original research report

Morphometry to identify subtypes of leukocytes

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INTRODUCTION: Recent studies in image cytometry evaluated the replacement of specific markers by morphological parameters. The aim of this study was to develop and evaluate a method to identify subtypes of leukocytes using morphometric data of the nuclei.

METHOD: The analyzed images were generated with a laser scanning cytometer. Two free programs were used for image analysis and statistical evaluation: Cellprofiler and Tanagra respectively. A sample of leukocytes with 200 sets of images (DAPI, CD45 and CD14) was analyzed. Using feature selection, the 20 best parameters were chosen to conduct cross-validation.

RESULTS: The morphometric data identified the subpopulations of the analyzed leukocytes with a sensitivity and specificity of 0.95 per sample.

CONCLUSION: The present study is the first that identifies subpopulations of leukocytes by nuclear morphology.

In 1969, flow cytometry became commercially available. Initially, the technique was of scientific interest rather than for public use. In 1975, Köhler and Milstein[1] reported on the production of monoclonal antibodies with high specificity for some antigens in different cellular compartments. Thereafter, flow cytometry evolved into an emerging resource in scientific and clinical research, enabling a fast, objective and quantitative analysis of cells in suspension.[2] The flow cytometric analysis is multiparametric, where physical and chemical characteristics of cells are measured. Single cells stained with fluorochrome-tagged monoclonal antibodies pass a laser beam and can be identified by size, granularity and fluorescence intensity.[3]

In 1991, a new type of cytometry, laser scanning cytometry (LSC), became commercially available.[4] LSC produces images during the cytometric analysis, thereby enabling a morphologic verification of the results. The possibility of morphological verification results in lower samples and reagents volume.[5–7] However, apart from size and granularity in both, flow cytometry and LSC do not generate morphological parameters of the analyzed cells.[8]

During the process of scanning, an image of the area of view is generated and converted to pixels that are related to wavelength and fluorescence intensity.[9] The data generated with LSC is similar to that of flow cytometry.[4,8]

Cellprofiler (CP), an open-source software for image analysis, was recently released.[10] Cellprofiler automatically identifies objects on images and quantifies a plethora of parameters of each identified cell. During the analysis, the software keeps records for each object, such as the location within the image, size, shape, and texture.[10,11]

The fluorescence signals are due to fluorochrome labeled antibodies bound to cell surface antigens that emit light with certain wavelength when excited by laser.[2] Among the applications within cytometry, immunophenotyping, that is, the identification of leukocyte subtypes based on membrane antigens, is considered.[12,13] Recently, studies were conducted employing image cytometry in order to replace specific markers for morphological data.[10,14] However, an identification of subtypes of leukocytes by nuclear morphology has not yet been conducted.

The combination of image cytometry and morphometry could substantially lower costs for

clinical exams, for example, in the case of HIV patients in South Africa. The present study aims to identify lymphocytes, monocytes and granulocytes by the nuclear morphology, thereby omitting the markers CD45 and CD14.

MATERIAL AND METHODS

Sample collection and preparation

Blood from healthy adult volunteers (n = 10) was collected into syringes coated with ethylenediaminetetraacetic acid and subsequently stained, adding 5µL of APC labeled anti-CD-14 and FITC labeled anti-CD45 in 40 µL blood respectively. DAPI was added to a final concentration of 0.5 µg/mL.

The samples were incubated for 20 min at room temperature in a dark environment. After antibody staining, the red blood cells were lysed (FACSLysis, BD Biosciences, Mountain View, CA), and specimens were washed in phosphate buffered saline (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) and then placed onto a conventional glass microscope slide (Menzel Gläser, Braunschweig, Germany).

The analyzed images were obtained using the cytometer iCys (Compucyte, Boston, MA, USA). Pictures were scanned 20× magnification. The dip depth of the image were 14, that is, pictures have a dynamic range of 65,536 pixel intensities. The iCys uses a photomultiplier to generate pictures, not a camera. Images were saved on hard disc in a Tiff file format. The analyzed sample consisted of 200 sets of images of DAPI, CD45 and CD14 (Fig. 1a, b and c, respectively), and a mean of 1,000 cells were analyzed per sample.

Analysis with CellProfiler

The CellProfiler software was programmed as depicted below: simultaneously, the images of DAPI, CD14and CD45 (Fig. 1a–c, respectively) were

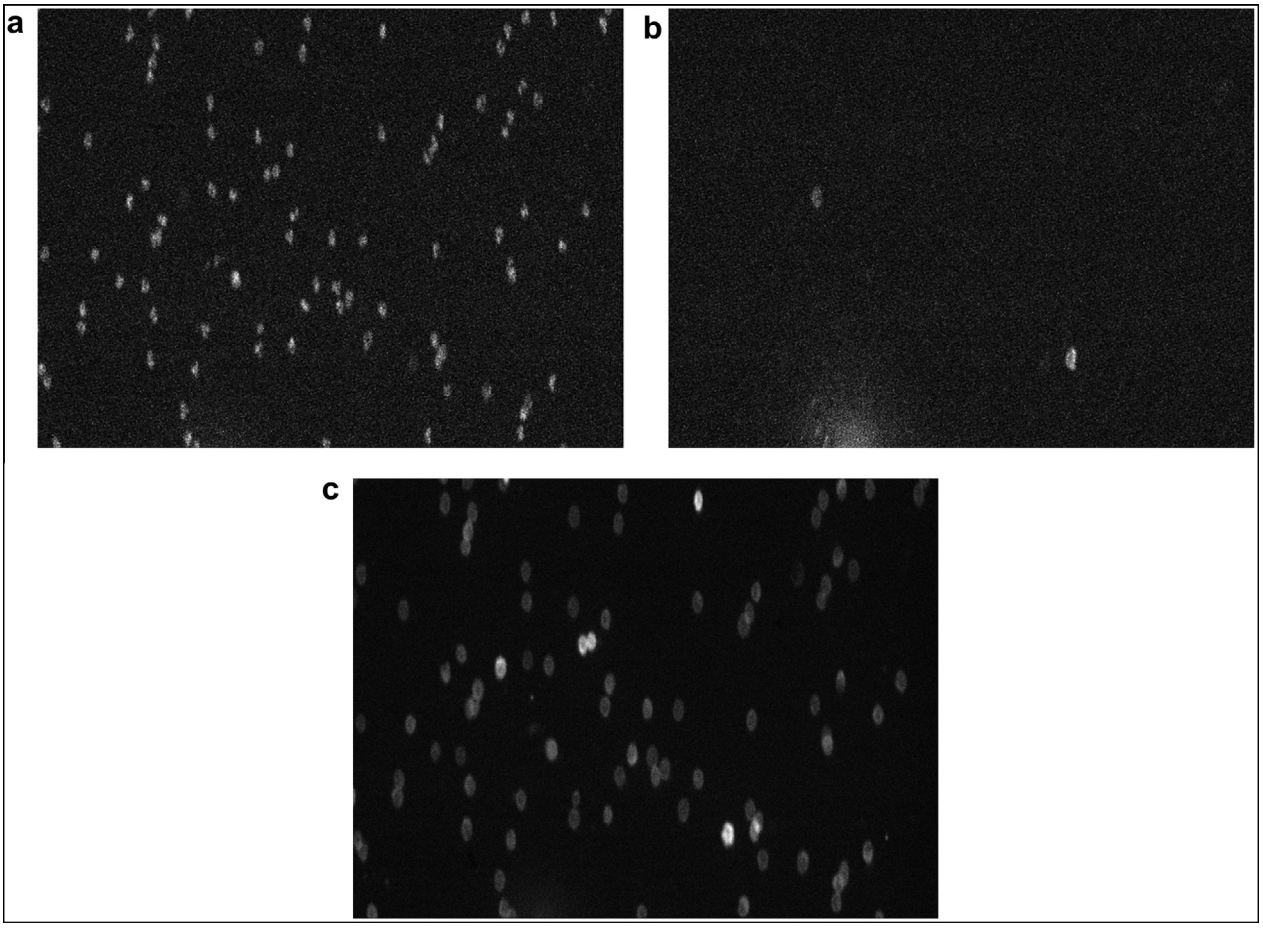

Fig. 1. (a) DAPI – nuclear signal as imaged by the cytometer. (b) CD14 – signal as imaged by the cytometer. (c) CD45 –signal as imaged by the cytometer.

loaded. Cells were identified automatically using the DAPI signal. For segmentation, the robust background adaptive algorithm was used. Minimum and maximum areas of 15 and 40 pixels were chosen. The identified objects were called nuclei (Fig. 2a). Within the objects called nuclei, intensity parameters of DAPI (e.g. minimal, maximal fluorescence intensity, integral intensity), as well as morphological parameters (e.g. area, perimeter, minor and major axis length, form factor, Euler number) were quantified. Subsequently, the objects called nuclei were expanded by three pixels. The resulting objects were called cells (Fig. 2b). Within the objects called cells, the fluorescence intensity of CD45 and CD14 was quantified. As a next step, the objects called cells were expanded infinitely; the resulting objects are a Voronoi diagram (Fig. 2c). Within the Voronoi diagram, texture parameters (e.g. eccentricity, granularity, angular second moment, Gabor X and Gabor Y) of the nuclei were quantified in different scales. After having finished the analysis, data were exported to Excel. A scatter plot was generated of the CD45 and CD14 intensities. Using this plot, cells were divided into lymphocytes, neutrophils and monocytes (Fig. 3).

Statistical processing

For statistical processing, the open-source software Tanagra[15] was used. It was first verified if the texture data of the nuclei was capable of replacing the markers CD45 and CD14. The cells were divided into three groups (lymphocytes, monocytes and neutrophils) and the morphological data of the nuclei such as parameters, feature selection and subsequent linear discriminant analysis (LDA) were taken. Linear discriminant analysis was performed using the free software Tanagra.[15] LDA is a machine learning process. In the present study, LDA was used to predict the origin of white cells formally identified by the CP. The origin of white cells was known by the authors, but not by the software. However, during

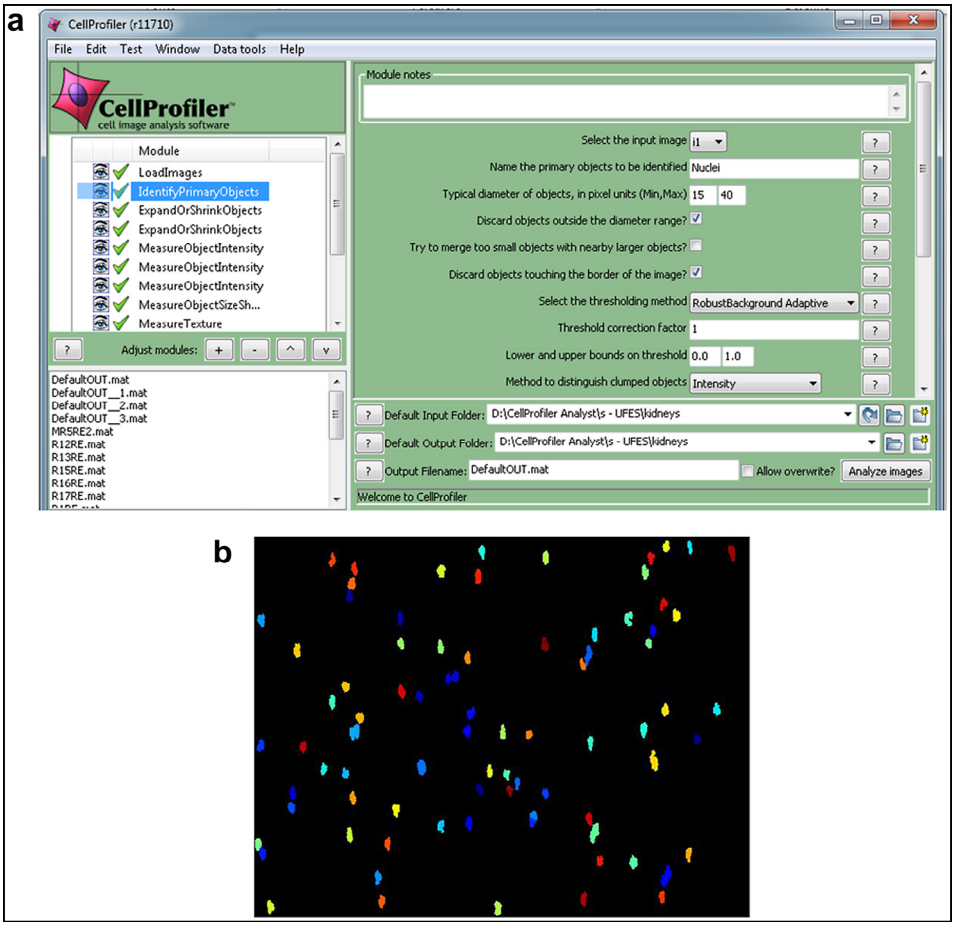

Fig. 2a. Objects called nuclei as automatically identified by the software.

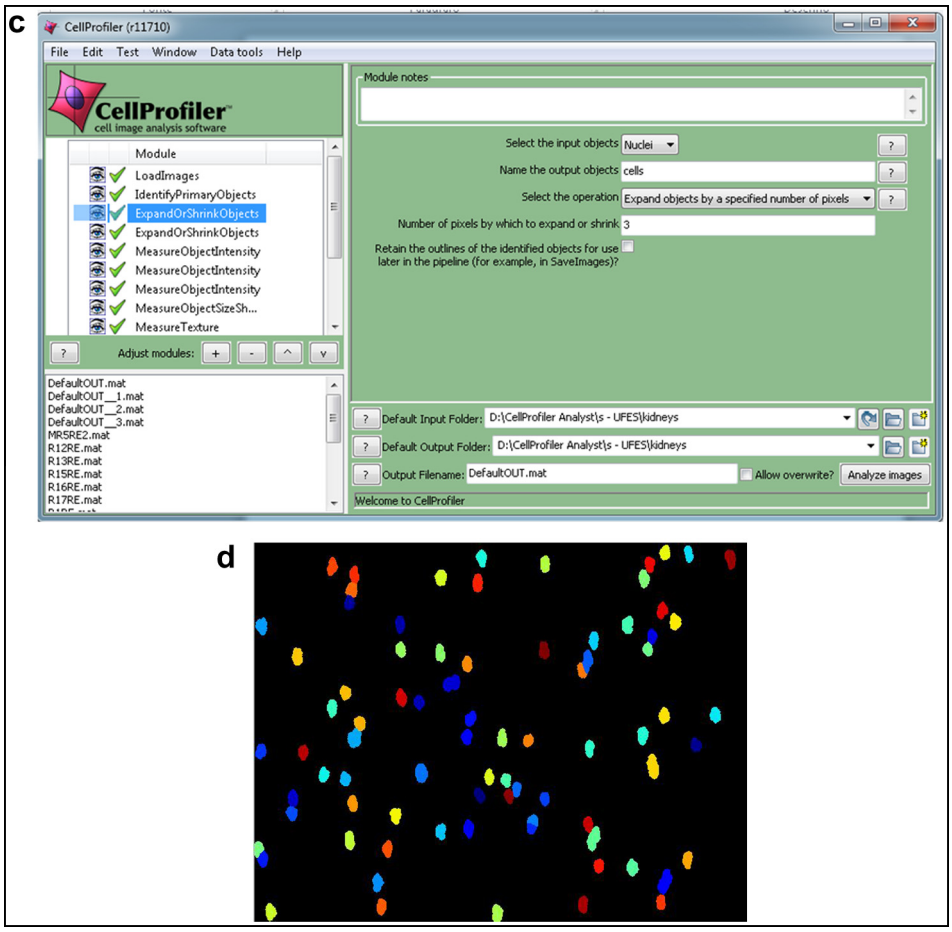

Fig. 2b. Objects called cells (i.e. nuclei expanded by three pixels respectively) as identified by the software.

the LDA, an origin for white cells was predicted. With the count of correctly predicted cells, false positives and false negatives, sensitivity and specificity for each population, (L, M, N) was calculated as described below.

$$\text{Sensitivity} = \text{true positive events}/(\text{true positive events} + \text{false negative events})$$

$$\text{Specificity} = \text{true negative events}/(\text{true negative events} + \text{false positive events}).$$

RESULTS

Using nuclear morphometry as given by the DAPI signal, lymphocytes, neutrophils and monocytes were identified. The identification with morphometry was compared to the identification by the CD45 and CD14 signal (Fig. 3) and finally, specificity and sensitivity were calculated. Values of sensitivity and specificity for each population are shown in Tables 1 and 2.

DISCUSSION

This is the first study that identifies subtypes of leukocytes by nuclear morphometry, thereby replacing the specific markers CD45 and CD14. Selinummi et al.[14] found out, that it is possible to replace markers for macrophages by morphological data. The authors also used the CellProfiler software. Lamprecht et al.[10] demonstrated a variety of applications for the program, such as yeast colony counts and the analysis of wound healing processes. CellProfiler is an open-source software that enables an automatic and reproducible image analysis. Each step of the image analysis can be morphologically verified. The morphometric parameters of the identified nuclei were obtained from the Voronoi diagram. Data gathering within the Voronoi diagram delivered better results than any other tested segmentation algorithm (data not shown). This type of diagram consists of an area with generating points; it is subsequently divided into polygons, and each polygon contains exactly one generating point. Each pixel

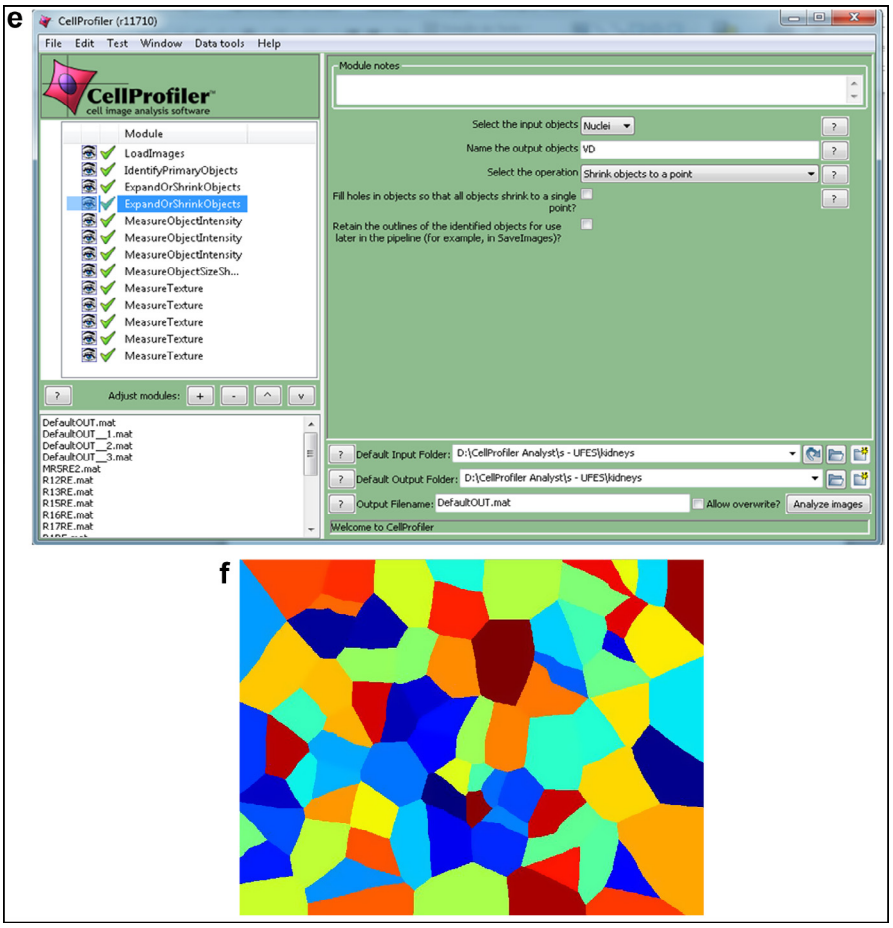

Fig. 2c. Voronoi diagram (i.e. objects called cells expanded infinitely).

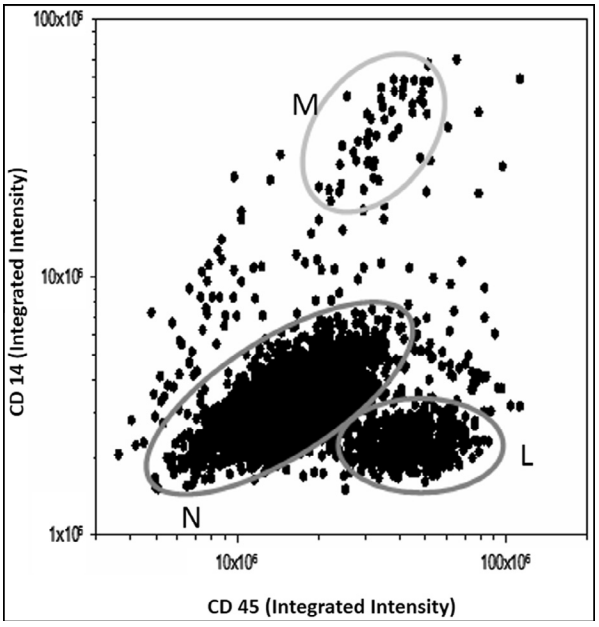

Fig. 3. Expression of CD45 (x-axis) and CD14 (y-axis) of all the cells of an analysis. Every dot represents one cell.

Table 1. Sensitivity.

	Lymphocytes	Neutrophils	Monocytes
Lymphocytes	–	0.77	0.85
Neutrophils	0.78	–	0.86
Monocytes	0.99	0.98	–

Table 2. Specificity.

	Lymphocytes	Neutrophils	Monocytes
Lymphocytes	–	0.78	0.99
Neutrophils	0.77	–	0.98
Monocytes	0.85	0.86	–

within a given polygon is closer to its point of generation than any other. There is no gap and no overlap in a Voronoi diagram.[16] Voronoi diagrams have been established in various studies about quantitative imaging.[11,17] Texture parameters used in this study have been used to detect changes in cellular physiology. It was discovered that changes in texture already appear if there is no visible difference. The consequences of this phenomenon are highly significant for diagnostic purposes.[18,19]

For statistical processing, the program Tanagra was used. With this software, the morphometric parameters were compared to the gold standard, CD45 and CD14. In the end, sensitivity and specificity were calculated to evaluate the predictive power of the parameters.[20]

The prediction of the different population yielded sensitivity and specificity of 0.95 in identifying subtypes of leukocytes per sample. Differentiation between lymphocytes and monocytes reached 99% certainty. The impact of its capability in identifying leukocytes could be of value for leukocyte counts in HIV patients, for example, in South Africa.[21]

Besides the reduction of costs for reagents, image cytometry is known to be performed with substantially less sample volume, thereby enabling studies in neonates suffering from congenital heart diseases,[22] or in animals like birds and small mammals.[23]

Image cytometry has changed areas of science by providing an automated, reproducible analysis where, in contrast to flow cytometry, the results can be verified visually. Furthermore, it is proven that image cytometry generates data similar to those of flow cytometry.[4,8]

CONCLUSION

It may be concluded that there is a strong correlation between identification of leukocytes employing specific markers, and the morphometric parameters used for the present study. To improve the results, further studies should be conducted using images with a higher magnification.

CONFLICT OF INTEREST

There is no conflict of interest to declare.

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