Semiautomated Segmentation and Measurement of Cytoplasmic Vacuoles in a Neutrophil With General-Purpose Image Analysis Software

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Background: Morphological observation of blood or marrow film is still described non-quantitatively. We developed a semiautomatic method for segmenting vacuoles from the cytoplasm using Photoshop (PS) and Image-J (IJ), called PS-IJ, and measured the relative entire cell area (rECA) and relative areas of vacuoles (rAV) in the cytoplasm of neutrophil with PS-IJ. **Methods:** Whole-blood samples were stored at 4°C with ethylenediaminetetraacetate and in two different preserving manners (P1 and P2). Color-tone intensity levels of neutrophil images were semiautomatically compensated using PS, and then vacuole portions were automatically segmented by IJ. The rAV and rECA were measured by counting pixels by IJ. For evaluating the accuracy in segmentations of vacuoles with PS-IJ, the rAV/rECA ratios calculated with results from PS-IJ were compared with those calculated with human eye and IJ (HE-IJ). **Results:** The rECA and rAV/ in P1 significantly ($P < 0.05$, $P < 0.05$) were enlarged and increased, but did not significantly ($P = 0.46$, $P = 0.21$) change in P2. The rAV/rECA ratios by PS-IJ were significantly correlated ($r = 0.90$, $P < 0.01$) with those by HE-IJ. **Conclusion:** PS-IJ method can successfully segment vacuoles and measure the rAV and rECA, becoming a useful tool for quantitative description of morphological observation of blood and marrow film. J. Clin. Lab. Anal. 30:918–923, 2016. © 2016 Wiley Periodicals, Inc.

Key words: quantitative; differentiation; area; Image-J; Photoshop

INTRODUCTION

Morphological observation of blood or marrow film and the differentiation of leukocyte is still one of the most fundamental laboratory tests. However, test results are still described nonquantitatively, and the standard of differentiation is also determined subjectively. Therefore, new tools or methods for quantitative and objective description of morphology are necessary to be developed. For image analysis of a blood cell, especially of a leukocyte, the segmentation of the nucleus from the cytoplasm has been reported (1), and the localization and segmentation of lymphoblastic cells from a peripheral blood film have also been reported (2). The sizes of each

cell and granules in cytoplasm and the color of the latter are listed higher as the valuable points in differentiation of leukocytes (3). The number of granules in the cytoplasm of neutrophil is one of the most valuable and pathognostic points for the diagnosis of myelo-dysplastic

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syndrome (MDS). However, the number of granules has only been evaluated subjectively and is still also lacking in quantitativity and reproductivity. We cannot find any report about tools or methods for quantitatively evaluating characteristics of organelles or small structures in cytoplasm, such as granules or vacuoles. However, we know how various numbers and sizes of vacuoles are produced in the cytoplasm of neutrophil (4). Therefore, we began with semiautomatically segmenting vacuoles in the cytoplasm, because vacuoles are easier to analyze than granule because of their bigger size and the uniformity of color tones inside. We developed a new quantitative image analysis method using two types of general-purpose image analysis software, Photoshop and Image-J, which is called PS-IJ. With this method, we semiautomatically segmented vacuoles from the cytoplasm in a neutrophil, and measured the area of every vacuole. In this article, we also discuss the usefulness of the method.

MATERIALS AND METHODS

Samples

Venous blood samples were collected from five healthy volunteers (males, 21~27 years old), after obtaining informed consent.

Method

Vacuoles formation in the cytoplasm of neutrophils

Vacuoles formation (VF) in the cytoplasm of neutrophils has been already observed after preservation of a whole blood sample (5). The VF in the cytoplasm increases with the time of preservation, of blood samples at 4°C (4). In this study, we quantitatively evaluate the process of increase in VF and the difference with PS-IJ method.

Blood collection and preservation

Each venous blood sample was stored at 4°C in Vacutainer blood-collecting tubes (Becton Dickenson, NJ) containing dipotassium ethylenediaminetetraacetate (EDTA).

Two different preservation manners (PMs) were set up for preserving the blood samples. The first PM (P1) involved preparing only one blood sample tube for smearing of blood films at four periods (0, 48, 96, and 120 hr), which was turned over and the cap was opened for every smearing, recapped, and stored again for the next. The second PM (P2) involved preparing six blood sample tubes for the same four periods of smearing blood films from 0 to 120 hr, in which the blood sample tubes were

neither turned over nor the caps were opened until the appointed time for smearing.

Preparation and observation of blood films

Four microliters of whole blood was smeared on a glass slide with the wedge method in every scheduled period after blood collection. These slides were dried with cold air and stained with May-Glünwald and Giemsa dyeing solution (Wako Pure Chemical Industries, Tokyo, Japan) according to the standard protocol (6). The morphology of a neutrophil in the blood film was observed at 600× magnification. Photographs of 20 cells from a blood film, which were not obviously determined as lymphocytes, monocytes, eosinophils, or basophiles, were taken consecutively (Fig. 1a) with a digital camera for microscopes (Dino-Eye, 1.35 megapixels, AnMo Electronics Corporation, New Taipei City, Taiwan).

Quantitative evaluation of morphology (PS-IJ method)

Color-tone compensation of each original neutrophil image (Fig. 1a) was done for increasing the contrast of color tones between vacuoles and the other portions of each cell (Fig. 1b) using “Image > Adjustment > Tone Curve” tool (Fig. 1e) in PS CS6 (v. 13.0, Adobe Systems Incorporated, San Jose, CA, USA). This procedure made it easy to binarize the images, and the vacuole portions were consequently easy to separate from the cell using IJ (v. 1.43, Freeware). The outer edge of the cytoplasm in every neutrophil was manually bordered using a mouse or a graphics tablet (Bamboo pen and touch, Wacom, Tokyo, Japan). The outside of the cell was cut away with the “Edit > Clear Outside” tool in IJ. After binarization, the vacuole portions were automatically painted with red (Fig. 1c). Then, the pixels of each whole cell were counted using the “Analyze > Histogram” tool (Fig. 1f) and the vacuoles were counted using the “Analyze > Analyze Particle” tool (Fig. 1d, g, and h) in IJ. A table of the histogram in “csv” formed (Fig. 1i) output by the “Analyze > Histogram” tool in IJ. With this table, the ratio of relative area for all the vacuole portions to the entire cell area (rAV/rECA ratio) was calculated using Excel 2007 (Microsoft, Redmond, WA, USA).

One blood film was respectively prepared for P1 or P2 on day 1 (0 hr), day 3 (48 hr), day 5 (96 hr), and day 6 (120 hr) during preservation. From each of the eight films, six typical ones were selected from a total of 20 neutrophil images as objects for quantitative evaluation with PS-IJ. A total of 48 neutrophil images were evaluated with PS-IJ. For all images, the total number of pixels of the ECA was counted and the rAV/rECA ratio was calculated.

Fig. 1. PS-IJ and object image for comparison with HE-IJ. (a) Original full-colored image of neutrophil, which was stained using May-Glünwald Giemsa stain at 600× magnification. (b) Image after compensation procedure with PS. (c) Image after cutting outside away, resolved into 256 graduations of gray scale with eight-bit analog-to-digital conversion then binarizing using “Image > Adjust > Threshold” tool in IJ. Higher-intensity (brighter or whiter) parts are displayed in red and lower (darker or blocky) intensity parts are displayed in blue. (d) Image of selecting vacuole portions with “Analyze > Analyze Particle” tool in IJ is added to original full-colored image. (e) Example of compensations for all three primary colors. Intensity of each pixel (input) is indicated on X-axis, and that (output) in resulting image after compensation procedure is indicated on Y-axis. Dull gray graph indicates histogram of image (Fig. 1a). Line of each primary color indicates relation between input intensity and output intensity for each primary color. In this case, input intensity of blue changed to brighter and narrower range of intensity than original one. Lower-intensity green and red parts changed to the darkest (blackest) intensity, and higher-intensity parts were displayed in wider range of intensity than original ones. (f) Histogram of intensity for each pixel of image (c). IJ outputs table of these results with “csv” form file, which is readable with Excel. Number of pixels except for white portion (intensity of 255 and neighbor values) of image, which corresponded to outer portion of neutrophil in image (c), is defined as relative area of entire cell (rECA). (g) Image indicating vacuole portions and the results of counting them in the cytoplasm of image (a).

Comparison of PS-IJ with human eye-IJ

For evaluating the equivalency of judgment on the vacuole portion with PS-IJ to that with the human eye (HE) and IJ, the ratios of rAV/rECA for an image of the same neutrophil, which are calculated with the two different methods, were compared. Five neutrophil images before processing with PS (Fig. 1a) were selected as objects for the analysis with HE. The rAV/rECA ratios of the neutrophils had been already calculated with PS-IJ; 9.31%, 17.49%, 18.36%, 19.47%, and 23.04%, respectively. The same set of five images, exhibiting varied rAV/rECA ratios from 9.31% to 23.04%, were presented to five volunteers who were fourth-year undergraduate students and had already studied laboratory hematology. The outline of each cell and the vacuole portions in the cytoplasm were determined with their eyes. The former were traced and the latter were hand-painted with black, respectively

(Fig. 1j), by putting tracing paper over an original image of a neutrophil. These traced and painted results (Fig. 1j) were used with IJ as the next objects for counting the total number of pixels of both the ECA and the vacuole portions in each neutrophil, and then the rAV/rECA ratio was calculated. These rAV/rECA ratios with HE-IJ (Fig. 1k) were compared with those with PS-IJ (Fig. 1g). The correlation coefficient between the two rAV/rECA ratio results was calculated.

Statistical analysis

Statistical analysis was conducted using EZR (v. 1.10, Free Software, Japan) with one-way layout analysis of variance (one-way ANOVA), multiway ANOVA, and Pearson's product-moment correlation (7). These comparisons were conducted using Dannett's method and Tukey's method; $P < 0.05$ was considered significant.

Fig. 2. Images of neutrophils from blood film of donor F (MGG stain). Six representative images of neutrophils on day 1, immediately after blood collection (0 hr), day 3 (after 48 hr), and day 6 (after 120 hr) are indicated in tables (a) for P1 and (b) for P2. Every blood film was stained with MGG $\times$ stain. Every neutrophil was observed at the magnification of 600 $\times$. Blood sample for smearing films for images in (a) was preserved in P1, and that in (b) was preserved in P2.

RESULTS

Observation on the Process of Morphological Changes in Neutrophils

We evaluated six typical images from the 20 neutrophil images in each blood smear after 0, 48, 96, and 120 hr of preservation (Fig. 2). The changes in the area and VF in the neutrophils were estimated subjectively.

For P1, the area of each neutrophil seemed to increase after preservation with EDTA. The VF in the cytoplasm seemed to increase after preservation with EDTA (Fig. 2a).

For P2, the area of each neutrophil did not seem to noticeably change after preservation with EDTA. The VF in

the cytoplasm also did not seem to increase after preservation (Fig. 2b).

Next, we compared the differences in both the area of each neutrophil and VF in the cytoplasm between P1 and P2. There did not seem to be any difference in the area between P1 and P2. The VF after preservation in P1 seemed to have increased more noticeably than that in P2.

Quantitative Evaluation of Morphology

We conducted quantitative evaluation using our PS-IJ (Fig. 1a–i) for both the relative areas and rAV/rECA ratios of the five neutrophils mentioned in the Materials and Methods section. The average relative area of these images

Fig. 3. The relative areas (a) and rAV/rECA ratios (b) of all neutrophils in the two different manners of preservation with EDTA. (a) Process of increasing average relative area of entire cell in neutrophils preserved in P1 and P2 with EDTA is indicated. (b) Process of increasing average ratio of vacuole portions to relative whole cell area (rAV/rECA ratio) of neutrophil preserved in P1 and P2 with EDTA is indicated.

for P1 (Fig. 3a) significantly ($P < 0.05$) increased, but did not significantly ($P = 0.46$) change for P2 (Fig. 3a). This increase did not significantly ($P = 0.07$) differ between P1 and P2 (Fig. 3a).

The average rAV/rECA ratio of the images for P1 (Fig. 3b) significantly ($P < 0.05$) increased, but did not significantly ($P = 0.21$) change for P2 (Fig. 3b). This increases significantly ($P < 0.05$) differ between P1 and P2 (Fig. 3b).

The average pixel count of the rECA of all neutrophils with EDTA for P1 (Fig. 3a) was $9,127 \pm 534$ immediately after blood collection and significantly ($P < 0.05$) increased 1.65 times to a maximum of $15,092 \pm 4,511$ 120 hr later. With EDTA for P2 (Fig. 3a), the count was $8,930 \pm 1,193$ immediately after blood collection and was not significantly ($P = 0.46$) increased 1.40 times to a maximum of $11,778 \pm 1,611$ 48 hr later. The enlargement of the average rECA for P2 was significantly ($P < 0.001$, two-way ANOVA) smaller than that for P1.

The average rAV/rECA ratio of all the neutrophils with EDTA for P1 (Fig. 3b) was $6.2 \pm 3.0\%$ immediately after blood collection and significantly ($P < 0.05$) increased 1.74 times to a maximum of $10.8 \pm 3.7\%$ 96 hr later. With EDTA for P2 (Fig. 3b), the ratio was $4.7 \pm 2.6\%$ immediately after blood collection and was not significantly ($P = 0.21$) increased 1.93 times to a maximum of $8.0 \pm 3.8\%$ 120 hr later.

Comparison of PS-IJ With HE-IJ

Five volunteers clearly traced the outline of each original image of a neutrophil and painted the inside of each vacuole in the cytoplasm of the neutrophils on tracing paper. A total of 25 images were processed with IJ, and the rAV/rECA ratios of the 25 images are indicated in Figure 4. The result, in which the rAV/rECA ratio was calculated to be 23.04% with PS-IJ, was excluded from the further analysis, because it was quite smaller than the other four results. The average rAV/rECA ratio for the 24 results was $20.9 \pm 6.20\%$ with HE-IJ and $17.30 \pm 4.57\%$ with PS-IJ. The ratio was a little higher with HE-IJ than with PS-IJ; however, the correlation coefficient for the 24 results was $r = 0.90$ ($P < 0.01$, Fig. 4).

DISCUSSION

The results of morphological observations of blood or marrow cells, which is one of the most fundamental tests for differentiating leukocyte, are still described nonquantitatively. The standard of the differentiation for normality is mostly determined subjectively by individual examiners. The sizes of each leukocyte and small organelles such as granules or vacuoles in cytoplasm and the color of the

Fig. 4. Correlation between HE-IJ and PS-IJ. rAV/rECA ratios of 25 neutrophils calculated with PS-IJ are indicated on X-axis. Those with HE-IJ are indicated on Y-axis.

latter are the valuable points in differentiation of leukocyte (3). So, we started with developing a new quantitative image analysis method. We used IJ for this purpose. However, IJ is designed basically for eight-bit gray-scale images. It is likely that important information in the original full-color image will be lost, by only simple and automatic conversion of full-color blood images to eight-bit gray-scale images. Consequently, the necessary or important parts for quantitative measurement in an original full-color image must be emphasized before the conversion. This procedure for each image is conducted using PS in this study. The input image of each primary color, that is, red, green, and blue, is converted to the output image by the functions, which are indicated by lines of each primary color (Fig. 1e). The procedure is repeated with little change in every line of the previous step until the output result is subjectively judged by an examiner as the most optimal image as objects for evaluation with IJ. This is one of the major causes for the increase in required time for image processing with PS-IJ because more than 5 min is required for making the most of optimal image.

The rAV/rECA ratios with PS-IJ demonstrate a very good correlation ($r = 0.90$, $P < 0.01$) to those with HE-IJ. This suggests that PS-IJ enables correct determination of the vacuole portions in every cell compared with that with HE-IJ. However, the rAV/rECA ratio calculated with HE-IJ is a little higher than that with PS-IJ. This difference might be the result of the microscope and digital camera performances used in this study, that is, the number of pixels, resolution, and sensitivity, which are insufficient at the boundary of each vacuole, are not clearly identified. If a high-quality microscope and digital camera are used, both values might be similar. We did not use higher-quality microscopes or digital cameras for this study due to a limited budget, which is also a limitation of this study.

We used PS-IJ for quantitative evaluation of leukocytes. It can be also used as a tool for quantitative evaluation of the differences in staining performances with either different staining agents or staining systems, and can also be applied to count or calculate the ratio of positive cells or granules in a cell on a slide of pathological specimens.

We clarified in this study that simple or primary rather than very complicated tools in PS or IJ can be a useful and powerful method for processing medical images; however, there has been only one report to the best of our knowledge concerning medical image processing using these two types of software (8). We also clarified that PS-IJ currently requires a large amount of time and much manual work, which is a limitation for systems using general-purpose software. Some practical algorithms or methods for semiautomated analysis of microscope images have been recently proposed, with which a user interactively outlines one or more margins of a blood cell image (9). These will enable to put simple or primary method or tools in PS-IJ to practical use with high-performance hardware, in the near future.

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