AM5019-ADVANCED BME LABORATORY

1. ZEBRAFISH EMBRYONIC DEVELOPMENT AND VISUALIZATION TECHNIQUES

Aim

To study about the development of zebrafish and to visualize zebrafish embryonic larvae and adult zebrafish using different staining methods.

Introduction:

Zebrafish have become an important model organism to study the development and disease of the skeleton in basic and preclinical research. The potential of these teleost fish lies in their small size, ease of care, genetic amenability, and high regenerative capacity. A major advantage of using zebrafish to probe the mechanism of bone homeostasis is that cell behavior can be visualized dynamically in vivo. Zebrafish skeleton is translucent and develops rapidly, and skeletal processes can be dynamically visualized. In pharmaceutical drug discovery, the number of compounds discovered in early target- or phenotypic-based screens for drug candidates far outweighs the number that advances as clinical candidates, of which relatively few to none progress to clinical trials. This nonclinical attrition is the result of diligent studies conducted in animal models designed to demonstrate bioavailability, efficacy, and safety. During this early phase, depending on the reporting source, between 40 and 80% of the compounds are halted in development due to safety concerns. 70% of protein-coding genes, and 84% of human disease genes are equivalent in zebra-fish. Completely sequenced genome, Easy genetic manipulation, High fecundity, external fertilization, and transparent embryos. The zebrafish genome has been sequenced in full. Zebrafish are a fast model for the study of genetic and de novo mutations. The genes can inactivate in vivo, simulate human phenotypes, and obtain information on human diseases with genetic backgrounds through genomic editing approaches, such as CRISPR/Cas9 or artificial site-specific nucleases, zinc-finger nucleases, and transcription activator-like nucleases. Comparatively, zebrafish have quicker developmental stages than mammalian models and majority of the organs develops within hours post fertilization (hpf).

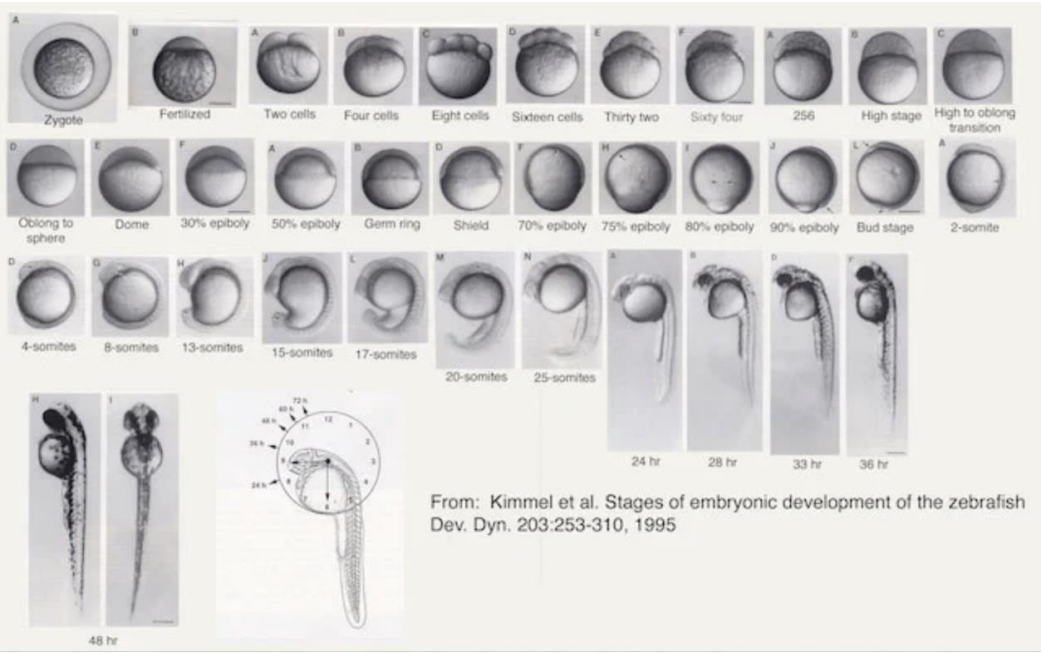

Figure 1: Stages of embryonic development of the Zebrafish

Figure.1 depicts the zebrafish embryonic development stages. At time zero of fertilization, single cell zygote form and within the egg, an animal pole is formed initially. After 45 minutes of fertilization, this cell forms partial cleavage and these duplicated cells proceed to form blastomeres. The next stage is blastula, which possess 128 cells. For every 15 minutes, these cells duplicate and reaches 1000 cells after 3 hpf. After 4 hours, a dome shape can be seen. An interface between yolk cell and blastodisc can be clearly seen, which represent the epiboly formation around the egg. During the Gastrulation phase of embryonic development, a process known as epiboly, which is characterized by cell movements, the form of the zebrafish embryo expands along the animal-vegetal axis. In addition to cells that will eventually create the notochord, axial somite-derived muscles, and certain neurons in the hindbrain, this stage also sees the formation of the neural plate, which represents a primordial brain. The yolk plug closes at the conclusion of the epiboly, causing the production of buds, including the tail bud, which will also help build the posterior trunk. During segmentation (10-24 hpf), somites form and fundamental organs are visible. Cells differentiate morphologically and bodily movements starts. At 18 hpf, 18 somites are present, they intended to form another two somites for every hour. Along the extension of zebrafish during embryonic development, the muscular segments and vertebral cartilage arranges along with the somites. After third somite pair, pronephric kidneys and ducts are formed. At third somite pair, the optic, tissues of ear, and olfactory tissues are formed, which are primary sensory tissues. The behavioural reflex and motor response to the light of the zebrafish depends on the sensory neurons and motoneurons. At larvae phases, these stimulus to dark and light are the key factors for behavioural studies after 5 hpf. On second day of zebrafish development is pharyngula period where the pharyngeal arches are developed. Mandibula and gills are developed from these pharyngeal arches. During pharyngula stage, fins, pigmentation of cells, circulatory system, heart beat and compact head is formed with saturation in lengthening of the embryo. The hatching period varies from fish to fish. Usually, the hatching occurs between 48-72 hours from fertilization. The fish is about 3- 3.5 mm in size after 3 days from hatching. All the organs are completely developed in this stage. The fish started to have feed after 5 days of fertilization, once they are out from the chorion. As per Animal welfare Legislation (EU Directive 2010/63/EU), they are considered as experimental animals at 5 dpf. After segmentation stages, different analysis like chemo- toxicity analysis, cardiotoxicity analysis, and behavioural reflex etc., can be studied using zebrafish. In bone tissue engineering, juvenile and adult fish are used to study bone regeneration, osteogenesis and fracture healing etc.

Different staining and visualization of zebrafish:

Various staining techniques are involved in visualization of zebrafish tissues. Some staining techniques are more specific. Common staining techniques includes, Hematoxylin and eosin (H&E) staining, Alcian blue staining, Alizarin red staining, Oil Red O staining, Toluidine blue staining, silver staining, Acid Schiff staining, and Calcein staining etc., H&E staining is used for histological examination, where hematoxylin stains nuclei blue, while eosin stains cytoplasm and extracellular matrix pink. Usually, Alcian blue and Alizarin red is used to visualize cartilage and bone visualization. Calcein staining is a fluorescent dye used for detecting calcium as an index of mineralization. Oil Red O staining is used to visualize the lipid droplets, that aids in studying lipid metabolism in zebrafish. In case of developmental studies, Gill's H&E staining is used, which is more specific. In nervous tissue visualization, toluidine blue stain is used. Acid-Schiff staining for glycogen and carbohydrate detection and Sudan black B for lipids, myclin and to visualize nerve fibers can be used. Different fluorescent dyes and markers can also be used [Example: DAPI, GFP etc.,]. Von kossa staining is used to visualize the mineralized area in tissues. It's important to choose staining methods based on the specific structures or molecules you want to visualize and the nature of your study (live imaging, fixed tissue, etc.). Additionally, consider the compatibility of staining methods with downstream analyses and the preservation of tissue morphology.

Materials:

Von Kossa staining:

- 1%-5% Silver nitrate solution
- 5% Sodium thiosulfate

- Distilled water

Alizarin Red stain:

- Alizarin red S dye powder
- Acetic acid/HCL
- Distilled water

a) Von Kossa staining:

One of the best techniques to visualize calcium deposit in bone tissue is Von kossa staining. The process of von Kossa staining depends on the capacity of silver ions to react with phosphate ions found in calcium deposits. When silver ions are exposed to ultraviolet (UV) radiation, they are reduced and precipitate dark brown to black at the sites of calcium deposition. This experiment should be carried out in dark environment, since it is light sensitive stain.

Procedure:

- Dissect the fish part using scalpel.
- Wash the fish part with distilled water.
- Now, immerse it in 1%-5% silver nitrate solution.
- Expose it to UV-light for 20 minutes.
- After 15 minutes, discard the silver nitrate solution.
- Now add 5% sodium thiosulfate and leave it for 5 minutes.
- Discard the sodium thiosulfate solution and give gentle rinse using distilled water.
- Using Wipes, just give gentle touch of the tissues to remove water. Now visualize under the Bright field microscope.

Observation:

The calcium deposited region appeared as black/Brown-black precipitate and the remaining tissues remains un-stained.

b) Alizarin red S staining:

One of the commonly used histological stain is alizarin red S stain. Alizarin red is an organic sodium salt, soluble in water or alcohol. It binds with calcium via sulfonic acid/ OH groups. It is used to visualize bone mineralization, osteogenesis, and bone formation. It is a fluorescent dye, which emits red under green light. The alizarin fluorescence was maximal between the wavelengths 490-550nm at excitation and 545-570 nm at emission. It can be viewed under optical microscope too.

Procedure:

- Dissect the fish part using scalpel.
- Wash the fish part with distilled water.
- Discard the distilled water and immerse the dissected portion in 0.2% alizarin red S solution.
- Incubate it for 10 minutes.
- Now, discard the solution and give gentle rinse with distilled water.
- Using Wipes, just give gentle touch of the tissues to remove water. Now visualize under the Optical/fluorescence microscope.

Observations:

Calcium deposited region appears as red under microscope.