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

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**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

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

2. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.

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.

4. Please add a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Reply- A summary has been added in the manuscript.

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Reply- All the appropriate abbreviations have been added.

6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Reply – Spaces have been added.

7. Please use centrifugal force (x g) for centrifuge speeds.

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.