**Araştırma Makalesi
Research Article**

Automatic Cells Counting in Natt-Herrick Stained Fish Blood

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Abstract

Monitoring of hematological values which provide important information about the health status of fish is considerably important in aquaculture. One of the most commonly used methods for detecting the hematological values in fish bloodis the usage of Natt-Herrick solution. Basically, in this approach, Natt-Herrick stained blood samples are examined with a microscope and the cells are counted. Nevertheless, the counting process is both tough and time-consuming. In this study, a technique in which cell countingin blood samples images is automatically performed has been presented. Natt-Herrick stained blood samples of *Oncorhynchus mykiss* and *Sparus aurata* were used for evaluation of the developed scheme. The outputs generated by automatic blood cells detection algorithm in 90 images were compared with results which were obtained by means of user's intervention. Consequently, an average f-score over 0.96 was achieved.

Öz

Natt-Herrick Boyalı Balık Kanı Hücrelerinin Otomatik Sayımı

Hematolojik değerlerin kontrolü balıkların sağlık durumları hakkında önemli bilgiler verdiğinden, bu kontrol balık yetiştiriciliğinde oldukça önemlidir. Hematolojik değerlerin kontrolünde en sık kullanılan yöntemlerden biri de Natt-Herrick solüsyonudur. Natt-Herrick solüsyonu yardımıyla boyanan kan örnekleri mikroskop ile incelenmekte ve hücre sayımı yapılmaktadır. Ancak bu sayım hem yorucu hem de zaman alıcıdır. Bu çalışmada kan örneklerine ait görüntüler üzerinden otomatik olarak hücre sayımı yapabilecek bir teknik sunulmuştur. Geliştirilen yöntemin değerlendirilmesinde Natt-Herrick solüsyonu ile boyanmış Gökkuşağı Alabalığı ve Çipura balıklarına ait örnekler kullanılmıştır. Bu örneklere ait 90 adet görüntüdeki kan hücrelerinin otomatik belirlenmesi sonuçları ile kullanıcılar tarafından belirlenen sonuçlarla karşılaştırılmıştır. Karşılaştırma sonucunda ortalama olarak 0,96'nın üzerinde f-skor değeri elde edilmiştir.

Introduction

The demand for seafood and especially fish is growing day by day whilethe resources inmarine and ocean are decreasing. Consequently, fish farming is gaining significance to meet the demand. Nevertheless, it is important to reduce the labor and other cost in aquaculture.

One of the most critical points in the fish farming is to increase healthy fish production with high efficiency. In fish farming, the regular checking of fish health is very important and it is traced through fish blood parameters.

Hematological data and blood parameters are some of the most important sources for early diagnosis (Luskova, 1997). Also, hematological parameters have become a standard for determination of fish health (Tavares-Dias and Moraes, 2007). These parameters and other diagnosis tools can be used for identification and assessment of problems that could affect the health status of fish (Pavlidis *et al.,* 2007). These data can also be used as important tools for environmental quality (Qiang J *et al.,* 2013). Natt-Herrick method is still being used as one of the basic technique for counting fish blood cells (Arnold *et al.,* 2014). However, cell counting process performed on microscopic images is difficult and takes a long time. It is also an error-prone process due to the human nature. The computer technologies can be used to shorten the process and reduce the error rate. Nevertheless, any study has seen that used computer technologies for Natt-Herrick stained fish blood images in the literature. Therefore, it is observed that there is a new technique that can automa-tically count the cells in Natt-Herrick stained fish blood images. In this study, we present a novel approach to count cells in Natt-Herrick stained fish blood images automatically.

Materials and Methods

In this study, we used blood samples of *Oncorhynchus mykiss* and *Sparus aurata* from Republic Of Turkey Ministry Of Food, Agriculture and Livestock, Mediterranean Fisheries Research, Production and Training Institute. Visual counting of the blood samples was performed by using the Natt-Herrick solution prepared according to (Konuk, 1975) on the Thoma hemocytometer. After the visual counting process, 90 images with size of 1200x1600 pixels were stored by means of Olympus BX53 trinocular light microscope with 100X zoom ratio and Olympus DP72 image capturing system. One of the attained images is shown in Figure 1a. Original size of black boxed section of the sample image is illustrated in Figure 1b.

a   b
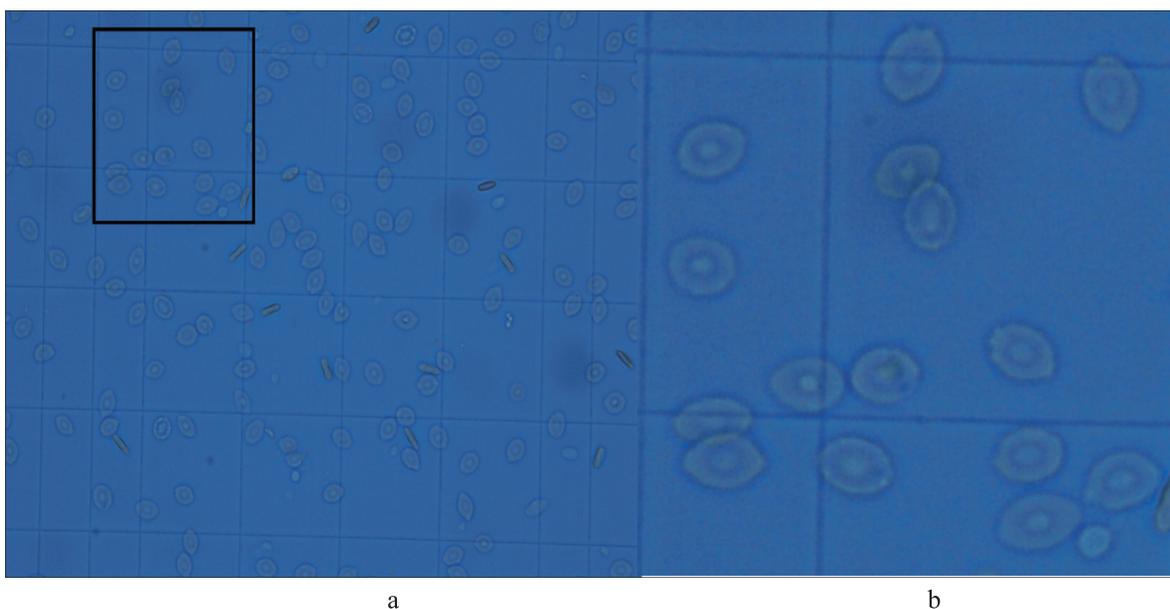
Figure 1. Natt-Herrick stained fish blood sample, a) Whole image, b) Original size of black boxed section in Figure 1a.

In general, very simple methods are useful for the segmentation of an image if color and gray level differences between objects and background on the image are fixed and distinctive. Nevertheless, it needs to be developed particular methods for complex images if the color differences are variable and indistinctive.

This requirement could be justified by plenty of scientificpapers about image segmentation in the literature. In this paper, a new method which is adapted by using different techniques has been developed for Natt-Herrick stained fish blood images. The proposed method consists of two main stages as shown in Figure 2.
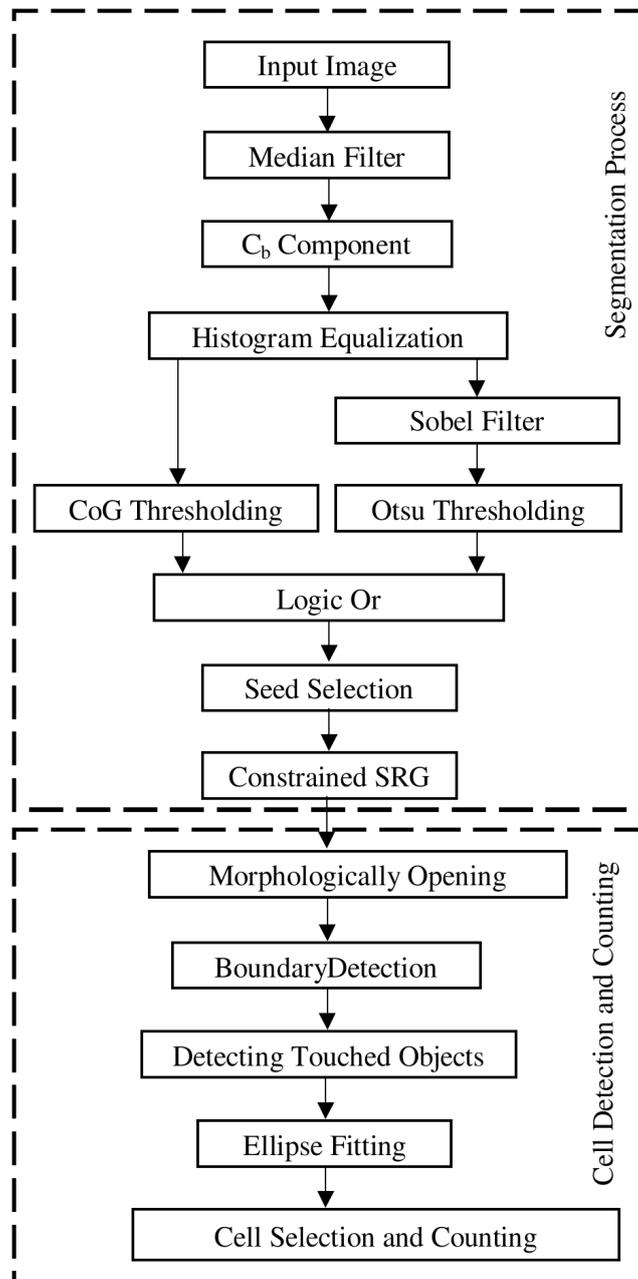

Figure 2. Block diagram of cell detection and counting.

The first stage is about detection of the objects in images by the segmentation process. Selection and counting process for the cells in distinguished objects is carried out in the second stage. Detection process of objects has been performed by using algorithm developed by author's previous approach (İncetas *et al.,* 2014). As shown in Figure 1b, cells have elliptical shapes and some of them touches each other. Therefore, the recognition of touched cells is very important forcounting the number of cells correctly. To achieve the aim, firstly, boundaries of the objects which are derived after segmentation process are determined. Edge parts of touched objects are distinguished with the determined boundaries.

After then, each of the boundary curves are fitted for an ellipse function and each object is shown as an ellipse. Consequently, the objects are labeled as cells as they satisfy the criteria as size and eccentricity. Finally, the number of cells is determined. All of these processes are shown in detail in following section.

Segmentation Process; İnitially, as shown in Figure 2, median filter is used to make input images more homogenous. Median filter removes the noises and softens the objects' edges but cell borders are preserved because of their thickness. Cell borders are thicker than a few pixels. Accordingly, more homogeneous images are achieved after the median filter process. Grayscale image are obtained with C_b component from the filtered image by using the conversion to YC_bC_r color space. Thus, differences between the blue and the other colors are revealed by removing the lightening information in the image. In next step, pixels with similar gray level values are grouped by applying the histogram equalization process to C_b image. C_b gray level image and histogram equalization result for the Figure 1b are shown in Figure 3.

a   b
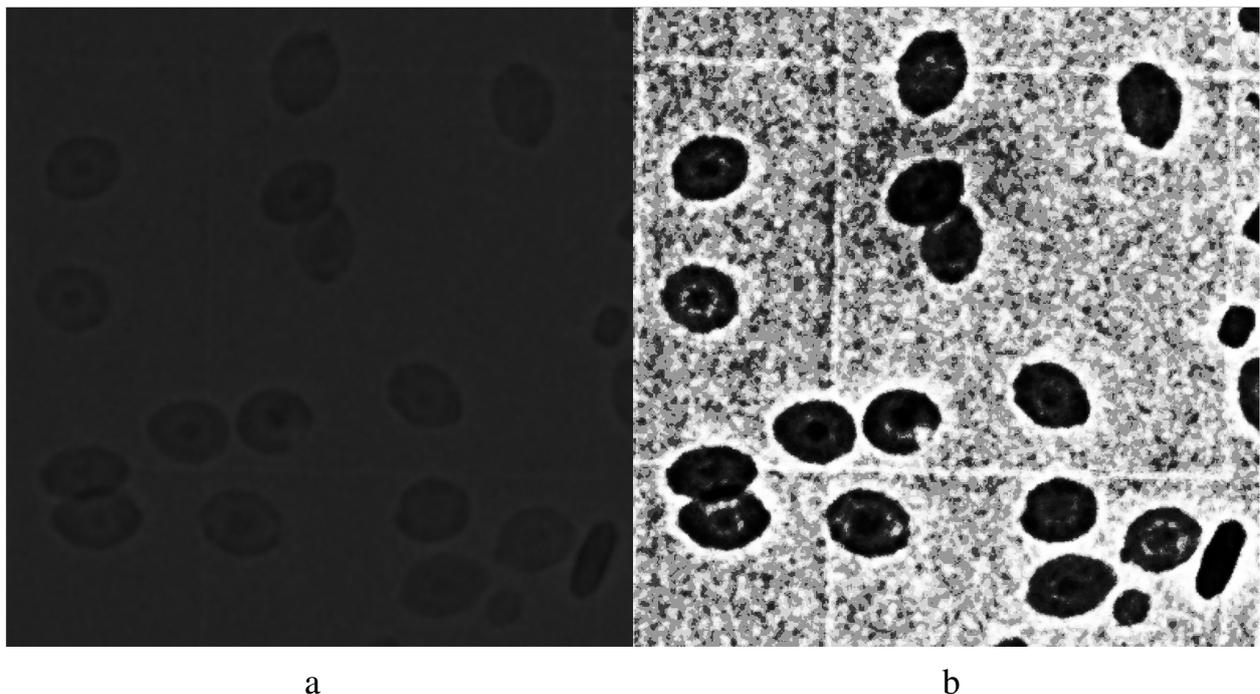
Figure 3. Color conversion results a) C_b component of YC_bC_r, b) Histogram equalization.
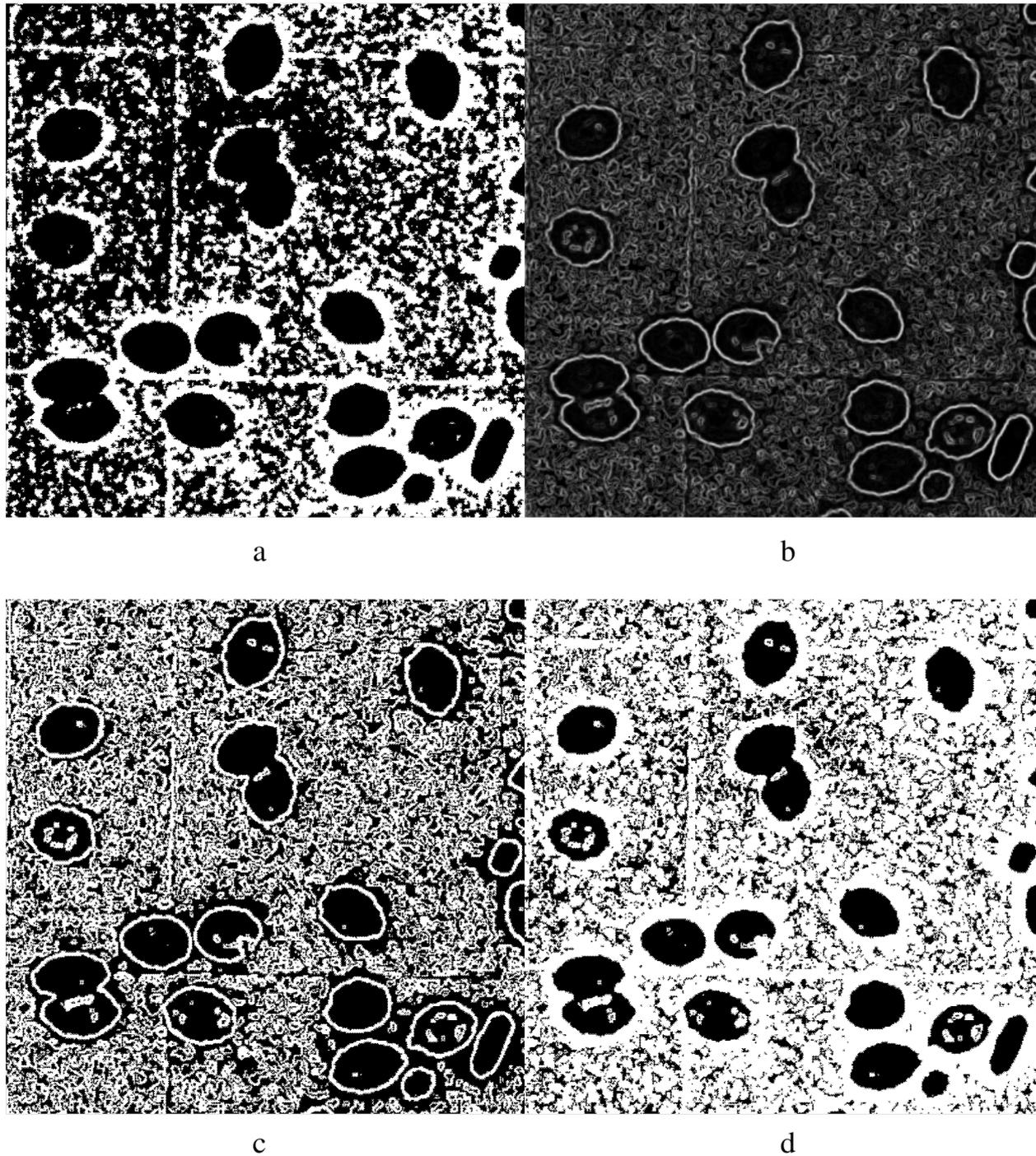
Figure 4. Binarization and filtering results, a) CoG Thresholding, b) Sobel filter c) Otsu thresholding of Sobel filter, d) Logic Or.

It seems in Figure 3a that gray level values of pixels in cell regions are lower than the values of background or line pixels. As shown in Figure 3b, the cells arise more significantly after the histogram equalization process. Figure 4 shows the output production stages for the image in Figure 3b. Binary image obtained by using thresholding technique called center of gravity (CoG) method is in Figure 4a (Demirci, 2010).

Although, it revealed the homogenous regions and cells are observed significantly, objects and background pixels are same color value and connected at some areas in the binary image. Figure 4b shows filtered image in the Figure 3b by using Sobel filter. It seems clear boundaries between the objects and the background in this step. As shown in Figure 4c, binary image obtained by another thresholding process using Otsu technique on Figure 4b.

As a result, the pixels in the interior of the objects in the Figure 4c have usually 0 (zero) value and these pixels are displayed as black. Moreover, the inner pixels of objects in the Figure 4a have usually 0 (zero) value and so black color. Finally, the image Figure 4d has been obtained by Logic OR process using binary values of pixels in Figure 4a and 4c. The pixels of objects have generally 0 value as black and the pixels of the background have 1 value as white in Figure 4d which is binary image.

Nevertheless, there are still some background pixels which have 0 value. Therefore, completely homogenous areas with 9x9 sized mask are labeled and the result of this labelling process is also shown in Figure 5a with green pixels.

The green pixels in the achievedimage are employed as seeds in constrained seeded region growing (SRG) scheme(Fan, 2001; Leyk and Boesch, 2010). The result of constrained SRG process performed only on the pixels with 0 value by using the seeds is shown in Figure 5b. As could be seen, objects are shown with white and the background has black color.

Cell Detection and Counting; After finding the object in the input image as shown in Figure 5b, detection and counting of cells is performed. Boundaries of the objects for this aim should be determined. First challenge for border detection is the protrusions in contours of objects as shown in Figure 5b. Morphological opening has been used to overcome the challenge(Gonzalez and Woods, 2008). A mask with size of 7x7 was employed for the morphological opening procedure.


a   b
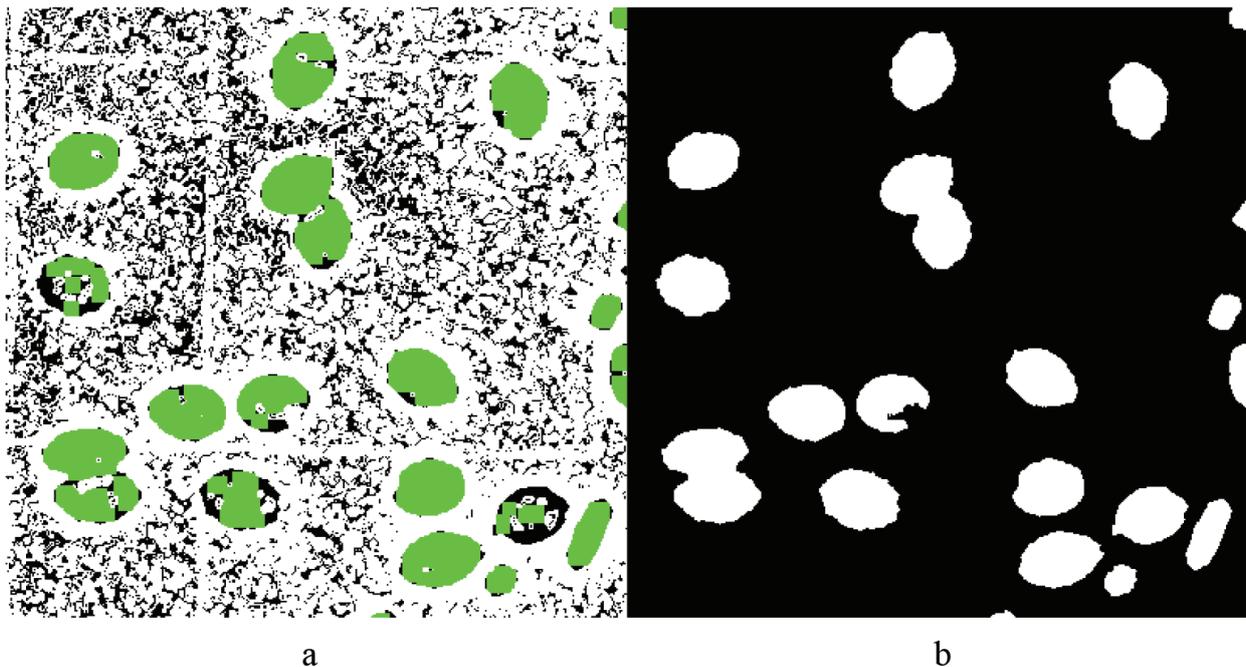
Figure 5. Constrained Seeded Region Growing Results a) Seed Selection, b) Constrained SRG.

Thus, small protrusions consisting of few pixels in the images are removed easily and result of this process is shown in Figure 6a. Then, object boundaries are determined by selecting pixels on the edge of the objects as shown in Figure 6b.

Touched cells are also another challenge in cell detection and the relevant cells can be found by using concave high curvature points (Chetverikov and Szabo, 1997). Concave high curvature points detected in object borders are shown in Figure 7a.

a   b
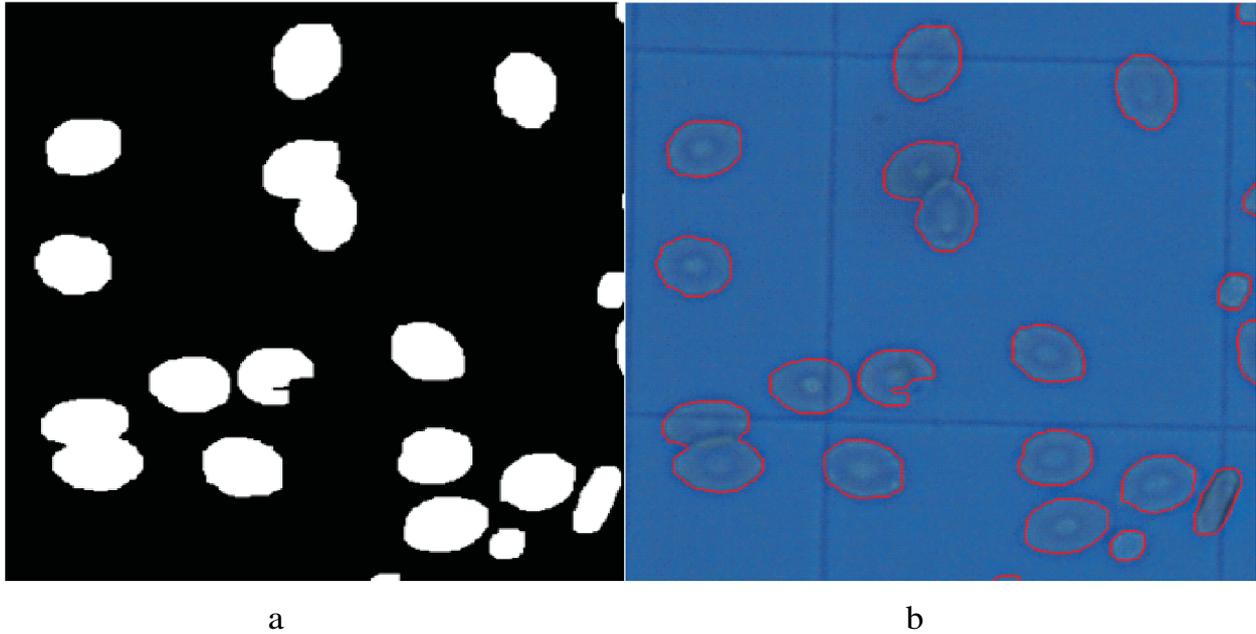
Figure 6. Detecting object borders, a) Morphological opening, b) Boundary detecting.

a   b
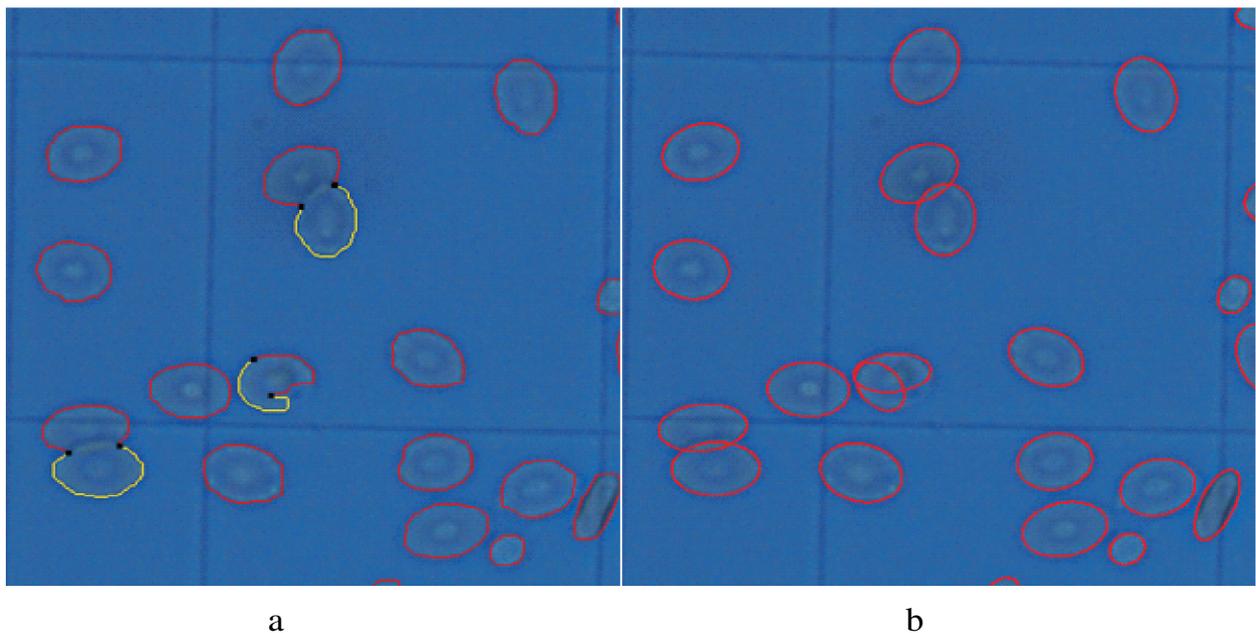
**Figure 7**. Detecting and fitting objects, a) Detecting touched objects b) Ellipse fitting.

One or more border sets are obtained with the points (Bai et al., 2009; Gonçalves and Bruno, 2012).When there are two or more border sets on an object, they are displayed with different colors in Figure 7a. Each of the border sets is fitted by an ellipse (Halir and Flusser, 1998). Consequently, cells are attained from all border sets of objects even if they are touched to each other. The result of the ellipse fitting performance is shown in Figure 7b.Finally, the determined cells are evaluated according to their eccentricity and fieldinfomation gained. The eccentricity and fieldvalues of an ellipse can show whether an object is a cell. Completed test results show that a cell typically has eccentricity value less than 0.60 and area value greater than *mean field - 2** (:standard deviation of cells field data). The results obtained by applying the specified criteria are shown in Figure 8.

Results

In this study, a new cell detection method has been developed and it has been applied 90

images obtained from fish blood samples. The cells in the images are also marked by users with a graphical tablet. Then, the marked cells are compared with the results obtained from the devised scheme. A quantitative measure of the performance of a new algorithm is almost a requirementof scientific research. Commonly, F-score (or F-measure) calculated by precision-recall data is intensively used to compare the results with new technique and ground truth information. For example, F-scorecriteria, which was employed for evaluating the edge detect algorithms was also used for evaluating the performance of background extraction process (Martin *et al.,* 2004; Guyon *et al.,* 2012). In fact, F-score is the harmonic average of precision and recall values. The term was used for the evaluation of a system or routine (Van Rijsbergen, 1979). In this study, precision represents the ratio between manually marked an object as celland detected cells by the algorithm. Subsequently, precision displays accurateness evidence of selected cells.

Recall provides the ratio between the objects which were selected as cell by algorithm

a   b
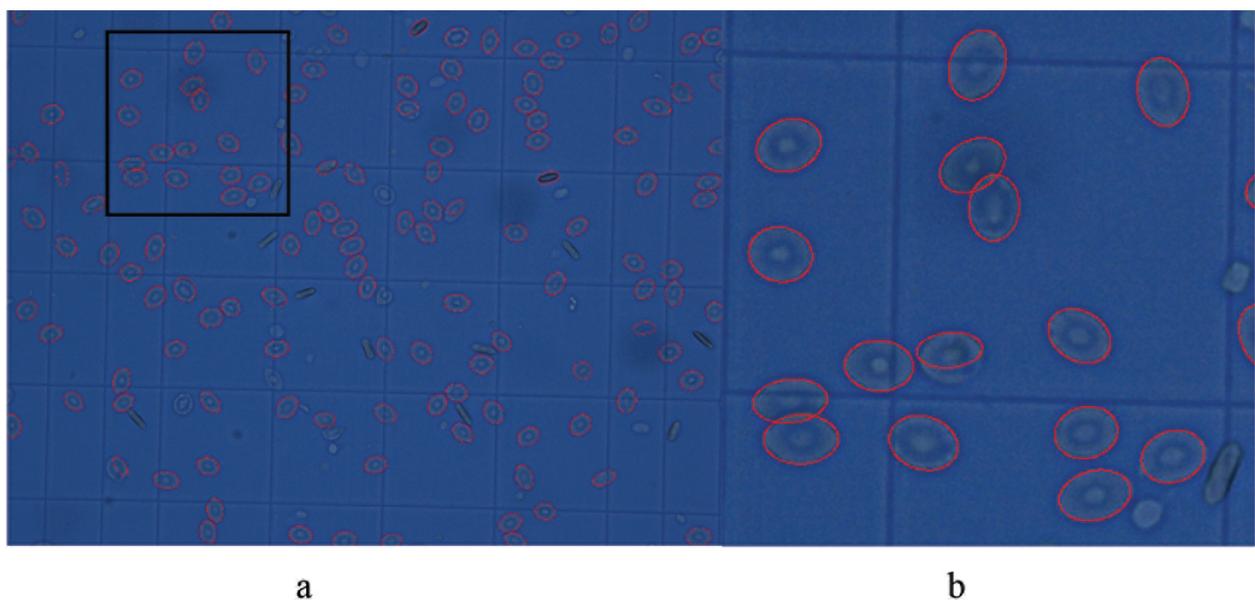
Figure 8. Cell detection results b) Whole image b) Original size of black boxed piece.

and manually marked cells. In other words, recall shows determining ratio of marked cells as tissue. Essential terms for the performance evaluation are shown in Table 1.

Table 1. Performance evaluation of cell detection

Evaluation		Algorithm result	
		Cell	Other
Ground	Cell	A	B
Truth	Other	C	D

In Table 1, Ground Truth represents the set of manually marked cells while Algorithm Results expression is used for the set of cellsdetected by algorithm. *A* is the number of True Positive cellswhich are selected as cells and *B* is the number of False Negative cells selected as non-cell by algorithm. Additionally, *C* is the number of False Positive non-cell areas as cellsand D is also the number of True Negative non-cell areas selected as non-cell areasp by algorithm. The basic formulas used for the F-score computation are as follows:

$$Precision = \frac{A}{A+C} \qquad (1)$$

$$Recall = \frac{A}{A+B} \quad (2)$$

$$F = \frac{2 \times Precision \times Recall}{Precision + Recall} \quad (3)$$

Where *F* is the F-score value which is the harmonic mean of Precision and Recall. All cellsin Ground Truth information for evaluation of F-score are marked by users in 90 images. The results of the technique for image in Fig. 1b, where Red marks show the cells, can be seen in Fig. 9a

Thus, the performance of the proposed method for detection of the cellsis realized as a percentage. Results for different cases are shown in Figure 9 b,c,d. Essentially, circled objects with ellipses are cells found by the developed method while the cells marked with red pointsin the middle are detected manually by users. A, B and C values in Equation 1 and 2 are attained from the marked images. The number of the ellipses marked in centreshows value of A, and not marked ones shows assessment of C. Furthermore the number of the cells not fitted an ellipse is defined as B value.

A, B, C values and evaluation results of the established technique are given in Table 2.

There are 7640 (A+B) cells marked manually in all 90 images. 7423 (A) of these cells are found correctly by the proposed technique and the recall value is found as 0.9715. In addition, only 298 (C) of the 7721 (A+C) cells which are identified by the developed scheme are faulty and then the precision value is found as 0.9614. Consequently, the F-score value is calculated as 0.9664. When the averages of the results achieved from the images are considered, the grades are revealed clearly. In other words, 83 of 85 cells in image are found correctly whereas just 3 objects are incorrectly labelled as cell. Average recall precision values have beenattained as 0.9709 and 0.9566, respectively. Finally, the F-score value with the recall and precision has been calculated as 0.9637.

Table2 also shows the minimum values for recall, precision and F-score. Only 67 of 74 cells were correctly identified in the image where the minimum recall value is obtained. In this imagewhere 70 cells were labelled, 3 cells were over-labelled and 0.9305 F-score value was obtained. In the image with which minimum precision value is estimated, there are 63 cells and the algorithm has marked 71 cells. Precision and F-score values for 8 over-labelled cells are calculated as 0.8873 and 0.9402, respectively. In other case, 85 cells were marked in the image with 88 cells and minimum F-score value is calculated as 0.9284.
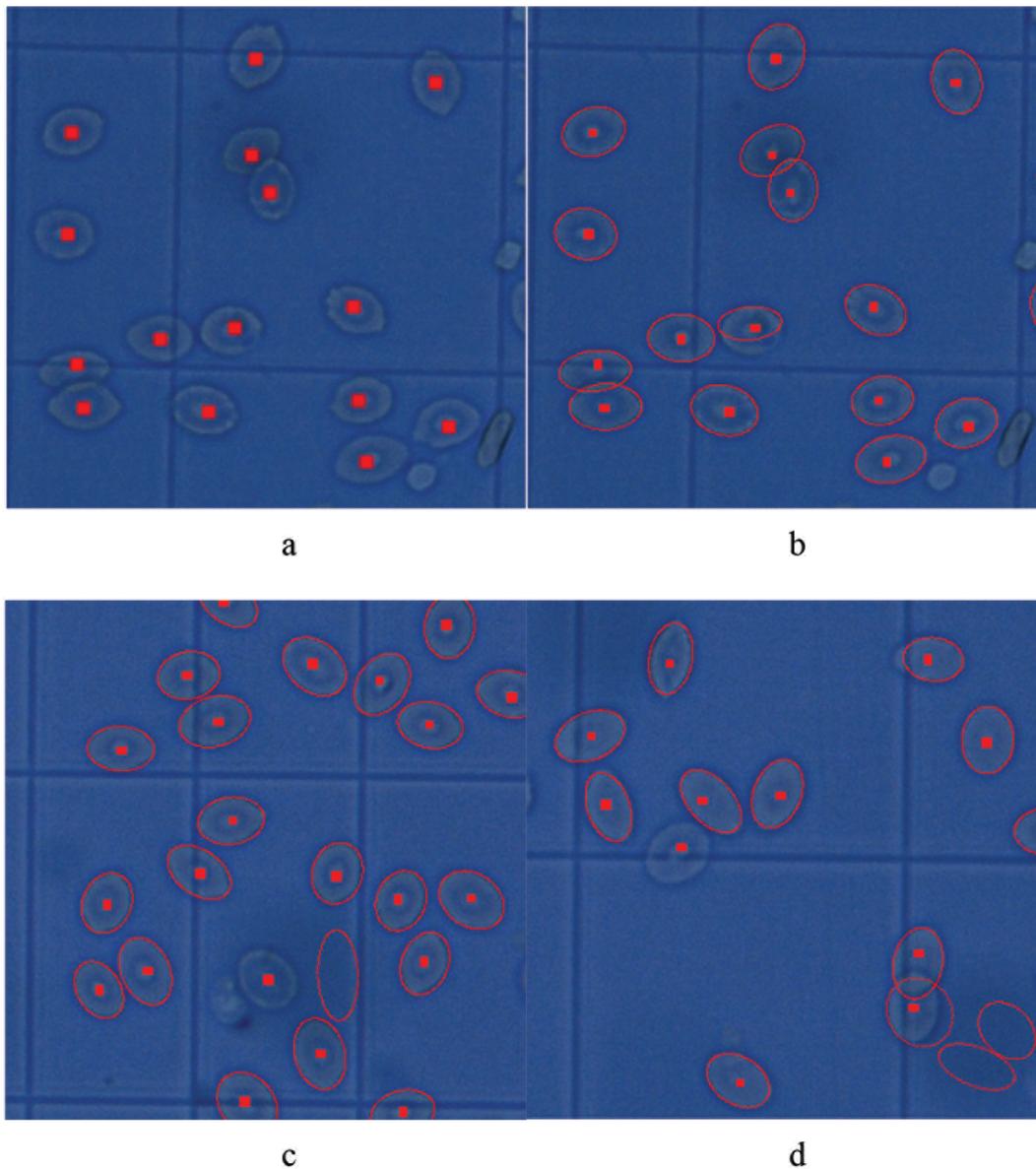
Figure 9. Manual marking and comparison results a) result of manual marking b-d) Manual marking and algorithm results.

Table 2. Performance evaluation of cell detection algorithm

	Object and Cell Counts			Performance Evaluation		
	A	B	C	Recall	Precision	F-score
All images	7423	217	298	0.9715	0.9614	0.9664
Average Values	82.47	2.41	3.31	0.9709	0.9566	0.9637
Min. Recall	67	7	3	0.9054	0.9571	0.9305
Min. Precision	63	0	8	1	0.8873	0.9402
Min. F-score	80	8	5	0.9090	0.9411	0.9284

Moreover, the algorithm incorrectly marked 5 objects as cell while 8 cells were not marked. Accordingly, the values of recall, precision and F-score values are calculated as 0.9090, 0.9411 and 0.9284, respectively. Additionally, both recall and precision values were1 so the F-score was 1 in 3 image samples. Apart from above examples, at least one of the recall or precision values is 1 in 17 different image samples. Furthermore, the F-score values are more than 0.95 in 71 images.

Discussion

Fish health plays a very important role in the fish farming which gainsbenefit day by day. Regulation of hematological value is one of the basic processes of monitoring fish health. Natt-Herrick solution is widely used to control for hematological standards of fish and obtained results are examined with the aid of a microscope. Nevertheless, the time required for theprocedure prevents to widespread practice. In this study, an automated technique has been presented for counting the cells in fish blood stained Natt-Herrick solution. Thus, cell counting on a microscope or image could be completed quickly, easily and correctly. The devisedsystem was tested on 90 images and high F-score valueover 0.96 has beenachieved. In other words, the error rate of the algorithm is quite low.

Nevertheless, when errors were being analysed during the study, it was observed that there were two types of faults. First fault is that some of the cells marked by users could not bedetected by the algorithm. It wasobserved as 2.8% with B/(A+B). The second disability is determination of non-cell areas as cells. Its rate was calculated as 3.8% from C/(A+C). When the images with the first error type were examined, some cells were not stained clearly and the quality of the related images was low. It was also observed that there were overlapping cells in some images. For the second error type, the light and color distribution on the images was not homogenous. Therefore, it is clearly realized that the preparation of the solution, the staining of the samples and the quality of the image obtaining considerably affect the performance of the cell counting system. The developed interface system gives flexibility to usersto compensate any improper markings as it is required. Nevertheless, it is possible to improve the system and to increase its performance with the help of new research with a larger number of images and different species.

Acknowledgments

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