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Quantitative assessment of cardiomyocyte mechanobiology through high-throughput cantilever-based functional well plate systems†

Jongyun Kim,^{a,d} Arunkumar Shanmugasundaram,^{a,d} Dong-Su Kim,^{a,d,e} Yun-Jin Jeong,^{a,d} Pooja P. Kanade,^{a,d} Eung-Sam Kim,^{id} Bong-Kee Lee^{id} ^{a,c} and Dong-Weon Lee^{id} ^{*a,c,d}

Proper regulation of the *in vitro* cell culture environment is essential for disease modelling and drug toxicity screening. The main limitation of well plates used for cell culture is that they cannot accurately maintain energy sources and compounds needed during cell growth. Herein, to understand the importance of perfusion in cardiomyocyte culture, changes in contractile force and heart rate during cardiomyocyte growth are systematically investigated, and the results are compared with those of a perfusion-free system. The proposed perfusion system consists of a Peltier refrigerator, a peristaltic pump, and a functional well plate. A functional well plate with 12 wells is made through injection moulding, with two tubes integrated in the cover for each well to continuously circulate the culture medium. The contractile force of cardiomyocytes growing on the cantilever surface is analysed through changes in cantilever displacement. The maturation of cardiomyocytes is evaluated through fluorescence staining and western blot; cardiomyocytes cultured in the perfusion system show greater maturity than those cultured in a manually replaced culture medium. The pH of the culture medium manually replaced at intervals of 3 days decreases to 6.8, resulting in an abnormal heartbeat, while cardiomyocytes cultured in the perfusion system maintained at pH 7.4 show improved contractility and a uniform heart rate. Two well-known ion channel blockers, verapamil and quinidine, are used to measure changes in the contractile force of cardiomyocytes from the two systems. Cardiomyocytes in the perfusion system show greater stability during drug toxicity screening, proving that the perfusion system provides a better environment for cell growth.

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1. Introduction

The *in vitro* cell culture platform has garnered significant attention across various scientific and technological fields, including organ-on-a-chip systems, regenerative tissue production, drug development, and drug-induced toxicity screening.¹ The growth and maturation of *in vitro* cells are greatly

impacted by several crucial factors, including the cell culture substrate, stimulation methods, and the composition of the culture medium. Among these factors, the presence of serum in the culture medium typically ranging from 5 to 20% is essential for facilitating rapid cell growth and maturation.^{2–4} Serum provides transport proteins that carry hormones, minerals, and fats in the cells. Additionally, serum acts as a pH buffer in the culture medium.⁵ The optimal pH for proper growth and maturation of cardiomyocytes in animal-based cell cultures is 7.4. However, numerous studies have demonstrated that a culture medium pH of 7.1–7.4 facilitates the maturation and proliferation of cardiomyocytes.^{6–9} The culture medium gradually becomes affected by the generation of large amounts of H⁺ and OH[−] ions during the culture period.⁷ The generation of H⁺ and OH[−] ions leads to a decrease in contractility^{10–14} and induces abnormal Ca²⁺ secretion^{14–16} and arrhythmia^{17–19} in the cultured cardiomyocytes. In addition, by-products of cellular metabolism such as lactic acid and ammonia are toxic components and alter the pH inside the cell, which can lead to physiological and morphological problems in cardiomyocytes.^{20–25}

^aMEMS and Nanotechnology Laboratory, School of Mechanical Engineering, Chonnam National University, Gwangju 61186, Republic of Korea.

E-mail: mems@jnu.ac.kr

^bDepartment of Biological Sciences, Chonnam National University, Gwangju, 61186, Republic of Korea

^cCenter for Next-Generation Sensor Research and Development, Chonnam National University, Gwangju 61186, Republic of Korea

^dAdvanced Medical Device Research Center for Cardiovascular Disease, Chonnam National University, Gwangju 61186, Republic of Korea

^eGreen Energy & Nano Technology R&D Group, Korea Institute of Industrial Technology (KITECH), Gwangju, 61012, Republic of Korea

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Therefore, there is a great desire for the development of a reliable *in vitro* cardiac cell-based platform capable of regulating the pH of the culture medium.

Over the years, several cell culture platforms with perfusion systems have been proposed for regulating pH and nutrient levels in culture media.^{26–30} Perfusion-based cell culture stimulates the maturation of cardiomyocytes by continuously supplying the culture medium and removing H⁺, OH[−], and other toxic elements. For instance, Chen *et al.* proposed a microfluidic cell array-based cell culture perfusion system in which the culture medium was replaced in the platform through the height difference between the inlet and outlet wells.²⁶ Furthermore, Goral *et al.* performed high-throughput screening of culture medium perfusion through a cellulose membrane in 96-well plates.²⁷ However, the developed perfusion-based cell culture platforms were unable to control the flow rate of the culture medium, and the volume capacity of the culture vessel prevented long-term cell culture. Additionally, Yoshimitsu *et al.* developed a perfusion cell culture platform based on a polydimethylsiloxane (PDMS) microfluidic chip in which pressure was applied to the inlet port of the culture medium. Kim *et al.* also developed a perfusion system using PDMS microfluidic well plates. The perfusion systems were tilted to the desired angle, and the culture medium was perfused through the cell culture platform by gravitational force.^{28,29} However, the mechanisms employed in the proposed perfusion systems tended to exert shear stress on cultured cardiomyocytes. The shear stress generated by the perfusion system reduced cell adhesion to the culture substrate, thereby significantly affecting the maturation and growth of cultured cardiomyocytes. Wei *et al.* solved this shear stress issue with a microfluidic cover on a 96-well plate built using the droplet technique.³⁰ This approach minimised the shear stress and controlled the flow rate of the culture medium through a pump. Additionally, various systems capable of circulating culture fluid have been reported; however, none of these systems have been able to accurately measure the contraction force of cardiomyocytes. Despite these advancements in perfusion-based platforms that can regulate nutrient levels and pH, accurately quantifying cardiomyocyte contractility in real time remains a significant challenge.

In the context of these challenges, three papers discuss and highlight the development and significance of organ-on-a-chip platforms for disease research and drug development.^{31–33} These papers emphasize the limitations of traditional 2D cell culture models and underscore the pressing need for more accurate and reliable 3D models that can faithfully mimic human physiology's complexity. Engineering challenges and advancements in constructing platforms that integrate multiple aspects of human organ systems are also emphasized. Furthermore, the papers delve into the specific attributes and capabilities of each platform, such as its capacity to model intricate environments such as the tumor microenvironment, support neurosphere culture, and enable high-throughput screening. As cardiomyocytes, like other mammalian cells, adhere to the substrate for growth in 3D cell culture, these

platforms present a potential avenue for their optimal development.

Herein, we propose a perfusion-based cell culture platform for real-time measurement of contractility in cultured cardiomyocytes and assessment of drug-induced cardiotoxicity. The perfusion system was implemented to address issues related to temperature fluctuations during medium replacement in cell culture and the lack of timely medium replacement, which could affect nutrient supply. The platform comprises functional well plates, an array of SU-8 cantilevers, and peristaltic pumps. The effect of perfusion on the growth and maturation of cultured cardiomyocytes was systematically investigated using immunocytochemistry (ICC) and western blot techniques. Moreover, the proposed platform was employed to evaluate drug-induced cardiotoxicity in cultured cardiomyocytes. Adverse effects of cardiovascular drugs, specifically verapamil and quinidine, were systematically investigated at different concentrations by measuring the contractile changes of cardiomyocytes cultured with and without the perfusion system. This novel approach plays a crucial role in the development of a reliable *in vitro* cardiac cell-based platform that can effectively regulate the pH of the culture medium and pave the way for improved disease modeling and drug testing.

2. Materials and methods

2.1. Development of the drug-screening platform with the perfusion system

The proposed drug-screening platform with the perfusion system is schematically illustrated in Fig. 1. The device consists of three components: (i) a Peltier-based refrigeration system for culture medium storage, (ii) a pump system for continuously feeding and replacing the culture medium, and (iii) an incubator system for cell culture. First, the Peltier refrigerator was constructed using a Peltier device to accommodate a 6 °C environment. A cooling pad was installed on the top side of the Peltier device to generate the cooling effect and a cooling fan was installed on the bottom to dissipate the heat generated inside the Peltier device, respectively. A custom-designed circuit board was used to supply the refrigeration system with a constant voltage of 5 V. The various parts of the developed Peltier refrigeration system are shown in Fig. S1.† The Peltier refrigeration system can store 150 mL of culture medium for 7 days of use.

The design and construction of the functional well plate cover for culture medium perfusion is shown in Fig. 2. The functional well plate cover was made up of polystyrene and consisted of three parts: the cover, an inlet channel, and an outlet channel. The functional well plate cover was designed to be attached to a commercial 12 D/C well plate (SPL Life Science, Korea). The culture medium in the well plates was replaced continuously through the inlet and outlet channels at a flow rate of 1.39 $\mu\text{L min}^{-1}$ using a peristaltic pump. The cell culture medium was dropped from the top to avoid any shear stress on the cardiomyocytes that could result in reduced

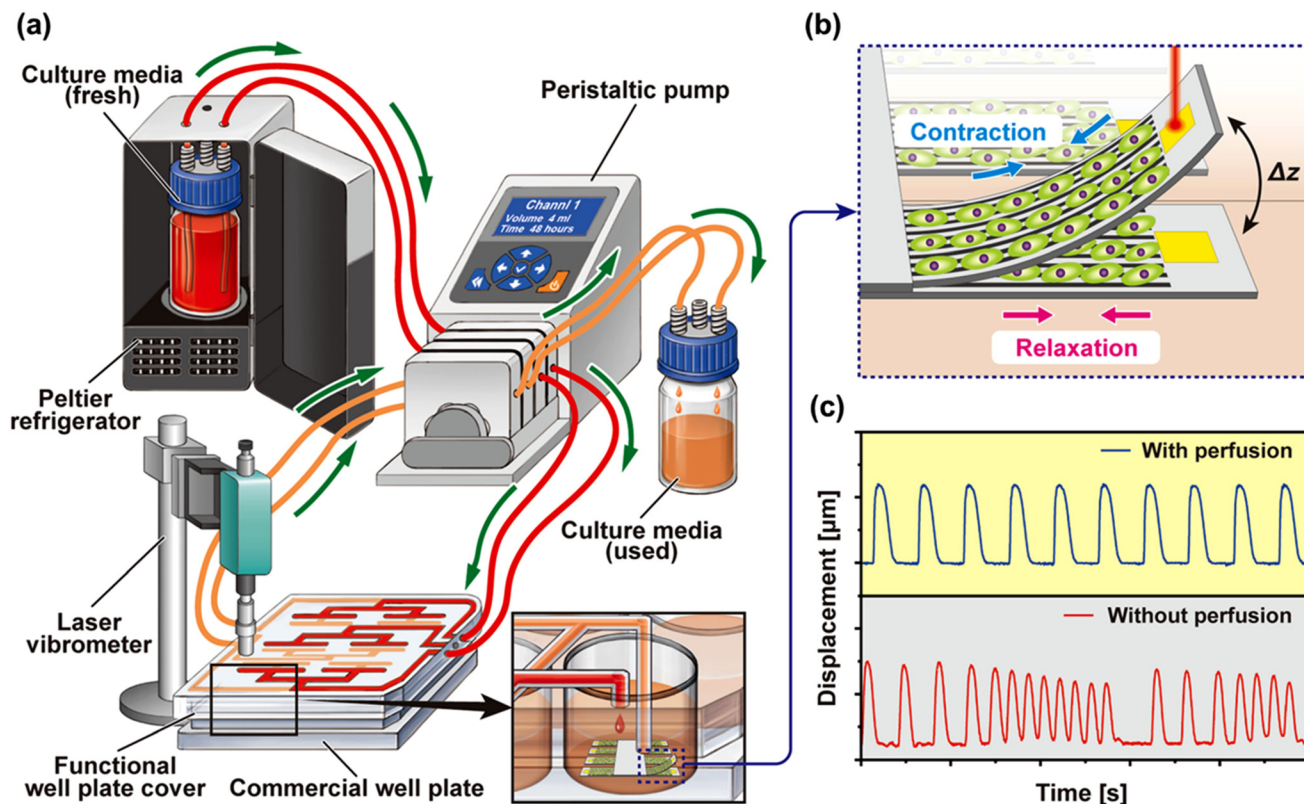


Fig. 1 Schematic illustrating the proposed drug screening platform with the culture medium perfusion system. (a) A Peltier-based refrigeration system for storage of the culture medium and a peristaltic pump for continuously feeding and replacing the culture medium. (b) Functional well plates with SU-8 cantilevers for cell culture and monitoring. (c) A laser vibrometer was used to measure the displacement of the SU-8 cantilever caused by the contraction and relaxation of the cultured cardiomyocytes with and without the perfusion system.

adhesion between the cells and the substrate. The culture medium was supplied to each well *via* the inlet channel and with the same glucose content inside the well through an independent inlet channel design. The by-products of cell activity were removed through the outlet channel located at 5 mm from the bottom surface of the functional well plates.

2.2. Fabrication of the SU-8 cantilever

Details of the fabrication process of the SU-8 cantilever are schematically illustrated in Fig. S2.† In a typical fabrication process, first, a 300 nm-thick silicon dioxide (SiO_2) sacrificial layer was deposited on a 4 inch n-type (100) silicon (Si) wafer through a wet oxidation method. Then, 16 μm -thick SU-8 3010 was spin-coated on the Si wafer at 1000 rpm for 40 s to form an 8-cantilever structure. Subsequently, the SU-8 3010-coated Si wafer was soft baked at 95 °C for 10 min using a preheated hot plate. The Si wafer was then exposed to ultraviolet light (UV) using a mask aligner for 10 s. Post-exposure bake was conducted at 65 °C for 1 min and at 95 °C for 4 min. The cantilever pattern was then developed for 6 min using an SU-8 developer and rinsed using isopropyl alcohol (IPA, Sigma). The dimensions of the cantilever were 6000 μm in length, 2000 μm in width, and 16 μm in thickness. A gold (Au) reflector pattern was coated at the free end of the cantilever using a lift-off process (AZ5214E, Sigma). To create a groove structure,

SU-8 2002 was coated at 3000 rpm for 40 s and soft baked at 95 °C for 1 min using a preheated hot plate. Subsequently, the substrate was exposed to UV for 7 s using a mask aligner. Post-exposure bake was performed at 95 °C for 2 min. Then, the groove pattern with 3 μm width, 3 μm pitch distance, and 1 μm thickness was developed using the SU-8 developer for 1 min and rinsed with IPA. The cantilever body was created by spin-coating SU-8 2050 at 1200 rpm for 40 s. The substrate was soft baked at 65 °C for 2 min and at 95 °C for 30 min using a preheated hot plate and then subjected to UV treatment for 16 s, followed by post-exposure bake at 65 °C for 5 min and at 95 °C for 12 min using a preheated plate. The pattern was developed with the SU-8 developer for 15 min. The final cantilever body thickness was ~ 150 μm . The prepared cantilever device was then released from the SiO_2 sacrificial layer using a buffered oxide etchant (6:1). The extracellular matrix fibronectin (50 mg mL^{-1} , Corning) was coated on the cell culture substrate to improve cardiomyocyte adhesion and was incubated at 37 °C for 1 h. The SU-8 cantilevers were washed three times with Dulbecco's phosphate buffered saline (Welgin). The eight SU-8 cantilevers were placed in a single well to improve the reliability of the measured displacement data and were bonded to a well plate using PDMS to minimise the movement caused by the flow between the perfusion experiments.

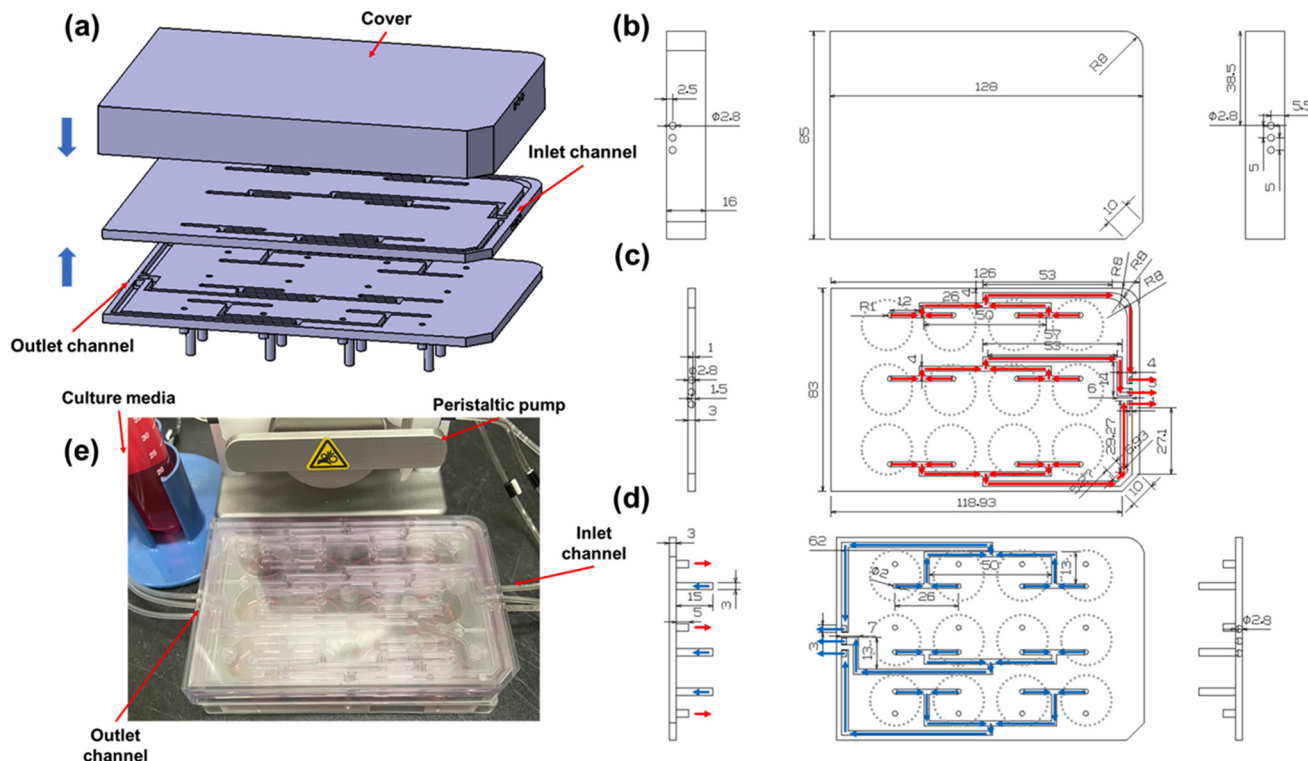


Fig. 2 Functional well plate for culture medium perfusion; (a) schematic of a functional well plate with perfusion lines, (b) cover, (c) inlet channel, (d) outlet channel, and (e) optical image of a manufactured functional well plate.

2.3. Neonatal rat ventricular myocyte isolation and cell culture

All animal experiments were performed following protocols approved by the Animal Ethics Committee of Chonnam National University in accordance with the Principles of Laboratory Animal Care and specific national laws (license number: CNU IACUC-YB-2022-29). The isolation of NRVM was performed as described previously.³⁴ Cardiomyocytes were isolated from a 1 day-old neonatal rat (Sprague-Dawley). The isolated cardiomyocytes were cut into small pieces and dispersed into single cells using enzyme solutions (collagenase 0.4 mg mL⁻¹, Northington; pancreatin 0.6 mg mL⁻¹, Sigma-Aldrich) for 2 h in a 37 °C shaking incubator. Cardiomyocytes were separated into single cells by centrifugation of the fibroblast and cardiomyocyte layers using Percoll solutions. The isolated cardiomyocyte layer was pre-plated to obtain high-purity cardiomyocytes. The cardiomyocytes were cultured in the SU-8 cantilever at a density of 800 mm⁻². The cultured cells were placed in an incubator for cell culture growth. For this purpose, a stage-top incubator (Live cell Instrument, Chamlyde WP) was used, and the temperature inside the incubator was maintained at 37 °C with 5% CO₂.

2.4. Immunocytochemistry staining and western blot analysis

Cardiomyocyte maturation was evaluated through ICC and western blot analysis. The cardiomyocytes cultured with and

without the perfusion system were treated with paraformaldehyde (3.7%, Sigma-Aldrich) and Triton X (0.2%, Sigma-Aldrich) at 25 °C for 10 min and then washed with a phosphate-buffered saline (Takara) solution. Subsequently, the cardiomyocytes were treated with a 3% bovine serum albumin (BSA, Sigma-Aldrich) solution at 25 °C for 40 min. A 1% BSA solution mixed with primary antibodies (monoclonal anti α -sarcomere actin and Cx43) was added to the cardiomyocytes and incubated at 25 °C for 120 min. Then, secondary antibodies (Alexa Fluor 488 and 568 goat anti-mouse IgG modified) were mixed with 1% BSA solution and added to the cardiomyocytes and incubated at 25 °C for 90 min. Finally, 4',6-diamidino-2-phenylindole (DAPI) solution was added to the cardiomyocytes and incubated at 37 °C for 15 min.

2.5. Measurement of the contractility of cultured cardiomyocytes

Cantilever displacement caused by the contraction and relaxation of the cultured cardiomyocytes was measured at the free end of the cultured cantilever using a laser vibrometer. The LabVIEW-assisted laser vibrometer measures the cantilever displacement at nanoscale accuracy. The laser vibrometer detects light reflected from the Au reflector patterned at the free end of the cantilever, which is subject to frequency changes proportional to the cantilever movement. From the detected frequency shift, the software calculates the cantilever displacement. The Cx43 protein was analyzed using stained

images obtained through a confocal microscope. The images were converted to grayscale using ImageJ software, and the resulting intensities were measured for signal values above a certain threshold. A control sample was created based on day 7, and intensity changes were measured for each day. Additionally, to minimize intensity variations due to differences in cell numbers, similar numbers of DAPI regions were selected and analyzed.

3. Results and discussion

3.1. Preliminary characteristics of the fabricated drug-screening platform

The Peltier refrigerator fabricated using a Peltier device was maintained at $\sim 7^\circ\text{C}$ for storing the culture medium. Therefore, we first investigated the temperature distribution inside the Peltier refrigerator as a function of time (Table S1†). The temperature distribution inside the Peltier refrigerator, such as in the central, left, right, bottom, and top sides, was measured over 48 h. The temperature of the lower part close to the inlet was higher than that of the surrounding area, and the centre of the cooling pad recorded the lowest temperature. The Peltier refrigerator reached the desired temperature ($6.0 \pm 0.57^\circ\text{C}$) in 48 h. Then, the effect of various environments such as warm bath, 5% CO_2 incubator, room temperature, and the proposed Peltier refrigerator on the pH of the culture medium was investigated as shown in Table S2.† The three most important conditions for culturing cardiomyocytes are as follows: (1) maintaining a temperature of 37 degree Celsius, (2) 100% humidity, and (3) 5% CO_2 concentration. The incubator system for cardiomyocyte culture satisfies all three conditions. CO_2 is supplied to the incubator through a gas cylinder consisting of 100% CO_2 , and its concentration is regulated to 5% using a CO_2 sensor. Apart from the 5% CO_2 , the remaining 95% consists of normal air, which includes oxygen and nitrogen, thereby avoiding the issue of hypoxia during cell culture. Initially, the culture medium was stored in specific environments, including a 37°C warm bath, a 37°C incubator with 5% CO_2 , the ambient environment (25°C), and a custom-developed Peltier refrigerator set at 6°C . These carefully selected storage conditions were essential to meet the required conditions for the culture medium, ensuring its optimal properties and supporting successful cell growth. The culture medium was stored in four different environments, including a 37°C warm bath, a 37°C incubator with 5% CO_2 , the ambient environment (25°C), and a developed Peltier refrigerator at 6°C . The culture medium stored in the warm bath showed an initial pH of 7.52, but it gradually increased to 7.7 after 48 h, while the pH of the culture medium maintained in the incubator decreased to 7.4 after 48 h. The pH of the culture medium stored under ambient conditions reached 7.66 after 48 h. Lastly, the pH of the culture medium stored in the developed Peltier refrigerator remained unchanged after 48 h, indicating that the culture medium can be stored in the fabricated Peltier refrigerator for a longer duration.

3.2. Effect of perfusion on cardiomyocyte maturation

The effect of perfusion on cardiomyocyte maturation was assessed with ICC and western blot experiments. The cardiomyocytes were cultured on an SU-8 substrate with and without culture medium perfusion, and the culture medium was replaced every 72 h for the cardiomyocytes without perfusion. The maturation of cardiomyocytes cultured on various SU-8 substrates was monitored with fluorescence staining analysis on days 7, 14, 21, and 28 of the culture periods, as shown in Fig. S3.† DAPI, α -actinin, and Cx43 are the key proteins whose expressions indicate cardiomyocyte maturation. The sarcomere length and Cx43 of cardiomyocytes at different culture periods are shown in Fig. 3a and b. The sarcomere lengths of cardiomyocytes on day 7 were 1.84 ± 0.09 and $1.86 \pm 0.07\ \mu\text{m}$ for without and with perfusion, respectively. The sarcomere lengths on days 14, 21, and 28 were found to be 1.88 ± 0.04 , 1.877 ± 0.05 , and $1.875 \pm 0.02\ \mu\text{m}$ for cells without perfusion and 1.9 ± 0.05 , 1.899 ± 0.03 , and $1.895 \pm 0.04\ \mu\text{m}$ for cells with perfusion. The cardiomyocytes obtained from both SU-8 substrates showed the maximum sarcomere length on day 14 after incubation. Cx43 is a protein that represents gap junction intercellular communication, which also represents cell death, proliferation, and differentiation. Cx43 of cardiomyocytes was also measured every 7 days to determine Cx43 protein expression in the cells. The intensities of Cx43 of cardiomyocytes cultured on the SU-8 surface without and with perfusion on days 7, 14, 21, and 28 were 1 ± 0.01 , 1.15 ± 0.02 , 1.12 ± 0.01 , and 1.1 ± 0.02 , and 1.03 ± 0.03 , 1.21 ± 0.01 , 1.2 ± 0.02 , and 1.198 ± 0.01 , respectively (Fig. 3b). Similar to sarcomere length, the maximum Cx43 intensity was measured on day 14 after incubation, following which the value decreased. Thus, sarcomere length and Cx43 protein expression were found to be greater in cardiomyocytes with culture medium perfusion.

Protein expression in the cardiomyocytes was further investigated with western blot analysis, as shown in Fig. 3c. The reference protein was β -actinin, and contractile-related proteins (α -actinin) and surface adhesion proteins (vinculin) were analyzed. α -Actin and vinculin protein expressions of cardiomyocytes cultured on the SU-8 substrate with perfusion increased by 10% and 50% compared with those of cardiomyocytes cultured without perfusion. Fluorescence staining and western blot analyses demonstrated that cardiomyocytes cultured on the SU-8 substrate with perfusion exhibited better maturation than those cultured without perfusion.

3.3. Effect of culture medium pH and glucose content on the beat rate of cultured cardiomyocytes

Changes in the pH of the culture medium were assessed as a function of culture day, as shown in Fig. 3d. The culture medium was manually replaced every 3 days for cardiomyocytes without perfusion (control state). The culture medium in the perfusion system was maintained at a pH of 7.41 ± 0.4 , whereas the pH of the culture medium without perfusion decreased as the culture day increased. The pH value of the cell culture medium would decrease according to the activity

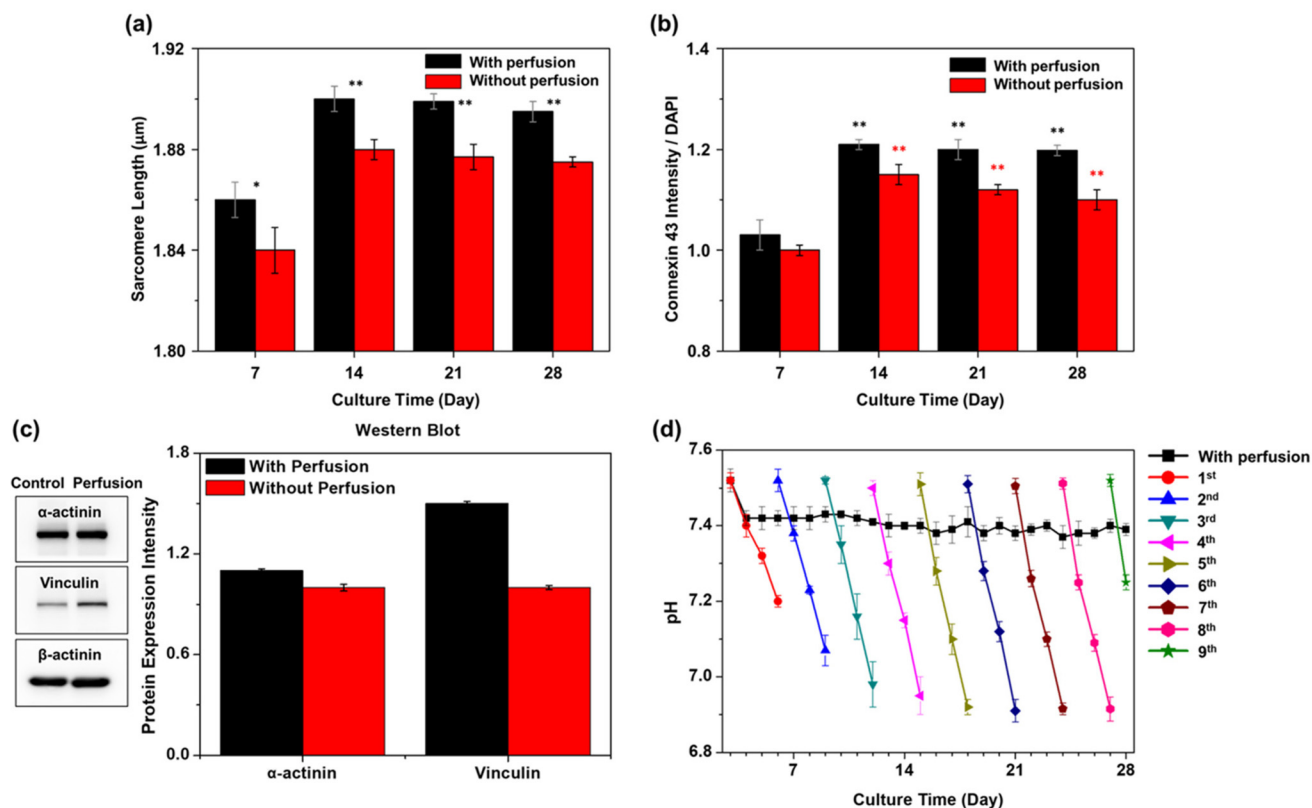


Fig. 3 Sarcomere length and Cx43 intensity changes of cardiomyocytes according to culture medium perfusion: (a) sarcomere length change according to perfusion and culture day; (b) Cx43 intensity change according to perfusion and culture day. (c) Western blot analysis according to the perfusion system; α -actinin and vinculin protein expression with the same intensity of β -actinin. (d) pH value changes with and without culture medium perfusion. The bars and error bars indicate the mean \pm s.d. ($n = 6$). ** $P < 0.01$.

and growth of cardiomyocytes and was the lowest (6.91 ± 0.03) on day 21 following cell culture. The glucose content in the culture media with and without the perfusion system was measured according to the culture day, as shown in Fig. S4.† The culture medium consisted of 340 mL of Dulbecco's Modified Eagle Medium (Welgin), 25 mL of foetal bovine serum (Gibco), 50 mL of horse serum (Gibco), and 5 mL of penicillin–streptomycin (Gibco). The initial glucose content in the culture medium was $\sim 25 \pm 1.3$ mM. The glucose concentration of the culture medium with perfusion showed the same value of 25 ± 1.3 mM as the initial state on days 6 and 9. The glucose concentration of the culture medium without the perfusion system decreased with the increase of incubation period. The glucose concentration on days 6 and 9 was found to be 7.7 ± 0.4 and 3.8 ± 1.9 mM, respectively. We also measured the beat rate of cardiomyocytes cultured on the SU-8 cantilever with and without the perfusion system, as shown in Fig. S5.† The cardiomyocytes cultured on the SU-8 cantilever without the perfusion system showed an irregular beat rate, whereas the beat pattern was regular in cardiomyocytes with the perfusion system, indicating that the pH of the culture medium has a significant effect on the maturation and beat rate of cultured cardiomyocytes. The nutrients and by-products required for cell activities increased as the culture day pro-

gressed. The manually replaced medium was unable to keep up with the increasing requirements, and as a result, cardiomyocytes cultured without perfusion showed less maturity and growth than those with the perfused medium (Fig. 4).

3.4. Relative contractile force changes with respect to culture medium perfusion

The cardiomyocytes were cultured on SU-8 cantilevers. The cantilevers were fixed on a commercial 12 D/C well plate. The cardiomyocytes started beating from day 1 after incubation and showed synchronised beats with adjacent cells by day 3. The cardiomyocytes cultured on the cantilever were fully synchronised from day 5, resulting in cantilever displacement. Cantilever displacement increased with the culture period and showed maximum displacement on day 12, following which it decreased with the culture day (Fig. 5a). The decrease in cantilever displacement may be attributable to the detachment and aggregation of cardiomyocytes, which prevents the cardiomyocytes from transmitting sufficient contractile force to generate cantilever displacement. The cardiomyocytes cultured without and with the perfusion system produced maximum cantilever displacement on day 12, which was 18 ± 0.5 and 18.5 ± 0.2 μ m, respectively. Cardiomyocytes cultured with perfusion on the SU-8 cantilever were continuously supplied with fresh medium

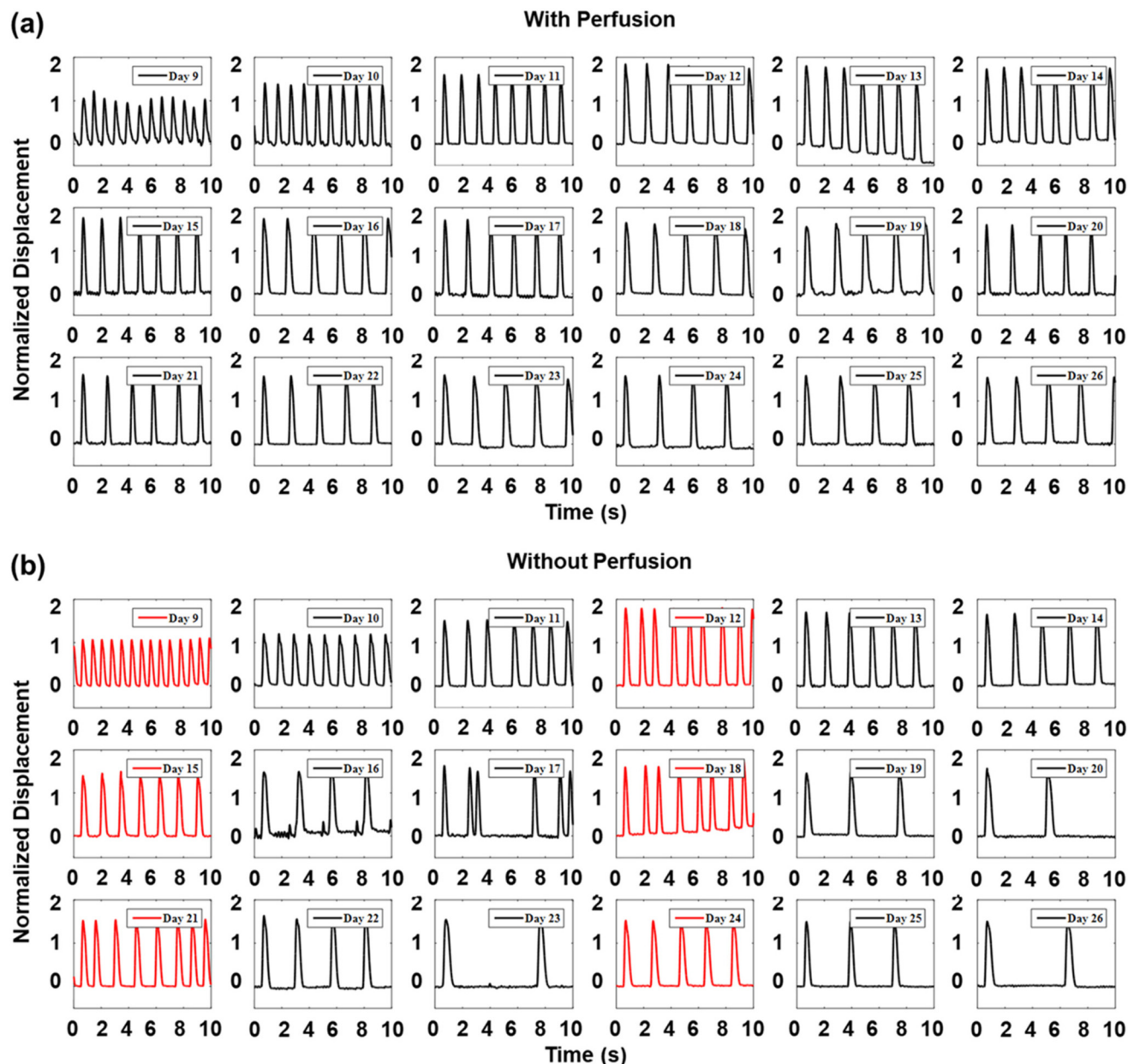


Fig. 4 Relative contractile force of cardiomyocytes cultured on the SU-8 cantilever with and without perfusion. (a and b) Real-time traces of cantilever displacement resulting from the contraction and relaxation of the cultured cardiomyocytes on different culture days. The contractile force of cardiomyocytes on the day the culture medium is changed is marked in red.

and nutrients, resulting in a higher contractile force than those cultured without perfusion. The beat rate of the cardiomyocytes according to the culture period is shown in Fig. 5b. The beat rate of the cardiomyocytes decreased with the culture period because of cardiomyocyte maturation. However, cardiomyocytes cultured on the SU-8 cantilever without perfusion exhibited significant fluctuations in the beat rate each time the culture medium was replaced, whereas the beat rate of the cardiomyocytes cultured with perfusion showed no significant variations and periodically decreased as the culture day progressed.

Fig. S6† shows the single beating characteristics of the cardiomyocytes. The cantilever displacement is determined by the contraction and relaxation characteristics of cultured cardiomyocytes. From the cantilever displacement, several parameters such as relative contractile force, beating duration, rise time, and decay time can be determined. The rise time of cardiomyocytes is defined as the time required for the cardiomyocytes to reach 90% of their maximum from the baseline, while the decay time is defined as the time required for the cardiomyocytes to return to 10% of their initial value. The beat rate is defined as the period taken by the cardiomyocytes to reach

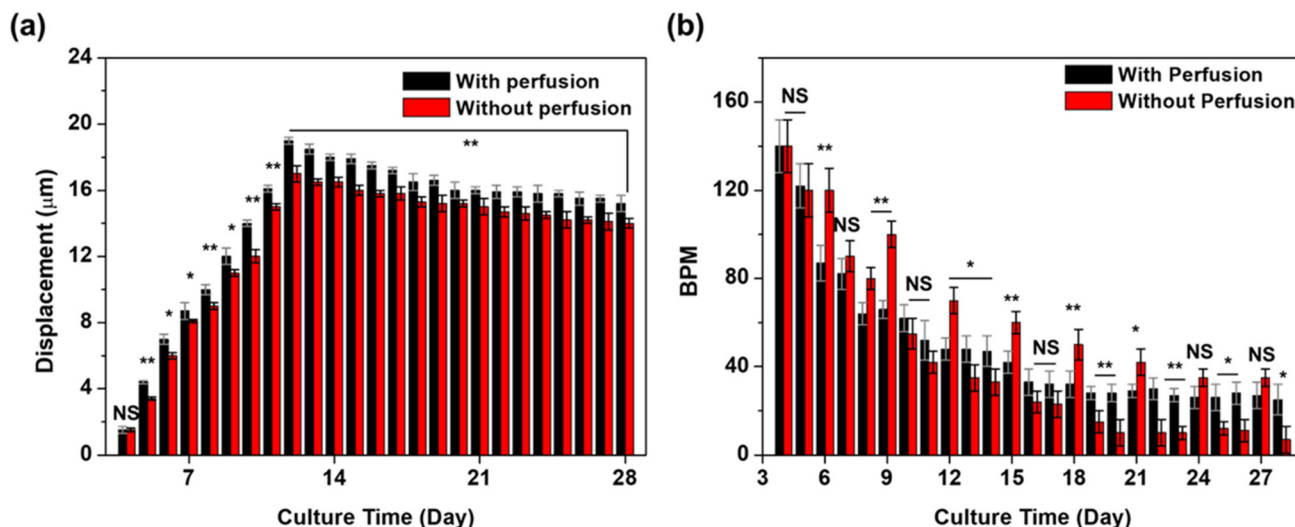


Fig. 5 Displacement and beat rate changes with culture day of samples with and without perfusion: (a) change in cantilever displacement; (b) change in beat rate. The bars and error bars indicate the mean \pm s.d. ($n = 6$). NS (non-significant), * $P < 0.05$, ** $P < 0.01$.

their maximum contractile force from the beginning of the contraction to its end point. Contraction characteristics of the cardiomyocytes with and without perfusion on day 16 are shown in Fig. S7.† The pH values of the culture medium with and without perfusion were 7.38 ± 0.03 and 7.28 ± 0.036 , respectively. The cardiomyocytes cultured with perfusion showed regular contractile characteristics, while those without perfusion showed irregular beat rates. Besides, the cardiomyocytes cultured on the SU-8 cantilever without perfusion showed significantly lower relative contractile force than those cultured with the perfusion system. The relative contractile force and beat rate of the cardiomyocytes on day 17 are shown in Fig. S8.† In the case of cardiomyocytes cultured without the perfusion system, measurements were taken 48 h after culture medium replacement. The pH of the culture medium with and without perfusion was 7.39 ± 0.036 and 7.1 ± 0.04 , respectively. Irregular beat rates were measured in the cardiomyocytes cultured without perfusion. These analyses demonstrated that maintaining the pH and glucose concentration of the culture medium regulates the beat rate and enhances the relative contractile force of cardiomyocytes.

3.5. Effect of ion channel-related drugs on cardiomyocytes with perfused culture medium

Finally, the proposed drug-screening platform was used to examine drug-induced adverse effects on cardiomyocytes cultured with and without the perfusion system. The adverse effect of two typical cardiovascular ion channel-related drugs, namely verapamil and quinidine, on the contractile properties of cardiomyocytes was assessed. The contractility of cardiomyocytes was determined by treating them with drugs in a concentration-dependent manner and measuring their response. Verapamil is an L-type calcium channel blocker that decreases cardiomyocyte contraction and heart rate as drug

concentration increases. The culture medium was changed at least 4 h prior to drug screening, and the contraction characteristics of cardiomyocytes cultured with and without perfusion were measured for five different drug concentrations, as shown in Fig. 6. Real-time traces of cardiomyocyte contractility at different verapamil concentrations ranging from 1 nM to 1 μM are shown in Fig. S9a and b.† The bar plot summarises the relative contractile force and beat rate of cardiomyocytes at different verapamil concentrations (Fig. 6a and b).

The relative contractile force of the cardiomyocytes decreased as the concentration of verapamil increased. Although verapamil concentration decreased the contractile force of both cardiomyocytes cultured with and without the perfusion system, cardiomyocytes cultured with the perfusion system responded better to verapamil and displayed a higher contractile force than those cultured without perfusion. The contractile force of the cardiomyocytes decreased by ~50% in those with perfusion and by 58% in those without perfusion. The initial contractile force of the cardiomyocytes cultured with the perfusion system was retained after culture medium replacement. In contrast, the contractile force of cardiomyocytes cultured without the perfusion system was not fully restored. The beat duration of the cardiomyocytes with and without perfusion at different verapamil concentrations is shown in Fig. 6b. The beat duration of the 100 nM-treated cardiomyocytes increased by ~240 ms in the samples with perfusion and ~296 ms in the samples without perfusion. As the drug concentration increased, the contractile force and contraction cycle of the cardiomyocytes were prolonged, and the cardiomyocytes cultured with the perfusion system responded to the drug more rapidly than those cultured without perfusion. Since the IC_{50} of verapamil was 136 nM, the contractile force and beat duration of the cardiomyocytes were considerably altered at verapamil concentrations of ≥ 100 nM. After

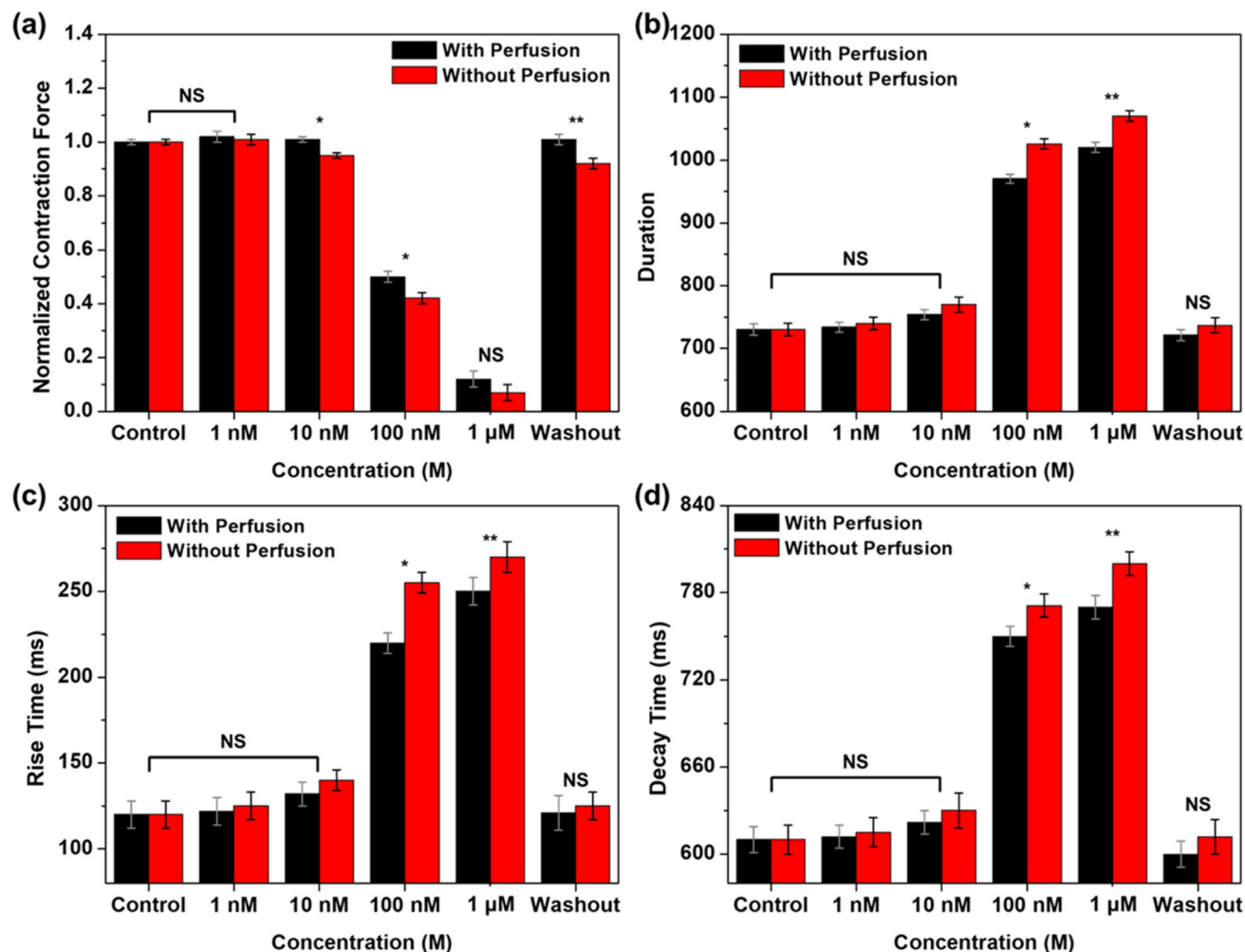


Fig. 6 Effect of the ion channel-related cardiac drug verapamil on the contractility of cardiomyocytes. (a and b) Relative contractile force and beat duration of cardiomyocytes cultured with and without perfusion at different verapamil concentrations. (c and d) Rise and decay times of the cardiomyocytes cultured with and without perfusion according to different concentrations of verapamil. The bars and error bars indicate the mean \pm s.d. ($n = 6$). NS (non-significant), * $P < 0.05$, ** $P < 0.01$.

culture medium replacement, the cardiomyocytes returned to their normal condition. The rise and decay times of the cardiomyocytes cultured with and without perfusion at different verapamil concentrations are shown in Fig. 6c and d. When the cardiomyocytes were treated with 100 nM verapamil, the rise time of the cardiomyocytes increased by ~ 100 ms in the sample with perfusion and by 135 ms in the sample without perfusion (Fig. 6c), whereas the decay time increased by 140 ms and 161 ms, respectively (Fig. 6d).

Quinidine is a sodium ion channel blocker that produces a negative inotropic and chronotropic effect in cardiomyocytes. The effect of different quinidine concentrations in the range of 10 nM–100 μ M on cardiomyocytes is shown in Fig. 7. Real-time traces with and without perfusion according to different quinidine concentrations are shown in Fig. S10a and b.† The relative contractile force of cardiomyocytes at different quinidine concentrations is shown in Fig. 7a. The relative contractile force of cardiomyocytes is not significantly altered with

increasing quinidine concentration until 10 μ M. Further increasing the quinidine concentration rapidly decreased the relative contractile force of the cardiomyocytes. The relative contractile force of the cardiomyocytes decreased by $\sim 60\%$ in the samples with perfusion and 75% in the samples without perfusion. After replacing the culture media, cardiomyocytes cultivated with and without perfusion regained their natural contractile characteristics. The beat duration of the cardiomyocytes according to different quinidine concentrations is shown in Fig. 7b. Nonetheless, increasing quinidine concentration has no discernible effect on the beat length of cardiomyocytes in either circumstance. The rise time and decay times of the cardiomyocytes cultured with and without the perfusion system are shown in Fig. 7c and d. The rise time of the cardiomyocytes decreased with increasing quinidine concentration. At a concentration of 1 μ M, the rise time of cardiomyocytes with and without perfusion decreased by 15 ms and 24 ms, respectively. The decay time of cardiomyocytes cultured with

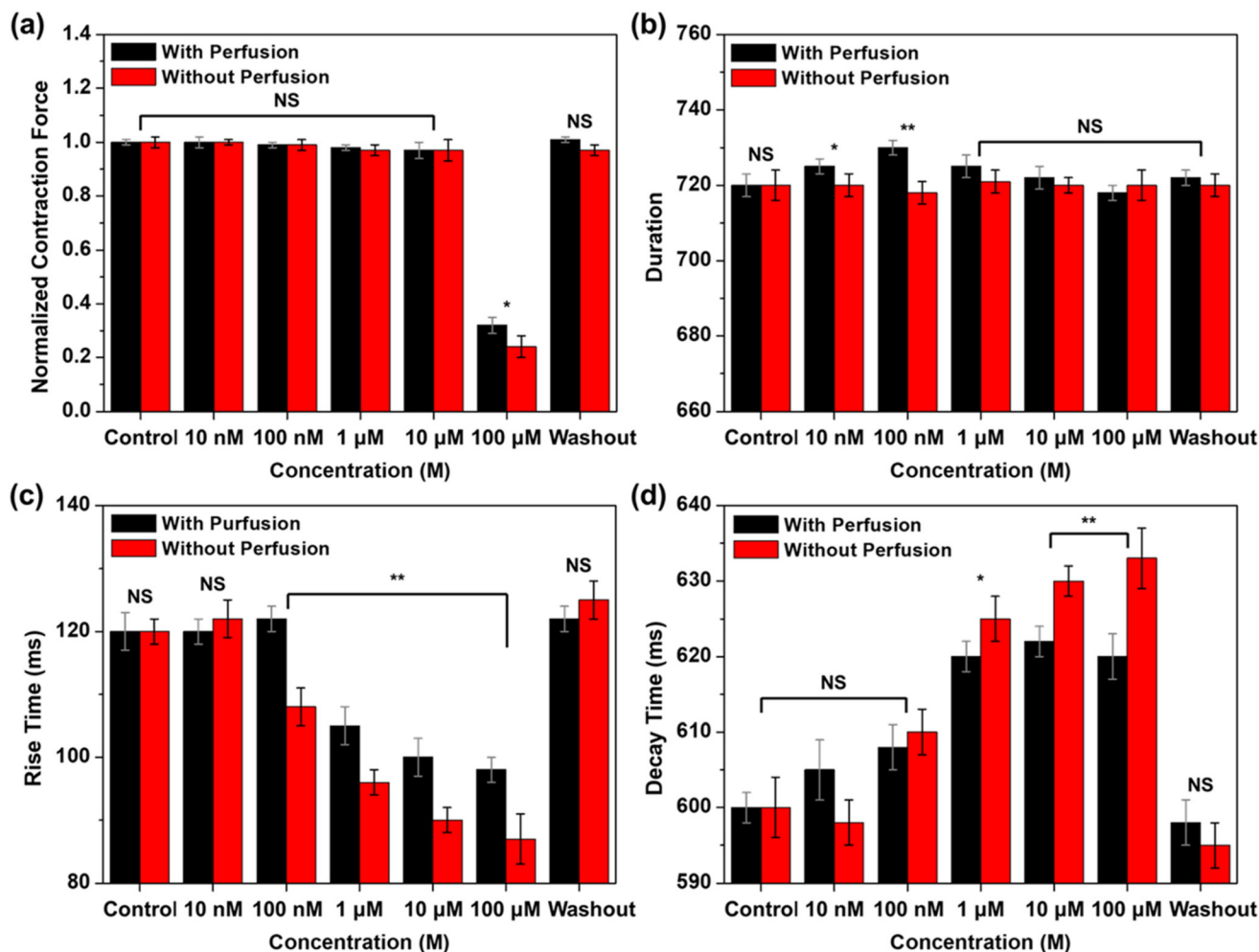


Fig. 7 Effect of the ion channel-related cardiac drug quinidine on the contractility of cardiomyocytes. (a and b) Relative contractile force and beat duration of cardiomyocytes cultured with and without perfusion at different quinidine concentrations. (c and d) Rise and decay times of cardiomyocytes cultured with and without perfusion at different concentrations of quinidine. The bars and error bars indicate the mean \pm s.d. ($n = 6$). NS (non-significant), * $P < 0.05$, ** $P < 0.01$.

and without perfusion increased with increasing quinidine concentration. The decay time of cardiomyocytes increased by 20 ms in the samples with perfusion and 25 ms in the samples without perfusion. Since the IC_{50} of quinidine was found to be $1.068 \mu\text{M}$, the rise and decay times of cardiomyocytes were considerably altered at quinidine concentrations of $\geq 1 \mu\text{M}$. These studies demonstrated that cardiomyocytes cultured with the perfusion system displayed better drug dose-response characteristics owing to the improved maturation, stable beating behaviour, and enhanced contractile force.

Differences between verapamil and quinidine include their effects on contractile properties, with verapamil impacting contractile force and beat duration and quinidine inducing negative inotropic and chronotropic effects. In terms of response, verapamil's impact is reversible upon medium replacement, while quinidine's effect persists. Additionally, verapamil's effect becomes apparent at lower concentrations compared to that of quinidine. Quinidine demonstrates a stronger

negative influence on contractile force, particularly notable in non-perfused cultures. Furthermore, while quinidine has a minimal effect on beat duration, verapamil significantly alters it. The trends in rise and decay times also vary between the two drugs.

4. Conclusion

In this study, we propose a perfusion system comprising an SU-8 cantilever for improved contractility and uniform beat rate of cardiomyocytes. The proposed perfusion system consisted of a Peltier refrigerator, peristaltic pumps, a functional well plate, a stage-top incubator, and a laser vibrometer-based contraction force measurement system. Fluorescence staining confirmed that cardiomyocytes cultured in the perfusion system showed increased sarcomere length by up to $0.02 \mu\text{m}$ and Cx43 intensity by 4.9%, and the western blot results

showed increased α -actinin and vinculin protein expression by 10% and 50%, respectively. Cardiomyocytes cultured in the perfusion system maintained a constant pH of the culture medium compared with the control group and showed higher contractility and a uniform heart rate. The reactivity testing of two drugs, verapamil and quinidine, showed at least 10% less change in contractility and heart rate in perfusion-cultured cardiomyocytes. The use of a cell culture medium perfusion system comprising the proposed SU-8 cantilever array can contribute to improved reliability of drug reactivity testing with regular heartbeat and biological maturation of cardiomyocytes.

Author contributions

Jongyun Kim: conceptualisation; investigation; formal analysis; data curation; visualisation; writing – original draft. Arunkumar Shanmugasundaram: investigation; writing – original draft; reviewing and editing. Dong-Su Kim: formal analysis; data curation. Yun-Jin Jeong: formal analysis; data curation. Pooja P. Kanade: investigation; writing – original draft; editing. Eung-Sam Kim: investigation; writing – original draft; editing. Bong-Kee Lee: investigation; writing – original draft; editing. Dong-Weon Lee: project supervision; funding acquisition; supervision; conceptualisation; methodology; software; validation; formal analysis; data curation; visualisation; writing – original draft, reviewing, and editing.

Conflicts of interest

There are no conflicts to declare.

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