**Ref: JoVE59150**

**Title manuscript: A High-Throughput High-Sensitive Assay to Assess and Quantify Neutrophil Extracellular Trap Formation**

**First of all we would like to thank the editor and reviewers for their valuable comments and suggestions.**

***Editorial comments:*** *Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*The manuscript has been proofread several times.

*2. Please define all abbreviations before use.*All abbreviations are defined before use.

*3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are:* Pipetboy, SYTOXgreen, Falcon.

Besides Pipetboy, SYTOXgreen and Falcon also PKH has been removed from the manuscript.

*4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).*Several sentences have been rephrased and “we” is removed from the manuscript.

*5. 3.1: Please provide composition of phenol red free RPMI 1640 2% FCS.*

RPMI is such a commonly used medium for in vitro experiments around the world that it seems superfluous to extensively explain its composition. Besides, the composition can be easily found online, for example at <https://www.fishersci.com/shop/products/gibco-rpmi-1640-medium-no-phenol-red-2/p-4919929>

*6. 3.4: Please specify the time selected in this protocol.*

The time has been specified.

*7. 3.5: What volume of diluted SYTOXgreen is needed?*

Paragraph 3.5 and 3.6 have been rephrased.

*8. Steps 4, 5, and substeps: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.*Chapter 4 and 5 of the protocol have been rephrased and extended according to this comment.

*9. 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. Please do not highlight any steps describing anesthetization and euthanasia.*

Step 1,2 and 3 are highlighted completely. The final part from step 4 and the final part of step 5 is highlighted so that all important steps fit 2,5 pages.

*10. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*We hope that the highlighted parts as they are now are as desired for filming.

*11. Figure 1: Please replace “SYTOX” with a generic term.*SYTOX and PKH have been replaced in all the figures.

*12. Discussion: Please discuss any limitation of the technique.*In our opinion all major limitations are addressed in this version of the discussion. Added is the use of FCS and the possible underrepresentation of NETs induced by crystals suggested by reviewer #1. The most important limitation, also addressed by reviewer #2 is addressed in line 378.

*13. References: Please do not abbreviate journal titles.*Journal titles have been adjusted.

*14. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.*The order has been adjusted.

***Reviewers' comments:*** *Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.****Reviewer #1:*** *Manuscript Summary:  
Arends and colleagues present a protocol for quantification of neutrophil-derived extracellular DNA via immunofluorescence on a confocal microscope.  
  
Comments and questions:  
-) The usage of the term "NETosis" has been discouraged (Galluzzi et al. Cell Death & Diff 2018) since it implies cell death, which however does not occur in all forms of NET formation.*We agree completely with this comment, in line 330 Netosis has been changed in Net formation.

*-) The authors state that physiological stimulators induce less NET formation than PMA. This might be the case for immune complexes, as the same group showed (Kraaij et al., Autoimm Rev 2018). However, other physiological stimuli (such as bacteria) are on par with PMA in their efficacy to trigger NET formation (see Kenny et al., eLIFE 2016). Please rephrase.*This is correct, we have rephrased the sentence in 69-72.

*-) Under certain conditions, particulate triggers of NET formation, such as crystals, induce larger agglomerates of NETs (Hahn et al, FASEB J 2018). These might be underrepresented by the method the authors describe. Please discuss.*It is indeed possible that in our assay not all forms of NETs are detected with the same efficiency and might contribute to an underrepresentation. Although it seems unlikely that large agglomerates are not detected. The assay has been validated for two different diseases SLE and AAV in which NETs are usually small and very large respectively and both are detected [Kraaij 2016 Autoimmunity Reviews, Kraaij 2018 J Autoimmunity and Kraaij 2018 Kidney international].

It is not complete clear what exactly is meant by this comment. The reviewer might point at the possible degradation of inflammatory mediators by crystal induced NETs. But with the possibility to measure at different time points also NET induction followed by rapid degradation could be detected and NET degradation could also be used as an outcome. Extensive validation of the assay in order to study this would be necessary.

*-) Please include characteristic purity for the neutrophil isolation procedure described. How many erythrocytes are still contained after two rounds of hypotonic lysis?*

The obtained purity of neutrophils by this methods is measured by FACS analysis from different isolations. The mean percentage of neutrophils in 14 different samples obtained after this isolation protocol is 98.7% with standard deviation (SD) of 1.10%. The mean percentage of red blood cells is 1.04% SD 1.10% and the mean percentage of monocytes is 0.085% SD 0.17%. This has been added to the results section.

*-) Please replace "water" with "distilled water", "aqua dest" or the like in sentences as ""…erythrocytes will be lysed by cold sterile water."*

This has been rephrased in line 146,148 and 150.

*-) The authors suggest to use 2% FCS during induction of NET formation. This might not be suitable for all triggers, since the antioxidant activity of serum suppresses NET formation (Fuchs et al, JCB 2007).*

The reviewer’s point is well-taken and we have added this notion to the discussion at line 434-438 with an appropriate reference.

*-) The legends to Figure 2 states that a 10x magnification is shown. Probably the authors mean 10x objective and 10x ocular, which would be a 100x total magnification? Better add scale bars to avoid confusion.*The reviewer is correct: Indeed 10x objective is meant, not 10x magnification. The size of the obtained image tiles are 1.39mmx1.39mm(x3um in depth). A scale bar is added as suggested by the reviewer.

***Reviewer #2:*** *Manuscript Summary:  
The authors present a novel protocol for in vitro quantification of NETs. They use the cell membrane stain PKH 26 to quantify the area of cells and Sytox Green for quantification of not membrane-bound DNA. The protocol uses a not-specified confocal microscope with hardware autofocus and automatic stage. The microscope is programmed to generate overlapping image stacks with a z extension of 30 µm. The cell area (PKH-positive) is taken from the focal area while the Sytox signal is quantified from the 2D projection of the Z-stack.  
  
Major Concerns:  
As the authors state in the introduction and discussion, Sytox staining is not indicative of NETosis but of free DNA. Since the cells are fixed after careful removal of the supernatant, traces of Sytox Green are still present in the samples when the cells are fixed and can stain DNA which is accessible after fixation. The isolation protocol includes erythrocyte lysis which as the authors state results in a pre-activation of neutrophils. Together with possible false positive Sytox Green staining this results in a rather high background in unstimulated samples (Fig. 2A). Quantification of the number of neutrophils using cell-permeant DNA dyes which is not sensitive to some erythrocyte contamination would allow more gentle PMN purification and less activation.*The reviewer’s points are well-taken and the reviewer’s concern is a high background Sytox staining of extracellular DNA that is unrelated to the process of NET formation. This is indeed an important issue to which we have paid extensive attention during the development of this assay. The present assay attempts to reduce background Sytox staining by analysing the plate directly after fixation with only 15 minutes of SYTOX in the well. The assay therefore needs to be performed with the use of an advanced confocal microscope that has enough speed and analytical power to capture the 96-well plate within 1 to 2 hours.. This approach reduces false positive staining to a minimum. Secondly, during the analysis of captured images the use of a pixel threshold and size threshold in the analysis program allows for a better selection of NET formation. We have added these notions to the revised manuscript.

Next, during the assay development, we have thoroughly tested several forms of neutrophil isolation methods which resulted in the currently described method with the lowest background of Sytox. We realize this might not exactly qualify as a measure for ‘the most gentle neutrophil handling’ but an isolation method optimized for this assay

Lastly, we believe figure 2a led to a misinterpretation of our purpose. We intended to show the different type and morphology of background Sytox staining which exhibits clear differences to the typical extracellular DNA staining of NETs by patient serum. We realize this is confusing to readers and have therefore changed figure 2A in the revised manuscript to represent the overall view of medium  *Minor Concerns:  
Since the confocal stacks are not used for 3D analysis, the protocol could more easily be performed on a wide field microscope with automated stage. No details are given for image analysis, preferably with a freeware software package.*

With widefield imaging the fluorescence from parts of the sample that are above or below the focal plane are included in the analysis. Confocal imaging excludes fluorescence from parts of the sample that are out of focus and by the possibility of using the Z-stacks a high percentage of the well can be measured without overlap. A normal widefield microscope with automated stage is not capable of performing this type of high quality analysis with images in focus with a low error rate and especially also not in a short period of time allowing for less background and more accurate results.

To address this issue, we have extended Chapter 5 of the protocol which explains the image analysis preformed.