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

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Abstract

19 Every infection is a battle for trace elements. Neutrophils migrate first to the infection

20 site and accumulate quickly to high numbers. They fight pathogens by phagocytosis

21 and intracellular toxication. Additionally, neutrophils form neutrophil extracellular traps

22 (NETs) to inhibit extracellular microbes. Yet, neutrophil trace element characteristics

23 are largely unexplored. We investigated unstimulated and phorbol myristate acetate-

24 stimulated neutrophils using synchrotron radiation X-ray fluorescence (SR-XRF) on

25 the sub-micron spatial resolution level. PMA activates pinocytosis, cytoskeletal

26 rearrangements and the release of NETs, all mechanisms deployed by neutrophils to

27 combat infection.

28 By analyzing Zn, Fe, Cu, Mn, P, S, and Ca, not only the nucleus but also vesicular

29 granules were identifiable in the elemental maps. Inductively Coupled Plasma Mass

30 Spectrometry (ICP-MS) revealed a neutrophil-specific composition of Zn, Fe, Cu, and

31 Mn in comparison with J774 and HeLa cells, indicating a neutrophil specific

32 metallome complying with their designated functions.

33 When investigating PMA-activated neutrophils, the SR-XRF analysis depicted typical

34 subcellular morphological changes: the transformation of nucleus and granules and

35 the emergence of void vacuoles. Mature NETs were evenly composed of Fe, P, S,

36 and Ca with occasional hot spots containing Zn, Fe, and Ca. An ICP-MS-based

37 quantification of NETs supernatants revealed a NETosis-induced decrease of soluble

38 Zn, whereas Fe, Cu, and Mn concentrations were only slightly affected.

39 In summary, we present a combination of SR-XRF and ICP-MS as a powerful tool to

40 analyze trace elements in human neutrophils. The approach will be applicable and

41 valuable to numerous aspects of nutritional immunity.

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Introduction

45 Metal ions play an important role in many biological processes. They catalyze
46 reactions, stabilize structures, and act during gene regulation or cell signaling [1-5].
47 Their usage is conserved in prokaryotes and eukaryotes [6]. During infections, when
48 the host encounters microbial invaders, metal ions are essential for survival of both
49 parties. The host therefore restricts the availability of metal ions in order to avoid
50 microbial growth – a strategy named "nutritional immunity" and described in animals
51 and plants [7]. In response, many pathogens have evolved sophisticated tools to
52 chelate metal ions from the host and these tools are targeted by the host in return.
53 This interplay illustrates co-evolution of host and pathogen and is best exemplified by
54 the well-studied fight for Fe [8]: In the host, Fe is mainly kept intracellular - free Fe is
55 essentially unavailable in blood or other extracellular liquids. It is bound to transferrin
56 in plasma and lactoferrin in external secretions. Microbial pathogens have two
57 mechanisms to cope with these limitations. In case of direct contact between the host
58 Fe source and the pathogen, host Fe-retaining proteins are modified to release their
59 Fe load into the extracellular environment for uptake. Secondly, many bacteria and
60 fungi secrete siderophores, small molecules with exceptionally high Fe affinity that
61 free Fe from host proteins followed by resorption into the pathogenic cell. As a
62 response, mammals produce lipocalin-2, to intercept siderophores. This can in turn
63 be counteracted by many pathogens which disguise their siderophores by
64 glycosylation [8].

65 Fe is not the only transition metal important in nutritional immunity. Zn, Mn and Cu
66 are also shown to be critical [9, 10]. Especially Zn has been shown to be crucial during
67 fungal pathogenicity [11]. This can be explained by its unique biochemical features
68 which are reflected in the utilization in different organisms. Zn is a strong Lewis-acid,
69 water-soluble at the oxidized state +II and also redox-stable under standard
70 physiological conditions [12]. About 7% of the entire proteome of archae, bacteria and
71 eukayotes are predicted to be Zn proteins[13]. In addition to the enzymatic function in
72 peptidases, amidases and esterases – conserved to similar extent in all kingdoms –
73 Zn functions also in Zn-finger proteins to mediate binding to DNA and RNA. Whereas
74 bacteria and archae have less than 0.5% of Zn-finger proteins, eukaryotes have
75 about 3% - so almost half of the entity of their Zn proteome [13]. In agreement with this,

fungi are especially susceptible to Zn depletion. Shortage of Zn has been shown to cause intracellular oxidative stress in yeast [14]. Also, the fungal pathogen *Candida albicans* was found to be completely growth arrested, if the surrounding concentration of Zn drops below 1 µM [15].

Neutrophils play a crucial role in the immune defense against fungal and bacterial pathogens. Once a pathogen passes the mechanical barrier formed by epithelial cells and penetrates into the underlying tissue, surrounding cells sense the pathogen and send signals that recruit neutrophils to the site of infection. Since neutrophils circulate in a ready-to-use state, influx into tissue can occur fast and exceedingly [16]. Upon contact with invaded microbes, neutrophils fight pathogens in a well-orchestrated sequence of recognition, phagocytosis, intra- and extracellular toxification and local retainment [17].

These functions are all facilitated by the unique morphology of neutrophils. The nucleus is lobulated, allowing the cell to squeeze through layers of tissue. High numbers of granules store antimicrobial peptides and proteins that can be either secreted into the surrounding of the neutrophils or into the phagolysosome [18]. The latter is a highly inhospitable cellular compartment that retains the phagocytosed pathogen. Inside, the pathogen encounters not only antimicrobial peptides and proteins, but also low pH and reactive oxygen and nitrogen species [19]. In addition to secretion and phagocytosis, neutrophils form also extracellular traps (NETs) [20]. During NET formation, the nuclear membrane followed by granule membranes and finally the cytoplasmic membrane lose integrity. This allows compartments to mix that are usually separated. NETs consist of DNA, histones, and cytoplasmic and granular proteins [21].

Calprotectin is one of the NET effector proteins [22]. It is the most abundant protein in the neutrophilic cytoplasm, usually not secreted by intact neutrophils, but released during NET formation. Calprotectin is crucial for the NETs efficacy towards bacterial and fungal pathogens, which is due to its Mn and Zn chelating properties [22-25]. It has been shown earlier that NET-mediated growth inhibition of *Candida albicans* and *Aspergillus nidulans* could be reversed by the addition of Zn. Whether NET formation, a process of complete cell burst, actually reduces the availability of Zn for the fungal pathogen remains unexplored.

108   On these grounds, we aimed to elucidate the trace elemental properties of human

109   neutrophils – resting and activated. To do so, we combined SR micro/nano-XRF,

110   ICP-MS, and live cell imaging. The sample preparation for the SR-XRF

111   measurements was adjusted to optimize the neutrophil analysis: In order to quantify

112   whole neutrophils and analyze filamentous NETs with optimally preserved metal

113   contents, entire cells were seeded on a Si_3N_4 wafer and freeze-dried. To investigate

114   neutrophils with the highest possible subcellular resolution and analyze intracellular

115   metal concentrations, neutrophils were high-pressure frozen (HPF), cryosubstituted

116   and sliced in 2 µm thin sections. Using ICP-MS, we quantified neutrophil

117   supernatants *post* NET formation and neutrophil full cell lysates for total metal

118   content. Lastly, we investigated the distribution of labile Zn in living cells by applying

119   the fluorophore FluoZin in activated neutrophils.

120   In summary, we successfully analyzed the trace element distributions of human

121   neutrophils down to nanoscopic levels using SR-XRF complemented with ICP-MS

122   analysis and live cell imaging. While ICP-MS is a broadly used technique, reports of

123   SR-XRF for analysis of biological samples are still scarce [26-28]. To our knowledge,

124   this is the first study to use SR-XRF at the micro- and nanoscopic level on individual

125   innate immune cells. Earlier work included for instance single neurons and

126   keratinocytes, but also a variety of other biological sample fluids, tissues and even

127   whole small organisms like mussel larvae or the water flea [29-35]. SR-XRF offers

128   spatially resolved (down to 50 nm resolution) and quantitative insights (sub-ppm

129   level) that can contribute to the in-depth understanding of the site of infection as a

130   nutritional niche. The approach presented will be applicable and valuable to

131   numerous aspects of nutritional immunity.

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Results

Trace-level metallome of human neutrophils determined by SR-XRF and ICP-MS

High influx of neutrophils to the site of infection very likely affects the local metal environment. In this context, transition metals are particularly important for nutritional immunity. We measured the neutrophil suspension diameter as 8.85 ± 0.44 μm and using this value calculated the total cellular concentration of metal ions assuming an even spherical shape of the cells, resulting in a cell volume of approx. 363 μm^3. We used ICP-MS to analyze the neutrophil's total content of Zn, Fe, Cu, and Mn. Neutrophil lysates from 10^7 cells per sample were conducted to ICP-MS, resulting in the following metallome: freshly isolated human neutrophils were found to contain $6.07 \pm 1.29 \times 10^7$ Zn atoms, $1.64 \pm 0.33 \times 10^8$ Fe atoms, $2.26 \pm 0.69 \times 10^6$ Cu atoms, and $2.49 \pm 0.69 \times 10^5$ Mn atoms (Tab. 1). The total cellular concentration was calculated similarly: [Zn] = 275.31 ± 58.61 μM, [Fe] = 743.91 ± 148.62 μM, [Cu] = 10.24 ± 3.11 μM, and [Mn] = 1.13 ± 0.31 μM (Tab. 1). To obtain information on the spatial distribution of trace-level metals within neutrophils we scanned whole cells using micro/nanobeam SR-XRF, fitted the individual point spectra and generated the elemental maps. In addition to the information on spatial distribution, XRF was used to quantitatively determine the neutrophil metallome using an ICP-MS-independent method. The total XRF spectrum of a single neutrophil was obtained by region-of-interest selection and summing up the individual XRF spectra belonging to the single cell, followed by quantification of the cell sum spectrum using a glass trace-element calibration standard. Based on the measurements of three individual freeze-dried neutrophils, an average neutrophil was determined to contain $6.94 \pm 0.86 \times 10^7$ Zn atoms, $9.38 \pm 1.11 \times 10^6$ Fe atoms, $1.38 \pm 0.18 \times 10^7$ Cu atoms, and $8.73 \pm 1.76 \times 10^5$ Mn atoms (Tab. S1). Accordingly, the total cellular concentrations were calculated: [Zn] = 317.35 ± 39.17 μM, [Fe] =42.91 ± 5.09 μM, [Cu] = 63.27 ± 8.17 μM, and [Mn] = 3.99 ± 0.80 μM (Tab. S1). In comparison, the two different techniques resulted in partially different neutrophil metallomes. The determined concentration of cellular Zn was consistent between the two techniques. Zn is known to have total cellular concentrations of 0.1-0.5 mM, so both neutrophil concentrations determined are also well in range with other mammalian cells [12]. SR-XRF determined total metal

167 contents were approximately 3 and 6-fold higher than ICP-MS for Mn and Cu,

168 respectively. Finally, the cellular Fe level ICP-MS quantification resulted in an

169 approximately 17-fold higher value than XRF. From this point on, we use and refer to

170 the ICP-MS metallome, due to a higher statistical certainty – we used 8 different

171 donors and 10^7 cells per sample.

172 To test the hypothesis that neutrophils might be purposely low in metal ions to

173 compensate for their high influx to infection sites, we included two other mammalian

174 cells frequently used in molecular infection research: macrophage-like J774 cells and

175 HeLa cells. Since both cells are larger than neutrophils, we were not surprised to find

176 a higher total metal content for J774 and HeLa cells. In suspension, J774 cells have

177 a diameter of 15.8 µm; HeLa cells have a diameter of 22 µm [36, 37]. Accordingly, the

178 volumes of these cells differ greatly. The calculated volumes were 363 µm^3, 2065

179 µm^3, and 5575 µm^3 for neutrophils, J774 and HeLa cells, respectively. When the

180 metal content was normalized to cell volume, we found J774 cells to be slightly richer

181 in Zn and Cu, much richer in Mn, and poorer in Fe. HeLa cells had a similar Zn and

182 Cu density, slightly increased Mn content, and a lower level of Fe as well (Fig. 1).

183 This data indicates that neutrophils are higher in Fe than the other two cell types

184 analyzed.

185

186 ***Distribution of P, S, Ca, Zn, Fe, Cu, and Mn in human resting neutrophils***

187 ***(obtained at P06 beamline, PETRA III)***

188 SR-XRF measurements were performed at the P06 Hard X-ray Microprobe (PETRA

189 III, Hamburg, Germany) providing at the time of the measurement a beam size of 0.5

190 µm (vert.) x 0.4 µm (hor.) FWHM. The scanning step size was typically 1 µm

191 (compromise between covering a large area for NET visualization and sufficient

192 resolution). Freshly isolated neutrophils were seeded onto Si_3N_4 wafers, plunge-

193 frozen in liquid ethane, and finally freeze-dried. Freeze-drying of whole cells was

194 chosen to preserve elemental integrity - at the expense of morphology collapse [33];

195 entire cells were analyzed in this 'whole-cell' set-up. Neutrophils of interest were

196 selected based on their light-microscopic appearance and then analyzed by SR-XRF

197 – representative images are discussed in the following (Fig. 2A). Strikingly, the most

198 prominent feature of neutrophils - the lobulated nucleus – stood out against the

199 cytoplasm by an increased signal from P and Zn. The cytoplasm displayed areal

200 concentrations above background level for the elements P, S, Zn, and Fe. Several

201 potential contaminations were found, one of them in close proximity to the nucleus,

202 slightly overlaying the neutrophil. In this and in randomly distributed 'speckles' on the

203 entire sample, we detected P, S, Ca, and Fe. To complement our observations, we

204 aimed to quantify the trace element content of nucleus and cytoplasm. Since surface-

205 attached neutrophils vary in thickness and total mass per area was not determined,

206 average concentrations of metals in nucleus and cytoplasm could only be determined

207 in approximation (Tab. S2). We found a higher average areal concentration of Zn and

208 Fe in the nucleus as compared to the cytoplasm. Cu had a very low, but almost equal

209 areal concentration in nucleus and cytoplasm (with the nucleus being thicker), while

210 Mn was found to show higher areal concentrations in the cytoplasm.

211

212 ***Distribution of P, S, Ca, Zn, Fe, Cu, and Mn in activated human neutrophils and***

213 ***NETs (obtained at P06 beamline, PETRA III)***

214 Similarly to the previous section ('whole-cell' set-up), freshly isolated neutrophils were

215 seeded directly onto Si_3N_4 wafers and stimulated with phorbol-myristate acetate

216 (PMA) to induce ROS production, internalization events and NET formation. After 2 h

217 and 4 h, the samples were plunge-frozen and freeze-dried to preserve morphology.

218 Unstimulated neutrophils were also included (see previous section). A 4 h stimulation

219 with PMA is sufficient to cause a nearly full conversion of neutrophils to NETs [21, 38].

220 The 2 h stimulation represents an intermediate stage (Fig. 2B). As mentioned, the

221 analysis of unstimulated neutrophils revealed clear differences between the nucleus

222 and the cytoplasm. After 2 h stimulation, the morphology of the neutrophils changed

223 considerably, but not in a perfectly synchronized fashion. Some neutrophils engaged

224 in the process of NET formation earlier than others. The progress of NET formation is

225 most apparent when following the nucleus. In early NET formation, the nucleus loses

226 its lobulation and shrinks to form a smaller and round shape (Supplement, S3, * + a).

227 The lower neutrophil #1 is representative of this stage (Fig. 2B). As in unstimulated

228 neutrophils, the nucleus contained a higher areal concentration of P and Zn, whereas

229 the cytoplasm featured a homogeneous distribution of P and S (higher areal

230 concentration than background level). Interestingly, the intact stimulated neutrophil

231 also showed the presence of an Fe rich region, distributed in a ring-like manner in the

232 cell periphery. The upper neutrophil #2 is representative of a later stage of NET

233 formation, characterized by the loss of membrane integrity, first of the nucleus, then

234 of the cytoplasmic membrane – the cell opens up and lyses (Supplement, S3, b + c).

235   When comparing the light microscope images of neutrophil #2 with neutrophil #1, we

236   observe that from the area initially covered by neutrophil #1, only cell residues in

237   neutrophil #2 remain visible. The SR-XRF elemental maps confirm that the upper cell

238   has likely lysed: within the area of the cell (shape of an inverted heart or leaf), we

239   could no longer observe the homogeneous distribution of P and S. In contrast, the

240   remaining cell debris was enriched in P, S, Ca, Fe, and Zn. A hotspot containing not

241   only these elements, but also Cu and Mn was found to be within the area of

242   neutrophil #2. This is most likely a non-biological contamination. Short fiber-like

243   structures, which we identified as NETs, are protruding out of the cell debris. Areal

244   concentrations for P, S, Zn, and Fe were found to be above background level. Similar

245   to the unstimulated sample, several 'hot-spots' (1-4 µm size) containing P, S and Ca

246   are present which could be cell remnants. After 4 h of stimulation, NET formation was

247   completed and no intact neutrophils were remaining (Fig. 2C). The typical orbital

248   patches of cellular debris were as prominent as long filamentous fibers of NETs with

249   a thickness at least below 1 µm (estimation of size was limited by the step size of the

250   scanning stages). The SR-XRF analysis clearly showed the NETs structure in the

251   elemental maps of P, S, Ca and Fe. No strong accumulation of Zn could be detected

252   in NETs, which is remarkable considering the high intracellular content of Zn in the

253   neutrophil. This indicates that freshly releases NETs, under the given cell culture

254   conditions with very low medium concentrations of Zn, are not loaded with Zn, yet. As

255   mentioned, we observed high local Ca and Fe concentrations in NETs which were

256   not detected within intact neutrophils. This finding correlates well with rather high

257   concentrations of Ca (400 µM Ca, according to manufacturer's information) and Fe

258   (0.7 µM according to ICP-MS quantification, data not shown) in the surrounding

259   medium. Several Ca, Fe and Zn rich spots having varying XRF intensity were

260   detected in the vicinity of the NET structures. Consulting the microscopy images,

261   these could be contaminations, cell debris or uneven twisting of numerous NETs from

262   numerous neutrophils. In summary, SR-XRF scans providing elemental distributions

263   reconstitute the typical architecture of neutrophils at the microscopic level and

264   allowed discrimination of nucleus, cytoplasm and far-reaching (up to 100 µm) NETs.

265   Strikingly, cellular morphology changes accompanying NET formation and even NET

266   filaments were visible in the element maps.

267

Trace level metal distribution of nucleus and cytoplasm in resting neutrophils
(obtained at ID22NI beamline, ESRF)

For the analysis of human neutrophils at the ID22NI (nano-imaging) beamline (European Synchrotron Radiation Facility (ESRF), Grenoble, France), cells were seeded onto sapphire disks, conducted to high pressure freezing (HPF) and cryo-substituted into resin before being sliced into 2 µm thin sections. We will refer to this as the 'sectioned' approach. In order to slice neutrophils at this scale, fixation and embedding were inevitable [31]. We minimized the procedure's impact on element integrity by including wash steps and reducing exposure time to potential contamination sources. The beam size was estimated to be 64 nm (vert.) by 54 nm (hor.) and the step size of the scans was 50 nm. Images of representative cells are discussed in the following.

As observed before, the neutrophil nucleus stood out due to its distinct increase in metal content relative to the cytoplasm. Remarkably, the higher resolution revealed more subcellular detail than observed in the previous analysis (Fig. 3A). As a morphology reference, we used corresponding sections from the same sample imaged with transmission electron microscopy. The typical lobulated nucleus of an unstimulated neutrophil was distinguishable by increased areal concentrations of P, Ca, Zn, Fe, and Mn. These elements were not evenly distributed within the nucleus, but seemed to be more abundant in the outer region, closer to the nuclear membrane. The pattern is reminiscent of hetero- and euchromatin. Additionally, the Fe distribution showed round patches with increased Fe intensity, which might be nucleoli, the largest structures within the nucleus. Interestingly, S and Cu displayed an inverted pattern compared to the other elements: lowest intensity in peripheral regions of the nucleus and presence in higher amounts within inner regions and cell cytoplasm. As we find both elements also in the surrounding matrix material, their presence in the cytoplasm must be considered very cautiously. Then again, a higher concentration of S in the cytoplasm is consistent with the high prevalence of reducing, S-containing, molecules and proteins in the cytosol [39]. The presence of Cu in the neutrophil cytoplasm appears likely, as Cu is an essential trace element [4]. In the periphery of the cells (at the edges of the scans), we observed a halo-like area of higher S and Cu concentration. This halo in the S and Cu maps very likely originates from S and Cu presence in the embedding resin. In areas with low biological mass, the beam-induced warm up of the samples probably caused the resin to shrink, and

302 therefore locally increased the concentration of elements in the resin. In the

303 cytoplasm, Ca was homogenously present, whereas Fe and Zn showed a speckled

304 pattern, most probably resulting from neutrophil-characteristic granules loaded with

305 antimicrobial proteins. The speckles had a average size of 200 nm, which matches

306 earlier studies that have shown granule size varying between 0.14 μm and 0.29 μm

307 [40]. The Zn speckles were generally smaller in size than those formed by Fe. An

308 overlay of the Zn and Fe elemental maps revealed that the granular speckles did not

309 always co-localize, which indicates the presence of a variety of granules. For our

310 comparative analysis of nucleus and cytoplasm (Tab. 2), the speckles (potentially

311 granular) or subnuclear structures were too small to be excluded from the cytoplasm.

312 Since 2 μm thin sections of single neutrophils were analyzed, the integration of the

313 total illuminated mass signal of the entire cell depth along the beam path can be

314 ruled out. Average areal concentrations of P, S, Ca, Zn, Fe, Cu, and Mn confirm our

315 previous observation that most elements were preferentially enriched in nucleus or

316 cytoplasm – only Fe seemed to be almost evenly distributed. In order to obtain

317 quantitative insight in the inhomogeneous presence of metals in the nucleus as

318 compared to the cytoplasm, we calculated a so-called 'enrichment factor': P (50.11 x)

319 > Zn (3.19 x) > Ca (2.83 x) > Mn (3.98 x) > Fe (1.29 x). For the less abundant

320 elements we found: S (0.47 x) < Cu (0.63 x). In summary, these data indicate higher

321 concentrations of P, Zn, Ca, and Mn, as well as lower concentrations of S and Cu in

322 the nucleus as compared to the cytoplasm.

323 Clearly, the measurements at ID22NI allowed the detection and spatial analysis with

324 higher sensitivity than SR-XRF measurements at PETRAIII. The major differences

325 between the two experiments and results in spatial distribution are mentioned in the

326 text where appropriate and were additionally summarized in a comparing table (Tab.

327 S4).

328

329 ***Nanoscale elemental distributions of P, S, Ca, Zn, Fe, Cu, and Mn in activated***

330 ***human neutrophils (obtained at ID22NI beamline, ESRF)***

331 Similar to unstimulated cell preparations, cells were seeded on sapphire disks and,

332 apart from PMA stimulation, prepared identically. However, more time points were

333 included in order to relate cellular changes during activation and NET formation with

334 differences in metal distribution.

335 Neutrophils activated for 1 h showed similar metal distributions of P, S, Ca, Zn, Fe,
336 Cu and Mn resulting in similar visibility of nucleus and cytoplasm (Fig. 3B). Fe
337 speckles were again rougher and more dominant than Zn speckles present in the
338 cytoplasm, and likewise low concentration of Ca could be detected in the cytoplasm.
339 Interestingly, the cytoplasm of these briefly stimulated neutrophils revealed void
340 vacuoles with visibly reduced concentrations of Ca, Fe, Zn, and Cu. Neutrophils are
341 known to generate cytoplasmic membrane vacuoles upon strong stimulation. These
342 vacuoles are formed by the internalization of extracellular liquid (pinocytosis) and
343 show similarities to phagosomes [41]. Thus, the low metal content in voids of PMA-
344 stimulated neutrophils are consistent with the metal composition of phagosomes,
345 membrane-surrounded compartments which contain engulfed microbes and are also
346 known to be low in Zn, Fe, and Mn [7]. At 2 h and 3 h *post stimulation*, the morphology
347 of the nucleus as well as its inner elemental distribution was altered tremendously
348 (Fig. 3C + 3D). The lobulated shape of the nucleus disappeared and yet, enrichment
349 of Ca, Mn, and Zn (and P) was still observed. The distribution of those elements
350 within the nucleus however, was more homogenous than in nuclei from resting cells.
351 This indicates that nuclei might have remained intact to this point, whereas
352 separation of inner and outer nuclear region was lost. The cytoplasmic membrane,
353 depicted by the Zn element map in resting cells, disappeared at these later stages.
354 Even after a stimulation of 3 h, void vacuoles characterized by the strongly reduced
355 intensity of Zn and Fe were still present, at these stages in close proximity to the
356 nuclei and smaller than during earlier stages. Finally, after 4 h of stimulation, it was
357 increasingly difficult to locate nuclei or whole cells (Fig. S5). The intensities in all
358 elemental maps were close to the background level probably caused by the cell burst
359 spreading the intracellular content over a larger volume and therefore decreasing the
360 areal elemental concentration. In summary, synchrotron XRF analysis with a spatial
361 resolution at the 50 nm level revealed the elemental composition of neutrophil
362 nucleus and cytoplasm and enabled detection of nuclear structures as well as
363 cytoplasmic granules based on distinct element signals.

364

365 ***Sample radiation damage and detection of NETs using SR-XRF***

366 We believe that the conditions for observing NETs in the high resolution (ID22NI)
367 elemental maps starting from 2 h *post activation* are particularly challenging due to
368 the following reasons. First of all, later stages of NET formation are accompanied by

369 the loss of cell integrity, resulting in decreased areal concentrations of metals,

370 resulting in higher signal-to-noise ratio. A second reason is the more pronounced

371 sample radiation damage due to photo and auger electrons, depositing their energy

372 locally. In Fig. 3D and S5, the sample radiation damage occurring in the embedding

373 resin was surprisingly observed as a flame-like pattern, especially in the Cu map, of

374 neutrophils stimulated for 3 h and 4 h. Third, as the sections used in this experiment

375 were only 2 µm thick, NETs are sectioned in a random plane and therefore easily

376 overlooked by 2D nano-XRF analysis, as their diameter would only encompass a few

377 pixels, randomly located within the elemental map. Fourth, the scanning area of

378 interest was defined by light microscopy, which is slightly biased in favor of visible

379 and integer looking cells.

380 In contrast, conditions for observing NETs in the freeze-dried samples (beamline

381 P06, PETRA III) scanned with 1 µm step size were more favourable due to the

382 following differences and changes: First, freeze-dried cells are denser than

383 embedded cells resulting in less deposited energy and sample radiation damage.

384 Second, deposition of the entire three-dimensional cell structure onto a two-

385 dimensional wafer gathered all available NETs. And finally, the 'whole-cell' set-up

386 increased the areal elemental concentrations of the sample allowing the beam to

387 excite elements over the entire depth of the sample. Disadvantages are however the

388 lower preservation of the ultrastructure and difficulties in comparing areal

389 concentrations (Tab. S4).

390 In general, we can conclude that the combination of the two sample preparation

391 approaches: 'whole cell' (freeze-dried whole cells analyzed at the microscale) and

392 'sectioned' (shock-frozen, embedded and sectioned cells) were crucial to meet the

393 very different needs of resting and activated neutrophils and NETs.

394

395 ***Presence of Co, Ni, Sr, and Pb in human neutrophils***

396 It is a major advantage of SR-XRF compared to ICP-MS that it is unbiased regarding

397 the investigated elements – no standard of individual elements is required prior to

398 quantification. In agreement with this notion, we detected the elements Co, Ni, Pb

399 and Sr which are less-well established as biologically relevant components (Fig. S5).

400 Co, Ni, and Pb were accumulated in the nucleus and showed a distribution pattern

401 similar to Ca. In contrast, the concentration of Sr was only above background level

402 near the cytoplasm border. While the presence of Sr in biological samples is

403 expected - due to its chemical equivalence with Ca and accumulation in bone tissue

404 – the presence and distribution of the other elements was less anticipated.

405 Secondary effects in X-ray fluorescence can often be a source for misinterpretation of

406 results. In our case, a sudden increase in areal density of the sample (e.g. due to a

407 more dense nucleus) could have caused a local increase in scattered photons, which

408 may in turn generate additional fluorescence from the sample environment,

409 potentially resulting in an increased signal above the expected value. However, we

410 found little or no variation in the Compton scattering map, indicating that the areal

411 mass throughout scanned area around the neutrophil is nearly constant. Therefore,

412 even with a much higher presence of trace level metals in the nucleus, secondary

413 effects erroneously enhancing the detection of these metals can be excluded in our

414 case.

415

416 ***Effects of NET formation on the availability of Zn, Fe, Cu, and Mn***

417 NETs inhibit growth of and kill microbes. The NET-mediated growth inhibition of the

418 fungus *Candida albicans* can be overcome by the addition of Zn^{2+} to the medium [22]. It

419 is known that NETs contain calprotectin that chelates Zn [42]. To verify whether NET

420 formation indeed decreases the availability of Zn, even though the entire cell opens

421 up during this process, we analyzed the liquid and protein-filtered supernatant of

422 NETs. Freshly isolated neutrophils were stimulated with PMA for 4 h in the presence

423 of increasing Zn^{2+} concentrations up to 33 µM and the Zn^{2+} concentration of the

424 medium was quantified with ICP-MS before and after NET formation (Fig. 4). We

425 found the remaining Zn^{2+} concentration to be depending on the initial Zn^{2+}

426 concentration, following a saturation curve. With low initial Zn^{2+} concentrations up to

427 ca. 6 µM, the resulting Zn concentration was in the range of 0-51 % of the initial

428 concentration. At initial concentration beyond this, the resulting concentration

429 reached a maximum of approximately 63 % (Fig. 4). We conclude that NET formation

430 indeed reduces the availability of Zn and that there is specific binding at low to

431 medium (< 3-6 µM) and unspecific association at high Zn concentrations (>6 µM). We

432 additionally measured Fe, Cu, and Mn concentrations before and after NET formation

433 and detected only slight reductions of 9 %, 5 %, and 7 %, respectively (Tab. S7).

434

435 ***FluoZin-labeling of labile Zn pool during neutrophil activation***

436   The analysis of activated neutrophils using SR-XRF under routine conditions (i.e.

437   without combining it with e.g. X-ray absorption spectroscopic techniques,

438   XANES/EXAFS) is only providing element information. Therefore, distinguishing

439   between strongly and loosely bound Zn is not possible. Strongly bound Zn exists in

440   proteins of the Zn proteome, e.g. superoxide dismutase and ribosomes. Loosely

441   bound Zn, also called labile or "free" Zn, is associated with all kinds of negatively

442   charged molecules like DNA, organic acids or glutathione [12]. An earlier study

443   analyzing the distribution of "soluble" and "particulated" Zn in fractions of HepG2 cells

444   found 65 % of the Zn to be soluble and 35 % to be in the pellet of a high-speed

445   centrifuged cell extract [43]. FluoZin can detect the labile Zn pool in living cells and was

446   therefore used to complement our findings about the Zn distribution in neutrophils

447   during activation of neutrophils and NET release [44]. FluoZin-stained neutrophils were

448   stimulated with PMA and followed by fluorescence-based live cell imaging over ca. 3

449   h (video V1). Four observations were most striking: First, the nucleus did not show a

450   stronger fluorescence signal compared to the cytoplasm, indicating that the labile Zn

451   is not higher concentrated in this organelle. Comparing this with the higher total areal

452   Zn concentration in the nucleus observed with SR-XRF, we can deduce that most of

453   the Zn is strongly bound and present in the nucleus. Second, void vacuoles with low

454   metal content, which were indicated by SR-XRF, were also visualized by FluoZin-

455   labeling, confirming the low Zn content of these membrane-surrounded vesicles.

456   Third, small round organelles high in labile Zn exclusively appeared after stimulation.

457   We cannot distinguish at this point what type of intracellular vesicles these are. They

458   also partially remained in the typical cell residues left after full NETosis and could

459   therefore additionally contribute to the reduced Zn level after NET formation (Fig. 4).

460   Lastly, major reshaping of the intracellular architecture occurred when the cells

461   opened up just before NET release. Once this happened, the evenly distributed

462   intracellular labile Zn disappeared, probably because Zn was diluted into the low-Zn

463   medium. As expected, NETs were not visible in this setting. The cell culture medium

464   used contained very low amounts of Zn to avoid background fluorescence, and NETs

465   were probably not saturated with Zn under these conditions. Similar effects were also

466   seen with strongly bound Zn in the SR-XRF images from PETRAIII (Fig. 2B + 2C).

467   Altogether, by FluoZin-labeling we were able to follow the major morphological

468   changes in activated neutrophils up to final NET release visualizing the labile Zn pool.

469   In contrast to SR-XRF, where Zn showed a higher total Zn areal concentration in the

470   nucleus, FluoZin-imaging of free Zn indicates a homogenous distribution of the labile

471   Zn pool in nucleus and cytoplasm.

472

Discussion
473

474   Neutrophils patrol in circulation until they quickly invade a site of infection in high

475   numbers and fulfill their antimicrobial task until they reach cell death. The neutrophil

476   life time is limited, since their weaponry is too dangerous for the host's own tissue to

477   be stored for too long. Apoptosis and NETosis are two important cellular death

478   mechanisms of neutrophils. During NETosis, the entire neutrophil opens up to

479   release its intracellular content. During apoptosis, the cell stays surrounded by an

480   intact membrane and is eventually removed by efferocytosis. A high influx of

481   neutrophils into the infectious site, where individual neutrophils end their life by either

482   NETosis or apoptosis, serves to remove the threat of invading microbes on the one

483   hand, but probably also delivers a sudden nutritional enrichment of the infection

484   niche. On these grounds, we performed an in-depth analysis of the trace element

485   landscape of human neutrophils. By applying SR-XRF for the first time on single

486   neutrophils, we obtained trace element distributions in resting and activated

487   neutrophils undergoing pinocytotic events, cytoskeletal rearrangements and NETosis.

488   We were able to detect intracellular structures at the size of cytoplasmic granules (≈

489   200 nm) and thin filamentous NETs (below 1 µm diameter, up to 100 µm length). We

490   identified the lobulated nucleus and subnuclear structures, compared element

491   distributions of nucleus and cytoplasm and followed neutrophil activation in a time

492   course until ultimately NETs were released. We quantified the total content of

493   biologically relevant metals (Zn, Fe, Cu, Mn) of neutrophil lysates by ICP-MS and

494   compared the resulting metallome with intact neutrophils analyzed by SR-XRF. The

495   neutrophil metallome was further compared to macrophage-like J774 cells and HeLa

496   cells revealing a cell type-specific composition of Zn, Fe, Cu, and Mn with an

497   exceptionally high Fe content. In addition, we experimentally demonstrated a specific

498   reduction in available Zn by the process of NETosis. Finally, we visualized 1) the

499   homogenously distributed labile Zn pool in activated neutrophils by FluoZin (implying

500   a nucleus mainly containing strongly bound Zn resulting from the high Zn areal

501   concentration maps in SR-XRF, and 2) the presence of 'void' vacuoles - low in metal

502   content - using FluoZin and SR-XRF in living and fixed cells respectively. Void

503 vacuoles share features with the early phagosome, since they are also derived from

504 pinocytotis, the uptake of extracellular liquids [7, 41].

505 Our metallome analysis of neutrophils in comparison to other mammalian cells

506 commonly used in *in vitro* infection studies indicated a high Fe content in this cell

507 type. This is also reflected by the ratios of the respective metals. For HeLa cells,

508 were determined the following ratios: Zn/Fe 2.0, Zn/Cu 16.7, and Fe/Cu 8.3. For

509 macrophage-like J774 cells, the ratios were notably different: Zn/Fe 1.8, Zn/Cu 40.2

510 and Fe/Cu 21.9. The ratios of metals in neutrophils were even more distinct: Zn/Fe

511 0.4, Fe/Cu 72.6, and Zn/Cu 26.9. Earlier quantifications of 7 different cell culture lines

512 by total reflection XRF (T-XRF) demonstrated that the ratio of cell metals can differ

513 from cell line to cell line, resulting in a range of ratios [43]. The cell lines investigated

514 originated from different tissues and mammals. Primary immune cells were not

515 included. Tapia *et al*. compared the concentration of Fe, Cu, and Zn in pelleted cells

516 normalized to the protein mass and determined the range of metal ratios to describe

517 the metal composition: Zn/Fe 1.4-2.4, Zn/Cu 5.5-21.1, and finally Fe/Cu 3.0-12-3. Mn

518 was not considered in this study. A comparison to these findings confirms that

519 neutrophils are very high in iron and rather low in Cu. Macrophage-like J774 cells are

520 low in Cu, and according to our data richer in Mn than neutrophils. HeLa cells are

521 closest to the types of cells analyzed by Tapia *et al.* – and their metal ratios are

522 indeed completely in agreement with the ratios described. A possible explanation for

523 the very high Fe content of human neutrophils is the high abundance of the enzyme

524 myeloperoxidase (MPO), up to 5 % of the cell mass [45]. It contains a heme cofactor

525 and produces hypochlorous acid during the oxidative burst. Additionally, neutrophils

526 and also macrophages contain the heme-containing enzyme catalase that is used to

527 protect against oxidative damage. Lastly, the relative low Mn content of neutrophils

528 could result from the low number of mitochondria in these cells [46]. All together, these

529 values demonstrate nicely that the metal content of neutrophils is cell type specific.

530 Our SR-XRF analysis revealed that typical morphological features of neutrophils are

531 also reflected in the spatial distribution of elements. The most striking features of

532 resting neutrophils were the lobulated nucleus and speckles that we identified as

533 granules. Those granules were enriched in Fe and Zn, and the signal from both

534 elements did not overlap completely. Indeed, neutrophils contain a number of

535 different granules with partially distinct and partially overlapping content [18].

536 Interestingly, the enzyme myeloperoxidase (MPO) is exclusively present in so-called

537 azurophil granules and contains a heme cofactor [45], while neutrophil matrix

538 metalloproteases are present in so-called specific granules, and both MMP8

539 (collagenase) and MMP9 (gelatinase B) are Zn-dependent. Iron-binding lactoferrin is

540 also found in specific granules, but since lactoferrin is secreted to sequester iron, it is

541 unlikely already loaded with Fe in the granules [8].

542 In NETs on the other hand, lactoferrin is exposed to the extracellular environment. It

543 is therefore likely that lactoferrin bound to NETs can chelate some Fe from the

544 surrounding. In our study, we found that the Fe concentration in the supernatant of

545 NETs was slightly decreased and we observed that NETs were enriched in Ca and

546 Fe. The high content of Fe in NETs is probably originating not only from lactoferrin,

547 but also from the heme-containing enzymes MPO and catalase, as they are also

548 known to be bound to NETs [22, 47]. Calprotectin is part of the NETome as well. This

549 protein can bind Ca, and on another binding site Zn and Mn [42]. It is most likely

550 responsible for the reduction of available Zn in the supernatant of NETs. Zn chelation

551 of NETs is important towards fungal pathogens. This is illustrated by the inability of

552 other metal ions than Zn^{2+} to rescue *Aspergillus nidulans* [23] and *Candida albicans*

553 (data not shown) from NET-mediated growth inhibition

554 These findings shape the view on the nutritional immunological importance of

555 neutrophils: Compared to other cells, the neutrophil is exceptionally high in Fe. This

556 is likely the result of a high MPO content in neutrophil granules, which matches our

557 findings of Fe speckles in the cytoplasm of neutrophils. Other metal-containing

558 enzymes in neutrophil granules are Zn-dependent metalloproteases. While these

559 enzymes are released by activated neutrophils, we did not find an increased Fe or Zn

560 concentration in the supernatant of activated neutrophils. On the contrary, we found

561 the Zn concentration to be reduced and the Fe concentration to be almost

562 unaffected. The analysis of NETs confirmed their capacity to bind Zn. Further, void

563 vacuoles were found in activated neutrophils that were low in Fe and Zn. Void

564 vacuoles are likely similar to the early phagosome. Taken together, our findings

565 shape the view that neutrophils are capable of starving microbial pathogens intra-

566 and extracellularly from key metal nutrients such as Zn and Fe, remarkably even if

567 they release metal containing proteins by bursting open during NETosis. Due to the

568 detection limitations and their lower abundance, we could not conclusively follow Mn

569 and Cu. Our data indicates though that void vacuoles are also low in Cu. On these

570 grounds, it would be worthwhile to investigate whether microbial pathogens target

571 secreted or NET-released metal containing proteins as micro nutrient sources. An

572 interplay similar to that of Fe in other settings seems very plausible [8].

573 In addition to Zn, Fe, Cu, and Mn, we also detected a group of metals that are less

574 renowned for their biological function or mostly described for their toxicity: Co, Ni, Pb,

575 and Sr. All metals besides Sr were found to be enriched in the nucleus of neutrophils;

576 Sr was present at higher concentration in the cytoplasm. Interestingly, Ca, which

577 behaves very similar to Sr, was enriched in the nucleus, and showed lower

578 abundance in the cytoplasm. Due to the similarities of Ca and Sr, Sr accumulated in

579 the bones of an exposed human. Co is an essential trace element, but also toxic at

580 high concentrations. It is only found in humans as part of the cofactor cobalamin or

581 vitamin B12 (VitB12), which is exclusively produced by commensal bacteria in the

582 gastro-intestinal tract. Amongst the small number of VitB12-binding proteins in

583 humans, transcobalamin I was identified in neutrophils [48]. The protein is localized in

584 secondary granules and can be secreted to bind VitB12. Thus, transcobalamin I is

585 not necessarily saturated with VitB12 in the granules. To our knowledge, no nuclear

586 cobalamin-containing enzymes have been described, yet. Nevertheless, it was

587 shown that human keratinocytes for instance accumulate Co in the nucleus and

588 perinuclear areas [32]. Ni, one of the most toxic of these metals, has no known

589 biological function in eukaryotes, but is found in a variety of prokaryotic enzymes. Pb

590 is a known environmental pollutant that accumulates in our bodies in many organs,

591 most persistently in the bones. Notably, all 4 elements have been detected in whole

592 blood of healthy individuals that were considered "free of exposure" to any heavy

593 metal contamination [49, 50]. Since the majority of all white blood cells are neutrophils, it

594 seems reasonable to assume that neutrophils contributed to this detection in whole

595 blood. As all blood cells are generated in the bone marrow, neutrophils are unlikely

596 the only blood cells to contain Ci, Ni, Pb, and Sr. In conclusion, we have detected Co,

597 Ni, Sr, and Pb in isolated neutrophils from two individuals living under supposedly

598 non-exposed conditions. This data indicates an accumulation of Co, Ni, and Pb in the

599 nucleus, whereas Sr is present in the vicinity of the outer cell membrane in

600 unstimulated neutrophils.

601 In the present study, we used two different approaches for SR-XRF with their

602 respective benefits and disadvantages: Sectioned samples analyzed at nanoscopic

603 resolution allowed us to have highly detailed insights into intact neutrophils and

604 compare the areal concentration in nucleus and cytoplasm. In contrast, the

605 microscopic approach with whole freeze-dried neutrophils/NETs enabled us to

606 quantify the neutrophil metallome and analyze element distributions of neutrophils

607 forming NETs (both intermediate and final stages of NETosis). Substantial benefits of

608 SR-XRF are 1) the extreme sensitivity, with the potential of single atom detection at

609 future nano-beam facilities for transition metals; 2) the minimal sample preparation

610 required: cells do not need to be lyzed and diluted – factors that might affect the

611 reliability - in any other way than the final fixation; 3) the potentially non-destructive

612 character: under right measuring conditions samples can be stored and re-analyzed

613 at later periods; and 4) the spatial information: individual cells meeting all

614 requirements can be selected and analyzed with submicron resolution. Artifacts

615 influencing the determined metal distributions are therefore, assuming an appropriate

616 sample preparation method and experimental design, rather unlikely. As described,

617 we utilized SR-XRF to determine the neutrophil metallome to complement our

618 measurement using ICP-MS. Due to the time consuming character of SR-XRF, we

619 only analyzed three individual cells, whereas for ICP-MS each sample contained 10^7

620 neutrophils. This is, together with the distinct sample preparations, most likely the

621 major reason for the deviations between the results of the two different methods.

622 Moreover, uncertainties in calibration and interfering background signals are factors

623 which might influence Fe concentration measurement using SR-XRF. Quantification

624 of Fe by ICP-MS on the contrary, might be hampered by spectral interferences.

625 Nevertheless, the usage of SR-XRF in actual *in vitro* infections or sections from *in*

626 *vivo* infection (e.g. as tissue sections) seems very promising. Earlier studies have

627 demonstrated, for instance, the cellular reduction of Fe and Zn in *Histoplasma*-

628 infected macrophages [51] or the specific enrichment of Cu in the phagolysosome

629 containing *Mycobacteria* [52]. These and similar phenomena are ideal candidates for

630 SR-XRF analysis at the nanoscale – adding new information about elemental

631 distribution and concentrations. Meticulously describing the trace elemental

632 composition during different stages of infection within affected organs might even

633 identify new targets for antimicrobial therapy. Trace elements are essential for

634 microbial growth and their removal could be a potential treatment for infections. The

635 lack of certain metals cannot be easily overcome by mutagenesis leading to

636 resistance, which makes it a promising target for further research.

637

638

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 x 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 x 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 x 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 lyzed. For each sample, 10^7 neutrophils were taken up in 100 µl HEPES-buffered RPMI and NaOH (99.99 % metals basis, semiconductor grade) was added to reach 0.25 M in 200 µl. This mixture was incubated for 2 h at 65 °C with vigorous shaking. Subsequently, lysates were sonicated for 1 h in a sonification bath. Samples were stored at -20 °C up to further use. J774 cells ($9*10^6$) and HeLa cells ($5*10^6$) were handled accordingly. A biological replicate was therefore either a different blood donor or a different flask and passage of the cultured cells.

NET supernatant preparation for ICP-MS

669 Neutrophils were diluted in HEPES-buffered RPMI with or without supplementation of

670 different concentrations of Zn sulfate (99.999% metals basis). To avoid variations,

671 RPMI from only one batch was used for all sample preparations for ICP-MS. Into

672 each well of a 24-well plate, 500 µl cell suspension containing 10^6 cells were seeded

673 and then stimulated with 100 nM PMA (Sigma-Aldrich). NET formation occurred

674 during a 4 h incubation at 37° C with 5 % CO_2. After this, 400 µl of the supernatant

675 were harvested and cell debris was pelleted by centrifugation for 10 min at 21,000 x

676 g. 300 µl of the supernatant were transferred onto a Vivaspin-500 centrifugal

677 column(Sartorius) with a molecular weight cut-off of 3 kDa (corresponding ca. 27

678 amino acids). The supernatants were filtered by centrifugation for 30 min at 10,000 x

679 g at 4 °C. 200 µl of the flow through were collected into new reaction tubes and

680 stored at -20 °C for further use.

681 *ICP-MS measurement*

682 The concentrations of trace metals were determined with inductively coupled plasma

683 mass spectrometry (ICP-MS, PerkinElmer/Sciex Elan DRC-e instrument) operated in

684 reaction cell mode with 0.6 ml/min oxygen and an RPq-value of 0.45. The instrument

685 was equipped with a microconcentric PFA nebulizer (Elemental Scientific Inc.)

686 combined with a quartz cyclonic spray chamber (Elemental Scientific Inc.) set to 4

687 °C. A nebulizer gas flow rate of 0.66 L min^{-1} and a plasma RF power of 1200 W were

688 used. The ^{55}Mn, ^{56}Fe, ^{57}Fe, ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn and ^{68}Zn isotopes were monitored

689 with a dwell time of 50 ms and a total measurement time of 1 min 48 s. The obtained

690 isotope distributions were compared with the theoretical patterns to verify the

691 absence of spectral interferences. Quantification was done by external calibration

692 using indium (monitoring isotope ^{115}In) as internal standard. Sample solutions were

693 typically diluted 40 times with Milli-Q water (> 18 MΩ.cm, Milli-Q Advantage A10

694 Ultrapure Water Purification System, Merck Millipore) containing 1 % HNO_3 prior to

695 analysis.

696 *Sample preparation for beamline P06, PETRA III*

697 Neutrophils were seeded directly on a 5x5 mm^2 silicon nitride window (Silson Ltd,

698 Northampton, UK, 1.5x1.5 mm^2 membrane size, 200 nm membrane thickness). 5 *

699 10^3 cells were added in a 10 µl drop of HEPES-buffered RPMI and NET formation

700 was induced with 100 nM PMA. Incubation occurred as described earlier. After the

701 incubation, cells were washed very briefly with ultraclean H_2O and plunge-frozen in

702   liquid ethane. After 1 h storage in liquid nitrogen, samples were transferred into a

703   home-built freeze dryer, based on conventional metal block cooled with liquid

704   nitrogen, and samples were lyophilized for 48 h.

Experimental set-up beamline P06, PETRA III

706   Measurements were performed at the P06 Hard X-ray Micro/Nanoprobe at PETRA

707   III, Hamburg, Germany. The primary beam is generated by a 2 m long spectroscopy

708   undulator U32 having 60 periods with 31.4 mm period length and output power of 3.8

709   kW. The beam was monochromatized by a Si(111) double crystal monochromator

710   having an energy resolution of 1.4×10^{-4} $\Delta E/E$, set to a beam energy of 20.5 keV. A

711   KB-system (JTEC Corporation, Osaka, Japan) was used to provide a beam size of

712   0.5 μm horizontally and 0.4 μm vertically at a working distance of 200 mm measured

713   from the second mirror, with an estimated flux density of approx. 10^{11} photons/s/μm^2.

Sample preparation for beamline ID22NI, ESRF

715   In a 45 μl droplet, $2.5 * 10^4$ neutrophils in HEPES-buffered RPMI were seeded onto a

716   1.4 mm sapphire disk (Leica consumables nr. 16706849). NET formation was

717   induced by adding 100 nM PMA or cells were left unstimulated. Stimulated cells were

718   incubated for up to 4 h at 37 °C with 5 % CO_2. Then, the medium was carefully

719   removed, cells were washed twice very briefly with a droplet of ultraclean H_2O (to

720   avoid osmotic burst) and 50 μl of 20 % w/V BSA in PBS (Sigma) were added onto each

721   sapphire disk as a cryoprotectant. Cells were in contact with the BSA-containing PBS

722   only very briefly to minimize this inevitable step affecting the element integrity of the

723   cells. The sapphire disc was then very quickly inserted in a membrane carrier (Leica

724   consumables nr. 16707898, 1.4mm diameter, 100 μm thickness) and frozen

725   immediately in a high-pressure freezer (EM PACT; Leica Microsystems, Vienna,

726   Austria). Freeze substitution was carried out using a Leica EM AFS2 (Leica

727   Microsystems) in dry acetone with 0.1 % glutaraldehyde over 4 days as follows: -90°

728   C per hour increase for 15 hours, and -30°C for 24 hours. Samples were then

729   washed 3 times in pure acetone and slowly warmed up to 4°C, infiltrated stepwise

730   over 3 days at 4°C in Spurr's resin (solution composed of NSA, ERL 4221, DER 736

731   and DMAE from emsdiasum.com, Hatfield) and embedded in capsules. The

732   polymerization was performed at 70°C for 16h. Ultrathin (~60 nm) sections intended

733   for EM were made using an ultramicrotome (Leica EM UC6) and post-stained in a

734   Leica EM AC20 for 40 min in uranyl acetate at 20°C and for 10 min in lead citrate at

735   20°C. Grids were viewed with a JEM 1010 transmission electron microscope (JEOL,

736   Tokyo, Japan) operating at 80 kV using Image Plate Technology from Ditabis

737   (Pforzheim, Germany). Thin sections (2 µm) were cut and deposited on square

738   silicon nitride (Si_3N_4) ultra-thin membranes in square silicon nitride supporting frames

739   from Silson Ltd, Northampton, UK (3.0 x 3.0 mm membrane size, 500 nm membrane

740   thickness, 7.5 x 7.5 mm frame size and 200 µm frame thickness).

741   *Experimental set-up beamline ID22NI, ESRF*

742   The scanning nano-XRF experiments were performed at the ID22NI XRF nanoprobe

743   at the European Synchrotron Radiation Facility (ESRF). This instrument was installed

744   at a high-β straight section equipped with two different undulators covering an energy

745   range of 6-70 keV. The ID22NI nanoprobe (currently replaced by NINA) was

746   dedicated to hard X-ray nanoanalysis allowing nano-XRF and absorption/phase

747   contrast nanotomography. X-ray focusing was obtained by a crossed elliptical Rh

748   coated graded-multilayer mirror-pair in the Kirkpatrick-Baez (KB) geometry. X-rays

749   are collected and focused in both vertical and horizontal axis at a glancing angle

750   (<3.5 mrad). The first mirror, coated with a graded multilayer plays both the role of

751   vertical focusing device and monochromator, resulting in a very high flux *(exceeding*

752   *10^{11} photons/s)* and a medium monochromaticity ($\Delta E/E \approx 10^{-2}$). In our case the beam

753   size was determined by knife-edge scans of a Au test pattern and determined to be

754   64 nm vertically and 54 nm horizontally at an excitation energy of 17 keV.

755   *Live cell imaging of Zn pool during NET formation*

756   Human neutrophils (5 * 10^6), stained with 20 µM FluoZin-3 (Life Technologies), were

757   seeded into a 35 mm glass bottom micro-well dish (MatTek, Ashland, MA, USA).

758   Neutrophils were stimulated with 100 nM PMA and kept at cell-culture conditions

759   throughout the entire measurement (5% CO_2 and 37° C). Pictures, phase contrast

760   and green fluorescence, were taken using a 60x objective every 2 min for a period of

761   2.5 h (beginning at 15 min *post stimulation)* using a Nikon eclipse Ti live-cell

762   microscope operated by NIS-Elements AR 3.2. software.

763   *Quantification of SR-XRF measurements and image segmentation*

764   All quantitative results shown were obtained by measuring NIST standard reference

765   materials (SRMs). For the ID22NI measurements (ESRF, Grenoble, France), a total

766   amount of 17.4 mg powder of NIST SRM 1577c (bovine liver) was pressed into a

767 self-supporting pellet of 13mm diameter resulting in an areal density of 13.11

768 mg/cm^2. The pellet was measured for 1100 s using a raster scan to compensate for

769 possible inhomogeneities. For the P06 beamline measurements (PETRA III,

770 Hamburg, Germany), a NIST SRM 613 (trace elements in glass) and a MPI DING

771 ATHO standard (both prepated as flat samples with a thickness of 100 µm) were

772 measured for 1100 seconds using the same measuring strategy described

773 previously. The obtained spectra were deconvoluted using Analysis of X-ray spectra

774 using Iterative Least squares AXIL [53] to obtain the net line intensities, which were

775 then normalised to the incoming photon flux, corrected for detector dead time and for

776 self absorption effects. Spectral data was then batch processed using an inhouse

777 developed software package called MICROXRF2. Manual image segmentation (or

778 clustering) was performed on the obtained element maps of the unstimulated

779 neutrophils, indicating nucleus, cytoplasm and cell. The individual point spectra

780 belonging to a single segment were then summed and net line intensities of the

781 segment were obtained using AXIL.

782 Quantification was based on a fundamental parameter approach which exploits the

783 theoretical relation between the net-line intensities and the elemental concentrations

784 (Sherman, 1955). The elemental yields of the elements of interest were calculated

785 from the measured standard reference material taking into account: 1) variations in

786 incoming beam intensity (related to varying synchrotron ring current and small

787 variations in alignment of beamline optical components) by using ionisation chamber

788 values, 2) the use of absorbers (3000 µm Al in case of NIST SRM 1577c) and 3)

789 detector dead time (registered for every scanned point).

790 The elemental intensity maps were converted into areal concentration distributions

791 (expressed in µg/cm^2) by dividing the pixel values in the normalised elemental maps

792 (normalised to dead time and storage ring current) by the areal elemental yields. In

793 order to determine the so-called "relative enrichment factors" for the relevant

794 elements in the nucleus versus the cytoplasm, the sum spectra of the nucleus and

795 the cytoplasm were first normalised to the Compton scattering peak and the ratios of

796 the relevant elemental intensities were taken.

797 *Statistical analysis*

798 Unless stated otherwise, all statistical calculations have been performed using

799 Microsoft Excel. Further statistical analysis was performed using Graphpad Prism

800   Software 5. For the quantification of NETs supernatants, data was fitted using a non-

801   linear Michaelis-Menten fit.

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Acknowledgments

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5   824 **References**
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Figure 1: Metallome comparison of human neutrophils with J774 and HeLa cells.
Cultured J774 and HeLa cells were alkaline lyzed and the resulting lysates were quantified
by ICP-MS. The average content was related to the average content of human neutrophils, n
= 3, and normalized by cell volume.

**Figure 2A: 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 µm x 78 µm, step size is 300 nm and 4 s dwell time/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 (2500x
magnification).

**Figure 2B: Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of two
activated human neutrophils (2 h stimulation) obtained at P06 beamline.** Freshly
isolated neutrophils were seeded on a Si_3N_4 membrane, stimulated with PMA for 2 h and
subsequently freeze-dried. Image size: 73 µm (hor.) x 97 µm (vert.), step size: 1 µm and 5 s
dwell time/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 (2500x magnification).

**Figure 2C: Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of human
neutrophils (4 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 h and
subsequently freeze-dried. Image size: 100 µm x 100 µm, step size: 1 µm and 3 s scanning
time/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 (2500x magnification).

**Figure 3A: Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of a single
resting human neutrophil obtained at ID22NI beamline (ESRF, Grenoble).** Freshly
isolated neutrophils were high pressure frozen, cryosubstiued in resin, sliced in 2 µm thin
sections before deposition onto a Si_3N_4 wafer. Image area is 9 µm (hor.) x 7.4 µm (vert.),
step size is 50 nm and 300 ms dwell time/point. The RGB image represents a colored
overlay of the elements Ca (red) – Zn (green) and Fe (blue). The additional TEM image
(lower right panel) was derived from the same sample and selected by similarity in
morphology (10,000x magnification).

**Figure 3B: Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of two
activated human neutrophils (1 h stimulation) obtained at ID22NI beamline (ESRF,
Grenoble).** Freshly isolated neutrophils were stimulated with PMA for 1 h, high pressure
frozen, cryosubstiued in resin and sliced in 2 µm thin sections before deposition onto a Si_3N_4
wafer. Image area is 15 µm (hor.) x 11.2 µm (vert.), step size is 50 nm and 400 ms dwell
time/point. The RGB image represents a colored overlay of the elemental intensities
corresponding to Ca (red) – Zn (green) and Fe (blue). The additional TEM image (lower right
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959 **Figure 3C: Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of**
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965 corresponding to Ca (red) – Zn (green) and Fe (blue). The additional TEM image (lower right
966 panel) was derived from the same sample and selected by similarity in morphology (2500x
967 magnification).
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970 **Figure 3D: Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of**
971 **activated human neutrophils (3 h stimulation) obtained at ID22NI beamline (ESRF,**
972 **Grenoble).** Freshly isolated neutrophils were stimulated with PMA for 3 h, high pressure
973 frozen, cryosubstitued in resin, sliced in 2 μm thin sections before deposition onto a Si_3N_4
974 wafer. Image area is 20 μm (hor.) x 14 μm (vert.), step size is 50 nm and 300 ms dwell
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976 (green) and Fe (blue). The additional TEM image (lower right panel) was derived from the
977 same sample and selected by similarity in morphology (2500x magnification).
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980 **Figure 4: NET-mediated reduction of Zn availability.** NET formation was induced in
981 neutrophils with PMA for 4 h in the presence of different Zn^{2+} concentrations. The remaining
982 Zn concentration was quantified by ICP-MS. Pooled data from three independent ICP-MS
983 experiments including samples from 8 healthy donors is shown.
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986 **Table 1: Total metal content of neutrophils determined by ICP-MS.** Freshly isolated
987 neutrophils from 8 healthy volunteers were alkaline lyzed and resulting lysates were
988 quantified by ICP-MS. The average and standard deviation of all 8 biological replicates are
989 displayed.

Element	Atoms per cell	Intracellular concentration (µM)
Zn	$6.07 \pm 1.29 \times 10^{7}$	275.31 ± 58.61
Fe	$1.64 \pm 0.33 \times 10^{8}$	743.91 ± 148.62
Cu	$2.26 \pm 0.69 \times 10^{6}$	10.24 ± 3.11
Mn	$2.49 \pm 0.69 \times 10^{5}$	1.13 ± 0.31

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992 **Table 2: Comparison and relation of areal concentrations in neutrophil nucleus and**
993 **cytoplasm.**
994 Unstimulated neutrophils were analyzed individually by SR-XRF (ID22NI beamline, ESRF)
995 and the areal concentration was determined and compared between nucleus and cytoplasm
996 in 2 µm thin slices. Trace-level metal content was determined using a NIST SRM 1577c
997 standard for quantification. The average and standard deviation of three individual cells are
998 displayed, as well as the corresponding mass-normalized 'enrichment factor' of the nucleus.

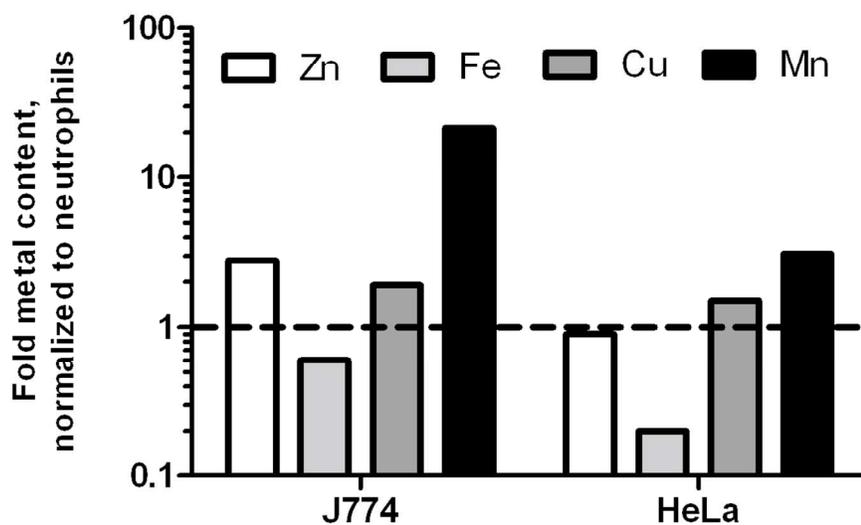| Element	Nucleus (µg/cm^2)	Cytoplasm (µg/cm^2)	Enrichment Factor
P	$8.81 \pm 0.33 \times 10^{0}$	$1.59 \pm 0.33 \times 10^{0}$	50.11 ± 27.41
S	$1.62 \pm 0.40 \times 10^{0}$	$3.02 \pm 0.31 \times 10^{0}$	0.47 ± 0.09
Ca	$8.80 \pm 0.58 \times 10^{-3}$	$2.90 \pm 0.25 \times 10^{-3}$	2.83 ± 0.26
Zn	$2.28 \pm 0.32 \times 10^{-3}$	$6.84 \pm 2.11 \times 10^{-4}$	3.19 ± 0.78
Fe	$1.14 \pm 0.15 \times 10^{-4}$	$8.83 \pm 0.81 \times 10^{-5}$	1.29 ± 0.18
Cu	$2.59 \pm 0.34 \times 10^{-3}$	$4.04 \pm 0.35 \times 10^{-3}$	0.63 ± 0.10
Mn	$2.40 \pm 0.19 \times 10^{-4}$	$1.00 \pm 0.04 \times 10^{-4}$	3.98 ± 0.10

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Figure 1: Metallome comparison of human neutrophils with J774 and HeLa cells.
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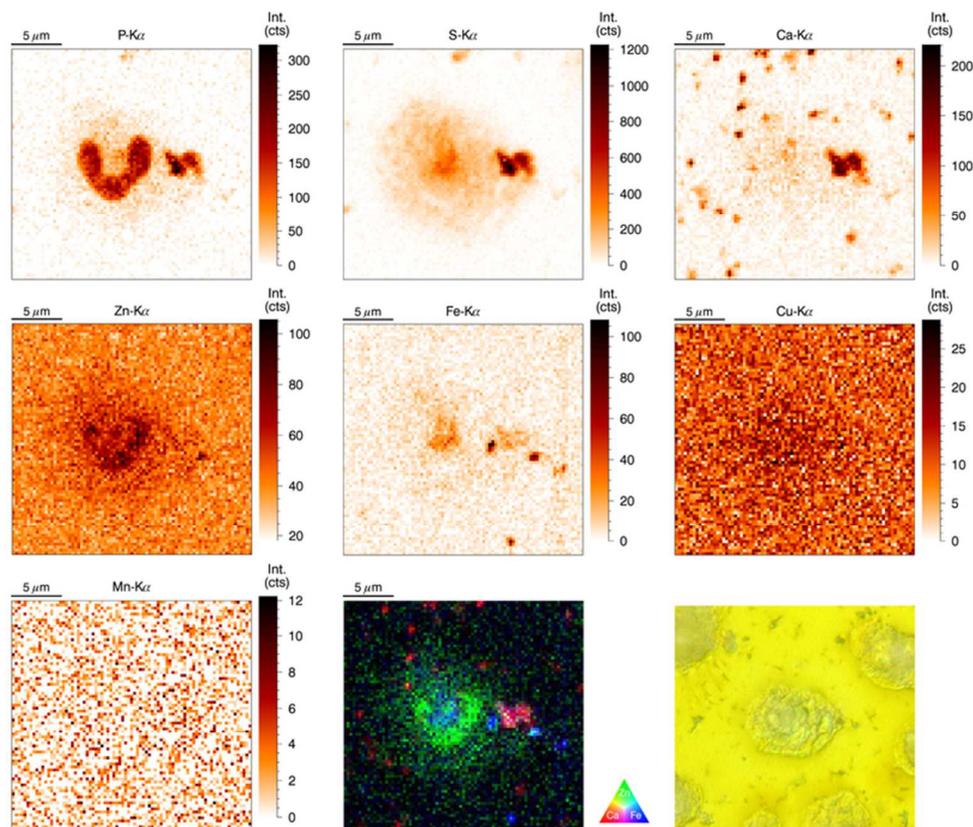112x67mm (300 x 300 DPI)

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70x58mm (300 x 300 DPI)

Figure 2B: Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of two activated human neutrophils (2 h stimulation) obtained at P06 beamline. Freshly isolated neutrophils were seeded on a Si3N4 membrane, stimulated with PMA for 2 h and subsequently freeze-dried. Image size: 73 μm (hor.) x 97 μm (vert.), step size: 1 μm and 5 s dwell time/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 (2500x magnification).
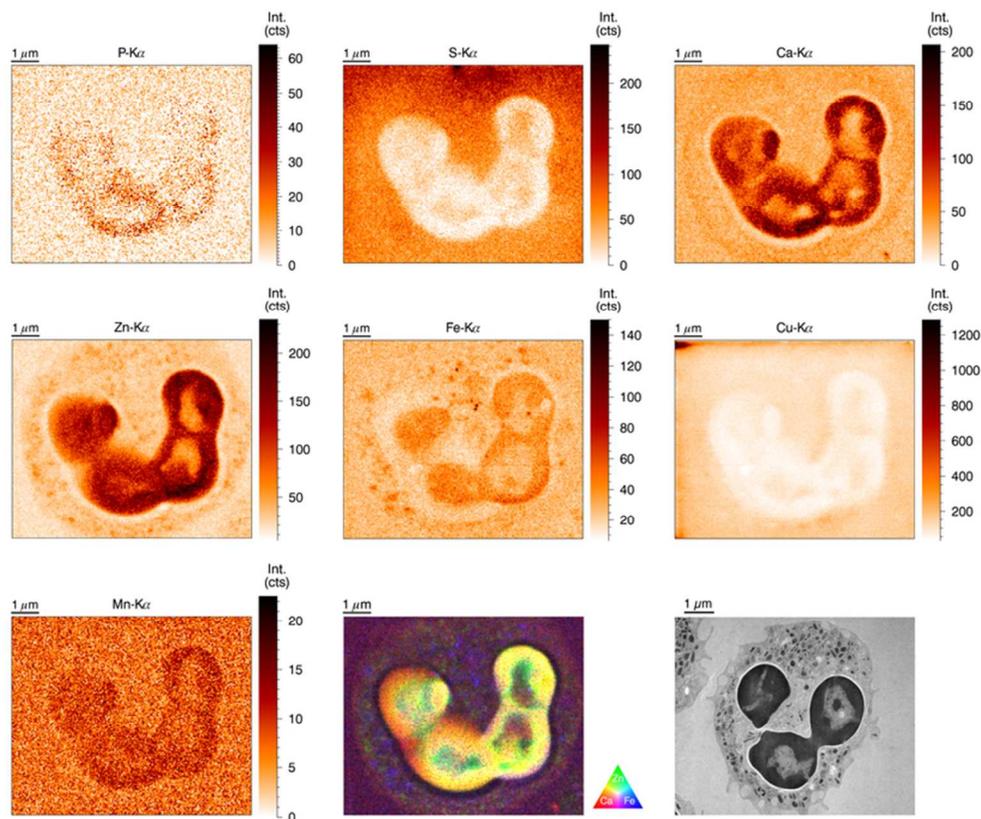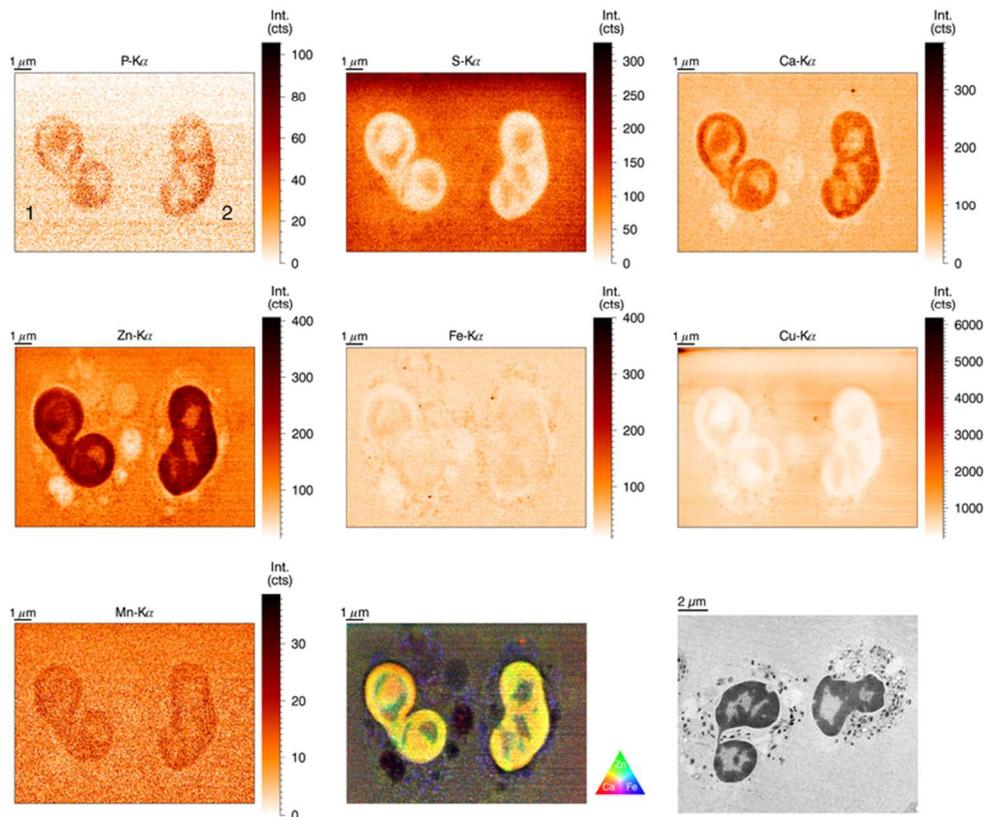70x58mm (300 x 300 DPI)

Figure 2C: Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of human neutrophils (4 h stimulation) obtained at the PETRA III P06 beamline. Freshly isolated neutrophils were seeded on a Si3N4 membrane, stimulated with PMA for 4 h and subsequently freeze-dried. Image size: 100 μm x 100 μm, step size: 1 μm and 3 s scanning time/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 (2500x magnification).
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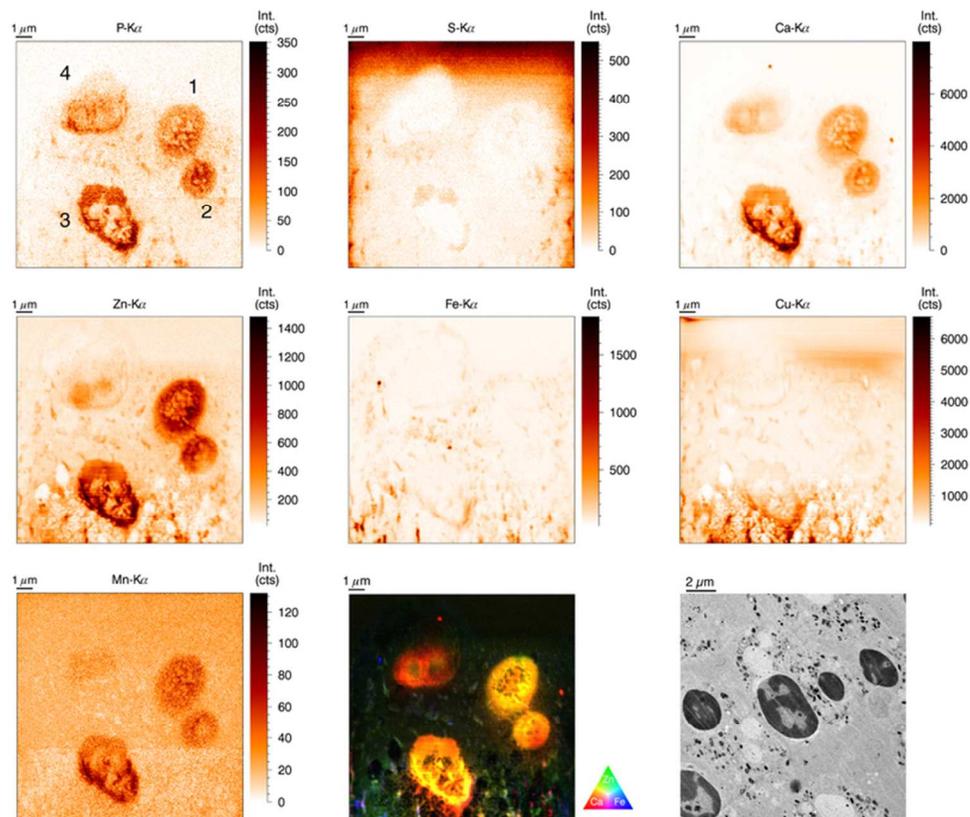70x58mm (300 x 300 DPI)

Figure 3B: Normalized elemental distributions (P, S, Ca, Zn, Fe, Cu, and Mn) of two activated human neutrophils (1 h stimulation) obtained at ID22NI beamline (ESRF, Grenoble). Freshly isolated neutrophils were stimulated with PMA for 1 h, high pressure frozen, cryosubstitued in resin and sliced in 2 μm thin sections before deposition onto a Si3N4 wafer. Image area is 15 μm (hor.) x 11.2 μm (vert.), step size is 50 nm and 400 ms dwell time/point. The RGB image represents a colored overlay of the elemental intensities corresponding to Ca (red) – Zn (green) and Fe (blue). The additional TEM image (lower right panel) was derived from the same sample and selected by similarity in morphology (2500x magnification).
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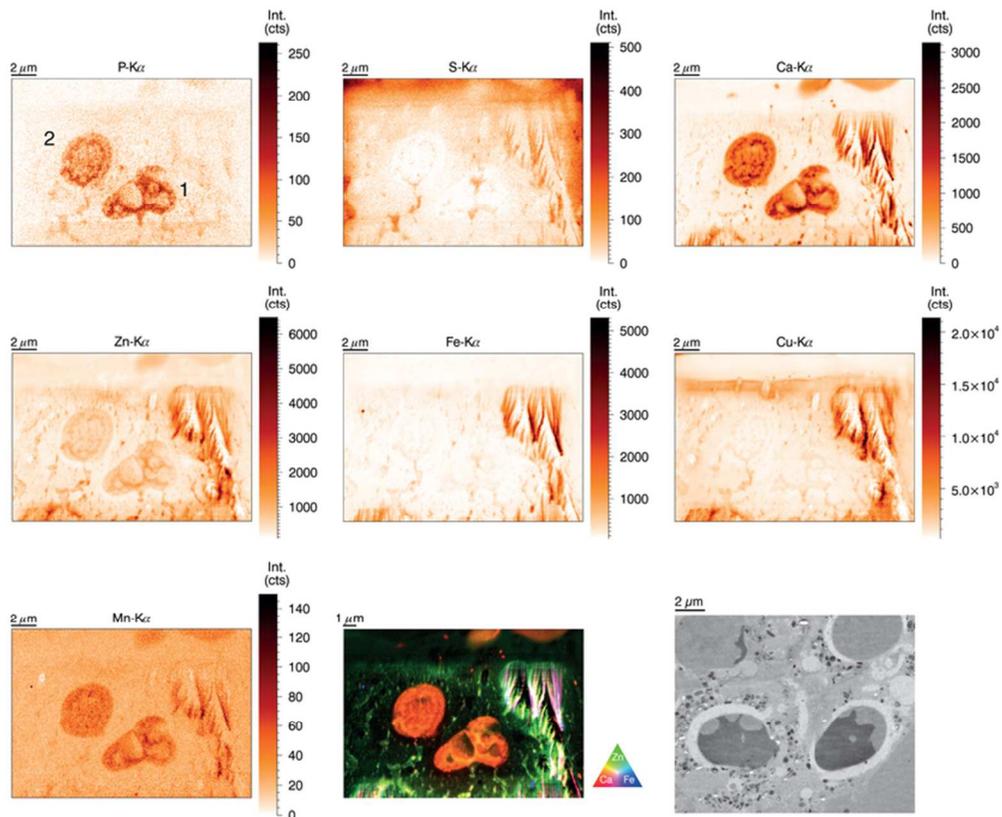70x58mm (300 x 300 DPI)

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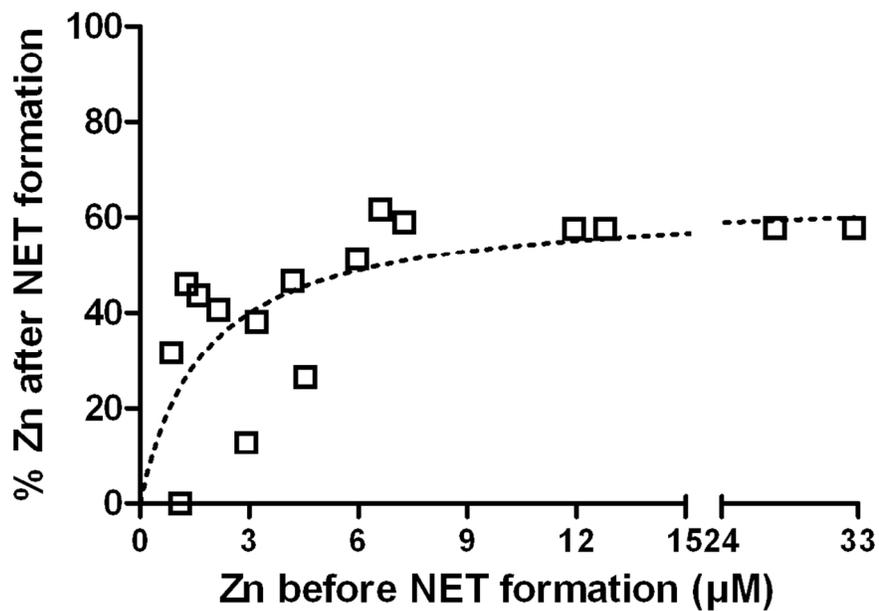70x58mm (300 x 300 DPI)

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