Opportunities and limits in imaging microorganisms and their activities in soil microhabitats

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Abstract

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

However, soil imaging techniques tend to be under-used, possibly due to a lack of awareness of the methods or due to a lack of access to the relevant instruments. In recent years, new methods have been developed, and continuously improved, offering new possibilities to decipher and describe soil physical, chemical and biological features of the soil microhabitat in 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

SEM: Scanning electron microscopy
SHIM: scanning helium ion microscopy
SIM: Structured illumination microscopy
SIMS: Secondary ionization mass spectrometry
SMLM: Single molecule localisation microscopy
SOM: Soil organic matter
STED: Stimulated-emission depletion microscopy
STMX: Scanning transmission X-ray microscopy
TEM: Transmission electron microscopy
TOF: Time of flight
TXM: Transmission X-ray microscopy
VNIR: Visible and near infrared
XANES: X-ray absorption near edge structure
X-ray CT: X-ray computed tomography
XRF: -ay fluorescence

1. Introduction

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

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

Baveye et al., 2018; Chenu and Stotzky, 2002). The access to resources and energy, the availability of water or O_2, the pH that microbial cells are exposed to, the other organisms in proximity for example, can all have dramatic effects on the activity of microbial cells. The environmental properties and the biological neighbourhood that microbial cells experience depend the physical structure of the soil's solid and pore phases across scales, from the micro-environment scale to the scales at which fluxes are regulated. However, we have little or no information on how microbial cells are distributed within this complex and heterogeneous environment, nor do we know much about their interactions with the physical environment or population pressures that they are subjected to. Our understanding of soil microbial functioning is therefore derived from measurements of means and gross trends (Vos et al., 2013) and, in the final analysis, is not built on a solid mechanistic foundation. It is clear that an exhaustive mapping soil microbes would be an arduous and, in view of the temporal variability inherent to soil conditions, futile endeavour. However, a clearer picture of the range of interactions that occur in soil, their prevalence and the effects they can have on microbial functioning would allow us to build models with sounder mechanistic basis. Ultimately, this can only be achieved after studying the spatial relations of soil's constituent parts, as spatial proximity is a strong modulator of the interactions that can occur.

New models, that account for microscale soil functioning, have begun to appear in recent years (Pot et al., 2015; Tecon and Or, 2017; Portell et al., 2018; Wilmoth et al., 2018; Kemgue et al., 2019). The need for data at relevant spatial scales, such as the physical structure and chemical characteristics of soil and the spatial distribution of microorganisms and their activities, for parametrising such models, is paramount if useful information is to be acquired from microscale modelling approaches (Baveye et al., 2018). In particular, visualising soil microorganisms in undisturbed soil samples is essential for understanding how they interact with their local environment, such as the local conditions experienced, their access to trophic resources or their interactions with other microorganisms, and the consequences these interactions have for soil functioning.

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

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

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

Despite the technological progress, imaging methods are still under-utilised in soil sciences because the scientific community is not always aware of them, and due to the fact that they are time consuming, expensive and not always easy to implement. Furthermore, several visualisation methods require access to instruments that are not widely available (e.g. NEXAFS, nanoSIMS). Many of the imaging methods that are useful for visualising microbial communities in their habitat have extremely small fields of view (e.g. the area covered by a nanoSIMS image is approximately 30 x 30 μm^2). The questions then arise, should targetted or random sampling be used (Brus, 2019) and what of the representativeness of the images acquired? Targetted imaging is useful for determining the types of situations that can be encountered, but there is a risk that it will provide a biased view as the areas of interest chosen may not reflect the soil more generally. In order to obtain a representative view, a degree of random sampling is required, though this can be reduced with a judicious use of spatial modelling (Brus, 2019). Whilst sampling considerations are clearly a drawback of these approaches, their exceptional analytical power means that they deserve more attention.

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

(iv) in situ information on the activities of microorganisms can be acquired. We systematically specify the spatial resolution of the methods, the preparation techniques and quantification possibilities. We address the limits of the different methods, the possibilities of combining them and discuss perspectives in the field.

2. Visualising microorganisms in their habitats

2.1. Localising microorganisms

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

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

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

Epi-fluorescence microscopy, combined with the use of stains, i.e. fluorochromes, makes it possible to distinguish the targeted organisms from the background and therefore locate and enumerate bacteria (Fig. 1a) (Fisk et al., 1998; Nunan et al., 2001; Juyal et al., 2020) and fungi (Baschien et al., 2001) in 2D. The staining needed to visualise microorganisms 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

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

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

3. Characteristics of microbial microhabitats

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

3.1. Physical characteristics of microhabitats

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

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

Schlüter et al. (2019b) incubated sand-based microcosms in which they placed microbial 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 microscopy 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

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

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

Fourier transform infrared micro-spectroscopy (micro-FTIR) can be used to characterise, quantify and locate organic molecules based on the bonds of functional groups (Singh and Gräfe, 2010). However, the resolution is generally low (Table 3). Several infrared spectro-microscopy methods using different wavelengths such as the visible and near infrared (VNIR) have enabled, using predictive models, the acquisition of images with a 53 µm resolution on dried soil, with detailed estimations of the organic carbon spatial distribution in a soil as a function of depth (Fig. 4) (Steffens and Buddenbaum, 2013; Hobley et al., 2018). This method requires almost no prior preparation except drying samples and ultrathin sectioning to 200nm using a cryo microtome (Hernandez-Soriano et al., 2018; Weng et al., 2021). Other micro-spectroscopy methods are still little used in soil science, such as Raman spectrometry with which organic matter can be characterised based on vibrational and rotational mo-

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

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

Elementary maps of samples can be established at high resolutions using Secondary Ion Mass Spectrometry (Bandara et al., 2021; Remusat et al., 2012; Schurig et al., 2015). There are several types of Secondary Ion Mass Spectrometry (SIMS): static SIMS or Time of flight SIMS (TOF-SIMS) and dynamic SIMS, CAMECA or nanoSIMS (Myrold et al., 2011). A primary ion beam (usually Cs^+ for samples with organic matter, or O^-) is used to sputter the sample surface and release secondary ions, which are collected, separated and analysed. These secondary ions are characterised using a mass spectrometer for dynamic SIMS and a time-of-flight mass spectrometer for static SIMS (Myrold et al., 2011). Scanning the sample gives access to elementary and isotopic map of the soil sample at very fine resolutions, i.e., less than 100 nm (Herrmann et al., 2007b). However, as with many recent techniques, SIMS suffer from heavy technical constraints, particularly in sample preparation: the samples must be dry, stable, conductive, flat and resistant to a very high vacuum (Herrmann et al., 2007b). Hence, samples are usually embedded in a carbon containing epoxy resin which contributes to a background signal that has to be removed (Mueller et al., 2013). In addition, the method is relatively destructive since the ion beam sputters the sample's surface. Quantitative elemental analyses are complicated to obtain because adequate standards are needed (Mueller et al., 2013). Finally, the observed field with nanoSIMS is very small (from 5x5 to 50x50 µm),

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

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

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

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

Micro-spectroscopy, µX-ray fluorescence (µXRF) tomography, where X-ray induce the reemission of a X-ray fluorescent radiation from the sample, is a promising method which provides information about the chemistry of 2D (Schlüter et al., 2022) or 3D samples. Its use is very rare in 3D, in particular because improvements still have to be made to circumvent problems of fluorescence attenuation in soils (Feng et al., 2021; Hapca et al., 2015; Roose et al., 2016). Fluorescent light can be absorbed by the sample, in particular in the case of thick samples, thus attenuating the fluorescent signal. It has already be used in 3D to analyse diatom 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

671 *Pinus* roots to mycorrhizae in rhizoboxes with ^{14}C (Leake et al., 2001), ^{32}P (Lindahl et al.,
672 1999) and ^{33}P (Wu, 2014), and to locate methanotrophs assimilating ^{14}C-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}N and ^{13}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}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}C-
693 CO_2 by a plant. However, as ^{11}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-

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-

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

5.1.3. Embedding

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

In addition, the choice of embedding resin should suit the study objectives. For example, nanoSIMS requires the use of special resins such as Aradite-502 (Mueller et al., 2012; Vidal et al., 2018) for their resistance to the applied pressures (Herrmann et al., 2007a; Mueller et al., 2012). Any study targeting the localisation and characterisation of soil carbon requires the use of resins that can be differentiated from the sample (Mueller et al., 2012; Vidal et al., 2018; Vergara Sosa et al., 2021). For the specific case of nanoSIMS, even though most samples are embedded, direct deposition of samples is also possible as long as they are very flat (< 1µm topography for natural abundance and 30 µm for stable isotopes enriched samples) (Mueller et al., 2013).

5.2. Image acquisition and processing

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

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

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

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

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

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

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

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

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

900 *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,

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

5.5. Perspectives

5.5.1. Methodological developments. Methods of the future.

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

Other methods, however, are underused, even though they would be appropriate for investigating soil microorganisms in their habitats. Super-resolution methods which give access to unprecedented resolutions in optical microscopy could be used, e.g. to study microorganisms and soil particles interactions. Super resolution techniques such as STED (stimulated-emission depletion microscopy), SIM (structured illumination microscopy) or SMLM (single molecule localisation microscopy) have resolutions down to a few tens of nanometers (Turkowyd et al., 2016), but have not yet been used to visualise soil microorganisms. With STED, the sample is scanned using two lasers. The first one stimulates the emission of fluorescence subjected to diffraction on a targeted zone and the second, in the form of a "donut", uses a de-excitation beam and comes to switch off a part of the emission of the fluorochrome leaving only its central emission source. This process counterbalances the effects of diffraction and means that resolutions of between 20-70 nm can be achieved. As this method is based on activation and successive repeated extinction of fluorochromes, all fluorochromes 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 functioning 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 microorganisms to their substrates and modulates biodegradation (Védère et al., 2020). X-ray computed tomography can allow to describe the organisation of the porosity in the vicinity of labelled plant residues and the porosity directly connected to it (distances to the residues, connectivity, tortuosity, direction…). Following the pores description, thin sections can be produced 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 microorganisms decomposing plant residues and the interplay between mineralisation and stabilization 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 microorganisms as habitats. Rhizosphere development and functioning is influenced by biochar addition (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 diffusion 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 epifluorescence microscopy. Such a workflow could address questions like: Is rhizosphere porosity influenced by biochar addition in soil? Do biochar particles provide new habitats for microorganisms and modify their spatial distribution? Are enzyme activities affected by biochar in the rhizosphere?

6. Conclusion

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

Conflict of interest

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

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References

Alexander, M., 1964. Biochemical Ecology of Soil Microorganisms. Annu. Rev. Microbiol. 18, 217–250. https://doi.org/10.1146/annurev.mi.18.100164.001245

Altemüller, H.-J., Van Vliet-Lanoe, B., 1990. Soil thin section fluorescence microscopy. Dev. Soil Sci. 19, 565–579.

Anderson, T.I., Vega, B., Kovscek, A.R., 2020. Multimodal imaging and machine learning to enhance microscope images of shale. Comput. Geosci. 145, 104593. https://doi.org/10.1016/j.cageo.2020.104593

Antipova, O., Kemner, K., Roehrig, C., Vogt, S., Li, L.X., Gursoy, D., 2018. Developments of X-ray Fluorescence Tomography at 2-ID-E at APS for Studies of Composite Samples. Microsc. Microanal. 24, 520–521. https://doi.org/10.1017/S1431927618014824

Arai, M., Uramoto, G.-I., Asano, M., Uematsu, K., Uesugi, K., Takeuchi, A., Morono, Y., Wagai, R., 2019. An improved method to identify osmium-stained organic matter within soil aggregate structure by electron microscopy and synchrotron X-ray micro-computed tomography. Soil Tillage Res. 191, 275–281. https://doi.org/10.1016/j.still.2019.04.010

Atkinson, C.J., Fitzgerald, J.D., Hipps, N.A., 2010. Potential mechanisms for achieving agricultural benefits from biochar application to temperate soils: a review. Plant Soil 337, 1–18. https://doi.org/10.1007/s11104-010-0464-5

Bandara, C.D., Schmidt, M., Davoudpour, Y., Stryhanyuk, H., Richnow, H.H., Musat, N., 2021. Microbial Identification, High-Resolution Microscopy and Spectrometry of the Rhizosphere in Its Native Spatial Context. Front. Plant Sci. 12, 1–18. https://doi.org/10.3389/fpls.2021.668929

Baschien, C., Manz, W., Neu, T.R., Szewzyk, U., 2001. Fluorescence in situ hybridization of freshwater fungi. Intern. Rev Hydrobiol 86, 371–381.

Baveye, P.C., Laba, M., Otten, W., Bouckaert, L., Dello Sterpaio, P., Goswami, R.R., Grinev, D., Houston, A., Hu, Y., Liu, J., Mooney, S., Pajor, R., Sleutel, S., Tarquis, A., Wang, W., Wei, Q., Sezgin, M., 2010. Observer-dependent variability of the thresholding step in the quantitative analysis of soil images and X-ray microtomography data. Geoderma 157, 51–63. https://doi.org/10.1016/j.geoderma.2010.03.015

Baveye, P.C., Otten, W., Kravchenko, A., Balseiro-Romero, M., Beckers, É., Chalhoub, M., Darnault, C., Eickhorst, T., Garnier, P., Hapca, S., Kiranyaz, S., Monga, O., Mueller, C.W., Nunan, N., Pot, V., Schlüter, S., Schmidt, H., Vogel, H.-J., 2018. Emergent Properties of Microbial Activity in Heterogeneous Soil Microenvironments: Different Research Approaches Are Slowly Converging, Yet Major Challenges Remain. Front. Microbiol. 9, 1929. https://doi.org/10.3389/fmicb.2018.01929

Becker, J.N., Holz, M., 2021. Hot or not? Connecting rhizosphere hotspots to total soil respiration. Plant Soil 464, 489–499. https://doi.org/10.1007/s11104-021-04963-4

Berg, S., Ott, H., Klapp, S.A., Schwing, A., Neiteler, R., Brussee, N., Makurat, A., Leu, L., Enzmann, F., Schwarz, J.-O., Kersten, M., Irvine, S., Stampanoni, M., 2013. Real-time 3D imaging of Haines jumps in porous media flow. Proc. Natl. Acad. Sci. 110, 3755–3759. https://doi.org/10.1073/pnas.1221373110

Berleman, J.E., Zemla, M., Remis, J.P., Liu, H., Davis, A.E., Worth, A.N., West, Z., Zhang, A., Park, H., Bosneaga, E., others, 2016. Exopolysaccharide microchannels direct bacterial motility and organize multicellular behavior. ISME J. 10, 2620–2632.

Bertola, M., Mattarozzi, M., Sanangelantoni, A.M., Careri, M., Visioli, G., 2019. PGPB Colonizing Three-Year Biochar-Amended Soil: Towards Biochar-Mediated Biofertilization. J. Soil Sci. Plant Nutr. 19, 841–850. https://doi.org/10.1007/s42729-019-00083-2

Bilyera, N., Kuzyakova, I., Guber, A., Razavi, B.S., Kuzyakov, Y., 2020. How “hot” are hotspots: Statistically localizing the high-activity areas on soil and rhizosphere images. Rhizosphere 16, 100259. https://doi.org/10.1016/j.rhisph.2020.100259

Binnig, G., Quate, C.F., Gerber, C., 1986. Atomic force microscope. Phys. Rev. Lett. 56, 930.

Blagodatskaya, E., Kuzyakov, Y., 2013. Active microorganisms in soil: Critical review of estimation criteria and approaches. Soil Biol. Biochem. 67, 192–211. https://doi.org/10.1016/j.soilbio.2013.08.024

Bleuet, P., Gergaud, P., Lemelle, L., Bleuet, P., Tucoulou, R., Cloetens, P., Susini, J., Delette, G., Simionovici, A., 2010. 3D chemical imaging based on a third-generation synchrotron source. TrAC Trends Anal. Chem. 29, 518–527. https://doi.org/10.1016/j.trac.2010.02.011

Borisov, S.M., Seifner, R., Klimant, I., 2011. A novel planar optical sensor for simultaneous monitoring of oxygen, carbon dioxide, pH and temperature. Anal. Bioanal. Chem. 400, 2463–2474. https://doi.org/10.1007/s00216-010-4617-4

Bouckaert, L., Van Loo, D., Ameloot, N., Buchan, D., Van Hoorebeke, L., Sleutel, S., 2013. Compatibility of X-ray micro-Computed Tomography with soil biological experiments. Soil Biol. Biochem. 56, 10–12. https://doi.org/10.1016/j.soilbio.2012.02.002

Bouvier, T., Del Giorgio, P.A., 2003. Factors influencing the detection of bacterial cells using fluorescence in situ hybridization (FISH): A quantitative review of published reports. FEMS Microbiol. Ecol. 44, 3–15. https://doi.org/10.1016/S0168-6496(02)00461-0

Brus, D.J., 2019. Sampling for digital soil mapping: A tutorial supported by R scripts. Geoderma 338, 464–480. https://doi.org/10.1016/j.geoderma.2018.07.036

Calvelo Pereira, R., Camps Arbestain, M., Kelliher, F.M., Theng, B.K.G., McNally, S.R., Macías, F., Guitián, F., 2019. Assessing the pore structure and surface area of allophane-rich and non-allophanic topsoils by supercritical drying and chemical treatment. Geoderma 337, 805–811. https://doi.org/10.1016/j.geoderma.2018.10.037

Cardinale, M., 2014. Scanning a microhabitat: plant-microbe interactions revealed by confocal laser microscopy. Front. Microbiol. 5. https://doi.org/10.3389/fmicb.2014.00094

Carminati, A., Kaestner, A., Ippisch, O., Koliji, A., Lehmann, P., Hassanein, R., Vontobel, P., Lehmann, E., Laloui, L., Vulliet, L., Flühler, H., 2007. Water flow between soil aggregates. Transp. Porous Media 68, 219–236. https://doi.org/10.1007/s11242-006-9041-z

Carminati, A., Moradi, A.B., Vetterlein, D., Vontobel, P., Lehmann, E., Weller, U., Vogel, H.-J., Oswald, S.E., 2010. Dynamics of soil water content in the rhizosphere. Plant Soil 332, 163–176. https://doi.org/10.1007/s11104-010-0283-8

Chen, M.-Y., Lee, D.-J., Tay, J.-H., Show, K.-Y., 2007. Staining of extracellular polymeric substances and cells in bioaggregates. Appl. Microbiol. Biotechnol. 75, 467–474. https://doi.org/10.1007/s00253-006-0816-5

Chenu, C., Hassink, J., Bloem, J., 2001. Short-term changes in the spatial distribution of microorganisms in soil aggregates as affected by glucose addition. Biol. Fertil. Soils 34, 349–356. https://doi.org/10.1007/s003740100419

Chenu, C., Jaunet, A.M., 1992. Cryoscanning electron microscopy of microbial extracellular polysaccharides and their association with minerals. Scanning 14, 360–364.

Chenu, C., Plante, A.F., 2006. Clay-sized organo-mineral complexes in a cultivation chronosequence: revisiting the concept of the “primary organo-mineral complex.” Eur. J. Soil Sci. 57, 596–607. https://doi.org/10.1111/j.1365-2389.2006.00834.x

Chenu, C., Rumpel, C., Lehmann, J., 2015. Methods for Studying Soil Organic Matter, in: Soil Microbiology, Ecology and Biochemistry. Elsevier, pp. 383–419. https://doi.org/10.1016/B978-0-12-415955-6.00013-X

Chenu, C., Stotzky, G., 2002. Interaction between microorganisms and soil particles: an overview, in: Huang, P.M., Bollag, P.M., Senesi, N. (Eds.), Interaction between Soil Particles and Microorganisms. Impact on the Terrestrial Ecosystem, IUPAC Series on Analytical and Physical Chemistry of Environmental Systems. John Wiley & Sons, New York, pp. 3–40.

Chenu, C., Tessier, D., 1995. Low temperature scanning electron microscopy of clay and organic constituents and their relevance to soil microstructures. Scanning Microsc. 9, 989–1010.

Cliff, J.B., Gaspar, D.J., Bottomley, P.J., Myrold, D.D., 2002. Exploration of Inorganic C and N Assimilation by Soil Microbes with Time-of-Flight Secondary Ion Mass Spectrometry. Appl. Environ. Microbiol. 68, 4067–4073. https://doi.org/10.1128/AEM.68.8.4067-4073.2002

Couradeau, E., Sasse, J., Goudeau, D., Nath, N., Hazen, T.C., Bowen, B.P., Chakraborty, R., Malmstrom, R.R., Northen, T.R., 2019. Probing the active fraction of soil microbiomes using BONCAT-FACS. Nat. Commun. 10, 2770. https://doi.org/10.1038/s41467-019-10542-0

Dal Cortivo, C., Barion, G., Visioli, G., Mattarozzi, M., Mosca, G., Vamerali, T., 2017. Increased root growth and nitrogen accumulation in common wheat following PGPR inoculation: Assessment of plant-microbe interactions by ESEM. Agric. Ecosyst. Environ. 247, 396–408. https://doi.org/10.1016/j.agee.2017.07.006

1200 Desbois, G., Urai, J.L., Houben, M.E., Sholokhova, Y., 2010. Typology, morphology and connectivity of
1201 pore space in claystones from reference site for research using BIB, FIB and cryo-SEM
1202 methods. EPJ Web Conf. 6, 22005. https://doi.org/10.1051/epjconf/20100622005
1203 Eickhorst, T., Tippkötter, R., 2008a. Detection of microorganisms in undisturbed soil by combining
1204 fluorescence in situ hybridization (FISH) and micropedological methods. Soil Biol. Biochem.
1205 40, 1284–1293. https://doi.org/10.1016/j.soilbio.2007.06.019
1206 Eickhorst, T., Tippkötter, R., 2008b. Improved detection of soil microorganisms using fluorescence in
1207 situ hybridization (FISH) and catalyzed reporter deposition (CARD-FISH). Soil Biol. Biochem.
1208 40, 1883–1891. https://doi.org/10.1016/j.soilbio.2008.03.024
1209 Elberling, B., Askaer, L., Jørgensen, C.J., Joensen, H.P., Kühl, M., Glud, R.N., Lauritsen, F.R., 2011.
1210 Linking Soil O_2, CO_2, and CH_4 Concentrations in a Wetland Soil: Implications for CO_2 and CH_4
1211 Fluxes. Environ. Sci. Technol. 45, 3393–3399. https://doi.org/10.1021/es103540k
1212 Elsass, F., Chenu, C., Tessier, D., 2008. Transmission Electron Microscopy for Soil Samples:
1213 Preparation Methods and Use, in: Ulery, A.L., Richard Drees, L. (Eds.), Methods of Soil
1214 Analysis Part 5—Mineralogical Methods. American Society of Agronomy and Soil Science
1215 Society of America, Madison, WI, USA, pp. 235–268.
1216 https://doi.org/10.2136/sssabookser5.5.c9
1217 Elyeznasni, N., Sellami, F., Pot, V., Benoit, P., Vieublé-Gonod, L., Young, I., Peth, S., 2012. Exploration
1218 of soil micromorphology to identify coarse-sized OM assemblages in X-ray CT images of
1219 undisturbed cultivated soil cores. Geoderma 179–180, 38–45.
1220 https://doi.org/10.1016/j.geoderma.2012.02.023
1221 Falconer, R.E., Battaia, G., Schmidt, S., Baveye, P., Chenu, C., Otten, W., 2015. Microscale
1222 Heterogeneity Explains Experimental Variability and Non-Linearity in Soil Organic Matter
1223 Mineralisation. PLOS ONE 10, e0123774. https://doi.org/10.1371/journal.pone.0123774
1224 Feng, X., Zhang, H., Yu, P., 2021. X-ray fluorescence application in food, feed, and agricultural science:
1225 a critical review. Crit. Rev. Food Sci. Nutr. 61, 2340–2350.
1226 https://doi.org/10.1080/10408398.2020.1776677
1227 Fisk, A.C., Murphy, S.L., Tate III, R.L., 1998. Microscopic observations of bacterial sorption in soil
1228 cores. Biol. Fertil. Soils 28, 111–116. https://doi.org/10.1007/s003740050470
1229 Foster, R.C., 1993. The ultramicromorphology of soil biota in situ in natural soils: a review, in:
1230 Developments in Soil Science. Elsevier, pp. 381–393. https://doi.org/10.1016/S0166-
1231 2481(08)70427-6
1232 Foster, R.C., 1988. Microenvironments of soil microorganisms. Biol. Fertil. Soils 6, 189–203.
1233 Frey, S.D., 2015. The Spatial Distribution of Soil Biota, in: Soil Microbiology, Ecology and
1234 Biochemistry. Eldor A. Paul, pp. 223–272.
1235 Gaboriaud, F., Dufrene, Y.F., 2007. Atomic force microscopy of microbial cells: Application to
1236 nanomechanical properties, surface forces and molecular recognition forces 10.
1237 Gaillard, V., Chenu, C., Recous, S., Richard, G., 1999. Carbon, nitrogen and microbial gradients
1238 induced by plant residues decomposing in soil. Eur. J. Soil Sci. 50, 567–578.
1239 Gao, W., Schlüter, S., Blaser, S.R.G.A., Shen, J., Vetterlein, D., 2019. A shape-based method for
1240 automatic and rapid segmentation of roots in soil from X-ray computed tomography images:
1241 Rootine. Plant Soil 441, 643–655. https://doi.org/10.1007/s11104-019-04053-6
1242 Garbout, A., Munkholm, L.J., Hansen, S.B., Petersen, B.M., Munk, O.L., Pajor, R., 2012. The use of
1243 PET/CT scanning technique for 3D visualization and quantification of real-time soil/plant
1244 interactions. Plant Soil 352, 113–127. https://doi.org/10.1007/s11104-011-0983-8
1245 Ghaderi, N., Guber, A., Schmidt, H., Blagodatskaya, E., 2020. Towards soil micro-zymography:
1246 comparison of staining and impregnation strategies (other). oral.
1247 https://doi.org/10.5194/egusphere-egu2020-5784
1248 Gleeson, D.B., Clipson, N., Melville, K., Gadd, G.M., McDermott, F.P., 2005. Characterization of Fungal
1249 Community Structure on a Weathered Pegmatitic Granite. Microb. Ecol. 50, 360–368.
1250 https://doi.org/10.1007/s00248-005-0198-8

Greuter, D., Loy, A., Horn, M., Rattei, T., 2016. probeBase—an online resource for rRNA-targeted oligonucleotide probes and primers: new features 2016. Nucleic Acids Res. 44, D586–D589. https://doi.org/10.1093/nar/gkv1232

Grundmann, G.L., 2004. Spatial scales of soil bacterial diversity – the size of a clone. FEMS Microbiol. Ecol. 48, 119–127. https://doi.org/10.1016/j.femsec.2004.01.010

Guber, A., Blagodatskaya, E., Juyal, A., Razavi, B.S., Kuzyakov, Y., Kravchenko, A., 2021. Time-lapse approach to correct deficiencies of 2D soil zymography. Soil Biol. Biochem. 157, 108225. https://doi.org/10.1016/j.soilbio.2021.108225

Guber, A., Kravchenko, A., Razavi, B.S., Uteau, D., Peth, S., Blagodatskaya, E., Kuzyakov, Y., 2018. Quantitative soil zymography: Mechanisms, processes of substrate and enzyme diffusion in porous media. Soil Biol. Biochem. 127, 156–167. https://doi.org/10.1016/j.soilbio.2018.09.030

Gutiérrez Castorena, E.V., Gutiérrez-Castorena, Ma. del C., González Vargas, T., Cajuste Bontemps, L., Delgadillo Martínez, J., Suástegui Méndez, E., Ortiz Solorio, C.A., 2016. Micromapping of microbial hotspots and biofilms from different crops using digital image mosaics of soil thin sections. Geoderma 279, 11–21. https://doi.org/10.1016/j.geoderma.2016.05.017

Hapca, S., Baveye, P.C., Wilson, C., Lark, R.M., Otten, W., 2015. Three-dimensional mapping of soil chemical characteristics at micrometric scale by combining 2D SEM-EDX data and 3D X-Ray CT images. PloS One 10, e0137205.

Hapca, S.M., Houston, A.N., Otten, W., Baveye, P.C., 2013. New Local Thresholding Method for Soil Images by Minimizing Grayscale Intra-Class Variance. Vadose Zone J. 12, 0. https://doi.org/10.2136/vzj2012.0172

Hatzenpichler, R., Scheller, S., Tavormina, P.L., Babin, B.M., Tirrell, D.A., Orphan, V.J., 2014. In situ visualization of newly synthesized proteins in environmental microbes using amino acid tagging and click chemistry. Environ. Microbiol. 16, 2568–2590. https://doi.org/10.1111/1462-2920.12436

Heitkötter, J., Marschner, B., 2018. Soil zymography as a powerful tool for exploring hotspots and substrate limitation in undisturbed subsoil. Soil Biol. Biochem. 124, 210–217. https://doi.org/10.1016/j.soilbio.2018.06.021

Helliwell, J.R., Miller, A.J., Whalley, W.R., Mooney, S.J., Sturrock, C.J., 2014. Quantifying the impact of microbes on soil structural development and behaviour in wet soils. Soil Biol. Biochem. 74, 138–147. https://doi.org/10.1016/j.soilbio.2014.03.009

Hemes, S., Desbois, G., Urai, J.L., Schröppel, B., Schwarz, J.-O., 2015. Multi-scale characterization of porosity in Boom Clay (HADES-level, Mol, Belgium) using a combination of X-ray µ-CT, 2D BIB-SEM and FIB-SEM tomography. Microporous Mesoporous Mater. 208, 1–20. https://doi.org/10.1016/j.micromeso.2015.01.022

Hernandez-Soriano, M.C., Dalal, R.C., Warren, F.J., Wang, P., Green, K., Tobin, M.J., Menzies, N.W., Kopittke, P.M., 2018. Soil Organic Carbon Stabilization: Mapping Carbon Speciation from Intact Microaggregates. Environ. Sci. Technol. 52, 12275–12284. https://doi.org/10.1021/acs.est.8b03095

Herrmann, A.M., Clode, P.L., Fletcher, I.R., Nunan, N., Stockdale, E.A., O'Donnell, A.G., Murphy, D.V., 2007a. A novel method for the study of the biophysical interface in soils using nano-scale secondary ion mass spectrometry. Rapid Commun. Mass Spectrom. 21, 29–34. https://doi.org/10.1002/rcm.2811

Herrmann, A.M., Ritz, K., Nunan, N., Clode, P.L., Pett-Ridge, J., Kilburn, M.R., Murphy, D.V., O'Donnell, A.G., Stockdale, E.A., 2007b. Nano-scale secondary ion mass spectrometry — A new analytical tool in biogeochemistry and soil ecology: A review article. Soil Biol. Biochem. 39, 1835–1850. https://doi.org/10.1016/j.soilbio.2007.03.011

Hobley, E., Steffens, M., Bauke, S.L., Kögel-Knabner, I., 2018. Hotspots of soil organic carbon storage revealed by laboratory hyperspectral imaging. Sci. Rep. 8. https://doi.org/10.1038/s41598-018-31776-w

Holman, H. ?Y. N., Martin, M.C., 2006. Synchrotron Radiation Infrared Spectromicroscopy: A Noninvasive Chemical Probe for Monitoring Biogeochemical Processes, in: Advances in Agronomy. Elsevier, pp. 79–127. https://doi.org/10.1016/S0065-2113(06)90003-0

Holz, M., Zarebanadkouki, M., Carminati, A., Kuzyakov, Y., 2019. Visualization and quantification of root exudation using 14C imaging: challenges and uncertainties. Plant Soil 437, 473–485. https://doi.org/10.1007/s11104-019-03956-8

Hoshino, T., Schramm, A., 2010. Detection of denitrification genes by in situ rolling circle amplification-fluorescence in situ hybridization to link metabolic potential with identity inside bacterial cells: In situ detection of denitrification genes by in situ RCA-FISH. Environ. Microbiol. 12, 2508–2517. https://doi.org/10.1111/j.1462-2920.2010.02224.x

Hota, S., 2021. Synchrotron based techniques in soil analysis: a modern approach, in: Ahmad, F. (Ed.), Technology in Agriculture.

Houben, M.E., Desbois, G., Urai, J.L., 2013. Pore morphology and distribution in the Shaly facies of Opalinus Clay (Mont Terri, Switzerland): Insights from representative 2D BIB–SEM investigations on mm to nm scale. Appl. Clay Sci. 71, 82–97. https://doi.org/10.1016/j.clay.2012.11.006

Houston, A.N., Schmidt, S., Tarquis, A.M., Otten, W., Baveye, P.C., Hapca, S.M., 2013. Effect of scanning and image reconstruction settings in X-ray computed microtomography on quality and segmentation of 3D soil images. Geoderma 207–208, 154–165. https://doi.org/10.1016/j.geoderma.2013.05.017

Huang, B., Bates, M., Zhuang, X., 2009. Super-Resolution Fluorescence Microscopy. Annu. Rev. Biochem. 78, 993–1016. https://doi.org/10.1146/annurev.biochem.77.061906.092014

Huang, Q., Wu, H., Cai, P., Fein, J.B., Chen, W., 2015. Atomic force microscopy measurements of bacterial adhesion and biofilm formation onto clay-sized particles. Sci. Rep. 5, 16857. https://doi.org/10.1038/srep16857

Iassonov, P., Gebrenegus, T., Tuller, M., 2009. Segmentation of X-ray computed tomography images of porous materials: A crucial step for characterization and quantitative analysis of pore structures: X-Ray CT image segmentation. Water Resour. Res. 45. https://doi.org/10.1029/2009WR008087

Jasinska, E., Wetzel, H., Baumgartl, T., Horn, R., 2006. Heterogeneity of Physico-Chemical Properties in Structured Soils and Its Consequences. Pedosphere 16, 284–296. https://doi.org/10.1016/S1002-0160(06)60054-4

Jeckel, H., Drescher, K., 2021. Advances and opportunities in image analysis of bacterial cells and communities. FEMS Microbiol. Rev. 45, fuaa062. https://doi.org/10.1093/femsre/fuaa062

Joens, M.S., Huynh, C., Kasuboski, J.M., Ferranti, D., Sigal, Y.J., Zeitvogel, F., Obst, M., Burkhardt, C.J., Curran, K.P., Chalasani, S.H., Stern, L.A., Goetze, B., Fitzpatrick, J.A.J., 2013. Helium Ion Microscopy (HIM) for the imaging of biological samples at sub-nanometer resolution. Sci. Rep. 3, 3514. https://doi.org/10.1038/srep03514

Joy, D.C., Pawley, J.B., 1992. High-resolution scanning electron microscopy. Ultramicroscopy 47, 80–100.

Juyal, A., Eickhorst, T., Falconer, R., Baveye, P.C., Spiers, A., Otten, W., 2018. Control of Pore Geometry in Soil Microcosms and Its Effect on the Growth and Spread of Pseudomonas and Bacillus sp. Front. Environ. Sci. 6, 73. https://doi.org/10.3389/fenvs.2018.00073

Juyal, A., Otten, W., Baveye, P.C., Eickhorst, T., 2020. Influence of soil structure on the spread of Pseudomonas fluorescens in soil at microscale. Eur. J. Soil Sci. 72, 141–153. https://doi.org/10.1111/ejss.12975

Juyal, A., Otten, W., Falconer, R., Hapca, S., Schmidt, H., Baveye, P.C., Eickhorst, T., 2019. Combination of techniques to quantify the distribution of bacteria in their soil microhabitats at different spatial scales. Geoderma 334, 165–174. https://doi.org/10.1016/j.geoderma.2018.07.031

Kaestner, A., Lehmann, E., Stampanoni, M., 2008. Imaging and image processing in porous media research. Adv. Water Resour. 31, 1174–1187. https://doi.org/10.1016/j.advwatres.2008.01.022

Karbin, S., Hagedorn, F., Hiltbrunner, D., Zimmermann, S., Niklaus, P.A., 2017. Spatial micro-distribution of methanotrophic activity along a 120-year afforestation chronosequence. Plant Soil 415, 13–23. https://doi.org/10.1007/s11104-016-3141-5

Karsanina, M.V., Gerke, K.M., Skvortsova, E.B., Ivanov, A.L., Mallants, D., 2018. Enhancing image resolution of soils by stochastic multiscale image fusion. Geoderma 314, 138–145. https://doi.org/10.1016/j.geoderma.2017.10.055

Keiluweit, M., Bougoure, J.J., Zeglin, L.H., Myrold, D.D., Weber, P.K., Pett-Ridge, J., Kleber, M., Nico, P.S., 2012. Nano-scale investigation of the association of microbial nitrogen residues with iron (hydr)oxides in a forest soil O-horizon. Geochim. Cosmochim. Acta 95, 213–226. https://doi.org/10.1016/j.gca.2012.07.001

Keiluweit, M., Wanzek, T., Kleber, M., Nico, P., Fendorf, S., 2017. Anaerobic microsites have an unaccounted role in soil carbon stabilization. Nat. Commun. 8, 1771. https://doi.org/10.1038/s41467-017-01406-6

Kemgue, A.T., Monga, O., Moto, S., Pot, V., Garnier, P., Baveye, P.C., Bouras, A., 2019. From spheres to ellipsoids: Speeding up considerably the morphological modeling of pore space and water retention in soils. Comput. Geosci. 123, 20–37. https://doi.org/10.1016/j.cageo.2018.11.006

Kenzaka, T., Ishidoshiro, A., Yamaguchi, N., Tani, K., Nasu, M., 2005. rRNA Sequence-Based Scanning Electron Microscopic Detection of Bacteria. Appl. Environ. Microbiol. 71, 5523–5531. https://doi.org/10.1128/AEM.71.9.5523-5531.2005

Kherlopian, A.R., Song, T., Duan, Q., Neimark, M.A., Po, M.J., Gohagan, J.K., Laine, A.F., 2008. A review of imaging techniques for systems biology. BMC Syst. Biol. 2, 74. https://doi.org/10.1186/1752-0509-2-74

Khosrozadeh, S., Guber, A., Kravchenko, A., Ghaderi, N., Blagodatskaya, E., 2022. Soil oxidoreductase zymography: Visualizing spatial distributions of peroxidase and phenol oxidase activities at the root-soil interface. Soil Biol. Biochem. 167, 108610. https://doi.org/10.1016/j.soilbio.2022.108610

Kim, H.J., Boedicker, J.Q., Choi, J.W., Ismagilov, R.F., 2008. Defined spatial structure stabilizes a synthetic multispecies bacterial community. Proc. Natl. Acad. Sci. 105, 18188–18193. https://doi.org/10.1073/pnas.0807935105

Kleber, M., Röner, J., Chenu, C., Glaser, B., Knicker, H., Jahn, R., 2003. Prehistoric alteration of soil properties in a central german chernozemic soil: in search of pedologic indicators for prehistoric activity. Soil Sci. 168, 292–306. https://doi.org/10.1097/01.ss.0000064892.94869.3a

Kobabe, S., Wagner, D., Pfeiffer, E.-M., 2004. Characterisation of microbial community composition of a Siberian tundra soil by fluorescence in situ hybridisation. FEMS Microbiol. Ecol. 50, 13–23. https://doi.org/10.1016/j.femsec.2004.05.003

Koliji, A., Vulliet, L., Laloui, L., 2010. Structural characterization of unsaturated aggregated soil. Can Geotech 47, 297–311.

Kravchenko, A.N., Guber, A.K., Razavi, B.S., Koestel, J., Blagodatskaya, E.V., Kuzyakov, Y., 2019. Spatial patterns of extracellular enzymes: Combining X-ray computed micro-tomography and 2D zymography. Soil Biol. Biochem. 135, 411–419. https://doi.org/10.1016/j.soilbio.2019.06.002

Kubota, K., 2013. CARD-FISH for Environmental Microorganisms: Technical Advancement and Future Applications. Microbes Environ. 28, 3–12. https://doi.org/10.1264/jsme2.ME12107

Kuzyakov, Y., Blagodatskaya, E., 2015. Microbial hotspots and hot moments in soil: Concept & review. Soil Biol. Biochem. 83, 184–199. https://doi.org/10.1016/j.soilbio.2015.01.025

Kuzyakov, Y., Mason-Jones, K., 2018. Viruses in soil: Nano-scale undead drivers of microbial life, biogeochemical turnover and ecosystem functions. Soil Biol. Biochem. 127, 305–317. https://doi.org/10.1016/j.soilbio.2018.09.032

Lammel, D.R., Arlt, T., Manke, I., Rillig, M.C., 2019. Testing Contrast Agents to Improve Micro Computerized Tomography (µCT) for Spatial Location of Organic Matter and Biological Material in Soil. Front. Environ. Sci. 7, 153. https://doi.org/10.3389/fenvs.2019.00153
Landis, E.N., Keane, D.T., 2010. X-ray microtomography. Mater. Charact. 61, 1305–1316. https://doi.org/10.1016/j.matchar.2010.09.012
Larsen, M., Borisov, S.M., Grunwald, B., Klimant, I., Glud, R.N., 2011. A simple and inexpensive high resolution color ratiometric planar optode imaging approach: application to oxygen and pH sensing. Limnol. Oceanogr. Methods 9, 348–360.
Leake, J.R., Donnelly, D.P., Saunders, E.M., Boddy, L., Read, D.J., 2001. Rates and quantities of carbon flux to ectomycorrhizal mycelium following 14C pulse labeling of Pinus sylvestris seedlings: effects of litter patches and interaction with a wood-decomposer fungus. Tree Physiol. 21, 71–82. https://doi.org/10.1093/treephys/21.2-3.71
Lee, J., Hestrin, R., Nuccio, E.E., Morrison, K.D., Ramon, C.E., Samo, T.J., Pett-Ridge, J., Ly, S.S., Laurence, T.A., Weber, P.K., 2022. Label-Free Multiphoton Imaging of Microbes in Root, Mineral, and Soil Matrices with Time-Gated Coherent Raman and Fluorescence Lifetime Imaging. Environ. Sci. Technol. 56, 1994–2008. https://doi.org/10.1021/acs.est.1c05818
Lee, N., Nielsen, P.H. er, Andreasen, K. er H., Juretschko, S., Nielsen, J.L., Schleifer, K.-H., Wagner, M., 1999. Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. 65, 1289–1297.
Lehmann, J., Hansel, C.M., Kaiser, C., Kleber, M., Maher, K., Manzoni, S., Nunan, N., Reichstein, M., Schimel, J.P., Torn, M.S., Wieder, W.R., Kögel-Knabner, I., 2020. Persistence of soil organic carbon caused by functional complexity. Nat. Geosci. 13, 529–534. https://doi.org/10.1038/s41561-020-0612-3
Lehmann, J., Liang, B., Solomon, D., Lerotic, M., Luizão, F., Kinyangi, J., Schäfer, T., Wirick, S., Jacobsen, C., 2005. Near-edge X-ray absorption fine structure (NEXAFS) spectroscopy for mapping nano-scale distribution of organic carbon forms in soil: Application to black carbon particles: carbon NEXAFS spectroscopy of black carbon particles. Glob. Biogeochem. Cycles 19. https://doi.org/10.1029/2004GB002435
Lehmann, J., Solomon, D., Kinyangi, J., Dathe, L., Wirick, S., Jacobsen, C., 2008. Spatial complexity of soil organic matter forms at nanometre scales. Nat. Geosci. 1, 238–242. https://doi.org/10.1038/ngeo155
Li, T., Wu, T.-D., Mazéas, L., Toffin, L., Guerquin-Kern, J.-L., Leblon, G., Bouchez, T., 2008. Simultaneous analysis of microbial identity and function using NanoSIMS. Environ. Microbiol. 10, 580–588. https://doi.org/10.1111/j.1462-2920.2007.01478.x
Li, Y., Tuovinen, O.H., Dick, W.A., 2004. Fluorescence microscopy for visualization of soil microorganisms: a review. Biol. Fertil. Soils 39, 301–311. https://doi.org/10.1007/s00374-004-0722-x
Li, Z., Liu, L., Lu, X., Zhao, L., Ji, J., Chen, J., 2021. Mineral foraging and etching by the fungus Talaromyces flavus to obtain structurally bound iron. Chem. Geol. 586, 120592. https://doi.org/10.1016/j.chemgeo.2021.120592
Lin, B., Cerato, A.B., 2014. Applications of SEM and ESEM in Microstructural Investigation of Shale-Weathered Expansive Soils along Swelling-Shrinkage Cycles. Eng. Geol. 177, 66–74. https://doi.org/10.1016/j.enggeo.2014.05.006
Lindahl, B., Stenlid, J., Olsson, S., Finlay, R., 1999. Translocation of 32P between interacting mycelia of a wood-decomposing fungus and ectomycorrhizal fungi in microcosm systems. New Phytol. 144, 183–193. https://doi.org/10.1046/j.1469-8137.1999.00502.x
Liu, K., Ke, Z., Chen, P., Zhu, S., Yin, H., Li, Z., Chen, Z., 2021. Classification of two species of Gram-positive bacteria through hyperspectral microscopy coupled with machine learning. Biomed. Opt. Express 12, 7906. https://doi.org/10.1364/BOE.445041
Liu, S., Razavi, B.S., Su, X., Maharjan, M., Zarebanadkouki, M., Blagodatskaya, E., Kuzyakov, Y., 2017. Spatio-temporal patterns of enzyme activities after manure application reflect mechanisms

of niche differentiation between plants and microorganisms. Soil Biol. Biochem. 112, 100–109. https://doi.org/10.1016/j.soilbio.2017.05.006
Lower, S.K., Hochella, M.F., Beveridge, T.J., 2001. Bacterial recognition of mineral surfaces: nanoscale interactions between shewanella and alpha-FeOOH. 2001 292, 1360–1363.
Lucas, M., Pihlap, E., Steffens, M., Vetterlein, D., Kögel-Knabner, I., 2020. Combination of Imaging Infrared Spectroscopy and X-ray Computed Microtomography for the Investigation of Bio- and Physicochemical Processes in Structured Soils. Front. Environ. Sci. 8, 42. https://doi.org/10.3389/fenvs.2020.00042
Ma, X., Razavi, B.S., Holz, M., Blagodatskaya, E., Kuzyakov, Y., 2017. Warming increases hotspot areas of enzyme activity and shortens the duration of hot moments in the root-detritusphere. Soil Biol. Biochem. 107, 226–233. https://doi.org/10.1016/j.soilbio.2017.01.009
Maenhout, P., De Neve, S., Wragg, J., Rawlins, B., De Pue, J., Van Hoorebeke, L., Cnudde, V., Sleutel, S., 2021. Chemical staining of particulate organic matter for improved contrast in soil X-ray µCT images. Sci. Rep. 11, 370. https://doi.org/10.1038/s41598-020-79681-5
Maraha, N., Backman, A., Jansson, J.K., 2004. Monitoring physiological status of GFP-tagged Pseudomonas fluorescens SBW25 under different nutrient conditions and in soil by flow cytometry. FEMS Microbiol. Ecol. 51, 123–132. https://doi.org/10.1016/j.femsec.2004.07.007
McMaster, T.J., 2012. Atomic Force Microscopy of the fungi–mineral interface: applications in mineral dissolution, weathering and biogeochemistry. Curr. Opin. Biotechnol. 23, 562–569. https://doi.org/10.1016/j.copbio.2012.05.006
Merino, C., Kuzyakov, Y., Godoy, K., Jofré, I., Nájera, F., Matus, F., 2021. Iron-reducing bacteria decompose lignin by electron transfer from soil organic matter. Sci. Total Environ. 761, 143194. https://doi.org/10.1016/j.scitotenv.2020.143194
Mizutani, R., Suzuki, Y., 2012. X-ray microtomography in biology. Micron 43, 104–115. https://doi.org/10.1016/j.micron.2011.10.002
Monga, O., Bousso, M., Garnier, P., Pot, V., 2008. 3D geometric structures and biological activity: Application to microbial soil organic matter decomposition in pore space. Ecol. Model. 216, 291–302. https://doi.org/10.1016/j.ecolmodel.2008.04.015
Mueller, C.W., Kölbl, A., Hoeschen, C., Hillion, F., Heister, K., Herrmann, A.M., Kögel-Knabner, I., 2012. Submicron scale imaging of soil organic matter dynamics using NanoSIMS – From single particles to intact aggregates. Org. Geochem. 42, 1476–1488. https://doi.org/10.1016/j.orggeochem.2011.06.003
Mueller, C.W., Weber, P.K., Kilburn, M.R., Hoeschen, C., Kleber, M., Pett-Ridge, J., 2013. Advances in the Analysis of Biogeochemical Interfaces, in: Advances in Agronomy. Elsevier, pp. 1–46. https://doi.org/10.1016/B978-0-12-407685-3.00001-3
Muggia, L., Klug, B., Berg, G., Grube, M., 2013. Localization of bacteria in lichens from Alpine soil crusts by fluorescence in situ hybridization. Appl. Soil Ecol. 68, 20–25. https://doi.org/10.1016/j.apsoil.2013.03.008
Musat, N., Foster, R., Vagner, T., Adam, B., Kuypers, M.M.M., 2012. Detecting metabolic activities in single cells, with emphasis on nanoSIMS. FEMS Microbiol. Rev. 36, 486–511. https://doi.org/10.1111/j.1574-6976.2011.00303.x
Myrold, D.D., Pett-Ridge, J., Bottomley, P.J., 2011. Nitrogen Mineralization and Assimilation at Millimeter Scales, in: Methods in Enzymology. Elsevier, pp. 91–114. https://doi.org/10.1016/B978-0-12-386489-5.00004-X
Nubel, U., Bateson, M.M., Vandieken, V., Wieland, A., Kuhl, M., Ward, D.M., 2002. Microscopic Examination of Distribution and Phenotypic Properties of Phylogenetically Diverse Chloroflexaceae-Related Bacteria in Hot Spring Microbial Mats. Appl. Environ. Microbiol. 68, 4593–4603. https://doi.org/10.1128/AEM.68.9.4593-4603.2002
Nunan, N., Ritz, K., Crabb, D., Harris, K., Wu, K., Crawford, J.W., Young, I.M., 2001. Quantification of the in situ distribution of soil bacteria by large-scale imaging of thin sections of undisturbed soil. FEMS Microbiol. Ecol. 36, 66–77.

Nunan, N., Wu, K., Young, I.M., Crawford, J.W., Ritz, K., 2002. In Situ Spatial Patterns of Soil Bacterial Populations, Mapped at Multiple Scales, in an Arable Soil. Microb. Ecol. 44, 296–305. https://doi.org/10.1007/s00248-002-2021-0

Otten, W., Gilligan, C.A., 1998. Effect of physical conditions on the spatial and temporal dynamics of the soil-borne fungal pathogen Rhizoctonia solani. New Phytol. 138, 629–637. https://doi.org/10.1046/j.1469-8137.1998.00154.x

Otten, W., Harris, K., Young, I.M., Ritz, K., Gilligan, C.A., 2004. Preferential spread of the pathogenic fungus Rhizoctonia solani through structured soil. Soil Biol. 8.

Ouverney, C.C., Fuhrman, J.A., 1999. Combined microautoradiography–16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl. Environ. Microbiol. 65, 1746–1752.

Pagel, H., Kriesche, B., Uksa, M., Poll, C., Kandeler, E., Schmidt, V., Streck, T., 2020. Spatial Control of Carbon Dynamics in Soil by Microbial Decomposer Communities. Front. Environ. Sci. 8, 2. https://doi.org/10.3389/fenvs.2020.00002

Pedersen, L.L., Smets, B.F., Dechesne, A., 2015. Measuring biogeochemical heterogeneity at the micro scale in soils and sediments. Soil Biol. Biochem. 90, 122–138. https://doi.org/10.1016/j.soilbio.2015.08.003

Peth, S., Chenu, C., Leblond, N., Mordhorst, A., Garnier, P., Nunan, N., Pot, V., Ogurreck, M., Beckmann, F., 2014. Localization of soil organic matter in soil aggregates using synchrotron-based X-ray microtomography. Soil Biol. Biochem. 78, 189–194. https://doi.org/10.1016/j.soilbio.2014.07.024

Pohlmeier, A., Oros-Peusquens, A., Javaux, M., Menzel, M.I., Vanderborght, J., Kaffanke, J., Romanzetti, S., Lindenmair, J., Vereecken, H., Shah, N.J., 2008. Changes in Soil Water Content Resulting from Root Uptake Monitored by Magnetic Resonance Imaging. Vadose Zone J. 7, 1010. https://doi.org/10.2136/vzj2007.0110

Portell, X., Pot, V., Garnier, P., Otten, W., Baveye, P.C., 2018. Microscale Heterogeneity of the Spatial Distribution of Organic Matter Can Promote Bacterial Biodiversity in Soils: Insights From Computer Simulations. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.01583

Possinger, A.R., Zachman, M.J., Enders, A., Levin, B.D.A., Muller, D.A., Kourkoutis, L.F., Lehmann, J., 2020. Organo–organic and organo–mineral interfaces in soil at the nanometer scale. Nat. Commun. 11, 6103. https://doi.org/10.1038/s41467-020-19792-9

Postma, J., Altemüller, H.-J., 1990. Bacteria in thin soil sections stained with the fluorescent brightener calcofluor white M2R. Soil Biol. Biochem. 22, 89–96. https://doi.org/10.1016/0038-0717(90)90065-8

Pot, V., Peth, S., Monga, O., Vogel, L.E., Genty, A., Garnier, P., Vieublé-Gonod, L., Ogurreck, M., Beckmann, F., Baveye, P.C., 2015. Three-dimensional distribution of water and air in soil pores: Comparison of two-phase two-relaxation-times lattice-Boltzmann and morphological model outputs with synchrotron X-ray computed tomography data. Adv. Water Resour. 84, 87–102. https://doi.org/10.1016/j.advwatres.2015.08.006

Pot, V., Portell, X., Otten, W., Garnier, P., Monga, O., Baveye, P.C., 2021. Accounting for soil architecture and microbial dynamics in microscale models: Current practices in soil science and the path ahead. Eur. J. Soil Sci. ejss.13142. https://doi.org/10.1111/ejss.13142

Pot, V., Zhong, X., Baveye, P.C., 2020. Effect of resolution, reconstruction settings, and segmentation methods on the numerical calculation of saturated soil hydraulic conductivity from 3D computed tomography images. Geoderma 362, 114089. https://doi.org/10.1016/j.geoderma.2019.114089

Pratscher, J., Stichternoth, C., Fichtl, K., Schleifer, K.-H., Braker, G., 2009. Application of Recognition of Individual Genes-Fluorescence In Situ Hybridization (RING-FISH) To Detect Nitrite Reductase Genes (*nirK*) of Denitrifiers in Pure Cultures and Environmental Samples. Appl. Environ. Microbiol. 75, 802–810. https://doi.org/10.1128/AEM.01992-08

Pushie, M.J., Pickering, I.J., Korbas, M., Hackett, M.J., George, G.N., 2014. Elemental and Chemically Specific X-ray Fluorescence Imaging of Biological Systems. Chem. Rev. 114, 8499–8541. https://doi.org/10.1021/cr4007297

Qafoku, O., Lybrand, R.A., Shutthanandan, V., Gallery, R.E., Austin, J.C., Schroeder, P.A., Fedenko, J., Rooney, E., Zaharescu, D.G., 2019. A Correlative Bimodal Surface Imaging Method to Assess Hyphae-Rock Interactions. Microsc. Microanal. 25, 2436–2437. https://doi.org/10.1017/S1431927619012911

Qu, C., Qian, S., Chen, L., Guan, Y., Zheng, L., Liu, S., Chen, W., Cai, P., Huang, Q., 2019. Size-Dependent Bacterial Toxicity of Hematite Particles. Environ. Sci. Technol. 53, 8147–8156. https://doi.org/10.1021/acs.est.9b00856

Quigley, M.Y., Rivers, M.L., Kravchenko, A.N., 2018. Patterns and Sources of Spatial Heterogeneity in Soil Matrix From Contrasting Long Term Management Practices. Front. Environ. Sci. 6, 28. https://doi.org/10.3389/fenvs.2018.00028

Ranjard, L., Richaume, A., 2001. Quantitative and qualitative microscale distribution of bacteria in soil. Res. Microbiol. 152, 707–716.

Rawlins, B.G., Wragg, J., Reinhard, C., Atwood, R.C., Houston, A., Lark, R.M., Rudolph, S., 2016. Three-dimensional soil organic matter distribution, accessibility and microbial respiration in macroaggregates using osmium staining and synchrotron X-ray computed tomography. SOIL 2, 659–671. https://doi.org/10.5194/soil-2-659-2016

Raynaud, X., Nunan, N., 2014. Spatial Ecology of Bacteria at the Microscale in Soil. PLoS ONE 9, e87217. https://doi.org/10.1371/journal.pone.0087217

Razavi, B.S., Zarebanadkouki, M., Blagodatskaya, E., Kuzyakov, Y., 2016. Rhizosphere shape of lentil and maize: Spatial distribution of enzyme activities. Soil Biol. Biochem. 96, 229–237. https://doi.org/10.1016/j.soilbio.2016.02.020

Razavi, B.S., Zhang, X., Bilyera, N., Guber, A., Zarebanadkouki, M., 2019. Soil zymography: Simple and reliable? Review of current knowledge and optimization of the method. Rhizosphere 11, 100161. https://doi.org/10.1016/j.rhisph.2019.100161

Remusat, L., Hatton, P.-J., Nico, P.S., Zeller, B., Kleber, M., Derrien, D., 2012. NanoSIMS Study of Organic Matter Associated with Soil Aggregates: Advantages, Limitations, and Combination with STXM. Environ. Sci. Technol. 46, 3943–3949. https://doi.org/10.1021/es203745k

Rodionov, A., Lehndorff, E., Stremtan, C.C., Brand, W.A., Königshoven, H.-P., Amelung, W., 2019. Spatial Microanalysis of Natural 13C/12C Abundance in Environmental Samples Using Laser Ablation-Isotope Ratio Mass Spectrometry. Anal. Chem. 91, 6225–6232. https://doi.org/10.1021/acs.analchem.9b00892

Rogers, S.W., Moorman, T.B., Ong, S.K., 2007. Fluorescent In Situ Hybridization and Micro-autoradiography Applied to Ecophysiology in Soil. Soil Sci. Soc. Am. J. 71, 620. https://doi.org/10.2136/sssaj2006.0105

Rohde, F., Braumann, U., Schmidt, M., 2020. *Correlia* : an *ImageJ* plug‐in to co‐register and visualise multimodal correlative micrographs. J. Microsc. 280, 3–11. https://doi.org/10.1111/jmi.12928

Rohe, L., Apelt, B., Vogel, H.-J., Well, R., Wu, G.-M., Schlüter, S., 2021. Denitrification in soil as a function of oxygen availability at the microscale. Biogeosciences 18, 1185–1201. https://doi.org/10.5194/bg-18-1185-2021

Roose, T., Keyes, S.D., Daly, K.R., Carminati, A., Otten, W., Vetterlein, D., Peth, S., 2016. Challenges in imaging and predictive modeling of rhizosphere processes. Plant Soil 407, 9–38. https://doi.org/10.1007/s11104-016-2872-7

Rudolph, N., Esser, H.G., Carminati, A., Moradi, A.B., Hilger, A., Kardjilov, N., Nagl, S., Oswald, S.E., 2012. Dynamic oxygen mapping in the root zone by fluorescence dye imaging combined with neutron radiography. J. Soils Sediments 12, 63–74. https://doi.org/10.1007/s11368-011-0407-7

Rudolph-Mohr, N., Gottfried, S., Lamshöft, M., Zühlke, S., Oswald, S.E., Spiteller, M., 2015. Non-invasive imaging techniques to study O2 micro-patterns around pesticide treated lupine roots. Geoderma 239–240, 257–264. https://doi.org/10.1016/j.geoderma.2014.10.022

Ruiz, S.A., McKay Fletcher, D.M., Boghi, A., Williams, K.A., Duncan, S.J., Scotson, C.P., Petroselli, C., Dias, T.G.S., Chadwick, D.R., Jones, D.L., Roose, T., 2020. Image-based quantification of soil microbial dead zones induced by nitrogen fertilization. Sci. Total Environ. 727, 138197. https://doi.org/10.1016/j.scitotenv.2020.138197

Sanaullah, M., Razavi, B.S., Blagodatskaya, E., Kuzyakov, Y., 2016. Spatial distribution and catalytic mechanisms of β-glucosidase activity at the root-soil interface. Biol. Fertil. Soils 52, 505–514. https://doi.org/10.1007/s00374-016-1094-8

Santner, J., Larsen, M., Kreuzeder, A., Glud, R.N., 2015. Two decades of chemical imaging of solutes in sediments and soils – a review. Anal. Chim. Acta 878, 9–42. https://doi.org/10.1016/j.aca.2015.02.006

Sauzet, O., Cammas, C., Gilliot, J.M., Bajard, M., Montagne, D., 2017. Development of a novel image analysis procedure to quantify biological porosity and illuvial clay in large soil thin sections. Geoderma 292, 135–148. https://doi.org/10.1016/j.geoderma.2017.01.004

Schlüter, S., Eickhorst, T., Mueller, C.W., 2019a. Correlative Imaging Reveals Holistic View of Soil Microenvironments. Environ. Sci. Technol. 53, 829–837. https://doi.org/10.1021/acs.est.8b05245

Schlüter, S., Leuther, F., Albrecht, L., Hoeschen, C., Kilian, R., Surey, R., Mikutta, R., Kaiser, K., Mueller, C.W., Vogel, H.-J., 2022. Microscale carbon distribution around pores and particulate organic matter varies with soil moisture regime. Nat. Commun. 13, 2098. https://doi.org/10.1038/s41467-022-29605-w

Schlüter, S., Sammartino, S., Koestel, J., 2020. Exploring the relationship between soil structure and soil functions via pore-scale imaging. Geoderma 370, 114370. https://doi.org/10.1016/j.geoderma.2020.114370

Schlüter, S., Sheppard, A., Brown, K., Wildenschild, D., 2014. Image processing of multiphase images obtained via X-ray microtomography: A review. Water Resour. Res. 50, 3615–3639. https://doi.org/10.1002/2014WR015256

Schlüter, S., Zawallich, J., Vogel, H.-J., Dörsch, P., 2019b. Physical constraints for respiration in microbial hotspots in soil and their importance for denitrification. Biogeosciences 16, 3665–3678. https://doi.org/10.5194/bg-16-3665-2019

Schmidt, H., Eickhorst, T., 2014. Detection and quantification of native microbial populations on soil-grown rice roots by catalyzed reporter deposition-fluorescence *in situ* hybridization. FEMS Microbiol. Ecol. 87, 390–402. https://doi.org/10.1111/1574-6941.12232

Schmidt, H., Eickhorst, T., Mußmann, M., 2012. Gold-FISH: A new approach for the in situ detection of single microbial cells combining fluorescence and scanning electron microscopy. Syst. Appl. Microbiol. 35, 518–525. https://doi.org/10.1016/j.syapm.2012.04.006

Schmidt, H., Nunan, N., Höck, A., Eickhorst, T., Kaiser, C., Woebken, D., Raynaud, X., 2018. Recognizing Patterns: Spatial Analysis of Observed Microbial Colonization on Root Surfaces. Front. Environ. Sci. 6, 61. https://doi.org/10.3389/fenvs.2018.00061

Schmidt, H., Vetterlein, D., Köhne, J.M., Eickhorst, T., 2015. Negligible effect of X-ray µ-CT scanning on archaea and bacteria in an agricultural soil. Soil Biol. Biochem. 84, 21–27. https://doi.org/10.1016/j.soilbio.2015.02.010

Schmidt, M., Byrne, J.M., Maasilta, I.J., 2021. Bio-imaging with the helium-ion microscope: A review. Beilstein J. Nanotechnol. 12, 1–23. https://doi.org/10.3762/bjnano.12.1

Schmidt, M.W.I., Knicker, H., Hatcher, P.G., Kogel-Knabner, I., 1997. Improvement of 13C and 15N CPMAS NMR spectra of bulk soils, particle size fractions and organic material by treatment with 10% hydrofluoric acid. Eur. J. Soil Sci. 48, 319–328. https://doi.org/10.1111/j.1365-2389.1997.tb00552.x

Schnepf, A., Carminati, A., Ahmed, M.A., Ani, M., Benard, P., Bentz, J., Bonkowski, M., Knott, M., Diehl, D., Duddek, P., Kröner, E., Javaux, M., Landl, M., Lehndorff, E., Lippold, E., Lieu, A.,

Mueller, C.W., Oburger, E., Otten, W., Portell, X., Phalempin, M., Prechtel, A., Schulz, R., Vanderborght, J., Vetterlein, D., 2022. Linking rhizosphere processes across scales: Opinion. Plant Soil. https://doi.org/10.1007/s11104-022-05306-7

Schurig, C., Mueller, C.W., Höschen, C., Prager, A., Kothe, E., Beck, H., Miltner, A., Kästner, M., 2015. Methods for visualising active microbial benzene degraders in in situ microcosms. Appl. Microbiol. Biotechnol. 99, 957–968. https://doi.org/10.1007/s00253-014-6037-4

Sharma, K., Palatinszky, M., Nikolov, G., Berry, D., Shank, E.A., 2020. Transparent soil microcosms for live-cell imaging and non-destructive stable isotope probing of soil microorganisms. eLife 9, e56275. https://doi.org/10.7554/eLife.56275

Shi, L., Günther, S., Hübschmann, T., Wick, L.Y., Harms, H., Müller, S., 2007. Limits of propidium iodide as a cell viability indicator for environmental bacteria. Cytometry A 71A, 592–598. https://doi.org/10.1002/cyto.a.20402

Singh, B., Gräfe, M. (Eds.), 2010. Synchrotron-based techniques in soils and sediments, Developments in soil science. Elsevier, Amsterdam.

Smith, D.J., 2008. Ultimate resolution in the electron microscope? Mater. Today 11, 30–38. https://doi.org/10.1016/S1369-7021(09)70005-7

Solomon, D., Lehmann, J., Wang, J., Kinyangi, J., Heymann, K., Lu, Y., Wirick, S., Jacobsen, C., 2012. Micro- and nano-environments of C sequestration in soil: A multi-elemental STXM–NEXAFS assessment of black C and organomineral associations. Sci. Total Environ. 438, 372–388. https://doi.org/10.1016/j.scitotenv.2012.08.071

Solovei, I., 2010. Fluorescence in situ Hybridization (FISH) on Tissue Cryosections, in: Bridger, J.M., Volpi, E.V. (Eds.), Fluorescence in Situ Hybridization (FISH), Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 71–82. https://doi.org/10.1007/978-1-60761-789-1_5

Spohn, M., Carminati, A., Kuzyakov, Y., 2013. Soil zymography – A novel in situ method for mapping distribution of enzyme activity in soil. Soil Biol. Biochem. 58, 275–280. https://doi.org/10.1016/j.soilbio.2012.12.004

Spohn, M., Kuzyakov, Y., 2014. Spatial and temporal dynamics of hotspots of enzyme activity in soil as affected by living and dead roots—a soil zymography analysis. Plant Soil 379, 67–77. https://doi.org/10.1007/s11104-014-2041-9

Spohn, M., Kuzyakov, Y., 2013. Distribution of microbial- and root-derived phosphatase activities in the rhizosphere depending on P availability and C allocation – Coupling soil zymography with 14C imaging. Soil Biol. Biochem. 67, 106–113. https://doi.org/10.1016/j.soilbio.2013.08.015

Steffens, M., Buddenbaum, H., 2013. Laboratory imaging spectroscopy of a stagnic Luvisol profile — High resolution soil characterisation, classification and mapping of elemental concentrations. Geoderma 195–196, 122–132. https://doi.org/10.1016/j.geoderma.2012.11.011

Steffens, M., Rogge, D.M., Mueller, C.W., Höschen, C., Lugmeier, J., Kölbl, A., Kögel-Knabner, I., 2017. Identification of Distinct Functional Microstructural Domains Controlling C Storage in Soil. Environ. Sci. Technol. 51, 12182–12189. https://doi.org/10.1021/acs.est.7b03715

Tecon, R., Or, D., 2017. Biophysical processes supporting the diversity of microbial life in soil. FEMS Microbiol. Rev. 41, 599–623. https://doi.org/10.1093/femsre/fux039

Thompson, I.A., Huber, D.M., Guest, C.A., Schulze, D.G., 2005. Fungal manganese oxidation in a reduced soil. Environ. Microbiol. 7, 1480–1487. https://doi.org/10.1111/j.1462-2920.2005.00842.x

Tippkötter, R., Eickhorst, T., Taubner, H., Gredner, B., Rademaker, G., 2009. Detection of soil water in macropores of undisturbed soil using microfocus X-ray tube computerized tomography (µCT). Soil Tillage Res. 105, 12–20. https://doi.org/10.1016/j.still.2009.05.001

Tippkötter, R., Ritz, K., 1996. Evaluation of polyester, epoxy and acrylic resins for suitability in preparation of soil thin sections for in situ biological studies. Geoderma 69, 31–57.

Tippkötter, R., Ritz, K., Darbyshire, J.F., 1986. The preparation of soil thin sections for biological studies. J. Soil Sci. 37, 681–690.

Torsvik, V., Øvreås, L., 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol. 5, 240–245.

Tötzke, C., Kardjilov, N., Hilger, A., Rudolph-Mohr, N., Manke, I., Oswald, S.E., 2021. Three-dimensional in vivo analysis of water uptake and translocation in maize roots by fast neutron tomography. Sci. Rep. 11, 10578. https://doi.org/10.1038/s41598-021-90062-4

Tracy, S.R., Daly, K.R., Sturrock, C.J., Crout, N.M.J., Mooney, S.J., Roose, T., 2015. Three-dimensional quantification of soil hydraulic properties using X-ray Computed Tomography and image-based modeling. Water Resour. Res. 51, 1006–1022. https://doi.org/10.1002/2014WR016020

Turkowyd, B., Virant, D., Endesfelder, U., 2016. From single molecules to life: microscopy at the nanoscale. Anal. Bioanal. Chem. 408, 6885–6911. https://doi.org/10.1007/s00216-016-9781-8

Van Loo, D., Bouckaert, L., Leroux, O., Pauwels, E., Dierick, M., Van Hoorebeke, L., Cnudde, V., De Neve, S., Sleutel, S., 2014. Contrast agents for soil investigation with X-ray computed tomography. Geoderma 213, 485–491. https://doi.org/10.1016/j.geoderma.2013.08.036

Védère, C., 2020. Impact du potentiel matriciel sur la biodégradation de résidus végétaux – Évolution spatio-temporelle de la détritusphère. Paris-Saclay, Grignon.

Védère, C., Vieublé Gonod, L., Pouteau, V., Girardin, C., Chenu, C., 2020. Spatial and temporal evolution of detritusphere hotspots at different soil moistures. Soil Biol. Biochem. 150, 107975. https://doi.org/10.1016/j.soilbio.2020.107975

Vergara Sosa, M., Lehndorff, E., Rodionov, A., Gocke, M., Sandhage-Hofmann, A., Amelung, W., 2021. Micro-scale resolution of carbon turnover in soil - Insights from laser ablation isotope ratio mass spectrometry on water-glass embedded aggregates. Soil Biol. Biochem. 159, 108279. https://doi.org/10.1016/j.soilbio.2021.108279

Vidal, A., Hirte, J., Bender, S.F., Mayer, J., Gattinger, A., Höschen, C., Schädler, S., Iqbal, T.M., Mueller, C.W., 2018. Linking 3D Soil Structure and Plant-Microbe-Soil Carbon Transfer in the Rhizosphere. Front. Environ. Sci. 6. https://doi.org/10.3389/fenvs.2018.00009

Vidal, A., Klöffel, T., Guigue, J., Angst, G., Steffens, M., Hoeschen, C., Mueller, C.W., 2021. Visualizing the transfer of organic matter from decaying plant residues to soil mineral surfaces controlled by microorganisms. Soil Biol. Biochem. 160, 108347. https://doi.org/10.1016/j.soilbio.2021.108347

Vidal, A., Remusat, L., Watteau, F., Derenne, S., Quenea, K., 2016. Incorporation of 13C labelled shoot residues in Lumbricus terrestris casts: A combination of transmission electron microscopy and nanoscale secondary ion mass spectrometry. Soil Biol. Biochem. 93, 8–16. https://doi.org/10.1016/j.soilbio.2015.10.018

Vidal, A., Watteau, F., Remusat, L., Mueller, C.W., Nguyen Tu, T.-T., Buegger, F., Derenne, S., Quenea, K., 2019. Earthworm Cast Formation and Development: A Shift From Plant Litter to Mineral Associated Organic Matter. Front. Environ. Sci. 7, 55. https://doi.org/10.3389/fenvs.2019.00055

Villemin, G., Mansot, J.L., Watteau, F., Ghanbaja, J., Toutain, F., 1995. Study of the biodegradation and humification of soil organic matter of plant origin by Electron Energy Loss Spectroscopy (EELS): distribution of carbon, nitrogen and C:N evaluation at ultrastructural level in situ. Comptes Rendus Acad. Sci. Ser. 2a Sci. Terre Planetes 321, 861–868.

Villemin, G., Toutain, F., 1987. Méthode de fixation d'échantillons organo-minéraux des sols pour la microscopie électronique à transmission. Presented at the N. Fedoroff, L.M. Bresson, M.A. Courty (Eds.), Soil Micromorphology. Proc. VIIth Int. Work. Meet. Soil Micromorphology. A.F.E.S., Plaisir, France, Association francaise étude sols, Plaisir, France, pp. 43–48.

Vos, M., Wolf, A.B., Jennings, S.J., Kowalchuk, G.A., 2013. Micro-scale determinants of bacterial diversity in soil. FEMS Microbiol. Rev. 37, 936–954. https://doi.org/10.1111/1574-6976.12023

Watteau, F., Villemin, G., 2018. Soil Microstructures Examined Through Transmission Electron Microscopy Reveal Soil-Microorganisms Interactions. Front. Environ. Sci. 6, 106. https://doi.org/10.3389/fenvs.2018.00106

Watteau, F., Villemin, G., Bartoli, F., Schwartz, C., Morel, J.L., 2012. 0–20 µm aggregate typology based on the nature of aggregative organic materials in a cultivated silty topsoil. Soil Biol. Biochem. 46, 103–114. https://doi.org/10.1016/j.soilbio.2011.11.021

Watteau, F., Villemin, G., Ghanbaja, J., Genet, P., Pargney, J.-C., 2002. In situ ageing of fine beech roots (Fagus sylvatica) assessed by transmission electron microscopy and electron energy loss spectroscopy: description of microsites and evolution of polyphenolic substances. Biol. Cell 94, 55–63.

Watteau, F., Villemin, G., Mansot, J.L., Ghanbaja, J., Toutain, F., 1996. Localization and characterization by electron energy loss spectroscopy (EELS) of the brown cellular substances of beech roots. Soil Biol Biochem 28, 1327–1332.

Weng, Z. (Han), Lehmann, J., Van Zwieten, L., Joseph, S., Archanjo, B.S., Cowie, B., Thomsen, L., Tobin, M.J., Vongsvivut, J., Klein, A., Doolette, C.L., Hou, H., Mueller, C.W., Lombi, E., Kopittke, P.M., 2021. Probing the nature of soil organic matter. Crit. Rev. Environ. Sci. Technol. 1–22. https://doi.org/10.1080/10643389.2021.1980346

Wilmoth, J.L., Doak, P.W., Timm, A., Halsted, M., Anderson, J.D., Ginovart, M., Prats, C., Portell, X., Retterer, S.T., Fuentes-Cabrera, M., 2018. A Microfluidics and Agent-Based Modeling Framework for Investigating Spatial Organization in Bacterial Colonies: The Case of Pseudomonas Aeruginosa and H1-Type VI Secretion Interactions. Front. Microbiol. 9, 33. https://doi.org/10.3389/fmicb.2018.00033

Withers, P.J., Bouman, C., Carmignato, S., Cnudde, V., Grimaldi, D., Hagen, C.K., Maire, E., Manley, M., Du Plessis, A., Stock, S.R., 2021. X-ray computed tomography. Nat. Rev. Methods Primer 1, 18. https://doi.org/10.1038/s43586-021-00015-4

Witzgall, K., Vidal, A., Schubert, D.I., Höschen, C., Schweizer, S.A., Buegger, F., Pouteau, V., Chenu, C., Mueller, C.W., 2021. Particulate organic matter as a functional soil component for persistent soil organic carbon. Nat. Commun. 12, 4115. https://doi.org/10.1038/s41467-021-24192-8

Woignier, T., Braudeau, E., Doumenc, H., Rangon, L., 2005. Supercritical Drying Applied to Natural "Gels": Allophanic Soils. J. Sol-Gel Sci. Technol. 36, 61–68. https://doi.org/10.1007/s10971-005-2659-4

Woignier, T., Primera, J., Duffours, L., Dieudonné, P., Raada, A., 2008. Preservation of the allophanic soils structure by supercritical drying. Microporous Mesoporous Mater. 109, 370–375. https://doi.org/10.1016/j.micromeso.2007.05.019

Wu, B., 2014. Visualization of nutrient translocation in ectomycorrhizal symbioses. Botany 92, 129–133. https://doi.org/10.1139/cjb-2013-0093

Wu, Y., Misra, S., Sondergeld, C., Curtis, M., Jernigen, J., 2019. Machine learning for locating organic matter and pores in scanning electron microscopy images of organic-rich shales. Fuel 253, 662–676. https://doi.org/10.1016/j.fuel.2019.05.017

Yang, J.Q., Zhang, X., Bourg, I.C., Stone, H.A., 2021. 4D imaging reveals mechanisms of clay-carbon protection and release. Nat. Commun. 12, 622. https://doi.org/10.1038/s41467-020-20798-6

Zappala, S., Helliwell, J.R., Tracy, S.R., Mairhofer, S., Sturrock, C.J., Pridmore, T., Bennett, M., Mooney, S.J., 2013. Effects of X-Ray Dose On Rhizosphere Studies Using X-Ray Computed Tomography. PLoS ONE 8, e67250. https://doi.org/10.1371/journal.pone.0067250

Zarda, B., Hahn, D., Chatzinotas, A., Schönhuber, W., Neef, A., Amann, R.I., Zeyer, J., 1997. Analysis of bacterial community structure in bulk soil by in situ hybridization. Arch. Microbiol. 168, 185–192. https://doi.org/10.1007/s002030050486

Zheng, H., Kim, K., Kravchenko, A., Rivers, M., Guber, A., 2020. Testing Os Staining Approach for Visualizing Soil Organic Matter Patterns in Intact Samples via X-ray Dual-Energy Tomography Scanning. Environ. Sci. Technol. 54, 8980–8989. https://doi.org/10.1021/acs.est.0c01028

Zumstein, M.T., Schintlmeister, A., Nelson, T.F., Baumgartner, R., Woebken, D., Wagner, M., Kohler, H.-P.E., McNeill, K., Sander, M., 2018. Biodegradation of synthetic polymers in soils: Tracking carbon into CO2 and microbial biomass. Sci. Adv. 4, eaas9024. https://doi.org/10.1126/sciadv.aas9024