bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1 # Electron microscopy characterization of minerals formed *in vitro* by
2 # human bone cells and vascular smooth muscle cells

3

4 **Elena Tsolaki[1], Louis Didierlaurent[2], Eike Müller[3], Markus Rottmar[3], Najma Latif[4],**
5 **Adrian H. Chester[4], Inge K. Herrmann[2*¶], Sergio Bertazzo[1*¶]**

6

7 [1]Department of Medical Physics & Biomedical Engineering, University College London,
8 London WC1E 6BT, UK
9 [2]Laboratory for Particles- Biology Interactions, Empa, Swiss Federal Laboratories for
10 Materials Science and Technology, Lerchenfeldstrasse 5, CH-9014, St. Gallen, Switzerland.
11 [3]Laboratory for Biointerfaces, Empa, Swiss Federal Laboratories for Materials Science and
12 Technology, Lerchenfeldstrasse 5, CH-9014, St. Gallen, Switzerland.
13 [4]Magdi Yacoub Institute, Imperial College London, Heart Science Centre, Harefield Hospital,
14 Harefield, Middlesex UB9 6JH, UK,

15

16 * Corresponding author
17 email: s.bertazzo@ucl.ac.uk (SB); inge.herrmann@empa.ch (IKH)

18

19 ¶These authors contributed equally to this work.

20

21

22

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

23 **Abstract**

24 Soft tissue mineralization has been found to be a major component of diseases such as aortic
25 valve stenosis and rheumatic heart disease. Cardiovascular mineralization has been suggested
26 to follow mechanisms similar to those of bone formation with several cell culture models
27 been developed over the years to provide mechanistic insights. These cell models have been
28 characterized by a wide range of biochemical and molecular methods, which identified the
29 presence of osteogenic markers and bone-like cells. However, there is a surprisingly small
30 number of studies where the mineral formed in these cell culture models has been
31 characterized by physico-chemical methods, and even fewer studies have compared this
32 mineral to the one produced by bone cells in cultures. Here we investigated the morphology
33 and composition of the minerals formed in cell cultures of vascular smooth muscle cells and
34 bone cells. Electron microscopy and traditional cell mineralization assays were applied,
35 revealing that vascular cells are indeed able to form calcified nodules of elemental
36 composition similar to bone, however with different morphology. Comparison of
37 morphologies of the two minerals to that found in cardiovascular tissue shows that some of
38 tissue calcification resembles the calcified fibers produced by bone cells *in vitro*. These
39 results suggest that the characterization of the mineral is of utmost importance and its
40 morphology and chemical properties can contribute an important piece of information in the
41 comprehensive analysis of soft tissue mineralization mechanisms, both in *in vitro* cell culture
42 as well as in clinical samples.

43

44 **Introduction**

45 Healthy hard tissues, including bone and teeth, are naturally created in the body by
46 mineralizing cells such as osteoblasts and odontoblasts. Mineralization of soft tissues has also
47 been extensively described in the literature and generally, if not always, it has been attributed

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

48 to pathological processes in humans. Diseases associated with calcification of the vascular
49 system are major contributors to patient morbidity and mortality worldwide [1]. Vascular
50 calcification is a wide spread phenomenon, with a third of all Americans over the age of 45
51 developing cardiovascular calcification complications [2, 3]. Mineral formation is a hallmark
52 of major diseases including atherosclerosis [4] and coronary heart disease [5], for both of
53 which there is currently no definitive treatment [5, 6]. Calcific cardiovascular diseases are
54 estimated to lead to up to 23.3 million of deaths per year worldwide in 2030 [7].

55 Despite its clinical importance and major scientific efforts to understand it, the
56 mechanisms that lead to formation of cardiovascular calcification are yet to be understood.
57 Several studies suggest that cardiovascular calcification is an active process [8], even though
58 for many years it was thought to be the direct result of degenerative processes [9]. Works in
59 the literature that associate cardiovascular calcification formation with bone [10, 11] are
60 mainly based on the identification of proteins such as osteonectin and Runx2 in calcific
61 cardiac lesions [10, 12-16]. On the other hand, more recent research on cardiovascular
62 calcification, showed that these calcifications are formed by three distinct forms of mineral;
63 calcified particles, calcified fibers and a compact calcification [1], all of which have a
64 structure significantly different from native bone [1, 17-19].

65 Cell cultures have been an integral part of cardiovascular research, since they provide
66 a high degree of control of cell environment and allow the induction of calcification [20]. For
67 example, it has been previously shown that the presence of calcium phosphate in cell cultures
68 of mesenchymal stem cells promotes bone formation [21] and that calcification can be
69 induced in vascular smooth muscle cells (VSMC) in the presence of inorganic phosphate [20,
70 22-24]. Several works suggest that the capability of VSMC to produce calcification *in vitro*
71 [25] is driven possibly by a transdifferention of VSMC to bone-like cells [11, 26, 27]. On the
72 other hand, recent studies demonstrated that the formation of calcification in the vascular

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

73 tissue or from VSMC can be related to the release of extracellular vesicles [28-30], in a
74 process different to the one observed in bone cells. In light of these seemingly conflicting
75 results, it becomes clear that cardiovascular calcification is a much more complex
76 phenomenon, possibly consisting of a mixture of osteogenic and non-osteogenic processes,
77 and that our current assays and endpoints may not necessarily be sufficient to characterize
78 calcifying cell cultures, as we have yet
79 to understand their underlying mechanisms.

80 Here, we used physico-chemical methods to characterize the morphology and
81 chemical composition of the minerals present in calcification produced by human bone cell
82 (HBC) and human vascular smooth muscle cell (HVSMC) cultures. We then compared the
83 morphology and composition of the minerals generated *in vitro* to minerals found in human
84 bone and in calcified vascular tissue. Our results show clear structural differences between
85 HBC and HVSMC mineralization, and similarities between the mineralized fibers formed in
86 HBC cultures to mineralised fibers found sometimes in late stage cardiovascular disease.

87

88 **Materials and Methods**

89

90 **Cell culture experiments**

91 Human vascular smooth muscle cells (HVSMC) were acquired from ATCC®. For the
92 different experiments (detailed below), the cells used were kept at -80°C and were used at
93 passage four to eight. Cells were cultivated in vascular cell basal medium (ATCC®)
94 complemented with vascular smooth muscle growth kit (ATCC®).

95 Bone marrow samples were harvested from patients undergoing surgical hip
96 replacement (ethical approval was obtained from the local ethics committee; EKSG
97 08/14). The detailed isolation procedure of the human bone cells (HBC) is described

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

98 elsewhere [31]. Human bone cells (HBC) were cultivated in a proliferation medium
99 consisting in α-MEM (Gibco®), 10% Fetal Calf Serum (FCS), 1% Penicillin-Streptomycin-
100 Neomycin (PSN) and 1 ng/ml basic fibroblast growth factor (FGF-2). Human dermal
101 fibroblast cells (HFC, primary cells, Sigma Aldrich, Buchs, Switzerland) were cultivated in
102 DMEM supplemented with 15% FCS and 1% PSN/L-glutamine. During the cell culture
103 experiments, Lactate Dehydrogenase (LDH) release was measured regularly (twice per week)
104 using a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega).

105 For experiments, cells were seeded on 24, 48 and 96 well-plates and grown to
106 confluence. Cells were treated with different media for 28 days. Medium was exchanged
107 every third day.

108 The following four media compositions were used: (i) α-MEM (*α-MEM*): containing
109 only α-MEM (ii) Proliferation medium (*Prolif*): α-MEM, 10% FCS, 1% PSN and 1 ng/ml
110 basic FGF-2. (iii) Differentiation medium (*Diff*): α-MEM, 10% FCS, 1% PSN, 10 nM 1.25
111 dihydroxy-vitamine D3 (VitD3), 50 µM ascorbic acid phosphate, 2 mM β-glycerophosphate
112 and 10 nM dexamethasone (Dex) (iv) Differentiation medium + Calcium (*DiffCa*): α-MEM,
113 15% FCS, 1% PSN, 6 mM calcium chloride , 10 mM sodium pyruvate, 10µM insulin,
114 50µg/ml ascorbic acid, 10 mM β-glycerophosphate and 1µM Dex. To exclude any influence
115 of compromised cell viability, lactate dehydrogenase content was monitored over the full
116 duration of the experiments and cell viability was measured by MTS assay at day 28.

117

118 **Scanning Electron microscopy (SEM)**

119 For SEM imaging, samples were fixed in 4% (w/v) formaldehyde, dehydrated through
120 a graded ethanol (Sigma, ACS reagent 99.5%) series (20, 30, 40, 50 70, 80, 90, 100 and
121 100% (v/v)) and air dried. All samples were carbon coated and silver painted. A Hitatchi S-
122 3499N, a Carl Zeiss VP and a LEO Gemini 1525 FEGSEM were used for SEM analysis,

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

123 which involved imaging of the samples using secondary electron (SE) and backscattering
124 electron (BSE) modes, for density-dependent colour SEM (DDC-SEM) to be acquired [1].
125 Energy Dispersive X-ray Spectroscopy (EDS) analysis was also carried out using Oxford
126 Instruments EDS detectors, integrated into the microscopes. For the analysis, accelerating
127 voltages of 5 kV and 10 kV were used.

128

129 **Alizarin Red staining**

130 Following paraformaldehyde fixation, cells were stained with Alizarin Red S to reveal
131 macroscopic calcifications. Alizarin Red S (2 g) was dissolved in 100 mL of distilled water
132 and pH was adjusted to 4.2. Cells were stained with freshly prepared Alizarin Red solution
133 for 2-3minutes.

134

135 **Calcium Assay**

136 After the samples were washed three times with PBS, cells were lysed in 1 M HCl for
137 3 h at 37 °C under constant agitation. Calcium concentrations in the solutions were measured
138 using a colorimetric QuantiChrom™ Calcium Assay Kit (BioAssay Systems) following the
139 protocol provided by the manufacturer. In Brief, 5 µl of each lysate was transferred to a 96-
140 well plate and 195 µl of working reagent (Quanti ChromTM Calcium Assay DICA-500,
141 Gentaur) were added. After 3 min of incubation, the absorbance of the obtained solution was
142 measured at 595 nm (Biotek Instruments Elx 800, Witec AG). All samples were analysed in
143 triplicates and calcium concentrations were calculated by means of a calibration curve using a
144 calcium standard.

145

146 **Human sample procurement and preparation**

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

147 All samples were collected under the guidelines of ethical approval, which included
148 informed consent that allowed us to anonymously analyse the tissues. Human femoral head
149 bone samples were obtained by A. Hart from Charing Cross Hospital (London) from patients
150 undergoing elective total hip arthroplasty procedures. Aortic valves from thirty-two patients
151 were provided by the Oxford Heart Valve Bank at John Radcliffe Hospital (Oxford) and the
152 Royal Brompton Hospital (London). These samples were obtained either after having been
153 rejected for use as homografts or from patients undergoing aortic valve replacement surgery.
154 All samples were fixed using 4% (w/v) formaldehyde (Sigma, BioReagent, ≥36.0%)
155 solution in phosphate buffered saline (PBS; Sigma) at room temperature immediately after
156 collection, for at least one day.
157
158 **Results and Discussion**
159 We investigated the mineralization of HBC and HVSMC in different cell culture
160 media. Pure α-MEM (α-MEM) and proliferation medium (*Prolif*) was used as base line
161 (negative control). Mineralizing media used by representative literature studies were included
162 in order to study *in vitro* mineralization with media containing 2 mM β-glycerophosphate
163 (*Diff*) [31], and media supplemented with 6 mM $CaCl_2$ and an additional 8 mM β-
164 glycerophosphate (*DiffCa*) [32].
165 Total calcium concentration was measured in the cell cultures following 28 days of
166 culturing with media changes every third day (Fig. 1). HBC accumulate significant amounts
167 of calcium irrespective of the mineralizing medium (*Diff* and *DiffCa*), while HVSMC only
168 show an increase in calcium concentration when exposed to calcium-supplemented
169 mineralization medium (*DiffCa*). Interestingly, human fibroblast cells (HFC), which were
170 included as non-vascular cells for reference, show similar calcium accumulation to the other
171 cell cultures when exposed to *DiffCa* for 28 days.

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

172 **Fig 1. Total calcium content per well at day 28.** Cultures of human bone cells (HBC),
173 human vascular smooth muscle cells (HVSMC) and human dermal fibroblasts (HFC), using
174 different media.
175
176 Alizarin Red staining was performed, in order to visualize calcium accumulation in
177 the cell cultures (Fig. 2). The results confirm mineral deposition in HBC cultures when
178 exposed to *Diff* and *DiffCa,* and in HVSMC (and HFC, not shown) cultures, only when
179 exposed to *DiffCa.*
180
181 **Fig 2. Alizarin Red stained cell cultures.** Calcium-rich regions appear in red. Magnification
182 (40x).
183
184 Considering that Alizarin Red results may be misleading when applied to cell cultures
185 and can stain positive even in the absence of mineral [33], we also performed high-resolution
186 electron microscopy with elemental analysis. As expected, no evidence of calcium phosphate
187 deposition was found in the HBC culture when exposed to α-MEM and *Prolif* medium *(Fig.*
188 *3(a, b))*. In agreement with the calcium assay and the Alizarin Red staining, HBC exposed to
189 *Diff* and *DiffCa* presented significant amounts of mineral deposits. Scanning electron
190 micrographs show fibre-like mineral deposits consisting of calcium phosphate *(Fig. 3(c, d))*
191 in HBC incubated with *Diff* medium. Strikingly, the HBC exposed to *DiffCa* also present
192 large amounts of calcium phosphate mineral deposits *(Fig. 3 (e, f))*, albeit with a completely
193 different morphology to the mineral formed in the *Diff* medium. In the case of *DiffCa*, large
194 chunks of mineral were detected with no evidence of fibrous structures, suggesting a rapid
195 mineral precipitation. While nominal amount and the composition of mineral was very
196 comparable in these two cell culture systems, the morphology of the mineral deposits is

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

197 strikingly different (see *Fig. 3(c, d)* vs. (*e, f*)).

198

199 **Fig 3. DDC-SEM micrographs of HBC (Human Bone Cells) calcification morphology**
200 **produced in different media, imaged at low and high magnifications, with**
201 **correspondent EDS spectra of indicated region. The organic material is represented as**
202 **green while the inorganic material as orange.** (a, b) *Prolif* medium where no deposition of
203 calcium phosphate is observed. (c, d) *Diff* medium where fibrous calcium phosphate
204 structures are observed. (e, f) *DiffCa* medium where large deposition of calcium phosphate is
205 observed. Scale bars = 20 μm, 2 μm.

206

207 For HVSMC, again, no evidence of calcium phosphate mineral deposition was found
208 for cell cultures exposed to α-MEM and *Prolif* medium *(Fig. 4 (a, b))*. In contrast to HBC,
209 no evidence of calcium phosphate formation was found for HVSMC exposed to *Diff (Fig. 4*
210 *(c, d))*. Calcium phosphate minerals were, however, observed in HVSMC cultures exposed to
211 *DiffCa (Fig. 3 (e, f))*. Similar results were also observed in the HFC culture with
212 mineralization only present in the *DiffCa* medium (results not shown). *DiffCa* medium is
213 indeed saturated for hydroxyapatite [34], and thus it comes as no surprise that rapid mineral
214 precipitation could form in an unstructured manner, similarly to the precipitate formed in the
215 HBC. However the structures observed by the different cell cultures, are significantly
216 different thus a cell involved rather than a simple supersaturation process might be taking
217 place (see *Fig. 3* (*e, f*) *vs. Fig.4 (e,f)*).

218

219 **Fig 4. DDC-SEM micrographs of HVSMC (Human Vascular Smooth Muscle Cells)**
220 **calcification morphology produced in different media, imaged at low and high**
221 **magnifications, with correspondent EDS spectra of indicated region. The organic**

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

222 **material is represented as green while the inorganic material as orange.** (a, b) *Prolif*
223 medium where no deposition of calcium phosphate is observed. (c, d) *Diff* medium where no
224 deposition of calcium phosphate is observed. (e, f) *DiffCa* medium where calcium phosphate
225 mineral is observed. Scale bars = 20 µm, 10 µm.

226

227 In order to compare the structure and morphology of the *in vitro* generated minerals to
228 minerals found in clinical samples, we analysed a series of calcified human tissues. First, we
229 imaged human bone samples by SEM. The characteristic calcified oriented and regular
230 structure can be easily recognized on the micrographs (*Fig. 5 (a)*). On the other hand, at least
231 three distinctly different characteristic structures, including fibers *(Fig. 5 (b)),* calcified
232 particles *(Fig. 5 (c))* and compact calcification *(Fig. 5 (d))* could be identified in human
233 cardiovascular tissues as previously described in the literature [1].

234

235 **Fig 5. DDC-SEM micrographs of the microstructure of human bone (a) and mineralised**
236 **structures observed in cardiovascular tissue (b, c and d).** (a) High magnification image,
237 showing calcified collagen fibers forming bone. Scale bar = 1 µm. DDC-SEM micrographs of
238 the calcified structures observed in human cardiovascular tissue (b) calcific fibres, (c) calcific
239 particles (white arrow), (d) large compact calcification (white arrow) with calcific particles
240 (red arrow). Scale bars = 1 µm.

241

242 There is a notable similarity between minerals formed in the *in vitro* cultures of HBC
243 in *Diff* media *(Fig. 2 (c, d))* and the fibers that can be sometimes found at the later stages of
244 calcification [1] present in the vascular tissue *(Fig. 5b and S1 Fig.)*. From the similarities
245 presented between the bone cell cultures calcific structures and the vascular structures, we
246 could speculate that in some cases there is indeed some form of calcification present in the

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

247 cardiovascular tissue which is similar to the material produced by bone cells, supporting
248 partially the many studies that report osteogenic differentiation of vascular cells. However, no
249 similarities are observed with the natural bone structure, suggesting that the calcified fibers
250 could be formed by a distinct mechanism to bone mineralization. Also, no morphological
251 similarities were found for the mineral produced by HVSMC, neither to the mineral found in
252 bone tissue nor in cardiac tissues, suggesting that our HVSMC model does not adequately
253 depict the *in vivo* mineralization mechanism.

254 Taken together, these results show that the culturing of human bone cells is indeed
255 able to form minerals similar to some of the structures found *in vivo*, and hence partially
256 support the hypothesis that some sort of osteogenic differentiation in vascular tissue could
257 happen sporadically. On the other hand, none of the cultures were able to produce any
258 structure similar to the calcified particles that are widespread in vascular tissue [1, 17, 30]
259 and are the first calcified structure that can be detected in those tissues [1] (*Fig. 5 (a)*). This
260 study indicates that a major component of vascular calcification still cannot be described by
261 the cell cultures tested here, and that there is likely an unaccounted player contributing to
262 mineral deposition in the cardiovascular system.

263

264 **Acknowledgement**

265 I.K.H. and S.B. acknowledge support from the Swiss Heart Foundation and the Swiss
266 National Science Foundation (grant no. 173077).

267

268 **References**

269 [1] Bertazzo S, Gentleman E, Cloyd KL, Chester AH, Yacoub MH, Stevens MM. Nano-analytical
270 electron microscopy reveals fundamental insights into human cardiovascular tissue calcification.
271 Nat Mater. 2013;12(6):576-83. doi: 10.1038/nmat3627.

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

272 [2] Jain T, Peshock R, McGuire DK, Willett D, Yu Z, Vega GL, et al. African Americans and Caucasians
273 have a similar prevalence of coronary calcium in the Dallas Heart Study. J Am Coll Cardiol.
274 2004;44(5):1011-7. doi: 10.1016/j.jacc.2004.05.0693.
275
276 [3] Bild DE, Detrano R, Peterson D, Guerci A, Liu K, Shahar E, et al. Ethnic differences in coronary
277 calcification: the Multi-Ethnic Study of Atherosclerosis (MESA). Circulation. 2005;111(10):1313-
278 20. doi: 10.1161/01.CIR.0000157730.94423.4B.
279
280 [4] Doherty TM, Asotra K, Fitzpatrick LA, Qiao J-H, Wilkin DJ, Detrano RC, et al. Calcification in
281 atherosclerosis: Bone biology and chronic inflammation at the arterial crossroads. Proc Natl
282 Acad Sci U S A. 2003;100(20):11201-6. doi: 10.1073/pnas.1932554100.
283
284 [5] Liu W, Zhang Y, Yu CM, Ji QW, Cai M, Zhao YX, et al. Current understanding of coronary artery
285 calcification. J Geriatr Cardiol. 2015;12(6):668-75. doi: 10.11909/j.issn.1671-5411.2015.06.012.
286
287 [6] Wu M, Rementer C, Giachelli C. Vascular Calcification: An Update on Mechanisms and
288 Challenges in Treatment. Calcif Tissue Int. 2013;93(4):365-73. doi: 10.1007/s00223-013-9712-z.
289
290 [7] Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030.
291 PLoS Med. 2006;3(11):e442. doi: 10.1371/journal.pmed.0030442.
292
293 [8] Karwowski W, Naumnik B, Szczepański M, Myśliwiec M. The mechanism of vascular calcification
294 – a systematic review. Med Sci Monit. 2012;18(1):RA1-RA11. doi: 10.12659/msm.882181.
295

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

296 [9] Proudfoot D, Skepper JN, Hegyi L, Bennett MR, Shanahan CM, Weissberg PL. Apoptosis Regulates
297 Human Vascular Calcification In Vitro: Evidence for Initiation of Vascular Calcification by
298 Apoptotic Bodies. Circ Res. 2000;87(11):1055-62. doi: 10.1161/01.res.87.11.1055.
299
300 [10]Dhore CR, Cleutjens JPM, Lutgens E, Cleutjens KBJM, Geusens PPM, Kitslaar PJEHM, et al.
301 Differential Expression of Bone Matrix Regulatory Proteins in Human Atherosclerotic Plaques.
302 Arteriosclerosis, Thrombosis, and Vascular Biology. 2001;21(12):1998-2003. doi:
303 10.1161/hq1201.100229.
304
305 [11]Abedin M, Tintut Y, Demer LL. Vascular calcification: mechanisms and clinical ramifications.
306 Arterioscler Thromb Vasc Biol. 2004;24(7):1161-70. doi: 10.1161/01.ATV.0000133194.94939.42.
307
308 [12]Trion A, van der Laarse A. Vascular smooth muscle cells and calcification in atherosclerosis. Am
309 Heart J. 2004;147(5):808-14. doi: 10.1016/j.ahj.2003.10.047.
310
311 [13]Thompson B, Towler DA. Arterial calcification and bone physiology: role of the bone-vascular
312 axis. Nat Rev Endocrinol. 2012;8(9):529-43. doi: 10.1038/nrendo.2012.36.
313
314 [14]Bostrom K, Watson KE, Horn S, Wortham C, Herman IM, Demer ll. Bone morphogenetic protein
315 expression in human atherosclerotic lesions. J Clin Invest. 1993;91(4):1800-9. Doi:
316 10.1172/JCI116391
317
318 [15]Shanahan CM, Cary NR, Metcalfe JC, Weissberg PL. High expression of genes for calcification-
319 regulating proteins in human atherosclerotic plaques. J Clin Invest. 1994;93(6):2393-402. doi:
320 10.1172/JCI117246.

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

321

322 [16]Fitzpatrick LA, Severson A, Edwards WD, Ingram RT. Diffuse calcification in human coronary
323 arteries. Association of osteopontin with atherosclerosis. J Clin Invest. 1994;94(4):1597-604. doi:
324 10.1172/JCI117501.

325

326 [17]Bertazzo S, Steele JAM, Chester AH, Yacoub MH, Stevens MM. Cardiovascular calcification violet
327 pearl. The Lancet. 2014. doi:https://doi.org/10.1016/S0140-6736(13)62369-7.

328

329 [18]Agarwal S, Bertazzo S. New paradigms in cardiovascular calcification. Comptes Rendus Chimie.
330 2016;19(11):1605-9. doi: https://doi.org/10.1016/j.crci.2015.09.013.

331

332 [19]Bertazzo S, Gentleman E. Aortic valve calcification: a bone of contention. Eur Heart J.
333 2017;38(16):1189-93. doi: 10.1093/eurheartj/ehw071.

334

335 [20]Akiyoshi T, Ota H, Iijima K, Son BK, Kahyo T, Setou M, et al. A novel organ culture model of aorta
336 for vascular calcification. Atherosclerosis. 2016;244:51-8. doi:
337 10.1016/j.atherosclerosis.2015.11.005.

338

339 [21]Castren E, Sillat T, Oja S, Noro A, Laitinen A, Konttinen YT, et al. Osteogenic differentiation of
340 mesenchymal stromal cells in two-dimensional and three-dimensional cultures without animal
341 serum. Stem Cell Res Ther. 2015;6:167. doi: 10.1186/s13287-015-0162-6.

342

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

343 [22]Rangrez AY, M'Baya-Moutoula E, Metzinger-Le Meuth V, Henaut L, Djelouat MS, Benchitrit J, et
344 al. Inorganic phosphate accelerates the migration of vascular smooth muscle cells: evidence for
345 the involvement of miR-223. PLoS One. 2012;7(10):e47807. doi: 10.1371/journal.pone.0047807.
346

347 [23]Shin MY, Kwun IS. Phosphate-induced rat vascular smooth muscle cell calcification and the
348 implication of zinc deficiency in a7r5 cell viability. Prev Nutr Food Sci. 2013;18(2):92-7. doi:
349 10.3746/pnf.2013.18.2.092.
350

351 [24]Lau WL, Pai A, Moe SM, Giachelli CM. Direct effects of phosphate on vascular cell function. Adv
352 Chronic Kidney Dis. 2011;18(2):105-12. doi: 10.1053/j.ackd.2010.12.002.
353

354 [25]Durham AL, Speer MY, Scatena M, Giachelli CM, Shanahan CM. Role of smooth muscle cells in
355 vascular calcification: implications in atherosclerosis and arterial stiffness. Cardiovasc Res.
356 2018;114(4):590-600. doi: 10.1093/cvr/cvy010.
357

358 [26]Shanahan CM, Crouthamel MH, Kapustin A, Giachelli CM. Arterial Calcification in Chronic Kidney
359 Disease: Key Roles for Calcium and Phosphate. Circ Res. 2011;109(6):697-711. doi:
360 10.1161/circresaha.110.234914.
361

362 [27]Latif N, Sarathchandra P, Chester AH, Yacoub MH. Expression of smooth muscle cell markers and
363 co-activators in calcified aortic valves. Eur Heart J. 2015;36(21): 1335-45. doi:
364 10.1093/eurheartj/eht547
365

bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

366 [28]Koenen RR, Aikawa E. Editorial: Extracellular Vesicle-Mediated Processes in Cardiovascular
367 Diseases. Front Cardiovasc Med. 2018;5:133. doi: 10.3389/fcvm.2018.00133.
368
369 [29]Bakhshian Nik A, Hutcheson JD, Aikawa E. Extracellular Vesicles As Mediators of Cardiovascular
370 Calcification. Front Cardiovasc Med. 2017;4:78. doi: 10.3389/fcvm.2017.00078.
371
372 [30]Hutcheson JD, Goettsch C, Bertazzo S, Maldonado N, Ruiz JL, Goh W, et al. Genesis and growth
373 of extracellular-vesicle-derived microcalcification in atherosclerotic plaques. Nat Mat.
374 2016;15:335. doi: 10.1038/nmat4519.
375
376 [31]Kopf BS, Schipanski A, Rottmar M, Berner S, Maniura-Weber K. Enhanced differentiation of
377 human osteoblasts on Ti surfaces pre-treated with human whole blood. Acta Biomat.
378 2015;19:180-90. doi: https://doi.org/10.1016/j.actbio.2015.03.022.
379
380 [32]Trion A, van der Laarse A. Vascular smooth muscle cells and calcification in atherosclerosis. Am
381 Heart J. 2004;147(5):808-14. doi: https://doi.org/10.1016/j.ahj.2003.10.047.
382
383 [33]Cloyd KL, El-Hamamsy I, Boonrungsiman S, Hedegaard M, Gentleman E, Sarathchandra P, et al.
384 Characterization of Porcine Aortic Valvular Interstitial Cell 'Calcified' Nodules. PLoS One.
385 2012;7(10):e48154. doi: 10.1371/journal.pone.0048154.
386
387 [34]Bertazzo, S, Zambuzzi, WF, Campos, DD, Ogeda, TL, Ferreira, CV, Bertran, CA., Hydroxyapatite
388 surface solubility and effect on cell adhesion, Colloids Surf B Biointerfaces. 2010;78(2): 177-84,
389 10.1016/j.colsurfb.2010.02.027

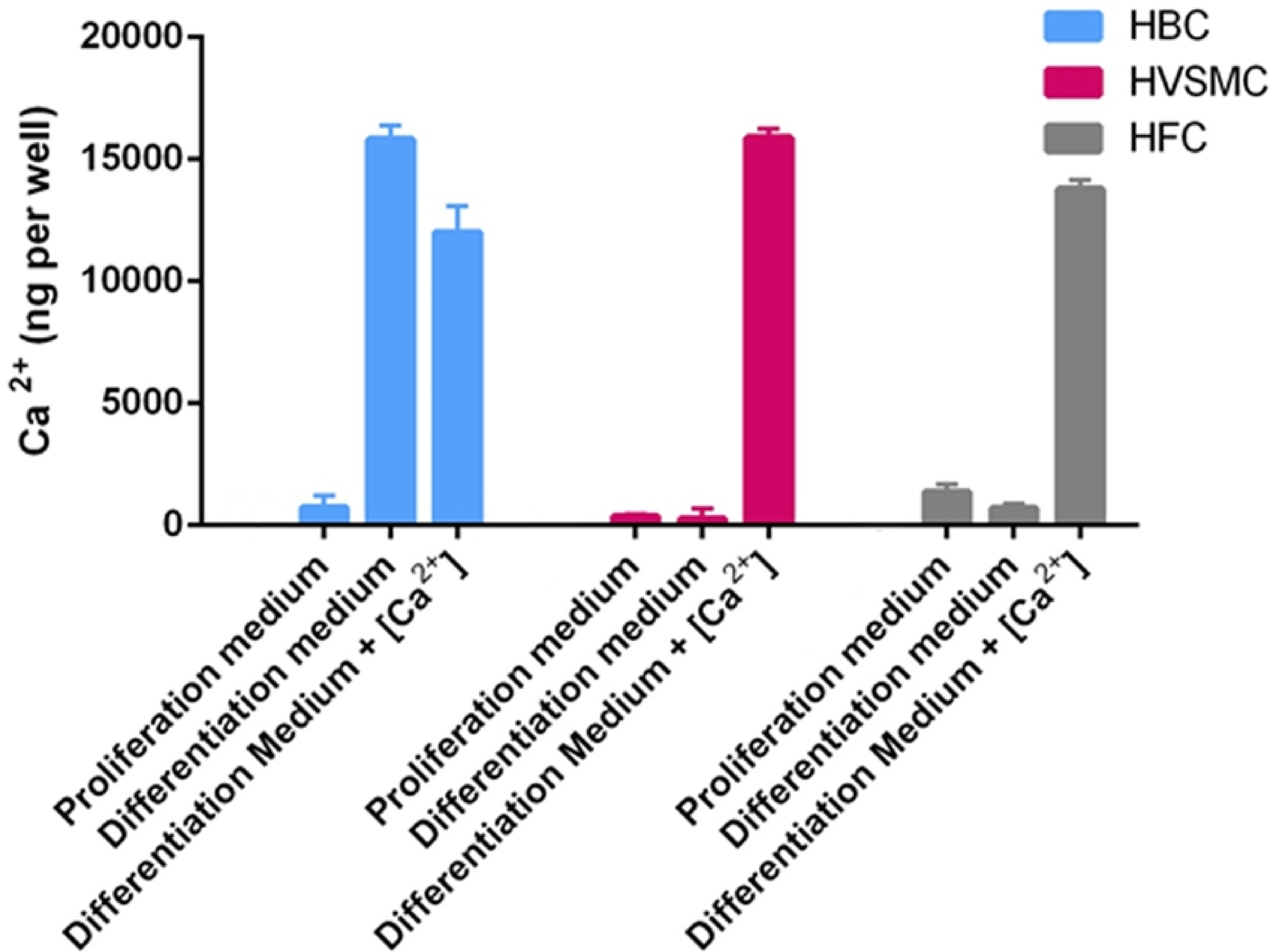
bioRxiv preprint doi: https://doi.org/10.1101/661785; this version posted June 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

390

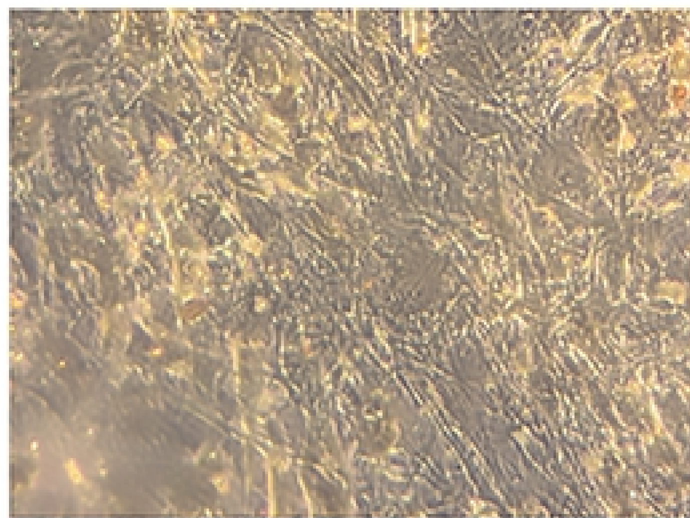
391

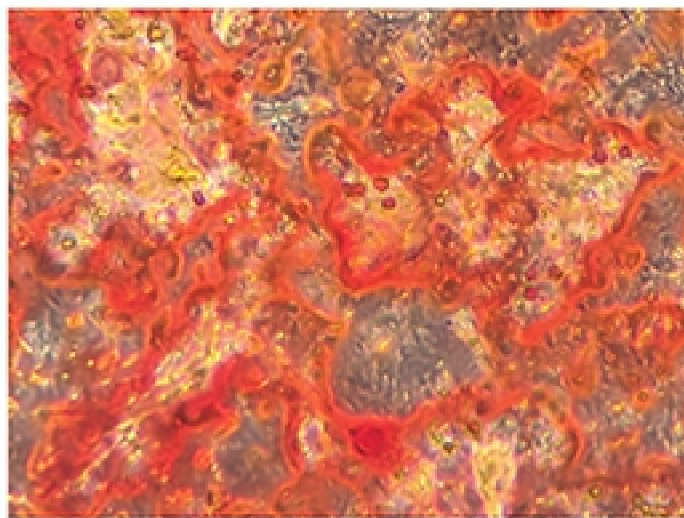
392 **Supporting Information**

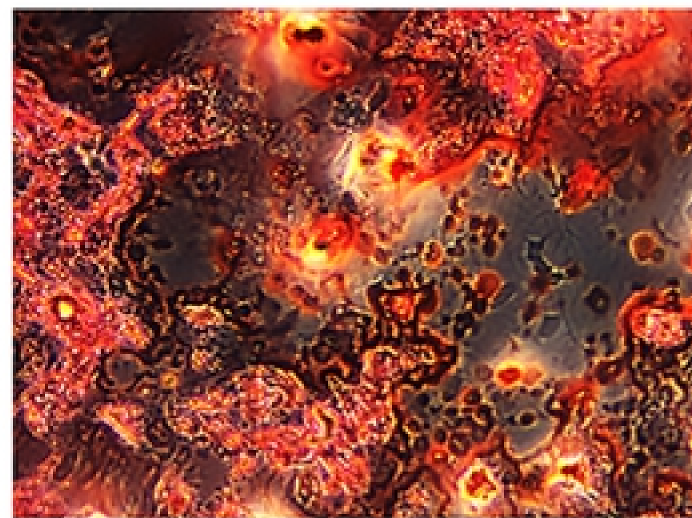
393 **S1 Figure. BSE electron micrographs of calcification observed in human cardiovascular**
394 **tissue and in the HBC culture exposed to Diff media.** a Low magnification of fibrous
395 calcification observed in human cardiovascular tissue. b Low magnification of fibrous
396 calcification produced in HBC culture exposed to Diff media. Scale bars = 10 µm. c Higher
397 magnification of calcification observed in human cardiovascular tissue where the calcified
398 fibers along with collagen fibers can be observed. d Higher magnification of calcification
399 produced in HBC culture exposed to Diff media. Scale bars = 1 µm.

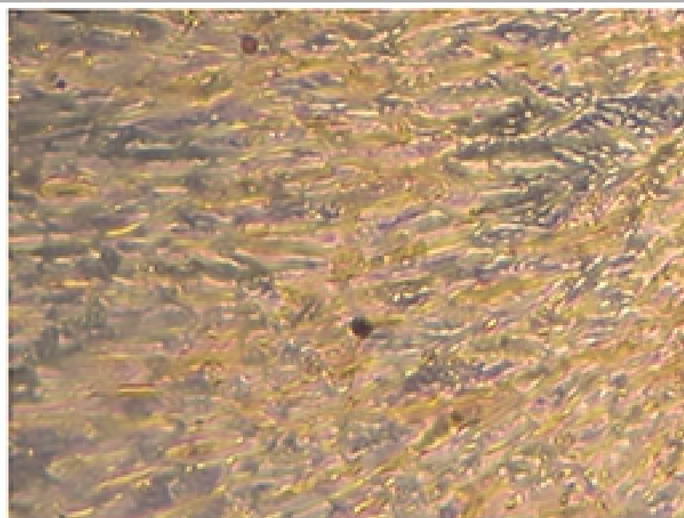


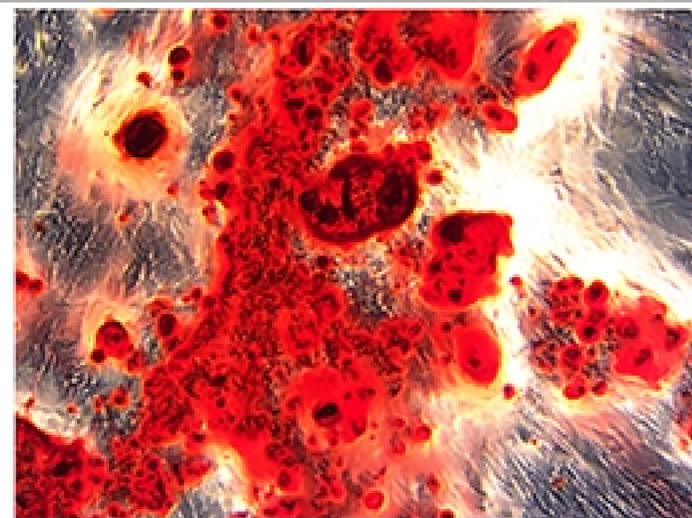
Figure



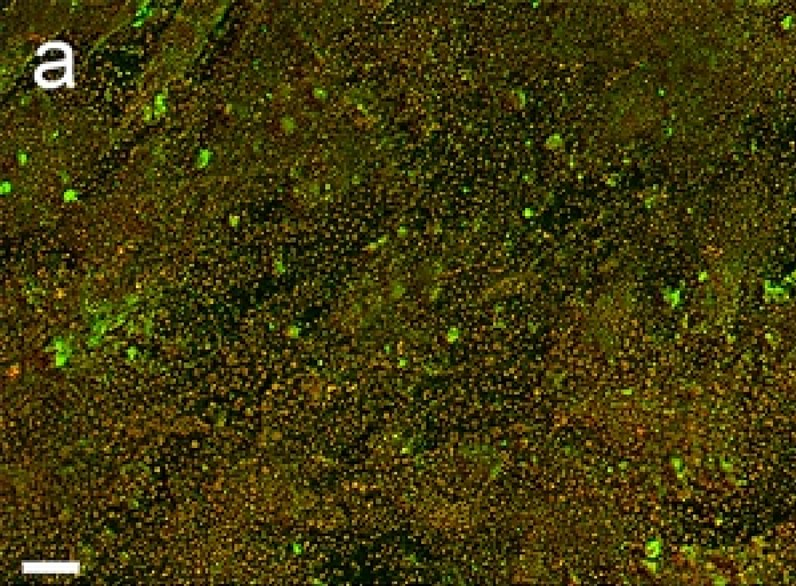


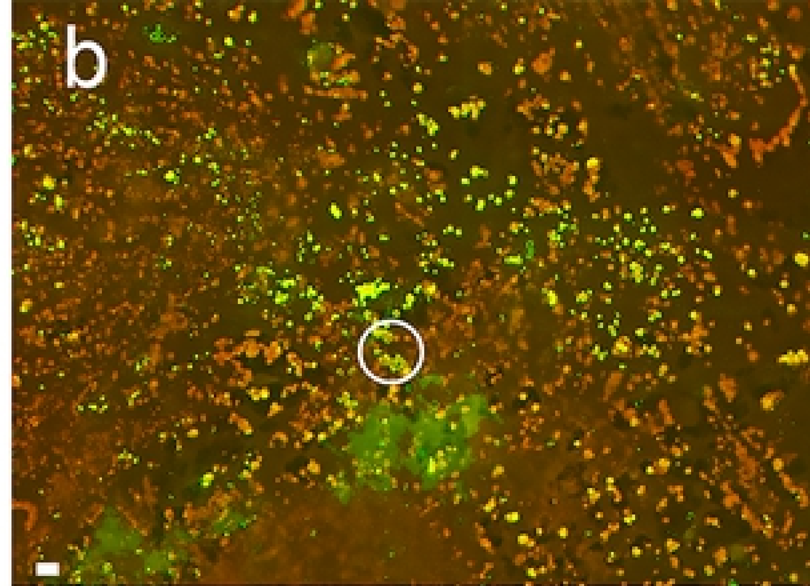


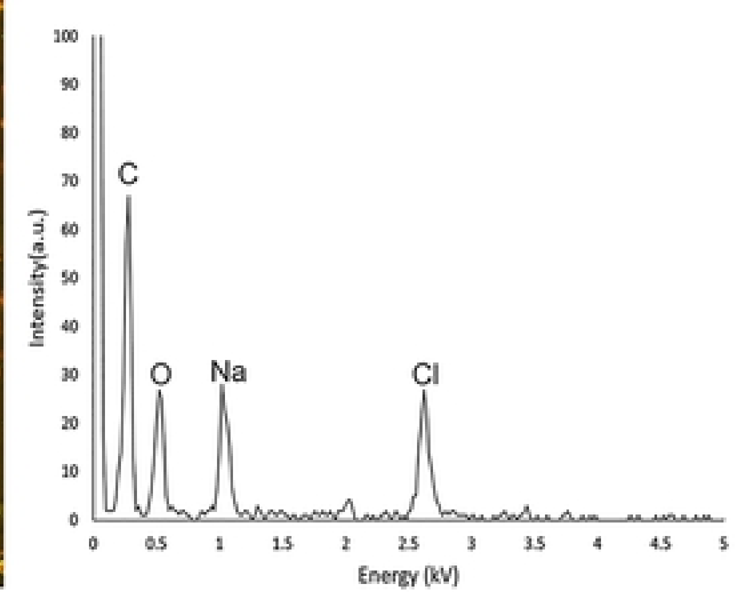


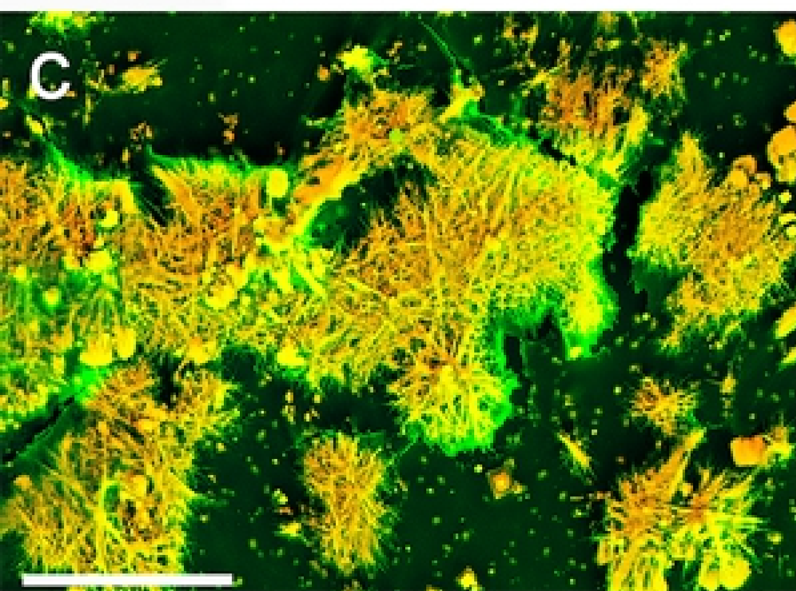


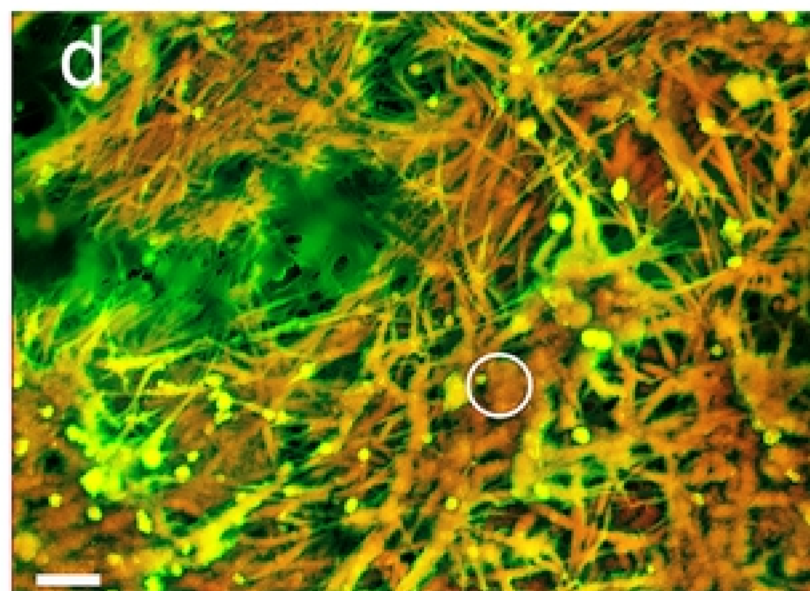
Figure

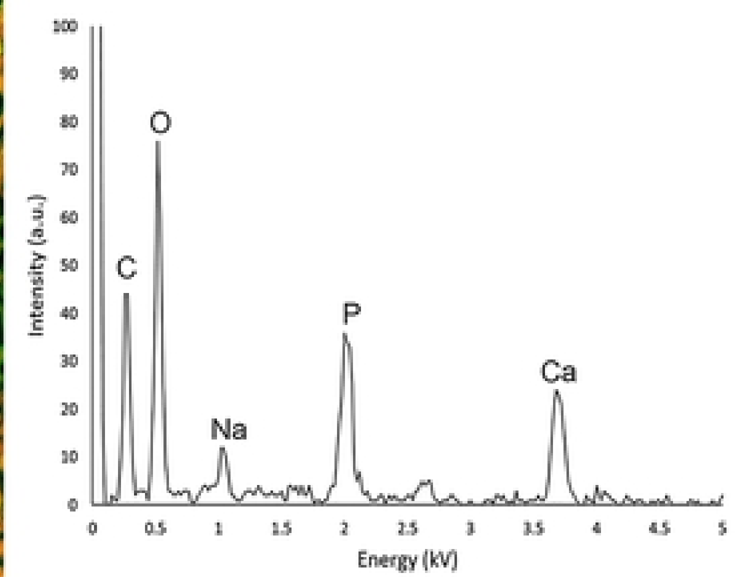


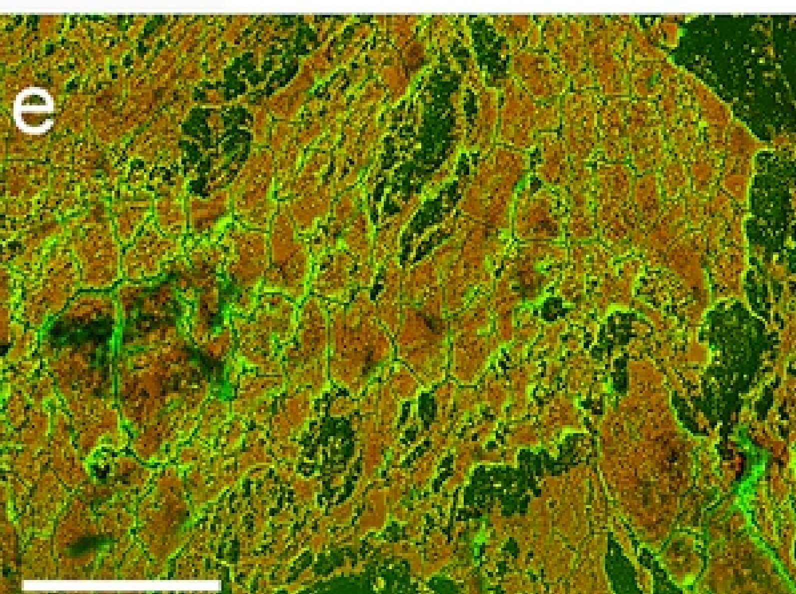


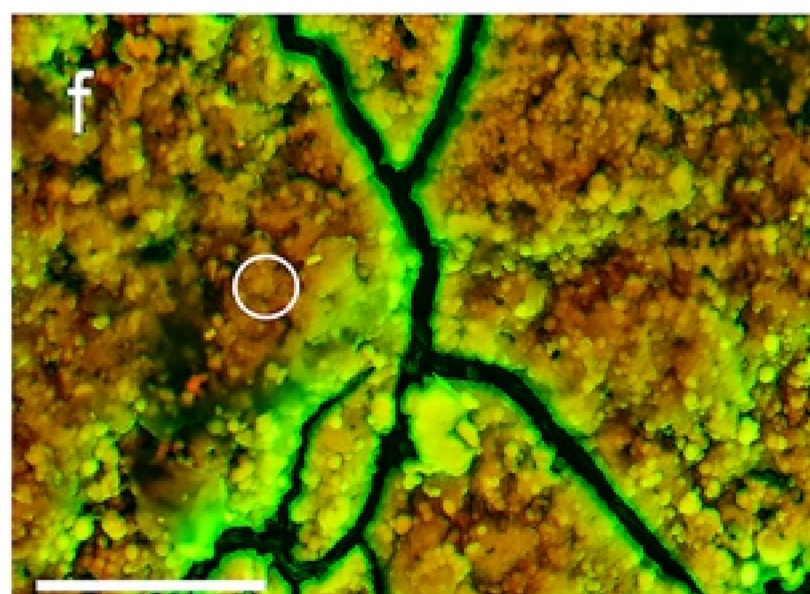


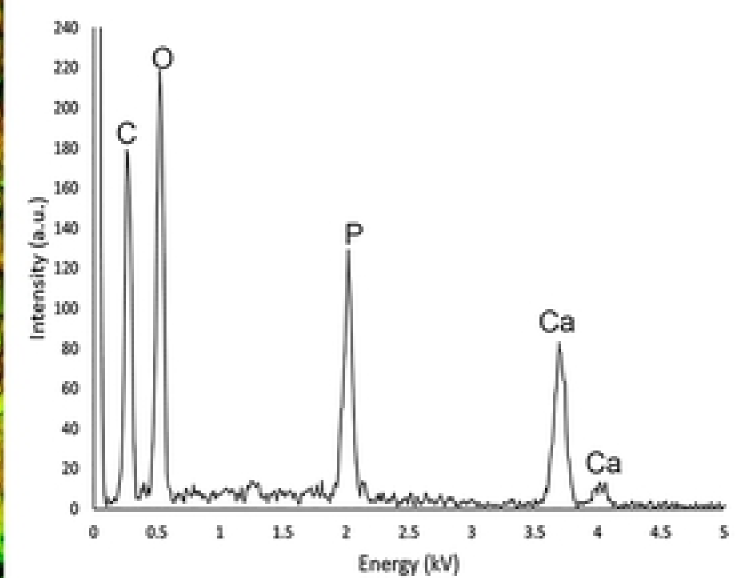




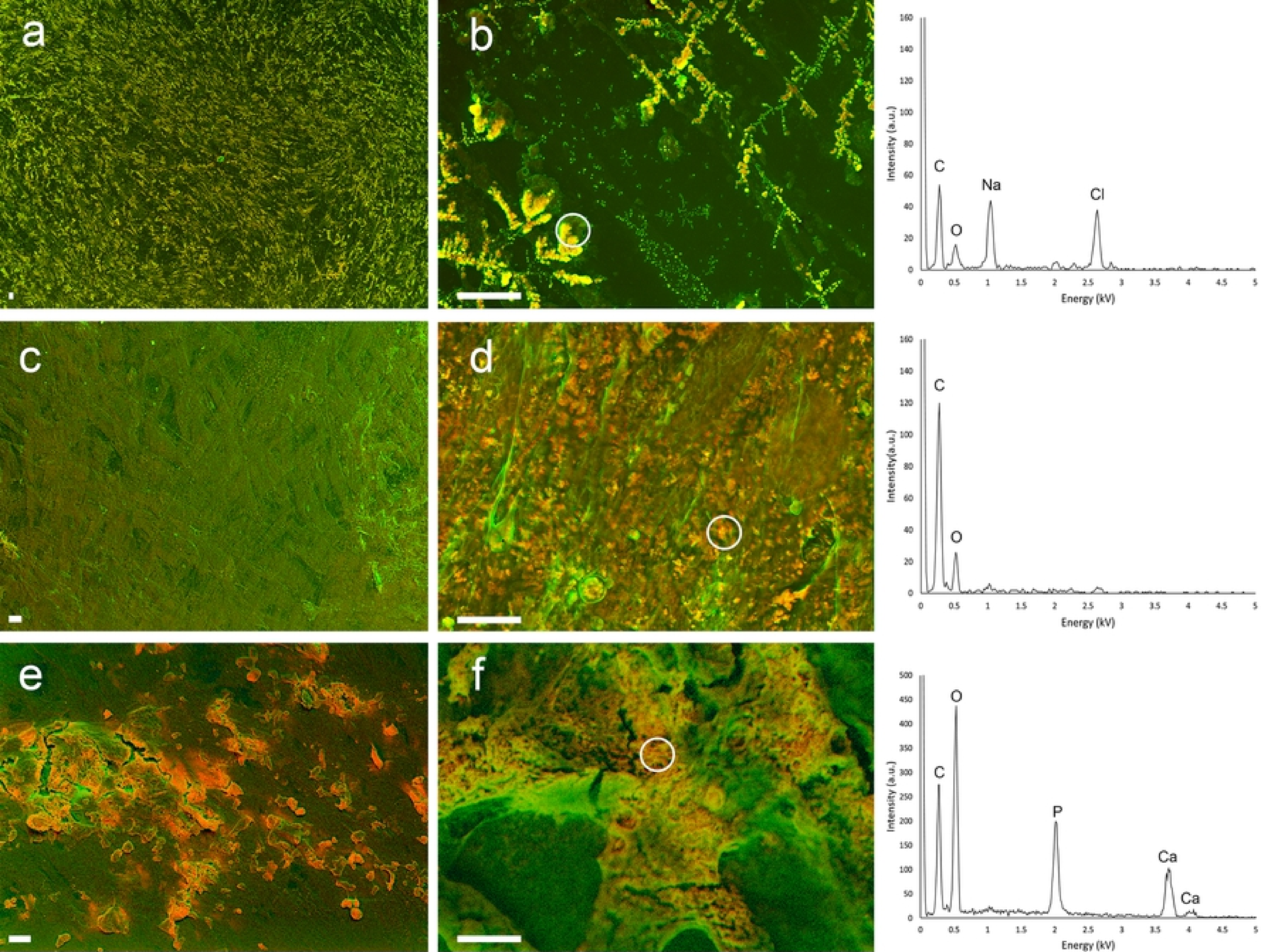




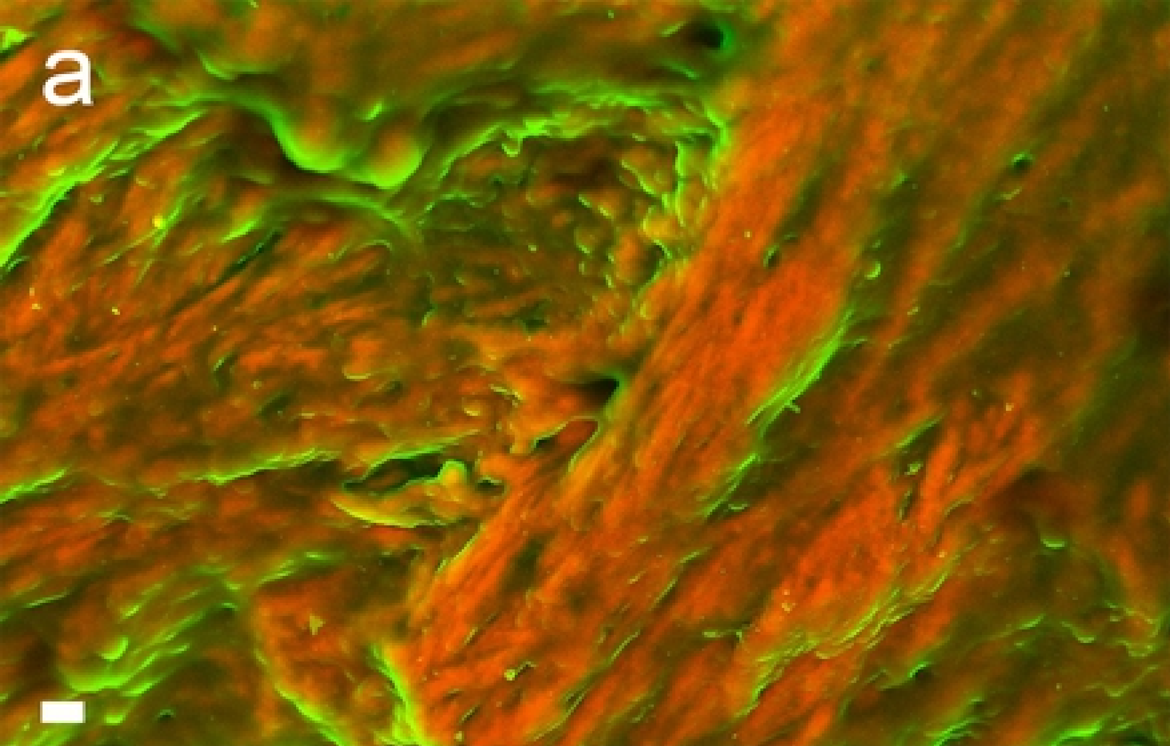


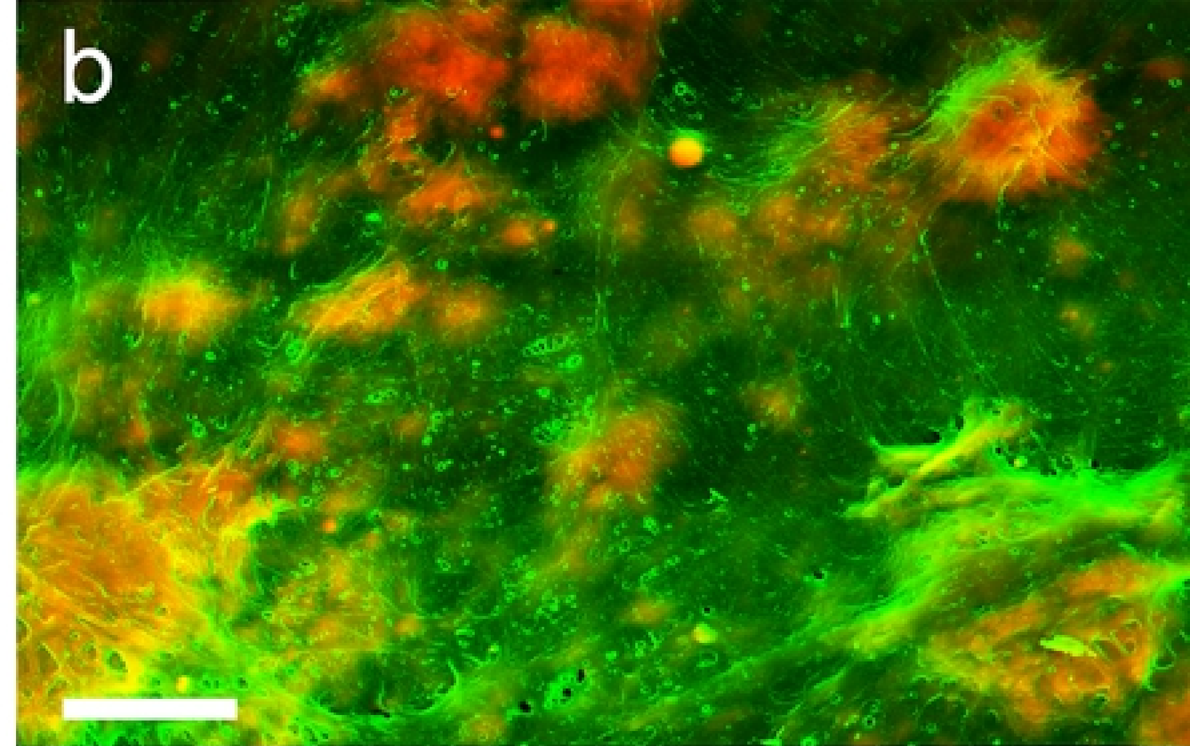


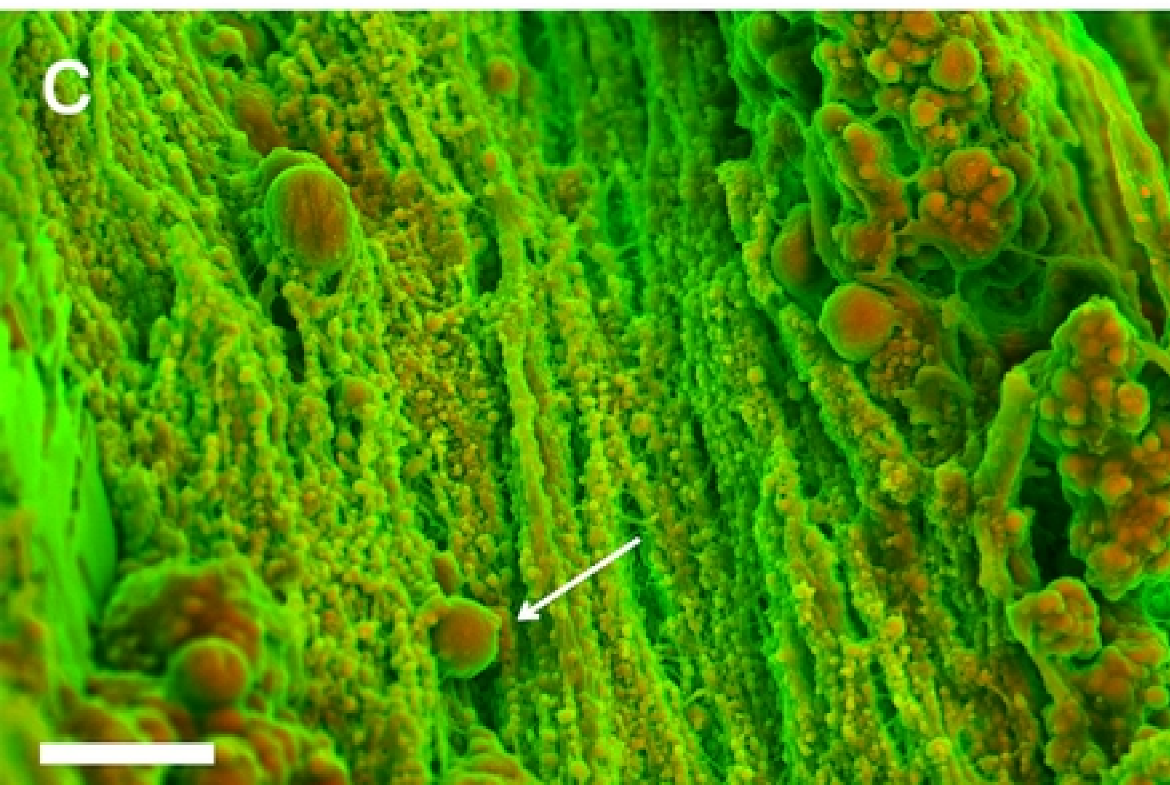
Figure

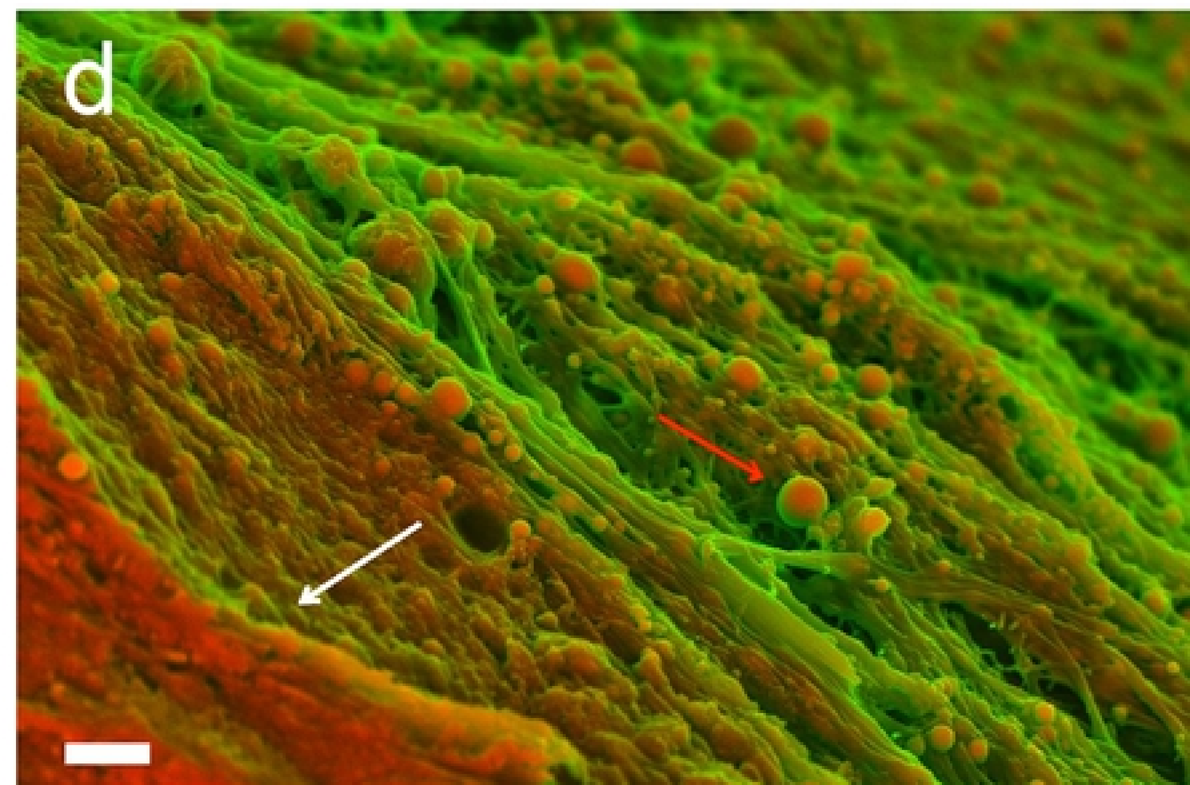


Figure









Figure