

Artefactual Suboptimal Fixation Effect to Nuclear Staining on Erythrocytes of *Lutjanus kasmira* (Forsskål 1775)

Do Huu-Quy^{1*}, Vo Van-Thanh², Nguyen Thi Le-Na³, Hoang Thi Thuy-Duong¹

¹Institute of Tropical Ecology, Vietnam-Russia Tropical Center, 63 Nguyen Van Huyen, Nghia Do Ward, Cau Giay District, Hanoi, Vietnam

²Biology Faculty, Ho Chi Minh City University of Education, 280 An Duong Vuong, Ward 4, District 5, Ho Chi Minh, Vietnam

³Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Thanh Xuan, Hanoi, Vietnam

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ABSTRACT

This research presents the unusual staining characteristic of the teleosts red blood cells—the common bluestripe snapper *Lutjanus kasmira*, caught at Toc Tan (Allison) Reef, Truong Sa Archipelago, Vietnam, due to side effects of the delayed staining procedure. In addition to the quantitative data measured, the results of microscopic image research showed that, on slides performed with the delayed Romanovsky staining procedure using Giemsa dye, the cell "nucleus" was not dye-stained as seen in the erythrocytes of much other fish. The AFM and SEM images of blood slides show that the structure of the nuclear zone and the nuclear membrane was destroyed. Although methanol is used as one of the common fixatives in biology, the delayed staining still causes artifactual side effects, disrupts the structure of the red blood cell nucleus and can cause confusion when studying cytogenetics.

1. Introduction

The erythrocytes of teleost are known as lower animals' nucleated red blood cells, which perform primary functions almost similar to other higher organisms; in addition to transporting CO₂ (Thomas and Egée 1998), such erythrocytes can participate in the immune response (Shen *et al.* 2018; Puente-Marin *et al.* 2019).

In haematology research, the Romanovsky stain, using Giemsa or Giemsa-Wright stain solution, is one of the standard methods. Microscopic images of blood slides of sea fish as well as freshwater fish are similar to those of other mammals. Specifically, their erythrocytes showed blue cytoplasm with dark blue nuclear (Campbell 2015).

In this article, we report on the first recognition of unusual cell structure characteristics in the erythrocytes of the common bluestripe snapper through microscopy after blood smear and application of Romanovsky stain with the delayed staining procedure. The findings showed that besides the cell immobilization ability,

methanol can still exert an artifactual effect on the erythrocyte nucleus of the four-line snapper.

2. Materials and Methods

2.1. Preparation of Ringer's Physiological Solution

Physiological solution for marine fish, also known as Ringer's solution (Hoar and Hickman 1975) for marine fish, is prepared 2 weeks before use. Dissolve the following ingredients in 1 litre of distilled water: 7.8 g NaCl, 0.18 g KCl, 0.166 g CaCl₂, 0.095 g MgSO₄, 0.084 g NaHCO₃, 0.06 g NaH₂PO₄.

2.2. Catching and Collecting Fish Blood

Six common bluestripe snappers, *Lutjanus kasmira*, were caught with a fishing rod at a depth of 20–35 m from 13 to 18 October 2020 at Toc Tan Reef, Truong Sa Archipelago, Vietnam (in the 100 m radius around coordinates of 8°49'050" and 113°57'763"—one of the research sites under the KCB-TS01 project of the Vietnam-Russia Tropical Centre in October–November 2020. After the fish had been caught, their length and weight were measured. The average length of six fish was 25±2.3 cm,

* Corresponding Author

E-mail Address: dhquyet@gmail.com

and their average weight was 185 ± 38 grams. Male and female fish were not distinguished when taking blood. Use a 5 ml syringe with one drop of Ringer's solution containing heparin (500 IU/ml) to take blood from the caudal vein of the common bluestripe snapper. Collect about 1.5 ml of blood into a dark test tube (with nebulized heparin). Shake gently so that the heparin anticoagulant is evenly distributed in the blood. Put the test tube in the refrigerator at $8-10^{\circ}\text{C}$ and experiment for 30-60 minutes.

2.3. Red Blood Cell Count and Blood Smear

Dilute a portion of blood 300 times and use Neubauer's chamber to count red blood cells.

Dilute another portion of blood 3 times for smear according to the traditional method. Fix the slides with pure methanol (Methanol AR, Xilong Scientific Co., Ltd.) for 3 minutes. Then, the slides were stored at room temperature for 5 days. Perform the manual staining with Giemsa's staining solution (Dilute Giemsa's staining solution, Merck) for 20 minutes. Then, rinse the slides with buffer solution ($\text{pH} = 7.2$) twice per minute. Finally, naturally, dry the blood slides into the air.

All blood slides are stored at room temperature in a container and shall be studied in the laboratory for 2 months after the field research trip. All of the slides, as mentioned earlier, are currently stored at the laboratory of the Vietnam-Russia Tropical Centre, Hanoi, Vietnam.

2.4. Research of Microscopic Structure

The above blood slides were captured with an Olympus CX43 microscope at the Vietnam-Russia Tropical Center, an Atomic Force Microscope Model NT-206, HMTI, Microtestmachines Co, Belarus, Center for Applied Physics and Scientific Instrument (Institute of Physics, Vietnam Academy of Science and Technology, Hanoi, Vietnam) and a NANOSEM 450 Scanning Electron Microscope (FEI, Hillsboro, OR, USA) at VNU University of Science (Vietnam).

Use the ImageJ, version 1.52r, to measure the size and area of the cells as well as the cell "nucleus" and Microsoft Excel to calculate the mean and standard deviation.

3. Results

The mean (Mean \pm SD) of erythrocytes of *Lutjanus kasmira* (Forsskål 1775) was $3.07 \pm 0.46 \times 10^6$ cells per mm^3 of blood.

On the microscopic image, we recognized the unusual staining of red blood cells of the common bluestripe snapper *Lutjanus kasmira* (Forsskål 1775) caught at Toc Tan Reef, Truong Sa Archipelago (Spratly Islands, Vietnam). The cytoplasmic portion of the red blood cell had the distinctive colour of Giemsa dye; however, the cell nucleus zone was not stained, although the boundary of the cytoplasmic membrane and surrounding environment also existed boundary of cell nucleus zone was clearly expressed. The cell has measured average size was $10.40 \pm 0.53 \times 8.33 \pm 0.47 \mu\text{m}$; the size of the "nucleus zone" was $4.19 \pm 0.26 \times 3.23 \pm 0.22 \mu\text{m}$; the cell's respective area was $72.34 \pm 9.42 \mu\text{m}^2$ and $9.75 \pm 1.52 \mu\text{m}^2$ (Figure 1).

The SEM micrograph of red blood cells from blood smears confirmed this exciting feature. The nucleus zone bulged in most of the blood cells of many teleosts, while the red blood cells of the common bluestripe snapper appeared "nucleated" (Figure 2). However, the cell centre, the zone considered to contain the cell nucleus, showed traces of damaged cellular structures.

Take images of blood slides on the atomic force microscopy (AFM), and the result showed that the nucleus zone of the erythrocytes of *Lutjanus kasmira* fish looked concave and lacked a nucleus. The image clearly showed the boundary between the "nucleus zone" and the cytoplasm, which was in the shape of a surrounding border (Figure 3).

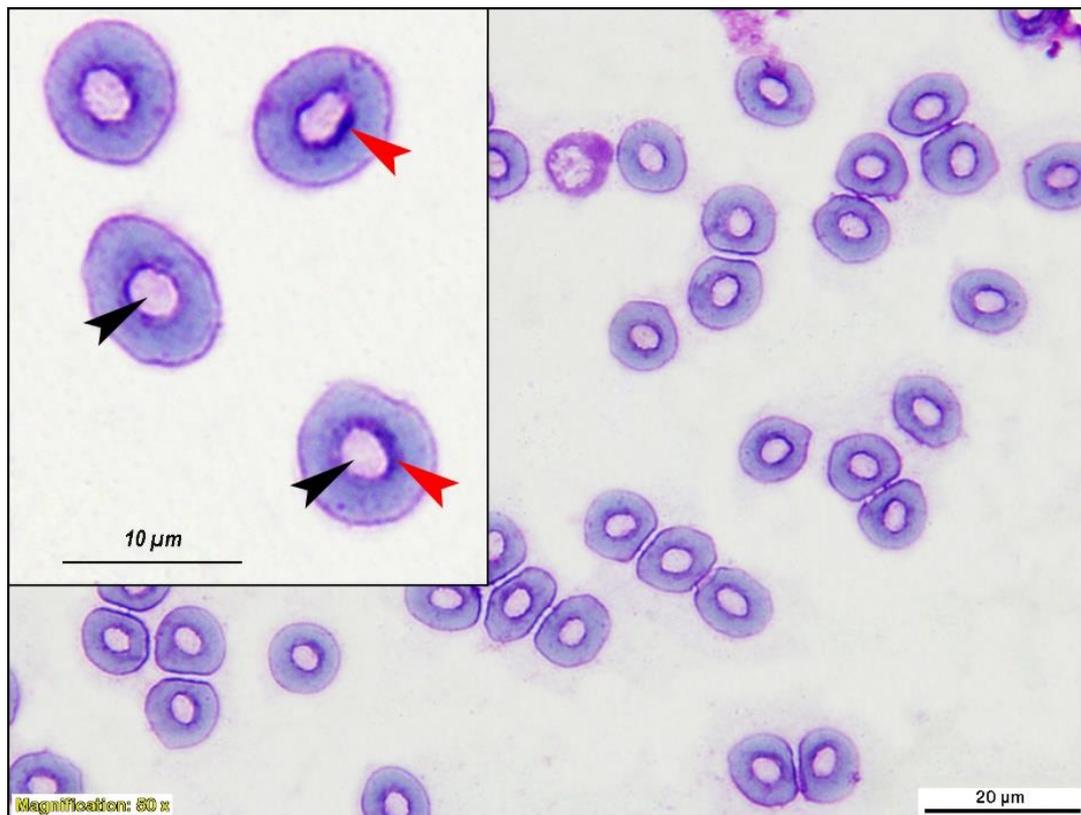


Figure 1. Erythrocytes of *Lutjanus kasmira* after the delayed Romanovsky staining procedure using Giemsa dye. Black arrows-nucleus, red arrows-nuclear membrane

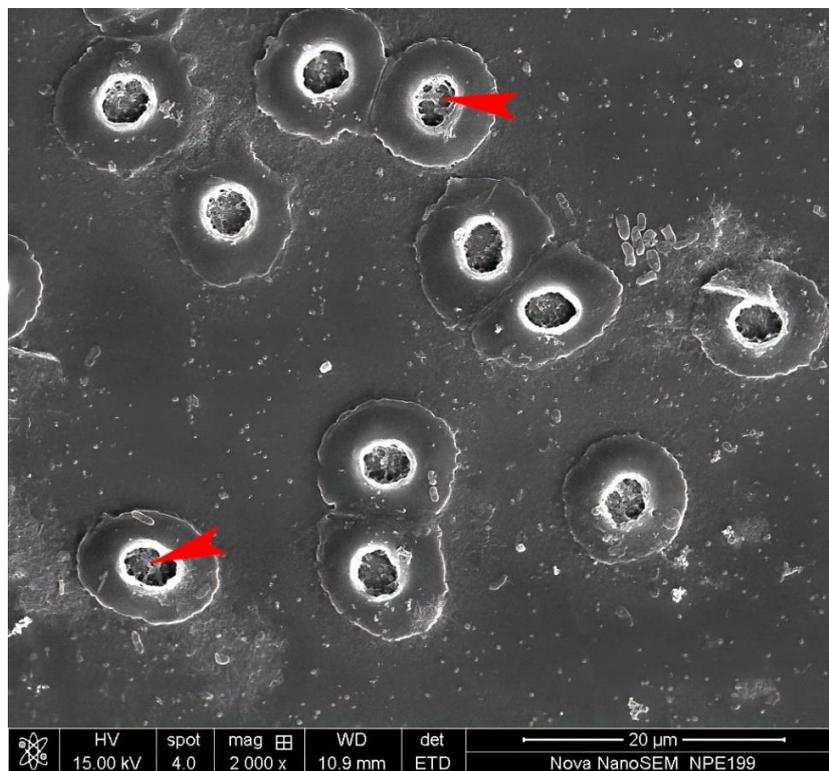


Figure 2. Erythrocytes of the bluestripe snapper *Lutjanus kasmira* through the SEM. The cell nucleus structure was destroyed due to the artefactual suboptimal fixation effect

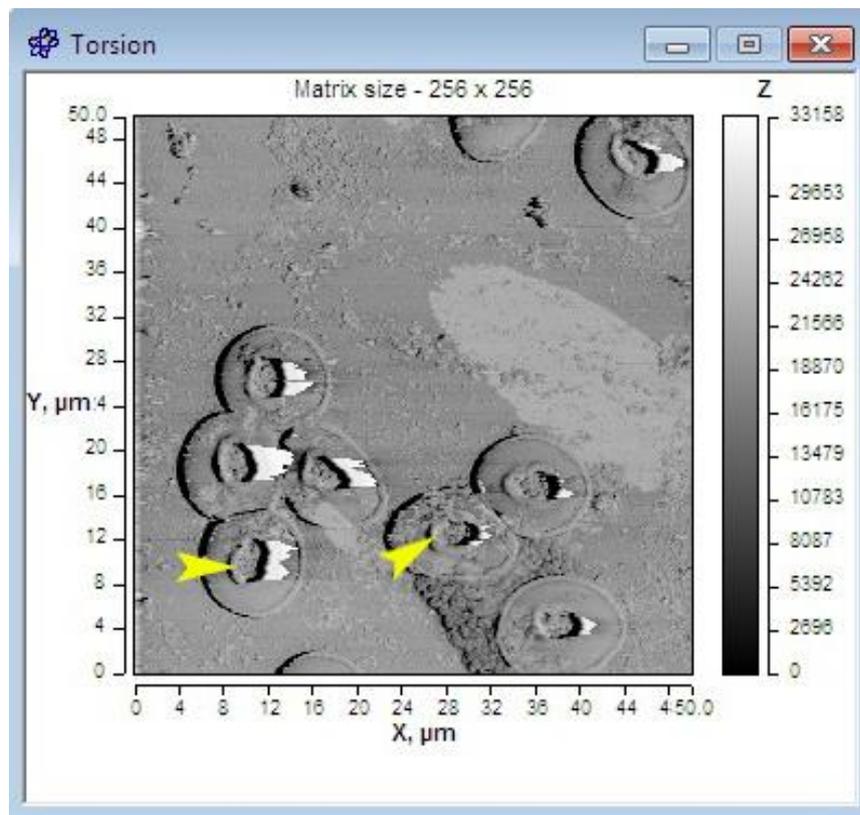


Figure 3. Erythrocytes of *Lutjanus kasmira* through the AFM. Yellow arrows-nuclear membrane

4. Discussion

Generally, fish haematology has been one of the topics of interest and research by many scientists, and it is incredibly complicated by systematic differences among groups (Fänge 1992) and differences in staining features of cells among species (Ellis 1977). It is abundant species and a comprehensive ecological environment around the earth that create different physiological and biochemical characteristics for each fish species. Even the same species in different regions are also different. That is why generalization about fish haematology becomes difficult.

In comparison with our findings about some other sea fish species (Filho *et al.* 1992), it is easy to see that the common bluestripe snapper belongs to the group of species which has a relatively high erythrocyte count. This fact is also recognized in the species of active sea fish (Witeska 2013).

In terms of morphology, the mature red blood cells of teleosts in many types of research where Giemsa or Wright-Giemsa dyes are used are nucleated cells with a light or dark blue colour (Clauss *et al.* 2008;

Campbell 2015), in the shape of double convex in the centre, corresponding to the location of the nucleus (Campbell 2015; Chernyavskikh *et al.* 2018; Quyet *et al.* 2015) while some species have concave and flat red blood cells (Hibiya 1985). Methanol used in the Giemsa staining technique is classified as a dehydrating fixative, structured by (CH₃OH), which is closer to the water molecule (H₂O) than ethanol (C₂H₅OH) and has long been used as a fixative in cytological and histological studies. The efficiency of this fixative compared to ethanol, acetone, formaldehyde, glutaraldehyde, Zenker's fixative, and Bouin's fixative was also mentioned at different levels (Eltoum *et al.* 2001; Kumarasinghe *et al.* 1997). Dehydrated fixatives displace water in tissue media, which disrupts hydrophobic and hydrogen bonds, thereby exposing internal hydrophobic protein groups and altering their tertiary structure and solubility in water. Under the effect of a group of dehydrating fixatives, including methanol-a fixative commonly used in blood smear fixation, the nucleic acid structure is relatively unchanged (Eltoum *et al.* 2001). However, in this study, the 5-day staining

delay after methanol fixation produced an interesting artefact in the red blood cells of *Lutjanus kasmira*, as above-mentioned-the structure of red blood cell nuclear, including the nucleic acids, was destroyed and was no longer able to stain the dye, although the cytoplasm is still stained typically. It means the red blood cell nucleus of *Lutjanus kasmira* was not preserved after the delayed staining.

In conclusion, in addition to the provision of some quantitative information on the red blood cell of four-striped red snapper *Lutjanus kasmira* (Forsskål, 1775), caught at Toc Tan Reef, Truong Sa Archipelago (Spratly Islands, Vietnam), this is the first research to discover artifactual effects of methanol fixative on red blood cell nuclei of this fish. It is also the basis for further studies of microstructure in fish erythrocytes and to learn more about the effects of methanol on fish red blood cell structure in the coming time.

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