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Hierarchical electrospun tendon-ligament bioinspired scaffolds induce changes in fibroblasts morphology under static and dynamic conditions

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1 **Summary**

2 The regeneration of injured tendons and ligaments is challenging since the scaffolds
3 needs proper mechanical properties and a biomimetic morphology. In particular, the
4 morphological arrangement of scaffolds is a key point to drive the cells growth to
5 properly regenerate the collagen extracellular matrix. Electrospinning is a promising
6 technique to produce hierarchically structured nanofibrous scaffolds able to guide cells
7 in the regeneration of the injured tissue. Moreover, the dynamic stretching in bioreactors
8 of electrospun scaffolds had demonstrated to speed up cell shape modifications *in vitro*.
9 The aim of the present study was to combine different imaging techniques such as high-
10 resolution x-ray tomography (XCT), scanning electron microscopy (SEM), fluorescence
11 microscopy and histology to investigate if hierarchically structured poly(L-lactic acid)
12 and collagen electrospun scaffolds can induce morphological modifications in human
13 fibroblasts, while cultured in static and dynamic conditions. After 7 days of parallel
14 cultures, the results assessed that fibroblasts had proliferated on the external nanofibrous
15 sheath of the static scaffolds, elongating themselves circumferentially. The dynamic
16 cultures revealed a preferential axial orientation of fibroblasts growth on the external
17 sheath. The aligned nanofiber bundles inside the hierarchical scaffolds instead, allowed
18 a physiological distribution of the fibroblasts along the nanofiber direction. Inside the
19 dynamic scaffolds, cells appeared thinner compared with the static counterpart. This
20 study had demonstrated that hierarchically structured electrospun scaffolds can induce
21 different fibroblasts morphological modifications during static and dynamic conditions,
22 modifying their shape in the direction of the applied loads.

23

24 **Keywords:**

25 Electrospinning, Hierarchical Scaffolds, High-Resolution X-Ray Tomography, Cell
26 Culture, Dynamic Cell Culture, Cell Morphology, Tissue Engineering, Tendons and
27 Ligaments.

28

29 **Introduction**

30 The challenge of the innovative three-dimensional scaffolds, suitable for tendon and
31 ligament regeneration, is to strictly reproduce the native tissue mechanical properties
32 and hierarchical morphology (Alshomer et al., 2018; Cheng et al., 2015; Goulet et al.,
33 2014; Kuo et al., 2010). The morphological arrangement of the scaffold is fundamental
34 to correctly drive cell proliferation and growth, during collagen extracellular matrix
35 regeneration. It has been shown that fibroblasts and tenocytes shape is strictly dependent
36 on the specific site of growth *in vivo*: cells that colonize tendon and ligament membranes
37 (made of randomly arranged collagen fibrils), tend to spread their bodies; conversely,
38 cells in the internal volume of these tissues appear elongated in the direction of the
39 axially oriented fibrils (Kannus, 2000; Kastelic et al., 1978; Murphy et al., 2016).
40 Several manufacturing approaches to produce fibrous scaffolds inspired to tendons or
41 ligaments have been investigated in literature, among these electrospinning technology
42 is the most promising (Sensini & Cristofolini, 2018). Thanks to the possibility to obtain
43 nanoscale fibers with different spatial arrangements, electrospun scaffolds have
44 demonstrated enhancement of cellular orientation in the fibers direction (Bosworth &
45 Downes, 2011; Denchai et al., 2018). Furthermore, several studies have confirmed the
46 possibility to speed up cell proliferation and elongation on the electrospun scaffolds with
47 a simplified shape, such as flat mats, bundles or yarns, by uniaxially stretching the
48 constructs in a bioreactor (Bosworth et al., 2014; Wu et al., 2017; Xu et al., 2014;
49 Youngstrom & Barrett, 2016). These simple designs allow for convenient
50 documentation of changes in cellular shape using standard techniques, such as scanning
51 electron microscopy (SEM), fluorescent microscopy or histology. Despite the high-
52 quality of images and the cellular information obtainable, these gold-standard methods
53 have shown some limitation when applied to the study of the cell morphology on

54 complex three-dimensional scaffolds (Leferink et al., 2016). SEM images can achieve a
55 high-resolution, but are limited to the surface of the structures. Fluorescent techniques,
56 such as fluorescent or confocal microscopy, allow an accurate identification of the shape
57 of cells, but are strongly limited by possible autofluorescent effects of the nanofibers,
58 especially if they are composed by natural polymers such as collagen (Sensini et al.,
59 2018). Moreover, these techniques do not allow easy visualization if the structure
60 investigated is not planar, making the investigation of three-dimensional scaffolds
61 challenging. On the other hand, histology allows a clear identification of the cellular
62 components, even in case of three-dimensional shapes, but it typically produces a bi-
63 dimensional view of the specimens and of the cells inside. This limits a correct definition
64 of the cellular shape. Moreover, during the slicing and washing procedure, in particular
65 for the electrospun materials, it is easy to damage parts of the scaffold losing the related
66 information. A possible solution to overcome these limitations is offered by high-
67 resolution x-ray computed tomography (XCT). However, due to the low X-ray
68 attenuation of the polymeric nanofibers, XCT investigation of electrospun materials is
69 particularly challenging. This problem is especially true in the case of collagenous
70 materials (Balint et al., 2016; Zidek et al., 2016). Recent studies have defined dedicated
71 protocols to overcome such limitations even in case of submicron voxel sizes (Bosworth
72 et al., 2014; Sensini et al., 2018). Furthermore, Bradley et al. have defined a procedure
73 to document, by using a laboratory XCT, cell infiltration inside electrospun mats of
74 random microfibers (Bradley et al., 2017). However, to the best of our knowledge, no
75 work has ever tried to investigate the cell growth and infiltration in complex three-
76 dimensional electrospun nanofibrous scaffolds by combining XCT and other different
77 imaging techniques. This approach could be fundamental to analyze how the different
78 elements of the scaffolds can induce cellular morphological modifications.

79 The aim of the present study was to compare the fibroblast morphological modifications
80 during static and dynamic culture protocols on complex electrospun scaffolds. Cells
81 were seeded on three-dimensional electrospun nanofibrous hierarchically structured
82 scaffolds made of a poly(*L*-lactic acid) (PLLA) and collagen (Coll) blend. Different
83 imaging techniques including high-resolution x-ray tomography (XCT), scanning
84 electron microscopy (SEM), fluorescent microscopy and histology were employed
85 confirming different cellular modifications in shape and orientation during static and
86 dynamic conditions of culture.

87 **Materials and methods**

88 In order to investigate the morphologically changes in the fibroblasts shape, electrospun
89 PLLA/Coll nanofibrous hierarchically structured scaffolds were produced (Figure
90 1(A)). The scaffolds were seeded with human fibroblasts and cultured in different
91 conditions for 7 days: two of each in static conditions, while the other two were stretched
92 two times in a bioreactor for 1 hour each (Figure 1(B)). At the end of the culture, the
93 specimens were cut in pieces and investigated with different imaging techniques (Figure
94 1(C)).

95 *Hierarchical electrospun scaffolds production*

96 In order to reproduce the morphology of tendon and ligament fibrils and fascicles
97 (Kannus, 2000; Murphy et al., 2016), electrospun bundles (cross-sectional diameter =
98 550-650 μm) of aligned nanofibers (cross-sectional diameter of the nanofibers =
99 $0.36\pm 0.06 \mu\text{m}$) of a PLLA/Coll-75/25 (w/w) blend were produced as previously
100 described (Sensini et al., 2017, 2018). To obtain the bundles the following
101 electrospinning parameters were used: a rotating drum collector (peripheral speed = 22.8
102 m s^{-1}); the polymer solution was delivered through two needles (internal diameter 0.51

103 mm); room temperature (RT) and relative humidity 20–30%; applied voltage = 22 kV;
104 feed rate = 0.5 mL h⁻¹, electrospinning time = 2 hours; needles-collector distance = 200
105 mm; the sliding spinneret with the two needles had an excursion of 120 mm, with a
106 sliding speed of 1200 mm min⁻¹.

107 To reproduce the structure of a whole tendon or ligament (Kastelic et al., 1978; Murphy
108 et al., 2016), each bundle was pulled out from the drum, obtaining a ring-shaped
109 structure that was twisted in the middle and bent over itself. Then, each assembly was
110 covered with an electrospun epitenon/epiligament-like sheath, as previously described
111 (WO 2018/229615 A1, 2018; Sensini et al., 2019; Sensini et al., 2019). The scaffolds
112 were finally crosslinked with a mixture of *N*-(3-dimethylaminopropyl)-*N'*-
113 ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) (Sigma-
114 Aldrich, USA) as previously described (Alberto Sensini et al., 2018) (cross-sectional
115 diameter = 1.46±0.08 mm; length of the scaffolds = 89.4±2.1 mm). Four hierarchical
116 scaffolds were produced (Figure 1).

117 *Cell seeding*

118 The four hierarchical scaffolds were sterilized by immersion in 70% (v/v) ethanol (Acros
119 Organics, Thermo Fisher Scientific, BEL) for 1 hour, washed in sterile PBS (Thermo
120 Fisher Scientific, USA) three times to remove any remaining ethanol and equilibrated in
121 complete medium for 24 hours. The complete medium was obtained by mixing
122 Dulbecco Modified Eagle Medium (DMEM) (i.e. 4.5 g/L D-Glucose, with
123 GlutaMAXTM and Pyruvate) (Thermo Fisher Scientific, USA), 10% foetal bovine serum
124 (Thermo Fisher Scientific, USA) and 1% (v/v) penicillin/streptomycin solution (Thermo
125 Fisher Scientific, USA).

126 Human foreskin fibroblasts (Hs27) were cultivated with complete medium at 37°C in a
127 humid atmosphere with 5% CO₂. Medium was refreshed three times a week and cells
128 were used between passage 4 and 6.

129 To perform the test, cells were seeded at 2.0 x 10⁵ cells/scaffold. In particular, cells were
130 suspended in 350 microliters of complete medium and seeded, using a syringe with a
131 25G needle: half volume was seeded on one side of the scaffold, then the scaffold was
132 turned 180° and the other half volume was seeded on the other side. The seeding was
133 carried out in a sterile petri dish. After 45 minutes in an incubator at 37°C and 5% CO₂,
134 each hierarchical scaffold was transferred into one low adherence T25 flask each and
135 covered with 5 ml of complete medium to allow for cell proliferation.

136 In order to avoid potential artefacts caused by the relevant amount of medium during the
137 dynamic cultures (see below) and the total length of the specimens, quantitative data
138 regarding the cell viability were not reported.

139 After 7 days of culture, the hierarchical scaffolds were fixed for 48 hours in 4%
140 paraformaldehyde (PFA, Sigma-Aldrich, Saint Louis, USA) in PBS (at 4°C). Then, each
141 specimen was cut in the center and divided in two equal sections: one half for the
142 SEM/XCT imaging; the other was cut in two additional pieces for fluorescence
143 microscopy and histology (Figure 1(C)).

144 *Dynamic cultures in bioreactor*

145 The dynamic culture was carried out on two hierarchical scaffolds by using a
146 commercial bioreactor (MCB1, CellScale, CAN). Before each stretching session, the
147 bioreactor was sterilized by washing the test chamber in ethanol 70% (v/v) and sterilized
148 by means UV radiations under a fume hood for an hour. To transmit a uniaxial
149 stretching, the hierarchical scaffolds were hooked between the stainless-steel actuator of

150 the bioreactor and a custom-made 3D printed pin of acrylonitrile butadiene styrene
151 (ABS) (ABS-M30, Stratasys, USA). During each session, the specimens were covered
152 with 150 ml of complete medium and stimulated for 1 hour with 4 mm of displacement
153 (corresponding at a strain of approximately 5%) at a frequency of 1 Hz (3600 cycles).
154 These parameters were chosen in accordance with the literature (Bosworth et al., 2014).
155 Each of the two scaffolds was stretched two times during the 7 days of culture (i.e. at
156 day three and day six of culture). After each bioreactor session, the dynamic specimens
157 were put in T25 flasks with 5 ml of medium and left in static conditions for two days.

158 *Static cultures*

159 Parallely, as a control for the dynamic specimens, two hierarchical scaffolds were
160 cultured for 7 days in T25 flasks with 5 ml of medium, changing the medium at day 3
161 and six of culture.

162 *High-resolution x-ray tomography*

163 To evaluate the full-field fibroblast distribution, morphology and the hierarchical
164 arrangement in the scaffolds, an XCT investigation was performed.

165 Firstly, after fixing with PFA, the scaffolds specimens for XCT were washed three times
166 in PBS. Specimens were post-fixed with osmium tetroxide (Sigma-Aldrich, USA) for 1
167 hour and then dehydrated in ethanol (v/v) 30%, 50%, 70%, 90%, 95% and 100% for 1
168 hour for each step (the 100% step was repeated twice). Then the specimens were
169 dehydrated in acetone for 20 minutes. The specimens were chemically dried using a
170 mixture of hexamethyldisilazane (HMDS) (Sigma-Aldrich, USA) and ethanol in
171 different (v/v) ratios: (i) HMDS:ethanol = 1:2 (v/v) and (ii) HMDS:ethanol = 2:1 (v/v)
172 for 20 minutes each. An additional step was performed in HMDS 100% until dry.

173 To avoid imaging artifacts resulting from micromovements, the specimens were fixed
174 in custom-made plastic masks adapted from (Sensini et al., 2018).

175 The two dynamic and static specimens of the hierarchical scaffolds were scanned with
176 a laboratory XCT system (Xradia 520 Versa, Zeiss X-ray Microscopy, USA), with the
177 following parameters:

178 (i) Voxel size = 1.6 micrometers (i.e. overview of the specimens): 40 kV voltage, 2
179 W power, 49 microampere tube current, 10 sec. exposure time.

180 (ii) Voxel size = 0.5 micrometers (i.e. zoom-in on the fibroblasts): 40 kV voltage, 2
181 W power, 50 microampere tube current, 30 sec. exposure time.

182 All the XCT images, were reconstructed using the Scout-and-Scan Reconstructor
183 software (Zeiss, USA), and were visualized using XM3DViewer1.2.8 software (Zeiss,
184 USA).

185 *SEM imaging*

186 After the XCT investigation, in order to confirm the fibroblasts presence, the XCT
187 specimens were removed from the masks and prepared for the SEM imaging. Each
188 specimen was cut in two pieces: one was longitudinally opened with a scalpel to
189 investigate the fibroblasts on the internal bundles, while the other was left intact to
190 investigate the fibroblasts on the electrospun sheath. Scanning Electron Microscopy
191 (SEM) (Philips 515 SEM, NL) observations were carried out using an accelerating
192 voltage of 15 kV and specimens were gold sputtered.

193 *Directionality analysis*

194 In order to quantify the orientation of the nanofibers of the scaffolds, the Directionality
195 plugin of ImageJ was used (Liu, 1991; Schindelin et al., 2012; Schneider et al., 2012).
196 This approach quantifies the distribution of nanofibers within a given angle from the
197 axis of the specimen. The analysis was performed using a Local Gradient Orientation
198 method following a procedure previously applied (Sensini et al., 2018).

199 To assess the orientation of the bundles inside the hierarchical assemblies, a full volume
200 investigation was performed applying the procedure to all the slices of the XCT stack
201 (voxel size = 1.6 micrometers), after reslicing. In order to list also the orientation of the
202 nanofibers in a single crosslinked PLLA/Coll-75/25 bundle, the Directionality analysis
203 data on a XCT scan from a previous study were reported (Sensini et al., 2018).

204 To quantify the orientation of the nanofibers in the electrospun sheath the Directionality
205 analysis was performed on a stack of 5 SEM surface images (magnification = 8000x)
206 derived from (Sensini et al., 2018).

207 In order to investigate the preferential orientation of the fibroblasts on the external
208 sheath, in static and dynamic conditions of culture, a Directionality investigation was
209 performed on a stack of 2 fluorescent images for each condition of culture (see below)
210 (magnification = 20x) derived and adapted from (Sensini et al., 2018; Tseng et al., 2013).

211 As the nuclei are better visible and are stretched in the same direction of the cell itself,
212 the analysis was based on the alignment of the nuclei. Firstly, to enhance visibility of
213 the cells nuclei the fluorescent images were segmented, using ImageJ. Over each
214 segmented image, a mask was produced onto which lines were drawn of the same length
215 and orientation of the longest axis of each nucleus. Finally, the masks were analyzed
216 with Directionality as described above.

217 *Fluorescent microscopy*

218 After PFA fixing, specimens were washed with PBS and put in 3 ml of Triton-X (Sigma-
219 Aldrich, USA) 0.1% (v/v) for 15 min. Then, the scaffolds were washed 3 times with
220 PBS, before being treated with 1% (v/v) bovine serum albumin (BSA) (Sigma-Aldrich,
221 USA) in PBS, for 1 hour. Then, the specimens were washed twice with sterile PBS.
222 Phalloidin Dylight 550 (Thermo Fisher Scientific, USA) (2 units/ml in PBS) was added
223 to each sample before incubation for 90 min at RT. Then the specimens were washed
224 two times with PBS and DAPI (Sigma Aldrich, USA) (2 µg/ml) was added and
225 incubated for 20 min in the dark, at RT. The specimens were stored at 4°C in petri dishes
226 containing sterile PBS to prevent specimen dehydration. Finally, the external surfaces
227 of the were imaged using a fluorescent microscope (Axio Imager Z1, Zeiss, USA)
228 equipped with a camera (Hamamatsu HR, Hamamatsu, JAP) and a color camera
229 (AxioCam MRc, Zeiss, USA) too. Images were processed by Volocity 6.3 software
230 (Quorum Technologies Inc, UK).

231 *Histology*

232 The specimens for the haematoxylin and eosin staining were fixed in 4% (v/v) PFA/PBS
233 overnight. PFA fixed specimens were processed into paraffin (Histosec®, Merck,
234 Darmstadt, GER), using a dedicated embedder (EG1150 H, Leica, Wetzlar, GER) and
235 sectioned (slices thickness = 5 µm) using a microtome (RM2235, Leica, GER). The
236 specimens were sectioned parallelly to their longitudinal axis. Sections were
237 deparaffinized and incubated in hematoxylin (Sigma-Aldrich, USA) and then in
238 alcoholic eosin (Sigma-Aldrich, USA) for 5 min respectively. Finally, sections were
239 differentiated, dehydrated in graded series of ethanol, and mounted in dibutyl phthalate
240 xylene (DPX) (Sigma-Aldrich, USA) using glass coverslips. The histological slices of
241 the scaffolds were imaged using a microscope (Diaplan, Leitz, GER) and processed with
242 the Image-Pro Plus 6 software (Media Cybernetics, UK).

243 *Cell morphology*

244 In order to quantify the dimensions of cells (the length, i.e. the preferential direction of
245 elongation of the cellular body; and the width and thickness), in the different conditions
246 of culture, measurements of cells bodies were performed using ImageJ on the different
247 images acquired. The cells length and width were estimated from XCT, fluorescence,
248 SEM and histological images (static cultures: n = 20 cells for the length; n = 27 cells for
249 the width; dynamic cultures: n = 8 cells for the length; n = 4 cells for the width), while
250 the cells thickness was measured using SEM and histological images (static cultures: n
251 = 7 cells; dynamic cultures: n = 5 cells). The mean (three measurements for each cell)
252 of each parameter was used to produce the final mean and standard deviation of each
253 dimension.

254 **Results**

255 *Morphological investigation of the hierarchical scaffolds*

256 To investigate the orientation of the nanofibers and bundles in the different levels of the
257 hierarchical scaffolds, a Directionality analysis was performed (Figure 2). The
258 Directionality analysis confirmed the preferential axial orientation of the nanofibers in
259 the bundles, with a predominant peak of $31.4 \pm 2.82\%$ in the range of 0° - 3° from the
260 bundle axis, and a decrescent distribution (Sensini et al., 2018). A small amount of
261 nanofibers ($0.55 \pm 0.08\%$) was perpendicular to the bundle (87° - 90°). The Directionality
262 investigation showed that the nanofibers of the sheaths for the hierarchical assemblies
263 had a slight preferential circumferential orientation: more than 31% of the nanofibers
264 fell in the range of 66° - 90° . The preferential axial of alignment of the bundles inside the

265 hierarchical scaffolds was confirmed by a predominant peak of $61.6\% \pm 9.43\%$ in the
266 range of 0° - 3° , and a decrescent distribution.

267 *Fibroblasts morphology from XCT investigation*

268 The specimens mounting setup for the XCT scans successfully prevented the artefacts
269 of micromovements, permitting to obtain high-resolution images after the three-
270 dimensional reconstruction (Figure 3). The hierarchical scaffolds were homogeneous,
271 and the internal bundles strongly grouped by the electrospun sheath. The cells fixation
272 and dehydration procedure enabled visualization of the fibroblasts growth on the
273 hierarchical scaffolds (Figure 3). The reconstructions with a $1.6 \mu\text{m}$ voxel size provided
274 an overview of the specimens (Figure 3(A)). Zooming on the sheath at $0.5 \mu\text{m}$ voxel
275 size, fibroblasts were clearly distinguishable (Figure 3(B)). On the sheath of static
276 specimens, fibroblasts were circumferentially oriented along the axis of the scaffolds,
277 **spreading their bodies also** along the scaffold longitudinal axis (Figures 3(AI, AII) and
278 3(BI, BII)). On the sheath of dynamic specimens, the fibroblasts were **thinner and less**
279 **wide (see below)**, with increasing axial orientation compared to the static ones (Figure
280 3(AIII, AIV) and 3(BIII, BIV)). In the internal bundles, due to the high-alignment of the
281 nanofibers and the elongated shape of the fibroblasts, cell detection was not possible.

282 *Fibroblasts morphology from SEM investigation*

283 The SEM images obtained for the same specimens used for the XCT scans and are
284 shown in Figure 4. Despite the preferential random arrangement of the sheath
285 nanofibers, the static fibroblasts showed a circumferential orientation with spread bodies
286 (Figure 4(AI, II)), while cells on the dynamic specimens were thinner and preferentially
287 elongated axially to the hierarchical scaffolds (Figure 4(BI, II)). The SEM investigation
288 also assessed the fibroblasts infiltration inside the hierarchical scaffolds, both in static

289 and dynamic conditions. In both test conditions the internal fibroblasts appeared
290 elongated and distributed axially aligned with the bundles nanofibers (Figure 4(AIII,
291 AIV) and (BIII, BIV)). In the dynamic specimens the fibroblast appeared thinner
292 compared to the static counterpart (Figure 4(BIII, IV)).

293 *Fibroblasts morphology from fluorescence microscopy investigation*

294 The fluorescence microscopy results are reported in Figure 5. On the static specimens,
295 fibroblasts were again circumferentially oriented on the nanofibrous sheath (the nuclei
296 were also ovalized in the transversal direction of the scaffolds), with spread bodies
297 (Figure 5(A)). In the sheath of dynamic specimens, the fibroblasts appeared more axially
298 aligned compared with the static ones (Figure 5(B)).

299 The Directionality analysis of the cell nuclei performed on the electrospun sheaths
300 revealed that, in static specimens, the 71.1% of cells were oriented in a range of 72°-90°
301 (Figure 6). In the dynamic specimens instead, the 53.4% of cells were oriented in the
302 range of 0°-18° (Figure 6).

303 The fluorescence investigation in the internal bundles was not possible due to a low
304 infiltration of the fluorescent reagents.

305 *Fibroblasts morphology from histological investigation*

306 The histological investigation outcomes are showed in Figure 6. The axial slices of the
307 hierarchical scaffolds obtained, had cut transversally the fibroblasts grown on the
308 electrospun sheaths (Figure 7(AI, II) and 7(BI, II)). In both the static and dynamic
309 specimens, the fibroblasts appeared preferentially circumferentially arranged, due to the
310 reduced axial elongation of their bodies. Moreover, on the dynamic specimens, the
311 fibroblasts were thinner than on the static ones (according to their progressive extension

312 in the axial direction). Cells also infiltrated inside the hierarchical scaffolds aligning
313 themselves in the nanofibers direction (Figure 7(AIII, IV) and 7(BIII, IV)). The
314 fibroblasts in the dynamic specimens appeared thinner compared with the static
315 counterpart.

316 *Quantification of cells morphology*

317 The quantification of cells morphology revealed that in the static specimens, the cells
318 on the electrospun sheaths had a length of 72.1 ± 27.9 micrometers, a width of 18.8 ± 15.3
319 micrometers and a thickness of 3.2 ± 0.8 micrometers; in the internal bundles, the cells
320 showed a length of 26.8 ± 9.9 micrometers, a width of 2.5 ± 1.6 micrometers and a
321 thickness of 1.5 ± 0.2 micrometers. In the dynamic specimens, the cells on the sheath had
322 a length of 77 ± 52.4 micrometers, a width of 8.9 ± 6.9 micrometers and a thickness of
323 1.9 ± 0.6 micrometers; on the internal bundles the cells had a length of 21.4 ± 8.5
324 micrometers, a width of 2.6 ± 1.3 micrometers and a thickness of 1.2 ± 0.8 micrometers.

325 **Discussion**

326 To produce an electrospun scaffold suitable for tendon and ligament tissue engineering,
327 proper mechanical properties need to be combined to a biomimetic hierarchical
328 structure. These properties are mandatory to transmit physiological loads to the cells,
329 enabling their proper infiltration and growth inside the scaffolds. The aim of this study
330 was to investigate an innovative electrospun PLLA/Coll-75/25 hierarchically structured
331 scaffold, using different imaging techniques, in order to evaluate its ability to guide the
332 fibroblasts growth in static and dynamic conditions. The hierarchical scaffolds were
333 assembled by wrapping a PLLA/Coll-75/25 ring-shaped bundles of axially aligned
334 nanofibers, with an electrospun PLLA/Coll-75/25 sheath of randomly oriented

335 nanofibers. The scaffolds nanofibers and bundles were in the same size range of collagen
336 fibrils and fascicles reported in literature (Kastelic et al., 1978). The Directionality
337 analysis confirmed that bundles nanofibers, as well as bundles themselves, were axially
338 aligned with the hierarchical scaffolds, while the nanofibers of the sheath showed a
339 slightly circumferential orientation (Figure 2). In this way the hierarchical structure of a
340 whole tendon or ligament was reproduced (Kastelic et al., 1978; Murphy et al., 2016).
341 In order to evaluate the morphological changes in the cell shape induced by the
342 hierarchical scaffolds, Hs27 fibroblasts were seeded on them for 7 days, comparing a
343 static culture with a dynamic one in a bioreactor. To reproduce a physiological
344 displacement configuration, the stretching parameters of the bioreactor were chosen
345 consistently with the previous literature (Bosworth et al., 2014). At the end of the
346 cultures, the full-field XCT investigation permitted to successfully visualize the
347 fibroblasts grown on the external sheaths (Figure 3). Considering such complex and
348 three-dimensional nanofibrous scaffolds, acquiring XCT images was challenging. In
349 their work, Bradley et al. (Bradley et al., 2017) were able to visualize human fibroblasts
350 seeded on electrospun poly(lactide-*co*-glycolide) (PLGA) random microfibrinous mats by
351 using a laboratory XCT scanner thanks to the micrometric cross-section of the fibers and
352 the different levels the X-rays attenuation between the PLGA and the cellular
353 component. In the case of the PLLA/Coll nanofibers instead, it is difficult to obtain
354 tomographic images fibers, due to the low absorption of the collagen of X-rays (Balint
355 et al., 2016; Zidek et al., 2016). This criticality is increased when the aim of the XCT
356 scan is to discriminate elements with a similar attenuation and dimensions, such as cells
357 and collagenous nanofibers. This aspect was fundamental for the XCT visualization of
358 fibroblasts. Due to their spread shape and the random arrangement of the nanofibers,
359 fibroblasts detection on the electrospun sheath was clearly visible (Figure 3(B)).

360 Conversely, the identification of cells inside the internal bundles was not distinguishable
361 (Figure 3(AII) and 3(AIV)). This was mainly caused by the axially aligned nanofibers
362 and the thinner and elongated shape of the cells. Further optimization, especially in the
363 thresholding phase would possibly to allow the XCT detection of cells along the bundles
364 aligned nanofibers.

365 In order to overcome this limitation and to validate the XCT results, additional imaging
366 techniques such as fluorescence microscopy, SEM and histology were performed. The
367 combination of these imaging protocols confirmed that fibroblasts on the electrospun
368 sheath of the scaffolds adopt a different shape depending on the culture conditions
369 employed. Both on the static and on the dynamic specimens, the cells had a length that
370 was one order of magnitude longer than the other two dimensions. On the electrospun
371 sheath of the static specimens, cells were elongated along the circumference of the
372 scaffolds with a spread body, while in the dynamic ones, a prevalent axial orientation
373 with thinner and slender morphology was observed (Figures 3-5 and Figure 7).

374 Moreover, the SEM and histological investigations showed that fibroblasts were able to
375 penetrate inside the electrospun sheath, growing and aligning themselves in the direction
376 of the axially aligned nanofibers. In the dynamic specimens, the cells bodies were
377 slightly thinner and shorter (length = 21.4 ± 8.5 micrometers; thickness = 1.2 ± 0.8
378 micrometers) compared to the static ones (length = 26.8 ± 9.9 micrometers; thickness =
379 1.5 ± 0.2 micrometers) (Figures 4, 5 and Figure 7). These results were in accordance with
380 the previous studies on cell cultures carried out on PLLA/Coll electrospun bundles of
381 aligned nanofibers (Sensini & Cristofolini, 2018; Sensini et al., 2018).

382 However, considering the different imaging investigations, the fibroblasts grown on the
383 sheath of the hierarchical scaffolds showed an unprecedented phenomenon compared to
384 previous cell studies (Alshomer et al., 2018; Hampson et al., 2008; Sensini &

385 Cristofolini, 2018). In fact, the circumferential alignment and elongation of cells grown
386 in the static condition was unexpected, even considering the slightly circumferential
387 alignment of the sheath nanofibers (Figure 2). Moreover, when cultured under dynamic
388 conditions, the sheath fibroblasts progressively elongated their shape trying to align
389 themselves to the axis of the hierarchical scaffolds. All these qualitative considerations
390 about the cellular orientation, were confirmed by the cellular Directionality analysis
391 performed on cells grown on the sheaths in the different conditions of culture (Figure
392 6). This behavior can be probably ascribed to the combination of three factors: the
393 electrospinning production process of the sheath, the hydration and mechanical
394 component, and the crosslinking of the nanofibers. Firstly, the mechanism to produce
395 the sheath was proved to tune the level of compacting of the internal bundles of the
396 hierarchical scaffolds (WO 2018/229615 A1, 2018; Sensini et al., 2019; Sensini et al.,
397 2019). This effect causes a pre-tensioning of the sheath nanofibers and of the internal
398 bundles. Secondly, after immersion in the culture medium, the scaffolds absorbed the
399 liquid which likely resulted in swelling of the internal bundles inducing additional
400 stretching of the sheath. The combination of these two effects can explain the presence
401 of circumferential stress, that could in turn drive the fibroblasts to change shape even in
402 static conditions. The progressive axial alignment of cells in the dynamic cultures
403 instead, could be explained by considering the effect of the collagen crosslinking. In
404 fact, it is possible that, during the crosslinking process, the nanofibers at the interface
405 between the sheath and the internal bundles could have been crosslinked together,
406 reducing their sliding. This could have caused a transmission of the axial load between
407 the bundles and sheath, producing an increment of the longitudinal stretch of the sheath
408 themselves, that induced the cells alignment. Both these effects, to the best of our

409 knowledge, were completely unexplored so far and they need further investigations in
410 the near future increasing the sample size of the hierarchical scaffolds tested.

411 **Conclusion**

412 In this study a preliminary investigation on the change in fibroblasts morphology was
413 assessed by culturing them on electrospun hierarchical scaffolds in static and dynamic
414 conditions. The integration between XCT scans and gold-standard techniques such as
415 SEM, fluorescence microscopy and histology allowed the detection of the modifications
416 in the cell morphology and orientation. Considering the results, these electrospun
417 hierarchical scaffolds could be suitable for future *in vivo* animal study, permitting an
418 axial orientation of cells both on the electrospun sheath and the internal bundles when
419 stimulated with axial loads. Moreover, the improvement of the imaging protocols
420 developed in this study will be useful for the future development of correlative
421 microscopy workflows dedicated to similar electrospun materials.

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436 useful suggestions and the use of the facilities during the histologic investigations.

437 **Figure captions**

438 **Fig. 1.** Workflow of the experiment. (A) Electrospun hierarchical scaffolds assembly
439 (scale bar = 1 mm). (B) Fibroblasts culture: two scaffolds were cultured in static
440 conditions, while other two with uniaxial sessions of stretching in a bioreactor. (C)
441 Scaffolds preparation for the different imaging investigations (scale bar = 1 mm).

442 **Fig. 2.** Directionality analysis at different levels of the hierarchical scaffolds. The
443 directionality histograms show the comparison between: the alignment of the bundles
444 inside the hierarchical scaffold (gray bars), the distribution of nanofibers in the different
445 directions for the bundle (green bars) and on the electrospun sheath (blue bars). An angle
446 of 0° means that the nanofibers were aligned with the longitudinal axis of the
447 hierarchical scaffold, an angle of 90° means that the nanofibers were perpendicular to it.
448 Mean and standard deviation between images of the same specimen are plotted.

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452 =1 mm). (BI, III) Overview of fibroblasts on the external sheath; (AII, IV) zoom on the
453 fibroblasts (voxel size = 0.5 micrometers; scale bar = 200 micrometers).

454 **Fig. 4.** SEM images of fibroblasts cultured onto the hierarchical scaffolds in static (A)
455 and dynamic (B) conditions (scale bar = 10 micrometers). (I-II) SEM images of the
456 fibroblasts on the electrospun sheath; (III-IV) SEM fibroblasts on the internal bundles.

457 **Fig. 5.** Fluorescence images of fibroblasts onto the hierarchical scaffolds sheath in static
458 (A) and dynamic (B) conditions (scale bar = 30 micrometers).

459 **Fig. 6.** Directionality analysis of cells grown on the electrospun sheaths (based on the
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462 scaffold, an angle of 90° means that the cells were perpendicular to it. Mean and standard
463 deviation between images of the static and dynamic specimens are plotted.

464 **Fig. 7.** Histological investigation on the hierarchical scaffolds cultured in static (A) and
465 dynamic (B) conditions (scale bar = 50 micrometers). (I-II) Zoom-in on the fibroblasts
466 on the electrospun sheath; (III-IV) images of the elongated fibroblasts on the aligned
467 nanofibers of the internal bundles.

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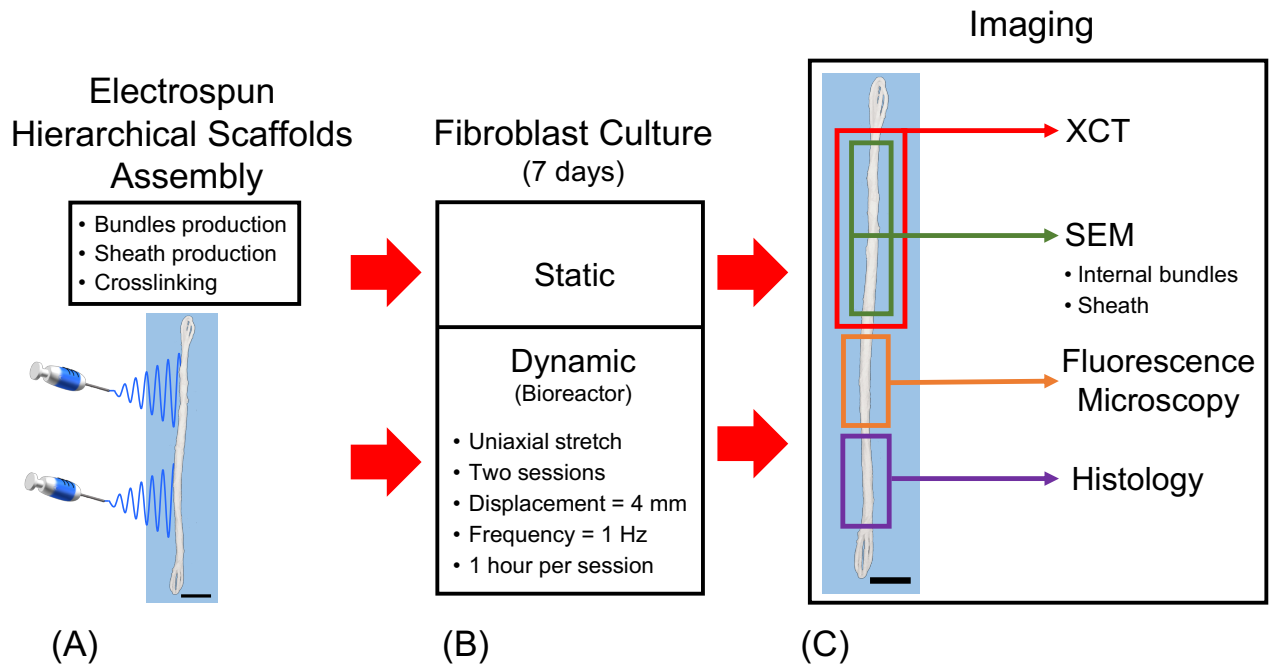
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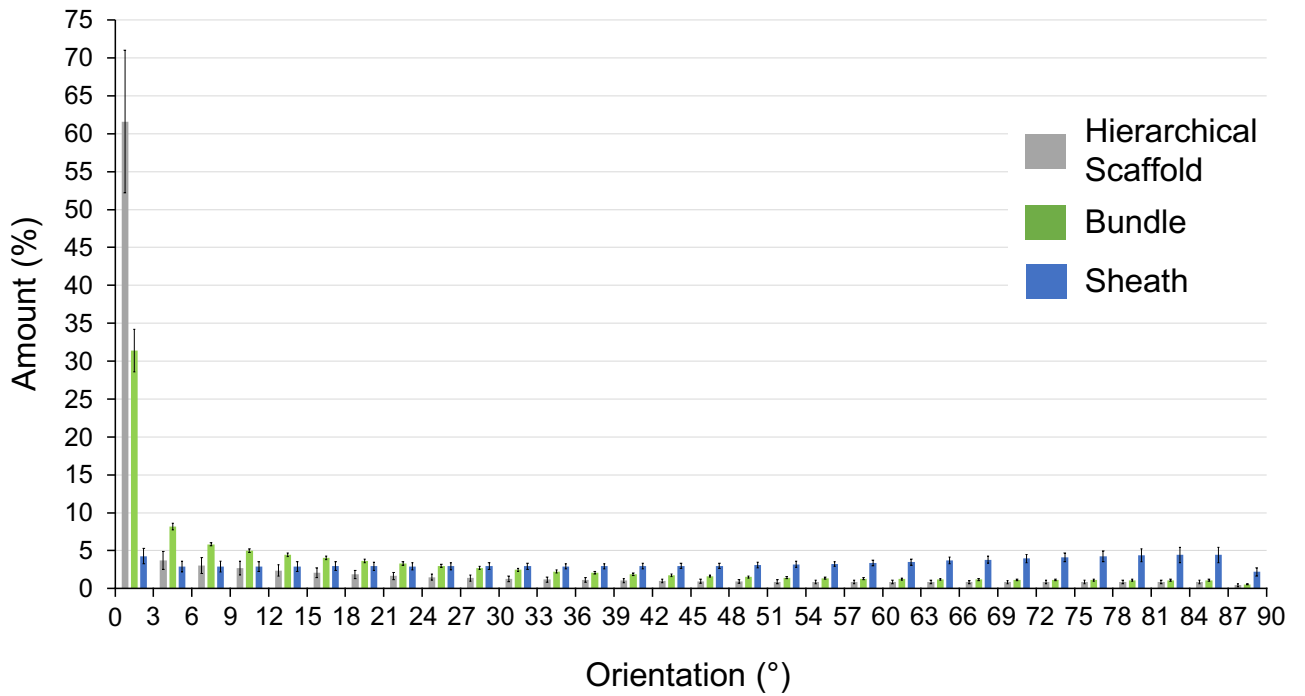
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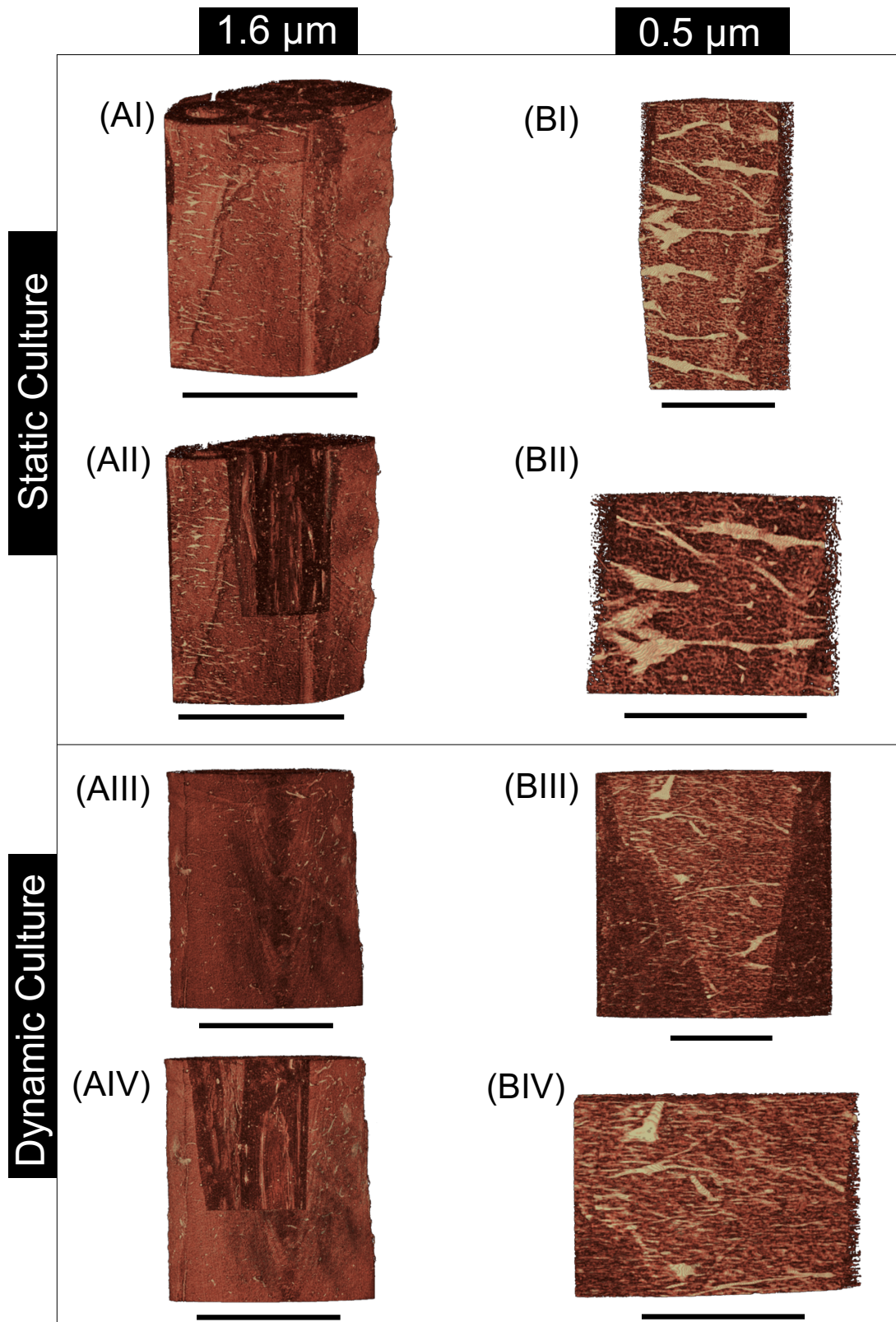
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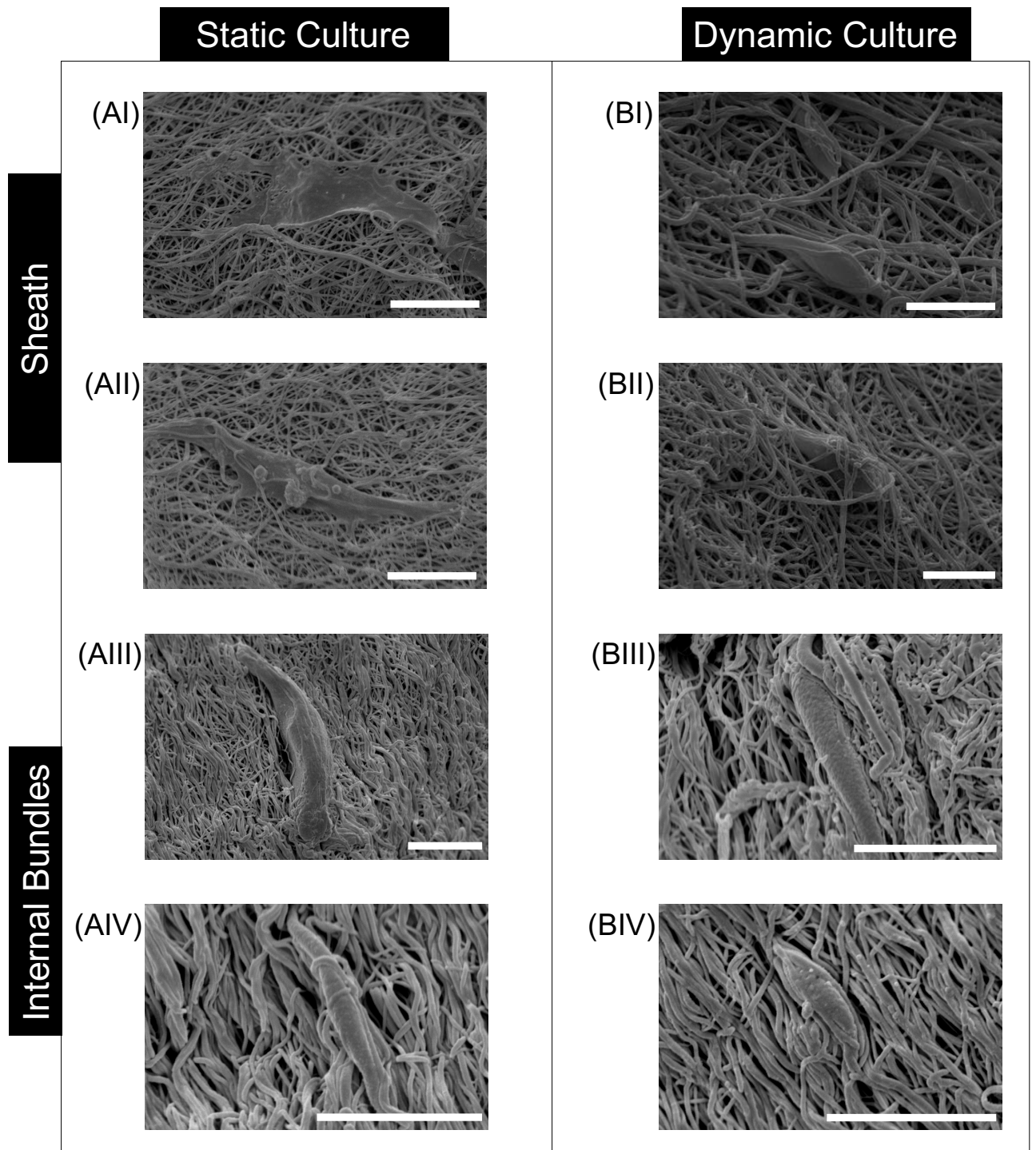
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Fig. 2. Directionality analysis at different levels of the hierarchical scaffolds. The directionality histograms show the comparison between: the alignment of the bundles inside the hierarchical scaffold (gray bars), the distribution of nanofibers in the different directions for the bundle (green bars) and on the electrospun sheath (blue bars). An angle of 0° means that the nanofibers were aligned with the longitudinal axis of the hierarchical scaffold, an angle of 90° means that the nanofibers were perpendicular to it. Mean and standard deviation between images of the same specimen are plotted.

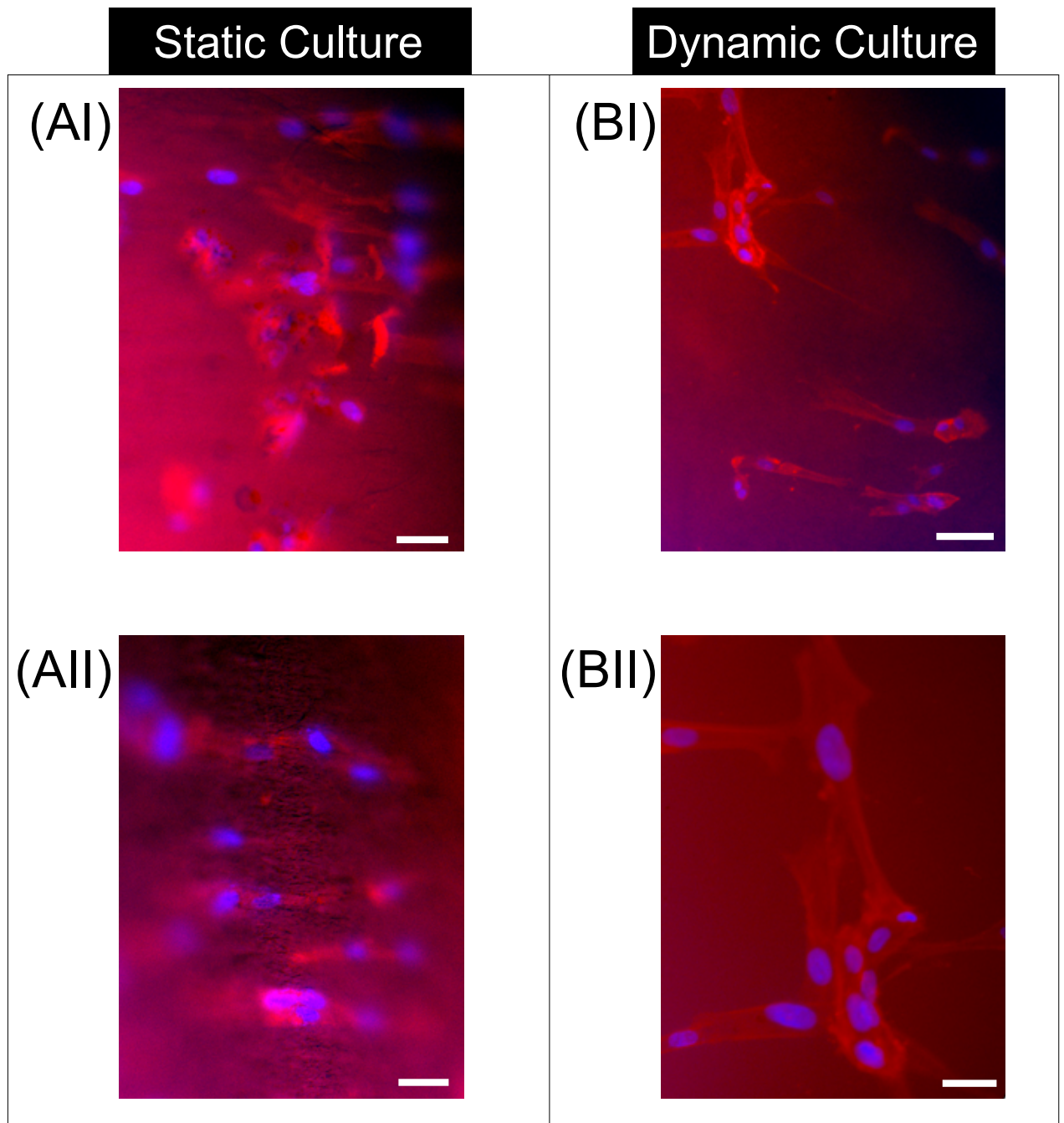


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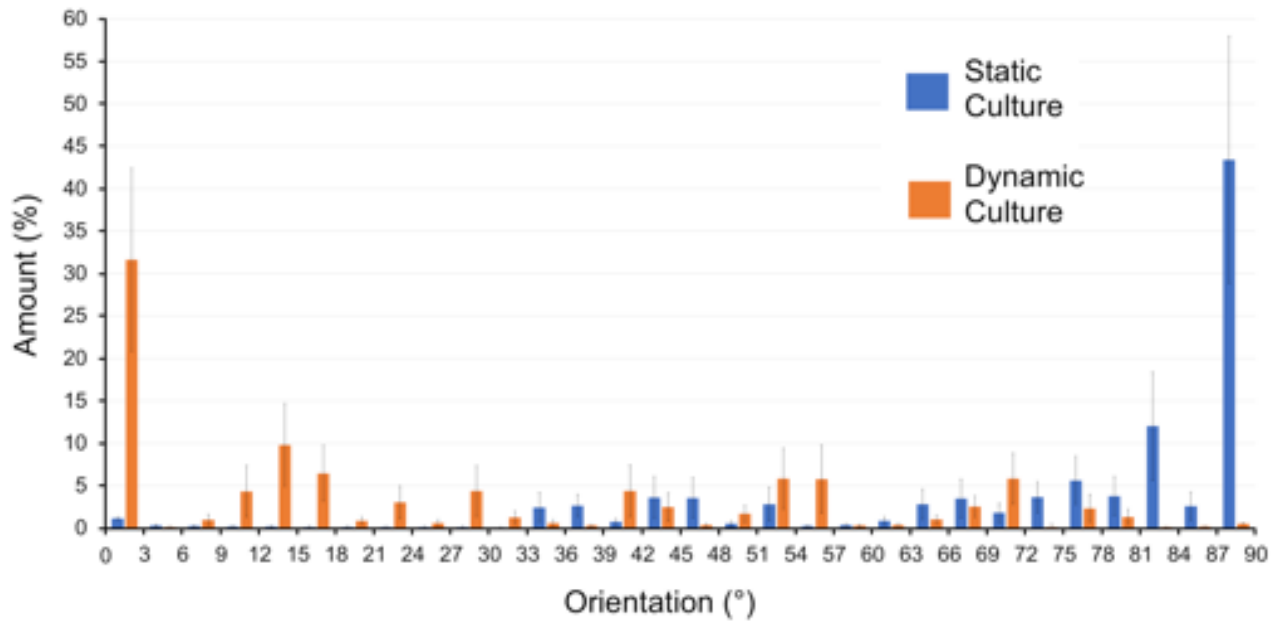


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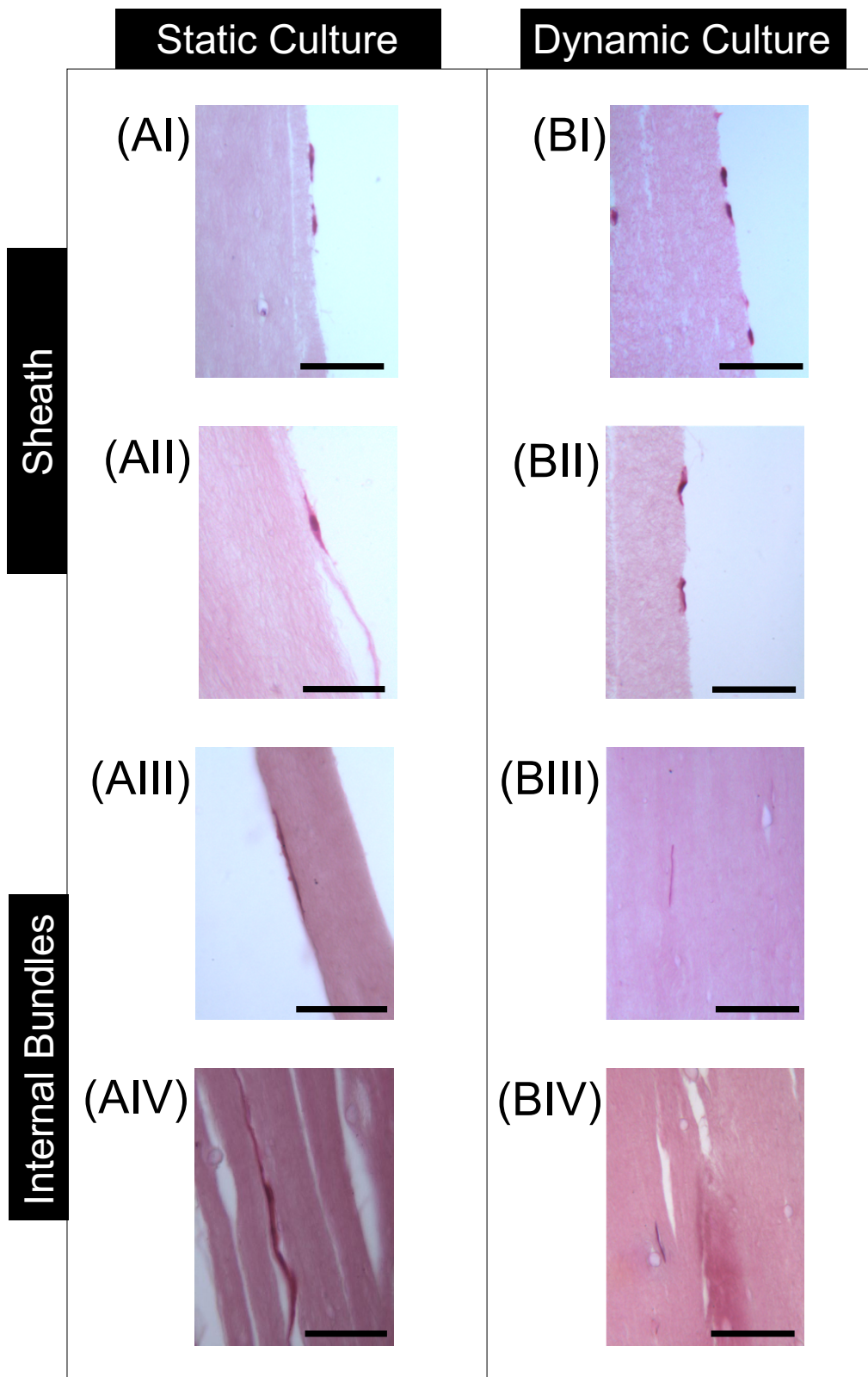
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