

Compact and tunable stretch bioreactor advancing tissue engineering implementation. Application to engineered cardiac constructs

*Original*

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1 **Compact and tunable stretch bioreactor advancing tissue engineering**  
2 **implementation. Application to engineered cardiac constructs**

3

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18 tissue maturation.

19

20

21

22 **Abstract**

23 Physical stimuli are crucial for the structural and functional maturation of tissues both *in vivo* and *in*  
24 *vitro*. In tissue engineering applications, bioreactors have become fundamental and effective tools  
25 for providing biomimetic culture conditions that recapitulate the native physical stimuli. In addition,  
26 bioreactors play a key role in assuring strict control, automation, and standardization in the  
27 production process of cell-based products for future clinical application. In this study, a compact,  
28 easy-to-use, tunable stretch bioreactor is proposed. Based on customizable and low-cost  
29 technological solutions, the bioreactor was designed for providing tunable mechanical stretch for  
30 biomimetic dynamic culture of different engineered tissues. In-house validation tests demonstrated  
31 the accuracy and repeatability of the imposed mechanical stimulation. Proof of concepts biological  
32 tests performed on engineered cardiac constructs, based on decellularized human skin scaffolds  
33 seeded with human cardiac progenitor cells, confirmed the bioreactor Good Laboratory Practice  
34 compliance and ease of use, and the effectiveness of the delivered cyclic stretch stimulation on the  
35 cardiac construct maturation.

## 36 1. Introduction

37 Tissue engineering (TE) is a multidisciplinary research field whose primary purpose is the *in vitro*  
38 development of functional tissue constructs used as models for basic research, drug testing, and  
39 disease investigations, or ultimately aimed at repairing injured tissues or even organs [1,2].

40 According to the TE paradigm, the bioprocess for generating functional constructs is based on three  
41 key elements: cells, scaffolds, and culture environmental cues [3,4].

42 Cells play a crucial role, since they generate the new tissue through proliferation, differentiation and  
43 maturation. In particular, the use of human stem or progenitor cells, which can differentiate into  
44 tissue-specific functional cell types, provides promising perspectives for patient-specific tissue  
45 models and personalized TE [5–10].

46 Scaffolds substantially serve as active biochemical and structural support for cell growth. In  
47 particular, decellularized extracellular matrix (ECM) is recognized as one of the most promising  
48 biological scaffolds, because of its native biochemical and biomechanical features, and its three-  
49 dimensional (3D) microarchitecture [11,12].

50 Lastly, biomimetic chemical and physical environmental cues have proven to be fundamental for  
51 defining the fate and the functionality of the engineered constructs [13–16]. Focusing on strategies  
52 for engineering tissues that *in vivo* are physiologically subjected to mechanical stimuli (e.g., tensile  
53 or compressive load), several studies demonstrated that the use of dynamic culture devices  
54 providing adequate *in vitro* mechanical stimuli leads to significant improvements in structural and  
55 functional tissue maturation [17–20]. For example, it was observed that the controlled exposure of  
56 engineered skeletal muscle tissues to mechanical cyclic stretch promotes their development, with  
57 improved morphological, contractile and myogenic properties [21–24]. Furthermore, stretch was  
58 successfully applied for cultivating *in vitro* tendon and ligament grafts, with several studies  
59 demonstrating that mechanical stimulation is crucial for promoting tenocyte differentiation, tendon  
60 matrix synthesis, and construct tensile strength [25–30]. Dynamic culture devices providing stretch  
61 stimuli were also used for generating skin tissue models characterized by thick epidermal layers  
62 with high levels of expressed basement membrane proteins [31], and for *ex vivo* expansion of skin  
63 grafts, promoting dermal ECM synthesis [32,33]. Cyclic stretch plays a fundamental role in  
64 bioprocesses designed for the *in vitro* maturation of cardiac tissue models. A large body of literature  
65 demonstrated that the provision of cyclic stretch stimulation mimicking the cyclic diastolic filling of  
66 the ventricles promotes cell proliferation, myocardium-like morphological arrangement and  
67 maturation, and contractile performance of engineered cardiac tissues [34–42].

68 The need of TE bioprocesses to provide biomimetic physical stimuli in a strictly controlled manner  
69 is faced using bioreactors. When equipped with advanced and programmable technological

70 solutions, these devices can guarantee control, automation, and standardization of the production  
71 process [43,44], fulfilling the rigorous requirements for clinical translation of cell-based products.  
72 Moreover, bioreactors represent useful platforms for generating *in vitro* tissue models, thus  
73 addressing the need for providing investigation methods alternative to animal-based  
74 experimentation.  
75 However, bioreactor-based approaches have to cope with a series of drawbacks limiting their wide  
76 spread. In particular, complex technology and high costs, often related to the high level of  
77 customization required by the specific application, represent relevant limiting factors [45].  
78 Moreover, difficulty of use is a critical aspect affecting both custom-made and commercial  
79 bioreactor platform diffusion [46,47].  
80 Nowadays, the availability of affordable open-source and low-cost electronic solutions for  
81 bioprocess monitoring and control purposes and the diffusion of low-cost 3D printing technologies  
82 give the opportunity to rethink the design phase as well as to develop highly customizable and  
83 flexible bioprocess platforms at limited implementation costs [48–52]. In this perspective, we  
84 present here a compact, easy-to-use, tunable stretch bioreactor platform for TE applications.  
85 Customizable and low-cost technological solutions are adopted for the platform implementation.  
86 Using a purpose-built test bench, in-house validation tests are performed to assess the motor motion  
87 accuracy and repeatability. To demonstrate the bioreactor platform performance in a cell culture  
88 laboratory and to investigate the impact of cyclic stretch on maturation of engineered cardiac  
89 tissues, explanatory biological experiments on decellularized human skin (d-HuSk) scaffolds seeded  
90 with human cardiac progenitor cells (hCPCs), performed within the bioreactor platform, are  
91 presented. The hCPC-seeded d-HuSk scaffolds are subjected to controlled cyclic stretch, and the  
92 effect of cyclic stretch conditioning is analyzed in terms of cell organization and gene expression of  
93 typical cardiac markers.

94

## 95 **2. Materials and methods**

### 96 **2.1 *Bioreactor platform***

97 The design of the bioreactor platform was guided by specific requirements. Firstly, the device  
98 should provide tunable mechanical stretch for biomimetic dynamic culture of different engineered  
99 tissues (e.g., myocardium, skeletal muscle, skin, tendon, and ligament tissue). Then, it should  
100 accomplish general specifications of a bioreactor for TE strategies [17], particularly Good  
101 Laboratory Practices (GLP) compliance in terms of ease of assembling, cleaning, and use in a cell  
102 culture laboratory and with conventional laboratory equipment. Moreover, the bioreactor platform

103 should be modular for facilitating assembling/disassembling/cleaning procedures and  
104 customization, and it should be characterized by small size, to be easily handled under laminar flow  
105 hood and within the incubator. Lastly, for promoting the use of the system, the bioreactor platform  
106 should be designed and produced with easy-to-use and low-cost hardware and software, and overall  
107 it should guarantee reliability for long-term experiments within the incubator (37°C, 5% CO<sub>2</sub>, and  
108 90-95% humidity).

109 Based on these requirements, the bioreactor platform is designed consisting of three main units (Fig.  
110 1A): (1) the culture unit, housing the constructs; (2) the stimulation unit, providing the biomimetic  
111 mechanical stimuli; (3) the control unit, devoted to the control of the stimulation unit. Both the  
112 culture unit and the stimulation unit are mounted on an aluminum planar base (342 mm x 128 mm)  
113 to be incubated, while the control unit is located outside the incubator.

114 In detail, the culture unit, adapted from a previously developed device [53], is composed of a  
115 polycarbonate culture chamber (140 x 80 x 75 mm<sup>3</sup> with a priming volume of ~100 ml) designed to  
116 house multiple constructs to be cultured simultaneously. Within the culture chamber, two opposite  
117 polyoxymethylene (POM) clamps allow grasping the constructs during stimulation. One clamp is  
118 mobile, coupled with a stainless steel through-shaft externally connected to the stimulation unit  
119 motor, while the opposite clamp is fixed (Fig. 1B). Silicone bellows (J-Flex rubber, Retford, UK)  
120 assure watertightness of the culture chamber. The culture chamber is inserted within an L-shaped  
121 chassis, previously developed for guaranteeing a correct positioning of the culture chamber on the  
122 planar base [54]. The stimulation unit consists of a watertight box (130 x 95 x 65 mm<sup>3</sup>), which  
123 houses a captive stepper motor (NEMA 14, Nanotec Electronic GmbH & Co. KG, Feldkirchen, DE)  
124 that generates a linear motion with a resolution of 10 µm/step. The motor provides the mechanical  
125 stimulation to the cultured constructs, controlled by the control unit. The latter is made of a compact  
126 box (170 x 150 x 60 mm<sup>3</sup>) containing a microcontroller board (Arduino Due, Arduino, Ivrea, IT),  
127 selected because it is an open-source and low-cost electronics platform, which is coupled with a  
128 small-sized motor driver (A4988, Allegro MicroSystems, Manchester, USA). The motor driver with  
129 built-in translator and current regulator acts as bridge component between the microcontroller and  
130 the motor, and enables motor control in open-loop configuration efficiently assuring the needed  
131 power supply. A user-friendly interface, based on push buttons and a 1.8" LCD screen (Arduino),  
132 allows the proper adjustment of the initial relative position between clamps and the setting of the  
133 stimulation parameters (i.e., stretching amplitude and frequency). A schematic diagram of the  
134 control unit implementation is reported in Figure 1C.

135 To perform the explanatory biological tests, dedicated to culture cardiac constructs under cardiac-  
136 like cyclic stretch, the microcontroller is programmed to generate a sinusoidal motor motion with

137 tunable stretching amplitudes (0.1-3.0 mm, by 0.1 mm steps) and frequencies (1-3 Hz, by 1 Hz  
 138 steps). Available combinations of stimulation parameters for culturing constructs are reported in  
 139 Table 1.  
 140

Amplitude range (mm)	Frequency (Hz)		
	1	2	3
2.1 - 3	•		
1.1 - 2	•	•	
0.1 - 1	•	•	•

141 **Table 1.** Stimulation parameter combinations. Black dots indicate the available combinations.  
 142

143 All culture chamber components in contact with medium or constructs are made of cytocompatible  
 144 and autoclavable materials [53,55]. The L-shaped chassis housing the culture chamber and the  
 145 stimulation unit box are manufactured in ABS thermoplastic material by fused deposition modelling  
 146 (FDM) for guaranteeing design flexibility and cost-efficiency [54].

## 147 **2.2 In-house tests**

148 The ease of use and the reliability of stimulation and control units were preliminarily tested in-  
 149 house. In detail, the motion accuracy of the stimulation unit operated by the control unit was  
 150 characterized using a purpose-built test bench. A linear variable displacement transducer (LVDT,  
 151 AML/EU/±5/S, Applied Measurements Ltd., Aldermaston, UK), mounted on a chassis and  
 152 connected to a dedicated data acquisition system (Personal computer equipped with a cDAQ-9174  
 153 coupled with a NI 9218 module, National Instruments, Austin, TX, USA), was put in contact with  
 154 the through-shaft connected to the stimulation unit motor (Supplementary Fig. S1), and all the 60  
 155 combinations of motor amplitude and frequency parameters were tested. In detail, for each possible  
 156 combination, the motor imparted displacement was acquired continuously over 30 cycles (sampling  
 157 rate = 1652 Hz). The measured LVDT signals were acquired, filtered (Butterworth low-pass filter,  
 158 order 8, cut-off frequency = 10 Hz), and analyzed in LabVIEW environment (LabVIEW, National  
 159 Instruments) to evaluate the peak-to-peak amplitude as well as the frequency of the recorded  
 160 displacement signals. All measurements were carried out in triplicate. The motor displacement  
 161 waveforms were characterized by comparing the measured waveforms with the prescribed ideal  
 162 sinusoidal waveforms. The mean percentage errors of measured amplitude and frequency values  
 163 with respect to the prescribed nominal values were expressed as mean ± standard deviation (SD).  
 164

## 165 **2.3 Biological tests**

### 166 *2.3.1 Bioreactor platform performance in a cell culture laboratory*

167 The bioreactor platform was then tested in a cell culture laboratory in order to assess its ease of use  
168 and compliance with GLP procedures. In detail, the components of the culture chamber were  
169 autoclaved and assembled under laminar flow hood, the culture chamber was filled with Dulbecco's  
170 Modified Eagle's Medium/Ham's Nutrient Mixture F12 culture medium (Sigma-Aldrich, St. Louis,  
171 MO, USA), and the assembled system was placed in incubator without constructs but with the  
172 mechanical stimulation (1 mm, 1 Hz) switched on for 5 days.

### 173 *2.3.2 Preparation and culture of cardiac constructs*

174 To investigate the influence of biomimetic cyclic stretch on the maturation of cardiac constructs,  
175 explanatory biological tests were carried out on decellularized human skin (d-HuSk) scaffolds  
176 seeded with human cardiac progenitor cells (hCPCs) and hCPC-derived early cardiac myocytes.  
177 Concerning the scaffold preparation, human skin samples were obtained from patients undergoing  
178 abdominoplasty (n = 4, mean age  $41.75 \pm 2.36$ ). Upon receipt, samples were washed in  
179 physiological saline solution, then subcutaneous tissue was removed and multiple specimens were  
180 cut (length = 20 mm, width = 10 mm) marking Langer's line orientation. For decellularization  
181 treatment, specimens were enclosed in embedding cassettes housed in a purpose-built sample-  
182 holder, put within a beaker filled with the decellularizing solution (700 ml) and placed on a  
183 magnetic stirrer, and kept under constant stirring (150 rpm) for 24 h [56]. The decellularizing  
184 solution contained 1% w/v sodium dodecyl sulfate (SDS) (Sigma-Aldrich) and 1% v/v Triton  
185 (Sigma-Aldrich). The specimens were then rinsed for 24 h in antibiotic solution containing 0.25  
186  $\mu\text{g/ml}$  Amphotericin B, 100 U/ml Penicillin, and 50 U/ml Streptomycin (all from Sigma-Aldrich) in  
187 PBS, and lastly in sterile bidistilled water for additional 30 min [57,58]. The d-HuSk specimens  
188 were snap-frozen, mounted on a cryostat chuck using Tissue Freezing Medium (Leica  
189 Microsystems, Wetzlar, Germany), and sliced into 600- $\mu\text{m}$ -thick sections by a Leica CM1950  
190 cryostat (Leica Microsystems). Cryosections of d-HuSk were sterilized by exposure to ultraviolet  
191 radiation for 40 min and rehydrated for one week with F12K medium in incubator ( $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ).  
192 The sterilized and rehydrated d-HuSk cryosections were then stored in standard culture conditions  
193 with the same medium until use.

194 As regards the hCPCs, they were isolated from cardiac specimens derived from macroscopically  
195 uninjured areas of the left ventricle of explanted hearts of patients undergoing heart transplant  
196 because of end-stage heart failure (n = 10, mean age  $49.5 \pm 4.7$ ). Specifically, following a  
197 previously described protocol [59] cardiac specimens were washed in physiological saline solution,



198 dissected, minced, and enzymatically disaggregated by incubation in 0.25% trypsin (Sigma-  
199 Aldrich) for 6 h at 4 °C and in 0.1% w/v collagenase II (Sigma-Aldrich) for 30 min at 37 °C. The  
200 digestion was stopped by adding double volume of Hanks' Balanced Salt solution (Sigma-Aldrich)  
201 supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich). Resulting fragments of tissue  
202 were further disaggregated by pipetting. Tissue debris and cardiomyocytes were then removed by  
203 sequential centrifugation at 100 g for 2 min, passage through a 40-µm cell strainer (BD Biosciences,  
204 Franklin Lakes, NJ, USA), and centrifugation at 400 g for 5 min. The obtained cell population was  
205 then incubated with anti-fibroblast MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to  
206 magnetically label fibroblasts that were then removed loading cells onto a MACS column (Miltenyi  
207 Biotec) placed in the magnetic field of a MACS separator (Miltenyi Biotec). The negative fraction  
208 of unlabeled hCPCs ran through the column was collected and plated at a density of  $4 \times 10^3$  cells per  
209  $\text{cm}^2$  in F12K medium, prepared from Nutrient Mixture F-12 Ham medium (Sigma-Aldrich)  
210 supplemented with 10% FBS (Sigma-Aldrich), basic fibroblast growth factor (Peprotech, Rocky  
211 Hill, NJ, USA), glutathione (Sigma-Aldrich), penicillin and streptomycin (Sigma-Aldrich). The  
212 hCPCs were cultured in incubator (37°C, 5% CO<sub>2</sub>) and observed daily by an inverted phase-contrast  
213 microscope (Olympus, Tokyo, Japan). Medium was replaced every 3 days until the 75% confluence  
214 was reached. Then, an unselected subpopulation of hCPCs was induced to differentiate towards  
215 cardiac myocytes by adding 50 µg/ml of ascorbic acid (Sigma-Aldrich) and 10 ng/ml of Vascular-  
216 Endothelial Growth Factor (Sigma-Aldrich) to the culture medium for 7 days.  
217 Successively, the d-HuSk scaffolds (n = 24) were seeded with  $2.5 \times 10^6$  hCPCs and  $2.5 \times 10^6$  hCPC-  
218 derived early cardiac myocytes. After 7 days of static culture in Petri dish, half of the constructs (n  
219 = 12) were transferred, in pairs, into the bioreactor culture chamber with F12K medium and  
220 subjected to dynamic conditions (i.e., sinusoidal cyclic stretch, 10% strain, 1 Hz) for additional 7  
221 days (see Supplementary Movie 1) for mimicking the cyclic diastolic filling of the ventricles [60–  
222 63]. As control experiment, the other half of the constructs (n = 12) were cultured statically in Petri  
223 dishes for the entire duration of 14 days (Fig. 2). Finally, constructs were cut into smaller specimens  
224 that were either fixed in 10% neutral-buffered formalin (Sigma-Aldrich) for morphological analyses  
225 or processed for RNA extraction for gene expression profiling.

### 226 2.3.3 *Histochemistry analysis*

227 Following standard protocols, subsets of constructs cultured in static conditions (control) or in  
228 bioreactor and fixed in 10% neutral-buffered formalin were dehydrated in a graded series of  
229 alcohols, embedded in paraffin and sliced into serial 5-µm-thick sections [57,58]. Sections were  
230 stained with Hematoxylin and Eosin (H&E) and with Mallory's trichrome staining using specific  
231 kits (both from Bio-Optica, Milan, Italy). Stained sections were observed by at least three

232 independent researchers using a light microscope DM2000 Led (Leica Microsystems) equipped  
233 with an ICC50HD camera (Leica Microsystems).

#### 234 *2.3.4 Gene expression profile analysis*

235 Total RNA was extracted from hCPCs seeded on d-HuSk scaffolds, cultured both in static and  
236 cyclic stretch conditions, using Trizol Reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA,  
237 USA), according to the manufacturer's instructions. RNA was dissolved in RNase-free water and its  
238 final concentration was quantified at the NanoDrop 1000 spectrophotometer (Thermo Scientific,  
239 Waltham, MA, USA). All RNA samples were checked for quality and resulted suitable for gene  
240 expression profiling analyses. Analysis was performed as previously described [64]. Briefly, RNA  
241 from each sample was reverse transcribed into cDNA with QuantiTect Reverse Transcription Kit  
242 (Qiagen, Hilden, Germany) and gene expression was quantified by real-time qPCR using Power  
243 SYBR Green PCR Master Mix (Applied Biosystem, Thermo Fisher Scientific). DNA amplification  
244 was carried out using QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific) and the  
245 detection was performed by measuring the binding of the fluorescent dye SYBR Green I to double-  
246 stranded DNA. The thermal cycling conditions included an initial enzyme activation at 95°C for 10  
247 min and 40 cycles consisting of a denaturation step at 95°C for 15 s and an annealing step at 60°C  
248 for 60 s. Melt curve analysis was performed to assess uniformity of product formation, primer  
249 dimer formation and amplification of non-specific products. Primers used in this study were  
250 designed with Primer3 software (<http://frodo.wi.mit.edu>) starting from the CDS (coding sequence)  
251 of mature mRNA available on GeneBank (Supplementary Table S1). All samples were tested in  
252 triplicate with the housekeeping gene (GAPDH) to correct for variations in RNA quality and  
253 quantity. Comparative quantification of target gene expression in the samples was performed based  
254 on cycle threshold (Ct) normalized to the housekeeping gene and using the  $2^{-\Delta\Delta C_t}$  method.

#### 255 *2.3.5 Statistical analysis*

256 Data from gene expression profiling were analyzed by GraphPad Prism 5.0 (GraphPad Software, La  
257 Jolla, CA, USA) using Student's two-tailed unpaired t-test. All experiments were performed in  
258 triplicate and data were averaged and expressed as the mean  $\pm$  standard error of the mean. The  
259 statistical significance is denoted as \* for p-value  $\leq$  0.05, \*\* for p-value  $\leq$  0.01, \*\*\* for p-value  $\leq$   
260 0.001.

261

### 262 **3. Results**

#### 263 **3.1 *In-house tests***

264 In-house tests confirmed the ease of use and the reliability of the stimulation and control units. In  
265 detail, tests on motor imposed displacement accuracy highlighted that real displacement waveforms  
266 agree with the prescribed ideal sinusoidal waveforms for all the available combinations of working  
267 conditions, as testified by the explanatory waveforms presented in Figure 3A. As regards the  
268 comparison between the measured stretching amplitude values and the nominal ones, mean error  
269 values up to  $13.3\% \pm 7.3\%$  were observed, with the largest deviation from the nominal curve  
270 corresponding to the combination characterized by minimum amplitude equal to 0.1 mm and  
271 frequency equal to 1 Hz. For nominal amplitude values higher than 0.4 mm the observed mean error  
272 values were lower than 4% (Fig. 3B). Concerning the nominal frequency, mean error values up to  
273 10.5% (corresponding to the combination characterized by amplitude equal to 1.3 mm and  
274 frequency equal to 1 Hz) were observed (Fig. 3C).

## 275 **3.2 Biological tests**

### 276 *3.2.1 Bioreactor platform performance in a cell culture laboratory*

277 Preliminary tests performed in a cell culture laboratory confirmed ease of use, sterility maintenance,  
278 and functionality of the bioreactor platform in a standard incubator. During the explanatory cyclic  
279 stimulation tests run for 5 days in incubator, the system did not present adverse issues, the  
280 watertightness of the culture chamber and the stimulation unit was confirmed, and the culture  
281 medium did not present any signs of contamination.

### 282 *3.2.2 Histochemistry*

283 The H&E and Mallory's trichrome staining of the cardiac constructs revealed that, under both static  
284 (control) and dynamic (sinusoidal cyclic stretch, 10% strain, 1 Hz) culture conditions, hCPCs  
285 organized into a structured multilayered tissue on the surface of the d-HuSk scaffolds (Fig. 4).  
286 Noteworthy, the histochemical analysis highlighted that the dynamic culture promoted hCPC  
287 migration towards the inner layers of the scaffolds (Figs. 4B and 4D).

### 288 *3.2.2 Gene expression profile analysis*

289 The gene expression profile analysis of hCPCs extracted from constructs cultured under either static  
290 or dynamic conditions included genes typical of main cardiac cell lineages. In particular, in  
291 bioreactor-cultured constructs, a significant up-regulation of cardiac alpha actin (ACTC1), a marker  
292 typical of late differentiating and mature cardiac myocytes, was observed. On the opposite, markers  
293 typical of undifferentiated hCPCs (like CD117) or of early stages of cardiac myocyte differentiation  
294 (like TBX3 and TBX5) were significantly down-regulated with respect to control constructs (Fig.  
295 5). The transcription of other markers typical of cardiac myocytes, like MEF2C, CX43, and  
296 GATA4, did not differ significantly among constructs cultured in static or dynamic conditions, and

297 similarly happened for the transcription of the mesenchymal cell marker CD105 and of genes  
298 typical of smooth muscle cells (GATA6, ACTA2) and endothelial cells (ETS1, FVIII)  
299 (Supplementary Fig. S2).

300

#### 301 **4. Discussion**

302 In TE research, a number of studies demonstrated that successful strategies for the *in vitro*  
303 generation of functional engineered tissues require a synergistic combination of appropriate cells,  
304 scaffolds, and biochemical and biophysical signals [65–67]. As specifically concerns mechanical  
305 cues, in the last two decades a plethora of custom-made bioreactors providing *in vitro* biomimetic  
306 mechanical stretch have been proposed [23,24,29–31,33,40,42]. In parallel, ready-to-use systems  
307 have been developed by commercial companies (e.g., Tissue Train 3D Culture System from  
308 FlexCell International, Hillsborough, USA; TC-3 from Ebers Medical Technology, Zaragoza,  
309 Spain; MCT6 from CellScale, Waterloo, Canada; BioDynamic 5100 from TA Instruments, New  
310 Castle, USA). All the developed culture devices substantially contributed to unravel the  
311 fundamental role that mechanical stretch has on structural and functional development of biological  
312 tissues and in regulating tissue homeostasis and pathophysiology. Moreover, their use increased the  
313 knowledge on sensitivity of cells to mechanical stimuli, to which cells react activating specific  
314 mechanotransduction pathways that can lead to phenotypic changes [68–71].

315 However, the proposed custom-made bioreactors were often based on complex technological  
316 solutions, difficult to use by non-trained operators in a cell culture laboratory and typically  
317 dedicated to highly specialized applications, while the commercial devices are generally expensive  
318 and not fully customizable.

319 Taking into account these limitations, in this study we developed a compact, easy-to-use, tunable  
320 stretch bioreactor platform for culturing *in vitro* 3D engineered constructs under biomimetic stretch  
321 conditions. Particular attention was paid in developing reliable and affordable stimulation and  
322 control units. As regards the stimulation unit, the use of a captive stepper motor enables the  
323 provision of linear motion adopting an open loop control strategy ensuring high displacement  
324 resolution without the need for additional and complex feedback sensing solutions. In combination,  
325 a compact control unit, based on low-cost open-source hardware and freeware software, avoids the  
326 use of cumbersome and expensive equipment (e.g., laptop, data acquisition module, and  
327 commercial software). Moreover, the integrated user-friendly interface allows ease-of-use to not  
328 experienced operators as well as system portability. In-house performance tests confirmed that the  
329 bioreactor platform is reliable in providing accurate and repeatable stimulation within a range of

330 physiological interest. For imposed motor displacement values higher than 0.4 mm, the mean error  
331 values between the measured amplitude values and the nominal ones were lower than 4%, thus  
332 negligible, for all available stimulation parameter combinations. Conversely, for motor  
333 displacement values in the range of 0.1 - 0.4 mm, higher amplitude errors were calculated (Fig. 3).  
334 However, it should be noted that such small displacement values are not commonly adopted for  
335 mechanical stimulation of macroscopic constructs. This inaccuracy could be ascribed to the axial  
336 play of the motor shaft, and to inertial and vibrational phenomena that are intrinsic to stepper  
337 motors. In addition, possible signal artefacts during the LVDT data acquisition, due to inductive and  
338 capacitive electrical interference, could not be excluded. As concerns the stimulation frequency,  
339 measurements revealed negligible errors, probably ascribable to intrinsic technical limitations of the  
340 adopted low-cost microcontroller.

341 Preliminary tests in a cell culture laboratory demonstrated that the device is easy-to-use with GLP  
342 compliant procedures, compact to handle and fit in a standard incubator, and guarantees  
343 watertightness, sterility maintenance and functionality.

344 For investigating the effect of cyclic stretch on cardiac construct maturation, the biological  
345 experiments were performed on decellularized human skin scaffolds seeded with hCPCs and  
346 cultured for 7 days in static conditions, and then transferred into the bioreactor (sinusoidal cyclic  
347 stretch, 10% strain, 1 Hz) for additional 7 days. The histochemical analysis showed cell engraftment  
348 on the scaffold surface in both controls and dynamically cultured constructs (Fig. 4), but only when  
349 subjected to cyclic stretch cells migrated towards the inner layers of the scaffolds, starting to  
350 colonize their 3D structure (Figs. 4B and 4D). The gene expression analysis highlighted a  
351 significant up-regulation of the ACTC1 marker, typical of late differentiating and mature cardiac  
352 myocytes, concomitantly with a marked down-regulation of CD117, TBX3 and TBX5 markers  
353 (Fig. 5), typical receptors for stem cells or early stage cardiac myocytes, suggesting that dynamic  
354 culture likely promoted hCPC differentiation towards mature cardiac myocytes, in accordance with  
355 previous studies [72–76].

356 Although further and longer experimental tests will be necessary for comprehensively  
357 characterizing the effect of cyclic stretch on the maturation of d-HuSk scaffolds seeded with  
358 hCPCs, the latter particularly sensitive to the microenvironment, the preliminary promising findings  
359 provided evidence of the bioreactor platform reliability and suitability for cardiac tissue engineering  
360 applications. In the future, the possibility to switch from stretching to compression mode will be  
361 implemented in the bioreactor platform, and the device will be adapted to be equipped with an  
362 electrical stimulation unit [77] to provide combinable mechanical and electrical stimulations for  
363 mimicking the complex native cardiac environment.

364 In conclusion, adopting customizable and low-cost technological solutions, a compact, easy-to-use,  
365 tunable stretch bioreactor platform for biomimetic dynamic culture of 3D engineered was  
366 developed. Based on modular components and providing tunable stimulation, the proposed device is  
367 versatile and adaptable for different tissue engineering applications. Moreover, the choice of the 3D  
368 printing technology and low-cost hardware coupled with free and open-source software,  
369 substantially limited the development costs and will support in the future the use of the system as  
370 valuable tool for *in vitro* investigation and for future production of functional engineered constructs.  
371

### 372 **Data Availability Statement**

373 Data associated with this study is available upon request to the corresponding author.  
374

### 375 **Conflict of Interest**

376 The authors declare that the research was conducted in the absence of any commercial or financial  
377 relationships that could be construed as a potential conflict of interest.  
378

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382 Ethical approval: Patients provided written informed consent and samples were collected without  
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386

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