Label-free automated neutropenia detection and grading using deep-ultraviolet microscopy

ASHKAN OJAGHI,1,4 © PALOMA CASTELEIRO COSTA,2,4 © CHRISTINA CARUSO,3 WILBUR A. LAM,1,3 AND FRANCISCO E. ROBLES1,2,*

1Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia, USA
2School of Electrical and Computer Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA
3Aflac Cancer and Blood Disorders Center of Children’s Healthcare of Atlanta and Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, USA
4These authors contributed equally
*robles@gatech.edu

Abstract: Neutropenia is a condition identified by an abnormally low number of neutrophils in the bloodstream and signifies an increased risk of severe infection. Cancer patients are particularly susceptible to this condition, which can be disruptive to their treatment and even life-threatening in severe cases. Thus, it is critical to routinely monitor neutrophil counts in cancer patients. However, the standard of care to assess neutropenia, the complete blood count (CBC), requires expensive and complex equipment, as well as cumbersome procedures, which precludes easy or timely access to critical hematological information, namely neutrophil counts. Here we present a simple, low-cost, fast, and robust technique to detect and grade neutropenia based on label-free multi-spectral deep-UV microscopy. Results show that the developed framework for automated segmentation and classification of live, unstained blood cells in a smear accurately differentiates patients with moderate and severe neutropenia from healthy samples in minutes. This work has significant implications towards the development of a low-cost and easy-to-use point-of-care device for tracking neutrophil counts, which can not only improve the quality of life and treatment-outcomes of many patients but can also be lifesaving.

© 2021 Optical Society of America under the terms of the OSA Open Access Publishing Agreement

1. Introduction

Neutropenia describes an abnormally low number of neutrophils in blood (<1500/µL) and can be graded as mild (1000–1500/µL), moderate (500–1000/µL), and severe (<500/µL) [1]. Cancer patients are particularly susceptible to this condition because cancer treatments, such as chemotherapy, suppress the hematopoietic system, which stymies the production of neutrophils [2]. These types of white blood cells (WBCs) are responsible for fighting infections; thus, neutropenia can put cancer patients at an increased risk of medical emergencies, which can not only compromise cancer treatments but may also lead to death [1,2]. The risk of severe infections and subsequent complications is directly related to the severity and duration of neutropenia [1]; thus, it is necessary to monitor this condition frequently. However, the standard of care to assess neutropenia, the complete blood count (CBC), requires complex equipment, multiple chemical reagents, laborious system calibration and procedures, and highly trained personnel for operation. This complexity prevents easy or timely access to lifesaving hematological information and forces cancer patients (among others susceptible to, or suffering from neutropenia) to make frequent trips to the few healthcare centers or laboratories with the available infrastructure to provide rapid feedback on neutrophil counts. This practice presents a significant burden to cancer patients and healthcare systems [3,4], especially in rural and low resource areas where clinical laboratories
are rare. Plus, this current standard-of-care fails to capture changes in blood counts that can occur between clinic visits.

Several methodologies have been proposed to facilitate point-of-care blood analysis. For instance, fluorescence-based techniques have been used for WBC identification based on low-cost assays along with image analysis methods [5,6] or flow cytometry [7,8]. Although these methods have shown promise, they suffer from phototoxicity and photobleaching, which can limit their use for quantitative analysis. Moreover, the degree of cellular differentiation is often achieved at the expense of more complex setups and staining procedures. Label-free imaging modalities have also been explored to enable blood cell analysis based on a combination of morphological and biochemical properties, including Raman imaging [9], hyperspectral imaging [10], fluorescence lifetime imaging microscopy (FLIM) [11], and quantitative phase imaging (QPI) [12–16]. Although these methods possess unique advantages, a trade-off still exists between the complexity of the optical system, cost, or speed of the approach vs. the information content provided, limiting their applicability for use in clinical and point-of-care settings. More recently, Bourquard et al. reported on a point-of-care device for severe neutropenia detection based on imaging WBCs flowing through nail fold capillaries. However, the approach is unable to uniquely identify neutrophils [17,18]. Consequently, this method is inherently limited to the detection of very severe cases of neutropenia where the total WBC count correlates well with the condition [17].

Here we demonstrate that deep-ultraviolet microscopy enables facile, robust, and fast detection and grading of neutropenia. The approach is based on a recently developed label-free, multi-spectral deep-UV assay, which leverages the rich molecular sensitivity and high spatial resolution provided by this spectral region [19–21], to enable detailed quantitative hematological analysis [22]. We also extend the deep-UV assay capabilities by developing a fast, automated cell segmentation algorithm and implementing a support vector machine (SVM) based classifier using structural and molecular/color features of the UV images. Results show robust automated identification and enumeration of neutrophils and accurate detection and grading of neutropenia in near real-time. The results of this work will pave the way for the development of a compact, label-free, low-cost, portable, and minimally invasive approach to monitoring different grades of neutropenia.

2. Methods

2.1. Whole blood samples collection and smear preparation

Peripheral venous whole blood was collected from a cohort of healthy donors (N = 4) and neutropenia patients (N = 6 severe, and N = 5 moderate). The neutropenia samples were collected from Leukemia patients who had been under chemotherapy and were diagnosed with moderate and severe grades of neutropenia based on CBC tests performed at the blood collection site. Anticoagulant solution (sodium citrate, Beckton Dickenson) was added after collection. All protocols used in this study were approved by the Institutional Review Boards of the Georgia Institute of Technology and Emory University. Informed consent was obtained from donors. Blood smears were made on un-coated quartz slides by using 10 μL of whole blood. After drying the samples in air for 5 minutes, UV imaging was performed.

2.2. Experimental setup

As reported in Ref. [22], the developed deep-UV microscopy system consisted of an incoherent broadband laser-driven plasma light source (EQ-99X LDLS, Energetiq Technology). The output light from the broadband source was collected through an off-axis parabolic mirror (Newport Corporation) and relayed to the sample using a short-pass dichroic mirror (Thorlabs, NJ, USA). Multi-spectral imaging was performed using UV band-pass filters (Chroma Technology Corp,
VT, USA) installed on a filter wheel, allowing acquisition of images at three wavelength regions centered at 260, 280, and 300 nm. The light intensity on the sample plane was measured to be 0.14, 4.5, and 0.22 mW at 260, 280, and 300 nm wavelengths, respectively. For imaging, we used a 40x UV microscope objective (NA 0.5) (LMU-40X, Thorlabs), which achieves an average theoretical spatial resolution of \( \sim 342 \) nm. Images were then recorded using a UV sensitive CCD (pc.o.ultraviolet, PCO AG, Kelheim, Germany) camera (integration time = 30-100 ms) with a pixel size of 4.65\( \times \)4.65 \( \mu \)m, each covering an approximate area of 0.165\( \times \)0.165 \( \mu \)m on the sample. Each smear sample was translated and adjusted for focusing via a three-axis high-precision motorized stage (MLS2031, Thorlabs). By raster scanning the sample, a series of UV images from a 1\( \times \)2 mm area were acquired at each wavelength. Imaging time is approximately 3 minutes per wavelength (currently limited by the translation stage). A schematic of the deep-UV microscopy system is illustrated in Fig. 1(b).

![Fig. 1. Overall workflow for deep-UV microscopy and automated WBC and neutrophil counting of whole blood smear samples. Whole blood smears were collected and prepared on quartz slides (a). Samples were imaged using the deep-UV microscopy system (schematic shown in b). The multi-spectral images were then scaled and mixed to form a pseudo-colorized RGB image (c) and transferred to our automatic segmentation and classification algorithms (d, e) from which WBC and neutrophil counts were obtained (f). Scale bars indicate 30 \( \mu \)m.](image-url)
2.3. Image processing, registration, and pseudo-RGB colorization

After obtaining the tile image series at the three wavelengths, each image was normalized by a reference background image acquired from a blank area on the sample at each wavelength to remove any illumination artifacts. Next, to obtain accurate colorization of UV images, an intensity-based image registration algorithm (based on imregister function, applying a similarity transform with a gradient decent optimizer), implemented in MATLAB (MathWorks), was used to co-register the corresponding images across all three wavelengths.

The registered intensity image stacks (260, 280, and 300 nm wavelength images) for each field of view (FOV) were used to obtain pseudo-RGB colorized images. To form the pseudo-colored images, each color channel (i.e., R, G, and B channels) was formed according to the weights ($W_R = 0.85$, $W_G = 0.9$, $W_B = 0.83$) and gamma ($\gamma_R = 1.4$, $\gamma_G = 1.9$, $\gamma_B = 1.2$) values based on $R = w_R \times I_{260}^R$, $G = w_G \times I_{280}^G$, and $B = w_B \times I_{300}^B$, where $I$ denotes the normalized intensity corresponding to each pixel of the image obtained at each wavelength. The weights and gamma values were optimized by comparing the colors of each blood cell type to their stained counterparts. Finally, the colorized images were transformed to the HSV color-space, a constant hue offset of +0.05 was applied, and converted back to RGB color-space [22]. The offset was applied to the hue channel which only affects the color properties of the image and was performed to ensure the highest level of color accuracy compared with Giemsa-stained images of blood smears.

Stitching of pseudo-colored images was performed using the Grid/Collection stitching plugin [23] of the Fiji [24] software, which calculates the overlap between each tile and linearly blends them into a single wide-field image. The obtained wide-field pseudo-colored images were then exported for manual counting of WBCs. This step is not necessary for the automated method.

2.4. Automated segmentation of RBCs and WBCs

The pseudo-colored images are analyzed to count and segment the white blood cells present. The segmentation process relies mostly on the color properties of each cell type as well as the textural differences among the different components available within our images. The two regions that are most characteristic, and therefore easiest to segment, are the WBC nuclei and the red blood cells. The WBC nuclei have noticeably higher levels of color saturation, as well as a blue tone. The RBCs, on the other hand, appear to have a softer red to violet hue. When the individual RGB channels are inspected, it becomes evident that the RBCs have higher red channel values, while the WBC nuclei appear to have higher blue tones. When the blue channel is normalized by the red, we can strongly enhance the contrast from cell nuclei, which enables their accurate segmentation (see supplementary Fig. S1). By binarizing the enhanced nuclei map and the saturation channel, we can identify all WBC nuclei with high precision. The RBC map is obtained similarly, but here we normalize the red channel by the green channel, which provides high separation between the RBCs and the WBC cytoplasm as well as the image background. A binary operation is then applied to the normalized map, followed by the removal of all previously detected nuclei to obtain the RBC map (Fig. S1(d)). The most challenging part of this segmentation is to accurately identify the cytoplasm of the WBCs and not confuse it with parts of the background. Color segmentation is not enough for this process, but the textural differences between the image background and the cytoplasm are clear enough to easily separate the two. To do this, two $9 \times 9$ pixels ($\sim 1.5 \times 1.5$ $\mu$m) moving entropy window filters are applied, one to the hue channel and one to the Luminance channel. The hue entropy map helps us identify the whole extent of white blood cells, while the luminance entropy map allows for a high precision map of the background (see supplementary Fig. S1(b)). The size of the entropy filters is chosen to ensure that the contribution of the subcellular structures within the cells to the entropy is also considered, and a high-resolution map is generated. Finally, a high precision mask of each detected WBC is obtained by subtracting the RBCs, the background, and local edges.
from the binarized entropy filtered Hue map. All binary conversions are done by thresholding using Otsu’s method, which identifies the global threshold to minimize intra-class variance [25].

Two main challenges were overcome during the design stage of the segmentation algorithm, which included (1) identifying and eliminating any dead cells present in our images and (2) ensuring a highly precise and robust cell mask generation (pink boundaries in Fig. 2 inserts). Dead white blood cells should be excluded from the total count to be consistent with existing clinical practice which exclude dead cells when assessing ANC via flow cytometry and manual white blood cell differentials, and when performing visual morphology analysis. Identification of dead cells was achieved by analyzing the nuclear saturation map, which is significantly lower for dead WBCs compared to live ones (see supplementary Fig. S2).

While the entropy maps gave us a good idea of where WBCs may be located, the separation between the cytoplasm of WBCs and background was not always clear, and thus obtaining a precise edge map was not straightforward. To improve the precision in our segmentation, we calculated the cellular edge maps from the RGB images as well as the hue channel of each image in HSV color-space. Both edge maps were obtained by calculating the image Laplacian, which measures the 2nd spatial derivative of the image, thus strongly enhancing the edges. These two edge maps were then combined to isolate the cell cytoplasm from the background. The cell and nucleus masks shown in Fig. 2 display the high precision at which segmentation was performed.

2.5. Feature extraction and WBC classification

Once the WBC nuclear and cytoplasm masks were identified and the total WBC counts were obtained, a classification step was performed to identify neutrophils amongst WBC subtypes. Only samples with detected WBCs went forward to the classification step. Otherwise, for patients with zero detected WBCs (1 in our data), the classification step is omitted, and zero neutrophils and WBCs are immediately reported without the additional step. To train and test the classifier, a total of 48 features (listed in supplementary Table S1) were calculated based on the shape, size, texture, and biomolecular absorption information of the segmented cells in images captured at 255 nm. These features were chosen to capture the visually apparent differences amongst the cells. To identify the most significant features that best classify neutrophils, we ran a Neighborhood Component Feature Selection (NCFS) algorithm on the feature set, which assigns specific weights to each feature depending on how big a role they play in maximizing the nearest neighbor classification accuracy [26]. Features with an assigned weight of at least 2% of the maximum weight were selected and used for the classification.

Once the most significant features were selected, we developed a classifier composed of a set of Gaussian Support Vector Machines (SVM) trained to identify neutrophils amongst all other white blood cell subtypes. A schematic of the classification model can be found in Fig. 1(e), in which the outputs of three individual SVM models are averaged to obtain one final classification output. The possible outcomes of the classifier are neutrophil (1) or not neutrophil (0). The first classifier was made from an SVM cascade ensemble with two steps. The first one learned the support vectors to separate granulocytes (i.e., neutrophils, eosinophils, and basophils) from monocytes and lymphocytes. The next step only included the detected granulocytes and learned to separate neutrophils from eosinophils and basophils. The second classifier in the model was a one vs. all SVM, which found the support vectors that best separate neutrophils from all other white blood cells. The last SVM in the model was a five-way classifier that learned the support vectors to differentiate amongst all WBC subtypes. This multi-class SVM was trained following a one vs. one strategy, which splits the multi-class problem into multiple binary problems, one for each cell type pair. The outcome of the five-way classifier was then simplified into a binary output by making anything other than a neutrophil, a non-neutrophil. All the SVMs in the model performed similarly when tested individually, but the performance routinely improved when combining all three outputs.
Fig. 2. Pseudo-colorized UV image segmentation. Automatic WBC segmentation of a wide-field pseudo-colorized UV image acquired from blood smear obtained from a healthy donor. Pink and green lines denote the algorithm-generated whole cell and cell nucleus masks, respectively.
The available data from smear samples obtained from 11 neutropenia patients as well as 4 healthy donors was separated into a series of training and testing sets. Given the limited amount of data available, we decided to perform a set of independent training and testing processes, each of which left out a different patient for testing and used the rest of the data for training. One of the severe neutropenia patients had a total white blood cell count of 0, therefore this patient was omitted for training and testing the cell classifiers, leading to a total of 15 training-testing processes. This architecture allowed us to measure our model’s performance while ensuring a leave-one-patient-out scheme. The NCFS algorithm was applied to the training data set, and the resulting features were put through all independent SVMs of the set. Each SVM was optimized using a 5-fold cross-validation scheme [27]. The final decision was made by averaging the output of all three individual classifiers.

3. Results

3.1. Deep-UV microscopy of unlabeled whole blood smears

Whole blood smear samples are imaged immediately after preparation on quartz microscope slides without any cell fixation, dilution, or staining (Fig. 1(a-b)). The system itself consists of a conventional transmission-based microscope geometry with Illumination based on a broadband, laser-driven plasma source and a set of band-pass filters that tune the imaging wavelength to 255, 280, or 300 nm [22]. These wavelengths are specifically chosen because they correspond to the absorption peak of important biomolecules (e.g., nucleic acids and proteins) [19–21,28] or can serve as a virtual counterstain for our pseudo-colorization [22]. After images are captured at these three distinct UV-wavelengths, the gain and compression of the individual images are adjusted and combined to produce a single pseudo-colorized RGB image that recapitulates the standard Giemsa stain [22], as shown in Fig. 1(c) (see pseudo-colorization method in the Methods section). Compared to the individual acquisitions, the enhanced contrast of the synthesized color image is critical in the automated segmentation process described below.

To ensure that a sufficient number of neutrophils are identified for robust neutropenia detection, we acquire 120 images per smear (for each wavelength), which spans an area of \( \sim 1\text{mm} \times 2\text{mm} \) and includes approximately 20,000 blood cells. With the current system, this process takes \(~3\) minutes per wavelength. In this work, we also stitch the 120 pseudo-colorized UV images together (e.g., Fig. 2), allowing us to easily count the neutrophils manually for comparison with the automated counts (see Methods section for details). Smears (one per donor or patient) were generated from peripheral whole blood collected from healthy donors (\( N = 4 \)) and neutropenia patients (\( N = 5 \) moderate, and \( N = 6 \) severe).

3.2. Automated cell segmentation and classification using pseudo-colorized UV microscopy images

To demonstrate the potential utility of our label-free assay as a facile, reliable, and accurate point-of-care neutropenia diagnosis and monitoring device, we develop an automated cell segmentation and classification algorithm. Our approach relies on the unique colors and textures of each blood cell type in the pseudo-colorized UV images. In this process, we first detect red blood cells (RBCs) using a color-based segmentation approach that leverages their relatively homogenous and unique red color in the pseudo-colorized UV images (more details provided in the Methods section). In parallel, WBC nuclei are identified based on the structures’ high saturation levels and expected purple hue. Following these steps, we search the remaining space for WBCs boundaries, achieved using a texture analysis. Specifically, we apply a moving window entropy filter of \( 9 \times 9 \) pixels (\( \sim 1.5\mu m \times 1.5\mu m \)) to the Hue and Luminance channels of the color images. Regions with high entropy and a nucleus present are designated as WBCs, cropped, and stored for feature extraction. As a final step in the segmentation, we refine the WBC edges
by computing the Laplacian of the cropped images. This process enables (1) high precision
contouring of the WBCs’ cytoplasm and nucleus and (2) extraction of structural features within
these structures for classification in a computationally efficient manner (see Methods section for
more details). Figure 2 shows representative results from the segmentation algorithm, which
clearly demonstrate the high precision segmentation of WBCs in a wide-field pseudo-colorized
UV image of a blood smear with a wide variety of cell types. Importantly, the segmentation
process takes \( \sim 0.35s \) per UV image (171\( \mu m \times 230\mu m; 1040 \times 1392 \) pixels). By applying the
automatic segmentation on the acquired pseudo-colorized UV images, we can obtain the total
WBC count by simply counting the number of unique WBC masks.

The segmented white blood cells are then transferred into a classification pipeline to identify
and count neutrophils. First, a total of 48 structural, textural, and biochemical features are
extracted from the nucleus and cytoplasm of the WBCs. Here we only use the 255nm image
of each detected WBC, as we have previously determined that this wavelength contains the
most relevant information to classify WBCs [22] (see Methods section and supplementary
Table S1 for details). Next, we develop a classification scheme (Fig. 1(e)) consisting of three
individual classifiers, each trained to identify whether a given WBC is a neutrophil using a slightly
different approach. The first comprises a two-step SVM cascade, where the first (SVM-1a)
learns to separate granulocytes (i.e., neutrophils, basophils, and eosinophils) from monocytes and
lymphocytes. The second SVM (SVM-1b), takes the detected granulocytes from the first stage
to then identify the neutrophils. The second classifier uses one SVM (SVM-2) which follows
a one vs. all approach; that is, it learns the support vectors necessary to separate neutrophils
from all other WBC subtypes in one step. The third and final classifier is treated as a multi-class
SVM, which learned a 5-way differential of all classes. The three SVM classifiers display similar
uncertainty levels, therefore their outputs are averaged with equal weights to obtain a final
decision. The combined classifier consistently outperformed each classifier.

To train the classifiers, data are separated into various training and testing sets following an
iterative leave-one-out method. Specifically, for each iteration, the entire data from a given
patient is left out of training, then those data are used solely for testing in that iteration. This
process continues for \( N = 15 \) iterations where \( N \) is the total number of patients. This allows for a
robust performance analysis of our classification scheme (Fig. 3) while maintaining the integrity
provided by a leave-one-(patient)-out testing model.

In the training process, we first apply a feature selection algorithm to reduce the data
dimensionality and classification complexity. This is done via a Neighborhood Component
Feature Selection (NCFS) algorithm, which ranks features based on their nearest neighbor
classification accuracy [26]. The feature selection algorithm is applied independently for each
individual SVM (see Fig. 1(e)), and it consistently selects 5–7 highly significant features. Some
of the most commonly selected features include nuclear and cell area difference, cytoplasm
pixel variance, and cellular homogeneity (defined by uniformity of the non-zero elements of the
co-occurrence matrix), which align well with our prior knowledge about the morphological and
textural differences between WBC subtypes. Then, the selected features were used in the training
and testing of each SVM.

Figure 3 shows the neutrophil classifier performance, including the training and testing
confusion matrices and ROC curves of all classifiers for the training data. Here the performance
of the classifiers to correctly identify neutrophils compared to manual counts is evaluated
cumulatively across the 15 independent training-testing sets. The total number of detected white
blood cells among all patients was 164, out of which 64 were labeled as neutrophils. The average
accuracy for training is 97.8%. The accuracy of correctly classifying neutrophils for all combined
testing sets is 91.9%. For the training datasets, the classification sensitivity is 99%, and the
specificity is 96%. For the testing data, the sensitivity and specificity are 91.7% and 92.5%,
respectively. The average area-under-the-curve (AUC) calculated from all independent training
sets was 0.99, with a standard deviation of 0.0088, and AUC of 0.93 for the cumulative test sets. These results show that the neutrophil classification SVMs converged well during training and remained successful throughout the testing process, making it a robust tool to identify neutrophils amongst the automatically segmented WBCs in our samples.

3.3. **Label-free automated WBC and neutrophil counting for neutropenia detection and staging**

Next, we assess our ability to correctly identify and grade neutropenia on individual patients (i.e., individual test sets). Here we also analyze how well WBC counts correlate with the presence and grade of neutropenia, and how results/conclusions may differ from those reached based on neutrophil counts (which is how neutropenia is diagnosed and graded). Figures 4(a-c) show the WBC and neutrophil counts obtained both manually and in an automated manner from smear samples collected from healthy donors (N = 4) as well as moderate (N = 5) and severe (N = 6) neutropenia patients. As shown in Fig. 4(a), although the total WBC counts reveal a statistically significant difference (based on student’s t-test) between the healthy and neutropenia samples ($p - val_{healthy vs moderate} = 0.0197$, $p - val_{healthy vs severe} = 0.0278$), no statistical significance was found between the two stages of neutropenia ($p_{moderate vs severe} = 0.4896$). The same trend can also be observed in the total WBC counts obtained via our automated counting algorithm in Fig. 4(b) ($p - val_{moderate vs severe} = 0.4537$). In contrast, the manual neutrophil counts in Fig. 4(c) show a high statistically significant difference between moderate and severe neutropenia samples ($p - val_{moderate vs severe} = 0.007$), as well as a significant difference for neutropenia vs healthy samples ($p - val_{healthy vs moderate} = 0.0221$, $p - val_{healthy vs severe} = 0.006$). The results of our automated neutrophil counting illustrated in Fig. 4(d), again show the same trend with a $p$-value of 0.0146 for healthy vs. moderate, a $p$-value of 0.0042 for healthy vs. severe neutropenia, and a $p$-value of 0.0062 for moderate vs. severe. The results in Fig. 4(a-d) clearly show the importance of specifically quantifying neutrophils, instead of just WBCs, for accurate
diagnosis and monitoring of different stages of neutropenia. Further, these results also shows that both the manual and automated neutrophils counts are nearly identical and thus yield the same classification power.

Finally, we analyze (1) the correlation between the automated and manual WBC and neutrophil counts from the blood smears, and (2) the relationship between absolute neutrophil count (ANC) values from CBCs and our automated neutrophil counts. Figures 4(e) and 4(f) show the results, revealing a high degree of linearity between the manual and automated counts with an $R^2$ value of 0.999 for WBC counts and 0.993 for neutrophil counts. This points to the fact that our automated
technique (segmentation plus classification) possesses equal diagnostic capability to conventional manual counting. Moreover, the color-coded patches that correspond to the three groups (i.e., healthy, moderate neutropenia, and severe neutropenia) in Fig. 4(f) display the high degree of separation between the three groups when neutrophil counting is performed. In contrast, the highly overlapping color-coded patches in Fig. 4(e) demonstrate a significantly lower separability between healthy and neutropenia samples, as well as the two condition stages, when counting WBC alone. Lastly, Fig. 5 show the relationship between the ANC counts (used as ground truth to assess neutropenia grade) and our automated counts from the blood smears collected from patients diagnosed with moderate and severe neutropenia. We note that since healthy volunteers were used for the healthy samples, ANC were not collected, and are thus not explicitly included in the graph. Nevertheless, the range of healthy ANC counts (2,000–7,000/µL [29]) and the range of neutrophils counted with our automated approach (3–22) are shaded in green in Fig. 5. Overall, we again observe good separation between the two grades of neutropenia (moderate vs. severe) when we plot the ANC values against our automated neutrophil counts (see color-coded patches in Fig. 5). However, given the low number of neutrophils detected in patients with severe neutropenia, it is not possible to assess a correlation for this group (all severe cases had zero neutrophils in the smear). Nevertheless, for the patients with moderate neutropenia, the correlation between ANC and the automated counts is relatively strong with $R^2 = 0.635$ (see dashed black line in Fig. 5). This indicates that neutropenia may be efficiently detected and graded with our current protocol (using 120 images spanning an area of 1mm × 2mm) but direct correlations to neutrophil counts from ANC may not be possible for severe cases of neutropenia, unless a larger area is scanned to obtain more robust counting statistics. This points to an engineering parameter that trades-off time of acquisition with correlation with ANC. The choice will depend on the application and intended purpose: these data suggest that 120 images indeed enable robust detection and grading of neutropenia, but if a detailed ANC is

![Fig. 5. Automated neutrophil counts and ANC values for severe and moderate neutropenia samples. Automated neutrophil counts obtained from our segmentation and classification algorithms applied to smear images from moderate and severe neutropenia samples plotted against the ANC values from CBC results obtained at the sample collection site. The color-coded dashed lines indicate the ANC and neutropenia count ranges for moderate and severe neutropenia as well as healthy blood. The color-coded patches indicate the bounding regions for the sample sets while the black dashed line indicates the fitted line to the moderate neutropenia samples’ data with an R2 of 0.635.](image-url)
desired, larger areas should be scanned. This could also be a dynamic parameter, where an initial analysis is performed on, for example, 120 images and if the analysis yields low counts, then more regions could be imaged to improve counting statistics.

4. Discussion

In this work, we have presented a robust, accurate, fast, label-free, and fixative-free automated technology to diagnose and grade neutropenia from a blood smear based on UV microscopy, image analysis, and machine learning-based cell classification. This novel assay gives access to information with similar diagnostic power to traditional hematology brightfield images [22]. Our UV-based pseudo-colorization scheme presents a substantially higher degree of consistency than conventional staining protocols used in hematological practice such as Giemsa stain. This method does not suffer from photobleaching as it only relies on the UV absorption of endogenous biomolecules (e.g., nucleic acid, proteins, hemoglobin, etc.), and does not rely on fluorescence. In addition, the brief UV light exposure here (30–100 ms) does not produce any damage nor alter intracellular morphology. UV induced morphological alterations have indeed been observed but only after ~10 minutes of continuous exposure to deep UV light [22], which is orders of magnitude greater than the exposure here. Thus, with our protocols, samples can be subsequently analyzed via conventional Giemsa or fluorescence stains without alterations to cellular structure. Given proper management of UV exposure, cells can be imaged with UV light contiguously for over 6 hours [19]. The UV images also hold quantitative information and give insight into the samples’ biomolecular properties, which are not accessible in traditional hematology techniques. This approach obviates the need for chemical processing, fixing, and staining, which makes this technique more accessible, inexpensive, and fast, suitable for use in clinical and point-of-care settings as well as in resource-limited environments.

With simple modifications (e.g., using low-cost LEDs), the overall cost of the system proposed here can be reduced to approximately 1/20th of the average cost of a hematology analyzer (which typically cost $80,000–120,000 USD). Plus, the developed assay does not require any reagents or stains, which significantly simplifies the sample preparation process while reducing the procedure’s overall cost with minimal maintenance compared to conventional hematology analyzers, which typically require multiple reagents (typically 7–14) and constant maintenance. The presented technique is also faster than the traditional blood smear hematology analysis, given the fact that it does not require any sample preparation (fixing and staining) and that each image can be segmented in less than a second.

The automatic WBC and neutrophil counts show a high correlation with manually counted cells, further validating the reliability of the developed framework for automated neutropenia detection and staging. The segmentation is fast (~0.35s per image), and the classification, using a pre-trained classifier set, can be done nearly instantly. This allows the system to offer near-real-time analysis of the pseudo-colorized images and, subsequently, monitor patients’ condition. It is also worth noting that, with a more extensive sample set, an automated five-part WBC differential count is possible [22] (omitted here due to low counts of basophils). This opens the door for the diagnosis of many other blood diseases for which regular monitoring of white blood cell morphology and population may be beneficial.

In conclusion, this novel label-free automatic neutropenia detection system based on deep-UV microscopy enables accurate, robust, and facile WBC and neutrophil enumeration that currently requires costly and time-consuming CBC and microscopic analysis. Our approach has the potential to improve the current standard-of-care for neutropenia diagnosis and monitoring owing to its simplicity, low-cost, and speed. The unique features of this UV microscopy-based assay can significantly improve the accessibility of hematological analysis for neutropenia patients that may benefit from regular monitoring of their neutrophil counts, with the potential of becoming a point-of-care or at-home device. Moreover, our approach’s fast feedback can be advantageous...
for many cloud-based telemedicine applications or low-resource settings where such a system can enable timely intervention for patients under severe conditions. Finally, this technique can be combined with active or passive microfluidic devices to have an accurate, quantitative ANC per unit volume measurement in a fully automated process.

**Funding.** The Massner Lane Family Foundation; Burroughs Wellcome Fund (CASI BWF 1014540); National Science Foundation (NSF CBET CAREER 1752011); Donaldson Charitable Trust Research Synergy Fund Award; Georgia Institute of Technology.

**Acknowledgments.** We greatly acknowledge support for this work by the Massner Lane Family Foundation; Burroughs Wellcome Fund (CASI BWF 1014540); National Science Foundation (NSF CBET CAREER 1752011); and the Donaldson Charitable Trust Research Synergy Fund Award, a philanthropic award provided by the Winship Cancer Institute of Emory University, the Aflac Cancer & Blood Disorders Center at Children’s Healthcare of Atlanta, and the Wallace H. Coulter Biomedical Engineering Department at Emory University and the Georgia Institute of Technology.

**Disclosures.** The authors declare no conflicts of interest.

**Data availability.** Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

**Supplemental document.** See Supplement I for supporting content.

**References**

1. L. S. Schwartzberg, “Neutropenia: etiology and pathogenesis,” Clin. Cornerstone 8(5), S5–S11 (2006).
2. A. S. Bhardwaj and S. C. Navada, “Management of chemotherapy-induced neutropenic fever,” Hosp. Pract. 41(1), 96–108 (2013).
3. S. L. Michels, R. L. Barron, M. W. Reynolds, K. Smoyer Tomic, J. Yu, and G. H. Lyman, “Costs associated with febrile neutropenia in the US,” Pharmacoeconomics 30(9), 809–823 (2012).
4. D. M. Courtney, A. Z. Aldeen, S. M. Gorman, J. A. Handler, S. M. Trifilio, J. P. Parada, P. R. Yarnold, and C. L. Bennett, “Cancer-associated neutropenic fever: clinical outcome and economic costs of emergency department care,” Oncologist 12(8), 1019–1026 (2007).
5. C. E. Majors, M. E. Pawlowski, T. Tkaczyk, and R. R. Richards-Kortum, “Low-cost disposable cartridge for performing a white blood cell count and partial differential at the point-of-care,” in 2014 IEEE Healthcare Innovation Conference, HIC 2014 (Institute of Electrical and Electronics Engineers Inc., 2014), pp. 10–13.
6. S. Zheng, J. Lin, H. Kasdan, and Y. Tai, “Fluorescent labeling, sensing, and differentiation of leukocytes from undiluted whole blood samples,” Sensors Actuators B Chem. 132(2), 558–567 (2008).
7. G. J. van de Geijn, V. van Rees, N. van Pul-Bom, E. Birnie, H. Janssen, H. Pegels, M. Beunis, and T. Njo, “Leukoflow: Multiparameter extended white blood cell differentiation for routine analysis by flow cytometry,” Cytometry 79(9), 694–706 (2011).
8. L. L. Chan, A. R. Wilkinson, B. D. Paradis, and N. Lai, “Rapid image-based cytometry for comparison of fluorescent viability staining methods,” (n.d.).
9. A. Ramoji, U. Neugebauer, T. Bocklitz, M. Foerster, M. Bauer, and J. Popp, “Toward a spectroscopic hemogram: Raman spectroscopic differentiation of the two most abundant leukocytes from peripheral blood,” Anal. Chem. 84(12), 5335–5342 (2012).
10. G. S. Verebes, M. Melchiorre, A. Garcia-Leis, C. Ferreri, C. Marzetti, and A. Torreggiani, “Hyperspectral enhanced dark field microscopy for imaging blood cells,” J. Biophotonics 6(11-12), 960–967 (2013).
11. B. P. Yakimov, M. A. Gogoleva, A. N. Semenov, S. A. Rodionov, M. V. Novoselova, A. V. Gayer, A. V. Kovalev, A. I. Bernakevich, V. V. Fadeev, A. G. Armaganov, V. P. Drachev, D. A. Gorin, M. E. Darvin, V. I. Shcheslavskiy, G. S. Budylin, A. V. Priezzhev, and E. A. Shirshin, “Label-free characterization of white blood cells using fluorescence lifetime imaging and flow-cytometry: molecular heterogeneity and erythrophagocytosis [Invited],” Biomed. Opt. Express 10(8), 4220 (2019).
12. J. Yoon, K. Kim, H. Park, C. Choi, S. Lang, and Y. Park, “Label-free characterization of white blood cells by measuring 3D refractive index maps,” Biomed. Opt. Express 6(10), 3865 (2015).
13. K. J. Chalut, A. E. Ekpenyong, W. L. Clegg, I. C. Melhuish, and J. Guck, “Quantifying cellular differentiation by physical phenotype using digital holographic microscopy,” Integr. Biol. 4(3), 280–284 (2012).
14. A. E. Ekpenyong, S. M. Man, S. Achouri, C. E. Bryant, J. Guck, and K. J. Chalut, “Bacterial infection of macrophages induces decrease in refractive index,” J. Biophotonics 6(5), 393–397 (2013).
15. T. A. Zangle, D. Burns, C. Mathis, O. N. Witte, and M. A. Teitell, “Quantifying biomass changes of single CD8+ T cells during antigen specific cytotoxicity,” PLoS One 8(7), e68916 (2013).
16. J. Yoon, Y. J. Jo, M. H. Kim, K. Kim, S. Y. Lee, S. J. Kang, and Y. K. Park, “Identification of non-activated lymphocytes using three-dimensional refractive index tomography and machine learning,” Sci. Rep. 7(1), 1–10 (2017).
17. A. Bourquard, A. Pablo-Trinidad, I. Butterworth, Á Sánchez-Ferro, C. Cerrato, K. Humala, M. Fabra Urdiola, C. Del Rio, B. Valles, J. M. Tucker-Schwartz, E. S. Lee, B. J. Vakoc, T. P. Padera, M. J. Ledesma-Carbayo, Y. Bin Chen, E.
P. Hochberg, M. L. Gray, and C. Castro-González, “Non-invasive detection of severe neutropenia in chemotherapy patients by optical imaging of nailfold microcirculation,” Sci. Rep. 8(1), 5301 (2018).

18. G. N. McKay, N. Mohan, I. Butterworth, A. Bourquard, Á Sánchez-Ferro, C. Castro-González, and N. J. Durr, “Visualization of blood cell contrast in nailfold capillaries with high-speed reverse lens mobile phone microscopy,” Biomed. Opt. Express 11(4), 2268 (2020).

19. B. J. Zeskind, C. D. Jordan, W. Timp, L. Trapani, G. Waller, V. Horodnicu, D. J. Ehrlich, and P. Matsudaira, “Nucleic acid and protein mass mapping by live-cell deep-ultraviolet microscopy,” Nat. Methods 4(7), 567–569 (2007).

20. A. Ojaghi, M. E. Fay, W. A. Lam, and F. E. Robles, “Ultraviolet hyperspectral interferometric microscopy,” Sci. Rep. 8(1), 9913 (2018).

21. S. Soltani, A. Ojaghi, and F. E. Robles, “Deep UV dispersion and absorption spectroscopy of biomolecules,” Biomed. Opt. Express 10(2), 487 (2019).

22. A. Ojaghi, G. Carrazana, C. Caruso, A. Abbas, D. R. Myers, W. A. Lam, and F. E. Robles, “Label-free hematology analysis using deep-ultraviolet microscopy,” Proc. Natl. Acad. Sci. 117(26), 14779–14789 (2020).

23. S. Preibisch, S. Saalfeld, and P. Tomancak, “Globally optimal stitching of tiled 3D microscopic image acquisitions,” Bioinformatics 25(11), 1463–1465 (2009).

24. J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, and A. Cardona, “Fiji: An open-source platform for biological-image analysis,” Nat. Methods 9(7), 676–682 (2012).

25. N. Otsu, “Threshold selection method from gray-level histograms,” IEEE Trans. Syst. Man Cybern. SMC 9(1), 62–66 (1979).

26. W. Yang, K. Wang, and W. Zuo, “Neighborhood component feature selection for high-dimensional data deep structured scene parsing by learning with image descriptions view project human recognition view project neighborhood component feature selection for high-dimensional data,” J. Comp. 7, 161–168 (2012).

27. M. S. Santos, J. P. Soares, P. H. Abreu, H. Araujo, and J. Santos, “Cross-validation for imbalanced datasets: Avoiding overoptimistic and overfitting approaches [Research Frontier],” IEEE Comput. Intell. Mag. 13(4), 59–76 (2018).

28. M. C. Cheung, J. G. Evans, B. McKenna, and D. J. Ehrlich, “Deep ultraviolet mapping of intracellular protein and nucleic acid in femtograms per pixel,” Cytometry 79A(11), 920–932 (2011).

29. J. Dacie, Dacie and Lewis Practical Haematology (Elsevier, 2006).