Morpho-Anatomical Analysis Of Zebra Fish Scale Melanocytes

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Abstract
The zebrafish (*Danio rerio*) is an excellent research model in biomedical sciences and other upcoming research areas. Despite the huge importance of an effective and high-throughput zebrafish aquaculture, little is known about morpho-anatomy of its scales and their embedded melanocytes. Here we have analysed the morpho-anatomical structure of the zebra fish scales and their melanocytes, along with their distribution, position and the variations in number and their physiological responsive states. It was found that the maximum number of melanocytes ~150-200 was present in the scales from the dorsal region of the zebrafish minimum being in the ventral region. These melanocytes had an average diameter, of 3.65±0.927 microns; corresponding to the intermediate state (neither aggregated nor dispersed) of the melanophore index. Other regions of the zebrafish, such as head, tail and ventral regions, had ~120-150, ~50-80, ~0-10 number of melanocytes in their scales respectively. Among the four regions of the zebrafish, the most uniform and intermediate state melanocytes were found in the scales of the dorsal region. Our analysis of zebrafish scales and physiological responsiveness of different region melanocytes opens new vistas for future use of these disguised type of smooth muscle cells.

Keywords: Scales, Zebrafish, Morpho-anatomy, Melanocytes, Mean melanophore size index.

1.1 INTRODUCTION

The zebrafish (*Danio rerio*) being genetically analogous to humans has emerged as a very useful vertebrate model, (Agalou et al., 2018). The zebrafish genome has been fully sequenced and has 80% resemblance to the human genome, making is the most widely used model organism globally, (Singh et al., 2019). Zebrafish have scales and melanin pigments present on their bodies surface, making it possible to study pigmentation without the need for a difficult experimental approach. Additionally, zebrafish melanogenesis is comparable to human melanogenesis, allowing for the investigation of protein interaction and functionality, (Kim et al., 2015). The characteristic external pigment pattern of zebrafish is generated by an array of three types of pigment cells i.e., melanophores (containing black pigment), xanthophores (containing yellow pigment), and iridophores (containing reflecting platelets) all of which are derived from the neural crest (Clancey et al., 2013, Yamanaka and Kondo., 2021).

With its emergence as a popular model system, the zebrafish scale melanocytes need accurate morpho-anatomical description, based on exterior localization (easy experimental access) and their functions in physiology and pharmacology leading to opening of new areas in biomedical research. Thus far, comprehensive studies have investigated only the anatomy and development of zebrafish scales, (Sire and Akimenko, 2004; Iwasaki et al., 2018; Hung et al., 2019). Information on the epidermal melanophores of scales present in different regions of the zebra fish and their variations in number, size, diameter and physiological responsiveness has been analysed in the present study.

1.2 MATERIAL AND METHODS

The present experimental work was carried out in the laboratory of the Biotechnology Department, Saifia Science College Bhopal, India, and approved by the Institutional Animal Ethics Committee (IAEC) under the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, Government of India letter No. 2154/PO/Re/S/22/CPCSEA.

1.2.1 Zebrafish housing and acclimatization:

8 months old adult zebrafish (n=~100) of both sexes (0.6 g±0.4 g; 3.5 cm ± 2 cm total lengthmean ± SD-proportion 1:1) were purchased from commercial dealer in Bhopal, Madhya Pradesh, India, and were acclimatized in aquarium for 15 days in laboratory conditions, in dechlorinated water providing photoperiod of 14 h light:10 h dark. Temperature range of 28 °C, pH 70±.5 and dissolved oxygen of 3 ppm were maintained. Fishes were fed micropellets twice a day, (Westerfeld et al., 2007; Kim et al., 2017).

1.2.2 Morphological Analysis of zebrafish scales: After acclimatization, 12 healthy male and female zebrafish were used in this study. The zebrafish scales were removed according to the methods of Spaeth (1917) modified by Ali et al. (2011). In this method zebrafish scales were removed from four regions of the fish: Head region (HR), Dorsal region (DR), Tail

region (TR), Ventral region (VR) (Fig: 1). For removal of scales the zebrafish were taken out from the tank and kept in a wet muslin cloth carefully in a tray filled with water. The scales were removed from the selected regions of the fish with the help of sterilised scalpel and forceps.
The basal region of the scales which is devoid of and pigment cells was carefully held by the fine tips of the forceps and scale was picked slowly. These scales were then immediately transferred in 0.7% fish saline in Petri plates, which were equilibrated for 15-20 min with frequent stirring after that the scales were transfer in the 1×10^{-8} g/ml KCl solution. As per the method of Brager and Moritz, (2016) the size of the scale was measured. The mean melanophore size index of melanocytes was recorded according to the method of Bhattacharya et al., (1976), using Leitz ocular micrometre (ERMA), calibrated previously with 10x10 magnification. The value thus obtained was then multiplied by the unit of the micrometre, which was 15 µ. The arithmetical mean was calculated. This was the mean melanophore size index (MMSI), (Bhattacharya et al., 1976).

1.2.3 Statistical analysis: Statistical data analysis is presented as mean ± standard deviation n=10 represents the number of scales isolated from different regions. Comparisons were made between the control and ionic solutions using student t-test. All data were analysed using GraphPad Prism software $p<0.001$ indicates a statistically significant difference.

1.3 RESULTS

1.3.1 Scale Topography: Following the protocol of Brager and Moritz, (2016) it was found that the epidermal scales of dorsal, head, tail and ventral regions of zebrafish are similar in shape i.e., circular and discoidal, having an average diameter of 7.5 mm (Fig 1).

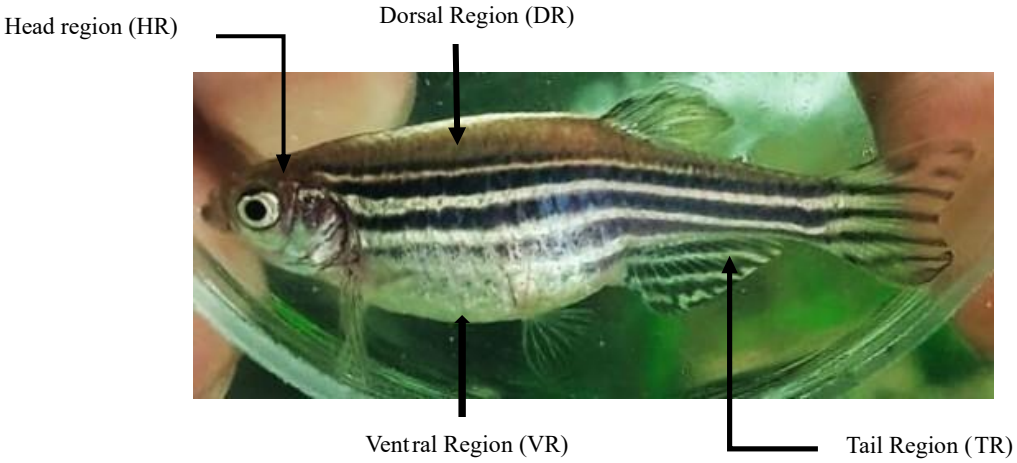
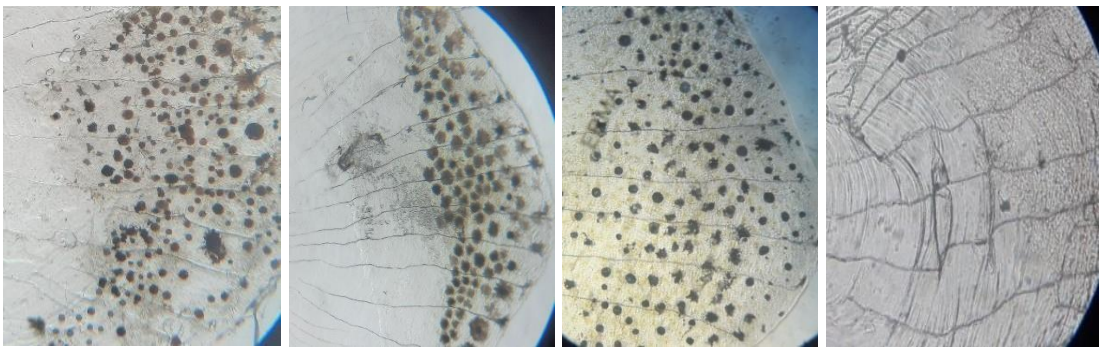


Figure 1- showing the lateral view of adult zebrafish displaying different regions of the body from where the scales were removed.

Structurally, all the scales were found to be polarized along the apical axis of the body, with the anterior part of the scale harbouring circular ridges and the posterior part of the scales showed radial grooves. These two ornamentations were found to originate from the central part of the scale (focus), from which the scale extended posteriorly. In addition, the episquamal surface of the fish scales exhibited concentric ridges called as circuli as well as grooves called as radii, which emerge out from the anterior part of the scale, (Fig 2).
On further examination of the scale topography in relation to morphology, distribution and number of the black pigment cells, the melanocytes present on the epidermal scale surface, following observations have been recorded.

1.3.2 Scales and their Number of Melanocytes from Different Regions of Zebrafish: The zebrafish have several distinct stripes on their body, having black melanophores, yellow xanthophores, and silver iridophores. The dark blue stripes contain black pigment cells, the melanophores or melanocytes. The dark stripes are found in the dorso-lateral region whereas faded stripes are in the ventral region running all along the length of the fish. It was observed that each stripe contained approximately 90±10 scales, of uniform size, being 7.5 mm in diameter.
In this study scales from four different regions head region (HR), dorsal region (DR), tail region (TR) and Ventral region (VR) were selected and isolated (Fig 1). Approximately 10-15 scales from each regions were removed and kept in 0.7% fish saline. The number of scale melanocytes was counted and it was found that each scale from the dorsal region of *Danio rerio* contained ~120-150 melanophores, whereas the head region scale had ~100-120, tail region ~50-80, and ventral region had devoid of pigment cells, (Fig 2, Table: 1).



(A) **(B)** **(C)** **(D)**

(A)- Melanocytes of zebrafish from the scales of head region (B**)-** dorsal region **(C)-** tail region **(D)-** ventral region scales devoid of any melanocytes (100X)

Figure 2 showing the morpho-anatomy of scales from different regions of zebrafish with their melanocytes

Table 1 showing the region-wise number of melanocytes present in scales of zebrafish

Region	No. of Melanocytes
Dorsal Region (DR)	~120-150
Head Region (HR)	~100-120
Tail region (TR)	~50-80
Ventral Region (VR)	No melanocytes were found

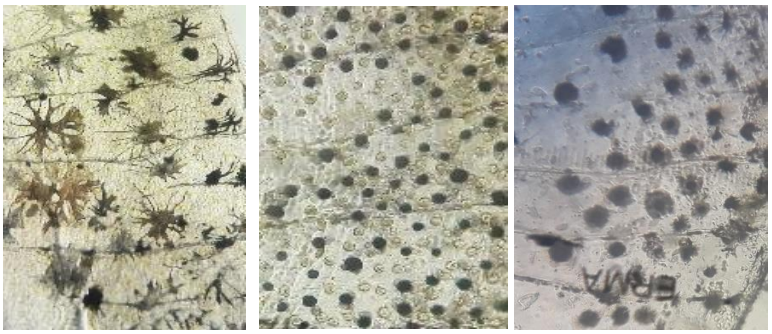
1.3.3 Mean melanophore size index (MMSI in µ) of the melanocytes from different regions of the zebrafish scales: The MMSI of the pigment cells was calculated from the scale melanocytes of different regions of the zebrafish. In the dorsal region scale melanocytes, the MMSI was found to be 3.65±0.92, whereas the MMSI of the head region melanocytes was 3.37±0.68. The MMSI of the tail region melanocytes was 3.15±0.71 (Table: 2).

Table 2 showing average MMSI (u) of melanocytes from different regions of scales

S. No.	Regions	MMSI (u) of Zebrafish Scale Melanocytes from different regions
1.	Dorsal Region	3.65±0.927
2.	Head Region	3.37±0.684
3.	Tail Region	3.15±0.716

n = 10, Values are Mean ± SD, p value<0.0001***

Employing the method of Hogben and Slome, (1931) the state of the melanocytes was studied in 0.7% fish saline, it was found that the melanocytes showed different states of aggregation and dispersion, which directly relates to their cellular responsiveness. The data on responsiveness of dorsal scale melanocytes of zebrafish showed that the melanocytes get fully dispersed (reticulate state where all finger like premises of the cell are fully extended) within 15-20 minutes, when scales were placed in 0.7% fish saline where the MMSI was found to be 7.03±0.83. As compared to this fully dispersed state, in the intermediate state of scale melanocytes i.e. (neither aggregated nor dispersed) the MMSI was 3.65±0.92 (Table 2).



A **B** **C**

A – 0.7 % Na (FS) **B-**0.7 % Na (FS) +1× 10^{-8} g/ml KCl **C-** 0.7 % NaCl (FS) washed scales Re-immersed in 0.7 % Na

Figure 3 showing the responsiveness of zebrafish scales melanocytes of the dorsal region (Magnification 400X). (FS= Fish saline)

However, when the scales with dispersed melanocytes were placed in 0.7% NaCl+1×10^{-8} g/ml of KCl solution, the melanocytes started to show aggregation. The dendritic of the cells began to retract and the melanocytes became ball like in structure, corresponding to the punctate state of the melanophore index. The MMSI had become 2.41±0.22 within 15-20 minutes as compared to the control (Fig 3 A).
When 0.7% NaCl+1×10^{-8} g/ml of KCl treated zebrafish scales were washed with 0.7% fish saline (FS), reimmersion was occurred and the melanocytes remained in an intermediate state of neither aggregation nor dispersion, where the MMSI was 4.12±0.41. From the above results it is concluded that the melanocytes are physiologically responsive and sensitive towards the effects of NaCl and KCl which are significant (p value ≤ 0.001) (Table 3, Fig 3).

Table 3: Responsiveness MMSI (μ) of melanocytes of the dorsal region scales of the zebrafish

S. No.	Incubating Solutions (10 Min)	MMSI (μ) of melanocytes measured after 15 minutes
1.	0.7 % Na (FS)	7.03±0.83 (Dispersed State)
2.	0.7 % Na (FS) +1×10^{-8} g/ml KCl	2.41±0.22 (Aggregated State)
3.	0.7 % NaCl (FS) washed scales Re-immersed in 0.7 % Na	4.12±0.41 (Intermediate state)

n = 10, Values are Mean ± SD, p value <0.04* FS = Fish Saline (0.7% Na)

1.4 DISCUSSION

This is the first report on the morpho-anatomical aspects of epidermal melanocytes of scales of various regions of the zebrafish *Danio rerio,* where studies on scale topography, their melanocyte distribution, position, number, size and variations in their responsive states have been carried out. Findings show that each stripe of the fish has about 90±10 scales having a diameter of 7.5 mm. Scales further consisted of concentric ridges the circuli and the grooves, which seem to emerge out from the basal region of the scale till the apex, harbouring different types of melanocytes in varied shape, size and numbers. Here we also report the details of the zebrafish scale melanocytes and their variations in physiological responsiveness of intracellular pigment granules.
Reviewing the literature on the morpho-anatomy of scales of several teleost fishes belonging to a large number of families, it becomes evident that there is little morpho-anatomical information on the melanocytes of zebrafish *Danio rerio*, conspicuously missing even in an extensive teleost fish-scale description, in the form of an atlas by Brager and Moritz., (2016), followed by Hung et al., (2019). Perusing an earlier paper by Sire et al., (1997) information on development of zebrafish scales and their importance as a model has been provided, but does not any offer any details of the morpho-anatomical and physiological responsiveness of melanocytes. Similarly, other earlier papers have also reported that data on zebrafish scales, appearance, size, responses, and structure with regard to melanocytes are poorly documented, (Waterman 1970, Armstrong, 1973).
With regard to the number of melanocytes, it is reported for the first-time trial zebrafish scales from different regions have distinct variations in their number as well as size. The dorsal region scales contain ~150-200 melanocytes of uniform size, whereas the head region scale had ~100120, tail region ~50-80, and ventral region had devoid of pigment cells, where their mean melanophore size index (MMSI) was found to be 3.65 μ ±0.92, whereas the MMSI of the head region melanocytes was 3.37±0.68. The MMSI of the tail region melanocytes was 3.15±0.71.
The results also demonstrated that all the epidermal scale melanocytes exhibit physiologically significant morphological changes in response to various ionic incubating media like 0.7% Na fish saline and 1×10^{-8} g/ml KCl solution. The stellate scale melanocytes of the intermediate state, changed to fully dispersed (reticulate state) within 15 to 20 min, when placed in the 0.7 % NaCl fish saline. However, when the scales with dispersed melanocytes were placed in fish saline with 1×10^{-8} g/ml of KCl, the melanocytes started to show dendritic contractions leading to pigment aggregation. These data of responsiveness and sensitivity of the scale melanocytes of zebrafish are similar to those described by the pioneering workers, Hogben and Slome (1931) for vertebrate melanocyte model of *Xenopus laevis,* leading to darkening and paling of the skin, which has pharmacological basis, i.e., relaxation and contraction.
In the light of these circumstances, our analysis on the morpho-anatomical structure of zebrafish scales and responsiveness of different melanocytes opens new vistas for further work. The study recommends the use of zebrafish, *Danio rerio* as an experimental model in studies pertaining to revealing of the cellular mechanisms of pigment cells in response to externally applied pharmacological agents.

1.5 CONCLUSION

Our analysis on the morpho-anatomical structure of zebrafish scales and responsiveness of different melanocytes opens new vistas for future use of these disguised type of smooth muscle cells. The study recommends the use of zebrafish scales as an experimental model in studies pertaining to revealing of the cellular mechanisms of pigment cells in response to externally applied agents. Thus, the dorsal region scale-melanocytes of zebrafish, which are disguised types of smooth muscle cells, can be employed for physiological and pharmacological studies.

1.6 ACKNOWLEDGEMENTS

The authors are thankful to the Secretary and Principal of Saifia Science College, Bhopal, India for providing the necessary facilities.

1.6.1 AUTHORS CONTRIBUTIONS: Sharique A Ali: Analyzed and interpreted the data and carried out the writing and final corrections of the manuscript, Tasneem Husain and Gulafsha Kassab: Performed the experimental work on morpho-anatomy of zebrafish, Writing and
Statistical Analysis. All authors have read and approved the manuscript

1.6.2 CONFLICT OF INTEREST: NA
1.6.3 FUNDING: NA
1.6.4 DATA AVAILABLITY STATEMENT: The data underlying this article are available in the article.
1.6.5 ABBREVIATIONS:
HR: Head region
DR: Dorsal region
TR: Tail region
VR: Ventral region
KCl: Potassium Chloride
NaCl: Sodium Chloride
MMSI: Mean Melanophore Size Index
FS: Fish Saline

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