Research Paper

Volume : 3 | Issue : 12 | December 2014 • ISSN No 2277 - 8179

Assessment of Flow Cytometry in Counting Blood of Whale Sharks as a Rapid and Reliable Method

Veterinary

KEYWORDS : Flow cytometry, CBC, hematological technique, Whale shark (Rhincodon typus)

Konomi Ito	Department of Veterinary Pathology, Faculty of Applied Biological Sciences, Gifu University
Sawsan Ghattas	Kafrelsheikh University, Faculty of Veterinary Medicine, Department of Veterinary histology and Cytology, 33516, Kafr, El-Sheikh, Egypt, Egypt
Makio Yanagisawa	Japan2Kafrelsheikh University, Faculty of Veterinary Medicine, Department of Veterinary Histology and Cytology, 33516, Kafr El-Sheikh
Senzo Uchida	Okinawa Churaumi Aquarium, Motobu Town, Okinawa Prefecture , Japan
Hiroki Sakail	Department of Veterinary Pathology, Faculty of Applied Biological Sciences, Gifu University
Tokuma Yanail	Department of Veterinary Pathology, Faculty of Applied Biological Sciences, Gifu University

ABSTRACT

Three captive whale sharks were used for blood collection. A CBC was performed manually based on Natt and Herrick's method and a Neubauer hemacytometer. Leukocyte differential counts were measured on M-G stained blood smear. Cytochemical stains for reukocytes were conducted with MPR, PAS and SBB. The CBC were established, total reukocyte count. Granulocytes were identified heterophilic, eosinophilic and basophilic granulocytes. Heterophilic granulocyte and lymphocyte were predominant leukocytes. Eosinophilic granulocytes were positive with all three cytochemical stains. Heterophilic granulocytes showed weak reactivity with MPR, and eccentrically-located reactivity with PAS reaction. Basophilic granulocytes were only positive with PAS. With DiOC stain, several blood cell populations were identified by different FL-1, FSC and SSC properties. FL-1 vs. SSC or FSC vs. SSC dot-plot of stained blood cells showed four separate cell populations, namely erythrocytes, a mixture of three types of granulocytes, lymphocytes plus thrombocytes and monocytes plus immature cells. Each leukocyte percentages counted by the FACS analysis had relatively good correlation with those counted microscopically. Finally, in whale sharks, blood examination based on flow cytometric analysis may be useful for clinical diagnose as quick and reliable hematological technique.

Introduction

Whale shark (Rhincodon typus) is the extent largest fish. This species is a highly migratory one (Forster et al., 1972). In Japanese waters, whale sharks have been recorded from areas in Okinawa to the Pacific side of Hokkaido, as well as the Sea of Japan off Niigata Prefecture (Compagno, 2001, Hrubec, et al.,1997). Japan had been a pioneer in captive rearing of whale sharks. In Okinawa Churaumi Aquarium, a hematological examination had been periodically conducted for health management of keeping animals, including whale sharks. The blood examination has been used routinely in the medical care of fishes. However, hematological studies of shark are very few (Macey, 1994, Mader, 2000). Recently , a flow cytometric method for automatic blood cell count has been developed (Martin et al.,1992.). This method is a rapid and easy to use for large number of samples, and it widely used on human samples for clinical and experimental works including hematology, immunology and pathology (Nishida, 2001). Although there have been a number of hematological studies and blood examination methods in mammals, avian, reptiles and teleost fishes, there is very limited information in sharks. The purpose of this study is to estimate hematological characteristic and develop a rapid and reliable methods for counting blood cells by using flow cytometry in whale sharks.

Materials and methods

Animals

Three whale sharks (Rhincodon typus) were used for blood collection, and it was conducted 3 or 4 times each between May and November in 2007. The sharks were communally housed in a 7500L semi-open circular tank at the Okinawa Churaumi Aquarium (Motobu, Okinawa prefecture, Japan) and fed euphausiid (Euphausia superb and Euphausia pucifica) and sergestidae (Sergia lucens) twice a day. Seawater was circulated per one and half hours and water temperature was changed according to outer sea temperature (23-30C). The sharks were immature males and each total length was 6.0-7.5m (Table 1).

Blood collection

Blood were collected from vessels at the axilla of the first dorsal fin, swimming with the shark, using a sterile 10 cm^3 syringe (TERUMO Co. Tokyo, Japan) with a 20 gauge needle (TERUMO Co. Tokyo, Japan) (Fig.1). Immediately, the samples were transferred to micro tubes containing dry EDTA (ERMA INC. Tokyo, Japan) and mixed gently by inversion. The samples were maintained at 4C on ice until analysis and transported to the Gifu University or Nihon University by the airplane.

Hematological analysis

Freshly blood was draw into a red-cell counting pipette and filled to the 0.5 mark and then to the 101 mark with seawater. The blood specimen, diluted 1:200, was shaken for one minute to ensure thorough distribution of the cells. A hemacytometer (Neubauer line, ERMA INC., Tokyo, Japan) was filled with the dilute solution in the routine manner. Erythrocytes were counted in one primary square and multiplied by 100,000 to calculate total RBC count per microliter. RBCs were counted in both chambers of the hemacytometer, and the mean was calculated.

Hematocrit value was determined by micro-hematocrit centrifugation. Microcapillary tubes (TERUMO Co., Tokyo, Japan) were filled with fresh blood, plugged with clay, and centrifuged at 11,000 rpm for 5 minutes.

Leukocyte count and morphological observation by microscope

For total leukocyte count, according to Natt and Herrick's diluent methods (Knoll 2000 , Pierson 2000) the blood was diluted by the diluent solution adding 3 times quantity of NaCl. Fresh blood was drawn into a leukocyte counting pipette and filled to the 1.0 mark and then to the 11 mark with the diluent. The specimen was shaken for 1 minute to ensure perfect distribution of the cells. A Burker-turk line (ERMA INC., Tokyo, Japan) was filled with the mixture solution gently. All leukocytes were counted in 4 primary squares. The total leukocyte count per microliter was calculated by multiplying the number of leukocytes observed in

the 4 primary squares times. To minimize analytical variability, the cells were counted in both chambers of the hemacytometer, and the mean was calculated.

Blood smears were made from each blood sample at the time of just before flow cytometric analysis and each smear was stained with May-Gruenwald Giemsa (M-G).

Blood cell classification and identification were conducted with M-G smears based on previous reports (Knoll 2000 , Mader 2000.). Under an optical microscope, each type of leukocyte (including thrombocytes and immature cells) was scored with 2 smears of each sample. Counting was ended when total 1,000 cells had been counted, respectively. The percentage mean of each blood cells was calculated.

Cytochemical staining procedure

Some of blood smears, made at the time of collection, were also stained with myeloperoxidase (PER), periodical acid-Schiff (PAS) and Sudan black B (SBB). PER stain was performed using a commercial kit (Muto Pure Chemicals, Tokyo, Japan), following the instruction manual. The smear was counterstained with Giemsa stain for 15 min.

$DiOC_5(3)$ stain

Stock solutions of $DiOC_5(3)$ (3,3'-dipentyloxacarbocyanine iodide, Molecular Probes, Eugene, OR, USA) were prepared in ethanol at 500μg/ml. The solution was stored in the dark at 4C until use.

To determine the optimal dilute solution for FACS analysis,

the blood samples were diluted at 1:100-1:200 (at 1×10^6 cells/ml) with five types of dilute solution. As one dilute solution, blood plasma of whale sharks and brownbanded bamboosharks were used after filtration with Millipore Swinnex Filter (μm;). RPMI (Sigma-Aldrich Company, UK), which was added with NaCl at 20 mg/ml (S-RPMI), was also used. Additionally, mixture of equal parts of each shark's blood plasma and S-RPMI were used as dilute solution. Two μl $DiOC_5(3)$ stock solution was added with 998 μl of blood suspension and incubated at room temperature for 10 min.

Since the number of leukocytes population was too small for cell-sorting from whole blood, the leukocytes including thrombocytes were separated from erythrocytes by centrifugation. Capillary glass tubes (Ringcaps 100/200μl, Hirschmann Laborgerate, Germany) were filled with blood samples, plugged with cray and centrifuged at 2500×g for 5 min at 4C. Buffy coat was obtained by cutting the tube 1 mm below the leukocyte layer and suspended in sterile whale shark's blood plasma. The cell number was then adjusted to 2-6 $\times10^6$ cells/ml. Two μl $DiOC_5$ (3) stock solution was added with 998μl of buffy coat suspension, incubated at room temperature for 10 min. After the staining, the buffy cells were used for the sorting procedure.

Flow cytometry and fluorescence microscopy

After staining with $DiOC_5$ (3), blood cells were analyzed using FACS (FACScalibur, Becton Dickinson, USA). Side light scatter (SSC) and green fluorescence (FL-1) of each sample were measured. FL-1 high population was selected by gating and indicated on the SSC vs. forward light scatter (FSC) dot-plot for detailed analysis.

Stained blood cells were also examined under a fluorescence microscope (BZ-800, KEYENCE Co., Osaka, Japan).

Sorting procedure

Each blood cell population identified by its typical location in a FL-1 v. SSC or FSC vs. SSC dot-plot was subjected to sorting procedures. Sorting was carried out by the Epics Altra (Beckman Coulter, CA, USA). Each cell population was selected by gating, 5,000 cells were sorted following the manufactures instructions. Collected cells were suspended in 500μl sterile whale shark blood plasma. After the collection, smear was made using a cytocentrifuge attachment (Centrifugal Cell Collector; Tomy Seiko, Tokyo,

Japan) at 1,000×g for 5 min. at 4C and stained with M-G. Each smear was observed under an optical microscope.

Blood cells count by FACS analysis

Each cell population was selected by gating and was identified by its typical location in a SSC vs. FSC dot-plot. The percentages of each cell population obtained by FACS analysis were compared with the percentages made from the microscopic count.

Results

Leucocyte count and morphological observation by microscope: Results shows that the total number of erythrocytes, total umber of leucocytes and hematocrit value of each specimen. The mean of total erythrocyte count and of hematocrit value were 17.2 ± 3.2 ($\times10^4$ cells/μl), 14.8 ± 2.3 (%), respectively. Both values of all sharks were fluctuated considerably on examinations, but there were some changes between two values. The mean of total leukocyte count was 4077.9±1544.8 cells/μl. In case of shark B (2nd, 3rd and 4th examinations), total numbers of leukocyte were markedly higher than those of other sharks.

Erythrocytes (including mature erythrocytes and immature erythrocytes), thrombocytes, lymphocytes and monocytes were identified readily (Fig. 2 A-E). Immature erythrocytes were distinguished from erythrocytes since they have pale blue or blue-gray cytoplasm (Fig. 2 A and B). They were also smaller in size, more round in shape and had round nuclei with higher nuclear: cytoplasmic (N/C) ratios than mature cells. Granulocytes were identified based on cytoplasmic characteristics. Fig. 2 F-H shows heterophilic granulocytes, eosinophilic granulocytes and basophilic granulocytes, respectively. Heterophilic granulocytes had a colorless cytoplasm contained fine and round-shaped granules that were stained as lightly eosinophilic. The cytoplasm of eosinophilic granulocytes was filled with rod-shaped granules that were considerably more intensely eosinophilic and appeared as more distinct shape than granules of the heterophilic granulocytes. Basophilic granulocytes had purple-staining and needle-shaped granules.

Results shows the percentages of each leukocyte, including thrombocyte and immature erythrocyte counted by microscopic observation. The most common cell was thrombocytes, and the sum of thrombocytes and lymphocytes percentage reached around 75-85%. The most common leukocytes except thrombocytes were heterophilic granulocytes or lymphocytes. In specimens of shark B, total leukocytes count was markedly high, and had a relatively high percentage of heterophilic and eosinophilic granulocytes. In case shark C with episode of anorexia (2nd and 3rd examinations), proportion of heterophilic granulocytes were markedly increased.

Cytochemical staining

The cytochemical staining patterns of leukocyte subtypes of whale sharks are shown in Table 2. Heterophilic granulocytes stained weakly positive with PER (Fig. 3-A), but shown no reaction with SBB (Fig. 5-A.). The margin of the cytoplasm, the other side of nuclei, were stained positively with PAS (Fig. 4-A.). Eosinophilic granulocytes were strongly positive with all stains (Fig. 3-A, 4-B and 5-A). In contrast, basophilic granulocytes were negative with all stains (Fig. 3-A, 4-C and 5-A). Monocytes and lymphocytes were also negative (Fig. 3-C, 4-D and 5-C). PAS-positive

Volume : 3 | Issue : 12 | December 2014 • ISSN No 2277 - 8179

granules were occasionally present in the cytoplasm of thrombocytes (Fig. 4-E). PER and SBB activities were not observed in thrombocytes (Fig. 3-B and 5-B).

$DiOC_5$ (3) staining

After $DiOC_5$(3) staining, the blood cells were observed with a fluorescence microscope (Fig. 6). $DiOC_5$(3) stained leukocytes and thrombocytes well, but erythrocytes were weakly stained. Relatively large leukocytes considered to be granulocytes or monocytes, which showed more intense staining than that in lymphocytes and thrombocytes.

FACS analysis

Fig. 7 shows typical FACS analyses of $DiOC_5$(3) stained specimens. When FL-1 vs. SSC was recorded, mainly three distinct cell populations termed R1, R2 and R3 were revealed according to the intensity of FL-1. The populations of R1, R2 and R3 were differentially sorted and morphologically determined. Fig. 8 shows blood cell smears of each population stained with M-G. Population of R1 was composed of broken cells and bare nucleus (Fig. 8-A). R2 was entirely composed of erythrocytes (Fig. 8-B), while R3 was composed of leucocytes, thrombocytes and immature cells (Fig. 8-C).

Population of R3 was selected by gating. A dot-plot of FSC v. SSC was obtained for the gated region (Fig. 9-B). When FSC v. SSC was recorded, population R3 was further subdivided into three populations, termed R3-1, R3-2 and R3-3. In cases of shark B (2nd, 3rd and 4th examinations: leukocyte number of the animal was more than twice high compared with the other shark's count), R3-1 was further subdivided into two populations by FSC vs. SSC properties, termed R3-1-1 and R3-1-2 (Fig. 10-A and B).

To determine each population, R3-1, R3-1-1, R3-1-2, R3-2 and R3-3 populations were differentially sorted from buffy coat suspension.

Fig. 11 shows cell smears of each leukocyte population. Population of R3-1 was mostly composed granulocytes, including heterophilic, eosinophilic and basophilic granulocytes (Fig. 11 A-1). In smear of R3-1-1 population, heterophilic and basophilic granulocytes were mainly observed, but eosinophilic granulocytes were rarely observed. In contrast, population of R3-1-2 was composed mainly eosinophilic (more than 50%) and heterophilic granulocytes (approximately 40%), basophilic granulocytes were rare.

Population of R3-2 was composed of mixture of thrombocytes plus lymphocytes, and R3-3 was composed of mixture monocytes plus immature cells (including immature errythlocytes and myeloblasts).

Comparison of FCM analysis and microscopic count for blood cell percentage

Each of leukocyte percentage calculated by FACS analysis was compared with those from microscopic counts (Fig. 12). Relatively good correlations were obtained for the granulocytes (r=0.641) and lymphocytes plus thrombocytes (r=0.482). Weak and negative correlation was obtained for the monocytes plus immature cells (r=0.287).

Determination of optimal dilute solution for FCM analysis

Fig. 13 shows typical FACS analyses of $DiOC_5$(3) stained specimens diluted by shark blood plasma, NaCl added RPMI and mixture of equal parts of the two solutions. In case of analysis of specimens diluted by shark blood plasma, R3-2 region (thrombocytes plus lymphocytes) was clearly observed by FL-1 and SSC properties (Fig. 13-A and B). But, in case of diluted by mixture, R2 region slid right-side and closed to R3 region (Fig. 13-C and D). In case of RPMI, R2 region slid moreover, R3-2 region was overlapped with R2 (Fig. 13-E). In contrast, the proportion of R1 population (region of broken cells and bare nucleus) was evidently larger for blood plasma analysis compared to mixture or RPMI analyses. By the RPMI method, the proportion of R1 was decreased obviously.

To obtain the R3-2 region, leukocyte region and a region of overlapped with R2 and R3-2 were selected by gating, and a dot-plot of FSC v. SSC was obtained (Fig. 14-B). R3-2 population was able to distinguish from erythrocytes population (R2'), but R3-3 region became unclearly because of overlapping with erythrocytes population (Fig. 14-B-2).In comparison of each leukocyte percentages counted by the FACS analysis had relatively good correlation with those counted microscopically (Fig.15).

Discussion

Hematological analysis

Hematologic reference intervals have not been established for elasmobranchs (sharks, skates and rays) (Macey 1994) .In this study, periodical data of CBC (complete blood count) over time of three whale sharks could be collected. The mean of total erythrocyte and leukocyte count were 17.2 ± 3.2 ($\times 10^4$ cells/μl), 4.0779 ± 1.5448 $\times 10^3$ cells/μl, respectively. These value were markedly lower than those in other sharks, e.g. lemon shark (66.5 $\times 10^4$ cells/μl, 25.9$\times 10^3$ cells/μl), nurse shark (36.6 $\times 10^4$ cells/μl, 27.8 $\times 10^3$ cells/μl) (Walsh and Luer, 1998) and sandtiger shark (27.6 $\times 10^4$ cells/μl, 16.1 $\times 10^3$ cells/μl) (Mader. 2000). However, the mean of hematocrit value, 14.8 ± 2.3 (%), close agree with the other shark, e.g. lemon shark (20%), nurse shark (10%) (Walsh and Luer1998) and portuguese shark (13%) (Stoskopf 2000). This might be resulted in that the whale shark blood cells were bigger than the others. Total erythrocytes count and hematocrit value of each shark were varying considerably depending on examinations, and total leukocyte counts differed from a shark to a shark or depending on examinations. These hematological values were affected by various intrinsic and extrinsic factors, e.g. age, breeding status, water temperature, oxygen content, and season (Raskin and Valenciano 2000, Rothamann et al.,2000, Sherburne 1973 , Smith, 1829). Furthermore, confinement stress and heavy metal toxicity also induce the changes of these values (Speckner 1989, Stevens 2007). Differences of sensitivity to the stress in blood drawing or discrepancy in health conditions might be other related factors for the fluctuation in hematological results. A tendency to maintain higher levels of total leukocytes count was observed in shark B (2nd, 3rd and 4th examinations), but it appeared normal without abnormal clinical conditions. On the other hand, shark C with some clinical abnormalities showed only minimum variation in total leukocytes counts, which was inconsistent with those in shark B. More cases will be needed to clarify the factors which influence hematological values in whale shark.

There have been some reports to identify and classify blood cells in several species of shark (Macey, 1994, Stokopf, 1993 and Stoskopf, 2000). Like other sharks, erythlocytes, thlombocytes, lymphocytes, monocytes and three types of granulocytes were observed in whale shark blood smears (Knoll, 2000). Heterophilic granulocytes and lymphocytes were most common leukocyte, excect thrombocytes like previous study (Mader, D. R. 2000). Although percentages of lymphocytes were kept a regular level, the levels of heterophilic granulocytes were varied frequently in case of shark C (2nd and 3rd examinations). There was a noteworthy finding in shark C that levels of heterophilic granulocytes had been increased markedly when the shark had developed anorexia. It was suggestive that monitoring of each leukocyte levels was important for health management of whale sharks like the other vertebrates. Heterophilic granulocytes of whale sharks were probably considered to be analogous to the mammalian neutrophil, judging from number and morphology of the cells. Heterophilic granulocytes of sharks were indicated that an active role played in phagocytosis like other vertebrates

(Svetina et al. 2002, Taylor et al.,1983and Terasaki, & Fujiwara, 1992) and level might increase in bacterial infections, diseases and stressful conditions (Knoll, 2000 and Macey, 1994). Other leukocytes were also performed a similar function as in other vertebrates (Macey, 1994). Monitoring the relationship between clinical features and leukocyte counts should be continued for some years periodically to clarify each leukocytes function or clinical diagnostic evaluation.

A relative weak PER activity was observed in heterophilic granulocytes, while an intense activity was seen in eosinophilic granulocytes. This stain is used as a marker for myeloid cells in mammals, but chicken eosinophils, channel catfish and koi (Cyprinus carpio) neutrophils were also positive (Torres et al.,1986). By SBB staining, lipids including phospholipids, neutral fats and sterols within neutrophils, eosinophils and occasional monocytes were identified in mammals, and chicken eosinophils, channel catfish and koi neutrophils showed positive reaction (Torres et al., 1986). In the present study, only eosinophilic granulocytes were positive with SBB stain in whale shark. Based on their reactivity, whale shark granulocytes could be classified by cytochemistry. PAS reactivity was observed in all granulocytes and thrombocytes in whale shark, but reactivity patterns or intensity were different among blood cells.

FCM analysis

DiOC has been used in several applications, such as differentiation of bone marrow cells of dogs (Tort, and Flos,1987), counting of reticulocytes in humans (Uchida, 1983), and counting of blood cells of avian, reptiles and fishes (Parish et al., 1986). DiOC is a lipophilic dye that selectively stains lipid bilayers (Uchida, 1995) , thus it appears that each blood cell were distinguished by difference of cytoplasmic structures. In the present study, DiOC5(3) staining was an useful for distinguishing whale sharks leukocytes from erythrocytes, in this study. DiOC5(3) stained leukocytes and thrombocytes well, while erythrocytes were only faintly stained, which was similar to those in $DiOC_6(3)$ staining in mammalian blood and fish blood (Parishet al.,. 1986 and Uchida, 1983).

FACS analysis of $DiOC_5(3)$ stained blood cells displayed mainly four separate blood cell populations (namely erythrocytes, granulocytes, lymphocytes plus thrombocytes and monocytes plus immature cells) by means of their typical FL-1, FSC and SSC properties, as described previously in carp. In case of shark B (the number of leukocyte was markedly high), the granulocytes population was further subdivided into two populations, composed of heterophilic granulocytes plus basophilic granulocytes population and composed of mainly eosinophilic granulocytes population. The population containing lymphocytes and thrombocytes, each granulocytes was not be able to differentiate by using $DiOC_5(3)$ staining in whale shark. Applications of other fluorescent dyes such as $DiOC_6(3)$ or acridine orange, or using monoclonal antibodies against thrombocytes and lymphocytes may differentiate these blood cells. FACS analysis using both monoclonal antibodies against thrombocytes and $DiOC_6(3)$ should be able to count all the cell types in blood (Valet,1984). Alternatively other method, for instance leukocytes differentiation by Percoll density centrifugation, may be useful to separate each cell population in FACS analysis (Parish et al,1986).

Five types of dilute solutions were used in this study, and whale sharks blood plasma was optical for FACS analysis. In analysis by brownbanded bambooshark plasma, R1 population became markedly larger, which was considered that the solution made blood cell breakable or a large amount of sediment and cell debris were contaminating the solution. Analysis by RPMI and RPMI mixture, R2 region slid right-side and disturbed R3-2 region, which reasons were remained unclear, but absence of solution elements, such as urea and glutamine were considered (Walsh and Luer 2004), and might metamorphose erythrocytes.

It is necessary to understand influences of diseases and stress in alternation of absolute leukocyte count of each leukocyte. However, the complicated nature of the microscopic count techniques made them impractical for processing a large number of samples. Thus flow cytometric analysis was applied on whale sharks blood cells because simple, rapid and practical for large numbers of samples with quantization. In the present study, relatively high-correlations were obtained between the FACS and the microscopic counts in granulocytes and lymphocytes plus thrombocytes populations. Thus, the FACS analysis is useful to examine changes in the composition of blood cells as well as clinical monitoring and diagnosis. This method may be able to apply for hematological analysis in other elasmobranch. However, it remains several problems to be solved, such as how to separate lymphocytes, thrombocytes and three granulocytes effectively. In addition, not only percentages of cell count but also absolute cell count should be needed. Further studies may be required to use FACS on hematological diagnosis and finding hematologic characteristic of whale shark.

Animal	Sex	S.M.*	T.L.**	C.P.***
Shark A	male	immature	7.5m	12 years
Shark B	male	immature	6.5m	10 years
Shark C	male	immature	6.0m	7 years

S.M.* Sexual maturity
T.L.** Total length
C.P.*** Captivity period, y: year

Table. 2 Cytochemical staining reactions of whale shark leucocytes (including thrombocyte)

	Heterophilic g.	Eosinophilic g.	Basophilic g.	Lymphocyte	Monocyte	Thrombocyte
Peroxidase	±	++	-	-	-	-
Periodic acid-Schiff	+	+	+	-	-	+*
Sudan black B	-	++	-	-	-	-

-: negative, ±: weak positive, +: positive, ++: strong posit
* : occationally positive

Fig.1. Blood collecting were performed, swimming with a shark, from vessels at the axilla of the first dorsal fin.

Volume : 3 | Issue : 12 | December 2014 • ISSN No 2277 - 8179

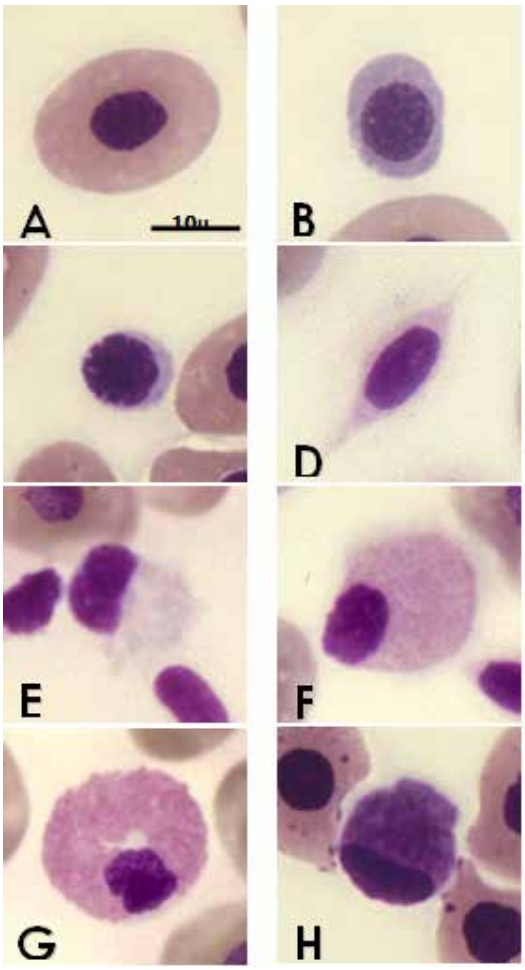


Fig. 2. Blood cells of whale shark. M-G stain.
A: Erythrocyte, B:Immature erythrocyte, C:Lymphocyte
D: Thrombocyte, D: Monocyte, E:Heterophilic granulocyte
F: Eosinophilic granulocyte, G:Basophilic granulocyte

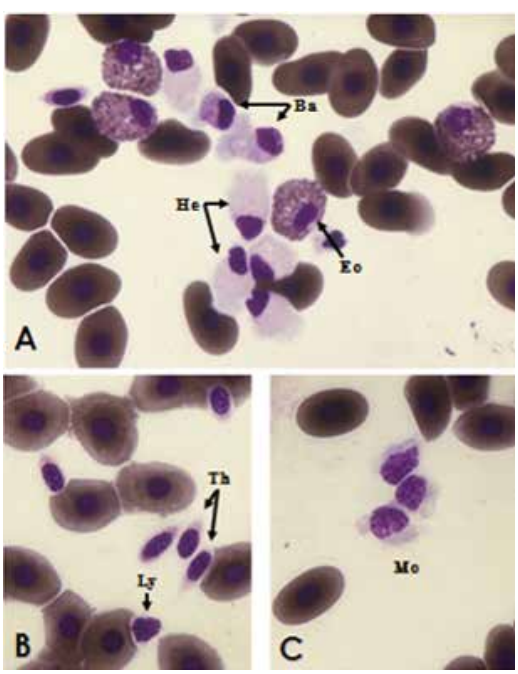


Fig. 3. Blood cells of whale shark. Peroxidase stain.
Eosinophilic granulocytes (Eo) were strongly positive with peroxidase stain, but heterophilic granulocytes (He) were weak positive and basophilic granulocytes (Ba) were negative (A). Lymphocytes (Ly), thrombocytes (Ly) and monocytes (Mo) were also negative (B,C).

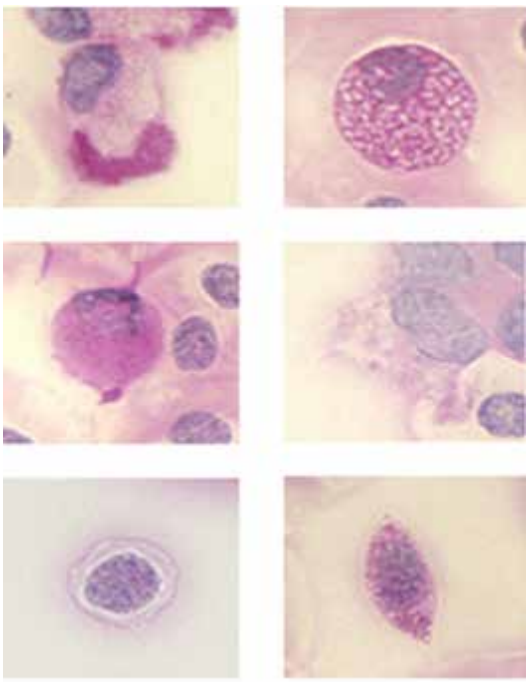


Fig. 4. Blood cells of whale shark. Periodical acid-Schiff (PAS) stain.

Heterrophilic granulocytes, confined to margin, were positive with PAS (A).

Eosinophilic granulocytes, surrounding area of granules, were strong positive (B), and basophilic granulocytes were uniformly positive in cytoplasm (C). Monocytes (D) and lymphocytes (E) were negative and thrombocytes were frequently observed positive granules in cytoplasm (F).

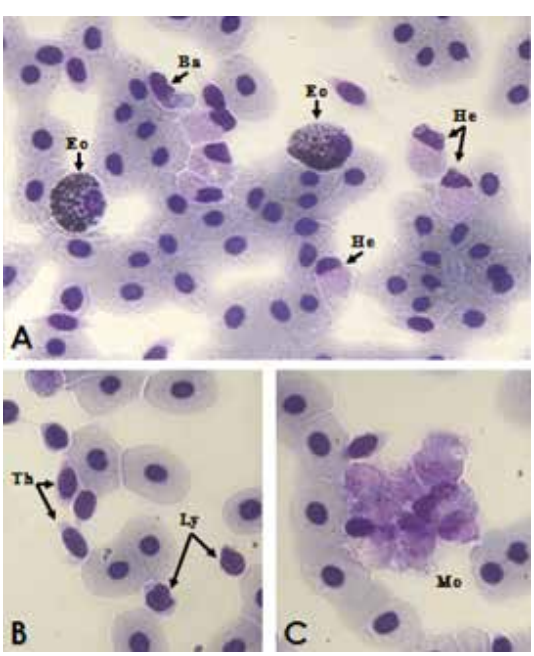


Fig. 5. Blood cells of whale shark. Sudan Black B stain.

Eosinophilic granulocytes were strongly positive with peroxidase stain,but the other cells were negative.

A: Heterophilic granulocytes (He), Eosinophilic granulocytes (Eo),Basophilic granulocytes (Ba)
B: Lymphocytes (Ly) and Thrombocytes (Th)
C: Monocytes (Mo).

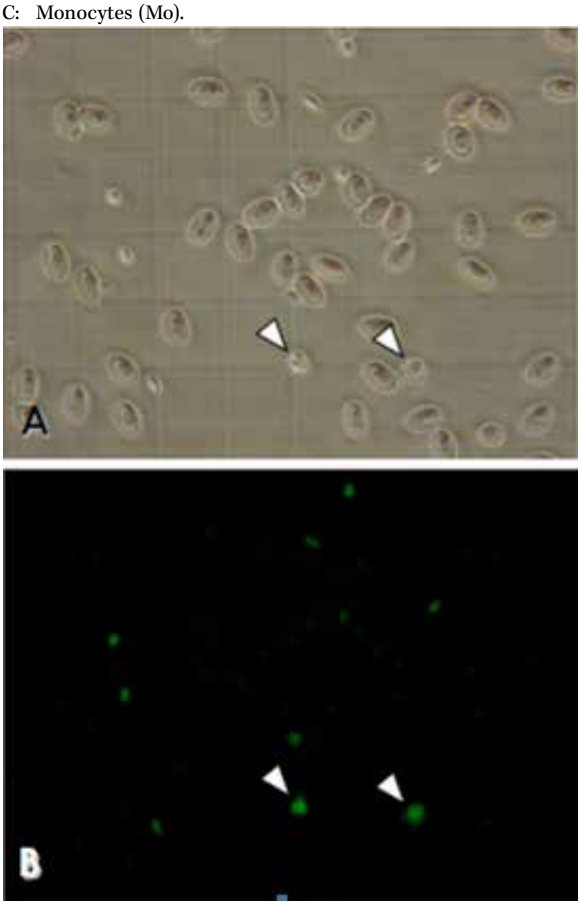


Fig. 6. Whale sharks blood cells stained with $DiOC_5(3)$.

Cells observed with visual light (A) and ultraviolet (B). Two large leukocytes

(granulocyte-like cells) (arrow head) and some small leukocytes (lymphocyte-like cells and thrombocyte-like cells) were present. Erythrocytes were faintly stained.

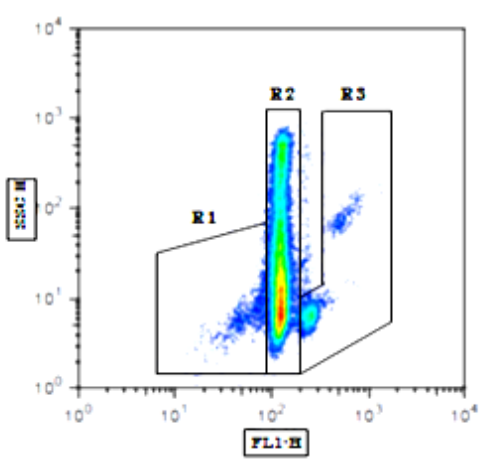


Fig. 7. Typical FACS analysis of whale shark blood cells stained with $DiOC_5(3)$and diluted by whale sharks serum. FL-1 v. SSC was recorded. Three distinct cell populations termed R1, R2 and R3 were revealed according to the intensity of FL-1.

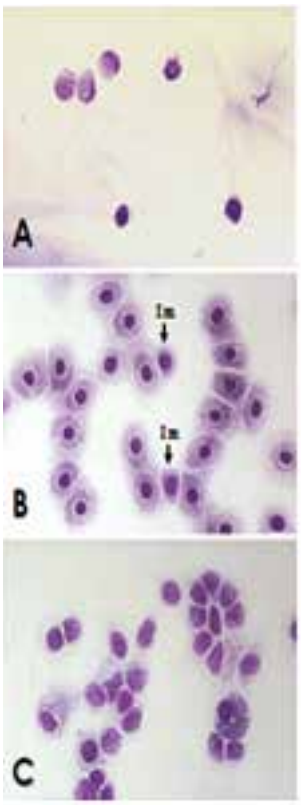


Fig. 8. Cell types of R1 (A), R2 (B) and R3 (C) region. M-G stain.

R1 was composed of broken cells or bare nuceleus (A). R2 was composed of erythrocytes and immature erythrocytes (Im) (B). R3 was mixed with all types of leukocyte, thrombocytes and immature cells (including immature erythrocytes) (C).

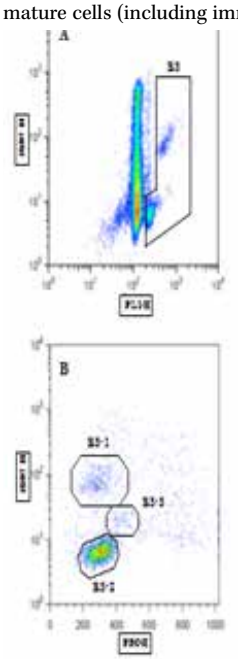


Fig. 9. Typical FACS analysis of $DiOC_5(3)$-stained blood cells.
A: Leukocytes could be divided into three populations by

Volume : 3 | Issue : 12 | December 2014 • ISSN No 2277 - 8179

FL-1 v. SSC properties.
B: The three population were also obtained by FSC v. SSC when the R3 region was recorded .

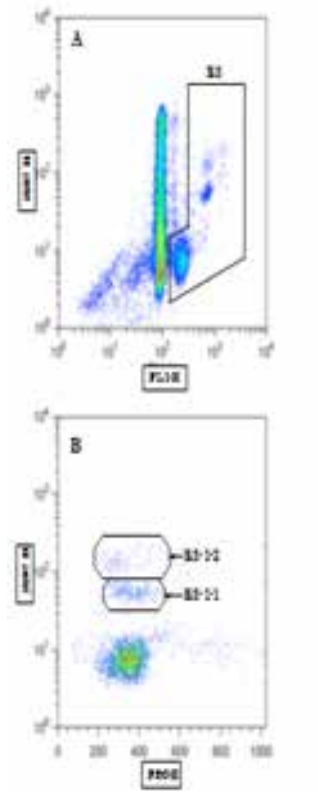


Fig. 10. FACS analysis of DiOC$_5$(3)-stained Shark B's blood cells.

R3-1 region was divided into two populations termed R3-1-1 and R3-1-2.
A: FL-1 v. SSC properties. B: FSC v. SSC properties.

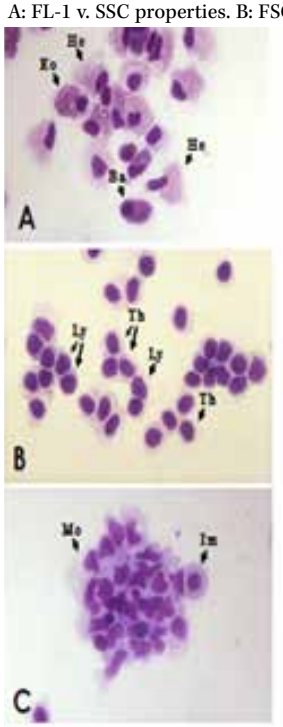


Fig. 11. Cell types of R3-1 (A), R3-2 (B), R3-3 (C) region. M-G stain.

R3-1 was composed of heterophilic granulocytes (He), eosinophilic granulocytes (Eo) and basophilic granulocytes (Ba). R3-2 was composed of lymphocytes (Ly) and thrombocytes (Th), R3-3 was composed of monocytes (Mo) and immature cells (Im).

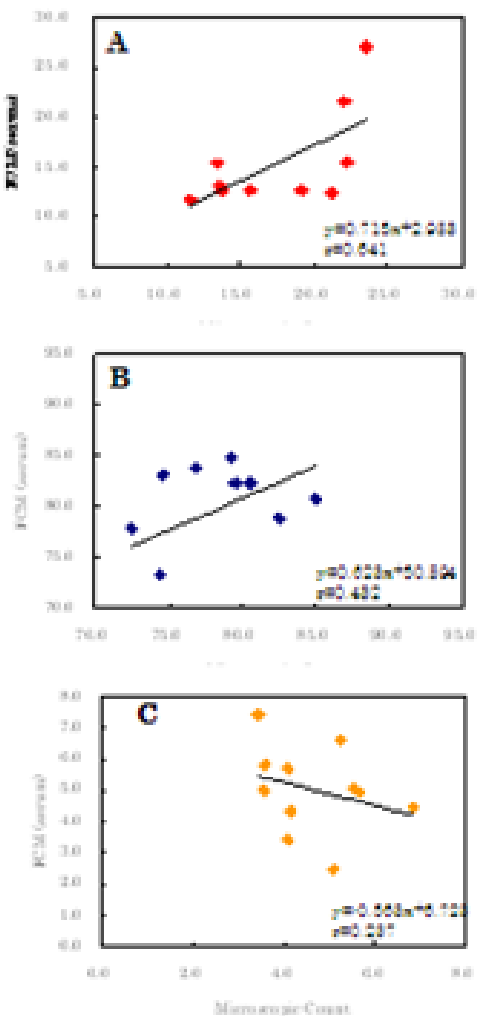
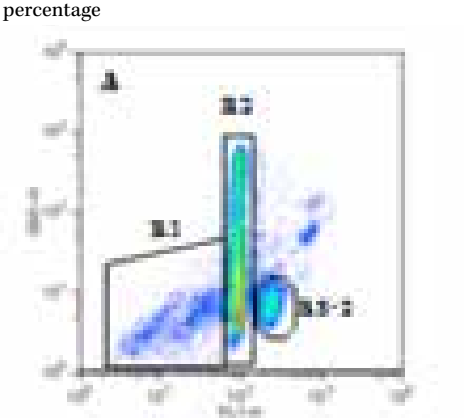


Fig. 12. Comparison of each blood cells percentage counted by microscopy and FACS analysis. Relatively good correlation were shown in granulocyte percentage (A) and lymphcyte and thrombocyte percentage (B) between the two methods. There was weak and negative correlation in monocyte and immature cell percentage



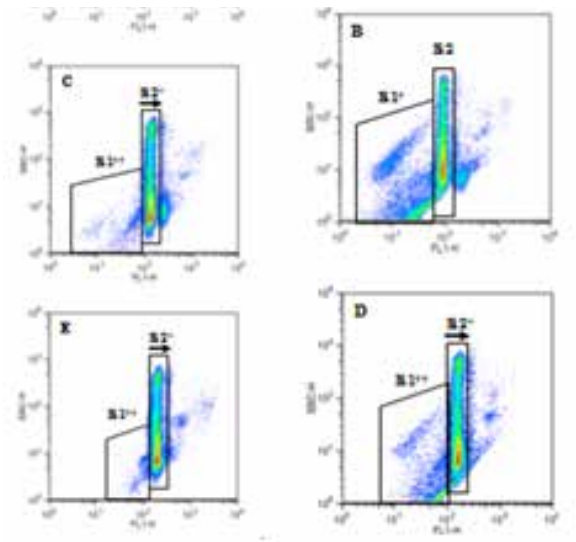


Fig. 13.FACS analysis of blood cells diluted with each solution.

A: whale shark's serum.

B: brownbanded bambooshark's serum.

C: whale shark's serum and RPMI.

D: brownbanded bambooshark's serum and RPMI.

E: RPMI

*: R2 region of specimens diluted with RPMI or mixture slid right-side and overlapped with R3-2 region, progressively.

#: In case diluted with brownbanded bambooshark serum, R1 region was enlarged and increased.

##: Diluted with RPMI or mixture, R1 regions were narrowed

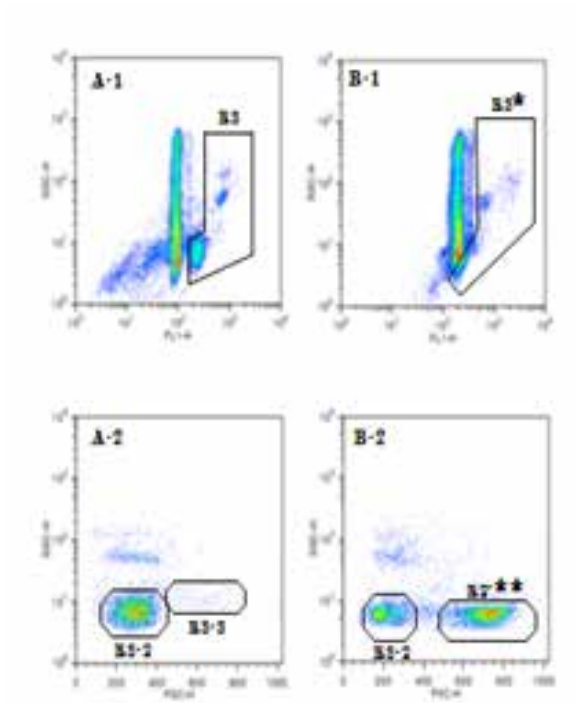


Fig. 14.FACS analysis of blood cells diluted with whale shark's serum and RPMI.

A-1: Diluted with whale shark's serum. FL-1 v. SSC recorded.

A-2: A dot-plot of FSC v. SSC obtained for the gated region (R3) ofA-1.

B-1: Diluted with RPMI. FL-1 v. SSC recorded.

B-2: A dot-plot of FSC v. SSC obtained for the gated region (R3) of B-1.

*: For obtain the R3-2 region (lymphocytes and thrombocytes), the region

which overlaped with R2 and R3-2 was also selected by gating. And a dot-plot of FSC v. SSC was obtained for the gated region.

**: Erythrocyte population (R2') appeared in case specimens were made with RPMI or RPMI mixture. So R3-2 region was able to obtain, but R3-3 region became unclearly.

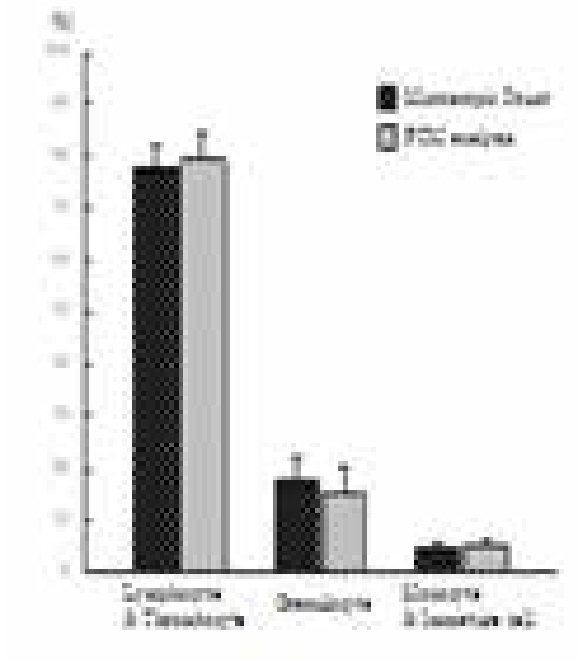


Fig.15 Comparison of each leucocyte percentages counted by microscopy and FCM analysis. There were no significant differences between two methods for counting each cell percentage.

Volume : 3 | Issue : 12 | December 2014 • ISSN No 2277 - 8179

REFERENCE

Compagno, L.J.V., 2001:Sharks of the World: An Annotated and Illustrated Catalogue of Shark Species Known to Date, vol. 2. Bullhead, mackerel, and carpet sharks (heterodontiformes, lamniformes and orectolobiformes) FAO species catalogue for fishery purposes, no. 1. FAO, Rome. | Forster, R. P., Goldstein, L. and Rosen, J. K. 1972: Intrarenal control of urea reabosorption by renal tubeles of the marine slasmobranch, Squalus acanthias. Comp Biochem Physiol A. 42(1):3-12. | Hrubec, T. C., Robertson, J. L., Smith, S. A. 1997: Effect of temperature on hematologic and serum biochemical profiles of hybrid striped bass (Morone chrysops X Morone saxatilis). Am J Vet Res. 58:126–130. | Knoll, J. S. 2000: Clinical Automated Hematology Systems. Pages 3-11 in Feldman, B. F., Zinkl, J. G. and Jain, N. C., Eds. Schalm's Veterinary Hematology. 5th ed. Lippincott Williams and Wilkins. | Macey, M. G. 1994: Introduction. Pages 1-44 in Macey, M. G. Eds. Flow Cytometry; Clinical Applications. Blackwell Scientific Publications. | Mader, D. R. 2000:Normal Hematology of Reptiles. Pages 1126-1132 in Feldman, B. F., Zinkl, J. G. and Jain, N. C., Eds. Schalm's Veterinary Hematology. 5th ed. Lippincott Williams and Wilkins. | Martin, R., Brott, D., Zandee, J. and Mckeel, J. 1992: Differential analysis of animal bone marrow by flow cytometry. Cytometry. 13(6):638-43. | Nishida, K. 2001:. Whale shark-the largest fish. Pages 20-26 in Nakabo, T., Machida, Y., Yamaoka, K. and Nishida, K. Eds. Fishes of Kuroshio Current, Japan. Osaka Aquarium KAIYUKAN. | Parish, N., Wrathmell, S., Hart, S. and Harris, J. E. 1986: Phagoyctic cells in the dogfish, Scyliorhinus canicula L. □. In vitro studies. Acta Zoologica (Stockholm) b; 67:215-224. | Parish, N., Wrathmell, S., Hart, S. and Harris, J. E. 1986: Phagoyctic cells in the peripheral blood of the dogfish, Scyliorhinus canicula L. □. In vivo studies. Acta Zoologica (Stockholm) b;67:225-234. | Pierson, F. W. 2000: Laboratory Techniques for avian. Pages 1145-1146 in Feldman, B. F., Zinkl, J. G. and Jain, N. C., Eds. Schalm's Veterinary Hematology. 5th ed. Lippincott Williams and Wilkins. | Raskin, R. E. and Valenciano, A. 2000: Cytochemistry of normal leukocytes. Pages 337-346. in Feldman, B. F., Zinkl, J. G. and Jain, N. C., Eds. Schalm's Veterinary Hematology. 5th ed. Lippincott Williams and Wilkins. | Rothamann, C., Levinshal, T., Timan, B., Avtalion, R. R., Malik, Z., 2000: Spectral imaging of red blood cells in experimental anemia of Cyprinus carpio. Comp Biochem Physiol A. 125:75–83. | Sherburne, S. W. 1973: Cell types, differential cell counts and blood cell measurements of a Portuguese shark (Centrocymnus coelolepsis) captured at 700 fathoms. Fish bull. 71:435-439. | Smith, A., 1829: Contributions to the natural history of South Africa. Zool. J. 16, 443-444. | Speckner, W, Schindler, J. F., Albers, C. 1989 : Age-dependent changes in volume and haemoglobin content of erythrocytes in the carp (Cyprinus carpio L.). J Exp Biol. 141:133-149. | Stevens, J. D. 2007 : Whale shark (Rhincodon typus) biology and ecology: A review of the primary literature. Fish. Res. 84, 4-9. | Stokopf, M. K. 1993 : Clinical pathology. Pages 113-757 in Stokopf, M. K. Eds. Fish Medicine. Philadelphia: WB Saunders. | Stoskopf, M. K. 2000 : Normal Hematology of Elasmobranchs. Pages 1174-1178 in Feldman, B. F., Zinkl, J. G. and Jain, N. C., Eds. Schalm's Veterinary Hematology. 5th ed. Lippincott Williams and Wilkins. | Svetina, A., Matasin, Z., Tofant, A., et al. 2002 : Haematology and some blood chemical parameters of young carp till the age of three years. Acta Vet Hung. 50:459–467. | Taylor, L.R., Compagno, L.J.V., Struhsaker, P.J., 1983 : Megamouth—a new species, genus and family of lamnoid shak (Megachasma pelagios), Family Megachasmidae from the Hawaiian Islands. Proc Calif Acad Sci 43, 87-110. | Terasaki, M. R. and Fujiwara, K. 1992 : Fluorescent labeling of membranes: Method for ER staining. Seimeinokagaku 43:632-637 (in Japanese). | Torres, P., Tort, L., Planas, J. and Flos, R. 1986 : Effects of confinement stress and additional Zinc treatment on some blood parameters in the dogfish, Scyliorhinus canicula Comp Biochem Physiol. 83C;89-92. | Tort, L., Torres, P. and Flos, R. 1987 : Effects on dogfish haematology and liver composition after acute copper exposure. Comp Biochem Physiol. 87C;349-353. | Uchida, S. 1983 : On the morphology of the whale shark, Rhincodon typus Smith. Aquabiology, 5(2):93-101. (In Japanese, English abstract and captions.) | Uchida, S. 1995 : Whale shark. Pages146-153 in Japan Fisheries Resource Conservation ed. Data Book of rare aquatic animals and plants of Japan (□). Japan Fisheries Resource Conservation, Tokyo. (In Japanese.) | Valet, G. 1984 : A new method for fast blood cell counting and partial differentiation by flow cytometry. Blut. 49:83-90. | Walsh, C. J. and Luer, C. A. 1998 : Comparative phagocytic and pinocytic activities of leukocytes from peripheral blood and lymphomyeloid tissues of the nurse shark (Ginglymostoma cirratum Bonaterre) and the clearnose skate (Raja eglanteria Bosc). Fish Shellfish Immnol. 8:197-215. | Walsh, C. J., Luer, C. A. 2004 : The Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays and their Relatives. Pages 307-323 in Mark, S., Doug, W., Dennis, t. and Robert, H. Eds. Elasmobranch Hematology: Identification of Cell Types and Practical Applications. Chapter 23. Ohio Biological Survey, Ohio state Univ., Ohio. |