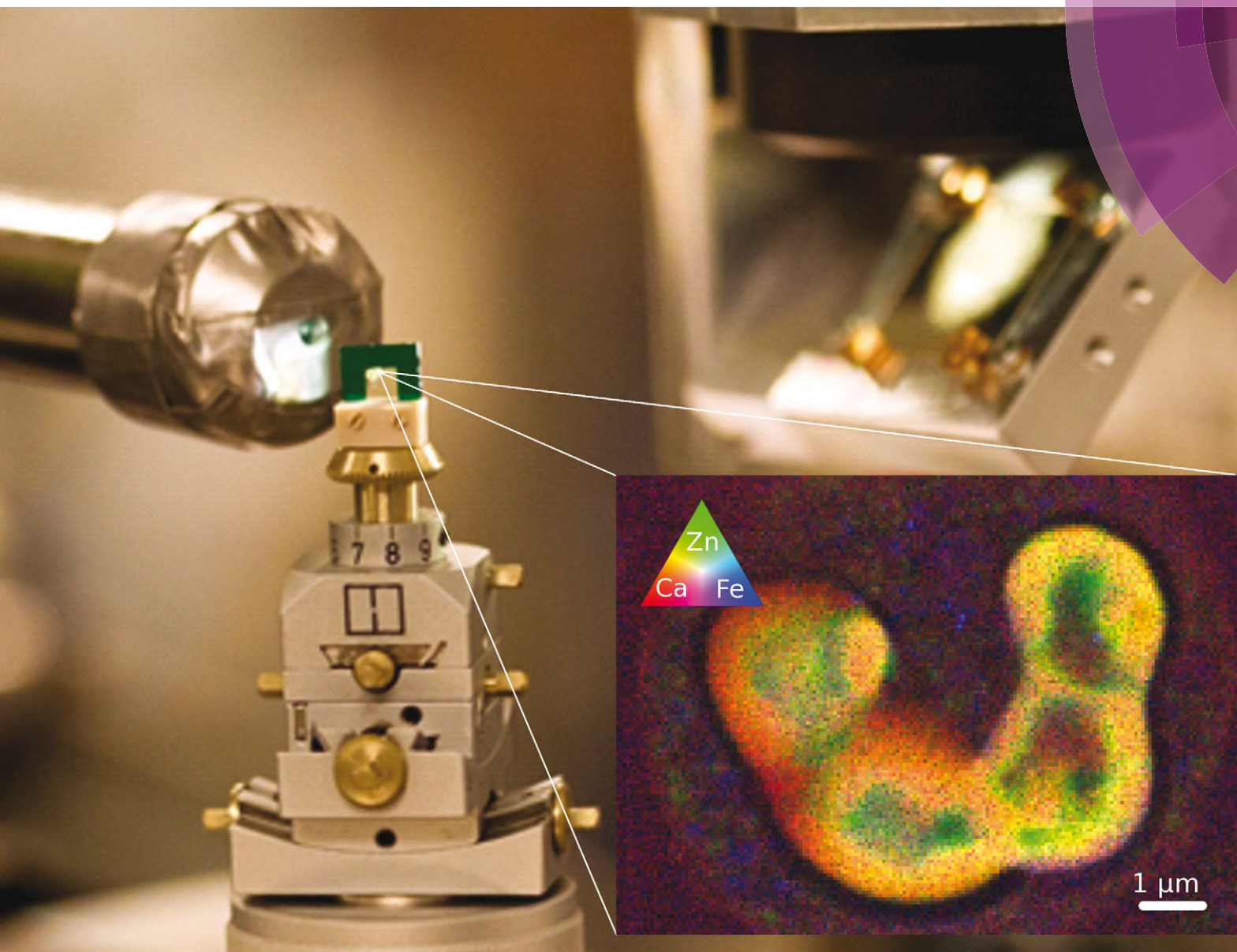


Metallomics

www.rsc.org/metallomics



ISSN 1756-5901



PAPER

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Trace element landscape of resting and activated human neutrophils on the sub-micrometer level

Indexed in
Medline!



Cite this: *Metallomics*, 2015, 7, 996

Trace element landscape of resting and activated human neutrophils on the sub-micrometer level†

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Every infection is a battle for trace elements. Neutrophils migrate first to the infection site and accumulate quickly to high numbers. They fight pathogens by phagocytosis and intracellular toxication. Additionally, neutrophils form neutrophil extracellular traps (NETs) to inhibit extracellular microbes. Yet, neutrophil trace element characteristics are largely unexplored. We investigated unstimulated and phorbol myristate acetate-stimulated neutrophils using synchrotron radiation X-ray fluorescence (SR-XRF) on the sub-micron spatial resolution level. PMA activates pinocytosis, cytoskeletal rearrangements and the release of NETs, all mechanisms deployed by neutrophils to combat infection. By analyzing Zn, Fe, Cu, Mn, P, S, and Ca, not only the nucleus but also vesicular granules were identifiable in the elemental maps. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) revealed a neutrophil-specific composition of Zn, Fe, Cu, and Mn in comparison with J774 and HeLa cells, indicating a neutrophil-specific metallome complying with their designated functions. When investigating PMA-activated neutrophils, the SR-XRF analysis depicted typical subcellular morphological changes: the transformation of nucleus and granules and the emergence of void vacuoles. Mature NETs were evenly composed of Fe, P, S, and Ca with occasional hot spots containing Zn, Fe, and Ca. An ICP-MS-based quantification of NET supernatants revealed a NETosis-induced decrease of soluble Zn, whereas Fe, Cu, and Mn concentrations were only slightly affected. In summary, we present a combination of SR-XRF and ICP-MS as a powerful tool to analyze trace elements in human neutrophils. The approach will be applicable and valuable to numerous aspects of nutritional immunity.

Received 22nd December 2014,
Accepted 20th March 2015

DOI: 10.1039/c4mt00346b

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Introduction

Metal ions play an important role in many biological processes. They catalyze reactions, stabilize structures, and act during gene regulation or cell signaling.^{1–5} Their usage is conserved in prokaryotes and eukaryotes.⁶ During infections, when the host encounters microbial invaders, metal ions are essential for survival of both parties. The host therefore restricts the availability of metal ions in order to avoid microbial growth – a strategy named “nutritional immunity” and described in animals and plants.⁷ In response,

many pathogens have evolved sophisticated tools to chelate metal ions from the host and these tools are targeted by the host in return. This interplay illustrates co-evolution of host and pathogen and is best exemplified by the well-studied fight for Fe.⁸ In the host, Fe is mainly kept intracellular – free Fe is essentially unavailable in blood or other extracellular liquids. It is bound to transferrin in plasma and lactoferrin in external secretions. Microbial pathogens have two mechanisms to cope with these limitations. In case of direct contact between the host Fe source and the pathogen, host Fe-retaining proteins are modified to release their Fe load into the extracellular environment for uptake. Secondly, many bacteria and fungi secrete siderophores, small molecules with exceptionally high Fe affinity that free Fe from host proteins followed by resorption into the pathogenic cell. As a response, mammals produce lipocalin-2, to intercept siderophores. This can in turn be counteracted by many pathogens which disguise their siderophores by glycosylation.⁸

Fe is not the only transition metal important in nutritional immunity. Zn, Mn and Cu are also shown to be critical.^{9,10} Especially Zn has been demonstrated to be crucial during fungal pathogenicity.¹¹ This can be explained by its unique biochemical

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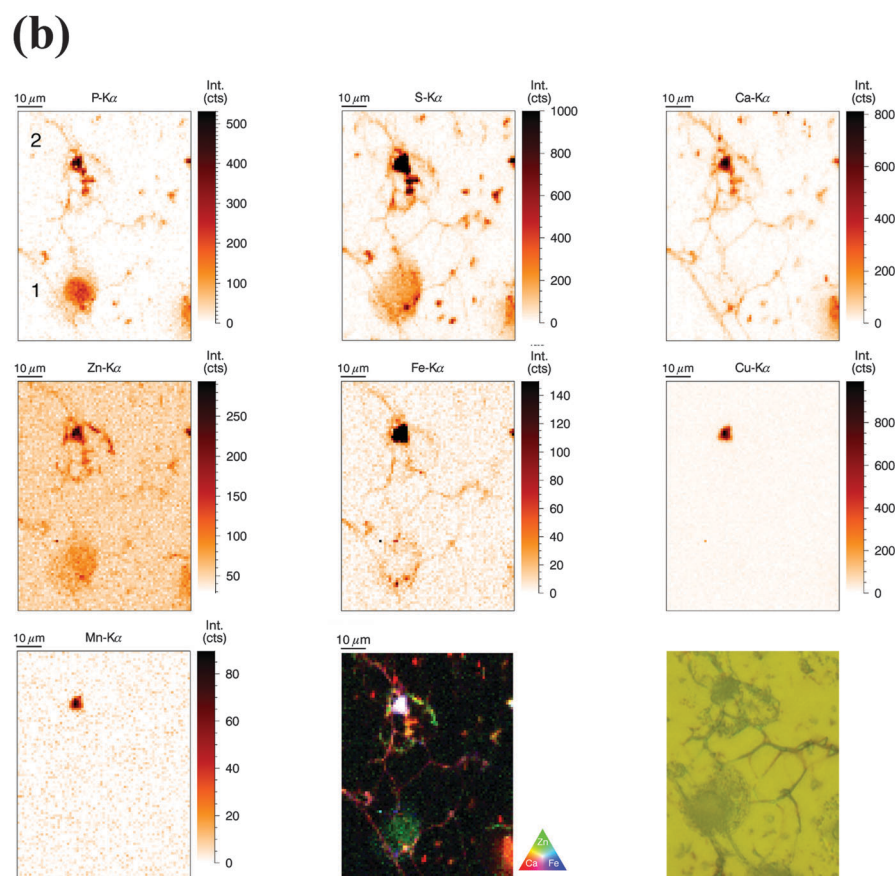
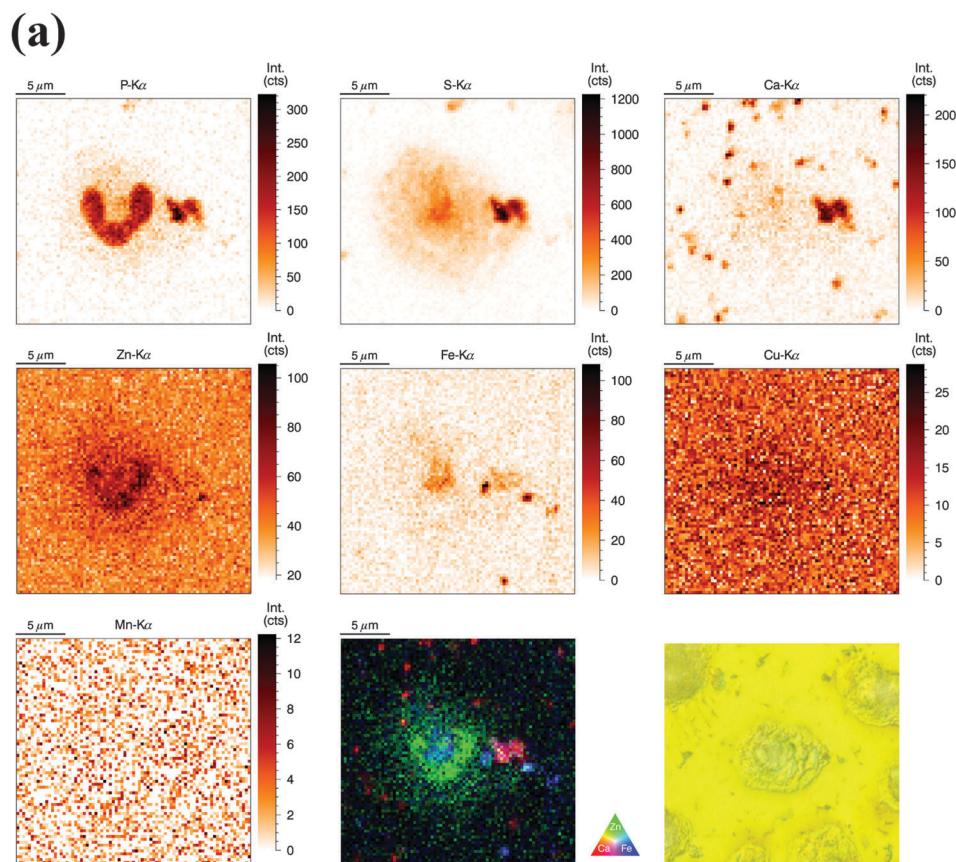
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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4mt00346b



Metallomics, 2015, 7, 996–1010 | 997



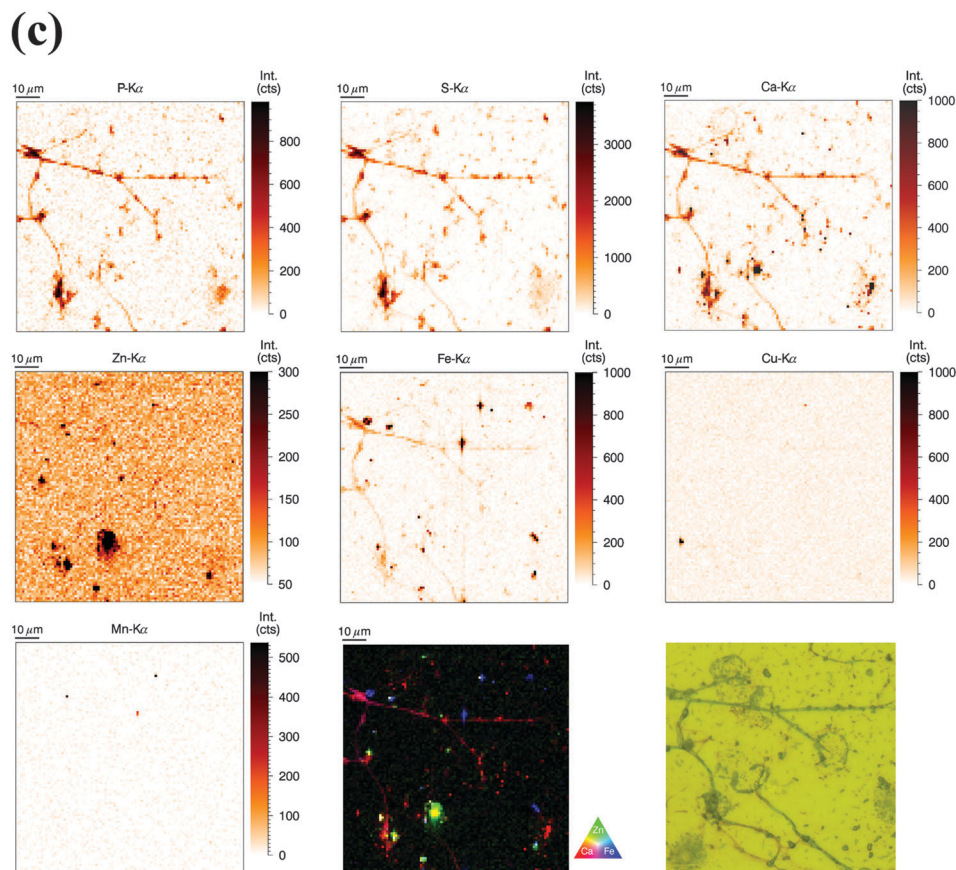


Fig. 2 (a) Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of a single resting human neutrophil obtained at P06 beamline (PETRA III, Hamburg). Freshly isolated neutrophils were seeded on a Si_3N_4 membrane and subsequently freeze-dried. Image size is $81\ \mu\text{m} \times 78\ \mu\text{m}$, step size is $300\ \text{nm}$ and $4\ \text{s}$ dwell time per point. The RGB image represents a colored overlay of the elemental intensities corresponding to Ca (red)–Zn (green) and Fe (blue). An optical image was taken by the beamline microscope ($2500\times$ magnification). (b) Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of two activated human neutrophils ($2\ \text{h}$ stimulation) obtained at P06 beamline. Freshly isolated neutrophils were seeded on a Si_3N_4 membrane, stimulated with PMA for $2\ \text{h}$ and subsequently freeze-dried. Image size: $73\ \mu\text{m}$ (hor.) $\times 97\ \mu\text{m}$ (vert.), step size: $1\ \mu\text{m}$ and $5\ \text{s}$ dwell time per point. The RGB image represents a colored overlay of the elemental intensities corresponding to Ca (red)–Zn (green) and Fe (blue). An optical image was taken by the beamline microscope ($2500\times$ magnification). (c) Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of human neutrophils ($4\ \text{h}$ stimulation) obtained at the PETRA III P06 beamline. Freshly isolated neutrophils were seeded on a Si_3N_4 membrane, stimulated with PMA for $4\ \text{h}$ and subsequently freeze-dried. Image size: $100\ \mu\text{m} \times 100\ \mu\text{m}$, step size: $1\ \mu\text{m}$ and $3\ \text{s}$ scanning time per point. The RGB image represents a colored overlay of the elemental intensities corresponding to Ca (red)–Zn (green) and Fe (blue). An optical image was taken by the beamline microscope ($2500\times$ magnification).

stage (Fig. 2b). As mentioned, the analysis of unstimulated neutrophils revealed clear differences between the nucleus and the cytoplasm. After $2\ \text{h}$ stimulation, the morphology of the neutrophils changed considerably, but not in a perfectly synchronized fashion. Some neutrophils engaged in the process of NET formation earlier than others. The progress of NET formation is most apparent when following the nucleus. In early NET formation, the nucleus loses its lobulation and shrinks to form a smaller and round shape (Fig. S3, * + a, ESI†). The lower neutrophil #1 is representative of this stage (Fig. 2b). As in unstimulated neutrophils, the nucleus contained a higher areal concentration of P and Zn, whereas the cytoplasm featured a homogeneous distribution of P and S (higher areal concentration than background level). Interestingly, the intact stimulated neutrophil also showed the presence of an Fe rich region, distributed in a ring-like manner in the cell periphery. The upper neutrophil #2 is representative of a later stage of NET formation,

characterized by the loss of membrane integrity, first of the nucleus, then of the cytoplasmic membrane – the cell opens up and lyses (Fig. S3, b + c, ESI†). When comparing the light microscope images of neutrophil #2 with neutrophil #1, we observe that from the area initially covered by neutrophil #1, only cell residues in neutrophil #2 remain visible. The SR-XRF element maps confirm that the upper cell has likely lysed: Within the area of the cell (shape of an inverted heart or leaf), we could no longer observe the homogeneous distribution of P and S. In contrast, the remaining cell debris was enriched in P, S, Ca, Fe, and Zn. A hotspot containing not only these elements, but also Cu and Mn was found to lie within the area of neutrophil #2. This is most likely a non-biological contamination. Short fiber-like structures, which we identified as NETs, are protruding out of the cell debris. Their areal concentrations for P, S, Zn, and Fe were found to be above background level. Similar to the unstimulated sample, several ‘hot-spots’ ($1\text{--}4\ \mu\text{m}$ size) containing P, S and Ca are present which could be cell remnants.



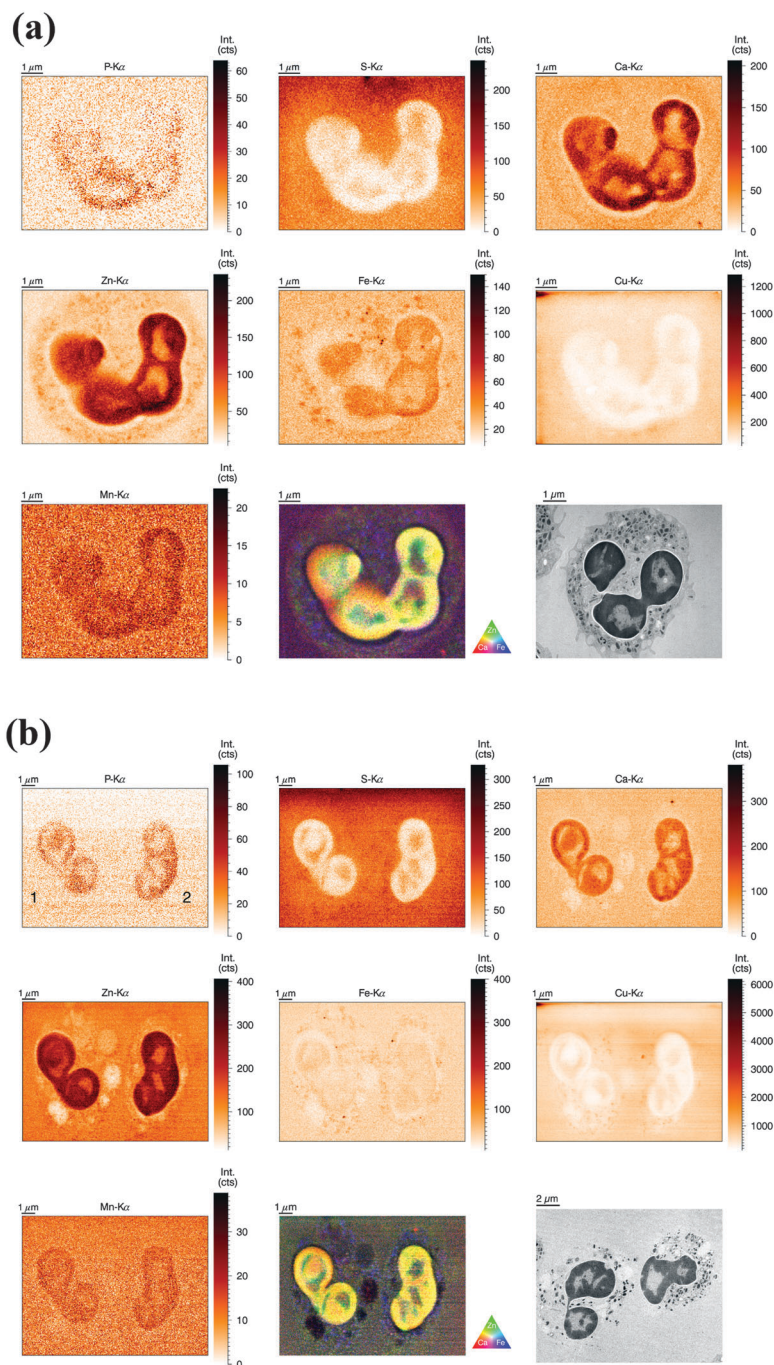
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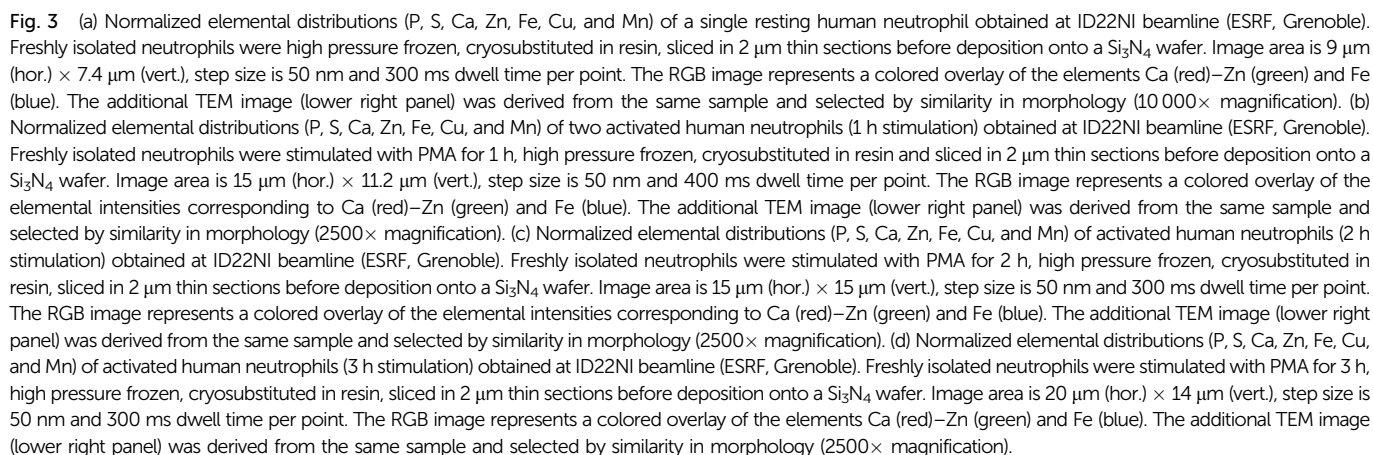
identically. However, more time points were included in order to relate cellular changes during activation and NET formation with differences in metal distribution.

Neutrophils activated for 1 h showed similar metal distributions of P, S, Ca, Zn, Fe, Cu and Mn resulting in similar visibility of nucleus and cytoplasm (Fig. 3b). Fe speckles were again rougher and more dominant than Zn speckles present in the cytoplasm, and likewise low concentration of Ca could be detected in the cytoplasm. Interestingly, the cytoplasm of these briefly stimulated neutrophils revealed void vacuoles with visibly reduced concentrations of Ca, Fe, Zn, and Cu. Neutrophils are known to generate

cytoplasmic membrane vacuoles upon strong stimulation. These vacuoles are formed by the internalization of extracellular liquid (pinocytosis) and show similarities to phagosomes.⁴¹ Thus, the low metal content in voids of PMA-stimulated neutrophils is consistent with the metal composition of phagosomes, membrane-surrounded compartments which contain engulfed microbes and are also known to be low in Zn, Fe, and Mn⁷.

At 2 h and 3 h *post* stimulation, the morphology of the nucleus as well as its inner elemental distribution was altered tremendously (Fig. 3c and d). The lobulated shape of the nucleus disappeared and yet, enrichment of Ca, Mn, and Zn









Our SR-XRF analysis revealed that typical morphological features of neutrophils are also reflected in the spatial distribution of elements. The most striking features of resting neutrophils were the lobulated nucleus and speckles that we identified as granules. Those granules were enriched in Fe and Zn, and the signal from both elements did not overlap completely. Indeed, neutrophils

In addition to Zn, Fe, Cu, and Mn, we also detected a group of metals that are less renowned for their biological function or mostly described for their toxicity: Co, Ni, Pb, and Sr. All metals besides Sr were found to be enriched in the nucleus of neutrophils; Sr was present at higher concentration in the cytoplasm. Interestingly, Ca, which behaves very similar to Sr,

was enriched in the nucleus, and showed lower abundance in the cytoplasm. Due to the similarities of Ca and Sr, Sr accumulates in bones of exposed humans. Co is an essential trace element, but also toxic at high concentrations. It is only found in humans as part of the cofactor cobalamin or vitamin B12 (VitB12), which is exclusively produced by commensal bacteria in the gastro-intestinal tract. Amongst the small number of VitB12-binding proteins in humans, transcobalamin I was identified in neutrophils.⁴⁸ The protein is localized in secondary granules and can be secreted to bind VitB12. Thus, transcobalamin I is not necessarily saturated with VitB12 in the granules. To our knowledge, no nuclear cobalamin-containing enzymes have been described, yet. Nevertheless, it was shown that human keratinocytes for instance accumulate Co in the nucleus and perinuclear areas.³² Ni, one of the most toxic of these metals, has no known biological function in eukaryotes, but is found in a variety of prokaryotic enzymes. Pb is a known environmental pollutant that accumulates in our bodies in many organs, most persistently in the bones. Notably, all 4 elements have been detected in whole blood of healthy individuals that were considered “free of exposure” to any heavy metal contamination.^{49,50} Since the majority of all white blood cells are neutrophils, it seems reasonable to assume that neutrophils contributed to this detection in whole blood. As all blood cells are generated in the bone marrow, neutrophils are unlikely the only blood cells to contain Co, Ni, Pb, and Sr. In conclusion, we have detected Co, Ni, Sr, and Pb in isolated neutrophils from two individuals living under supposedly non-exposed conditions. This data indicates an accumulation of Co, Ni, and Pb in the nucleus, whereas Sr is present in the vicinity of the outer cell membrane in unstimulated neutrophils.

In the present study, we used two different approaches for SR-XRF with their respective benefits and disadvantages: Sectioned samples analyzed at nanoscopic resolution allowed us to have highly detailed insights into intact neutrophils and compare the areal concentration in nucleus and cytoplasm. In contrast, the microscopic-scaled ‘whole-cell’ approach enabled us to quantify the neutrophil metallome and analyze element distributions of neutrophils forming NETs (both intermediate and final stages of NETosis). Substantial benefits of SR-XRF are (1) the extreme sensitivity, with the potential of single atom detection at future nano-beam facilities for transition metals; (2) the minimal sample preparation required: cells do not need to be lysed and diluted – factors that might affect the reliability – in any other way than the final fixation; (3) the potentially non-destructive character: under right measuring conditions samples can be stored and re-analyzed at later periods; and (4) the spatial information: individual cells meeting all requirements can be selected and analyzed with submicron resolution. Artifacts influencing the determined metal distributions are therefore, assuming an appropriate sample preparation method and experimental design, rather unlikely. As described, we utilized SR-XRF to determine the neutrophil metallome to complement our measurement using ICP-MS. Due to the time consuming character of SR-XRF, we only analyzed three individual cells, whereas for ICP-MS each sample contained 10^7 neutrophils. This is, together with the distinct sample preparations, most likely

the major reason for the deviations between the results of the two different methods. Moreover, uncertainties in calibration and interfering background signals are factors which might influence Fe concentration measurement using SR-XRF. Quantification of Fe by ICP-MS on the contrary, might be hampered by spectral interferences.

Nevertheless, the usage of SR-XRF in actual *in vitro* infections or sections from *in vivo* infection (e.g. as tissue sections) seems very promising. Earlier studies have demonstrated, for instance, the cellular reduction of Fe and Zn in *Histoplasma*-infected macrophages⁵¹ or the specific enrichment of Cu in the phagolysosome containing *Mycobacteria*.⁵² These and similar phenomena are ideal candidates for SR-XRF analysis at the nanoscale – adding new information about elemental distribution and concentrations. Meticulously describing the trace element composition during different stages of infection within affected organs might even identify new targets for antimicrobial therapy. Trace elements are essential for microbial growth and their removal could be a potential treatment for infections. The lack of certain metals cannot be easily overcome by mutagenesis leading to resistance, which makes it a promising target for further research.

Materials & methods

Chemicals and solutions

Unless stated otherwise, chemicals and solutions were purchased from Sigma-Aldrich.

Isolation of neutrophils from venous blood

Neutrophils were isolated from human venous blood by using an isolation technique that combines separation on Histopaque-1119 (Sigma-Aldrich) and Percoll (GE Healthcare). Venous blood from human donors was received upon fully informed written consent according to the guidelines of the local ethical committee (Regionala etikprövningsnämnden i Umeå). Briefly, fresh blood was layered in a 1:1 ratio on top of Histopaque-1119, followed by centrifugation at $800 \times g$ for 20 min. The resulting white-blood-cell containing phase was demounted and cells were washed by adding 5–6-fold volume of PBS with 0.5% human serum albumin (HSA). Cells were pelleted by centrifugation for 5 min at $300 \times g$. The cell pellet was resuspended and applied onto a discontinuous gradient of phosphate-buffered Percoll with the concentrations 85%, 80%, 75%, 70% and 65%. Cell separation occurred on the gradient during centrifugation at $800 \times g$ for 20 min. Cells accumulated at the interphase between 75% and 70% were collected and washed as described before. Finally, cells were taken up in RPMI medium (Lonza).

Neutrophil lysate preparation for ICP-MS

Freshly isolated neutrophils from 8 different healthy donors were lysed. For each sample, 10^7 neutrophils were taken up in 100 μ l HEPES-buffered RPMI and NaOH (99.99% metals basis, semiconductor grade) was added to reach 0.25 M in 200 μ l. This mixture was incubated for 2 h at 65 °C with vigorous shaking. Subsequently, lysates were sonicated for 1 h in a sonification bath.



X-ray focusing was obtained by a crossed elliptical Rh coated graded-multilayer mirror-pair in the Kirkpatrick–Baez (KB) geometry. X-rays are collected and focused in both vertical and horizontal axis at a glancing angle (<3.5 mrad). The first mirror, coated with a graded multilayer plays both the role of vertical focusing device and monochromator, resulting in a very high flux (exceeding 10^{11} photons per s) and a medium monochromaticity ($\Delta E/E \approx 10^{-2}$). In our case the beam size was determined by knife-edge scans of a Au test pattern and determined to be 64 nm vertically and 54 nm horizontally at an excitation energy of 17 keV.

Live cell imaging of Zn pool during NET formation

Human neutrophils (5×10^6), stained with 20 μM FluoZin-3 (Life Technologies), were seeded into a 35 mm glass bottom micro-well dish (MatTek, Ashland, MA, USA). Neutrophils were stimulated with 100 nM PMA and kept at cell-culture conditions throughout the entire measurement (5% CO_2 and 37 $^\circ\text{C}$). Pictures, phase contrast and green fluorescence, were taken using a 60 $\times$ objective every 2 min for a period of 2.5 h (beginning at 15 min post stimulation) using a Nikon eclipse Ti live-cell microscope operated by NIS-Elements AR 3.2. software.

Quantification of SR-XRF measurements and image segmentation

All quantitative results shown were obtained by measuring NIST standard reference materials (SRMs). For the ID22NI measurements (ESRF, Grenoble, France), a total amount of 17.4 mg powder of NIST SRM 1577c (bovine liver) was pressed into a self-supporting pellet of 13 mm diameter resulting in an areal density of 13.11 mg cm^{-2} . The pellet was measured for 1100 s using a raster scan to compensate for possible inhomogeneities. For the P06 beamline measurements (PETRA III, Hamburg, Germany), a NIST SRM 613 (trace elements in glass) and a MPI DING ATHO standard (both prepared as flat samples with a thickness of 100 μm) were measured for 1100 seconds using the same measuring strategy described previously. The obtained spectra were deconvoluted using analysis of X-ray spectra using Iterative Least squares AXIL⁵³ to obtain the net line intensities, which were then normalised to the incoming photon flux, corrected for detector dead time and for self absorption effects. Spectral data was then batch processed using an inhouse developed software package called MICROXRF2. Manual image segmentation (or clustering) was performed on the obtained element maps of the unstimulated neutrophils, indicating nucleus, cytoplasm and cell. The individual point spectra belonging to a single segment were then summed and net line intensities of the segment were obtained using AXIL.

Quantification was based on a fundamental parameter approach which exploits the theoretical relation between the net-line intensities and the elemental concentrations (Sherman, 1955). The elemental yields of the elements of interest were calculated from the measured standard reference material taking into account: (1) variations in incoming beam intensity (related to varying synchrotron ring current and small variations in alignment of beamline optical components) by using ionisation chamber

values, (2) the use of absorbers (1500 μm Al in case of NIST SRM 1577c) and (3) detector dead time (registered for every scanned point).

The elemental intensity maps were converted into areal concentration distributions (expressed in $\mu\text{g cm}^{-2}$) by dividing the pixel values in the normalised elemental maps (normalised to dead time and storage ring current) by the areal elemental yields. In order to determine the so-called “relative enrichment factors” for the relevant elements in the nucleus *versus* the cytoplasm, the sum spectra of the nucleus and the cytoplasm were first normalised to the Compton scattering peak and the ratios of the relevant elemental intensities were taken.

Statistical analysis

Unless stated otherwise, all statistical calculations have been performed using Microsoft Excel. Further statistical analysis was performed using Graphpad Prism Software 5. For the quantification of NETs supernatants, data was fitted using a non-linear Michaelis–Menten fit.

Acknowledgements

We would like to thank Quinten Remijnsen for the help during the neutrophil stimulation experiment (Postdoctoral fellow FWO, VIB Department of Molecular Biomedical Research, Ghent University), Jaime Segura, Gema Martinez-Criado (ID22NI beam-line staff, ESRF) and Ulrike Boesenberg (P06, Petra III, DESY) for their excellent technical support during the measurements, Maximillian Popp for support during ICP-MS analyses, Roland Nordfeldt for technical support, and also Lena Meyer and Patrick Engström for providing cultured cells. This work was supported by grants for C. F. Urban from the Swedish Research Council VR-M (2011-2393), the Laboratory for Molecular Medicine Sweden (MIMS), the Medial Faculty Umeå (316-886-10) and the Åke Wiberg Foundation (3772734). M. J. Niemiec acknowledges financial support from the J. C. Kempe Memorial Fund. B. De Samber receives a postdoctoral grant from the Fund for Scientific Research Flanders (FWO Vlaanderen, application number 12B3313N). Both E. Vergucht and J. Garrovoet are funded by PhD grants from the Flemish Institute for the Promotion of Scientific and Technological Research in Industry (IWT, Flanders, Belgium). Financial support by the Fund for Scientific Research Flanders (FWO Vlaanderen, Belgium) contract no. G.0395.11 & Big Science program G.0C12.13 is acknowledged.

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