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Visualization of Extracellular Trap by Light Microscopy

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Abstract

Aim: Neutrophil extracellular traps (N(ET)) are structures made of DNA studded with antimicrobial peptides, that are ejected out of many types of white blood cells including neutrophils. They are released as a defense strategy to trap/kill pathogens. NET-DNA is usually observed after staining with fluorescent stains specific to DNA or NET-proteins. As this method is costly and requires expertise, this study focused on visualizing NET using light microscopy. **Method:** Neutrophils were isolated by density gradient centrifugation. The cells were adhered on a gelatin-coated coverslip and stimulated using *S. aureus* cell-free culture supernatant for 10 or 30 minutes for N(ET) release. The slides were stained with a combination of Hoechst and Giemsa stains and viewed under the light microscope. **Result:** NET-DNA was observed more clearly and prominently by this double staining method using a light microscope. **Conclusion:** This method helped in seeing NET-DNA with more resolution under the light microscope. Since the dyes used for staining are cost effective, this method can be employed for preliminary NET research in labs with limited resources.

Keywords
Neutrophils; N(ET)osis; Microscopy; Staining; Hoechst; Giemsa

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1. INTRODUCTION

Extracellular trap (ET) formation or N(ET)osis is a defense strategy displayed by neutrophils, macrophages, eosinophils, and mast cells. This phenomenon is characterized by the release of chromosomal or mitochondrial DNA [1, 2, 3, 4] studded with antimicrobial molecules in the form of a web. Pathogens are trapped in this N(ET) and are eliminated by N(ET)-associated antimicrobials. Depending on the size and type of stimuli encountered by the cell, variants of N(ET)osis have been described. In classical N(ET) release, DNA is ejected out upon exposure to stimulants like phorbol myristate acetate and microbial/parasitic pathogens [1, 5]. It is characterized by chromosomal unwinding and blending of cytoplasmic and nuclear contents. This is followed by the release of DNA from the cell in conjunction with the loss of integrity of cell membrane and cell viability [1, 2]. Hence, this process was given the name suicidal N(ET)osis. On the contrary, cells may stay alive while casting N(ET)s and this type of N(ET) release was given the name vital N(ET)osis. This is formed when they are encountered with stimulants like lipopolysaccharide and Gram-positive bacteria via the involvement of Toll-like receptors and complements [4, 6]. Pilsczek

et al. (2010) reported another variant of vital N(ET) release in response to *Staphylococcus aureus* [7]. Here, nuclear DNA packed into vesicles of different sizes may bud off from neutrophils, that may release their content similar to conventional N(ET) release. Curiosity in understanding these types of N(ET), their antimicrobial property against various bacteria, fungi, virus and protozoan, N(ET)-associated pathways and the possibility of developing therapeutic strategies to overcome the N(ET)-associated complications seen in many inflammatory diseases, resulted in the involvement of many research groups in N(ET)osis-related research.

Researchers rely on microscopic, flow cytometric, fluorimetric and immunofluorescent based approaches to visualize or characterize N(ET)osis. Among microscopic methods, fluorescent microscopy is the most opted method in visualizing N(ET)s. It requires fluorescent tagged antibodies against N(ET) proteins [1, 2, 8] or fluorescent DNA dyes like Sytox green, Sytox orange and MitoSOX [2, 4, 6]. Hoechst 33342/33258, Propidium iodide and DAPI stains are also used to stain N(ET)-DNA [2, 9, 10]. Fluorescent microscopy needs sophisticated instrumentation and with fluorescent dyes, imaging should be carried out immediately to avoid fluorescence fading. Fluorescent tagged antibodies and stains used in fluorescent microscopy are very expensive and possess only a very short half-life. Considering these limitations, introducing a light microscopic method with improved staining techniques can make N(ET) research more accessible, particularly to labs having limited resources. Light microscopic techniques can make N(ET) research cost effective and this would be greatly useful for preliminary studies. Therefore, we attempted visualization of N(ET) with a modified double staining method using Hoechst 33258 and Giemsa stain.

2. MATERIALS AND METHODS

2.1 Isolation of neutrophils

Neutrophils were isolated from blood of healthy human volunteers after obtaining informed consent. Experiments were conducted in accordance with the declaration of Helsinki with the approval of the Institutional Human Ethics Committee (IEC/IRB No: 05 / IHEC20082015). Neutrophils were freshly isolated by dextran sedimentation followed by Ficoll hypaque density gradient centrifugation [11]**.** The isolated cells were checked for viability and purity by trypan blue dye exclusion method and Giemsa staining respectively.

2.2 Preparation of bacterial filtrate

S. aureus (MTCC 1430) strains were inoculated in sterile nutrient broth and incubated for 6-12 hours at 37°C. After incubation, the culture was diluted with an equal amount of PBS and centrifuged at 12000 rpm for 30 minutes to prepare *S. aureus* cell-free culture supernatant (SCS). SCS was collected in another sterile tube without disturbing the pellet and was used for stimulating N(ET) release.

2.3 Stimulation of extracellular trap

2 X 10^6 cells were added to a coverslip coated with gelatin and incubated at 37°C for 30 minutes. Non-adherent cells were removed by washing with PBS. Cells adhered on the coverslip were stimulated with SCS for 10 or 30 minutes at 37^0C. After 10 or 30 minutes, cells were washed with PBS and fixed using 4% formaldehyde for 10 minutes. Coverslips were washed with PBS and allowed to dry.

2.4 Staining of extracellular trap for light and fluorescent microscopy

Cells were stained using Hoechst 33258 for 15 minutes in presence of sunlight. After washing the coverslips, Giemsa stain was added and kept for 1 hour. The coverslips were washed, dried and observed under the light microscope at 1000 X magnification. Cell stained with Giemsa alone was used as the control.

For fluorescent microscopy, after incubation with Hoechst, coverslips were washed with PBS and fixed with formaldehyde. Coverslips were then stained with Hoechst and observed under the fluorescent microscope at 400 X magnification.

Notes

1. Conduct all experiments under sterile condition.
2. The working solution of Hoechst (1:50000 dilution) should be protected from light.
3. The working solution of Giemsa (1:1 dilution) should be freshly prepared.
4. Gelatin (0.1 %) coated slides should be freshly prepared.
5. Propidium iodide can be used in the place of Hoechst for light microscopy. However, when it was replaced by Sytox green, the clarity of the image was found to be reduced.

3. RESULT AND DISCUSSION:

Combination of multiple stains is generally used to differentiate between cell structures or components of the cell. Application of fluorescent DNA-specific dyes counter-stained with Giemsa has been described for the differential staining of sister chromatids [12]. Since N(ET)s are also made up of unwound double stranded DNA; we checked whether this staining is suitable to observe N(ET) in

light microscopy. This method is almost similar to that described by Goto *et al*. (1975) [12] and involves primary staining with Hoechst, followed by light exposure and counter-staining with Giemsa.

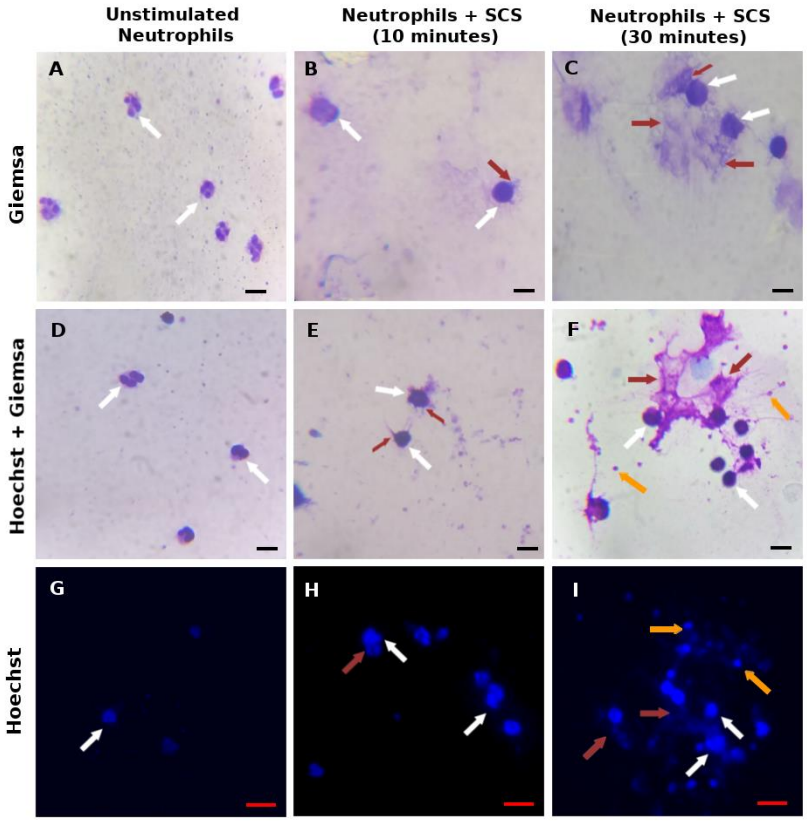

Fig 1. Light microscopy (1000 X) and fluorescent microscopy (400 X) of neutrophils stimulated with SCS for 10 and 30 minutes. Unstimulated neutrophils with multi-lobed nucleus (A, D, G). Neutrophils (white arrow) stimulated with SCS for 10 minutes under light microscopy (B, E). Fibre like structure releasing out of the cell (red arrow) were clearly visible in Hoechst+Giemsa stained cells compared to the cells stained alone with Giemsa stain. Neutrophils started to release N(ET) upon stimulation with SCS for 10 minutes (B, E, H). Extensive N(ET) release in neutrophils stimulated for 30 minutes. An extended fibre (red arrow) and vesicular (orange arrow) N(ET) could be seen in Hoechst+Giemsa staining (F) With more clarity when compared to cells stained with Giemsa alone (C). Increase in fluorescence around the cells indicating N(ET) released as fibre (red arrow) and as vesicles (orange arrow) (I)

By this staining, the multi-lobed nucleus of neutrophils could be clearly seen (Fig. 1 A, D, G) in unstimulated cells. Cells stimulated with *S. aureus* were reported to induce N(ET)osis in neutrophils within 5-40 minutes [13] with the aid of staphylococcal proteins [7,13]. With this stimulation, neutrophils were shown to eject out both classical and vesicular type of N(ET) release [7]. In this study also, neutrophils released N(ET)s rapidly upon 10 minutes stimulation with SCS. In cells stimulated with SCS, after 10 minutes, the multi-lobed structure of neutrophil nucleus was found to get disorganized. These cells lost the integrity of their nuclear membrane and nuclear material was found to occupy the entire cytoplasm (Fig. 1 B, E, and H). Disorganized nuclear material observed by this staining could be due to the chromosomal unwinding and nuclear-cytoplasmic mixing up, that are typically observed during N(ET)osis [2].

With Hoechst+Giemsa staining, released N(ET)s could be seen as thin fibres ejecting out of the cell (Fig. 1 E). The fibrous nature of N(ET) was quite visible in light microscopy. However, with fluorescent microscopy, N(ET) release was observed only as bright fluorescence coming out of cells (Fig. 1 H). In short, N(ET) release was more clearly observed in Hoechst+Giemsa stain (Fig. 1 E) rather than in Giemsa-alone stained cells (Fig. 1 B). Extended N(ET) release was observed, as the stimulation was increased from 10 minutes to 30 minutes. More cells

released N(ET), covering a vast area and instead of beaded strings of N(ET), more extended N(ET) structures were observed. At this point, we also observed N(ET)-fibres of adjacent cells getting interlinked to form mesh like structures (Fig. 1 F). In cells stained with Giemsa alone (Fig. 1 C), these fibres could be seen only as hazy structures dispersed around the cells. However, in cells stained with Hoechst+Giemsa, the purple colored fibres could be seen as a prominent structure with more clarity (Fig. 1 F). The vesicular form of N(ET) release could also be observed in all SCS-stimulated neutrophils.

Hoechst 33258 is a derivative of bi-benzimidazole and is cell-permeable. Compared to other fluorescent dyes, Hoechst binds to the base pair on DNA rather than intercalating into the DNA. The increase in fluorescence with increase in AT base pair shows its affinity towards this base pair compared to GC rich region [14] and binding of Hoechst 33258 on DNA showed a very little alteration in the helical structure [15]. Hoechst undergoes photo-bleaching upon light-exposure and Hoechst shows strong affinity to DNA even after photo-bleaching [16]. As Giemsa can bind only to areas that have not been stained by Hoechst, this masking of Hoechst provides the required contrast to the images, thereby enhancing its clarity. The photo-bleaching of Hoechst using sunlight had already been reported to increase the clarity of the banding pattern in sister chromatids during staining by Giemsa, subsequent to Hoechst staining [12]. Giemsa stain is commonly used by cytogeneticists in staining chromatids. Unlike Hoechst, Giemsa stain intercalates with the DNA base pair AT in the DNA. The stain is made up of cationic azure B and anionic eosin Y dyes. The azure B molecules in Giemsa stain are small in size and penetrate the cell membrane easily. It binds to DNA by hydrophobic and van der Waals interactions. Azure B also form a hydrogen bond with dAMP which helps in stabilizing the binding [17, 18]. Azure B stains the nucleus blue. Then, the larger eosine molecule binds with azure by hydrophobic interaction. The eosin-azure complex then gets precipitated out staining nucleus purple [18].

Previous studies have demonstrated the staining of N(ET) using Giemsa alone [19, 20] or Hoechst alone [9, 21]. However, a combination of these stains has not been used in staining N(ET) DNA till date. However, Hoechst 33258 was reported to be employed in other co-staining techniques [22]. Dasari *et al*. (2010) reported the use of Hoechst in conjugation with an infrared probe for the detection of extracellular DNA from the necrotic cell [23]. In another study, Crissman *et al*. (1990) had reported the use of Hoechst in combination with DiOC5 (3) (Iodide [3,3-Dipentyloxacarbocyanine Iodide]) for increasing the resolution of DNA staining in live cells [24]. Moreover, during the early 1970s, the combination of Hoechst with Giemsa to study the banding patterns in sister chromatids was developed [12,14].

The major advantage of this double staining method is the visualization of N(ET) using less-expensive stains, even without the requirement of a fluorescent microscope. Also, this method improved the clarity of the images and increased the storage period of these slides to one month by avoiding quick fading. However, this staining method does not establish that the fibres you see is N(ET) and for that, confirmation of N(ET)-components has to be established by other means. This could be performed by demonstrating the presence of MPO/PAD4/Elastate activity or citrullinated histones in them. Nonetheless, this technique is well-suited for preliminary experiments. In conclusion, by this staining technique, N(ET) could be visualized more clearly and this cost-effective method may pave the way to the involvement of labs of low-income countries in elaborating N(ET) research.

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