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A High-Throughput Assay to Assess and Quantify Neutrophil Extracellular Trap Formation

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Dear Editor,

Thank you for the positive review and detailed comments on our manuscript entitled “A High-Throughput High-Sensitive Assay to Assess and Quantify Neutrophil Extracellular Trap Formation”. We submit the revised version of the paper with the changes highlighted by track changes and a revised version without changes. Enclosed is also a detailed response to the editor and reviewers in the rebuttal letter.

We hope that this version will be considered suitable for video and manuscript publication in JoVe.

Sincerely, on behalf of the other authors,

Drs. E.J. Mlejnek, MSc
Dr. Y.K.O. Teng, MD

TITLE:

A High-Throughput Assay to Assess and Quantify Neutrophil Extracellular Trap Formation

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KEYWORDS:

Neutrophil extracellular traps (NETs), neutrophils, immunofluorescence microscopy, immunology, systemic autoimmune disease, ANCA-associated vasculitis, systemic lupus erythematosus, lupus nephritis

SUMMARY:

This protocol describes a highly sensitive and high throughput neutrophil extracellular trap (NET) assay for the semi-automated quantification of *ex vivo* NET formation by immunofluorescence three-dimensional confocal microscopy. This protocol can be used to evaluate NET formation and degradation after different stimuli and can be used to study potential NET-targeted therapies.

ABSTRACT:

Neutrophil extracellular traps (NETs) are immunogenic extracellular DNA structures that can be released by neutrophils upon a wide variety of triggers. NETs have been demonstrated to serve as an important host defense mechanism that traps and kills microorganisms. On the other hand, they have been implicated in diverse systemic autoimmune diseases. NETs are immunogenic and toxic structures that contain a pool of relevant autoantigens including anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) and systemic lupus erythematosus (SLE). Different forms of NETs can be induced depending on the stimulus. The amount of NETs can be quantified using different techniques including measuring DNA release in supernatants, measuring DNA-complexed with NET-molecules like myeloperoxidase (MPO) or neutrophil elastase (NE), measuring the presence of citrullinated histones by fluorescence

microscopy, or flow cytometric detection of NET-components which all have different features regarding their specificity, sensitivity, objectivity, and quantity. Here is a protocol to quantify *ex vivo* NET formation in a highly-sensitive, high-throughput manner by using three-dimensional immunofluorescence confocal microscopy. This protocol can be applied to address various research questions about NET formation and degradation in health and disease.

INTRODUCTION:

The formation of neutrophil extracellular traps (NETs) is the process in which neutrophils release their DNA in an extracellular three-dimensional (3D) web like structure, complexed with a wide range of antimicrobial and dangerous molecules, granular and cytoplasmic enzymes, peptides and proteins. These immunogenic and toxic structures have an important physiological role in the innate immune defense of healthy individuals by trapping and killing infectious pathogens[1]. However, they have also been demonstrated to be involved in thrombosis[2] and various systemic autoimmune diseases, including anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV)[3], systemic lupus erythematosus (SLE)[4,5], antiphospholipid syndrome (APS)[2,6], rheumatoid arthritis (RA), psoriasis, and gout[7-9].

In vitro NET formation has been widely studied with the chemical compound phorbol 12-myristate 13-acetate (PMA), which induces massive NET formation. However most physiological stimuli induce much lower levels of NET formation[10]. To study NET-triggers in, for example, an autoimmune disease setting, a standardized, sensitive, high-throughput quantitative assay is needed to detect and quantify NET formation. Quantification of NETs has proven to be challenging and is currently performed by different methods, each with their own advantages and limitations[11]. A commonly used method is the detection of DNA in the supernatants[12], which is objective but does not discriminate between the origin of the DNA (apoptotic, necrotic, NETs), and therefore is not very specific for NETs. Secondly, enzyme-linked immunosorbent assays (ELISAs) of DNA-complexed with NET-specific proteins, for example, myeloperoxidase (MPO) or neutrophil elastase (NE), are a more specific approach to detect NETs and were demonstrated to correlate well with citrullinated histone-3 (CitH3) positive NETs[13]. However, it is not known whether this method is sensitive enough to pick up all NETs (*e.g.*, MPO, NE, and CitH3 negative NETs). A third approach is immunofluorescence microscopy that is used to detect co-localization of NET-associated molecules (NE, MPO, CitH3) with extracellular DNA to quantify NETs. This method is generally specific for NETs, but it cannot be applied as a high-throughput method and is not objective due to observer bias. Additionally, this method does not take into account MPO-, NE-, CitH3-negative NETs that are frequently present depending on the used NET-trigger[14,15]. Flow cytometry approaches detect NETs through a changed forward/sideward scatter (FSC/SSC) indicating swelling of the nucleus in NET-ting neutrophils[16]. This method does not take into account the different forms of NET formation that have been identified, which might not involve swelling of the nucleus, such as vital NET formation[17]. Lastly, immunofluorescence confocal microscopy has been applied to visualize and quantify NET formation by directly staining extracellular DNA with a cell-impermeable dye that stains extracellular DNA[12,18]. Generally, 5 to 10 high-power fields are manually picked and assessed, which covers 1-5% of each well of a 96-well plate[11,17]. Manual selection of images is not always objective, prone to bias and not attractive for high-throughput analysis. An automated, high-

throughput NET quantification assay was recently developed, which imaged 11% of the well in a 3D manner covering 13 µm through Z-stacked immunofluorescence confocal microscopy, thereby leading to a highly sensitive technique to assess NETs compared to the conventional methods[10]. The current report describes the most recent protocol to quantify NET formation through an automated, highly sensitive assay using 3D confocal microscopy, which achieves a total imaged area of 45% of each well and covers 27 µm through Z-stacks. This protocol is suitable to quantify, with a high sensitivity, low levels of NET formation in an objective and unbiased manner.

PROTOCOL:

All patients and healthy controls consented to participate in the LUMC biobank. Both biobanking studies were approved by the LUMC ethical committee.

1 Isolation of Healthy Neutrophils

1.1 Obtain 20 mL of peripheral blood from a healthy donor in two 10 mL EDTA-coated tubes.

1.2 Put 10 mL of blood in a sterile 50 mL tube and add phosphate buffered saline (PBS) up to 32.5 mL.

1.3 Add density gradient (*e.g.*, Ficoll-amidotrizoaat) under the cells.

1.3.1 Take up 14 mL of density gradient with a 10 mL pipet and pipet controller.

1.3.2 Place the pipet on the bottom of the 50 mL tube.

1.3.3 Take the pipet controller off the pipet, allowing the density gradient to flow out of the pipet by gravity until the maximum is reached by capillary effect (1-2 mL will be left), without using the motor.

1.3.4 Remove pipet by placing a thumb on top of the pipet, thereby preventing density gradient from leaking out during removal of the pipet.

1.4 Spin the tubes for 20 min at 912 x *g* and room temperature (RT) without acceleration or brake.

NOTE: Red blood cells (RBC) and neutrophils have a high density and are at the bottom of the 50 mL tube. Peripheral blood mononuclear cells (PBMCs) are separated and on top of the density gradient as a white ring. PBS-diluted plasma will be above the PBMCs. If needed, PBMCs can be isolated by transferring the white ring to a new 50 mL tube with additional washing steps with PBS.

1.5 Carefully remove the white ring containing PBMCs first, followed by removal of the PBS-diluted plasma and lastly the density gradient layer as much as possible.

1.6 To isolate neutrophils from the neutrophil/erythrocytes mix, lyse erythrocytes by cold sterile distilled water.

1.6.1 Take cold sterile distilled water bottle and a 10x concentrated PBS flask from the fridge.

1.6.2 Work quickly for this step. Add 36 mL of cold sterile distilled water directly on top of the pellet and mix once carefully. Add 4 mL of 10x PBS after 20 s to make an isotonic solution. Mix once carefully.

1.6.3 Spin tube for 5 min at 739 x *g* and 4 °C (for the removal of RBCs). Neutrophils will be in the white pellet.

1.6.4 Carefully discard the supernatant. Perform step 1.6.2 again and make sure the pellet is suspended properly.

1.6.5 Spin tube for 5 min at 328 x *g* and 4 °C.

1.6.6 Carefully remove the supernatant and resuspend the pellet in 5 mL of PBS. Count the neutrophils and keep them on ice.

NOTE: Expected yield from 1 tube of blood (10 mL) is approximately 15-75 million neutrophils.

2 Red Fluorescent Cell Labelling of Neutrophils

2.1 Make a neutrophil suspension of 10-20 million neutrophils in 2 mL of PBS in a 15 mL tube.

2.2 Make a solution of 2 mL PBS with 4 µL of 2 µM red fluorescent cell linker (see **Table of Materials**) in a different 15 mL tube. Add this gently to the neutrophil suspension and mix carefully.

2.3 Incubate in the dark for exactly 25 min at 37 °C to label the neutrophils with the red fluorescent cell linker.

2.4 Inactivate the labeling by adding RPMI 1640 medium containing 10% heat inactivated fetal bovine serum (FCS) and 10% penicillin/streptomycin (P/S) at RT up to 15 mL and mix once carefully. Make sure that the pellet is carefully resuspended if a pellet has formed.

2.5 Spin tube for 5 min at 328 x *g* and RT.

2.6 Remove the supernatant and resuspend pellet in 5 mL of phenol red-free RPMI 1640 medium containing 2% FCS and 10% P/S at RT. Count the neutrophils.

NOTE: A cell loss of 50% can occur after red fluorescent cell labelling.

3 Induction of Neutrophil Extracellular Trap Formation

3.1 Make a cell suspension of 0.42 x 10^6 cells/mL in phenol red free RPMI 1640 medium containing 2% FCS and 10% P/S.

3.2 Add 37,500 neutrophils in 90 µL per well in a black 96-well, flat bottom plate.

3.3 Add 10 µL of the chosen stimulus (*e.g.*, sera of patient) in triplicate to reach a concentration of 10% in the well. Always include a negative control (medium) in triplicate.

3.4 Incubate in the dark at 37 °C for the desired time, ranging from 30 min to 2, 4 or 6 h. Incubating for 4 h is suggested.

3.5 Calculate the volume needed to add 25 µL of 5 µM impermeable DNA dye (see **Table of Materials**), to reach a final concentration of 1 µM in the well. Make a predilution if necessary in RPMI 1640 medium containing 2% FCS and 10% P/S to obtain a 5 µM concentration.

3.6 Add 25 µL of 5 µM impermeable DNA dye 15 min before the end of the incubation time. Continue incubation for another 15 min at 37 °C in the dark.

3.7 Remove the supernatant (~125 µL) very carefully and store if needed. Add 100 µL of 4% paraformaldehyde (PFA). Keep in the dark, and immediately continue with step 4.

4 NET Visualization with 3D High-content, High-resolution Immunofluorescence Confocal Microscopy

4.1 Configure the settings on the immunofluorescence confocal microscope by clicking on the **Acquisition** setup.

4.1.1 Click on the **Configure** tab.

4.1.2 Select the objective and the camera. Choose the 10X Apo Lambda objective with an acquisition mode of a confocal 60 µm pinhole.

4.1.3 Click on **Plate** and choose the 96-well plastic plate. Select the sites to visit and choose a fixed number of sites. Fill in 3 columns and 3 rows without overlap (0 µm), which cover a total of 45% of the well.

4.1.4 Click on **Acquisition**. Select **Enable Laser-Based Focusing**. Select **Acquire Z Series/Time Series** if needed.

4.1.5 Click on **Site Autofocus**.

4.1.5.1 Click on **Focus on Plate Bottom** | **Off-Set by Bottom Thickness**. For the initial well to find the sample, choose the first well acquired. For the site autofocus, choose all sites.

4.1.6 Click on **Wavelengths**.

4.1.6.1 For the number of wavelengths, choose 2. For the TL shading correction refinement level, choose 2.

4.1.6.2 For Wavelength 1, select Texas Red.

4.1.6.2.1 For the autofocus options, select laser with Z offset, post laser offset 1.1 µm. Use Z-stack with a custom range of 200-10.

4.1.6.3 For Wavelength 2, select FITC.

4.1.6.3.1 For the autofocus options, select laser with Z offset from w1 0 µm. Use Z-stack with a custom range of 200-10.

4.1.6.3.2 For the acquisition options, select Z series and 2D projection image maximum. For the acquisition options, select **Shading Correction | Off**.

4.1.7 Select **Z Series**. Select the number of steps: 10. Select the step size: 3 µm (total range will be 27 µm).

4.2 Put the plate in the immunofluorescence confocal microscopy.

4.2.1 Click on the **Run** tab.

4.2.1.1 Fill out plate name and description and choose the storage location.

4.2.1.2 Select the wells that need to be acquired.

4.3 Choose exposure time for Texas Red and FITC.

4.4 Click on **Acquire Plate** to start the acquisition, which will take approximately 1 h per plate.

5 Analysis of NET Formation

5.1 Use an image processing program designed for analysis of scientific multidimensional images (see **Table of Materials**) to analyze NET formation.

5.2 Transfer the acquired image data to a separate hard drive.

5.3 Select the color adding tool.

5.3.1 Select w1 and choose the folder in the hard drive where the data is stored.

5.3.2 Select w2 and choose the folder in the hard drive where the data is stored.

NOTE: Use a standard macro that uses w1 in the file name to add red color to the Texas Red images and uses w2 to add green color to the FITC images.

5.4 Select **Analysis Macro**.

5.4.1 Select **w1**, choose the threshold value (intensity threshold), which is usually 10. Select the desired pixel value (size threshold, *e.g.*, 100).

5.4.2 Select **w2**, choose the threshold value (intensity threshold), which is usually 10. Select the desired pixel value (size threshold, *e.g.*, 500).

5.4.3 Choose the destination for the spreadsheet file, run analysis, and save log files afterwards.

5.5 Analyze data in a spreadsheet.

REPRESENTATIVE RESULTS:

Neutrophil extracellular trap (NET) formation is quantified in a 3D manner by quantifying stained extracellular DNA over 10 Z-stacks with 3 µm distance starting off at the focal plane in each well. By measuring the cumulative area, the sensitivity of the assay increases (**Figure 1A**). The isolated neutrophils have a mean purity of 98.7% with standard deviation (SD) of 1.10% measured in 14 different samples from different isolations. The mean percentage of red blood cells is 1.04% ± 1.1% SD and the mean percentage of monocytes is 0.085% ± 0.17% SD (data not shown). The total area of stained neutrophils imaged are quantified only in the focal plane in each well, which correlate significantly with the total neutrophil count in each well with a Pearson correlation coefficient of 0.99 (95% confidence interval [CI] 0.985-0.997, $p<0.0001$) (**Figure 1B**). The representative outcome of quantification of NET formation in neutrophils stimulated with 10% sera of AAV patients or medium (MED) as a negative control, expressed as cumulative stained extracellular DNA area over 10 Z-stacks per imaged neutrophil (**Figure 1C**). Snapshots of representative images of unstimulated neutrophils (**Figure 2A**) and of NETs in AAV-stimulated neutrophils (**Figure 2B**) are shown.

FIGURE LEGENDS:

Figure 1: Quantification of NET formation by measuring extracellular DNA and neutrophil count. (**A**) Area is cumulatively quantified over the 10 Z-stacks for each well for unstimulated neutrophils (MED) and for neutrophils stimulated with 10% serum of ANCA-associated vasculitis (AAV) patients (n = 4) quantified with an image processing program. Each stimulus was tested in triplicate, each point represents the median value. (**B**) Red fluorescent labelled cell area and cell count were quantified in the focal plane of each well by the image processing program (R^2 = 0.99, p<0.0001). (**C**) NET formation is expressed as cumulative area per imaged neutrophil (cell area). The mean ± standard error of the mean (SEM) of each triplicate is plotted per stimulus.

Figure 2: Snapshots of NET quantification assay. Fluorescent labelled neutrophils are shown in red, and stained extracellular DNA is shown in green. 10X Plan Apo Lambda objective. (**A**) Unstimulated neutrophils. (**B**) Neutrophils stimulated with AAV serum.

DISCUSSION:

The most critical part of this assay is the need to use freshly isolated neutrophils for each experiment because neutrophils are short-lived and die when frozen. This requires a healthy donor each time, which could implicate some variations due to donor characteristics. One of these variations is the activation status of the neutrophils. Neutrophils could be activated already *in vivo* prior to isolation. In addition, neutrophils can be activated throughout the isolation steps notably during lysis of erythrocytes, therefore an experienced handler of neutrophils is required to minimize the activation of neutrophils. In general, isolation of neutrophils should be performed as soon as possible after blood drawing and the experiment should not be paused to avoid excessive spontaneous activation. Secondly, rough handling of neutrophils should be avoided. As such, the notable advantage of the described protocol is the minimal pipetting interventions once neutrophils are seeded in a 96-well plate. Importantly, the activation status of the neutrophils is best assessed in the unstimulated condition, in which this assay can sensitively detect low levels of NET formation. Another factor that could influence the assay is the use of FCS in the medium. The percentage of FCS has been decreased from 10% to 2% to avoid the possible suppression of NET formation by antioxidant activity[19,20] or the possible activation of the neutrophils despite heat inactivation. Culture without FCS or with the use different types of media has not been tried. An unstimulated or medium control is always taken along when performing the assay to have an indication of the background signal (*e.g.,* activation status of the neutrophils). The fold increase for each stimulus compared to the unstimulated sample is displayed to achieve consistent results over different experiments using the same stimulus.

An important factor for a possible high background is staining of extracellular DNA that is unrelated to the process of NET formation. The present assay attempts to reduce this by removing the extracellular DNA staining immediately after the short incubation period of 15 minutes and by analyzing the plate directly after fixation. Therefore, it is essential to use an advanced confocal microscope that has enough speed and analytical power to capture the 96-well plate within 1 to 2 hours. Automated setting of the exposure time and focus is recommended. As such, the microscope setting can vary between each sample and experiment with respect to the color intensity threshold which is necessary for overall optimal picture

quality. The latter influences the eventual capability to correctly quantify neutrophils and NETs and the optimal setting should therefore be confirmed by using multiple control samples (*e.g.*, serum of healthy controls). 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.

Extracellular DNA derived from NET formation can be the result of distinct death pathways, including NETosis, necroptosis, pyroptosis, ferroptosis or even non-lytic process of vital NET formation[1]. As such, a limitation of the present assay is that by staining only for extracellular DNA there is no differentiation possible between NET formation and other relevant cell death pathways. It is possible to achieve this by using either selective inhibitors of distinct death pathways to discriminate between the different forms underpinning NET formation or confirm by separate immunostainings the presence of specific NET markers, such as citH3 and NE, co-localized with DNA. The co-localization of extracellular DNA with citH3 and NE has recently been confirmed for this assay[10]. The advantage of avoiding NET specific markers in this assay, allows to assess all forms of NET formation leading to the extrusion of DNA by neutrophils as complete and as objective as possible with the potential of high-throughput screening. The applicability of this protocol has been shown in studying low level NET induction mediated by immune complexes in auto-immune disease in which the ability to detect qualitative and quantitative differences might be more important than the type of process involved[10,21,22]. Illustrating that this novel NET quantification assay can be of added value for different researchers to investigate various aspects of NET formation. Small adjustments to the assay are easily implemented: adjustment of the stimulation period, the use of a favorite NET marker to focus on one specific death pathway leading to NET formation, the use of a different magnification or the use of different NET criteria in the quantification and analysis.

In conclusion, the protocol provided is a highly-sensitive broadly applicable assay for the semi-automated quantification of NET formation for the evaluation of *ex vivo* induction of NETs upon different stimuli.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure

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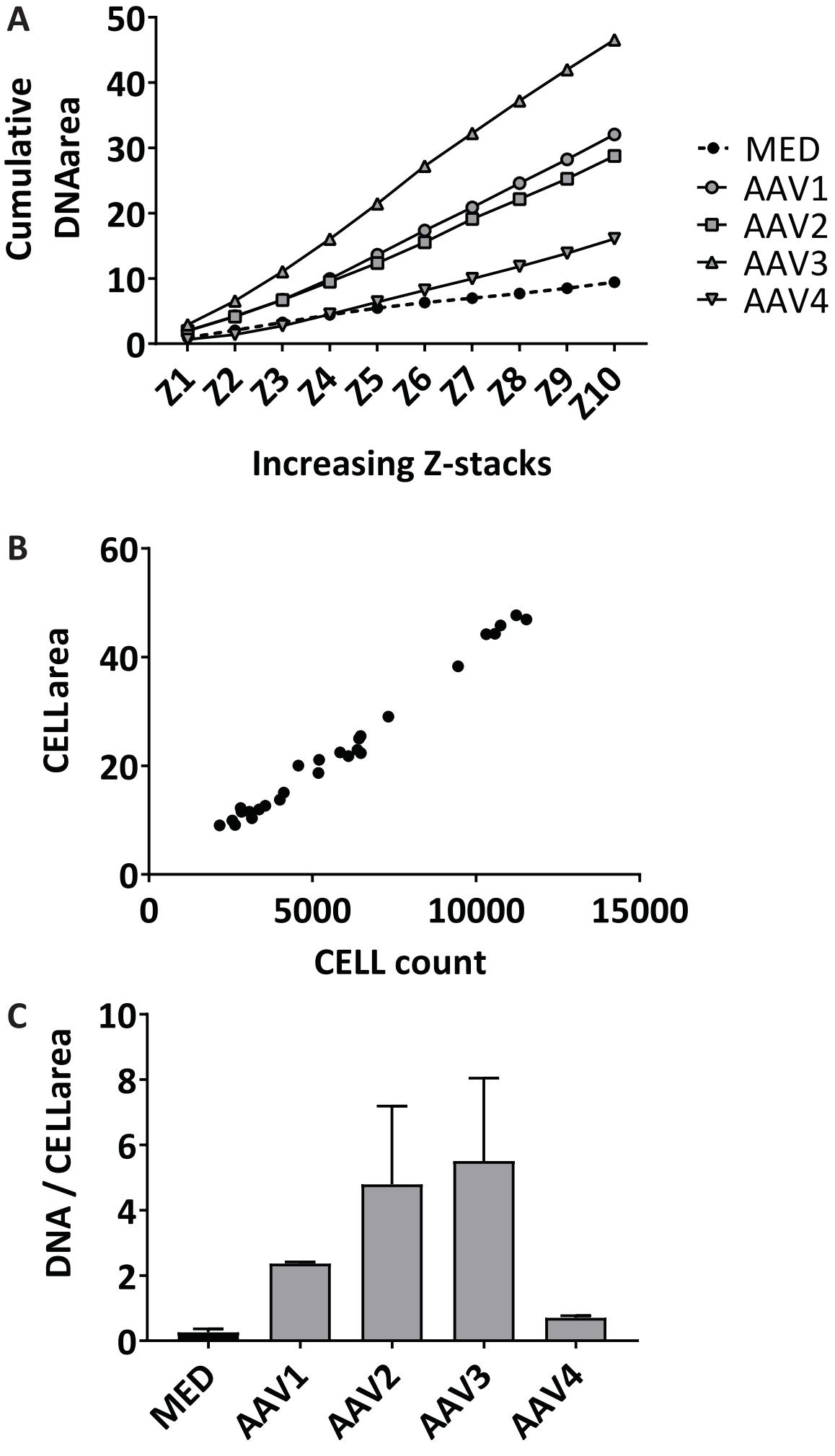

Figure

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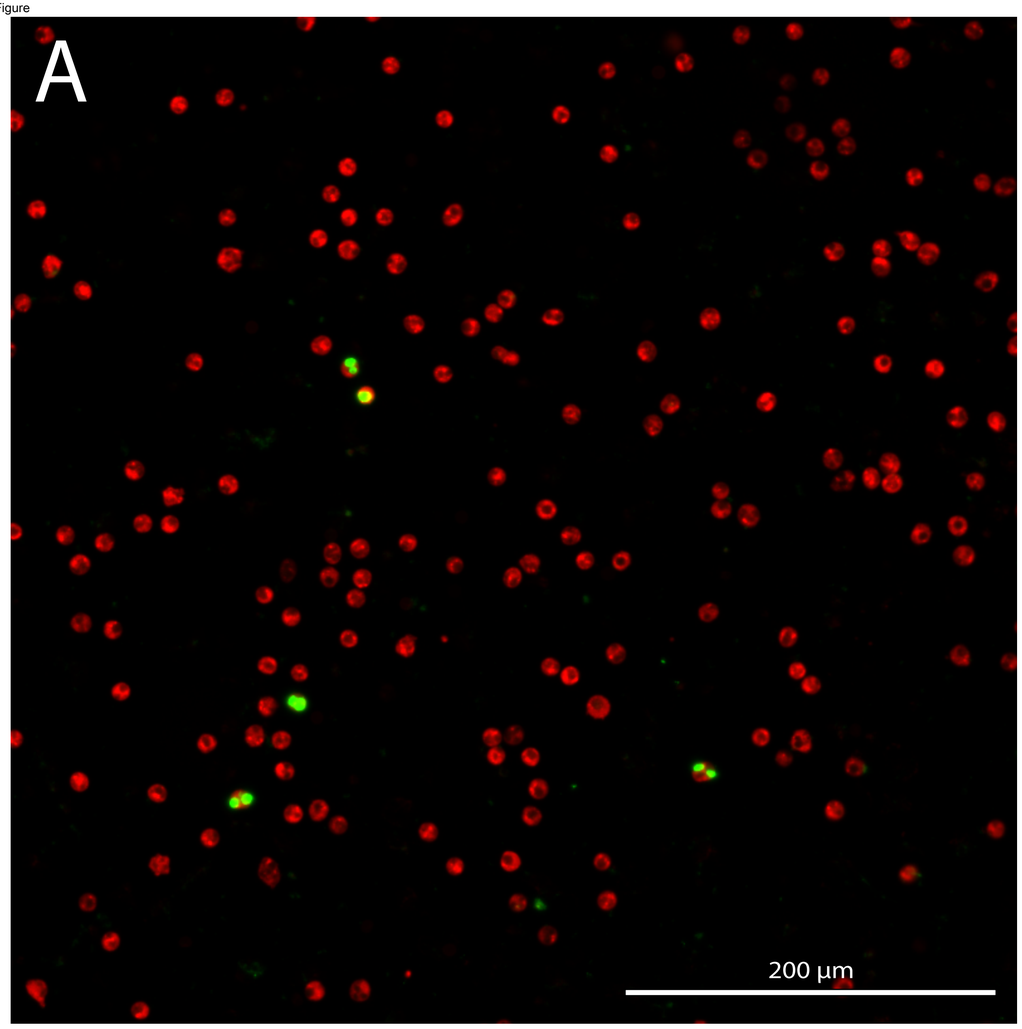

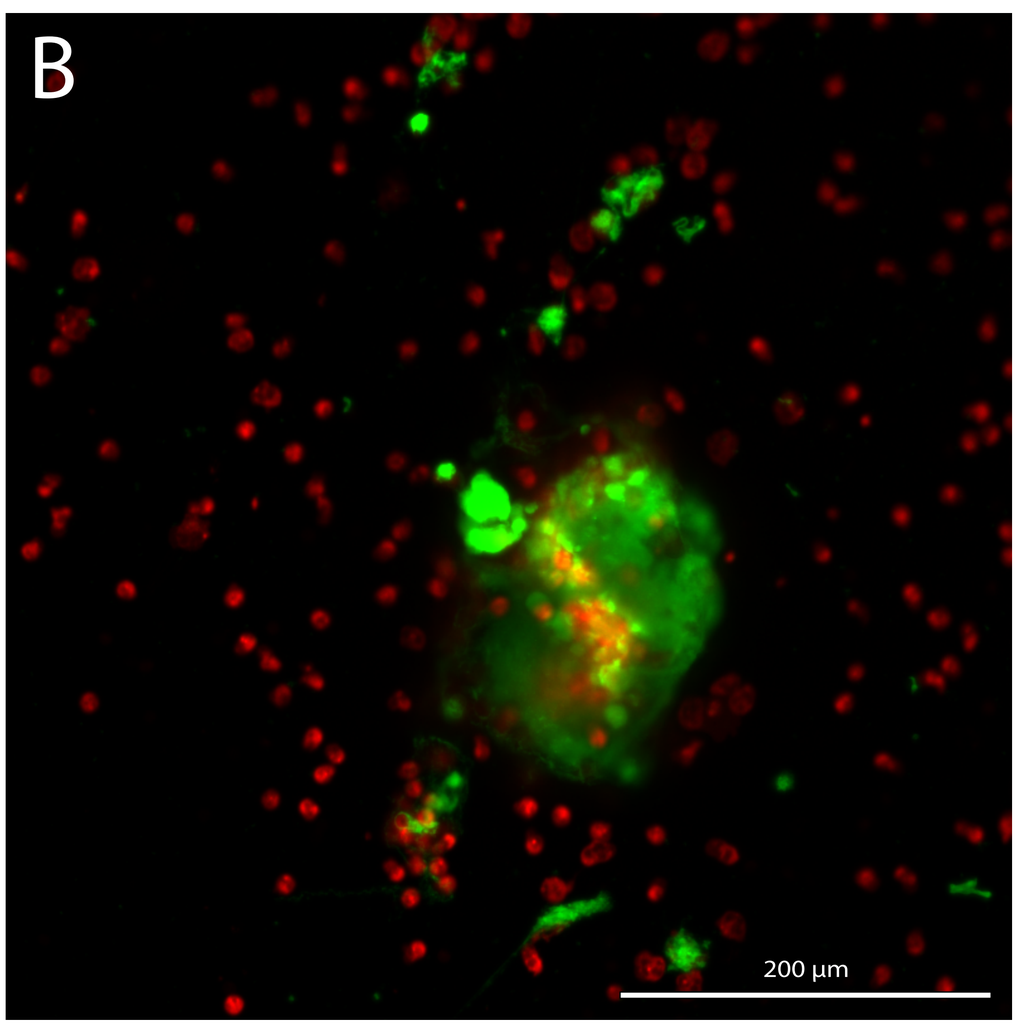

Name of Material/ Equipment
Aqua Sterile H2O
Fetal bovine serum (FCS)
Ficoll 5,7% - amidotrizoaat 9% density 1,077 g / mL
Immunofluorescence confocal microscope
Neutralization PBS (10x)
Penicillin / streptomycin (p/s)
Phenol red free RPMI 1640 (1x)
Phosphate-buffered saline (PBS)
PKH26 2 uM Red fluorescent cell linker
Program for scientific multidimensional images analysis
RPMI medium 1640 (1x)
Sytox green 5 mM Impermeable DNA dye
Trypan blue stain 0,4%
96 well, black, flat bottom, tissue culture treated plate

Company	Catalog Number
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Gibco, Paisley, UK	11835-063
B. Braun, Melsungen, Germany	174628062
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Falcon, NY, USA	353219

	Comments/Description
Used in high concentrations it could influcence NET formation	
Phenol red can interfere with the immunofluorescence signal	
PKH are patented fluorescent dyes and a cell labeling technology named after their discoverer Paul Karl Horan	

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Title of Article: A HIGH-THROUGHPUT HIGH-SENSITIVE ASSAY TO ASSESS AND QUANTIFY NEUTROPHIL EXTRACELLULAR TRAP FORMATION

Author(s): E.J. ARENDS, L.S. VAN OAM, T. KRAAJ, S.W.A. KAMERLING, T.J. RABELINK, C. VAN KOOTEN, Y.K.O. TENG

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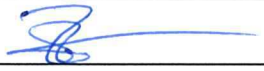
Signature:	
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Ref: JoVE59150
Title manuscript: A High-Throughput High-Sensitive Assay to Assess and Quantify Neutrophil Extracellular Trap Formation

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.