Organo–organic and organo–mineral interfaces in soil at the nanometer scale

Angela R. Possinger1,6, Michael J. Zachman2,7, Akio Enders1,8, Barnaby D. A. Levin2, David A. Muller2,3, Lena F. Kourkoutis2,3 & Johannes Lehmann1,4,5✉

The capacity of soil as a carbon (C) sink is mediated by interactions between organic matter and mineral phases. However, previously proposed layered accumulation of organic matter within aggregate organo–mineral microstructures has not yet been confirmed by direct visualization at the necessary nanometer-scale spatial resolution. Here, we identify disordered micrometer-size organic phases rather than previously reported ordered gradients in C functional groups. Using cryo-electron microscopy with electron energy loss spectroscopy (EELS), we show organo–organic interfaces in contrast to exclusively organo–mineral interfaces. Single-digit nanometer-size layers of C forms were detected at the organo–organic interface, showing alkyl C and nitrogen (N) enrichment (by 4 and 7%, respectively). At the organo–mineral interface, 88% (72–92%) and 33% (16–53%) enrichment of N and oxidized C, respectively, indicate different stabilization processes than at organo–organic interfaces. However, N enrichment at both interface types points towards the importance of N-rich residues for greater C sequestration.
Soil organic carbon (SOC) constitutes a critical reservoir in the global C cycle, which highlights the importance of understanding the processes that drive soil organic matter (OM) persistence, ranging from global (e.g., climate) to very fine scales (e.g., organo–mineral surface interactions)\textsuperscript{11–14}. Improving the ability to describe drivers of SOM persistence, including mechanisms of SOM protection, enables better prediction of changes in the soil C reservoir in light of global environmental change\textsuperscript{2}.

Soil OM is a mixture of organic compounds subject to organo–mineral interactions\textsuperscript{6–10}. Such interactions between SOM and mineral phases result in lower microbial accessibility and availability for decomposition, which is seen as a dominant process for SOM stabilization\textsuperscript{15,16}. The spatial and chemical heterogeneity of SOM, soil physical structures, and microbial distribution at the scale of soil micro- and macroaggregates and pore structures are relatively well studied\textsuperscript{2,11–16} (Fig. 1a). In contrast to microaggregate-scale heterogeneity, previous imaging and spectroscopy of SOM coatings within micrometer-scale organo–mineral assemblages have shown relatively uniform, ordered layers with distinct OM composition as a function of distance from mineral surfaces at smaller spatial scales of micrometers\textsuperscript{15–19} (Fig. 1b). The spatial architecture of layered SOM accumulation is consistent with the concept of preferential association of nitrogen (N)-bearing and oxidized functional groups at the mineral surface, acting as a backbone for additional layers of OM accumulation with different composition (i.e., the zonal-structure model)\textsuperscript{20,21}. While monolayer adsorption of SOM on mineral surfaces and the maximum OM accumulation (i.e., saturation) could be limited by mineral surface area and chemistry, layering of OM may circumvent these limitations and therefore be effective for maximizing soil organic C sequestration\textsuperscript{13,22}.

However, the suite of interactions among OM constituents (e.g., biomolecules, microbial cell membranes, extracellular products, and small soluble compounds)\textsuperscript{23} in addition to mineral associations challenges previous observations of ordered layers of OM accumulation. The resolution of previously used imaging and spectroscopy techniques (e.g., 30–50 nm in ref. 17) may be too coarse to resolve or describe the interfaces among OM constituents embedded within an organo–mineral assemblage (Fig. 1b). Despite the potentially critical role of interactions between OM forms in shifting the current view of the spatial arrangement of SOM accumulation on mineral surfaces, the chemical composition of these interfaces has not been directly visualized or described in natural soil samples at the relevant nanometer scale.

Associations between SOM and semicrystalline reactive iron (Fe) and aluminum (Al) mineral surfaces are recognized to contribute to long-term SOM persistence and accumulation across widely variable soil types\textsuperscript{24,25}. The formation of associations between reactive Fe and Al and SOM has been linked to preferential reactions with oxidized functional groups\textsuperscript{26} and N-containing biomolecules\textsuperscript{27–29}. However, given the submicrometer spatial scale of OM distribution and its chemical complexity\textsuperscript{2}, little spatially explicit evidence exists to understand these hypothesized mechanisms of interactions in natural soils at the scale of single (<10) nanometers. Further, analysis of the spatial architecture at the necessary resolution to detect the potential effect of interactions among OM phases with different compositions is lacking. Single-digit nanometer-scale imaging and spectroscopy techniques may enable us to confirm the existence of previously hypothesized nanoscale interactions, and generate novel hypotheses to be tested at larger scales based on observations of previously inaccessible structures.

Fig. 1 Conceptual summary of organo-organic and organo-mineral interactions. Shifting conceptual view of the spatial architecture and composition of organo-mineral and organo-organic interactions. a At the scale of aggregate thin-sections, heterogeneous distribution of organic matter (OM) and microbial populations is well-known. b However, previous imaging and spectroscopic characterization of organo-mineral assemblages using a 10–50 μm field of view has shown organized OM layers of different composition. The concept of layered OM accumulation (i.e., the zonal model) has often been linked to nitrogen (N) enrichment at the mineral surface\textsuperscript{20,21}. c-f The images and spectroscopic data presented in this study (conceptualized in c-f) show an order of magnitude smaller spatial scale. e Enrichment of N was detected at an organo-mineral interface in a volcanic soil sample (Fig. 2). d-f Irregular organic structures and the organo-organic interface between them were also detected in (a separate volcanic soil sample) (Figs. 3–5). These observations directly reveal heterogeneous patches that contradict previous assumptions of ordered and unidirectional layering of OM forms, generating new insights into OM composition at mineral-organic and organo-organic interfaces. Note: scale bars are for illustrative purposes only.
The goal of this study was to examine the hypothesized zonal-structure model of interaction and identify the functional groups at the organo–mineral interface. To gain further insight into the properties of organo–mineral interactions in natural soil samples and to ascertain the presence of what we call organo–organic interactions, we developed a novel approach for cryogenic sample preparation in conjunction with analytical cryogenic scanning transmission electron microscopy and electron energy-loss spectroscopy (cryo-STEM-EELS). This approach pairs single-digit nanometer spatial resolution with the ability to resolve variations in the C bonding environment across organo–mineral and organo–organic interfaces. This approach also avoids the use of C-based stabilizing resins that typically make interpretation of the native soil C content and bonding environments difficult, and enables direct visualization and analysis of the interfaces between organic and mineral phases in soil without alteration of the spatial architecture. We applied cryo-STEM-EELS to probe organo–organic and organo–mineral interfaces in archetypical high-reactive Fe and Al volcanic soils (Supplementary Table 1) with a known capacity for long-term C accumulation\(^8,26,30\).

### Results

**Organo–mineral interface: enrichment of N and oxidized C.** Our observations of an organo–mineral interface at the nanometer scale provide direct evidence for the role of N-containing functional groups of polar C compounds in interactions at aluminum (Al) mineral surfaces. Using cryo-STEM-EELS, we show enrichment of C K-edge EELS features at 288.1 eV, indicative of oxidized carboxylic/carbonyl C with substituted or adjacent N\(^{31,32}\) (Fig. 2a, b). The average intensity of oxidized C (286.6–289.0 eV) relative to lower-energy C (284.0–286.5 eV) intensity increased by approximately 33% (range: 16–53%) at the organo–mineral interface compared to the adjacent OM (Fig. 2, Supplementary Table 2). Relative enrichment of N was indicated by a decrease in the C/N signal intensity (approximately 88% decrease, range: 72–92%) closer to the Al mineral surface compared to an adjacent OM region (Fig. 2, Supplementary Table 2; Al content and structures in Supplementary Figs. 1 and 2).

The presented data provide unambiguous evidence of co-location between an Al mineral and N-rich oxidized organic matter but do not directly probe the mechanism of interaction. However, the presence of N-rich oxidized OM is consistent with

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**Fig. 2 Nitrogen and oxidized carbon enrichment at the organo-mineral interface.** High-resolution cryo-electron imaging and spectroscopy of an organo–mineral interface in a volcanic soil sample. **a** Annular dark-field (ADF) scanning transmission electron microscopy (STEM) image of organo–mineral interface and adjacent organic matter region, showing the location of electron energy-loss spectroscopy (EELS) data collection across and adjacent to an aluminum (Al) mineral with layered structure. **b, c** Average carbon (**b**) and nitrogen (**c**) K-edge EELS (black line) and individual spectra (gray lines) for either five points (A–E) across the organo-mineral interface or three EELS point scans within the adjacent OM region. For both **b** and **c**, spectra are shown unsmoothed and normalized to the maximum carbon intensity. In **b**, dotted vertical line indicates peak at 288.1 eV, putatively identified as carboxylic/carbonyl groups with substituted or adjacent N\(^{31,32}\). Lower (284.0–286.5 eV) (y) and higher (286.6–289.0 eV) (x) energy regions are indicated by shaded boxes. Ratio x/y indicates the ratio of integrated EELS intensity within each region, normalized to total carbon integrated area (280.0–315.0 eV). Between the adjacent region and interface, the x/y ratio increased by an average 33% (Supplementary Table 2), indicating an increase in oxidized C at the organo-mineral interface compared to the adjacent C region. In addition, the ratio of integrated total carbon (280.0–315.0 eV) to total nitrogen (395.0–430.0 eV) decreased by an average 88% between the adjacent C region and the organo-mineral interface region.
the preferential retention of N-containing functional groups and oxidized (e.g., carboxylic acid) OM in soils with short-range ordered (SRO) Fe and Al mineralogy. Aromatic and carboxylic acids have been identified as key C forms in stabilizing interactions with reactive metals not only in archetypical soils with SRO mineralogy (e.g., the studied Andisols), but also in broadly distributed forest soils. While our data do not address the frequency or distribution of organo-reactive metal interactions, reactive metal phases are present in most soil types as surface coatings on silicate clays and primary minerals, Fe precipitates, and Fe–OM co-precipitates under fluctuating redox conditions. In addition, spatial associations of N-rich OM on reactive metal surfaces have been identified both within SRO-rich soils and temperate soil organic horizons. More broadly, the role of N in organo–mineral associations is consistent with the role of microbially derived residues for the accumulation of OM, a foundation of the microbial efficiency-matrix stabilization (MEMS) framework and mineral–microbe biogeochemical models. Multiple mechanisms of N-rich OM interaction with reactive Fe and Al minerals have been proposed, including indirect association via reactive phosphate groups in phosphorylated proteins and hydrogen bond formation.

While inner-sphere ligand exchange is well documented for oxidized carboxylic and aromatic acids, ligand exchange mechanisms specific to N-substituted carboxylic groups are less well-known. In this study, the very high enrichment of total N (88% lower C/N intensity ratio) at the interface also suggests that the N-rich OM association may co-exist with the accumulation of inorganic N (e.g., electrostatic retention of NH₄⁺). The potential for either competitive or synergistic interactions between organic and inorganic N at reactive metal surfaces provides a new direction for the evaluation of N in OM stabilization. In addition, interactions between N-rich OM and reactive Al (as identified here) have received less attention than N-rich OM and reactive Fe. With an emerging focus on Al-mediated OM stabilization under conditions of Fe depletion, further experiments should target possible divergence in physicochemical mineral–organic mechanisms between N-rich OM and reactive Al in contrast to Fe.

**Hierarchy of spatial heterogeneity in OM composition.** Cryo-STEM-EELS analysis of an OM-rich region in a soil aggregate with high OM content (Supplementary Table 1) revealed 0.1–1 μm-size features of distinct OM composition using nanometer-scale spatial resolution (Fig. 3a–c, Supplementary Fig. 3). The presence of irregular aromatic-rich C, lower-N, and higher-O organic features with distinct C composition embedded in a more

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**Fig. 3 Composition of organic features and the organo–organic interface.** High-resolution imaging and spectroscopy of organo–organic features and their interface by cryo-scanning transmission electron microscopy and electron energy-loss spectroscopy (cryo-STEM–EELS). a Overview low-magnification (5 kx) annular dark-field (ADF) STEM image of cryo-focused ion beam (cryo-FIB) thin section of organic-rich volcanic soil. The approximate region indicated by a box was used for subsequent electron energy-loss spectroscopy (EELS) mapping of carbon (C) and nitrogen (N). b EELS elemental map showing the patchy distribution of low-N features in the high-N matrix. c Patchy distribution of organic matter (OM) features microimeters in size, shown with EELS maps of the two distinct carbon (C) bonding environments, as revealed by carbon K-edge multivariate curve resolution (MCR) component fits (component spectra are shown in f). d Nanometer-scale EELS C K-edge MCR component map of the interface between two OM patches (component spectra shown in g). e Raw EELS data for representative lower N (top spectrum) and higher N (bottom spectrum) regions in (b). Spectra show the high C K-edge (~280.0 eV) intensity relative to N (~400.0 eV) and O (~530.0 eV) intensities. f, g Normalized (maximum = 1) C K-edge MCR component spectra corresponding to (c, d), with a distinctive transition from ~285.0 eV in component 1 (assigned to aromatic C=C bonds) to higher-energy features (~286.3–287.0 eV) in component 2. Features in spectra are shown by dotted vertical lines. Spectra are shown as raw (unsmoothed) MCR fit outputs normalized to the maximum output value.
alkyl-rich C with higher-N and lower-O organic matrix contra-
dicts previous observations of micrometer-sized ordered gradi-
ents as a function of distance to mineral surfaces15–19 (Fig. 1b).

EELS C K-edge fine structure varied greatly between the
features and the surrounding matrix, which were identified as
discrete C forms using multivariate curve resolution (MCR)
(Fig. 3c, f). Within the observed patchy features, the EELS
fine structure is dominated by a low-energy feature at ~285 eV, which
is assigned to the C 1s→π* C=C transition of C=C bonds associated
with aromatic structures32. In contrast, the EELS
fine structure of the surrounding matrix distinctly shifts from ~285 eV to higher
energy (Fig. 3f, g). At higher spatial resolution (approximately 2
nm) (Fig. 3d), the higher-energy feature is at ~287 eV (Fig. 3g,
Fig. 4d). While many overlapping transitions occur in this region,
features can be broadly linked to alkyl C-H bonds with a variety of
transitions (e.g., C 1s→π* C–H)32,37.

By observing C/N, C/O, and aromatic/alkyl C ratios across the
boundaries of the OM patches, the improved spatial resolution
of cryo-STEM-EELS over previously utilized techniques (such as
scanning transmission X-ray spectroscopy with near-edge X-ray
absorption fine structure15–19) enabled the discovery of
nanometer-scale ordered gradients of layered OM at the boundary

Fig. 4 Gradient in organic composition across the organo–organic interface. Distinct combinations of carbon/nitrogen (C/N), carbon/oxygen (C/O), and aromatic/alkyl C ratio across the organo–organic interface shows a gradient of organic matter (OM) composition at the ~50 nm scale. a  Annular dark-field (ADF) scanning transmission electron microscopy (STEM) detail of the interface between organic phases in a soil thin section. Boxes 1 through 5 indicate regions used to compute average electron energy-loss (EEL) spectra across the interface (lateral distance). b  Ratios of normalized (maximum = 1 unit) elemental integrated area for C/N, C/O, and aromatic/alkyl C across the interface. The aromatic/alkyl C ratio is the ratio of integrated intensity in EELS spectral regions (defined here as aromatic from 284.25 to 285.75 eV and alkyl from 286.0 to 287.5 eV) normalized to total C integrated area (280.0–315.0 eV). Each location (1–5) across the interface shows a different combination of C/N, C/O, and aromatic/alkyl C. At point 4, alkyl C is enriched relative to the high-alkyl C matrix. The calculations for each point are from ~540 individual spectra (1 spectrum per pixel) in each 11 × 44 nm box. c  Rate of change for C/N and aromatic/alkyl ratios across the organo–organic interface relative to box 1. The C/N ratio decreases more rapidly than the aromatic/alkyl ratio for the transitions from boxes 1 to 2 and 1 to 3, which suggests trends in C composition and N quantity are decoupled across the interface. d  Average EEL spectra (spline curve, an average of ~540 individual spectra) for boxes 1–5 for the carbon, nitrogen, and oxygen K-edges. Carbon K-edge EELS show similar intensity but a change in fine structure. For carbon, vertical lines at 285.0 and 287.0 eV are putatively associated with aromatic and alkyl C, respectively. Nitrogen and oxygen K-edge EELS show an increase and decrease in intensity across the interface, respectively.
consistent in size with small biomolecules or associations\textsuperscript{23,38} of between layers. The observed layers of distinct OM composition improved spectrometer detection (e.g., direct electron detection) gradients of C composition may occur even at a scale of <5 nm, but between adjacent 2 × 44 nm regions also suggests that ordered fl...lines at 285.0 and 287.0 eV, putatively associated with aromatic and alkyl C, respectively. The presence of clear alkyl C was gradual, and overall, tracked the 11 × 44 nm analysis across the interface (Fig. 5). The transition between aromatic and examined changes in C forms using ~2 × 44 nm analysis regions...of micrometer-sized organic features, in addition to micrometer-scale patchiness (Figs. 4 and 5). Along the edge of the interface, the change in aromatic/alkyl C ratios constrains the homogeneity of the interface to ~25 nm in the vertical direction (Supplementary Fig. 4).

To discern the presence of ordered gradients at this fine scale, we examined changes in C forms using ~2 × 44 nm analysis regions across the interface (Fig. 5). The transition between aromatic and alkyl C was gradual, and overall, tracked the 11 × 44 nm analysis region EELS data (Supplementary Fig. 5). The presence of clear fluctuations in aromatic/alkyl, C/N, and C/O signal intensity between adjacent 2 × 44 nm regions also suggests that ordered gradients of C composition may occur even at a scale of <5 nm, but improved spectrometer detection (e.g., direct electron detection) may be needed to definitively identify the points of separation between layers. The observed layers of distinct OM composition are consistent in size with small biomolecules or associations\textsuperscript{23,38} of small biomolecules, such as many proteins. However, even the single-digit nanometer resolution does not allow for subnanometer spatial mapping of individual functional groups or bonds.

**Organo–organic interface: enrichment of N and alkyl C.** We identified hitherto undescribed organo–organic interfaces as part of OM composition at a very fine scale. Within aromatic-rich patches, N contents increased towards the interface with the alkyl phase (Fig. 4). The shift from low to high N contents was spatially separate from the shift from aromatic to alkyl C, with N contents beginning to increase further from the interface (Fig. 4c). In addition, the C/N ratio was 7% lower directly at the interface (Box 4) compared to the adjacent higher-N phase (Box 5 in Fig. 4a, b, Supplementary Table 3). This suggests N-containing components of the aromatic C phase may be preferentially involved in an interaction. Aromatic C=C groups are only thought to be responsible for interactions between organic and mineral phases in specific situations\textsuperscript{20}. In contrast, N groups in OM are expected to associate readily with other organic constituents via electrostatic interactions or covalent bonds, due to the potential for their positive charge and electron-withdrawing character, respectively. In contrast to the mineral interface analyzed in this study, enrichment at the edge of the aromatic organic phase occurred without the presence of oxidized OM (e.g., in carboxylic C of amino acids), suggesting that N-containing aromatic compounds with a relatively high C/O ratio and alkyl constituents (e.g., cytokinins) may facilitate interactions between organic phases. However, additional characterization of the N bonding environment is needed to further refine the physicochemical mechanism of interaction.

The ratio of aromatic C (integrated 284.25–285.75 eV signal) or alkyl C (integrated 286.0–287.5 eV signal) to the total integrated C edge intensity showed enrichment in alkyl C (by 4%) occurring directly at the organo–organic interface (Box 4 vs. Box 5 in Fig. 4, Supplementary Table 3). No unique third C component at the interface was present, indicating a shifting aromatic/alkyl C ratio rather than the appearance of a new C feature (Supplementary Fig. 6). The overall trends in C/N and C/O across the interface show mixing between OM forms across a boundary of ~50 nm or less across (Fig. 4). For this analysis, we achieved a single-digit
nanometer spatial resolution (see “Methods”), suggesting that the size of the overall boundary represents a property of the interface itself rather than the instrument resolution. However, the dimension of the boundary may be overestimated due to the unknown orientation of the boundary within the <200 nm-thick thin section. The transition in C EELS fine structure (regions of interest (ROI) 1–4 in Fig. 4a) from more aromatic (~285 eV) to alkyl C (~287 eV) was sharper than the gradual change in N and O composition (Fig. 4c).

Discussion

Taken together, these observations provide evidence for a shifting view (Fig. 1) of the hierarchy of OM spatial distribution in soil and the possible role of OM accumulation facilitated by organo–organic interactions. At the scale of a microaggregate and soil pore, OM is inherently heterogeneous in spatial distribution and composition\(^7,11–16\). The spatial separation of OM composition within an OM-rich region observed here underscores the need to consider nonlinear organic-inorganic-mineral interaction models due to patchy, disordered OM accumulation at a very fine scale. At such high resolution, we show evidence for <5–11 nm-thick layers of OM forms (Figs. 4 and 5), approaching a meaningful scale to consider the implications for the zonal model\(^20\), though still larger than the size of individual functional groups or bonds. At this scale, our observations do not contradict the existence of discrete zones (i.e., layers of OM composition). However, we highlight that the arrangement in space may not only extend in ordered layers away from a mineral surface but also at the boundaries of irregularly shaped organic patches (Fig. 1d–f). In addition, enrichment of N co-occurred with oxidized C at the mineral interface, in contrast to alkyl C at the organo–organic boundary. This distinction suggests the need to consider a combination of convergent and unique interaction pathways of organo–organic interactions in addition to the current focus only on the variations among organo–mineral interaction mechanisms.

The existence of organic patches and organo–organic interfaces with different organic C composition than those at organo–mineral interfaces generates important information for sequestering soil organic C and predicting its changes. The described organo–organic interactions may occur at a distance to or separately from mineral surfaces, pointing toward the need to consider soil organic C stabilization mechanisms that are independent of soil minerals (de-emphasizing variables such as mineral surface area or surface chemistry), and involve N-rich surfaces\(^13\). In litter decomposition, organo–organic interactions between aromatic and N-rich compounds have been linked to slower litter turnover\(^39\). Here, we suggest interactions between relatively low and high-N OM forms may also be relevant for soil OM.

The demonstrated existence of organo–organic in addition to organo–mineral interactions may also provide an avenue to help explain that saturation of soil organic C is often not clearly predictable in the field, even though SOM saturation is a theoretically reasonable and experimentally sound expectation\(^80,41\). Despite the emerging evidence that N-rich OM promotes SOM accumulation\(^35,42\), major Earth system models (e.g., the Community Land Model 5.0) treat soil N availability as primarily a limitation on decomposition\(^43\). Further, long-term N fertilization experiments in the field have shown mixed effects on SOM, ranging from decreased SOM decomposition rates and facilitation of occlusion under N fertilization\(^42\) to recent work\(^44\) showing no or inconsistent effects of N addition. Further, the distinction between SOM accumulation (i.e., amount) and persistence (i.e., turnover time) with respect to N involvement in stabilization is not well-known. The potential for system-specific (i.e., dependent on soil physicochemical properties) N effects on SOM accumulation and decomposition\(^44\) emphasizes the need to further interrogate the biophysical mechanism of SOM persistence that may be conferred by N.

The characteristics of the identified organo–mineral and organo–organic interfaces provide an incentive to perform targeted experiments testing the biogeochemical importance of organo–organic interactions in soils, including the implications of input composition varying in N functional group composition (e.g., plant and animal manures, soil fungal and bacterial necromass or metabolites). These observations also provide motivation to expand a one-dimensional model of SOM stabilization of changing OM composition with distance to mineral surfaces to a multi-dimensional model\(^45–46\), even at a very small scale as investigated here, recognizing that multi-phase organic matter does not align at a predictable gradient with respect to the soil mineral matrix.

However, the presented data on their own are not sufficient to quantify the persistence that N-facilitated organo–mineral or organo–organic interactions may confer to SOM. The link between reactive Fe and Al mineral abundance and increased persistence of bulk SOM is built on the assumption that mineral bonding limits microbial access to the substrate and slows OM desorption\(^7\). In our study, we cannot resolve the age of the OM observed (e.g., via \(^14\)C), though previous soil \(^14\)C measurements indicate that bulk OM is very old at the studied site, typically more than 6500 radiocarbon years\(^47–49\). To demonstrate a difference in persistence of OM at the mineral surface requires further methodological advancement to pair high-resolution imaging with quantification of C composition and natural abundance \(^14\)C measurements (e.g., by the advancement of monochromated EELS techniques\(^50,51\) for damage-sensitive materials). Additionally, our results provide direct evidence only of interactions between the studied mineral and organic materials, but these techniques can in the future be applied to other soil types to evaluate their importance in other systems. Future research should also include cryo-electron tomography and the development of methods that pair ultrahigh-resolution measurements with complementary imaging at the scale of aggregate cross-sections. Further work to reveal the full three-dimensional structure and spatial context of both organo–organic and organo–mineral interfaces may ultimately inform a multi-dimensional spatial model of SOM accumulation.

Methods

Overview. In this study, we identified and analyzed naturally occurring interfaces between mineral and organic phases in a natural soil sample. Our approach employed a combination of very high spatial resolution imaging by STEM and compositional analysis of organic carbon by EELS under cryogenic conditions (cryo-STEM-EELS). Due to the targeted nature of our analysis, we emphasized: (1) the likelihood of encountering such interfaces within a very small (micrometer-scale) analytical field of view (Supplementary Fig. 7), (2) how to prevent changes to the mineral–organic interface architecture due to drying or formation of crystalline ice, and (3) a method for effective preparation of the electron-transparent (~200 nm thick) thin sections necessary for STEM-EELS.

Study site and sample collection. Cryo-STEM-EELS procedures to reveal organo–organic and organo–mineral interactions were conducted using subsols with high carbon (C) and iron (Fe) content derived from volcanic parent materials (Andisols). While Andisols occupy a relatively small land area (0.7% globally), this soil type stores a disproportionately large amount of SOC (1.3% of global SOC to a depth of 1 m)\(^32\). Andisols have been studied as an archetype of reactive Fe and Al control on SOM persistence for several decades. The oldest SOM in this study system is expected between 20 and 1500 kyr of soil development\(^8\). For this study, subsols were collected from the Pololu Flow on Kohala, HI, with approximately 350 kyr of soil development from tholeiitic lavas, and contain high C, Fe, and Al (Supplementary Table 1, Supplementary Figs. 1–3)\(^34\). Subsoils were chosen for analysis due to their expected increased mineral-associated SOM content, older radiocarbon ages, the lower contribution of particulate OM, and reduced signal of land use or vegetation\(^23,53–55\). Soil pits were excavated by hand to ~1 m and sampled by the pedogenic horizon (in 2014) as described in previous
Cryogenic thin-section preparation. Background. The heterogeneity of soil provides a methodological challenge for preparing soil samples for high-resolution transmission electron microscopy, particularly for C, N, and other light elements easily damaged by high-energy analysis techniques. Specifically, sample preparation approaches are needed that: (1) maintain mineral–organic, organic–organic, and pore space spatial distribution, (2) eliminate the use of C-based resins that preclude the interpretation of C spectroscopic data, (3) reduce the risk of beam damage, (4) result in appropriate sample thicknesses (~100–200 nm) for electron transparency, and (5) avoid freeze-thaw and wet-dry fluctuations that alter mineral–OM interactions.

Performing sample thin-sectioning under cryogenic conditions—a common way to reduce sample damage for microscopy and spectroscopy of biological materials—is an approach to stabilize hydrated soil samples that precludes the need for either C-based resins or elemental S. Cryo-thin-sectioning followed by room-temperature imaging and spectroscopy is a common approach, although sample thawing and drying is often required. To address the methodological challenges above, we developed an integrated sample preparation procedure that maintained the soil aggregate in cryogenic conditions for initial sectioning, thinning to electron transparency, and STEM-EELS mapping.

Aggregate cryo-ultramicrotome pre-thinning. Cryogenic thin-sectioning (to approximately 1–5 μm thickness) was undertaken to improve efficiency and selection of regions for subsequent cryo-focused ion beam (FIB) milling to electron transparency. Subsamples (~100 g) of bulk soils from two subsoil horizons were sieved to 63 μm (dry) and 0.63 mm (wet) and added pressure to form aggregate microaggregates. This size fraction was selected because microaggregates are expected to represent the aggregate fraction with the greatest contribution to persistent carbon.

Cryogenic STEM-EELS. With the ability to converge ion or electron beams onto sample areas of approximately 100 nm × 100 nm in size, STEM provides high spatial resolution by use of a nano-beam or low-loss electrons. This is especially useful for particle size analysis using elemental mapping. In this study, STEM sample preparation and imaging (conducted in 2014–2017) included cryogenic field-emission ion microscopy (cryo-FIB) for sample transfer and thinning and cryo-electron microscopy (cryo-TEM) for sample preparation and imaging.

Cryo-FIB transfer: Pre-thinned samples were transferred from storage to the cryo-FIB stage at temperatures near that of liquid N2 and maintained in the cryo-FIB at ~80 °C. Further details describing the cryo-transfer system are published in ref. 64.

Cryo-FIB Milling: Preparation of <100–200 nm sections for STEM-EELS was completed using an FEI Strata 400 STEM DualBeam FIB (FEI Company, Hillsboro, OR) operated at 120 kV. During experiments on both instruments, the samples were maintained at an approximate temperature of ~180 °C. ROI for high-resolution imaging and analysis were selected based on the sample position (over vacuum) and electron transparency. Given these criteria were met, the analysis was conducted on visible features with contrast differences and a detectable C signal. The approach to selecting the analysis region is described in detail in Supplementary Fig. 7 (analysis workflow).

Cryo-STEM-EELS spatial resolution. Spatially, the spatial resolution of the STEM probe size, ranging from sub-Å (Titanium instrument) to ~2 Å (F20 instrument). However, when performing EELS mapping over relatively large fields of view (Figs. 3–5)—important for capturing relevant features in the soil specimen—the spatial sampling, i.e., the step size, sets the effective lower limit of spatial resolution relevant to statistical analysis of EELS data. In this study, step sizes used were an order of magnitude larger than the STEM probe size to minimize sample damage. The smallest step size used in this study was ~1 nm (10 Å), resulting in an effective spatial resolution of 2 nm by set by the Nyquist limit (Figs. 3d, 4, and 5).

Cryo-STEM-EELS damage assessment. We assessed evidence of artifacts in the C K-edge spectra (eliminating from the EELS analysis) for the organo–organic interface sample (Titanium instrument), potential sample damage from cryo-STEM-EELS was assessed by repeated measurements of a semicrystalline iron oxide.
organic material serving as reference material for the volcanic semicrystalline soils analyzed. The reference material was prepared by precipitation of ferrithrydite (nominal Fe₂FeO₅·0.5H₂O) in the presence of water-extractable organic matter (WEMO) derived from a soil organic horizon (Oa) at a 10:1 C:Fe ratio (WEMO extraction methods described in ref. 3). Precipitation of ferrithrydite was completed using a low-concentration modification of standard laboratory ferrithrydite synthesis38 and purified by dialysis (1000 D molecular weight cut-off). Ferrithrydite-WEMO suspensions were applied to Cu grids and air-dried. Samples were controlled by loading into the instrument for cryo-STEM–EELS to analyze. The reference material was prepared by precipitation of ferrithrydite in the presence of water-extractable organic matter (WEMO) derived from a soil organic horizon (Oa) at a 10:1 C:Fe ratio (WEMO extraction methods described in ref. 3). Precipitation of ferrithrydite was completed

Data availability

The analytical microscopy data that support the findings of this study are available in the Cornell University eCommons Repository with the identifier https://doi.org/10.7298/6ntt-66867.

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Author contributions

A.R.P and J.L. developed the research question and scope and wrote the paper. D.A.M., L.F.K., B.D.A.L., and M.J.Z. provided guidance in method development and data interpretations. A.E. conducted sample preparation using the cryo-ultramicrotome and provided general technical assistance and method development. B.D.A.L., M.J.Z., and A.R.P. collected microscopy and spectroscopy data, conducted data analysis, and generated plots, figures, and images in the manuscript. All authors contributed to paper revisions.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to J.L.

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