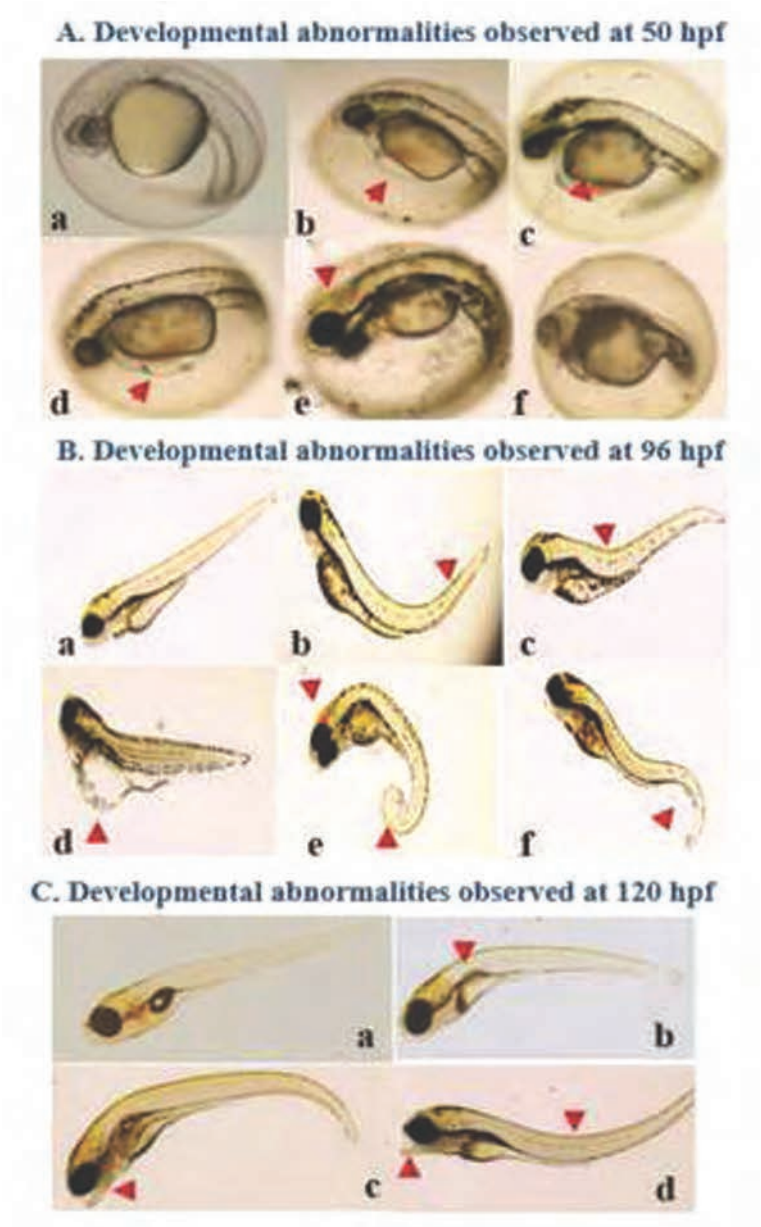

Figure 3. Developmental deformities observed in zebrafish larvae.
(A) Developmental abnormalities observed at 50 hpf: a. Control embryo in fish water; b. Yolk sac edema; c. Blood accumulation; d. Yolk and pericardial edema; e. Head edema; f. Deformed embryo. Images were taken at 10X.
(B) Developmental abnormalities observed at 96 hpf: a. Control embryo in fish water; b. Tail curvature; c. Body curvature; d. Pericardial sac edema and Yolk sac edema; e. Blood pooling and tail curl; f. Tail bent. Images were taken at 4X.
(C) Developmental abnormalities observed at 120 hpf: a. Control embryo in fish water with inflated swim bladder; b. Un-inflated swim bladder; c. Mouth deformity with inflated swim bladder; d. Mouth deformity, un-inflated swim bladder, and body curvature. Images were taken at 10X.
(Pictures: Zebrafish lab facility, Daulat Ram College)

a mouse. In addition, zebrafish embryos form complete organ systems, including the heart, intestine, and blood vessels, within 2 days post fertilization (2 dpf). More than 10,000 mutants in protein-coding genes have been generated and several transgenic

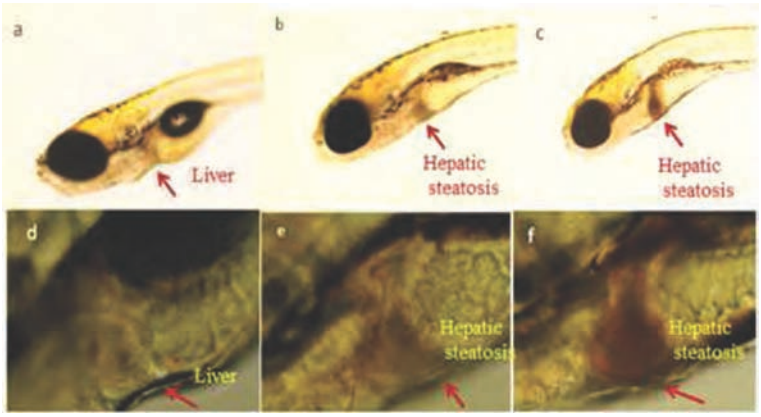

Figure 4. Hepatic steatosis in zebrafish larvae at 165 hpf. **(a)** Control larvae (4X). **(b, c)** Larvae with varying degrees of hepatic steatosis (4X). **(d)** Control larvae (10X). **(e, f)** Larvae with varying degrees of hepatic steatosis (10X).
(Picture: Zebrafish lab facility, Daulat Ram College)

lines of zebrafish have been created to study human diseases. Some of the common human diseases that can easily be modeled in zebrafish in the lab are—diet/chemical-induced diseases, such as metabolic disorders, hepatic ailments, intestinal diseases, and neurological disorders. Zebrafish is an excellent model to study metabolic dysfunction as it has the key organs important for the regulation of energy homeostasis and metabolism in mammals, including digestive organs, adipose tissues, and skeletal muscle. Additionally, the key regulatory pathways such as appetite regulation, insulin regulation, and lipid storage are also well conserved [9]. Non-alcoholic fatty liver disease (NAFLD) can be modeled in zebrafish larvae by feeding high cholesterol (HC), high fructose (HF) and extra feeding (EF) diets for 10 days. The larvae display varying degrees of steatosis, which can be identified phenotypically [10] (*Figure* 4). Glucose metabolism can be studied in zebrafish larvae by exposing 96 hpf larvae to antidiabetic drugs such as metformin (MET: 10 μM) and glipizide (GLIP: 100–250 μM). Whole larvae extract can be used to study the activity of PEPCK (phosphoenolpyruvate carboxykinase), the key regulatory enzyme of gluconeogenesis and glucose concentration. Inflammatory bowel disease (IBD), which results from dysfunctional interactions between the intestinal immune system and microbiota, can be modeled in larval zebrafish by exposure to the haptenizing agent trinitrobenzene sulfonic acid (TNBS). Subsequently, intestinal inflammation and intestinal epithelial dam-

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age can be observed [11]. The list of disease models in zebrafish is expanding every day.

Behavioral Model

The structural organization, cellular morphology, and neurochemistry of the zebrafish brain are very similar to that of humans. Zebrafish also possess a well-developed neuroendocrine system homologous to that in mammals. The zebrafish stress neuroendocrine (hypothalamic–pituitary–interrenal, HPI) axis is similar to the human hypothalamic–pituitary–adrenal (HPA) axis, and releases cortisol following stress exposure [12].

Behavioral responses are the most complex product of central nervous system (CNS) activity. Zebrafish larvae show similarity to adult zebrafish and other vertebrates in key behaviors such as anxiety, stress, learning, memory, and social defense. Locomotion or swimming behavior of zebrafish larvae as an indicator to measure changes in behavior is increasingly gaining popularity in behavioral science and neuropharmacology.

Zebrafish larvae are observed to be sensitive to a variety of stimuli, such as touch, smell, chemicals, sound, heat, and light, which are used as modulators for studying behavior. Some of the commonly exploited behaviors of zebrafish larvae, which are easily studied and quantitated are described below [13].

The wall-hugging behavior or the tendency of the zebrafish larvae to stay near the wall of multi-well plates or petri dishes is a valid index of the anxiety response. The wall-hugging behavior is evolutionarily conserved across species including rodents and humans.

Thigmotaxis: The wall-hugging behavior or the tendency of the zebrafish larvae to stay near the wall of multi-well plates or petri dishes is a valid index of the anxiety response. The wall-hugging behavior is evolutionarily conserved across species including rodents and humans. An analogy can be drawn with a baby hugging close to her mother under threat or fear. Thigmotaxis can be observed in zebrafish larvae as early as 5 dpf. Students can be encouraged to explore the anxiogenic or anxiolytic activity of common food items consumed, such as caffeine, energy drinks, green tea, and milk.

Scotophobia: Zebrafish larvae have a natural preference for light

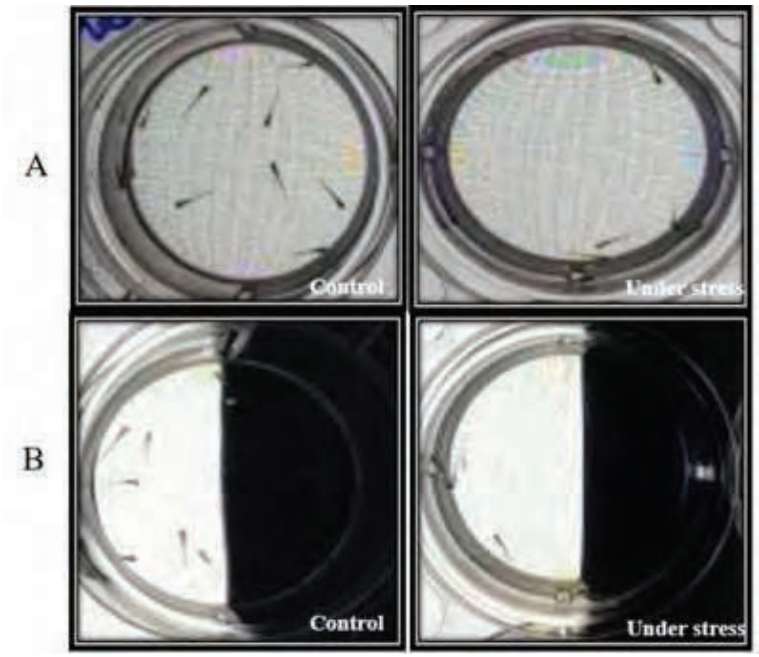

Figure 5. Behavioural assays. **(A)** Thigmotaxis assay: Stress-induced zebrafish larvae show wall-hugging behavior. **(B)** Scotophobia assay: Stress-induced zebrafish larvae show a preference for dark.

(or avoidance of dark). When exposed to stress, the larvae prefer to spend more time in the dark. Scotophobia can easily be quantitated by designing special dark/light well plates (*Figure* 5).

Regeneration Model

The zebrafish model system has been used to identify the molecular mechanisms involved in regeneration in multiple tissues such as the heart, kidney, retina, brain, fin, spinal cord, hair cells (lateral line), pancreas, liver, and skin.

Maintenance of tissue morphology and function is essential for the survival of multicellular organisms. Regeneration is the regrowth of lost tissues or organs post-injury into a fully functional state and shape. Organisms have different regenerative capacities depending on the species and the type of tissues. In humans, regeneration is observed only in the adult liver and infant's fingertips. In most vertebrates, tissue repair is restricted to wound healing, which results in the formation of scar tissue without complete recovery of morphology and function.

Lower vertebrates such as zebrafish, salamanders, and frog tadpoles exhibit the fascinating potential to regenerate lost or damaged tissues. The zebrafish model system has been used to identify the molecular mechanisms involved in regeneration in mul-

tiple tissues such as the heart, kidney, retina, brain, fin, spinal cord, hair cells (lateral line), pancreas, liver, and skin. Several injury models using different approaches have been established in zebrafish to study regeneration, for example, fin and spinal cord injury can be made by transection, cryoinjury can induce lesions in the heart, retina, and fins, gentamicin can induce kidney or lateral line injuries, and acetaminophen is used to induce liver lesions [14, 15]. Genetic ablation can be used to create tissue/cell-specific lesions. Most of the studies on organ regeneration require adult zebrafish except zebrafish larval tail fin, which is a simple yet attractive classroom regeneration model for studying mechanisms of regeneration.

The larval fin-fold has a simple architecture comprising two layers of skin that encloses undifferentiated mesenchyme. The tail fin of zebrafish larva regenerates in a short span of three days post-amputation. Thus, it is easy to carry out regeneration assays in a short experimental time frame. Moreover, zebrafish larvae are amenable to transient genetic manipulations, chemical treatments, and live imaging [16]. Studying zebrafish larvae tail fin regeneration would introduce students to various molecular mechanisms and pathways underlying regeneration. At the injury site, reactive oxygen species (ROS), specifically, hydrogen peroxide (H_2O_2), is generated, which then mediates the initial recruitment of immune cells to damaged tissues and initiates an inflammatory response. Neutrophils are the first cells to migrate to the site of inflammation, and once the infectious threat is neutralized, reverse migration of neutrophils occurs for successful inflammation resolution. Transgenic fluorescent zebrafish *Tg* (*mpx:EGFP*) with EGFP (enhanced green fluorescent protein) sequence inserted downstream of the myeloperoxidase (*mpx*) promoter is available for live visualization of migration and reverse migration of neutrophil cells [17]. In the absence of the availability of transgenic fluorescent zebrafish, neutrophil migration can also be studied using Sudan black staining.

The tail fin of zebrafish larva regenerates in a short span of three days post-amputation. Thus, it is easy to carry out regeneration assays in a short experimental time frame.

Angiogenesis Model

Angiogenesis is a complex process by which new blood vessels are formed from preexisting ones. It plays a critical role in embryonic development, vascular development, wound healing, tissue repair, organ regeneration, and disease. Angiogenesis is mainly accomplished through vessel sprouting, which involves tip cell formation, tubule morphogenesis and lumen creation, and maturation of the newly formed vessels [18]. Angiogenesis is a result of signals from the surrounding milieu to the endothelial cells, secretion of extracellular matrix-degrading enzymes such as matrix metalloproteinases (MMPs), and mobilization of the endothelial cells that finally leads to the sprouting of new blood vessels. Inhibition of angiogenesis may result in impaired wound healing, whereas, excessive angiogenesis is associated with human diseases such as tumor growth and metastasis.

At the cellular level, angiogenesis involves localized endothelial cell proliferation and migration, followed by remodeling of the nascent vessel, and then by growth or subdivision of the new channels. Several growth factors have been shown to play a crucial role in normal angiogenesis. Vascular endothelial growth factor, *VEGF*, is expressed in the vicinity of sprouting vessels, and its receptor (*VEGF-R2/Flk-1/kdr*) on the angioblasts and new vessels, and both are required for vasculogenesis and angiogenesis [19].

The transgenic zebrafish line *Tg* (*fli1:EGFP*), in which the promoter for the endothelial marker *fli1* drives the specific expression of EGFP in blood vessels is used for the live visualization of blood vessels in zebrafish larvae.

The transgenic zebrafish line *Tg* (*fli1:EGFP*), in which the promoter for the endothelial marker *fli1* drives the specific expression of EGFP in blood vessels is used for the live visualization of blood vessels in zebrafish larvae [20]. The patterning of blood vessels of the zebrafish larvae is distinct, in addition to the major blood vessels (dorsal aorta–DA, dorsal longitudinal anastomotic vessel–DLAV, and posterior cardinal vein–PCV), 26 pairs of intersegmental vessels (ISVs), originates from the dorsal aorta, running vertically and parallel to each other in between adjacent segments from trunk to tail that merge into the DLAV (*Figure* 6). Other blood vessels, which are characteristic and clearly visible in

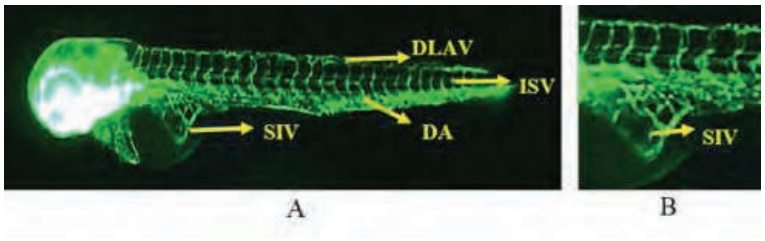

Figure 6. **(A)** Visualization of blood vessels in zebrafish larvae using *Tg (fli1: EGFP)* line at 4X. Main vessels include dorsal aorta–DA, dorsal longitudinal anastomotic vessel–DLAV, 26 pairs of intersegmental vessels–ISVs, and sub intestinal vein–SIV. **(B)** Sub intestinal vein–SIV (10X).
(Picture: Zebrafish lab facility, Daulat Ram College)

the zebrafish embryos and larvae are the intersegmental vessels, which emerge from the dorsal aorta into the embryonic trunk and tail. A number of experiments or assays may be carried out in the *Tg* (*fli1:EGFP*) background for testing the modulators of the VEGF pathway and other chemical effectors that may affect angiogenesis. Imaging of blood vessels can also be done by whole-mount staining for endogenous alkaline phosphatase (ALP) [21].

Stem Cell and Cancer Research Model

Owing to their optical transparency, zebrafish larvae have been widely used for *in vivo* stem cell studies. To understand the mechanistic details of stem cell proliferation, tumor formation, and metastasis, adult fish is a better option but suffers the limitation of opacity. Stem cell scientists have created an adult zebrafish model called 'Casper', which is transparent and allows the easy study of stem cell proliferation and tumor progression in adult engraftment models [22]. GFP labeled stem cells, or tumors can be easily transplanted in Casper fish, and *in vivo* imaging facilitates the tracking of hematopoietic stem cells or tumor progression in space and time-dependent manner. Casper fish is a double mutant of *nacre*, a mutation in *mitfa* gene, which results in the loss of melanophores, and *roy orbison* a mutant in *mpv17* gene, which codes for a mitochondrial protein and regulates pigmentation. Three types of pigment cells—melanophores (blackish color), iridophores (reflective shine), and xanthophores (yellowish tinge) give rise to the signature striped pattern in zebrafish. Several pigmentation mutants have been described in zebrafish.

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Genetics

Zebrafish is an established model for studying gene functions and creating mutations using both forward and reverse genetic approaches. Transgenic zebrafish lines with the *Tol2* transposon have been used to study real-time gene expression and genome.

Zebrafish is an established model for studying gene functions and creating mutations using both forward and reverse genetic approaches. Transgenic zebrafish lines with the *Tol2* transposon have been used to study real-time gene expression and genome [23]. Besides research, transgenic zebrafish have been utilized as a teaching tool. Glofish, a transgenic ornamental fish, has been made publicly available in the USA and is utilized for teaching Mendelian genetics, statistics, and scientific methods to high school and undergraduate students.

2. Protocols

Zebrafish Husbandry

Materials required:
Fish tanks (made up of transparent plastic such as polycarbonate), sea salt, fishnets, and fish feed.

Adult zebrafish can be procured from a local pet shop. Male fish (thin streamlined body and gold or yellow in colour) and female fish (white protruding belly and prominent silver stripes in between blue stripes) can be distinguished easily (*Figure* 1). Male and female fish are to be kept separately in manually maintained tanks in water supplemented with sea salt (0.06 g ocean sea salt/litre of RO water) (*Figure* 7A). Adult fish to be maintained at 28 ± 2^{o}C in a light-controlled room (14 hours light and 10 hours dark cycle). The fish water (FW) is changed daily, and the adult fishes are fed dry flake fish feed (Tetramin, Germany) twice a day (*Figure* 7B). While handling the fish using fishnets (*Figure* 7C), utmost care must be taken to minimize animal suffering.

Study objectives:
(a) Fish handling and care.
(b) Sexual dimorphism in zebrafish, identification of the morphological differences between male and female zebrafish.

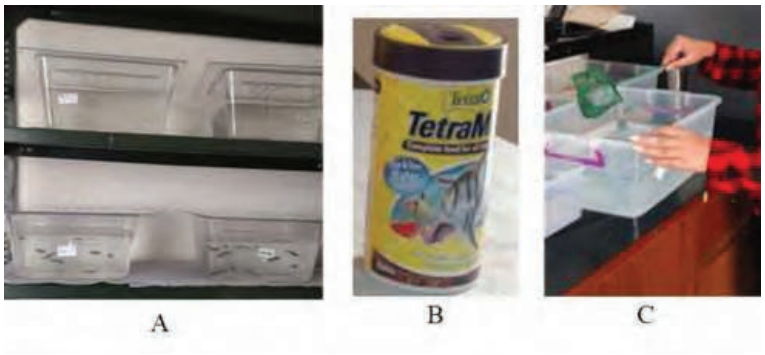

Figure 7. **(A)** Zebrafish reared in tanks maintained at 28 ± 2°C **(B)** Adult fish feed (Tetramin™) **(C)** Zebrafish handling with fishnets.

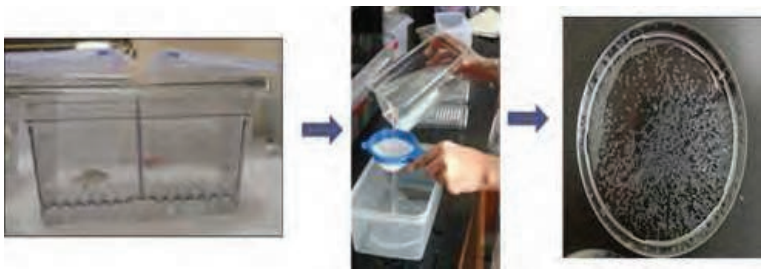

Figure 8. Zebrafish male and female pair is set for mating in a breeding tank. Eggs are collected after spawning with the help of a strainer and transferred into a Petri dish. .

Zebrafish Breeding and Egg Collection

Material required:
Breeding tanks, fishnets, tea strainer, Petri plates, plastic Pasteur pipettes, fish water.

Breeding tanks are set up in the evening, a day before, for egg collection. A plastic separator is placed in the middle of the breeding tank to separate male and female fish. Male and female fish are transferred into the breeding tanks on either side of the separator an hour after feeding. The breeding tanks are left undisturbed overnight. The separator is removed as soon as the lights are turned on the next morning. The fish are allowed to mate and spawn, and fertilized eggs are collected. The viable, fertilized eggs are then transferred into Petri plates containing fish water (0.06 g ocean sea salt/litre of RO water) and are grown in the incubator at 28 ± 2°C (*Figure* 8).

Study objectives:

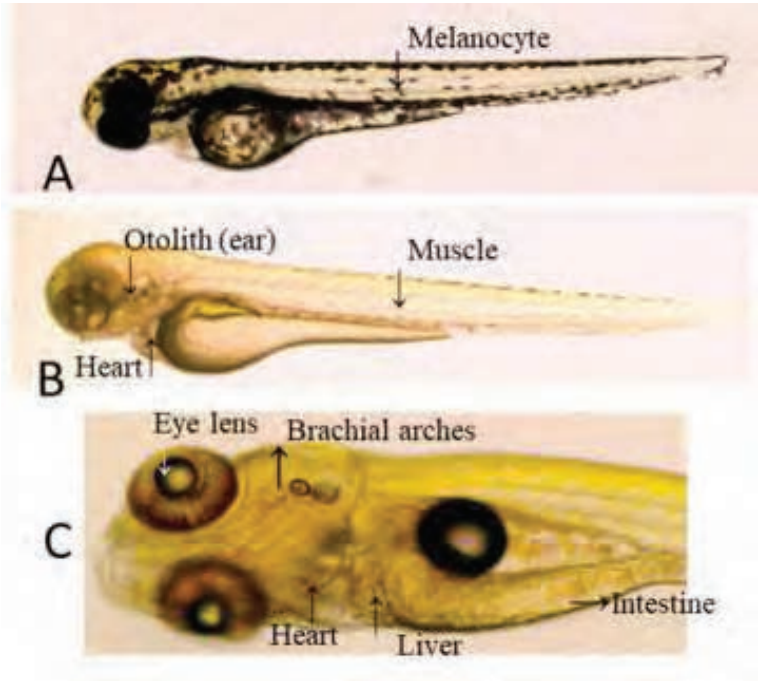

Figure 9. **(A)** Pigmented zebrafish larvae at 4 dpf. **(B)** Poly thiourea (PTU) treated larvae (4 dpf) exhibiting inhibition of melanin synthesis in melanocytes. **(C)** Visualization of internal organs in 5 dpf PTU treated zebrafish larvae at 10X. (Picture: Zebrafish lab facility, Daulat Ram College)

(a) Observe the breeding behaviour in adult fish.
(b) Understand the concept of external fertilization.

Study of Developmental Stages

Materials required:
Fertilized eggs, dissecting stereomicroscopes, Petri plates, plastic Pasteur pipettes, 1-phenyl-2-thiourea (PTU), fish water.

(a) After collection, the fertilized eggs are transferred into Petri plates containing fish water with 0.003% PTU. PTU inhibits melanin synthesis, hence prevents pigmentation and helps in better visualization.
(b) The students can immediately start observing the embryos under the microscope and study different stages of development (*Figure* 2). (c) Students can spend two practical lab periods for 5 days observing zebrafish embryos transform into larvae. PTU treatment enables visualization of fully developed internal organs of zebrafish larvae (*Figure* 9).

Study objectives:
(a) Identify the animal and vegetal pole in the zygote as the blastodisc segregates from the yolk cytoplasm (One cell stage).
(b) Understand the concept of incomplete meroblastic cleavage and differentiate it from cell division.
(c) Understand the concept of axis specification and gastrulation (cell movements).
(d) Observe heartbeat, blood flow, eye movement, tail, and pectoral fin movement and identify different organ systems in the larva.

Toxicology Assays

Materials required:
Embryos, water, PTU, 6-well plates, compounds to be tested, stereo microscopes.

Fertilized eggs are collected and maintained at 28°C as described previously. Embryos that have progressed till 1000 cell stage (3.5 hpf) are transferred into 6-well experimental plates with a density of 25 embryos/8 ml/well. The fish water is taken as control, and the chemicals to be tested are taken at different concentrations. All the wells are labelled. After every 24 hours, the respective water or solution is changed (water/solution samples are brought to 28°C before transferring it to the embryos in the well plates), and dead embryos, if any, are removed. The embryos are then observed under a stereomicroscope and photographed using a digital camera (*Figure* 10). The embryos are to be monitored up to 120 hpf/5 dpf, and various parameters such as mortality rate, hatching rate, heartbeat rate, and other phenotypic, morphological, and developmental anomalies are scored.

Antioxidant Assay/SOD Assay

25 embryos aged 96 hpf are taken in 100 μl of embryo water (EW) and homogenized at 8,000 rpm for 30 seconds. After centrifugation at 12,000 rpm for 10 min, the clear supernatant (crude lysate) is collected. In a 1 ml reaction mix, the final concentrations of 50

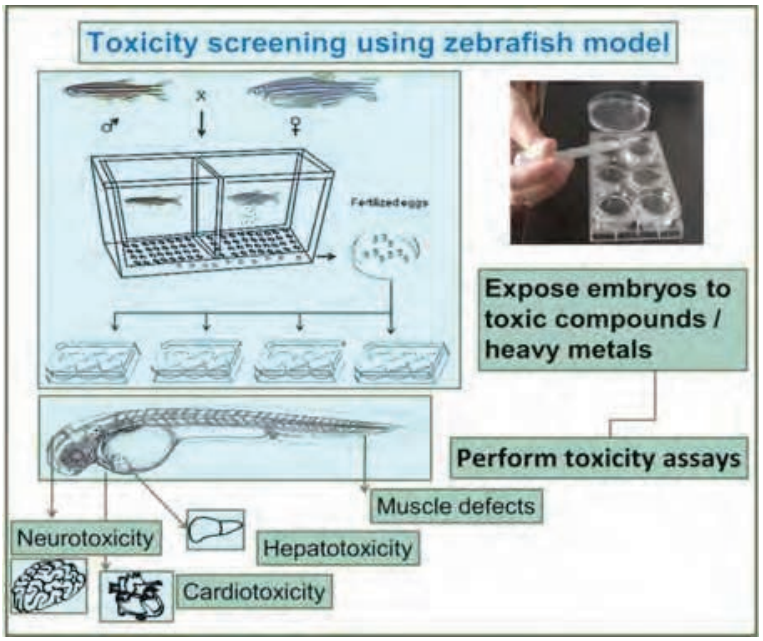

Figure 10. Toxicology assay performed using zebrafish embryos. (Figure adapted and remade using various sources from the web)

mM potassium phosphate, 0.1 mM ethylenediamine tetra-acetic acid, 0.01 mM cytochrome C, 0.05 mM xanthine, 0.005 units xanthine oxidase and 50 μl of crude lysate are mixed. The increase in absorbance can be noted at 550 nm for approximately 5 minutes. The change in absorbance is used as the measure of SOD activity. The experiment is repeated in triplicates. Untreated embryos are used as controls.

Study objectives:
(a) Compare the SOD levels of control larvae with treated larvae.
(b) Identify the morphological and phenotypic defects.

Study/Observe Physiological Processes

Materials required:
Slides, tricaine (stock: 400 mg tricaine powder dissolved in 97.9 ml of double-distilled water and add ~2.1 ml 1 M Tris pH 9. Adjust pH to ~7.
Working solution: Take 1 ml stock and dilute to 25 ml with fish water).

Figure 11. Tail transection of the fin fold is done using a sterilized sharp blade at the caudal position posterior end of the notochord (the red dotted line shows the transection site).

Tricaine acts as an anaesthetic. Larvae are taken on a slide and anaesthetized with tricaine. Processes such as heartbeat count, blood circulation (tracking RBCs), intestinal movements, and excretion can be observed in zebrafish larvae.

Study of Leukocyte Migration Post Tail Fin Transection in Zebrafish Larvae

The leukocyte migration assay is carried out at 4 dpf in zebrafish larvae that have been treated with PTU, as mentioned earlier. Larvae are taken on a slide and anaesthetized with tricaine. Tail transection (cut) of fin fold is done using a sterilized sharp razor blade at the caudal position near the posterior end of the notochord (*Figure* 11).

Live visualization of forward and reverse migration of leukocytes can be observed under a fluorescent microscope after performing tail transection of transgenic fluorescent zebrafish, *Tg* (*mpx:EGFP*), which have the leukocytes labelled with GFP. First, the uncut larvae are observed, and then the transected larvae are observed every hour up to 5 hours post transection (5 hpt) (*Figure* 12).

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Laboratories that lack a fluorescent microscope can study leukocyte migration by using the Sudan black stain for staining the leukocytes. The protocol of Sudan black staining is given below:

(a) Take a 6-well culture plate. Transfer fish water to all the wells. Transfer uncut 24 dpf larvae (control animals) to one well. Transfer the transected (tailfin-cut) larvae to other wells (2 larvae per well). Every hour post transection, fish water in the well containing transected larvae is to be replaced by a 4% paraformaldehyde fixing solution, such that the last transected larvae observed will be at 5 hours post transection. Fixing is to be done for 2 h at room

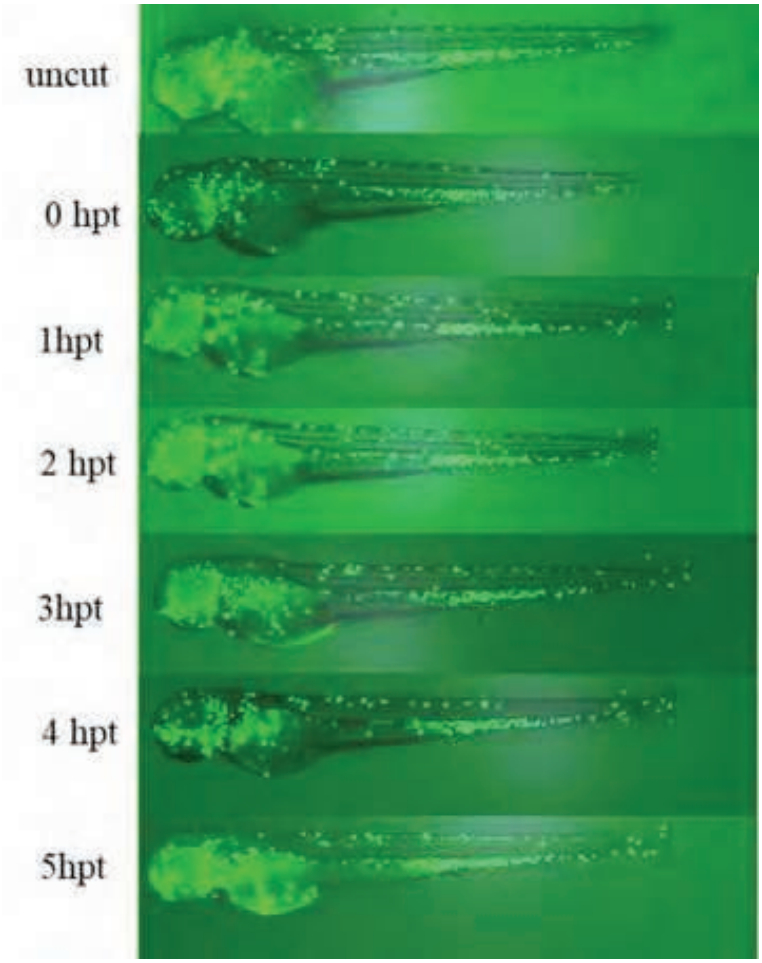

Figure 12. Neutrophil migration to the injury site. Both forward and reverse migration were monitored up to 5 hours post transection (hpt) using zebrafish transgenic line *Tg* (*mpx:GFP*).
(Picture: Zebrafish lab facility, Daulat Ram College)

temperature.

(b) Then, wash the embryos thrice with PBS Tween-20 (PBST).

(c) Next, incubate the embryos in 35% ethanol, 65% PBST and 70% ethanol, each for 5 min.

(d) Afterwards, transfer the embryos to a 0.18% Sudan black staining solution and incubate for 20 min, followed by two short washing steps in 70% ethanol before they are progressively rehydrated. Subsequently, replace the PBST with a 30% glycerol solution. Repeat this step with a 60% and 90% glycerol solution.

(e) For the analysis of the Sudan black staining, transfer the embryos in a 90% glycerol solution onto a slide and bring it into

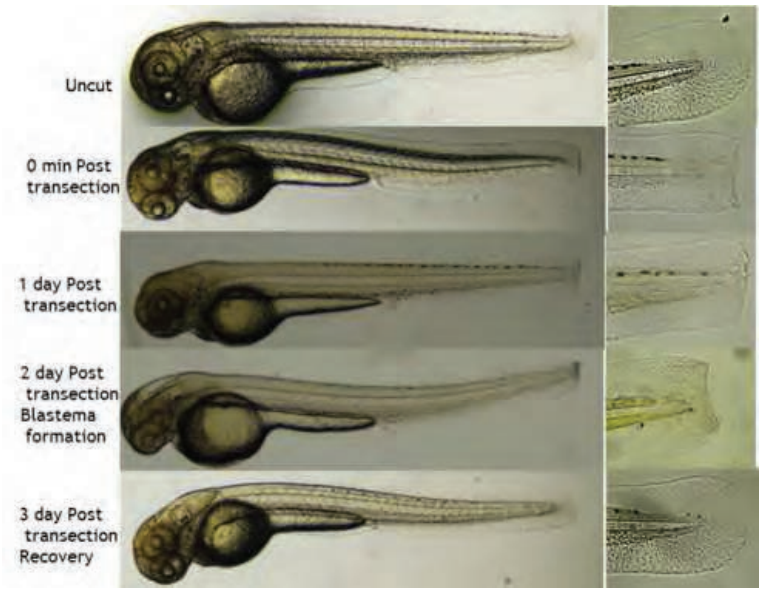

Figure 13. Stages of regeneration in zebrafish larvae tail fin up to 3 days post transection (3 dpt). The transected tail fin completely regenerates by 3 dpt.
(Picture: Zebrafish lab facility, Daulat Ram College)

a suitable position using a fine needle. Then analyze the embryos using a stereomicroscope and photograph them with a digital camera.

Regeneration Assays

Tail fin fold transection is performed on 4 dpf PTU treated zebrafish larvae as mentioned earlier. The larvae are observed under a simple stereomicroscope and digital photographs were taken at 5 hpt (hours post transection), 24 hpt, 48 hpt, and 72 hpt. The larvae regenerate the lost fin fold by 72 hpt (*Figure* 13).

Study Angiogenesis

(a) Whole-mount staining for endogenous alkaline phosphatase (ALP)

At 72 hpf, the zebrafish embryos are processed for endogenous alkaline phosphatase (ALP) staining. Embryos are fixed in 4% PFA for 3 hrs. and incubated in 3% H_2O_2 and 0.5% KOH at room temperature for 30 to 60 minutes until pigmentation disap-

pears completely. Embryos are then washed 5–6 times in PBS (pH 7.4) with 0.1% Tween-20 (Sigma-Aldrich, St Louis, USA) and subjected for dehydration and rehydration process (15 min) in methanol—PBST gradation (25%, 50%, 75%, 100%, 75%, 50%, 25%) and acetone for 30 min at -20°C and finally suspended in PBST for 10–15 min. These embryos are then equilibrated in alkaline phosphatase (ALP) buffer with 100 mM Tris HCl (pH 9.5), 50 mM $MgCl_2$, 100 mM NaCl and 0.1% Tween-20 for 45 mins followed by incubation in staining solution: 110 μg/mL NBT (nitro blue tetrazolium), and 55 μg/mL BCIP (5-bromo-4-chloro-3-indolyl phosphate), in alkaline phosphatase buffer at room temperature for 15–20 min. The staining is terminated using a stop buffer, which is prepared with 0.25 mM EDTA (ethylene diamine tetra acetic acid) in PBST, pH 5.5 for 5 min. After completion of staining, embryos are fixed in 4% PFA overnight at 4°C, stored in 80% glycerol, and are observed and photographed.

(b) Live fluorescent imaging of transgenic Tg (fli1:EGFP) zebrafish

Tg (fli1:EGFP) fishes are set up for breeding, and fertilized eggs are collected. The embryos are allowed to develop at 28°C in the incubator for 48 h. PTU (0.003%) is added to avoid melanin synthesis and maintain transparency. The *fli1* gene expression is visible clearly by 48 hpf. The embryos are then transferred to a slide and observed using a fluorescent microscope to study angiogenesis. Images of the developing blood vessels are taken using a digital camera attached to the microscope (*Figure* 5).

Behavioral Assays

Take a 12-well culture plate. Add 3 ml fish water to each well and transfer ten (n=10) 4 dpf larvae to each well. Keep the plate in an incubator at 28 ± 2°C for acclimatization overnight. The next day, prepare another well plate and expose the 5 dpf larvae to various stress inducers. Students can divide themselves into groups to study the effects of multiple stress factors such as osmotic stress (31.6 mM, 100 mM, or 316 mM concentration NaCl), cold stress

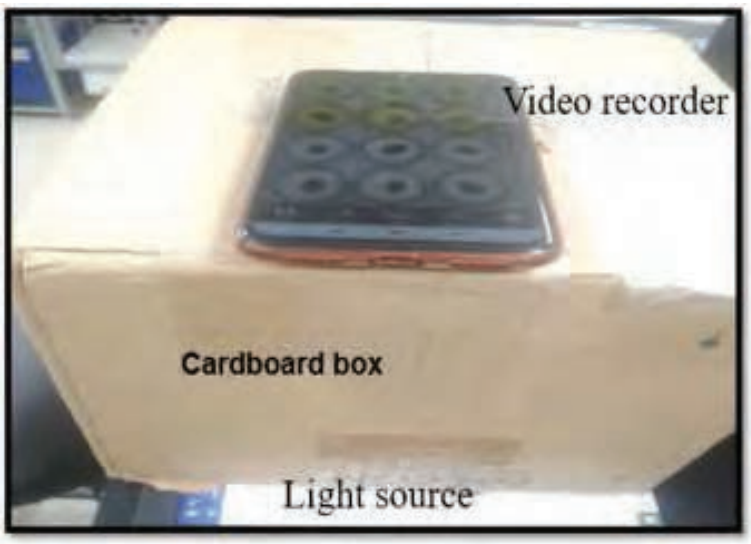

Figure 14. Experimental setup for behavioural assay: The experimental well plate is kept over an iPad, which works as a light source in switch on position. A cardboard box covers the plate. A mobile phone is placed on top of the cardboard, where a small cut has been made for capturing the video in real-time.

(20°C, 10°C and 4°C), heat stress (30°C, 35°C and 40°C), angiogenic chemicals such as caffeine (10 mg/l and 100 mg/l).

The well plate is kept on an iPad which is kept switched on for use as a light source to enhance the visibility of the larvae. The plate on the iPad is covered with a cardboard box to prevent any outside disturbance. A small square or rectangular cut is made on top of the cardboard box to allow the phone camera to record the proceedings as shown in *Figure* 14. Zebrafish larvae (5 dpf) are exposed to stress (5 dpf days post-fertilization) for 10 minutes, and their behaviour is tracked for a 10-minute interval using video recording with a 12MP digital camera using the 'Lapse It' application. The behavioural response of the stressed larvae is observed in picture frames taken every 30 seconds by the 'Free Studio' application.

In the thigmotaxis assay, the number of embryos in the centre are counted in 20 frames taken over 10 minutes. The number of embryos is plotted against time, and the control plots are compared with those under stress.

For the scotophobia assay, half of the well is covered with black paper to prevent light exposure (*Figure* 5). The number of em-

bryos in light are counted in 20 frames taken over 10 minutes. The number of embryos is plotted against time, and the control plots are compared with those under stress.

To study locomotor or swimming behaviour, grids are drawn at the base of the wells to track the movement of zebrafish larvae. If automated tracking software is not available, students can trace the path using a grid.

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