1 # Opportunities and limits in imaging microorganisms and their

2 # activities in soil microhabitats

3 Védère Charlotte[a], Vieublé Gonod Laure[a], Nunan Naoise[b,c], Chenu Claire[a].

4 [a] UMR Ecosys, INRAE, AgroParisTech, Université Paris-Saclay, Thiverval Grignon, 78850,

5 France

6 [b] Institute of Ecology and Environmental Sciences – Paris, Sorbonne Université, CNRS, IRD,

7 INRAe, P7, UPEC, Paris, France

8 [c] Department of Soil and Environment, Swedish University of Agricultural Sciences; P.O. Box

9 7014, SE-75007, Uppsala, Sweden

10

11 **Corresponding author:**

12 Claire Chenu claire.chenu@inrae.fr

13 **E-mail addresses:**

14 charlotte.vedere@inrae.fr (C. Védère).

15 naoise.nunan@cnrs.fr (N. Nunan)

16 laure.vieuble@agroparistech.fr (L. Vieublé Gonod)

17 claire.chenu@inrae.fr (C. Chenu)

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.

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

Keywords

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

Abbreviations

AFM: Atomic force microscopy

AHA: L-azidohomoalanine

BIB: Broad ion beam

BONCAT: Bioorthogonal non-canonical amino acid tagging

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

DAPI: Di Aminido Phenyl lndol

DTAF: Dichlorotriazinylaminofluorescein

EDX: Energy dispersive X-ray spectroscopy

EELS: Electron energy loss spectroscopy

ESEM: Environmental scanning electron microscopy

FDA: Fluorescein diacetate

FIB: Focused ion beam

FISH: Fluorescence in situ hybridization

FITC: fluorescein isothiocyanate

FTIR: Fourier transform infrared micro-spectroscopy

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

LTSEM: Low temperature scanning electron microscopy

MALDI: Matrix assisted laser desorption ionisation

MRI: Magnetic resonance imaging

NEXAFS: Near edge X-ray fine structure spectroscopy

NMR: Nuclear magnetic resonance

PET: Positron emission tomography

PI: Propium iodide

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_2, 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 envir-

103   onmental 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 (Kuzyakov 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 detritusphere, 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-
135 plementary microbial taxa being co-located (Kim et al., 2008). To answer these questions, we
136 must 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-
139 erogeneous organo-mineral matrices, composed essentially of very small particles in the sub-
140 mm range that are variably hydrated, and the spatial organisation of which is strongly affected
141 by moisture state. A range of visualisation methods has been used over the last 30 years, but
142 there have been a number of recent methodological developments and a concomitant increase
143 in the number of studies in this area. New methods are now able to provide much more in-
144 formation than before: not only are the resolutions of images higher but it is also possible to
145 obtain spatial information on soil physical, chemical and biological characteristics in three di-
146 mensions. Furthermore, image processing and analysis tools have become more efficient, al-
147 lowing for better correction, segmentation of areas of interest or even predictions of chemical
148 composition (Hapca et al., 2015; Anderson et al., 2020).

149 Despite the technological progress, imaging methods are still under-utilised in soil sci-
150 ences because the scientific community is not always aware of them, and due to the fact that
151 they are time consuming, expensive and not always easy to implement. Furthermore, several
152 visualisation methods require access to instruments that are not widely available (e.g. NEX-
153 AFS, nanoSIMS). Many of the imaging methods that are useful for visualising microbial
154 communities in their habitat have extremely small fields of view (e.g. the area covered by a
155 nanoSIMS image is approximately 30 x 30 μm²). The questions then arise, should targetted or
156 random sampling be used (Brus, 2019) and what of the representativeness of the images ac-
157 quired? Targetted imaging is useful for determining the types of situations that can be en-
158 countered, but there is a risk that it will provide a biased view as the areas of interest chosen
159 may not reflect the soil more generally. In order to obtain a representative view, a degree of
160 random sampling is required, though this can be reduced with a judicious use of spatial mod-
161 elling (Brus, 2019). Whilst sampling considerations are clearly a drawback of these ap-
162 proaches, 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
164 that can be used to obtain information on soil microorganisms and their activities in their mi-
165 cro-environment. We review and present different methods in which (i) microorganisms can
166 be located in their habitats, (ii) different types of microorganisms can be identified, (iii) the
167 characteristics 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-

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

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

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

7

vacuum to which the sample is exposed in the microscope is a challenge and requires the use of specific preparation methods. Samples are generally air-dried before SEM analyses. However, drying strongly affects all hydrated structures and damages biological features (e.g. bacterial cells as well as sheaths of extracellular polysaccharides appear flattened) so that conventional SEM is not recommended (Chenu and Jaunet, 1992). Samples can be observed directly in a moist state without any conducive coating using an Environmental Scanning Electron Microscope (ESEM) (Gleeson et al., 2005; Lin and Cerato, 2014), though it gives poorer quality images in terms of brightness and contrast (Dal Cortivo et al., 2017; Bertola et al., 2019). In order to avoid the damage that is associated with drying, samples can also be observed with a Low Temperature Scanning Electron Microscope (LTSEM) after cryo-fixation (Chenu and Tessier, 1995). To our knowledge, with SEM, image analysis is hardly possible given the 3D surface rendering of the images and the fact that soil microorganisms are only identified based on shape, which is a difficult task. SEM has been used for example to show the presence of fungi in the detritusphere extending between plants cells and adhering soil aggregates (Gaillard et al., 1999) and more recently to study the colonisation of fungi and unicellular organisms involved in the biodegradation of plant residues (Witzgall et al., 2021) or plastic materials in soil (Zumstein et al., 2018).

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

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

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

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

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

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

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

2.2. Identifying microorganisms

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

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

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

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.

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 (μ 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).

Schlüter et al. (2019b) incubated sand-based microcosms in which they placed micro-bial hotspots either randomly or in dense layers and found that both the spatial distribution of the hotspots and the water saturation critically affected NO and N_2O emission rates. Determining air connectivity, pores tortuosity and diffusion lengths with X-ray μCT, they concluded that local oxygen supply was the driving variable, paving the way to use soil structural attributes to predict denitrification via parametrized models.

Light and transmission electron microscopy may also be used to describe the structure of a soil, but these methods provide far less complete information, since they are in two dimensions. However, they can be adapted when better resolutions are needed (Table 3). For example, 3D acquisitions can be done on small samples such a soil aggregates using FIB-SEM (e.g., Vidal et al. 2018). Microscale biogeochemical models with an explicit representation of soil structure use X-ray μCT images as input information, but are limited by the lack of information on sub-resolution pores in which many microorganisms reside (Pot et al., 2021). 3D high resolution methods such as FIB-SEM might help to overcome these limitations.

3.2. Chemical characteristics of microhabitats

3.2.1. Localisation of organic matter in soil

Being the trophic resource of heterotrophic microorganisms, localizing and characterizing in-situ soil organic matter is crucial. At the moment, even in microscale biogeochemical models, SOM spatial distribution is not described from soil imaging, but is prescribed assuming either homogeneous or heterogeneous distributions (Pot et al., 2021).
Organic matter can be observed in soil at different resolutions (Fig. 1 and 2); first with optical microscopy on the basis of its shape and colour. It is possible to visualise organic matter after staining, as in a recent study of Merino et al. (2021), who used confocal laser micro-scopy to measure the decomposition of lignin that was stained with safranin-O.

With electron microscopy, organic matter can be identified using shape criteria, in the case of SEM and TEM, and by electron density in the case of TEM. It is also possible to stain SOM using heavy metals in order to amplify the contrast with the rest of the matrix (Foster, 1988; Elsass et al., 2008; Chenu et al., 2015) (Fig. 2). The use of electron microscopy coupled 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 μm 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; Hobley 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 5x5 to 50x50 μm),

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 μm 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}C or ^{15}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}C/^{12}C abundance of soil organic matter, which was found to be hetero-

475 geneous, at a 10μm 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, μX-ray fluorescence (μXRF) 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_2 distributions in soil*

507

As oxygen consumption and carbon dioxide emissions are directly related to the respiratory activity of soil organisms (Kuzyakov and Blagodatskaya, 2015), they are often monitored quantitatively in the atmosphere around the soil sample but are rarely monitored at the microhabitat scale. Methods are being developed to visualise the distribution of partial gas pressures in soils using probes, called needle-type or planar optodes (Santner et al., 2015) (Table 4). The former are needles, often a set, pinched in the soil with which gas partial pressures are measured (Elberling et al., 2011) ; it can bring additional image information if combined with other methods like X-ray computed tomography (Rohe et al., 2021). There are different types of planar optodes, but they generally involve a combination of gels and optical sensors (Pedersen et al., 2015). They are based on the use of fluorochromes that are sensitive to the presence of a gas, which acts as an exciter or an extinguisher of fluorescence, causing variations in brightness which is recorded by a camera (Rudolph et al., 2012; Pedersen et al., 2015). These fluorescent chemical probes are very sensitive and the reactions are reversible (Rudolph et al., 2012). The associated resolutions vary depending on the cameras used and the experimental device (Santner et al., 2015), but resolutions of a few tens of μm have already been reached (Larsen et al., 2011; Pedersen et al., 2015).

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

3.2.3. Water distribution in soil

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

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

Neutron computed tomography is based on the absorption of neutrons by the sample, providing three-dimensional images similar to X-ray CT images (Koliji et al., 2010). It has been used by Carminati et al. (2007) to study water flows in soil. Furthermore, neutron radiography, in which neutrons transmitted through a sample are analysed, also gives information on the distribution of water, but in two dimensions and only at resolution of a few tens of μm (Carminati et al., 2010; Rudolph et al., 2012) (Fig. 7). Recently, fast neutron tomography has been used to visualise and monitor in 3D the root uptake of water, as 3D images can be acquired in few seconds (Tötzke et al., 2021), and determine which roots are preferentially at the origin of water uptake in soil.

The resolution of these methods explains why they are mainly used at the rhizosphere scale and are not adapted for studies at the microbial scale. Finally, X-ray CT, delivering greyscale images that are characteristic of the different phases of soil, including water, can also provide information on the spatial distribution of water. A segmentation treatment makes it possible to distinguish water from soil, in 3D and with a resolution which may be less than a mm (Landis and Keane, 2010). As with the mapping of organic matter, a number of studies have used contrast agents (heavy elements dissolved in water like $CdSO_4$, KI or $AgNO_3$) in order to increase the water signal (Van Loo et al., 2014). Others have attempted to combine images of a dry and wet soil to subtract the "dry soil" signal from the "wet soil" signal in order to isolate the "water" signal (Tracy et al., 2015). However, this approach is not suitable for clay soils, because of the risk of shrinking and swelling of clays with soil moisture changes (Baveye et al., 2018).

3.2.4. Other soil chemical characteristics

Planar optodes, described in section 3.2.2., also allow for the visualisation of other molecules such as NH_4^+ or PO_4^{3-} (Pedersen et al., 2015), and for the mapping of pH and redox potential (Eh) (Pedersen et al., 2015; Roose et al., 2016). Reagents sensitive to acid-base variations have, for example, been used by Rudolph-Mohr et al. (2015) to describe pH variations within a soil after the addition of pesticides.

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

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

4.1. Physiological state of soil microorganisms

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

These different physiological states can be directly or indirectly observed *in situ* by microscopy using fluorescent markers targeting microbial nucleic acids or proteins. Specific markers, such as Fluorescein Diacetate (FDA), 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC), make the direct visualisation of active microorganisms possible because they are subjected to enzymatic oxidation or hydrolysis by active cells (Table 5).
Recently, a promising method for identifying the active fraction of microorganisms *in situ*, called bioorthogonal non-canonical amino acid tagging (BONCAT), has been introduced to soil science (Couradeau et al., 2019). The method is based upon the incorporation *of* an ad-

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

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

These methods generally allow for a good estimate of dead, active and dormant microorganisms (Blagodatskaya and Kuzyakov, 2013) but present some limitations. For example, intact dead cells are not labelled by PI (Maraha et al., 2004). These labelling techniques also present limits, in particular within a soil matrix, in which, in addition to unspecific staining (Li et al., 2004), accessibility may be limited for the marker to reach its target.

4.2. Enzymatic activities

Finally, a third way to characterise the activity of microorganisms *in situ* is to visualise their production of extracellular enzymes. Zymography has been developed quite recently (Pedersen et al., 2015) and is used by only a limited number of soil research teams (Spohn and Kuzyakov, 2013; Spohn et al., 2013; Spohn and Kuzyakov, 2014; Razavi et al., 2016; Sanaullah et al., 2016; Ma et al., 2017; Guber et al., 2018; Bilyera et al., 2020). Zymography produces images of the spatial distribution of enzymes on the surface of a soil sample. A gel or membrane containing a substrate that changes colour when it comes into contact and reacts with a specific enzyme is placed on the surface of a soil sample. Colour zones signal the presence of the targeted enzyme in the sample (Guber et al., 2018; Razavi et al., 2019). The resolution of zymography (~ tens of μm and often used at the mm scale) is often lower than the

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

There are few examples of correlative imaging using zymography with other methods, even if it has been used with X-ray tomography and ^{14}C imaging (Kravchenko et al., 2019; Becker and Holz, 2021). However, this technique has enabled the identification of hotspots of activity of numerous enzymes (β-glucosidase, α-glucosidase, xylanase, phosphatase, chitinase, peroxidase etc.) (Kuzyakov and Blagodatskaya, 2015; Heitkötter and Marschner, 2018; Razavi et al., 2019).

4.3. Assimilation of substrates

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

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

Pinus roots to mycorrhizae in rhizoboxes with ^{14}C (Leake et al., 2001), ^{32}P (Lindahl et al., 1999) and ^{33}P (Wu, 2014), and to locate methanotrophs assimilating ^{14}C-CH_4 according to soil depth and aggregates size in an afforestation chronosequence on subalpine pasture (Karbin et al., 2017). Autoradiography can be applied at much smaller scales when combined with electron microscopy, i.e. micro-autoradiography, but this has seldom been used with soils (Lee et al., 1999; Rogers et al., 2007). Radioisotope based methods are very sensitive, meaning that low substrate concentrations can be used, but they demand specific safety procedures, unlike stable isotopes.

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

No technique can visualise microorganisms and their activities in 3D at present. However, positron emission tomography (PET) is a promising approach, as it can locate isotopes in 3D in a sample. Garbout et al. (2012) used this method to visualise the assimilation of ^{11}C-CO_2 by a plant. However, as ^{11}C is very unstable (half-life of 20.4 min), the observation time was short. Although commonly used in the medical sciences field, this technique, which does not require any particular sample preparation, is nearly un-used in soil science. In addition, the resolutions obtained so far are very low, i.e. in the order of a mm, which mean that this methodology not really suitable for investigating microorganisms in soils.

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

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

5. Methodological challenges and solutions

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

5.1. Sample preparation methods

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

5.1.1. Fixation and dehydratation

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

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

5.1.2. Staining

Stains (fluorochromes or contrast agents) used in particular with epi-fluorescence, confocal microscopy and electron microscopy for observing microorganisms may have limited efficiency. Many stains, and particularly in undisturbed soil, may have difficulty penetrating 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 (< 1μm topography for natural abundance and 30 μm 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

5.3. Image representativeness

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

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

5.4. Combination of observation methods

5.4.1. Compatibility of the different methods

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

Indeed, the methods previously described require specific preparations and are more or less destructive, such as SEM or SIMS, which require the samples to be covered with a gold layer or which sputter the sample, respectively. These methods should therefore be implemented in the final stages of observation. Other methods such as tomography or zymography 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 pluggin developed for imageJ, help correlating images

898 from different methods (Bandara et al., 2021; Rohde et al., 2020).

899

5.4.2. Upscaling issues

901

902   Focusing on microorganisms in their habitat results in considering a very small field

903 of observation compared to the global functioning of the soil and raises the question of the up-

904 scaling. How can we account for these observations on larger scales? Are microscale observa-

905 tions representative of the phenomena described on a larger scale? Some studies recommend

906 the creation of mosaics of high-resolution images in order to obtain a wide image of the

907 samples. Others advice to work with an average volume which consists of averaging micro-

908 scopic description into a Representative Elementary Volume (Baveye et al., 2018) or a homo-

909 genisation which assumes that structure is sufficiently periodic to be considered as composed

910 of repeated units for variables as porous structure (Roose et al., 2016). The use of these op-

911 tions is still under discussion considering the trade-off between their potential and the consid-

912 erable loss of data that they can involve (Baveye et al., 2018).

913   The combination of observation methods can also address this problem with comple-

914 mentary observations at different spatial scales. Such studies have already been carried out,

915 such as that of Schlüter et al. (2019), who used correlative imaging with X-ray tomography at

916 a centimetre scale, optical microscopy and fluorescence at a mm scale, then electron micro-

917 scopy on the scale of a few hundred microns and finally nanoSIMS on the tens of microns

918 scale in order to describe the microhabitat in a decaying leaf detritusphere combining struc-

919 tural, geochemical and biological data. They showed that if bacteria were mainly present in

920 pores < 10 μm, they were preferentially concentrated near macropores and organic matter.

921 Juyal et al. (2019, 2020) also combined observations at mm (X-ray tomography, light and

922 fluorescence microscopy) and micrometric (light and fluorescence microscopy) scales to

923 study the localisation and spread of microorganisms in the soil structure and in particular as a

924 function of porosity indicators. Keiluweit et al. (2012) characterised the soil-microorganisms

925 interaction by combining nanoSIMS and NEXAFS with STMX allowing them to propose a

926 conceptual model of the fate and transformation of fungal cell wall compounds in soil and its

927 relation with mineral particles. Bandara et al. (2021) acquired images allowing description of

928 the rhizosphere using six different imaging methods, i.e.. light microscopy, epifluorescence

929 microscopy, HIM microscopy, Tof-SIMS, nanoSIMS, SEM-BSE and -EDX and confocal Ra-

930 man spectroscopy.

931   2D (SEM-EDX) and 3D (X-ray CT) approaches may be combined to build 3D chem-

932 ical maps of soil samples based on a statistical approach such as that proposed by Hapca et al.

933 (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*
941

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. µX-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 (Turkowyd 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 (Turkowyd et al., 2016).

The SIM method uses a mask containing a pattern of lines regularly spaced with a frequency, orientation and phase known, rotating above the sample to achieve a controlled illumination of the fluorochrome. The exploitation of the "moiré" effect obtained by this approach is used to reconstruct the structure of the observed object at a resolution of approximately 80-100 nm. One advantage is that it does not require any staining (Turkowyd et al., 2016).

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

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

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

5.5.2. Dynamic observations

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

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

5.5.3. Precious experimental data for modelling

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

5.5.4. Imaging soils at the microscale for soil microbial ecology

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

different workflows providing valuable data and an overall better understanding of soil func-

tioning at the microscale and will present two exemples.

A first example concerns the detritusphere. In the detritusphere, soil moisture controls

the transfers of organic solutes, enzymes and microorganisms, the accessibility of microor-

ganisms to their substrates and modulates biodegradation (Védère et al., 2020). X-ray com-

puted tomography can allow to describe the organisation of the porosity in the vicinity of la-

belled plant residues and the porosity directly connected to it (distances to the residues, con-

nectivity, tortuosity, direction…). Following the pores description, thin sections can be pro-

duced on the same samples and the spatial distribution of microorganisms in the vicinity of

previously targeted porosity and at increasing distances from the residues could be assessed

using epifluorescence microscopy. Finally, nanoSIMS can allow to observe C and N transfers

from the residue to the soil and to locate microbial assimilation hotspots at this interface.

Combining several imaging methods can address questions such as the influence of plant

residues on soil porosity, whether soil moisture modifies the spatial distribution of microor-

ganisms decomposing plant residues and the interplay between mineralisation and stabiliza-

tion of carbon from these plant residues.

A second example addresses the possible influence of biochar addition of rhizosphere

functioning. Biochars are increasingly used to improve soil properties like water retention or

cation exchange capacity of soil. They present a high porosity that can be used by micro-

organisms as habitats. Rhizosphere development and functioning is influenced by biochar ad-

dition (Atkinson et al., 2010). Unfortunately no clear description of microorganisms spatial

distribution relative biochar particle exists. X-ray computed tomography can describe soil

porosity inside the rhizosphere artificially produced in a rhizobox. Then, the surface of the

box could be exposed to direct zymography in order to give information about enzymes diffu-

sion in the porosity. Once those measurements being done, thin sections of soil at the vicinity

of root and biochar particles could be prepared to localize microorganisms using epifluores-

cence microscopy. Such a workflow could address questions like: Is rhizosphere porosity in-

fluenced by biochar addition in soil? Do biochar particles provide new habitats for micro-

organisms and modify their spatial distribution? Are enzyme activities affected by biochar in

the rhizosphere?

6. Conclusion

1066

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

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1089

1090 **References**

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