Immunology of Infection

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7 Visualization and Functional Evaluation of Phagocyte Extracellular Traps

Maren von Köckritz-Blickwede[1,2], Ohn Chow[1], Mariam Ghochani[3] and Victor Nizet[1,4,5]

[1] Department of Pediatrics, University of California, San Diego School of Medicine, La Jolla, CA, USA; [2] Department of Physiological Chemistry, University of Veterinary Medicine Hannover, Germany; [3] Department of Physics and Biology, San Diego State University, San Diego, CA, USA; [4] Skaggs School of Pharmacy and Pharmaceutical Science, University of California San Diego, La Jolla, CA, USA; [5] Rady Children's Hospital, San Diego, CA, USA

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♦♦♦♦♦♦ **I. INTRODUCTION**

In 2004, Brinkmann *et al.* (2004) discovered that neutrophils can produce extracellular traps (ETs) as a mechanism for bacterial clearance. This novel phenomenon has forced a reappraisal of the principal means by which granulocytes function in innate immune defence. ETs are DNA-based net like fibres that mediate an antimicrobial function outside the cell. These structures bind microorganisms, preventing their spread and ensuring a high local concentration of antimicrobial agents capable of inhibiting or killing the invading pathogens extracellularly [reviewed by von Köckritz-Blickwede and Nizet (2009)]. ETs are complexes of nuclear or mitochondrial DNA (Brinkmann *et al.*, 2004; Yousefi *et al.*, 2009) together with proteins such as histones, cell-specific enzymes (e.g. myeloperoxidase or elastase) and antimicrobial peptides (e.g. cathelicidins). DNA is the

major structural component of ETs, since treatment of cells with nucleases leads to their dissolution (Fuchs *et al.*, 2007). However, the DNA itself is not antimicrobial, and it is the host proteins bound to the DNA scaffold that give ETs their antimicrobial activity. Using a proteomic approach, Urban *et al.* (2009) identified a total of 24 ET-associated proteins in neutrophils (Urban *et al.*, 2009).

The cellular processes that lead to the formation of ETs are not completely understood. It has been shown that the production of reactive oxygen species (ROS), such as superoxide (O_2^-) or H_2O_2, are essential signalling molecules leading to the induction of a unique cell death program and the elaboration of ETs (Fuchs *et al.*, 2007). Interference with ROS generation using diphenyleneiodonium (DPI), an inhibitor of NADPH-oxidase enzymes, blocks the formation of ETs. Since this cell death process is morphologically distinct from the classical cell death processes of apoptosis and necrosis, it was named 'ETosis' (Wartha and Henriques-Normark, 2008). In ETosis, global chromatin decondensation and disintegration of the nuclear membrane occur concomitantly with cytoplasmic granule dissolution, allowing the ET components to mix in the cytoplasm prior to their extracellular release.

Formation of ETs was first thought to be restricted to neutrophils, as stimulation of peripheral blood mononuclear cells did not induce the release of similar DNA-based structures (Fuchs *et al.*, 2007). However, confocal fluorescent and scanning electron microscopic studies have also demonstrated the formation of ETs by mast cells (von Köckritz-Blickwede *et al.*, 2008) and eosinophils (Yousefi *et al.*, 2008) in response to bacteria or proinflammatory stimuli. It still remains to be shown whether ET formation by additional cell types might also be detected using techniques which are specifically aimed to analyse this novel immune function.

Here we present methods that facilitate the visualization and/or quantification of ETs. These methods comprise different *in vitro* cell culture techniques as well as *in vivo* histological techniques. Furthermore, we discuss techniques that can be used to evaluate the antimicrobial function of ETs against bacterial pathogens. *Staphylococcus aureus* and *Streptococcus pyogenes* are used as examples. Both are important human pathogens responsible for a wide spectrum of localized and invasive disease conditions. Each pathogen can produce infections in essentially every human organ or tissue, including severe life-threatening conditions such as necrotizing fasciitis, endocarditis, sepsis and toxic shock syndrome. The propensities of *S. aureus* and *S. pyogenes* to produce systemic infections, often in otherwise healthy children and adults, define a capacity of each pathogen to resist host innate immune clearance mechanisms that normally function to prevent microbial dissemination beyond epithelial surface [reviewed in Nizet (2007)]. The innate defences overcome by an invasive pathogen are now known to include antimicrobial ETs.

♦♦♦♦♦♦ II. PREPARATION OF NEUTROPHILS AND BACTERIA

This chapter describes techniques based on use of human blood-derived neutrophils as a prototype. Similar techniques can be used and have already been

proven to be fruitful for other cell types, including human or murine bone-marrow derived mast cells (von Köckritz-Blickwede *et al.*, 2008) and eosinophils (Yousefi *et al.*, 2008). However, efficiency of ET induction may vary depending on the cell type, source or differentiation status of the cells (Martinelli *et al.*, 2004). For example, a longer period of stimulation with Phorbol myristate acetate (PMA) is necessary to induce similar amounts of ETs from murine bone-marrow-derived neutrophils compared to human blood-derived neutrophils (Ermert *et al.*, 2008a). Furthermore, neutrophils derived from several individual mouse strains produce neutrophil ETs (NETs) at different rates (Ermert *et al.*, 2008a).

A. Isolation of Neutrophils

Human neutrophils are isolated and purified from venous blood by density gradient centrifugation using the Polymorphprep™ system (Axis-Shield, Fisher Scientific, #AN1114683). Twenty millilitre of whole blood is sufficient to isolate approximately $2–4 \times 10^7$ neutrophils according to the following protocol:

1. Draw 20 ml venous blood using a 30 ml heparinized syringe (100 µl heparin in a 30 ml syringe).
2. Slowly layer 20 ml blood on top of 20 ml Polymorphprep™ in a 50 ml Falcon tube, taking care to avoid mixing.
3. Centrifuge at $512 \times g$ (without brake) for 30 min at room temperature (Figure 1).
4. Aspirate 5 ml plasma and mononuclear cells from the top layer (This plasma can be used as medium supplement during the assays. Therefore it needs to be heat inactivated at 56°C (to inhibit complement) or 70°C (to completely inhibit serum nucleases) for 30 min and centrifuged at $3000 \times g$ for 10 min to remove remaining proteins).
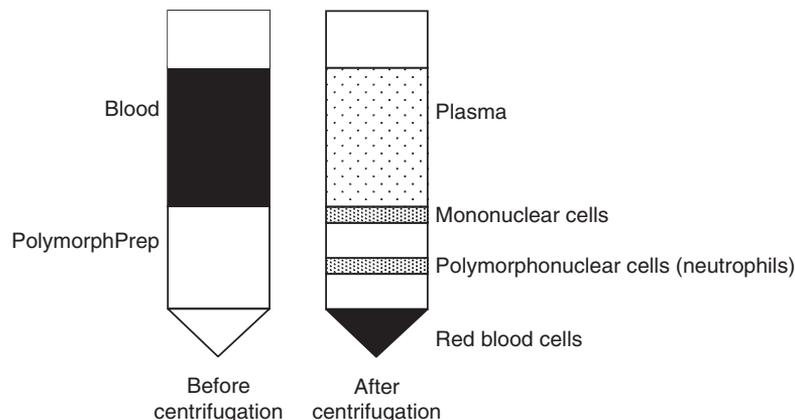

Figure 1. Density gradient before and after centrifugation of Polymorphprep™ with blood.

5. Collect the neutrophil layer (~5–10 ml) into a 50 ml Falcon tube.
6. Add sterile phosphate-buffered saline (PBS, Mediatech, #21-031-CV) to a volume of 50 ml and spin at $512 \times g$ for 10 min.
7. Remove the supernatant, add 5 ml of sterile molecular grade water (Cellgro, #46-000-CM) and mix by pipetting up and down for 5 s to lyse erythrocytes.
8. Immediately add 45 ml of PBS and spin at $512 \times g$ for 10 min.
9. Repeat the above step once again until complete lysis of erythrocytes. The neutrophil pellet should be white in colour.
10. After the final wash, discard supernatant and resuspend the neutrophils in 1000 µl PBS.
11. For the haemocytometer count, dilute neutrophils 1:100 with 0.4% trypan blue (Invitrogen, #15250) and count at least 100 cells. Score for both blue (dead) cells and the total number of cells. Calculate the percentage of dead cells and the total number of cells present. Neutrophils should have >95% viability when used in the following assays.
12. Finally, the cells are resuspended in RPMI-1640 (Invitrogen, #11875) containing desired serum supplements (e.g. autologous heat-inactivated human plasma, foetal calf serum (FCS) or serum albumin). Since serum has been shown to block the formation of ETs based on its antioxidant properties and the presence of serum nucleases, a maximum of 2% serum should be added to the medium. See the following *Note* for further information about the presence of nucleases in serum.

Note: Degradation of ETs by serum nucleases!

We previously showed that serum contains heat-stable nucleases that can degrade non-fixed or paraformaldehyde-fixed neutrophil-derived ETs (von Köckritz-Blickwede et al., 2009). Serum nucleases can be inhibited by heat inactivation of serum for 30 min above 66°C (Segal et al., 1992). Nuclease activity has also been shown to be present in aged solution of bovine plasma albumin Fraction V (Anai et al., 1972), which is widely used in culture experiments as an alternative to FCS.

    The presence of serum nucleases should be avoided and can simply be tested by a functional DNA-degradation assay: 7.5 µl of calf thymus DNA (1 mg/ml, Sigma, #D3664), 40 µl Tris-buffer (5 mM $MgCl_2$, 5 mM $CaCl_2$, 300 mM Tris; pH 7.4) and 10 µl serum are mixed and incubated for 18 h at 37°C. Then, 12.5 µl or 0.33 M EDTA (pH 8.0) is added to stop the reaction. After addition of 12.5 µl of 6× loading dye, the DNA degradation can be visualized by agarose gel electrophoresis (100 mV for 30 min) using a 1% agarose gel containing 0.5 µg/ml ethidium bromide (Figure 2).

B. *Staphylococcus aureus* and *Streptococcus pyogenes*

S. aureus and *S. pyogenes* are both Gram-positive, non-motile, non-spore-forming cocci, 0.6–1.0 µm in diameter. However, the arrangement of cells is different
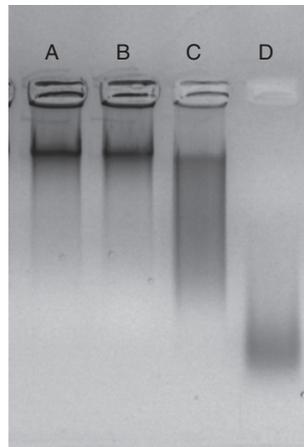
Figure 2. Representative agarose gel demonstrating nuclease activity in different serum charges. (A) DNA control, (B) nuclease-free serum, (C) nuclease-containing serum, (D) micrococcal nuclease as positive control.

between those two organisms due to a different pattern of binary fission: streptococci form a chain of round cells as a result of their linear division, whereas staphylococci divide in various directions forming grape-like clusters. Both are facultative anaerobes that grow by aerobic respiration or by fermentation that mainly yields lactic acid. However, in contrast to *S. pyogenes, S. aureus* shows more robust growth under aerobic than anaerobic conditions due to its catalase activity. Therefore *S. aureus* is grown under shaking conditions (at 180 rpm).

Before starting the assay, dilute overnight cultures of the bacteria 1:50 in fresh Todd–Hewitt broth and grow to the logarithmic phase [$OD_{600\,nm} = 0.4$ corresponding to $\sim 2 \times 10^8$ colony forming units (cfu)/ml]. Centrifuge the bacteria at $512 \times g$ for 10 min and wash with PBS to remove released toxins, proteases and nucleases. Finally, resuspend the bacteria in their respective cell culture media at the desired concentration.

C. ET-Inducing Agents to Serve as Positive Controls

Various agents have been shown to induce the formation of ETs in neutrophils or other cell types. Table 1 resumes those agents that can be used as positive controls for induction of ETs.

♦♦♦♦♦♦ III. *IN VITRO* VISUALIZATION OF ETs

Different techniques can be used to visualize and quantify the formation of ETs in response to a bacterial infection *in vitro* by fluorescence microscopy. Since DNA is

Table 1. Factors inducing formation of ETs in neutrophils

Factor	Concentration	Reference
Phorbol myristate acetate	25 nM (30–240 min)	Brinkmann *et al.* (2004)
H_2O_2-producing glucose oxidase	100 mU/ml (30–60 min)	Fuchs *et al.* (2007)
Interleukin 8	100 ng/ml (30–60 min)	Brinkmann *et al.* (2004); Fuchs *et al.* (2007)
Lipopolysaccharide (LPS)	100 ng/ml	Brinkmann *et al.* (2004)
Interferon (IFN) α + C5a	500 units/ml (30 min) IFNα + 10^{-7} M C5a (10 min)	Martinelli *et al.* (2004)
GM-CSF + LPS	25 ng/ml (20 min) GM-CSF + 0.3 μg/ml LPS (15 min)	Yousefi *et al.* (2009)
GM-CSF + C5a	25 ng/ml (20 min) GM-CSF + 10^{-7} M C5a (15 min)	Yousefi *et al.* (2009)

the major backbone of ETs, different DNA-intercalating dyes are sufficient to visualize ETs. However, since cell-specific enzymes, such as neutrophil-specific myeloperoxidase in NETs and mast cell-specific tryptase in mast cell ETs, have been shown to be present at high amounts within ETs, additional immunostaining of those enzymes can help to visualize ETs.

For all staining techniques, neutrophils are seeded onto poly-L-lysine-coated glass coverslides using the following protocol:

1. Place one glass coverslide per well into non-treated suspension culture plates (Cellstar, Greiner Bioone, #677102). Use 12 mm glass slides (Fisherbrand microscope cover glass 1.5 thickness, Fisher Scientific, #12-545-81) for a 24-well plate and 8 mm glass slides (Electron Microscopy Research, #72296-08) for a 48-well plate. In contrast to tissue culture-treated plates, non-treated (suspension culture) plates prevent unspecific attachment of bacteria to the bottom of the plates.
2. To coat the glass slides, add 100 μl (12 mm) and 50 μl (8 mm) of 0.01% poly-L-lysine (Sigma, #P4707) to the centre of each cover slide. Be careful not to put poly-L-lysine to close to the border of the glass slide. Do not allow the poly-L-lysine to run beside or below the glass slide. Otherwise cells may attach below the glass slide! Incubate for 10–30 min at room temperature.
3. Wash the wells twice with PBS to remove excessive poly-L-lysine.
4. Immediately add the cells to the wells. Seed 5×10^5 cells/well in 24-well plates (with 12 mm glass slides) or 2 × 10^5 cell/well in 48-well plates (with 8 mm glass slides) with 500 or 250 μl medium per well, respectively.
5. Add bacteria at a multiplicity of infection (MOI) of 0.1. Higher MOIs (1, 2, 5, 10, 25) can be used to analyse concentration-dependent ET induction. Always include a negative control of unstimulated cells to detect unspecific

ET formation that may be induced spontaneously during the procedure or that may occur due to fixation artefacts.
6. Centrifuge the plates for 10 min at 800 rpm and further incubate at 37°C and 5% CO_2 for 30 min to 3 h before visualization of ETs.

A. Staining of ETs with DNA-Intercalating Dyes

Different DNA-intercalating dyes can be used for the visualization of extracellular DNA. The cells can be stained with the cell-permeable fluorescent DNA-staining dye SYTO 13 (0.5 µM, Invitrogen, #S7575) and/or with a fluorescent dye unable to enter intact cells (Sytox Orange, 5 µM, Invitrogen, #S11368). A combination of both dyes can be used to distinguish between ETs released by living or dead cells (Yousefi *et al.*, 2009). After staining the cells (for 10 min at room temperature in the dark), washing with PBS can reduce unspecific background staining. (Make sure to never let the slides dry out during washing steps to avoid undesired unspecific background fluorescence. After soaking off the supernatant, immediately add back respective buffers. This is also important during immunostaining of paraformaldehyde-fixed cells or tissue.)

A successfully used alternative to these dyes is the usage of the LIVE/DEAD® Viability/Cytotoxicity Assay Kit for mammalian cells (Invitrogen, #L3224). This kit provides a two-colour fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with the two probes calcein acetoxymethyl (AM) and ethidium homodimer (EthD-1) that measure recognized parameters of cell viability (intracellular esterase activity) and plasma membrane integrity. Background fluorescence levels are inherently low with this assay technique, since the dyes are virtually non-fluorescent before interacting with cells. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby not only producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm) but also staining the formation of ETs. EthD-1 is excluded by the intact plasma membrane of live cells.

LIVE/DEAD® Viability/Cytotoxicity Assay Kit for mammalian cells (Invitrogen)

1. Prepare the dye solution provided within the kit: 20 µl of component B + 1 µl of component A in 10 ml PBS (for cells on poly-L-lysine-coated glass slides). If glass-bottom-plates are used, only ⅓ of the amount of both dye components should be used.
2. Before starting the staining procedure, wash the cells twice with PBS.
3. Add 150 µl of the preliminary prepared dye solution to each well.

4. Incubate for approximately 30 min in the dark.
5. Visualize the samples immediately without fixation by adding 10 µl of staining solution to a microscope slide and placing the glass cover slide with attached cells 'face down' into the staining solution.
6. This staining needs to be visualized immediately. Staining characteristics are lost after paraformaldehyde fixation. Thus, immediately after staining take $n=5$ representative images of each sample. Subsequently count the total amount of cells (green), the amount of dead cells (red) and the amount of ET-forming cells.

Using the above-mentioned method for ET visualization and quantification, an individual bacterial protein, the surface anchored and soluble M1 protein of *S. pyogenes*, was recently found to induce ET formation by neutrophils and mast cells (Lauth *et al.*, 2008). The authors show that ET induction was significantly reduced with an isogenic M1 protein-deficient mutant strain compared to the wild-type *S. pyogenes* parent strain. Furthermore, complementation of the mutant strain with a plasmid expressing M1 protein restored the ET formation (Figure 3).

B. Immunostaining of ETs

Since DNA is not the only ET component, immunostaining of specific additional ET elements may help to better visualize these unique structures and characterized possible antimicrobial factors that are embedded within them. Furthermore,
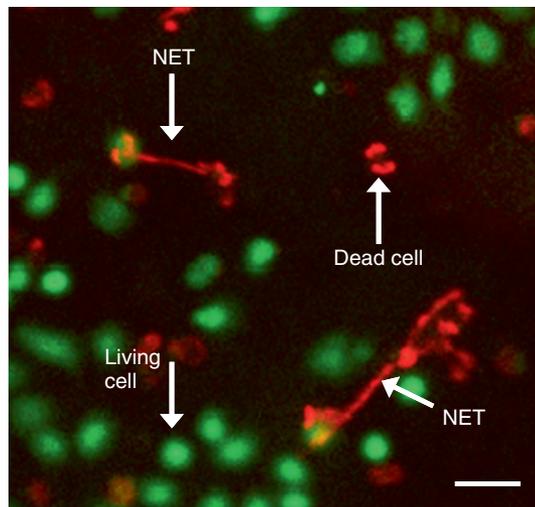

Figure 3. Representative fluorescent image of ETs stained with Live/dead viability/cytotoxicity kit for mammalian cells (Cytoplasm of viable cells is stained in green and dead cells and extracellular DNA are stained in red). Bar 10 µm. Unfixed samples were recorded using a Zeiss Axiolab microscope (Zeiss 20×/0.5 Plan-Neofluor objective) with an attached Sony Digital Photo Camera DKC-5000 at calibrated magnifications. (See color plate section).

immunostaining techniques offer the possibility of using fixed cells and/or tissue that can be kept cool for up to 6 months.

For immunostainings, fix stimulated/infected cells by adding 16% paraformaldehyde (Electron Microscopy Science, #15710) to each well at a final concentration of 4% PFA for 10 min at room temperature. After that, the slides can be kept at 4°C before starting the immunostaining. For storage, wrap parafilm around the plate to avoid evaporation.

Use the following protocol for myeloperoxidase staining of fixed samples:

1. Wash the fixed glass slides three times with PBS.
2. Block by adding 2% BSA–PBS+2% goat serum for 45 min at room temperature. (To additionally visualize intracellular protein expression, the blocking step can be combined with the permeabilization of the cells by adding 0.25% Triton X-100 to the blocking buffer.)
3. Wash three times with PBS.
4. Add rabbit anti-human MPO (Dako, #A0398) 1:300 diluted in 2% PBS–BSA for 1 h at room temperature.
5. Wash three times with PBS.
6. Add the secondary antibody Alexa fluor 488 goat anti-rabbit IgG (Invitrogen, #A11070) 1:500 diluted in 2% BSA–PBS for 45 min at room temperature in dark.
7. Wash three times with PBS and embed the samples in 5 µl ProlongGold antifade + Dapi (Invitrogen, #P36931). For embedding, add 5 µl of the ProlongGold to a microscope slide, and place the glass cover slide with attached cells 'face down' into the embedding solution. Let it dry in the dark at room temperature over night. The following day, seal the border with nail polish to avoid evaporation. Keep at 4°C in dark.
8. Take $n=5$ representative images per sample and count the number of ET-releasing cells versus non-ET-forming cells (Figure 4).
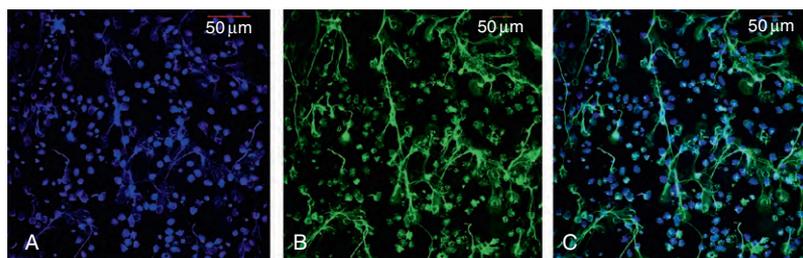
Visualization and Functional Evaluation of ETs

Figure 4. Representative immunofluorescence micrograph of neutrophils ETs (NETs) after stimulation for 3 h with 25 nM PMA. NETs were visualized using a rabbit anti-myeloperoxidase-antibody followed by a secondary goat anti-rabbit Alexa 488 antibody; samples were embedded in ProlongGold+Dapi to counterstain DNA in blue. A) DNA (blue), B) myeloperoxidase (green), c) overlay. Mounted samples were examined using an inverted confocal laser scanning 2-photon microscope Olympus Fluoview FV1000 with Fluoview TM Spectral Scanning Technology (Olympus) and a 20×/0.75 UPlanSApo Olympus objective. (See color plate section).

For the identification of a new component within ETs, a similar protocol can be used. The following controls need to be included to validate the specificity of respective antibodies:

1. Cells that are known to be positive for the respective target (positive control).
2. Cells that do not express the protein of interest (negative control).
3. Isotype control antibody or pre-immune serum, which is used instead of the primary antibody to exclude possible unspecific binding of the antibodies.

Using an antibody against histone–DNA complexes (Losman *et al.*, 1992), different stages of NET-formation can be identified based on characteristic morphological changes of the nucleus upon stimulation and before release of ETs. The process that leads to ET formation has been shown to be neither apoptosis nor necrosis, but rather a new form of cell death termed 'ETosis'. During this process, disintegration of the nuclear membrane occurs concomitantly with cytoplasmic granule dissolution, allowing NET components to mix in the cytoplasm. The normal lobulated nuclear structure is then broken and a delobulated nuclear form can be found in those cells that are in the early stages of Etosis (Figure 5).
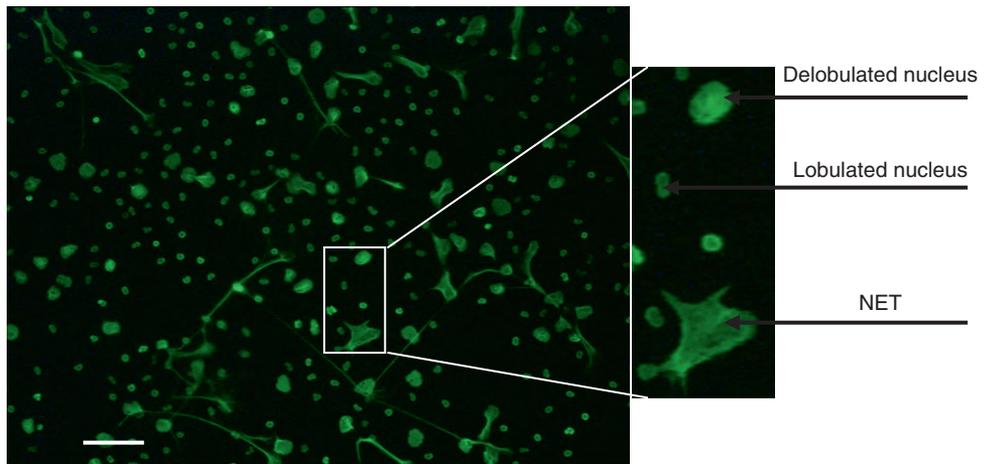

Figure 5. Representative immunofluorescence micrograph of neutrophils ETs (NETs) after stimulation for 2h with 25 nM PMA. NETs were visualized using a mouse anti-H2A–H2B–DNA complex antibody (Losman *et al.*, 1992) followed by a secondary goat anti-mouse Alexa 488 antibody (Invitrogen). Samples were embedded in ProlongGold+Dapi (Invitrogen) to counterstain DNA in blue. Bar 30 μm. Mounted samples were examined using an inverted confocal laser scanning 2-photon microscope Olympus Fluoview FV1000 with Fluoview TM Spectral Scanning Technology (Olympus) and a 20×/0.75 UPlanSApo Olympus objective. (See color plate section).

Use the following protocol for staining H2A–H2B–DNA complexes:

1. Wash the glass slides three times with PBS.
2. Block and permeabilise the cells by adding 2% BSA–PBS +2% rabbit + 0.2% Triton X-100 (MP-Biomedicals, #807423) for 45 min at room temperature.
3. Wash three times with PBS.
4. Add mouse monoclonal anti-H2A–H2B–DNA complex (1 µg/ml; Losman et al., 1992) diluted in 2% PBS–BSA + 0.2% Triton X-100 overnight at 4°C. Isotype control: mouse IgG2b (Thermo Scientific, # NC1391)
5. Wash three times with PBS.
6. Add the secondary antibody Alexa fluor 488 goat anti-mouse IgG (Invitrogen, #A11017) 1:500 diluted in 2% BSA–PBS + 0.2% Triton X-100 for 45 min at room temperature in dark.
7. Wash three times with PBS and embed in 5 µl ProlongGold antifade + Dapi (Invitrogen, #P36931).

C. Quantification of ETs

In addition to the visual assessment of ET formation by fluorescence microscopy, another spectrofluorometric method can be used to quantify ETs. This method is based on the fact that DNA is the major backbone of ETs. Micrococcal nuclease is used to disrupt the ETs and to release the DNA of ETs into the supernatant. The amount of extracellular DNA can then be quantified in the supernatant of cells by using the Quant-iT^{TM} Picogreen®-dsDNA kit (Invitrogen, #P11496). The percentage of ET-DNA is calculated using total cell DNA as 100%.

Although this kit is an ultrasensitive fluorescent nucleic stain for quantifying double-stranded DNA (dsDNA) in solution, the Picogreen assay is not sensitive enough to detect a relatively small amount of cells (<than 20% within 1 h after stimulation) that are releasing ETs. In this case, the microscopic evaluation of ET release is more sensitive. However, the Picogreen assay is a useful tool to investigate the formation of ETs in a high-throughput format. Nevertheless, a microscopic confirmation of the results is always necessary to exclude necrotic release of cellular DNA and to thus confirm specificity of the assay (Figure 6).

Spectro-(fluoro)-metric quantification of ETs

1. Seed 2×10^5 cells per well (each sample in triplicate) into a 96-well tissue culture plate (BD Bioscience, #353072) in RPMI without Phenol red (Mediatech, #17-105-CV).
2. Stimulate with selected ET inducer as positive control (see Table 1) or with different MOIs of bacteria in 200 µl volume. Use untreated cells as background control.
3. Incubate for 1–4 h.
4. Add 500 mU/ml micrococcal nuclease in a volume of 50 µl per well (stock: 50 kU/ml, dilute 1:20.000; Worthington, #NFCP) for 10 min at 37°C.
5. Stop with 5 mM EDTA (stock: 0.33M pH 8).
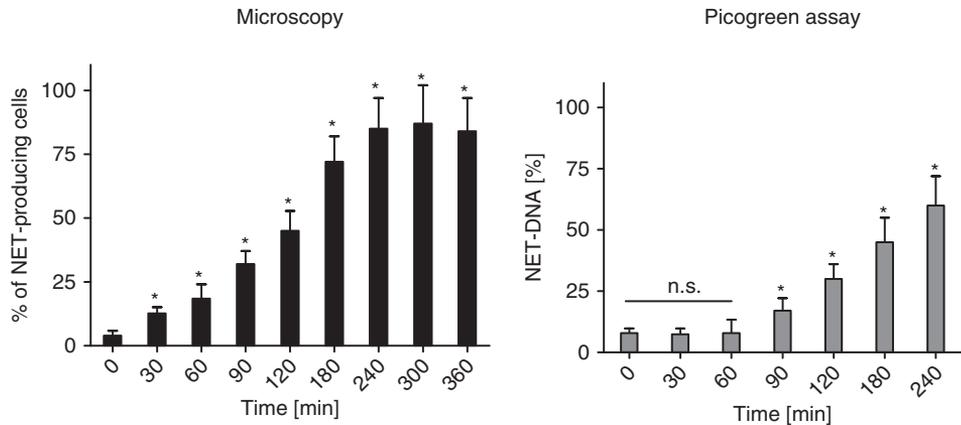6. Centrifuge at $200 \times g$ for 8 min.

Microscopy

Picogreen assay

Figure 6. Determination of percentage (%) of NET-release by human blood-derived neutrophils after stimulation with 25 nM PMA (incubated in RPMI without serum supplements) using two different methods: microscopic evaluation versus spectrofluorometric quantification with the Quant-iT^{TM} Picogreen$^{®}$-dsDNA assay. Comparison of zero-time point with later time points was made by use of Student's t-test. P values of .05 or less were considered significant. Note that the microscopic evaluation is more sensitive to detect significant difference at 30 and 60 min upon stimulation.

7. Transfer 100 μl supernatant to a separate 96-well plates (flat bottom) for Quant-iT Picogreen (Invitrogen, #P11496) assay.
8. Dilute Picogreen reagent 1:200 in TE buffer (freshly made).
9. Mix reagent 1:1 with samples (add 100 μl working solution to 100 μl samples in 96-well plate).
10. Incubate 2–5 min at room temperature in dark.
11. Measure excitation 480 nm, emission 520 nm (fluorescein).
12. Quantify the amount of extracellular DNA in respect to a Lambda DNA standard curve (10–0.1 μg/ml).
13. Calculate the percentage compared to total DNA of 2×10^5 cells.

Isolation of total DNA as 100% control:

1. Add 250 μl DNazol (MRS, #DN127) +2.5 μl Polyacryl Carrier (Molecular Research Center, #PC152) to 2×10^5 cells and lyse cells by pipetting up and down.
2. Centrifugate 10 min at $10,000 \times g$.
3. Transfer viscous supernatant to a new tube.
4. Add 125 μl of 100% ethanol.
5. Mix samples by inverting the tube five to eight times and incubate at room temperature for 1–3 min.
6. Centrifuge $5000 \times g$ for 5 min.
7. Wash pellet twice with 75% ethanol by inverting the tubes three to six times.
8. Centrifuge $1000 \times g$ for 1–2 min.
9. Resuspend the DNA in water.

D. Electron Microscopy

Several different electron microscopy protocols have been used to understand the formation of ETs. Whereas scanning electron microscopy (SEM) can be used to visualize the overall release of ETs and its three-dimensional structure, transmission electron microscopy (TEM) can be used to visualize the morphological changes within a cell.

Thus, high-resolution SEM showed that ETs contain smooth stretches with a diameter of 15–17 nm and globular domains of around 25 nm that aggregates into larger threads with diameters of up to 50 nm (Brinkmann *et al.*, 2004). Analysis of cross-sections of the ETs by TEM additionally revealed that ETs are not surrounded by membranes. Furthermore, TEM analysis was used to identify the morphological changes that lead to the formation of ETs: first, the nuclei start to lose their lobules, and the chromatin begins to decondense. The space between the inner and outer nuclear membrane dilates and the nuclear envelope finally completely disintegrates. With the loss of nuclear and granule membranes, the decondensed chromatin comes into contact with cytoplasmic and granule components (Fuchs *et al.*, 2007).

Here, selected methods for SEM as well as TEM will be presented. For both processes, 2×10^7 neutrophils are incubated with bacteria (MOI of 1) in 250 μl HBSS + Ca/Mg (Invitrogen, #14065) in a 1.5 ml Eppendorf tube under constant rotation at 37°C for 20 min. Then the cells are centrifuged for 10 min at $512 \times g$ and washed twice with PBS before fixation (Figure 7).
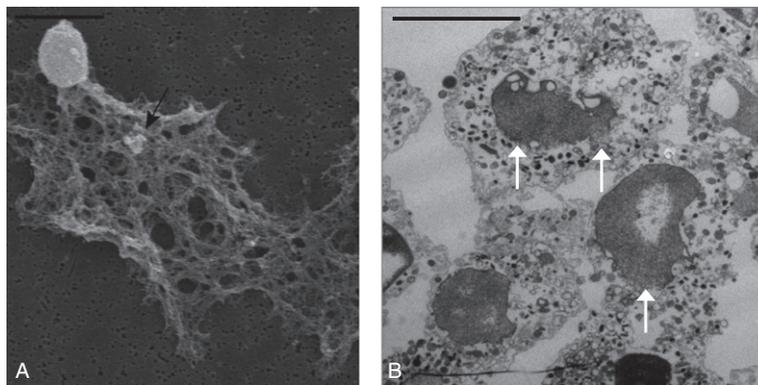
Figure 7. Electron micrograph showing neutrophils that are releasing extracellular DNA-traps in response to *S. pyogenes* infection. (A) Scanning electron micrograph using a Hitachi S-2700 scanning electron microscope in a 60:60 ratio at an accelerating voltage of 10 kV. Note the bacterium entrapped within the ET (black arrow). Bar, 10 μm. (B) Transmission electron micrograph (TEM) using an FEI Tecnai 12 transmission electron microscope operated at 120 kV. Bar, 5 μm. Images were recorded on a Tietz 214 CCD camera after which images were assembled in Adobe Photoshop CS version 8 with only linear adjustments in brightness and contrast. Note the disintegration of the nuclear membrane with subsequent release of nuclear DNA and mixing with cytoplasm (white arrow).

Transmission electron microscopy

1. After incubation, the cells are immediately fixed with 3.0% formaldehyde (Ted Pella, #18505)+1.5% glutaraldehyde (Ted Pella, #18420)+0.1 M sodium cacodylate trihydrate (Sigma-Aldrich, #C0250)+5 mM calcium chloride (J.T. Baker, #1-1332)+2.5% sucrose (Sigma-Aldrich, #24761-8) at pH 7.4 for 1 h at room temperature.
2. Cells are then washed three times for 10 min each in ice-cold 0.1 M sodium cacodylate buffer containing 2.5% sucrose.
3. In accordance with the general procedure of Perkins and McCaffery (2007), the primary fixed cells are then incubated with 1% osmium tetroxide (Ted Pella, #18463)+0.02 N hydrochloric acid (J.T. Baker, #6011) in 0.056 M acetate-veronal solution (0.028 M sodium acetate anhydrous (J.T. Baker, #1-3470)+0.028 M sodium barbital (Merck, #6921)) for 1 h on ice in the dark.
4. Wash with 0.056 M acetate-veronal solution-0.028 N hydrochloric acid three times for 10 min each.
5. Fixed cells are stained and stabilized en bloc with 0.5% uranyl acetate (Ted Pella, #19481)+0.056 M acetate-veronal solution+0.028 N hydrochloric acid solution, pH 6, over night at room temperature in the dark.
6. After one rinse with ddH$_2$O and one rinse with 50% ethanol (4°C), the cells are dehydrated at 4°C through a series of 70, 95 and 100% ethanol successively for 15 min each.
7. Wash three times for 15 min each in fresh 100% ethanol (Pharmco-AAPER, #E200) at room temperature.
8. Cells are then infiltrated in well-mixed Epon (Ted Pella, #18010)-ethanol resin series of 33% for 7 h, 66% for 7 h followed by 100% at least overnight with agitation at room temperature.
9. The samples are allowed to polymerize in 100% Epon blocks at 60°C for 24 h.
10. For conventional electron microscopy, 70-nm sections are cut using a Diatome diamond knife on a Leica EM UC6 ultramicrotome.
11. Mount the samples on 100 mesh copper grids (Ted Pella, #12414-CU).
12. Stain the cells with 2% uranyl acetate and Reynolds lead citrate before examination.

Scanning electron microscopy

1. Cells are immediately fixed with 3.0% formaldehyde (Ted Pella, # 18505)+1.5% glutaraldehyde (Ted Pella, #18420)+0.1 M sodium cacodylate trihydrate (Sigma-Aldrich, #C0250)+5 mM calcium chloride (J.T. Baker, #1-1332)+2.5% sucrose (Aldrich Chem. Comp., #24761-8) at pH 7.4, for 1 h at room temperature.

2. Cells are washed three times for 10 min each in ice-cold 0.1 M sodium cacodylate buffer containing 2.5% sucrose.

3. To post-fix the cells with Palade's OsO_4 (Palade, 1952), they are then incubated with 1% osmium tetroxide (Ted Pella, #18463) + 0.02 N hydrochloric acid (J.T. Baker, #6011) in 0.056 M acetate-veronal solution [0.028 M sodium acetate anhydrous (J.T. Baker, #1-3470) + 0.028 M sodium barbital (Merck, #6921)] for 30 min on ice in the dark.

4. Wash three times with 0.056 M acetate-veronal solution-0.028 N hydrochloric acid for 10 min each.

5. The cells are then filtered through 0.4 μm HTTP Isopore membrane filters (Millipore, #HTTP01300).

6. Wash with ddH_2O and dehydrate in graded series of ethanol (30, 50, 70, 95 and 100%) (Pharmco-AAPER, #E200) for 15 min each at room temperature.

7. The cells in the filter are then critical-point dried with liquid CO_2 in a Tousimis Samdri 790 drier.

8. Mount the cells with an adhesive carbon conductive tab onto an aluminium sample stub (Ted Pella) and coat with Au-Pd (60:40w/w) film on a Denton Desk II sputter coater before examination in a SEM.

Visualization and Functional Evaluation of ETs

♦♦♦♦♦♦ IV. *IN VIVO* VISUALIZATION OF ETs

Most of the antibodies in Table 2 that have been used for the visualization of ETs *in vitro* can also be used to identify ETs *in vivo* by using formalin-fixed and paraffin-embedded tissue sections. The most commonly used fixatives are 10% buffered formalin (Fisher Scientific, #SF93-4) or 4% paraformaldehyde. However, it must be noted that the fixative penetrates into the tissue very slowly (approximately 1 mm per hour). Thus, the tissue needs to remain in the fixative for approximately 24 h. Note that the lung morphology is best examined after perfusion of fixative through the trachea to inflate all lobes. After fixation and subsequent paraffin embedding, 3–7 μm thick sections should be used for subsequent immunostaining using the following described protocol. Besides, haematoxylin-eosin-staining can be useful to detect additional pathological changes within the tissue (Figure 8).

Immunostaining of paraffin-embedded tissue

1. Deparafinize sample with xylene (three times 10 min; Fisher Scientific, #X3P-1GAL), 1000% alcohol (two times, 5 min each; Fisher Scientific, #A962P-4), 95% alcohol (two times, 5 min each), 70% alcohol (two times, 5 min each).

2. Wash with PBS (three times 10 dips).

3. Microwave (high level) the sections for 2×5 min in target retrieval solution, citrate buffer pH 6 (Dako, #S2369), with lid to break cross-links formed during formalin fixation.

4. Let stand for 20 min in citrate buffer solution without lid at room temperature.

5. Wash with PBS three times with 10 dips/wash.

6. Block with 2% BSA–PBS + 2% goat serum for 45 min at room temperature. [Since paraffin sections are usually very thin (3–7 µm), most of antigens/epitopes are already exposed, making detergent-permeabilization unnecessary.]

7. Add the primary antibody (rabbit anti-mouse CRAMP; Dorschner *et al.*, 2001) in the presence of 2% BSA–PBS covered with parafilm, overnight at 4°C in humid chamber.

8. Wash with PBS four times.

9. Add the secondary antibody (Alexa 488 goat anti-rabbit, Invitrogen #A11070) 1:500 and incubate for 45 min at room temperature, in humid chamber covered with parafilm.

10. Wash with PBS four times.

11. Put on coverslides using ProlongGold+Dapi (Invitrogen, #P36931) as embedding solution.

Table 2. Antibodies for visualization of ETs

| Target protein	Antibody	Target tissue/cells	Reference
Myeloperoxidase	Rabbit anti-human MPO (Dako, # A0398)	Murine and human neutrophils	Ermert *et al.* (2008a); Von Köckritz-Blickwede *et al.* (2009)
H2A–H2B–DNA complex	Mouse anti-H2A–H2B–DNA complex (Losman *et al.*, 1992)	Murine and human neutrophils	Brinkmann *et al.* (2004); Ermert *et al.* (2008a)
Histone H4cit3	Rabbit anti-H4cit3 (Millipore, #07-596)	HL-60 cells	Wang *et al.* (2009)
Cathelicidins (CRAMP)	Rabbit anti-CRAMP (Dorschner *et al.*, 2001)	Mouse lung tissue	See this chapter '*In vivo* visualization of ETs'
Elastase	Urban *et al.* (2006)	Human neutrophils	Urban *et al.* (2006)
Tryptase	Mouse anti-human tryptase (clone 4A1, Dako, #M7052)	Mast cells (HMC-1)	Von Köckritz-Blickwede *et al.* (2008)
Calprotectin	Urban *et al.* (2009)	Human neutrophils, mouse lung tissue	Urban *et al.* (2009)
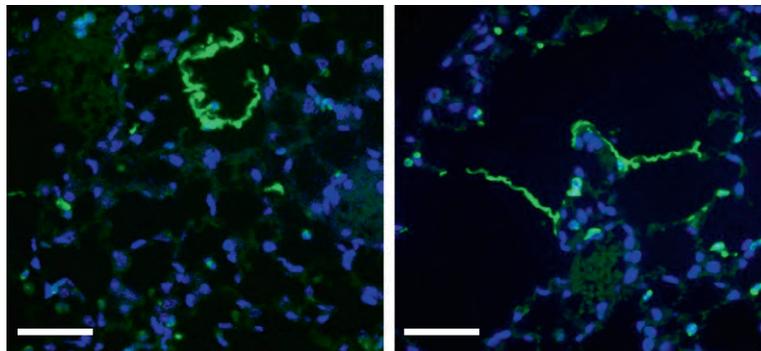
Figure 8. Representative fluorescent images of extracellular trap formation (visualized by Alexa 488 (green)-labelled CRAMP production and counterstained with Dapi; see protocol on the next page) in 3 μm thick paraffin-embedded lung sections of mice intranasally infected with 2×10^8 cfu of *S. aureus* strain Newman for 48 h. Bars represent 25 μm. Images were recorded using an Olympus Spinning Disc Confocal IX81 microscope (40×/1.0 oil UPlanApo objective) with a Xenon DG5 illumination source driven by SlideBook software (Intelligent Imaging Innovations). (See color plate section).

♦♦♦♦♦♦ V. FUNCTIONAL ASSAYS

ETs have been discovered to exhibit an important antimicrobial role during host immune defence by entrapment of an invading pathogen to (a) prevent its further spread and/or (b) to directly kill the pathogen. Ermert *et al*. (2008b) described methods that can be used to measure killing of microbes by ETs and further to differentiate whether phagocyte killing is occurring through ETs, phagocytosis or granule extracts. The methods are based on quantification of surviving cfu as parameter for the calculated antimicrobial activities. Here we present additional fluorescence-based methods that can be used to quantify entrapment and to visualize killing of bacteria within ETs.

A. Quantification of Bacterial Entrapment by ETs

For the quantification of bacterial entrapment, the bacteria are labelled with fluoresceinisothiocyanat (FITC) and then added to neutrophils, which have been stimulated for 4 h with PMA for maximal ET formation and abolishment of phagocytic activity (Fuchs *et al.*, 2007). Finally the entrapment of fluorescent bacteria can be measured spectrofluorometrically. The following protocol can be used:

1. Seed 2×10^5 neutrophils per well in 96-well plate (each sample in triplicate) in RPMI without Phenol red (100 μl per well). Use similar amounts of respective control wells without cells.
2. Stimulate cells with 25 nM PMA (Sigma, #P1585) for 4 h at 37°C + 5% CO_2.
3. Meanwhile incubate logarithmic phase bacteria with 0.2 mg/ml FITC (stock 10 mg/ml in DMSO, Invitrogen, #F1906) for 30 min on ice in the dark (Goldmann *et al.*, 2004).

4. After incubation, centrifuge the bacteria at $3000 \times g$ for 10 min and wash twice with PBS to remove unbound FITC.
5. Infect the PMA-stimulated neutrophils with FITC-labelled bacteria at an MOI of 10, 25, 50 and 100 (by adding 100 µl medium). Include control wells without bacteria.
6. Include control wells without neutrophils for bacterial-FITC standard curve.
7. Centrifuge at $512 \times g$ for 10 min.
8. Incubate for 30 min at 37°C.
9. Wash cells carefully two times with 200 µl RPMI (without Phenol red).
10. Measure green fluorescence at 485/538 nm (SpectraMax® Gemini XS Spectro-fluorometer (Molecular Devices).
11. Percent entrapment of GAS can be calculated as ([A485/538 nm experimental well]/[A485/538 nm control well without neutrophils]) × 100%.
12. Control wells containing serial dilutions of FITC-labelled bacteria are used for standard curve construction and subsequent quantification of entrapped bacteria.

B. Visualization of Bacterial Killing by ETs

To visualize the bacterial killing by ETs, the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen, #L7012) can be used in combination with Dapi Prolong-Gold. The kit utilizes a mixture of Syto 9-green fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. Syto 9 stain generally labels all bacteria in a population – those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the Syto 9 stain fluorescence. Thus, with an appropriate mixture of the Syto 9 and propidium iodide stains, living bacteria stain fluorescent green, whereas dead bacteria stain red.

1. Seed neutrophils at cell density of 5×10^5 cells per 500 µl in RPMI in a 24-well plate with poly-L-lysine-coated glass cover slides (as described above).
2. Add bacteria at an MOI of at least 2.
3. Centrifuge for 10 min at $512 \times g$.
4. Incubate at 37°C, 5% CO_2 for 20 min.
5. After incubation, wash attached cells twice with PBS (to remove serum which may interfere with the staining).
6. Add 150 µl of the dye components A + B (each 1.5 µl in 1 ml PBS) to each well.
7. Incubate for 15 min in the dark.
8. Wash three times with PBS to remove unbound dye.
9. Fixation with 1% paraformaldehyde for 5 min at room temperature.
10. Wash three times with PBS.
11. Embedding in 5 µl ProlongGold + Dapi.
12. Let it dry in the dark at room temperature over night. Then seal the border with clear nail gloss to avoid evaporation and drying of the samples. Keep samples at 4°C in dark (Figure 9).
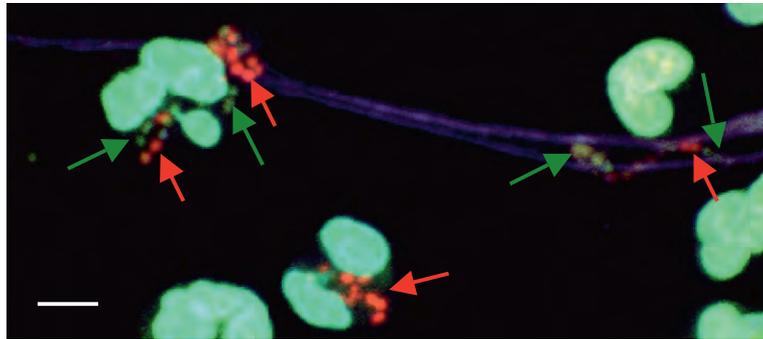
Figure 9. Representative immunofluorescence image of viable (green) versus dead (red) bacteria entrapped by NETs or phagocytosed by neutrophils. Samples were embedded in ProlongGold+Dapi to counterstain DNA and visualize ETs in blue. Mounted samples were examined using an inverted confocal laser scanning 2-photon microscope Olympus Fluoview FV1000 with Fluoview TM Spectral Scanning Technology (Olympus) and a 60×/1.42 PlanApo Olympus objective. (See color plate section).

♦♦♦♦♦♦ VI. COMMERCIAL SUPPLIERS

- BD Biosciences, 2350 Qume Drive, San Jose, CA 95131, USA
  Phone: 877-232-8995
  Fax: 410-316-4770
  Website: http://www.bdbiosciences.com/home.jsp
  E-Mail: Industrial_CS@bd.com
- Dako Denmark A/S
  Produktionsvej 42, DK-2600 Glostrup, Denmark
  Phone: +45-44-859500
  Fax: +45-44-859595
  Website: www.dako.com
  E-mail: contact@dako.com
- Electron Microscopy Science, P.O. Box 550, 1560 Industry Road, Hatfield, PA 19440, USA
  Phone: 800-523-5874
  Fax: 215-412-8450
  Website: http://www.emsdiasum.com
  E-Mail: sgkcck@aol.com
- Fisher Scientific, 2000 Park Lane Drive, Pittsburgh, PA 15275, USA
  Phone: 800-766-7000
  Fax: 800-926-1166
  Website: http://www.fishersci.com
  E-Mail: Access via Website
- Greiner Bio-One North America Inc., 4238 Capital Drive, Monroe, NC 28110, USA
  Phone: 1-800-884-4703
  Fax: 1-800-726-0052

Website: http://www.greinerbioone.com
E-Mail: info@us.gbo.com
- Invitrogen Corporation, 5791 Van Allen Way, PO Box 6482, Carlsbad, CA 92008, USA
Phone: 760-603-7200
Fax: 760-602-6500
Website: http://www.invitrogen.com
E-mail: catalog@invitrogen.com
- J.T. Baker, Mallinckrodt Baker, Inc., 222 Red School Lane, Phillipsburg, NJ 08865, USA
Phone: 908-859-2151
Fax: 908-859-9318
Website: http://www.solvitcenter.com
E-Mail: Access via Website
- MatTek Corporation, 200 Homer Avenue, Ashland, MA 01721, USA
Phone: 800-634-9018
Fax: 508-879-1532
Website: http://www.mattek.com
E-Mail: information@mattek.com
- Mediatech, Inc., 9345 Discovery Blvd., Manassas, VA 20109, USA
Phone: 800-235-5476
Fax: 703-471-0363
Website: http://www.cellgro.com/shop/customer/home.php
E-Mail: custserv@cellgro.com
- Merck & Co., One Merck Drive, P.O. Box 100, Whitehouse Station, NJ 08889, USA
Phone: 908-423-1000
Website: http://www.merck.com/
- Millipore, 290 Concord Road, Billerica, MA 01821, USA
Phone: 800-645-5476
Website: http://www.millipore.com
E-Mail: Access via Website
- Molecular Research Center, Inc. (MRC), 5645 Montgomery Road, Cincinnati, OH 45212, USA
Phone: 800-462-9868
Fax: 513-841-0080
Website: http://www.mrcgene.com/
E-Mail: mrc@mrcgene.com
- MP-Biomedicals, 29525 Fountain Pkwy., Solon, OH 44139, USA
Phone: 800-854-0530
Fax: 800-334-6999
Website: http://www.mpbio.com/
E-Mail: Access via Website
- Pharmaco-AAPER, 58 Vale Road, Brookfield, CT 06804, USA
Phone: 800-243-5360
Fax: 203-740-3481
Website: http://www.pharmco-prod.com

E-Mail: j.perez@pharmcoaaper.com
- Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178, USA
  Phone: 800-325-3010
  Fax: 800-325-5052
  Website: http://www.sigmaaldrich.com/united-states.html
  E-Mail: OC_DOM_HC@sial.com
- Ted Pella Inc., P.O. Box 492477, Redding, CA 96049, USA
  Phone: 530-243-2200
  Fax: 530-243-3761
  Website: http://www.tedpella.com/
  Email: sales@tedpella.com
- Thermo Scientific, Thermo Fisher Scientific, 120 Bishops Way, Brookfield, WI 53008, USA
  Phone: 800-532-4752
  Website: http://www.thermo.com
  E-Mail: Access via Website

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Acknowledgements

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