

1 Title

2 **Relating microfeatures of soil organic matter to C stabilisation: optical microscopy,**
3 **SEM-EDS, abiotic oxidation**

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Abstract

We investigated the relationships between microscale distribution of soil organic matter (SOM) features and their stability by combining optical microscopy, SEM-EDS analysis and NaClO oxidation of soil thin sections on five soils from Harwood Forest in Northumberland (UK) differently affected by water stagnation.

Plant organs at different stages of decomposition and amorphous organic matter were observed by optical microscopy in all samples. SOM microfeature distribution, size of SOM features and the relation with the C-to-N ratio suggested that amorphous features could be the end-products of organ transformation. SEM-EDS elemental analysis showed that amorphous material had higher Si:C, Al:C and Fe:C molar ratios than organs, clearly pointing to interactions with the soil inorganic phases, which contributed to SOM stabilisation. Soil porosity coupled with water stagnation seemed to affect the Fe-SOM interactions as a greater proportion of small water retention pores (10-50 μm) was associated with higher abundance of Fe-rich amorphous organic features. The higher chemical stability of amorphous features was confirmed by oxidation. After NaClO treatment organs were almost totally removed, while amorphous organic material was less affected both morphologically and chemically.

Our results demonstrate that in water-affected soils local environment defined by the pore system affects the distribution of SOM microfeatures and that the highest resistance to oxidation of the amorphous features is attributable to the formation of organic-inorganic associations. The proposed combined approach seems to be a promising means to investigate SOM dynamics by relating features to stability.

1 **Introduction**

2 The capacity of soil to act as a carbon sink and counteract the increasing concentration of
3 CO₂ in the atmosphere is widely recognized. In soil, fresh organic material is stabilized at
4 varying rates and to a varying degree, thus organic C mineralization is limited and various
5 pools of soil organic matter (SOM) accumulate. SOM biodegradation is strongly dependent
6 upon the interactions between the microbial community and its local environment that
7 regulate many of the soil functions carried out by microorganisms (Hassink 1992; Strong et
8 al. 2004; Chaparro et al. 2012). In particular, adequate quantities of available water and
9 oxygen are required to optimize the process of SOM transformation. Since the pore space and
10 the pore distribution of the soil matrix control the availability of fluids, soil architectural
11 properties exert a control over SOM decomposition and mineralization (Baldock and
12 Skjenstad 2000). The interactions between organic matter and inorganic soil components
13 further alter the rate of decomposition, as the associations with the inorganic soil phase
14 increase the energy required to break chemical bonds (Sollins et al. 1996). On the other hand,
15 C stabilisation is also linked to SOM quality and to the presence of primary or secondary
16 recalcitrant substances that contribute to active and passive pools of SOM (von Lützow et al.
17 2008).

18 Detailed analysis and micromorphological description of soil thin sections can allow
19 the direct study of different features of SOM in undisturbed soil samples at microscopic scales
20 (Stolt and Lindbo 2010). According to Babel (1975), the organic constituents visible in soil
21 thin sections can be categorized as either organ, tissue or amorphous in form. SOM fragments
22 can be further described according to the extent of their decomposition following
23 FitzPatrick's classification (1984) or based on their colour (Bullock et al. 1985). The
24 micromorphological classification of organic components is based solely on the morphology
25 of the particles and ignores other criteria such as chemistry. However, the combination of

micromorphological observations with other techniques may be useful for developing functionally different morphologic classes of soil C characterized by different turnover times (Blazejewski et al. 2005).

In disturbed soil samples, several methods have been used to fractionate or isolate SOM into functionally different pools. Among the oxidative methods, the use of NaClO was proposed to remove younger SOM and to leave behind the stable residue (as reviewed by von Lützow et al. 2007). Recently, Mikutta and Kaiser (2011) suggested that the C fraction that resisted to abiotic oxidation consisted of the organic compounds with greater affinity for mineral surfaces. By treating the samples with NaClO, Bonifacio et al. (2013) indeed found an increasing trend in the interactions between surfaces and organo-metallic associations with increasing podzolisation.

To the best of our knowledge, NaClO has never been employed on thin sections to specifically isolate and study stable SOM, although it has been used as an effective bleaching agent to distinguish brown-dark amorphous organic materials from other amorphous constituents in micromorphological studies (Babel 1964). Furthermore, Glab (2007) applied NaClO treatment during soil section preparation in order to increase the image contrast. Babel (1975) stated that in 20 to 30 μm thick sections the organic parts were nearly completely decolorized by NaClO treatment at room temperature.

In this paper, we investigate the relationships between microscale distribution of SOM features and their stability in undisturbed soil samples through the combination of optical microscopy, SEM-EDS analysis and NaClO oxidation of soil thin sections. More specifically we discussed the suitability of morphologically defined SOM fractions to describe functional SOM pools and tested the suitability of this new methodological approach by evaluating if; a) the resistance to NaClO oxidation differed among SOM features, and b) SOM features and stability were linked to interactions with the mineral soil phases. We also tried to relate

morphologically defined SOM features to the soil pore system as an indicator of the complex environment in which SOM degradation occurs.

Materials and Methods

Soil samples

The study area is located in the Harwood Forest in Northumberland (55°12'N, 02°01'W, UK). Depending on geomorphological position, the soils differently suffered from water saturation, which is expected to influence soil C dynamics. Soils on gentle slopes (<10°) were water saturated at surface (down to +40 cm) because of a stagnant perched water table, while those located in a geomorphological basin were saturated with a seasonal groundwater and the topsoil was less affected by water saturation, as the upper limit of saturation was at least 20 cm below the surface (Payton and Palmer 1990). Soils on gentle slopes showed a stagnic colour pattern, while those located in the basin showed a gleyic colour pattern and, consequently, both Stagnosols and Gleysols (IUSS Working Group WRB 2006) occurred. Three Histic Stagnosols (P2, P4 and P5) and two Haplic Gleysols (P7 and P9) were selected. The soil pits for each soil type were dug at a distance of around 10-20 m, and the two soil types were 4.5 km apart. Both soil types developed in glacial till deposits overlying Carboniferous sandstone, mudstone and limestone belonging to the Alston Formation (British Geological Survey 2012) within grassy clearances in mature (>40 year) Sitka spruce (*Picea sitchensis* (Bong.) Carr.) plantations, thus the sites were dominated by low-growing vegetation of grasses, mosses and herbs. Soil texture was sandy loam in the surface horizons, whereas in the deeper horizons the amount of silt increased and soil texture was silt loam.

Undisturbed soil samples of A surface horizons were taken from each soil profile at a depth of 5-15 cm from the mineral soil surface. Bulk samples for soil characterization were

1 taken from alongside the undisturbed samples (Smith 2010), and their main chemical
2 properties are reported in Table 1. As evident from the Al and Fe fractionation, in the 5-15 cm
3 layer of Stagnosols poorly crystalline Al and Fe forms (Al_O and Fe_O) constitute almost the
4 totality of the pedogenic oxides (Al_D and Fe_D) further supporting the differentiation between
5 saturated and unsaturated conditions at soil surface.

6 From intact soil blocks, two thin sections of approximately 7x5 cm were produced at
7 the University of Stirling following a modified protocol (Murphy 1986). Water was removed
8 from the soil samples by freeze-drying under vacuum conditions to avoid C losses during the
9 acetone replacement drying process. No accommodating crack voids were identified in the
10 vacuum dried thin sections, suggesting that shrinkage and cracking during the drying process
11 had been minimal. Epoxy resin was used for soil sample impregnation. Epoxy resin cures at
12 normal laboratory temperatures, reducing the danger of reaction between resin and the organic
13 components of the sample due to heating (López-Buendía 1998).

14 *Micromorphology observations, image analysis of soil pores and organic components*

15 Soil thin sections were observed using a polarizing microscope (Olympus BX50) under plane
16 (PPL), crossed polarized light (XPL) and oblique incident light (OIL). Conventional
17 descriptions of thin sections were made at 40x following the guidelines of Stoops (2003).

18 High resolution mosaic images were captured at 40x using a three-chip video camera
19 (HV-C10, Hitachi Densai, Japan) and a motorized stage (Lang, Hüttenberg). Two images
20 were stitched using the multiple image alignment function of the AnalySIS software (Soft
21 Imaging System, Münster) as described in Adderley et al. (2002). Mosaic images were
22 produced to form a contiguous area of interest (AOI) of 12 mm² as required to meet the
23 representative elemental area (REA; VandenBygaart and Protz 1999). To determine the size
24 of the REA, a preliminary investigation was carried out by measuring the pore area and
25

1 perimeter in three successive areas of different size and verifying that the parameters did not
2 change $\pm 10\%$ relative to the next greater area of measurement. For each thin section, at least
3 two AOI were studied.

4 Binary pore images were obtained based on the RGB threshold, with pores represented
5 by white and soil matrix by black pixels. The minimum size for pore detection was set at 100
6 μm^2 . Pore distribution was measured according to pore shape and equivalent diameter. Pore
7 shape was expressed by a shape factor, calculated as $(\text{perimeter}^2/4\pi \times \text{area})$ and pores divided
8 into rounded (shape factor 1-2), irregular (shape factor 2-5) and elongated (shape factor >5) as
9 proposed by Pagliai et al. (2003). Pores of each shape group were further subdivided into
10 equivalent diameter classes (Greenland 1977).

11 The soil organic components were classified using the procedure described in Smith
12 (2010). Briefly, once organic features were classified according to their form following the
13 recommendation of Babel (1975), the extent of their decomposition was identified using the
14 classification proposed by FitzPatrick (1984). Dark organic features and other dark
15 components, typically rich in Fe or Mn oxides, were distinguished by the elemental
16 composition obtained using the SEM-EDS. No charcoal or coal fragments were detected
17 during optical observations.

18 On the same AOI studied for soil porosity, image analysis was used to manually
19 delimit the different SOM features within PPL images. Organic fragments were selected, and
20 the frequency and area of classes of organic fragments were measured. By exporting images
21 into an image manipulation program (GIMP 2.6) each organic feature was represented by
22 differently coloured pixels and stacked upon the binary pore image thereby forming a map
23 showing the distribution of organic matter in relation to soil pores (electronic supplement 1a-
24 b).

SEM and EDS analysis

Soil thin sections were analyzed using an environmental scanning-electron-microscope (ESEM) and elemental data were collected by energy-dispersive spectroscopy (EDS) detector using ZEISS SEM systems (EVO MA15) linked to an Oxford Instruments INCA X-max detector with a 80 mm² SDD. Since C was the principal element of interest, low vacuum conditions (>30 kPa) were used to control charging without C coating the sample. The obtained backscattered electron scanning images (BESI) were of mediocre quality, therefore the location of features was checked using OM-pore maps generated by image analysis. Optimal C detection was ensured using an accelerating voltage of 5-15 keV (Goldstein et al. 2003), a working distance of 8.5 mm, and a probe current of ~ 700 pA. To minimize any topographic effects impairing X-ray detection, the thin sections were polished (Goldstein et al. 2003) and EDS analysis was performed at high magnifications (500-1000x). Data were normalized to 100% giving a semi-quantitative measure of elemental concentrations, thus elemental ratios are discussed in this paper rather than absolute concentrations. To verify the absence of any resin effect on the sensitivity to oxidation of organic matter, the elemental composition of five organs and six dark organic features was measured by point analysis close to the feature edge (where the resin effect should be the greatest) and inside, approximately in the feature core. The measurement was then repeated after the NaClO treatment. Both before and after NaClO treatment, each pair of measurements had similar values of O:C ratio (see electronic supplement 2). Overall no relation between the variation of O:C value after the NaClO treatment and the distance from the feature edge was found. This preliminary data confirmed the absence of resin-organic matter interactions at the edge of organic features and that any effect on the response to NaClO treatment was avoided.

NaClO treatment

We applied the standardized NaClO treatment for disturbed soil samples (i.e. sieved) suggested by Siregar et al. (2005) to thin sections. Babel (1964) suggested 90 minutes of NaClO treatment for bleaching thin sections, while Siregar et al. (2005) treated sieved soil samples for 6 hours for three times, thus different treatment durations were previously tested to check and optimize the efficiency of organic C removal by NaClO on two test thin sections collected from soils different in texture (electronic supplementary material 3). After 90 minutes, decolourisation of slightly decomposed organs was already visible by microscopic observation. After repeating the treatment for longer periods (4.5, 12 and 18 hours) some additional C removal occurred, as clearly visible (especially for strongly decomposed organs) checking from morphological changes. However, prolonged treatment time did not further affected the SOM features. Therefore, we fixed the treatment time to 18 hours (i.e., 6 hours for three times).

As a consequence of the preliminary tests, the following routine procedure was applied: after microscopic observation of the soil thin sections, the samples were immersed in a beaker containing 6% NaClO solution (wt/wt) adjusted at pH 8 and stirred for 6 hours. The amount of solution was sufficient to cover completely the surface of the thin section. The treatment was repeated two additional times. After the last treatment, the soil thin sections were removed from the beakers, washed until electrical conductivity was lower than 40 $\mu\text{S cm}^{-1}$ and air dried.

Results and Discussion

Micromorphology and image analysis

Pore shape and equivalent diameter distribution measured by image analysis are reported in Figure 1. Water saturated soil samples, especially P4 and P5, had a high amount of rounded

1 and irregular pores ranging from 10 to 50 μm . They act as a water reservoir for plants and
2 microorganisms according to Greenland (1977). In contrast the samples less affected by water
3 saturation, especially P7, had a high proportion of large elongated pores ($>200\text{ }\mu\text{m}$). These are
4 transmission pores, important in maintaining good soil structure conditions and in soil
5 drainage (e.g. Greenland 1977; Pagliai et al. 2003). Therefore, in P4, P5 and P7 especially, the
6 pore distribution further emphasized the different water flow and saturated conditions already
7 discriminated by the occurrence of water saturation. The contrasting pore distribution could
8 affect the processes of transformation of organic C because with increasing water content
9 water becomes more available to decomposer organisms, but oxygen availability decreases
10 (Smiles 1988; Baldock and Skjemstad 2000). This means that in P4 and P5, but also in P7,
11 although for opposite reasons in pore distribution that could cause an unbalance between
12 oxygen and water availability, the process of decomposition and mineralization of organic C
13 may not be optimized, slowing down the turnover of SOM.

14 Plant materials showed evidence of decomposition through the loss of cell structure
15 and the fragmentation, ranging from slightly to strongly decomposed organs of size $>100\text{ }\mu\text{m}$
16 (Figure 2). The amorphous components were present as separated aggregates in a dense
17 groundmass or within the void spaces between coarse grains (Figure 2). These organic
18 features were identified by their isotropism and strong brown or red colour. Both brown- and
19 red-coloured amorphous organic matter had smooth surfaces and ranged in size from 20 to 50
20 μm . They corresponded to the organic masses described by Blazejewski et al. (2005).

21 The slightly decomposed organs were present in all the samples, with the exception of
22 P9 (Figure 3). However, this sample (P9) showed the highest content of strongly decomposed
23 plant residues matching the organ content of the other samples. Black amorphous organic
24 material was more frequent in the samples less affected by water, i.e. P7 and P9 samples;

1 while the reddish amorphous organic matter was the dominant feature in P5, but lower
2 amounts were also observed in P4 and P7.

3 The occurrence of both organs and amorphous organic matter in the same samples
4 suggested that the latter could be the end-product of transformation processes of SOM and
5 therefore, it should likely be less labile than the initial C forms. A common origin of organs
6 and amorphous material was indicated also by their size (always $>20\ \mu\text{m}$), in fact the sand-
7 silt fractions of temperate soils are dominated by SOM deriving from plant material, in
8 contrast to the clay fraction which is dominated by microbially derived metabolites (von
9 Lützow et al. 2007). The C:N ratio is a simple and rather common indicator of the whole
10 SOM pool turnover (Bronick and Lal 2005), and showed a positive relationship with the ratio
11 between the area occupied by organs and that by amorphous C features (TOC/TN vs. area
12 organs/amorphous C, $r_s=0.975$, $p=0.005$; Figure 3). P4, P5 and P7 had a lower
13 organs/amorphous features area ratio corresponding to lower TOC/TN ratio than P2 and P9.
14 This means that in P4, P5 and P7 SOM is more transformed and that a greater accumulation
15 of transformed or partly transformed organic compounds (i.e. amorphous) occurred than in P2
16 and P9. However, even if the TOC content (Table 1) was rather similar and the soil samples
17 were collected under similar vegetation cover, we cannot exclude that other factors, such as
18 different amounts of root derived C or physical disconnection between decomposers and
19 organic matter (Schmidt et al. 2011), could contribute to the high proportion of organs in P2
20 and P9.

21 As SOM decomposes, it becomes depleted in labile fractions, and the most recalcitrant
22 C remains, therefore we expect a variation in C stability related to organic features
23 discriminated through micromorphology (Blazejewski et al. 2005).

24
25 *SEM and EDS analysis*

1 The backscattered electron scanning images (BESI) were compared to the OM-pore maps
2 generated by image analysis to verify the location of organic features (electronic
3 supplementary material 2). The elemental analysis showed that neither the O:C nor the Si:C
4 molar ratios of each organic feature differed between soil water saturation conditions or soil
5 samples (ANOVA p level always >0.10 ; Table 2). The elemental data allowed instead the
6 separation of the SOM and resin as the O:C molar ratio was significantly lower in the resin
7 than in any other organic component (Table 2). Using the OM-pore maps and checking the
8 O:C ratio, we can be confident that an organic feature other than resin has been analyzed.

9 Slightly and strongly decomposed organs had similar mean O:C ratios of 0.30-0.29
10 (Table 2). Amorphous red organic matter had a similar O:C ratio to organs while black
11 amorphous features had a higher mean O:C ratio, though not significantly different from the
12 reddish features. Therefore, this criterion (O:C ratio) appeared to be too weak to discriminate
13 among organic features. Higher values of the O:C ratio were expected in the amorphous
14 features as a result of SOM transformation processes. However, besides being influenced by
15 the increase in oxidation of organic matter during decomposition and humification processes,
16 the O:C ratios also strongly depend on the source of the organic residues. For instance, the
17 O:C ratio reported for lignin and cellulose, two of the most abundant biopolymers in soil
18 (Kögel-Knabner 2002), were 0.37 and 0.83 (Stoffyn-Egli et al. 1997). Differences in their
19 proportion in slightly and strongly decomposed OM could strongly affect the O:C ratio of the
20 derived organic features.

21 In contrast to O:C ratio, significant differences in mean Si:C molar ratio were found,
22 providing a differentiation between organs and amorphous features which was related neither
23 to the soil water saturation condition nor to the soil sample (Table 2). Amorphous features
24 also showed the highest Al:C and, as visible in Figure 4a, strong significant correlations
25 between Al:C and Si:C ratios were found for amorphous black and red organic matter ($r=$

0.743 and 0.794, respectively), but not for resin and organs. These data suggested that some interactions between amorphous features and mineral particles, likely Al-silicates, have occurred. Consequently, these organic features could be further stabilized due to binding to minerals and organic-inorganic interactions, in addition to the transformations of organic compounds depicted by morphological evaluation.

No relationship between Si:C and Fe:C ratio was found (Figure 4b), suggesting that low amounts of Fe-bearing silicates occurred and that Fe was concentrated in (hydr)oxides. Moreover, in contrast to Si:C, in the Fe:C molar ratio significantly varied among the soil samples with P4 and P5 having the highest Fe:C ratio for black and red amorphous materials, respectively (Figure 5a). The Fe-enrichment of amorphous SOM features in P4 and P5 suggested that the soil water conditions affected the Fe-SOM interactions. However, water stagnation did not seem to be the only factor affecting Fe-SOM interactions. In fact, the amorphous SOM features of P2 were not Fe-enriched and no relationships were found between the Fe:C molar ratios and the content of poorly crystalline Fe (hydr)oxides of the bulk soil (Fe_O ; Table 1). Several mechanisms may be responsible for Fe-SOM interactions. The importance of sorption of SOM on poorly crystalline Fe (hydr)oxides in soils with stagnant and gleyic colour patterns has been suggested by Wiseman and Püttmann (2005), and a large adsorption, and consequent stabilisation, of organic matter on iron (hydr)oxides is indeed expected at the acidic pH of the investigated soils (Table 1) as stated by Schwertmann and Taylor (1989). Co-precipitation of organic matter and Fe can also occur especially if interchanging redox conditions prevail (Schwertmann et al. 2005; Kalbitz and Kaiser 2008). Reduction processes require access to surface sites on the oxide; therefore, binding of ligands to Fe(III) oxides can potentially decrease the rate of Fe(II) production by limiting access to surface sites (O'Loughlin et al. 2010). Adsorption and co-precipitation therefore may on one side stabilize organic matter through the increase of the energy required to break chemical

bonds, on the other hand, the presence of organic coverings on oxide surfaces may make them inaccessible to microorganisms, thus preserving Fe(III) even in water saturated soils. Under reducing conditions at more neutral pH values than those found in our samples, Henneberry et al. (2012) motivated the lack of release of dissolved organic matter from organo-Fe complexes by a re-conformation of OM around the positively charged Fe. A mutual stabilisation process may therefore act between organic matter and Fe oxides also in our samples.

Iron mobilization was expected to be more intense in the water saturated samples because of oxygen deficiency resulting from stagnating water at the soil surface. In P4 and P5, water stagnation was coupled with a high proportion of water retention pore class (i.e. 10-50 μm) thus affecting redox processes (e.g., Taina et al. 2010). Therefore, only upon the simultaneous presence of water stagnation and a high amount of small-sized pores could Fe dynamics be affected and the mutual preservation of both inorganic and organic phases be enhanced.

However, independent of the specific mechanism, the stabilisation of SOM by interaction with the mineral-metal phase should contribute to preserve it against the NaClO treatment. Differences in the resistance to chemical oxidation between organs and amorphous features were therefore expected as a result of both the selective preservation of recalcitrant compounds due to the SOM transformation process, and the interaction with the inorganic phase.

Analysis on NaClO-treated thin sections

After treating with NaClO all the organs, both slightly and strongly decomposed, were almost totally bleached (Figure 2d-e and electronic supplement 2) without any visible effect on the soil groundmass. In contrast, the effect of the NaClO on the amorphous features was clearly

1 less marked. Only a slight fading of the contour of some amorphous black organic masses was
2 noticed, while the most part of both black and red amorphous features did not show any
3 differences before and after the NaClO treatment (Figure 2d-e and electronic supplement 1).

4 Optical observations were confirmed by the SEM-EDS analysis. The organic material
5 was removed from organs and the O:C molar ratio following treatment no longer
6 discriminated between the bleached features and resin (Table 3). Conversely, organic matter
7 of the amorphous features still showed a significantly higher O:C molar ratio than resin.

8 Although the amorphous organic features were only very slightly affected by the NaClO
9 treatment, and indeed the ratio between O and C did not change, the range of this molar ratio
10 was much wider (Tables 2 and 3), particularly with respect to the maximum values. This
11 finding suggests that, within the amorphous compounds some parts may be slightly affected
12 by NaClO treatment, in agreement with the contour fading identified by optical investigations
13 that could represent transformation in more oxidized compounds. The alkaline pH of NaClO
14 treatment could have induced a partial desorption of SOM from mineral surfaces in our
15 undisturbed soil samples. However, amorphous features were generally a chemically stable C
16 pool, and this was confirmed by the values of the Si:C and Fe:C ratios, which were little
17 affected by the NaClO treatment (Table 2 vs 3 and Figure 5a vs 5b, respectively).

18 Mikutta and Kaiser (2011), studying some preparations of organic-mineral
19 associations, observed that the NaClO labile organic C did not represent the SOM available
20 for microbial degradation, and that the NaClO oxidation resulted in an enrichment of C bound
21 to the mineral phase. Our results confirmed this observation and indicated the suitability of
22 the oxidation treatment to isolate stabilized SOM in undisturbed samples. Furthermore the
23 lack of variations in the Si:C and Fe:C molar ratios points to the importance of the organo-
24 mineral interactions as an important SOM stabilisation mechanism in the studied soils.

Conclusions

The micromorphological observations of SOM indicated that various mechanisms must be considered for C stabilisation, such as the transformation process of SOM and/or the formation of organic-inorganic associations. The NaClO treatment enabled us to relate differences in C stability to SOM features, and to assess that the highest resistance to oxidation of soil carbon in the amorphous features was related to organo-mineral interactions. At microscopic scales of investigation, the expression of water saturation conditions indicated by the morphological characteristics of soil horizons is insufficient to predict C dynamics, whereas porosity data contributed to a better understanding of the distribution of stabilized SOM features, particularly when interactions with Fe forms are considered. The proposed combined procedure seems to be a very promising approach to investigate soil C dynamics in undisturbed samples, isolating SOM stabilized by the interactions with the mineral soil phases, and in future research it could provide new insights into the complexity of soil physical, chemical and biological processes.

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