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Identification and characterization of neutrophil extracellular trap shapes in flow cytometry

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ABSTRACT

Neutrophil extracellular trap (NET) formation is an alternate immunologic weapon used mainly by neutrophils. Chromatin backbones fused with proteins derived from granules are shot like projectiles onto foreign invaders. It is thought that this mechanism is highly anti-microbial, aids in preventing bacterial dissemination, is used to break down structures several sizes larger than neutrophils themselves, and may have several more uses yet unknown. NETs have been implied to be involved in a wide array of systemic host immune defenses, including sepsis, autoimmune diseases, and cancer. Existing methods used to visually quantify NETotic versus non-NETotic shapes are extremely time-consuming and subject to user bias. These limitations are obstacles to developing NETs as prognostic biomarkers and therapeutic targets. We propose an automated pipeline for quantitatively detecting neutrophil and NET shapes captured using a flow cytometry-imaging system. Our method uses contrast limited adaptive histogram equalization to improve signal intensity in dimly illuminated NETs. From the contrast improved image, fixed value thresholding is applied to convert the image to binary. Feature extraction is performed on the resulting binary image, by calculating region properties of the resulting foreground structures. Classification of the resulting features is performed using Support Vector Machine. Our method classifies NETs from neutrophils without traps at 0.97/0.96 sensitivity/specificity on $n = 387$ images, and is 1500X faster than manual classification, per sample. Our method can be extended to rapidly analyze whole-slide immunofluorescence tissue images for NET classification, and has potential to streamline the quantification of NETs for patients with diseases associated with cancer and autoimmunity.

Keywords: Neutrophil extracellular trap, support vector machine, flow cytometry, image analysis.

1. INTRODUCTION

Neutrophils are a type of white blood cell that protect the body from infections. Neutrophils engulf microbes via phagocytosis to provide the first level of defense to our body. Such phagocytosis is achieved via various cellular signaling mechanisms[1]. Neutrophil extracellular trap (NET) formation is an alternative method used by neutrophils to aid in controlling infection of extracellular (non-phagocytosed) pathogens[2]. NETs are composed of chromatin backbones and

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neutrophil granular constituents that kill and/or limit the spread of microbes, including bacteria and fungi[3]. NETosis can also result in tissue injury and thrombosis, underscoring the importance of regulation of NETs and response to microbes and non-infectious stimuli, including the release of pro-inflammatory products from cellular injury[4]. Moreover, NETs have been implicated in the pathogenesis of a wide array of diseases caused by inflammatory injury, including sepsis, autoimmune diseases, and cancer[5, 6]. There is considerable interest in developing new therapeutic approaches that target NETs, including the development of small molecule inhibitors of pathways required for NETosis. Conversely, there is also substantial interest in exploiting NET constituents to augment host defense in patients with immune impairment.

Proper exploration of NETs as therapeutic agents requires high throughput analysis of neutrophils under varying biological conditions. Existing methods used to visually quantify NETotic versus non-NETotic neutrophil shapes are extremely time-consuming and subject to user bias. The sole computational method in the literature pertaining to NET imaging explores quantifying NET fractions in *in vitro* microscopic images of mixture of neutrophils and NETs[7]. However, this method does not consider estimating the NETs' morphology, quantification of which is important to understand the implications and functions of NETs, allowing identification of early digital biomarkers to slow down disease progression. The lack of an efficient and reproducible automatic NET characterization scheme hinders the development of NETs as prognostic biomarkers and therapeutic targets for diverse diseases. We have therefore developed an automated method to automatically detect and quantify the shapes of NETs in flow cytometry images, to aid in clinical assessment. Our method is simple to implement and has potential to rapidly identify and characterize the fraction of a neutrophil population that has formed a NET.

In the current manuscript, our method first starts with raw fluorescence images of neutrophils stained with DRAQ5 and imaged using a flow cytometry system. Each individual image is contrast enhanced, binarized, and fit with a convex hull[8]. Next, the area, average intensity, and eccentricity of the convex region are quantified. Shape features are classified using a support vector machine with radial basis kernel and generalizability is analyzed with 10-fold cross validation[9]. The end output is images classified as NETotic or non-NETotic. In the next sections, we summarize our results and methods. Our future work will explore quantifying NETotic features from immunofluorescence images of murine tissue slices.

2. METHODS

This section contains details on the preparation of neutrophils, neutrophil images, and imaging conditions and setup. Subsequently, processing of neutrophil fluorescence images and characterization of NET structures are discussed. We conclude the section by discussing the classification method of extracted structural features along with our analysis in evaluating the proposed classifier's performance.

2.1. Image preparation

Neutrophils were isolated from normal donor blood and stimulated *in vitro* for two hours with phorbol myristate acetate (Sigma-Aldrich, St. Louis, MO) as a positive control for NET generation. The procedure followed a protocol approved by Institutional Review Board at Roswell Park Cancer Institute, Buffalo, NY. The cells were then stained with DRAQ5 (ex/em (nm): 681/697; ThermoFisher Scientific, Waltham, MA), and imaged using ImageStream® flow-cytometry system (Millipore Sigma, Darmstadt, Germany). The pixel resolution of the captured images was 0.17 µm per pixel. In this resolution, a typical NET shape varies from 7-20 µm along the major axis and 1-5 µm along the minor axis, assuming that a typical NET shape is elliptical. In contrast, a neutrophil shape is typically circular with 6-7 µm along the diameter.

2.2. Image preprocessing

Contrast limited adaptive histogram equalization (CLAHE) method[8] locally adapts the contrast of the input fluorescence image of NET or non-NET so that its resulting gray-scale intensity histogram matches a specified probability distribution[10]. MATLAB has implemented this method in the function *adapthisteq*[11]. In our pipeline, we used the target distribution to be Rayleigh[8], and all other parameters are left as MATLAB in-built default. The normalized contrast improved image is thresholded at 0.5. This threshold value ensures capturing the chromatin tail (white arrow; Fig. 1A) in the NET positive images analyzed in this manuscript. The resulting binary image is then passed for feature extraction.

2.3. Feature extraction

We first compute the convex hull of the binarized image. The convex hull of a set of points in Euclidean space is the smallest convex set which contains all the points. Convex hull accentuates the long, elliptical morphology of NET shapes. Area of the input image is defined as the sum of pixels within the convex hull. Normalized convex intensity is defined as

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the mean intensity of the raw image within the convex hull divided by the area of the convex hull. The convex hull is then fit with an ellipse having the same second moments as the region. Eccentricity of this fitted ellipse is used as the third feature of the input NETotic or non-NETotic image to be classified. Implementation was done using MATLAB.

2.4. Feature classification

The features extracted from each object are passed to a support vector machine (SVM) classifier[9]. To train the SVM, MATLAB's in-built command *fitcsvm* is used. All parameters are left to default, except for the *KernelFunction* and *BoxConstraint* parameters. For *KernelFunction*, we used radial basis function, and the *BoxConstraint* value is set to be 100. The former parameter determines the type of kernel function to discriminate the feature space. The latter controls the amount of leniency that is allowed when placing the decision boundary between samples of opposite classes that are close together in space. The sister function *predict* is used to perform classification using the generated SVM[12]. Independent testing for performance generalizability was performed using 10-fold cross validation. In this technique, 10 successive test phases are performed, each time randomly holding out $1/10^{th}$ of the dataset and training on the rest of the dataset. The classifier performance is obtained upon averaging the respective classifier performances over all the 10 phases of the cross-validation process.

3. RESULTS

This section discusses first our computational pipeline for extracting features from *in vitro* NETotic and non-NETotic neutrophil images. We then summarize the feature scores (area, eccentricity, and normalized convex intensity) estimated from 143 NETotic and 244 non-NETotic neutrophil images. We discuss the result of SVM classification next, and conclude this section by providing a brief overview of an end-user interface software built in MATLAB for *in vitro* NETs classification.

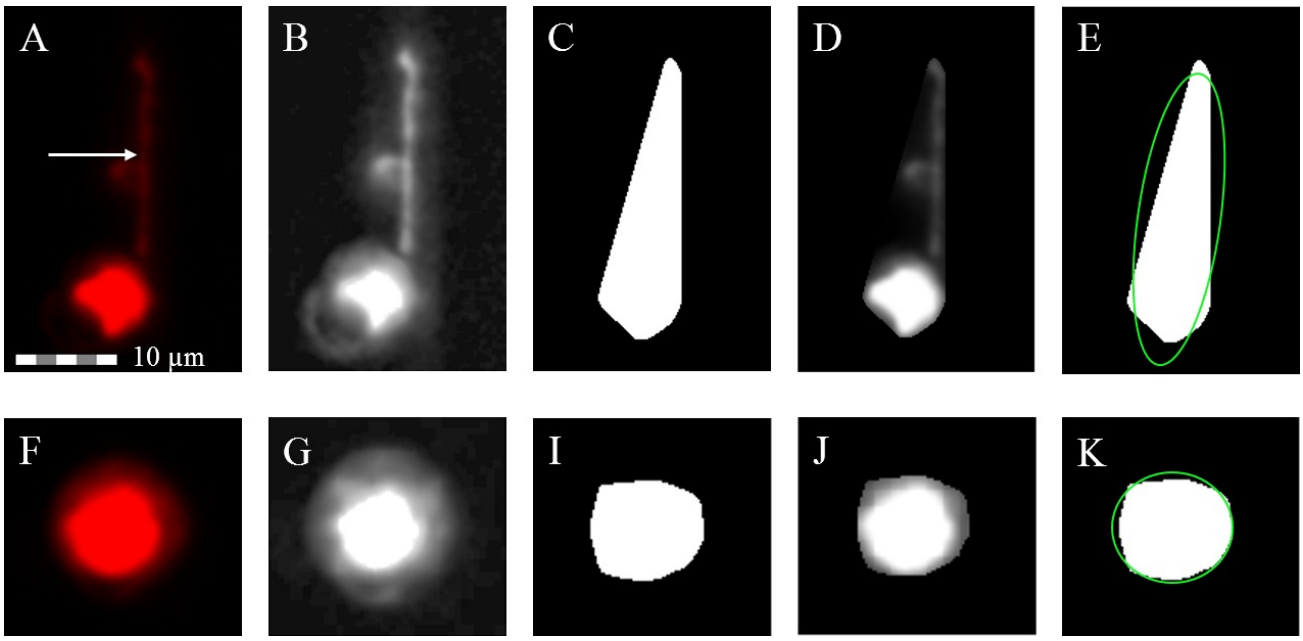

Fig. 1. Demonstration of the computational feature extraction method for flow cytometry images containing neutrophil extracellular traps (NETs). (A) Fluorescence image of a NETotic neutrophil. White arrow shows extracellular content which constitutes a NET. (B) Gray-scale contrast enhanced version of the image shown in (A). (C) Convex hull after thresholding of the image shown in (B). (D) Raw intensity of the NET inside the convex hull region obtained in (C). (E) Ellipse fitting of the structure shown in (C). (F-K) Identical example of what is shown in (A-E), for a non-NETotic neutrophil image.

3.1. Computational pipeline

Fig. 1 demonstrates the computational feature extraction pipeline from flow cytometry images containing NETs and neutrophils (non-NETs). Fig. 1A shows a fluorescence image of a neutrophil with its corresponding NET. The trail of chromatin wrapped in cytoplasmic content ejected from the cell is marked using a white arrow. First each image is

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normalized to have value 0-1. Adapting the initial raw intensity distribution to a Rayleigh distribution using contrast limited adaptive histogram equalization (CLAHE) implemented in MATLAB[12] improves contrast of the NET images. We noted that this contrast enhancement is critical to illuminate NETs which do not demonstrate high intensity and would otherwise be lost to the background. Next the convex hull of each binary image is computed (Fig. 1C). Normalized convex intensity is defined as sum of raw intensity values within the convex hull divided by its area. NET negative images display a much higher value than positive images. This is biologically motivated because the average pixel intensity corresponds to the average level of DNA within the convex boundary of an object; further, highly concave objects like NETs enclose more negative space within their convex hull than highly convex objects like cell bodies, and thus have a lower average DNA content per convex area. The convex hull region shown in Fig. 1C is also fitted with an ellipse, and its major and minor axes and eccentricity are taken as features (Fig. 1E). Figs. 1F-1K show feature extraction for an image of neutrophil with no extracellular content. As can be seen in Figs. 1E and 1K, the convex shape of a NET is typically larger and more elliptical than its sole neutrophil counterpart.

Table 1. Distribution of extracted features for the proposed pipeline under the specific imaging conditions described in Section 2.1. Features shown for both NET positive distributions and NET negative distributions, where the feature separation is evident.

	Convex area (pixels)	Eccentricity (a.u.)	Normalized convex intensity (a.u.)
NET	4770 ± 1660	0.89 ± 0.09	0.43 ± 0.12
Non-NET	1900 ± 300	0.54 ± 0.13	0.79 ± 0.06

3.2. Feature distribution

Using the feature extraction method described above, we computationally quantified convex area, eccentricity, and normalized convex intensity for 143 NETotic and 244 non-NETotic manually annotated images. Manual annotation was performed by the authors Mr. Brandon Ginley and Ms. Tiffany Emmons under the supervision of Dr. Brahm Segal. For the image resolution described in Section 2.1, we report the respective NETotic features in Table 1. Fig. 2A shows the binary classification task as a 3D scatter representation of these features. Owing to the excellent separability between NET vs non-NET shape features, we employed support vector machine (SVM)[9] to automatically classify them from their flow cytometry images.

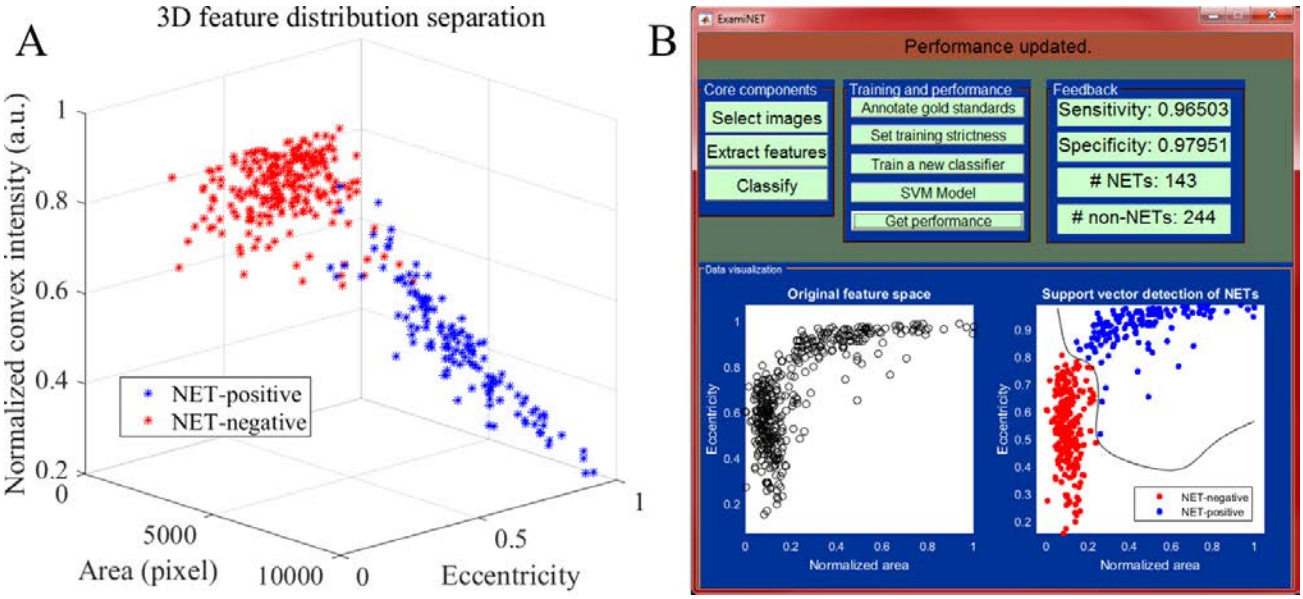

Fig. 2. Classification of NETs' shapes. (A) Scatter of convex area, eccentricity, and normalized convex intensity of NETs and non-NETs. (B) Classification of NETs using support vector machine, packaged as an end user graphical interface.

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3.3. Support vector machine classification

The SVM classification method is able to achieve 0.96/0.97 sensitivity/specificity in our limited dataset of 387 images, quantified using 10-fold cross validation. See Section 2.4 for more information on the cross-validation performance analysis described herein. While no other commercially available automated method exists for NET morphology classification, the IDEAS® software, a commercial software package that comes with ImageStream® flow-cytometer, is trained for automatic identification of NETs[13]. Our automated method outperforms the ImageStream® based method; this latter method achieves 0.92/0.93 sensitivity/specificity. Further, ImageStream® cannot assign a class label to 13% of the $n = 387$ images, while our method labels 100% of the NETs images. The computational speed of our method implemented in MATLAB was found to be >1500X faster than manual classification of NETs conducted by co-author Ms. Tiffany Emmons. Specifically, our method requires 0.00269 seconds to classify a NET from non-NET, while manual classification process requires 4.13 sec for the same task.

3.4. End user interface

Fig. 2B shows an end user graphical interface developed for the proposed method. Users need to provide the folder of input images to analyze, and the features of every image can be calculated. Once the features are calculated, classifying the images will generate two new folders with the NET objects separated from the non-NET ones. Classification is done using the SVM classifier trained on the manually labeled 387 images. Users also have the option of training new SVM classifiers, provided they have manually annotated their data set into two populations. Additionally, the SVM training function allows users to decide how strictly the separating hyperplane is drawn. Too high of a strictness allows minimum samples to be misclassified, but is too specific and not generalized. Strictness too low may yield sub-optimal performance.

4. DISCUSSION

Throughout this manuscript, we claim that our method supersedes the performance of the IDEAS® classifier bundled with ImageStream®. Though we found this to be true for our 387 images, they stem from one common dataset. It is noteworthy to stress that the current study is a pilot study with simulated *in vitro* data and limited scope just to demonstrate the feasibility of our proposed pipeline. A detailed analysis on statistical significance as compared to the IDEAS® software using multiple datasets will be conducted in future works. As we analyze the performance of our method in more unique datasets, we will use further independent testing to objectively compare both methods, and generate receiver operating characteristic curves for both methods.

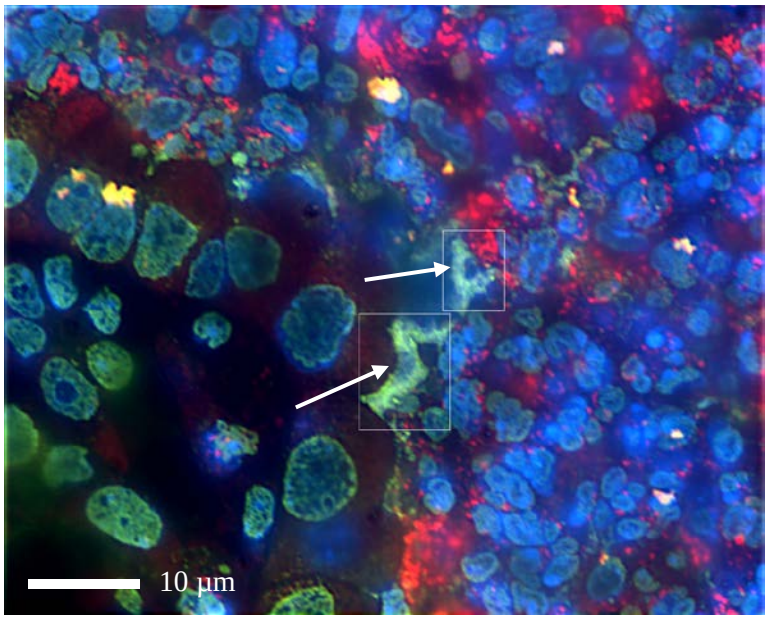

Fig. 3. ***In vivo* immunofluorescence image of NETosis in a mouse model of aspergillus fumigatus pneumonia.** Blue color depicts DNA tagged with DAPI stain (ex/em (nm): 358/461). Red color is Alexa Fluor® 568 (ex/em (nm): 578/603) marking myeloperoxidase. Green color marks histone H1 using Alexa Fluor® 488 (ex/em (nm): 490/525). NETosis is marked with white arrows. The amorphous, abstract shape of NETs *in vivo* is much more heterogeneous than the *in vitro* case discussed in this manuscript. Details of the *in vivo* data preparation method as depicted in this figure are discussed in a previous publication by co-author Dr. Urban[1].

As discussed in Section 1, NETs are implicated to be involved in a wide array of diseases. However, inaccessibility of an automated method to identify and quantify NETs hinders the research community from rapidly unraveling the molecular mechanisms of NETs in large *in vitro* and *in vivo* samples. We have developed a high throughput computational method to quantify NETs *in vitro* that preserves NET morphology. This method leverages the inherent morphological dissimilarity of neutrophils and NETs to assign a class label. High throughput is crucial for rapid analysis of large neutrophilic populations and their response to a variety of biological perturbations. Knowing the fraction of a neutrophil population which undergoes NET formation will be extremely beneficial in developing NETs as prognostic markers and therapeutic targets. This has already been

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accomplished by authors in the past[7], however, this method does not preserve morphology. We believe that the morphology of NETs is fundamental to fully perceive its biological mechanism, function, and response to environmental influence. Our method is simple, intuitive, and biologically motivated by the morphology of neutrophils and NETs. Its simplicity increases receptiveness for all researchers, and allows derivation of computationally inspired biological hypotheses.

Though simple morphological operations can derive efficient features to identify NETs *in vitro*, there is a much greater challenge working with *in vivo* data. Fig. 3 depicts an immunofluorescent section of mouse lung challenged with *aspergillus fumigatus* pneumonia. This image data was provided by the co-author Dr. Urban, and prepared under the protocol described in Ref. [1]. NETosis is marked in white arrows. Due to the extreme heterogeneity and amorphous nature of *in vivo* NETs, identification of *in vivo* NETosis will require an extension to our current method. Further, NETs are closely entangled with other structures in the cellular microenvironment which will require separation. We envision a method which derives both fluorescent marker based features in combination with morphological features will provide sufficient information to identify *in vivo* NETosis using popular machine learning methods. Finally, we will make a correlation between different stages of NETs and understand how they are impacted under various pathological perturbations.

5. CONCLUSION

We present an automated and reproducible classification method for neutrophil extracellular traps *in vitro* using flow cytometry. Our method can be extended to rapidly analyze whole-slide immunofluorescence tissue images comprising trillions of pixels for NET shape classification and for deriving structural motif distributions of these immunological features. Future computational advancement will seek to analyze the proposed method's performance in multiple datasets, and will extend to identify NETs *in vivo*. Future health science advancement will evaluate this technology in identifying and quantifying NETs samples from patients with NET associated diseases, such as cancer and autoimmunity.

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REFERENCES

[1] Rohm, M., Grimm, M. J., D'Auria, A. C. *et al.*, "NADPH oxidase promotes neutrophil extracellular trap formation in pulmonary aspergillosis," Infect Immun, 82(5), 1766-77 (2014).

[2] Brinkmann, V., Reichard, U., Goosmann, C. *et al.*, "Neutrophil extracellular traps kill bacteria," Science, 303(5663), 1532-5 (2004).

[3] Halverson, T. W., Wilton, M., Poon, K. K. *et al.*, "DNA is an antimicrobial component of neutrophil extracellular traps," PLoS Pathog, 11(1), e1004593 (2015).

[4] Martinod, K. and Wagner, D. D., "Thrombosis: tangled up in NETs," Blood, 123(18), 2768-76 (2014).

[5] Demers, M. and Wagner, D. D., "NETosis: a new factor in tumor progression and cancer-associated thrombosis," Semin Thromb Hemost, 40(3), 277-83 (2014).

[6] Berthelot, J. M., Le Goff, B., Neel, A. *et al.*, "NETosis: At the crossroads of rheumatoid arthritis, lupus, and vasculitis," Joint Bone Spine, (2016).

[7] Coelho, L. P., Pato, C., Friaes, A. *et al.*, "Automatic determination of NET (neutrophil extracellular traps) coverage in fluorescent microscopy images," Bioinformatics, 31(14), 2364-70 (2015).

[8] Gonzalez, R. C. and Woods, R. E., [Digital Image Processing], Prentice Hall, 1-976 (2007).

[9] Hastie, T., Tibshirani, R., Friedman, J.H., [The Elements of Statistical Learning: Data Mining, Inference, and Prediction], Springer, 768 (2009).

[10] Hogg, R. V., Craig, A. and McKean, J. W., [Introduction to Mathematical Statistics], Prentice Hall, Upper Saddle River, New Jersey, 1-692 (2004).

[11] Zuiderveld, K., [Contrast limited adaptive histogram equalization] Academic Press Professional, Inc, San Diego, CA, USA (1994).

[12] MATLAB, The Language of Technical Computing, Available from: http://it.mathworks.com/help/matlab/

[13] Zhao, W., Fogg, D. K. and Kaplan, M. J., "A novel image-based quantitative method for the characterization of NETosis," J Immunol Methods, 423, 104-10 (2015).

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