Semi-automatic Image Processing Algorithm for Cell Counting in Neubauer Chambers

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Abstract— Cell counting is an essential field of cytometry that has many applications in biology and medicine. Neubauer chamber is often used in laboratories to count cells for research of different types of cell cultures. Manual (visual) counting is a tedious and slow process that requires a previously trained professional with full visual capability. This work proposed a semi-automatic algorithm for cell counting in Neubauer chambers based on digital image processing. Different from related works that have focused on small cells (such as red blood cells), this work focuses on big cells that are counted in the quadrants at the corners of the Neubauer chamber. Results for digital images obtained from Neubauer chambers from NIH/3T3 Fibroblasts cell cultures show an average error of 4.24% of our algorithm compared to a manual (visual) counting conducted by a biochemical specialist.

Keywords— Image processing, cell counting, Neubauer chamber, Hemocytometer, 3T3.

I. Introduction

Cytometry is the field of biology that studies the measurement of the characteristics of cells in quantitative terms. Cell counting is an essential subfield of cytometry that has widespread use in medicine and biology for research and medical diagnosis of various diseases. For example, complete blood count is frequently requested by medical doctors because it gives a detailed report of cell count for each cell type and also the concentration of proteins and minerals contained in blood, which helps the diagnosis of different diseases.

Early techniques to count blood cells were developed in the 19th century [1]. The best-known technique is based in a thick flat piece of glass with a rectangular indentation that creates a chamber, so-called Neubauer chamber (also known as Hemocytometer). The cells to be analyzed are placed in the top of Neubauer chamber, immersed in a specific volume of fluid, and an optical microscope is employed to assist the specialist to count the cells in a visual way. We referred this visual counting as "manual cell counting". An overview of the manual cell counting using Neubauer chamber is shown in Section II.

Nowadays, it is available on the market many advanced and high-cost equipment capable of counting cells in an automated way. The Flow Cytometer can count cells in a short time and with high precision so that it is used in most clinic analysis laboratories for blood cells counting. In developed countries, in which the budget for scientific research is substantial, the use of this equipment is also a reality in research laboratories. However, in developing countries like Brazil, in which the budget for scientific research is scarce, acquire and maintain that equipment for automatic cell counting is impractical. Unfortunately, the reality of those research laboratories in developing countries indicates that most of them do not have even optical microscopes with a digital camera coupled to them for digital image capturing.

Considering the current situation of scientific research laboratories in Brazil and other developing countries, there is a demand for low-cost techniques to automate cell counting. Related works [2]–[4] propose techniques for automatic and semi-automatic cell counting based on digital image processing. However, they considered only small cells, such as red blood cells, that are deposited in the center quadrant of the Neubauer chamber. No related work can be found targeting big cells that are deposited in the corner quadrants of the Neubauer chamber.

This work proposes a semi-automatic algorithm for cell counting based on digital image processing. A regular smartphone digital camera captures the image from an inverted optical microscope, and the proposed algorithm processes the image and provides the cell counting. Results using images of big cells from NIH/3T3 Fibroblasts cell cultures show an average error of 4.24% compared to manual (visual) counting conducted by a specialist.

The paper is organized as follows. Section II presents an overview of manual (visual) cell counting in Neubauer chambers. Section III details our proposed algorithm. Section IV shows results and discussion. Section V concludes the work.

II. Cell Counting in Neubauer Chamber

Initially, the Neubauer chamber, also known as Hemocytometer, was developed to identify the concentration of blood cells. Nowadays the counting of blood cells is done with modern equipment, and the Neubauer chamber is used in research laboratories to determine the number of cells in prepared suspension for scientific research.

It is important to mention that the manual counting depends on many aspects, for example, the characteristics of the deposited fluid between the chamber and the blade. This fluid must be homogeneous and must have an adequate concentration, to avoid the superposition of cells that may be caused by a high quantity of cells. On the other side, the limited quantity of cells must be avoided, because it can create and overestimate the statistical error. Not less important, the manual counting requires a previously trained professional with full visual capability.
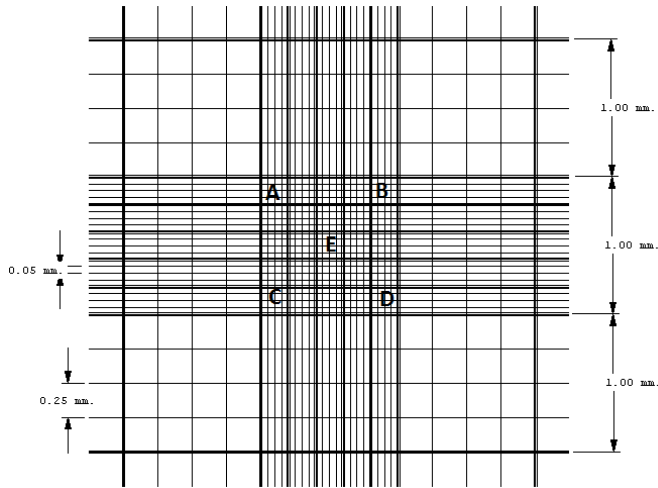
Fig. 1.   Neubauer chamber.

The Neubauer chamber is a square with a gridded area of nine 1 x 1 mm squares (shown in Fig. 1). The manual method used for big cells counting consists of adding the number of cells found in each one of the four outer quadrants of the Neubauer chamber. Each quadrant is subdivided into 16 compartments. There are many techniques of manual counting inside each quadrant, e.g., zig-zag technique, that helps to obtain exact values, and techniques that divide the quadrant into two-by-two compartments, among others. However, although there are mechanisms to improve manual cell counting, it is very imprecise and demands much time of the researcher, delaying the process of manipulating the cells and incurring in prejudices of cell viability due to that time wasted.

Some procedures must be followed to obtain an accurate count of cells in the Neubauer chamber. Such procedure is divided into the five following steps.

Step 1. Sample preparation

First, the cell suspension should be homogenized and transferred in a sterile environment to a microtube with 0.1 ml concentration. Afterward, it should be added 0.3 ml of 0.2% trypan blue dye in the test tube together with the cell suspension, thus obtaining a 1/4 dilution. Depending on the type of sample, the dilution preparation can be altered to increase or decrease the concentration for better counting of the cells.

Typically, the concentration ranges from 250.000 cells/ml to 2.5 million cells/ml on counting using the Neubauer chamber.

Step 2. Introducing the sample into the Neubauer chamber

We must withdraw $10\mu l$ with the pipette and place the tip on the edge of the cover, carefully filling the Neubauer chamber. The suspension must fill only one side of the chamber.

Step 3. Microscope set up and focus

After insertion of the suspension, the Neubauer chamber is introduced under a microscope, allowing visualization of the grid of the quadrants. The focus and intensity of the light should be adjusted in the microscope for better visualization. The markings of the quadrants have different dimensions, allowing counts of cells of different sizes, large or small.

Step 4. Cell counting

Different laboratories have different methods for counting cells. However, one of the most commonly used patterns is zig-zag. For larger cells, one should count the four big quadrants and record the values obtained. The cells presented above or outside the outer lines of each quadrant of the Neubauer chamber should be discarded from the counting.

Step 5. Concentration calculation

In this step, the formula for the calculation of the concentration is applied, as shown in Eq. 1.

$$Concentration\ (cell/ml) = \frac{Number\ of\ Cells}{Volume\ (in\ ml)} \quad (1)$$

The number of cells will be the sum of all the counted cells in all squares counted. The volume will be the total volume of all the squares counted. Since the volume will be of 1 big square is:

0.1 cm x 0.1 cm = 0.01 cm^2 of area counted.

Since the depth of the chamber is 0.1 mm:

0.1 mm = 0.01cm

0.01 cm^2 $*$ 0.01 cm = 0.0001 cm^2 = 0.0001 ml = 0.1 μl

So, for the Neubauer chamber, the formula used when counting in the big squares as shown in Eq. 2.

$$Concentration = \frac{Number\ of\ cells\ \times\ 10000}{Number\ of\ squares} \quad (2)$$

In case a dilution was applied, the concentration obtained should be converted to the original concentration before the dilution. In this case, the concentration should be divided by the dilution applied, as shown in Eq. 3. Depending on the type of sample, a dilution with a suitable concentration should be prepared for cell counting.

$$Concentration = \frac{Number\ of\ cells\ \times\ 10.000}{Number\ of\ square\ \times\ dilution} \quad (3)$$

III. RELATED WORKS

There are some works in the literature about automatic and semi-automatic cell counting algorithms based on digital image processing. The work in [2] proposes a semi-automatic method to count cells in Neubauer chamber. The user must identify the region of interest (ROI) and inform the algorithm to start the counting process that is conducted through 4-p connected algorithm. The method was tested by counting yeasts in the prepared suspension. The work in [3] proposes an automatic red blood cells (RBC) counting system for wild animals blood analysis. The algorithm employs the use of morphology and segmentation operations. The algorithm was tested with RBC of three animal species (Leopardus pardalis, Cebus apella and Nasua nasua). The work in [4] introduces an automatic method that uses edge identification to isolate the chamber grid and define ROI to count the cells of each grid. The algorithm was tested by counting spores of Clonostachys rosea fungus.

The related works [2]–[4] proposed algorithms to count small cells, which are counted in the quadrant in the center

Fig. 2.   Experimental setup.

Fig. 3.   Original Image (RGB format).

of the Neubauer chamber. No other method was found in the literature to count big cells, that must be counted in the four quadrants on the corners of the Neubauer chamber. It is important to mention that the quadrants in the corner are more suitable to the distortion of the lens of optical microscopes. This work focuses on proposing an algorithm to count big cells, that are counted in the four quadrants in the corner.

IV. PROPOSED ALGORITHM AND EXPERIMENTAL SETUP

A. Experimental Setup

The image acquisition was performed by using an inverted optical microscope (Nikon TS100-F) with 40X objective lens. An Asus Zenfone 3 smartphone with 16 MPixel digital camera (4656 x 2620 pixels resolution) was coupled to the microscope with a holder that was built with a 3D printer, as shown in Fig. 2. The work uses images from 7 experimental samples of NIH/3T3 Fibroblasts cell cultures. The digital images captured by the smartphone's camera are stored in compressed form in the Joint Photographic Experts Group (JPEG) format. The contrast control and image focus were kept in automatic mode to obtain a better sharpness of the cells. The microscope uses a green filter in order to obtain better contrast from to cells to the background, allowing a better precision in the automatic cell counting. An example of an image captured from this setup is shown in Fig. 3. The image of that quadrant of interest of the Neubauer chamber is captured and imported in the MATrix LABoratory (MATLAB) software that is equipped with Image Processing Toolbox.

B. Proposed Algorithm

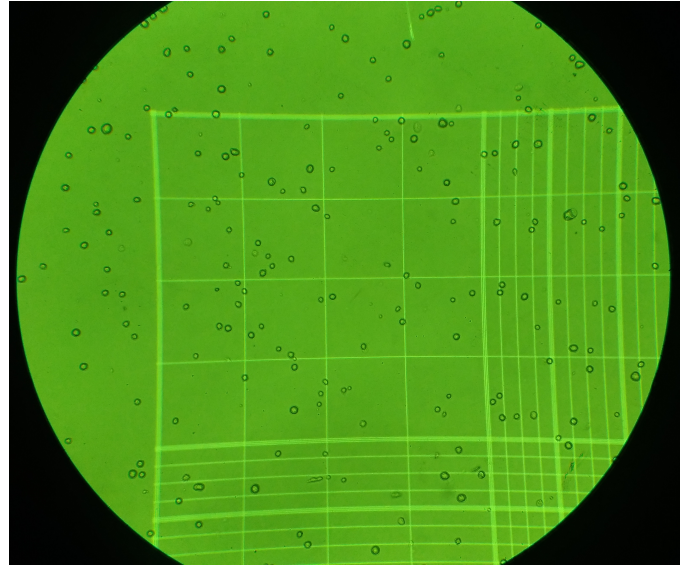The proposed algorithm is composed of some steps from the image acquisition to the cell labeling algorithm. The block diagram of the proposed algorithm is shown in Fig. 4. First, due to the distortion of the microscope lens, the user must select eight points of the input image to form the region of the interest (ROI) that is an adjusted image that corrects the lens distortion. Fig. 5 shows the selection of the eight points on the input image and Fig. 6 shows the output ROI adjusted image. This image is then converted to grayscale, and then it is binarized with the threshold determined by the Otsu's method from the image's histogram [5].

After the binarization process, morphological operators are applied to remove small points in the image that do not represent cells. The erode morphological operator reduces the size of the cells, disconnecting some cells from the others and reducing them to the size of one pixel. To identify in the image the cells that are counted from the ones that are not counted by the algorithm, the dilatation morphological operation that transforms the cells, previously represented by one pixel, to a disk of 4-pixel size. Finally, an 8-connected objects labeling algorithm is applied to that image to identify cells and count them.

V. RESULTS AND DISCUSSION

Fig. 7 presents the resulting image, with the cells highlighted with the orange color that represent the cells that are counted by the proposed algorithm. Some cells are not counted due to the threshold used for binarization and also to the contrast of the image obtained by the smartphone camera.

Table I presents the results of cell counting of 7 samples of images of quadrants of the Neubauer chamber from NIH/3T3 Fibroblasts cell cultures. The table compares the manual counting that is done by a biochemist with experience in manual counting. The manual counting is used as a reference to compare with our proposed algorithm (what we have referred to as automatic counting). Table I shows an average error of 4.24% of automatic cell counting (our algorithm) compared to manual (visual) counting conducted by the biochemical specialist. Moreover, the time for counting is reduced from
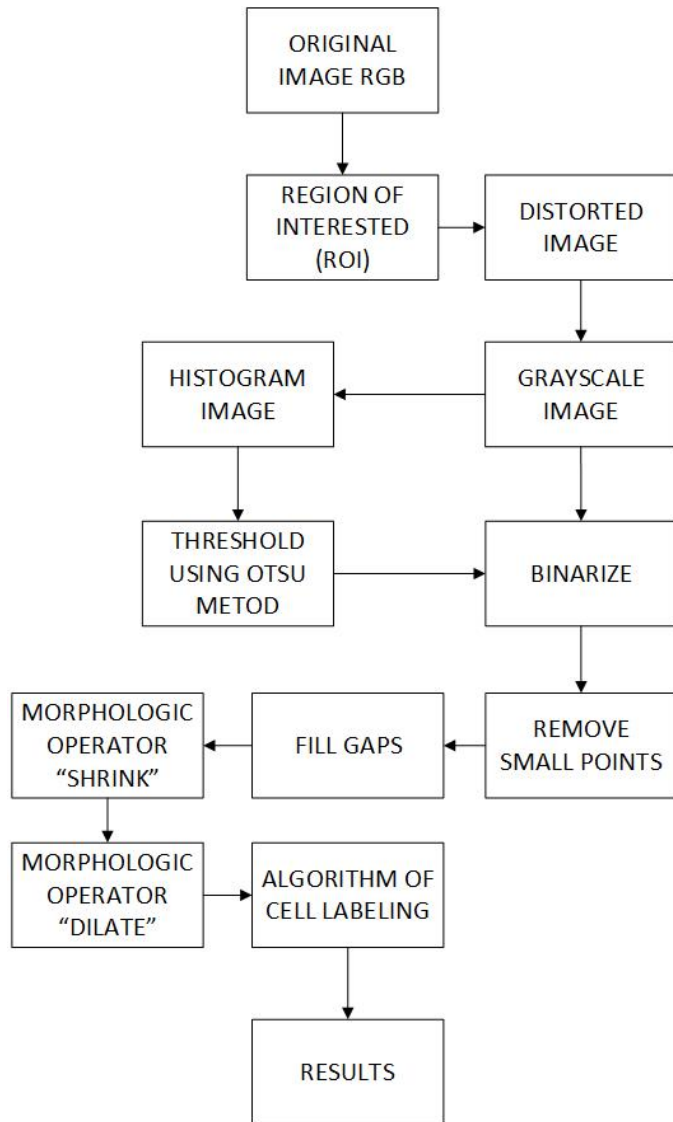
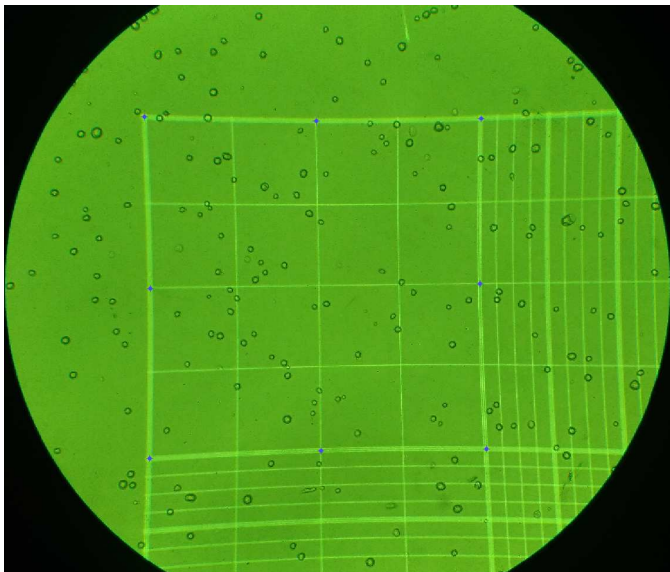Fig. 4.   Block diagram of the proposed algorithm

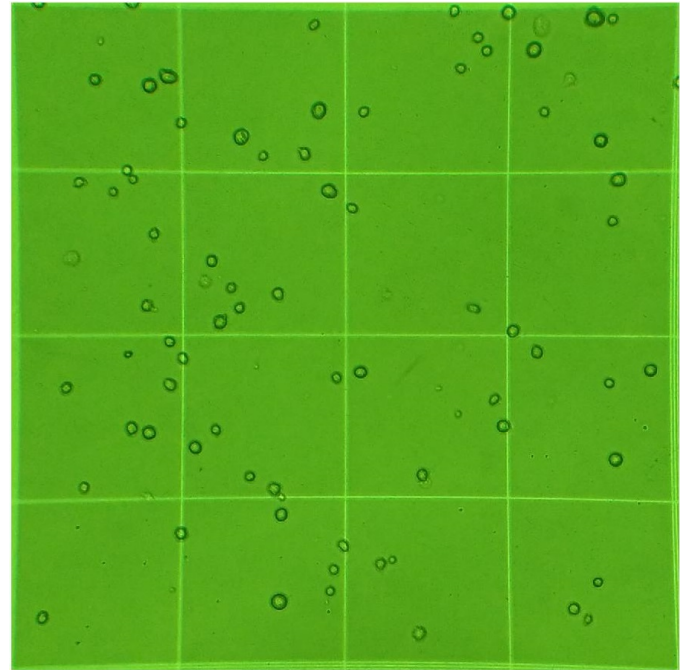Fig. 5.   Region of interest (ROI) selection (eight points in blue).

Fig. 6.   Region of interest (ROI)

TABLE I

CELL COUNTING RESULTS WITH THE PROPOSED ALGORITHM COMPARED
TO VISUAL COUNTING

	Number of cells		Error	Error %
	Manual	Automatic		
Sample 1	185	185	0	-
Sample 2	164	154	10	6.1%
Sample 3	137	135	2	1.5%
Sample 4	69	71	2	2.9%
Sample 5	60	56	4	6.7%
Sample 6	39	42	3	7.7%
Sample 7	62	65	3	4.8%

some minutes (for manual cell counting) to less than 1 second, when using our method.

It is not possible to compare the obtained error between automatic and manual cell counting with related works [2]–[4], since their methods are based on small cells that are deposited at the center of the Neubauer chamber and are not prone to errors such as the lens distortion. Reports also indicate that there is a typical error of 10% in manual cell counting [6]. Therefore, errors below 10% are tolerable by biochemists when counting cells using the Neubauer chamber.

VI. CONCLUSION

This work proposed a semi-automatic algorithm for cell counting in Neubauer chambers based on digital image processing. Different from related works, that have focused on small cells (such as red blood cells), this work focused on big cells, in which cells are counted in the quadrants at the corners of Neubauer chamber, where lens distortion problems difficult the automatic image processing. Results for images from NIH/3T3 Fibroblasts cell cultures show an average error of 4.24% of our algorithm compared to a manual (visual)
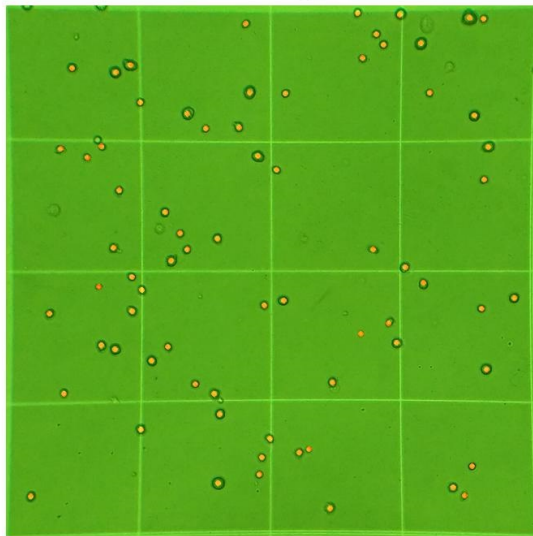
Fig. 7. Output image. Orange marks highlight the cells identified by the algorithm.

counting conducted by a biochemical specialist. As future works we will research techniques for automatic cell counting.

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