Chapter 5

Quantification and Visualization of Neutrophil Extracellular Traps (NETs) from Murine Bone Marrow-Derived Neutrophils

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Abstract

Neutrophils are one of the first cells to respond to an inflammatory stimulus, and are equipped with an assortment of antimicrobial and proteolytic enzymes to disarm and degrade bacterial pathogens. A novel mechanism of bacterial trapping, termed neutrophil extracellular traps (NETs), was recently described whereby neutrophils were shown to cast out web-like structures of chromatin, capturing and immobilizing invading pathogens. Herein we describe protocols to isolate murine bone marrow-derived neutrophils, and spectrophotometrically quantify, immunolabel, and visualize NET structures in vitro.

Key words Neutrophil extracellular trap, Bone marrow neutrophil, Nucleic acid stain, Histone H3, Elastase, Fluorescence microscopy

1 Introduction

Neutrophils mature in the bone marrow, where they synthesize and package enzymes and antimicrobial proteins into an assortment of granules [1]. The formation of neutrophil extracellular traps (NET), a type of novel cell death, is characterized by the externalization of web-like strands of decondensed chromatin (DNA and histones) that is highly decorated with antimicrobial and proteolytic enzymes [2–4]. Such a defined composition can be utilized to specifically label for NETs.

Unlike human bone marrow, mouse bone marrow contains a large number of functionally competent neutrophils, which survive much longer ex vivo than do blood neutrophils [5]. Here, we describe protocols to harvest mature murine bone marrow-derived neutrophils (BMDN) from mouse tibia and femur, and purify by discontinuous Percoll density gradient centrifugation [6]. BMDN collected from the 80/65 % Percoll interface contain >85 % BMDN, as confirmed by FACS analysis using antibodies against

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GR-1. To quantify NETs, BMDN are incubated with Sytox Green, a cell-impermeable fluorescent DNA dye. This ensures measurement of extracellular DNA from cells with compromised membrane integrity (typical during the formation of NETs), and not from viable, membrane-intact cells. Fluorescence emission is monitored following a 3-h incubation with the potent NET inducer phorbol 12-myristate 13-acetate (PMA) [7]. The percentage of NET formation can then be determined by subtracting the background fluorescence (determined with the addition of DNase), and dividing by the maximal fluorescence signal detected from lysed BMDN (during incubation with the detergent Triton X-100). NETs can also be visualized directly by first plating BMDN onto poly-L-lysine-coated coverslips, and then staining the fixed cells (resting or activated) with antibodies for the NET components histone H3 [7] or elastase [8], as well as DNA. Together, the protocols described are a complementary approach to quantify and visualize NETs.

2 Materials

2.1 Bone Marrow Neutrophil Isolation

1. 8–9-Week-old male or female C57BL/6 mice.
2. Laminar flow hood.
3. Small dissection scissors.
4. Forceps.
5. Lint-free wipes.
6. Sterile polyethylene disposable transfer pipettes.
7. 60 × 15 mm sterile polystyrene petri dishes.
8. 50 mL conical tubes.
9. 15 mL conical tubes.
10. 10 mL syringes.
11. 25G$^{5/8}$ needles.
12. 20G needles.
13. 70 % ethanol.
14. Ice-cold deionized water.
15. Ice, ice-bucket.
16. MEM alpha cell culture medium 1× (Gibco). Store at 4 °C.
17. Phosphate-buffered saline (PBS) 1×, pH 7.4 without calcium chloride/magnesium chloride. Store at 4 °C.
18. Hank's Balanced Saline Solution (HBSS) with calcium chloride/magnesium chloride. Store at 4 °C.
19. Percoll density gradients: Prepare 100 % Percoll stock by mixing 90 mL of Percoll (pH 8.5–8.9) with 10 mL of 10× Dulbecco's phosphate-buffered saline. In a 50 mL conical

tube, prepare 80 % (mix 40 mL of 100 % Percoll with 10 mL of 1× PBS), 65 % (mix 32.5 mL of 100 % Percoll with 17.5 mL of 1× PBS), and 55 % (mix 27.5 mL of 100 % Percoll with 22.5 mL of 1× PBS) Percoll gradient solutions. Store at 4 °C.

20. 3.6 % (w/v) NaCl: Dissolve 3.6 g of NaCl in deionized water. Store at 4 °C.
21. Turk's solution: Dissolve 0.1 % crystal violet in 3 % acetic acid (prepared in sterile water). Shake vigorously. Store at room temperature.

2.2 Quantification of Neutrophil Extracellular DNA

1. BMDN (1×10^6 cells/mL).
2. Fluorescence microplate reader equipped with filters to detect excitation/emission maxima: 485/520 nm.
3. Humidified CO_2 incubator.
4. Black 96-well microplate.
5. 96-well microplate lids.
6. Microplate-sealing tape.
7. HBSS with calcium chloride/magnesium chloride. Store at 4 °C.
8. Sytox Green nucleic acid stain, 5 mM stock (Invitrogen). Protect from light and store at −20 °C. Just prior to addition to wells, prepare a 10× working solution (50 μM) by diluting 5 mM stock solution 1:100 with HBSS, into a foil-wrapped conical tube.
9. DNase 1 (RNase-free), 2 Units/μL. Store at −20 °C.
10. PMA, 1 mM stock (dissolve 1 mg of PMA in 1.62 mL dimethyl sulfoxide). Aliquot and store at −20 °C.
11. 10 % Triton X-100 (stock).

2.3 Immunofluorescence Visualization of NETs

1. BMDN (1×10^6 cells/mL).
2. Epi-fluorescence or confocal microscope equipped with filters to detect excitation/emission maxima: 358/461 nm (DAPI), 550/570 nm (TRITC), 495/519 nm (Alexa Fluor 488).
3. Humidified CO_2 incubator.
4. Sterile 12-well cell culture plates. Store at room temperature.
5. 12 mm round poly-L-lysine-coated glass coverslips. Store at 4 °C.
6. $75 \times 25 \times 1$ mm microscope slides. Store at room temperature.
7. 1 mL microcentrifuge tubes.
8. Ice-cold methanol. Store at −20 °C.
9. PBS 1×, pH 7.4. Store at 4 °C.
10. PBS supplemented with Tween-20 (PBS-Tween). Mix 1 L PBS with 0.5 mL Tween-20. Store at room temperature.
11. 1 mM PMA stock. Dissolve 1 mg of PMA in 1.62 mL of dimethyl sulfoxide. Aliquot and store at −20 °C. Just prior to

use, prepare a 1 μM working stock solution by diluting 1:1,000-fold into HBSS. Store on ice until ready for use.

12. Fluorescent mounting medium. Store at 4 °C.
13. 4′,6-Diamidino-2-phenylindole, diacetate (DAPI; Invitrogen). Prepare a 5 mg/mL stock by dissolving 10 mg in 2 mL of deionized water. Solution may take some time to dissolve completely and may require sonication. For long-term storage, aliquot and store at –20 °C. For short-term storage store at 4 °C (stable for at least 6 months).
14. Histone H3 (D1H2) XP Rabbit monoclonal antibody (Cell Signaling). Store at –20 °C.
15. Neutrophil elastase polyclonal antibody (Abcam). Aliquot and store at -20 °C.
16. Goat anti-rabbit TRITC secondary antibody (Abcam). Aliquot and store at –20 °C.
17. Goat anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen). Store at 4 °C.
18. Blocking buffer: 3 % bovine serum albumin (BSA) prepared in PBS. Dissolve 0.3 g BSA in 10 mL of PBS. Store at 4 °C.

3 Methods

3.1 Bone Marrow-Derived Neutrophil Isolation

1. Sacrifice mouse by cervical dislocation (alternate methods such as CO_2 asphyxiation may also be utilized—refer to institutional guidelines).
2. In a laminar flow hood, spray the front (ventral) side of the mouse with 70 % ethanol, and make a lateral incision at the midline. Strip away the fur to expose the lower abdomen, soft tissue, and bone of the hind limbs (*see* **Note 1**).
3. Use scissors to make a cut above the hip joint, detach, and transfer intact hind limb to a 50 mL conical tube containing 20 mL of MEM alpha medium. Repeat with the second hind limb.
4. Gently cut away and remove soft tissue from the tibia and femur using scissors and lint-free wipes (*see* **Note 2**). Separate the tibia from the femur and transfer to a 60 × 15 mm petri dish containing MEM alpha medium. Repeat with the second hind limb.
5. Transfer tibias and femurs to a second petri dish containing 70 % ethanol. Soak the bones for ~30 s and then allow them to dry.
6. Use scissors to cut the proximal and distal ends off the tibia/femur, and flush the marrow into a third petri dish using a 10 mL syringe (containing 8 mL of MEM alpha medium) with a 25G$^{5/8}$ needle attached (*see* **Note 3**).

7. Using a fresh syringe, attach a 20G needle and very gently aspirate the bone marrow to separate any clumps. This process should be repeated approximately four to five times. Repeat this procedure with the remaining bones.
8. Transfer to a 15 mL conical tube and centrifuge at 400 × *g* for 10 min at room temperature.
9. Gently pour off the supernatant and resuspend the cell pellet with 1 mL of PBS (without calcium chloride/magnesium chloride).
10. Prepare a Percoll density gradient. In a 15 mL conical tube, carefully add 4 mL of 80 % Percoll. Gently overlay this first layer with 3 mL of 65 % Percoll, followed by 3 mL of 55 % Percoll. Care should be taken to avoid mixing or disturbing the gradient solutions as they are added to the tube (*see* **Note 4**). Allow the Percoll tubes to stand for 5 min and then carefully add the cell suspension, prepared in **step 9**, to the top of the density gradient. Centrifuge at 1,000 × *g* for 30 min at room temperature, without braking.
11. Remove the centrifuge tube and visually inspect the gradient. The bone marrow neutrophils will have separated into the 80 %/65 % Percoll interface.
12. Gently dispose of the uppermost serum and 55 % Percoll layers using a disposable sterile transfer pipette. In a fresh 15 mL conical tube, collect the upper portion of the 65 % Percoll gradient and cells at the 80 %/65 % gradient interface. Top up volume to 14 mL with 1× PBS (without calcium chloride/magnesium chloride) and centrifuge at 400 × *g* for 10 min at 4 °C.
13. Pour off the supernatant and lyse the remaining red blood cells by gently resuspending the cell pellet in 3 mL of ice-cold deionized water. Leave undisturbed for 30 s. Add 1 mL of 3.6 % NaCl and mix gently. Centrifuge at 400 × *g* for 5 min at 4 °C.
14. Pour off the supernatant and gently resuspend the bone marrow-derived neutrophil cell pellet with 1 mL of HBSS (with calcium chloride/magnesium chloride). Determine the concentration using a neubauer hemocytometer. Mix 10 μL of cell suspension with 90 μL of HBSS and 5 μL of Turk's solution. Load 10 μL onto a hemocytometer.
15. Dilute bone marrow neutrophils to a concentration of 1×10^6 cells/mL using HBSS. Typically, ~6×10^6 bone marrow-derived neutrophils can be harvested per mouse.

3.2 Quantification of Extracellular DNA

The following protocol outlines the procedures for measuring neutrophil extracellular DNA, an index for the formation of NETs. BMDN (isolated in Subheading 3.1) or neutrophils from other

Table 1
Treatment conditions used for the quantification of neutrophil extracellular DNA (NETs)

	HBSS (μL)	BMDN (μL) (1×10^6/μL)	Triton X-100 (μL) (10 %)	PMA (μL) (1 μM)	DNase (μL) (2 U/μL)	Sytox green (μL) (50 μM)
BMDN	170	100	–	–	–	30
BMDN + Triton X-100	160	100	10	–	–	30
BMDN + PMA (100 nM)	140	100	–	30	–	30
BMDN + PMA (100 nM) + DNase	137.5	100	–	30	2.5	30
BMDN + DNase	167.5	100	–	–	2.5	30

sources (such as cell lines or peripheral blood) are plated at a density of 1×10^5 cells per well, and activated with PMA. The cell-impermeable DNA-binding dye, Sytox Green, is then added and the resulting fluorescence quantified on a fluorescence microplate reader. This protocol can be modified to incorporate other cell activators or additional inhibitors, and measurements made at variable time-points to monitor the kinetics of NET formation.

1. In a black 96-well microplate (*see* **Note 5**), prepare duplicates of the treatment wells shown in Table 1.
2. Using a pipette, add HBSS, 1×10^5 BMDN, and Triton X-100 (to determine total DNA content) or PMA (an activator of NET formation), as appropriate. Cover the microplate with a lid, and transfer to a humidified incubator (37 °C, 5 % CO_2).
3. After 2 h, add DNase to appropriate wells, and transfer back to the humidified incubator (37 °C, 5 % CO_2) (*see* **Note 6**).
4. After a further 45 min, carefully add 30 μL of SYTOX Green (10× working stock; 50 μM) to each well, mix, and transfer back to the humidified incubator. Allow to stand for a further 15 min, after which the plate can be sealed with microplate-sealing tape, and fluorescence quantified on a fluorescence microplate reader (*see* **Note 7**).
5. To quantify the amount of extracellular DNA (as a percentage of total DNA), subtract the fluorescence intensity of the DNase-containing wells from the comparative control, and divide by the fluorescence intensity emitted from "BMDN + Triton X-100" wells (total DNA present).
   Example:

$$\text{Percentage total DNA (induced by PMA)} = \frac{\text{Fluorescence Intensity(BMDN + PMA)} - \text{Fluorescence Intensity(BMDN + PMA + DNase)}}{\text{Fluorescence Intensity(BMDN + Triton X} - 100)}$$

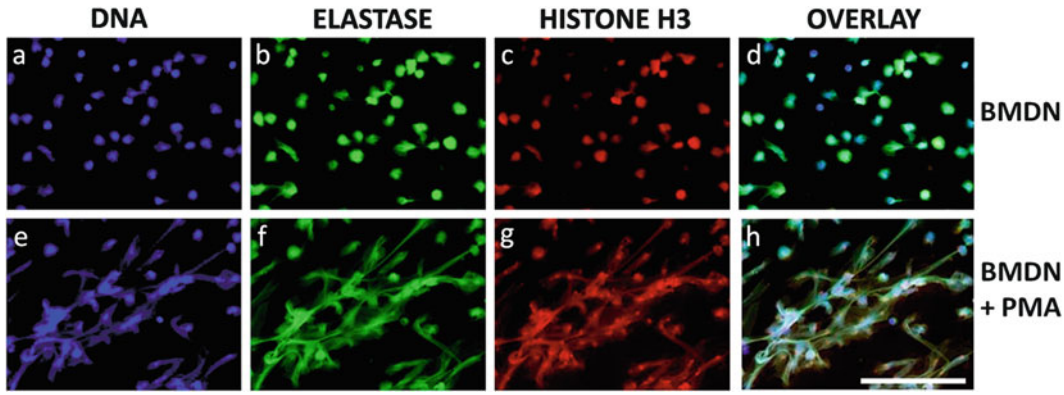

Fig. 1 Visualization of murine bone marrow-derived NETs by immunofluorescence microscopy. Resting BMDN or BMDN activated with PMA (100 μM, 3 h at 37 °C) were fixed and immunostained for DNA (DAPI; panels **a**, **e**), elastase (panels **b**, **f**), or histone H3 (panels **c**, **g**). Overlay of the three channels is shown in *panels* (**d**) and (**h**) for resting and PMA-activated BMDN, respectively. Scale bar = 100 μm

3.3 Immunofluorescence Visualization of NETs (See Fig. 1)

Visualization of NETs by immunofluorescence offers a complementary measure of extracellular DNA quantification, described in Subheading 3.2. While there are many markers for NETs, this protocol uses DAPI (to stain for DNA), as well as histone H3 and the serine protease elastase to label bone marrow NETs.

1. Place one poly-L-lysine-coated glass coverslip into each well of a sterile 12-well cell culture plate (*see* **Note 8**).
2. Plate BMDN onto the center of the poly-L-lysine-coated coverslips at a density of 1×10^5 cells, by gently pipetting 100 μL of BMDN cell suspension (1×10^6 cells/mL). To induce NETs with PMA (100 nM), mix 100 μL of BMDN cell suspension (1×10^6 cells/mL) with 15 μL of 10× PMA working stock solution (1 μM) and 35 μL of HBSS, in a separate microcentrifuge tube, before plating onto poly-L-lysine-coated coverslips. Replace the lid of the cell culture plate, and transfer to a humidified incubator (37 °C, 5 % CO_2) (*see* **Note 9**).
3. Incubate for 3 h.
4. Gently wash away cells that have not adhered with PBS. Aspirate with a pipette tip, and discard (*see* **Note 10**).
5. Transfer the cell culture plate, containing coverslips, to a fume hood and fix adherent cells by adding 400 μL of neutral-buffered formalin. Allow to stand for 15 min at room temperature.
6. Gently aspirate neutral-buffered formalin using a pipette tip, discard, and replace wells with 800 μL of PBS.
7. Repeat a further three times.
8. Transfer coverslips containing adherent BMDN to a new cell culture plate, containing 800 μL of PBS per well. Store at 4 °C (overnight) until ready to perform immunofluorescence labeling.

9. For immunofluorescence labeling of NETs, permeabilize adherent cells by transferring the prepared coverslips to a new cell culture plate, and add enough ice-cold 100 % methanol to sufficiently cover the surface (to a depth of 3.5 mm, ensuring that the cells do not dry out). Transfer to a –20 °C freezer and incubate for 10 min.
10. Aspirate with a pipette tip, and gently wash coverslips with PBS for 5 min.
11. Block nonspecific binding sites with blocking buffer (3 % BSA prepared in PBS) for 1 h at room temperature (*see* **Note 11**).
12. Aspirate with a pipette tip, and gently wash coverslips with PBS for 5 min.
13. To stain for histone H3, prepare primary histone H3 antibody (1:200 dilution) in blocking buffer supplemented with 0.3 % Triton X-100. Allow for 200 μL of diluted primary antibody per coverslip. For example, for 1 mL of diluted primary antibody, mix the following: 5 μL of histone H3 antibody, 33 μL of 10 % Triton X-100 (prepared with deionized water), and 962 μL of blocking buffer (3 % BSA prepared in PBS).
14. Transfer coverslips to a humidified chamber and incubate at 4 °C overnight, with gentle rotation (*see* **Note 12**).
15. Aspirate with a pipette tip, and gently wash coverslips with PBS-Tween for 5 min. Repeat three times.
16. Prepare goat anti-rabbit TRITC secondary antibody (1:400 dilution) in blocking buffer supplemented with 0.3 % Triton X-100. Pipette diluted antibody onto coverslips and incubate in a humidified chamber (protected from light) for 1 h at room temperature. For all subsequent steps, protect coverslips from light.
17. Aspirate with a pipette tip, and gently wash coverslips with PBS-Tween for 10 min. Repeat three times.
18. Incubate coverslips with blocking buffer (3 % BSA prepared in PBS) for 1 h at room temperature.
19. To stain for elastase, prepare primary elastase antibody (1:200 dilution) in blocking buffer supplemented with 0.3 % Triton X-100. Pipette diluted antibody onto coverslips, and incubate in a humidified chamber (protected from light) for 1 h at room temperature.
20. Aspirate with a pipette tip, and gently wash coverslips with PBS-Tween for 5 min. Repeat three times.
21. Prepare goat anti-rabbit Alexa Fluor 488 secondary antibody (1:400 dilution) in blocking buffer supplemented with 0.3 % Triton X-100. Transfer coverslips to a humidified chamber (protected from light), and incubate for 1 h at room temperature.

22. Aspirate with a pipette tip, and gently wash coverslips with PBS-Tween for 10 min. Repeat three times.
23. To counterstain for DNA, incubate coverslips with 1:12,500 dilution of DAPI (mix 0.4 μL of DAPI stock solution with 5 mL of PBS), for 5 min at room temperature.
24. Aspirate with a pipette tip, and gently wash coverslips with PBS. Repeat several times.
25. Mount coverslips using a small drop of fluorescent mounting medium per microscope slide (*see* **Note 13**). Allow the preparation to dry overnight by storing the slides in a slide holder at room temperature, protected from light. Thereafter, transfer to 4 °C for storage.
26. Visualize the DNA, histone H3, and elastase staining on a confocal or epi-fluorescence microscope, equipped with filters suitable for DAPI (excitation/emission: 358/461 nm), TRITC (excitation/emission: 550/570 nm), and Alexa Fluor 488 (excitation/emission: 495/519 nm).

4 Notes

1. Spraying with ethanol helps to reduce the amount of fur that sticks to exposed tissue. Make a shallow cut or incision with scissors so as to not pierce the intestinal tract, and continue cutting laterally to completely remove skin from the lower abdomen to hind paws.
2. Hold the tibia/femur between your thumb and forefinger, and use lint-free wipes (providing more friction) to remove the surrounding soft tissue.
3. Flush the bone marrow out, using about 2 mL of media per tibia/femur. The bones should appear transparent afterwards.
4. Percoll gradients should be slowly added to the conical tube, with the tip of disposable transfer pipette touching the tube wall. If added too quickly, the separation of bone marrow cells between the discontinuous gradients will be less effective.
5. Black microplates are used for fluorescence assays as they reduce the level of autofluorescence and therefore background signal.
6. DNase can be added to the assay 60 min before quantification of extracellular DNA. If increasing or reducing the overall incubation period (i.e., from 3 h), adjust accordingly.
7. Sytox green is added to the assay 15 min before the quantification of extracellular DNA. If increasing or reducing the overall incubation period (i.e., from 3 h), adjust the timepoint at which the DNA stain is added accordingly. Prior to measurement,

briefly shake the plate to mix the well contents. This option is available on most microplate readers.

8. 12-Well cell culture plates are used to contain the coverslips, although other holders such as petri dishes can also be used. Ensure that, if using 24-well plates, the coverslips can be removed with forceps without breaking.
9. At least 2–3 replicates of each treatment should be prepared, as the cell suspension occasionally leaks from the coverslip (onto the cell culture plate surface), and can then no longer be used.
10. NETs are very fragile and can easily be dislodged. Sufficient care should be taken during all washes. Only use a pipette, and gently aspirate.
11. Blocking buffer can be prepared the day before, and stored at 4 °C for at least a week.
12. An easy alternative to commercially available but expensive chambers is to line a shallow plastic container (large enough to hold the tissue culture plate, containing coverslips) with moistened paper towels, and replace the lid.
13. To mount the coverslips, place a small drop of fluorescent mounting medium onto the surface of a glass microscope slide. Use forceps to pick up the coverslip, and gently dry the underside on paper towel or lint-free wipes. Place one edge of the coverslip just outside of the mounting medium, and lower until it comes into contact with the medium. Release the remainder of the coverslip to allow the mounting medium to distribute evenly.

References

1. Borregaard N (2010) Neutrophils, from marrow to microbes. Immunity 33:657–670
2. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A (2004) Neutrophil extracellular traps kill bacteria. Science 303:1532–1535
3. Papayannopoulos V, Metzler K, Hakkim A, Zychlinksky A (2010) Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol 191: 677–691
4. Urban C, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, Brinkmann V, Jungblut PR, Zychlinsky A (2009) Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog 5:e1000639
5. Boxio R, Bossenmeyer-Pourie C, Steinckwich N, Dournon C, Nusse O (2004) Mouse bone marrow contains large numbers of functionally competent neutrophils. J Leukoc Biol 75:604–611
6. Chervenick PA, Boggs DR, Marsh JC, Cartwright GE, Wintrobe MM (1968) Quantitative studies of blood and bone marrow neutrophils in normal mice. Am J Physiol 215:353–360
7. Fuchs TA, Abed C, Goosmann R, Hurwitz R, Schulze I, Wahn V, Weinrauch Y, Brinkmann V, Zychlinsky A (2007) Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 176:231–241
8. Lim MB, Kuiper JW, Katchky A, Goldberg H, Glogauer M (2011) Rac2 is required for the formation of neutrophil extracellular traps. J Leukoc Biol 90:771–776