Computers in Biology and Medicine 96 (2018) 147–156

Contents lists available at ScienceDirect

Computers in Biology and Medicine

journal homepage: www.elsevier.com/locate/compbiomed

Accurate label-free 3-part leukocyte recognition with single cell lens-free imaging flow cytometry

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ARTICLE INFO

Keywords:
White blood cell
Lens-free imaging
Hologram
Flow cytometry
Three-part differential

ABSTRACT

Three-part white blood cell differentials which are key to routine blood workups are typically performed in centralized laboratories on conventional hematology analyzers operated by highly trained staff. With the trend of developing miniaturized blood analysis tool for point-of-need in order to accelerate turnaround times and move routine blood testing away from centralized facilities on the rise, our group has developed a highly miniaturized holographic imaging system for generating lens-free images of white blood cells in suspension. Analysis and classification of its output data, constitutes the final crucial step ensuring appropriate accuracy of the system. In this work, we implement reference holographic images of single white blood cells in suspension, in order to establish an accurate ground truth to increase classification accuracy. We also automate the entire workflow for analyzing the output and demonstrate clear improvement in the accuracy of the 3-part classification. High-dimensional optical and morphological features are extracted from reconstructed digital holograms of single cells using the ground-truth images and advanced machine learning algorithms are investigated and implemented to obtain 99% classification accuracy. Representative features of the three white blood cell subtypes are selected and give comparable results, with a focus on rapid cell recognition and decreased computational cost.

1. Introduction

White Blood Cells (WBC), or leukocytes, are the principal cells of the immune system and their main function is to protect and defend the body against foreign pathogens. Having precise information on the relative proportions of each of the three main white blood cell subtypes (i.e. lymphocyte, granulocyte and monocyte), a parameter called a three-part differential, constitutes important evidence in diagnostics of diseases as diverse as leukemia, viral or bacterial infections [1].

Conventional blood analysis is usually performed using hematology analyzers and elaborate microscopes, which are typically expensive and bulky, and require highly trained personnel to operate and accurately analyze results. Efforts are being undertaken to develop novel blood cell imaging technologies that can be integrated on-chip and bring about potential miniaturization of the current tools, rendering them amenable to the point-of-need [2–4].

Several studies demonstrated new imaging technologies that provide both spatial and spectral information to support cell identification and can also potentially be integrated into on-chip blood analysis systems. Examples include hyperspectral imaging [5–9], multispectral imaging [10] or Raman spectroscopic imaging [11]. However, these studies were limited to the examination of blood smear samples, the preparation of which has been shown to yield different cell morphologies when compared to cells in suspension [12]. Imaging flow cytometers [13–16] can provide optical and morphological information of cells in suspension beyond the single feature of a fluorescent label, but most systems are built with very complex optical components. Recently, novel lens-free imaging technologies have been described which are characterized by the absence of complex optical systems, offering advantages in terms of portability, scalability, and cost-effectiveness, and thereby bringing new opportunities for in-flow blood analysis [17].

Our group has developed a lens-free flow cytometer based on Digital Holographic Microscopy (DHM) for single leukocyte analysis [18]. The system captures holographic images of suspended single cells flowing by in a microfluidic channel. Earlier results on a three-part differential have been demonstrated by extracting two basic image features, diameter as

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https://doi.org/10.1016/j.compbiomed.2018.03.008
Received 9 November 2017; Received in revised form 7 March 2018; Accepted 11 March 2018

the measure for cell size and the internal complexity of a cell quantified using ridge detection based on scale-space analysis. In order to extend and improve this analysis, automation of a stable workflow for leukocyte recognition is required. Automated cell recognition usually requires several essential processing steps including feature extraction [15,16,19–22], feature selection [15,23–26] and classification [15,16,19–22]. However, simply borrowing the existing methods does not guarantee good results due to the differences in the image modalities or the differences in cell morphology. Therefore, a comprehensive feature study and machine learning algorithm comparison is needed to enhance the accuracy of 3-part leukocytes classification and bring it to the level of accuracy required in the clinic.

In this work, we focus on the image processing and cell recognition to improve the 3-part leukocytes classification using an extension of our lens-free imaging flow cytometer, which provides a reliable ground-truth for supervised classification. We propose an automated and stable workflow, built around a high-dimensional feature space, that yields high classification accuracy. Features are analyzed and selected for dimensionality reduction. Several machine learning algorithms are compared and used for classification. The rest of this paper is structured as follows: The cell preparation and experimental setup are described in Section 2. In Section 3, we present the entire automated data processing pipeline for 3-part leukocyte recognition, including image preprocessing, improved auto-focus hologram reconstruction, feature extraction based on both optical and morphologic characteristics, feature selection and classification. In Section 4, we investigate the performance of the extracted features as well as feature subsets by evaluating their classification accuracy using different classifiers. We discuss the advantages and limitations of this technology and some remarks about our experiments in Section 5. The concluding remarks are summarized in the final section of the paper.

2. Materials and data acquisition

2.1. Cell preparation

All experiments were performed in compliance with the Belgian (7th May 2004 law related to experiments on human persons), international regulations (Directive 2001/20/EC) and institutional guidelines for medical research.

Blood samples were obtained from healthy donors having consented to the goals and objectives of the study after signing a written informed consent. The study protocol was approved by the Medical Ethical Committee of University Hospital of the University of Leuven (UZ Gasthuisberg), Ref. S57599.

Two milliliter aliquots of whole blood were incubated with Phycoerythrin-conjugated anti-CD3, anti-CD14 and anti-CD15 antibodies (all from BD Biosciences) to specifically label lymphocytes, monocytes and granulocytes, respectively. Samples were then washed, re-suspended in BD FACS Lyse solution in order to lyse the red blood cells, washed again and finally re-suspended in running buffer (PBS + 0.5%BSA +2 mM EDTA). Cell concentrations of each sample were measured with a Scepter Cell Counter (Merck Millipore) and adjusted to 3×106 cells/mL.

Each fluorescently labeled cell sample was individually loaded into the system and analyzed in flow. Hologram acquisition of single cells was triggered by detection of the fluorescence signal emitted by each labeled cell, ensuring only cells of interest were imaged. At least 5000 holograms were acquired for each white blood cell subtype, which were subsequently reconstructed and analyzed.

2.2. Acquisition of holographic images

A schematic of the imaging system is depicted in Fig. 1. The acquisition of the single cell images is triggered by the detection of a fluorescence signal emitted by each labeled cell. This procedure ensures only cells of interest are imaged. The holographic imaging was performed on our lens-free microscopic system adapted for fluorescent signal triggering and recording. At least 5000 holograms were acquired for each leukocyte subtype and the holographic images of the cells are subsequently reconstructed and analyzed.

A CMOS camera is connected to a transparent glass microchip, in which microfluidic components hydrodynamically focus a stream of cells in a focusing channel. This stack of components is suspended on a manual stage which controls linear, vertical and tilt motion above a static optical stack. The optical stand redirects a 488 nm laser and a 532 nm laser for fluorescent excitation and imaging respectively. These are both focused in a 20× objective through the pinhole array at the top of the stand, which contains a small pinhole for imaging illumination and a large window for fluorescent excitation and emission. These lasers are redirected with mirrors in the optical stand, which include one for redirecting emitted fluorescent signals to a photomultiplier tube to trigger the CMOS camera.

Fluorescent signal values are read by a Field Programmable Gate Array (FPGA) and displayed to the user. Values surpassing a user-set threshold are triggered with the rising edge, which initiates fluorescent signal saving and subsequent cell imaging. After imaging is initiated, the cell passes through a detection region, which records the fluorescent signal on the FPGA. The excitation laser is subsequently turned off for imaging and a 532 nm ns laser is used for illumination. Therefore, only fluorescently detected cells are imaged; generated image files are saved on the PC hard drive, along with two consecutively-timed images used later to remove the background from the image. The fluorescent signal is

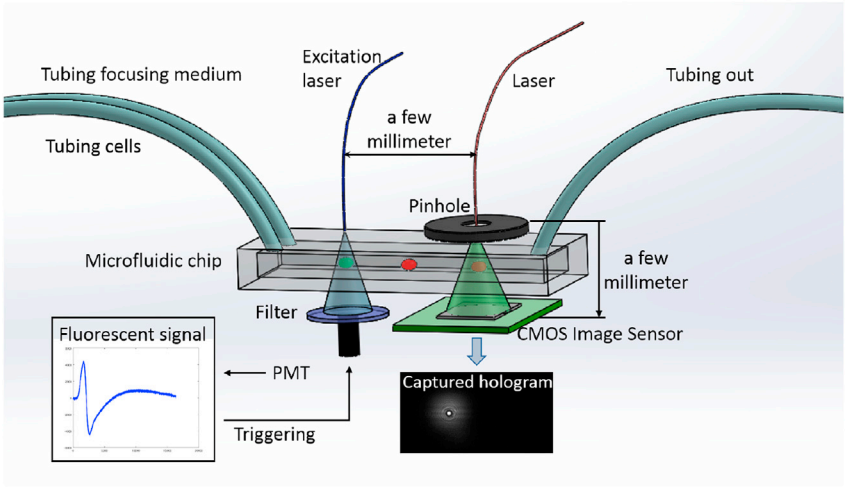

Fig. 1. Schematic drawing of the imaging set up. The inset is the detected fluorescent signal for camera triggering.