

# Journal of Visualized Experiments

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

--Manuscript Draft--

<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE58934R2
<b>Full Title:</b>	Simultaneous Electrical and Mechanical Stimulation to Enhance Cells Cardiomyogenic Potential
<b>Keywords:</b>	Electromechanical stimulation; Electrical Stimulation; mechanical stimulation; electromechanical system; Cardiac Progenitor Cells; cardiac ATDPCs; cell conditioning; cardiac tissue engineering; cell maturation; disease modeling; drug screening
<b>Corresponding Author:</b>	Aida Lluçia-Valldeperas VU medisch centrum Amsterdam, Holland NETHERLANDS
<b>Corresponding Author's Institution:</b>	VU medisch centrum
<b>Corresponding Author E-Mail:</b>	lluciavalldeperas@gmail.com
<b>Order of Authors:</b>	Aida Lluçia-Valldeperas Ramon Bragos Antoni Bayes-Genis
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Badalona. Barcelona, Spain

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.**

ICREC Research Program, Health Science Research Institute Germans Trias i Pujol.  
Amsterdam UMC, Pulmonology and Physiology, Amsterdam Cardiovascular Sciences.  
E-mail: [llucia.valldeperas@gmail.com](mailto:llucia.valldeperas@gmail.com), [a.lluciavalldeperas@vumc.nl](mailto:a.lluciavalldeperas@vumc.nl).



**TITLE:**

Simultaneous Electrical and Mechanical Stimulation to Enhance Cells' Cardiomyogenic Potential

**AUTHORS AND AFFILIATIONS:**

Aida Llucilà-Valldeperas<sup>1,2</sup>, Ramon Bragós<sup>3</sup>, Antoni Bayés-Genís<sup>1,4,5,6</sup>

<sup>1</sup>Insuficiencia Cardíaca y Regeneración Cardíaca (ICREC) Research Program, Health Science Research Institute Germans Trias i Pujol, Badalona, Spain

<sup>2</sup>Amsterdam Universitair Medisch Centrum (UMC), Vrije Universiteit Amsterdam, Pulmonology and Physiology, Amsterdam Cardiovascular Sciences, Amsterdam, The Netherlands

<sup>3</sup>Electronic and Biomedical Instrumentation Group, Departament d'Enginyeria Electrònica, Universitat Politècnica de Catalunya, Barcelona, Spain

<sup>4</sup>Cardiology Service, Germans Trias i Pujol University Hospital, Badalona, Spain

<sup>5</sup>Department of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain

<sup>6</sup>Centro de Investigación Biomédica en Red (CIBER) Cardiovascular, Instituto de Salud Carlos III, Madrid, Spain

**Corresponding Author:**

Aida Llucilà-Valldeperas (a.lluciavalldeperas@vumc.nl)

**Email Addresses of Co-authors:**

Ramon Bragós (rbb@eel.upc.edu)

Antoni Bayés-Genís (abayes.germanstrias@gencat.cat)

**KEYWORDS:**

Electromechanical stimulation; electrical stimulation; mechanical stimulation; electromechanical system; cardiac progenitor cells; cardiac ATDPs; 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-Valldeperas *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.

#### ACKNOWLEDGMENTS:

The authors want to thank the members of the ICREC Research Program (IGTP, Badalona) and the Electronic and Biomedical Instrumentation Group (UPC, Barcelona), especially Prof. J. Rosell-Ferrer. In addition, the authors acknowledge STEM CELLS Translational Medicine journal and AlphaMed Press for permitting the adaptation of previously published figures (Llucà-Valldeperas, *et al.*<sup>30</sup>). The development of this prototype and the design of the protocol were supported by Ministerio de Educación y Ciencia (SAF 2008-05144), Ministerio de Economía y Competitividad (SAF 2014-59892), the European Commission 7th Framework Programme (RECATABI, NMP3-SL-2009-229239), Fundació La Marató de TV3 (080330, 201516, 201502), and Fundación para la Innovación y la Prospectiva en Salud en España (FIPSE; 06-00001396-15).

#### DISCLOSURES:

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

#### REFERENCES:

573 1. McDonough, P.M., Glembotski, C.C. Induction of atrial natriuretic factor and myosin light  
574 chain-2 gene expression in cultured ventricular myocytes by electrical stimulation of contraction.  
575 *Journal of Biological Chemistry*. **267**, 11665-11668 (1992).

576 2. Tandon, N. *et al.* Electrical stimulation systems for cardiac tissue engineering. *Nature*  
577 *Protocols*. **4**, 155-173 (2009).

578 3. Serena, E. *et al.* Electrical stimulation of human embryonic stem cells: cardiac differentiation  
579 and the generation of reactive oxygen species. *Experimental Cell Research*. **315**, 3611-3619  
580 (2009).

581 4. Tandon, N. *et al.* Optimization of electrical stimulation parameters for cardiac tissue  
582 engineering. *Journal of Tissue Engineering and Regenerative Medicine*. **5**, E115-E125 (2011).

583 5. Zhang, X., Wang, Q., Gablaski, B., Lucchesi, P., Zhao, Y. A microdevice for studying intercellular  
584 electromechanical transduction in adult cardiac myocytes. *Lab on a Chip*. **13**, 3090-3097 (2013).

585 6. Chan, Y.C. *et al.* Electrical stimulation promotes maturation of cardiomyocytes derived from  
586 human embryonic stem cells. *Journal of Cardiovascular Translational Research*. **6**, 989-999  
587 (2013).

588 7. Pietronave, S. *et al.* Monophasic and biphasic electrical stimulation induces a precardiac  
589 differentiation in progenitor cells isolated from human heart. *Stem Cells and Development*. **23**,  
590 888-898 (2014).

591 8. Pavesi, A. *et al.* Electrical conditioning of adipose-derived stem cells in a multi-chamber culture  
592 platform. *Biotechnology and Bioengineering*. **111**, 1452-1463 (2014).

593 9. Baumgartner, S. *et al.* Electrophysiological and morphological maturation of murine fetal  
594 cardiomyocytes during electrical stimulation *in vitro*. *Journal of Cardiovascular Pharmacology*  
595 *and Therapeutics*. **20**, 104-112 (2015).

596 10. Lluc  -Valledeperas, A. *et al.* Electrical stimulation of cardiac adipose tissue-derived progenitor  
597 cells modulates cell phenotype and genetic machinery. *Journal of Tissue Engineering and*  
598 *Regenerative Medicine*. **9** (11), E76-E83 (2015).

599 11. Lluc  -Valledeperas, A. *et al.* Physiological conditioning by electric field stimulation promotes  
600 cardiomyogenic gene expression in human cardiomyocyte progenitor cells. *Stem Cell Research*  
601 *and Therapy*. **5**, 93 (2014).

602 12. Radisic, M. *et al.* Functional assembly of engineered myocardium by electrical stimulation of  
603 cardiac myocytes cultured on scaffolds. *Proceedings of the National Academy of Sciences of the*  
604 *United States of America*. **101** (52), 18129-34 (2004).

605 13. Fink, C. *et al.* Chronic stretch of engineered heart tissue induces hypertrophy and functional  
606 improvement. *FASEB Journal*. **14**, 669-679 (2000).

607 14. Zimmermann, W.H. *et al.* Engineered heart tissue grafts improve systolic and diastolic  
608 function in infarcted rat hearts. *Nature Medicine*. **12** (4), 452-8 (2006).

609 15. Birla, R.K., Huang, Y.C., Dennis, R.G. Development of a novel bioreactor for the mechanical  
610 loading of tissue-engineered heart muscle. *Tissue Engineering*. **13**, 2239-2248 (2007).

611 16. Salameh, A. *et al.* Cyclic mechanical stretch induces cardiomyocyte orientation and  
612 polarization of the gap junction protein connexin43. *Circulation Research*. **106**, 1592-1602 (2010).

613 17. Galie, P.A., Stegemann, J.P. Simultaneous application of interstitial flow and cyclic mechanical  
614 strain to a three-dimensional cell-seeded hydrogel. *Tissue Engineering Part C: Methods*. **17** (5),  
615 527-536 (2011).

616 18. Leychenko, A., Konorev, E., Jijiwa, M., Matter, M.L. Stretch-induced hypertrophy activates

NFkB-mediated VEGF secretion in adult cardiomyocytes. *PLoS One*. **6**, e29055 (2011).

19. Tulloch, N.L. *et al.* Growth of engineered human myocardium with mechanical loading and vascular coculture. *Circulation Research*. **109**, 47-59 (2011).

20. Mihic, A. *et al.* The effect of cyclic stretch on maturation and 3D tissue formation of human embryonic stem cell-derived cardiomyocytes. *Biomaterials*. **35**, 2798-2808 (2014).

21. Lluçà-Valldeperas, A. *et al.* Unravelling the effects of mechanical physiological conditioning on cardiac adipose tissue-derived progenitor cells *in vitro* and *in silico*. *Scientific Reports*. **8**, 499 (2018).

22. Stoppel, W.L., Kaplan, D.L., Black 3rd, L.D. Electrical and mechanical stimulation of cardiac cells and tissue constructs. *Advanced Drug Delivery Reviews*. **96**, 135-155 (2016).

23. Nunes, S.S. *et al.* Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes. *Nature Methods*. **10**, 781-787 (2013).

24. Barash, Y. *et al.* Electric field stimulation integrated into perfusion bioreactor for cardiac tissue engineering. *Tissue Engineering Part C: Methods*. **16**, 1417-1426 (2010).

25. Maidhof, R. *et al.* Biomimetic perfusion and electrical stimulation applied in concert improved the assembly of engineered cardiac tissue. *Journal of Tissue Engineering and Regenerative Medicine*. **6**, e12-e23 (2012).

26. Feng, Z. *et al.* An electro-tensile bioreactor for 3-D culturing of cardiomyocytes. A bioreactor system that simulates the myocardium's electrical and mechanical response *in vivo*. *IEEE Engineering in Medicine and Biology Magazine*. **24** (4), 73-79 (2005).

27. Wang, B. *et al.* Myocardial scaffold-based cardiac tissue engineering: application of coordinated mechanical and electrical stimulations. *Langmuir*. **29** (35), 11109-11117 (2013).

28. Morgan, K.Y., Black 3rd, L.D. Mimicking isovolumic contraction with combined electromechanical stimulation improves the development of engineered cardiac constructs. *Tissue Engineering Part A*. **20** (11-12), 1654-1667 (2014).

29. Godier-Furnémont, A.F. *et al.* Physiologic force-frequency response in engineered heart muscle by electromechanical stimulation. *Biomaterials*. **60**, 82-91 (2015).

30. Lluçà-Valldeperas, A. *et al.* Electromechanical Conditioning of Adult Progenitor Cells Improves Recovery of Cardiac Function After Myocardial Infarction. *Stem Cell Translational Medicine*. **6** (3), 970-981 (2017).

31. Ronaldson-Bouchard, K. *et al.* Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature*. **556** (7700), 239-243 (2018).

32. Gelmi, A. *et al.* Direct Mechanical Stimulation of Stem Cells: A Beating Electromechanically Active Scaffold for Cardiac Tissue Engineering. *Advanced Healthcare Materials*. **5** (12), 1471-1480 (2016).

33. Poulin, A. *et al.* An ultra-fast mechanically active cell culture substrate. *Scientific Reports*. **8** (1), 9895 (2018).

34. Bayes-Genis, A. *et al.* Human progenitor cells derived from cardiac adipose tissue ameliorate myocardial infarction in rodents. *Journal of Molecular and Cellular Cardiology*. **49** (5), 771-780 (2010).

35. Bagó, J.R. *et al.* Bioluminescence imaging of cardiomyogenic and vascular differentiation of cardiac and subcutaneous adipose tissue-derived progenitor cells in fibrin patches in a myocardium infarct model. *International Journal of Cardiology*. **169**, 288-295 (2013).

660 36. Zimmermann, W.H. *et al.* Tissue engineering of a differentiated cardiac muscle construct.  
661 *Circulation Research*. **90** (2), 223-230 (2002).

662 37. Rosell Ferrer, F.X., Sánchez Terrones, B., Bragós Bardia, R., Bayés Genís, A., Lluçà Valldeperas,  
663 A. Methods and devices for mechanical and electrical stimulation of stem cell monolayer and 3d  
664 cultures for tissue engineering applications. WO/2013/185818. Barcelano, Spain (2013).

665 38. Bayés Genís, A., Lluçà Valldeperas, A., Soler Botija, C., Bragós Bardia, R., Rosell Ferrer, F.X.  
666 Method for Conditioning Stem Cells. WO/2017/125159. Barcelano, Spain (2017).

667 39. Roura, S., Gálvez-Montón, C., Bayes-Genis, A. Myocardial healing using cardiac fat. *Expert*  
668 *Review of Cardiovascular Therapy*. **16** (4), 305-311 (2018).

669 40. Zhang, Y.M., Hartzell, C., Narlow, M., Dudley Jr., S.C. Stem cell-derived cardiomyocytes  
670 demonstrate arrhythmic potential. *Circulation*. **106** (10), 1294-1299 (2002).

671 41. Liu, J., Fu, J.D., Siu, C.W., Li, R.A. Functional sarcoplasmic reticulum for calcium handling of  
672 human embryonic stem cell-derived cardiomyocytes: insights for driven maturation. *Stem Cells*.  
673 **25** (12), 3038-3044 (2007).

674 42. Wipff, P.J. *et al.* The covalent attachment of adhesion molecules to silicone membranes for  
675 cell stretching applications. *Biomaterials*. **30** (9), 1781-1789 (2009).

676 43. Kim, C. iPSC technology--Powerful hand for disease modeling and therapeutic screen.  
677 *Biochemistry and Molecular Biology Reports*. **48** (5), 256-265 (2015).

678 44. Ronaldson-Bouchard, K., Vunjak-Novakovic, G. Organs-on-a-Chip: A Fast Track for Engineered  
679 Human Tissues in Drug Development. *Cell Stem Cell*. **22** (3), 310-324 (2018).

680 45. Bruyneel, A.A., McKeithan, W.L., Feyen, D.A., Mercola, M. Will iPSC-cardiomyocytes  
681 revolutionize the discovery of drugs for heart disease? *Current Opinion in Pharmacology*. **42**, 55-  
682 61 (2018).

683 46. Farley, A., Johnstone, C., Hendry, C., McLafferty, E. Nervous system: part 1. *Nursing Standard*.  
684 **28** (31), 46-51 (2014).

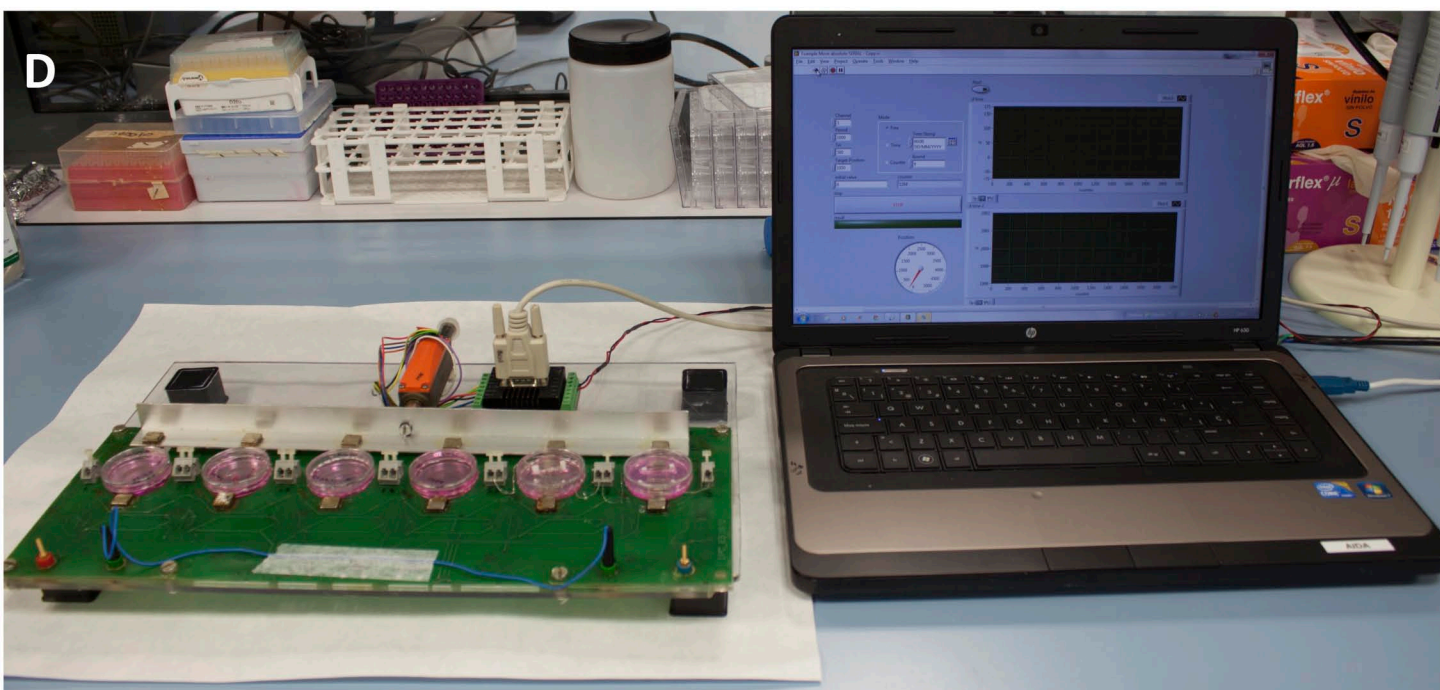
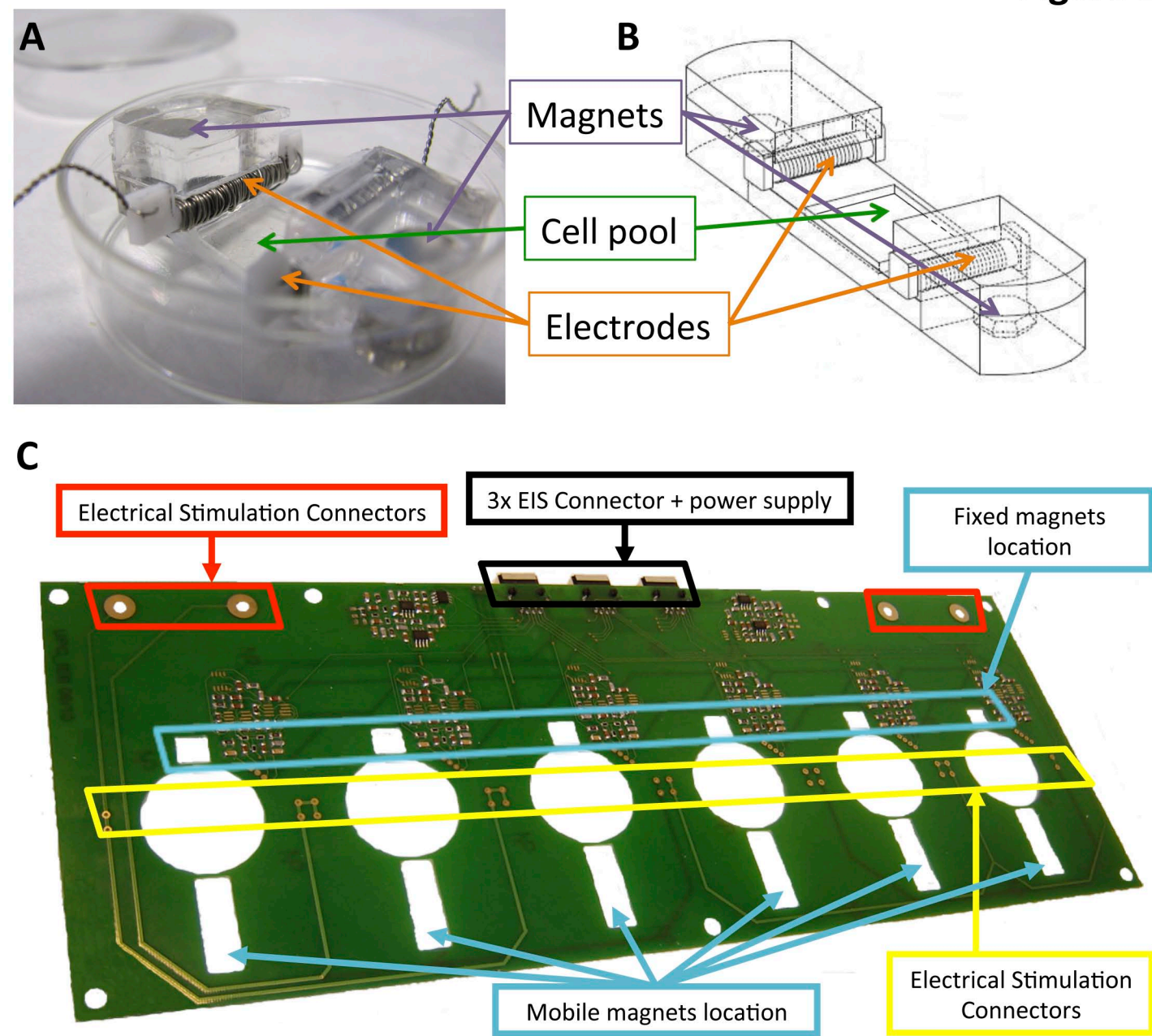
685 47. Brotto, M., Bonewald, L. Bone and muscle: Interactions beyond mechanical. *Bone*. **80**, 109-  
686 114 (2015).

687 48. Park, S.J. *et al.* Neurogenesis Is Induced by Electrical Stimulation of Human Mesenchymal  
688 Stem Cells Co-Cultured With Mature Neuronal Cells. *Macromolecular Bioscience*. **15** (11), 1586-  
689 1594 (2015).

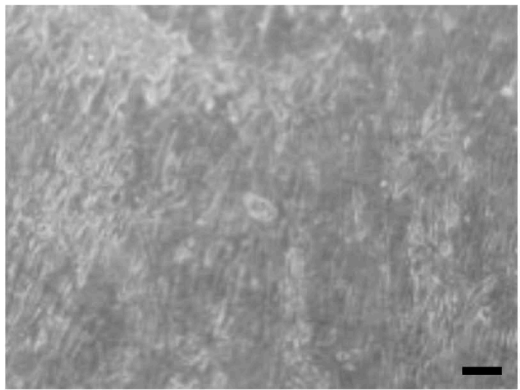
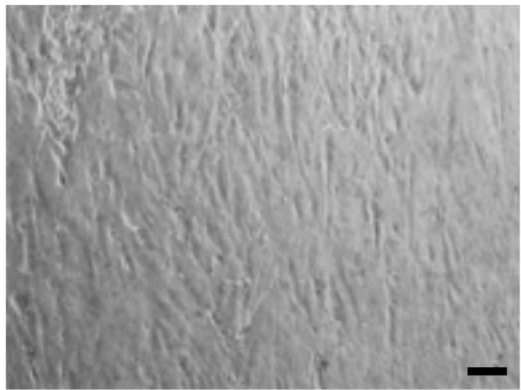
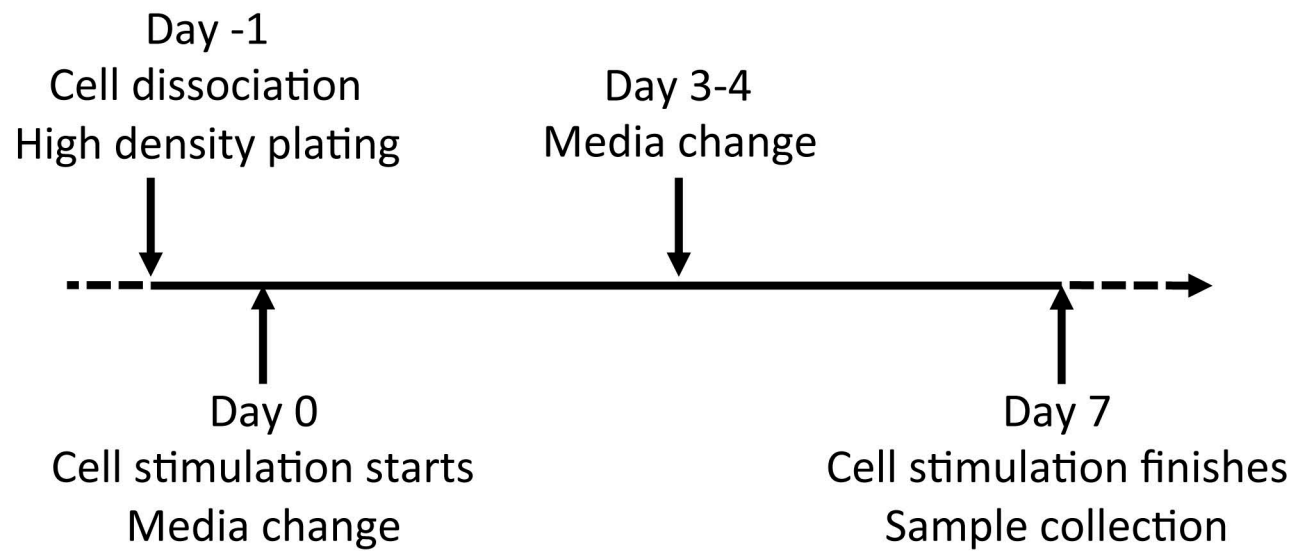
690 49. Vianney, J.M., Miller, D.A., Spitsbergen, J.M. Effects of acetylcholine and electrical stimulation  
691 on glial cell line-derived neurotrophic factor production in skeletal muscle cells. *Brain Research*.  
692 **1588**, 47-54 (2014).

693 50. Shima, A., Morimoto, Y., Sweeney, H.L., Takeuchi, S. Three-dimensional contractile muscle  
694 tissue consisting of human skeletal myocyte cell line. *Experimental Cell Research*. **370** (1), 168-  
695 173 (2018).

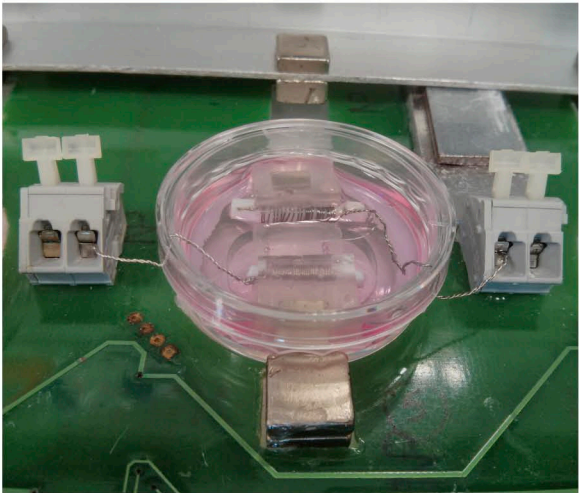




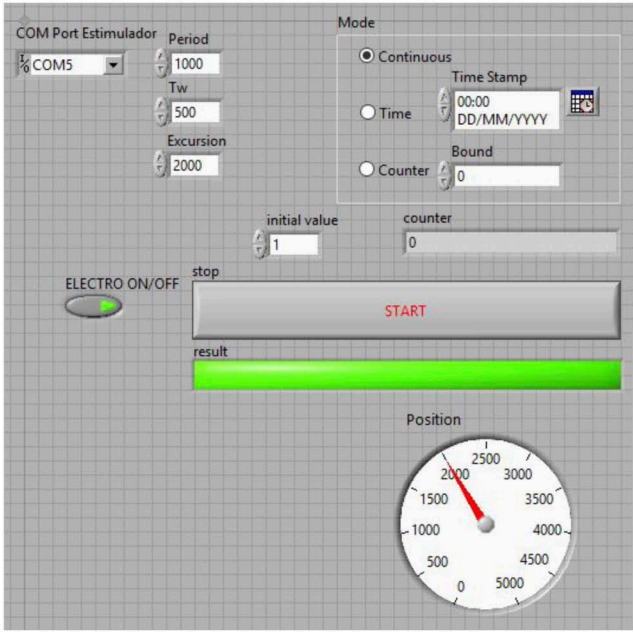
A Electromechanical stimulation schema



B Electromechanical stimulation unit

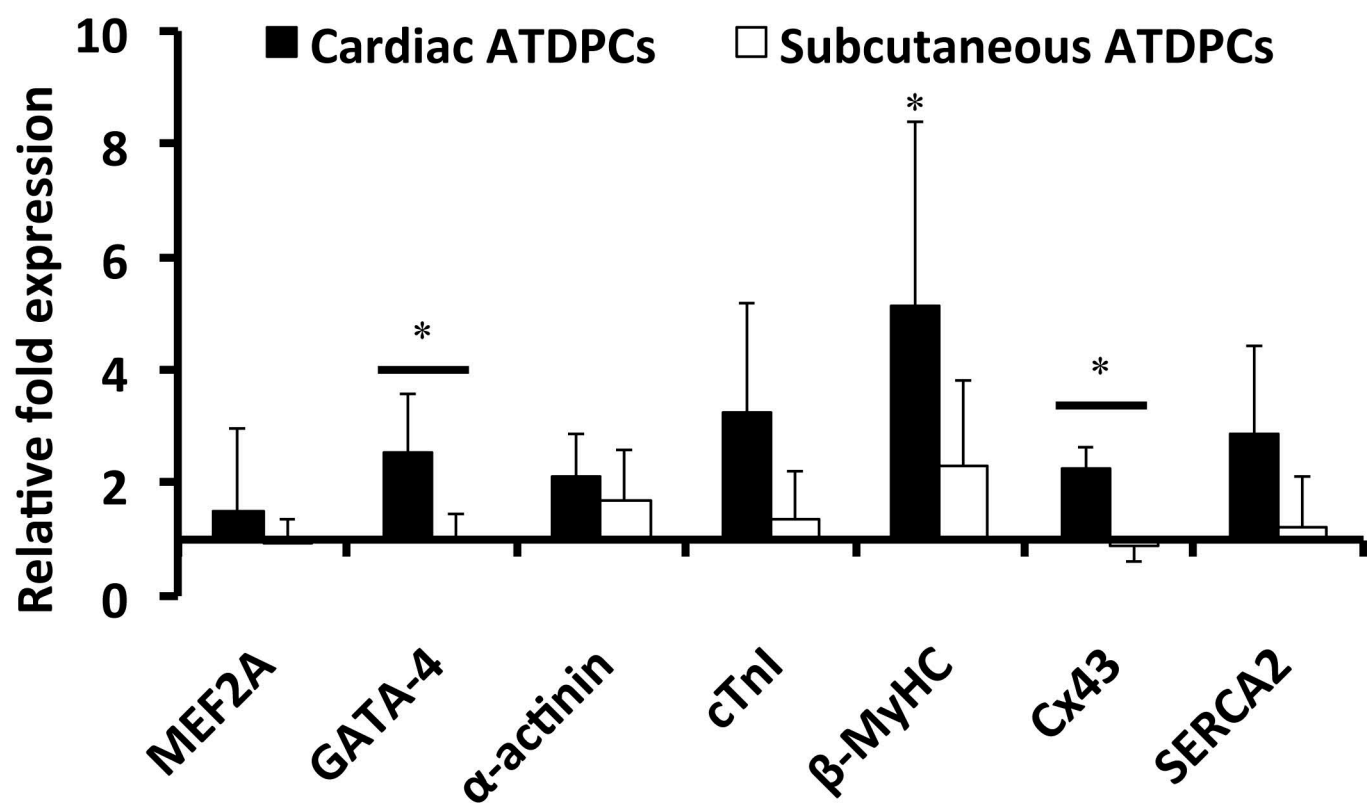


C Electromechanical stimulation user interface

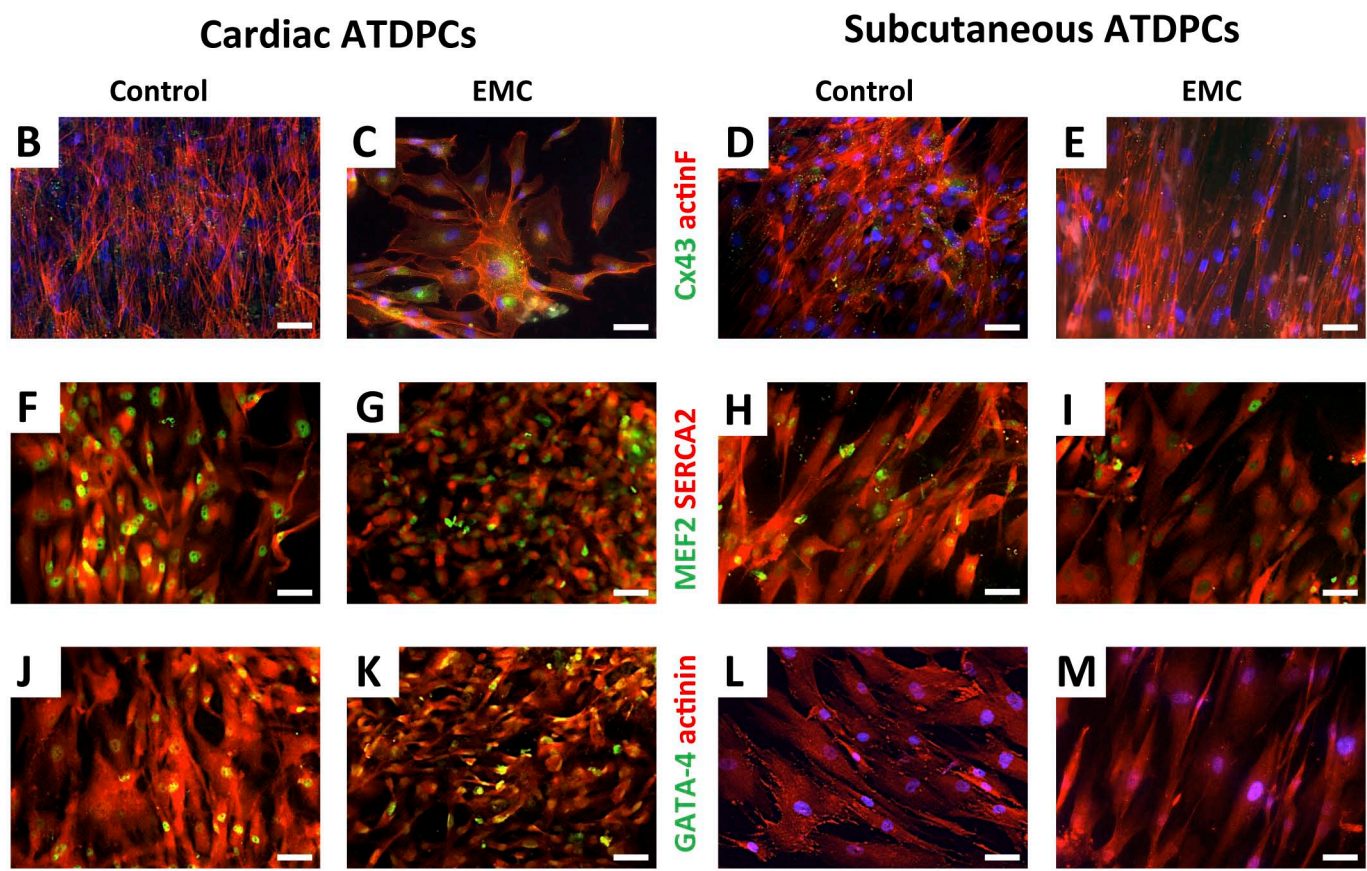




A Electromechanical stimulation effects at the gene level



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/Streptomycine, 10.000 U/mL	Gibco	15140-122
Minimum essential medium eagle (alfa-MEM)	Sigma	M4526-24x500ML
Protein & RNA analyses		
protease inhibitor cocktail	Sigma	P8340
QIAzol Lysis Reagent	Qiagen	79306
AllPrep 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 Albumina (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
Jackson ImmunoResearch	711-225-152
Jackson ImmunoResearch	705-225-147



## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

Cell electromechanical stimulation

Author(s):

Aida Llucià-Valldeperas, Ramon Bragós, Antoni Bayés-Genís.

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☒ Standard Access

☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

### ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: **"Agreement"** means this Article and Video License Agreement; **"Article"** means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; **"Author"** means the author who is a signatory to this Agreement; **"Collective Work"** means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; **"CRC License"** means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; **"Derivative Work"** means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; **"Institution"** means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; **"JoVE"** means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; **"Materials"** means the Article and / or the Video; **"Parties"** means the Author and JoVE; **"Video"** means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

## ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole



## ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to

the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

### CORRESPONDING AUTHOR

Name:

Aida Lluçia-Valdeperas

Department:

Cardiology (IGTP). Pulmonology and Physiology (Amsterdam UMC).


Institution:

ICREC Research Program (IGTP). Amsterdam UMC (Vrije Universiteit Amsterdam).

Title:

PhD

Signature:



Date:

11/08/2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140



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.**

ICREC Research Program, Health Science Research Institute Germans Trias i Pujol.  
Amsterdam UMC, Pulmonology and Physiology, Amsterdam Cardiovascular Sciences.  
E-mail: [llucia.valldeperas@gmail.com](mailto:llucia.valldeperas@gmail.com), [a.lluciavalldeperas@vumc.nl](mailto:a.lluciavalldeperas@vumc.nl).

**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.**

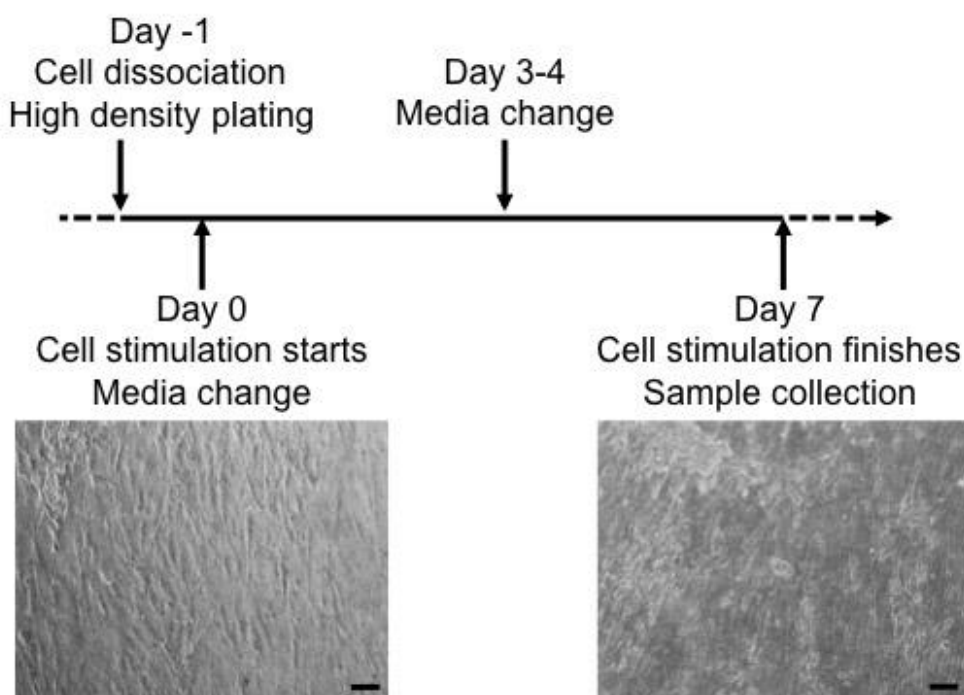
Clarification and more details were added to some steps.

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



**Figure 2**

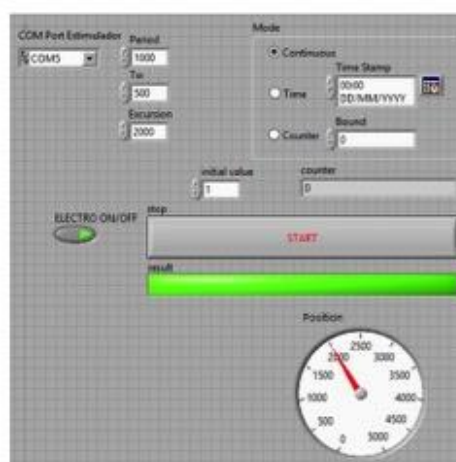
**A Electromechanical stimulation schema**



**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