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Advanced analytical techniques for studying the morphology and chemistry of Proterozoic microfossils

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Abstract: This contribution outlines the suite of advanced multiscalar techniques in the toolkit of the modern Proterozoic palaeobiologist. These include non-intrusive and non-destructive optical, laser and X-ray based techniques, plus more destructive ion beam and electron beam based methods. Together, these provide morphological, mineralogical and biochemical data at flexible spatial scales from that of an individual atom up to that of the largest of Proterozoic microfossils. Here we provide a description of each technique, followed by a case study from the exceptionally preserved Torridonian biota of Northwest Scotland. This microfossil assemblage was first recognized over a century ago, but its great

32   diversity and evolutionary importance has only recently come to light, due in no small part to

33   the research efforts of Martin Brasier.

34

35   **Running Title:** Proterozoic microfossil techniques

36

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39   TRANSMISSION ELECTRON MICROSCOPY–X-RAY TOMOGRAPHY–FOCUSSED

40   ION BEAM–LASER RAMAN–CONFOCAL LASER SCANNING MICROSCOPY–

41   SECONDARY ION MASS SPECTROMETRY–3D VISUALISATION

42

43   **INTRODUCTION**

44   Modern palaeobiology primarily exists to discover, describe, and decode the ancient

45   biosphere, and understand the course of global evolutionary change. Stemming from its roots

46   in Victorian natural history, palaeobiology has made good use of technological advances to

47   shed light on new discoveries (see Sutton *et al.* 2014, Wacey, 2014 and references therein),

48   and to reveal previously unimagined details in historical material (Brasier *et al.* 2015). As

49   with any modern field of science, palaeobiological research must continually look forwards

50   to the next potential discovery, utilizing all available tools and techniques.

51

52   Historically, major discoveries have predominantly dated from the Phanerozoic, owing to the

53   relatively well-preserved and easily recovered fossils of the macroscopic organisms alive

54   during this time. In the search for life's origins and early record, attention has inevitably

55   turned to the more poorly understood Proterozoic and Archaean fossil records. The

56   evolutionary history of these expanses of time is much less well established, as a result of the

57 shortage of exposed rock of appropriate age, relative paucity of fossil material, and

58 limitations in extracting relevant information. Fossils from these times are typically

59 microscopic, enigmatic, and poorly preserved, although a number of exceptionally preserved

60 deposits have come to characterize the Proterozoic fossil record (e.g. Torridonian biota,

61 Strother *et al.* 2011; Doushantuo biota, Yin & Li 1978). In both 'traditional' and

62 'exceptional' preservation cases, our understanding remains limited by the observational and

63 analytical techniques used to characterize these important specimens.

64

65 The approaches traditionally used to study early fossil material are essentially borrowed and

66 adapted from methods used in the study of Palaeozoic fossils, and are best suited to hard-

67 bodied macroscopic fossils or compressed organic material extracted by acid maceration.

68 However, as our understanding of Precambrian environments is fundamentally improving, it

69 has become clear that entirely different preservational styles are possible, some of which

70 require novel analytical approaches. Although many Proterozoic carbonaceous fossils can

71 still be found compressed within shales (Javaux *et al.* 2004; Agic *et al.* 2015), and extracted

72 for study by palynological acid maceration techniques, microfossil material can be hosted in

73 a variety of other media, including chert (e.g. Barghoorn & Tyler 1965), pyrite (e.g.

74 Rasmussen 2000), authigenic aluminosilicates (e.g. Wacey *et al.* 2014), and cryptocrystalline

75 phosphate (e.g. Strother *et al.* 2011). These alternative preservational styles originate from

76 the biogeochemical conditions that prevailed in specific environments or across specific

77 periods of time, and are able to exceptionally preserve microfossils of a wide range of

78 affinities in their original spatial context, often in three dimensions, and reflecting a broad

79 spectrum of taphonomic decay. In these cellular lagerstätte, challenges are posed by the small

80 scale, enigmatic nature, and relative scarcity of Proterozoic fossils, as well as by their

81 complex taphonomic and metamorphic histories. Thus, a thorough understanding of

82   Proterozoic and Archaean life necessarily calls for state-of-the-art high-spatial-resolution,

83   and holistic imaging and analysis techniques.

84

85   An increasing number of researchers are now making use of such techniques to study both

86   Proterozoic and Archaean material, revealing unprecedented levels of detail and allowing the

87   reconstruction of the complex Precambrian biosphere. It is still common, however, for these

88   different approaches to be attempted separately, often by different individual research groups,

89   which can partially preclude the synthesis of information and an overall understanding of

90   local, regional, or even global palaeoecologies. Here we present a holistic methodology for

91   studying Proterozoic fossil deposits, with a consideration of their unique preservational styles

92   and histories. A set of complementary microanalysis techniques have already been presented

93   with respect to Archaean material (Wacey 2014). However, with the expansion of the

94   biosphere (Knoll 1994), the evolution of Eukaryotic cells (Knoll *et al*. 2006), and the advent

95   of various metabolic pathways and trophic tiering (Knoll 2015), the Proterozoic fossil record

96   is more complex and - owing to its younger age (approximately 2500–540 Ma) - arguably

97   better preserved. Thus, a greater potential wealth of information might be gleaned from such

98   deposits, necessitating their study on a variety of spatial scales, as well as assessing both

99   morphology and chemistry.

100

101   The following sections detail, in a logical order for practical investigation, multiple

102   approaches for examining a Proterozoic microfossil assemblage, including: 'traditional' field

103   study and optical microscopy; X-ray based techniques including X-ray tomography and X-

104   ray spectroscopy; laser-based techniques including Raman spectroscopy and confocal laser

105   scanning microscopy; infrared spectroscopy; electron based techniques including scanning

106   electron microscopy and transmission electron microscopy; and ion based techniques

107   including focused ion beam milling and secondary ion mass spectrometry. The combination

108   of several of these techniques when investigating a single fossil deposit provides the best

109   opportunity to fully reveal the palaeocology of the Proterozoic biosphere. An example of

110   their application to the microfossiliferous rocks of the 1200-1000 Ma Torridonian

111   Supergroup of northwest Scotland is subsequently presented as a demonstrative case study.

112

113   **STANDARD PALAEOBIOLOGICAL TECHNIQUES**

114   **Field Study and Optical Microscopy**

115   A critical starting point for any palaeobiological investigation remains a comprehensive field

116   study and the preparation of candidate material for optical microscopy. As a preliminary

117   investigation, this can provide important palaeoenvironmental context, and enable the

118   quantification of the richness, morphology, and spatial distribution of fossils, plus the

119   depositional setting and taphonomic history of the fossil deposit.

120

121   Initially, a detailed sedimentological and stratigraphic study should be made of the

122   fossiliferous rocks, and those associated with them, to permit accurate palaeoenvironmental,

123   metamorphic, and tectonic interpretation. Such a study will provide regional, local, and fine

124   scale information pertaining to the location, type, and energy of the environment of

125   deposition, as well as any subsequent chemical or structural changes that may have taken

126   place since lithification. Fine scale field observations will also permit the identification of

127   candidate fossiliferous material. This may be related to specific preservational mineralogies,

128   such as cherts (e.g. ~1900 Ma Gunflint Formation; Barghoorn & Tyler 1965) or phosphates

129   (e.g. ~600 Ma Doushantuo Formation; She *et al*. 2013), or be found in association with

130   macroscopic fossil structures including siliceous and phosphatic stromatolites (e.g. ~1900 Ma

131   Belcher Supergroup; Hofmann 1976) and microbially induced sedimentary structures (e.g.

132 ~1000 Ma Diabaig Formation; Callow *et al.* 2011). Collection and documentation of

133 candidate material should be methodical, and include GPS localities, orientation data and

134 specific relationships to larger-scale structures.

135

136 From the collected samples, polished, uncovered (which are more useful than covered for

137 subsequent techniques) petrographic thin sections can be prepared for analysis using optical

138 microscopy. Ideally, thin sections should be prepared both perpendicular and parallel to the

139 bedding direction in order to capture the full spatial distribution of microscopic fossils. While

140 sections 30 µm thick are required for mineral identification using cross-polarized light, the

141 detection of fossil material may be facilitated by the use of sections up to ~150 µm-thick,

142 provided the encasing medium is sufficiently light-coloured and free of dark impurities. This

143 increases the chances of capturing entire cellular material and in-situ relationships between

144 different fossil taxa.

145

146 The primary purpose of optical microscopy is to locate and identify fossil material, and to

147 document its spatial distribution and relationship with non-biological minerals. For the

148 majority of Proterozoic carbonaceous fossil deposits, examination and imaging at all

149 magnifications up to 1000x is needed to provide a complete context. This can allow the

150 observation of fine structural details up to ~0.2 µm across, but note at highest resolution oil

151 immersion is required to increase clarity, which may be detrimental to some subsequent

152 techniques. The position of fossil material can be identified and recorded for future reference

153 using standard graticules, When fossil material is preserved with some degree of three-

154 dimensionality, focusing through the thickness of the slide can reveal its shape, organization,

155 and extent. A range of different photomicrography suites are now available for capturing

156 images of such samples (e.g., *Synchroscopy Auto-Montage* as demonstrated by Brasier *et al.*

157 2005). Many packages contain algorithms for stacking focused images from different depths

158 within a section to produce a single, focused image, or for stitching together images of

159 adjacent fields of view to produce a high-resolution 'map' of a thin section.

160

161 Using a variety of optical micrographic tools, preliminary identification and quantification of

162 fossil material may be carried out, larger-scale spatial relationships determined, and candidate

163 fossils selected for further analysis. This work is vital for the initial study of a fossil deposit

164 but the intrinsic limitations of this approach preclude its use for further, finer-scale analyses.

165 Furthermore, certain media may be unsuitable for investigation by optical microscopy. Dark

166 coloured material, or enclosing media with many impurities, for example, may mask fossil

167 details and reduce their visibility, especially through thick sections. Larger microfossils may

168 be cross cut by the sectioning process, limiting interpretation. Another limitation is that the

169 identification of chemical constituents of samples is limited to that which can be determined

170 by standard petrographic methods, and may not be sufficient for fine-grained or finely-

171 crystalline material. Since carbonaceous fossils are frequently dark-coloured, optical analyses

172 will also only be able to resolve their surface shape and structure, with the fossils themselves

173 masking any underlying ultrastructure or interior features. Thus, for a better understanding of

174 both fossil material and its preservational medium, more versatile high-spatial-resolution

175 techniques are required.

176

177 **Non-destructive moderate to high spatial resolution techniques**

178 Non-destructive techniques are here classified as those which can be applied to a standard

179 geological thin section, rock chip or rock hand sample with minimal further sample

180 preparation and do not consume or alter the specimen of interest during the analysis. Hence,

181   they can be applied to both type specimens (including holotypes on loan from museums) and

182   can be utilized as a precursor to more destructive techniques on newly discovered material.

183

184   *X-ray Computed Tomography (X-ray CT)*

185   X-ray CT maps the X-ray attenuation within a rotating sample. Data is captured as a series of

186   projections that can then be reconstructed as two-dimensional slices and then three-

187   dimensional visualisations (see Kak & Slaney 2001; Cnudde & Boone 2013 for overviews).

188   X-ray attenuation is dictated by factors such as elemental composition and density, hence X-

189   ray CT can often detect variations in the style of fossil preservation and mineralization, as

190   well as building up 3D models of entire specimens (Conroy & Vannier 1984; Haubitz *et al*.

191   1988; Sutton *et al*. 2001). The high resolution form of X-ray CT used for fossils is known as

192   X-ray microtomography (μCT), which has been utilized in palaeobiology for almost two

193   decades (Rowe *et al*. 2001; Sutton 2008). It is now routinely applied to Phanerozoic

194   vertebrate and invertebrate fossils, ranging for example from echinoderms (Rahman and

195   Zamora, 2009) to dinosaurs (Brasier *et al*. 2016, this volume), and from plants (Spencer *et al*.

196   2013) to arthropods (Garwood & Sutton 2010). The study of microfossils using CT has

197   become viable in recent years, with the use of synchrotron-based systems where more

198   intense, monochromatic X-rays, can allow improved contrast and greater spatial resolution

199   (Donoghue *et al*. 2006; Huldtgren *et al*. 2011). Recent years have also seen improvements in

200   the spatial resolution of lab-based 'micro-CT' and 'nano-CT' systems where sub-μm

201   resolutions are now possible (Hagadorn *et al*. 2006; Schiffbauer *et al*. 2012; Sutton *et al*.

202   2014).

203

204   Despite these technological advances, configuring the correct instrumental parameters for

205   μCT scanning a given microfossil specimen is challenging, and some specimens will not be

206   suited to µCT due to lack of X-ray attenuation contrast between specimen and matrix and/or

207   presence of X-ray opaque minerals. In general, µCT is applied to small rock chips. It is not

208   suited to geological thin sections because of their highly anisotopic nature, although thin

209   sections can be cut down to a more isotropic shape if permitted by the owner, or fossils

210   liberated using a micro-corer. Elsewhere in this volume, Hickman-Lewis *et al.* (2016)

211   provide several case studies of the µCT scanning of Precambrian microfossil bearing rocks

212   using two lab-based CT scanners with spatial resolutions (minimum voxel sizes) of about

213   5µm and 0.5µm respectively. They show that µCT can be a valuable tool to decode the 3D

214   petrographic context of such biological material, for example, by highlighting potential

215   organic grains and laminations, fractures within the matrix, assemblages of detrital heavy

216   minerals, and replacement of silica by carbonate rhombs (which are known to reduce the

217   quality of microfossil preservation). Detecting individual microfossils using lab-based CT

218   remains challenging unless the preservation window is particularly favourable (e.g. pyritised

219   microfossils in a silica matrix, see Hickman-Lewis *et al.* 2016, this volume). The use of a

220   synchrotron-based CT (or lab-based nano-CT) system can improve matters by providing

221   more intense X-rays and improved spatial resolution, but this requires more specialist sample

222   preparation (e.g., micro-coring) to obtain sub-mm pieces of fossiliferous rock, meaning that it

223   can no longer realistically be classified as a non-destructive technique, and could seldom be

224   applied to holotype material.

225

226   *X-ray Spectroscopy*

227   A logical extension to examining the morphology of microfossils using X-ray

228   microtomography is to investigate their chemistry using X-ray spectroscopy. A range of X-

229   ray techniques are available to characterize fossiliferous rocks, mostly performed on a

230   synchrotron beamline (see Templeton & Knowles 2009; Fenter *et al.* 2002 for overviews),

231   and utilising both hard X-rays (more penetrating with wavelengths of 1-20 angstroms and

232   photon energies over about 5-10 keV) and soft X-rays (less penetrating with wavelengths of

233   20-200 angstroms and photon energies below about 5 keV). X-ray fluorescence (XRF)

234   mapping provides semi-quantitative element-specific maps over flexible spatial scales (µm to

235   mm; e.g. Edwards *et al*. 2014). Near edge X-ray absorption fine structure (NEXAFS) and X-

236   ray absorption near edge structure (XANES) are spectroscopic techniques, using soft (low

237   energy) and hard (high energy) X-rays respectively, that excite core electrons in an element

238   (Templeton & Knowles 2009). The resulting spectra provide information on both the

239   coordination chemistry and valence of the element of interest. Scanning Transmission X-ray

240   microscopy (STXM) uses soft X-rays to obtain both spectral data and images of this spectral

241   data (e.g., maps of the spatial distribution of specific elements, valence states, or functional

242   groups) at the nm-scale, created by rastering samples through an X-ray beam at stepwise-

243   increasing incident X-ray energies to cover the absorption edges of the elements of interest

244   (e.g., Lawrence *et al*. 2003). Although these types of analyses do not destroy the specimen,

245   specialist sample preparation (e.g., micro-cored rock chips; doubly polished thin sections no

246   more than about 100 µm thick) means that permission for holotype specimens to be analysed

247   in this way is unlikely to be granted. Beam damage can also affect subsequent chemical

248   analyses.

249

250   In terms of Proterozoic microfossils, much of the interest in X-ray spectroscopy surrounds

251   the chemical bonding of carbon. The energy resolution of NEXAFS/XANES is excellent

252   (~0.1eV) so closely spaced peaks can be resolved. Hence, carbon bound, for example, in

253   aromatic groups, aliphatic groups, ketones, peptides, carbonyls, carboxyls and carbonate can

254   all be distinguished from one another (Bernard *et al.* 2007). Such spectra may help

255   characterise cellular versus extracellular organic components, while interfering signals from

256   carbonate minerals can be subtracted. De Gregorio *et al*. (2009) applied this methodology to

257   powders of organic material from the 1878 Ma Gunflint Formation, showing that

258   polyaromatic carbon, carboxyl and phenol groups had all been preserved in this ancient

259   kerogen. Similarly, the bonding characteristics of other elements common in organic material

260   (e.g., S, N, P, O) may help to determine whether they are present as organic or inorganic

261   forms in ancient fossiliferous rocks. For example, Lemelle *et al*. (2008) used XRF to quantify

262   the amounts of sulfur within the cell walls of coccoid microfossils from the ~750 Ma Draken

263   Formation, Svalbard, before using XANES to determine sulfur speciation. They showed that

264   the sulfur was a reduced organic form most likely belonging to a thiophene-like compound.

265

266   *Confocal Laser Scanning Microscopy (CLSM)*

267   CLSM provides high-spatial-resolution morphological data (<100 nm is possible) permitting

268   the visualisation of microfossils in three dimensions (see Sutton *et al*. 2014; Halbhuber &

269   Konig 2003 for overviews). Under ideal conditions data collection from standard polished or

270   unpolished geological thin sections is rapid and CLSM is able to resolve tiny morphological

271   features that may be unclear or hidden when viewed under light microscopy, as well as

272   giving a true 3D perspective to the distribution of microfossils (Schopf *et al*. 2006; Cavalazzi

273   *et al*. 2011). However, natural samples are rarely ideal for the application of this technique.

274   CLSM relies on the fact that organic material auto-fluoresces when excited by a laser of a

275   specific wavelength. The system can accurately focus and scan at different depths within a

276   microfossil specimen, and exclude the fluorescence from outside the plane of focus; 3D

277   images are then built up combining data acquired from successive planes of focus (see Amos

278   & White 2003). Hence, anything that interferes with the transmission or detection of this

279   signal severely degrades the quality of the final images obtained. For example, specimens

280   situated a long way below the surface of a thin section, or having thick opaque walls will not

281   provide sharp CLSM images. Similarly a specimen surrounded by plentiful fluorescing

282   organic detritus, or embedded in a mineral that internally reflects the fluorescence signal,

283   may be problematic. The maturity of the organic material also affects the quality of the data,

284   with the auto-fluorescence signal dissipating as the organic material becomes more

285   geochemically mature and loses more of its heteroatoms (i.e. evolves towards the structure of

286   graphite). Hence, CLSM is of greatest use when applied to thin-walled organic microfossils,

287   preserved in silica (and to a lesser extent phosphate), housed in rocks of low-metamorphic

288   grade. In these cases significant insights into the three-dimensional morphology and

289   taphonomic preservation of Proterozoic microfossils may be obtained. For example in the

290   Neoproterozoic Buxa Formation, CLSM was able to demonstrate the three dimensional

291   organisation of groups of filamentous microfossils (Schopf *et al*. 2008). In the 850 Ma Bitter

292   Springs Formation and the 650 Ma Chichkan Formation notches, tears, grooves and surface

293   ornamentation were all detected in microfossils using CLSM (Schopf *et al*. 2006), while in

294   the ~580 Ma Doushantuo Formation CLSM revealed parts of fibrous tissues and cell walls

295   within fossil alga that were not visible by other means (Chi *et al*. 2006).

296

297   *Laser Raman Microspectroscopy and Imagery*

298   Raman is a versatile, non-intrusive and non-destructive *in situ* technique. It can be used to

299   identify the mineralogy of microfossils and their host rocks, and is particularly sensitive to

300   the molecular structure and geochemical maturity of carbonaceous phases such as kerogen -

301   the prime constituent of organic walled microfossils (see Beyssac *et al*. 2002 and Fries &

302   Steele 2011 for details). In addition, when utilized in confocal imaging mode, Raman can

303   provide 2D and 3D chemical and structural maps of microfossils at moderate spatial

304   resolution (potentially < 1 μm). Raman can be applied to rock chips and standard uncovered

305   geological thin sections. Data is acquired via laser excitation of the chemical bonds within the

306   sample. This excitation produces characteristic spectra depending on the minerals and

307   compounds present, and maps can be constructed of the spatial distribution of various

308   spectral parameters, including the intensity of a given peak (also sometime referred to as a

309   band), or ratios of two given peaks.

310

311   For the field of Proterozoic palaeobiology, the peaks of interest are often associated with

312   carbon. In perfectly crystalline graphite a single first order peak occurs at 1582 cm^{-1},

313   attributed to stretching of the C-C bonds in basal graphite planes (known as the 'G' or

314   'graphite' peak) (Jehlicka *et al*. 2003). Second order peaks occur at ~2695 cm^{-1} and 2735 cm^{-}

315   ^{1}. Imperfectly crystallised graphitic carbons including kerogens have additional peaks at

316   ~1355 cm^{-1} (known as the 'D1' or 'disordered' peak) and ~1620 cm^{-1} ('D2'; occurring as a

317   shoulder to the 'G' peak), and a single broad second order peak around 2700 cm^{-1}. The

318   specific position, width and relative intensities of these peaks vary somewhat depending on

319   the degree of ordering of the carbon, and these parameters have been characterised in carbon

320   of varying metamorphic grade in an attempt to use Raman as an indicator of the antiquity of

321   carbon in ancient rocks (Tice *et al*. 2004). This is by no means an exact science, since the

322   starting composition of organic material in different metamorphic terrains, both

323   geographically and temporally, may differ. Putative carbonaceous microfossils should,

324   however, exhibit very similar Raman spectral features as other carbonaceous material in the

325   same rock specimen because both should have undergone the same maturation processes.

326   Raman spectra cannot be used to unequivocally determine the biogenicity of an ancient

327   carbonaceous object because similar spectra to those of biogenic kerogens are seen in

328   laboratory synthesised abiological disordered carbonaceous material (Pasteris & Wopenka

329   2003). However, the co-occurrence of kerogenous composition with features that optically

330   resemble cellular material provides promising preliminary data regarding biogenicity that can

331   be further tested using higher spatial resolution techniques (see below).

332

333   As with CLSM, the highest quality data is obtained from specimens close to the surface of a

334   thin section, and it has been suggested that for viable 3D maps of kerogen to be produced, the

335   entire specimen of interest should be no more than 6-8 µm below the surface (Marshall &

336   Olcott Marshall 2013). The best data will come from specimens lying under translucent

337   minerals such as quartz, ~1-5 µm below the surface of a thin section; microfossils associated

338   with phases that fluoresce strongly under the laser excitation beam may not provide usable

339   spectra. Care must also be taken not to confuse the carbon signature of interest with that

340   produced by: (i) the polymer used to attach the thin section to the glass slide; (ii) any coating

341   that may have been applied to the section during previous analyses; (iii) overlapping peaks in

342   the vicinity of carbon peaks – of particular note here is the 1320 cm^{-1} peak of hematite

343   (Marshall *et al*. 2011). The carbon spectrum can also be artificially modified by using too

344   high a laser power, or by analyzing right at the surface of a thin section that has been polished

345   (Fries & Steele 2011). Both of these should always be avoided. Raman can also be used to

346   elucidate some structural information from the minerals that host putative microfossils.

347   Several minerals produce Raman spectral peaks that vary in intensity depending on their

348   crystallographic orientation relative to the incoming laser. This feature can be used, for

349   example, to image the distribution of quartz crystallographic axes to see whether putative

350   microfossil material occurs between grain boundaries, is enclosed by entire grains, or occurs

351   in cracks (Fries & Steele 2011).

352

353   Examples of Raman applied to Proterozoic microfossils include a study by Fries and Steele

354   (2011) who mapped the carbon D/carbon G peak intensity ratio (an indicator of graphite

355   domain size) to show micron-sized variation in the structure of kerogen within and around

356   examples of *Huroniospora* from the 1878 Ma Gunflint Formation. This potentially reflects

357   initial heterogeneities in the biological material. Also within the Gunflint Formation, Wacey

358   *et al.* (2013) used Raman to demonstrate that *Gunflintia* microfossils were dominantly

359   carbonaceous in composition, but were preserved as pyrite in microenvironments where

360   anoxia had permitted pyrite formation via the metabolic activity of sulfate reducing bacteria.

361   Raman has been used extensively by Schopf and colleagues to characterize Proterozoic

362   microfossils (Schopf *et al.* 2005, 2008; Schopf & Kudryavtsev 2005, 2009), culminating in

363   the Raman Index of Preservation (RIP). This correlates the geochemical maturity of the

364   kerogen, the fidelity of microfossil preservation, H/C and N/C ratios of organic material, and

365   the metamorphic grade of the rocks, and includes examples from 22 chert units ranging in

366   age from 400 Ma to 2100 Ma (Schopf *et al.* 2005).

367

368   *Micro-Fourier Transform Infrared Spectroscopy (micro-FTIR)*

369   Micro-FTIR is a vibrational spectroscopy technique that provides complementary

370   information to that obtained from organic material using Raman. In particular, it provides

371   data pertaining to the functional groups attached to carbon chains and their bonding

372   environment within organic material (Mayo *et al.* 2004; Dutta *et al.* 2013; Chen *et al.* 2015).

373   Different peaks in an IR spectrum arise due to different vibrational behaviour in the bonds of

374   groups such as CH_2, CH_3, C-N, C=O and others. FTIR can be applied non-destructively but

375   requires doubly polished thin sections, and the main drawback is currently the limited spatial

376   resolution obtainable, with recent studies reporting only a ~15 μm^2 spot size in transmission

377   mode (Qu *et al.* 2015). This is sufficient to characterize larger Proterozoic acritarchs in

378   palynological extracts (Arouri *et al.* 1999; Marshall *et al.* 2005) and groups of smaller

379   filamentous and coccoid microfossils (Igisu *et al.* 2009), but is insufficient to determine the

380   difference between, for example, wall chemistry and internal chemistry of most Proterozoic

381   organisms. The spatial resolution problem may be circumvented somewhat by using micro-

382   FTIR attached to a synchrotron beamline, where spot sizes of < 5μm have been achieved for

383   some parts of the spectra (Bambery 2016). However, this may require more specialist, often

384   extremely difficult, sample preparation (e.g. <20 μm thickness, unglued slice).

385

386   Of particular interest are data from extant microorganisms suggesting that FTIR may provide

387   'domain specific' information, whereby specific components (e.g., lipids) of different

388   domains of life (i.e., prokaryote, eukaryote, and archaea) may possess characteristic ratios of

389   CH_2 and CH_3 groups in their IR spectra (Igisu $et\ al$. 2009, 2012). This has led to FTIR being

390   used in Proterozoic assemblages in an attempt to decode the phylogenetic affinity of

391   microfossils (Igisu $et\ al$. 2009, 2014). The study of Igisu $et\ al$. (2009) analysed microfossils

392   in their mineral matrix and thus concentrated on the CHx (2500-3100 cm^{-1}) region of the

393   spectrum. This type of research is very much in its infancy and a better understanding, both

394   of the changes in CH_2/CH_3 during postmortem alteration processes, and of the spectral

395   parameters of differentiated cells in multicellular organisms, is required in order for these

396   data to become a robust domain level signature. Insufficient data currently exist for

397   comparisons of organic material from different terranes and of different metamorphic grades

398   using this technique. Nevertheless, FTIR analyses from the 850 Ma Bitter Springs Formation,

399   Australia, and 1878 Ma Gunflint Formation, Canada, suggest that organisms in these fossil

400   assemblages belong to Bacteria rather than Archaea or Eukarya (Igisu $et\ al$. 2009). Likewise,

401   combined FTIR and Raman data from the 1485 Ma Wumishan Formation, China (Qu $et\ al$.

402   2015), suggests that the organic material here is derived from prokaryote cyanobacteria, and

403   is characterised by a rather homogenous and low CH_3/CH_2. Finally, FTIR data from

404   acritarchs from the ~575 Ma Tanana Formation, Australia, suggest that $Tanarium$ are likely

405   eukaryotic micro-algae but *Leiosphaeridia* may be Bacteria (Igisu *et al*. 2009, based on data

406   presented in Marshall *et al*. 2005).

407

408   **Destructive high spatial resolution techniques**

409   *Focused Ion Beam Milling and Scanning Electron Microscopy (FIB-SEM)*

410   SEM has traditionally been of limited use in characterizing Proterozoic microfossils in

411   geological thin sections since the majority of microfossils are embedded within the thin

412   section, and below the reach of this surface-based technique. SEM has, however, provided

413   high spatial resolution morphological data from the surfaces of individual microfossils in acid

414   etched rocks, or those extracted from their host rock using acid maceration. This has

415   revealed, for example, delicate wall ultrastructure that could not be resolved under the light

416   microscope (Javaux *et al*. 2004; Moczydlowska & Willman 2009; Agic *et al*. 2015).

417

418   Recently, the use of SEM in Precambrian palaeobiology has been reinvigorated by a new

419   generation of dual beam instrument, where the user has access to both a focused ion beam

420   (FIB) and an electron beam (see Young & Moore, 2005 for overview). Thus, a highly

421   focused beam of heavy ions (usually Ga^+) can be used to sputter ions from the sample

422   surface, essentially cutting into the sample with very high (nano-scale) precision (see Wirth

423   2009 for details). The electron beam can be used to image the results. Additional detectors

424   can be inserted to image backscattered electrons (BSE) as well as secondary ones, permit

425   elemental analysis (using an energy dispersive X-ray spectroscopy (EDS) detector), or even

426   phase detection and crystallographic mapping (using an electron backscatter diffraction

427   (EBSD) detector). FIB milling can be used to cut into or through specific features in a thin

428   section or rock chip, allowing the structure perpendicular to the surface to be better visualized

429   (Westall *et al*. 2006). Furthermore, a number of sequential slices can be milled through an

430 object, with images or other data acquired after each slice is milled. The latter is termed FIB-

431 SEM nano-tomography and permits the 3D reconstruction and visualisation of microfossils at

432 very high spatial resolution (see Wacey *et al*. 2012 for details). The resolution attainable is

433 essentially dictated by the size of the object to be analysed in 3D, plus the available time,

434 although instrumental resolution limits may come into play for very small objects. Slice

435 thicknesses are set by the user and can be smaller than 50 nm, however, for practical reasons

436 100-200 nm slices have commonly been used. Proterozoic microfossils have been visualized

437 using FIB-SEM nano-tomography from the 1878 Ma Gunflint Formation (Wacey *et al*. 2012,

438 2013), ~1700 Ma Ruyang Group (Schiffbauer & Xiao 2009; Pang *et al*. 2013) and ~1000 Ma

439 Torridon Group (see below). In the former, FIB-SEM data were key in revealing

440 heterotrophic bacteria attached to, and fossilized in the act of decomposing, larger organisms

441 (Wacey *et al*. 2013). Drawbacks of FIB-SEM nano-tomography include its destructive nature

442 – the analysed specimen is completely consumed and only a digital record of its existence

443 will remain – plus the restrictive timescales involved in both analyzing objects larger than

444 about 30 μm in diameter (24 hours or more beam-time required), and in processing and

445 reconstructing the data. A number of options exist for processing and visualizing such data

446 (and data from other 3D techniques such as X-ray CT), ranging from freeware products such

447 as *SPIERS* (serial palaeontological image editing and rendering system; Sutton *et al*. 2012),

448 *Drishti* (Limaye 2012), and *Blender* (Garwood & Dunlop 2014) to more advanced (but

449 expensive) products such as *AVIZO* (www.vsg3d.com). The choice of software will depend

450 on budget, time constraints, quality of the raw data, and whether one is interested in

451 producing just images, or images plus movies (see Sutton *et al*. 2014 for an overview of the

452 options).

453

454 *Transmission Electron Microscopy (TEM)*

455 TEM covers a number of separate sub-techniques that can all be performed in a transmission

456 electron microscope. Most simply, TEM is a very high spatial resolution imaging technique,

457 capable of resolving objects separated by as little as ~0.1 nm. A standard TEM image results

458 from variable electron scattering as a beam of electrons is accelerated at high voltage through

459 an ultrathin (ideally ≤100 nm) sample; a true high-resolution image (HRTEM) is a phase

460 contrast image with atomic-scale resolution, allowing the visualisation of the arrangement of

461 atoms within a sample (Wiliams & Carter 2009). This provides information about the

462 crystallinity of a sample, its lattice structure and any defects it may have.

463

464 Sample preparation is key to obtaining high quality data, and in this regard FIB has

465 revolutionized the use of TEM in Precambrian palaeobiology. Before the advent of FIB,

466 sample preparation for TEM involved either grinding up a rock, extracting organic material

467 by acid maceration, or using ion polishing, meaning that the context of the putative

468 microfossils was often lost, it was very difficult to obtain samples of uniform (and ultrathin)

469 thickness, and contamination was widespread. FIB-milling now allows individual

470 microfossils, or even specific parts of individual microfossils to be targeted with great

471 accuracy in their host thin section, then ultrathin wafers (typically about 15 µm x 10 µm x

472 100 nm) can be extracted from below the surface of the thin section (hence eliminating the

473 possibility of contamination) and mounted on a TEM grid (see Wacey *et al*. 2012 for an

474 overview).

475

476 In addition to morphology, a number of other parameters can also be analysed on many

477 TEMs, including elemental composition, bonding and oxidation state, crystal structure

478 (leading to mineral identification), and crystal orientation. The elemental composition of a

479 sample can be determined at the nano-scale using either energy-dispersive X-ray

480   spectroscopy (EDS) or by isolating and mapping specific energy windows from an electron

481   energy loss spectroscopy (EELS) spectrum. The fine structure of peaks within an EELS

482   spectrum can also be used to shed light on the bonding and oxidation state of the element of

483   interest, for example distinguishing disordered carbon from graphite (Buseck *et al*. 1988),

484   and Fe^{2+} from Fe^{3+}(Calvert *et al*. 2005). For advanced crystallography and mineral

485   identification, selected area electron diffraction (SAED) in the TEM provides quantitative

486   information on the distances between atomic planes in crystalline materials, and permits the

487   orientation of several grains of the same mineral to be compared to one another.

488

489   In Proterozoic palaeobiology, TEM has been used for several decades with early images of

490   microfossils extracted from their host rock in the ~850 Ma Bitter Springs Formation,

491   Australia, shown in Oehler (1977). A number of studies have investigated the wall

492   architecture of Proterozoic acritarchs in an attempt to decode their taxonomic affinities,

493   because TEM can detect variations in electron density and texture of different layers within

494   cell walls at nm-scale resolution. These include studies from: the ~575 Ma Tanana

495   Formation, Australia (Arouri *et al*. 1999; Moczydlowska & Willman 2009) where recognition

496   of a trilaminar sheath structure was part of a suite of evidence suggesting the microfossils

497   were chlorophyte algae; the 650 Ma Chichkan Formation, Kazakhstan (Kempe *et al*. 2005)

498   where TEM helped to elucidate the nanostructure of carbon particles making up the cell wall;

499   and the ~1450 Ma Roper and Ruyang Groups of Australia and China respectively (Javaux *et*

500   *al*. 2004) where at least four different types of wall ultrastructure suggested a greater

501   diversity of eukaryote clades in these deposits than could have been recognised by standard

502   optical techniques. TEM has also been used to investigate the interplay of microfossil walls

503   with the minerals in which they have been preserved, with studies from the 1878 Ma Gunflint

504   Formation showing how nano-grains of silica disrupt the carbonaceous walls of bacteria as

505   they are fossilized (Moreau & Sharp 2004; Wacey *et al.* 2012), and data from the ~750 Ma

506   Draken Formation, Svalbard, showing both the cell membrane and cytoplasm of the coccoid

507   microfossil *Myxococcoides* embedded within nano-grains of silica (Foucher & Westall 2013).

508   Finally, TEM data from the ~580 Ma Doushantuo Formation, China, helped to decode the

509   relationships between preserved microfossils and the phosphate granules in which they are

510   contained and suggested that phosphate precipitation was likely to have been microbially

511   mediated (She *et al.* 2013).

512

513   *Secondary Ion Mass Spectrometry (SIMS)*

514   SIMS as applied to the field of Proterozoic palaeobiology is a surface analysis technique,

515   whereby the elemental or isotopic composition of a sample can be determined at moderate to

516   high spatial resolution and with great sensitivity (i.e. many elements can be detected even

517   when present only at the parts-per-billion level). The surface of a sample is sputtered with an

518   ion beam and the secondary ions ejected from the sample are collected and analysed using a

519   mass spectrometer (see Ireland 1995 for details). Two different types of SIMS instruments

520   are commonly used in in palaeobiological investigations:

521   1) The large radius SIMS, used to accurately determine the stable isotope ratios of key

522   biogenic elements (e.g., carbon, sulfur), plus ratios of radiogenic isotopes in order to date

523   rock formations containing microfossils (see for example Stern *et al.* 2009; Williford *et al.*

524   2013; Farquhar *et al.* 2013). Such instruments can analyse objects as small as ~10-20 µm

525   diameter and the isotopic data can have a precision better than 0.5 parts per thousand (‰).

526   2) The NanoSIMS, which has a different geometry and is thus capable of element (ion)

527   mapping with a lateral resolution down to ~50 nm (see Kilburn & Wacey 2015 for details).

528   The NanoSIMS can also make accurate isotopic measurements from objects smaller than 5

529   µm, albeit with poorer precision (generally >1 ‰) than the large radius SIMS.

530   Both forms of SIMS can be applied to surface features in standard geological thin sections

531   and rock chips, although some specialist sample preparation is needed in order that the

532   sample and appropriate standards can be correctly mounted together within the instrument.

533   This generally involves mounting pieces of thin sections or rock chip alongside analytical

534   standards in resin discs. SIMS is partially destructive in that layers of surface material (as

535   deep as ~200 nm during isotope analysis with large radius SIMS) are consumed during the

536   analysis. Small specimens may be entirely consumed by the analysis, whereas larger

537   specimens can be repolished post analysis to look like new!

538

539   A number of Proterozoic microfossils have been analysed by SIMS in the last 15 years.

540   House *et al*. (2000) were the first to determine the carbon isotope composition of individual

541   microfossils using material from the ~850 Ma Bitter Springs and 1878 Ma Gunflint

542   Formations, finding $\delta^{13}C$ signatures (-21 to -45 ‰) consistent within specific metabolic

543   pathways (namely the Calvin Cycle and acetyl-CoA). This work was recently refined by

544   Williford *et al*. (2013) who analysed microfossils from four Proterozoic assemblages

545   (Gunflint, Bitter Springs, plus ~650 Ma Chichkan Formation and ~740 Ma Min'yar

546   Formation) with greater precision and reproducibility. They were able to show considerable

547   variability of $\delta^{13}C$ within individual assemblages that may reflect the preservation of original

548   metabolic differences between different components of each biota, and also potential

549   heterogeneities in molecular preservation in single microfossils. It must be noted at this stage

550   that non-biological reactions are able to produce similar $\delta^{13}C$ fractionations (McCollom &

551   Seewald 2006), so a $\delta^{13}C$ value must be supported by a definitive biological morphology in

552   order to prove the biogenicity of ancient carbonaceous objects.

553

554   SIMS has also been used to investigate metabolic pathways involving sulfur in Proterozoic

555   organisms. Wacey *et al*. (2013) determined the δ^{34}S composition of pyritised microfossils

556   from the 1878 Ma Gunflint Formation, finding sulfur fractionations (δ^{34}S = +7 to +22 ‰)

557   consistent with pyrite formation via the activity of sulfate reducing bacteria in sulfate starved

558   sediment porewaters. In the same study, Wacey *et al*. (2013) used NanoSIMS to map the

559   residual carbon and nitrogen associated with the pyritised microfossils and found

560   reproducible differences in the preservation of organic material between two different types

561   of organism (*Huroniospora* versus *Gunflintia*). *Gunflintia* was poorly preserved which

562   suggests that it was more prone to decay by heterotrophic bacteria (that also mediated pyrite

563   formation) than *Huroniospora*. NanoSIMS mapping of organic microfossils in the ~850 Ma

564   Bitter Springs Formation has shown the co-occurrence of carbon, nitrogen and sulfur in such

565   microstructures (Oehler *et al*. 2006) and attempts have been made to quantify the ratios of

566   nitrogen and carbon (N/C) to distinguish different components of microbial communities, or

567   to distinguish biology from co-occurring abiotic organic material (Oehler *et al*. 2009;

568   Thomen *et al*. 2014) although the SIMS community has yet to agree upon the robustness of

569   these methods.

570

571   **A PROTEROZOIC CASE STUDY: THE 1200-1000 MA TORRIDONIAN LAKES**

572   The effectiveness of combining multiple high-spatial-resolution, *in-situ* techniques is

573   demonstrated here using a case study of microfossils from the 1200-1000 Ma Torridonian

574   Supergroup of Northwest Scotland. Not all techniques described above were applied to the

575   Torridonian material in order to avoid duplication of data and in order to keep costs and

576   processing time to reasonable levels. For example, we felt in this case that higher quality 3D

577   morphological data could be acquired using FIB-SEM rather than CLSM, and that detailed

578   chemistry could be better (and more cheaply) determined using TEM rather than X-ray

579   spectroscopy. Below we present data obtained from light microscopy, SEM, µCT, laser

580   Raman, NanoSIMS, TEM and FIB-SEM nano-tomography which together provide a detailed

581   characterisation of a number of components of the Torridonian biota.

582

583   **Methods**

584   *Optical microscopy*

585   Polished and uncovered petrographic thin sections of 30 µm and 100 µm thickness were

586   examined under *Nikon Optiphot-Pol* and *Nikon Optiphot-2* microscopes with 4x, 10x, 20x,

587   40x and 100x (oil immersion) lenses at the Department of Earth Sciences, University of

588   Oxford, and with a *Leica DM2500M* microscope with 4x, 10x, 20x and 50x lenses at the

589   Centre for Microscopy Characterisation and Analysis (CMCA), The University of Western

590   Australia. Images were captured using *Synchroscopy* imaging software (*Acquis* and *Auto-*

591   *montage*) at Oxford, and using *Toupview* imaging software at CMCA. Post processing, for

592   example colouring of cells in Figures 2 and 3, was carried out in *Adobe Photoshop* (*GIMP* is

593   an open source alternative).

594

595   *SEM analysis of palynological specimens*

596   Palynological samples were prepared at the Department of Animal and Plant Sciences,

597   University of Sheffield, using conventional acid maceration techniques (Grey 1999).

598   Following HCl-HF-HCl acid maceration, the residues were sieved using a 10 µm mesh. They

599   were then treated to a heavy liquid separation using zinc chloride, followed by further sieving

600   at 10 µm. The organic residues were mounted directly onto glass slides using epoxy resin.

601   SEM imaging was carried out using a *JEOL JSM-840A* SEM located at the Department of

602   Earth Sciences, University of Oxford.

603

604   *X-ray μCT*

605   CT scans were performed at the Manchester X-ray Imaging Facility using: a *Nikon Metris*

606   225/320 kV X-ray CT system in a customized bay (tungsten reflection target; current/voltage

607   of 130μA/80kV; no filtration; 3142 projections of 708 ms exposure collected with a 2000 ×

608   2000 detector; reconstructed dataset 5.1μm voxels); and a *Zeiss Xradia Versa 520* (standard

609   transmission target; current/voltage of 62μA/160kV; standard in-built, high energy 2 Zeiss

610   filter; 4x optical magnification, 501–1001 projections of exposures between 0.5 and 2

611   seconds, collected with 4x binning using a 2000 x 2000 detector; reconstructed datasets with

612   1–2μm voxel size). Additional propagation-based phase-contrast scans were performed at the

613   TOMCAT beamline of the Swiss Light Source (Paul Scherrer Institut, Villigen, Switzerland;

614   1001 projections of 700ms exposure; 37 KeV monochromatic beam; 4x objective; a LAG:Ce

615   100μm scintillator; reconstructions based on both attenuation and phase used to create

616   datasets with 1.625μm voxels). Datasets were reconstructed using the *SPIERS* software suite

617   (Sutton *et al*. 2012) following the methods of Garwood *et al*. (2012), and *Drishti* (Limaye

618   2012) following the methods of Streng *et al*. (in press).

619

620   *Laser Raman*

621   Laser Raman analyses were carried out at the University of Bergen using a *Horiba LabRAM*

622   *HR800* integrated confocal Raman system and *LabSpec5* acquisition and analysis software.

623   Samples were standard uncovered geological thin sections allowing optical and chemical

624   maps to be superimposed. All analyses were carried out using a 514.5 nm laser, 100 μm

625   confocal hole, 1800 grating and 50x objective lens. The laser was focused at least 1 μm

626   below the surface of the thin sections to avoid surface polishing effects. For mineral

627   identification from Raman spectra, dual acquisitions were taken from each analysis point,

628   each with an acquisition time of 4 s. Raman maps were acquired with a 1.5 µm spatial

629   resolution.

630

631   *TEM of FIB-milled wafers*

632   TEM wafers were prepared using two dual-beam FIB system (*FEI Nova NanoLab*) at the

633   Electron Microscopy Unit (EMU) of the University of New South Wales (UNSW), and

634   Adelaide Microscopy at the University of Adelaide. Electron beam imaging was used to

635   identify microfossils of interest in standard polished thin sections coated with c. 30 nm of

636   gold, allowing site-specific TEM samples to be prepared. The TEM sections were prepared

637   by a series of steps involving different Ga^+ ion beam energies and currents (see Wacey *et al*.

638   2012), resulting in ultrathin wafers of c. 100 nm thickness. These TEM wafers were either

639   attached to *Omniprobe* copper TEM holders or deposited on continuous-carbon copper TEM

640   grids. TEM data were obtained using a *FEI Titan G2 80-200* TEM/STEM with *ChemiSTEM*

641   *Technology* operating at 200 kV, plus a *JEOL 2100* LaB_6 TEM operating at 200 kV equipped

642   with a *Gatan Orius* CCD camera and *Tridiem* energy filter. Both instruments are located in

643   CMCA.

644

645   *NanoSIMS*

646   Ion mapping was performed using a CAMECA NanoSIMS 50 at CMCA, with instrument

647   parameters optimized as described in Wacey *et al*. (2011). Analysis areas were between 12 x

648   12 µm and 25 x 25 µm with a resolution of 256 x 256 pixels (so each pixel measures between

649   47 nm and 98 nm), with a dwell time of 5-15 ms per pixel, and a primary beam current of

650   ~2.5 pA. Secondary ions mapped were $^{24}C_2^-$, $^{12}C^{14}N^-$, $^{28}Si^-$, $^{32}S^-$, and $^{56}Fe^{16}O^-$, and charge

651   compensation was achieved by using the electron flood gun.

652

653   *FIB-SEM nano-tomography*

654   Sequential FIB milling and SEM imaging was carried out on a *Zeiss Auriga Crossbeam*

655   instrument at the Electron Microscopy Unit of UNSW, using the method of Wacey *et al*.

656   (2012, 2014). Key parameters were adjusted to suit the specific size and nature of each

657   sample of interest. In summary: initial trenches were milled using a 9 nA beam current; the

658   imaged face was cleaned using a 2 nA beam current; ion beam current for slice milling was 2

659   nA; electron beam voltage for imaging varied between about 800 V and 5 kV; step sizes

660   between slices were between 75 nm and 200 nm; and image capture times were around 30

661   seconds per frame. In some cases, dedicated trenches were milled in order to obtain elemental

662   (EDS) maps of microfossils that were not subsequently milled for 3D analysis.

663

664   In order to visualize the data, FIB-SEM images were stacked, aligned and cropped using

665   *SPIERSalign* (Sutton *et al*. 2012). The resultant stacks were imported into *SPIERSedit*

666   (Sutton *et al*. 2012) where a number of masks were added to segment individual components

667   (e.g., cell walls, cell contents) of the microfossil assemblage. The resulting files were

668   exported and loaded into *SPIERSview* (Sutton *et al*. 2012) to generate the 3D surface

669   renderings.

670

671   **Results**

672   Critically, before the Torridonian microfossils were subjected to the high-spatial-resolution,

673   *in situ* microanalysis described here, multiple seasons of fieldwork had been completed in

674   order to gain a firm understanding of the geological context of the host rocks. In addition,

675   over 100 thin sections and hand samples had been studied in order to understand the

676   depositional context and post-depositional history of the rocks, and to isolate only the very

677   best and most promising samples for further study. A large amount of optical microscopy

678   work had also been completed in order to form an estimate of the morphological diversity of

679   the biota. This work has all been peer reviewed and published (Strother *et al*. 2011; Callow *et*

680   *al*. 2011; Battison & Brasier 2012; Strother & Wellman, 2015) thus giving a firm platform on

681   which to build with this high-resolution work. A summary of some of the most common

682   components of the Torridonian biota as observed by optical microscopy is given in Figure 1

683   for reference.

684

685   *SEM data*

686   As may be expected, the range of morphologies visible in SEM analysis (Fig. 2) is broadly

687   comparable to those observed within thin sections of the phosphate (Fig.1, plus Battison &

688   Brasier 2012). Many simple vesicles and tubular morphotypes are observed, with SEM

689   imaging affording enhanced resolution of their shape and wall structure. In particular,

690   differences in the physical responses of structures to compression hint at differences in cell

691   wall architecture. Two principal wall responses are observed. Thicker walled (wall at least 1

692   µm thick) specimens accommodate flattening with broad rounded velvet-like folds, or large

693   creases (Fig. 2a). In contrast, thin-walled vesicles (<0.5 µm) accommodate compression with

694   fine wrinkles irregularly distributed across the surface, and are apparently more prone to

695   small tears (Fig. 2b). The flattening of these walls during preparation does not permit

696   resolution of any ultrastructural lamination, but synthesis of taphonomic response and wall

697   thickness may be used to enhance interpretation of microfossils studied by optical

698   microscopy.

699

700   A number of unique forms of microfossils are also observed in SEM. This is likely due to the

701   processing of larger quantities of material during preparation by acid maceration, as well as

702   the enhanced resolution afforded by SEM imaging. Of note are two morphotypes, the first

703 (Fig. 2c) comprising a vesicle around 50 µm in diameter, ornamented with regular pits

704 around 10 µm across, with each pit possessing a raised `collar' approximately 2 µm wide and

705 2 µm high. This form bears some resemblance to the basal vesicle of *Cheilofilum*

706 *hysteriopsis* Butterfield (see Butterfield, 2005 fig. 8 and fig. 10) or the freshwater green

707 microalga Botryococcus braunii (see Vandenbroucke & Largeau 2007 plate e) in its

708 possession of flanged openings. The second form (Fig. 2d) is a spherical hollow vesicle

709 around 20 µm in diameter, with a spongy textured wall, and irregularly distributed, rounded

710 or sub circular holes ~1-3 µm across. This morphotype is particularly notable for its retention

711 of three-dimensional structure following maceration, indicating significant rigidity of the

712 wall. In addition, distributed abundantly amongst the structurally distinguishable vesicles and

713 sheaths, is non-vesicular membranous organic matter, with an irregularly pustulate and pitted

714 texture, and an amorphous architecture. The size and nature of this material is likened to the

715 amorphous extra-polymeric substances (EPS) secreted by mat-forming organisms in modern

716 microbial ecosystems (cf. Pacton *et al*. 2007), but could also be amorphous kerogen. This

717 material is occasionally seen contained within thin sections as a light-walled membrane, but

718 its texture and extent is clearer under SEM analysis.

719

720 Of particular note, amongst the vesicles, sheaths and putative EPS are small coccoid or

721 baccilate forms seen to be colonizing, to varying degrees, some of the larger fossil structures.

722 These are associated with pits within those larger structures, and are apparently embedded

723 within a membrane that links them to the host fossil (Fig. 3). We interpret these forms as

724 fossils of heterotrophic bacteria preserved feeding on the larger Torridonian microbial flora,

725 and this interpretation reinforces observations made previously using light microscopy (see

726 Battison and Brasier, 2012, fig.9, where evidence for heterotrophy includes roughly circular

727 holes in large microfossil vesicles and inferred clumps of heterotrophic bacteria

728 pseudomorphing decayed vesicles).

729

730 *X-ray μCT data*

731 Microtomography was explored as a method to investigate the petrographic context of

732 cellular material and was also tested in order to determine whether individual microfossils

733 could be detected and their 3D morphology characterized. Scans of rock chips from the

734 Cailleach Head Formation using a *Nikon Metris* 225/320 kV X-ray CT system with 5.1 μm

735 voxels revealed phosphate nodules as a slightly denser phase that could be distinguished from

736 the surrounding matrix sediment (Fig 4a, purple). It also suggested that phosphate was

737 present in small quantities close to, but exterior to the main nodule. Rounded concentrations

738 of a very dense phase, most likely to be an iron rich mineral such as pyrite or iron oxide, were

739 shown to be present both within and outside the nodule (Fig. 4a, gold). Hence, CT could be

740 employed in future investigations as a pre-screen of rock fragments in order to determine the

741 best position within the rock to cut thin sections. Within the phosphate nodules, the *Nikon* CT

742 scans detected phases of lower density that may be organic microfossils. However, the spatial

743 resolution of this instrument was insufficient to determine if these lower density objects were

744 indeed microfossils or simply lower density sediment grains (e.g. quartz) scattered through

745 the phosphate nodules. Higher resolution scans of a different rock chip (with 1.625 μm

746 voxels) conducted at the Swiss Light Source demonstrate a complex sedimentary texture -

747 here both phosphate and other dense phases are present in the form of evenly spaced

748 rounded- to angular- fragments within the scanned rock chips (Fig. 4b), with no evidence of

749 well-formed nodules of phosphate. The lack of evidence for nodules suggest that this rock

750 chip would not be a promising target for further investigation of microfossils.

751

752 CT scans of a sub-portion of the sample examined in the *Nikon* instrument, performed using a

753 *Zeiss Xradia Versa 520* with voxels of c. 1.5 µm detected a small number of low density

754 objects that strongly resemble microfossils observed in thin sections (Fig. 4c-d, f-g). These

755 objects are analogous to some of the largest and darkest-walled vesicles seen in thin sections

756 (Fig. 4e, h) and CT permits their viewing from multiple orientations in 3D space. These

757 putative fossils are also frequently found close to the very high density phases (presumably

758 iron oxide or pyrite). The combined evidence suggests that µCT at this resolution is only

759 capable of detecting the largest and thickest-walled components of the Torridonian biota. We

760 also suggest that the increased density contrast when such fossils occur in close proximity to

761 iron oxide or pyrite aids detection by CT. The remaining components of the biota (e.g.

762 examples shown in Fig. 1) are essentially invisible on X-ray CT scans conducted at these

763 resolutions. The biggest challenge for future work will be identifying workflows to isolate

764 known microfossils for future scanning.

765

766 *Raman data*

767 Raman data inform upon the dominant mineralogy of the Torridonian microfossils and their

768 surrounding matrix, plus the structure and thermal history of any organic carbon present.

769 Raman maps from the Cailleach Head Formation (Fig. 5a-c) demonstrate that the

770 microfossils are indeed carbonaceous (Fig. 5b) and that the dominant fossilizing phase is

771 apatite (Fig. 5c). Raman also shows that intracellular inclusions (Fig. 5a arrows), common in

772 many of the spheroidal fossils from this formation, are also carbonaceous in composition.

773 Hence, these inclusions likely represent plasmolysed (shrunken) cell contents or, in some

774 cases, could represent a fossilised cell nucleus. Raman spectrum in the first order region of

775 carbon, show the two main bands (D1 at about 1350 cm^{-1} and G at about 1600 cm^{-1})

776 characteristic of disordered carbonaceous material. The D1 band is very broad (full width at

777   half peak maximum (FWHM) of ~120 cm^{-1}) with a shoulder at its low wavenumber side.

778   This shoulder is caused by a small band at ~1150 cm^{-1} which is only observed in very

779   disordered carbonaceous material (Marshall *et al*. 2005). The G band appears to have been

780   shifted considerably from its value in crystalline graphite (1582 cm^{-1}) to a value of ~1610 cm^{-1}

781   . This reflects an overlap of the G band with a well-developed disorder band (D2) at ~1620

782   cm^{-1}. The spectrum indicates that the carbonaceous material has very weak structural

783   organization, has experienced little or no metamorphism (cf. Wopenka & Pasteris 1993), and

784   is consistent with previously suggested maximum heating of only ~100 °C (Stewart & Parker

785   1979).

786

787   Not all microfossils are preserved purely as carbon. In the Stoer Group, Raman reveals that

788   significant portions of microfossil walls have been pyritised, although some carbonaceous

789   signal remains (Fig. 5d-f). The matrix mineralogy is also different here, with typical phases

790   including calcite and albitic feldspar (Fig. 5g-h). These data indicate that different suites of

791   lakes within the Torridonian had different chemistries, with those of the Stoer Group being

792   sulphate-rich and phosphate-poor compared to those of the Cailleach Head and Diabaig

793   Formations (see Parnell *et al*. 2016, this volume, for further details on contrasting fossil

794   preservation in these lakes).

795

796   *NanoSIMS data*

797   NanoSIMS was used as an additional tool to determine whether the microfossils were

798   composed of carbonaceous material and then to determine if any additional elements of

799   biological interest were preserved within their cell walls or intracellular space. NanoSIMS

800   uniquely revealed significant (but not quantifiable) amounts of nitrogen and sulfur within

801   cellular material from the Diabaig Formation (Fig. 6). These data were collected from FIB-

802 milled wafers and so the nitrogen and sulfur come from cell walls located below the surface

803 of a thin section; this negates the possibility that these biological signals come from surface

804 contamination and provides an improvement on previous NanoSIMS methodology where

805 such ion mapping was performed on surface features (e.g. Oehler *et al*. 2006, 2009). The co-

806 occurrence of C, N and S in microstructures that have cellular morphology is strong evidence

807 of the biogenicity of such structures; while this is less relevant to the Torridonian material

808 whose biogenicity is well accepted, it is a very useful tool for the investigation of older

809 and/or more controversial fossil material. Building up a database of the C, N and S

810 concentrations of different types of organic material may also be useful in the future to help

811 determine if different components of cells (i.e. wall, membrane, nucleus, cytoplasm) can be

812 preserved in exceptional circumstances. NanoSIMS also revealed the nature of some non-

813 carbonaceous intracellular inclusions within the Diabaig Formation; in optical microscopy

814 these inclusions are ruby red in colour (Fig. 6a), and NanoSIMS shows that they are iron

815 oxides (Fig. 6e-f) and at least some occur in direct contact with the inner cell wall. These

816 inclusions are rare, found in <1% of Torridonian microfossils, but may indicate a unique

817 intracellular chemistry in this small proportion of specimens.

818

819 *TEM data*

820 TEM data reveal the chemistry of the fossilizing mineral phases and the ultrastructure of the

821 microfossils at a spatial scale (nm) unattainable by any other technique. For example,

822 ChemiSTEM (STEM-EDS) elemental mapping combined with selected area electron

823 diffraction has shown that phosphate is not necessarily the dominant mineral responsible for

824 exceptional microfossil preservation in the Cailleach Head and Diabaig Formations (cf.

825 Raman and optical data). In fact, the minerals immediately adjacent to most vesicle walls are

826 Fe-rich clay minerals of the chlorite group or K-rich clay minerals approximating illite (Fig.

827 7; see Wacey *et al.* 2014 for details on clay mineral identification); phosphate only dominates

828 at some distance (tens to hundreds of nm) away from the cellular material. The interior of

829 many microfossils are also filled with K-rich clay minerals (Fig. 7), although phosphate

830 grains are also common in many cell interiors (e.g., Wacey *et al.* 2014, fig. 8). STEM-EDS in

831 the TEM detects small C and F peaks in the phosphate spectra confirming that the phosphate

832 is the common low temperature form often associated with fossils, francolite (carbonate

833 fluorapatite).

834

835 TEM imaging reveals sub-components of microfossil walls that were not previously

836 recognized. In many cases a presumed single, thick vesicle wall is shown to comprise

837 multiple components. These can include a thicker inner wall sitting within a thinner outer

838 wall, perhaps suggesting a cyst housed within a vegetative cell, or even more complex

839 arrangements of up to four distinct layers within a 'wall zone' (Fig. 7). Such arrangements

840 are too complex for simple prokaryote cells. Hence, this strongly suggests a eukaryotic

841 component to the biota. These complex layered walls are also preserved in clay minerals.

842 Hence, the combined data suggest that the fidelity of microfossil preservation may be

843 enhanced by early precipitation of clay minerals, and that microfossil preservation in clay

844 minerals may be of even higher quality than in phosphate.

845

846 *FIB-SEM data*

847 Two types of data were acquired using FIB-SEM, chemical and 3D morphological. Chemical

848 data were acquired by simply slicing into a microfossil using a FIB and then analyzing the

849 chemistry of a cross section through the microfossil using SEM-EDS. This provided similar

850 data to STEM-EDS in the TEM but at a more flexible spatial scale (i.e. could be applied to

851 larger fossils, albeit at lower spatial resolution). These data reinforced those acquired using

852   TEM, showing that in fossils with complex walls (interpreted as eukaryotes) clay minerals

853   occurred in direct contact with microfossil walls, in between multiple walls, and in

854   microfossil interiors, while calcium phosphate tended to occur exterior to the fossil (Fig. 8).

855   In simpler prokaryote fossils, the pattern was less defined, with phosphate mixed with clay

856   minerals typically occurring both exterior and interior to the cell (Fig. 9a-b).

857

858   Morphological data in three dimensions were acquired using FIB-SEM nano-tomography

859   whereby sequential FIB slicing was followed by imaging using the SEM (see methods

860   above). This provided an excellent visualization of cellular material located below the surface

861   of the thin section (Fig. 9b) that would otherwise have been hidden by overlying fossil

862   material (Fig. 9a). In addition, individual cells and cell contents could be visualized from

863   multiple orientations in 3D space (Fig. 9c-f). This is particularly useful for accurately

864   locating the position of organic intracellular inclusions (Fig. 9c-f). In the example presented

865   here these inclusions are most likely shrunken remnants of the cytoplasm of simple

866   prokaryote cells, but in future it may be possible to detect the remnants of eukaryotic nuclei

867   or organelles, if preserved using such methods.

868

869   **CONCLUSIONS**

870   Here we have provided an overview of the types of high-resolution techniques currently

871   available to those interested in characterizing Proterozoic microfossils and their associated

872   minerals and fabrics. Techniques have been classified either as non-destructive, hence

873   applicable to all material including holotypes, or destructive, hence applicable in cases where

874   conservation of the specimen is not a requirement. Non-destructive techniques include laser

875   Raman, CLSM, SEM, infrared spectroscopy, X-ray CT and X-ray spectroscopy, although to

876   obtain the highest spatial resolution data using the latter two methods, specialized (and partly

877   destructive) sample preparation is required. Destructive techniques include SIMS where the

878   surface layers of a microfossil are sputtered away during analysis, TEM where an ultrathin

879   slice must be extracted from the microfossil, and FIB-SEM nano-tomography which

880   consumes the entire specimen during analysis.

881

882   Maximum information is gained by the consilience of multiple approaches to a microfossil

883   assemblage, but in reality there will be some tradeoff between time and budget constraints,

884   efforts to conserve the best specimens, and the spatial resolution required. The destructive

885   techniques of TEM and FIB-SEM provide the greatest spatial resolution while SIMS

886   uniquely provides isotopic data. A sensible workflow would involve analysis of petrographic

887   context and a significant number of representative specimens using non-destructive avenues,

888   followed by focused analysis of fewer specimens by destructive means.

889

890   A case study from the Torridonian of northwest Scotland, a microfossil assemblage whose

891   importance has recently been highlighted by work led by Martin Brasier, demonstrated the

892   additional insights that these high resolution techniques can offer. Microtomography

893   provided a rapid way to determine the locality of phosphate nodules that house microfossils,

894   and other petrological details. SEM revealed a number of new morphotyes not previously

895   recognized in optical work and hinted at different taphonomic responses by different types of

896   cell and vesicle walls. TEM revealed the fine scale distribution of mineral phases in and

897   around cellular material, and showed that clay minerals played an important part in the

898   exceptional preservation of this biota. Raman together with NanoSIMS revealed details of the

899   organic material making up the cells, including its thermal maturity and biochemistry in

900   terms of C, N and S content. Finally, FIB-SEM nano-tomography provided a detailed 3D

901 view of a number of fossilized cells, including the location of the remains of organic cell

902 contents.

903

924

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FIGURE CAPTIONS

Fig 1. Optical microscopy of Torridonian microfossils, demonstrating common morphotypes

present in the assemblage. (**a**) Highly degraded dark-walled vesicle. (**b**) Pristine dark-walled

vesicle. (**c**) Light-walled vesicle, potentially possessing a double wall. (**d**) Cluster of light-

walled spheroidal unicells, most with a dark spot indicating potential preservation of cell

contents. (**e**) Cluster of light-walled cells with mutually adpressed cell walls. (**f**) Pair of

spheroidal unicells with very prominent dark inner sphere. (**g**) Partially decomposed

filamentous sheath. (**h**) Filamentous sheath with bulbous termination housing potential

spheroidal cell. (**i**) Colony of light-walled elliptical cells comparable to *Eohalothece*

lacustrina described by Strother & Wellman (2015). (**j**) Pair of cells that may have divided

shortly before fossilisation, each containing a dark spot. Scale bars are 20 µm for (**a-i**) and 10

µm for (**j**).

Fig. 2. Torridonian microfossils imaged and analysed by SEM-SE, coloured for easier

interpretation. (**a**) Large, thick-walled vesicle, showing `velvet-like' folds. (**b**) Smaller, thin-

walled vesicles with a crinkled surface and finely irregular outline. (**c**) Vesicle with large

hemispherical pits bounded by raised rims or `collars'. (**d**) Subspherical rigid vesicle retaining

a 3D structure, and bearing many irregular rounded holes. Pink coccoid structures attached to

the vesicles in (**a-b**) are potential fossil heterotrophs (see also Fig. 3). Sample CAI-7,

macerated from phosphate from Cailleach Head. All scale bars are 10 µm.

Fig. 3. Evidence of bacterial heterotrophy in SEM images. (**a-b**) Rounded pits and occasional

holes, irregularly distributed on the surface of larger vesicles' walls, (b) is enlargement of

boxed area in (a). (**c-d**) Collapsed coccoid or baccilate cells ~5 µm across, occupying pits in

Fig. 4. X-ray microtomography analysis of Torridonian rock chips. (**a**) Reconstruction of a

CT scan of a rock chip using the *Nikon* instrument (voxels c. 5 µm), highlighting part of a

phosphate nodule (purple) within a quartz-rich sediment (grey), plus a number of higher

density grains that are likely pyrite or iron oxide (gold). (**b**) Reconstruction of an X-ray scan

of a second rock chip using the Swiss Light Source Synchrotron (voxels 1.625 µm). This

shows a mixture of phosphate and other denser phases rather evenly distributed through the

rock chip with no distinct phosphate nodule. (**c-d** and **f-g**) Reconstruction of two putative

vesicles identified in a higher resolution CT scan using the *Zeiss Xradia Versa* instrument

(voxels c. 1.5 µm). The light micrograph images (**e** and **h**) show specimens observed in thin

sections that may be analogous to those identified using CT. Scale bar is 2 mm for (**a**) 500

µm for (**b**) and 20 µm for (**c-h**).

Fig. 5. Raman analysis of microfossils from the Torridonian Supergroup. (**a**) Optical

photomicrograph of two coccoid microfossils from the Cailleach Head Formation, each

containing dark interior spheroids (arrows). (**b**) Raman map of the carbon G ~1600 cm^{-1} peak

showing that the microfossils have carbonaceous walls and the dark interior spheroids are

also carbonaceous. This suggests that they are clumps of degraded cellular material or

1373 remnants of a cell nucleus. (**c**) Raman map of the major calcium phosphate (apatite) ~960 cm^{-1}

1374 peak showing that a large proportion of the mineralising phase is apatite. The patchy

1375 appearance of the apatite suggests the presence of further mineral phases, interpreted to be

1376 clay minerals as detected in higher resolution SEM and TEM analyses (see Figs 7-8). (**d**)

1377 Optical photomicrograph of a microfossil from the Stoer Group. Raman maps of the carbon

1378 G ~1600 cm^{-1} peak (**e**), pyrite ~380 cm^{-1} peak (**f**), calcite ~1090 cm^{-1} peak (**g**), and albite

1379 ~510 cm^{-1} peak (**h**) demonstrating that the microfossil is partially pyritised but some

1380 carbonaceous composition remains, and that the sediment is dominantly calcite and feldspar.

1381 Scale bars are 10 µm.

1382

1383 **Fig. 6.** NanoSIMS analysis of a microfossil from the Diabaig Formation. (**a**) Optical

1384 photomicrograph of a light-walled spheroidal cell with ruby red intracellular particles. (**b**)

1385 Overview of a FIB-milled wafer prepared for NanoSIMS from the region indicated by the

1386 yellow line in (**a**). Note the contrast between the large dark-grey grains, which equate to the

1387 ruby red grains in (**a**), and the remainder of the wafer, plus holes in the wafer likely induced

1388 by excessive FIB-milling. (**c**) NanoSIMS ion map of nitrogen measured as CN$^-$. (**d**)

1389 NanoSIMS ion map of sulfur measured as S$^-$. (**e**) NanoSIMS ion map of iron oxide measured

1390 as FeO$^-$. (**f**) Three colour overlay of nitrogen (blue), iron oxide (red) and silicon (green)

1391 showing that the large dark grains are iron oxides and they are located just inside the cell wall

1392 (intracellular). The other mineral phases are dominantly clays and quartz. Scale bar is 20 µm

1393 in (**a**), and 5 µm for (**b-f**). Note scale bar in (**c**) also applies to (**d-f**).

1394

1395 **Fig. 7.** TEM analysis of a FIB milled wafer extracted from a Torridonian microfossil. (**a**)

1396 Optical photomicrograph of a dark-walled spheroidal microfossil from the Cailleach Head

1397 Formation. (**b**) Overview of the FIB-milled wafer extracted from the region marked by the

1398   yellow line in (**a**) showing a complex wall structure and different mineral phases (indicated

1399   by different levels of grey within the image) inside and outside of the microfossil (from

1400   Wacey *et al*. 2014). (**c**) Three colour overlay of ChemiSTEM elemental maps of carbon

1401   (blue), aluminium (orange) and calcium (pink) from the region indicated by the dashed box in

1402   (**b**). Carbon represents the organic material of the microfossil walls, and at least four separate

1403   walls (or wall layers) can be seen. Calcium represents apatite, the dominant mineral phase

1404   outside of the microfossil. Aluminium represents clay minerals that infill the microfossil,

1405   occur between the walls of the microfossil and occur in minor amounts outside of the

1406   microfossil. Black areas are holes in the TEM wafer. Scale bar is 10 μm in (**a**), 2 μm in (**b**),
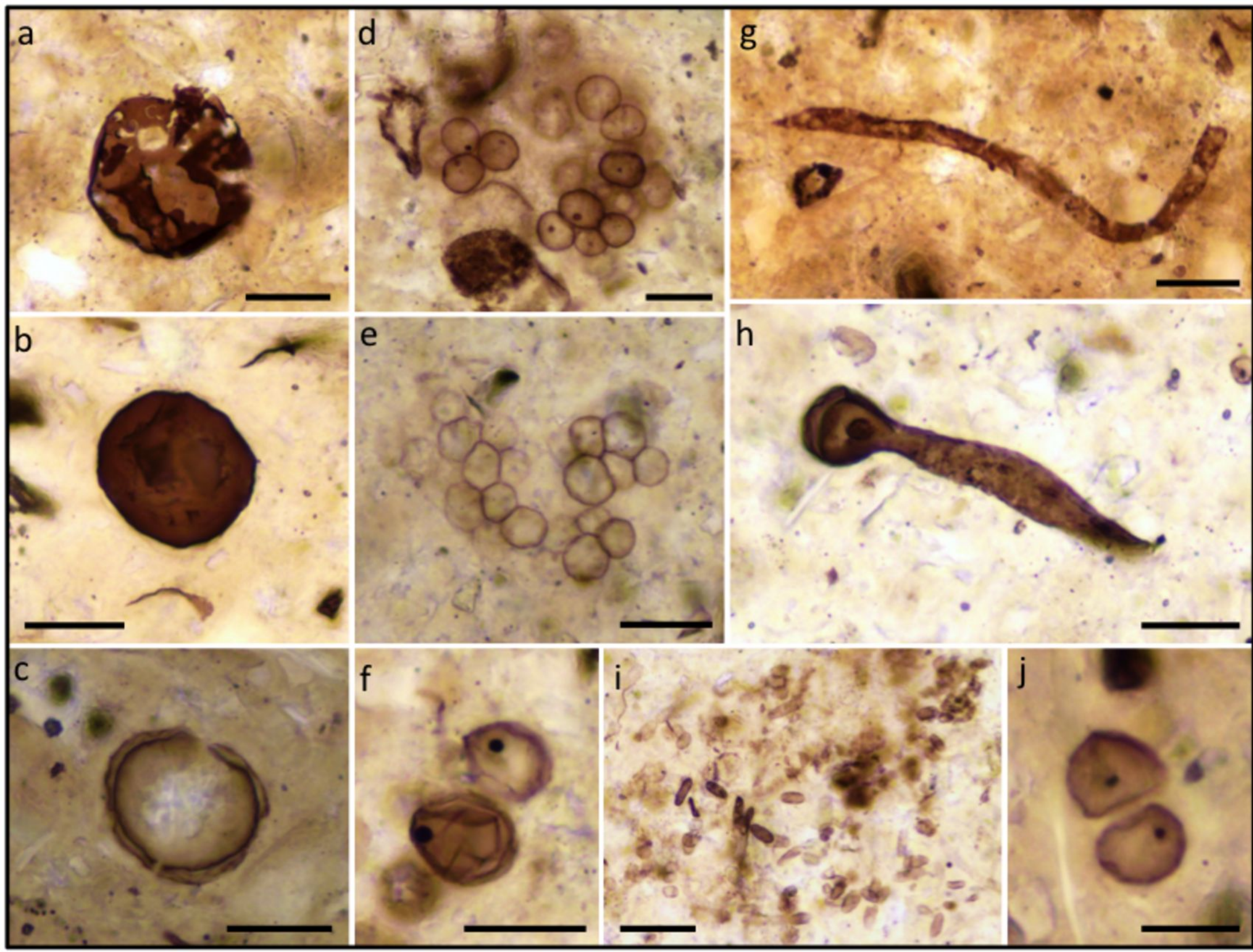
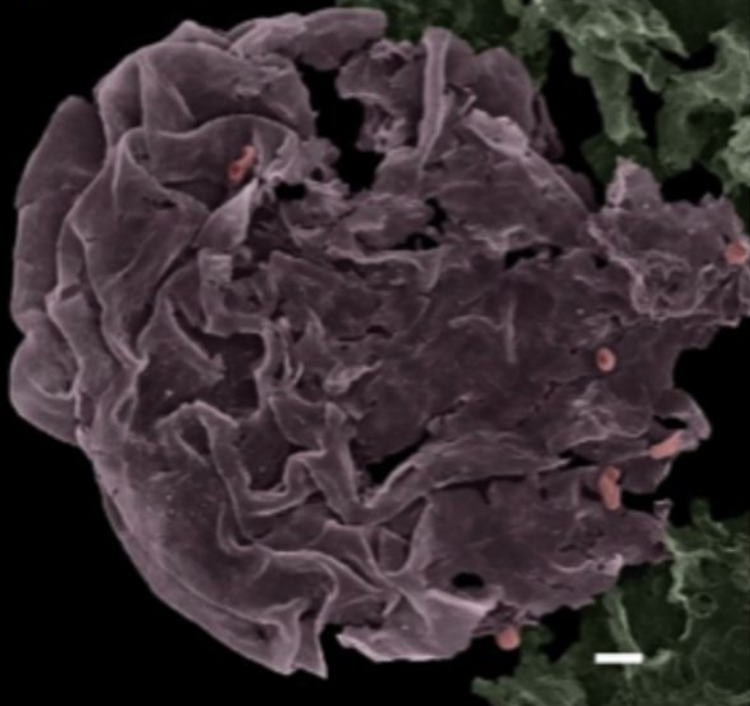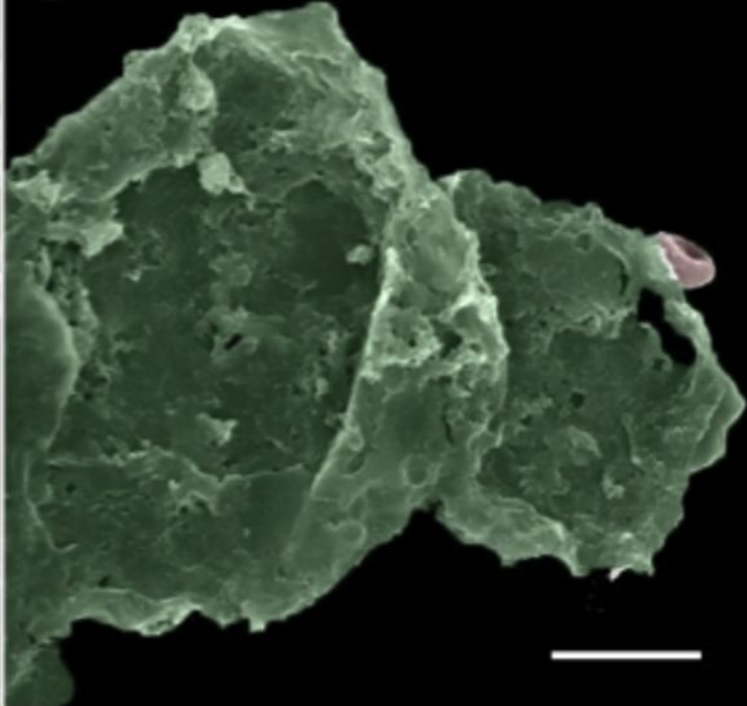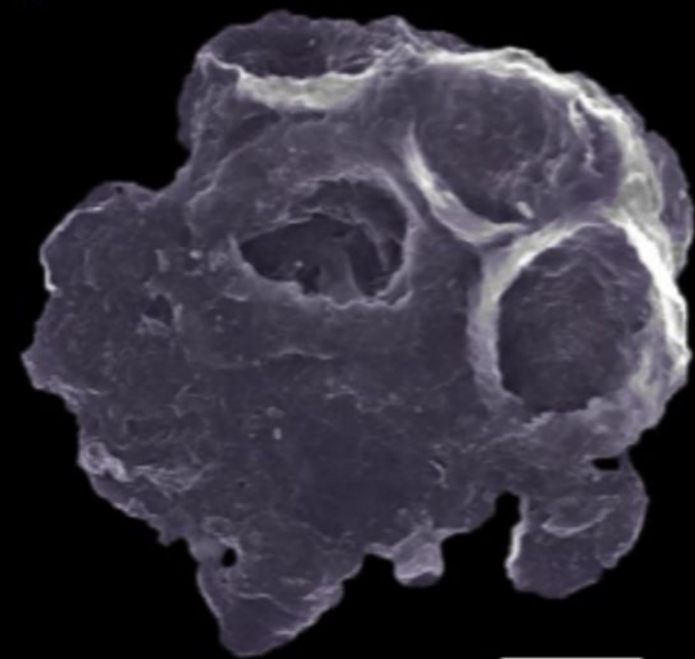1407   and 1 μm in (**c**).

1408

1409   **Fig. 8.** FIB-SEM-EDS of a microfossil from the Cailleach Head Formation. (**a**) Optical

1410   photomicrograph of a dark walled spheroidal vesicle showing the location of the FIB-milled

1411   area and direction of view for the other panels in the figure (from Wacey *et al*. 2014). (**b**)

1412   Secondary electron image showing the FIB-milled face below the surface of the thin section.

1413   Shown below are energy-dispersive X-ray (EDS) elemental maps of the FIB-milled face

1414   shown in (**b**). Carbon (light blue) represents the organic microfossil walls, highlighting a

1415   thick inner cyst wall and thinner outer vegetative cell wall. Phosphorus (red), calcium (pink)

1416   and moderate levels of oxygen (green) represent apatite, the dominant fossilising mineral

1417   outside of the microfossil. Iron (blue), plus moderate amounts of silicon (turquoise),

1418   aluminium (orange) and oxygen represents Fe-rich clay, occurring between the two

1419   microfossil walls, replacing parts of the outer wall, and continuing for 1–2 μm outside the

1420   outer wall. Potassium (yellow), plus silicon, aluminium and oxygen represents K-rich clay

1421   restricted to the interior of the vesicle.

1422   Scale bars are 5 μm.

1423

1424 **Fig. 9.** 3D FIB-SEM nano-tomography of a Torridon microfossil. (**a**) Optical

1425 photomicrograph of a cluster of light-walled spheroidal cells from the Cailleach Head

1426 Formation (from Wacey 2014). (**b**) Example of a FIB-milled slice through the cluster of

1427 microfossils in the region indicated by the dashed line in (**a**). Note that portions of at least 8

1428 cells can be seen in this image, some of which are hidden from view below other cells in the

1429 optical photomicrograph. Note also dark material inside the upper central cell (dashed arrow)

1430 (**c-f**) 3D model of the cell indicated by the solid arrow in (**b**) viewed from four different

1431 orientations, showing the location of preserved cell contents (blue) with respect to the cell

1432 wall (yellow). Note that in (f) part of the cell wall has been removed to better visualize the

1433 cell contents. Scale bar is 10 µm in (**a**), and 5 µm for (**b-f**). Note scale bar in (**c**) also applies

1434 to (**d-f**).

1435

a

b

Protective Pt coat

hole

c   CN⁻

d   S⁻

e   FeO⁻

f   CN⁻
     FeO⁻
     Si⁻

a

b
Protective Pt coat
Microfossil interior
Exterior
Wall region

c
C
Al
Ca

a

b   Protective Pt strip

C   Microfossil walls

P

Ca

Si

Fe

K

Al

O