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Visualization of *ex vivo* Neutrophil Extracellular Traps by Fluorescence Microscopy

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[Abstract] Neutrophil extracellular traps (NETs) are extracellular DNAs decorated with nuclear and granular proteins such as histones, neutrophil elastase or myeloperoxidase. They exhibit fibrous mesh-like, web-like, or string-like structures. Here, we describe our protocol regarding visualization of *ex vivo* NETs released from neutrophils activated by lipopolysaccharide (LPS) using fluorescence microscopy.

Materials and Reagents

1. Whole blood from wild-type C57/BL6 mice (Japan SLC, Inc.)
2. Whole blood from human volunteers
3. LPS (*Escherichia coli*, serotype 0111:B4) (Sigma-Aldrich, catalog number: L4391)
4. Polymorphprep™ (Axis Shield PoC AS, catalog number: 1114683)
5. RPMI 1640 medium (no phenol red) (Life Technologies, catalog number: 32404-014)
6. Fetal bovine serum (Life Technologies, catalog number: 12483-020)
7. ACK (Ammonium-chloride-potassium) lysing buffer (Lonza, catalog number: 10-548E)
8. SYTOX Green (Life Technologies, Invitrogen™, catalog number: S7020)

Equipment

1. Glass Pasteur pipets (Iwaki brand, Asahi Techno Glass Corporation)
2. 96-well plates (TPP Techno Plastic Products AG)
3. CO_2 Incubator (SANYO)
4. Fluorescence microscopy (Olympus, model: IX71)

Procedure

A. Isolation of human neutrophils
   1. Venous blood (6 ml each) was obtained from healthy human volunteers.
   2. Neutrophils were isolated by density gradient centrifugation using Polymorphprep™ according to the manufacturer's instructions.
   3. EDTA anti-coagulated blood (the optimal concentration is 1.5 mg per ml of blood) was

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layered onto 6 ml Polymorphprep solution and centrifuged at 500 *x g* for 30 min.

4. The granulocyte fraction was carefully harvested using a glass Pasteur pipette.
5. Wash the pellet containing granulocytes with 2 ml phosphate buffered saline (PBS).
6. Centrifuge at 400 *x g* for 10 min, and resuspend the pellet in 1 ml PBS.
7. When present, erythrocytes were lysed with ACK lysing buffer.
8. 1 ml ACK lysing buffer was added to the pellet with residual erythrocytes.
9. Incubate at room temperature for 5 min with occasional pipetting.
10. Wash the pellet containing granulocytes with 2 ml PBS.
11. Centrifuge at 400 *x g* for 10 min.
12. Neutrophils were resuspended in 1 ml of RPMI 1640 without phenol red supplemented with 1% fetal bovine serum.
13. Final neutrophil concentration was determined by hemacytometer. Approximately 0.5~1.0 x 10^7/ml of neutrophils will be obtained.
14. Neutrophil purity was confirmed to be routinely >90%, as assessed by May-Grünwald Giemsa staining on the blood smear.
15. In brief, immerse the air-dried smear slide in 1 ml May-Grunwald solution for 1 min.
16. Add an equal part of phosphate buffer and incubate for 3 min.
17. Pour off the stain and wash the slide with tap water.
18. Immerse in the 8% Giemsa solution for 20 min.
19. Wash the slide with tap water and air-dry.

B. Isolation of murine leukocytes

1. Heparinized blood was withdrawn from the inferior vena cava of anesthetized wild-type C57/BL6 mice.
2. In brief, open the abdomen and identify the inferior vena cava between the kidneys.
3. Use a 25 gauge needle and a 1 ml syringe filled with 50 µl heparin for the prevention of blood coagulation.
4. Insert the needle into the vein and draw blood slowly until the vein collapses.
5. Approximately 500 µl blood will be obtained.
6. ACK lysing buffer was used to lyse erythrocytes.
7. 5 ml ACK lysing buffer was added to 500 µl of murine whole blood.
8. Incubate at room temperature for 5 min with occasional gentle shaking.
9. Centrifuge at 400 *x g* for 10 min.
10. Discard the supernatant containing lysed erythrocytes carefully.
11. (If necessary, repeat steps B6-10.)
12. Wash the pellet with 2 ml PBS.
13. Centrifuge at 400 *x g* for 10 min, and resuspend the pellet in the 1 ml of the above mentioned medium (step A12).
14. After ACK treatment, the blood cells that remained included white blood cells (leukocytes) and platelets.

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15. Final leukocyte concentration was determined by hemacytometer. Approximately 1.0~2.0 x 10^6/ml of neutrophils will be obtained.

C. Neutrophil activation by LPS

1. Human neutrophils or murine leukocytes obtained from wild-type C57/BL6 mice were suspended in the above-mentioned medium (step A12).
2. They were seeded to the 96-well plate (first plate) at a density of 1 x 10^4 cells per well (100 µl).
3. They were stimulated with LPS at indicated concentrations (2, 20, 100, and 200 µg/ml). The plates were placed in a humidified incubator at 37 °C with CO_2 (5%) for 6 h.

D. Visualization of *ex vivo* NETs by fluorescence microscopy

1. Cell fixation is not performed for *ex vivo* live cell imaging.
2. A cell-impermeable DNA binding dye, SYTOX Green (excitation 504/emission 523) is a 5 mM solution.
3. To make a 10 µM SYTOX Green solution, mix 2 µl of a 5mM SYTOX Green solution with 1,000 µl PBS.
4. 100 µl of a 10 µM SYTOX Green solution was added to each well (containing 100 µl).
5. SYTOX Green (final concentration is 5 µM) can only get into the cell when the cell membrane was compromised. Therefore, SYTOX Green should stain both extracellular DNAs and nuclei of non-viable neutrophils.
6. After adding SYTOX Green, both round and fibrous structures can be observed by fluorescence microscopy. SYTOX Green stained round structures are considered as nuclei of non-viable neutrophils. On the other hand, SYTOX Green stained fibrous structures are considered as extracellular DNAs which are almost identical to NETs in this protocol.

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Representative data

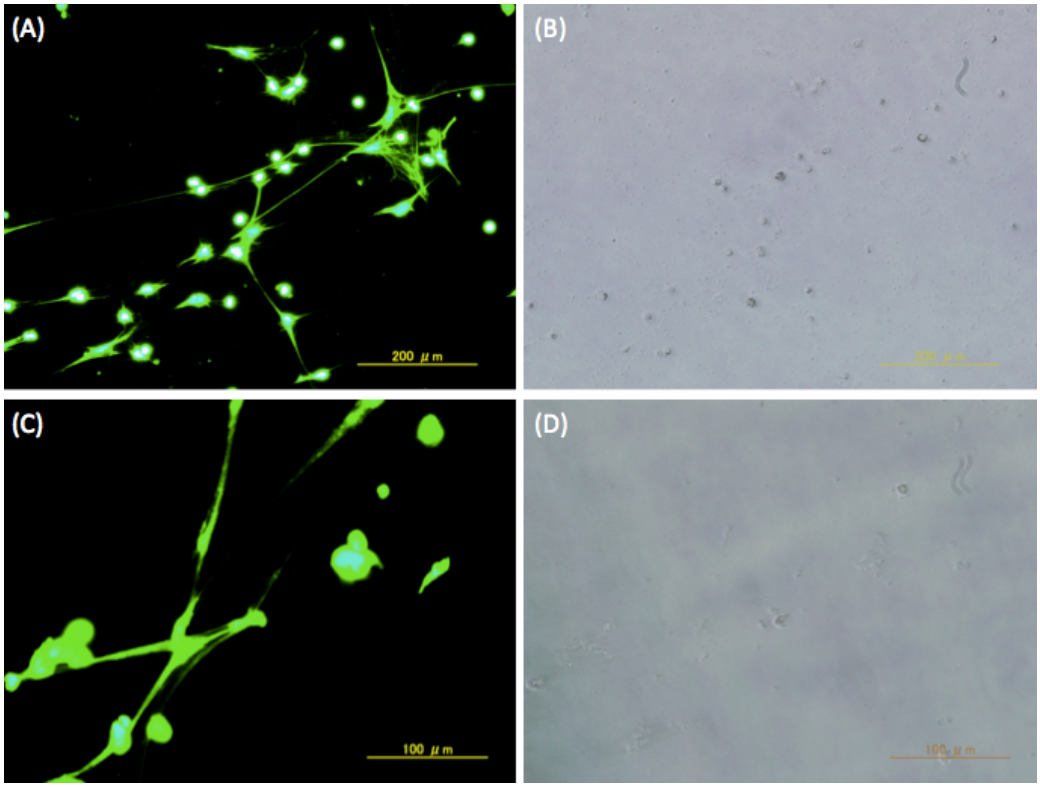

Figure 1. Images (A, C) under fluorescence microscopy and images (B, D) under light microscopy. SYTOX Green stained NETs were released from *ex vivo* murine leukocytes (neutrophils) activated by LPS (A). The image of its counterpart under light microscopy (B). SYTOX Green stained NETs were released from *ex vivo* human neutrophils activated by LPS (C). The image of its counterpart under light microscopy (D).

Notes

1. LPS (*Escherichia coli*, serotype 0111:B4) is used ad NETs inducer. Among the various serotypes (such as B5, B12, and so on), B4 would be the best with regard to the incubation time and the results obtained.
2. To distinguish NETs from non-viable neutrophils, a staining with anti- histone, anti-neutrophil elastase or anti-myeloperoxidase antibody would be useful.

Acknowledgments

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