# Effect of electrical stimulation on the contractile performance of engineered heart tissues (EHT)

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## Abstract

Three dimensional models like engineered heart tissues (EHT) have the advantage of resembling human physiology; in terms of cell structure, mechanical factors and contractile performance. However, they are made from human pluripotent stem cell derived cardiomyocytes (hPSC-CM), that exhibit an immature phenotype. By conditioning hPSC-CMs electromechanically, it is possible to observe improvements in contractile performance and expression of biological markers, found in mature CMs . This research focuses on the effect of continuous electrical stimulation on the contractile properties and the biological markers of the EHTs, and the pH of the cell culture media. Previous researches have shown that ramp stimulation of EHTs has the most effect on improvements in contractile performance and bio-marker expression. However, they lack the determination of the most optimum electrical conditions for the survival and maturation of the CMs, and the effect of continuous electrical stimulation on pH. A previously designed EHT platform, that allows electrical stimulation, is used to make EHTs in a 12 well plate format. A mathematical model and a simulation of the EHT platform were made to identify the well resistance and current distribution by the tissues and those results were compared with the experimental setup. As a result, there was high correlation between the model and the experiment, which indicates for a design of an accurate model. The most suitable electric field was determined to ensure cell survival throughout continuous stimulation of 30 days. The effect of continuous stimulation was then analysed in terms of pH, contractile performance and expression of biological markers- Connexin 43 (Cx-43) and Cardiac Troponin T (cTnT). Results present the change in these parameters and possible causes of change, in addition to continuous stimulation, were discussed.

## Introduction

Drugs have to go through several expensive and extensive validation during their development before they can be introduced to the market [1].These include preclinical trials, which involve two-dimensional (2D) *in vitro* cell cultures and animals [1]. However, these models do not represent the human body accurately; as 2D cell cultures are not able to outline the micro environment of living tissues completely and animals are physiologically different from humans [1]. This causes high failure rates during clinical studies.

In addition, the Dutch government is aiming to stop animal testing by 2025, which creates a need for advanced human-based models to evaluate the effect of drugs [2].

Three-dimensional (3D) *in vitro* tissues have been developed in order to represent physiological conditions of the human body [1]. They have the advantage to display improved cell morphology and structural complexity [3].

Engineered heart tissue (EHT) is one of the in vitro 3D models that have shown promising results for cardiac drug screenings [4]. They are made from human pluripotent stem cells derived cardiomyocytes (hPSC-CM), which allows for the creation of tissues that resemble human physiology [4]. Despite of its advantage to resemble human physiology, the behavior of hPSC-CMs is restricted to an immature phenotype, that is similar to the behavior of a fetal heart [5]. This greatly limits the disease modelling on these tissues, which requires a development of a method to induce maturation of hPSC-CMs [6].

Electromechanical conditioning is one of the methods used for maturation of hPSC-CMs. It has shown the advantage to induce maturation, which is recognized by improvement of contractile properties and expression of markers that are found in mature CMs, such as Connexin 43 (Cx-43) and Cardiac Troponin T (cTnT) for cell development and sarcomere organization [5] [7] [8] [9]. To have the highest impact on EHTs, the most effective method of electromechanical conditioning has to be determined.

Previous researches have shown that electrical ramp stimulation of EHTs is the most effective method for conditioning [7] [10]. Nevertheless, the most suitable electrical conditions for the maturation and survival of EHTs, have not been determined yet.

This paper focuses in analysing the effect of continuous electrical stimulation on the contractile performance of EHTs by using a versatile platform that allows electrical stimulation. Here we analysed the effect of two different protocols in the contractile output and the tissue formation after 30 days of continuous stimulation.

# Materials and methods

## EHT platform

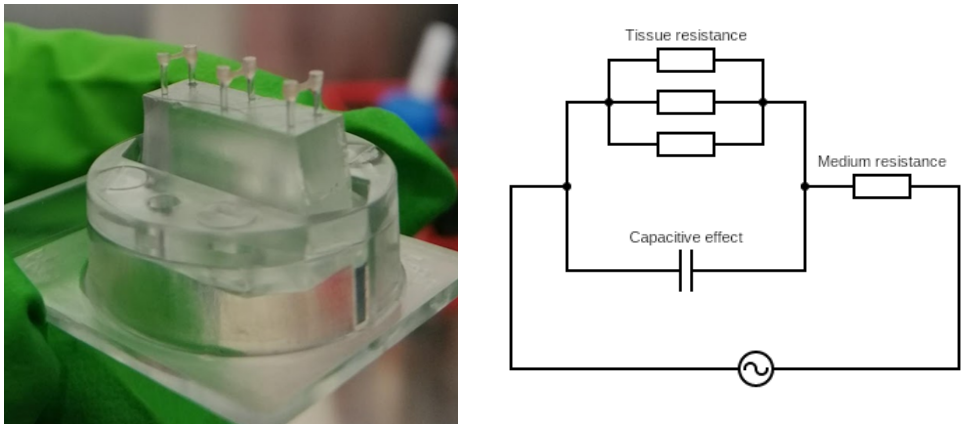
A previously designed EHT platform for a 12 well plate was used to make and electrically stimulate EHTs [11]. The platform consists of 12 holders made from polymethylmethacrylate (PMMA) that fit into each well of the well-plate, and thermoplastic elastomer (TPE) pieces with three sets of pillars. For each set of pillars an EHT can be made, which means 3 EHTs per well. The distance between the pillars within a set is 3mm; and each pillar has a length of 3mm and a diameter of 0.5mm [11].

For electrical stimulation of the tissues; two holes are present on the holders, where carbon electrodes were placed perpendicular to the tissues with a distance of 15.5mm [11]. They were chosen to be used for stimulation as they are highly resistant to chemical reactions and corrosion compared to other types of electrodes, such as platinum [12]. One holder of the platform, with 3 parallel EHTs anchored to the pillars, is presented in Fig. 1a.

## Electrical modelling of wells

### Calculation of electrical parameters

To analyse the current distribution and to determine the tissue resistance within the well, electrical modelling of the EHT platform was done [11]. Initially, to simplify the modelling of the platform, one well was analysed as a circuit, which is presented in Fig 1b.




**(a)** **(b)**

**Fig 1.** Modelling of EHT platform based on one well. a) One holder of the EHT platform with 3 parallel EHTs, b) Representation of one well of the EHT platform as an electrical circuit.

The circuit was designed as an adaptation from Tandon et al. [12]: There were three parallel resistors to represent each tissue in the platform. In parallel to that, a capacitor was added to represent the effect of ion movement and the cell culture media in between the parallel electrodes. Then, a resistor in series with the capacitor and tissue resistances was included to symbolize the resistance of the cell culture media. Finally, an alternating current (AC) source was connected to the system to show the input from the electrodes. In order to calculate the corresponding values for these parameters, the electrodes, tissues and the cell culture media were classified as conductive, and the pillars of the holders as non-conductive [12].

With this classification, it was possible to encompass different types of conductive and non-conductive materials in the calculation. Therefore, there were few assumptions made: The material of the electrode was not included in the calculation, the electrodes were assumed to be covering the whole side of the tissues and the TPE pillars were assumed to have no effect on the distribution of the currents, as the tissue is covering the pillars.

In order to calculate the tissue resistance, that is the resistance per tissue, the tissue dimensions were taken into consideration. The length of the tissue is the same as the distance between pillars (3mm) and the width is the diameter of the pillars (0.5mm). The height of the tissues was taken from the data previously published (0.3mm) [ [11]].

Additionally, the resistivity of human heart was found to be $175\Omega cm$ [13]. The resistance of tissues were then calculated using the length (L), cross sectional area (A) and resistivity ($\rho$).

$$R = \rho \frac{L}{A} \tag{1}$$

The cell culture media resistance was also calculated using Eq.1, but instead of resistance, the conductivity of the media ($\sigma = 1.5 \frac{S}{m}$ [14]) was used, which is the reciprocal of the resistivity. The length of the media is the same as the distance between the electrodes (15.5mm) as the electric field, therefore the capacitance and resistance effects, is only present in between the electrodes. The cross-sectional area was calculated as the multiplication of the diameter of the well (22.3mm) and the height of the media (5.1mm), since the flow of current in between the electrodes is perpendicular to this

area [11]. The height was calculated using the volume of the media (2mL) that was kept constant throughout the experiments.

Lastly, the capacitance of the cell culture media was calculated using the dielectric permittivity of the media ($\epsilon_r = 80.1$ [14]), the vacuum permittivity ($\epsilon_0$), the distance between the electrodes ($d = 15.5mm$) and the surface area of the electrode that is in contact with the cell culture media (A).

$$C = \frac{\epsilon_r \epsilon_0 A}{d} \tag{2}$$

### Simulations and measurements

After calculations, a simulation of the circuit in LTSpice XVII (x64) (17.0.32.0) was created in order to simulate the current and the well resistance. There were two simulations done in order to analyse this behavior: Using the parameters calculated before the simulation to determine the current and using the current values determined by a custom-made pacing device during the stimulation of the EHTs per well while being stimulated with different input voltages.

To compare the simulation results with an experiment, a setup to determine the well resistance and the current was used (Fig. 2). A resistance of 150Ω was connected in series to a well of a 12 well plate and DC voltage was applied to the system [12]. In order to determine the current and well resistance of the setup; first, the voltage across the 150Ω resistor and across the well were measured with a digital multimeter. Then, the current across 150Ω resistor was calculated. By using the calculated current, the well resistance was determined [12].

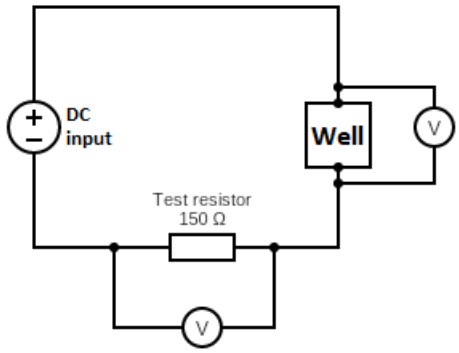



**Fig 2.** Circuit design for current and well resistance measurements

## Fabrication and maintenance of EHTs

Three different batches of cells were used to make EHTs for the experiment. The EHTs were made using hPSC-CMs from WTC Line (GM25256*G0002) [15], and 3% human adult cardiac fibroblasts (HCF)-obtained from Promocell (C-12375) [16]. Initially, the CMs were mixed with HCF and the amount of cells were counted to a final amount of 250,000 cells per tissue [11]. Then, the cell mixture was transferred to the 12 well plate with the EHT platform, and was kept in the incubator with 2mL serum free maturation media (MM) at 37°C and 5% $CO_2$ [11]. After this, 1mL of the cell culture media was refreshed daily.

## Electrical stimulation of EHTs

### Determination of Excitation Threshold

To determine the excitation threshold (ET), the tissues were stimulated, by Zhao et al., with 2ms, 1Hz, monophasic square pulses. A range of electric fields were applied, starting from $1\frac{V}{cm}$ and increasing with steps of 0.1 $\frac{V}{cm}$. The ET was then determined to be the electric field at which the tissues beat synchronously [7].

For the 12 well plate EHT platform; the stimulation was done separately for each well, using a custom-made device that generates 10ms, 2Hz, biphasic square pulses. A range of electric fields starting from 3.23 $\frac{V}{cm}$ up to $32.23\frac{V}{cm}$ was applied with increments of $3.23\frac{V}{cm}$. The electric field at which the tissue starts beating with a frequency of 2Hz, that is the frequency of the input, was determined to be the ET.

### Determination of optimum electric field

To determine the most optimal electric field for continuous stimulation, the average of the excitation threshold, within the EHT platform, was determined. To be able to make all the tissues follow the frequency of the input signal, twice the ET was also taken into consideration [7]. In addition, based on the recommendation of Tandon et al., a field that was lower than 8 $\frac{V}{cm}$ was used for stimulation. Overall there were three pre-determined magnitudes of electric fields respectively: $10.32\frac{V}{cm}$, $20.64\frac{V}{cm}$ and $3.23\frac{V}{cm}$. The tissues were continuously stimulated with 10ms, 2Hz, monophasic square pulses, using Multi Channel Systems STG 4008 stimulus generator [17].

### Experimental setup

The experiment was conducted on a 12-well plate with three tissues per well [11]. Four conditions were defined as : No stimulation, electrical stimulation every 5 days (5-day stimulation) , continuous stimulation with increase of frequency from 2Hz to 6Hz with steps of 1Hz/week (ramp stimulation with 1Hz/week) [7] and continuous stimulation with increase of frequency from 2Hz to 6Hz with steps of 0.33Hz/day (ramp stimulation with 0.33Hz/day) [10]. This resulted in three wells (9 tissues) with three different batches per condition. The 5-day stimulation was started on day 5 and the stimulation was done for a period of 5 minutes, using the custom-made device that generates 10ms, 2Hz, biphasic square pulses. The magnitude of the stimulation was at the ET of the well, which changed throughout the experiment. The continuous stimulations were started on day 4 of the experiment, with 10ms, monophasic square pulses with a pre-determined electric field of $3.23\frac{V}{cm}$, using Multi Channel Systems STG 4008 stimulus generator [17]. The ramp stimulation with 1Hz/week was adapted to 1Hz per 5-days, so that it can be done in 30 days and the duration of the ramp stimulation with 0.33Hz/day was extended to 30 days by keeping the frequency at 2Hz after 6Hz was reached.

The planning of the experiment is presented, as a timeline, in Fig. 3.

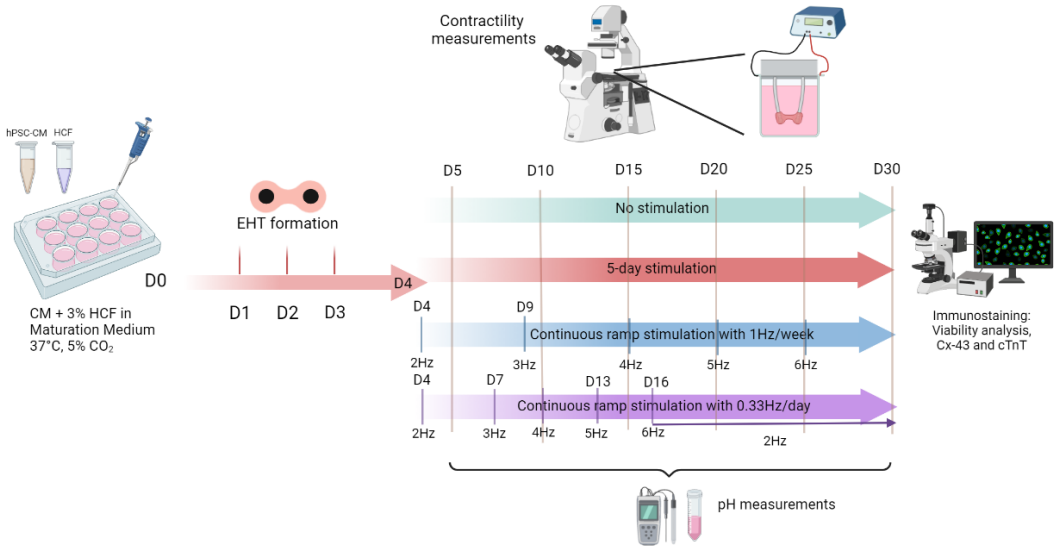



**Fig 3.** Timeline of continuous stimulation experiment. The EHTs were made up of hPSC-CM and HCF and they were kept at $37°C$, with 5% $CO_2$ in maturation medium. The EHT formation took 3 days (D1-D3). The four conditions of the experiment are presented as four arrows: No stimulation (cyan), 5-day stimulation (red), continuous ramp stimulation with 1Hz/week (blue) and continuous ramp stimulation with 0.33Hz/day. Continuous stimulations were started on day 4 (D4). The contractility measurements were done every 5 days (D5, D10, D15, D20, D25, D30) and the frequency change of the continuous stimulation is presented as a timeline for each condition. The pH was measured daily, for each condition, by collecting the media in a 50mL tube. After day 30 (D30), immunostaining was done for viability, Connexin-43(Cx-43) and Cardiac Troponin T (cTnT). hPSC-CM = human pluripotent stem cell derived cardiomyocytes, HCF = Human adult cardiac fibroblast, EHT = Engineered heart tissue, D = day, Cx-43 = Connexin-43 and cTnT = Cardiac Troponin T. Created with Biorender.com.

### Analysis of electrical and contractile performance

To analyse the contractile performance; the force generated by the tissues, spontaneous frequency, contraction and relaxation velocity, and the ET, were measured every 5 days. At least three hours before the measurements, the continuously stimulated tissues were disconnected from electrical stimulation and the cell culture media was refreshed. The measurements were done using a custom-made pacer generating 10ms, 2Hz, biphasic square pulses with a voltage range from 5 to 50 volts. During the measurements, the 12 well plate was preserved at $37°C$ in 5% $CO_2$ [16]. In order to measure contractile properties, first, the excitation threshold was determined per tissue by increasing the input voltage from 5 to 50 volts, with increments of 5. Then, the tissues were continued to be stimulated at their ET. During this stimulation, 5-second videos of the tissues were taken by Nikon Ti2-E inverted microscope with a Prime BSI high-speed camera (Photometrics) at 100 fps with 2X magnification. Using the videos, the rest of the contractile properties were analysed for each condition with a custom-made stand-alone application. [16]. Furthermore, by using ET values from the measurements, the average current and resistance per well were calculated.

## pH measurements

To track the effect of continuous electrical stimulation on the pH of the media, the pH of each condition was measured daily, using Mettler Toledo FiveEasy Plus (FEP20) pH benchtop meter [18]. The pH values were then compared with the pH of the input media and the control to detect any changes.

In addition, a fresh media with a pH of 7.4 was prepared to track the effect of environmental conditions on the pH of the media. For this experiment, the media was stored in the fridge for 1 hour, in the incubator for 1 and 2 hours, outside in the LAF hood for 1 hour and in the freezer overnight. The pH for each condition was then measured with Mettler Toledo FiveEasy Plus (FEP20) pH benchtop meter [18].

## Immunostaining

To observe the effect of electrical stimulation on the survival and the maturation of the EHTs, the cell viability and the expression of Connexin-43 (Cx-43) and cardiac Troponin T (cTnT) were analysed respectively, at the end of the experiment (day 30) [8].

The viability analysis was done by using LIVE/DEAD$^{TM}$ Viability/Cytotoxicity Kit, for mammalian cells, based on the protocol given by ThermoFisher [19]. The kit uses calcein AM for the detection of alive cells and ethidium homodimer for dead cells [19].

To analyse the expression of Cx-43 and cTnT, the tissues were initially isolated from the cell culture media, and washed with PBS+ (phosphate buffered saline (PBS) with magnesium and calcium). Then, they were fixated with 4% Paraformaldehyde (PFA) for one hour. After this, they were transferred into 96 wells plate, and washed with permeabilization buffer (0.3% Triton-X-100 in PBS+) for 3 times with 20 minute intervals. Before adding the primary antibodies, the tissues were kept in blocking buffer (3% bovine serum albumin (BSA), 0.3% Triton-X-100,0.1% Tween in PBS+) for 7 hours at room temperature.

The primary antibodies for the staining were Cx-43 (1:200, Sigma, C6219) rabbit [20] and cTnT (1:250, ThermoFisher, MA512960) mouse [21]. A solution with the antibodies was prepared in antibody buffer (0.1% BSA, 0.3% Triton-X-100,0.1% Tween in PBS+). After the addition of the primary antibody solution, they were kept at $4°C$ for two days.

After two days, the secondary antibody mix was made with antibody buffer, consisting of Alexa Fluor 488 chicken anti rabbit (1:500, Molecular probes, A21411) [22], Alexa Fluor 647 donkey anti mouse (1:500, ThermoFisher, A31571) [23] and DAPI (1:4000, ThermoFisher, D1306) [24]. Before the addition of the mix, the tissues were washed with permeabilization buffer (3 times, 20 minute intervals). After the addition of the mix, they were kept in dark and stored at $4°C$.

Before imaging, they were washed with PBS (3 times, 20 minute intervals) and transferred to a glass slide. The imaging was done with Zeiss LSM 880 confocal microscope [25].

# Results

## Well resistance and current analysis

### Calculations

To model the EHT platform as a circuit (Fig. 1b), the values for the electrical components were calculated based on the dimensions of the EHT platform. There were few assumptions made before the calculation, which allowed for a more comprehensive realization that could be applied to all types of experiments, especially about the materials

of electrodes and pillars. Moreover, the assumption of the size of the electrode allowed for a more ideal calculation.

Using Eq.1, the resistance of the tissues and the resistance of the cell culture media were calculated to be $35k\Omega$ and $90.85\Omega$.

After the calculation of the contact area of the electrode from media height (5.1mm) and from the radius of the electrode (1mm); the capacitance, using Eq. 2, was calculated to be 1.6pF.

In addition, to compare the calculated value with the measurements, the tissue resistance was varied in the simulation and the current values were matched with the custom-made pacing device accordingly, as it was used throughout the measurements. Using this method, the tissue resistance was determined to be $1.1k\Omega$.

### Simulation and measurements

The results of simulation and measurements were calculated from voltages across the $150\Omega$ resistor and the well. The reason for measuring voltages was to have a parallel connection to the system instead of a series connection of the ammeter, which simplifies the measurement procedure (Fig. 2). The system was tested with different input voltages, with and without tissues. The range of input for the tests with media was from 5 to 30V with increments of 5 and with tissues it was from 5 to 15V, as there is a possibility of harming the tissues at higher voltages. As a result, the maximum current for the measurements with tissues is less than the measurements with media and simulation.

For simulation results, it was observed that the well resistance had a constant value of approximately $457\Omega$, whereas in the measurements with DC voltage, there was an exponential decrease of well resistance with increasing voltage. For the case with only cell culture media, the resistance was approaching to a value close to $280\Omega$ and for the case with 3 tissues per well, it was approaching to $390\Omega$. Furthermore, the results from the continuous stimulation experiment showed that the well resistance was ranging between 200 and 600 $\Omega$. The well resistance plots of LTSpice simulation and measurements are presented in Fig. 4, and the current plots are presented in Fig. 5.

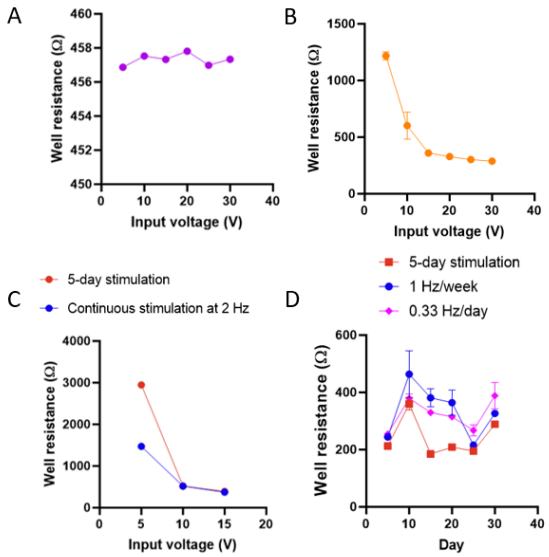



**Fig 4.** Simulation and measurement results of well resistance: A) LTSpice simulation of well resistance, B) DC voltage well resistance measurements with only media, C) DC voltage well resistance measurements with tissues (red: 5-day stimulation, blue: continuous stimulation at 2Hz), D) Well resistance measurement every 5 days from day 5 until day 30 (red: 5-day stimulation, blue: ramp stimulation at 1Hz/week, purple: ramp stimulation at 0.33Hz/day).Data presented as mean ± s.e.m.

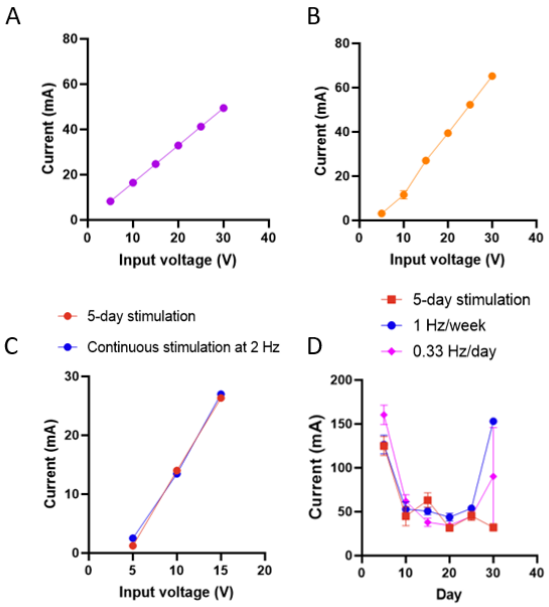



**Fig 5.** Simulation and measurement results of current: A)LTSpice simulation of current, B) DC voltage current measurements with only media, C) DC voltage current measurements with tissues (red: 5-day stimulation, blue: continuous stimulation at 2Hz) , D) Current measurement every 5 days from day 5 until day 30 (red: 5-day stimulation, blue: ramp stimulation at 1Hz/week, purple: ramp stimulation at 0.33Hz/day) Data presented as mean ± s.e.m.

## Electrical stimulation

Continuous electrical stimulation of EHTs induces maturation of hPSC-CMs, which results in more improved contractile properties [8]. To determine the effect of electrical stimulation on EHTs, the most suitable electric field had to be found. Zhao et al. suggests that by an electrical conditioning, that is double the ET, it is possible to make most of the tissues follow the frequency of the input [7]. The ET that was able to make all the tissues follow in the EHT platform, was determined to be $10.32\frac{V}{cm}$. The tissues were then continuously stimulated with twice the ET ($20.64\frac{V}{cm}$), according to Zhao et al., and the ET ($10.32\frac{V}{cm}$). After approximately 24 hours, the cell culture media was oxidized and the tissues were not able to survive for both cases. This indicated that the electric field was too high for the tissues.

Since, the continuous stimulation with ET and twice the ET showed that the values are significantly high for survival, a lower electric field was required for stimulation. Tandon et al. suggests that for continuous electrical stimulation, the electric field has to be kept lower than $8\frac{V}{cm}$ to ensure cell survival [12]. That's why , an electric field with a magnitude of $3.23\frac{V}{cm}$ was chosen to be experimented on the tissues. The results showed that the tissues were able to survive at this electric field. As a result, $3.23\frac{V}{cm}$ was used throughout the rest of the experiments. Additionally, the tissues were stimulated with 10ms, 2Hz monophasic waves as monophasic waves are more compatible with carbon electrodes and the possibility of interfering with the initiation of action potential is less compared to biphasic waves [12].

To observe the effect of continuous stimulation on EHTs, the most effective methods presented in literature were chosen (Table S1 and Table S2) . There were four criteria for choosing the best method of stimulation: Positive force-frequency relationship (FFR), post-rest potentiation (PRP), increased contractility and detailed description of the method. FFR is the response of the tissue with increasing frequency and PRP is the force generated by the tissue at its first beat, when the stimulation is initiated [26]. It is expected that the force of the tissue will increase with increasing frequency and the PRP force will increase compared to the control case with no stimulation, as the tissue gets more mature [7]. In addition, increase in contractility performance also indicates maturation [7]. Based on these criteria, two stimulation methods were chosen as the best and second best method: Continuous electrical stimulation with weekly increase of frequency from 2 to 6Hz with steps of 1Hz [7] and with daily increase from 2 to 6Hz with increments of 0.33Hz [10].

The results of contractility measurements present the relative change of day 20 according to day 5. Day 20 was chosen to be the final day of the experiment, as after day 20, a significant decrease in tissue performance was observed. The results showed that the spontaneous frequency was decreasing for all of the conditions. For 5-day stimulation and for ramp stimulation with 1Hz/week, the force generated while stimulation increased; being the tissues with ramp stimulation the ones with a higher contractile force. The contraction velocity increased for all of the electrically stimulated conditions and the relaxation velocity also increased for all cases except for ramp stimulation with 0.33Hz/day. In addition, the ET decreased for all of the conditions. In particular, a more pronounced decrease in the ET was observed in the condition of 5-day stimulation. The plots of the contractility measurements are presented in Fig. 6.

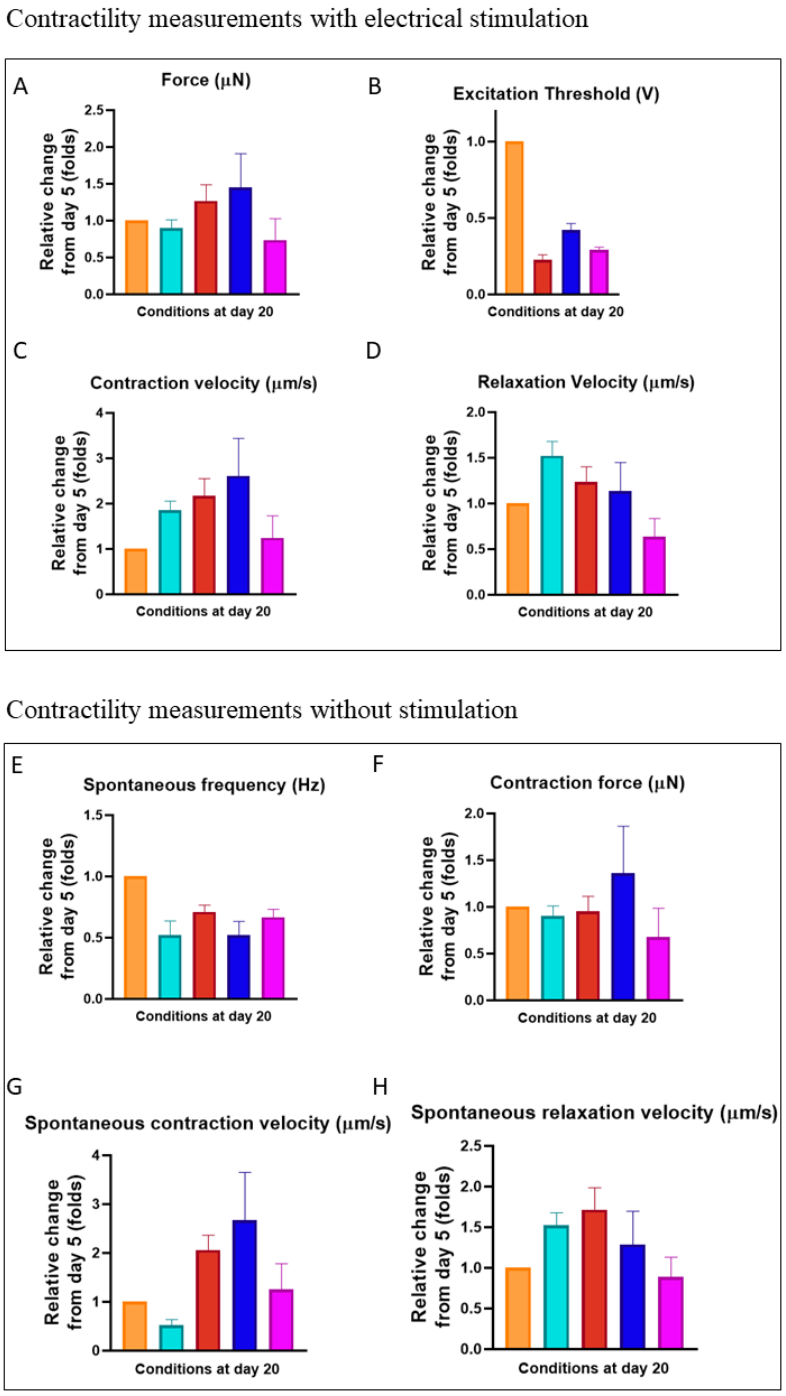



**Fig 6.** Continuous electrical stimulation results at day 5 and at day 20 of stimulation (orange: day 5, cyan: control, red: 5-day stimulation, blue: 1Hz/week, purple: 0.33Hz/day). A)Force of contraction during stimulation, B) Excitation threshold, C)Contraction and D) Relaxation velocity during stimulation, E)Spontaneous frequency, F)Contraction force with no stimulation, G)Contraction and H)Relaxation velocity with no stimulation. The data is presented as two way ANOVA plus Tukey's test. The results are presented as the relative change of day 20 according to day 5 of each condition, which have similar values. Day 5 is normalized to 1 for simplicity. Values are expressed as mean ± SEM : $* = p < 0.01$; $** = p < 0.001$ and $*** = p < 0.0001$.

## pH measurements

Throughout the electrical stimulation experiment, the pH of each condition was measured daily. The results from these measurements showed that the pH was approximately 8.5 for all conditions (Fig. S3), which was also the pH of the cell culture media used for daily media refreshments.

Additionally, the experiment on the pH change of the fresh cell culture media showed an increase of pH for all of the environmental conditions. The most increase was observed when the media was stored in the freezer overnight. The pH values for each environmental condition is presented in Table S3.

## Immunostaining

To determine the effect of continuous electrical stimulation on the viability and the expression of bio-markers, immunostainings were performed at day 30 of the experiment. Initially, a viability assay was done to determine the survival of cells, based on the amount of alive (green) and dead (red) cells per condition. As expected, the control condition had high amount of alive cells, whereas the continuously stimulated conditions had more dead cells than alive cells. The 5-day stimulation condition had less alive cells than the control but more than the continuously stimulated conditions.

Then, the expression of Cx-43 (green) and cTnT (red) were analysed. For all of the conditions, the Cx-43 was not observed clearly. On the other hand, cardiac troponin showed an alignment on the force axis in the control and 5-day stimulation conditions. However,in the continuous stimulation conditions, no organization of sarcomeres was observed. The results of immunostaining are presented in Fig. 7.

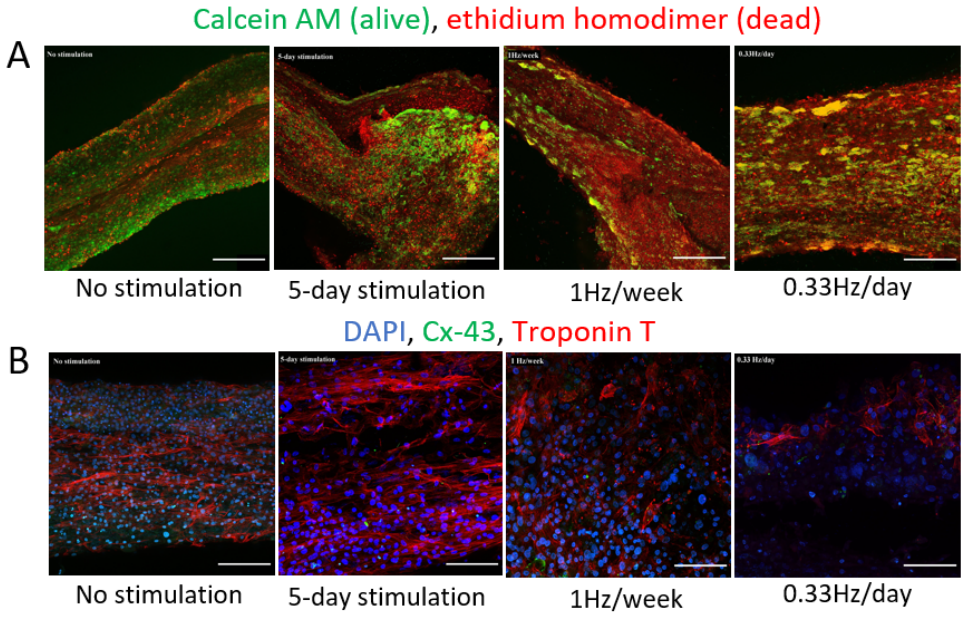



**Fig 7.** Immunostaining results at day 30 of the experiment. A)Viability analysis with calcein AM (alive) and ethidium homodimer (dead) and B) Connexin-43 (Cx-43) (green) and Troponin T (cTnT) (red) confocal microscope images for all conditions. DAPI (blue) is used to identify the nuclei of the cells. The images are presented with a 2mm scale bar.

# Discussion

An electrical model of one well of the EHT platform was done to determine the tissue resistance. The tissue resistance was calculated using the dimensions of the tissues (Eq. 1) and the model was simulated in LTSpice. The results showed that there was significant difference between the resistance values from calculations and simulation. The difference between the tissue resistances could be because of the resistivity value used in the calculation. That value was determined, by Faes et al., as an average of resistivities from various *in vivo* and *in vitro* mammal heart tissues, which are mature [13]. During maturation, the sarcomere organization develops, which changes the mechanical stress and the resistivity of the tissue [27] [28]. This organization cannot be observed in hPSC-CMs at the beginning of the experiment, as they exhibit immature phenotype, which results in a different resistivity compared mature phenotypes. In addition, there are physiological differences in mammals, so the resistivities used for approximation do not reflect human tissues completely.

Since, the current values from the pacing device were obtained by the stimulation of the EHTs, the calculation of resistance per well from the currents determined by the device were more valid to the measurements, than using the resistivity value of the heart muscle. From the simulation results, it was possible to calculate the resistivity of the EHT. Using Eq.1, the resistivity of the tissue was calculated to be $5.5\Omega cm$, which is 3.14% of the resistivity of the heart [13].

All in all, the first approach for the modelling of the resistivity of 3D cardiac tissues on a 12 well plate EHT platform was done, however, additional analysis of resistivity is needed to achieve a more comprehensive result.

Unlike the other conditions, current measurements at every 5 days (Fig. 5D) showed an exponential decrease of current until day 20 and then the current started increasing. This is because, the value of current was calculated based on the ET at each time point, which decreased over time with electrical stimulation until day 20 (Fig.S1B). After day 20, it was observed that the tissues with continuous stimulation had a decrease in the contractile performance, which resulted in weaker contractions and higher ETs for stimulation. Therefore, the current started to increase with increasing ET. The decrease in current was still observed for the case of 5-day stimulation, which could indicate that there was less decrease in contractile performance and less tissue death in this condition compared to continuous stimulation conditions. This was also observed with viability analysis( Fig. 7A).

There was an exponential decrease of well resistance for the measurements with DC voltage (Fig. 4), since the cell culture media is a polar liquid with many ions, such as Potassium, Calcium and Magnesium [29]. Due to the electrical input from the electrodes, an ion flow is generated which acts similar to an electrolysis reaction. As a result, with increasing voltage, the flow of ions (current) increases, which increases the electrolysis [30]. Increase in electrolysis then results in a decrease of ions at the center of the media as they accumulate more on the surroundings of the electrodes, which causes a decrease in the resistance of the media. This decrease was not observed in the simulation as the effect of electrolysis was not considered in the model.

The well resistance of the 5-day measurements changed based on the conditions of cells over time (dead or alive) and the volume of media inside the well-that is ideally 2mL but can have minor variations. However, it was still observed that for continuous stimulation, the well resistance had a value that was approximately $450\Omega$ from day 10 to day 20, which was close to the value determined by the simulation ($457\Omega$).

The effect of continuous electrical stimulation on contractile properties of EHTs was analysed. The results show that ramp stimulation with 1Hz/week had the most improvement in contractile performance, followed by 5-day stimulation (Fig. 6). There was immediate improvement observed, especially for force of contraction, contraction velocity and relaxation velocity, at day 10 - that is presented in Fig. S1. After day 10, the performance of tissues decreased, and they started to die after day 20. That's why, the contractile properties started to behave unexpectedly.

In addition to the changes in the contractile performance over time, there were large deviations within the same conditions for each time point (Fig. 6). This was because of using different batches of cells, which resulted in variations within wells. The variations are shown in Fig. S2 for different conditions at day 20. There was also a variability within a well, which could be because of the surface area of the electrodes.

The carbon electrodes had cylindrical shape with a diameter of $2mm$, which did not cover the whole side of the tissues. This suggests a non-uniform electric field, since to have a uniform electric field, the sides of the stimulated area has to be covered completely with the electrodes [31]. The non-uniformity of the electric field could decrease the effect of conditioning, as the maximum electric field do not cover the well, and cause variation. To have uniform electric field, the surface area of the electrodes could be increased; which could increase the effect of electrical conditioning on all of the tissues and decrease variability. A pilot experiment was conducted to observe the effect of change of the surface area of the electrode.

Additionally, for the control, the contraction and relaxation velocities were improved even though there was no electrical stimulation (Fig. 6C and Fig. 6D) . This could be because of daily media replacements. MM can induce improvements in contractile properties [11] and with daily replacement of media, there was continuous supplementation of nutrients to the tissues.

The cell culture media, used for refreshments, and the electrically stimulated conditions had similar pH values. This suggests that the electrical stimulation has no significant effect on the pH. However, the pH was too high compared to the optimal pH for mammalian cells, that is approximately 7.4 [32]. In spite of an higher pH, the cells were still able to survive but the pH effect on cellular performance is not known. To analyse the effect on performance, an experiment is suggested.

In addition, as the cell culture media used for refreshments also had a pH higher than the optimal value at the time when the media was replaced, the environmental conditions affecting the pH of the media had to be tested.

For all of the cases, pH was increased and storage in the freezer overnight had the most effect on the increase, which increased the pH to 8.28 (Table S3). This suggests that in order to have the optimal pH, the cell culture media has to be set to 7.4, just before the media refreshments.

The viability analysis, done at day 30, showed that with continuous stimulation; there was more tissue death compared to no stimulation and 5-day stimulation. During the experiment, it was observed after 20 days that the tissue performance, especially for continuous stimulation, was decreasing and cell death occurred, which can also be observed from the microscope images (Fig. 7). This suggests that, continuous electrical stimulation could have detrimental effects after 20 days of stimulation. This was not observed in other papers, where the continuous stimulation was continued for at least 30 days [7]. The reason for this could be due to the composition of the cell culture media or the type of cells. Zhao et al. used human embryonic stem cells (hESC), instead of hPSC [7], which originates from embryos. On the other hand, hPSCs are from adult

tissues and cells [33]. In addition, the compositions of media, used by Zhao et al. [7] and Ronaldson-Bouchard et al. [10], are different from the composition of maturation medium [11]; which could influence the behaviour and survival of cells.

The images for Cx-43 and cTnT showed disorganized sarcomeres for continuous stimulation and aligned sarcomeres for no stimulation and 5-day stimulation. The decrease in cellular performance after day 20, especially for continuous stimulation conditions, affected the expression of Cx-43 and sarcomere organization. As a result, it is not possible to observe and comment on the effect of continuous electrical stimulation on Cx-43 concentration and the sarcomere organization at day 30 of the experiment. The organization observed for the other conditions could be due to the stiffness of the pillars (TPE), which could generate a pulling force on the tissues as TPE is resistant to bending [34]. This force might have caused the organization of the sarcomeres as it was observed in the case of no stimulation.

Based on the results from the previous experiment, a pilot experiment with wider electrodes and pillars with different stiffness was performed. To test these effects, three conditions were chosen : 5-day pacing, ramp stimulation with 1Hz/week and with 0.33Hz/day. Polydimethylsiloxane (PDMS) was chosen for the material of the pillars as it is softer and more malleable than TPE [34].

However, due to time constraints, the experiment was conducted until day 5, with an electrical stimulation of a day. The contraction of EHTs was observed more clearly with PDMS pillars and the ET was lower with wider electrodes, compared to the ETs measured with thinner electrodes.

Moreover, the ET of tissues on PDMS pillars was lower than the tissues on TPE pillars. Due to limited data from this experiment, it is not possible to conclude on the effect of pillars and electrodes. A follow-up experiment is suggested.

In summary, a model of 3 parallel tissues on one well of the 12 well plate EHT platform was designed as a circuit and the well resistance was calculated. This resistance had a similar value to the well resistance, determined by the measurements of excitation threshold at every 5 days. This correlation was useful to determine the resistivity of one EHT. In addition, the distribution of currents with different input voltages was simulated with LTSpice and compared with the measurements. There was high correlation between the model and the measurements, which was useful to determine one of the electrical parameters affecting electrical stimulation, that is the electric field. The most optimal value of electric field was determined to ensure cellular survival and was used throughout continuous electrical stimulation experiments. The best and second best methods in literature was determined to have the most efficient way of stimulation. They were determined to be continuous stimulation of EHTs with a frequency change from 2 to 6Hz, with steps of 1Hz/week and 0.33Hz/day. Four conditions were defined for the experiment: No stimulation, 5-day stimulation, ramp stimulation with 1Hz/week and 0.33Hz/day. At the end of the experiment, the ramp stimulation with 1Hz/week showed the most improvement in contractile performance. In other conditions, an improvement was also observed. However, a large variability was present in the data, which could be due to the sizes of electrodes and using different batches. The pH measurements showed that electrical stimulation does not have any effect on the pH of the media; but the affect of higher pH on the cell performance has to be analysed as the pH values were higher than the optimal pH for all conditions, including the fresh cell culture media. Furthermore, a viability analysis was conducted, which showed a decrease in viability for continuous stimulation, and the analysis for the expression of bio-markers showed an

organization of sarcomeres for control, which could be due to the material of the pillars of the EHT platform. A pilot experiment was conducted to test the effect of the sizes of electrodes and the material of pillars on the maturation and organization of the EHTs. Nevertheless, it was not possible to complete this experiment. Follow-up experiments are suggested to observe the effect of surface area of the electrodes and the material of the pillars on EHT maturation and organization.

# Conclusion

In this research, the goal was to model and simulate EHTs electrically, and analyse the effect of continuous electrical stimulation on the contractile performance. In the end, an electrical model of the EHT platform [11] was developed as a circuit and two continuous stimulation protocols were tested with EHTs. The effect of contractile performance on the EHTs were analysed with measurements and immunostaining. This analysis will be useful for the development of an electrical stimulation protocol to induce maturation of EHTs on the 12 well plate EHT platform, which will be valuable for the modelling of human body in cardiac drug screenings.

# Supporting information



| Paper | Setup | Protocol of pacing |
|---|---|---|
| Zhao et al. [7] | • Biowire 2<br>• 8 rectangular wells<br>• Polystyrene+polymer wires<br>• human embryonic stem cell derived cardiomyocytes | • Start on day 7<br>• 1Hz/week (2-6Hz) and 0.2Hz/day (1-6Hz)<br>• ET: 1Hz, 2ms, 1V/cm, 0.1V/cm steps<br>• MCR: 2xET, 0.1Hz from 1Hz |
| Ronaldson-Bouchard et al. [10] | • early stage hiPSC-CM<br>• 12 well plate<br>• 48 well plate spacing | 4 weeks:<br>• Start on day 7<br>• Monophasic (2ms), 3.5-4mV/cm<br>• Continuous<br>• Ramp (2-6Hz, 0.33/day) then 1week 2Hz |
| Tamargo et al. [26] | • PDMS<br>• 6 wells, 4 platforms, 24 tissues<br>• hPSC-CM + stromal cells<br>• 400 $\mu$L well | 21 days:<br>• 5V/cm (2.5V) biphasic(2ms)<br>• Continuous<br>• ramp stimulation (2-6Hz, 0.33 steps), 6Hz 3 days, 2Hz rest<br>• Start on day 7 |
| Schneider et al. [35] | • hiPSC-CM+FB<br>• dogbone shaped chambers<br>• spheroids<br>• 6 well plate (d=150$\mu$m)<br>• 180$\mu$m channels and 30$\mu$m height<br>• PDMS | • Biphasic (1ms)<br>• 10V , 0.3-2Hz, 0.1Hz steps, 10s for each frequency<br>• E= 0.8V/cm |
| Thavandiran et al. [36] | • hPSC-CM cardiac microrings + hPSC-EC<br>• PDMS<br>• 96-well, 1 tissue<br>• different input cells<br>• tests with drugs | Not given |
| Nunes et al. [37] | • PDMS<br>• Biowire<br>• hPSC-CM | 40-44 days:<br>• Rectangular, biphasic (1ms), 3-4V/cm<br>• low(1-3Hz) and high(1-6Hz) frequency regime<br>• 4 day ramp up then constant<br>• Start on day 7 |
| Dostanic et al. [38] | • micropillars+elliptic microwell<br>• 1-3$\mu$L volume<br>• PDMS<br>• 96 well single unit<br>• hPSC-CM+FB | • Biphasic (20V)<br>• 0.5-3.0Hz |
| Hirt et al. [27] | • rat heart cells vs. hPSC-CM<br>• 24-well<br>• 6 silicone racks(4 EHT per rack) | rat:<br>• biphasic, continuous 0.5Hz<br>hPSC:<br>• 1week 2Hz, 1.5Hz after, biphasic (4ms), 2V/cm<br>• 60-65mA per unit<br>• Start on day 4 |
| Chiu et al. [39] | • rat heart ventricles<br>• addition of FB and EC<br>• Enriched CM, FB, EC | Biphasic:<br>• 2.5V/cm,1Hz, 1ms<br>Monophasic:<br>• 5V/cm, 1Hz, 2ms<br>• Start on day 2 |
| Visone et al. [14] | • Lateral medium chamber<br>• integrated electrical system<br>• pressure chamber<br>• PDMS<br>• neonatal rat CM<br>• microscale biomimetic platform | • 2 days<br>Controlled E-field:<br>• 5V/cm, 2ms, 1Hz, biphasic<br>Controlled current:<br>• 74.4mA/cm2,2ms, 1Hz, biphasic |
| Tandon et al. (2009) [12] | • 3D EHT vs. cell monolayer<br>• rat CM<br>• collagen scaffolds<br>• 6 wells<br>• electrodes 1cm apart | • Monophasic (2ms)<br>• square<br>• 5V, 1Hz<br>• 5 days |
| Cannizzaro et al. [40] | • Scaffolds<br>• Bioreactors<br>• electrodes 1cm apart<br>• PDMS<br>• 6 wells | • suprathreshold stimulation<br>• 5 days |
| Tandon et al. (2006) [41] | 4 different electrodes:<br>• stainless steel<br>• Carbon<br>• Titanium<br>• titanium nitride<br>• neonatal rat CM | • Square<br>• Biphasic (2ms)<br>5V, 1Hz |

**Table S1.** Setup and the protocol of the experiments, found in the literature review. Literature review was done to determine the best and the second best method for electrical stimulation of EHTs. The setup and the protocol of electrical pacing was collected from 13 different papers for this table.

| Paper | Biological outcomes | Contractile parameters |
|---|---|---|
| Zhao et al. [7] | • more structured proteins with collagen+fibrin<br>• 50mln/mL cells: better alignment<br>• no collagen islands | • Increased FFR and PRP<br>• Higher active forces |
| Ronaldson-Bouchard et al. [10] | • mature sarcomere lengths and organizations<br>• mature % area of mitochondria (30%)<br>• robust T-tubules<br>• uniform cell densities<br>• Calcium response adult like gene expression | increased FFR<br>• -70mV resting membrane potential<br>• contractile force: 44mN/mm2 |
| Tamargo et al. [26] | • cellular alignment<br>• pronounced $\alpha$-actinin striations<br>• shorter calcium transients | • FFR: increase<br>• PRP: increase<br>• Force: 3.22mN/mm2<br>• contraction velocity: 622$\mu$m/s<br>• relaxation velocity: 911$\mu$m/s |
| Schneider et al. [35] | Not applicable | Not applicable |
| Thavandiran et al. [36] | • Best: 90% CM, 2.0mg/mL collagen<br>• cardiac troponin alignment (strongest with 90% CM) | • Similar contraction frequencies<br>• highest mean micro-cantilever deflection with 90% CM<br>• more force with EC |
| Nunes et al. [37] | • high response to caffeine (calcium)<br>• increased mitochondria<br>• improved sacromeric structure | • higher cell capacitance<br>• longer action potential<br>• more negative resting membrane potential<br>• higher MCR, lower ET and higher conduction velocity |
| Dostanic et al. [38] | Not given | All EHTs followed until 2.4Hz |
| Hirt et al. [27] | • thinner rat EHT after 3 weeks<br>• densely packed cells<br>• mature cross striation<br>• less cell density gradient<br>• Increasing Cx-43 and sarcomere organization<br>• increasing mitochondria<br>• organized myofibrils<br>• calcium and isoprenaline response | paced EHT:<br>• lost spontaneous beating<br>• Increasing contractile force (2.2x rat 0.26mN, 1.5x human 0.08mN) |
| Chiu et al. [39] | • increased Cx-43 (most for biphasic, enriched CM)<br>• same amount of T1<br>• 3D organization<br>• higher cell density | • Decrease in ET (lowest in monophasic)<br>• increased MCR |
| Visone et al. [14] | All:<br>• cellular elongation<br>• cytoskeletal striation<br>controlled E-field (best):<br>• higher Ca transient | Controlled E-field (best):<br>• lower ET (2.9V)<br>• higher MCR<br>• increased peak velocity |
| Tandon et al. (2009) [12] | • Aligned myofibers<br>• increased mitochondria and glycogen<br>• well aligned sarcomeres<br>• increased Cx-43<br>$\beta$MHC, Tn-1,<br>creatine kinase MM<br>• developed intercalated discs and gap junctions | • 7x higher amplitude contractions<br>• higher MCR<br>• not significantly but lower ET |
| Cannizzaro et al. [40] | • enhanced cell proliferation<br>• increased Cx-43<br>• well developed myofibrils with parallel sacromeres and intercalated discs | • 7x contaction amplitude |
| Tandon et al. (2006) [41] | Not applicable | Not applicable |

**Table S2.** Biological outcomes and change in contractile properties of the experiments, presented in literature. The results of 13 different papers were collected for this table. The results were used to determine the best and the second best electrical stimulation method in literature.

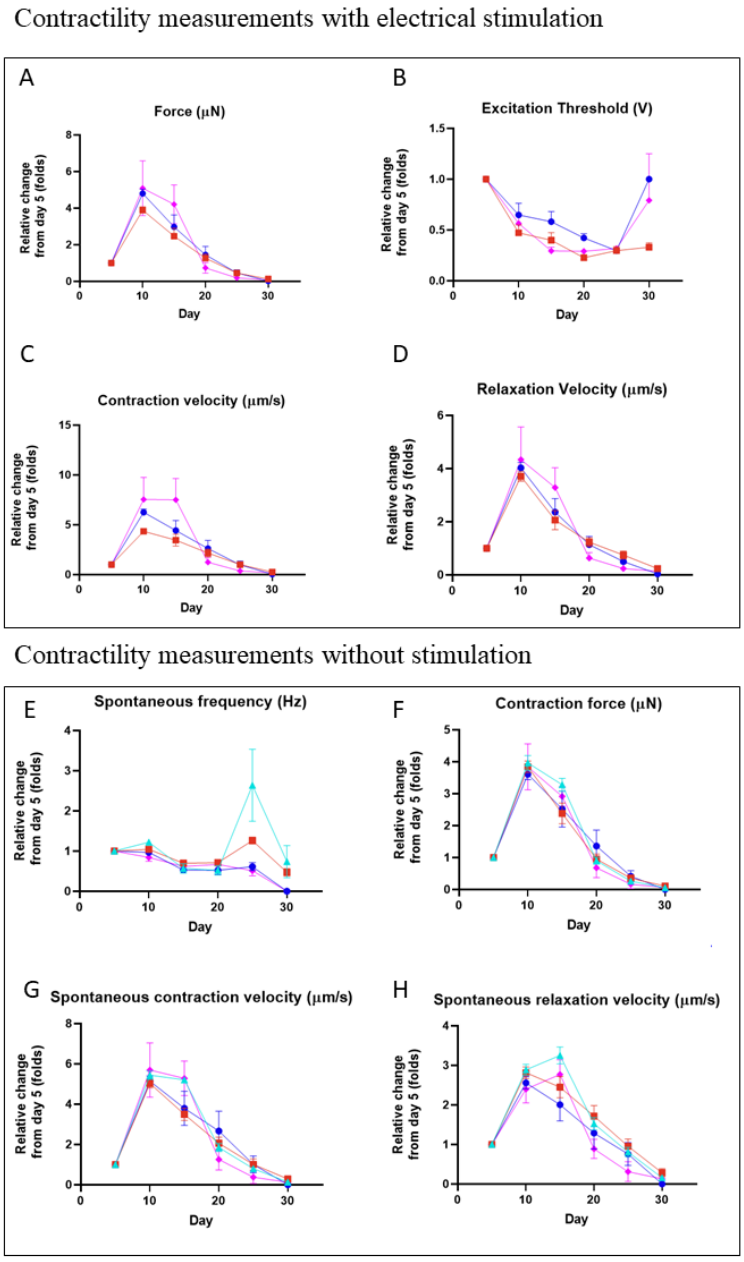



**Fig S1.** Change in contractile properties over time (cyan: control, red: 5-day stimulation, blue: 1Hz/week, purple: 0.33Hz/day). The measurements were done every 5 days for each condition. A)Force of contraction during stimulation, B) Excitation threshold, C)Contraction and D) Relaxation velocity during stimulation, E)Spontaneous frequency, F)Contraction force with no stimulation, G)Contraction and H)Relaxation velocity with no stimulation. The data is presented as mean ± SEM, day 5 of each condition normalized to 1.

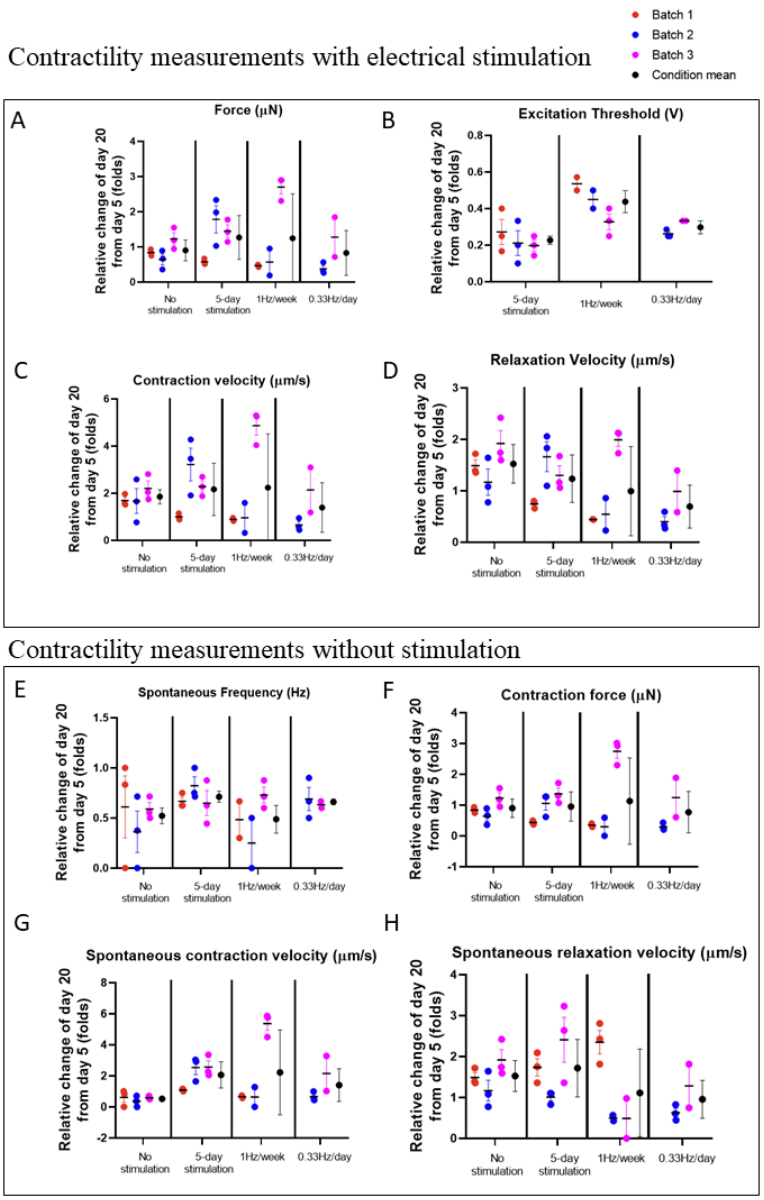



**Fig S2.** The variability within each batches of cells and within wells per condition at day 20 (red: batch 1, blue: batch 2, purple: batch 3, black: condition mean). A)Force of contraction during stimulation, B) Excitation threshold, C)Contraction and D) Relaxation velocity during stimulation, E)Spontaneous frequency, F)Contraction force with no stimulation, G)Contraction and H)Relaxation velocity with no stimulation. The results show the relative change of day 20 with respect to day 5. The data is presented as mean ± SEM and day 5 of each condition, normalized to 1. Values show the change from day 5 as folds.

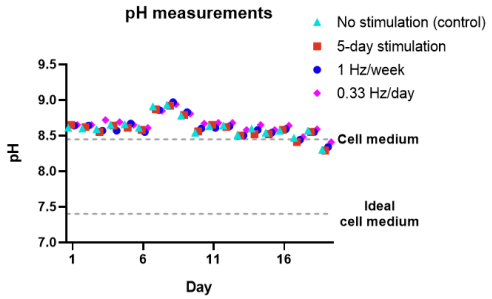



**Fig S3.** Daily pH measurements. The measurements are conducted using Mettler Toledo FiveEasy Plus (FEP20) pH benchtop meter [18]. To test the accuracy of the device before each measurement, two buffers with pH values of 4 and 9 are used.

| Condition | pH |
|---|---|
| Fresh media | 7.4 |
| Fridge (1 hour) | 8.1 |
| Incubator (1 hour) | 8.24 |
| Incubator (2 hours) | 8.17 |
| LAF hood (1 hour) | 7.99 |
| Freezer (overnight) | 8.28 |

**Table S3.** Cell culture media pH measurements under different environmental conditions.

# References


1. Mittal R, Woo FW, Castro CS, Cohen MA, Karanxha J, Mittal J, et al. Organ-on-chip models: Implications in drug discovery and clinical applications. Journal of Cellular Physiology. 2019;234(6):8352–8380. doi:10.1002/JCP.27729.

2. Dutch government plans to stop animal testing by 2025;. Available from: `https://chemicalwatch.com/51958/dutch-government-plans-to-stop-animal-testing-by-2025`.

3. 2D Versus 3D Cell Cultures: Advantages and Disadvantages;. Available from: `https://www.mimetas.com/en/blogs/315/2d-versus-3d-cell-cultures-advantages-and-disadvantages.html`.

4. Tzatzalos E, Abilez OJ, Shukla P, Wu JC. Engineered heart tissues and induced pluripotent stem cells: macro- and microstructures for disease modeling, drug screening, and translational studies. Advanced drug delivery reviews. 2016;96:234. doi:10.1016/J.ADDR.2015.09.010.

5. Dhahri W, Romagnuolo R, Laflamme MA. Training heart tissue to mature. Nature Biomedical Engineering. 2018;doi:10.1038/s41551-018-0253-7.

6. Stein JM, Mummery CL, Bellin M. Engineered models of the human heart: Directions and challenges. Stem Cell Reports. 2021;16(9):2049. doi:10.1016/J.STEMCR.2020.11.013.

7. Zhao Y, Rafatian N, Wang EY, Feric NT, Lai BFL, Knee-Walden EJ, et al. Engineering microenvironment for human cardiac tissue assembly in heart-on-a-chip platform. Matrix biology : journal of the International Society for Matrix Biology. 2020;85-86:189–204. doi:10.1016/J.MATBIO.2019.04.001.

8. Barash Y, Dvir T, Tandeitnik P, Ruvinov E, Guterman H, Cohen S. Electric Field Stimulation Integrated into Perfusion Bioreactor for Cardiac Tissue Engineering; 2010. Available from: https://pubmed.ncbi.nlm.nih.gov/20367291/.

9. Sehnert AJ, Huq A, Weinstein BM, Walker C, Fishman M, Stainier DYR. Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. Nature Genetics 2002 31:1. 2002;31(1):106–110. doi:10.1038/ng875.

10. Ronaldson-Bouchard K, Ma SP, Yeager K, Chen T, Song LJ, Sirabella D, et al. Advanced maturation of human cardiac tissue grown from pluripotent stem cells. Nature. 2018;556(7700):239–243. doi:10.1038/S41586-018-0016-3.

11. Ribeiro MC, Rivera-Arbeláez JM, Cofiño-Fabres C, Schwach V, Slaats RH, Ten Den SA, et al. A New Versatile Platform for Assessment of Improved Cardiac Performance in Human-Engineered Heart Tissues. Journal of Personalized Medicine 2022, Vol 12, Page 214. 2022;12(2):214. doi:10.3390/JPM12020214.

12. Tandon N, Cannizzaro C, Chao PHG, Maidhof R, Marsano A, Au HTH, et al. Electrical stimulation systems for cardiac tissue engineering. Nature Protocols 2009 4:2. 2009;4(2):155–173. doi:10.1038/nprot.2008.183.

13. Faes TJC, Van Der Meij HA, De Munck JC, Heethaar RM. Physiological Measurement The electric resistivity of human tissues (100 Hz-10 MHz): a meta-analysis of review studies You may also like The electric resistivity of human tissues (100 Hz-10 MHz): a meta-analysis of review studies. Physiol Meas. 1999;20:1–10.

14. Visone R, Talò G, Occhetta P, Cruz-Moreira D, Lopa S, Pappalardo OA, et al. A microscale biomimetic platform for generation and electro-mechanical stimulation of 3D cardiac microtissues. APL Bioengineering. 2018;2(4):046102. doi:10.1063/1.5037968.

15. UCSFi001-A · Cell Line · hPSCreg;. Available from: https://hpscreg.eu/cell-line/UCSFi001-A.

16. Rivera-Arbeláez JM, Cofiño-Fabres C, Schwach V, Boonen T, ten Den SA, Vermeul K, et al. Contractility analysis of human engineered 3D heart tissues by an automatic tracking technique using a standalone application. PLOS ONE. 2022;17(4):e0266834. doi:10.1371/JOURNAL.PONE.0266834.

17. Stimulus Generator Manual STG 4004 and STG 4008. 2021;.

18. Mettler Toledo FiveEasy Plus™ pH benchtop meter, FEP20 MICRO Kit including LE electrode, AC/DC input 230 V AC, universal plug set — Sigma-Aldrich;. Available from: https://www.sigmaaldrich.com/NL/en/product/aldrich/mt30089749.

19. LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells;. Available from: https://www.thermofisher.com/order/catalog/product/L3224?SID=srch-srp-L3224.

20. Anti-Connexin 43 Antibody, clone 5H23, ZooMAb® Rabbit Monoclonal recombinant, expressed in HEK 293 cells — Sigma-Aldrich;. Available from: https://www.sigmaaldrich.com/NL/en/product/sigma/zrb1179?gclid=CjwKCAjwwdWVBhA4EiwAjcYJEJDA6HzQZR4D7WEG0h_Qv3PqbyJSNenurnxz584NSTIklAk3ku1ZJhoC5l8QAvD_BwE.

21. Cardiac Troponin T Antibody (MA5-12960);. Available from: https://www.thermofisher.com/antibody/product/Cardiac-Troponin-T-Antibody-clone-13-11-Monoclonal/MA5-12960.

22. Chicken anti-Rabbit IgG (H+L) Cross-Adsorbed, Alexa Fluor™ 488 (A-21441);. Available from: https://www.thermofisher.com/antibody/product/Chicken-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21441.

23. Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor™ 647 (A-31571);. Available from: https://www.thermofisher.com/antibody/product/A-31571.html?ef_id=CjwKCAjwwdWVBhA4EiwAjcYJEPOvDYL7tZ1cNwJYKACIBsZSaKNoDYZmQ00zRyYxskaFOKaQBFBwE:G:s&s_kwcid=AL!3652!3!516608152470!!!g!!&cid=bid_pca_aus_r01_co_cp1359_pjt0000_bid00000_0se_gaw_dy_pur_con&gclid=CjwKCAjwwdWVBhA4EiwAjcYJEPOvDYL7tZ1cNwJYKACIBsZSaKNoDYZmQ00zRyYxskaFOKaQBFBwE.

24. DAPI (4',6-diamidino-2-phenylindole) — Thermo Fisher Scientific - NL;. Available from: https://www.thermofisher.com/nl/en/home/life-science/cell-analysis/fluorophores/dapi-stain.html.

25. Zeiss LSM 880 Confocal Laser Scanning Microscope — Biomed Core Facilities I Brown University;. Available from: https://biomedcorefacilities.brown.edu/bioimaging-facility/zeiss-lsm-880-confocal-laser-scanning-microscope.

26. Tamargo MA, Nash TR, Fleischer S, Kim Y, Vila OF, Yeager K, et al. milliPillar: A Platform for the Generation and Real-Time Assessment of Human Engineered Cardiac Tissues. ACS biomaterials science & engineering. 2021;7(11):5215–5229. doi:10.1021/ACSBIOMATERIALS.1C01006.

27. Hirt MN, Boeddinghaus J, Mitchell A, Schaaf S, Börnchen C, Müller C, et al. Functional improvement and maturation of rat and human engineered heart tissue by chronic electrical stimulation. Journal of Molecular and Cellular Cardiology. 2014;74:151–161. doi:10.1016/J.YJMCC.2014.05.009.

28. Factors Effecting the Resistivity of Electrical Materials — Electrical4U;. Available from: https://www.electrical4u.com/factors-effecting-the-resistivity-of-electrical-materials/.

29. Saad AIM, Elshahed AM. Plant Tissue Culture Media. 2012;doi:10.5772/50569.

30. Factors that affect an electrolysis reaction - Easy-Chem Australia;. Available from: https://easychem.com.au/shipwrecks-and-salvage/3-electrolytic-cells/factors-that-affect-an-electrolysis-reaction/.

31. A Dictionary of Physics. A Dictionary of Physics. 2009;doi:10.1093/ACREF/9780199233991.001.0001.

32. BINDER GmbH: The pH value is a key factor in cell and tissue cultures;. Available from: https://www.binder-world.com/int-en/knowledge/detail/the-ph-value-is-a-key-factor-in-cell-and-tissue-cultures.

33. Comparison of the two most common types of Human Pluripotent Stem Cells (hPSC)-Pluripotent Human Embryonic Stem Cells (hESC) versus Human Induced Pluripotent Stem Cells (hiPSC);. Available from: https://scholars.direct/Articles/regenerative-medicine/rmt-1-001table1.html.

34. Busek M, Nøvik S, Aizenshtadt A, Amirola-Martinez M, Combriat T, Grünzner S, et al. Thermoplastic elastomer (Tpe)–poly(methyl methacrylate) (pmma) hybrid devices for active pumping pdms-free organ-on-a-chip systems. Biosensors. 2021;11(5). doi:10.3390/BIOS11050162.

35. Schneider O, Moruzzi A, Fuchs S, Grobel A, Schulze HS, Mayr T, et al. Fusing spheroids to aligned $\mu$-tissues in a heart-on-chip featuring oxygen sensing and electrical pacing capabilities. Materials Today Bio. 2022;15:100280. doi:10.1016/J.MTBIO.2022.100280.

36. Thavandiran N, Hale C, Blit P, Sandberg ML, McElvain ME, Gagliardi M, et al. Functional arrays of human pluripotent stem cell-derived cardiac microtissues. Scientific Reports 2020 10:1. 2020;10(1):1–13. doi:10.1038/s41598-020-62955-3.

37. Nunes SS, Miklas JW, Liu J, Aschar-Sobbi R, Xiao Y, Zhang B, et al. Biowire: a platform for maturation of human pluripotent stem cell–derived cardiomyocytes. 2013;10(11):12. doi:10.1038/nMeth.2524.

38. Dostanić M, Windt LM, Stein JM, Van Meer BJ, Bellin M, Orlova V, et al. A Miniaturized EHT Platform for Accurate Measurements of Tissue Contractile Properties. Journal of Microelectromechanical Systems. 2020;29(5):881–887. doi:10.1109/JMEMS.2020.3011196.

39. Chiu LLY, Iyer RK, King JP, Radisic M. Biphasic Electrical Field Stimulation Aids in Tissue Engineering of Multicell-Type Cardiac Organoids; 2011. Available from: https://pubmed.ncbi.nlm.nih.gov/18783322/#:~:text=Biphasic%20field%20stimulation%20was%20also,and%20enhancing%20Connexin%2D43%20presence.

40. Cannizzaro C, Tandon N, Figallo E, Park H, Gerecht S, Radisic M, et al. Practical aspects of cardiac tissue engineering with electrical stimulation. Methods in molecular medicine. 2007;140:291–307. doi:10.1007/978-1-59745-443-8_16/COVER/.

41. Tandon N, Cannizzaro C, Figallo E, Voldman J, Vunjak-Novakovic G. Characterization of electrical stimulation electrodes for cardiac tissue engineering. Annual International Conference of the IEEE Engineering in Medicine and Biology - Proceedings. 2006; p. 845–848. doi:10.1109/IEMBS.2006.259747.