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### ► To cite this version:

Charlotte Védère, L. Vieublé-Gonod, Naoise Nunan, Claire Chenu. Opportunities and limits in imaging microorganisms and their activities in soil microhabitats. *Soil Biology and Biochemistry*, 2022, 174, pp.108807. 10.1016/j.soilbio.2022.108807. hal-03775657

**HAL Id: hal-03775657**

**<https://hal.science/hal-03775657>**

Submitted on 21 Sep 2022

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# 1 **Opportunities and limits in imaging microorganisms and their** 2 **activities in soil microhabitats**

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18

## 19 **Abstract**

20 The soil microhabitat is a heterogeneous and complex environment where local variations can  
21 modulate phenomena observed at the plot scale. Most of the current methods used to describe  
22 soil functioning are bulk soil analyses which do not account for fine-scale spatial variability  
23 and cannot fully account for the processes that occur under the influence of the 3D organisa-  
24 tion of soil. A good representation of spatial heterogeneities is necessary for the parametrisa-  
25 tion of new models, which aim to represent pore-scale processes that affect microbial activity.  
26 The visualization of soil at the scale of the microhabitat can be used to extract descriptors and  
27 reveal the nature of the relationships between the fine-scale organisation of soil's constituent  
28 parts and soil functioning.

29 However, soil imaging techniques tend to be under-used, possibly due to a lack of awareness  
30 of the methods or due to a lack of access to the relevant instruments. In recent years, new  
31 methods have been developed, and continuously improved, offering new possibilities to de-  
32 cipher and describe soil physical, chemical and biological features of the soil microhabitat in  
33 evermore exquisite detail.

34 This review is structured into several parts in which first imaging methods that are useful for  
35 describing the distribution of microorganisms and microbial activities, followed by methods  
36 for characterising the physical organisation of the microhabitat and, finally, methods for char-  
37 acterising the distribution of soil chemical features, including soil organic matter, are de-  
38 scribed. Special attention is given to the preparation steps that are required for the proper use  
39 of the methods, either alone or in combination.

40

#### 41 **Keywords**

42 Imaging, microhabitat, microscale, microorganisms, soil characterisation, soil organic matter

#### 43 **Abbreviations**

44 AFM: Atomic force microscopy

45 AHA: L-azidohomoalanine

46 BIB: Broad ion beam

47 BONCAT: Bioorthogonal non-canonical amino acid tagging

48 CTC: 5-cyano-2,3-ditolyl-tetrazolium chloride

49 DAPI: Di Aminido Phenyl Indol

50 DTAF: Dichlorotriazinylaminofluorescein

51 EDX: Energy dispersive X-ray spectroscopy

52 EELS: Electron energy loss spectroscopy

53 ESEM: Environmental scanning electron microscopy

54 FDA: Fluorescein diacetate

55 FIB: Focused ion beam

56 FISH: Fluorescence in situ hybridization

57 FITC: fluorescein isothiocyanate

58 FTIR: Fourier transform infrared micro-spectroscopy

59 INT: 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride

60 LTSEM: Low temperature scanning electron microscopy

61 MALDI: Matrix assisted laser desorption ionisation

62 MRI: Magnetic resonance imaging

63 NEXAFS: Near edge X-ray fine structure spectroscopy

64 NMR: Nuclear magnetic resonance

65 PET: Positron emission tomography

66 PI: Propium iodide

67 SBF: Serial block face

68 SEM: Scanning electron microscopy  
69 SHIM: scanning helium ion microscopy  
70 SIM: Structured illumination microscopy  
71 SIMS: Secondary ionization mass spectrometry  
72 SMLM: Single molecule localisation microscopy  
73 SOM: Soil organic matter  
74 STED: Stimulated-emission depletion microscopy  
75 STMX: Scanning transmission X-ray microscopy  
76 TEM: Transmission electron microscopy  
77 TOF: Time of flight  
78 TXM: Transmission X-ray microscopy  
79 VNIR: Visible and near infrared  
80 XANES: X-ray absorption near edge structure  
81 X-ray CT: X-ray computed tomography  
82 XRF: -ay fluorescence

## 83 **1. Introduction**

84 Soils are extremely complex and heterogeneous environments and many properties  
85 observed at the profile or at the plot scale are, in fact, determined by microscale conditions  
86 and processes (e.g., Falconer et al., 2015; Keiluweit et al., 2017; Steffens et al., 2017). In  
87 soils, the local environment can differ dramatically across millimetres, or even less, and these  
88 variations can control the spatial distribution of microorganisms and their activities (Chenu et  
89 al., 2001; Ranjard and Richaume, 2001; Chenu and Stotzky, 2002; Grundmann, 2004; Jasin-  
90 ska et al., 2006; Raynaud and Nunan, 2014; Frey, 2015; Juyal et al., 2020). The majority of  
91 the models that are currently used to describe or predict soil functioning are based on bulk soil  
92 characteristics, thus implicitly assuming that microscale interactions and processes do not af-  
93 fect the higher scale properties. This view has been vigorously challenged recently (Baveye et  
94 al., 2018).

95 Soils exhibit heterogeneities along spatial, temporal, chemical, physical and biological dimen-  
96 sions (Lehmann et al., 2020). The combinations and interactions among these dimensions  
97 mean that soils are made up of a myriad of micro-environments with unique combinations of  
98 properties. The microbiological functioning of soils is highly dependent on how microbial  
99 cells interact and are affected by the properties of their local environment (Alexander, 1964;

100 Baveye et al., 2018; Chenu and Stotzky, 2002). The access to resources and energy, the avail-  
101 ability of water or O<sub>2</sub>, the pH that microbial cells are exposed to, the other organisms in prox-  
102 imity for example, can all have dramatic effects on the activity of microbial cells. The enviro-  
103 nmental properties and the biological neighbourhood that microbial cells experience depend  
104 the physical structure of the soil's solid and pore phases across scales, from the micro-envir-  
105 onment scale to the scales at which fluxes are regulated. However, we have little or no in-  
106 formation on how microbial cells are distributed within this complex and heterogeneous en-  
107 vironment, nor do we know much about their interactions with the physical environment or  
108 population pressures that they are subjected to. Our understanding of soil microbial function-  
109 ing is therefore derived from measurements of means and gross trends (Vos et al., 2013) and,  
110 in the final analysis, is not built on a solid mechanistic foundation. It is clear that an exhaust-  
111 ive mapping soil microbes would be an arduous and, in view of the temporal variability inher-  
112 ent to soil conditions, futile endeavour. However, a clearer picture of the range of interactions  
113 that occur in soil, their prevalence and the effects they can have on microbial functioning  
114 would allow us to build models with sounder mechanistic basis. Ultimately, this can only be  
115 achieved after studying the spatial relations of soil's constituent parts, as spatial proximity is a  
116 strong modulator of the interactions that can occur.

117         New models, that account for microscale soil functioning, have begun to appear in re-  
118 cent years (Pot et al., 2015; Tecon and Or, 2017; Portell et al., 2018; Wilmoth et al., 2018;  
119 Kemgue et al., 2019). The need for data at relevant spatial scales, such as the physical struc-  
120 ture and chemical characteristics of soil and the spatial distribution of microorganisms and  
121 their activities, for parametrising such models, is paramount if useful information is to be ac-  
122 quired from microscale modelling approaches (Baveye et al., 2018). In particular, visualising  
123 soil microorganisms in undisturbed soil samples is essential for understanding how they inter-  
124 act with their local environment, such as the local conditions experienced, their access to  
125 trophic resources or their interactions with other microorganisms, and the consequences these  
126 interactions have for soil functioning.

127         For example, it has been suggested that microbial activity hotspots account for a major  
128 part of total microbial activity in soils, despite being found in only a small portion of the soil  
129 volume (Kuz'yakov and Blagodatskaya, 2015). A better understanding of the functioning of  
130 such hotspots, in particular of those outside well defined "spheres" such as the rhizosphere or  
131 the detritosphere, would contribute greatly to our capacity to predict the response of microbial  
132 activity to changing environmental conditions or soil management. Yet, we know close to  
133 nothing about such hotspots nor about what causes them to occur. Are they more likely to oc-

134 cur in the presence of certain types of pore architecture or is it the happy coincidence of com-  
135plementary microbial taxa being co-located (Kim et al., 2008). To answer these questions, we  
136must first know who is where and how they are organised.

137

138 However, visualisation methods of soils at fine scales are challenging, as soils are het-  
139erogeneous organo-mineral matrices, composed essentially of very small particles in the sub-  
140mm range that are variably hydrated, and the spatial organisation of which is strongly affected  
141by moisture state. A range of visualisation methods has been used over the last 30 years, but  
142there have been a number of recent methodological developments and a concomitant increase  
143in the number of studies in this area. New methods are now able to provide much more in-  
144formation than before: not only are the resolutions of images higher but it is also possible to  
145obtain spatial information on soil physical, chemical and biological characteristics in three di-  
146mensions. Furthermore, image processing and analysis tools have become more efficient, al-  
147lowing for better correction, segmentation of areas of interest or even predictions of chemical  
148composition (Hapca et al., 2015; Anderson et al., 2020).

149 Despite the technological progress, imaging methods are still under-utilised in soil sci-  
150ences because the scientific community is not always aware of them, and due to the fact that  
151they are time consuming, expensive and not always easy to implement. Furthermore, several  
152visualisation methods require access to instruments that are not widely available (e.g. NEX-  
153AFS, nanoSIMS). Many of the imaging methods that are useful for visualising microbial  
154communities in their habitat have extremely small fields of view (e.g. the area covered by a  
155nanoSIMS image is approximately  $30 \times 30 \mu\text{m}^2$ ). The questions then arise, should targeted or  
156random sampling be used (Brus, 2019) and what of the representativeness of the images ac-  
157quired? Targetted imaging is useful for determining the types of situations that can be en-  
158countered, but there is a risk that it will provide a biased view as the areas of interest chosen  
159may not reflect the soil more generally. In order to obtain a representative view, a degree of  
160random sampling is required, though this can be reduced with a judicious use of spatial mod-  
161elling (Brus, 2019). Whilst sampling considerations are clearly a drawback of these ap-  
162proaches, their exceptional analytical power means that they deserve more attention.

163 The aim of this review is to give a broad overview of the methods currently available  
164that can be used to obtain information on soil microorganisms and their activities in their mi-  
165cro-environment. We review and present different methods in which (i) microorganisms can  
166be located in their habitats, (ii) different types of microorganisms can be identified, (iii) the  
167characteristics of the immediate environment of microbial communities can be described and

168 (iv) in situ information on the activities of microorganisms can be acquired. We systematic-  
169 ally specify the spatial resolution of the methods, the preparation techniques and quantifica-  
170 tion possibilities. We address the limits of the different methods, the possibilities of combin-  
171 ing them and discuss perspectives in the field.

## 173 **2. Visualising microorganisms in their habitats**

### 174 *2.1. Localising microorganisms*

175

176           There are different methods for localising microorganisms in soil samples and for  
177 identifying them as bacteria, fungi, archaea etc. (Table 1). Distinguishing microorganisms  
178 from soil particles is not always straightforward, but there are a range of useful criteria based  
179 on size and shape for facilitating this: rounded shapes, filamentous forms, or the structure of  
180 the cell. These may also be combined with general or specific stains in order to increase the  
181 contrast between objects of interest and the background.

182           At the extremes in terms of resolution, stereomicroscopes allow for the observation of  
183 fungi (Otten and Gilligan, 1998; Otten et al., 2004; Thompson et al., 2005), identified as such  
184 based on criteria such as shape, size or even colour, while bacteria and archea are too small to  
185 be visible. Fresh soil samples can be observed directly without any special preparation but  
186 thin sections should be prepared if the spatial organisation or spatial relations are of interest.  
187 With the greater magnification of light microscopy, both uni- and pluri-cellular organisms can  
188 be visualised. Nonetheless, in soil, microorganisms do not show great contrast and it is diffi-  
189 cult to identify them precisely based on shape, size and natural colour alone.

190           If the spatial relations of microbial communities with constituents of their micro-en-  
191 vironment is of interest, then the physical structure of samples must be preserved intact, as is  
192 done in resin embedded, thin sections of soil (Tippkötter et al., 1986; Tippkötter and Ritz,  
193 1996; Li et al., 2004). In this way, the spatial integrity of samples and the integrity of biolo-  
194 gical cells are preserved.

195           Epi-fluorescence microscopy, combined with the use of stains, i.e. fluorochromes,  
196 makes it possible to distinguish the targeted organisms from the background and therefore  
197 locate and enumerate bacteria (Fig. 1a) (Fisk et al., 1998; Nunan et al., 2001; Juyal et al.,  
198 2020) and fungi (Baschien et al., 2001) in 2D. The staining needed to visualise microorgan-  
199 isms can be carried out either before the impregnation, by immersion of the sample in a stain-

200 ing bath (Nunan et al., 2001), often after a fixation step, or applied after thin section prepara-  
201 tion (Juyal et al., 2020). However, only microorganisms situated at the surface of the thin sec-  
202 tion can be stained and visualised in the latter case. Several fluorochromes specifically stain  
203 cell constituents (Table 2). Provided their excitation spectra are not superimposed, and no in-  
204 terferences occur, it is possible to use several staining agents simultaneously (Chen et al.,  
205 2007). The main difficulties encountered with the use of fluorochromes are related to unspe-  
206 cific staining, in particular with positively charged fluorochromes such as acridine orange,  
207 and to background auto-fluorescence of soil organic particles (Altemüller and Van Vliet-  
208 Lanoe, 1990; Li et al., 2004). Figure 1a shows an example of auto-fluorescence (red arrow)  
209 with similar shape and size characteristics as bacterial cells, making the identification of bac-  
210 teria more complicated. In addition, the quality of the staining can be affected by the presence  
211 of clay particles, as stains tend to adsorb to clay surfaces resulting in a fluorescence which can  
212 hinder the observation of microorganisms (Li et al., 2004). Other factors may also interfere  
213 with the staining such as the stain concentration, soil pH and the type of resin used (Al-  
214 temüller and Van Vliet-Lanoe, 1990; Postma and Altemüller, 1990). It is possible to distin-  
215 guish microorganisms from organic compounds by collecting visual information on microbes  
216 and organic matter auto-fluorescence in different channels and subtracting the signal in one  
217 from that in the other (Cardinale, 2014; Schmidt et al., 2018). Differences in signal intensity  
218 between dyed and auto-fluorescent objects can also be used to distinguish objects of interest  
219 from the background. Finally, new methods based on two-photon excitation fluorescence can  
220 be used to take advantage of the native auto-fluorescence of soil and microorganisms to locate  
221 fungi and bacteria in soil without using any stain (Lee et al., 2022). Using methods such as  
222 those described above, Nunan et al. (2002) measured the spatial organisation of bacteria at the  
223 micrometre scale and showed that bacteria were more strongly aggregated in the subsoil than  
224 in the topsoil. Juyal et al. (2020) showed that lower soil bulk densities favor the the dispersion  
225 of inoculated bacteria in soil.

226         The resolution limit of light microscopy, imposed by the diffraction of light, is around  
227 200 nm, which allows for the observation of objects between  $10^{-3}$  and  $10^{-7}$  meters (Ranjard  
228 and Richaume, 2001) in preparations between slide and coverslip or in thin sections after in-  
229 clusion in a resin.

230         The resolution of scanning and transmission electron microscopy (SEM & TEM) is  
231 much higher (Table 1, Fig. 1b and c). It is possible to reach resolutions of circa 1 nm with  
232 SEM (Joy and Pawley, 1992) and 0.05 nm with TEM (Smith, 2008). Preserving the original  
233 soil microstructures and the integrity of organisms and organic constituents despite the high



234 vacuum to which the sample is exposed in the microscope is a challenge and requires the use  
235 of specific preparation methods. Samples are generally air-dried before SEM analyses. How-  
236 ever, drying strongly affects all hydrated structures and damages biological features (e.g. bac-  
237 terial cells as well as sheaths of extracellular polysaccharides appear flattened) so that conven-  
238 tional SEM is not recommended (Chenu and Jaunet, 1992). Samples can be observed directly  
239 in a moist state without any conductive coating using an Environmental Scanning Electron Mi-  
240 croscope (ESEM) (Gleeson et al., 2005; Lin and Cerato, 2014), though it gives poorer quality  
241 images in terms of brightness and contrast (Dal Cortivo et al., 2017; Bertola et al., 2019). In  
242 order to avoid the damage that is associated with drying, samples can also be observed with a  
243 Low Temperature Scanning Electron Microscope (LTSEM) after cryo-fixation (Chenu and  
244 Tessier, 1995). To our knowledge, with SEM, image analysis is hardly possible given the 3D  
245 surface rendering of the images and the fact that soil microorganisms are only identified based  
246 on shape, which is a difficult task. SEM has been used for example to show the presence of  
247 fungi in the detritosphere extending between plants cells and adhering soil aggregates (Gail-  
248 lard et al., 1999) and more recently to study the colonisation of fungi and unicellular organ-  
249 isms involved in the biodegradation of plant residues (Witzgall et al., 2021) or plastic materi-  
250 als in soil (Zumstein et al., 2018).

251         Transmission electron microscopy can be used to visualise soil microorganisms in  
252 their habitats (Vidal et al., 2016; Watteau and Villemin, 2018). In order to do this, however,  
253 samples cannot be simply deposited on TEM grids as a suspension but rather have to be em-  
254 bedded in a resin, from which ultrathin sections are prepared (Watteau et al., 2002, 2012;  
255 Elsass et al. 2008). The soil samples are fixed, dehydrated and impregnated with a resin prior  
256 to the ultrathin sectioning. These methods have been adapted from biology to soils to ac-  
257 count for the sensitivity of soil organic matter to the electron beam (Villemin and Toutain,  
258 1987; Villemin et al., 1995; Elsass et al., 2008). In soil thin sections prepared for TEM, the  
259 use of contrasting agents and stains, such as osmium tetroxide (Villemin et al., 1995; Arai et  
260 al., 2019), uranium acetate, lead citrate (Foster, 1988; Chenu and Plante, 2006; Elsass et al.,  
261 2008) or more specific stains, helps with shape and structure based identification by adding  
262 contrast between cells wall structure and the surrounding environment. TEM allows manual  
263 quantification if studied structures are recognisable (Chenu and Plante, 2006; Watteau and  
264 Villemin, 2018) but the method is most often used qualitatively to characterise proximity and  
265 thus potential interactions between microorganisms and the surrounding minerals or aggreg-  
266 ates (Fig 1c) (Vidal et al., 2019).

267         The surface of a soil sample and microbial cells can be visualised with scanning he-

268 lium ion microscopy (SHIM or HIM) (Qafoku et al., 2019). This relatively new method is still  
269 rarely used in soil science, even though the ion beam is less destructive for the surface of the  
270 sample than the electron beam of SEM (Bandara et al., 2021; Schmidt et al., 2021), samples  
271 do not need coating prior to analysis and resolutions of 5 Å are attainable (Joens et al., 2013),  
272 which makes it very promising for studying soils.

273         Microorganisms can also be identified with Atomic Force Microscopy (AFM) by visu-  
274 alising their topography at very fine scales. This is achieved with a stylus that moves vertic-  
275 ally with a vertical resolution of a few Å (Binnig et al., 1986). It requires fewer sample pre-  
276 preparation treatments than electron microscopy (Kherlopian et al., 2008); however it requires a  
277 flat surface at the base and the scale of the study objects (cell surfaces) is necessarily small  
278 (Gaboriaud and Dufrene, 2007). It has therefore not been used on soil samples but rather on  
279 model systems, e.g. to study the adhesion of bacteria to clay mineral surfaces with kaolinite,  
280 montmorillonite, goethite or hematite particles (Lower et al., 2001; Huang et al., 2015; Qu et  
281 al., 2019) and to study the weathering of biotites, chlorites or serpentine minerals by mycor-  
282 rhizae and other fungi (McMaster, 2012; Li et al., 2021).

283         Several of the methods presented here only allow the visualisation of soil microorgan-  
284 isms in 2D. The localisation of microbial cells in 3D is possible either (i) by using the depth  
285 of field of the microscope (stereomicroscope, SEM, HIM, AFM), (ii) by reconstituting the  
286 sample in a non-destructive way using confocal laser scanning microscopy (Li et al., 2004),  
287 but due to the opacity of soil this is mainly useful in the analysis of transparent artificial soil  
288 models as in Sharma et al. (2020), (iii) by progressively abrading the sample with a focused  
289 ion probe (FIB) (Berleman et al., 2016; Vidal et al., 2018), (iv) by reconstitution from thin  
290 sections: superimposed serial block face (SBF) sections or broad ion beam (BIB). However,  
291 the latter have been mainly used to study geo-materials (Desbois et al., 2010; Houben et al.,  
292 2013; Hemes et al., 2015).

293         The data derived from the imaging of microbial distributions, such as those in Nunan  
294 et al. (2001), have been used to develop a statistical model of the spatial distribution of bac-  
295 teria in soil (Raynaud and Nunan, 2014). Schnepf et al. (2022) have advocated that such an  
296 approach should be implemented more widely to analyse the distribution and organisation of  
297 microbial communities in the rhizosphere. Indeed, such data are slowly becoming available,  
298 e.g. data on the distribution of microbes around root cells (Schmidt et al., 2018), or spatial  
299 distributions of inoculated microorganisms in the soil porosity across time (Juyal et al., 2020),  
300 and start being used in biogeochemical models (Pagel et al., 2020).

301 In addition, electron microscopy is one of the few methods with which viruses can be visuali-  
302 sed, their size ranging between 30 and 80 nm (Kuzyakov and Mason-Jones, 2018). Viruses  
303 are believed to influence the assembly of bacterial communities, even if this is still poorly un-  
304 derstood for soils.

305

## 306 2.2. Identifying microorganisms

307

308 Prokaryotes can be distinguished from fungi on the basis of shape and size with SEM and  
309 fluorescence microscopy and on the basis of the cell wall structure with TEM. However, these  
310 tools do not allow to differentiate archaea or actinomycetes from bacteria (Foster, 1993), nor a  
311 finer differentiation within bacterial, archeal or fungal groups.

312

313 Fluorescence *in situ* hybridization (FISH) can be used for the *in situ* identification of  
314 microorganisms. The general principle of FISH is to use an oligonucleotide probe coupled to  
315 a fluorescent marker which binds to a specific sequence of RNA or DNA within the microbial  
316 cell. It is used in conjunction with epi-fluorescence microscopy (Bandara et al., 2021) or with  
317 confocal microscopy (Muggia et al., 2013). A database of probes, called probeBase, can be  
318 used to search for, and identify, relevant probes (Greuter et al., 2016). More or less specific  
319 probes can be used to target microorganisms at different taxonomic levels: archaea, crenara-  
320 chaea, bacteria and fungi, bacterial phyla, or identifying gram positive bacteria (Baschien et  
321 al., 2001; Eickhorst and Tippkötter, 2008a, 2008b; Zarda et al., 1997; Kobabe et al., 2004).  
322 The FISH methods can also be used to target certain functional groups of microorganisms,  
323 e.g. those involved in denitrification (Pratscher et al., 2009; Hoshino and Schramm, 2010), ni-  
324 trification and methane oxidation (Torsvik and Øvreås, 2002).

325 The main limitations of FISH are the percentage of successfully detected cells among  
326 the total targeted cells (Bouvier and Del Giorgio, 2003), as well as the high levels of back-  
327 ground noise due to non-specific fixation of the stains on soil particles. It explains why FISH  
328 has been used so infrequently on soil samples. Different probes have been developed, making  
329 it possible (i) to amplify the signal, e.g. catalysed reporter deposition FISH or CARD-FISH  
330 (Kubota, 2013; Schmidt and Eickhorst, 2014; Juyal et al., 2018; Schmidt et al., 2018; Bandara  
331 et al., 2021), (ii) to increase the resolution by allowing observations *via* SEM using GOLD-  
332 FISH (Kenzaka et al., 2005; Schmidt et al., 2012) (Fig. 1d, e, f and g), and (iii) to limit unspe-  
333 cific staining or at least detect it. NON338 or NONEUB probe complementary to the

334 EUB338 probe and serving as a negative control for non-specific labelling) (Eickhorst and  
335 Tippkötter, 2008a).

### 337 **3. Characteristics of microbial microhabitats**

338           Understanding the drivers of microbial activity requires that the soil habitat be well  
339 described. The soil matrix determines the physical accessibility of microorganisms to re-  
340 sources and water, the local conditions that control microbial activities and also regulates  
341 trophic relations (predation, competition etc.). Unravelling the distribution of microorganisms  
342 and resources, as well as their probabilities of encounter, will likely improve our understand-  
343 ing of soil microbial activity.

#### 345 *3.1. Physical characteristics of microhabitats*

346

347           Soil structure affects the spatial distribution of microorganisms and trophic resources  
348 and consequently the access that microorganisms have to substrate and controls the transport  
349 of oxygen and water, key factors for microbial growth and activity (Schlüter et al., 2020). Soil  
350 structure can be heterogeneous at the microscale and must therefore be studied at this scale.  
351 The most frequently used method to describe the 3D structure of a soil is X-ray computed  
352 tomography (X-ray CT) or X-ray micro computed tomography ( $\mu$  X-ray CT) (Fig. 2). It has  
353 been used to study the heterogeneity of soil organisation (Elyeznasni et al., 2012; Hapca et al.,  
354 2015; Lucas et al., 2020) and its relation with soil biotic organisation and functioning (Helli-  
355 well et al., 2014; Kravchenko et al., 2019; Schlüter et al., 2019a, 2019b; Rohe et al., 2021).  
356 Samples used in these studies vary in size from cm, with a voxel resolution of tens of micro-  
357 metres (Elyeznasni et al., 2012; Hapca et al., 2015; Rohe et al., 2021), to mm sized aggreg-  
358 ates, where resolutions can be in the micrometre range.

359           The variation in absorption of the X-rays by different soil materials makes it possible  
360 to obtain images, in Hounsfield units (represented in gray levels), that reveal the different  
361 phases of the soil. As it is based on differences in density, it is mainly used to distinguish  
362 solids from voids, but the water phase can also be detected if located in pores that are suffi-  
363 ciently large relative to the image resolution) (Landis and Keane, 2010; Tippkötter et al.,  
364 2009) (Table 3).

365 Schlüter et al. (2019b) incubated sand-based microcosms in which they placed micro-  
366 bial hotspots either randomly or in dense layers and found that both the spatial distribution of  
367 the hotspots and the water saturation critically affected NO and N<sub>2</sub>O emission rates. Determ-  
368 ining air connectivity, pores tortuosity and diffusion lengths with X-ray  $\mu$ CT, they concluded  
369 that local oxygen supply was the driving variable, paving the way to use soil structural attrib-  
370 utes to predict denitrification via parametrized models.

371 Light and transmission electron microscopy may also be used to describe the structure  
372 of a soil, but these methods provide far less complete information, since they are in two di-  
373 mensions. However, they can be adapted when better resolutions are needed (Table 3). For  
374 example, 3D acquisitions can be done on small samples such a soil aggregates using FIB-  
375 SEM (e.g., Vidal et al. 2018). Microscale biogeochemical models with an explicit representa-  
376 tion of soil structure use X-ray  $\mu$ CT images as input information, but are limited by the lack  
377 of information on sub-resolution pores in which many microorganisms reside (Pot et al.,  
378 2021). 3D high resolution methods such as FIB-SEM might help to overcome these limita-  
379 tions.

## 381 *3.2. Chemical characteristics of microhabitats*

382

### 383 *3.2.1. Localisation of organic matter in soil*

384

385 Being the trophic resource of heterotrophic microorganisms, localizing and character-  
386 izing in-situ soil organic matter is crucial. At the moment, even in microscale biogeochemical  
387 models, SOM spatial distribution is not described from soil imaging, but is prescribed assum-  
388 ing either homogeneous or heterogeneous distributions (Pot et al., 2021).

389 Organic matter can be observed in soil at different resolutions (Fig. 1 and 2); first with op-  
390 tical microscopy on the basis of its shape and colour. It is possible to visualise organic matter  
391 after staining, as in a recent study of Merino et al. (2021), who used confocal laser micro-  
392 scopy to measure the decomposition of lignin that was stained with safranin-O.

393 With electron microscopy, organic matter can be identified using shape criteria, in the  
394 case of SEM and TEM, and by electron density in the case of TEM. It is also possible to stain  
395 SOM using heavy metals in order to amplify the contrast with the rest of the matrix (Foster,  
396 1988; Elsass et al., 2008; Chenu et al., 2015) (Fig. 2). The use of electron microscopy coupled  
397 with energy dispersive X-ray spectroscopy (EDX), enabled by the genesis of X-ray photons

398 following the interaction of electrons with atoms, has been used to determine the elementary  
399 composition of OM (Table 3) (e.g., Chenu and Plante, 2006; Hapca et al., 2015). Similarly,  
400 electron energy loss spectroscopy (EELS) can provide information on the elementary com-  
401 position and the chemical bonds of OM (Watteau et al., 1996, 2002; Watteau and Villemin,  
402 2018) allowing, for example, an elemental (C and N) characterisation of the polyphenolic  
403 substances during root senescence and biodegradation (Watteau et al., 2002). More recently it  
404 was used to characterize contrasting N and oxidized C contents between organo-organic and  
405 organo-mineral interfaces in a soil and suggested different organic matter stabilization pro-  
406 cesses at these interfaces, at the nanometre scale (Possinger et al., 2020).

407 Scanning transmission X-ray microscopy (STXM) provides an image of thin objects  
408 by transmission, as does TEM, but in this case from X-rays. The distribution of elements can  
409 be mapped based on the absorption of X-rays at different energy levels (Table 3). The  
410 samples must be very thin, but the method has the advantage that ultrathin sections of hy-  
411 drated materials can be used (Solomon et al., 2012). Information on functional groups can be  
412 derived from X-ray absorption near edge structure (XANES) by coupling the STXM with  
413 Near-edge X-ray fine structure spectroscopy (NEXAFS) (Keiluweit et al., 2012; Remusat et  
414 al., 2012) at nanometre resolutions (Table 3, Fig. 3). This method was used by Lehmann et al.  
415 (2005) to map the distribution of different forms of organic carbon at the nanometer scale in  
416 soil micro-aggregates. Using ultrathin sections of rapidly frozen samples obviates the need for  
417 inclusion in a resin (Lehmann et al., 2008; Solomon et al., 2012). Otherwise, the resin used  
418 must be distinguishable from the mapped elements and for distribution maps of carbon must  
419 be carbon-free, such as polymerized elemental sulphur (Lehmann et al., 2005) or with a dif-  
420 ferentiable signature (Vidal et al., 2018; Vergara Sosa et al., 2021).

421 Fourier transform infrared micro-spectroscopy (micro-FTIR) can be used to character-  
422 ise, quantify and locate organic molecules based on the bonds of functional groups (Singh and  
423 Gräfe, 2010). However, the resolution is generally low (Table 3). Several infrared spectro-mi-  
424 croscopy methods using different wavelengths such as the visible and near infrared (VNIR)  
425 have enabled, using predictive models, the acquisition of images with a 53  $\mu\text{m}$  resolution on  
426 dried soil, with detailed estimations of the organic carbon spatial distribution in a soil as a  
427 function of depth (Fig. 4) (Steffens and Buddenbaum, 2013; Hobbey et al., 2018). This  
428 method requires almost no prior preparation except drying samples and ultrathin sectioning  
429 to 200nm using a cryo microtome (Hernandez-Soriano et al., 2018; Weng et al., 2021).  
430 Other micro-spectroscopy methods are still little used in soil science, such as Raman spectro-  
431 metry with which organic matter can be characterised based on vibrational and rotational mo-

432 lecular analyses (Bandara et al., 2021; Lee et al., 2022; Musat et al., 2012).

433

434 Spectro-microscopy based on synchrotron radiation (SR), which is brighter and,  
435 above all, better focused than other sources of radiation, gives a finer OM identification than  
436 that obtained by spectroscopy used with conventional sources of radiations (Singh and Gräfe,  
437 2010; Hota, 2021). It also provides information on the location and nature of the OM con-  
438 tained in a sample at finer scales than with conventional radiations. These methods are, for the  
439 most part, non-destructive and with higher resolutions than the same methods used with con-  
440 ventional sources of radiations (Weng et al., 2021), which makes it possible to obtain inform-  
441 ation on various molecular species of OM (depending of their absorption characteristics at a  
442 given wavelength) at the scale of microbial habitat (Singh and Gräfe, 2010; Hernandez-Sori-  
443 ano et al., 2018). Depending the photon energies used for image acquisition, some methods  
444 can be more harmful than others. SR based X-ray spectromicroscopy can harm and kill mi-  
445 croorganisms, because of the high levels of electron volts applied to the sample. Lower en-  
446 ergy levels are used with SR-based infrared spectromicroscopy, meaning that measurement  
447 can be repeated more easily and time sequences can be recorded (Holman and Martin, 2006).

448

449 Elementary maps of samples can be established at high resolutions using Secondary Ion Mass  
450 Spectrometry (Bandara et al., 2021; Remusat et al., 2012; Schurig et al., 2015). There are sev-  
451 eral types of Secondary Ion Mass Spectrometry (SIMS): static SIMS or Time of flight SIMS  
452 (TOF-SIMS) and dynamic SIMS, CAMECA or nanoSIMS (Myrold et al., 2011). A primary  
453 ion beam (usually  $Cs^+$  for samples with organic matter, or  $O^-$ ) is used to sputter the sample  
454 surface and release secondary ions, which are collected, separated and analysed. These sec-  
455 ondary ions are characterised using a mass spectrometer for dynamic SIMS and a time-of-  
456 flight mass spectrometer for static SIMS (Myrold et al., 2011). Scanning the sample gives ac-  
457 cess to elementary and isotopic map of the soil sample at very fine resolutions, i.e., less than  
458 100 nm (Herrmann et al., 2007b). However, as with many recent techniques, SIMS suffer  
459 from heavy technical constraints, particularly in sample preparation: the samples must be dry,  
460 stable, conductive, flat and resistant to a very high vacuum (Herrmann et al., 2007b). Hence,  
461 samples are usually embedded in a carbon containing epoxy resin which contributes to a  
462 background signal that has to be removed (Mueller et al., 2013). In addition, the method is re-  
463 latively destructive since the ion beam sputters the sample's surface. Quantitative elemental  
464 analyses are complicated to obtain because adequate standards are needed (Mueller et al.,  
465 2013). Finally, the observed field with nanoSIMS is very small (from  $5 \times 5$  to  $50 \times 50 \mu m$ ),

466 which makes it very difficult to identify the areas of interest (Herrmann et al., 2007b). This  
467 suggests that when the spatial scales targeted are between 100  $\mu\text{m}$  and 1 cm, TOF-SIMS may  
468 be more appropriate (Myrold et al., 2011; Bandara et al., 2021), although it is rarely utilised  
469 for soils due to a lower sputtering rate of the surface samples (impacting a thinner part of the  
470 sample surface) and is therefore more exposed to problems with surface contamination. Nev-  
471 ertheless, the method is still worthwhile as it is one of the only methods that can be used to  
472 trace isotopic elements, such as  $^{13}\text{C}$  or  $^{15}\text{N}$  labelling, in a spatially explicit manner.

473 Recently, laser ablation-isotope ratio mass spectrometry (LA-IRMS) has been used to  
474 characterize natural  $^{13}\text{C}/^{12}\text{C}$  abundance of soil organic matter, which was found to be hetero-  
475 geneous, at a  $10\mu\text{m}$  spatial resolution, in soil aggregates of the rhizosphere (Rodionov et al.,  
476 2019).

477 Finally, Matrix assisted laser desorption ionisation (MALDI), with which various ana-  
478 lytes (sugars, lipids, amino acids, metabolites etc.) may be mapped on roots after their extrac-  
479 tion from the soil, is worthy of consideration, as shown by Rudolph-Mohr et al. (2015).

480

481 Is it possible to localize organic matter un 3-D soil samples ? X-ray CT is appropriate  
482 as samples are subjected to an X-ray beam from different angles and this is followed by a 3D  
483 reconstruction of the object (Roose et al., 2016). With X-ray CT, as with transmission micro-  
484 scopy, SOM is distinguished on a density basis but this remains difficult given the density  
485 similarity between SOM and other soil constituents (Roose et al., 2016). A number of heavy  
486 metal stains have been tested in order to accentuate the contrast (Peth et al., 2014; Van Loo et  
487 al., 2014; Maenhout et al., 2021). So far, only osmium (Peth et al., 2014; Zheng et al., 2020;  
488 Maenhout et al., 2021) and iodine (Lammel et al., 2019) stains have proved to specifically  
489 stain organic matter (though Schlüter et al. (2022) suggest that Osmium binds to some miner-  
490 als), to provide a detectable staining and to diffuse through the soil matrix. Osmium was suc-  
491 cessfully used to map soil organic matter (Rawlins et al., 2016).

492 Micro-spectroscopy,  $\mu\text{X}$ -ray fluorescence ( $\mu\text{XRF}$ ) tomography, where X-ray induce  
493 the reemission of a X-ray fluorescent radiation from the sample, is a promising method which  
494 provides information about the chemistry of 2D (Schlüter et al., 2022) or 3D samples. Its use  
495 is very rare in 3D, in particular because improvements still have to be made to circumvent  
496 problems of fluorescence attenuation in soils (Feng et al., 2021; Hapca et al., 2015; Roose et  
497 al., 2016). Fluorescent light can be absorbed by the sample, in particular in the case of thick  
498 samples, thus attenuating the fluorescent signal. It has already be used in 3D to analyse di-  
499 atom cells at the micrometre scale, or plant seedling (Pushie et al., 2014). Attempts have been



500 made to use the method with soil aggregates (Antipova et al., 2018).

501 Another option to go from 2D to 3D images is to use one of the methods described  
502 above and to carry out 3D reconstructions using statistical approaches. For example, Hapca et  
503 al. (2015) obtained several elementary 2D maps of a soil in SEM-EDX and extrapolated the  
504 chemical characteristics after combination with 3D images from X-ray tomography (Fig. 5).  
505

506 *3.2.2. Oxygen and CO<sub>2</sub> distributions in soil*

507

508 As oxygen consumption and carbon dioxide emissions are directly related to the res-  
509 piratory activity of soil organisms (Kuzyakov and Blagodatskaya, 2015), they are often mon-  
510 itored quantitatively in the atmosphere around the soil sample but are rarely monitored at the  
511 microhabitat scale. Methods are being developed to visualise the distribution of partial gas  
512 pressures in soils using probes, called needle-type or planar optodes (Santner et al., 2015)  
513 (Table 4). The former are needles, often a set, pinched in the soil with which gas partial pres-  
514 sures are measured (Elberling et al., 2011) ; it can bring additional image information if com-  
515 bined with other methods like X-ray computed tomography (Rohe et al., 2021). There are dif-  
516 ferent types of planar optodes, but they generally involve a combination of gels and optical  
517 sensors (Pedersen et al., 2015). They are based on the use of fluorochromes that are sensitive  
518 to the presence of a gas, which acts as an exciter or an extinguisher of fluorescence, causing  
519 variations in brightness which is recorded by a camera (Rudolph et al., 2012; Pedersen et al.,  
520 2015). These fluorescent chemical probes are very sensitive and the reactions are reversible  
521 (Rudolph et al., 2012). The associated resolutions vary depending on the cameras used and  
522 the experimental device (Santner et al., 2015), but resolutions of a few tens of  $\mu\text{m}$  have  
523 already been reached (Larsen et al., 2011; Pedersen et al., 2015).

524 Most of these probes allow for the simultaneous visualisation of several parameters,  
525 such as oxygen, carbon dioxide, pH (Cf paragraphe 3.2.4.) and temperature (Borisov et al.,  
526 2011). However, this technique still requires some improvement. Indeed, measuring simultan-  
527 eously different parameters can result in interferences which affect the quality of the results  
528 (Borisov et al., 2011; Pedersen et al., 2015). The dynamics of oxygen and water were studied  
529 non-invasively using this approach, thus providing new understanding of the activation of  
530 root systems regarding root respiration in a rhizosphere soil (Rudolph et al., 2012), and in a  
531 soil treated with pesticides (Rudolph-Mohr et al., 2015) with respective resolutions of 0.21  
532 mm and 50  $\mu\text{m}$  per pixel (Fig. 6).

### 534 3.2.3. *Water distribution in soil*

535

536 Water is a major factor driving microbial activity in soil and can be visualised with a  
537 few methods (Table 4). Magnetic resonance imaging (MRI) can be used to visualise water dy-  
538 namics in soil (Roose et al., 2016), but rarely is. It has been tested at the rhizosphere scale (0.6  
539 mm resolution) by Pohlmeier et al. (2008), who observed changes in soil water content as a

540 consequence of water uptake by roots. This method has the advantage of offering 3D images,  
541 which few other methods currently offer.

542 Neutron computed tomography is based on the absorption of neutrons by the sample,  
543 providing three-dimensional images similar to X-ray CT images (Koliji et al., 2010). It has  
544 been used by Carminati et al. (2007) to study water flows in soil. Furthermore, neutron radio-  
545 graphy, in which neutrons transmitted through a sample are analysed, also gives information  
546 on the distribution of water, but in two dimensions and only at resolution of a few tens of  $\mu\text{m}$   
547 (Carminati et al., 2010; Rudolph et al., 2012) (Fig. 7). Recently, fast neutron tomography has  
548 been used to visualise and monitor in 3D the root uptake of water, as 3D images can be ac-  
549 quired in few seconds (Tötzke et al., 2021), and determine which roots are preferentially at  
550 the origin of water uptake in soil.

551

552 The resolution of these methods explains why they are mainly used at the rhizosphere  
553 scale and are not adapted for studies at the microbial scale. Finally, X-ray CT, delivering  
554 greyscale images that are characteristic of the different phases of soil, including water, can  
555 also provide information on the spatial distribution of water. A segmentation treatment makes  
556 it possible to distinguish water from soil, in 3D and with a resolution which may be less than  
557 a mm (Landis and Keane, 2010). As with the mapping of organic matter, a number of studies  
558 have used contrast agents (heavy elements dissolved in water like  $\text{CdSO}_4$ , KI or  $\text{AgNO}_3$ ) in  
559 order to increase the water signal (Van Loo et al., 2014). Others have attempted to combine  
560 images of a dry and wet soil to subtract the "dry soil" signal from the "wet soil" signal in or-  
561 der to isolate the "water" signal (Tracy et al., 2015). However, this approach is not suitable for  
562 clay soils, because of the risk of shrinking and swelling of clays with soil moisture changes  
563 (Baveye et al., 2018).

#### 565 *3.2.4. Other soil chemical characteristics*

566

567 Planar optodes, described in section 3.2.2., also allow for the visualisation of other  
568 molecules such as  $\text{NH}_4^+$  or  $\text{PO}_4^{3-}$  (Pedersen et al., 2015), and for the mapping of pH and redox  
569 potential (Eh) (Pedersen et al., 2015; Roose et al., 2016). Reagents sensitive to acid-base vari-  
570 ations have, for example, been used by Rudolph-Mohr et al. (2015) to describe pH variations  
571 within a soil after the addition of pesticides.

572

#### 573 4. *In situ* information on the physiological state and activities of microorganisms

574

575 Visualizing the microstructure of soil, localising microorganisms in the pore network,  
576 localising organic constituents which are potential substrates sets the scene for microbial  
577 activity. This also needs to be assessed directly and a range of methods have been developed  
578 to gain information on the *in situ* activities of soil microorganisms. Here, we focus on those  
579 that are compatible with visual observations of soils. The *in situ* activities of soil microorgan-  
580 isms at the micro-scale can be studied using methods that (i) differentiate the physiological  
581 state of microbial cells, using markers, (ii) demonstrate *in situ* substrate uptake, using tracers,  
582 and (iii) visualise potential activities of extracellular enzymes.

583

##### 584 4.1. *Physiological state of soil microorganisms*

585

586 Microorganisms can be present in different physiological states in soil. While dead  
587 microorganisms are in an irreversible state in which no growth, cell elongation, nor protein  
588 synthesis can take place, active microorganisms are, as defined by Blagodatskaya and  
589 Kuzyakov (2013), « the portion of total microbial biomass that i) is involved in current utiliz-  
590 ation of substrates, ii) readily responds to substrate input e.g., by respiration, producing en-  
591 zymes, or iii) is growing and reproducing ». The dormant state of soil microorganisms is that  
592 of microbial cells exhibiting strongly reduced physiological activity, e.g., resting cells form-  
593 ing spores or cysts. Dormant microorganisms may switch more or less rapidly from inactive/  
594 dormant state to activity (Blagodatskaya and Kuzyakov (2013).

595

596 These different physiological states can be directly or indirectly observed *in situ* by  
597 microscopy using fluorescent markers targeting microbial nucleic acids or proteins. Specific  
598 markers, such as Fluorescein Diacetate (FDA), 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl  
599 tetrazolium chloride (INT), 5-cyano-2,3-ditoly-tetrazolium chloride (CTC), make the direct  
600 visualisation of active microorganisms possible because they are subjected to enzymatic oxid-  
601 ation or hydrolysis by active cells (Table 5).

602 Recently, a promising method for identifying the active fraction of microorganisms *in situ*,  
603 called bioorthogonal non-canonical amino acid tagging (BONCAT), has been introduced to  
604 soil science (Couradeau et al., 2019). The method is based upon the incorporation of an ad-

605 ded L-methionine analog, L-azidohomoalanine (AHA) combined with a chemically reactive  
606 azide group, during protein synthesis. A biorthogonal azide-alkyne click reaction is then used  
607 to tag the molecule with a fluorophore which renders the active fraction of the microbiome  
608 detectable (Hatzenpichler et al., 2014). Although this method holds much promise, it has not  
609 yet been used on undisturbed samples. Nevertheless, BONCAT has already shown that a sur-  
610 prisingly high proportion of cells extracted from a soil at two depths was active (25-70%)  
611 Couradeau et al. (2019).

612

613 While its reliability is sometimes questioned (Shi et al., 2007), propidium iodide (PI)  
614 allows the visualisation of dead microorganisms because damaged cells-walls are PI per-  
615 meable, contrary to living cells (Table 5). These stains may be combined with fluorochromes  
616 such as DAPI or Calcofluor White (Table 2), to both locate the total microorganisms and infer  
617 their physiological state. Dormant microorganisms are estimated by deducting active and dead  
618 microorganisms from the total microorganisms (Maraha et al., 2004).

619

620 These methods generally allow for a good estimate of dead, active and dormant mi-  
621 croorganisms (Blagodatskaya and Kuzyakov, 2013) but present some limitations. For ex-  
622 ample, intact dead cells are not labelled by PI (Maraha et al., 2004). These labelling tech-  
623 niques also present limits, in particular within a soil matrix, in which, in addition to unspecific  
624 staining (Li et al., 2004), accessibility may be limited for the marker to reach its target.

625

#### 626 4.2. Enzymatic activities

627

628 Finally, a third way to characterise the activity of microorganisms *in situ* is to visual-  
629 ise their production of extracellular enzymes. Zymography has been developed quite recently  
630 (Pedersen et al., 2015) and is used by only a limited number of soil research teams (Spohn  
631 and Kuzyakov, 2013; Spohn et al., 2013; Spohn and Kuzyakov, 2014; Razavi et al., 2016; Sa-  
632 naullah et al., 2016; Ma et al., 2017; Guber et al., 2018; Bilyera et al., 2020). Zymography  
633 produces images of the spatial distribution of enzymes on the surface of a soil sample. A gel  
634 or membrane containing a substrate that changes colour when it comes into contact and reacts  
635 with a specific enzyme is placed on the surface of a soil sample. Colour zones signal the pres-  
636 ence of the targeted enzyme in the sample (Guber et al., 2018; Razavi et al., 2019). The resol-  
637 ution of zymography (~ tens of  $\mu\text{m}$  and often used at the mm scale) is often lower than the

638 techniques described in the previous two paragraphs. Nevertheless, research is under way to  
639 adapt zymography to the micro-scale environment and resolutions ( $< 100\mu\text{m}$ ), by preparing  
640 thin sections from resin impregnated samples and analysing these with epifluorescence micro-  
641 scopy (Ghaderi et al., 2020). Recently, zymography has been adapted to also visualise oxido-  
642 reductases (Khosrozadeh et al., 2022), and time lapse imaging has been implemented allowed  
643 to better describe enzyme activities by accounting diffusion losses and the kinetics of signa  
644 development (Guber et al., 2021). It has, for example, allowed the visualisation of the distri-  
645 bution of phosphatases at the rhizobox scale (Spohn and Kuzyakov, 2013; Spohn et al., 2013;  
646 Razavi et al., 2016; Liu et al., 2017) (Fig. 9). Heitkötter and Marschner (2018) demonstrated  
647 that microbial hotspots of activity, revealed by zymography, represented less than 3% of cores  
648 surface area, but after spraying glucose onto the surfaces of interest, the enzymatic activity  
649 dramatically increased outside of these initial hotspots, demonstrating that apparently dormant  
650 areas of soil are easily stimulated.

651         There are few examples of correlative imaging using zymography with other methods,  
652 even if it has been used with X-ray tomography and  $^{14}\text{C}$  imaging (Kravchenko et al., 2019;  
653 Becker and Holz, 2021). However, this technique has enabled the identification of hotspots of  
654 activity of numerous enzymes ( $\beta$ -glucosidase,  $\alpha$ -glucosidase, xylanase, phosphatase, chit-  
655 inase, peroxidase etc.) (Kuzyakov and Blagodatskaya, 2015; Heitkötter and Marschner, 2018;  
656 Razavi et al., 2019).

#### 658 *4.3. Assimilation of substrates*

659

660         Physiologically active heterotrophic microorganisms assimilate organic substrates and,  
661 therefore, isotopically labelled organic substrates can be used to label and visualise active mi-  
662 croorganisms within the soil architecture. It requires a combination of methods for detecting  
663 both the microorganisms and the isotopically labelled constituents, the superposition of which  
664 can reveal active cells, as described below.

665         Radioisotope labelled organic substrates in combination with autoradiography have  
666 been used to measure the spatial distribution of substrate assimilation activities. When placed  
667 in contact with an emulsion or a photographic film, the distribution of radioactive source is re-  
668 corded, such as zones of root exudation (Holz et al., 2019), rhizosphere hotspots (Becker and  
669 Holz, 2021) and assimilation zones (Torsvik and Øvreås, 2002) at scales ranging from cm-  
670 mm. It has allowed, for example, the visualisation of the transfer of  $^{14}\text{C}$  photosynthates from

671 *Pinus* roots to mycorrhizae in rhizoboxes with  $^{14}\text{C}$  (Leake et al., 2001),  $^{32}\text{P}$  (Lindahl et al.,  
672 1999) and  $^{33}\text{P}$  (Wu, 2014), and to locate methanotrophs assimilating  $^{14}\text{C}\text{-CH}_4$  according to soil  
673 depth and aggregates size in an afforestation chronosequence on subalpine pasture (Karbin et  
674 al., 2017). Autoradiography can be applied at much smaller scales when combined with elec-  
675 tron microscopy, i.e. micro-autoradiography, but this has seldom been used with soils (Lee et  
676 al., 1999; Rogers et al., 2007). Radioisotope based methods are very sensitive, meaning that  
677 low substrate concentrations can be used, but they demand specific safety procedures, unlike  
678 stable isotopes.

679

680 Similar information can be obtained using substrates labelled with stable isotopes and  
681 SIMS. Cliff et al. (2002) visualised the assimilation of  $^{15}\text{N}$  and  $^{13}\text{C}$  by bacteria and fungi  
682 grown in a model soil system made of kaolinite with TOF-SIMS. SIMS techniques have been  
683 used to monitor the fate of labelled organic root exudates, thus highlighting microbial hot-  
684 spots in the rhizosphere and shedding light on rhizosphere functioning through the visualisa-  
685 tion of the transfer of organic carbon from the plant to rhizosphere microorganisms (e. g.  
686 Vidal et al., 2018). They have also been used to visualise microorganisms that have assimil-  
687 ated N after  $^{15}\text{N}$  nitrogen fertilizers addition to soil, as shown in Fig. 8 (Herrmann et al.,  
688 2007b).

689

690 No technique can visualise microorganisms and their activities in 3D at present. How-  
691 ever, positron emission tomography (PET) is a promising approach, as it can locate isotopes  
692 in 3D in a sample. Garbout et al. (2012) used this method to visualise the assimilation of  $^{11}\text{C}\text{-}$   
693  $\text{CO}_2$  by a plant. However, as  $^{11}\text{C}$  is very unstable (half-life of 20.4 min), the observation time  
694 was short. Although commonly used in the medical sciences field, this technique, which does  
695 not require any particular sample preparation, is nearly un-used in soil science. In addition,  
696 the resolutions obtained so far are very low, i.e. in the order of a mm, which mean that this  
697 methodology not really suitable for investigating microorganisms in soils.

698

699 The methods presented so far target assimilation sites. In order to establish links  
700 between the spatial distribution of microorganisms and the distribution of their activities in  
701 the soil structure, several methods need to be combined. In some studies microorganisms  
702 were located with FISH labels and their activities with micro-autoradiography FISH (MAR-  
703 FISH) (Lee et al., 1999; Ouverney and Fuhrman, 1999; Torsvik and Øvreås, 2002; Nubel et  
704 al., 2002; Musat et al., 2012). In others, such as in Schlüter et al. (2019a), fluorescence micro-

705 scopy and electron microscopy were used to locate microorganisms in a soil structure that was  
706 visualized with computed microtomography. In this study nanoSIMS was also used to  
707 identify microorganisms that had assimilated the organic substrate. Similarly, the use of FISH  
708 followed by the use of nanoSIMS (FISH-SIMS) made visualising microorganisms that had as-  
709 similated stable isotopes possible (Musat et al., 2012; Mueller et al., 2013; Schurig et al.,  
710 2015). The FISH methods can be adapted using very electronegative halogen markers (I, Br)  
711 (HISH-SIMS) which can be detected in SIMS directly (Li et al., 2008; Musat et al., 2012;  
712 Mueller et al., 2013). However, to our knowledge, this technique has not yet been applied to  
713 soil samples. Finally, the cross-use of SIMS with electron microscopy is frequent (Watteau  
714 and Villemin, 2018). It facilitates the prior identification of areas of interest and makes it pos-  
715 sible to obtain information related to microhabitat (Mueller et al., 2013).  
716

## 717 **5. Methodological challenges and solutions**

718

719 All observation methods have different advantages and disadvantages and the choice  
720 of one rather than another will depend on the scientific question to be answered. Different  
721 critical points must be considered before choosing the most relevant method: the sample pre-  
722 preparation, possible combination of different methods, existing imaging treatments and repres-  
723 entativeness of these images with regards to the scientific question.

### 725 *5.1. Sample preparation methods*

726

727 The sample preparation impose a number of constraints, which need to be taken into  
728 account when choosing a method of analysis. First, observation methods often, though not  
729 systematically, require the production of thin sections and/or impregnation for the soil struc-  
730 ture to remain undisturbed. The procedure used must be adapted to the sample and the object-  
731 ive of the study, and all samples and controls should be prepared in equivalent conditions and  
732 technical controls to assess the quality of preparations.

#### 734 *5.1.1. Fixation and dehydration*

735



736           When fragile objects need to be kept in their original state or microorganisms activity  
737 needs to be stopped, a fixation can be performed, by cross linking proteins. Fixatives often are  
738 toxic compounds, which require to take precautions to ensure user safety. Glutaraldehyde  
739 solutions are frequently used for this purpose (Tippkötter et al., 1986; Altemüller and Van  
740 Vliet-Lanoe, 1990; Nunan et al., 2001; Elsass et al., 2008; Vidal et al., 2018) but avoided with  
741 FISH because glutaraldehyde impedes hybridization of nucleic acids (Solovei, 2010), making  
742 formaldehyde the fixative of choice with FISH (Schmidt et al., 2012; Schmidt and Eickhorst,  
743 2014). Fixatives can also have substantial influence on the samples' chemistry, which is sus-  
744 ceptible to modify the information acquired with Raman spectra for example (Bandara et al.,  
745 2021). Thus, control samples are needed to assess their effect. Cryo-fixation, using liquid ni-  
746 trogen, propane or ethane for rapid freezing without ice crystal formation is another fixation  
747 option. Also, rewetting soil samples with fixatives should be performed as carefully as pos-  
748 sible as it may displace the microorganisms and modify particles organisation.

749           Dehydration, which is essential for some methods (TEM, SEM and nanoSIMS for ex-  
750 ample) can alter soil structure (Tippkötter and Ritz, 1996) because of clay shrinkage. Differ-  
751 ent protocols exist, such as air drying (Gutiérrez Castorena et al., 2016), freeze-drying (Tipp-  
752 kötter et al., 1986) or dehydration with water-ethanol or water-acetone gradients (Tippkötter  
753 et al., 1986; Nunan et al., 2001; Elsass et al., 2008; Mueller et al., 2012). Water-acetone ex-  
754 changes have proven their efficiency in limiting impacts on soil structure (Altemüller and Van  
755 Vliet-Lanoe, 1990). However, biological material must be fixed prior dehydration with water  
756 -acetone gradients in order to avoid cell damage (Elsass et al. 2008). Water-ethanol exchanges  
757 are believed to minimize cells damage because ethanol is a weaker solvent than acetone  
758 (Bandara et al., 2021). Following cryo-fixation, freeze-substitution allows dehydration by  
759 substituting frozen water with a solvent at sub-zero temperatures. Supercritical drying is also  
760 efficient for drying soil containing natural “gels” (allophanic soils) (Woignier et al., 2005,  
761 2008; Calvelo Pereira et al., 2019).

### 763           5.1.2. *Staining*

764

765           Stains (fluorochromes or contrast agents) used in particular with epi-fluorescence,  
766 confocal microscopy and electron microscopy for observing microorganisms may have lim-  
767 ited efficiency. Many stains, and particularly in undisturbed soil, may have difficulty penetrat-  
768 ing the sample and reach their target (Peth et al., 2014). Testing this by quantifying the gradi-

769 ents of the stain in soil aggregates as performed by Zheng et al. (2020) is hence very useful. In  
770 addition, non-specific staining, as well as autofluorescence can also occur, depending on soil  
771 characteristics. Therefore, controls should be considered whenever possible with unstained  
772 thin sections or samples. Stains can be applied on the surface of previously prepared thin sec-  
773 tions (Elsass et al., 2008; Gutiérrez Castorena et al., 2016; Juyal et al., 2019; Schlüter et al.,  
774 2019a), which is useful when used in combination with surface imagery methods.

### 776 *5.1.3. Embedding*

777

778 The embedding step can present some difficulties with ensuring that the resins saturate  
779 the entire porosity and with avoiding the presence of air bubbles, which lead to fragile  
780 samples for subsequent processing (cutting, polishing). The relatively high viscosity is at  
781 cause here. These problems are particularly true for large samples, which require long periods  
782 under high vacuum conditions. A solution is to perform multiple additions of resins at differ-  
783 ent dilutions with acetone making it less viscous, thus ensuring that the porosity is better filled  
784 (Nunan et al., 2001; Elsass et al., 2008; Mueller et al., 2012; Vidal et al., 2018).

785 In addition, the choice of embedding resin should suit the study objectives. For ex-  
786 ample, nanoSIMS requires the use of special resins such as Aradite-502 (Mueller et al., 2012;  
787 Vidal et al., 2018) for their resistance to the applied pressures (Herrmann et al., 2007a; Muel-  
788 ler et al., 2012). Any study targeting the localisation and characterisation of soil carbon re-  
789 quires the use of resins that can be differentiated from the sample (Mueller et al., 2012; Vidal  
790 et al., 2018; Vergara Sosa et al., 2021). For the specific case of nanoSIMS, even though most  
791 samples are embedded, direct deposition of samples is also possible as long as they are very  
792 flat ( $< 1\mu\text{m}$  topography for natural abundance and  $30\mu\text{m}$  for stable isotopes enriched  
793 samples) (Mueller et al., 2013).

794

### 795 *5.2. Image acquisition and processing*

796

797 When acquiring images, it is important to note that very often the operator will have  
798 an impact on the quality of the final image. Indeed, parameters such as time of exposure or  
799 aperture, that are generally set manually, are very important and should be taken into account.  
800 Regardless of the choices made when establishing a protocol, the different steps should be  
801 systematically recorded as, failing that, variations in choices from one operator to another and

802 from a day to another may have an impact on the results.

803         The image processing steps are also of fundamental importance for the extraction of  
804 relevant information. It allows analysing the image and thus distinguishing objects, enumerat-  
805 ing them, but also extracting data about their size and shape and characterizing their spatial  
806 distribution. The images obtained with the different methods present characteristics which dif-  
807 fer according to the modes of acquisition (size and depth fields, resolutions, voxels or pixels,  
808 number of channels, bit depth) and which require different image processing and analysis  
809 strategies. Starting from the raw image, the identification of different soil constituents can re-  
810 quire significant expertise for choosing the optimal processing procedure. Indeed, observation  
811 methods based on shapes and colours for example can generate errors and also lead to differ-  
812 ent conclusions from one observer to another (Baveye et al., 2010). Replications of observa-  
813 tions at different dates and by several observers may be useful (Kleber et al., 2003; Chenu and  
814 Plante, 2006), but it is costly and time consuming..

815         Image processing often requires segmentation, correction and filtering steps, amongst  
816 others (Schlüter et al., 2014; Roose et al., 2016; Withers et al., 2021; Jeckel and Drescher,  
817 2021). To avoid operator effects, image analyses should be automated as much as possible.  
818 The choice of a segmentation threshold is subjective and depends on the observer. Therefore,  
819 most studies recommend the use of a fully automatic thresholding. However, thresholds must  
820 be chosen correctly depending on the targeted object characteristics and the type of sample  
821 (Iassonov et al., 2009; Hapca et al., 2013; Bilyera et al., 2020; Pot et al., 2020). The same is  
822 true for all stages of image processing (Kaestner et al., 2008; Houston et al., 2013) and this  
823 should always be carefully described in any scientific publication. There are more and more  
824 methodological publications describing specific methods to perform total or locally adaptative  
825 methods (Sauzet et al., 2017; Gao et al., 2019; Bilyera et al., 2020). Machine learning and es-  
826 pecially supervised pixel classification have been increasingly found to provide reliable clas-  
827 sification of different minerals and organic matter in nanoSIMS images (Steffens et al., 2017;  
828 Vidal et al., 2021), in SEM microscopy of shales (Wu et al., 2019) and in hyperspectral mi-  
829 croscopy where bacteria were classified *ex situ* (Liu et al., 2021). We can thus expect that it  
830 will help future research to extract more easily the images interest objects.

831         All samples, including controls, should be acquired in the exact same conditions with  
832 the same parameters and the image processing should follow the same steps, otherwise they  
833 cannot be considered equivalent and compared.

834

### 835 *5.3. Image representativeness*

836

837         As the representativeness of images is linked to their size, the question of the repres-  
838         entativeness of the images arises when the imaging resolution is high. The study areas must  
839         be chosen with care and otherwise it can lead to focusing on exceptional areas, which can res-  
840         ult in incorrect conclusions being drawn. Then it may be difficult to link observation results to  
841         the overall functioning of the soil. Microbial densities are much higher in hot-spots than un-  
842         bulk, which has consequences on the choice of imaging methods because the necessary field  
843         to describe processes inside these environments has to be considered. NanoSIMS is adapted to  
844         the study of the rhizosphere and detritosphere as the probability of encountering microorgan-  
845         isms that have assimilated isotopically-labelled resource is relatively high, contrary to the  
846         bulk soil (Védère, 2020).

847         Furthermore, it should not be forgotten that many of the techniques available provide a  
848         2D image of a 3D object. For example, the organisation of porosity in 2D images is not rep-  
849         resentative of the 3D pore network. To overcome this, correlative imaging can help to reach a  
850         more complete understanding of the spatial organisation of soil constituents soil functioning,  
851         as was done by Schlüter et al. (2019a).

852

### 853 *5.4. Combination of observation methods*

#### 854 *5.4.1. Compatibility of the different methods*

855

856         Reviewing the literature shows that there is no single method to study both the spatial  
857         and temporal fate of soil microorganisms and their activities in their microhabitats. This is  
858         why understanding the functioning of the soil as a whole requires a combination of different  
859         methods and correlative-imaging. Recent publications demonstrate the great potential of this  
860         approach. The first step is to check that the methods are compatible. When the analyses can  
861         be carried out on the same samples, then the question of the order of the methods to be used  
862         must be considered.

863         Indeed, the methods previously described require specific preparations and are more  
864         or less destructive, such as SEM or SIMS, which require the samples to be covered with a  
865         gold layer or which sputter the sample, respectively. These methods should therefore be im-  
866         plemented in the final stages of observation. Other methods such as tomography or zymo-  
867         graphy are advantageous since, not requiring specific prior soil preparations, they allow for

868 multiple observations of the same object without such concerns. Moreover, X-ray computed  
869 tomography has negligible effects on root growth (Zappala et al., 2013), archaea and bacteria  
870 and their functioning in soil (Bouckaert et al., 2013; Schmidt et al., 2015).

871 Altogether, we suggest to favour first the methods which do not require any particular  
872 preparation (X-ray or neutron tomography for example), secondly the observations using flat  
873 probes or gels (zymography or planar optodes) and requiring only a cut in the material, then  
874 the methods requiring the preparation of thin sections and finally all the methods whose oper-  
875 ation consists in bombarding the surface of the object and which can irreversibly damage the  
876 surface of the objects (SEM, nanoSIMS). Other methods, such as Raman scanning can be  
877 harmful for the resin (Bandara et al., 2021). All these particularities should be considered in  
878 order to define the best pathway to do correlative imaging. For example, Bandara et al. (2021)  
879 successfully applied a thin section protocol in order to combine and correlate images from  
880 nothing less than six different imaging methods by using LR white resin. Otherwise, the  
881 methods can be applied to different samples prepared under the same conditions, but the num-  
882 ber of samples to be prepared can quickly become huge.

883 A challenge to overcome with correlative imaging is related to the superimposition of  
884 two images of the same exact zone acquired with different techniques. Solutions are to use a  
885 single same sample holder including a coordinate system (Bandara et al., 2021) or to target  
886 landmarks directly present in the samples (Juyal et al., 2020; Schlüter et al., 2019a) to find  
887 back the region of interest. For surface imaging methods, it is also possible to artificially mark  
888 the sample surface to create references helping at finding previously targeted region of in-  
889 terest using the electron microscopy beam (Bandara et al., 2021). Moreover, the difficulty in-  
890 creases when one wants to combine optical techniques, which imply a field depth, and surface  
891 techniques which have none. Indeed, some features below the sample surface may be visual-  
892 ized with field depth techniques but not with surface techniques. For example, if the zone of  
893 interest is not on the sample surface, although visible by light microscopy, then, it will not be  
894 possible to reach it with surface techniques such as scanning electron microscopy or  
895 nanoSIMS (except if the object is very close to the surface where you can expect to reach it  
896 by an abrasion of surface). This limitation makes the correlative imaging not trivial. How-  
897 ever, powerful tools, such as correlia, a plugin developed for imageJ, help correlating images  
898 from different methods (Bandara et al., 2021; Rohde et al., 2020).

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#### 5.4.2. Upscaling issues

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Focusing on microorganisms in their habitat results in considering a very small field of observation compared to the global functioning of the soil and raises the question of the upscaling. How can we account for these observations on larger scales? Are microscale observations representative of the phenomena described on a larger scale? Some studies recommend the creation of mosaics of high-resolution images in order to obtain a wide image of the samples. Others advice to work with an average volume which consists of averaging microscopic description into a Representative Elementary Volume (Baveye et al., 2018) or a homogenisation which assumes that structure is sufficiently periodic to be considered as composed of repeated units for variables as porous structure (Roose et al., 2016). The use of these options is still under discussion considering the trade-off between their potential and the considerable loss of data that they can involve (Baveye et al., 2018).

The combination of observation methods can also address this problem with complementary observations at different spatial scales. Such studies have already been carried out, such as that of Schlüter et al. (2019), who used correlative imaging with X-ray tomography at a centimetre scale, optical microscopy and fluorescence at a mm scale, then electron microscopy on the scale of a few hundred microns and finally nanoSIMS on the tens of microns scale in order to describe the microhabitat in a decaying leaf detritusphere combining structural, geochemical and biological data. They showed that if bacteria were mainly present in pores  $< 10 \mu\text{m}$ , they were preferentially concentrated near macropores and organic matter. Juyal et al. (2019, 2020) also combined observations at mm (X-ray tomography, light and fluorescence microscopy) and micrometric (light and fluorescence microscopy) scales to study the localisation and spread of microorganisms in the soil structure and in particular as a function of porosity indicators. Keiluweit et al. (2012) characterised the soil-microorganisms interaction by combining nanoSIMS and NEXAFS with STMX allowing them to propose a conceptual model of the fate and transformation of fungal cell wall compounds in soil and its relation with mineral particles. Bandara et al. (2021) acquired images allowing description of the rhizosphere using six different imaging methods, i.e.. light microscopy, epifluorescence microscopy, HIM microscopy, Tof-SIMS, nanoSIMS, SEM-BSE and -EDX and confocal Raman spectroscopy.

2D (SEM-EDX) and 3D (X-ray CT) approaches may be combined to build 3D chemical maps of soil samples based on a statistical approach such as that proposed by Hapca et al. (2015). Similarly, Anderson et al. (2020) combined 2D (FIB-SEM) with 3D images (TXM,

934 transmission X-ray microscopy) using machine learning on sediment rock materials. Another  
935 approach is to merge 2D images acquired at 3 different scales to create one final image with a  
936 better resolution and multiscale porosity information (Karsanina et al., 2018). The develop-  
937 ment of computer calculation capacity also allows thinking that reconstruction from 2D to 3D  
938 images will be facilitated in the future.

## 940 5.5. Perspectives

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### 942 5.5.1. Methodological developments. Methods of the future.

943

944 There are a number of potentially useful methods that are not or rarely used in soil sci-  
945 ence for a variety reasons.  $\mu$ X-ray fluorescence CT for example (Fig 2) is seldom used be-  
946 cause of necessary developments and improvements regarding the issue of energy attenuation  
947 (Bleuet et al., 2010; Hapca et al., 2015; Roose et al., 2016). Nuclear magnetic resonance  
948 (NMR) is little compatible with soil samples that contain paramagnetic particles (Schmidt et  
949 al., 1997; Baveye et al., 2018). Neutron radiography and positron emission tomography (PET)  
950 scanning do not yet have sufficient spatial resolutions for the study of soils at the scale of mi-  
951 croorganisms. A number of methods therefore still have technical limitations for applying  
952 them to the soil environment and to the microorganisms' spatial scales.

953 Other methods, however, are underused, even though they would be appropriate for  
954 investigating soil microorganisms in their habitats. Super-resolution methods which give ac-  
955 cess to unprecedented resolutions in optical microscopy could be used, e.g. to study microor-  
956 ganisms and soil particles interactions. Super resolution techniques such as STED (stimu-  
957 lated-emission depletion microscopy), SIM (structured illumination microscopy) or SMLM  
958 (single molecule localisation microscopy) have resolutions down to a few tens of nanometers  
959 (Turkowsky et al., 2016), but have not yet been used to visualise soil microorganisms. With  
960 STED, the sample is scanned using two lasers. The first one stimulates the emission of fluor-  
961 escence subjected to diffraction on a targeted zone and the second, in the form of a "donut",  
962 uses a de-excitation beam and comes to switch off a part of the emission of the fluorochrome  
963 leaving only its central emission source. This process counterbalances the effects of diffrac-  
964 tion and means that resolutions of between 20-70 nm can be achieved. As this method is  
965 based on activation and successive repeated extinction of fluorochromes, all fluorochromes  
966 must be photo-stable (Turkowsky et al., 2016).

967 The SIM method uses a mask containing a pattern of lines regularly spaced with a fre-  
968 quency, orientation and phase known, rotating above the sample to achieve a controlled illu-  
969 mination of the fluorochrome. The exploitation of the “moiré” effect obtained by this ap-  
970 proach is used to reconstruct the structure of the observed object at a resolution of approxim-  
971 ately 80-100 nm. One advantage is that it does not require any staining (Turkowyd et al.,  
972 2016).

973 Finally, the SMLM (single molecule localisation microscopy) methods use the blink-  
974 ing of certain fluorochromes to isolate them one by one via their isolated emissions, allowing  
975 very small resolutions to be obtained between 10 and 50 nm. Here the fluorochromes must be  
976 photo-convertible and photo-stable (Turkowyd et al., 2016).

977 These last methods are very sophisticated and the density of the staining, the type and  
978 the size of the fluorochrome used strongly impact the final result (Huang et al., 2009). The  
979 SIM and SMLM methods also require a time-consuming post-processing of images that  
980 hinders their use. These methods are used in cell biology and chemistry but not yet in soil sci-  
981 ence.

982 Similarly, FIB, BIB and SBF are rarely used while they could provide much 3D in-  
983 formation on objects not accessible to the Xray CT resolutions. Other methods of the future  
984 are to use transparent soil media to overcome soil’s opacity and thereafter use 3D imaging  
985 methods not previously suitable to study undisturbed soil. Sharma et al.(2020) tested different  
986 artificial transparent soils to obtain 3D images using CLSM and Raman microscopy. They re-  
987 corded microorganisms position and carbon uptake and were able to observe a higher activity  
988 of bacteria at the vicinity of dead fungal hyphae following a drying/ rewetting cycle. Yang et  
989 al. (2021) by using such transparent model of soil could observe under confocal microscopy  
990 the dynamics of organic matter sorption and protection by clay and the effect of microbial en-  
991 zyme activities on this protection.

992

### 993 5.5.2. *Dynamic observations*

994

995 While the importance of hot moments is recognized for soil biogeochemical processes,  
996 still few visualisation studies address temporal dynamics. The first reason is that visualisation  
997 methods for soil microorganisms in their microhabitat are time consuming and expensive.  
998 Further, if the visualisation methods are destructive, it requires to prepare and process a large  
999 number of replicate samples. Nevertheless, a few methods allow non-destructive time monit-



1000 oring visualisation of soil. X-ray tomography, neutron radiography and zymography allow re-  
1001 peated observations on the same sample at different dates. However, the scanning time of  
1002 these methods is sometimes too long for dynamics to be captured. For example the direct  
1003 monitoring of water flow through the porosity remains difficult to study because CT tomo-  
1004 graphy could not scan fast enough to capture the water flow (Baveye et al., 2018). Neutron  
1005 tomography has improved enough to allow fast imaging and monitoring of water uptake in  
1006 soil with 1 minutes iterations as recently demonstrated by Tötze et al. (2021) and high speed  
1007 synchrotron-based X-ray computed tomography allowed few seconds iteration scanning but it  
1008 is currently not used in soil sciences (Berg et al., 2013).

### 1010 *5.5.3. Precious experimental data for modelling*

1011

1012 It is now acknowledged that the spatial distribution of microorganisms, trophic re-  
1013 sources, air and water in the soil architecture at the microscale largely determine the biogeo-  
1014 chemical fluxes at the macroscale. A new generation of biogeochemical models is emerging  
1015 that are based on an explicit description of soil structure and water distribution at the micro-  
1016 scale (Pot et al., 2015; Kemgue et al., 2019; Ruiz et al., 2020; Pot et al., 2021), in order to  
1017 more accurately forecast the dynamics of organic matter in soil under a wide range of climate  
1018 and management conditions (Monga et al., 2008; Portell et al., 2018; Ruiz et al., 2020). In  
1019 these microscale models, the distribution of microorganisms and trophic resources are up to  
1020 now ascribed a priori (Pot et al. 2021), and not based on experimental data. However experi-  
1021 mental data, such as the distribution of microorganisms (Raynaud and Nunan, 2014; Juyal et  
1022 al., 2019; Schlüter et al., 2019a) or organic matter (Peth et al., 2014; Quigley et al., 2018;  
1023 Schlüter et al., 2022), are necessary input data for calibrating the models and assessing their  
1024 performance. The many technological advances and developments in the field should allow  
1025 for rapid progress in this area.

### 1026 *5.5.4. Imaging soils at the microscale for soil microbial ecology*

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1028 Despite the numerous constraints and difficulties, these methods, when combined, of-  
1029 fer major perspectives for characterizing the location of total and active microorganisms in  
1030 their environment and better understanding soil functioning. Considering the scant available  
1031 knowledge on soil microorganisms distribution and activities in their microhabitats, there is  
1032 an open field for microbial ecology research using imaging at the microscale. We can imagine

1033 different workflows providing valuable data and an overall better understanding of soil func-  
1034 tioning at the microscale and will present two exemples.

1035         A first example concerns the detritosphere. In the detritosphere, soil moisture controls  
1036 the transfers of organic solutes, enzymes and microorganisms, the accessibility of microor-  
1037 ganisms to their substrates and modulates biodegradation (Védère et al., 2020). X-ray com-  
1038 puted tomography can allow to describe the organisation of the porosity in the vicinity of la-  
1039 belled plant residues and the porosity directly connected to it (distances to the residues, con-  
1040 nectivity, tortuosity, direction...). Following the pores description, thin sections can be pro-  
1041 duced on the same samples and the spatial distribution of microorganisms in the vicinity of  
1042 previously targeted porosity and at increasing distances from the residues could be assessed  
1043 using epifluorescence microscopy. Finally, nanoSIMS can allow to observe C and N transfers  
1044 from the residue to the soil and to locate microbial assimilation hotspots at this interface.  
1045 Combining several imaging methods can address questions such as the influence of plant  
1046 residues on soil porosity, whether soil moisture modifies the spatial distribution of microor-  
1047 ganisms decomposing plant residues and the interplay between mineralisation and stabiliza-  
1048 tion of carbon from these plant residues.

1049         A second example addresses the possible influence of biochar addition of rhizosphere  
1050 functioning. Biochars are increasingly used to improve soil properties like water retention or  
1051 cation exchange capacity of soil. They present a high porosity that can be used by micro-  
1052 organisms as habitats. Rhizosphere development and functioning is influenced by biochar ad-  
1053 dition (Atkinson et al., 2010). Unfortunately no clear description of microorganisms spatial  
1054 distribution relative biochar particle exists. X-ray computed tomography can describe soil  
1055 porosity inside the rhizosphere artificially produced in a rhizobox. Then, the surface of the  
1056 box could be exposed to direct zymography in order to give information about enzymes diffu-  
1057 sion in the porosity. Once those measurements being done, thin sections of soil at the vicinity  
1058 of root and biochar particles could be prepared to localize microorganisms using epifluores-  
1059 cence microscopy. Such a workflow could address questions like: Is rhizosphere porosity in-  
1060 fluenced by biochar addition in soil? Do biochar particles provide new habitats for micro-  
1061 organisms and modify their spatial distribution? Are enzyme activities affected by biochar in  
1062 the rhizosphere?

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1065 **6. Conclusion**

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1067           Imaging techniques have developed significantly in recent years, have become in-  
1068 creasingly accessible and are attracting unprecedented attention in the soil's scientific com-  
1069 munity. Microorganisms can be located and identified at fine scales with more accuracy and  
1070 confidence using modern visualisation methods. The mapping of certain microbial activities,  
1071 such as enzyme activities and substrate assimilation, in the soil structure is also possible. The  
1072 improvement of existing techniques has made detailed descriptions, at better resolutions, pos-  
1073 sible and the development of new technologies opens new horizons. However, technical de-  
1074 velopment is still necessary, particularly to reduce analytical time and costs and to optimise  
1075 the combination of different methods. Moreover, imaging techniques often need a high-level  
1076 expertise to be used properly and these are rarely present at a single location. Therefore, a bet-  
1077 ter understanding of soil microhabitats will involve multi-disciplinarity and collaborative  
1078 studies. The information obtained on the spatio-temporal evolution of microorganisms and  
1079 their activities in the soil structure should make it possible to improve knowledge and lead to  
1080 a better understanding of soil functioning.

1081

## 1082 **Conflict of interest**

1083 The authors declare that they have no known competing financial interests or personal rela-  
1084 tionships that could have appeared to influence the work reported in this paper.

1085

## 1086 **Acknowledgements**

1087 This work was supported by a grant from the French ANR to project Soil $\mu$ -3D (ANR-15-  
1088 CE01-0006).

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