1 Advances in the Analysis of Biogeochemical Interfaces: NanoSIMS to

2 Investigate Soil Microenvironments

3 Review article for "Advances in Agronomy"

4 Carsten W. Mueller[1], Peter K. Weber[2], Matt R. Kilburn[3], Carmen Hoeschen[1], Markus Kleber[4]

5 and Jennifer Pett-Ridge[2]

6 [1]Lehrstuhl für Bodenkunde, TU München, Freising, Germany

7 [2]Chemical Sciences Division, Lawrence Livermore National Laboratory, Livermore,

8 California, USA

9 [3]Centre for Microscopy, Characterisation and Analysis, The University of Western Australia,

10 Crawley, Australia

11 [4]Department of Crop and Soil Science, Oregon State University, Corvallis, USA

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13 Running head: NanoSIMS to Investigate Soil Microenvironments

14 Corresponding author: Carsten W. Mueller, carsten.mueller@wzw.tum.de,

15   phone: +49(0)8161-714423, FAX: +49(0)8161-714466

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50 **Abstract**

51 Since a NanoSIMS high-resolution secondary ion mass spectrometry instrument was first

52 used for cosmochemistry investigtions over a decade ago, both interest in NanoSIMS and

53 the number of instruments available have significantly increased. However, SIMS comes with

54 a set of challenges that are of both technical and conceptual nature, particularly for complex

55 samples such as soils. Here, we synthesize existing research and provide conceptual and

56 technical guidance to those who wish to investigate soil processes at the sub-micron scale

57 using secondary ion mass spectrometry, specifically with NanoSIMS. Our review offers

58 advice resulting from our own operational experience but also intends to promote synergistic

59 research on yet unresolved methodological issues. We identify and describe the basic setup

60 of a NanoSIMS instrument and important issues that may arise as a soil sample specimen is

61 prepared for NanoSIMS analysis. This is complemented by discussions of experimental

62 design, data analysis and data representation. Next to experimental design, sample

63 preparation is the most crucial prerequisite for successful NanoSIMS analyses. We discuss

64 the requirements and limitations for sample preparation over the size range from individual

65 soil particles to intact soil structures such as macroaggregates or intact soil cores. For robust

66 interpretation of data obtained by NanoSIMS, parallel spatial, textural (scanning electron

67 microscopy, atomic force microscopy) or compositional analyses (scanning transmission X-

68 ray microscopy) are often necessary to provide necessary context. We suggest that

69 NanoSIMS analysis is most valuable when applied in concert with other analytical

70 procedures and can provide powerful inference about small scale processes that can be

71 traced via isotopic labeling or elemental mapping.

72

1. Introduction

1.1. The importance of nano-scale processes in soils research

Soil is often described as one of the most complex media on earth (Schulze and Freibauer, 2005). This complexity extends from the ecosystem-scale to individual microaggregates, where nanometer-scale interactions between microbiota, organic matter and mineral particles are thought to control the long-term fate of soil carbon, nutrients and pollutants (Lehmann et al., 2007; Schmidt et al., 2011). Processes that have a major impact at the landscape- or global-scale are determined by events occurring at the micro- and nanometer scales. For example, entrapment of soil organic matter (SOM) within microaggregates with a diameter of less than 250 μm and SOM sorption onto even smaller clay and iron oxides is a vital mechanism for long term preservation of organic carbon (OC) in soils (Lehmann et al., 2007; von Lützow et al., 2006). Release of nutrients in the rhizosphere is driven by root exudation at highly active micron-scale biogeochemical interfaces between roots, microbes and minerals (Breland and Bakken, 1991; Hinsinger et al., 2009; Norton and Firestone, 1996). Microbial activity occurs mostly in micro-habitats (Dechesne et al., 2007; Müller and Defago, 2006; Nunan et al., 2007) and involves mineralization of SOM and organic pollutants. Hydrologic processes at the field-scale are also influenced by fine-scale interactions as preferential flow paths may create localized zones of altered water and nutrient flow and thereby impact microbial abundance, community structure and SOM turnover (Chabbi et al., 2009; Morales et al., 2010). Preferential flow zones are themselves heterogeneous at the micro-scale, with a heterogeneous supply of oxygen, water and nutrients driving "hot spots" of microbial growth directly adjacent to areas of lesser microbial activity (Bundt et al., 2001). In all of these cases, activities at nano- to micron-scale soil biogeochemical interfaces determine the expression of higher level ecosystem functions. The majority of soil research, however, is conducted on bulk (> 1 g) samples, which are often significantly altered prior to analysis. Pretreatments and analytical side effects include drying at varying temperatures, sieving/homogenization for process or elemental analysis, thermal alteration (as in pyrolysis GC/MS) or chemical alteration (as in alkaline extraction of "humic"

101   substances or in cupric oxide oxidation for lignin analyses). With the advent of novel micro-

102   spectroscopy and spectrometry techniques that allow for the study of micro- to nano-scale

103   molecular, isotopic, and elemental patterns, it is now possible to make process-oriented

104   observations (e.g. the stabilization of organic matter, sorption of pollutants and mineral

105   weathering) at the micron or sub-micron scale.

106   Elemental and isotopic imaging conducted via secondary ion mass spectrometry (SIMS) is a

107   particularly promising technique for small-scale soil process research. SIMS uses a high-

108   energy ion beam to sputter material from a sample surface, which can then be analyzed in a

109   mass-spectrometer. With high resolution SIMS instruments (Cameca NanoSIMS 50, 50L,

110   Gennevilliers, France), the distribution of elements and isotopes can be visualized with up to

111   50-150 nm lateral resolution within soil samples ranging from primary particles to sub-regions

112   of intact soil cores. For this reason, NanoSIMS has the potential to provide quantitative

113   measures of organic matter-mineral-microbial interactions and biogeochemical processing at

114   the macro- and microaggregate or single-cell scale.

115   Relatively few SIMS experiments have been conducted to date in soil science. In one of the

116   first, Cliff *et al.*, (2002b) used time of flight-SIMS (ToF-SIMS) and additions of ^{15}N-labeled

117   and ^{13}C-labeled compounds to study small-scale differences in N assimilation as a function of

118   C vs. N limitation. When they compared SIMS values with bulk-measured microbial biomass

119   N assimilation, they found substantial spatial heterogeneity in ^{15}N distribution that was not

120   apparent through bulk analysis (Cliff et al., 2007). More recently, studies using SIMS and

121   NanoSIMS analysis have revealed effects at even finer scales within individual

122   microaggregates, mineral surfaces, microbes, and root hairs (Blair et al., 2006; Cliff et al.,

123   2002a; Clode et al., 2009; DeRito et al., 2005; Herrmann et al., 2007a; Herrmann et al.,

124   2007b; Keiluweit et al., 2012; Pumphrey et al., 2009). An early review paper by Herrmann

125   and co-authors (Herrmann et al., 2007b) focused on potential applications for soil ecology

126   and included the first application of the NanoSIMS technique with an intact soil

127   microaggregate. Subsequent publications have addressed the technical aspects (sample

128 preparation) and investigations of organo-mineral associations at scales ranging from clay
129 size mineral grain to intact soil cores (Keiluweit et al., 2012; Mueller et al., 2012b; Remusat
130 et al., 2012).

131 In this article, our goal is to provide insight into the range of potential NanoSIMS applications
132 in soil system research, discussing technical capabilities and limitations, major sample
133 requirements, and important complementary micro-spectrometry techniques. As NanoSIMS
134 applications in closely related fields, such as plant science and microbiology, have been
135 reviewed recently (Moore et al., 2011a; Musat et al., 2012), we focus on the use of
136 NanoSIMS in soil research.

137   1.2. Fundamentals of Secondary Ion Mass Spectrometry

138 SIMS is a surface analysis technique for solid samples. Primary ions, with a kinetic energy
139 ranging from a few hundred electron volts to tens of thousands of electron volts, are focused
140 on the sample surface, ejecting atoms and molecules in a process called sputtering (see
141 Figure 1). A small fraction of the ejected atoms and molecules are ionized, and can be
142 extracted with an electrostatic field into a mass spectrometer. The fraction of the sputtered
143 material that is ionized is determined by the ionization efficiency of the element in the sample
144 matrix, and is referred to as the secondary ion yield. For different elements, secondary ion
145 yields vary over many orders of magnitude, and also strongly depend on the physico-
146 chemical nature of the sample (Storms et al., 1977; Wilson et al., 1989). Within the mass
147 spectrometer, secondary ions can be separated according to their mass to charge ratio in a
148 quadrupole, magnetic-sector, or time-of-flight (TOF) mass analyzer. These analyzers differ in
149 terms of detectable mass range, sensitivity, ion transmission, and cost. As NanoSIMS has
150 both high sensitivity and spatial resolution at high mass resolving power, this particular SIMS
151 instrument meets many of the specific requirements for micro-scale elemental and isotopic
152 mapping analyses in soil science.

153

154   1.2.1. NanoSIMS

155 The NanoSIMS is optimized for SIMS imaging with sub-micron lateral resolution. The

156 NanoSIMS 50 and 50L instruments, conceived by George Slodzian (Slodzian, 1987;

157 Slodzian et al., 1992) was designed by Bernard Daigne, François Girard and François Hillion

158 (Hillion et al., 1993), and manufactured by Cameca France under a license from the Office

159 National d'Études et de Recherches Aérospatiales at Université Paris-Sud (UPS ONERA).

160 There are now more than thirty NanoSIMS instruments installed world-wide, working on a

161 wide range of applications ranging from geology and cosmochemistry (Floss et al., 2006;

162 Hoppe, 2006; Stadermann et al., 1999; Wacey et al., 2010a), to biology (Finzi-Hart et al.,

163 2009; Kraft et al., 2006; Lechene et al., 2006), material science (Valle et al., 2011) and soil

164 science (Herrmann et al., 2007a; Keiluweit et al., 2012; Mueller et al., 2012b).

165 The key innovation of the NanoSIMS is the coaxial lens (Figure 1) which focuses the primary

166 ion beam and extracts and focuses the secondary ion beam as well. This configuration

167 minimizes the distance between the sample surface and primary focusing lens, allowing the

168 primary beam to be focused to a much smaller diameter than in conventional SIMS

169 instruments. In addition, the secondary mass spectrometer is optimized for high-transmission

170 at high (>3000) mass resolving power. The NanoSIMS comes equipped with a Cs^+ primary

171 ion source for analysis of negative secondary ion species (e.g. $^{12}C^-$, $^{13}C^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$,

172 $^{28}Si^-$, $^{27}Al^{16}O^-$ and $^{56}Fe^{16}O^-$), and an O^- primary beam source, for analysis of positive

173 secondary ions (e.g. $^{23}Na^+$,$^{39}K^+$, $^{44}Ca^+$, ^{56}Fe). Due to the coaxial lens setup (Figure 1 B),

174 secondary ions must have the opposite charge from primary ions to enable extraction to the

175 mass spectrometer. A ~150 nm diameter Cs^+ primary ion beam with a beam current of 1 to 2

176 pico Ampere can routinely be achieved. While an even smaller beam diameter is possible,

177 there are trade-offs between high-resolution (with reduced beam current) and secondary ion

178 count rates. Higher currents and thus beam diameters are often crucial to yield significant

179 amounts of secondary ions (e.g. $^{13}C^-$, $^{12}C^{15}N^-$, $^{56}Fe^{16}O^-$) when analyzing soil samples. With an

180 O^- beam, a diameter of ~400 nm is typical.

181   The advantage of the NanoSIMS instrument lies in the coupling of a continuous, high spatial

182   resolution analysis beam with high mass resolving power, resulting in high sensitivity and

183   specificity with relatively short integration times. Users should also be fully aware that the

184   NanoSIMS 50 and 50L are both 'dynamic' SIMS instruments where the sample is actively

185   eroded during the sputtering process and molecular bonds are broken by the primary ion

186   beam. Up to five (NanoSIMS 50) or seven (NanoSIMS 50L) secondary ions can be detected

187   simultaneously. Additionally, if operated in Cs^+ mode, secondary electrons produced by the

188   collision cascade can be detected by a photo-multiplier, providing a secondary electron

189   image that can provide structural and textural information that is comparable to a low-

190   resolution SEM micrograph.

191   1.2.2. Basic requirements for NanoSIMS samples

192   A wide range of solid samples are compatible with SIMS, provided they are dry and stable

193   under high vacuum (< 10^{-9} mbar), relatively flat (< 2-30 microns of relief), and conductive.

194   Sample out-gassing can be caused by absorbed water, other volatiles, hydrocarbons or

195   samples prepared via resin embedding. Pre-treatment in a vacuum oven under low heat can

196   reduce out-gassing within the analysis chamber. Out-gassing may degrade analysis

197   conditions by elevating chamber pressure, reducing ion transmission and generating

198   molecular interferences or physical contamination, which will all lead to poor analysis quality.

199   Sample flatness is also important as any surface roughness may influence sample

200   sputtering, ion extraction and mass spectrometer tuning. For natural abundance isotopic ratio

201   measurements, 2-4 per mil level external precision can be achieved with repeated analyses

202   of bacterial spores with ~1 micron of relief (P.K. Weber, unpublished results). For isotopic

203   tracer experiments, more topographic relief (10 to 20 microns) can be tolerated (Woebken et

204   al., 2012). Even higher topographic relief (~30 microns) may also be viable in some

205   applications (P.K. Weber, unpublished results) but with significant loss in precision and the

206   need for careful monitoring of measurement quality. Finally, because SIMS uses an ion

207   beam to eject charged ions from the sample's upper atomic layers, a mechanism to dissipate

208 charge from the analysis location is critical. In our experience, most soil samples are semi-

209 insulating, and typically must be coated in an evaporator or sputter coater with a 2 – 20 nm

210 layer of gold (or carbon, iridium, gold-palladium, or platinum) to minimize charging during

211 analysis. Sample flatness can also interact with charge dissipation characteristics. As a

212 general rule, the more topography a sample has, the thicker a conductive coat needs to be to

213 bridge topographic gaps. Metal coating and sample flatness become particularly important if

214 an electron flood gun is to be used for charge compensation (see NanoSIMS soil preparation

215 details below).

216 ## 2. Experimental approaches for the study of soil microenvironments using

217 ## NanoSIMS

218 ### 2.1. Lessons learned from geology and microbiology

219 Perhaps one of the most appealing aspects of NanoSIMS analysis for many soil scientists is

220 the instrument's potential to quantitatively localize stable isotopes at a previously unresolved

221 spatial scale. Since the fields of geology, cosmochemistry, and geomicrobiology have a more

222 extensive tradition with SIMS and NanoSIMS applications, that literature is a logical source of

223 illustrative models for soils research. Cosmochemists were first to use NanoSIMS for isotopic

224 measurements, taking advantage of its high spatial resolution and simultaneous imaging

225 capabilities. Using NanoSIMS, Messenger et al. (2003) located rare isotopically anomalous

226 (> 100 ‰) micron-sized pre-solar grains within meteoritic samples that are too small to have

227 been analyzed by bulk measurements or even conventional SIMS (e.g. Cameca SIMS 1280).

228 NanoSIMS has also been used to measure large isotopic fractionation in biominerals (e.g.

229 carbonates from mollusk shells and corals) and deduce metabolic pathways such as

230 methanotrophy (Rasmussen et al., 2009) or sulfate reduction and sulfur disproportionation

231 (Philippot et al., 2007; Wacey et al., 2010b). In geologic samples, NanoSIMS imaging was

232 employed to support a microbial origin for Ooids - concentrically-laminated sedimentary

233 grains found in turbulent marine and freshwater environments (Pacton et al., 2012), and a

234 microbial role in Fe-mineralization within spheroidal iron oxide concretions associated with

235 palaeoaquifers (Weber et al., 2012).

236 All of the studies mentioned above capitalized upon natural isotope fractionation effects that

237 can shift natural abundance values by 10s to 100 per mil (e.g. for biological sulfur cycling -

238 8.5‰ to +19‰ $\delta^{34}S_{CDT}$ (CDT – Canyon Diablo troilite); for methanotrophy -55‰ to -43‰

239 $\delta^{13}C_{PDB}$ (PDB – Pee Dee belemnite) (Rasmussen et al., 2009). These effects are in many

240 cases significantly larger than those found in soil/sediment systems, where prominent

241 isotopic effects range from ~ 1 to 11‰ $\delta^{13}C$ (litter decomposition, C3-C4 plant shifts,

242 (Ehleringer et al., 2000)), ~7‰ $\delta^{15}N$ (soil depth gradients, (Billings and Richter, 2006)) or as

243 little as 0.8‰ $\delta^{56}Fe$ (soil iron pools, (Kiczka et al., 2011)). One exception is the work of

244 Orphan and colleagues (2001) who have used SIMS and NanoSIMS to image isotopic

245 fractionation in modern anoxic sediment cores of the Eel river basin in the Pacific Ocean.

246 There, the authors report isotopic fractionations of $\delta^{13}C$ of up to -96‰ in the interior of

247 bacterial aggregates, indicating consumption of isotopically light methane by methanotrophic

248 bacteria.

249 For most soil process-level questions, the best approach may be to use stable isotope

250 labeling as a way to document transformation pathways in soil micro-environments over time.

251 This approach, like many of the published examples in cosmo- and geo-chemistry, would

252 take full advantage of the NanoSIMS's spatial resolution while improving detection of the

253 isotopic species of interest. In the past decade, stable isotope labeling (often with ^{13}C or ^{15}N)

254 and NanoSIMS analyses have been widely used in environmental microbiology, supporting

255 research on the metabolism of single microbial cells (Pett-Ridge and Weber, 2012) both in

256 pure culture and in natural environmental samples ranging from marine bacterial and

257 archaeal communities (Dekas et al., 2009; Halm et al., 2009; Musat et al., 2008; Ploug et al.,

258 2010), to acid mine drainage biofilms (Moreau et al., 2007), to ^{13}C and ^{15}N fixation in

259 diazotrophic cyanobacteria (Finzi-Hart et al., 2009; Popa et al., 2007; Woebken et al., 2012)

260 and eukaryotes (Lechene et al., 2006; Lechene et al., 2007).

261 An idealized stable isotope labeling experiment in soil might proceed as follows: (1) add an

262 organic compound of interest, labeled for instance with ^{13}C or ^{15}N, to a model system that

263 contains reactive mineral surfaces and an active microbial decomposer community; (2)

264 incubate under controlled conditions, varying an edaphic variable of interest (moisture,

265 temperature, pH); (3) prepare and analyze samples via NanoSIMS imaging to determine the

266 physical fate of the target compound – whether it becomes metabolized (and the label is

267 transferred to microbial decomposers) or whether it is adsorbed to mineral surfaces.

268 Enrichment of the label above a background value could then be used to support inference

269 about the fate of the compound of interest. This type of application could potentially

270 contribute to both studies of soil carbon turnover dynamics as well as investigations of

271 contaminant fate in soils.

272 Previous NanoSIMS studies of biotic (microorganisms and plants) and abiotic materials

273 (minerals, fossils) represent endmember models of the soil system, with its inherent

274 combination of geologic and microbiological aspects. This substantial literature serves as a

275 valuable resource for soil scientists interested in designing micro-scale soil research,

276 particularly as a resource for experimental concepts and sample preparation protocols. In the

277 following section we discuss examples from soil science where NanoSIMS has been

278 successfully applied, as well as additional areas where soil interface research could

279 significantly benefit from high-resolution isotopic imaging in the future. As we point out

280 potential applications, we also mention pitfalls and methodological constraints.

281 2.1.1 Investigating mineral-organic associations

282 Historically, studies of mineral-organic associations have employed bulk analysis procedures

283 performed on operationally defined physical fractions (Balesdent et al., 2000; Christensen,

284 2001; Eusterhues et al., 2005; Schöning et al., 2005). The goal of such procedures is to

285 isolate mineral-organic associations of given characteristics, such as an increasing

286 proportion of microbially processed organic matter (OM) in fractions of increasing density

287 (Derrien et al., 2006; Grandy and Neff, 2008; von Lützow et al., 2007). In contrast to bulk

288 analysis, NanoSIMS offers the possibility to examine organo-mineral assemblages in the

289 context of intact spatial structures. If a stable isotope labeling experiment (see above) is

290 used, NanoSIMS images can potentially reveal the spatial distribution and dilution of a tracer

291 material as it moves into the soil matrix. They can also reveal whether preferential

292 associations of certain OM types predictably associate with certain mineral phases. This is

293 possible because of three main advantages NanoSIMS has over other microscopic

294 techniques: (1) elemental mapping can be done with better lateral resolution, (2) the low

295 depth penetration (~ 10 to 20 nm) of the NanoSIMS primary beam allows thin surface layers

296 to be examined, (3) highly accurate isotope detection allows the operator to track OM^{13}C and

297 OM^{15}N onto distinct minerals in an intact micro-environment, and thus enables process-level

298 studies. In a proof-of concept example, these three advantages were exploited by Heister et

299 al. (2012), who showed that in artificial soil mixtures, soil minerals and organic materials can

300 be distinguished in NanoSIMS images, using the distinction between organic material

301 derived ions (^{12}C$^-$ and ^{12}C^{14}N$^-$) and mineral derived ions (^{28}Si$^-$, ^{27}Al^{16}O$^-$, and ^{56}Fe^{16}O$^-$). The

302 authors used NanoSIMS in this study as a tool for micro-scale elemental mapping of organic

303 matter on mineral surfaces. They showed that organic matter tended to be attached to

304 phyllosilicate clays in the form of isolated patches, while continuous coatings of organic

305 matter enveloped small ferrihydrite particles. Such micro-scale heterogeneities could not

306 have been resolved by SEM/EDX measurements. In another example, Mueller et al. (2012b),

307 working with resin-embedded soil macroaggregates, found heterogeneous isotopic

308 enrichment at the micro-scale following the application of a ^{13}C/^{15}N label (amino acid mixture

309 of algal origin) to natural soils. They speculated that microbial activity may have lead to the

310 increased utilization of freshly added organic matter, or that soil components have different

311 sorption capacities. The NanoSIMS's unique capacity to detect stable isotope tracers at the

312 micro-scale enabled both these studies to confirm the predicted physical dimensions of

313 organo-mineral associations.

314 By combining isotopic and elemental imaging, NanoSIMS analysis can also reveal whether

315 certain OM types predictably associate with certain mineral phases. This particular capacity

316   of the NanoSIMS was used by Keiluweit et al (2012) in a study where $^{13}C/^{15}N$ enriched fungal

317   hyphal extracts were incubated with organic horizon soil. NanoSIMS images of ^{15}N

318   enrichment and iron distribution suggest that nitrogen from fungal cell walls was rapidly and

319   preferentially deposited as thin organic coatings onto Fe (hydr)oxide surfaces (Keiluweit et

320   al., 2012). Further analysis of these samples by scanning transmission X-ray microscope in

321   combination with near edge X-ray absorption fine structure spectrometry (STXM-NEXAFS)

322   revealed these soil microstructures were enriched in aliphatic C and amide N, suggesting

323   that a concentration of microbial lipids and proteins had quickly become associated with Fe

324   (hydr)oxide surfaces. Remusat et al. (2012) used a similar approach to image intact soil

325   particles with low levels of isotopic enrichment sampled 12 years after a ^{15}N litter labeling

326   experiment in a temperate forest. They describe microsites of isotopic enrichment ("^{15}N hot

327   spots") on mineral surfaces, and in one microsite, suggest that ^{15}N enrichment was also

328   linked to the presence of microbial metabolites. This kind of combined approach, NanoSIMS

329   image analysis joined to complementary microscopy (SEM-EDX, STXM/NEXAFS), may be a

330   particularly profitable means to infer the molecular and spatial fate of labeled organic

331   materials in a mineral matrix, and has the potential to contribute to a mechanistic

332   understanding of sorption, occlusion, and decomposition processes that operate at fine

333   spatial scales.

334   A recent quantitative analysis of organo-mineral assemblages by Hatton et al. (2012) used a

335   combination of macro- and micro-scale analyses for an internal calibration of C/N and $^{15}N/^{14}N$

336   ratios in sequentially separated soil density fractions. This approach is based on the

337   assumption that macroscopic features, visible under reflectance light microscope and

338   analyzable by elemental analyzer isotope ratio mass spectrometry (EA-IRMS), are also

339   found at the micro-scale as detected in SEM or NanoSIMS images. The authors collected

340   NanoSIMS images over 500 μm^2 for each density SOM fraction, and corrected these using

341   EA-IRMS data from macroscopic features. Because matrix-matching SIMS standards for soil

342   organic matter do not yet exist, this calibration approach is a promising step towards a better

343   quantification of data derived from SIMS images. While significant procedural challenges

344 remain, the examples presented above illustrate how well-designed experiments can benefit

345 from NanoSIMS information to help decipher chemical and microbiological processes in soil

346 microenvironments.

347   2.1.2 Investigating intact three dimensional micro-structures

348 NanoSIMS imaging may also be profitable in studies of micro-scale soil architecture. The first

349 systematic approach to the study of in-situ soil features was established by the

350 micropedological work of Kubiena (1938). Whole intact soil clods were impregnated with

351 epoxy resin, thin sections were produced and small scale pedological features were studied

352 using transmitted light microscopy. A large range of soils have been studied using this

353 technique, combining different light sources ranging from polarized light to fluorescent

354 staining of microbial cells (Bullock and Murphy, 1980; Eickhorst and Tippkoetter, 2008; Fisk

355 et al., 1999; Li et al., 2004; Pulleman et al., 2005). With the rise of analytical techniques that

356 can resolve soil features at the nano- to micro-scale (e.g. TEM, AFM, NanoSIMS), the

357 micromorphological examination of soils is experiencing a renaissance. However, mineral

358 particles pose a challenge to elemental mapping and isotope tracing experiments in intact

359 soil matrices because they make embedding and thin-sectioning more difficult, and can

360 cause electrical charging effects (Cliff et al., 2002b; Pett-Ridge et al., 2012). Still, a number

361 of proof-of-concept studies have successfully shown that ^{15}N and ^{13}C isotope additions can

362 be imaged by NanoSIMS in two dimensions within a natural or synthetic soil matrix

363 (Herrmann et al., 2007b; Keiluweit et al., 2012; Mueller et al., 2012b; Pett-Ridge et al., 2012;

364 Remusat et al., 2012).

365 Sample preparation is the most important issue to be resolved prior to micro-scale studies of

366 soil three dimensional structures. This is particularly true for soil macroaggregates (> 250 μm

367 in diameter) which have topography too large for reliable NanoSIMS measurements. To

368 maintain adequate flatness and integrity in friable samples, larger soil aggregates and intact

369 soil cores will typically require embedding and subsequent sectioning. However, simply

370 cutting large aggregates into sections can affect structural integrity. One solution is thin

371   sectioning, although the approach used must be chosen with the target ions in mind. The

372   most important considerations include:

373   Does the sample contain both mineral and organic phases?

374   Might the embedding medium dilute the signal of the target species (e.g. ^{13}C)?

375   Is *in situ* hybridization to be used, and are diffusible ions or molecules of interest?

376   We discuss the finer details of these concerns in Sections 3.3.2 and 3.3.3. In general, our

377   experience has shown that for smaller macroaggregates (~250 µm) cryosectioning is a

378   laborious but worthwhile technique to obtain cross sections while avoiding contamination with

379   any artificial C or N sources. For examination of whole intact soil cores or macroaggregates

380   (several mm in diameter) resin embedding is currently the best option, although it introduces

381   an artificial C and N source, which can interfere with both isotopic analyses and techniques

382   to determine the chemical structure of OM (e.g. STXM). If target ions include C and N, resin

383   embedding should thus be used only for larger volume soil specimens consisting of a friable

384   porous network of organic and mineral particles that have to be tightly bound together in

385   order to allow cross sectioning and polishing. However, for some resins (e.g. Araldite 502)

386   the ^{12}C^{14}N/^{12}C ratio allows to distinguish between sample OM and embedding agent (Weber

387   et al., 2012). The resin embedding approach has been used to prepare slices of intact soil

388   cores for elemental mapping of in-situ interfaces in a buried Oa horizon originating from a

389   permafrost-affected soil in Northern Alaska (Typic Aquiturbel, coastal plain near Barrow)

390   (Figure 2). In this case, NanoSIMS imaging was used for elemental mapping of natural

391   micro-scale features at a scale which could not be resolved by comparable techniques such

392   as SEM-EDX. This example shows how NanoSIMS can illustrate the patterning of distinct

393   phases (organic matter (^{12}C^{14}N$^-$), organo-mineral interfaces, plant cells) via elemental

394   mapping of such friable and highly heterogeneous intact soil structures.

395   2.1.3 Investigating plant – soil processes

396   The interfaces between plant roots and soil (rhizosphere) or fungal hyphae and minerals

397   (hyphaesphere) are extremely biologically active and important sites for mineral weathering

398 (Finlay et al., 2009). Hinsinger et al. (2009) suggest that a lack of suitable observational tools

399 stands in the way of a better understanding of micro-scale elemental distributions in the

400 rhizosphere. Here NanoSIMS might well fill the gap between reflectance light microscopic

401 (e.g. epifluorescence, polarized light) and x-ray techniques (e.g. x-ray tomography) to trace

402 C, N and nutrient transfers between roots, microbes and soil. For the biotic side of the plant-

403 soil system, Gea et al. (1994) showed the utility of SIMS by imaging Ca in ectomycorrhizal

404 fungi (*Hebeloma cylindrosporum*) associated with pine trees (*Pinus pinaster*). Figure 3 is a

405 proof-of-concept of how NanoSIMS may be used to explore an intact plant-soil system at the

406 micro-scale. In this example, a French oak (*Quercus robur*) seedling was grown in a

407 vermiculite / soil mixture with a mycorrhizal fungi *Piloderma croceum* in order to track

408 interfaces between mineral constituents and the plant root. NanoSIMS images of an

409 embedded oak root tip illustrate that clay minerals may be distinguished from root cells and

410 mycorrhizal cells within the vermiculite layers, revealing the interfaces between the mineral

411 soil compartment, roots, and mycorrhizal fungi. This example demonstrates how NanoSIMS

412 images might contribute to the exploration of intact plant-soil-microbe interfaces.

413 Part of the difficulty associated with attempts to image the interfaces between plants,

414 microbes and mineral particles has to do with preparing samples in a manner that adequately

415 preserves these interfaces. A challenging but promising approach to preserve intact soil

416 architecture was demonstrated by Clode et al. (2009) who prepared 100 nm thick cross-

417 sections of ^{15}N labeled wheat roots and associated bacteria by slowly infiltrating with araldite

418 epoxy over the course of several days. The resulting epoxy blocks were thin-sectioned and

419 then observed by both TEM and NanoSIMS at the University of Western Australia. The TEM

420 images clearly identified bacteria attached to the cortical cell walls, while NanoSIMS imaging

421 revealed that not all of the bacteria had incorporated the ^{15}N label (Figure 4). While it is

422 possible that some cells were not metabolically active or dead, it is equally possible that

423 some of the ^{15}N 'hotspots' were actually remnant effects of salts derived from the enriched

424 precursor material ((^{15}NH$_4$)$_2$SO$_4$). This is a case where a complementary technique, for

425 example fluorescent in situ hybridization (FISH) or a live/dead or DAPI stain (see section

426 4.4.), might be useful to corroborate whether enriched features in NanoSIMS images truly

427 are bacterial cells.

428 2.1.4 Tracking organic and inorganic pollutants

429 Organic and inorganic pollutants span a wide range of molecular properties and may be

430 involved in a host of mechanistically different interactions with soil solids. Important inorganic

431 pollutants are metals and metalloids (e.g. Pb, As) (Bradl, 2004; Wilson et al., 2010; Zimmer

432 et al., 2011), including radioactive particles from nuclear accidents (Carbol et al., 2003;

433 Spezzano, 2005). Organic pollutants are inherently more diverse, encompassing the full

434 range from nonpolar polycyclic aromatic hydrocarbons to relatively polar chlorinated

435 hydrocarbons and polychlorinated biphenyls. To date, SIMS has been used to study the

436 micro-scale distribution of metals (e.g. Cd, Cr), metalloids (e.g. As), and halogens and

437 organic pollutants in microbial cells (Eybe et al., 2008), plants (Lombi et al., 2011;

438 Mangabeira et al., 2006; Martin et al., 2001; Migeon et al., 2009; Moore et al., 2010; Moore

439 et al., 2011b; Tartivel et al., 2012), animals (Eybe et al., 2009) and human tissues (Audinot et

440 al., 2004). NanoSIMS has been used to examine plutonium transport in the subsurface of

441 heavily contaminated sites (parts per million levels) (Kips et al., 2012; Novikov et al., 2006).

442 When there is substantial contamination, Pu can be directly imaged in situ and the

443 association of Pu with specific minerals can be determined to constrain transport

444 mechanisms. An intriguing example of a system comparable to primary soil particles (e.g.

445 clay minerals, OM particles) was presented by Krein et al. (2007), who located heavy metal

446 accumulation in aerosols using NanoSIMS by imaging $^{63}Cu^-$, $^{75}As^-$, $^{118}Sn^-$ and $^{123}Sb^-$. This

447 work suggests that it is possible to determine spatial dependencies between organic matter

448 and inorganic pollutants and evaluate 'hot spots' on micron-scale particles.

449 The primary limitations for NanoSIMS studies on organic pollutants are the vapor pressure of

450 the target pollutants (e.g. non-volatile organic compounds), the concentration of the target,

451 and incorporating a tracer for the target. Eybe et al. (2008) embedded *Anabaena* sp. cells

452 grown on the pesticide deltamethrin in an epoxy resin. To trace the pesticide within the

453 embedded cells, ^{81}Br- was imaged in the NanoSIMS, illustrating how halogen containing

454 pollutants may be used as tracers within biological samples. Another example is the work of

455 Tartivel et al. (2012), who traced bromotoluene by the imaging of ^{81}Br$^-$ in chemically fixed

456 plant roots (*Hedera helix*) and resin embedded soil cross sections.

457 **3.   NanoSIMS requirements for soil related studies**

458 3.1.   Technical considerations for soil samples

459 With its improved primary ion optics and secondary ion transmission at high mass resolving

460 power the NanoSIMS 50 and 50L enable SIMS analysis at the nanometer scale. However,

461 there are specific technical limitations that the potential user must consider, especially for

462 soils applications. While primary beams smaller than 50 nm are possible with idealized

463 samples, the number of ions collected from the impacted volume starts to fall below the

464 useful level in soils. NanoSIMS is a high vacuum (~10^{-10} Torr) instrument that requires

465 samples to be dehydrated, conductive and have low topography (ideally submicron for

466 natural abundance and < 30 micron-scale for isotopic enrichment experiments). As a result,

467 live microbial cells cannot be tracked, and to measure process effects over time, one must

468 rely on sub-sampling and replication. Samples should be fixed and can be thin-sectioned to

469 achieve a flat surface, ideally without re-arranging the locale of target elements or molecules.

470 While not currently available, a cryogenic stage might allow frozen samples to be analyzed,

471 thereby preserving them in a more natural state.

472 Though analyses of natural abundance ^{13}C and ^{15}N are widely used in soil science, the

473 NanoSIMS is capable of measuring isotope ratios with a precision of ~ 1‰ only in very

474 favorable cases, and even a level of ~10‰ precision is likely to be very challenging to

475 achieve in most complex soil samples. To obtain such a high precision (<1‰), relatively large

476 amounts of material must be extracted from the sample surface (nanograms), requiring a

477 large primary spot size ~20 – 30 μm. Such a spot size might itself exceed the micro-scale

478 structures of interest (e.g. bacterial cells, clay minerals). Also, the much smaller primary

479 beam used in NanoSIMS imaging generates a smaller number of secondary ions,

480 necessitating the use of electron multiplier (EM) detectors. EMs have a faster response time

481 than faraday cup detectors (FC) (commonly used in larger beam SIMS instruments), and are

482 thus both fast enough and provide sufficient dynamic range for imaging. EM detectors are,

483 however, subject to a number of artefacts (limited count rates and detector aging) that

484 effectively limit the precision to about 1‰. On top of this, the extraction conditions from

485 location to location can be hard to maintain at a level that yields precision better than one

486 part in a thousand, especially with heterogeneous samples such as soils. The limitations on

487 precision mean that in most soil systems, natural abundance measurements will not produce

488 useful data. Isotopic measurements with higher precision can be achieved using the Cameca

489 IMS1280, a large-geometry magnetic sector ion probe, combining high transmission, high

490 abundance sensitivity and high density of the primary beam with thermally insulated FC

491 electronics. This instrument could potentially be used in complementary studies with a

492 NanoSIMS to record images of high spatial resolution as well as per mil level precision.

493 In our experience, tracking of isotopically labeled tracers is probably the most practical way

494 to explore micro-scale soil processes using NanoSIMS (Herrmann et al., 2007a; Keiluweit et

495 al., 2012). As C and N are the key elements in organic matter studies, substrates enriched in

496 ^{13}C and/or ^{15}N are regularly used for general investigations of microbial metabolism in soils

497 as well as for the more specific purpose of following the fate of organic compounds as they

498 cycle through soils (Kuzyakov et al., 2000; Ruetting et al., 2011). Of critical importance in

499 tracer studies is whether the labeled substrate becomes chemically modified or is otherwise

500 affected by the sample preparation. The potential user is reminded that NanoSIMS is not well

501 suited for the identification of molecules or characteristic molecular fragments and so will

502 rarely be able to address this kind of problem directly.

503 While quantitative NanoSIMS isotopic analyses are relatively straightforward, quantitative

504 analyses of elemental abundances are considerably more challenging. In the fields of

505 material science and mineralogy, SIMS users usually employ standards to correct for

506 differences in yields and quantity for defined element-matrix combinations. The inherent

507 complexity and variability of soil matrices can complicate this approach, as appropriate

508 standards are harder to obtain or manufacture. The C to N elemental ratio in soil OM, for

509 example, is of general interest to soil scientists. The measurement of this ratio is inherently

510 challenging because $^{12}C^-$ has a different formation mechanism than the $^{12}C^{14}N^-$ ion (N is

511 detected as CN), as atomic N ionization is very poor. As a result, the yield of the two species

512 can change relative to each other during the course of an analysis. C to N ratio

513 measurements are therefore very challenging, requiring matrix-matched standards and

514 method optimization, and ultimately may result in measurements of low accuracy and

515 precision. It is an open question whether the heterogeneity of soil material makes such

516 measurements additionally challenging.

517 ## 3.2. Sample documentation

518 To facilitate the analyses, it is best to determine regions of interest on the sample prior to

519 performing NanoSIMS measurements (Herrmann et al., 2007a; Moore et al., 2011a; Weber

520 and Holt, 2008). Sample mapping can greatly enhance the efficiency of the analyses and is

521 often critical to interpretation of results. Most SIMS instruments have the equivalent of an epi-

522 illumination microscope for sample navigation, and therefore epi-illumination micrographs

523 provide the best reference images for general navigation. Electron microscopy can also

524 positively identify targets for analysis, and these images are often easily comparable to the

525 secondary electron or ion images generated during NanoSIMS analyses. Ideal mapping

526 images range from the whole sample scale to the individual target scale, with reference

527 points that can be used to translate from one image scale to the next.

528

529

530 ## 3.3. Instrument tuning and quality control

531 Here we present a brief introduction to issues that may be encountered during the tuning of a

532 NanoSIMS 50 or 50L; more detailed instructions have been previously published(Pett-Ridge

533 and Weber, 2012). The central aspects of SIMS instrument tuning are mass selection,

534 resolving isobaric interferences and peak shape.

535   a) **Mass selection**: To obtain accurate measurements, the secondary ion mass

536   spectrometer must be tuned and aligned to collect ion masses of interest. Choosing

537   masses will depend entirely upon the question being asked, what isotopically labeled

538   compounds have been added, and whether operation is proceeding in Cs^+ or O^-

539   mode. Often this necessitates choosing between analyzing common bio-elements

540   (e.g. C, N, O, S, P) with a Cs^+ beam, or metals/metalloids (e.g. Fe, Al, Mn, Mo) with a

541 O^- beam. If the ion yield is sufficient, some creative solutions exist, for example Fe

542   can be detected as FeO^- in Cs^+ mode. Alternatively, subsequent O^- mode

543   measurement of the same spot is possible.

544   b) **Isobaric interferences**: When selecting ion masses it is critical to consider and

545   exclude isobaric interferences, which are species with near-identical masses to the

546   species of interest. Isobaric interferences can be problematic for SIMS because the

547   sputtering process generates molecules. Therefore, in addition to isotopes with the

548   same mass (e.g., ^{48}Ti and ^{48}Ca), users must consider isobaric interferences that are

549   clusters of atoms, many of which are artificial (e.g., $^{23}Na^{24}Mg^1H^+$). To identify

550   potentially significant isobaric interferences, the first step is to determine the major

551   elemental composition of the sample (here, previous SIMS studies may be helpful).

552   Blanks and control samples can be used to determine if interfering molecules are

553   produced at significant levels. If this turns out to be the case, the exact masses of

554   significant isobaric interferences need to be calculated relative to the exact mass of

555   the target species to determine difference in mass (ΔM) and the mass resolving

556   power ($MRP = M/\Delta M$) required to resolve the target species. MRP is a metric of peak

557   shape and the tuning of the mass spectrometer.

558   c) **Peak shape**: The scan of the mass peak should be both flat-topped and steep-sided.

559   The shape of the peak is the cumulative result of everything from the primary beam

560   location and size to the gain on the detector. A tightly focused and well centered

561   primary beam reduces angular aberration and minimizes potential distortions. The

562   secondary ion beam should be aligned relative to all the lenses, slits and apertures in

563   the secondary mass spectrometer in order to maximize transmission and minimize

564   distortion. The entrance slit effectively sets the nominal MRP of the mass

565   spectrometer, and the aperture slit and energy slit are used to steepen the peak side

566   slopes and reach the target MRP by reducing angular and chromatic aberrations.

567   Peak top flatness is important to measurement stability and indicates that 100% of

568   the target species is collected to the exclusion of isobaric interferences. In addition to

569   tuning and alignment issues, peak top flatness is also affected by the electron

570   multiplier gain, high voltage, threshold and deflector settings.

571 Standards are used during the process of setting up the mass spectrometer; they are used

572 for mass selection, quantification, and session to session comparison of transmission, MRP,

573 and elemental or isotopic ratios. Reference materials can be simple (e.g., iron), multi-element

574 standards like the NIST glass standard NBS610 (500 μg/g of most elements), or

575 generated/characterized 'in-house' by characterizing samples through bulk methods and

576 verifying high resolution homogeneity by replicate SIMS analyses. One example is the

577 *Bacillus subtilis* spore sample used by the LLNL NanoSIMS group as a reference standard

578 for C and N isotopic measurements ($^{13}C/^{12}C = 0.0110$; $^{15}N/^{14}N = 0.00370$). For this standard,

579 isotopic enrichments were determined by bulk analysis at the University of Utah (Finzi-Hart et

580 al., 2009); measurement precision, σ (internal), is ~4 % (2σ) for individual $^{13}C/^{12}C$ and

581 $^{15}N/^{15}N$. For many soil science applications, the co-occurrence of both mineral and organic

582 phases requires that both a multi-element (NBS 610) and organic phase standard be used.

583 However, when studying complex organo-mineral interfaces, the aim should always be to

584 simultaneously record mineral and organic derived ions to obtain a complete view on the

585 studied mineral surfaces. Caution must also be exercised when applying SIMS to identify

586 specific mineral phases. The count rate of an ion is not directly proportional to the

587 concentration in the mineral. For example, the content of iron influences the secondary ion

588 yield of other elements that are present in the sample. A linear interpolation based on the

589 elemental concentration of the mineral is, therefore, not reasonable (Lehmann, 2003). It is

590 thus advantageous to confirm mineral identity using X-ray techniques or other methods. In

591 cases where quantification of an element is desired, mineral specific standards are

592 necessary.

593 Minerals in soil pose a particular challenge because mineral grains may accumulate an

594 electrical charge under sustained Cs^+ primary ion beam sputtering. The phenomenon can

595 readily be observed in the NanoSIMS because charged regions generate very low secondary

596 electron yields. The area of charge accumulation may still yield secondary ions, but their

597 trajectories through the secondary mass spectrometer will deviate from those of other ions

598 when they have significantly different energy (>10 V difference). As a result, some species

599 may not be detected with the same relative efficiency, resulting in inaccurate measurements.

600 This is primarily an issue for the analysis of negative secondary ions because large numbers

601 of electrons are extracted when the sample surface is bombarded by a beam of Cs^+ ions. At

602 the initiation of analysis, while the metal coating is still intact, charging may not be obvious. In

603 some cases, minerals (e.g., magnetite) or organic material in the sample may provide

604 sufficient charge conductivity to allow analysis. Most organic matter, while insulating in its

605 natural state, becomes conducting under ion bombardment. However, soil particles, even

606 those initially coated with Au, often begin to show evidence of sample charging after ~ 20

607 minutes of analysis, with significantly diminished total ion counts (Pett-Ridge et al., 2012).

608 This effect has been observed in a NanoSIMS study of microaggregates deposited on

609 silicon-nitride windows, where regions free of organic material appeared to undergo the most

610 obvious charging (Remusat et al., 2012).

611 The best way to overcome sample charging is to use a device called an electron flood gun,

612 commonly referred to as the e-gun. Tuning of the e-gun requires significant experience in

613 aligning the electrons with the primary beam on the sample, and in adjusting the voltage to

614 the charge status of the sample. Another challenge is that secondary electron imaging is not

615 possible while the e-gun is in use. Often it is easiest to initially locate particles of interest with

616 secondary electron imaging, and then turn on the e-gun for ion image data collection. When

617 the e-gun is tuned correctly, nonconductive minerals will not charge and the mass peaks will

618 maintain the same level of mass resolution as achieved on a conducting sample. We note

619 that the sample must be covered with a conductive coating for the e-gun to work; electrons

620 that reach the sample outside the analysis area must have a path to ground. The e-gun is not

621 likely to work with samples with significant topography and is not a solution in cases where

622 topography is the proximal cause of charging and ion shadowing. In tests of different mineral

623 types performed at LLNL, using the e-gun increased the ion yield for C and N by as much as

624 a factor of 10. Additional tests showed that isotope ratios of standard reference materials

625 analyzed both with and without the e-gun were not significantly different (Pett-Ridge et al.,

626 2012). Caution is always advisable when interpreting results were charging is possible.

627 ## 3.4. Sample preparation – from single particles to intact soil

628 In the next paragraphs we discuss possible ways to prepare soil samples ranging from

629 primary particles and aggregates to complex intact samples containing microbial and plant

630 tissues.

631 ### 3.4.1. Direct deposition of individual particles and microaggregates

632 Individual soil particles (e.g. clay minerals, particulate OM) as well as microaggregates can

633 be deposited directly onto an analysis support (e.g. Si-wafer, polished metal stubs) and

634 imaged via NanoSIMS provided they have limited topography and remain adhered to the

635 sample support under high vacuum.

636 Such materials may be derived from physical soil fractionation (Eusterhues et al., 2005) or

637 taken directly from a soil suspension (Keiluweit et al., 2012). The application of ultrasound

638 during soil fractionation may destroy natural soil structure and also re-distribute both organic

639 compounds as well as individual mineral particles between physical fractions (Amelung and

640 Zech, 1999; Mueller et al., 2012a). While pretreatments needed to isolate small particles may

641 introduce experimental bias, it may still be desirable to attempt direct observations of small

642 particles or microaggregates, especially when the nature of the research question prohibits

643 the use of C and N-containing fixatives. Samples can be deposited on flat surfaces like

644 polished metal stubs, Si-wafers, gold foil or directly on SEM or TEM grids (Keiluweit et al.,

645 2012; Mueller et al., 2012b). Alternatively, particles can be collected from soil suspensions

646 on gold coated filter discs, a widely used preparation technique in microbiology (Musat et al.,

647 2012). For obvious reasons, the sample support must be chosen with the elements of

648 interest in mind (Si-wafers are not advisable when Si is a target element). In the direct

649 deposition preparation technique, a single layer of small particles/microaggregates should be

650 deposited on the sample support to minimize topography. If the goal is to simultaneously

651 explore both mineral and microbial constituents, fixation of the biological tissues will be

652 necessary (see below for detailed description).

653   3.4.2. Fixation and preparation of organic materials

654 A variety of biological sample types may be of interest to soil scientists, ranging from root or

655 hyphal tissues to bacterial and archaeal cells, protists and microfauna, or decaying plant

656 litter. For all these sample types, preservation of cell structure is important for target

657 identification and to contain mobile organic and inorganic constituents. Sample preparation

658 typically involves stabilization of biological components (fixation), removal of water

659 (dehydration) and salts (derived from growth media or sea/soil water), deposition of the

660 sample on a conductive support (Si wafer, TEM grid) and subsequent whole cell analysis or

661 thin sectioning after embedding. Fixation of biological tissues is a process used to preserve

662 cell morphology from decay and immobilize analytes of interest by increasing molecular

663 mechanical strength or stability. Chemical fixation for imaging analysis is typically performed

664 with aldehydes (e.g., glutaraldehyde, paraformaldehyde, formaldehyde) that cross-link

665 proteins to hold cell structure together during dehydration, (Kuo, 2007; Nunan et al., 2001;

666 Tippkötter and Ritz, 1996). Low temperature methods like flash freezing and high pressure

667 freezing (Chandra and Morrison, 1992; Dykstra and Reuss, 2003; Echlin, 1992) are an

668 alternative in some circumstances if it is critical to preserve the distribution of small

669 molecules and diffusible ions. For biological samples that are to be analyzed intact, collection

670 on a filter is very efficient, and nucleopore or polycarbonate filters can be used as a sample

671 support for SIMS analysis if they are kept flat at the micron scale. Samples grown on a solid

672 sample support can be immersed in fixative and gently washed by repeated immersion in

673 deionized water.

674 If subcellular elemental or isotopic distributions are of interest in soil microbial or plant tissue

675 samples, fixation is often followed by embedding (impregnating the sample with a hard,

676 vacuum stable matrix) and sectioning (cutting the sample into cross-sections) prior to

677 NanoSIMS analysis. Samples can be embedded in a number of polymers for room

678 temperature sectioning (e.g., epoxy, acrylic, paraffin (Dykstra and Reuss, 2003)). Cryogenic

679 sectioning can be performed with sucrose, OCT or similar compounds. Cryosectioning of

680 frozen (water/ice embedded) samples is also an option, but is more technically challenging

681 and requires much practice. Sectioning can be performed with an ultramicrotome, a standard

682 histological microtome or cryostat, or even with a razor blade, depending on the type of pre-

683 NanoSIMS imaging that is desired. An adhesive surface coating (e.g. poly-L-lysine, vector

684 bond, egg white) can be used to retain sections during washing or staining. Such surface

685 coatings can also be used for the fixation of other thin sections or micro-scale single particles

686 on sample mounts (e.g. Me-stubs, Si-wafers). Contrast stains used for TEM imaging of

687 biological ultrastructure (e.g. uranyl acetate) typically are compatible with SIMS because

688 mass spectrometry can easily resolve the stain components from many species of interest.

689 However, there may be cases where contrast stains would be a source of unwanted

690 background counts, particularly for metal analysis. Standard TEM-grade ultrathin sections

691 (~100 nm) can be analyzed by high resolution NanoSIMS, but with a limited analysis time to

692 avoid section breakage or consumption. To extend the SIMS analysis time and collect more

693 data while still allowing correlated TEM-SIMS analysis, thicker sections (up to 500 nm) can

694 be prepared, though with a loss in TEM image quality. Thicker sections are also desirable if

695 large areas (mm^2) need to be analyzed. Focused ion beam (FIB) milling can also be used as

696 an alternative to embedding and sectioning (Weber et al., 2010), particularly where the user

697 needs to have precise control over the location and orientation of the sample thin-section.

698 Thin sections can be laid on a TEM grid or directly on a solid substrate prior to SIMS

699 analysis. For example, this technique was used by Bonneville et al. (2011) to prepare cross

700 sections of a micro-scale fungal hyphae / biotite interface for subsequent TEM analyzes. If

701 transmission light imaging is necessary for sample mapping, indium tin oxide (ITO) coated

702 glass slides are preferable to uncoated glass slides because the ITO coating does not

703 charge under ion bombardment.

704 ### 3.4.3. Preparation of aggregated soil structure and intact plant-soil systems

705 Many soil researchers would like to be able to image cross-sections of both biological and

706 mineral phases in intact plant-soil samples. However, three dimensional multiphase objects

707 often require additional preparation steps that go beyond what is required for a purely

708 organic or purely mineral sample. Essentially, the object of interest needs to be embedded in

709 a solid, vacuum-compatible medium and subsequently cut and or polished to generate a flat

710 surface. The primary distinction between preparation of organic materials (see above) and

711 preparing soil samples, is that cutting heterogeneous samples must accommodate both

712 'hard' (quartz, Fe oxides) and 'soft' components (plant tissues).

713 The most useful approaches for NanoSIMS soil sample preparation fall into three general

714 categories: 1) embedding with resin (epoxy, acrylic, polyester), sucrose or paraffin, 2)

715 embedding in molten elemental sulphur (S), or 3) cryo-techniques. The appropriateness of

716 these three techniques depends on the target elements which are to be analyzed and the

717 size of the sample (microaggregate vs. intact soil core). In general, resin embedding is the

718 most useful approach for larger aggregates and soil cores, whereas cryo-

719 preservation/sectioning and S embedding are the best choices for small (~100 µm)

720 microaggregates. Cryo and S embedding have the added benefit that no artificial C or N is

721 added as background material. We note that it may be possible to subtract a background

722 resin signal from the sample signal using the difference in the CN/C ratio, (e.g. the biological

723 from the non-biological material). The CN/C ratio of resin is quite distinct from that of

724 biological material, so regions representing resin within analysis images can be omitted at

725 the data processing stage (Weber et al., 2012).

726 For larger specimens like macroaggregates (>2 mm) or intact soil cores containing both hard

727 mineral constituents and soft tissues such as plant residues (e.g. roots in Figure 5), microbes

728 and plant roots, resin embedded sections are typically created and then polished for further

729 analyses. One epoxy resin approach was described by (Herrmann et al., 2007a), who

730 developed a technique for the preservation of microbial communities in quartz-based soils

731 that was later modified by Clode et al. (2009) to include plant roots. Infiltration of samples in

732 acetone-epoxy resin mixtures was conducted over a period of days, with the concentration of

733 'Araldite' (epoxy resin) gradually increased until 100%. At a larger spatial scale, at TU

734 München this procedure was successfully applied to prepare natural intact soil cores for

735 NanoSIMS analyses from the permafrost layer of cryoturbated soils (see Figure 2). Previous

736 studies show that the abundance of nitrogeneous compounds was not adversely affected by

737 this procedure (Herrmann et al., 2007a; Peteranderl and Lechene, 2004).

738 An alternative to resin embedding is sulfur embedding (De Gregorio et al., 2010; Flynn et al.,

739 2004; Lehmann et al., 2005) which has been successfully used to section heterogeneous soil

740 aggregates (Herrmann et al., 2007a; Lehmann et al., 2005). The most significant benefit of S

741 embedding is that aggregates can be embedded in a non-C based, room temperature

742 sectionable medium. In the DeGregorio et al. (2010) approach, elemental sulphur is heated

743 to its molten state (> 100ºC), then a soil aggregate is inserted and allowed to cool. The

744 resulting material can be glued to a metal stub and sectioned using an ultramicrotome.

745 Unfortunately, the sulphur may provide only limited structural integrity for the sections

746 because it tends not to impregnate samples extensively. Also, the high temperature could

747 potentially alter organic materials, particularly those on the surface of the aggregate. A low-

748 temperature alternative, the S embedding approach described by Lehman et al. (2005),

749 consists of S heated until molten and rapidly cooled in liquid N_2. As the S is slowly warmed to

750 room temperature, it goes through a phase of high viscosity and at this stage (~20 seconds)

751 aggregates may be inserted into the S. The resulting S block remains amorphous or plastic

752 for a limited amount of time, during which sectioning should be carried out.

753   A final option for fixation and sectioning is the cryopreservation/sectioning approach used by

754   researchers at LLNL: high-pressure freezing (Leica EMPACT2) of soil aggregates

755   surrounded by ultrapure H_2O in small copper tubes (16.6mm) with an internal diameter of

756   350μm, followed by cryosectioning and freeze drying. This approach results in samples of

757   regular thickness (<400 nm) and minimal chemical changes (Figure 6). This method, though

758   challenging, preserves the distribution of diffusible ions and molecules. Because it involves

759   fast freezing but no cyro-protectant, no background material is introduced that might dilute

760   the C or N isotopic signal (Figure 6).

761   Once a soil sample is embedded, it must then be cut to expose a cross-section for

762   NanoSIMS analysis. The presence of mineral grains significantly increases the difficulty of

763   sectioning samples. While ultramicrotomy with a diamond knife is possible, it is significantly

764   easier to cut embedded soil aggregates and cores with a wafer saw and then polish them as

765   per a geological sample. This is, however, not ideal for some softer biological materials and

766   resins, which can abrade more easily, resulting in topographical differences across the

767   sample surface. This abrasion effect can be particularly pronounced at the edges of mineral

768   grains, where inorganic-organic interfaces are located.

769   Sectioning via ultramicrotomy is the primary alternative option to saw cutting, and has proven

770   highly effective for combined TEM and NanoSIMS stable isotope measurements of plant-soil

771   systems (Clode et al., 2009). In Figure 5, a SEM image of three embedded roots illustrates

772   how the heterogeneous distribution of soft and hard soil compartments can both be

773   preserved in a thin section (Clode et al., 2009). In this procedure, both plant tissues and

774   microorganisms appeared to be well preserved (Figure 4 and Figure 5). We note that the

775   sectioning of embedded soil aggregates with heterogeneous density requires a highly skilled

776   operator; it is easy to chip a diamond knife. A final option for sectioning fixed samples is FIB

777   sectioning. This approach is a particularly good option for preserving the distribution of

778   diffusible species because a fully dry sample can be sectioned, however the method requires

779 specialized equipment and limited sample material can be processed. If the samples are only

780 destined for SIMS analysis, top-cutting is a more rapid FIB option (Weber et al., 2010).

781 3.5. Data acquisition and analysis

782 There are two main data collection modes possible with the NanoSIMS 50; spot

783 measurements and ion images. The collection of secondary ions in the spot mode is usually

784 used for the direct recording of isotopic ratio data for a discrete location. However, the main

785 data collection mode for soil related studies is likely to be ion imaging because users working

786 with highly complex samples are usually interested in spatially explicit isotope ratios and

787 contextual information. NanoSIMS ion image files consist of individual images for each scan

788 (plane) for each given mass, along with metadata describing the conditions of the analysis.

789 There are a number of image processing software options available to analyze the ion image

790 data. WinImage is Cameca's Windows-based image processing package that comes pre-

791 installed with the Windows-based NanoSIMS software. The previous Solaris-based

792 NanoSIMS software featured a somewhat rudimentary ion image processing application

793 called simply, Image. Another software package for SIMS image processing is the Windows

794 based LIMAGE program developed by Larry Nittler at the Carnegie Institution of Washington

795 (independent from Cameca). In addition to these commercial programs there are two open

796 source packages. The NRIMS (National Resource for Imaging Mass Spectrometry) group at

797 Harvard University has developed a plugin for the java-based freeware ImageJ. The so-

798 called MIMS plugin provides a number of specific data processing options in addition to the

799 full range of ImageJ capabilities. Most notable is the ability to express ratio images on a Hue-

800 Saturation-Intensity (HSI) scale, where the scale parameters can be set by the user. Finally,

801 a very recently developed software is the Matlab based application Look@NanoSIMS

802 developed by Lubos Polerecky at the Max-Planck Institute for Marine Microbiology in Bremen

803 (Polerecky et al., 2012). No matter what software is used there are some basic steps for the

804 processing of NanoSIMS images (the sequence of steps may vary slightly for some specific

805 data sets or research questions).

806 Briefly, the first step of image analysis is to examine all obtained images for possible flaws

807 (e.g. spots of charging, unrecorded spots or lines within images). When multiple cycles are

808 recorded, the analyst has to decide whether any cycles should be excluded from further

809 analysis. Individual cycles should be aligned to correct for primary beam drift, and also dead-

810 time corrected. This dead time is the period when the detector has counted a single

811 secondary ion but is not ready to count another (usually tens of nanoseconds). Next, the

812 species of interest are normalized to major element species (e.g. $^{13}C^-/^{12}C^-$, or $^{12}C^-/^{12}C^{14}N^-$);

813 ratio images can be used efficiently to evaluate the quality of the ratio data and the

814 distribution of normalized data. Typically, quantitative data are obtained by defining regions

815 of interest (ROIs), which consist of a group of pixels bounding a particular feature. In most

816 image processing applications a ROI can be drawn directly onto the image, and the software

817 then records the sum of the counts from the pixels within the ROI and thus also ratio data.

818 Defining ROIs is a critical step. Without objective criteria, it is possible to change the isotopic

819 enrichments just by changing the size of an ROI. The best ROI selection criteria are external

820 images (e.g. SEM image or fluorescence image) or single ion images (e.g. $^{56}Fe^{16}O^-$ for iron

821 oxide particles). Alignment with an external image is possible when using the

822 Look@NanoSIMS software. In many cases, it is necessary to visually evaluate the spatial

823 distribution of several ions to interpret and properly define ROI's. Unfortunately, there are no

824 standard procedures for feature selection in complex matrices like soils.

825 After ROIs are defined, the data can be extracted and interpreted. One promising way to

826 evaluate micro-scale ion images might be use of geostatistics, which are often used for

827 larger plot or ecosystem analyses (Steffens et al., 2008). Geostatistics can be used to

828 identify dependencies within any spatial data (e.g. NanoSIMS ion images). For example it

829 might be possible to use this approach to evaluate the spatial distribution of Fe within a

830 complex soil matrix. Figure 7 illustrates the application of a statistical approach (R 2.13.1 (R

831 Development Core Team, 2011) in combination with the g-stat 2.4.0 package (Pebesma,

832 2004) to evaluate spatial dependencies between organic matter ($^{12}C^{14}N^-$) and particles

833 containing iron ($^{56}Fe^{16}O$). The analysis is based on data extracted from a 32 µm line scan

834 drawn on the NanoSIMS images (Figure 7 A and B). The sample is a Cambisol soil

835 aggregate embedded in Araldite, originally from Höglwald, Bavaria, Germany. Both

836 semivariograms for $^{12}C^{14}N^-$ and $^{56}Fe^{16}O^-$ (Figure 7 C and D) show a comparable periodic

837 behavior, where the semivariance is oscillating (y-axis). The semivariance is a measure of

838 spatial dissimilarity. Thus the wave like appearance of the semivariance demonstrates a

839 distinct spatial pattern of similar ion counts ($^{12}C^{14}N^-$ and $^{56}Fe^{16}O^-$) and thus a regularity in the

840 aggregate architecture (organic and mineral spheres). But as the spatial frequency (x-axis) of

841 the maxima and minima of the semivariance is different between $^{12}C^{14}N^-$ and $^{56}Fe^{16}O^-$, this

842 indicates differences in spatial structures and different pattern sizes. This analysis indicated

843 that iron clusters ($^{56}Fe^{16}O^-$) are spatially independent from organic matter ($^{12}C^{14}N^-$). Similar

844 analyses may be particularly useful to reveal spatial inter-dependencies between mineral and

845 organic soil constituents or even between areas enriched or depleted in specific stable

846 isotopes. Another approach to NanoSIMS data analysis is the upscaling approach used by

847 Fike and colleagues (Fike et al., 2008). They collected numerous single spot (6 µm x 6 µm)

848 NanoSIMS measurements (>300) and measured the isotopic variability of sulfide within the

849 oxycline of a cyanobacterial mat (sampling grids: 3500 µm by 450 µm and 3000 µm by 500

850 µm, spatial increment of 50-200 µm). Analyzing the $\delta^{34}S$ abundance and isotopic

851 fractionation, they observed fine scale laminations (1 to 400 µm) and clear zonation. This

852 work suggests that single spot NanoSIMS measurements, collected on up to a millimeter

853 scale and linearly interpolated, could be applied for upscaling micrometer spot data in soils.

854 ## 4. Combination with other micro-scale techniques

855 ### 4.1. Scanning and transmission electron microscopy

856 Prior to the development of NanoSIMS, electron microscopy was the approach of choice for

857 the observation of soil particle arrangements (Gillott, 1970; Gray, 1967; Howard et al., 1996;

858 Kowalkowski and Mycielskadowgiallo, 1985). Electron microscopy has evolved to achieve

859 very high resolution, touching on 1 nm in scanning electron microscopy (SEM) and 0.05 nm

860 in transmission electron microscopy (TEM). Similar to NanoSIMS, electron microscopy

861   techniques can be used to obtain information about the material properties of a sample, in

862   addition to providing an image. In a scanning electron microscope (SEM), a high energy

863   focused beam of electrons is produced. The electrons interact with electrons in the sample,

864   producing secondary electrons, back scattered electrons (BSE), and characteristic X-rays

865   (Energy-dispersive X-Ray Spectroscopy EDX/EDS/EDXS) that can be detected and that

866   deliver information about the sample's surface topography and composition. An SEM-BSE

867   image is a good tool to differentiate between organic and mineral spheres. This can be useful

868   in samples without topography such as polished soil cross sections. The crystalline state of a

869   sample can be determined by recording patterns of the diffracted backscattered electrons

870   (EBSD) and matching them with a data base for crystallographic structures. An EDX scan

871   yields information on the localization of minerals versus organic matter and is useful for

872   identifying regions of interest within the sample.

873   It should be noted however, that in contrast to NanoSIMS, where information is gathered only

874   from secondary ions sputtered from the uppermost atomic layers, the interaction volume of

875   secondary ions in SEM extends in a pear shaped fashion between 100 nm and 5 µm deep

876   into the sample (depending on the energy of the electron beam, atomic number and density

877   of the specimen). EDX is therefore not a surface technique *sensu strictu* and it has been

878   shown that TEM maps do not always compare well with subsequently obtained NanoSIMS

879   images (Badro et al., 2007).

880   4.2. Synchrotron based techniques

881   To study soil process dynamics as a function of location within aggregates and

882   microaggregates, simultaneous information on (a) localization, (b) identification and (c)

883   transformation of organic matter and mineral phases is required with very high spatial

884   resolution. Conventional electron microscopes can visualize basic elemental composition but

885   are unable to speciate carbon compounds. A number of techniques can yield data on C

886   composition (ion microprobe laser desorption, laser ionization mass spectrometry, Raman

887   microscopy, ^{13}C-NMR, FTIR (Lehmann et al., 2005; Lehmann et al., 2009), though few can

888   provide molecular or elemental characterization with the sub-micron resolution necessary to

889   study mineral-organic interactions in microaggregates. These goals can be approximated

890   with the combination of NanoSIMS and Scanning Transmission X-ray Microscopy, Near

891   Edge X-ray Absorption Fine Structure Spectroscopy (STXM/NEXAFS). The resolution of

892   synchrotron based X-ray microscopes can approach 50 nm, comparable to the NanoSIMS

893   spot size. Raster scan images can be obtained at energy spacings of ~0.1 eV across 100's

894   of eV energy ranges. The spatially resolved NEXAFS spectra extracted from the respective

895   image 'stacks' reveal the bonding environment of the element of interest, allowing the

896   speciation of organic matter forms and elemental redox states. STXM/NEXAFS has been

897   used successfully to describe spatial patterns and speciation of soil organic matter

898   associated carbon (Bardgett et al., 2007; Cheng et al., 2008a; Cheng et al., 2008b; Lehmann

899   et al., 2005), and nitrogen (Gillespie et al., 2009; Kögel-Knabner et al., 2008; Sleutel et al.) in

900   a broad range of environments, including marine systems (Brandes et al., 2004).

901   A combined 'STXM-SIMS' approach allows precise, quantitative measurement of molecular

902   and isotopic patterns in undisturbed samples, at high resolution (Keiluweit et al., 2012; Pett-

903   Ridge et al., 2012). By combining NanoSIMS with STXM/NEXAFS it is possible to map

904   organic C distribution, to image associations of organics with specific mineral types, and to

905   trace organic matter of variable origin into the soil matrix (Lehmann et al., 2007; Wan et al.,

906   2007). If combined with isotope tracer experiments, the images acquired through this

907   approach can document the forms of C that become stored in soil aggregates and

908   simultaneously track microbial debris and other organic polymers into the soil matrix.

909   Technical challenges to the combined application of STXM and NanoSIMS have recently

910   been summarized by Pett-Ridge et al. (2012). They point out that experimental activities

911   have to be planned in a way such that STXM analysis precedes NanoSIMS, as the latter

912   method has a much greater potential for destructive interference with the sample. Sample

913   holders commonly used in synchrotron spectroscopy may contain N (Si_3N_4) or C (TEM Cu

914   grids with C lacey) and must therefore be chosen to avoid unwanted secondary ion species

915 (e.g. $^{12}C^-$, $^{12}C^{14}N^-$) in subsequent NanoSIMS applications. Samples cannot be significantly

916 thicker than 150-200 nm for STXM/NEXAFS or they will be impenetrable for soft X-rays. This

917 limits the type of microstructures that can be observed with both methods at the same time to

918 thin platelet–like objects. Taking these restrictions into account, SEM, NanoSIMS and STXM

919 may be applied to the identical soil sample specimen, providing information that is

920 complementary: SEM may be used to generate a mesoscale surface image of the region of

921 interest (ROI), STXM/NEXAFS imaging can obtain information about whole sample

922 chemistry, and NanoSIMS analysis can yield elemental/isotopic data on either surface

923 characteristics or from a depth profile (Pett-Ridge et al., 2012) (Figures 6, 8). To allow for

924 high-resolution SIMS imaging and STXM/NEXAFS spectromicroscopic analysis of the same

925 sample, a specimen must be prepared with limited topography, able to withstand high

926 vacuum, be dry, conductive, thin enough to allow photon transmission (<1 μm), and prepared

927 without carbon-based reagents.

928 An example for the synergistic application of NanoSIMS and STXM/NEXAFS was recently

929 presented by Keiluweit et al. (2012). These authors employed NanoSIMS to follow the fate of

930 isotopically labeled amino sugars from fungal cell walls as they became metabolized or

931 bound to minerals and SOM. Concurrently, STXM/NEXAFS spectromicroscopy was used to

932 determine the chemical transformations of substrate C and N functionalities during the

933 process (Figure 8). The authors determined that ^{15}N-labeled amide N derived from fungal cell

934 wall material preferentially associated with Fe (hydr)oxide surfaces or Fe-OM coprecipitates

935 on the surface of other minerals. Through the combination of NanoSIMS and STXM/NEXAFS

936 it was further possible to determine that amide N found on mineral surfaces originated from

937 bacterial protein rather than from the original amino sugars or from nucleotides. This

938 example illustrates that combined applications of synergistic imaging techniques such as

939 NanoSIMS and STXM have the potential to provide information about organic matter-

940 mineral-microbial relationships while avoiding the artifacts inevitably generated by chemical

941 or physical fractionation procedures.

4.3. Atomic force microscopy

The atomic force microscope (AFM) is another tool for imaging, measuring, and manipulating matter at the nanoscale. An AFM consists of a cantilever with a sharp tip that is used to scan the specimen surface. When the tip is brought into proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever. The deflection of the cantilever is measured and converted to an image of the sample surface. Reports on AFM applications in soil science are increasing (Cheng et al., 2008c; Rennert et al., 2012; Schaumann and Mouvenchery, 2012; Totsche et al., 2010) and typically provide information about local topography and properties of minerals and soil aggregates surfaces at very high spatial resolution. To date, the focus of soil related AFM work has been on interactions between microorganisms and minerals, mainly iron oxides and hydroxides (Maurice, 1996; Maurice et al., 2000). Balogh-Brunstad et al. (2008) combined SEM and AFM to investigate fungal weathering of biotite in a batch liquid culture.

One potential combined AFM-NanoSIMS application could be the use of AFM to correct for topography effects in NanoSIMS analyses. Here AFM would be applied both before and after SIMS, allowing the user to convert SIMS data into a true three-dimensional representation of the analyzed species (Fleming et al., 2011). Wirtz et al. (2012a; 2012b) report the development of an integrated SIMS- scanning probe microscope (SPM). In this instrument, a specially developed SPM system was integrated in a Cameca NanoSIMS 50, allowing the user to record topographical *in situ* images of the sample surface before, in between and after SIMS analysis.

4.4. In situ single-cell labeling

Environmental microbiologists see particular value in the combination of *in situ* phylogenetic labeling and NanoSIMS in order to link metabolic function with taxonomic identity (Kuypers and Jorgensen, 2007). This capacity was first demonstrated using a combination of conventional SIMS and fluorescence *in situ* hybridisation (FISH) to analyze an archaeal-

968 bacterial consortium in anoxic marine sediments (Orphan et al., 2001). The FISH-SIMS

969 combination allowed Orphan et al. (2001) to demonstrate that cell aggregates binding a

970 specific archeal probe were strongly depleted in ^{13}C, indicating a methane-based

971 metabolism. In many natural microbial systems, this approach may be even better suited to

972 NanoSIMS, as it can provide this information at the length-scale of an individual bacterium

973 (~1 μm). Two variations of this approach are currently used: element-labeled fluorescent *in*

974 *situ* hybridization (EL-FISH) where catalyzed reporter deposition FISH (CARD-FISH) is used

975 to deposit high concentrations of fluorine-containing fluorophores in target cells (Behrens et

976 al., 2008); or halogen-labeled *in situ* hybridisation (HISH) where a standard FISH protocol is

977 used in combination with halogen (I, Br) tagged probes (Li et al., 2008). NanoSIMS can then

978 be used to visualize the labeled cells by acquiring a signal for ^{19}F, which is not naturally

979 present at high concentrations in most environmental cells (Behrens et al., 2008; Halm et al.,

980 2009; Musat et al., 2008). In these approaches, a phylogentic probe is linked to a highly

981 electronegative elemental label (fluorine, iodine, gold, selenium, or bromine) instead of the

982 typical fluorophore, which can be detected in concert with ^{13}C and ^{15}N isotopes for functional

983 characterization. These approaches enable simultaneous localization of the tag and chemical

984 mapping in the NanoSIMS. It may even be possible to use FISH-SIMS approaches in

985 embedded samples (Woebken et al., 2012). The work of Lemaire et al. (2008), where fixed

986 samples were embedded in TissueTek® (Sakura Finetek Labware & Accessories) and then

987 cryosectioned and FISH labeled, suggests this may be possible. However, its usefulness in

988 soil may be limited unless a means to overcome soil's natural background fluorescence is

989 developed. Also, care must be taken with quantitative interpretation of FISH-SIMS results,

990 since these approaches may reduce the original cell enrichment by 60-80% for ^{13}C and 30-

991 60% for ^{15}N (J. Pett-Ridge and S. Behrens, unpublished data).

992 **5. Conclusion**

993 Over the last decades soil scientists have gained a working knowledge of fundamental soil

994 processes ranging from the stabilization of organic matter to microbial diversity in soils. We

995 are now able to track the fate of specific molecular plant biomarkers into organo-mineral

996 associations and to determine the microbial communities responsible for the turnover of

997 specific organic compounds. However, commonly used bulk analyses average over a vast

998 swath of microbial and mineral landscapes, and can miss micro-scale phenomena caused by

999 specific micro-habitats or distinct spatial heterogeneities in the formation of organo-mineral

1000 assemblages. Thus, while our knowledge of bulk scale biogeochemical soil processes is

1001 expanding, we have lacked the high resolution techniques needed to illustrate the

1002 mechanistic underpinnings such processes in intact soil structures.

1003 The emerging class of nano- and microscale spectroscopy and spectrometry techniques has

1004 opened a new frontier in the effort to develop a fundamental understanding of soil processes.

1005 Though the first review of NanoSIMS in soil ecology and biogeochemistry by Herrmann and

1006 colleagues was in 2007 (Herrmann et al., 2007b), this field is still in its early phases as soil

1007 scientists grapple with the many technical challenges. Looking forward, the challenge will be

1008 upscaling from micro-scale analyses to scales from the soil horizon or even to the pedon or

1009 ecosystem scale. These leaps may not come soon, but for now the ability to image elements

1010 and stable isotopes at a previously unresolved spatial scale permits the combination of

1011 established isotopic enrichment techniques with the description of their spatial distribution in

1012 soil microenvironments. Recently, several new NanoSIMS instruments have been installed

1013 and the access for soil scientists is increasing. NanoSIMS analyses have the potential to

1014 contribute to a fundamentally new understanding of soil processes, one that is rooted at the

1015 relevant scale of microbial, mineral and organic matter interactions.

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Figure captions

Figure 1: A) coaxial setup of the NanoSIMS, indicating the primary and secondary ion beam in relation to the sample surface. Due to the coaxial setup, the secondary ions must have the opposite charge from the primary ions to enable extraction to the mass spectrometer. B) schematic of the NanoSIMS, with the primary ion beam in blue and the secondary ion beam in red. Courtesy of Cameca (Gennevilliers, France), adapted from Myrold et al. (2011).

Figure 2: Micrograph and microanalysis of an embedded cross section derived from a Cryosol soil core (Oa horizon, Typic Aquiturbel) from Barrow, Northern Alaska. A) back scatter electron image recorded with a SEM; B, C) NanoSIMS images ($^{12}C^{14}N^-$ and $^{56}Fe^{16}O^-$) recorded with a NanoSIMS 50 L at TU München. The back scatter SEM image shows collapsing plant cells of particulate OM in the center surrounded by mineral spheres. The red square in the SEM image indicates the area analyzed by NanoSIMS, the green line indicates the interface between particulate organic matter and mineral phase, the blue line depicts the boundary between totally and partly collapsed plant cell structures. The NanoSIMS images indicate the distribution of organic matter ($^{12}C^{14}N^-$) and the iron distribution ($^{56}Fe^{16}O^-$) within the plant cell region, and suggest organo-mineral interfaces in the early stages of formation. D) line scan data derived from analysis of NanoSIMS $^{12}C^{14}N^-$ and $^{56}Fe^{16}O^-$ secondary ion images. The line scans demonstrate the spatial distribution of both secondary ion species along a transect, illustrating the iron clusters within the organic matter region. An area of 0.5 to 0.5 µm (square #2 in images) in size was scanned along the transect (Mueller, unpublished data).

Figure 3: Back scattered secondary electron micrograph and NanoSIMS ion images ($^{16}O^-$; $^{12}C^{14}N^-$) of an embedded root tip cross-section prepared from a French oak root (*Quercus robur,* clone DF159 infected with mycorrhizal fungi *Piloderma croceum*, courtesy of F. Buscot, UFZ Halle, Germany and T. Grams, TU München, Germany) grown in a vermiculite / soil mixture. The root and adhering rhizosphere soil was fixed according to Karnovsky (1965), embedded in an epoxy resin, cross sectioned, polished and imaged via NanoSIMS. The $^{16}O^-$ NanoSIMS images illustrate thin clay mineral layers, whereas the $^{12}C^{14}N^-$ ion images

indicate the location of root cells. The row of yellow squares in the SEM image show where NanoSIMS analyses occured, in a transect across the interfaces between root, mycorrhizal fungi and mineral particles (Mueller, unpublished data).

Figure 4: TEM and NanoSIMS images of wheat roots (*Triticum aestivum*) exposed to ^{15}N for 24 h. The samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffered saline and dehydrated in a graded series of acetone. The roots were infiltrated in acetone araldite mixtures over several days, using a gradually increasing araldite concentration. Final embedding in Araldite 502 was done according to Herrmann et al. (2007a). Embedded samples were cut into slices, re-embedded in 10 mm mounts and polished using silicon carbide paper and finally diamond paste. TEM images (A, D) show the presence of microorganisms in the rhizosphere (rh) and extracellular mucilage matrix (e) adjacent to the root cells (c). NanoSIMS images (B, E) of the same regions show organic matter distribution recorded as $^{12}C^{14}N^-$. NanoSIMS ratio images (E, F) of $^{15}N/^{14}N$ (natural abundance at 0.004), confirmed the ^{15}N enrichment of some microorganisms. Linescan data from the regions between the arrows (in C, F) is shown in G (from C) and H (from F). (Figure adapted from Clode et al. 2009) (Copyright American Society of Biologists www.plantphysiology.org).

Figure 5: SEM micrograph (Figure 1 adapted from Clode et al. 2009) of three roots (*Triticum aestivum*) in soil, showing harder materials as quartz (light areas) and softer materials including roots (dark grey areas). The loamy-sand textured soil (Ap horizon from Meckering, Western Australia) containing the roots was infiltrated in acetone araldite mixtures over several days, using a gradually increasing araldite concentration. Final embedding in Araldite 502 was done according to Herrmann et al. (2007a). Embedded samples were cut in slices, re-embedded in 10 mm mounts and polished using silicon carbide paper and finally diamond paste. (Copyright American Society of Biologists www.plantphysiology.org).

Figure 6: A soil thin section prepared by 'cryo' techniques: high pressure freezing followed by cryosectioning and then freeze drying. The soil is from a clay-rich forest soil from the H.J. Andrews Experiment Station, Oregon, USA, and was exposed in a controlled microcosm to a low-concentration ^{13}C glucose label. A) SEM micrograph collected at 2 kV; B) NanoSIMS δ^{13}C image showing what appear to be isotopically enriched microbial cells (white arrows) flanked by plant residue particles; C) STXM transmission image collected at 300 eV of the region annotated by the red/white box in (A,B), scale = 1 µm; D) false color overlay of a STXM C difference map (ratio of scans collected above and below the C edge) and the

transmission map shown in (C);   E, F) NEXAFS spectra extracted from the regions of interest circled in (D) are plotted against the standard spectra for (E) ligno-cellulose and (F) protein and substantiate plant vs. microbe particle definitions. (M. Keiluweit, J. Bougoure, P. Weber, P. Nico, M. Kleber, J. Pett-Ridge, unpublished data).

Figure 7: NanoSIMS images and semivariograms (lower panel) of an embedded cross section of organic matter derived from a aggregate of Cambisol topsoil (Ah horizon) from Höglwald, Bavaria, Germany. A) NanoSIMS $^{12}C^{14}N^-$ ion image, B) NanoSIMS $^{56}Fe^{16}O^-$ ion image. The white arrow in the NanoSIMS images indicate the line scan which was used to generate the semivariograms in C-D. (Mueller, unpublished data).

Figure 8: Microstructures associated with fungal hyphae in an organic forest soil investigated by both NanoSIMS imaging and STXM/NEXAFS spectromicroscopy. A) STXM optical density map recorded at 300 eV; B, C) NanoSIMS isotope ratio images for $^{12}C^{15}N^-/^{13}C^{14}N^-$ and Fe concentrations ($^{56}Fe^{16}O^-/^{12}C^-$, normalized to carbon) of the same feature shown in the STXM image. Brighter colors reflect high enrichment/concentration. D) Optical density map of hyphal-associated microstructures with colored regions of interest (ROIs) from which NEXAFS spectra at the C 1s absorption edge and the N 1s absorption edge were collected. ROIs are color-coded according to the spectral types extracted from them: intact fungal hyphae (grey), decomposing hyphal residue (brown), microbial residue (green) and mineral surfaces (blue). E) Average NEXAFS spectra representing the major carbon forms encountered in the regions of interest defined in D. Carbon 1s absorption edge peaks are identified as C=C 1s-π^* transition of aromatic C at 285.1 eV (a), 1s-π^* transition of C=C in ene-ketone at 286.7 eV (b), 1s-3p/ σ^* transition of aliphatic C at 287.4 eV (c), 1s-π^* transition of carboxylic and/or amide C at 288.3 eV (d), the 1s-3p/σ^* transition of alcohol C-OH at 289.4 eV (e), and the 1s-π^* transition of carbonyl C at 290.3 eV (f). Reproduced with permission from Geochimica and Cosmochimica Acta.

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