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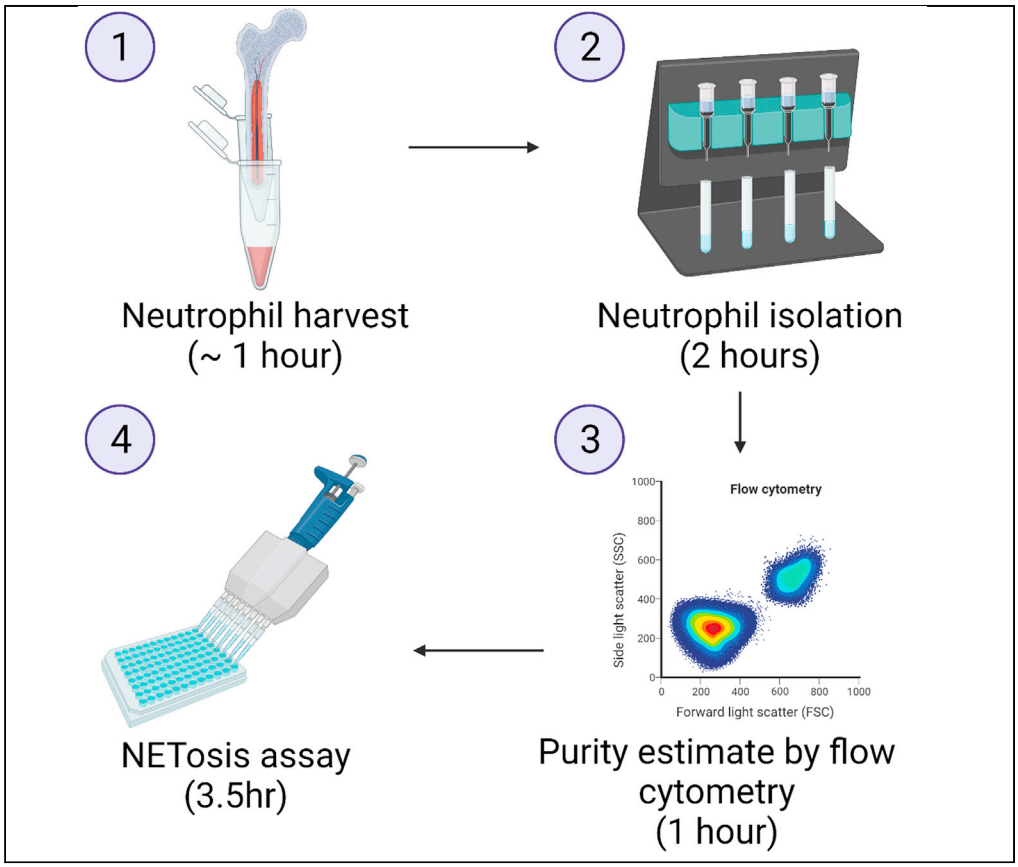
Protocol for analysis of mouse neutrophil NETosis by flow cytometry



Studies involving neutrophils are steadily increasing, thus creating a need for more optimized and thorough protocols for studying neutrophil function. Here, we present our protocol for extracting mouse bone marrow neutrophils, estimating the purity of isolated neutrophils, and assessing their ability to induce NETosis upon an external cue. We test two isolation protocols that can be used to attain neutrophils to assess NETosis induction. This approach allows for the parallel assessment of NETosis induction in cohorts larger than 10 samples.

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Highlights

Primary neutrophil isolation and functional analysis can be completed within a day

Neutrophil NETosis variations can be examined across sex, age, genotype, and treatment

NETosis induction can be assessed in many biological samples (>10) in parallel

McGill et al., STAR Protocols 2, 100948
December 17, 2021
https://doi.org/10.1016/j.xpro.2021.100948

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Protocol for analysis of mouse neutrophil NETosis by flow cytometry

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https://doi.org/10.1016/j.xpro.2021.100948

SUMMARY

Studies involving neutrophils are steadily increasing, thus creating a need for more optimized and thorough protocols for studying neutrophil function. Here, we present our protocol for extracting mouse bone marrow neutrophils, estimating the purity of isolated neutrophils, and assessing their ability to induce NETosis upon an external cue. We test two isolation protocols that can be used to attain neutrophils to assess NETosis induction. This approach allows for the parallel assessment of NETosis induction in cohorts larger than 10 samples.
For complete details on the use and execution of this protocol, please refer to Lu et al., 2021.

BEFORE YOU BEGIN

The protocol below describes the specific steps and timing for extracting primary cells from a cohort of 10 mice. Times listed will increase or decrease if mouse cohorts are larger or smaller, or if additional tissues are or are not harvested. This protocol has been successfully applied with both male and female mice, genetically modified mice, as well as mice having received treatments (e.g., intraperitoneal injections), between the ages of 3–24 months. Thus far, we have not found a condition where this protocol does not work.

Before starting, prepare the necessary solutions for the ''Bone Marrow Collection'' step and autoclave tweezers, razor blades, scissors, and any other tools that will be used to handle samples. Refer to the key resources table and materials and equipment section for the necessary recipes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Ly6G-APC (for purity estimate)	Invitrogen	Cat#17-9668-80
CD11b-Vioblue (for purity estimate)	Miltenyi Biotec	Cat#130-113-238

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's PBS (DPBS), without calcium and magnesium	Corning	Cat#21-031-CV
Antibiotic antimycotic 100×	Gibco	Cat#15240-062
Penicillin-streptomycin-L-glutamine 100×	Corning	Cat#30-009-CI
Red Blood Cell Lysis buffer	BioLegend	Cat# 420301
Neutrophil Isolation Kit	Miltenyi Biotec	Cat#130-097-658
EasySep Mouse Neutrophil Enrichment Kit	STEMCELL Technologies	Cat#19762
Mouse Fc-blocking reagent	Miltenyi Biotec	Cat#130-092-575
SYTOX Green	Thermo Fisher Scientific	Cat# S7020
RPMI 1640 without phenol red	Hyclone	Cat#SH3060501
BSA	Akron Biotechnology	Cat#AK1391
DMSO	Sigma	Cat# 20-139
Phorbol 12-myristate 13-acetate (PMA)	Sigma	Cat#P1565
autoMACS Rinsing Solution	Miltenyi Biotec	Cat#130-091-222
MACS BSA Stock Solution	Miltenyi Biotec	Cat#130-091-376
QuadroMacs Separator	Miltenyi Biotec	Cat#130-090-976
LS Columns	Miltenyi Biotec	Cat#130-042-401
EasyEights Easy Sep Magnet	Stemcell	Cat#18103
Deposited data		
Purity and NETosis flow cytometry data	This paper	https://doi.org/10.6084/m9.figshare.15072024
NETosis flow cytometry data (reanalysis)	(Lu et al., 2021) Aging Cohort 30 (young male and female groups only)	https://doi.org/10.6084/m9.figshare.14043923.v1
Experimental models: Organisms/strains		
Mouse: C57BL/6NTac, Nia or J: wild type (3–5 months)	Taconic farms, Charles River or Jackson laboratories	N/A
Software and algorithms		
Flowlogic v8	Miltenyi Biotec	Cat#160-002-087
Other		
6-Well Suspension Culture Plates	Genesee Scientific	Cat#25-100
0.5mL Tubes	Fisher Scientific	Cat#13-698-793
1.5mL Tubes	Fisher Scientific	Cat#13-698-794
20g needles	BD	Cat# 305175
70 mm MACS SmartStrainers	Miltenyi Biotec	Cat#130-110-916 or Cat#130-098-462
96-well plate	Greiner Bio-One	Cat#655090
Countess Cell Counting Chamber Slides (includes 0.4% Trypan blue solution)	Invitrogen	Cat#C10228
Countess II FL Automated Cell Counter	Invitrogen/Applied Biosystems	Cat#AMQAF1000
MACSQuant Analyzer 10	Miltenyi Biotec	Cat#130-096-343

MATERIALS AND EQUIPMENT

Note: Reagents from alternative suppliers may alter the efficiency of neutrophil extractions and should be validated prior to use in this protocol.

Bone collection buffer	
Reagent	Amount
D-PBS	500mL
Antibiotic Antimycotic 100×	5mL
The solution can be prepared in advance and stored at 4°C. We recommend storing the bone collection buffer for no longer than 6 months.	

Red blood cell lysis buffer	
Reagent	Amount
Red Blood Cell Lysis Buffer 10×	100mL
Deionized Water	900mL
The solution can be prepared in advance and stored between 20°C-25°C. We recommend storing Red Blood Cell Lysis buffer for no longer than 6 months.	

Resuspension buffer	
Reagent	Amount
autoMACS Rinsing Solution	475 mL
MACS BSA Stock Solution	25 mL
The final buffer composition corresponds to phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA. We filter sterilize the resuspension buffer. We recommend storing resuspension buffer for no longer than 6 months at 4°C.	

Flow cytometry staining buffer	
Reagent	Amount
Resuspension buffer	900 uL
Fc blocking reagent	100 uL
The flow cytometry staining buffer should be made immediately before use.	

Neutrophil culture medium	
Reagent	Amount
RPMI 1640 without phenol red	500mL
Penicillin/Streptomycin	5mL
BSA	50mg
The solution can be prepared in advance and stored at 4°C. We recommend storing neutrophil culture medium for no longer than 6 months. FBS is not used as it can promote spurious neutrophil activation.	

STEP-BY-STEP METHOD DETAILS

Set up reagents and materials

Timing: 30 min

1. Prepare all necessary buffers (Bone collection buffer, red blood cell lysis buffer, resuspension buffer, neutrophil culture medium).
2. Sterilize all the tools for dissection by soaking them in 70% EtOH.
3. Using a 20-gauge needle, poke three holes into the bottom of a 0.5mL microcentrifuge tube. Place this tube into a clean 1.5mL microcentrifuge tube. Prepare one set of microcentrifuge tubes for each sample (Amend et al., 2016).

4. Label 15mL conical tubes, one for each sample.
5. Prepare 1.5mL microcentrifuge tubes with 15uL of trypan blue, two for each sample.

Bone marrow neutrophil collection

Timing: 3 h (to process 10 animals)

Note: For the bone marrow neutrophil purification we use a magnetic purification with the Miltenyi negative selection bone marrow neutrophil purification kit, a well-supported method in the field (Kimmey et al., 2015; Mishra et al., 2017; Ponzetta et al., 2019; Spolski et al., 2019; Yee et al., 2020; Koss et al., 2021). Alternative magnetic-based isolation kits exist (*e.g.* Stemcell's EasySep Mouse Neutrophil Enrichment Kit, Biolegend's MojoSort Mouse Neutrophil Isolation Kit). Flow cytometry-based cell sorting [FACS] is of course another option for isolation of primary neutrophils. However, FACS can be impractical when large animal cohorts (>10) are processed, due to increased time for processing, which may lead to circadian differences and other transcriptional alterations between mice processed earlier versus later in the cohort.

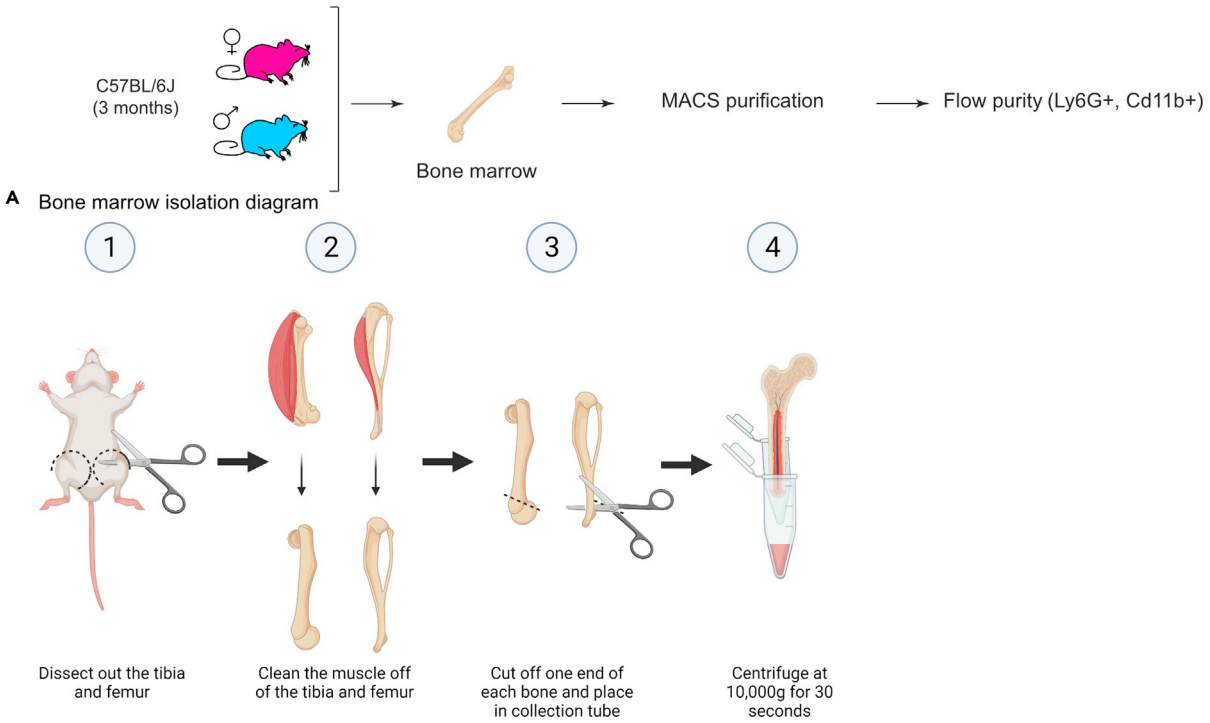
Note: Here, we provide quantification examples for a cohort of 10 mice. We have successfully applied this protocol to both sexes across a range of ages.

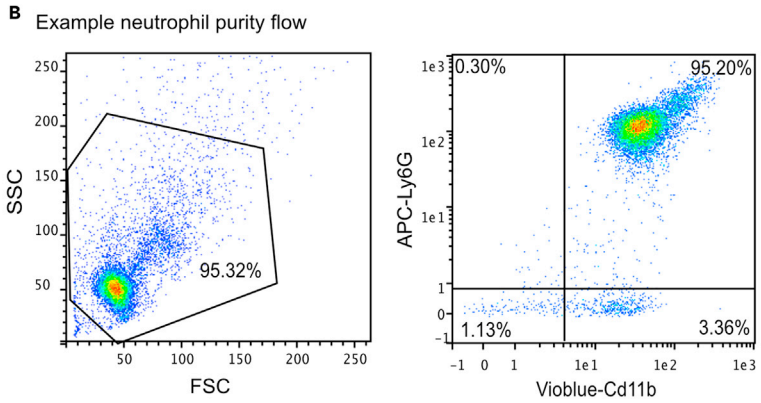
This step details how to collect and isolate mouse bone marrow neutrophils.

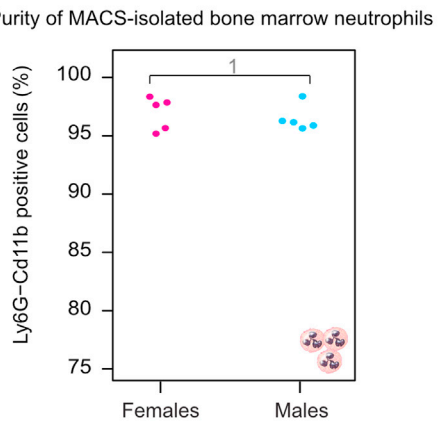
6. Aliquot 3mL of bone collection buffer into one well of a 6-well plate for each sample. Keep the plate on ice.
7. Euthanize each mouse according to the procedures approved by your Institutional Animal Care and Use Committee (IACUC). In our lab, mice are euthanized by CO_2 asphyxiation followed by cervical dislocation.
8. After ensuring euthanasia, place the mouse in a prone position and extract the femurs and tibias. Remove all skin, muscle, and tendons using sterile tools and clean paper towels (Figure 1A).
9. Put the cleaned bones into one well of the 6-well plate containing bone collection buffer on ice for each animal.
10. After collection and cleaning of all bones, cut the epiphyses of the bones with small scissors and place the bones cut side down into the 0.5mL/1.5mL collection tube.
11. Flush the bone marrow out by centrifuging for 30 s at 10,000g between 20°C-25°C.
12. Discard the bones and resuspend the collected sample in 1mL of resuspension buffer while minimizing air bubbles which can activate neutrophils. Carefully transfer the 1mL suspension to a 15mL conical tube.
13. Lyse red blood cells using 10mL of Red Blood Cell Lysis buffer for 1mL of suspension. Carefully invert to mix and incubate for 2 min between 20°C-25°C.

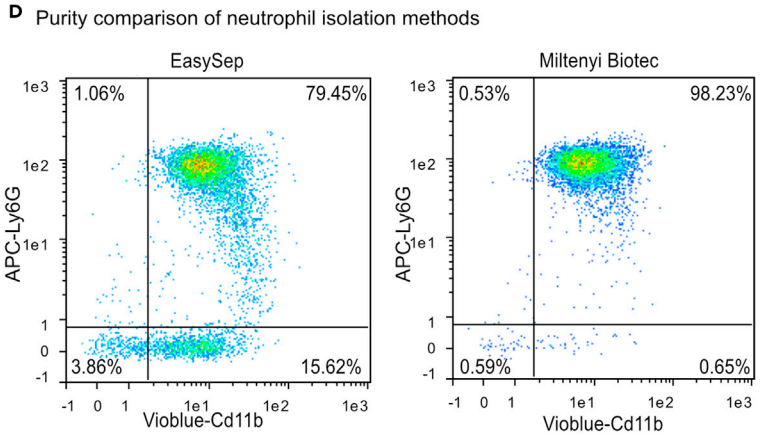
Note: DO NOT vortex the cells, as mechanical stress will cause spurious neutrophil activation.

14. Centrifuge at 300×*g* for 10 min at 4°C.
15. Prepare a 70 μM filter by rinsing it with 2mL of resuspension buffer.
16. Remove the supernatant by aspiration and resuspend the pellet in 5mL of resuspension buffer.
17. Filter the 5mL suspension on 70 μM mesh filters to retain only single cells for downstream processing.
18. Add an additional 5mL of resuspension buffer onto the filter.
19. Centrifuge the sample at 300×*g* for 10 min at 4°C.
20. Remove the supernatant and resuspend pellet in 1mL of resuspension buffer.
21. Take 15 μL of the 1mL suspension and add this to one of the 15 μL of trypan blue in 1.5mL microcentrifuge tubes. Slowly pipette up and down to mix. Repeat for each sample.


C57BL/6J
(3 months)
Bone marrow
MACS purification
Flow purity (Ly6G+, Cd11b+)
A
Bone marrow isolation diagram
1
2
3
4
Dissect out the tibia and femur
Clean the muscle off of the tibia and femur
Cut off one end of each bone and place in collection tube
Centrifuge at 10,000g for 30 seconds


B
Example neutrophil purity flow
SSC
FSC
95.32%
APC-Ly6G
Vioblue-Cd11b
0.30%
95.20%
1.13%
3.36%


C
Purity of MACS-isolated bone marrow neutrophils
Ly6G−Cd11b positive cells (%)
Females
Males


D
Purity comparison of neutrophil isolation methods
EasySep
Miltenyi Biotec
APC-Ly6G
Vioblue-Cd11b
1.06%
79.45%
3.86%
15.62%
0.53%
98.23%
0.59%
0.65%

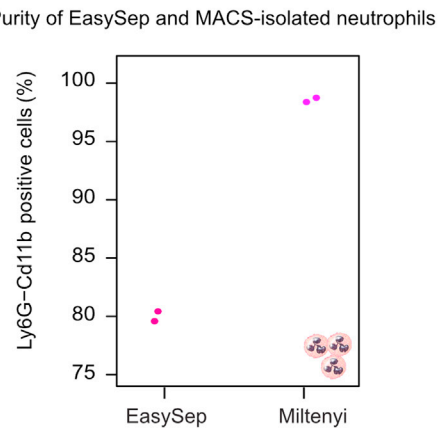

E
Purity of EasySep and MACS-isolated neutrophils
Ly6G−Cd11b positive cells (%)
EasySep
Miltenyi

Figure 1. Isolation and purity check of bone marrow neutrophils

(A) Diagram displaying the process of dissecting the tibia and femur from a mouse, cleaning the muscle off the bones, and flushing the marrow out using centrifugation.

(B and C) Bone marrow neutrophils from 3-months-old female C57BL/6J mice were then purified using the Miltenyi Biotec Neutrophil Isolation kit and checked for purity using CD11b and Ly6G antibodies.

(D and E) Bone marrow neutrophils were then purified from two 5-months-old female mice using either the Stemcell EasySep Neutrophil Enrichment kit or the Miltenyi Biotec Neutrophil Isolation kit and checked for purity using CD11b and Ly6G antibodies. To perform the comparison reported in (D and E), we used mice carrying a floxed allele for Foxl2 on the C57BL/6 background from our colony at USC. Flow cytometry data was analyzed using Flowlogic v8.

22. Take 15 μL from the trypan blue/sample mix and insert this into one of the wells of the COUNTESS chamber slide. Count the cells and check for viability using the COUNTESS.

Note: In the case that a COUNTESS machine is not available, other cell counting methods can be used (*e.g.* using other automated cell counters, or manual counts with hemocytometers).

23. Isolate the neutrophils from the suspension using the Miltenyi Biotec Neutrophil Isolation Kit, LS columns and quadroMACS magnets, following the manufacturer's directions.

Note: Be sure to minimize mechanical stress and bubbles during the entire isolation step to maximize viable neutrophil recovery.

24. Take 15 μL of the isolated neutrophils and add this to the remaining 1.5mL microcentrifuge tube with 15 μL of trypan blue. Slowly pipette up and down to mix. Repeat for each sample.
25. Take 15 μL from the trypan blue/sample mix and insert this into one of the wells of the COUNTESS chamber slide. Assess purified primary neutrophil viability and yield using the automated COUNTESS cell counter.

Neutrophil purity estimate by flow cytometry (optional)

Timing: 1.5 h (to process 10 samples)

This optional step details how to estimate neutrophil purity from the MACS purification step by flow cytometry using a MACSQuant Analyzer 10 benchtop flow cytometer.

Note: We assess purity by staining for Ly6G, a differentiation antigen expressed by myeloid-derived cells (Lee et al., 2013) and CD11b, a cell surface antigen of neutrophils (Biffl et al., 1996). We define neutrophils as CD11b+ Ly6G+ cells. See Figures 1B and 1C for an overview of the gating strategy and the results for a representative cohort.

26. Aliquot 250,000 cells per sample into 5 mL polystyrene round-bottom tubes for staining. Additionally, reserve cells for the unstained and single-stained controls to draw the gates.

Note: Make three equi-cellular mixes (250,000 cells in each 5 mL polystyrene round-bottom tube; control sample) of all samples for the one blank and two single-stained controls.

Note: Keep the remaining cells on ice during this time.

27. Pellet cells at 300×*g* at 4°C for 10 min in a refrigerated centrifuge. Remove supernatant without disturbing the pellet. Resuspend cells in 50 μL of flow cytometry staining buffer, which contains mouse Fc receptor blocker. Block cells by incubating at 4°C for 10 min.
28. For each sample that will be stained, prepare 50 μL of a 2× antibody solution in flow cytometry staining buffer (Table 1).
29. Add the 50 μL of 2× antibody solution to the 50 μL of blocked cells.

Table 1. Antibody dilutions for neutrophil markers

Antibody	Stock conc.	Dilution factor for a 2× solution	Final dilution factor	Final conc.
APC anti-mouse Ly6G	0.2 mg/mL	1:50	1:100	2 μg/mL
Vioblue anti-mouse CD11b	0.2 mg/mL	1:50	1:100	2 μg/mL

Note: Leave one control sample unstained and perform a single stain for each antibody using the other two 5 mL polystyrene round-bottom tubes. These will be used to draw the flow cytometry gates.

30. Stain the cells by incubating at 4°C for 20 min. Protect from light.
31. Add 1mL of resuspension buffer to wash away excess antibody. Pellet cells by centrifuging at 300×*g* at 4°C for 10 min.
32. Repeat step 31.
33. Resuspend cells in 250 μL of resuspension buffer (50,000-100,000 cells per 100 μL of resuspension buffer).
34. Run samples on a MACSQuant10 flow cytometer. First, use the unstained and single-stained samples to set up appropriate gates that encompass clear positive and negative populations. Adjust the scatter signals to exclude debris, dead cells, and doublets.

Note: If the MACSQuant10 flow cytometer is not available, other flow cytometry equipment with the appropriate lasers (e.g. BD Aria flow cytometry) can be used for similar results.

Note: An example of the purity obtained from a cohort of male and female 3-months-old C57BL/6J mice is reported in Figures 1B and 1C. We routinely obtain purities >90% with the Miltenyi Biotec method, without significant differences across biological groups (Lu et al., 2021).

Note: We performed a small-scale comparison of neutrophil purities using the Miltenyi Biotec Neutrophil Isolation Kit and the alternative Stemcell EasySep Neutrophil Enrichment Kit on two 5-months-old female mice (Figures 1D and 1E). Notably, the EasySep method seems to result in a lower purity of neutrophils derived from bone marrow.

Neutrophil NETosis assay

Timing: 3 h (to process 10 samples)

This step details how to quantify NETosis induction from MACS purified neutrophils by flow cytometry using a MACSQuant Analyzer 10 benchtop flow cytometer.

Note: The following protocol has been adapted from a flow cytometry-based protocol (Masuda et al., 2017) and a 96-well plate plate-reader protocol (Carmona-Rivera and Kaplan 2016), to assess NETosis induction for neutrophils in suspension culture.

Note: SYTOX Green stains extra-cellularized DNA, which is a phenotype of cells that have NETosed or are NETosing. If the neutrophil preparation has poor viability, non-NETosing dead cells may also by SYTOX positive. If desired, although lowering the throughput of the protocol, cells may be co-stained with H3-Cit, MPO, or ELANE to confirm NETosis status. In general, these markers correlate very well with SYTOX Green staining in viable cell preps (Gupta et al., 2014; Carmona-Rivera and Kaplan 2016; Masuda et al., 2017; Zhou et al., 2020).

Note: Phorbol 12-myristate 13-acetate (PMA) is a known activator of NETosis. To induce NETosis in purified primary neutrophils, alternatives to PMA include fMLP (Torres et al., 1993),

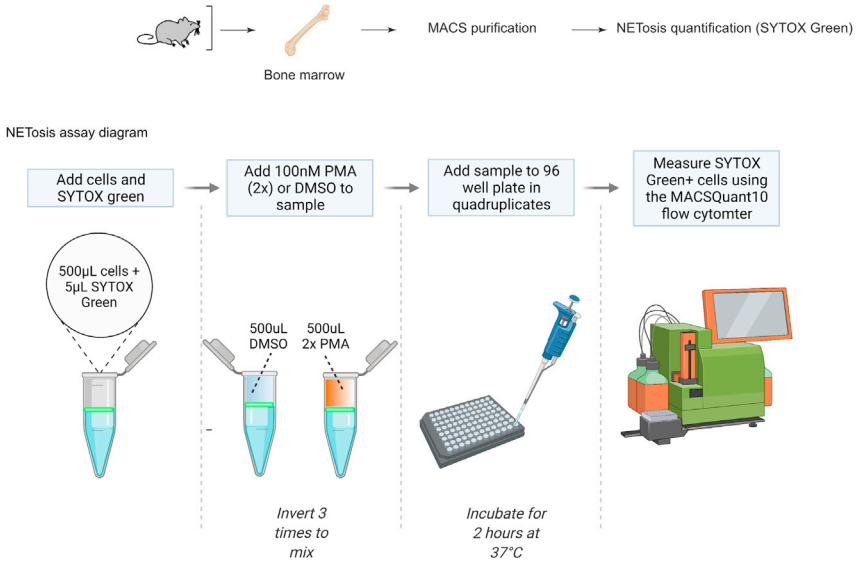


Figure 2. Assay to measure NETosis induction using SYTOX Green

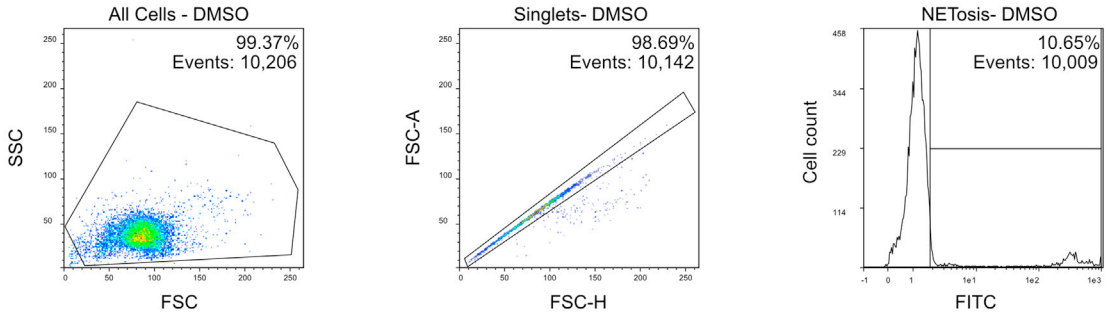
Diagram showing the steps to perform the NETosis measurement assay. 1 × 10^6 cells resuspended in 500 µL of neutrophil culture medium is added to a 1.5mL microcentrifuge tube. 5 µL of diluted SYTOX Green is then added to the cell suspension, followed by 500 µL of either DMSO or 2× PMA. The samples are then added to a 96-well plate in quadruplicates and incubated for 2 h at 37°C. After incubation, SYTOX Green+ cells are measured using the MACSQuant10 flow cytometer. Results are analyzed using Flowlogic v8.

TLR agonists such as LPS (Soler-Rodriguez et al., 2000), and the calcium ionophore A23187 (Kenny et al., 2017).

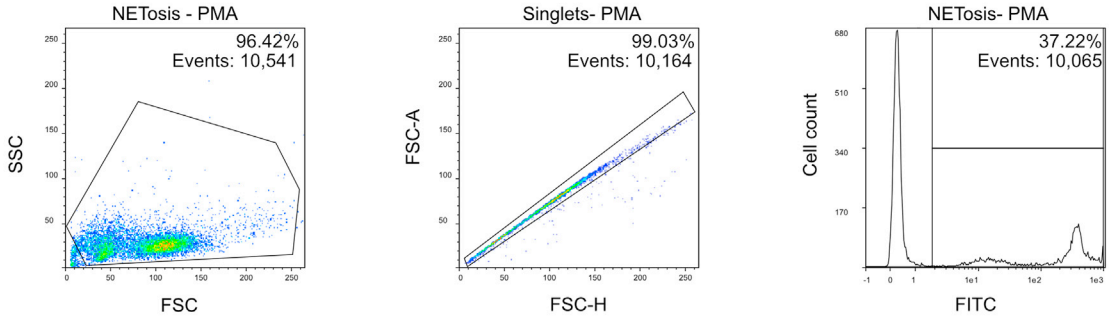
Note: For this protocol, we use a 2-hour incubation period to induce NETosis, a timeframe well supported by the literature (Brinkmann et al., 2012; Hu et al., 2016; Moussavi-Harami et al., 2016; Mercer et al., 2018; Neubert et al., 2018; Carmona-Rivera et al., 2019; Vaidya et al., 2021). However, an incubation period ranging from 1-4 h can also be used, as multiple studies have found robust levels of PMA-induced NETosis as early as 1 hour post PMA exposure (Gupta et al., 2014; Hoppenbrouwers et al., 2017; Masuda et al., 2017; Zharkova et al., 2019).

35. Resuspend the remaining cells in 15mL conical tubes to a concentration of 2 × 10^6 cells/mL.
36. Prepare two 1.5 mL microcentrifuge tubes for each sample. One will be for the DMSO [Vehicle] treated aliquot and the other for Phorbol 12-myristate 13-acetate (PMA).
37. Aliquot 500 µL of cells (1 × 10^6 cells total) into the 1.5 mL microcentrifuge tubes.
38. Prepare a 1:250 dilution of the SYTOX Green stock solution in the neutrophil culture medium. Protect from light.
39. Add 5 µL of the diluted SYTOX Green to each tube (Figure 2). Protect from light.
40. Prepare the DMSO and PMA working stocks. Supplement neutrophil culture media with DMSO [Vehicle] or 100nM PMA (2× concentration).
41. Add 500 µL of the DMSO or PMA supplemented media to the respective microcentrifuge tubes (for a final concentration of 50nM PMA).
42. Slowly invert three times to mix.
43. Seed 200 µL (2 × 10^5 cells) in technical quadruplicates in wells of a sterile black 96-well suspension plate.
44. Incubate in a humidified incubator with 5% CO_2 at 37°C for 2 h.
45. Measure the fraction of cells positive for SYTOX Green in each well using the MACSQuant10 flow cytometer. See Figures 3A and 3B for gating examples.

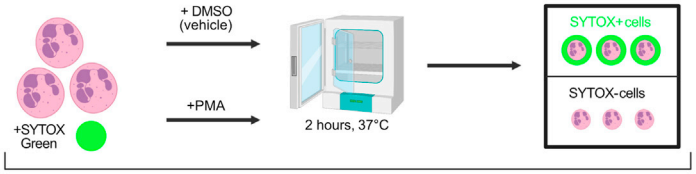
A DMSO-treated neutrophil activation



B PMA-treated neutrophil activation

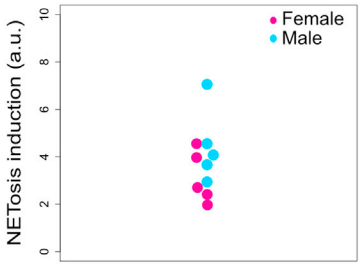


C NETosis induction calculation



$$\text{NETosis induction by PMA} = \frac{\text{PMA (\% SYTOX+cells)}}{\text{DMSO (\% SYTOX+cells)}}$$

D Quantification of NETosis induction



E Example of spurious neutrophil activation

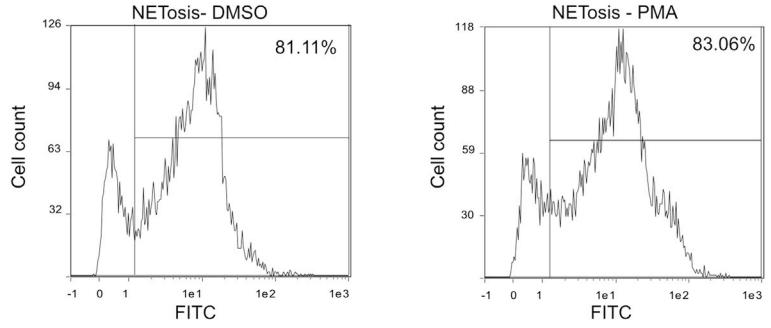


Figure 3. Flow cytometry results of the NETosis assay

Data for this figure was reanalyzed from Lu et al., 2021, using the publicly deposited raw data on Figshare (Cohort 30, young animals only).

(A and B)The percent of SYTOX Green+ staining cells is low in the DMSO treated group (A) compared to that of PMA (B).

(C) NETosis induction is calculated by dividing the % parent SYTOX Green+ median of PMA technical quadruplicates over that of DMSO (C).

(D) NETosis induction values in ten 4-months-old mice is quantified in panel D (half females, half males). For experiments in (A–D), we used C57BL/6Nia mice.

(E) Under unfavorable conditions, neutrophils will activate spuriously, which can lead to high background SYTOX Green staining and little no apparent difference between DMSO and PMA groups (E).

Note: If the MACSQuant10 flow cytometer is not available, other flow cytometry equipment with the appropriate lasers can be used for similar results.

46. Analyze the data using Flowlogic V8 (see quantification and statistical analysis section for more details).

EXPECTED OUTCOMES

Using the Miltenyi Biotec Neutrophil isolation kit, the expected yield is 5–10 million neutrophils per mouse (depending on age and sex of animal), with a proportion of CD11b+Ly6G+ stained cells > 90% (Figures 1B and 1C). If neutrophils are not accidentally activated, the differences in NETosis induction between DMSO and PMA treatment of the same sample are clear (Figures 3A and 3B). In the case of accidental neutrophil activation, the NETosis induction of DMSO will appear extremely high, as seen in Figure 3E. Neutrophil purity and viability should be checked prior to beginning the NETosis assay. Viability lower than 80% will be problematic and the NETosis assay will not yield accurate results.

Using alternative methods of bone marrow neutrophil purification may lead to varying results. Notably, the EasySep Neutrophil Enrichment Kit results in both lower purity compared to that of the Miltenyi MACS isolated neutrophils (Figures 1D and 1E). It is important to note that results from different isolation methods are not comparable and it is imperative to choose a single isolation method for experiments under the same project to be comparable with each other.

QUANTIFICATION AND STATISTICAL ANALYSIS

To quantify the fraction of cells positive for SYTOX Green in each well, follow the gating recommendations seen in Figure 3A. To account for differences in basal levels of NETosis across samples, NETosis is expressed as induction of NETosis: (median % SYTOX Green+ singlets of PMA technical quadruplicates) / (median % of DMSO technical quadruplicates). Figures 3C and 3D exemplifies quantification of NETosis induction in an example dataset with 5 female and 5 male 4-months-old mice.

The raw flow cytometry data for the example is available on Figshare (see key resources table).

LIMITATIONS

While our protocol has proven successful, neutrophils are extremely delicate and activate upon seemingly minor disturbances, such as the generation of bubbles or harsh pipetting. Thus, it is possible for this protocol to yield variable results depending on the experimenter. If running more than 1 96-well plate at a time, ensure to stagger the experiments to avoid additional non-standard incubation times while waiting to use the flow cytometer.

When treated with PMA, neutrophils become stickier, and may become harder to get into the flow cytometry buffer, causing differences in the number of events between DMSO and PMA. To avoid potential discrepancies across samples and/or replicates, ensure to set a gate on the flow cytometer to a max of 10,000 events.

For this protocol, we chose a 2-h window of PMA exposure to induce NETosis; however, NETosis may continue beyond this 2-h window. While several published time course analyses for NETosis have shown robust levels of PMA-induced NETosis as early as 1 h post exposure, all show clear detectable NETosis induction at 2 h (Gupta et al., 2014; Hoppenbrouwers et al., 2017; Masuda et al., 2017; Zharkova et al., 2019). Despite this, additional information about the dynamics of NETosis induction may be obtained by modifying the protocol to perform a time course analysis from 0-4 h as an alternative approach.

Additionally, aged female mice have decreased bone density compared to young female and aged males (Somerville et al., 2004). This leads to an increased possibility of breaking the tibia and/or femur during dissection and potential loss of bone marrow. Special care should be given to avoid breaking the more brittle bones.

TROUBLESHOOTING

Problem 1

Low yield of bone marrow neutrophils (step 23).

Potential solution

Bone marrow neutrophil yield can be maximized by not breaking the long bones of the mice during harvesting. If the bones do break, ensure to collect all pieces for the next step. Additionally, thoroughly cleaning the muscle tissue from the bones will maximize neutrophil yield as excess muscle issue can clog the hole by which the cells flow through into the new clean tube. If needed, bone marrow from the radius, ulna, and humerus can be used to increase bone marrow yield and ultimately, neutrophil yield.

Low yield is also frequently the result of premature neutrophil activation during isolation. This can be avoided through careful pipetting of bone marrow (see next problem and possible solution).

Problem 2

Neutrophil viability is low (step 25).

Potential solution

Neutrophils are easily activated by shear or mechanical stress, and extreme care should be taken when working with them. Avoid creating bubbles when pipetting, pipetting too fast, and shaking the tubes containing live neutrophils.

Problem 3

There is no increase in activation after exposure with PMA (step 45).

Potential solution

Unprimed neutrophils may be less prone to enter NETosis upon external activation. Consider analyzing the impact of PMA exposure in neutrophils primed with TNFα. TNFα is a pro-inflammatory cytokine whose levels increase in aging and infection. For this purpose, you may pre-treat the neutrophils with 10ng/mL TNFα (PeproTech Cat#315-01A-20UG) prior to PMA exposure for 15 min at 37°C. Prewarm the cells in a 37°C incubator for 15 min prior to TNFα exposure.

Problem 4

Low to no SYTOX Green signal is detected (step 45).

Potential solution

SYTOX Green is light sensitive. Ensure that the samples are protected from light. When first receiving the vial of SYTOX green, it is best practice to aliquot it into smaller amounts to avoid repeated freeze-thaw cycles.

Problem 5

Upon analysis of the NETosis assay flow cytometry data, it appears the DMSO group has a high background of NETosis (step 46).

Potential solution

As neutrophils are easily activated, activation can occur at any step of the protocol. Therefore, it is imperative to remain cautious throughout the procedure to avoid any bubble formation. See problems 1 and 2.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bérénice A. Benayoun (berenice.benayoun@usc.edu).

Materials availability

This study did not generate new or unique reagents.

Data and code availability

The example raw and processed flow cytometry data generated during this study are available at figshare: https://doi.org/10.6084/m9.figshare.14699619 and figshare: https://doi.org/10.6084/m9.figshare.15072024. The example flow cytometry data for the NETosis assays was reanalyzed from (Lu et al., 2021) and are available at figshare: https://doi.org/10.6084/m9.figshare.14043923.v1.

This study did not generate any code.

ACKNOWLEDGMENTS

The graphical abstract and diagrams were created using BioRender.com. This work was supported by NIA T32 AG052374 predoctoral fellowship to C.J.M., NIA T32 AG052374 predoctoral fellowship and a Diana Jacobs Kalman/AFAR Scholarships for Research in the Biology of Aging to R.J.L., NIA R00 AG049934, Pew Biomedical Scholar award #00034120, an innovator grant from the Rose Hills foundation, and the Kathleen Gilmore Biology of Aging research award to B.A.B.

AUTHOR CONTRIBUTIONS

Conceptualization, C.J.M., R.J.L., and B.A.B.; investigation, C.J.M., R.J.L., and B.A.B.; writing - original draft, C.J.M. and R.J.L.; writing - review and editing, C.J.M., R.J.L., and B.A.B.; funding acquisition, B.A.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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