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Label-free sensor for automatic identification of erythrocytes using digital in-line holographic microscopy and machine learning

Taesik Go, Hyeokjun Byeon, Sang Joon Lee*

Department of Mechanical Engineering, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea

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ABSTRACT

Cell types of erythrocytes should be identified because they are closely related to their functionality and viability. Conventional methods for classifying erythrocytes are time consuming and labor intensive. Therefore, an automatic and accurate erythrocyte classification system is indispensable in healthcare and biomedical fields. In this study, we proposed a new label-free sensor for automatic identification of erythrocyte cell types using a digital in-line holographic microscopy (DIHM) combined with machine learning algorithms. A total of 12 features, including information on intensity distributions, morphological descriptors, and optical focusing characteristics, is quantitatively obtained from numerically reconstructed holographic images. All individual features for discocytes, echinocytes, and spherocytes are statistically different. To improve the performance of cell type identification, we adopted several machine learning algorithms, such as decision tree model, support vector machine, linear discriminant classification, and *k*-nearest neighbor classification. With the aid of these machine learning algorithms, the extracted features are effectively utilized to distinguish erythrocytes. Among the four tested algorithms, the decision tree model exhibits the best identification performance for the training sets (n = 440, 98.18%) and test sets (n = 190, 97.37%). This proposed methodology, which smartly combined DIHM and machine learning, would be helpful for sensing abnormal erythrocytes and computer-aided diagnosis of hematological diseases in clinic.

1. Introduction

Blood plays an important role in the diagnosis of human diseases. Abnormalities of erythrocytes are cardinal symptoms used in identification of health disorders. The biochemical and mechanical properties of erythrocytes are highly sensitive to the types and stages of various hematological diseases (Tomaiuolo, 2014). Specifically, several hematological diseases, such as malaria (Park et al., 2008), sickle cell anemia (Barabino et al., 2010), and spherocytosis (Da Costa et al., 2013), modify the morphology of erythrocytes. Erythrocyte morphology is a valuable diagnostic element because additional expensive labeling procedures are not required. Recent studies revealed that the rheological and morphological properties of erythrocytes are adversely altered due to long storage period of blood (Moon et al., 2013; Park et al., 2016a). The increase of storage period induces alterations in biochemical properties of blood, such as decrease of pH and adenosine triphosphate (ATP), and increase of reactive carbonyl species and oxidative stress. These alterations damage membrane proteins of erythrocytes and induce a series of structural transitions erythrocytes (Bennett-Guerrero et al., 2007; Kriebardis et al., 2007; Li et al., 2010). With the lapse of storage period, the normal discocyte (DC) is changed to echinocyte (EC). When storage period is further increased, echinocyte (EC) is changed to spherocyte (SC) (Berezina et al., 2002; Hess, 2010; Moon et al., 2013; Park et al., 2016a, 2010). When DC is changed to EC and SC with the lapse of blood storage time, the surface-to-volume ratio and deformability are gradually decreased (Park et al., 2016a, 2010). These changes give rise to decrease of perfusion in the oxygen supply to the surrounding tissues. It is also difficult for rigid erythrocytes to pass through the small conduits of microcapillaries (Baskurt and Meiselman, 2003; Hess, 2010). Furthermore, flow streamlines are more distorted in the presence of rigid erythrocytes, compared with those of normal DCs. Therefore, the viscosity of blood increases, when the proportion of EC and SC, which are less deformable than DC, increases. The increase of blood viscosity induces to increase flow resistance in blood vessels, which is one of the crucial factors in microcirculation (LeVeen et al., 1980). These alterations lead to decrease of microvascular flow and oxygen delivery (Baskurt and Meiselman, 2003; LeVeen et al., 1980; Rebel et al., 2001). They are ultimately associated with mortality, infection, and multi-organ failure of patients (Grimshaw et al., 2011; Hod et al., 2010; Koch et al., 2008).

* Corresponding author.
E-mail address: sjlee@postech.ac.kr (S.J. Lee).

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Therefore, a simple and fast classification algorithm modality that can classify various types of erythrocytes accurately should be developed. In addition, accurate erythrocyte classification will be valuable for clinical status evaluation because the morphology and functionality of erythrocytes are modified by specific drugs (Reinhart and Felix, 2003; Reinhart et al., 2014).

Several techniques have been introduced for recognition or identifying biochemicals and biosamples. For example, Pb^{2+} ions in aqueous solution were electrochemically detected with chronocoulometric aptasensor using a DNA probe functionalized gold nanoparticle (AuNPs) (Zhang et al., 2016). Antibiotic kanamycin was visually detected using color change of AuNPs (Lai et al., 2017; Qin et al., 2017). However, preparation and labeling procedures of AuNPs are required for these techniques. A high-throughput identification method for circulating tumor cells in the background image of blood cells was introduced (Singh et al., 2017). Digital holograms of tumor cells flowing a microchannel were captured using digital in-line holographic microscopy (DIHM) technique. Three features extracted from intensity information of focal plane images were used to identify tumor cells. Thus, extraction of more advanced features is required to characterize the inter-type differences of erythrocytes. Conventional methods used for identifying erythrocyte types include scanning electron microscopy (SEM) (Blasi et al., 2012), optical microscopy (Das et al., 2013; Lee and Chen, 2014), and quantitative phase imaging (QPI) (Moon et al., 2012; Park et al., 2016a, 2016b; Yi et al., 2016, 2015). However, SEM requires complicated sample preparation and manual counting procedures for cell classification. Since images obtained by optical microscopy contain only two-dimensional (2D) information, the classification accuracy is not so high. QPI technique provides valuable phase information related with three-dimensional (3D) morphology and hemoglobin contents. Thus, it has been applied to classify various cells, including erythrocytes and microorganisms (Park et al., 2016b; Shin et al., 2010; Yi et al., 2016, 2015) and track a targeted erythrocyte with combination of mean-shift algorithm and Kalman filter (Moon et al., 2016). However, QPI requires a complex optical set-up with off-axis geometry. Considering that QPI requires digital image processing for retrieval of 3D morphological information based on single cell analysis by a trained expert, obtaining statistically significant data with high throughput is difficult. Recently, a new DIHM method which can identify geometrical shape (Zakrisson et al., 2015). However, the experimental and simulated results extracted from only the real part of back reconstructed amplitudes of spherical or ellipsoidal particles, and normal DCs were compared. Therefore, it is hard to predict the types of irregularly modified erythrocytes by using this method.

In our recent study, temporal variations of viscosity and optical focusing characteristics of stored blood were experimentally investigated by using a H-shaped microchannel and DIHM technique (Park et al., 2017). In the previous study, we proposed criteria for manually determining whether the stored blood is suitable for transfusion or not. Since the real and virtual focal lengths extracted from light scattering pattern of an erythrocyte did not fully provide the characteristics of the erythrocyte, erythrocytes were roughly classified. Thus, in this study, we seek to further improve the identification performance of erythrocyte types greatly by adopting advanced means for multiparametric characterization of individual erythrocytes based on several descriptors extracted from holograms of unstained erythrocytes.

In the present study, we proposed a fully automatic sensing for precisely classifying cell types of erythrocytes without human interpretation by combining DIHM technique and a proper machine learning algorithm. This combined technique can unbiasedly identify erythrocytes in a sensitive and accurate manner. To design the classifier, three typical erythrocytes, such as DC, EC, and SC, are used for training and testing. Compared with QPI techniques, DIHM does not require a separated reference beam path, and it has a simple optical set-up. DIHM can obtain 3D volumetric information from a single-shot holograms image (Choi and Lee, 2009; Katz and Sheng, 2010; Memmolo et al., 2015). The recorded holograms of test objects are numerically reconstructed along depth (z) direction. From these numerically reconstructed images, projection images, focal plane images, and light scattering patterns are acquired.

To characterize eminently different cell types of erythrocytes, 12 features are extracted. Among them, 7 features containing information on geometry and intensity distribution are extracted from projection images and focal plane images. On the other hand, 5 features which contain information on morphology and optical focusing characteristics are extracted from light scattering patterns derived from wavefront analysis. Erythrocyte classifiers are constructed by using the extracted 12 features and several machine learning algorithms. The supervised machine learning algorithms construct a predictive model based on user-labeled inputs as training datasets, and then classify new datasets using features extracted from each new datum. If a classification model were once established with training datasets, new test sets could be quickly classified. The performance of established classifiers is also examined to select the best classifier. As a result, the selected classifier based on the decision tree model exhibited excellent performance in cell type classification of erythrocytes ($> 97\%$) for the training and test sets.

2. Material and methods

2.1. Blood sample preparation

Whole blood was extracted from male Sprague–Dawley rats (11 weeks old, 350–400 g) through abdominal aortic puncture. Blood was first collected into an EDTA vacuum tube (Vacutainer K3 EDTA, BD, USA) to prevent blood coagulation, and then CPDA-1 (CPDA-1, C4431, Sigma-Aldrich, USA) was added to an EDTA-treated blood sample with a v/v ratio of 1:7. According to the current protocol for blood storage, blood can be stored up to 42 days at a low temperature in CPDA-1 solution which provides glucose and ATP to blood (Shields, 1969). The resultant mixture was stored in the dark at 4 °C for 42 days. DIHM measurements were performed several days after blood storage. We collected sufficient amounts of training and test datasets with observing temporal morphological variations of erythrocytes for 42 days. For each experiment, 5 μL of blood sample was collected from the mixture. The collected blood sample was diluted in 1x phosphate-buffered saline (PBS, Gibco™ PBS, USA) to make a hematocrit of 0.5%. The pH of the PBS solution is 7.4 at 25 °C. In this study, experiments were conducted at low hematocrit to minimize unexpected noise caused by cell-to-cell interference. A total of 3 μL diluted blood was placed between the top and bottom cover slips. The two cover slips were separated by two sheets of paper with about 50 μm thickness. Each measurement was conducted at 25 °C within 15 mins to prevent additional alterations in blood sample. The experimental procedures were approved by the Animal Care and Ethics Committee of Pohang University of Science and Technology (POSTECH), and all experiments were conducted according to the approved guidelines (POSTECH-2016-0007).

2.2. Experimental set-up

A single-beam DIHM system was employed to capture holograms and extract various features of erythrocytes. Fig. 1(a) illustrates the experimental set-up of the DIHM system. A continuous Nd: Yag laser ($\lambda = 532$ nm, 100 mW, Crystal Laser, USA) was used as a light source. The laser beam passed through a spatial filter and a collimating lens. Erythrocytes positioned between two cover slips were illuminated by the laser beam. A water-immersion objective lens (60×, Nikon, Japan) was used to magnify hologram images of erythrocytes. The magnified holograms were recorded by a high-speed CMOS camera (pco. 1200 hs, PCO, Germany) with 1k × 1k spatial resolution. The spatial resolution in the image plane was 0.2 μm/pixel. Bright-field microscopic images of erythrocytes were also simultaneously captured to supervise the