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Label-free Identification of Neutropenia Using Deep-Ultraviolet Microscopy

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ABSTRACT

Polymorphonuclear leukocyte (PMNL) count is employed as an immune status indicator for diagnosis of numerous medical conditions. Currently, assessment of PMNLs (i.e., neutrophils, eosinophils, basophils) is a part of complete blood count (CBC) that is performed by trained technicians at healthcare centers and involves sample preparation which is costly and time consuming, both of which limits monitoring frequency. A prominent application of PMNL counting is in identification of neutropenia—a condition describing an abnormally low number of neutrophils in the bloodstream (<1500/μL)—common among cancer patients receiving chemotherapy. Susceptibility to infections in neutropenia patients puts them at an increased risk for medical emergencies, and thus requires constant monitoring of their neutrophil count. Therefore, a portable and easy-to-use, in-home device can potentially circumvent these requirements and enable neutropenia diagnosis.

In this work, we demonstrate the feasibility of accurately identifying PMNL subtypes using deep-ultraviolet (UV) microscopy as label-free molecular imaging technique. Our approach benefits from quantitative endogenous molecular information provided by deep-UV imaging, to enable assessment of different cell types based on their molecular and structural signatures. We show the ability of our system to measure neutrophil count in samples containing a mixture of PMNL subtypes as well as whole blood samples by extracting various features from deep-UV images and performing classification to obtain cell count for each subtype. Finally, we will discuss the potential of this technology to empower cancer patients and improve their quality of life via a simple and relatively inexpensive device for point-of-care neutropenia assessment.

Keywords: Ultraviolet microscopy, Multispectral imaging, Neutropenia, White blood cells, Cell classification, point-of-care diagnosis

1. INTRODUCTION

Polymorphonuclear leukocytes (PMNLs)/granulocytes are a subgroup of white blood cells (WBCs) often characterized by their lobular nuclear structure as well as presence of granules in their cytoplasm. These cells are derived from differentiation of pluripotent hematopoietic stem cells in the bone marrow and form a lobular nucleus as they undergo maturation process[1]. Granulocytes are categorized into three main subtypes (i.e., neutrophils, eosinophils, and basophils) and named based on their staining characteristics in histological (e.g., hematoxylin and eosin (H&E)) or cytological (e.g., Giemsa) preparations. For instance, the cytoplasmic granules in neutrophils stain neutral pink while basophilic granules in basophils take up basic stains and appear deep blue, often obscuring the cell nucleus. In contrast, the acidophilic granules in eosinophils absorb acidic dyes such as eosin and appear bright red[1].

Granulocytes are the most abundant WBC subtype, constituting up to 70% of total of the total circulating WBCs. They play a key role in fighting infections and are responsible for inflammatory reactions during immune response such as chemotaxis, phagocytosis, and degranulation. As a result, assessment of the population and morphology of PMNLs is employed as an immunological status indicator for diagnosis of numerous medical conditions such as cancer, infectious diseases, sepsis, autoimmune disorders, and in the use of immunosuppressant drugs[2]. Another prominent application of PMNL assessment is in identification of neutropenia—a condition describing an abnormally low number of neutrophils in the bloodstream (<1500/μL)—common among cancer patients receiving chemotherapy. Chemotherapy suppresses the

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hematopoietic system, thus stymying the production of neutrophils which puts patients at an increased risk of infection and can cause delays in chemotherapy, compromising treatment outcomes, and in more severe cases, can lead to death[3]. For instance, fever in combination with severe neutropenia (i.e., febrile neutropenia) is considered a medical emergency and must be treated immediately[4]. Susceptibility to infections in neutropenia patients along with the increased risk for medical emergencies, requires constant monitoring of their neutrophil count. Therefore, a portable and easy-to-use, in-home device can potentially circumvent these requirements and enable point-of-care neutropenia diagnosis.

Currently, PMNL assessment is performed as a part of complete blood count (CBC) at healthcare centers by trained personnel and requires sample preparation and is costly and time consuming. In addition, PMNL assessment via CBC requires patients to visit the healthcare centers which puts an increased burden on patients and their families, reduces the applicability of this procedure in resource-limited areas, and limits the monitoring frequency.

To address the aforementioned limitations, in this study, demonstrate the feasibility of accurately identifying PMNL subtypes using deep-ultraviolet (UV) microscopy as label-free molecular imaging technique. The use of UV light for microscopy offers many advantages over traditional microscopy methods, including higher spatial resolution due to the light's shorter wavelength (240-450nm). Furthermore, our approach provides insight into structural and biochemical properties of live cells by allowing access to quantitative information from endogenous molecules (e.g., nucleic acids and proteins) that play an important role in cell function and structure. We show the ability of our system to identify PMNLs in samples containing different cell subtypes as well as whole blood samples by extracting various features from deep-UV images and performing classification. Finally, we demonstrate the ability of our method to serve as a powerful tool for visual assessment of cells in blood smear samples.

2. EXPERIMENTAL SETUP AND METHODS

2.1 Experimental setup

The developed deep-UV multi-spectral microscopy system consists of an incoherent broadband laser-driven plasma light source (EQ-99X LDLS, Energetiq Technology). The output light from the broadband source is collimated through a set of off-axis parabolic mirrors (Newport Corporation) and relayed to the sample using a 300 mm lens. Multi-spectral imaging is done using UV bandpass filters installed on a filter wheel, allowing acquisition of images at six wavelength regions at 255, 280, 300, 340, 380, and 415nm. For imaging, we use 40X (NA 0.5) (LMU-40X, Thorlabs), UV microscope objectives and achieve an average spatial resolution of ~300 nm. Images are then recorded using a UV sensitive CCD camera (integration time is set at 30-100ms range) while sample is translated and adjusted for focusing via a three-axis high-precision motorized stage (MLS2031, Thorlabs).

2.2 Preparation of isolated granulocyte samples

Live granulocytes are isolated from whole blood by negative magnetic antibody-based selection as described in ref 5 and resuspended in RPMI media with L-glutamine and HEPES (Life Technologies). In order to induce and maintain a normal cell adhesion and spreading[5] on microscope slide surface, quartz slides are coated with a 1 nM solution of N-Formylmethionine-leucyl-phenylalanine (fMLP, Sigma-Aldrich) for 60 minutes, then rinsed with distilled water and PBS. The cell suspension is pipetted onto the coated slide and incubated for 30 minutes to ensure that the cells are adhered and spread on the slide. In the next step, the slides are washed with PBS to remove the floating cells and air-dried to mimic a blood smear. The live cells are imaged using the multi-spectral UV microscope setup. Finally, the cells are fixed using methanol for 15 minutes and then stained using Giemsa stain (Sigma-Aldrich).

2.3 Preparation whole blood smear samples

Whole blood is collected from healthy donors and added to an anticoagulant solution (sodium citrate, Beckton Dickenson) according to approved protocols by Institutional Review Board of Georgia Institute of Technology. Informed consent is obtained from healthy donors. Blood smears are made on quartz slides by using 10 µL of whole blood. After drying the samples in air for 5 minutes, UV imaging is performed.

2.4 Quantitative nucleic acid and protein mass mapping

We quantify the nucleic acid and protein mass based on calculation of 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 260 nm and 280 nm 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. 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.

3. RESULTS AND DISCUSSION

The developed deep-UV multi-spectral microscopy setup allows us to obtain images from live cells at different wavelengths in the 240-450 nm range. Using the cell isolation technique described in section 2.2, we imaged the three granulocyte subtypes (i.e., neutrophils, eosinophils, and basophils) at six wavelengths. As depicted in Fig 1, the multi-spectral image stacks reveal many structural and biochemical details about these cells. For example, the images obtained at 255 nm which correspond to the well-known absorption peak for nucleic acids shows the highest absorption in cell nuclei with densely packed nucleic acids, revealing lobular structure of the granulocytes. Images at 280nm correspond to the absorption peak of proteins and show contrast from proteins present in the cell nucleus periphery as well as the cytoplasm. However, the images taken at 300-415 nm wavelengths show the highest contrast from cytoplasm and little contrast from the nucleus.

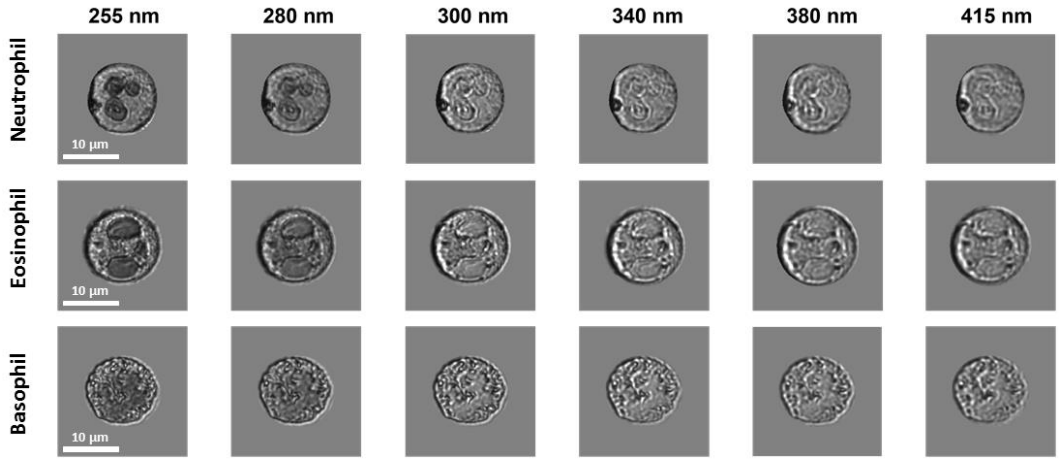

Figure 1. Deep-UV multispectral microscopy images of neutrophils, eosinophils, and basophils

The acquired deep-UV multi-spectral image stack is rich with information about the biochemical properties of the sample. The deep-UV microscopy technique allows us to access quantitative information about nucleic acid and protein masses in live cells by imaging them at 255 and 280 nm and calculating the mass maps according to the method described in section 2.4. Figure 2(a) shows an example of the obtained optical density (OD) map at 255 nm from a live neutrophil along with the nucleic acid and protein mass maps. The resulting nucleic acid mass map (depicted in Fig 2(b)) clearly shows the lobes of the nucleus, while the protein mass map (Fig 2(c)) highlights regions in the periphery of the lobes and throughout the cytoplasm.

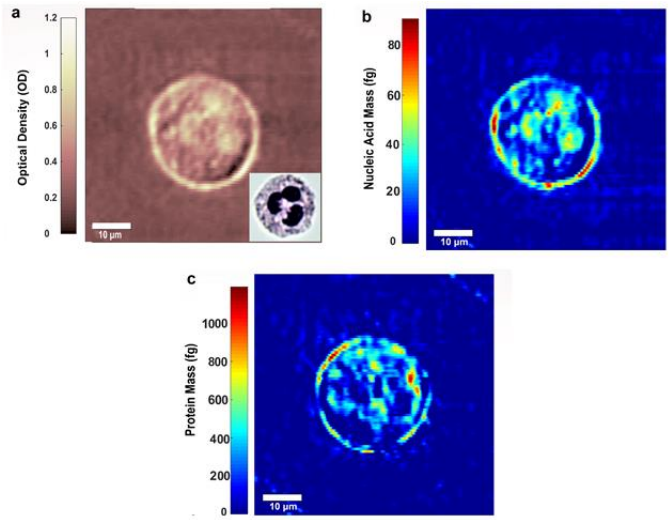

Figure 2. (a) Optical density (OD) map of a live neutrophil obtained at 260 nm. Inset depicting a bright-field microscopy image of the same neutrophil after Giemsa staining. (b) Nucleic acid mass map of a neutrophil (c) Protein mass map of the imaged cell. Adapted from Ref 5.

3.1 Classification of PMNL subtypes

Using the obtained multi-spectral images, we can extract several structural and biochemical features that potentially can enable us to uniquely identify the PMNL subtypes. In addition, images taken at 255 nm provide enough contrast to enable segmentation of the cell nucleus from the cytoplasm which allows us to obtain features from both nucleus and the cytoplasm. Figure 3 shows an overlay of the obtained mask after segmentation of the cell nucleus which is done by defining a threshold to identify the nucleus.

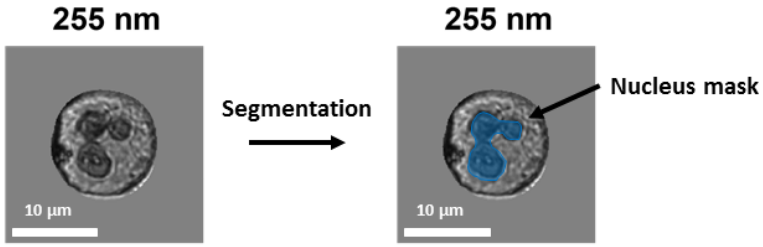

Figure 3. Segmentation of the cell nucleus using 255 nm images

In order to train our unsupervised cell classifiers, we extracted 58 different features (summarized in Table 1) from whole cells, cell nuclei and their cytoplasm. We extracted features based on morphology, statistical properties, textural features based on the gray-level co-occurrence matrix (GLCM), and average biochemical masses from 100 granulocytes. Next, we used the extracted feature set to train two types of supervised learning algorithms (support-vector machine (SVM) and linear discriminant analysis (LDA)). After the training the classifiers we obtained the performance (accuracy, sensitivity, and specificity) parameters for each algorithm to identify the best classifier.

By comparing the different classifiers, we found that our SVM model outperformed the LDA classifier yielding an accuracy of 98.24 %, sensitivity of 0.94 and specificity of 1. The classifier performance results demonstrate the capabilities of our approach for accurate identification of PMNL subtypes which can enable automatic neutrophil population monitoring.

Table 1. Extracted feature from multi-spectral UV microscopy images for cell classification

Morphological	Statistical	GLCM	Mass
Area	Mean	Contrast	Protein
Perimeter	Skewness	Correlation	Nucleic acid
Eccentricity	Kurtosis	Energy	
Circularity	Entropy	Homogeneity	
Major/Minor axis length	Standard deviation		
Extent			
Solidity			
Momentum			

3.2 Deep-UV multispectral microscopy of whole blood smears

PMNL assessment using whole blood smears is currently employed as the standard clinical procedure for visual population estimation and diagnosis of cell abnormalities. However, this procedure can only be performed by trained technicians at healthcare centers and requires standard fixing and staining procedures in a sterile environment. Our approach aims to obviate the need for sample preparation to enable point-of-care PMNL assessment and population monitoring in a simple and easy way. To this end, we prepared whole blood smear samples on quartz slides according to the standard clinical procedures without any cell fixation or staining. Then we imaged the live cell smear using the deep-UV microscopy setup to obtain a multi-spectral image stack. Figure 4 demonstrates the image stack at different wavelengths showing a granulocyte (a magnified view shown in the Figure inset) as well as the red blood cells (RBCs). The morphological properties of the granulocyte nucleus can be identified clearly in the 255 nm image while other wavelengths show contrast from cytoplasm. Additionally, the presence of hemoglobin in RBCs is apparent in the 415 nm image which correspond to the well-known soret absorption peak of hemoglobin[5, 7].

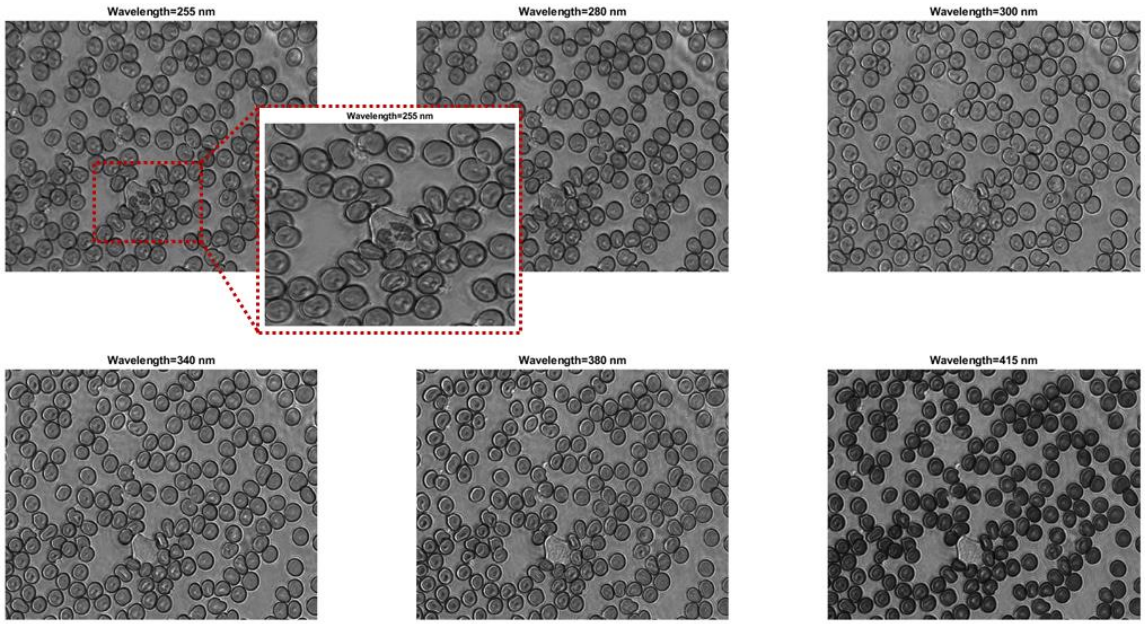

Figure 4. Deep-UV multi-spectral images of a whole blood smear sample

In order to facilitate the visual inspection of whole blood samples we applied a pseudo-colorization scheme based on mixing of images at three different wavelengths (i.e., 255, 280, and 300 nm). This approach enables us to produce pseudo-RGB images that mimic the colors produced by Giemsa staining. As depicted in Fig 5, we form an RGB image by 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 biochemical in the cells give rise to color contrast between the nucleus and cytoplasm of the granulocytes (shown in RGB image in Fig 5). The results of our colorization scheme further signify the potential of our approach as a powerful PMNL screening tool which enables visual inspection of blood cells without the need for standard sample preparation protocols. This simple and easy method have significant potential to aid clinicians in diagnosis of cell abnormalities and estimation of PMNL population.

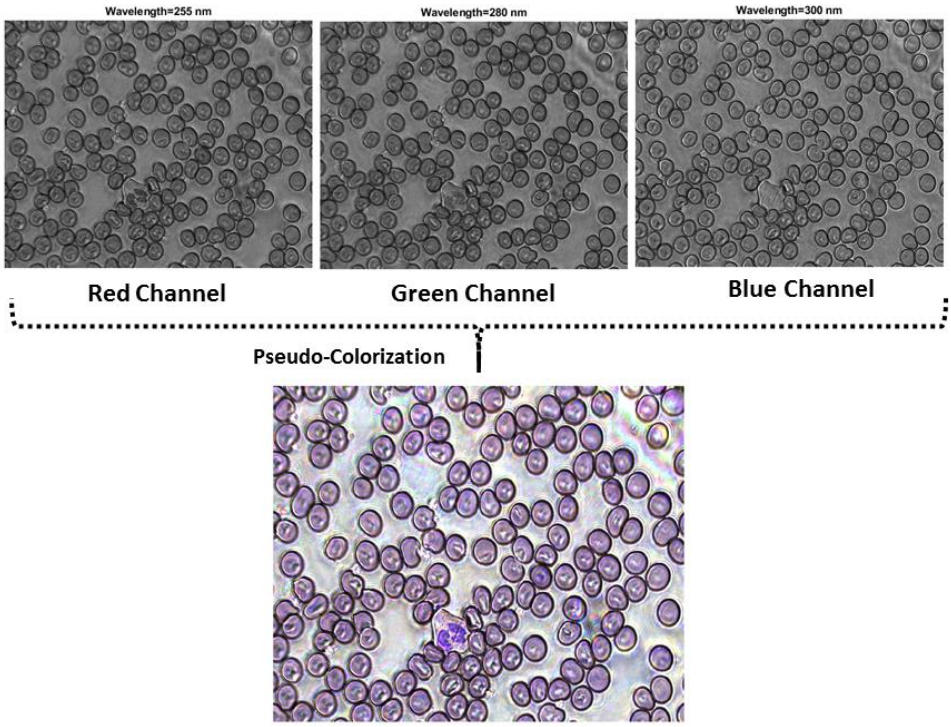

Figure 5. Pseudo-colorization scheme for assessment of PMNLs in blood smears

4. CONCLUSION

In this work, we demonstrate the feasibility of accurately identifying the PMNL subtypes using label-free molecular imaging, thus paving the way for developing a small, portable, easy-to-use, and relatively inexpensive device to assess neutropenia. Our approach 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, thus enabling facile assessment of different cell types. 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. Moreover, our results demonstrate a fast and simple colorization scheme to felicitate visual inspection of cell in whole blood smears. Unlike the standard method for blood cell assessment (e.g., complete blood count) our approach will not require any sample preparation, reagents, or a technician to run the test/analysis. Our approach can potentially lead to development of medical devices that will empower patients and improve quality of life via key advantages for in-home continuous PMNL screening for chemotherapy patients to determine if medical intervention is needed and how urgently. Continuous monitoring of the PMNL count and morphology will enable more personalized care, enable swifter action if severe medical conditions such as neutropenia persists for a long time, and it will significantly improve risk models to determine who would be a better candidate to receive preventative treatments.

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