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## Simultaneous Electrical and Mechanical Stimulation to Enhance Cells Cardiomyogenic Potential

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Dear Dr. Bajaj,

Please, find enclosed the revised version of our manuscript entitled “**Simultaneous Electrical and Mechanical Stimulation to Enhance Cells Cardiomyogenic Potential**” by Lluçia-Valldeperas *et al.*

We deeply appreciate the second review of our manuscript submitted to *Journal of Visualized Experiments*. Based on editor’s comments, we have revised the manuscript as described in the enclosed point-by-point response. We have also prepared a second version of Figure 2 to clarify some steps of the Protocol section. We could include this new Figure upon Editor’s decision.

We hope that the revised version of our manuscript meets with your approval and is suitable for publication. We wish to thank the editor for the comments to improve our paper. I look forward to hearing from you.

Sincerely,



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**TITLE:**

Simultaneous Electrical and Mechanical Stimulation to Enhance Cells' Cardiomyogenic Potential

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**KEYWORDS:**

Electromechanical stimulation; electrical stimulation; mechanical stimulation; electromechanical system; cardiac progenitor cells; cardiac ATDPCs; cell conditioning; cardiac tissue engineering; cell maturation; disease modeling; drug screening

**SUMMARY:**

Here we present a protocol for training a cell population using electrical and mechanical stimuli emulating cardiac physiology. This electromechanical stimulation enhances the cardiomyogenic potential of the treated cells and is a promising strategy for further cell therapy, disease modeling, and drug screening.

**ABSTRACT:**

Cardiovascular diseases are the leading cause of death in developed countries. Consequently, the demand for effective cardiac cell therapies has motivated researchers in the stem cell and bioengineering fields to develop *in vitro* high-fidelity human myocardium for both basic research and clinical applications. However, the immature phenotype of cardiac cells is a limitation on obtaining tissues that functionally mimic the adult myocardium, which is mainly characterized by mechanical and electrical signals. Thus, the purpose of this protocol is to prepare and mature the target cell population through electromechanical stimulation, recapitulating physiological parameters. Cardiac tissue engineering is evolving toward more biological approaches, and

strategies based on biophysical stimuli, thus, are gaining momentum. The device developed for this purpose is unique and allows individual or simultaneous electrical and mechanical stimulation, carefully characterized and validated. In addition, although the methodology has been optimized for this stimulator and a specific cell population, it can easily be adapted to other devices and cell lines. The results here offer evidence of the increased cardiac commitment of the cell population after electromechanical stimulation. Electromechanically stimulated cells show an increased expression of main cardiac markers, including early, structural, and calcium-regulating genes. This cell conditioning could be useful for further regenerative cell therapy, disease modeling, and high-throughput drug screening.

## INTRODUCTION:

Heart function is based on the coupling of electrical excitation and mechanical contraction. Briefly, cardiomyocyte intercellular junctions permit electrical signal propagation to produce almost synchronous contractions of the heart that pump blood systemically and through the pulmonary system. Cardiac cells, thus, undergo both electrical and mechanical forces that regulate gene expression and cellular function. Accordingly, many groups have attempted to develop culture platforms that mimic the cardiac physiological environment to understand the role of mechanical and electrical stimulation on cardiac development, function, and maturation. *In vitro* electrical and mechanical stimulations individually have been applied extensively in cardiac tissue engineering to enhance functional properties, increase cell maturation, or improve cell–cell coupling and calcium handling<sup>1-21</sup>. Nevertheless, synchronous electromechanical conditioning remains unexploited because of the challenge of developing a stimulator and protocol, and because of the mandatory optimization<sup>22</sup>.

Preliminary work addressed electromechanical stimulation as a combination of electrical stimulation and media perfusion; however, the flow does not involve the strain-based deformation typical of ventricular filling<sup>23-25</sup>. Later, more physiological approaches combined electrical stimuli with physical deformation or stretch to mimic the isovolumetric contraction<sup>26-31</sup>. Feng *et al.* described the first demonstration of electromechanical stimulation in 2005, reporting enhanced cardiomyocyte size and contractile properties<sup>26</sup>. Wang *et al.* pretreated mesenchymal stem cells with 5-azacytidine and applied simultaneous electrical and mechanical conditioning, improving recellularization, cell viability, cardiac differentiation, and tissue remodeling<sup>27</sup>. Since those publications, more groups have reported on electromechanical stimulation of cell monolayers or engineered tissues (*e.g.*, Black<sup>28</sup>, Vunjak-Novakovic<sup>29,31</sup>, and our group<sup>30</sup>) with the first conditioned cells tested *in vivo*<sup>30</sup>. Briefly, Morgan and Black tested several combinations of electrical and mechanical stimuli, reporting that the timing between stimulations was crucial because delayed combined electromechanical stimulation yielded the best results<sup>28</sup>. Next, Godier-Furnémont and collaborators optimized an electromechanical stimulation protocol for engineered heart muscle constructs from neonatal rat heart cells and achieved, for the first time, a positive force–frequency relationship<sup>29</sup>. Afterward, our group reported that electromechanically preconditioned cells increased the expression of main cardiac markers *in vitro* and broad beneficial effects *in vivo*, such as improved cardiac function or increased vessel density in the infarct border region<sup>30</sup>. The most recent publication demonstrated that cardiac tissues from stem-cell-derived cardiomyocytes subjected to electromechanical

conditioning reached a maturation level closer to human adult cardiac structure and function<sup>31</sup>. Additionally, alternative three-dimensional stimulation platforms comprise electroactive scaffolds that provide electrical, mechanical, and topographical cues to the cells attached<sup>32</sup>. Moreover, mechanical deformation (cell monolayer stretching and compression) can also be induced with stretchable electrodes mimicking normal physiological conditions, as well as extreme conditions<sup>33</sup>.

Therefore, the rationale is that *in vitro* electromechanical stimuli based on physiological conditions could enhance the cardiomyogenic potential of a cell. Indeed, this stimulation could benefit further integrations of therapeutic cells into the myocardium in a clinical scenario or increase tissue maturation for drug-screening applications.

In addition, we isolated and characterized a population of human adipose tissue-derived progenitor cells of cardiac origin (cardiac ATDPCs)<sup>34</sup>. These cells are located in the epicardial fat. These cells display beneficial histopathological and functional effects in the treatment of myocardial infarction and also maintain cardiac and endothelial differentiation potential.<sup>30,35</sup>. We hypothesized that these benefits would increase after biophysical stimulation.

Consequently, we developed a device and a stimulation regime for the cell population of interest and investigated the effects. This electromechanical protocol is a new strategy to induce active cell stretching in a sterile manner and noninvasively compared to previous publications<sup>36</sup>, in combination with electric field stimulation. The technique reported here explains in detail the device and method used for the electrical, mechanical, and electromechanical stimulation of cells.

This device can provide both electrical and mechanical stimulation, independently or simultaneously. The stimulation is performed with a noninvasive and aseptic novel approach that includes presterilized cell support, electrodes placed inside a standard culture plate, and a platform that induces the mechanical and electrical forces (**Figure 1**).

The platform can hold up to six culture plates and consists of a sandwich structure of laser-cut poly(methyl methacrylate) and printed circuit-board pieces. The platform prototype relies on a combination of a monophasic programmable computer-controlled electrical stimulator, a printed circuit board for the robust connection of the electrodes, and six 10 mm x 10 mm x 5 mm nickel-plated neodymium-fixed magnets placed near one side of the culture plates. There is also an aluminum bar with six driving magnets (same model) placed in front of the other side of the culture plates and moved with a linear servomotor. The motor is driven by a motor controller, operated through an RS-232 port by commercial software (see the **Table of Materials**). Through the user interface and programmable stimulator, it is possible to program the electrical intensity, the pulse duration and frequency, the frequency of mechanical stimulation, its duty cycle, the number of pulses, the pulse amplitude (magnet excursion), and the slope.

[Place **Figure 1** here.]

Both the stimulator and the method for electromechanical conditioning are fully described in two international patents, WO-2013185818-A1<sup>37</sup> and WO-2017125159-A1<sup>38</sup>.

The biocompatible silicone constructs designed to provide structural support to cells, electrodes, and magnets have been described previously<sup>10,21</sup>. Briefly, they consist of polydimethylsiloxane (PDMS), molded and cured at room temperature, with a Young's modulus of 1.3 MPa, close to physiological levels. The construct contains a cell culture pool in a flexible area (10 mm x 10 mm x 2 mm), two inner transverse slots to hold the electrodes, and two embedded 6 mm x 2 mm x 4 mm nickel-plated neodymium magnets. The electrodes are built with 0.2 mm platinum wire twisted around a 2 mm x 3 mm x 12 mm polytetrafluoroethylene (PTFE) core bar (21 cm per electrode, approximately 23 turns) and placed at opposite sides of the flexible area to create an electric field for inducing electrical stimulation. Mechanical stretching is achieved through magnetic attraction between magnets embedded in the support and external magnets placed next to the culture plate and on the moving aluminum arm. In this way, the cell support can be extended without breaking the sterile barrier. This approach is suitable for a cell monolayer but could be adapted to three-dimensional constructs, as well.

In addition, a regular pattern could be imprinted where the cells are seeded, using a ruled diffraction grating (1,250 grooves/mm). The direct visualization of cells cultured on the PDMS construct under brightfield and fluorescent microscopes is possible because of its transparency and 0.5 mm thickness. In the current case, the PDMS culture pool has a vertical surface pattern, perpendicular to the stretching force, to align the cells perpendicularly to the electric field, which minimizes the electric field gradient across the cell.

**Figure 1** shows a detailed description of the construct and device used for the stimulation. The PDMS construct and characteristics are optimized for cell stretching (**Figure 1A,B**). The stimulator is developed and validated for the effective application of the desired electrical and mechanical stimulation to cells attached to the PDMS construct. This process includes ensuring good connectivity and user operability through the software interface (**Figure 1C,D**).

The procedure for cell stimulation using this custom-made device is described in the protocol section.

## **PROTOCOL:**

This study uses human cardiac ATDPCs from patient samples. Their use has been approved by the local ethics committee, and all patients gave informed consent. The study protocol conforms to the principles outlined in the Declaration of Helsinki.

### **1. Preparations**

1.1. Autoclave two tweezers, 12 platinum PTFE electrodes for electrical stimulation, and some paper towels, at 121 °C for 20 min.

1.2. Sterilize 12 PDMS custom-made constructs (**Figure 1A**).

1.2.1. Wash each construct with 5 mL of sterile, distilled water with magnetic agitation at room temperature for 15 min.

1.2.2. Wash 1x with 5 mL of 70% ethanol with magnetic agitation at room temperature for 5 min.

1.2.3. Wash 5x with 5 mL of sterile, distilled water with magnetic agitation at room temperature for 10 min per wash, to remove alcoholic residues.

1.2.4. Dry the constructs on sterile paper towels inside the flow cabinet overnight.

1.2.5. Store them in sterile 50 mL centrifuge tubes until use.

## **2. Cell Seeding (Day -1)**

2.1. Before cell seeding, transfer the cleaned PDMS constructs to sterile plates and expose them to ultraviolet light for 5 min to ensure complete sterilization.

2.2. Transfer each construct to a 35 mm cell culture plate for immediate cell seeding.

2.3. Trypsinize a confluent T75 flask of cardiac ATDPCs.

2.3.1. Wash the T75 flask with 5 mL of 1x phosphate-buffered saline (PBS).

2.3.2. Add 1 mL of 0.05% trypsin-EDTA and incubate at 37 °C for 5 min to detach the cells.

2.3.3. Add 5 mL of complete medium to inactivate the trypsin-EDTA.

2.3.4. Collect all cells in a 15 mL tube and wash the flask 2x with 5 mL of PBS to collect any remaining cells.

2.3.5. Centrifuge at 230 x *g* for 5 min at 22 °C, remove the supernatant, and resuspend the cells in 2 mL of complete medium to count them with the hemocytometer chamber.

2.4. Seed 200 µL of cardiac ATDPCs ( $2.5 \times 10^5$  cells/mL) into the cell pool of the 12 PDMS constructs (**Figure 1A,B**) to have ~80% of the seeding surface covered by cells the day after, and incubate at 37 °C and 5% CO<sub>2</sub>.

NOTE: After 2 - 4 h, the cells should be attached. Cell inoculum is prepared according to the cell size and growth. For smaller cells, the seeding density should be increased.

2.5. Gently add 2 mL of prewarmed complete medium ( $\alpha$ -MEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin–streptomycin) per plate.

221 2.6. Incubate the constructs at culture conditions (usually 37 °C and 5% CO<sub>2</sub>) overnight.

### 223 3. Electromechanical Stimulation Setup (Day 0)

225 3.1. Before starting the procedure, take six constructs for electromechanical stimulation and six  
226 as nonstimulated controls. With fewer than six plates per stimulation, use empty constructs with  
227 the same medium volume to ensure proper electric field stimulation.

229 3.2. Clean the stimulation unit with 70% ethanol and place it in the flow cabinet.

231 3.3. Bring the sterile electrodes and tweezers inside the flow cabinet.

233 3.4. Remove 90% of the media from the culture plate to easily manipulate the electrodes and  
234 constructs. First, place the PDMS constructs in the right position to ensure magnetic attraction  
235 (PDMS displacement within the culture plate toward the magnet) between both fixed and mobile  
236 magnets. Next, connect the platinum wire to the electrode connectors and the PTFE part in its  
237 designated space in the PDMS construct.

239 3.5. Add 2.5 mL of fresh prewarmed complete medium to each construct.

241 NOTE: Maintain the sterility throughout the procedure and operate on one construct at a time.  
242 Maintain the rest of the constructs in the incubator at 37 °C and 5% CO<sub>2</sub> until use.

244 3.6. Once all PDMS constructs are placed and electrically connected to the platform, bring the  
245 platform back into the incubator at 37 °C and 5% CO<sub>2</sub>.

247 3.7. Connect the electrical and mechanical source.

249 3.8. Configure the stimulation program. Specify electrical and mechanical stimulation regimes  
250 through the user interfaces of the electrical stimulator and the application, which controls the  
251 mechanical stimulation. Set the synchronism as follows.

253 3.8.1. Switch on the electrical stimulator. Wait for the main menu to appear in the display.

255 3.8.1.1. Then, select **Option 2: Edit sequence + Enter**.

257 3.8.1.2. Edit the sequence **Menu** as follows.

259 3.8.1.2.1. Use the **Mode** tab to select either voltage or current. Select current by clicking + and  
260 press **Enter**.

262 3.8.1.2.2. For the **Amplitude** tab, select **1 (mA)** with +/- and press **Enter**.

264 3.8.1.2.3. For the **Period (T)**, select **1000 (ms)** with +/- and press **Enter**.

3.8.1.2.4. Set the **Pulse duration (Tw)** to 2 (ms) with +/- and press **Enter**.

3.8.1.2.5. For the **Trigger mode** tab, select **External by software** and press **Enter**.

3.8.1.2.6. Back in the main menu, select **Option 4: Generate sequence** and press **Enter**.

NOTE: The electrical stimulator rests in the standby until it receives a trigger command from the mechanical stimulator application through the serial port.

3.8.2. Perform the following steps in the mechanical stimulation section of the control application panel (**Figure 2C**).

3.8.2.1. Write 1000 (ms) in the **Pulse Period** text control.

3.8.2.2. Write 500 (ms) in the **ON time (Tw)** text control to set the mechanical pulse duration.

3.8.2.3. Write 2000 (AU) in the **Excursion** text control to deliver a 10% construct elongation. This is the number of steps in the linear control motor.

NOTE: The stimulation protocol applied here consists of alternating-current 2 ms monophasic square-wave pulses of 50 mV/cm at 1 Hz and 10% stretching for 7 days. The rise and fall times of the mechanical pulse are set at 100 ms, to roughly imitate the shape of the hearth pressure pulse. Also, the repetition mode is set to **Continuous** and there is a counter displaying the number of pulses.

3.9. Change the media 2x a week (Monday and Thursday afternoon). First, remove the old media; next, add the warm media on the sides of the PDMS support, never directly on the cell pool.

NOTE: If the cells have a high growth rate, the media should be changed 3x a week (e.g., Monday, Wednesday, and Friday). It is necessary to disconnect and reconnect all cables, but there is no need to remove the culture plates and electrodes from their place.

3.10. Collect the samples after the experimentation is performed.

## 4. Sample Collection at the End of the Experimentation (Day 7)

### 4.1. For RNA analyses

4.1.1. Wash the construct 2x with 3 mL of 1x PBS for 5 min at room temperature.

4.1.2. Add 3 mL of 0.05% trypsin-EDTA to each plate (enough to cover the whole construct) and wait for 5 min at 37 °C.

4.1.3. After the cells are detached, add 2 mL of complete medium to inactivate the trypsin-EDTA.

4.1.4. Collect all cells in a 15 mL tube and wash the construct 2x with 3 mL of PBS to collect any remaining cells.

4.1.5. Centrifuge at 230 x *g* for 5 min at 22 °C.

4.1.6. Remove the supernatant and resuspend the pellet in 1 mL of PBS.

4.1.7. Transfer the cell solution to a 1.5 mL tube and centrifuge at 230 x *g* for 5 min.

4.1.8. Remove the supernatant and store the pellet at -80 °C in 700 µL of lysis reagent for further RNA isolation.

4.1.9. Isolate RNA using a commercial kit, following the manufacturer's instructions.

4.1.10. Reverse-transcribe the isolated RNA using the kit and random hexamers, according to the manufacturer's protocol.

4.1.11. For small RNA concentrations, preamplify and, then, dilute 1:5 with RNase-free water before subsequent real-time reverse transcription polymerase chain reaction (RT-PCR) is performed. Continue with the standard protocol for real-time RT-PCR and check main cardiac markers.

NOTE: Typical cardiac markers comprise early and late markers from different categories, such as cardiac transcription factors (myocyte-specific enhancer factor 2A [MEF2A], GATA-binding protein 4 [GATA-4]) and structural (cardiac troponin I [cTnI], cardiac troponin T [cTnT], α-actinin) and calcium regulation (Connexin43 [Cx43], sarco-/endoplasmic reticulum Ca<sup>2+</sup>-ATPase [SERCA2])<sup>30</sup>. Protein isolation can also be performed if needed. Simultaneous RNA and protein isolation can be performed with the same sample, using commercially available reagents and kits (**Table of Materials**) if the sample quantity is less.

## 4.2. For immunostainings

NOTE: This is performed directly on the cells attached to the cell pool of the PDMS construct. Therefore, we recommend placing, every time, 1 cm x 1 cm of paraffin film on the top of the cell pool, apart from the plate lid, to minimize the evaporation of the incubation solutions.

4.2.1. Wash the construct 2x with 3 mL of PBS for 5 min at room temperature.

4.2.2. Fix the cells attached to the construct with 2 mL of 10% formalin for 15 min at room temperature.

4.2.3. Wash the cells 3x with 3 mL of PBS for 5 min at room temperature. For long-term storage,



leave the samples in PBS with 0.1% sodium azide at 4 °C.

4.2.4. Permeabilize the cells with 3 mL of PBS + 0.5% detergent (3x, each time 5 - 10 min, at room temperature).

4.2.5. Incubate the cells with 100 µL of PBS + 10% horse serum + 0.2% detergent + 1% bovine serum albumin at room temperature for 1 h, to block nonspecific antibody binding.

4.2.6. Incubate the cells with 100 µL of PBS + 10% horse serum + 0.2% detergent + 1% bovine serum albumin + primary antibody at room temperature for 1 h. For example, primary antibodies against Cx43 (1:100), sarcomeric  $\alpha$ -actinin (1:100), GATA-4 (1:50), MEF2 (1:25), and SERCA2 (1:50).

4.2.7. Wash 3x with 3 mL of PBS at room temperature for 5 min.

4.2.8. Incubate the cells with 100 µL of PBS + secondary antibody at room temperature in the dark for 1 h.

NOTE: Secondary antibodies conjugated with different fluorophores and a counterstaining agent were used.

4.2.9. Wash them 3x with 3 mL of PBS at room temperature in the dark for 5 min.

4.2.10. Incubate the cells with 100 µL of nuclear staining (0.1 µg/mL) in PBS at room temperature in the dark for 15 min.

4.2.11. Wash them 3x with 3 mL of PBS at room temperature in the dark for 5 min.

4.2.12. Store the samples in 3 mL of PBS with 0.1% sodium azide at 4 °C until the acquisition.

NOTE: Microscope acquisition is possible on inverted fluorescent and confocal microscopes with long-working distance objectives because the construct thickness is about 0.5 mm.

## REPRESENTATIVE RESULTS:

**Figure 2** represents the general schema followed for the cell stimulation. Briefly, cells were seeded on the PDMS construct and subjected to electromechanical stimulation, with a media change performed twice a week. Nonstimulated cells were used as a control for the electromechanical conditioning. Additionally, we added an extra control to the experiment, and subcutaneous ATDPCs were used as a control for cardiac ATDPCs. Subcutaneous ATDPCs are obtained from subcutaneous adipose tissue, following the same isolation and culture procedures as for cardiac ATDPCs<sup>33</sup>. Cells attached to the PDMS construct evidenced a typical fusiform phenotype before and after stimulation. Moreover, the cells aligned according to the patterned surface, in this case following the vertical pattern.

Electrical and mechanical stimulations were first optimized individually. First, electrostimulation was based on a previous study in which alternating-current 2 ms monophasic square-wave pulses of 50 mV/cm at 1 Hz were found to be best for cardiac ATDPCs<sup>10</sup>. We reported that electrical stimulation increased the expression of early cardiac markers in cardiac ATDPCs, such as MEF2A ( $P = 0.050$ ) and GATA-4 ( $P = 0.031$ ), but no effects on structural and calcium-handling genes were observed (data not shown).

Second, the mechanostimulation protocol consisted of 10% stretching and a 1 Hz trapezoidal waveform with a 50% duty cycle, with 100 ms rise and fall times to imitate the pressure cycle in the heart, as described in full before<sup>21</sup>. Mechanically stimulated cardiac ATDPCs augmented the expression of structural genes, such as  $\alpha$ -actinin ( $P = 0.001$ ) or cTnI ( $P = 0.044$ ), and showed an increasing trend for early cardiac markers, GATA-4 ( $P = 0.068$ ) and T-box transcription factor 5 (Tbx5;  $P = 0.065$ ) (data not shown). Effects derived from mechanical stimulation were strongly dependent on the patterned surface.

Afterward, both protocols were combined for an efficient electromechanical stimulation of cardiac ATDPCs, resembling ventricle-filling by blood. The resulting protocol comprised alternating-current 2 ms monophasic square-wave pulses of 50 mV/cm at 1 Hz and 10% stretching for 7 days<sup>30</sup>. Overall, electromechanically stimulated cardiac ATDPCs enhanced their cardiomyogenic potential. Stimulated cardiac ATDPCs increased the expression of early and late cardiac genes (**Figure 3A**), namely, the cardiac transcription factor GATA-4 ( $P = 0.050$ ), the structural marker  $\beta$ -myosin heavy chain ( $\beta$ -MHC;  $P = 0.000$ ), and the calcium-related gene Cx43 ( $P = 0.025$ ). Gene modulations resulting from the electromechanical stimulation were also translated at the protein level (**Figure 3B-M**). Phalloidin staining against actin fibers showed that the majority of cells aligned according to the vertical pattern and that the Cx43 distribution was mostly in the cytoplasm and at the plasma membrane, to contribute to intercellular communication through gap junctions (**Figure 3B-E**). MEF2 and GATA-4 transcription factors were located at the nuclei in cardiac ATDPCs; however, GATA-4 was not detected in subcutaneous ATDPCs (**Figure 3F-M**). The cytoplasmic markers SERCA2 and sarcomeric  $\alpha$ -actinin did not show a mature sarcomere organization typical for cardiomyocytes, and beating was not observed in control and stimulated cell populations (**Figure 3F-M**).

#### FIGURE LEGENDS:

**Figure 1: Electromechanical stimulator.** (A) PDMS construct used for the cell conditioning. (B) Drawing of the PDMS construct, including electrodes and magnets. (C) Detail of the printed circuit board (platform) used to perform the electromechanical conditioning. This panel has been modified from Lluçà-Valldeperas *et al.*<sup>30</sup>. (D) Picture of the electromechanical stimulation platform and user interface (computer).

**Figure 2: Electromechanical stimulation procedure, unit, and user interface.** (A) Electromechanical stimulation schema with representative images of nonstimulated cells at day 0 and stimulated cells at day 7, both on constructs with the vertical surface pattern. The scale bars = 100  $\mu$ m. (B) Electromechanical stimulation unit: seeded PDMS construct, electrodes, and

fixed/mobile magnets. (C) Control application panel for the mechanical stimulation.

**Figure 3: Gene and protein expression of the main cardiac markers after the electromechanical stimulation of cardiac and subcutaneous ATDPCs.** (A) Real-time PCR of main cardiac genes in cardiac and subcutaneous ATDPCs. The relative expression of cardiomyogenic markers in stimulated *versus* nonconditioned controls is shown for cardiac and subcutaneous ATDPCs. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase expression and are shown as mean  $\pm$  SEM for six independent experiments. \*  $P < 0.05$  (significance). (B - M) Protein expression in cardiac and subcutaneous ATDPCs on a vertical-patterned surface expression of main cardiac markers for control and stimulated cells. Phalloidin staining (actinF; red) and Cx43 expression (green), SERCA2 (red), MEF2 (green), sarcomeric  $\alpha$ -actinin (red), and GATA-4 (green) expression in control (in panels B, D, F, H, J, and L) and stimulated (in panels C, E, G, I, K, and M) cardiac (left) and subcutaneous (right) ATDPCs. Nuclei were counterstained with DAPI (blue; panels B - E, L, and M). The scale bars = 50  $\mu$ m. This figure has been modified from Lluc  a-Valledeperas *et al.*<sup>30</sup>.

#### DISCUSSION:

Electromechanical stimulation appears to be a safe alternative for preparing cells for a hostile cardiac environment and enhancing their cardiac commitment. Here, a protocol described for cardiac progenitor cells increased the expression of main cardiac markers and was reported to be beneficial for their next implantation on infarcted murine myocardium<sup>30</sup>. In general, electromechanically stimulated cardiac ATDPCs increased the expression of genes related to early, structural, and calcium regulation, which has never been achieved with previous electrical or mechanical stimulations individually. In fact, electromechanically stimulated cardiac ATDPCs show a more complete profile and seemed to be more committed to the cardiac lineage than previously reported.

Cardiac gene expression was modulated in both cell types after electromechanical stimulation, especially after the notable augmentation of cardiac ATDPCs compared to subcutaneous ATDPCs. This result may have been a consequence of the origin of the adipose tissue used for cell isolation, namely, epicardial or subcutaneous fat, respectively. The epicardial adipose tissue surrounding both heart and pericardium is a metabolically active organ and a source of progenitor cells. Of interest, the epicardial fat has anatomical and functional continuity with the myocardium. Under normal circumstances, the epicardial fat has biochemical and thermogenic cardioprotective properties; conversely, under pathological conditions, it can secrete proinflammatory cytokines to affect the heart<sup>39</sup>. Indeed, cardiac ATDPCs have an inherent cardiac-like phenotype and exhibit a constitutive expression of cardiac markers, such as Cx43, sarcomeric  $\alpha$ -actinin, SERCA2, and GATA-4, compared to subcutaneous ATDPCs, which are not that adapted to the cardiac environment<sup>34</sup>. Thus, effects derived from electromechanical stimulation might be greater for cardiac progenitor cells, whose niche is close to or within the myocardial milieu.

From the last observation in which gene modulation strongly depends on the cell population, a protocol optimization for each cell population is advisable, and approximate values can be extracted from those described in this protocol. The most critical steps in cell electromechanical

stimulation are cell attachment and stimulation regimes (intensity, duration, frequency).

For example, the electric parameters are crucial. Indeed, the electric field intensity applied was optimal for a nonelectric cell, so increased values would be more appropriate for cells with electrical activity, such as immature pluripotent stem-cell-derived cardiomyocytes<sup>40,41</sup>. Preliminary experimentation for cardiac ATDPC electrostimulation demonstrated that a direct current and a high voltage induced cell growth arrest and cell death<sup>10</sup>. From these results, alternating-current and low-voltage protocols were adopted for further experimentation.

Additionally, some cell lineages present a low attachment to the PDMS surface, so a coating or a plasma treatment is suggested to enrich the seeding efficiency<sup>42</sup>. Moreover, cell seeding should be adapted to each cell size and growth, decreasing the values for the highly proliferative cells or increasing them for smaller cells; thus, a few trials seeding several densities on the PDMS construct are recommended. Finally, the protocol was performed on a cell monolayer, and values may be slightly different for a three-dimensional scenario (*i.e.*, the cell density).

Furthermore, the surface pattern plays a key role in mechanical cell training. It was shown that the cells aligned according to the pattern, and their performance after mechanical stimulation was not the same among different surfaces. For instance, mechanically stimulated cardiac ATDPCs seeded on vertical-patterned surfaces (perpendicular to the stretching force) secreted proteins associated with myocardial infarction and extracellular matrix remodeling motifs. Mechanostimulated cardiac ATDPCs seeded on nonpatterned surfaces secreted proteins associated with cardiac regeneration<sup>21</sup>.

The main features of this method have been described above. However, it is important to highlight the noninvasive approach of the cell stretching as one of the unique methods to achieve these features. Of interest, because the same device can submit electrical and/or mechanical stimuli, a direct comparison of the resulting effects from different stimulations and cells is possible. In addition, the visualization of the cells seeded on the construct, thanks to transparency and thinness, is a clear advantage to evaluate cell status before, during, and after stimulation.

The device and the protocol have some limitations, some of them already noted. First, it was designed and optimized for a monolayer cell culture and not for a three-dimensional cell culture. Specifically, only adherent cells can be used in the monolayer setting. For nonadherent cells, a three-dimensional approach should be implemented. Second, the construct size limits the seeding surface; thus, the number of stimulated cells is small, and every experimental set requires several replicates to collect enough samples for further gene or protein analyses. A scale-up is mandatory for large animal experimentation or clinical translation, in which higher cell doses are required; in such cases, bigger stimulation surfaces can be considered.

The main applications of this protocol include cell conditioning, disease modeling, and drug screening. This stimulation enhances cell maturation, and its application would be useful for achieving and maintaining a more mature phenotype. On one hand, cells that mimic the

functional phenotype present in the adult myocardium are crucial for disease modeling and organ-on-a-chip experimentation. Indeed, human biopsies cannot represent the progression of the disease because they are usually end-stage or post-mortem samples, while animal models do not always recapitulate human physiology and symptomatology. Nevertheless, the use of human-induced pluripotent stem cells has emerged as an alternative modeling method for unraveling the mechanism underlying pathology development<sup>43</sup>. In addition, organs-on-a-chip are miniature tissues and organs grown *in vitro* that allow the modeling of human (patho)physiology and mimic the three-dimensional structures. Their aim is to establish a minimally functional unit that can recapitulate certain aspects of human physiology and disease in a controlled and straightforward manner and address the limitations of existing cell and animal models<sup>44</sup>.

On the other hand, drug screening requires culture platforms that mimic the biophysical environment present *in vivo* in a high-throughput, miniaturized system for testing the safety and efficacy of drugs *via* cardiomyocyte response. Mature cells could be used to recapitulate a clinically relevant readout (*i.e.*, contractile kinetics) in a high-throughput assay, to elucidate disease mechanisms and identify novel therapeutic targets<sup>45</sup>.

The cardiac field was the main scope of this protocol, but it could easily be adapted to neuronal or skeletal domains because these environments are characterized by electrical and mechanical stimuli. Previous studies with physical stimulations have indicated beneficial outcomes<sup>46-50</sup>.

In conclusion, a new protocol for the synchronous electromechanical conditioning of cardiac ATDPCs has been described. The resulting protocol and device have been extensively tested and validated for individual and synchronized stimuli. Synchronous electromechanical conditioning of ATDPCs boosts their cardiomyogenic potential and emerges as a promising strategy for cell therapy, disease modeling, and drug screening.

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#### **DISCLOSURES:**

The authors have nothing to disclose, except that the stimulation device and protocol were previously patented (WO-2013185818-A1, WO-2017125159-A1).

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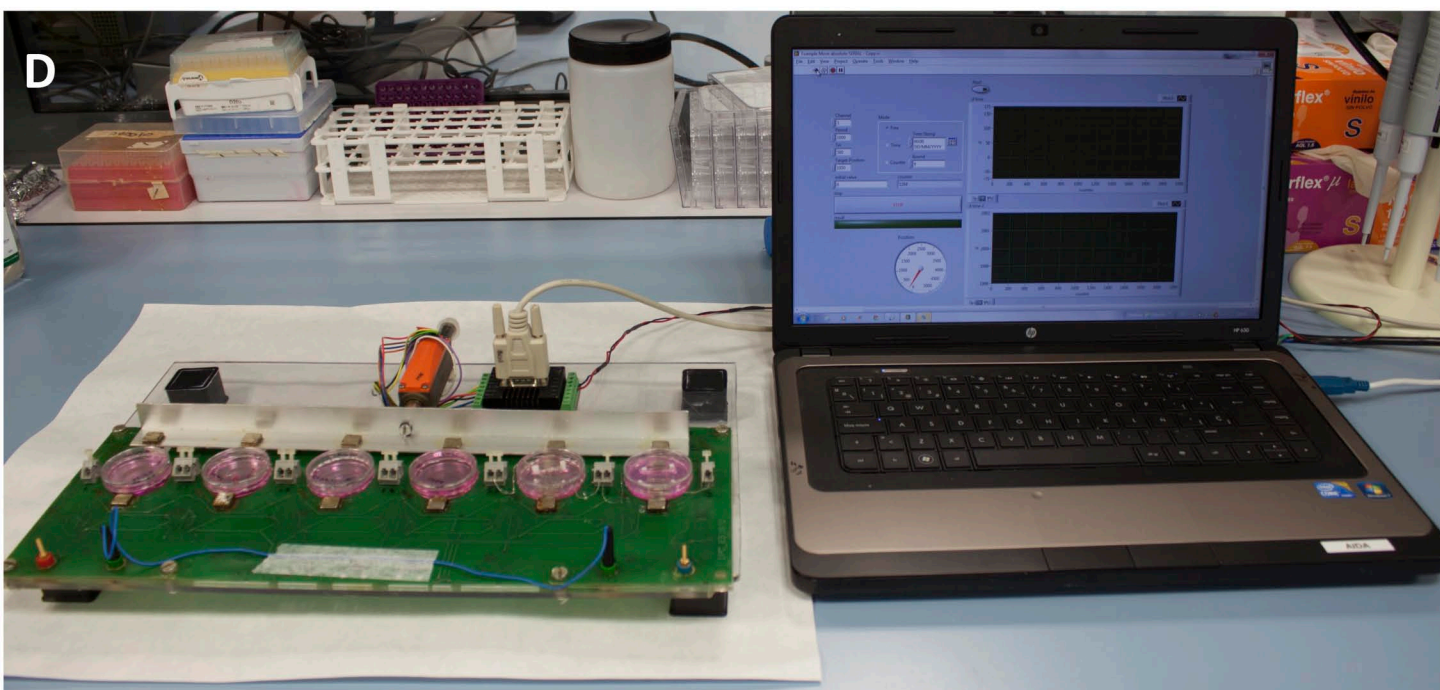
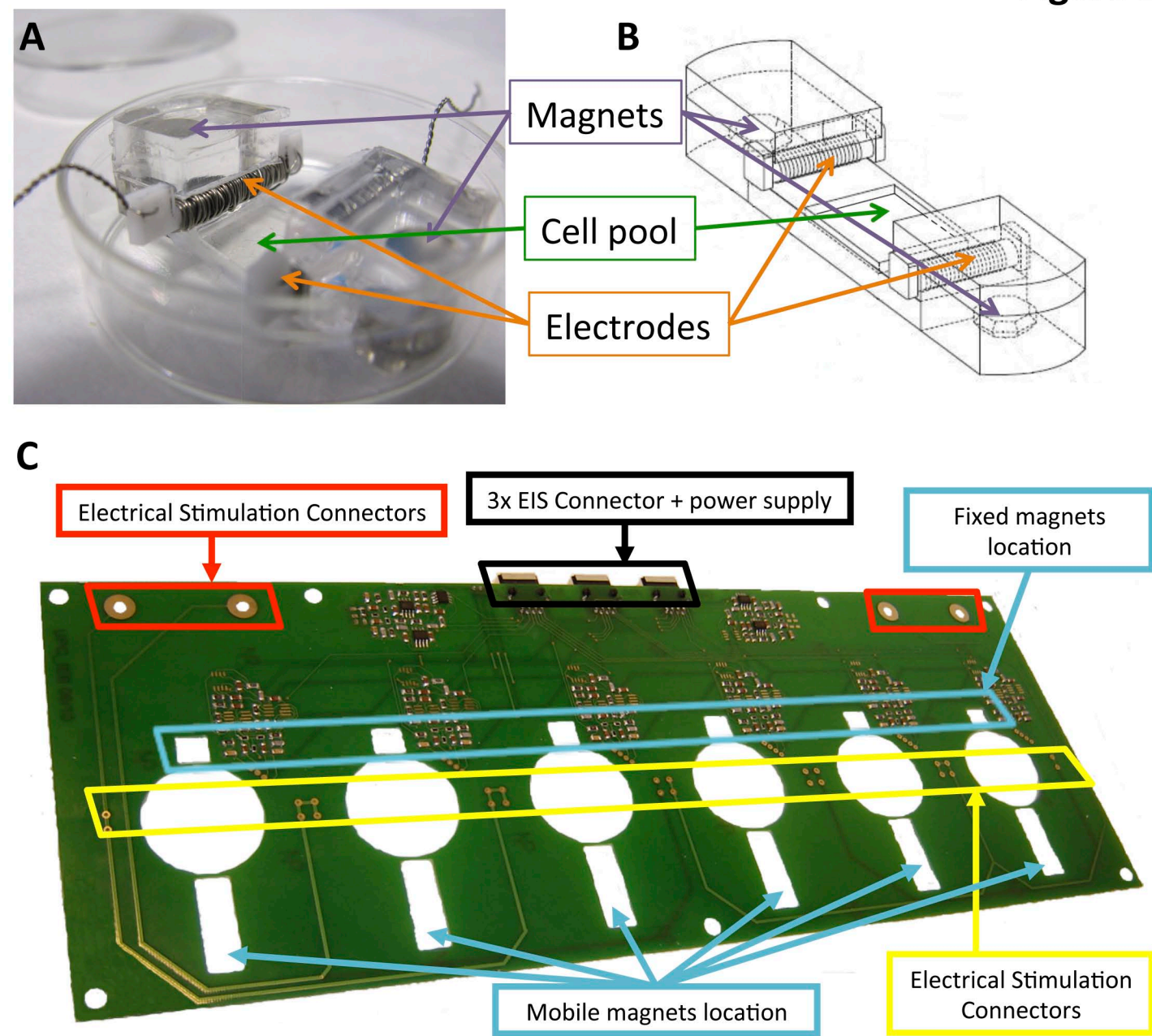
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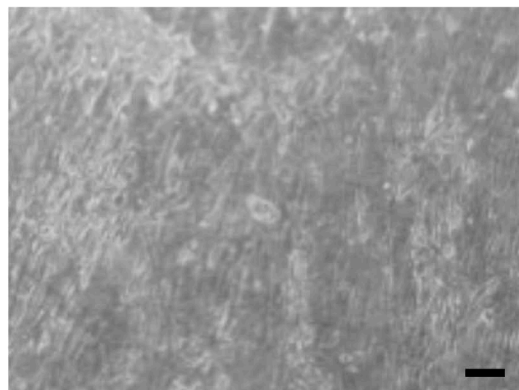
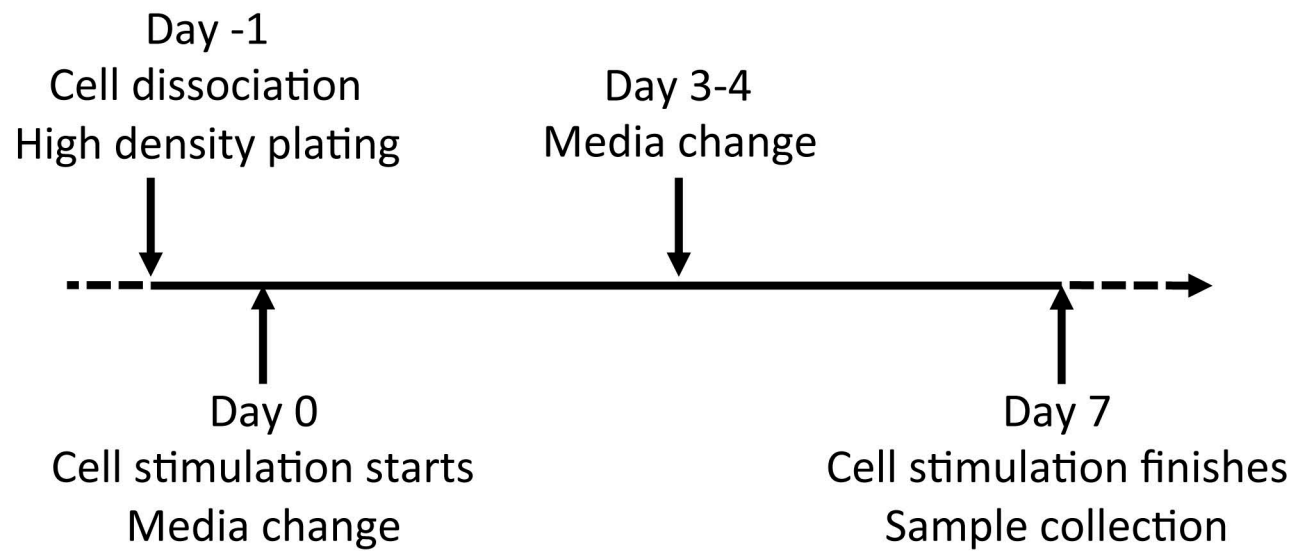
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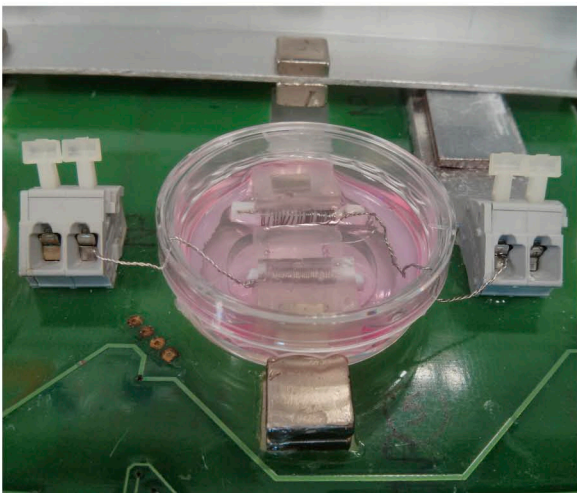




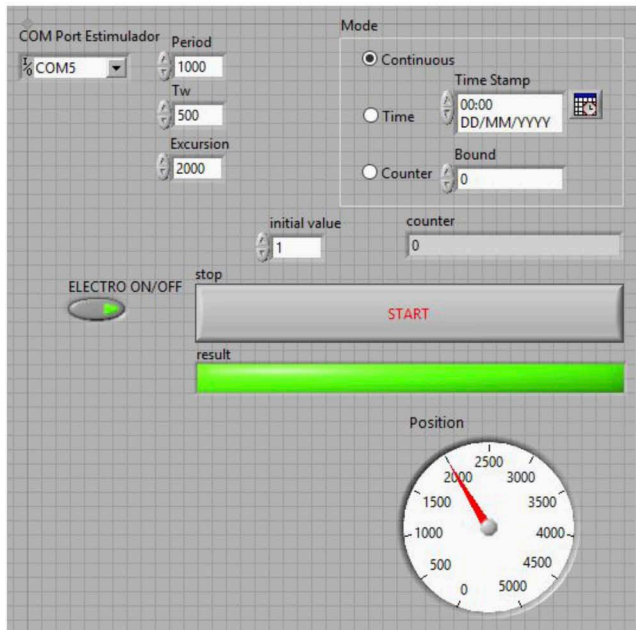
A Electromechanical stimulation schema



B Electromechanical stimulation unit

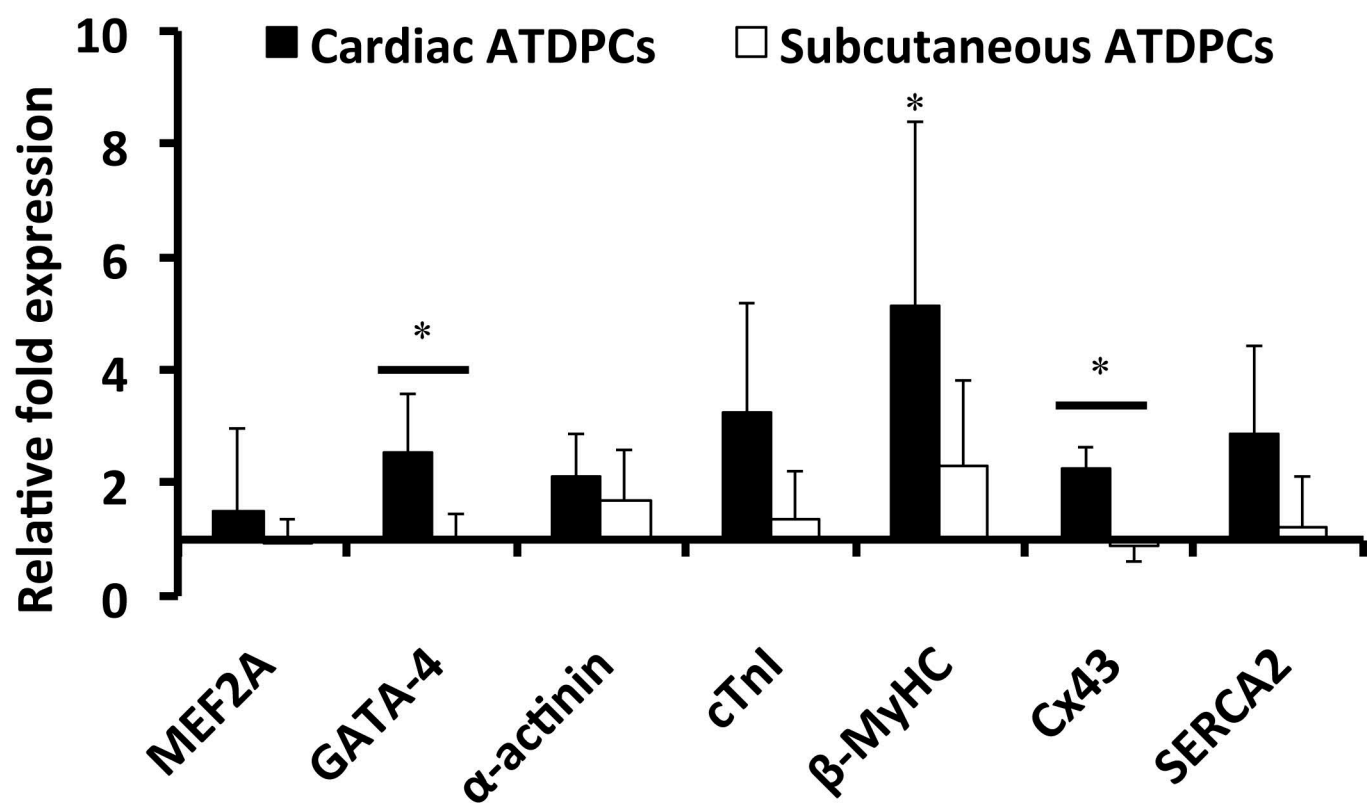


C Electromechanical stimulation user interface

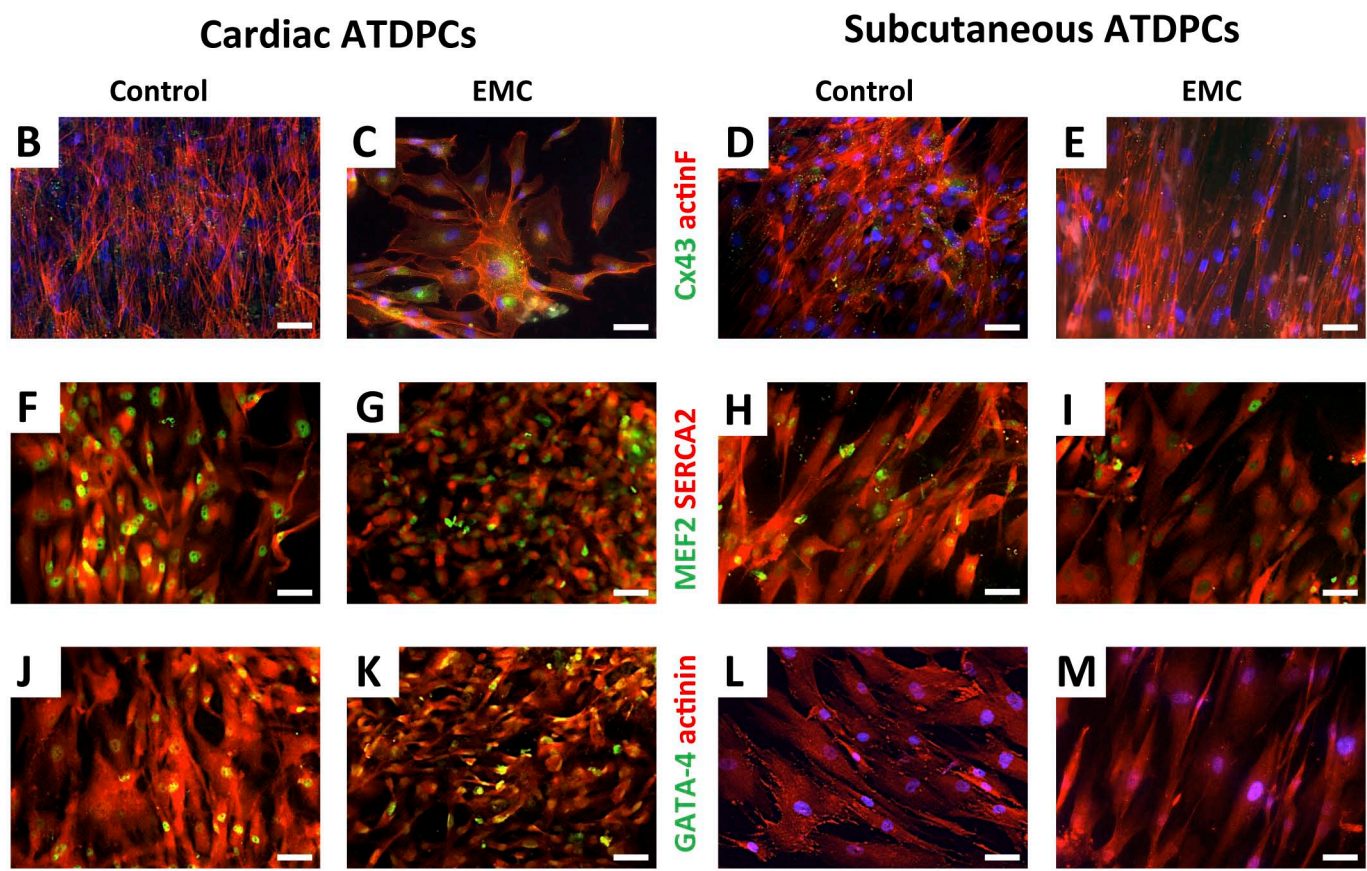




A Electromechanical stimulation effects at the gene level



Electromechanical stimulation effects at the protein level



Stimulator		
nickel plated neodymium magnets	Supermagnete	Q-10-10-05-N
nickel-plated neodymium magnets	Supermagnete	Q-06-04-02-HN
polydimethylsiloxane (PDMS) SYLGAR 184 Silicone Elastomer	Dow Corning Corp	184
ruled diffraction grating (1250 grooves/mm)	Newport	05RG150-1250-2
Motor controller	Faulhaber	MCLM-3006-S
Labview	National Instruments	
Cell culture		
phosphate-buffered saline (PBS)	Gibco	70013-065
0.05% trypsin-EDTA	Gibco	25300-120
35 mm cell culture dish	BD Falcon	45353001
fetal bovine serum (FBS)	Gibco	10270-106
L-Glutamine 200 mM, 100x	Gibco	25030-024
Penicilina/Streptomycin, 10.000 U/mL	Gibco	15140-122
Minimum essential medium eagle (alpha-MEM)	Sigma	M4526-24x500ML
Protein & RNA analyses		
protease inhibitor cocktail	Sigma	P8340
QIAzol Lysis Reagent	Qiagen	79306
RIPL Prep RNA/Protein Kit	Qiagen	50980404
RNeasy mini kit	Qiagen	74104
iTaq Universal Probes One-Step Kit	Bio-Rad Laboratories	172-5140
Random hexamers	Qiagen	79236
TaqMan PreAmp MasterMix 2X	Applied Biosystems	4391128
TaqMan Universal PCR MasterMix	Applied Biosystems	4324018
Immunostaining		
10% formalin	Sigma	HT-501128-4L
horse serum	Sigma	H1138
Triton X-100	Sigma	X100-500ML
Bovine Serum Albumin (BSA)	Sigma	A7906-100G
PARAFILM	Sigma	P6543
4',6-diamidino-2-phenylindole (DAPI)	Sigma	D9542
Phalloidin Alexa 568	Invitrogen	A12380
sodium azide	Sigma	S8032-100g

Hoechst 33342  
Connexin-43 rabbit primary antibody  
sarcomeric  $\alpha$ -actinin mouse primary antibody  
GATA-4 goat primary antibody  
MEF2 rabbit primary antibody  
SERCA2 goat primary antibody  
Cy3 secondary antibody  
Cy3 secondary antibody  
Cy3 secondary antibody  
Cy2 secondary antibody  
Cy2 secondary antibody  
Cy2 secondary antibody

Sigma	14533
Sigma	C6219 lot#061M4823
Sigma	A7811 lot#080M4864
R&D	AF2606 VAZ0515101
Santa Cruz	sc-313 lot#E0611
Santa Cruz	sc-8095 lot#D2709
Jackson ImmunoResearch	711-165-152
Jackson ImmunoResearch	715-165-151
Jackson ImmunoResearch	712-165-150
Jackson ImmunoResearch	715-225-150
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Jackson ImmunoResearch	705-225-147





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Badalona, October 22<sup>nd</sup> 2018

Dear Dr. Bajaj,

Please, find enclosed the revised version of our manuscript entitled “**Simultaneous Electrical and Mechanical Stimulation to Enhance Cells Cardiomyogenic Potential**” by Lluçia-Valldeperas *et al.*

We deeply appreciate the second review of our manuscript submitted to *Journal of Visualized Experiments*. Based on editor’s comments, we have revised the manuscript as described in the enclosed point-by-point response. We have also prepared a second version of Figure 2 to clarify some steps of the Protocol section. We could include this new Figure upon Editor’s decision.

We hope that the revised version of our manuscript meets with your approval and is suitable for publication. We wish to thank the editor for the comments to improve our paper. I look forward to hearing from you.

Sincerely,

**Dr. Aida Lluçia-Valldeperas, PhD.**

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**Editorial comments:**

**1. The manuscript is poorly written, please use a professional copyediting service or proofread it very well to bring out the clarity and removal of redundancy throughout.**

The comment is well received and the manuscript was sent to a professional copyediting service.

**2. The editor has formatted the manuscript to match the journal's style. Please retain the same.**

**3. Please address all the specific comments marked in the manuscript.**

All comments were addressed. However, we want to explain some of the changes to the following comments for their relevance:

**Comment 1:**

The stimulation could be simultaneous or not, so we slightly modified the title according to the comment but maintaining the word “Simultaneous” as it is important for the stimulation regime.

**Comment 10:**

Figure 1 citation has been added on line 212.

**Comment 20:**

The PDMS construct characteristics were described in the Introduction section and it has been shown in Figure 1A,B.

**Comment 28:**

The cell pool has been described before and also shown in Figure 1A,B.

**Comment 49:**

The paraffin film is added above the cell pool to minimize the evaporation during incubations. Additionally, the culture plate is always placed throughout culture and immunostainings.

**Comment 54:**

Transcriptional data is shown in Figure 3, as well as protein data (immunostaining). In that publication we did not perform westernblot analyses after electromechanical stimulation, so we cannot include this data. However, the technique allows us to collect protein samples for western blot.

**4. For the introduction section, Please revise to include all of the following:**

- a) A clear statement of the overall goal of this method**
- b) The rationale behind the development and/or use of this technique**
- c) The advantages over alternative techniques with applicable references to previous studies**



- d) A description of the context of the technique in the wider body of literature**
- e) Information to help readers to determine whether the method is appropriate for their application**

All this information has been addressed in the Introduction.

- 5. For the protocol section, Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.).**

The whole protocol has been revised to ensure the imperative tense was used.

- 6. Please ensure you answer the “how” question, i.e., how is the step performed? Please provide all button clicks, graphical user interface, the knob turns etc. wherever needed.**

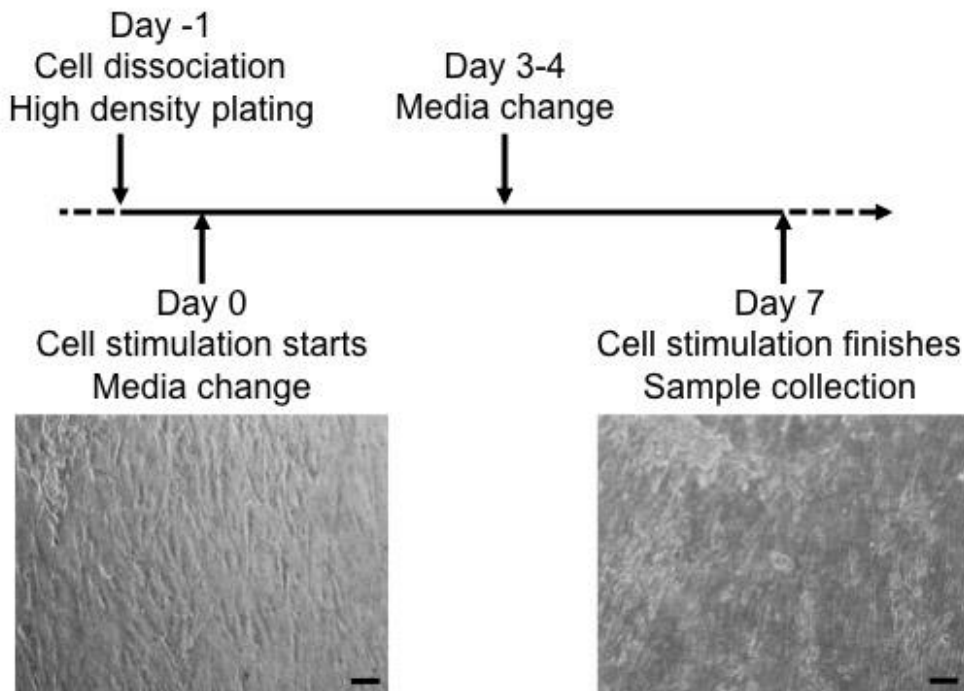
Clarification and more details were added to some steps.

Regarding section 3.8 a new Figure 2 has been prepared with a detail of the mechanical stimulation user interface:



**Figure 2**

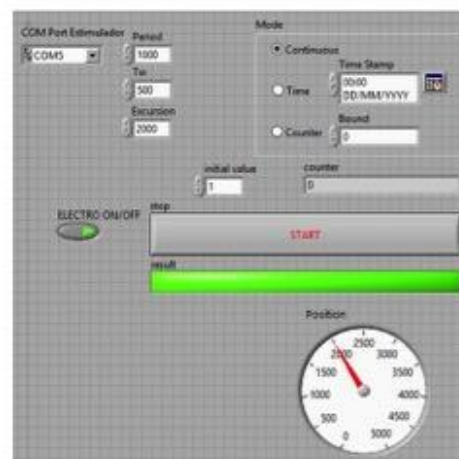
**A Electromechanical stimulation schema**



**B Electromechanical stimulation unit**



**C Electromechanical stimulation user interface**



If the Editor prefers the revised version of Figure 2, it could be incorporated instead of the original Figure 2. Both Figures will be uploaded.

**7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.**

The protocol was rewritten into discrete steps.



---

**8. Once formatted please ensure that the protocol is no more than 10 pages long and the highlighted section is no more than 2.75 pages including headings and spacings.**

The new revised protocol fits in the page limits.

**8. Results: Please ensure that you have representative results for all the sections described in the protocol.**

Representative results for immunostainings and PCR data are shown. However, we do not show western blot results after electromechanical stimulation of cardiac ATDPCs, as we did not perform it. The westernblot analyses section was added because the technique is allowed by the protocol. However, if results are needed we can omit this section.

**9. Please reword lines 102-105, 148-151 as it matches with the previously published protocols.**

We appreciate the comment and they were rewritten in the revised version of the manuscript.

Dear Dr. Lluçia-Valldeperas,

Permission is hereby granted to adapt figures 1 and 2 from your published article “Electromechanical Conditioning of Adult Progenitor Cells Improves Recovery of Cardiac Function After Myocardial Infarction” subject to the usual acknowledgements (author, title of material, title of the journal, ourselves as publisher) and a copy of the copyright notice. Any third party material is expressly excluded from this permission.

Thank you for your interest and continued support of STEM CELLS Translational Medicine.

Please let me know if I may be of further assistance.

Kind regards,

Tina Belle  
Editorial Staff  
STEM CELLS Translational Medicine