An Imaging Flow Cytometry Method to Measure Citrullination of H4 Histone as a Read-out for Neutrophil Extracellular Traps Formation

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[Abstract] The formation of neutrophil extracellular traps (NETs) is thought to play a critical role in infections and propagating sterile inflammation. Histone citrullination is an essential and early step in NETs formation, detectable prior to the formation of the hallmark extracellular DNA-scaffolded strands. In addition to the classical microscopy method, new technologies are being developed for studies of NETs and their detection, both for research and clinical purposes. Classical microscopy studies of NETs are subjective, low throughput and semi-quantitative, and limited in their ability to capture the early steps. We have developed this novel Imaging Flow Cytometry (IFC) method that specifically identifies and quantifies citrullination of histone H4 as a NETs marker and its relationship with other alterations at nuclear and cellular level. These include nuclear decondensation and super-condensation, multi-lobulated nuclei versus 1-lobe nuclei and cell membrane damage. NETs markers can be quantified following variable periods of treatment with NETs inducers, prior to the formation of the specific extracellular DNA-scaffolded strands. Because these high throughput image-based cell analysis features can be performed with statistical rigor, this protocol is suited for both experimental and clinical applications as well as clinical evaluations of NETosis as a biomarker.

Keywords: NETs detection, NETs quantification, Imaging flow cytometry, NETosis, Histone H4 citrullination, Nuclear decondensation

[Background] Activated neutrophils are rapidly recruited to sites of infection and injury; they contribute to host defense against pathogens and inflammation. About two decades ago, it was observed for the first time that in response to pathogens, a fraction of the neutrophil population can undergo a distinct type of cellular death, different from apoptosis or necrosis. This involves early chromatin decondensation, co-localization of the nuclear and granular compartments and finally, release of this mix into the environment. The extracellular DNA-scaffolded strands anchoring nuclear components (such as histones) or cellular constituents, some with strong anti-pathogen properties like elastase or myeloperoxidase (MPO), are known as "neutrophil extracellular traps" or NETs and have effective antimicrobial activity (Brinkmann *et al.*, 2004). More recent evidence shows that NETs also associate with non-infectious conditions including cancers, systemic lupus erythematosus, sickle cell disease, atherosclerosis, and thrombosis (Jorch and Kubes, 2017). It is yet not understood whether the NETs, or any of their components (cell-free DNA or histones) contribute to the pathology of these disorders as

drivers, or whether they are by-products of an unbalanced immune response. NETs might also function as biomarkers in certain diseases and provide information regarding the efficacy of a treatment regimen (Barnado *et al.*, 2016). NETs-associated components such as elastase or MPO are present in the plasma of individuals with autoimmune diseases and also in infections, which complicates the specificity of these molecules as biomarkers. In this light there is a great need for new methodologies for NETs characterization that offers consistent criteria between studies, and are reliable for both mechanistic and clinical applications.

Other NETs detection and quantification methods are being developed in addition to the classical widely used immunofluorescence microscopy technique. These include use of confocal microscopy that provides information on the structure of the NETs (Santos *et al.*, 2018), ELISA-based assay to quantify the citrullinated histone 3 (H3cit) in human plasma (Thalin *et al.*, 2017), high throuput live detection of NETosis- and apoptosis-related nuclear changes using membrane-permeable and -impermeable nuclear dyes in human neutrophils (Gupta *et al.*, 2018), quantification of free chromatin (predominantly associated with elastase) in human whole blood by using microfluidics (Muldur *et al.*, 2018). Several studies monitored NETs components (H3cit, MPO and extracellular DNA) in human and mice by using conventional flow cytometry (Gavillet *et al.*, 2015; Masuda *et al.*, 2017; Lee *et al.*, 2018; Zharkova *et al.*, 2019). While this technique allows high-throughput analysis it also does have noteworthy limitations, regarding the choice of the probes, the preparation of the samples and the way gating impacts final conclusions (Manda-Handzlik *et al.*, 2016; Masuda *et al.*, 2017). On the other hand, while Imaging Flow Cytometry (IFC) does allow high-throughput analysis, it also combines visualization and analysis features from both conventional flow cytometry and fluorescence microscopy (Basiji, 2016). NETs-associated morphological nuclear changes (*i.e.*, chromatin decondensation and DNA trails) and whether and to what degree MPO co-localized with the nuclear compartment were investigated with IFC (Zhao *et al.*, 2015; Ginley *et al.*, 2017; Pelletier *et al.*, 2017). Thus, different markers and different methods are currently employed to detect and quantify NETs *in vitro*. PAD4-mediated histone citrullination has been long designated as a hallmark of NETs formation, and thus a desirable marker to follow. More recent reports, however, predominantly in the mouse model, suggest that PAD4-mediated citrullination of histone 3 (H3cit) is stimulus-dependent (Neeli and Radic, 2013; Claushuis *et al.*, 2018; Guiducci *et al.*, 2018; Liang *et al.*, 2018) and therefore the utility of this site as a NETs marker might be limited.

Here, we describe in detail a novel IFC protocol that allows specific detection and quantification of citrullination of histone 4 (H4cit3) as a NETs marker in whole neutrophils, prior to the release of the DNA and the cytoplasmic protein strands into the extracellular space. Other analysis parameters that look at nuclear and cellular morphological changes (nuclear decondensation and supercondensation, multi-lobulated nuclei and cell membrane damage) can bring additional information on the behavior of the analyzed neutrophil population. To establish our methodology we determined the responses of healthy human neutrophils treated for 5 different periods of time (between 2 min and 60 min) with NETs agonists: pharmacological inducers, PMA and calcium ionophore (a well-known inducer of histone citrullination, and for our experimental conditions, the positive control), Hemin (an inducer present under hemolytic conditions) and LPS and IL-8 (inducers associated with infectious pathogens). We used *in vitro*

fluorescence microscopy to confirm the formation of DNA-elastase-MPO strands in healthy neutrophils treated with the stimuli used for the IFC tests (Barbu *et al.*, 2020). We further validated this technique in untreated and Hemin-treated neutrophils from healthy donors and patients with sickle cell disease at steady state (Barbu *et al.*, 2019).

The increased sensitivity and objective analysis make this methodology highly suitable for NETs detection in both research and clinical studies, that explore mechanistic answers or possible therapeutic strategies.

This protocol has 4 major parts: one-step neutrophil purification, induction of NETosis, specific staining, IFC acquisition and analysis (as highlighted in Figures 1A and 1C), with the first 3 steps requiring 3 days and up to one week to complete all steps.
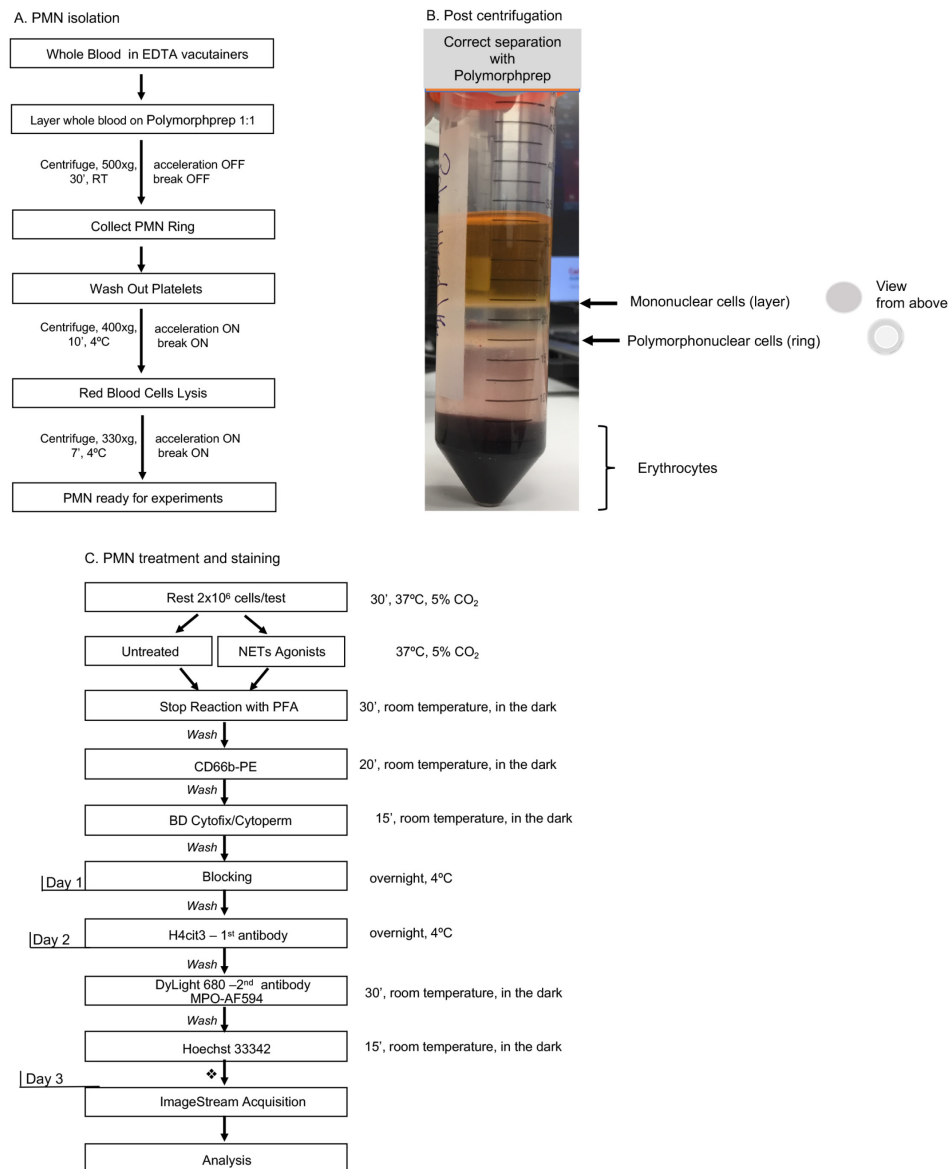
Figure 1. Day-by-day description of the major steps for specific detection of NETosis markers by Imaging Flow Cytometry. A. One-step polymorphoponuclear (PMN) cells isolation from whole blood with Polymorphprep medium; with this method neutrophils purity should be consistently above 80%. Total processing time: 90 min. **B.** An example of correct separation of whole blood by using Polymorphprep gradient medium. **C.** Detailed description of polymorphonuclear cells treatment and staining procedure. A time recommendation is not included for the neutrophil stimulation step as researchers should choose an appropriate treatment time according to their experimental purpose. Each washing step might need up to 20 min to complete, depending on the number of samples processed. This time should be taken into account while calculating total time required to complete all steps per day. ❖Short final wash followed by samples storage at 4 °C. Total stimulation and staining processing time for Day 1: 4+ h.

Materials and Reagents

1. See Through, Lavender, 10.0 V, 16 × 100 mm, Plastic Tube with BD Hemograd Closure (BD, catalog number: BD366643)
2. Polypropylene Conical Tubes, 50 ml (Fisher Scientific, Corning, catalog number: 07-203-510)
3. Centrifuge tubes, 5 ml, conical bottom, sterile (Benchmark Scientific, catalog number: C1005-T5-ST)
4. Centrifuge tubes 1.5 ml, mixed neon colors (USA Scientific, catalog number: 1415-1448)
5. Rapid flow sterile disposable filter units with PES membrane, capacity 500 ml, pore size 0.45 μm (Thermo Scientific Nalgene, catalog number: 09-740-63E)
6. Aluminum foil
7. Polymorphprep gradient medium (Cosmo Bio USA, catalog number: AXS-1114683)
   Note: Polymorphprep should be stored at room temperature, in the dark. Long term exposure to light affects its efficacy.
8. RPMI-1640 without L-glutamine (Lonza, catalog number: 12-167F)
9. L-glutamine 200 mM (Thermo Fisher, catalog number: 25030-081)
   Note: Aliquot in one-time use fractions and store at -80 °C. Thaw in water bath at 37 °C to dissolve the white sediment, then add to the RPMI to make the complete neutrophil medium.
10. Stock 32% Paraformaldehyde (PFA) Aqueous Solution, EM Grade (Science Services, catalog number: E15714). Once open use within days
11. Ultrapure 0.5 M EDTA pH 8.0 (Thermo Fisher, Invitrogen, catalog number: 15575020)
12. DPBS 1×, no calcium chloride, no magnesium chloride (Gibco, catalog number: 141190-136)
13. Cell Culture Grade Water (Corning, catalog number: 25-055-CV)
14. Bovine Serum Albumin, heat shock fraction, protease free, pH 7.0, ≥98% (Millipore Sigma, catalog number A3294)
15. Potassium Chloride Granular (Mallinckrodt, catalog number: 6858)

16. Dimethyl Sulfoxide (DMSO), ≥99.5% (GC), plant cell culture tested (Sigma-Aldrich, catalog number: D4540)

17. Hemin from bovine, ≥90% (Millipore Sigma, catalog number: 9039)
    Note: Prepare a 40 mM stock in DMSO, aliquot in single use fractions and store at at -20 °C. Mix well when preparing the working solutions. Add a DMSO-only control with the tested samples.

18. PMA, for use in molecular biology applications, ≥99%, HPLC (Millipore Sigma, catalog number: P1585)
    Note: Prepare a stock solution in DMSO and freeze single use aliquots at -20 °C.

19. LPS-EB, Ultrapure, *E. coli* 0111:B4 (InvivoGen, catalog number: tlrl-3pelps)
    Note: Prepare a stock solution in ddH$_2$O, aliquot and freeze at -20 °C. Limit the thaw-freeze cycles to three.

20. Recombinant Human IL-8 (CXCL7, 77aa) (Peprotech, catalog number: 200-08)

21. Calcium Ionophore A23187 ≥98% (TLC), powder (Millipore Sigma, catalog number: C7522)

22. Anti-Human CD66b-PE, clone G10F5 (Biolegend, catalog number: 305106)

23. Rabbit polyclonal anti-histone H4, citrulline 3 (H4cit3, Millipore Sigma, catalog number: 07-596) (see Notes)

24. Goat anti-Rabbit IgG (H+L) Secondary Antibody, DyLight 680 (Thermo Fisher, catalog number: 35568) (see Notes)

25. MPO Polyclonal Antibody, AlexaFluor 594 conjugated (Bioss Antibodies, catalog number: bs-4943R-A594)

26. Hoechst 33342 (BD Pharmingen, catalog number: 561908)

27. Gelatin from Porcine Skin, powder, Type A, suitable for cell culture (Millipore Sigma, catalog number: G1890)
    Note: Prepare 2% stock and keep it at 4 °C for up to 6 months. Open in sterile hood only. Monitor for signs of bacterial or fungal contamination.

28. BD Cytofix/Cytoperm kit (BD Biosciences, catalog number: 554722). Use only the Cytofix/Cytoperm solution in this protocol

29. Wash Buffer (see Recipes)

30. Blocking buffer (see Recipes)

31. Paraformaldehyde 8% working solution (see Recipes)

32. Porcine skin gelatin stock 2% (see Recipes)

33. 0.6 M KCl (see Recipes)

34. Neutrophils complete medium (see Recipes)

Equipment

1. Water bath at 37 °C
2. LabGard® ES, Class II, Type A2, Biological Safety Cabinet (NuAire, Plymouth, MN)

3. Benchtop Centrifuge with swinging bucket rotor (Beckman Coulter, model: Allegra X-14R)

4. Centrifuge (Thermo Fisher Scientific, model: Sorvall ST 16R)

5. CO_2 Incubator (Thermo Fisher Scientific, model: Heracell VIOS 160i)

6. Amnis ImageStream Mark II imaging flow cytometer (Luminex Corporation, Austin, TX, USA)

Software

1. Amnis INSPIRE (Luminex Corporation, Austin, TX, USA) for data acquisition

2. Amnis IDEAS (Luminex Corporation, Austin, TX, USA) for data analysis, available for download on the company's website

Procedure

A. Neutrophil solation using Polymorphprep medium
   Notes:
   a. *Choose a high yield one step neutrophil purification method as this NETs detection protocol requires relatively high number of neutrophils (2×10^6 per sample) with minimal background activation.*
   b. *The centrifugation step must be performed in a swing bucket rotor as the $500 \times g$ speed has to be reached in the middle of the tube, at the interface between the blood and the Polymorphprep medium.*
   c. *It is not uncommon that the Polymorphprep separation fails when using blood from female donors. In our experience this separation works consistently with donors of both sexes that have a MHCH (mean corpuscular hemoglobin concentration) value within the normal range.*
   d. *Keep the purified neutrophils on ice at all times and use them for experiments within 2 h.*

1. Collect whole blood in 10 ml EDTA (purple top) vacutainers and start neutrophil isolation within 1 h of the blood draw.

2. Equilibrate rotors and adaptors of the centrifuge to room temperature (18 °C to 22 °C). Set "Acceleration" to one; set "Deceleration" to zero. Set centrifugation to $500 \times g$ for 30 min.

3. Layer, very gently, undiluted whole blood on to the Polymorphprep medium always kept at room temperature, at 1:1 ratio and proceed to centrifugation. Centrifuged tubes will have the following layers from top to bottom: plasma, peripheral blood mononuclear cells – polymorphonuclear cells (neutrophils) – red blood cells (as shown in Figure 1B).

4. Collect the neutrophil ring and gently re-suspend in 50 ml ice-cold DPBS. Centrifuge at $400 \times g$ for 10 min at 4 °C, with acceleration and deceleration set at maximum. Remove and discard platelets-containing supernatant.

5. Flick the pellet to resuspend, then add 3 ml ice-cold sterile water and make sure the pellet is fully re-suspended by gently pipetting up-and-down a few times. After 30 s restore osmolarity

with 6 ml sterile ice-cold 0.6 M KCl. Fill up with ice-cold DPBS and centrifuge at 330 × *g*, 7 min at 4 °C.

6. Discard the supernatant and re-suspend the pellet in complete neutrophil medium equilibrated at room temperature (RPMI supplemented with 2 mM L-glutamine) at 4 × 10^6 cells/ml.

B. Induction of NETosis

Notes:

a. *Adapt the choice of NETs inducers and the length of the treatment according to the experimental requirements.*

b. *Distinctive NETs agonists induce optimal responses at different lengths of the treatment period. Five time points (2, 7, 15, 30 and 60 min) and 5 different agonists (calcium ionophore, Hemin, LPS, IL-8 and PMA) have been tested in this protocol.*

c. *Allow purified neutrophils to rest before treatment with the NETs inducers to reduce their background activation.*

d. *For each experiment prepare fresh 8% PFA dilution in 1× DPBS and keep it cold and in the dark until needed (this will be further diluted to a final concentration of 4% in a further step. Do not store for second use).*

e. *All steps below can be performed directly in 1.5 ml Eppendorf tubes if so chosen. However, vigorously mixing the PFA into the cell suspension by pipetting can result in splashing and loss of cells. Using 5 ml tubes provides more space for thorough mixing and to prevent loss of cells.*

1. Add purified neutrophils, 2 × 10^6 cells (in 500 μl of complete neutrophil medium) in a 5-ml sterile tube and allow the purified neutrophils to equilibrate at room temperature.

2. Transfer tube to a 37 °C incubator at 5% CO_2 and allow them to rest for at least 30 min without closing the cap.

3. Add 100 μl of complete RPMI for the untreated control or the RPMI containing the NETs inducers of choice, mix gently the same number of times in all tubes and return tubes to incubator for the desired periods of time. Do not close the caps of the tubes during this incubation period.

4. Remove from incubator and stop reaction by adding 600 μl of freshly made 8% PFA in 1× DPBS for a final concentration of 4% PFA, mix at least 5 times by pipetting, and allow to stand at least 30 min at room temperature, cover with aluminum foil.

5. Add 1ml Wash Buffer (WB) at room temperature and centrifuge at 1,120 × *g* for 5 min, at 4 °C. Discard the PFA-containing supernatant into the designated chemical waste.
   Note: To ensure that the PFA is removed, repeat this wash step one more time.

6. Gently re-suspended the pellet with 500 μl of WB and transfer it into 1.5 ml Eppendorf tube for subsequent staining steps.

7. Repeat the washing of the 5-ml tube with another 500 μl of WB to make sure all cells have been collected for staining.

C. NETosis staining technique for imaging flow cytometry

![bio-protocol logo]

Notes:

a. *Remove the supernatant completely from cells at all steps, while paying attention not to lose cells in the process.*

b. *Determine the optimal working dilutions for all the antibodies used in the staining panel by titrating with a range of dilutions above and below of those recommended by the vendors (for example, for a recommended dilution of 1:1,000, up to 5 different dilutions should be tested – 1:250; 1:500, 1:1,000, 1:2,000, 1:3,000). This is required as the lasers on different ImageStream cytometers might have different powers.*

c. *Optimal H4cit3 dilution is defined as the concentration that provides the greatest separation of the positive staining against the background (and has highest index score when antibody titration is performed). Because the concentration of the antibody stock provided by the company might vary between lots, <u>the optimal H4cit4 dilution has to be determined for each new lot of antibody</u>.*

d. *Acquire the samples within 72 h of the end of the staining protocol. Longer wait times might interfere with the strength of the signal, particularly for Hoechst (which leaks freely from the stained cells with time). Our recommendation is to acquire them immediately.*

e. *Store stained cells at 4 °C in the dark and keep them on ice and in the dark during acquisition on the ImageStream cytometer.*

1. In the 1.5 ml Eppendorf tubes re-suspend fixed and thoroughly washed cells in 100 µl of 2% BSA containing CD66b-PE, mix vigorously at least 5 times by pipetting and incubate for 20 min, at room temperature, in the dark.

2. Washing step: Add 500 µl WB and centrifuge at 1,120 × *g* for 5 min, at 4 °C. Remove the supernatant and repeat washing two more times.

3. Re-suspend washed pellet in 300 µl of BD Cytofix/Cytoperm, mix by pipetting up and down at least 5 times, incubate 15 min at room temperature, in the dark.

4. Washing (step #2).

5. Re-suspend washed pellet in 500 µl of blocking buffer, mix by pipetting up and down at least 5 times, incubate overnight at 4 °C, in the dark.

6. Washing (step #2).

7. Re-suspend in 100 µl of 2% BSA containing anti-histone H4 citrulline 3 (H4cit3) primary antibody at the optimal determined dilution. Pipet up and down at least 10 times and incubate overnight at 4 °C.
   Note: The concentration of this primary antibody is the limiting factor for the final strength of the fluorescence signal (see note b above).

8. Washing (step #2).

9. Re-suspend pellet in 100 µl of 2% BSA containing secondary antibody DyLight 680, at the determined optimal dilution and the anti-MPO-AlexaFluor 594 conjugated. Pipet up and down at least 10 times and incubate for 30 min, at room temperature, in the dark.

![bio-protocol logo]

10. Washing (step #2).

11. Re-suspend in 200 μl of 2% BSA containing Hoechst diluted as suggested by the vendor (1:1,000). Mix thoroughly by pipetting and incubate for 15 min, at room temperature, in the dark.

12. Add 500 μl WB and centrifuge at 1,120 × *g* for 5 min, at 4 °C. Remove supernatant.

13. Re-suspend in 50 μl WB for ImageStream (Imaging Flow cytometer) acquisition (see notes d and e above).

D. Imaging flow cytometry acquisition.

Within the INSPIRE software, establish the acquisition settings and create a template to be used with all experimental repeats.

1. Under the "Illumination" tab turn on the 785 laser (side scatter laser – SSC) and all other lasers to be used in the experiment, based on the fluorochromes panel (a chart showing the laser excitation wavelength and its corresponding dyes is available in the company's website).

2. Under the "Magnification" section, adjust magnification to 60×.

3. Under the "Fluidics" section, slide the Speed/Sensitivity bar to the left (to ensure acquisition with low speed and high sensitivity).

4. Under "File Acquisition", create a new folder to save the acquired files (with a .rif extension – *raw image file*).

5. Load a sample stained with all the colors and expected to yield the brightest signals for all the dyes used (stimulated with PMA as an example – the rational here is to ensure the positive fluorescent signal is within range and will not be saturating the channel).

6. Adjust the laser voltages based on the maximum of fluorescence without channel saturation of each fluorochrome according to their correspondent laser excitation.
Note: When setting the channel-specific laser power, the optimal voltage of the laser is the one that does not saturate the detectors (less than 4096 pixels in the generated image) and also promotes a clear distinction between negative and positive population. A 'Raw Max Pixel' feature of each of the used channels should be applied. This feature provides the highest value of the pixels contained in the input mask.

7. Once the laser voltages are set, "Return" the sample and set it aside to re-acquire it after the single color compensation control tubes have been acquired for spillover matrix calculation.

8. To generate a compensation matrix file, start the compensation wizard on Inspire and acquire at least 1,000 events from each of the compensation control tubes.
Note: The customers portal on the Amnis ImageStream website provides detailed instructions of how to run and validate compensation matrix using the Wizard in the INSPIRE (acquisition software). A compensation matrix has to be applied to every acquired .rif file when first opened for analysis in the IDEAS software (even when a compensation matrix was also applied during the acquisition of that file with INSPIRE). Spillover compensation can be done during acquisition or post-acquisition of data files. If the stop gates on acquisition of the files are based on fluorescent signals, for example, acquisition based on 5,000 positive cells for CD66b, then it is

advisable to perform compensation on INSPIRE (use the compensation wizard) to ensure that the fluorescent signal is corrected for spillover in other fluorescent channels.

9. Create a dot plot of Area of the Brightfield (BF) versus intensity of Side Scatter (SSC) channels and by clicking around the dots (clusters of populations) and evaluating the correspondent imagery displayed in the gallery, the single cells can be found and boundaries determined. In this protocol, for purified neutrophils, the gate of single cells should be in the range of 50 μm^2 to 200 μm^2 area. This should exclude most of the debris, small particles (to the bottom and left) and aggregates (slightly upper and right – please refer to Figure 2A).

    Note: Due to the nature of neutrophils, we thought it would be easier to identify singlets by the use of Area versus SSC; however, one can also use Area versus Aspect Ratio (AR) of BF to determine single cells. Aspect Ratio is the ratio of width by the height, events closer to AR of 1 will be where the single events should be located.

10. From the gate above, create a histogram using the gradient RMS (root mean square) feature of the BF (Channel 1 or 9), to select the cells in focus. Because this feature measures the sharpness quality of an image, changes of pixel values of 40 and above, usually corresponds to the cells in best focus. Selecting the bin and visualizing the correspondent cells within the bin in the image gallery will help determine where to begin the focused gate in this histogram.

11. Set the "Acquisition" tab to acquire 20,000 single and focused cells (first and second plots created, respectively).

12. Save this template to be used for subsequent acquisitions with the same settings for laser powers, magnification and number of events acquired.

13. Type the name of the sample into the "Acquisition" tab.

14. "Load" the sample, confirm cells are centered and in focus and click "Acquire".

    Note: Follow one of the two available brightfield channels (channel 1 or channel 9) to confirm the cells are centered (i.e., the cells are flowing in the middle of the channel) and in focus (the images of the flowing cells are clear and crisp).

Data analysis

Notes:

a. *Representative images for steps 3 to 5 are shown in Figure 2A.*

b. *For a full set of experimental results with our tested NETs agonist refer to our previous publication (Barbu et al., 2020).*

1. In the IDEAS analysis software, open the .rif extension file to be analyzed.

2. Apply the compensation matrix to be used.

    Note: A previously made compensation matrix is stored as a .ctm file. IDEAS software will prompt for selection of a compensation matrix for every .rif file that needs to be analyzed. Navigate to the stored file and load it by using the instructions from the analysis software.

3. Create a dot plot of area of the BF image (M01 – default mask) versus side scatter (SSC – channel 6).

   Note: Single neutrophils with high SSC and relatively big cellular area will appear as a distinct clustered population. Check a few of the single cells to define the boundaries of the singlets gate and exclude debris and mononuclear cells. The best way to determine and validate the gate boundaries, besides the visible cluster of events in the graph, is to click on some dots in the plot and visualize the correspondent imagery. When looking on Area axis, one should expect single events to the right of the population of debris or smaller cells and to the left of doublets, larger aggregates or even bigger cells, if the cell suspension may contain such cells.

4. Select the focused cells by using the focus quality feature in brightfield, Gradient RMS (Root Mean Square).

5. Create a dot plot of signal intensity for granulocyte-specific marker CD66b versus Hoechst and gate double positive events (CD66b$^+$Hoechst$^+$) for further analysis.

6. In the CD66b$^+$Hoechst$^+$ gate create a histogram for the intensity of the H4cit3 signal and evaluate percent of H4cit3$^+$ cells and/or mean/median intensity fluorescence for this marker (Figure 2B).

   Note: We recommend the use of median intensity fluorescence for this parameter.

7. On the nuclear channel (Hoechst) the CD66b$^+$Hoechst$^+$ gate create a dot plot of 'Area' versus the specific texture feature 'Bright Detail Intensity_R3" (BDI_R3) to identify neutrophils with decondensed nuclei from those with normal nuclei in the CD66b$^+$Hoechst$^+$ gate (Figure 2C).

   Note: BDI is a feature that calculates intensity of localized bright spots within the masked area in the image (the description of this and other IDEAS features used in this protocol can be found in the downloadable IDEAS ImageStream Analysis Software User's Manual).
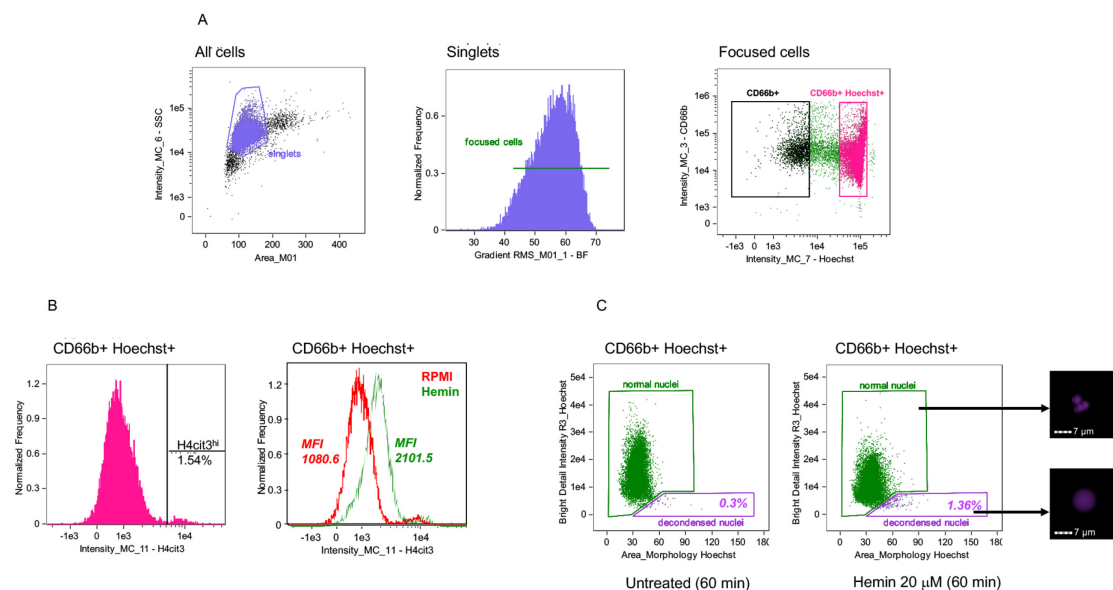

Figure 2. Gating strategy for identification of neutrophils and their nuclear NETosis markers by using IDEAS software. A. Single cells identified with two-parameter dot-plot of

area of Brightfield (BF – X axis) versus Intensity of Side Scatter on Channel 6 (SSC – Y axis) (left panel). Focused singlets cells selected by using Gradient RMS (Root Mean Square) in Brightfield (middle panel). Neutrophils (CD66b$^+$Hoechst$^+$, in strawberry red) identified from singlet focused cells in a two-parameter dot-plot of fluorescence of Hoechst on Channel 7 (X axis) and CD66b on Channel 3 (Y axis) (right panel). **B.** Representative histograms showing the percentage of H4cit3^{hi} cells in the CD66b$^+$Hoechst$^+$ gate and H4cit3 median fluorescence intensity (MFI). In the right panel the neutrophils were left untreated in RPMI (red) or were treated with 20 uM Hemin for 15 min (green). **C.** Decondensed nuclei (purple) and normal nuclei (green) identified with a dot-plot of area of nucleus (morphology mask function of Hoechst staining on Channel 7) versus Bright Detail Intensity R3 of the Hoechst staining (examples of nuclei from the two gates are included on the right side of the panel). Neutrophils were left untreated (left) or were treated with 20 µM Hemin for 60 min (right). Percentage of cells with decondensed nuclei is shown. Scale bars = 7 µm.

8. Use Similarity feature on the nuclear dye channel (Hoechst) and MPO channel to determine the degree to which the two images are linearly correlated within the masked nuclear area in cells with normal and decondensed nuclei (Figure 3A).
    Note: When located in the cytoplasm MPO staining has a dissimilar distribution compared to the Hoechst-stained nuclear mask (i.e., low Similarity score, no co-localization). When the intensity of both dyes at the nuclear location is high, the high Similarity values indicate co-localization.

9. Use the customized "Level Set" mask on the nuclear dye channel and the 'LobeCount' feature to detect reduction in the percentage of cells with multi-lobulated nuclei and separately quantify cells with variable number of lobes (1-lobe to 4-lobes) (Figure 3B).

10. In the 1-lobe gate create a dot plot of 'Area of nucleus' versus 'Ratio of nuclear by the whole cell areas' to quantify nuclear supercondensation. Supercondensed nuclei have low values for both parameters; (Figure 3C).
    Note: See the stand-alone Notes chapter for instructions on how to construct this custom feature. For better resolution of the nuclear area, custom masks should be used for the above-mentioned parameters.

11. In the normal nuclei gate construct a dot plot of 'Standard deviation' (X axis) and 'Modulation' (Y axis) to assess changes in cell membrane texture. High values for Standard deviation in darkfield (SSC channel) and for Modulation in brightfield indicate neutrophils with damaged membranes (Figure 3D).
    Note: The Standard Deviation Feature provides information on the complexity of an object. Modulation Feature can quantify image quality and thus characterize cells' texture.
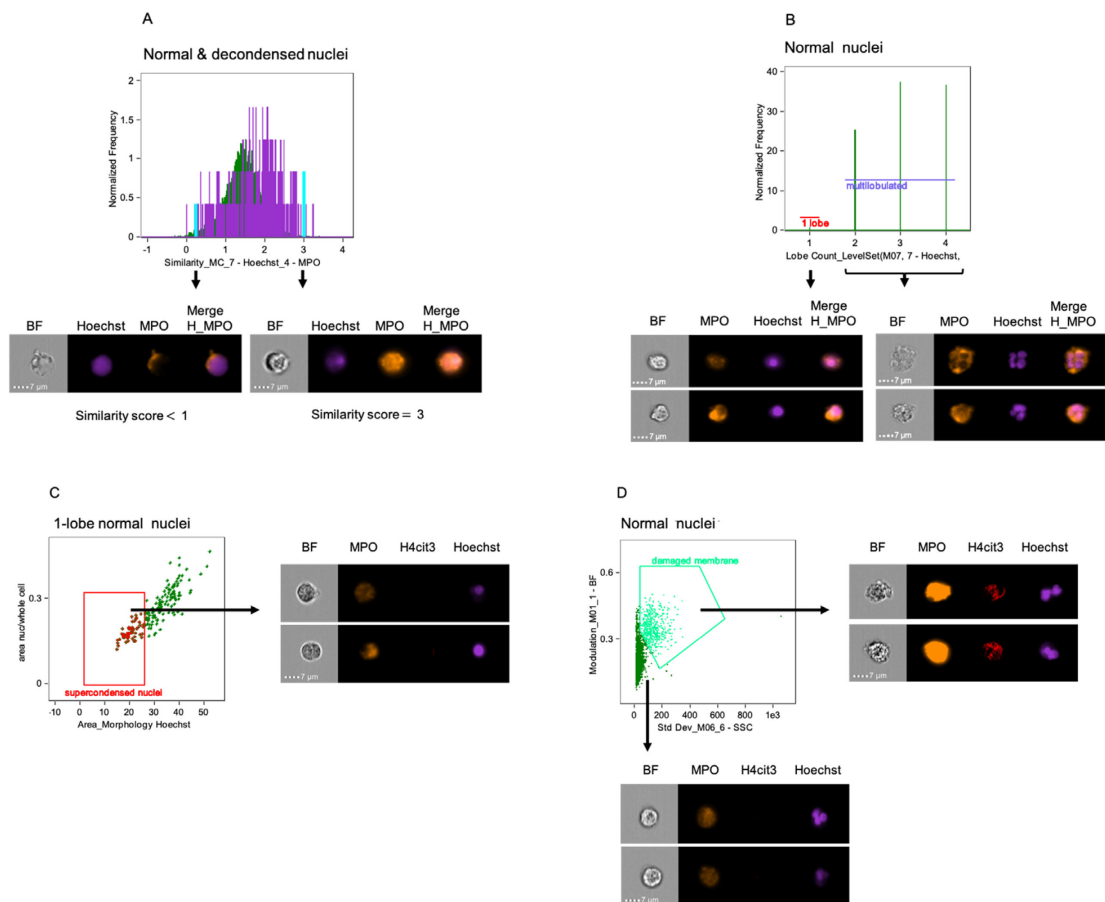
www.bio-protocol.org/e3927

Figure 3. IDEAS software analysis features to identify and quantify additional nuclear and cellular alterations. A. Similarity analysis feature for DNA (Hoechst – channel 7) and MPO (AF-594 – channel 4) indicates co-localization of the nuclear compartment with type I granules in neutrophils with normal nuclei (green) and decondensed nuclei (purple). Examples of cells with low (< 1) and high (3) similarity scores, indicating low and high DNA-MPO co-localization, respectively, are included (the position of their specific bins on the graph is highlighted in aqua). **B.** Lobe Count feature identifies changes in the number of nuclear lobes based on nuclear imagery and quantify the number of cells in each category. Representative images of neutrophils with 1 lobe or multilobed nuclei are included. **C.** Dot-plot of Area of Nucleus (morphology mask of Hoechst staining) on X axis versus ratio of the nucleus by the whole cell areas in the 1 lobe nucleus gate identifies supercondensed nuclei with low nuclear area and consequently low ratio when divided by the whole cell area. Representative images of cells with supercondensed nuclei are presented. **D.** Neutrophils with damaged membranes (bright green) are distinguished with two-parameter dot-plot of Standard Deviation Side Scatter on Channel 6 (X axis) versus Modulation of Brightfield (Y axis). High scores for both features indicate complex and textured objects. Included, representative imagery of cells within both gates. (BF = Brightfield; H = Hoechst). Scale bars = 7 μm.

12. Save file once all the analysis parameters have been established. For each .rif acquisition file, two additional files (.daf and .cif) are created in the analysis step. Use the .daf for analysis. Do not remove the .cif one (this is where all analysis data is stored).

13. Use "Batch Data File" under the "Tools" tab to assign all saved analysis parameters to the remaining .rif file to be analyzed.

14. In the .daf analysis file use "Reports" to define a statistic report for all parameters of interest in the target subpopulations and then "Generate Statistic Report" to apply it to all analyzed sample files (Figure 4).
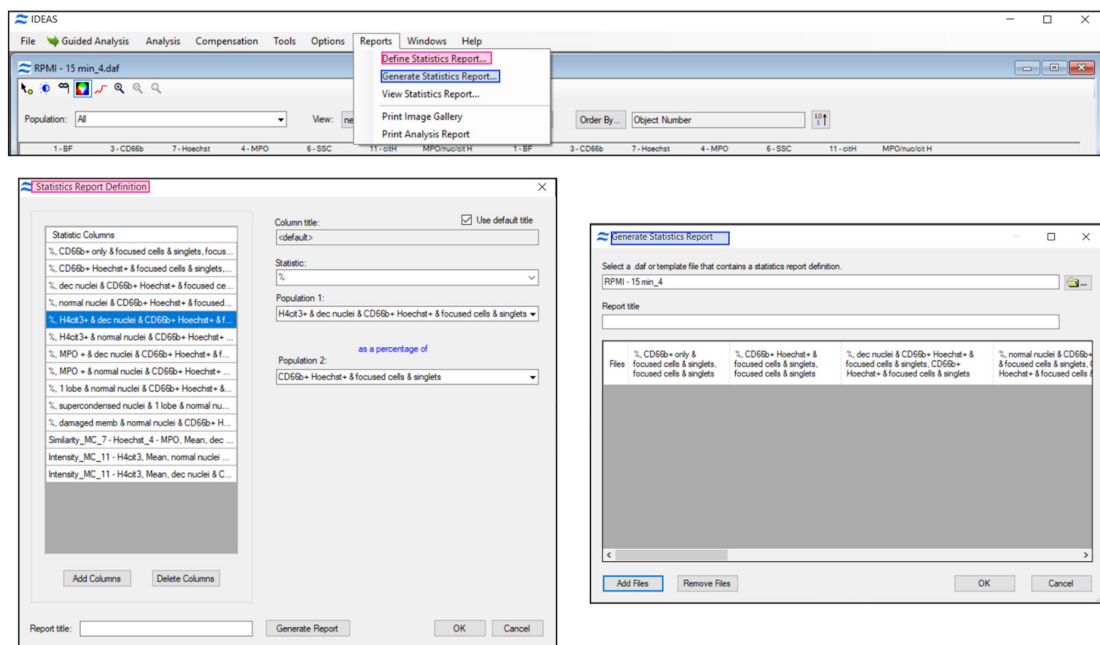

Figure 4. Define and generate a statistic report with all analysis parameters of interest. Use "Add Files" function to apply all defined stats to the .daf analysis files. IDEAS software generates and stores a .txt statistics file that can be opened with Excel for further processing.

Notes

1. In this protocol all steps (neutrophils isolation and stimulation, staining, detection and NETs quantification) can critically impact the data quality.

2. Neutrophils are notorious for being easily activated during purification. Rest the purified population for 30 min in the incubator prior to the NETs stimulation to reduce false positive data.

3. Confirm purification of neutrophil population by additional methods (*e.g.*, cytospin).

4. Stop the NETs challenge step by adding PFA into the reaction mix, rather than spinning the tube and removing the supernatant before adding the PFA to reduce activation by other means.

5. Confirm the lack of non-specific binding for the secondary antibody (DyLight 680) by running a control tube with cells and secondary DyLight 680 antibody but no H4cit3 primary antibody.

6. Use custom masks for the data analysis with the IDEAS software, as they represent more accurately the region of interest. Validate the custom masks for total cell area and nucleus, by confirming that the masks are indeed masking the region(s) of interest (*i.e.*, not missing parts of the target region or, on the opposite, masking 'ghost' regions outside of what is relevant for the features) (Dominical *et al.*, 2017).

7. Note that this protocol allows the identification of NETosis markers in whole neutrophils, prior to the release of the DNA and the cytoplasmic proteins strings into the extracellular space. This detail is crucial for deciding the length of the challenge with the NETs inducers for a peak number of cells that respond to stimulus and can be acquired with the ImageStream cytometer. Lengthy stimulation periods might lead to false negative results due to the fact that the cells have already lysed and released their content and thus are no longer "visible" to the flow cytometer. This case can be confirmed by independent assessments: for example, by the size of the pellet observed in the staining tube, and the number of events in the CD66b$^+$Hoechst$^+$ gate actively acquired by the flow cytometer in the stimulated tube being reduced as compared to the untreated control.

8. To generate a custom feature to evaluate nuclear supercondensation:

   a. In the IDEAS software, open a *.daf* extension file, select a Hoechst$^+$CD66b$^+$ double positive cells.

   b. On the top menu in IDEAS, select 'Analysis', then 'Masks'. In the open pop-up window select 'New', then 'Function'.

   c. To create a mask for the nuclear staining: select the fluorescent channel for the nuclear imagery for both the image and the channel boxes. In the drop-down menu of the function masks, test which mask function fits best with the nuclear image by looking at the changes of masking in the imaging box (usually "Morphology" works best as nuclear mask, as it covers more accurately the nuclear stained region) (Dominical *et al.,* 2017). Once the new function mask is chosen, Click 'Ok'.

   d. Create a name for that mask or use the default name suggested by the software.

   e. Click 'Ok' again to generate the mask.

   f. To create a new mask press 'New'.

   g. Repeat steps b through e to mask the whole cell area by using CD66b imagery or BF channel. "Object" or "Erode" function masks are good choices for masking the cell region.

   h. Click 'Close' to close the mask window.

   i. To apply new features on these new custom masks created, go back to 'Analysis' in the top menu bar and select 'Features'. Select "New" in the pop-up window.

   j. 'Feature Type' box is available for choosing an option. Choose 'Single' and in the drop-down menu in the side, choose the 'Area' feature.

   k. Below the 'Feature Type' box, in the mask box, select the nuclear mask newly created. 'Set a Default Name' or create a new name. Click 'Ok' when done.

   l. Repeat steps i to k. Next use the 'whole cell' mask generated to input in the mask box of the new area feature to create the new 'Area of the whole cell' feature.

m. To calculate the ratio of these new features created, make a combined feature by selecting 'New' in that same feature window.

n. In the 'Feature Type' box select 'Combined'.

o. In the 'Features Box' on the left, select the 'nuclear area' feature created.

p. Highlight the feature and on the right side of the window select the 'arrow down' to insert that feature in the box.

q. Select "/" (forward slash) to calculate the ratio.

r. Highlight the 'Area of the whole cell' new feature created and press the arrow down to insert this feature in the box after the ratio sign. The nuclear area is now divided by the whole cell area.

s. Name the new feature. Click 'Ok' when done.

t. Click 'Close' to close the Features window. This new feature can be now applied in histograms or two-parameters dot plot.

Recipes

1. 8% Paraformaldehyde (PFA) working solution
   2.5 ml PFA 32% stock
   Add 10 ml 1× DPBS, no calcium, no magnesium

2. Wash Buffer (WB)
   10 g BSA (final concentration 2%)
   2 ml EDTA from 0.5 M stock (final concentration 2 mM)
   Add 500 ml 1× DPBS, no calcium, no magnesium
   Filter through 0.45 µm pores

3. Blocking Buffer (BB) (keep sterile)
   15 g BSA (final concentration 3%)
   5 ml porcine skin gelatin stock (final concentration 0.2%)
   Add 50 ml 1× DPBS, no calcium, no magnesium
   Filter through 0.45 µm pores

4. Porcine skin gelatin stock 2%
   2 g porcine skin gelatin
   100 ml ddH$_2$O

5. 0.6 M KCl (keep ice-cold)
   37.28 g KCl granular
   1,000 ml ddH$_2$O
   Filter through 0.45 µm pores

6. Neutrophils complete medium (keep sterile)
   5 ml L-glutamine from 200 mM stock (final concentration 2 mM)
   500 ml RPMI-1640 without L-glutamine

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Competing interests

The authors declare no competing financial or non-financial interests.

Ethics

Informed consent was obtained from all subjects who provided blood samples enrolled in the study protocol NCT0004799, approved by the NHLBI Institutional Review Board.

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