Chapter 31

Neutrophil Extracellular Traps

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Abstract

Neutrophil extracellular traps (NETs) are made of a network of extracellular strings of DNA that bind pathogenic microbes. Histones and several neutrophil granule proteins associated with the DNA framework damage entrapped microorganisms. Reactive oxygen species generated by the neutrophil NADPH oxidase have been shown to be essential to mediate NET release by several stimuli including numerous pathogenic bacteria. Although several methods have been used in the literature to detect NETs in vitro and in vivo, a consensus is urgently needed on the field to standardize the best NET-specific assays. In this chapter, two methods are described in details that can be used to detect NETs and to distinguish them from other mechanisms of neutrophil cell death. While NET-specific, these assays are also relatively simple and straightforward enabling their potential use by a wide audience.

Key words NETs, Neutrophil, NADPH oxidase, MPO-DNA complex, NE-DNA complex, Citrullinated histone, Peptidylarginine deiminase 4, Nuclear decondensation

1 Introduction

Neutrophil extracellular traps (NETs) represent an antimicrobial mechanism of neutrophilic granulocytes (Fig. 1) [1]. The core structure of NETs is extracellular DNA associated with antimicrobial proteins originating from neutrophil granules and nucleus [1]. The major form of NET formation, called suicidal NETosis, leads to the death of neutrophils and is characterized by subsequent morphological changes: disintegration of nuclear membrane, chromatin decondensation, disappearance of plasma membrane, and finally the spill of DNA-based NETs into the extracellular space [2]. On the contrary to this mechanism leading to neutrophil death, vital NETosis has also been described during which process neutrophils remain live and release only parts of their nuclear or mitochondrial DNA [3–6]. NETs have been shown to entrap a wide variety of microbes and provide a crucial innate immune mechanism. Excessive NET release has, however, been associated with numerous diseases [7, 8]. Although NETs have been discovered 14 years ago, specific signaling events leading to NET release

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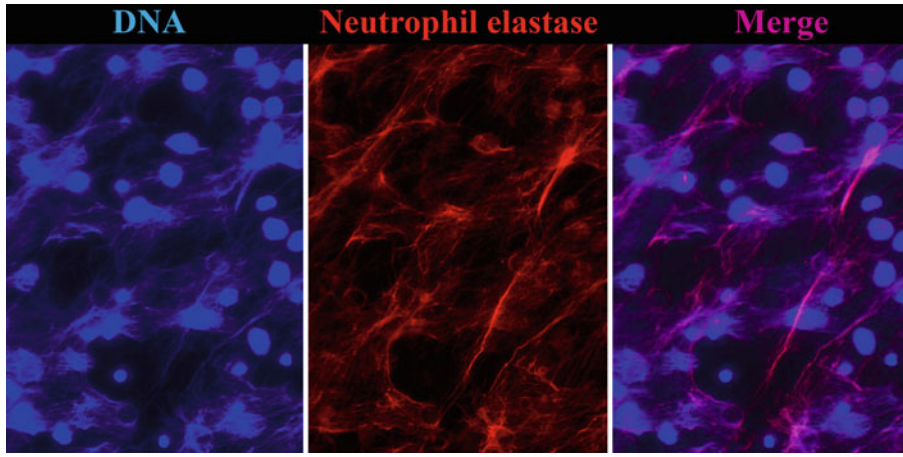

Fig. 1 Human neutrophils were stimulated with 100 nM PMA for 5 h, and immunofluorescence of formed NETs is shown by co-localization of neutrophil elastase (red) with extracellular DNA (blue) (merged image is purple)

are still largely unclear. The phagocytic NADPH oxidase has been early implicated in the process [2]. Pretreatment of neutrophils stimulated with phorbol myristate acetate (PMA) or *Staphylococcus aureus* with a NADPH oxidase inhibitor, diphenylene iodonium (DPI), prevented NET release [2]. Addition of an extracellular source of reactive oxygen species (ROS), glucose oxidase and glucose, circumvented the need for NADPH oxidase-produced ROS and triggered DPI-independent NET formation in human neutrophils [2]. The best evidence for the need of the NADPH oxidase for NET extrusion was provided by experiments done on human neutrophils derived from chronic granulomatous disease (CGD) patients [2]. CGD patients have mutations in one of the subunits of the NADPH oxidase enzyme complex leading to absent or significantly diminished respiratory burst of neutrophils [9]. CGD neutrophils do not expel NETs in response to PMA or *S. aureus*, but do release NETs when an NADPH oxidase-independent ROS source is used [2]. Restoration of NET formation in an X-linked CGD patient by $gp91^{phox}$-based gene therapy leads to improved clearance of *Aspergillus nidulans*, emphasizing the human clinical importance of NADPH oxidase-mediated NETs in antifungal defense [10]. Although originally the NADPH oxidase had been considered indispensable for the induction of NETs, accumulating evidence later showed that NETs can be triggered in an NADPH oxidase-independent fashion, as well. Broadly speaking, suicidal NET release stimulated by certain bacteria and fungi requires the NADPH oxidase, while extrusion of NETs induced by other bacteria, parasites, and most inflammatory microcrystals does not involve ROS generated by the oxidase [1, 11–14]. Since the NADPH oxidase is also required for intracellular, phagocytic killing

of several microbes by neutrophils, the NADPH oxidase cannot be considered a NET-specific protein.

Citrullination of histones by the enzyme peptidylarginine deaminase 4 (PAD4) has been shown to be involved in NET release [15, 16]. PAD4 is highly expressed in neutrophils, translocates to the nucleus upon rise in cytosolic calcium concentrations, and mediates the conversion of arginine residues to citrulline in target proteins, primarily in histones, that was proposed to lead to chromatin decondensation [15, 16]. Although PAD4 had been originally suggested as a specific and universal mediator of NET formation, recent findings suggest that NETs can be released in a PAD4-independent manner, as well. Irrespective of the molecular mechanism of NET formation, the following features are specific for NETs and provide the bases for NET-specific detection methods.

The characteristic morphological changes in the shape of neutrophil nucleus during NET formation can be followed, quantified, and distinguished from other neutrophil death mechanisms by microplate-based fluorescence microscopy [17–19]. Although such methods are NET-specific and can scan large amounts of neutrophils in vitro, they require sophisticated instrumentation and analytical methods that are more suitable for laboratories with neutrophil expertise than with interest in other fields of immunology. Although nuclear swelling at the early stage of NET formation can also be detected by image-based flow cytometry, this method is not capable of recording later stages of NET formation when cellular morphology is not intact anymore [20, 21]. Citrullinated histones can be detected by several methods including immunofluorescence, Western blotting and ELISA. Although citrullinated histones have originally been used as NET markers, they are only specific to PAD4-mediated NET formation and do not inform about PAD4-independent NET extrusion. The most specific NET markers are complexes of extracellular DNA and neutrophil granule proteins which can be detected by their co-localization in the weblike structures of NETs using immunofluorescence (Fig. 1). The same protein-DNA complexes can be also detected as NET remnants in biological biospecimen using hybrid ELISA assays that use a capture antibody specific for the protein component and a dsDNA detection antibody linked to horseradish peroxidase (Fig. 2) [7, 22, 23]. Two primary granule proteins have been mainly used in these assays: myeloperoxidase (MPO) and neutrophil elastase (NE) [7, 22].

In this chapter, these two latter assays (NET detection by immunofluorescence and ELISA) are described in detail because they provide NET specificity, are relatively easy, and can be performed in most laboratories.

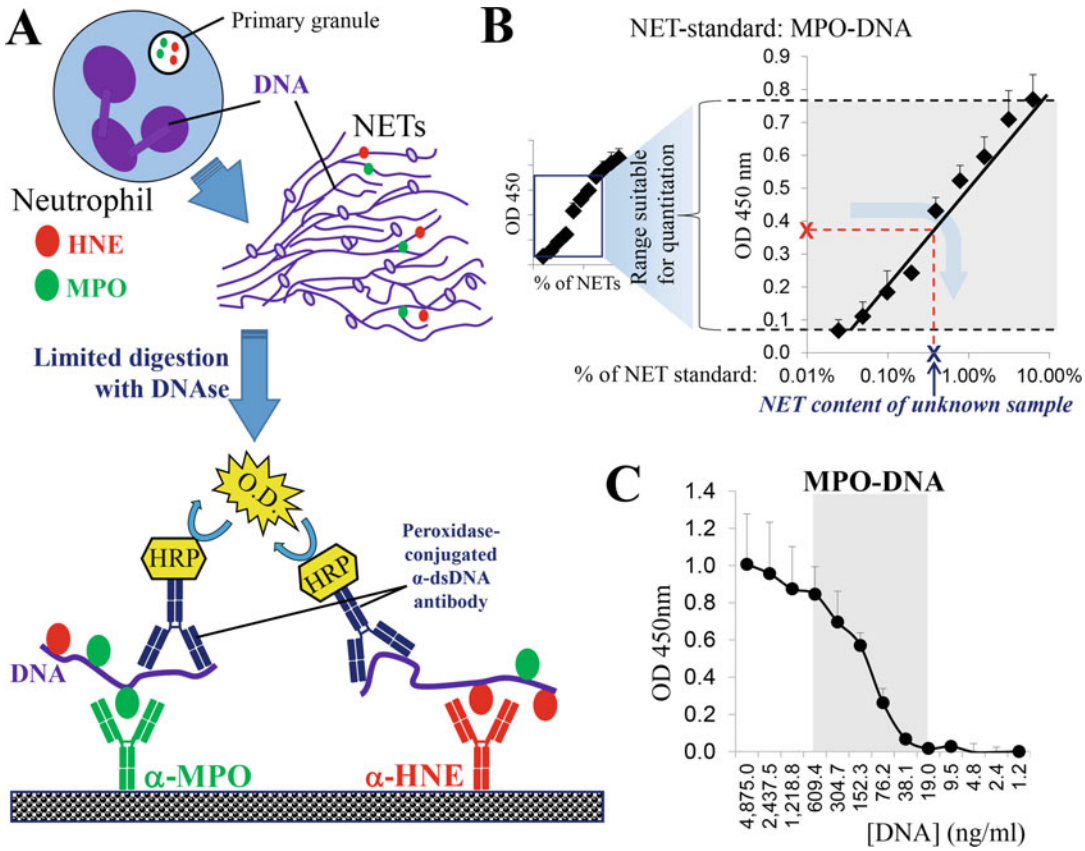

Fig. 2 NET-specific ELISA assays: principles and semiquantitative standard. (**A**) Scheme explaining how the MPO-DNA and HNE-DNA ELISA assays work. (**B**) Serial dilutions (twofold) of the NET standard were prepared and subjected to MPO-DNA ELISA. Measured OD values are plotted against % of NET-standard content. Red "X" indicates a measured OD value of an unknown sample. Gray arrow indicates how the "NET concentration" (blue "X") of the unknown sample is to be determined using the central range of the fitted standard curve. (**C**) DNA concentrations in serially diluted NET-standard samples were determined and plotted against the measured OD values of the MPO-DNA or HNE-DNA ELISA assays. Gray areas indicate the dynamic ranges of the assays

2 Materials

2.1 NET Detection by Immunofluorescence

1. Coverslips.
2. Hank's balanced salt solution (HBSS, 1×).
3. 5 mM D-glucose in HBSS.
4. Assay medium (HBSS + 5 mM glucose + 10 mM HEPES).
5. 4% paraformaldehyde (PFA, PBS).
6. 24-well tissue culture plates.
7. Phosphate-buffered saline (PBS, 1×).

8. Blocking solution (5% bovine serum albumin +5% normal donkey serum + 0.05% Triton X-100).
9. Tween 20.
10. FITC-conjugated antihuman myeloperoxidase (MPO) antibody (mouse, 1:200, Dako, Clone MPO-7, in PBS containing 1% BSA + 1% normal donkey serum + 0.05% Triton X-100).
11. Anti-histone H4 (citrulline 3) antibody (rabbit, 1:500, Millipore in PBS containing 1% BSA + 1% normal donkey serum + 0.05% Triton X-100).
12. ALEXA fluorochrome, anti-rabbit, 594 nm (1:2000 dilution, Invitrogen, donkey anti-rabbit IgG (H + L), in PBS containing 1% BSA + 1% normal donkey serum + 0.05% Triton X-100).
13. Antihuman neutrophil elastase antibody (rabbit, Calbiochem, 1:2000 dilution, in PBS containing 1% BSA + 1% normal donkey serum + 0.05% Triton X-100).
14. 4′,6-Diamidino-2-phenylindole (DAPI) is a fluorescent DNA stain. Prepare a stock solution of 5 mg/mL DAPI dissolved in DMSO, and use it in a 20,000-fold diluted concentration in PBS to stain neutrophil nuclear DNA and NET-DNA.
15. ProLong Antifade Kit (Molecular Probes, Grand Island, NY).
16. Fluorescence microscope (Zeiss AxioCam HRM microscope with Axioplan2 imaging software).

2.2 NET Detection by ELISA

1. Antihuman neutrophil elastase antibody (rabbit, Calbiochem, 1:2000 dilution, PBS).
2. Anti-myeloperoxidase antibody (Rabbit, Millipore, 1:2000 dilution, PBS).
3. 1 μg/mL DNase-1 (Roche, final concentration used for digestion).
4. 20 mM EGTA/PBS.
5. 0.05% (v/v) PBS-Tween 20.
6. Horseradish peroxidase-labelled anti-dsDNA antibody in the cell death detection kit (Roche, 1:500 dilution, PBS).
7. Eon microplate spectrophotometer (BioTek, Gen5 software) or equivalent.
8. 0.2% SYTOX Orange membrane-impermeable DNA-binding dye (Life Technologies, cat#: S11368, 0.2% final concentration/volume).
9. 10 mM HEPES and 5 mM glucose in HBSS (Corning).
10. Varioskan Flash Ver.2.4.3 combined microplate fluorimeter (Thermo Fisher Scientific) or equivalent.
11. 600 nM PMA (Sigma, 6× stock) in HBSS with HEPES and glucose.

12. 1 μM ionomycin.
13. High-binding capacity ELISA plates (Greiner Bio-One).

3 Methods

3.1 NET Detection by Immunofluorescence

1. Place microscope coverslips in wells of a 24-well plate (*see* **Note 1**). Neutrophils will be seeded, stimulated, and exposed to NET-inducing stimuli on the coverslips in the 24-well plate. Once NETs formed and are fixed, coverslips will be taken out from the 24-well plate for further processing.
2. Seed neutrophils at a concentration of 4,000,000 cells/mL in 250 μL assay medium on coverslips. The coverslips must be covered entirely by the medium; otherwise neutrophil density will not be equal throughout the coverslip (*see* **Note 1**).
3. Incubate plate with coverslips for 15 min in cell culture incubator at 37 °C to allow neutrophil adherence. Double check in light microscope that at the end of the incubation time, neutrophils are indeed attached to coverslips. If not, incubate them for another 15 min.
4. Add NET-inducing stimulants to neutrophils in a volume of 50 μL containing stimulants or their solvents at concentrations six times higher than the final one. Fifty microliters of the stimulant's volume will be diluted to a final volume of 300 μL per wells, hence the need to apply higher doses ($300/50 = 6$).
5. Use 100 nM PMA or 1 μM ionomycin, as positive controls of NET release. PMA is a better choice for studies on NADPH oxidase-mediated NET formation, while ionomycin is more appropriate for works focusing on NET release fueled by calcium and PAD4 activation.
6. For neutrophils without stimulation, add 50 μL assay medium or the solvent of the stimulators per well. Make sure all samples have a final volume of 300 μL.
7. Incubate neutrophils for 4–6 h at 37 °C in cell culture incubator to allow NET formation to take place. When the incubation time is over, handle samples as gently as possible, since formed NETs are very fragile and require special care. Coverslips will next undergo a series of sequential washing steps (*see* **Note 2**).
8. Fix NETs with 4% PFA and incubate for 10 min at room temperature. PFA is the best solution to fix NETs as methanol or ethanol can alter NET structures and even enhance NET release.
9. Wash samples twice with 300 μL sterile PBS for 10 min each time at room temperature.

10. Permeabilize and block samples using 300 μL blocking solution (*see* **Note 3**). Incubate for 30 min at room temperature.
11. Add primary (antihuman MPO-FITC or antihuman neutrophil elastase) antibodies into samples and incubate overnight at 4 °C in dark. To detect PAD4-dependent NET release, anti-citrullinated histone H3 antibody can also be used.
12. While the MPO staining does not require a secondary antibody due to the direct labeling of the primary MPO antibody by FITC, the neutrophil elastase or citrullinated histone H3 staining does need a secondary antibody. Wash coverslips three times in PBS for 5 min at room temperature, and incubate samples with fluorochrome-labelled secondary antibody in PBS for 1 h at room temperature in the dark.
13. Aspirate antibody solutions, and incubate cells in 20,0000-fold diluted DAPI (PBS) for 2 min (*see* **Note 4**).
14. Wash samples two more times in PBS for 5 min each time at room temperature to remove unbound secondary antibodies and DNA stain.
15. Let the coverslips dry on a paper towel, and drop 10 μL mounting medium supplemented with anti-fade solution per coverslip in the middle of the sample (*see* **Notes 5** and **6**). Carefully drop the coverslip on clean, degreased microscope slide with the sample and mounting medium facing down, toward the slide. Wait until the viscous mounting medium slowly spreads and covers the entire sample. Again, NETs are very fragile so be gentle (*see* **Note 7**).
16. Wait for 30 min to let the excess fluid evaporate. Seal the edges of the coverslip with clean nail polish or commercially available sealing liquid to prevent further damage to the sample. The immunofluorescence staining is ready. Store samples at 4 °C in dark to preserve them for later analysis.

3.2 NET Detection by ELISA

1. Apply capture antibodies (anti-MPO or anti-NE, 1:2000 dilution in sterile PBS) to 96-well high-binding capacity ELISA microplates.
2. Incubate ELISA plates overnight at 4 °C to allow binding of capture antibodies (*see* **Note 8**).
3. Apply three repeated washes with PBS-Tween 20 the next day.
4. Block ELISA plates with 5% BSA (200 μL/well).
5. Incubate plates for 2 h at room temperature.
6. Apply again repeated washes with PBS-Tween 20 (200 μL/well).

7. The ELISA plates are now ready to accept the experimental samples. Apply NETs collected from neutrophils in vitro or diluted biological fluids to ELISA plates.
8. Incubate ELISA plates with samples overnight at 4 °C to allow the protein component of NETs to bind to capture antibodies.
9. Wash plates again three times with PBS-Tween20 (200 μL/well) to remove unbound NETs and other components of the samples.
10. Apply detection antibody to ELISA plates (HRP-labelled anti-dsDNA antibody, 1:500, mouse, 50 μL/well), and incubate for 1 h in dark at room temperature.
11. Repeat washes four times with PBS-Tween 20 (200 μL/well).
12. Add TMB peroxidase substrate to each well for 30 min (50 μL/well).
13. Stop reaction by adding 1 M HCl to each well (50 μL/well).
14. Read absorbance at 450 nm using a microplate photometer.
15. Analyze and present results either as absorbance values (relative quantitation) or as percentage of the NET standard (semi-absolute quantitation; *see* **Note 9**).
16. The NET standard consists of pooled supernatants of DNase-digested NETs released from PMA-stimulated neutrophils.
17. To prepare DNAse-digested NETs for the NET standard from each neutrophil donor, stimulate human neutrophils in vitro with 100 nM PMA for 5–6 h, apply 1 U/mL DNAse I to the supernatants for 15 min, stop the action of DNAse by adding 2.5 mM PBS-EGTA, centrifuge the samples (10,000 × *g*. 4 min, 4 °C), and collect supernatants as DNAseI-digested NETs (*see* **Notes 10–12**).

4 Notes

1. Before each step (seeding neutrophils or start of the incubation time to form NETs), make sure that coverslips are at the bottom of the wells on the 24-well microplate as they can easily come up to the surface that will result in unanalyzable samples. If coverslips float on the surface of the 300 μL assay medium, use clean forceps to carefully push the coverslips back to the bottom of the well. Pay attention not to scrape off attached neutrophils as the forceps might remove cells from the coverslip or introduce artifacts in your immunostaining images.
2. Although washing the coverslips following fixation can be done in the wells of the 24-well plate, it requires the researcher to be extra careful as NETs can be easily washed off of the coverslips

by too strong pipetting. Better results can be achieved when coverslips are processed on top of 300 μL PBS droplets using forceps. This way of processing the samples has been described by *Brinkmann* et al. [24] Briefly, stretch a parafilm sheet on top of a 15 mL conical tube rack, and create indentations by pressing your thumb into the parafilm where holes are located [24]. Pipette 300 μL volumes of the solutions the coverslips will be exposed to into the parafilm dents according to the protocol (PBS, PBS with 4% PFA, PBS + Triton X-100). Coverslips will be washed by carefully transferring them between these droplets and dipping them into the solutions in an upside-down position (NETs facing down, into the liquid) [24].

3. Make sure to vortex the normal donkey serum well before applying it to the samples. Well-mixed serum will result in nice and uniform blocking of the NETs.
4. Be accurate with the time of DAPI staining as too long exposure will overstain DNA in your samples prohibiting recording of sharp, publishable images.
5. When drying NET-containing samples on slide before addition of the mounting medium, make sure not to let them dry completely. The best way to do this is one coverslip at a time. You might want to add the mounting medium to the slide before you take the coverslip off of the PBS. Take each coverslip off of PBS, carefully drag its edge on a paper towel (to get rid of excess liquid), and flip coverslips. Make sure you flip them, or you'll contaminate your slides by facing the neutrophil/NET side down on the paper towel.
6. Thaw both of the mounting medium and the anti-fade solutions way ahead of the time of their use to allow enough time for their warm-up. Keep in mind that the mounting medium is extremely viscous.
7. Before turning the coverslips onto the mounting medium, wet a KimTech wipe with ethanol, and gently wipe off the opposite side of the coverslip (facing down on paper towel). This will help you getting better pictures, since cells or debris adhered to the wrong side of the coverslip will appear blurry (in a different plane) on the microscope and give you lesser-quality images. If you want to take pictures that same day, then you won't be able to wipe with ethanol once the coverslips are mounted as they will not be fully dried. However, if you take pictures the next day, then you can wipe the tops of the mounted coverslips with ethanol before taking pictures of the slides.
8. For each incubation step during the ELISA, make sure to use a plate cover to prevent evaporation of the samples.

9. To analyze the data obtained using the NET ELISA assays, calculate the amount of MPO-DNA or HNE-DNA complexes present compared to the "NET standard" (Fig. 2). First, subtract the background absorbance value of the assay medium alone from all your unknown and calibration samples. Correlate the optical density values of the standard samples with their relative concentrations NETs, and establish a linear trend line using the unsaturated range of the standard. The received equation provides the means to convert absorption results into quantitative amounts of NETs. Calculate mean of replicates, and show final results as "MPO-DNA or HNE-DNA complexes (% of standard)."
10. When performing DNAse I digestion of NETs to prepare them for the ELISA assay, make sure that the content of each well is mixed thoroughly to provide an easy access of the DNAse I to expelled NETs.
11. Similarly, mix the contents of each well thoroughly when stopping DNAse I by adding EGTA.
12. NETs generated in vitro in human neutrophils have to be diluted in sterile PBS several-fold to be in the measurement range of the ELISA assays. The degree of dilutions has to be optimized, but diluting the DNAse I-digested NETs 100-fold is a good start in our experience.

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