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Automated image-based quantification of neutrophil extracellular traps using NETQUANT

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To the Editor
Journal of Visual Experiments

Lund, 2018

Dear Jaydev Upponi, Ph.D, Science Editor,

Please consider the enclosed manuscript "***Automated image-based quantification of neutrophil extracellular traps using NETQUANT"*** by Pontus Nordenfelt, and myself for publication as an article in the *Journal of Visual Experiments*.

Neutrophils are known to produce neutrophil extracellular traps (NETs) in response to a wide range of infectious and sterile agents. NETs have been implicated in several diseases including infections, cancer and autoimmune disorders[1]. This has resulted in an increase in interest, spanning across multiple fields and disciplines to study NETs. Detection of NETs by immunofluorescence microscopy and subsequent image-based quantification remains a prevalent choice among researchers to assess NET formation. However, manual image-based quantification methods have drawbacks in being either subjective and error prone, especially for non-trained users. Currently available software rooted options are either semi-automatic or require training prior to operation. Hence, there is a genuine need for developing novel, easy-to-use and reproducible software/approaches to quantify NET formation.

In the manuscript, we demonstrate the use of a fully automated immunofluorescence-based image quantification method to quantify NET formation called NETQUANT[2]. NETQUANT has been designed as a freely available app for MATLAB. It is easy to use, with programmable parameters and a user friendly graphic user interface (GUI). The quantification is based on biologically relevant parameters such as an increase in surface area and DNA:NET marker protein ratio, and loss of nuclear morphology to stringently distinguish between extruded NETs from cells that have only underwent nuclear decondensation. Furthermore, NETQUANT also performs analysis and quantification at a single-cell level, which is the highest achievable resolution.

NETQUANT can be potentially used for rapidly quantifying NET formation by various stimuli and to screen for NET enhancing/inhibiting drugs in a high throughput fashion, while also allowing for single quantification and analysis. Based on these points, we believe that the software solution presented in the manuscript represents a significant advancement in immunofluorescence image-based NET quantification and therefore could be of interest to the broad readership of *Journal of Visual Experiments*.

Yours sincerely,

Tirthankar Mohanty, Ph.D

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To the Editor
Journal of Visual Experiments

Lund, 2018

Dear Alisha Dsouza, Ph.D., Senior Review Editor,

Please find attached the revised version of the manuscript, "***Automated image-based quantification of neutrophil extracellular traps using NETQUANT"***, which had been assigned the id JoVE58528. We would like to express our gratitude to the referees for their time and insightful comments. This has certainly improved the overall depth and clarity of the manuscript. A detailed point-by-point explanation addressing the referees' comments has also been included. Apart from this, an updated version of the software (NETQUANT version 1.2) and manual has been made available for the referees on the NETQUANT homepage.

I would also like to draw your attention to the change of order in the author list. In the initial submission, I was listed as the senior and corresponding author, while Dr. Nordenfelt was listed as the first author. After internal discussion we have decided to switch roles and now I am listed as first and corresponding author, and Dr. Nordenfelt as the senior author.

Yours sincerely,

Tirthankar Mohanty, Ph.D

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TITLE:

Automated Image-Based Quantification of Neutrophil Extracellular Traps Using NETQUANT

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Keywords:

Immunology, Neutrophils, Neutrophil extracellular traps, NETs, Immunofluorescence microscopy, Automatic image-quantification, graphical user interface, GUI, NETQUANT

Summary:

Here, we present a protocol for generating neutrophil extracellular traps (NETs) and operating NETQUANT, a fully automatic software option for quantification of NETs in immunofluorescence images.

Abstract:

Neutrophil extracellular traps (NETs) are web-like antimicrobial structures consisting of DNA and granule derived antimicrobial proteins. Immunofluorescence microscopy and image-based quantification methods remain important tools to quantitate neutrophil extracellular trap formation. However, there are key limitations to the immunofluorescence-based methods that are currently available for quantifying NETs. Manual methods of image-based NET quantification are often subjective, prone to error and tedious for users, especially non-experienced users. Also, presently available software options for quantification are either semi-automatic or require training prior to operation. Here, we demonstrate the implementation of an automated immunofluorescence-based image quantification method to evaluate NET formation called NETQUANT. The software is easy to use and has a user-friendly graphical user interface (GUI). It considers biologically relevant parameters such as an increase in the surface area and DNA:NET marker protein ratio, and nuclear deformation to define NET formation. Furthermore, this tool is built as a freely available app, and allows for single-cell resolution quantification and analysis.

Introduction:

Neutrophils are crucial mediators of innate host defense responses against a wide variety of microbial pathogens[1]. They execute their antimicrobial functions by releasing their granules

containing a wide array of antimicrobial proteins[2], producing reactive oxygen species (ROS) and hypochlorite[1], and through phagocytosis[3]. In addition, Brinkmann *et al.*[4] described neutrophil extracellular traps (NETs) as a novel mechanism by which neutrophils trap and eliminate invading pathogens. Since their discovery a little over a decade ago[4], NETs have been implicated in a wide variety of infectious[5,6] and non-infectious[7] morbidities. NET formation is an active process and results in the extrusion of chromatin DNA coated with granule-derived antimicrobial proteins[8]. Some of the key changes in cellular and nuclear morphology associated with NET formation include the loss of nuclear morphology, chromatin decondensation, mobilization of granule proteins from cytoplasm to the nucleus and an increase in the nuclear and cellular diameter[8,9].

The web-like NETs, which may appear as diffuse structures slightly larger than the cell or as structures several times larger than a single neutrophil are considered as indicators of NETosis[5,10]. Using fluorescence microscopy, NETs can be detected by probing DNA with a fluorescent probe such as 4',6-diamidino-2-phenylindole (DAPI) and by immunofluorescence staining against NET-bound proteins such as neutrophil elastase. Quantification of overlapping areas of staining for DNA and NET-bound proteins determines the total area under NETs in an image[11].

A number of image analysis options are available to perform fluorescence image-based quantification of NETs[11,12]. But these software options present limitations in not being user-friendly and/or fully automated. In this article, we demonstrate the operation of NETQUANT[13], a freely available app that can perform unbiased fully automated immunofluorescence microscopy image-based NET quantification. The app has a user friendly graphical interface (GUI) and can perform single-cell analysis. The software quantifies NETosis in an image by detecting the morphological changes in the area of DNA-NET-bound marker, chromatin decondensation associated deformation of the nucleus and increase in the DNA:NET-bound protein ratio. Taken together, the multiple NET definition criteria allows for stringent NET quantification across several data sets in an unbiased fashion.

Protocol:

The ethics committee of Lund University approved the collection of venous blood from healthy volunteers in accordance with the Declaration of Helsinki (2013/728). All volunteers provided their written informed consent.

1. Isolation of Peripheral Blood Neutrophils using Density-Gradient Centrifugation

1.1 Collect human venous blood in tubes containing heparin and allow the tubes to reach room temperature.

Note: A minimum of 16 mL of blood from a healthy donor is required to yield a sufficiently large cell pellet.

1.2 Mix the blood with one volume of 2% dextran in saline (0.9% NaCl) and allow to sediment at room temperature for 30 min in a sterile 50 mL conical centrifuge tube.

1.3 Aspirate the supernatant into a sterile 50 mL conical centrifuge tube and centrifuge at 200 x g for 10 min at 4 °C.

1.4 From this step onwards continue isolation at 4 °C or on ice.

1.5 Resuspend the pellet in 5 mL of ice cold saline and layer on top of 5 mL of leukocyte isolation gradient (9.1% sodium diatrizoate with 5.7% dextran, w/v) in a sterile 15 mL conical centrifuge tube.

1.6 Centrifuge for 30 min at 400 x g at 4 °C.

1.7 Aspirate the supernatant and discard it.

1.8 Lyse red blood cells by resuspending the pellet in 3 mL of ice-cold water for 30 s. Immediately add 1 mL of 3.6% NaCl and then fill up with 10 mL of ice cold saline.

1.9 Centrifuge the cell suspension for 10 min at 350 x g.

1.10 Remove the supernatant, collect the cell pellet and resuspend it in 1 mL of saline. Set aside 10 µL in a microcentrifuge tube for assessment of the cell number and viability using trypan blue in a Bürker chamber.

1.11 Add 10 µL of cell suspension to 90 µL of 0.4% trypan blue solution. Take 10 µL of the cell suspension in a Bürker chamber. Count the cells in the 4 squares bound by 3 lines at each corner of the chamber. Cells that appear dark blue to the uptake of dye are non-viable, exclude them from the total cell number.

1.12 Express the cell number as cells/mL as defined by the equation below.

$$\text{Cells/mL} = \frac{(\text{Total cells} - \text{Trypan blue positive cells}) \times \text{Chamber factor} \times \text{Dilution factor}}{\text{Total number of Squares}}$$

Note: Here chamber factor was 10,000, dilution factor was 10 and the total number of squares was 4.

1.12 Dilute the remaining cell suspension to 10 mL for a final washing step.

1.13 Centrifuge for 5 min at 200 x g.

1.14 Resuspend the neutrophils in RPMI-1640 with 2 mg/mL heat-inactivated human serum albumin (HSA) at a concentration of 5 x 10^5 cells/mL.

2. Preparation of Coverslips and Stimulation of Neutrophils

2.1 Place one coverslip (10 mm, #1) in each well of a 12-well plate and coat the coverslip by adding 200 µL of 0.01% poly-L-lysine solution and leave it at 37 °C overnight.

2.2 Wash coverslips with 300 µL phosphate buffer saline (PBS) once and leave to dry.

2.3 Add 400 µL of 5 x 10^5 neutrophils/mL to each well and incubate at room temperature for 15 min.

2.4 Move the plate containing neutrophils to an incubator at 37 °C with 5% CO_2 for 15 min.

2.5 Remove the supernatant. Add 400 µL of prewarmed RPMI-1640 medium with 2 mg/mL HSA to controls. Add 300 µL pre-warmed RPMI with 20 nM phorbol 12-myristate 13-acetate (PMA) for stimulation.

2.6 Stimulate neutrophils for 150 min at 37 °C with 5% CO_2.

3. Visualization of NETs

3.1 Remove the supernatant and wash the samples 2x with 200 µL of PBS.

3.2 Fix samples by adding 200 µL of 4% paraformaldehyde (PFA) in PBS for 20 min at 37 °C.

Note: PFA is toxic and must be handled with care.

3.3 Wash samples 3x with 200 µL of PBS.

3.4 Permeabilize the samples by adding 50 µL of 0.5% Triton X-100 for 30 s.

3.5 Wash the samples 3x with 200 µL of PBS.

3.6 Block the samples with 5% goat serum in PBS for 1 h at 37 °C.

3.7 Add 300 µL of primary rabbit anti-human neutrophil elastase in blocking solution at a dilution of 1:500 for 90 min at 37 °C.

3.8 Wash the samples 3x with 300 µL of PBS.

3.9 Add 300 µL of secondary goat anti-rabbit fluorescent antibody at a dilution of 1:1000 for 90 min at 37 °C.

3.10 Wash the coverslips 3x with 300 µL of PBS.

3.11 Remove the coverslips from the wells and mount the coverslip with 10 µL mounting medium containing DAPI. Store overnight at room temperature in the dark to dry the samples.

Note: The staining of DNA with DAPI is certainly a critical step in the method. The users can also troubleshoot by adding exogenous DAPI solution at a final concentration range of 0.1–0.5 µg/mL for 2–3 min, followed by 3 washing steps with 300 µL PBS.

3.12 Acquire images with a wide-field fluorescence microscope using a 20X objective.

4. Analysis and Quantification of NETs using NETQUANT

Note: NETQUANT can be downloaded by clicking the installation file found on the website (https://nordlab.med.lu.se/?page_id=34).

4.1 Importing datasets for analysis, naming of channels and conversion of images

4.1.1 Open the **Setup** tab in NETQUANT.

4.1.2 Choose the source folder for analysis by clicking the **Get path** option in the source menu and select the folder containing the image sequences to be analyzed.

4.1.3 Click on the **Get Path** option in the target menu and select the folder for saving the data following the image analysis.

4.1.4 Name the channels so that 'DNA channel' corresponds with the DNA staining (*e.g*, DNA or DAPI) and 'NET-channel' depicts the NET-bound protein staining (*e.g*, NET, neutrophil elastase) in the images. For the smooth functioning of the software, (recommended) name the folder containing the control image files as 'control'.

Note: The NET channel refers to the NET-bound granule protein marker staining only.

4.1.5 Feed the image metadata into the software by clicking the **Load image information** button on the **Image information** sub-menu.

4.1.6 Select the correct channel order contained in the images in the **Channel order** sub-menu. This option has been included as a fail-safe to prevent accidental mismatches.

4.1.7 Acquire primary image properties from the raw data and convert the images by clicking the **Prepare data button**. The converted images appear in the **Sample type** sub-menu. Click on the **Sample type** menu for displaying and selecting all datasets acquired for analysis.

4.1.8 Select an image from the **Sample type** sub menu and click on the **Display image data** button to display the images split into the DNA and NET channel respectively.

4.2 Segmentation of Cells in the DNA channel and the NET channel

4.2.1 Select the segmentation method by clicking on the **Method** sub menu in both the DNA channel and the NET channel.

Note: The default method of segmentation is set to **adaptive** and is the recommended setting. Other options are also available including global, edge and Chan-Vese. A watershed option is also included to help distinguish between closely placed cells or NETs.

4.2.2 Enter the **Segmentation** tab to segment control cells first in both channels by clicking on the **Segment control samples** option.

4.2.3 Select PMA from the sample type sub-menu and click on the **Batch** option (recommended) to begin segmentation of all images included in the data set. Select the images in the sample type menu and click on the **Display image data** button to visualize and validate the binary image masks (DNA mask and NET mask) generated post-segmentation.

4.3 Single-cell Analysis of Identifiable Properties

4.3.1 Enter the analysis tab and analyze the control samples by clicking on the **Determine threshold** button.

4.3.2 Change the sample type to PMA and click on the **Get cell properties** button to complete the analysis of stimulated samples.

4.3.3 Select an image from the **Sample type** sub-menu and click on the **Display image data** button to display the overlay and the number of cells and NET forming cells in the image.

4.4 Comparison of Cell properties to Identify NET-forming Cells

4.4.1 Select sample from the sample type sub-menu and click on the **Analyze NETs** button to complete the analysis. Individual images can be selected from the sample type sub-menu for analysis or the entire batch of images can be analyzed by selecting the batch option (recommended).

4.4.2 Adjust NET criteria manually to yield optimal results for a given sample. Compare identified NETs with the original images to assess the quality of identification.

Note: The NET criteria can then be used across all images in the data set. Any changes in the NET-criteria are applied simultaneously across all control samples. This limits the possibility of any potential differences that may arise due to over-fitting of the NET parameters. The settings in the NET criteria can be adjusted according to the user's requirements. The relationship between false discovery rates and NETQUANT has been explored previously[13]. Typical ranges for area increase are 2–4, circularity to be 0.7–0.9 and the DNA/NET ratio to be 0.6–2.0.

4.4.3 Inspect the data summary in the **Cell data** sub-menu where the number of images, cell count per image and percentage of NETs per image are displayed.

Note: The total percentage of NETs in the entire dataset is displayed by the 'NET-gauge'. The total image count, cell count, percentage of NETs in the sample (NETs%) and NETs% in the control sample are displayed in the summary statistics table below the NET-gauge. We recommend that the control data are reported alongside the data obtained from stimulated samples.

4.5 Result Outputs

4.5.1 Enter the **Output** tab to select and view result outputs.

4.5.2 Explore and compare the various data outputs generated from the analysis of control and PMA by selecting the form of the output and clicking on the **Output results** button.

Note: All data generated post-analysis for both controls and stimulations is saved in the analysis folder as chosen in the target sub-menu. Data are saved in either .csv or .pdf formats.

4.5.3 Launch the **Method file** to obtain the version of the software and NET-criteria used for the analysis (to be included in methods section for publication purposes).

4.5.4 Click on the **Results data table** to visualize the individual data points in a given sample.

4.5.5 Visualize the NET area distribution and DNA:NET ratio in the samples. The red line indicates the threshold value in the graphs.

4.5.6 Determine the NET area versus shape of DNA by clicking on the **Bivariate distribution** file.

4.6 Loading Previous Analysis and Batch All Steps

4.6.1 Load previously successful analysis settings into NETQUANT using the **Load previous analysis** button.

4.6.2 Use the **Batch all steps** button included in the **Setup** menu to run steps 5–12 (**Figures 1–5**) directly to obtain the final output.

Representative results:

5 x 10^5 neutrophils/mL were seeded onto coverslips placed in a 12-well plate and stimulated with either 20 nM PMA or left unstimulated for 150 min. The samples were then stained using primary rabbit anti-human neutrophil elastase antibodies, secondary goat anti-rabbit fluorophore conjugated antibodies and DAPI — a fluorescently labelled dye that stains DNA (See the **Table of Materials** for details). A minimum of 5 images were then acquired using an epifluorescence microscope and a 20X (NA = 0.75) objective. Sample data used for the test analysis in the

manuscript can be downloaded by following the link https://nordlab.med.lu.se/?page_id=34 and clicking on the sample dataset icon.

Here we describe the ability of the NETQUANT workflow (**Figure 1**) to quantify NET formation in a given sample. The software tool can be installed as an app in MATLAB and can be used without any previous knowledge of this interface. The GUI of NETQUANT was used to analyze the datasets as described above. All images used for analysis by NETQUANT are always left unaltered in the parent directory. The GUI is divided into 4 tabs — setup tab, segmentation tab, analysis tab and results output tab. The samples were prepared for analysis in the setup tab (**Figure 2**). The file paths (1), naming conventions (2), image information (3) and channel orders (4) are defined by the user. The prepare data option (5) ensures that the conversion and organization in a standardized fashion throughout the experiment. The segmentation parameters were the set in the segmentation tab (**Figure 3**) to identify individual cells in the samples (6). The control samples are always segmented first (7) prior to the segmentation of stimulated samples (8). All recommended values for segmentation are indicated in the software tab. The properties that define unstimulated control cells are first acquired (9) and then compared to PMA stimulated cells (10) (**Figure 4**). NETs are then analyzed in the samples based upon the NET criteria (11) and displayed with total image counts, cell counts and corresponding NETs% in the control samples. The final data outputs from the analysis were selected and then visualized (**Figure 5**). An option for loading and using a previously successful analysis, and a batch-all option to process groups of steps 5-12 (**Figures 1–5**) have also been included in the setup tab.

During the analysis, a total of 2619 cells were analyzed in the experiment. The PMA stimulated neutrophils were able to generate 90.59% NETs (NETs%) in contrast to 25.87 NETs% in the control samples (**Figure 6**). The image analysis was completed within 10 min of initiating the workflow on the software. In summary, NETQUANT provides a rapid and convenient option to quantify NET formation in images that have been acquired using immunofluorescence microscopy.

Figure legends:

Figure 1: Overview of the NETQUANT workflow. Fluorescent micrographs of human neutrophils double stained with anti-elastase and DAPI (DNA) are first processed and converted. The images are then segmented, followed by analysis of cell properties, detection of NETs and result outputs.

Figure 2: Setup tab options in NETQUANT. File paths for the datasets to be analyzed and data output folders are entered in the setup tab first (1). The naming conventions are then filled in to define channel names and the control folder name (2). The image information is then acquired (3) followed by naming the correct channel order (4). The proper setup of all fields is recommended prior to the processing of the datasets (5) for standardized image conversion.

Figure 3: Segmentation tab parameters in NETQUANT. The parameters used for segmentation of both DNA channel and NET-protein marker include method, sensitivity, iterations, minimum area and watershed (6). The use of an adaptive segmentation method and watershed is recommended. Also, other settings provided as presets in the software can be used without

modification for most purposes. Control samples are first segmented (7) and then the stimulated samples are segmented (8).

Figure 4: Detection of NETs in the analysis tab. The cell properties defining unstimulated cells are analyzed and acquired from control samples (9). This is followed by the analysis of cell properties in the experimental samples (10). The NET-definition criteria (fold increase in cellular area, nuclear deformation (values 0 to 1) and DNA staining area/NET-marker staining area) are applied to both control and stimulated samples to define NET formation (11). The quantity of NETs, total cell count and number of images in both samples is displayed in the summary statistics section.

Figure 5: Visualization of result output files in NETQUANT. Results obtained post-analysis can be selected by the individual user. The result outputs that can be generated with NETQUANT includes a .csv file containing data points from individual cells. Also, histograms depicting distribution of cells undergoing increase in NET (cellular) area, deformation of nucleus due to chromatin decondensation and DNA:NET-marker ratio, and a 3D graph of NET area *versus* nuclear deformation are also generated in the analysis. All outputs are automatically saved in the analysis folder chosen in the setup tab as .csv, .pdf and .txt files.

Figure 6: Analysis of NET formation. (A) The NETs%, total cell count and number of images from the test data set as observed in the summary statistics for controls and PMA-stimulated (20 nM) neutrophils. **(B)** A side-by-side comparison of histograms depicting increase in the area, nuclear deformation (values 0 to 1) and DNA/NET ratio in control and PMA-stimulated samples. The red line in the histograms threshold values for the NET-definition criteria. **(C)** Comparison of 3D graph of NET area versus nuclear deformation generated by NETQUANT analysis of control and PMA-stimulated samples.

Discussion:

NET formation is a relatively recent addition to the diverse neutrophil armamentarium[4] and there has been a noticeable surge of interest to study the implication of NETs in a wide array of research areas[5,7,14,15]. Acquisition of images using Immunofluorescence microscopy and subsequent image-based quantification is a widely used method to quantify NETs. This approach has the advantage of being able to detect cells forming NETs at a single-cell level and can therefore better reduce the background signal. Manual methods of quantification can be exhaustive, slow and subject to user bias. A few semi-automatic options exist that have provided users with reliable analysis[11,12]. However, they might require significant technical skill for operation or may require several manual steps for quantification. This is challenging especially to previously non-experienced users. In a recent publication, a fully automatic computational method has been used to quantify NET formation using flow cytometry-based imaging as well as thin tissue sections. However, it can only be applied to specific scenarios and also requires a significant knowledge of programming for operation[16]. Presently, fully automated image quantification software dedicated towards general NET quantification that is easy to use and possesses single-cell resolution capacity is unavailable to researchers.

Here we present NETQUANT, a fully automated NET quantification software that is freely available. This software has been developed specifically to quantify NET formation with high stringency using immunofluorescence microscopy images, and with the ability to perform single-cell analysis and quantification. Apart from considering the increase in the area of staining under NETs, NETQUANT also takes into account nuclear deformation due to chromatin decondensation and an increase in the DNA/NET-marker protein co-localization to define NET formation in a given sample. This allows for distinguishing cells that have only undergone nuclear decondensation from cells that have undergone complete NET formation. The NET definition criteria included in the NETQUANT algorithm allows for higher stringency in quantification in comparison to many earlier quantification approaches that rely solely upon an increase in the area of NET staining.

The GUI is aimed to be user-friendly and simple to use. Each step of the analysis is numbered to ensure that users can follow the workflow. One of the key features provided in the software is the segmentation tool/tab that allows NETQUANT to perform single-cell analysis in images, which is the highest possible sensitivity that can be achieved. This makes NETQUANT one of the few available options that can perform single-cell quantification to quantitate NET formation. In addition, Mohanty *et al.* have demonstrated that the software can also adapt to image variations and acquisition parameters[13]. Due to the flexibility offered in the segmentation and NET-definition criteria, donor and plate variations can be accommodated. NETQUANT can handle large data sets adequately and can be used for reliable high-throughput image analysis[13].

Although we found the workflow to yield robust results from multiple donors, we recommend that the user should decide on the appropriate values based on the individual samples. In theory, the data generated post-analysis could also be influenced by high cell density. Also, excessive aggregation of NETs can result in the reduction of the number of NETs detected. This is because the segmentation algorithm cannot distinguish entangled NETs into separate events. A similar problem with segmentation may also arise when unstimulated neutrophils are trapped within the NETs. The use of images acquired at 20X and 40X is currently recommended as NETQUANT's performance has not been tested at other magnification levels. Still, as long as the pixel size is set correctly (either manually or from metadata in the load image info section) the software should adapt area calculations accurately to higher or lower magnifications. Proper staining of the DNA and the granule marker is a critical factor. Poor staining or poor image quality in either channel will result in suboptimal results. Therefore, it is recommended that the user should determine a suitable cell number, staining procedure and antibody titer to yield optimal results. NETQUANT has only been tested on NETs derived from human neutrophils *in vitro.* NETs from other species can also quantified with proper corrections in the criteria. NET quantification can be performed on fixed samples in chamber slides or multi-well glass bottom plates. High quality images of samples stained for DNA and granule protein markers are critical. NETQUANT can also be adapted to quantify other forms of extracellular traps (ET)-osis, *e.g.*, as exhibited by eosinophils. Quantifying ETs in non-granulocytes such as monocytes is currently not possible as cytoplasmic granule staining area is required for the segmentation algorithm.

In conclusion, NETQUANT is a simple, automatic and rapid tool for accurately identifying NET formation that allows for single-cell analysis and quantification. We believe this software may

lower the barrier for performing automatic NET quantification and that the research community will benefit from this freely available app.

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Disclosures:
TM and PN have a patent pending related to the algorithms used in NETQUANT.

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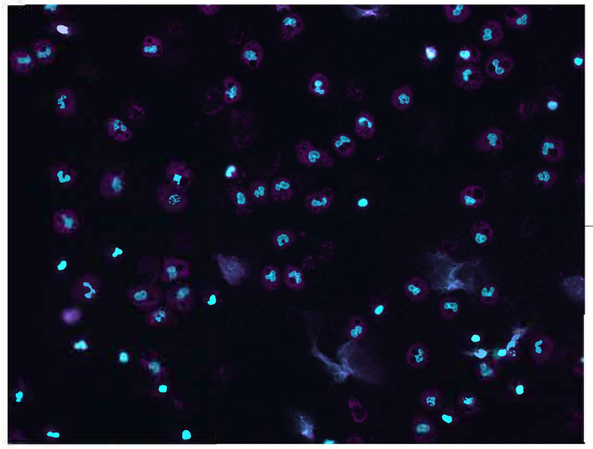
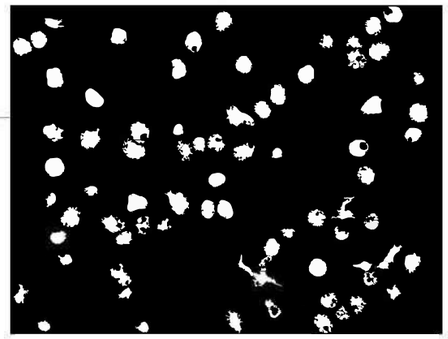
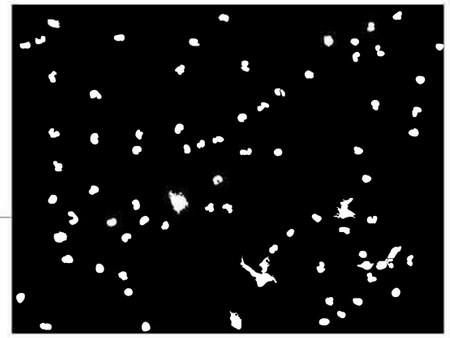
Figure 1

IMAGE PROCESSING → IMAGE SEGMENTATION → SINGLE-CELL ANALYSIS → NET DETECTION

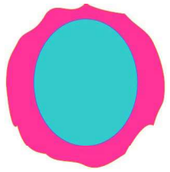


NET

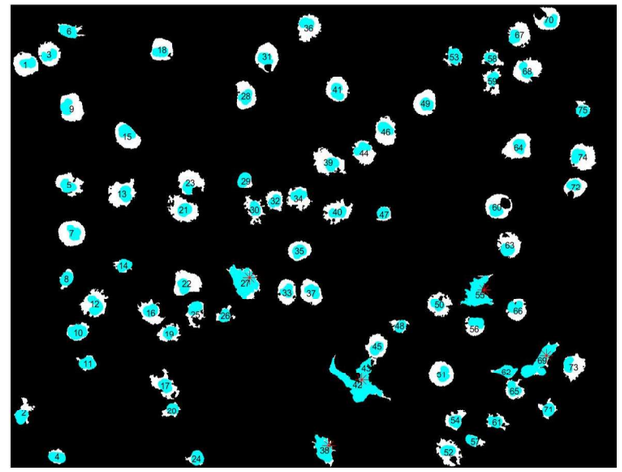




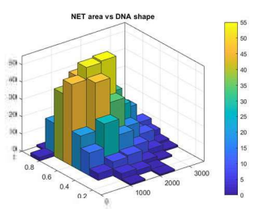
DNA



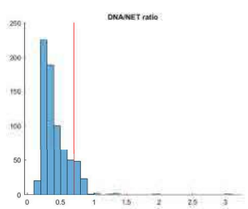
NET-criteria
1. NET area
2. Nuclear deformation
3. DNA/NET area ratio

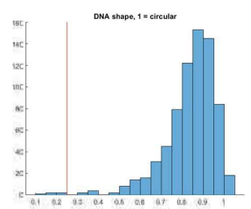


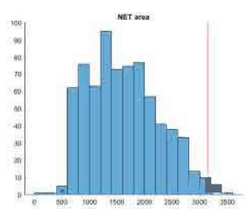
RESULT OUTPUTS



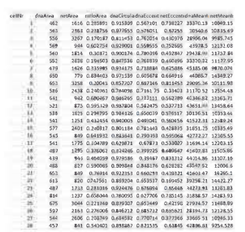
3D Graph







Histograms



.csv file

Figure 2

Click here to download Figure fig 2.pdf

Setup | Segmentation | Analysis | Output

1 File paths

source: C:\Users\guest\Desktop\All donors — get path

target: C:\Users\guest\Desktop\Analysis — get path

load previous analysis

2 Naming convention

DNA channel name: dna

NET channel name: net

control folder name: control

3 Image information

load image information — *will load first image from control data set*

image type: stack

image extension: .nd2

size X	1344	*pixels*
size Y	1024	*pixels*
bit depth	12	*bits*
pixel size	0.3225	*µm*

channel 1 name: TxRed

channel 2 name: DAPI

4 Channel order

DNA channel: DAPI

NET channel: TxRed

process all steps

5 prepare data

5-12 batch all

sample type: PMA

PMA_don1_20x001.nd2
PMA_don1_20x002.nd2
PMA_don1_20x003.nd2
PMA_don1_20x004.nd2
PMA_don1_20x005.nd2
PMA_don2_20x001.nd2
PMA_don2_20x002.nd2
PMA_don2_20x003.nd2
PMA_don2_20x004.nd2
PMA_don2_20x005.nd2
PMA_don3_20x001.nd2
PMA_don3_20x002.nd2
PMA_don3_20x003.nd2
PMA_don3_20x004.nd2
PMA_don3_20x005.nd2

display image data

Figure 3

Click here to download Figure fig 3.pdf

Setup | Segmentation | Analysis | Output

6 **Segmentation and label settings**

DNA channel

method	adaptive	
sensitivity	0.3	*0 to 1, higher value can lead to higher background*
iterations	100	*100 or more, higher value increase computation time*
minimum area	60	*µm2*
calculated	577	*pixels*
use watershed	Off On	*to separate close objects*

NET channel

method	adaptive	
sensitivity	0.2	*0 to 1, higher value can lead to higher background*
iterations	100	*100 or more, higher value increase computation time*
minimum area	80	*µm2*
calculated	769	*pixels*
use watershed	Off On	*to separate close objects*

7 *segment control samples* segment image(s)

batch

8 Off On segment image(s)

sample type PMA

PMA_don1_20x001.nd2
PMA_don1_20x002.nd2
PMA_don1_20x003.nd2
PMA_don1_20x004.nd2
PMA_don1_20x005.nd2
PMA_don2_20x001.nd2
PMA_don2_20x002.nd2
PMA_don2_20x003.nd2
PMA_don2_20x004.nd2
PMA_don2_20x005.nd2
PMA_don3_20x001.nd2
PMA_don3_20x002.nd2
PMA_don3_20x003.nd2
PMA_don3_20x004.nd2
PMA_don3_20x005.nd2

display image data

Figure 4

Click here to download Figure fig 4.pdf

Setup | Segmentation | Analysis | Output

Cell properties

9 *analysis of control samples* determine threshold

10 Off On get cell properties

batch

NET criteria

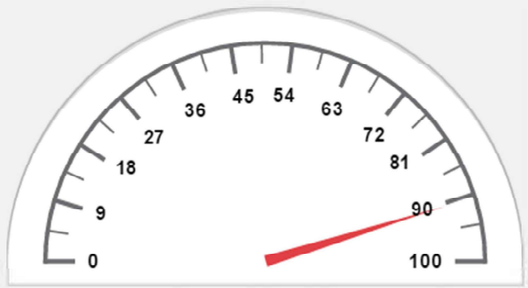
cell area fold increase	3
nuclei deformation	0.25
DNA/NET area	0.7

batch

11 Off On analyze NETs

Cell data

image nr	cell count	NETs (%)
1	81	93.8272
2	53	90.5660
3	64	90.6250
4	61	93.4426
5	64	90.6250
6	7	71.4286
7	7	71.4286
8	25	100
9	32	87.5000
10	37	78.3784
11	81	93.8272
12	53	90.5660
13	64	90.6250
14	37	78.3784
15	67	95.5224



NETs (%)

Summary statistics

image count	15
cell count	733
NETs (%)	90.59
control NETs (%)	25.87

sample type PMA

PMA_don1_20x001.nd2
PMA_don1_20x002.nd2
PMA_don1_20x003.nd2
PMA_don1_20x004.nd2
PMA_don1_20x005.nd2
PMA_don2_20x001.nd2
PMA_don2_20x002.nd2
PMA_don2_20x003.nd2
PMA_don2_20x004.nd2
PMA_don2_20x005.nd2
PMA_don3_20x001.nd2
PMA_don3_20x002.nd2
PMA_don3_20x003.nd2
PMA_don3_20x004.nd2
PMA_don3_20x005.nd2

display image data

Figure 5

Click here to download Figure fig 5.pdf

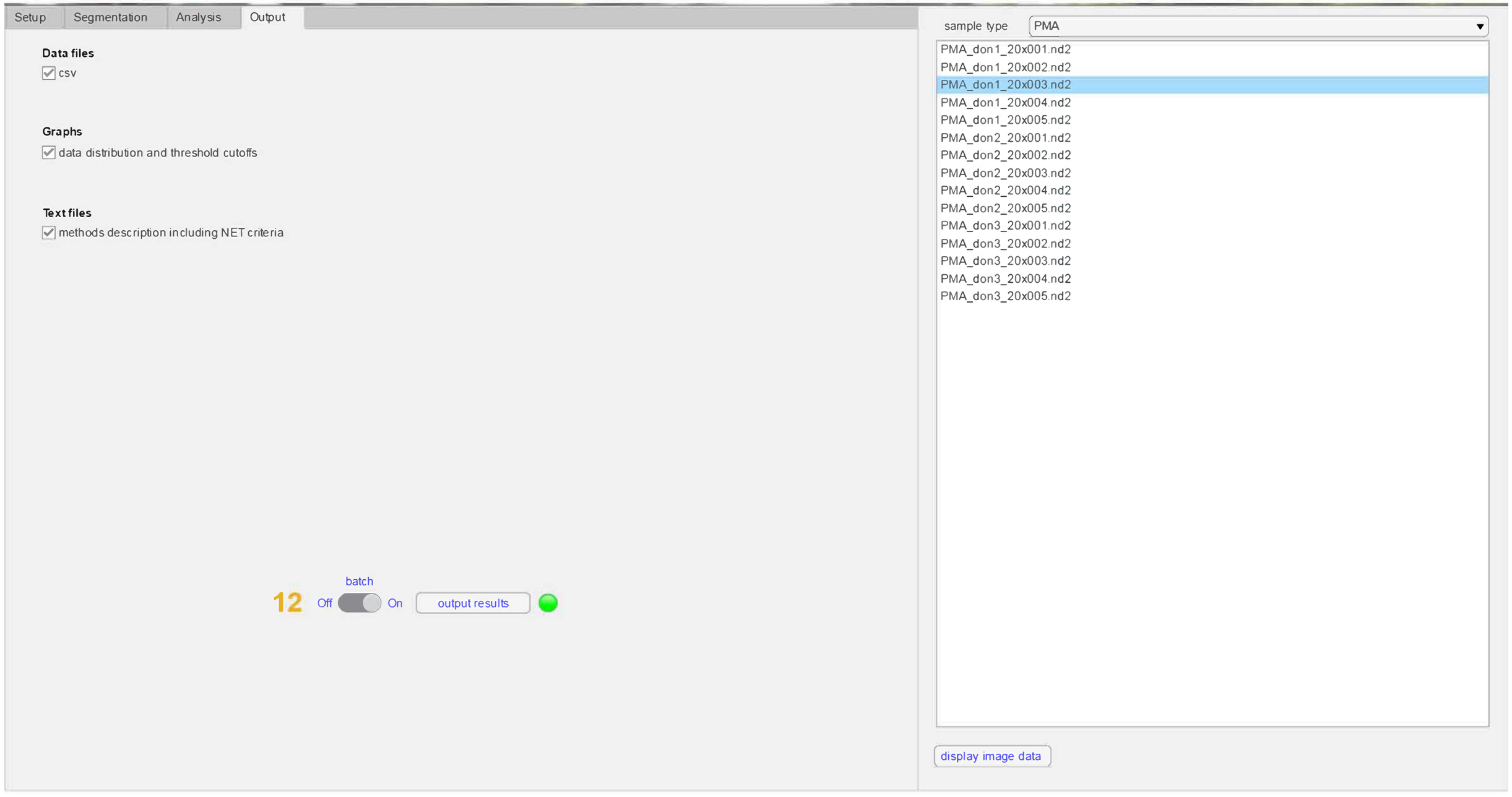
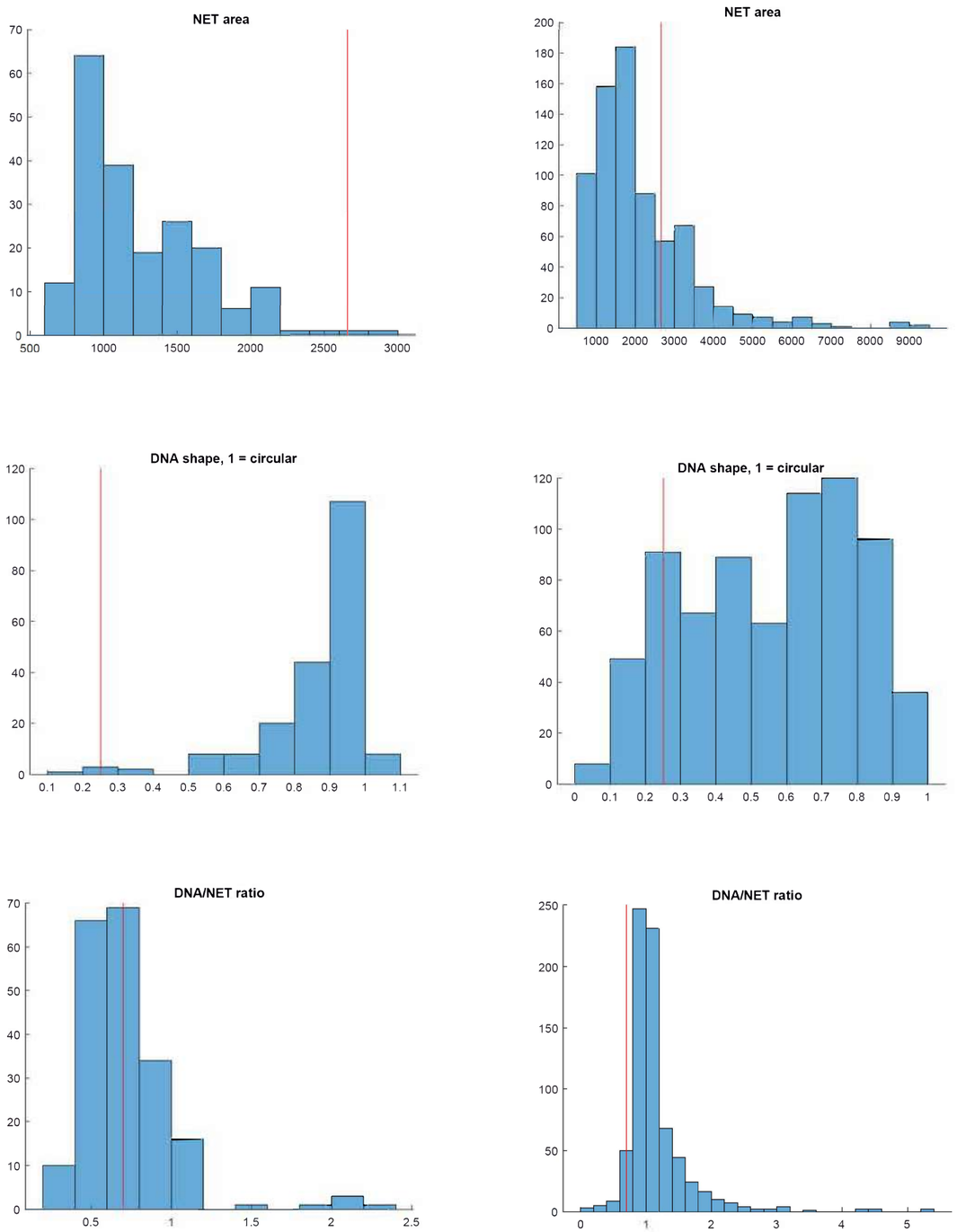


Figure 6

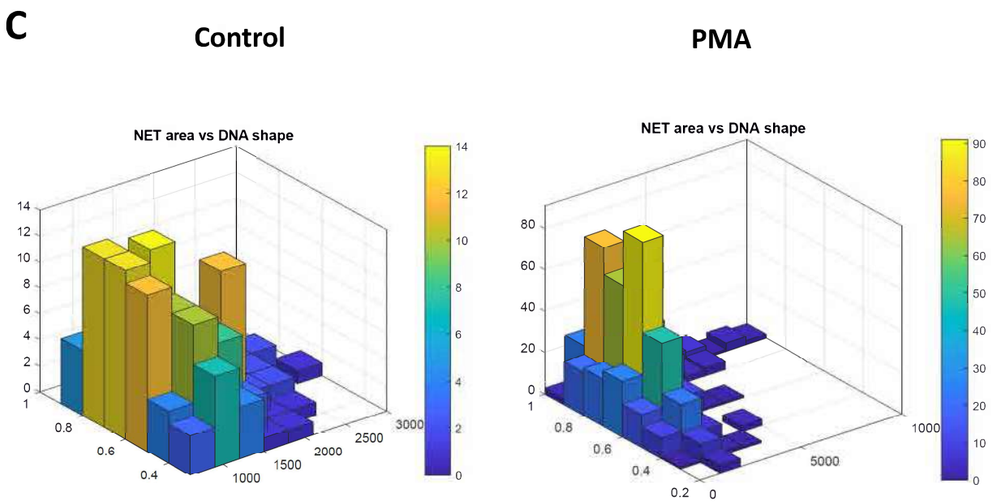
A

	Control	PMA
Total Images	15	15
Total cell count	1886	733
NETs %	25.87	90.59

B



C



Materials Table

Click here to download Table of Materials Table of materials.xls

Name of Material/ Equipment	Company
BD Vacutainer Heparinised plastic tubes	BD Biosciences
Lymphoprep	Axis-Shield
RPMI-1640 with L-Glutamine	Gibco
50mL conical flasks	Sarstedt
15mL conical flasks	Sarstedt
12-well Tissue culture plates	Falcon
#1 Coverslips 10mm	Menzel Glaser
Glass slides	Menzel Glaser
Primary anti-human elastase	DAKO
Secondary fluorophore conjugated goat anti-rabbit	Life technologies
PROLONG-Gold Antifade reagent with DAPI	Life technologies
Goat serum	Sigma-Aldrich
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Paraformaldehyde	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Nikon Ti-E Epifluorescence microscope	Nikon
CCD camera	Andor Zyla
Plan Apochromat 20x, 40x objectives	Nikon
Windows 10	Microsoft
macOS Sierra 10.12	Apple
MATLAB	Mathworks

Catalog Number	Comments/Description
367885	
114547	
11835-030	
62.547.004	
62.554.002	
10626491	
CS10100	
631-0098	
DAKO rabbit 1373, contract immunization	
A-11072, A-11070	
P36930	Mounting medium
G9023	
79346	
158127	
T8787	
	Operating system
	Operating system

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Author(s): Pontus Nordenfelt, Tirthankar Mohanty

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Institution:	Lund University
Article Title:	Automated image-based quantification of neutrophil extracellular traps using NETQUANT
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To the Editor Lund, 2018

Journal of Visual Experiments

Dear Alisha Dsouza, Ph.D., Senior Review Editor,

Please find attached the revised version of the manuscript, "***Automated image-based quantification of neutrophil extracellular traps using NETQUANT"***, which had been assigned the id JoVE58528. We would like to express our gratitude to the referees for their time and insightful comments. This has certainly improved the overall depth and clarity of the manuscript. A detailed point-by-point explanation addressing the referees' comments has also been included. Apart from this, an updated version of the software (NETQUANT version 1.2) and manual has been made available for the referees on the NETQUANT homepage.

I would also like to draw your attention to the change of order in the author list. In the initial submission, I was listed as the senior and corresponding author, while Dr. Nordenfelt was listed as the first author. After internal discussion we have decided to switch roles and now I am listed as first and corresponding author, and Dr. Nordenfelt as the senior author.

Yours sincerely,

Tirthankar Mohanty, Ph.D

Division of Infection medicine

Department of clinical sciences, Lund

Lund University, Sweden

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Reply – The manuscript has been checked thoroughly for mistakes in spelling or grammar.

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Reply- All individual high-resolution figures have been uploaded.

3. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

Reply- All legend text from the figures has been removed.

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Reply- A summary has been added in the manuscript.

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Reply- All the appropriate abbreviations have been added.

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Reply – Spaces have been added.

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Reply – Correct notations have been added.

8. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: MATLAB, Windows 10, macOS Sierra 10.12, MathWorks, etc.

Reply – All commercial names have been substituted with generic names in the manuscript. The name ‘MATLAB’ must be included in the discussion as NETQUANT has been developed as a stand-alone app for it.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Reply – Protocol have been numbered properly.

10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Reply – These have been removed from the protocol.

11. Line 105: Please mention the volume of blood collected.

Reply – The minimum volume used for the protocol has been mentioned.

12. Line 114: What does discharge mean here?

Reply – This has been substituted with discard.

13. Lines 118 and 119: Please describe how to assess cell number and viability using trypan blue.

Reply – This is now described in detail. The equation used for calculating cell number has also been added to the text.

14. Line 122: What is the container used in this step?

Reply – The container remains same from step 1.5 and is a 15 mL conical centrifuge tube.

15. Line 149: What volume of PBS is used to wash?

Reply - This has been added.

16. Line 151: What temperature is considered cool?

Reply – This has been added to the text.

17. Lines 156-161: Please write the text in the imperative tense in complete sentences. Any text that cannot be written in the imperative tense may be added as a "Note."

Reply – The paragraph has been modified accordingly.

18. Please include single-line spaces between all paragraphs, headings, steps, etc. After that, please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Reply –

19. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Reply –

20. Discussion: Please also discuss any limitations of the technique.

Reply – The limitations of the method have been added to the discussion.

21. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Reply – The references have been formatted according to the requirements of JoVE.

Reviewers' comments:

Reviewer #1:

The methods article "Automated image-based quantification of neutrophil extracellular traps using NETQUANT" outlines a program and methodology to image and analyze NETs, allowing for single-cell resolution. Drs Mohanty and Nordenfelt have adequately delineated the NETs associated applications for the methodology and protocol - could the authors speculate whether this methodology would apply to other new ETosis processes, e.g. those of eosinophils?

Reply – In theory, NETQUANT can be used to assess ETosis in other types of granulocytes. Appropriate corrections in the NET criteria for granule protein distribution in unstimulated cells and nuclear diameter have to be made first in NETQUANT as eosinophils, for example, have larger cellular and nuclear diameter than neutrophils. NETQUANT may also be used to

determine non-granulocytic ETosis, which involves DAPI staining and nuclear proteins such as histones. A short description of this has been added to the discussion.

As the methodology is heavily reliant on 20X imaging, emphasizing this in the introduction would improve the clarity of the manuscript. As Brinkmann et al. Front Immunol 2012 uses 100X images, area and pixel variability might need to be clarified for the readers.

Reply – NETQUANT has been tested for 20X and 40X images as described in the original article 'NETQUANT: automated quantification of neutrophil extracellular traps' (https://doi.org/10.3389/fimmu.2017.01999). This has been added to the discussion.

The materials and equipment are appropriately listed in the table. In part 3 of the methodology (visualization of NETs), 3.2 it is uncertain the percentage of paraformaldehyde. Throughout the methods, whether the incubation steps are in degrees Celsius or Fahrenheit needs to be clarified, as Celsius is the standard. Part 3 needs to include a dilution range of DAPI in the mounting medium since the imaging platform in part depends heavily on DNA staining. Line 171 includes measuring myeloperoxidase (MPO) staining which is otherwise not mentioned in the manuscript.

Reply – All necessary additions and corrections addressing the temperature and paraformaldehyde have been added. The reference for MPO is made in the context of using NET or granule markers other than neutrophil elastase. This is now added in the discussion section. The staining of DNA with DAPI is certainly a critical step in the method. The DAPI content in the prolong antifade reagent is undisclosed. However, proper DNA staining of the samples can be ensured by users by adding exogenous DAPI solution at a final concentration

range of about 0.1-0.5 µg/mL for 2-3 minutes. This been added as a note in step 3.11.

It would be helpful for the readership if in Part 4 there was a list of approved image formats for loading into the MATLAB program. Once the MATLAB application is being described in the methodology, more clearly marking the button labels in the text, potential with single quotes will improve the clarity of the text, as a user would presumably use the video and the methodology as they use the application to analyze their NETs samples. Some of the word choice in the methods and discussion ought to be more scientific, e.g. correction versus tweaking. In the representative results, why do the concentrations of neutrophils per mL vary from that written in line 128? Figure 6B has no labelled axis or at minimum, units, but it does look like all the graphs are giving different values for assessing NETs. Several groups besides those referenced have attempted to quantify NETs in images obtained from microscopy systems and more specifically using a MATLAB-based program, they need to be included in addition to the references currently listed in the manuscript.

Reply – We extensively tested NETQUANT with .nd2 and .tif files and recommend the use of these formats. In particular, .tif image format is universally accessible and files in other formats can be easily converted prior to use with NETQUANT. Apart from this, any Bio-Format should work in theory. More details are provided in the manual that can be downloaded from the NETQUANT page -(https://nordlab.med.lu.se/?page_id=34).
The buttons have been put in single quotations. The neutrophil concentration in line 128 was a typo and has been rectified.
NETQUANT combines all 3 criteria for the final output. The graphs depict the readouts from the individual NET criteria and hence, there are differences in them. A .csv file is also

generated post-analysis, this can be used further advanced analysis based on the individual's requirement.

The axes in figure 6 Have been added.

Other publications using MATLAB based NET quantification has been added to the discussion and references.

Overall, the authors have clearly and concisely presented a new platform for the analysis of images of NETs using a MATLAB-based program, especially on a per cell basis. This methodology has the potential to help provide more unbiased quantification of NETs images across the field as investigators interrogate regulation of NETs formation, this tool will be beneficial.

Reviewer #2:

Manuscript Summary:

The authors present an automated method for the quantification of NETs generated from isolated neutrophils in cell culture dishes. The cells are stained with a fluorescent DNA-intercalating dye and fluorescent immunodetection of granular proteins like NE or MPO. Images of both channels are automatically analyzed with a MATLAB app called "NETQUANT".

The analysis incorporates three parameters:

1 Segmentation of DNA and NE/MPO-channel. In unstimulated cells this results in a rather small nuclear region, while the NE/MPO signal comprises the entire cell. In NETs, DNA- and NE/MPO area are mostly identical

2 Circularity of DNA signal: while the circumference of the nuclear area in unstimulated cells

is rather small, it is enlarged drastically after the formation of NETs.

3 Ratio of cellular area positive for DNA vs. cellular area positive for NE/MPO.

Major Concerns:

The software could not be tested with images other than the provided ones due to the required file format.

Reply – Yes, we have realized that were critical bugs in handling separate files as well. We have now addressed this and uploaded a new version of NETQUANT (version 1.2). We have also written specific guidelines (see manual version 1.2 included on the NETQUANT download page) for how images should be prepared so it should be easier for users to get started.

The segmentation of the NE/MPO channel in unstimulated cells leads to an overestimated area since the nuclear area is included.

Reply – The systematic increase in the area due to the inclusion of nuclear area may be the case.

In several images, NETs that have originated from several cells have been counted as a single structure underestimating the NET percentage.

Reply – This is a potential drawback of the method as clumping of the NETs can prevent the segmentation from estimating all events. The users can try and reduce this source of uncertainty using the watershed option.

Minor Concerns:

The mask for the granular signal is designated "NET" which I find misleading.

Reply – The reviewer has a good point, and we did consider other designations, but in the end, we believe that it is quite clear that it refers to NET-related markers, such as granular proteins. It is mostly a practical thing, since NET is shorter and easier to handle than “granular” or a similar term, both in the graphical interface and naming of files. We have added clarifications at several places as to not cause confusion.

Reviewer #3:

Manuscript Summary:

The authors describe a new method to automatically and objectively quantify extracellular DNA derived from neutrophils, so called neutrophil extracellular traps. Because NETs have varying properties, successful quantification of NETs rely heavily on morphological characteristics. The authors here present an automated method for quantification. The obvious added value is that automatic quantification facilitates, i.e augments, the number of quantifiable neutrophils increasing the reliability of study results investigating NET formation.

Comments:

* Introduction

Because the authors describe a new way to quantify NETs, they should be advised to expand on the limitations in the current studies of the literature. It will help readers and listeners to assess the relevance of the tool the authors wish to bring forward.

* Protocol

- NETQUANT is a software tool for which it is unclear from the manuscript whether it can only be used with neutrophils imaged on coverslips or that it can also be applied on images acquired on a different way (for example culturing in 48-well plate) or for example staining with different antibodies. The authors should explain the broadness or narrowness of the NETQUANT application

Reply – In principle, NETQUANT can be used with fixed samples either on coverslips, chamber slides and 48-well glass-bottom plates with antibodies that detect NET markers such as myeloperoxidase. However, high quality images are critical for analysis. Images acquired at magnifications of 20 X and 40 X is also recommended, as the performance of NETQUANT has been monitored at these magnifications. This has been added to the discussion, highlighting the limitations in relation to currently tested scenarios.

- The neutrophil handling is well explained. I have some concerns however with the many washing steps in the protocol which usually lead to a loss of neutrophils which might be biased by the amount of NET formation. Second, I don't understand why the cell membrane is made permeable. The two staining antibodies used are Dapi, which is able to penetrate the cell and nucleus membrane, and elastase is found physiologically inside the neutrophil with or without NETosis. In my opinion there is more background when also the physiological elastase is measured inside the neutrophil. I think the programme corrects for this by only counting NET when there is co-localisation of DNA and elastase but also this assumption is not clearly explained. The authors should elaborate more on the "inside" of the program and

consider to add a limitation section or a section on troubleshooting that addresses potential hurdles for investigators that try to adapt their technique.

Reply – The washing steps included in the protocol can lead to loss of neutrophils. But this also facilitates a low background, making the washing steps difficult to bypass. The permeabilization and measurement of elastase in the cell interior is important as the program uses the staining for the segmentation.

-In 4.4.2. the authors suggest to investigators to adjust NET criteria manually to yield 'optimal' results. The authors should expand here on which criteria they refer to and logically would agree to adapt and which one not (if applicable). Furthermore, the authors should expand on their experiences and add data on the effects of varying criteria on sensitivity of the quantification analysis. For example, is there a way to set criteria on negative samples, is there a way to assess background DAPI staining from true DAPI positivity, how to deal with 'shotnoise'?

Reply – A detailed description of NET criteria, false discovery rates and adjustments used have been detailed in the publication - ‘NETQUANT: automated quantification of neutrophil extracellular traps’. Apart from this, the recommended ranges for the various NET criteria have been added as a note in the protocol.
Regarding noise, typically any signal that occupy less space than the dimensions of an unstimulated neutrophil will be filtered out during image processing of segmented images. Also, any ‘shot-noise’ is handled during the segmentation step, and we have so far not seen any issues with regards to this.

We agree that appreciable noise may due to poor staining protocols and low-quality images. Therefore, it has been recommended in the discussion that the researcher should ensure optimal staining protocols, cell number, antibody titres and high-quality images for obtaining reliable results.

- in general PMA is a very strong NET trigger and I am curious if and how the authors have addressed the sensitivity of their quantification method. In other words, if another stimulus than PMA is used that induces lesser amounts of NETs and how is the performance of this method?

Reply – This has been addressed in detail with weaker stimuli compared to PMA like *E. coli* and *S. aureus* in the original publication titled 'NETQUANT: automated quantification of neutrophil extracellular traps'(https://doi.org/10.3389/fimmu.2017.01999).

- Lastly, the authors do not give any detail as to how the program qualifies decondensating nuclei in neutrophils from undecondensated nuceli? From figure 1 and 6 it seems to be analyzed with the circularity of the nucleus but as the nucleus of the neutrophil is not circular from origin this should be explained in detail to be able to judge the accuracy. Also, once a neutrophil has extruded DNA it is unclear how the program counts this cell, the single cell analysis described and the end-result being %NET-ting suggests that it is discarded while NETs usually localize outside of the cell. What if the neutrophil died? In line with this, what if the NET that is casted outside cells covers an area of several neutrophils surrounding this event, how is then %NETs calculated?

Reply – NETQUANT performs segmentation on unstimulated controls first. During the process of segmentation leads to relatively circular nuclei in control cells. Therefore, a loss in the circularity can be used to estimate decondensation. NETQUANT only considers events where both the DNA and NET marker protein have increased over the set threshold. It does not consider decondensing nuclei alone, or extracellular DNA without elastase staining as a NET. Diffuse NETs that only exceed the set criteria will be considered as a NET. Again, due to the shrinkage observed in a dead or apoptotic cell, it will be not considered. Cells observed post NET formation will only be considered if they fulfil all three of the NET criteria. This has been added to the discussion section.

The clumping of NETs into single large event with several other trapped nuclei is a potential challenge for the program. The trapped cells and tangled NETs may not be segmented properly, and this represents a drawback of the program. A correction may be attempted by using the watershed option present in the segmentation tab of the program.

The data provided in the .csv file after analysis can be used for more advanced analysis by the user, such as utilise the area column to calculate the total area under NETs.