A microphysiological setup to mimic, test and analyze myocardial tissue for cardiovascular diseases

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Abstract

Creating cardiac tissue models for drug testing before clinical trials is still a major challenge in drug discovery. This is mainly because it is difficult to replicate the complex nature of cardiac tissue in a lab setting. One of the biggest challenges is accurately mimicking the functional features of the heart muscle. This is largely due to the immaturity of the cells used in these models, which makes it difficult to get reliable results that can be translated to human patients. Currently, in vivo models are the gold standard for evaluating new treatments. However, it is widely accepted that these animal models cannot fully reproduce human physiological responses. As a result, this mismatch often leads to failures in subsequent clinical trials. In this work, we designed two cardiac culture experiments, using GCampp6-infected cardiomyocytes, on a microfluidic chip. One of them is an aligned 2D model, and the other is a 3D tissue grown in a hydrogel of methacrylated gelatin (GelMa) and methacrylated hyaluronic acid (HaMa), the calcium traces of both were analysed at the network level, being able to obtain parameters that determine the degree of maturity and connectivity of the culture. In this way, we can ensure that we have a reproducible environment for future drug testing.

1. Introduction

Cardiovascular diseases (CVD) rank as the foremost cause of death on a global scale. Available therapeutic options consist of lifestyle and dietary modifications, medication, or surgical interventions. Since none of these interventions will completely regenerate the myocardium, it is vitally important to develop regenerative therapies to mitigate damage. Currently, the construction of *in vitro* models using innovative microfluidic techniques is gaining significance for testing these therapies during the preclinical stage. Among these diseases, our work focuses on those affecting the myocardium, which is a complex tissue with specialized cells (fibroblasts, smooth muscle cells, and cardiomyocytes) and an extracellular matrix (ECM) of collagen, elastin, fibronectin, laminin, and proteoglycans.

In this study, we designed a microfluidic chip and built two tissue culture models using mouse cardiomyocyte cell line infected for us with A calcium indicator (GCaMP6). The first model is a 2D aligned tissue with PLA fibers, and the other is a 3D model that uses an interpenetrated hydrogel based on GelMa and HaMa to mimic the ECM. Both cultures were recorded to extract maturation information from the calcium traces.

2. Materials and methods

2.1. Electrospinning

The polymer chosen for electrospinning was polylactic acid (PLA) 70/30 due to its good adhesion and proliferation properties [1]. An 8% solution of PLA 70/30 in 2,2,2-trifluoroethanol was used, which was left overnight on a three-dimensional orbital shaker at 50 rpm.

It was chosen to electrospin the coverslips before the plasma process to attach them to the PDMS for the manufacturing of PLA fiber-based devices. The coverslips were first cleaned with ethanol before being taped on sheets of aluminum foil measuring 20 x 32 cm. Three coverslips were centered on each of these sheets, with three centimeters between each one. Finally, using adhesive tape, the aluminum foil was applied to the collector. The PLA solution was loaded into a 10 ml syringe and attached to a pump (*NE-1000, New Era Instruments*) which delivered a flow rate of 0.5 ml/h. A high-voltage power supply (*NanoNC-403010, NanoNC*) was used to provide a potential of 18 kV between the syringe needle and the grounded collector. To obtain aligned fibres, the collector used was a 90 cm diameter *NanoNC DC90,* placed 20 cm from the syringe needle, rotating at 1000 rpm for 10 minutes.

2.2. Microfluidic platform

The microfluidic platform is based on a system created by *Funamoto et al. 2012* that allows us to study cell survival, proliferation, migration, morphogenesis, and differentiation under controlled conditions [2]. The device is composed of 3 parts: a main cell culture chamber (900 µm wide and, 8300µm long, and 150 µm high), flanked by two side channels (500 µm wide and 150 µm high), and two sets of optimized posts (200 µm wide and 150 µm high) each located between the cell culture chamber and the side channels. The chip design was made in AutoCAD and sent to CAD Art (Output City), where the mask was fabricated in acetate. The mask design was then transferred using a 4" silicon wafer as substrate and SU-8 2100 resin.

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The fabrication process of the chip involves the utilization of soft lithography, a technique that employs stamps, molds, and flexible photomasks to create or replicate structures. Initially, a mixture of polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) was prepared by combining PDMS base and curing agent in a weight ratio of 10:1. This PDMS mixture was then poured onto a Petri dish containing the wafer with the desired microfluidic chip design. To eliminate air bubbles, the mixture underwent a vacuum-assisted degassing process inside a desiccator for 45 minutes. Subsequently, it was cured by placing it at 65°C for a minimum of 4 hours, allowing the polymer to solidify. The cured PDMS was carefully separated from the mold, and the desired chip designs were cut using a scalpel. Biopsy punches were used to create 1 mm holes for the cell chamber and 6 mm holes for the cell culture medium reservoirs.

Following the fabrication steps, the PDMS chips underwent a thorough cleaning process with soap, water, and compressed air to remove any contaminants. Then, in a controlled clean room environment, the chips were attached to glass covers that were previously patterned with electrospun fibers, as described earlier. Oxygen plasma was employed to activate both the PDMS chips and the glass covers for 30 seconds. By applying gentle pressure with fingers, the activated parts were carefully joined together. Subsequently, the assembled chips were placed on a hotplate at 85°C for 2 hours to allow water evaporation and enhance the bonding strength. Finally, the chips underwent sterilization by exposure to UV light for a minimum of 15 minutes to ensure a sterile working environment.

2.3. Hydrogel synthesis

The pre-crosslinked polymer ECM slurry (HAMA and GelMA) was mixed in Claycomb medium overnight at 41ºC to obtain a solution with a final concentration of 4,5% GelMA and 3,5% HAMA. 0,1% w/v LAP photoinitiator solution was prepared and mixed with the polymer solution using a vortex to ensure homogeneity. To cross-link this solution, it uses UV light with a wavelength of 365 nm at 4cm, 15s and 10% lamp power giving an irradiance of 90mW/cm^2. To study the compression modulus of the hydrogel, uniaxial compression tests were carried out with a preload of 0.1mN and a deformation rate of 20%/min. The equipment used was an Zwick Z0.5 TN equipment (Zwick-Roell, Germany) with a 5N cell.

2.4. Cell culture

The cardiac cells used in this work are HL-1. The HL-1 cell line is a type of cardiac muscle cell line that was derived from the atrial tissue of mouse hearts. The HL-1 cell line has become a widely used tool in cardiac research because it closely mimics the properties of primary cardiac myocytes and is capable of spontaneous rhythmic contractions [3].

Cells were maintained according to the protocol provided by the Claycomb laboratory. Claycomb medium (Sigma) was supplemented with 10% FBS, 100 µg/ml penicillin/ streptomycin, 0.1 mM norepinephrine and 2 mM L-glutamine. Norepinephrine supplementation is key to the maintenance of the contractile phenotype. Cells from passages 7 to 14 were seeded at a density of 1,3x10^5/cm^2 in 25 ml flasks (T25) coated with fibronectin/gelatin (5µg/ml). 1 ml of medium per 5cm^2 of culture area was supplied daily and divided 1:3 every 4 days. HL-1 monolayers were contractile at day 3 after passaging.

Cells were lifted at 80-90% confluence, using 0.05% EDTA trypsin, then 0.25% soybean inhibitor solution to stop the effect of trypsin and centrifuged at 500G for 5 minutes.

Furthermore, HL-1 cardiomyocytes were infected with a calcium indicator (GCaMP6). GCaMP6s is a genetically encoded calcium indicator used in neuroscience research to monitor neuronal activity [4]. GCaMP6s and other genetically encoded calcium indicators can also be applied to cardiomyocytes to monitor calcium dynamics, important for understanding the electrical and mechanical activity of the heart and studying cardiac function. The genetic construct encoding GCaMP6s can be introduced into cardiomyocytes using techniques such as viral vectors allowing for the expression of the calcium indicator in these cells [5].

2.5. Chip seeding

For cell culture both in 2D and 3D, all the chips used had a layer of electrospinning fibers to achieve better adhesive and proliferative properties. The chips were previously irradiated with UV for 15 minutes to sterilize them. For the lifting of the cells, we waited for 80-90% confluence. 0.05% EDTA trypsin was used, then 0.25% soybean inhibitor solution was used to stop the effect of trypsin and centrifuged at 500G for 5 minutes.

For 2D assays, HL-1 cardiac cells are centrifuged and resuspended in a medium to have a concentration of 12x10^6 cells/ml. Then 10 µg/ml of the medium with the resuspended cells is introduced into the culture chamber of the chip. The chip is left in the incubator for 1 hour to finally hydrate the channels. For hydration of the channels, one reservoir is filled with medium and aspirated from the other. medium and aspirate from the other end with a 1 ml tip cut to fit the diameter of the reservoir. diameter of the reservoir. The same process is repeated for the other parallel channel. Finally, all reservoirs are filled with 100 µl and the medium is changed daily.

For 3D assays, 960,000 cells were centrifuged in an Eppendorf and resuspended in 72µl of hydrogel mixed with 8µl of LAP at a final concentration of 3x10^7 cells/ml. Finally, 10 µl of the solution loaded with cells was injected through the chamber inlet and UV was irradiated at a 4cm distance, during 10s, with a power of 9mW/cm^2. For hydration of the channels, was performed the same way as explained above in the 2D cultures, with 100 µl of medium in each reservoir and completely changed every day.

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2.6. Immunofluorescence analysis of tissues

Immunofluorescence was performed using Phalloidin (Cytoskeleton Inc.), a fluorescent marker that selectively binds to actin filaments in cells, specifically targeting F-actin. To label the cell nucleus, DAPI (Sigma Aldrich), a marker that binds to DNA's adenine-thymine-rich regions, was used.

The experimental procedure began by rinsing the cells with sterile PBS for 5 minutes. Two reservoirs on one side of the chip were filled with 60 µl of sterile PBS, allowing the liquid to flow across the chamber for the distribution of reagents. Subsequently, the cells were fixed with 4% paraformaldehyde for 15 minutes. A second wash was performed using PBS for 5 minutes. To enable the entry of staining agents, the cells were permeabilized with 0.1% Triton X in PBS for 10 minutes. Afterward, the cells were rinsed again with PBS for 5 minutes.

For staining, a solution containing DAPI at a 1:1000 dilution and Alexa Fluor 488-conjugated phalloidin at a 1:150 dilution in PBS was applied to the cells and incubated at room temperature for 1 hour. Finally, the cells were imaged using a confocal microscope, specifically the Nikon eclipse inverted model.

2.7. Alignment analysis

Images obtained by confocal microscopy and SEM were used for alignment analysis. The images were processed with ImageJ software (NIH, Maryland, USA, http://rsb.info.nih.gov/ij). Fast Fourier transform (FFT) analysis was used as it allows morphological quantification of the microscopic structural alignment [6]. Subsequently, using the Oval Profile Plot plugin an oval projection was placed on the FFT image and a radial summation of the pixel intensities for each angle between 0º and 360º was performed in increments of 1 [7]. Since the FFT data are symmetric, a pixel sum was performed between 0 and 180º. The degree of alignment that was present in the original image was reflected in the height and overall shape of the peak. The position of the peak in the graph reported the principal angle of the orientation. The FFT data were normalized to a reference value of 0 and plotted in arbitrary units ranging from 0 to 1 [8].

2.8. Calcium analysis

All data were preprocessed using ImageJ and self-developed scripts in Matlab and Python. The analysis was applied at the individual case and network level, both using adapted and optimized code from C. Südhof [9].

3. Results

3.1. Electrospinning and fabrication of microfluidic device

The creation of an aligned PLA mesh was successful achieving a density of 1160 fibres per mm, a fibre thickness of 712 ± 93 nm and an alignment at 94°. The mesh was correctly integrated into the base of the central culture channel of the chip. The main problems of culture medium leakage from the sides of the chip were solved fitting the mash as close as possible to the size of the central channel.

3.2. Hydrogel synthesis

The results of the mechanical tests show that the hydrogel reached a stiffness of 7.4k ± 0.96kPa, optimal for use as a cardiac scaffold. Experimentally, the loading of the pre-crosslinked polymer onto the chip was successful, preventing the hydrogel from leaking through the side channels of the chip, thus remaining crosslinked and confined in the central chamber after UV exposure.

3.3. 2D anisotropic cardiac tissue

The culture was grown directly on PLA70/30 without any coating. As a result, HL-1 adhered to the fibres without any problems, and an alignment in the direction of the fibres was evident, as can be seen in Figure 1(A), where the F-actin is represented in green (marked by the phalloidin) and in blue the nucleus of the HL-1. To quantify the alignment of the cells, the same process was used as for the fibres. The result is reflected in Figure 1(B), where the intensity of the cells is mostly at 87º.

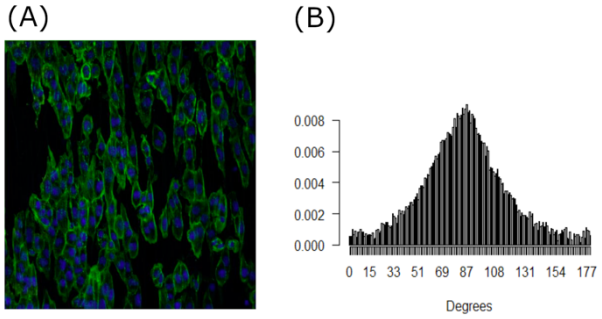

Figura 1. *A) DAPI (blue) and phalloidin (green) staining at 20x; B) Radial sum of phalloidin intensities.*

In another experimental case, with GCamp6-infected cells, a 30-second video was recorded, Figure 2 (A), from which network-level information could be extracted. It detected 15 synchronous network peaks, then 28.9 peaks/min and an amplitude variation coefficient of 0.05, meaning that the tissue beats with the same intensity at each synchronous contraction, Figure 2(B).

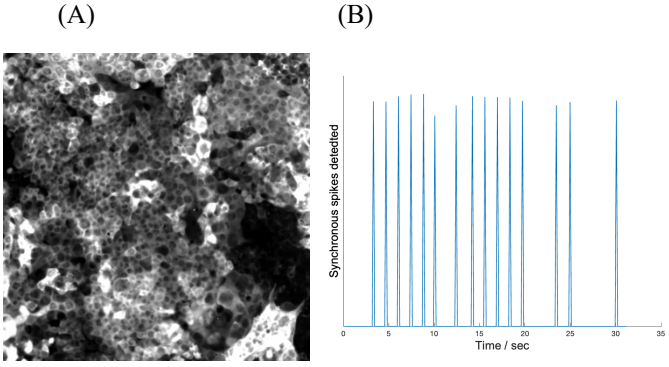

Figura 2. *A) 2D HL-1 GCamp6 culture, maximum intensity projection; B) Synchronous spikes detected*

3.4. 3D cardiac tissue

A 3D cardiac culture was obtained, where HL-1 cells formed spheroids in most cases, but also showed areas where cells were able to form larger aggregates, Figure 3 (A), thus enabling an interconnectivity study to be performed. The study was applied at the network level, and as a result, parameters such as a synchronous firing rate of 8.14 spikes/min, an amplitude variation coefficient of 0.01, and an inter-spike time of 7.85ms were obtained, Figure 3 (B).

(A) (B)

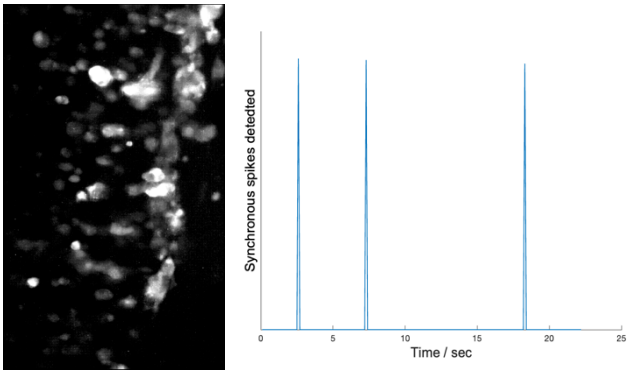

Figura 3.*A) 3D HL-1 GCamp6 culture, maximum intensiy projection; B) Synchronous spikes detected*

The degree of cell viability of this culture was also measured using a life/death test, and the result was 80.9% for the first day.

4. Discussion and Conclusions

We achieved our two objectives, on the one hand, a cell culture reproducing a 2D anisotropic tissue, using PLA fibers aligned by electrospinning , which implies a more mature tissue, [10] where we were able to apply calcium analysis and obtain important parameters for a cellular network synchronization. On the other hand, the results of the hydrogel synthesis show that we have achieved a stiffness of 7.4k ± 0.96kPa, which is optimal for use as a cardiac scaffold, subsequently, a 3D myocardial culture was also achieved, this model is currently being optimized to obtain the correct cell density for good growth. In the first 3D assays, cell viability of 80.9% was obtained on the first day, which then decreased; however, in areas with high cell density on the third day, high Ca2+ transient connectivity was observed. Based on our results, we are working on inducing a 3D topographic signal to generate 3D-aligned tissue.

The improved results would benefit cardiac research by providing a robust and reproducible microphysiological model for testing regenerative treatments and analyzing them by calcium tracings.

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References

[1] Z. álvarez et al., “The effect of the composition of PLA films and lactate release on glial and neuronal maturation and the maintenance of the neuronal progenitor niche,” *Biomaterials, vol. 34, no. 9, pp. 2221–2233, 2013*.

[2] Kenichi Funamoto, Ioannis K Zervantonakis, Yuchun Liu, Christopher J Ochs, Choong Kim, and Roger D Kamm. A novel microfluidic platform for high-resolution imaging of a three-dimensional cell culture under a controlled hypoxic environment. *Lab Chip, 12(22):4855–4863*, November 2012.

[3] WCClaycomb,NALanson,Jr,BSStallworth,DBEgeland,JBD elcarpio,ABahin- ski, and N J Izzo, Jr. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc. Natl. Acad. Sci. U. S. A., 95(6):2979–2984,* March 1998.

[4] Tsai-Wen Chen, Trevor J Wardill, Yi Sun, Stefan R Pulver, Sabine L Renninger, Amy Baohan, Eric R Schreiter, Rex A Kerr, Michael B Orger, Vivek Jayaraman, Loren L Looger, Karel Svoboda, and Douglas S Kim. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature, 499(7458):295–300,* July 2013.

[5] YuqianJiang,YuxiaoZhou,XiaopingBao,ChuanxinChen,La urenNRandolph,Jing Du, and Xiaojun Lance Lian. An ultrasensitive calcium reporter system via CRISPR- Cas9-mediated genome editing in human pluripotent stem cells. *iScience, 9:27–35*, November 2018.

[6] C. Ayres et al., “Modulation of anisotropy in electrospun tissue-engineering scaffolds: Analysis of fiber alignment by the fast Fourier transform.,” *vol. 27, no. 32, pp. 5524–5534*, 2010.

[7] S. E. Taylor, T. Cao, P. M. Talauliker, and J. Lifshitz, “Objective morphological quantification of microscopic images using a Fast Fourier Transform (FFT) analysis,” *Curr. Protoc. Essent. Lab. Tech., vol. 2013, no. SUPPL.7, pp. 1–12*, 2013.

[8] Y. Orlova, N. Magome, L. Liu, Y. Chen, and K. Agladze, “Electrospun nanofibers as a tool for architecture control in engineered cardiac tissue,” *Biomaterials, vol. 32, no. 24, pp. 5615–5624*, 2011.

[9] Zijun Sun, and Thomas C. Südhof, “A simple Ca2+-imaging approach to neural network analyses in cultured neurons” doi: 10.1016/j.jneumeth.2020.109041

[10] López-Canosa, Adrián, et al. "A microphysiological system combining electrospun fibers and electrical stimulation for the maturation of highly anisotropic cardiac tissue." *Biofabrication 13.3 (2021):* 035047.