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Deep-Ultraviolet Microscopy for Label-free Hematological Analysis

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ABSTRACT

Clinical hematological practice often relies on analysis of the peripheral blood based on microscopic evaluation of blood smears and complete blood count (CBC). Accurate examination of blood cell abnormalities using such methods necessitates complex, time-consuming, and expensive sample preparation as well as instruments which require a many reagents and intensive maintenance. Further, hematology analysis is performed at healthcare centers by trained personnel which significantly limits monitoring frequency for patients with severe conditions and can compromise the treatment outcome. Therefore, a portable, easy-to-use, and inexpensive hematology analysis device can potentially improve quality of life for patients with blood diseases and allow point-of-care monitoring and diagnosis. In this work, we demonstrate label-free blood cell assessment based on deep-ultraviolet (UV) microscopy. Our approach provides quantitative endogenous molecular information from live cells and enables assessment and differentiation of blood cell types based on their molecular and structural signatures. We show the ability of our method by performing classification of polymorphonuclear leukocyte (PMNL) subtypes based on features extracted from deep-UV images. In addition, we demonstrate a pseudo-colorization scheme which accurately mimics the colors produced by standard Giemsa staining and enable visual examination of blood smears. The results of our work paves the way for development of a low-cost and easy-to-use hematological analysis device that can be used for point-of-care applications.

Keywords: Deep-ultraviolet microscopy, Label-free cell classification, Molecular imaging, Hematology analysis, Point-of-care diagnosis, Thrombocytopenia, Sickle cell anemia

1. INTRODUCTION

Hematological analysis of blood cells has been the standard method for routine clinical diagnosis and monitoring of many blood diseases such as sickle cell anemia, neutropenia, and thrombocytopenia. Currently, the hematological practice relies on assessing alterations in morphology, population, and molecular or cytogenetic properties of blood cells to diagnose disease[1,2]. To this end, peripheral blood is collected and analyzed using a hematology analyzer to obtain a complete blood count (CBC). Modern hematology analyzers use a combination of multiple techniques such as absorption spectroscopy, impedance measurement, and flow cytometry to measure red blood cell and platelet counts as well as white blood cell (WBC) differentials (i.e., polymorphonuclear leukocytes (PMNLs) including neutrophils, eosinophils, and basophils, along with lymphocyte, and monocyte counts)[1]. Although hematology analyzers are capable of automated and rapid analysis of several samples, they are costly and require a many reagents and intensive maintenance which limit their application for point-of-care monitoring and diagnosis. In addition, in the case of abnormal and pathological samples which produce atypical results, manual microscopic examination of blood smears is often required. Microscopic analysis of peripheral blood smears is performed by staining the sample using Giemsa stain which is composed of a blue dye (i.e., methylene blue) and an acidic dye, (i.e., eosin), resulting in pink erythrocytes, purple leukocyte nuclei and platelets with dark blue cytoplasm containing red-purple granules[1]. Accurate evaluation of peripheral blood smears requires well-stained samples and time-consuming microscopic analysis performed by trained personnel and is prone to inter-observer variation. Thus, a low-cost, portable hematology analyzer can circumvent these issues and enable point-of-care diagnosis and continuous monitoring of blood cells.

To address the aforementioned limitations, we demonstrate a label-free method for blood cell assessment using deep-ultraviolet (UV) microscopy. Our approach provides quantitative endogenous molecular information from live unstained cells and enables cell classification based on their molecular and structural signatures. We developed a pseudo-colorization

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scheme which accurately mimics the colors produced by standard blood cell staining methods and can enable clinicians to visually analyze blood smears. Our results provide information that can assist clinicians in screening and diagnosis of many blood conditions without the need for expensive and time-consuming sample preparation and staining procedures.

2. EXPERIMENTAL SETUP AND METHODS

2.1 Experimental setup

We developed a label-free imaging system based on deep-UV multi-spectral microscopy which consisted of an incoherent broadband laser-driven plasma light source (EQ-99X LDLS, Energetiq Technology). The output light from the broadband source was collimated through a set of off-axis parabolic mirrors (Newport Corporation) and relayed to the sample using a 300 mm lens. Multi-spectral imaging was done using UV band-pass filters (~ 10nm bandwidth) installed on a filter wheel, allowing acquisition of images at three wavelength regions at 255, 280, and 300nm. For imaging, we used 40X (NA 0.5) (LMU-40X, Thorlabs), UV microscope objectives and achieve an average spatial resolution of ~300 nm. Images were then recorded using a UV sensitive CCD camera (integration time is set at 30-100ms range) while sample was translated and adjusted for focusing via a three-axis high-precision motorized stage (MLS2031, Thorlabs). A schematic of the developed setup is depicted in Fig 1(a).

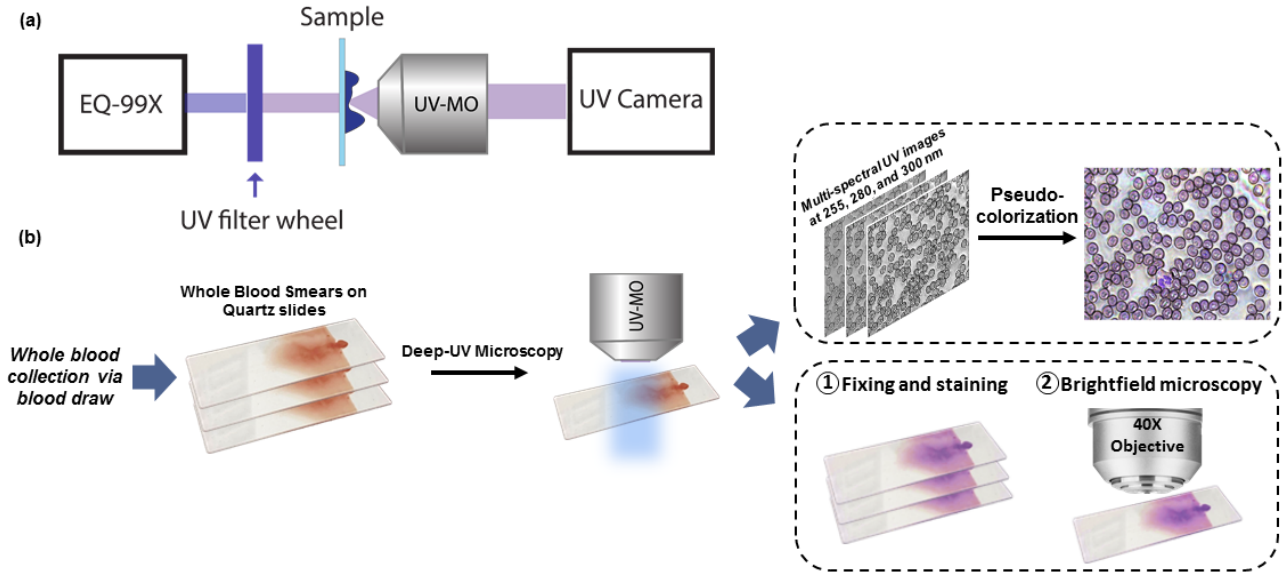

Figure 1. (a) Schematic of the developed deep-UV microscopy setup. (b) Sample preparation, UV and brightfield microscopy, and pseudo-colorization workflow.

2.2 Preparation peripheral blood smear samples

Whole blood was drawn from healthy donors as well as patients diagnosed with thrombocytopenia and sickle cell anemia according to approved protocols by Institutional Review Board (IRB) of Georgia Institute of Technology. Blood smears were made on quartz slides by using 10 µL of whole blood. After drying the samples in air for 5 minutes, UV imaging was performed. Lastly, the slides were fixed in methanol for 7 minutes, stained using Giemsa stain for 15-20 minutes, and imaged using a conventional brightfield microscope for comparison. The sample preparation procedure is shown in Fig 1(b).

2.3 Quantitative nucleic acid and protein mass mapping

The nucleic acid and protein mass maps were calculated based on the optical density ($OD = -ln(\tilde{I}/\tilde{I}_0)$) which is obtained by normalizing each UV image ($\tilde{I}$) by a reference background image taken from an empty area on the sample at each wavelength. The OD maps obtained at 260nm and 280nm wavelengths are then used to calculate the mass maps assuming a linear contribution of species at each wavelength according to Eq (1).

$$OD^{wavelength\ n} = \left(\varepsilon_{nuc.\ acid}^{wavelength\ n}\right) lc_{nucleic\ acid} + \left(\varepsilon_{protein}^{wavelength\ n}\right) lc_{protein} \quad (1)$$

where ε is the extinction coefficient, l is the optical path length, and c is the species concentration[6]. Based on OD values for the two wavelengths, we can generate a set of equations and solve for the concentration-pathlength products (lc terms) at each pixel. In our calculations we use average extinction coefficients at 260 nm and 280 nm for nucleic acid ($\varepsilon_{260} = 7000\, M^{-1}cm^{-1}, \varepsilon_{280} = 3500\, M^{-1}cm^{-1}$) and protein ($\varepsilon_{260} = 36{,}057\, M^{-1}cm^{-1}, \varepsilon_{280} = 54{,}129\, M^{-1}cm^{-1}$)[6] and average OD values over the bandwidth of our UV filters[3, 4]. Further, we assume an average molar mass of 52,728 Da for protein and of 330 Da for nucleic acids to obtain mass values at each pixel[4]. Example deep-UV image stack along with the corresponding nucleic acid and protein mass maps are depicted for an isolated eosinophil and a basophil are shown in Fig 2.

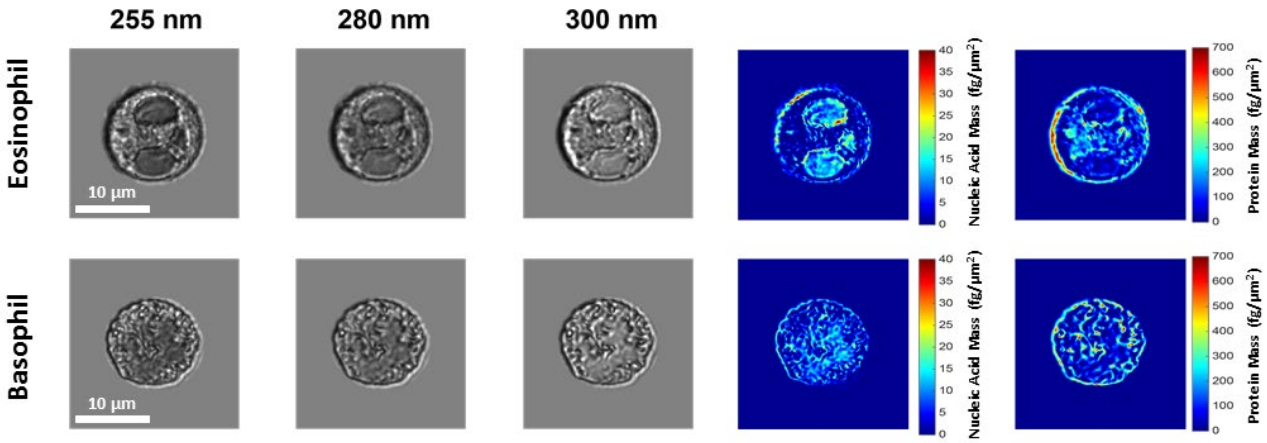

Figure 2. Multi-spectral UV-images obtained from isolated an Eosinophil and a Basophil along with their nucleic acid and protein mass maps.

3. RESULTS AND DISCUSSION

3.1 Classification of PMNL subtypes

The multi-spectral images obtained using our deep-UV microscopy provide insight into many structural and biochemical features that potentially can enable us to uniquely classify the PMNL subtypes. To this end, we extracted 58 different features from live cell UV-images. The extracted features were calculated based on morphology, statistical properties, textural features based on the gray-level co-occurrence matrix (GLCM), and average biochemical masses (examples shown in Fig 2) from 100 PMNLs. We used the extracted features to train a support-vector machine (SVM) and assessed the performance parameters for each PMNL subtype using a 6-fold cross validation algorithm.

The results of our classification revealed a high accuracy (~ 98%) in subtyping of PMNLs by our trained SVM model. The classifier was able to correctly identify each PMNL subtype, misclassifying only one cell within our testing dataset. The performance results for our SVM model demonstrate the ability of our approach for accurate identification of PMNL subtypes which can enable automatic WBC population monitoring in whole blood smears.

3.2 Deep-UV multispectral microscopy and pseudo-colorization of whole blood smears

Visual PMNL assessment using whole blood smears is currently employed as the standard clinical procedure for blood cell population estimation and diagnosis of blood abnormalities which is performed by trained technicians at healthcare centers, requiring expensive and time-consuming procedures and complex equipment. Our approach aims to obviate the need for sample preparation, reagents, and staining to enable clinical and point-of-care blood assessment in a cheap, simple, and easy way. In our experiments, whole blood smear samples were prepared on quartz slides without any cell fixation or staining and then imaged immediately using the deep-UV microscopy setup to obtain a multi-spectral image stack. In order to facilitate the visual inspection of whole blood samples with our label-free approach, we developed a pseudo-colorization scheme based on mixing images at three different wavelengths: 255, 280, and 300 nm. This approach enables us to produce pseudo-RGB images that mimic the colors produced by standard Giemsa staining. We form an RGB image by simply taking the 255 nm image as the red channel, the 280 nm image as the green channel, and the 300 nm image as the blue

channel. The contrast from absorption of different biochemicals within the cells give rise to color contrast between the nucleus and cytoplasm of the white blood cells (WBCs) (shown in the RGB image in Fig 3 inset) as well as enucleated RBCs. As depicted in Fig 3, many unique features of blood cells such as size, population, and morphology of red and white blood cells as well as platelet size and population can be evaluated from the UV images. Moreover, we generate a wide-field pseudo-colorized UV image by scanning a 2x1 mm area on the sample which enabled the clinicians to perform a thorough evaluation on a large number of blood cells.

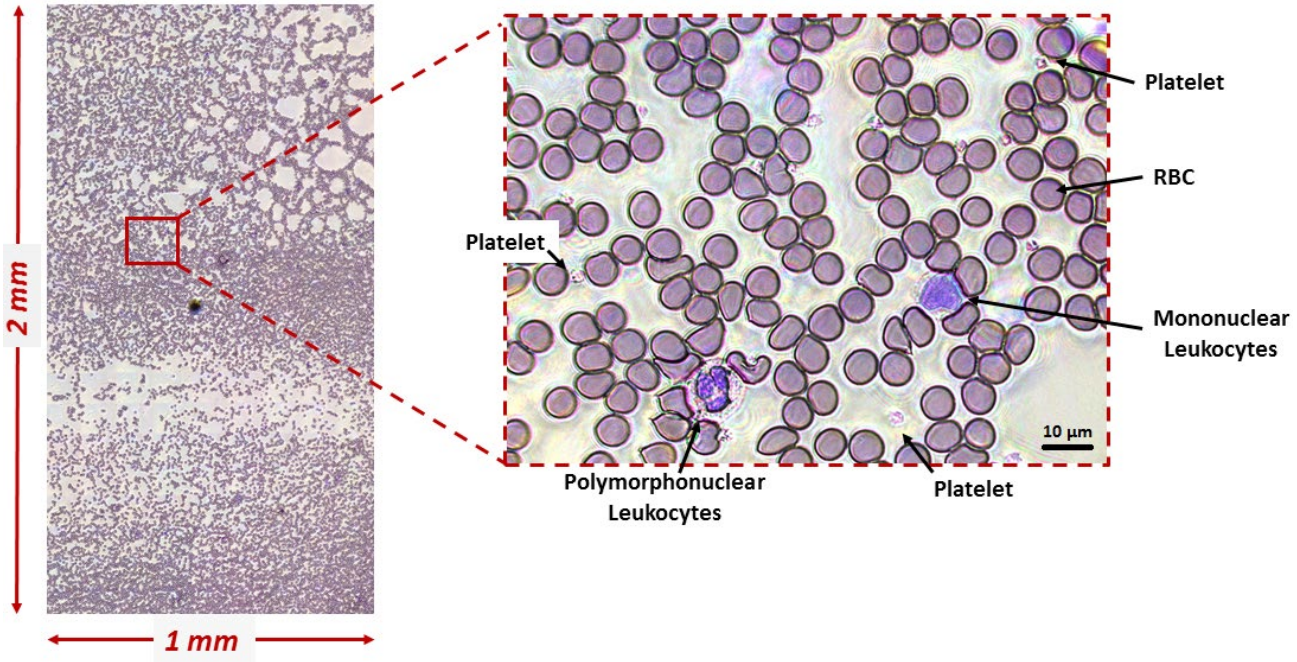

Figure 3. Pseudo-colorized UV image of a blood smear sample from a healthy donor showing various blood cell types. Inset scale bar is

Next, we prepared a whole blood smears obtained from a patient diagnosed with sickle cell anemia and imaged them using the deep-UV microscope. We applied the pseudo-colorization scheme to the multi-spectral UV image stack and compared those images to brightfield microscope images after Giemsa staining. An example pseudo-colorized UV image along with its corresponding brightfield image is depicted in Fig 4. As shown in Fig 4, several key pathological features can be observed in cells which match very well with the stained image. The pseudo-colorized image shows several sickled RBCs with the well-known crescent/elongated shape, commonly seen in smear samples collected from patients diagnosed with sickle cell anemia. This further verifies the capability of our approach in assisting clinicians to visually assess the morphology and population of blood cells in unstained and unfixed blood smears without the need for standard sample preparation procedures.

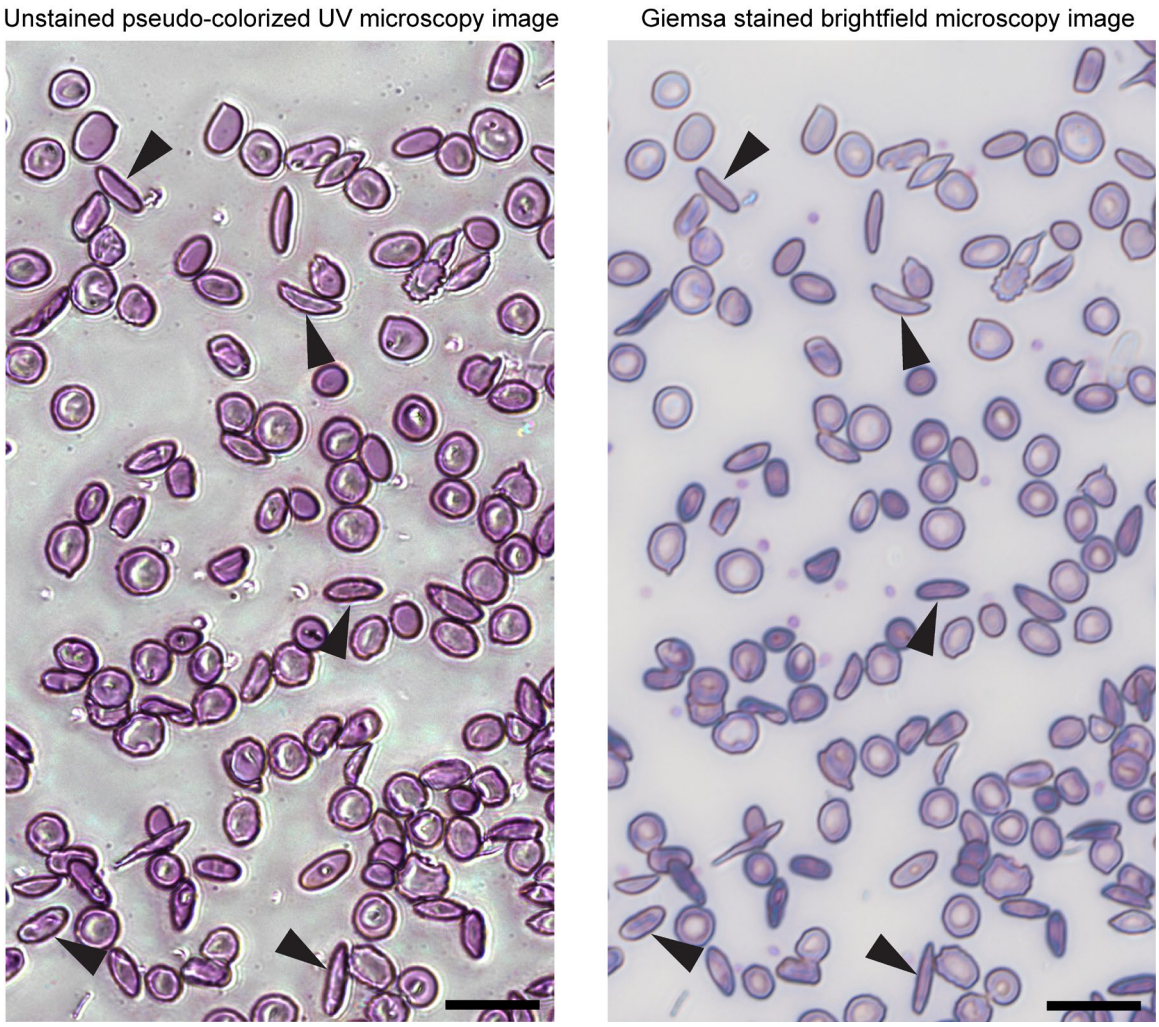

Figure 4. Pseudo-colorized UV and brightfield images obtained from a whole blood smear sample collected from a sickle cell anemia patient. Black arrowheads point to sickled RBCs. Scale bar is 20 μm.

4. CONCLUSION

The results of this study signify the potential of our approach as a powerful point-of-care diagnostic and screening tool which enables quantitative and qualitative analysis of blood cells without the need for standard sample preparation protocols. Our simple and cost-effective method benefits from advantages offered by microscopy in the deep-UV region, where many endogenous biochemical that play important roles in cell structure and function have signature absorption properties[5], thus enabling facile assessment of different cell types has significant potential to aid clinicians in diagnosis and monitoring of blood disorders. We demonstrate the ability of our method to enable accurate identification of different PMNL subtypes using the structural and biochemical information provided by deep-UV multi-spectral images and classification algorithms. In addition, our results demonstrate a fast and simple colorization scheme that mimics the colors produced by standard Giemsa staining to felicitate visual evaluation of blood cell morphology. Unlike the standard method for blood cell evaluation which requires reagents and laborious and time-consuming protocols our approach will not require any sample preparation or a technician to run the test/analysis. This has work significant implications towards the development of a hematology analysis device with improved capabilities for use in clinical, point-of-care, and low-resource settings.

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