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Surface-patterned SU-8 cantilever arrays for preliminary screening of cardiac toxicity

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ABSTRACT

Arrays of a μgrooved SU-8 cantilever were utilized to analyze changes in the contraction force and beating frequency of cardiomyocytes *in vitro*. The longitudinally patterned μgrooves facilitates alignment of cardiomyocytes on top of the SU-8 cantilever, which increases the contraction force of cardiomyocytes by a factor of about 2.5. The bending displacement of the SU-8 cantilever was precisely measured in nanoscale using a laser-based measurement system combined with a motorized *xyz* stage. The cantilever displacement due to contraction of the cardiomyocytes showed the maximum on day 8 after their cultivation. Following preliminary experiments, Isoproterenol, Verapamil, and Astemizole were used to investigate the effect of drug toxicity on the physiology of cardiomyocytes. The experimental results indicated that 1 μM of Isoproterenol treatment increased contraction force and beating frequencies of cardiomyocytes by 30% and 200%, respectively, whereas 500 nM of Verapamil treatment decreased contraction force and beating frequencies of cardiomyocytes by 56% and 42%, respectively. A concentration of less than 5 nM of the hERG channel suppression drug Astemizole did not change the contraction forces in the displacement but slightly decreased the beating frequencies. However, irregular or abnormal heartbeats were observed at Astemizole concentrations of 5 nM and higher. We experimentally conformed that the proposed SU-8 cantilever arrays combined with the laser-based measurement systems has the great potential for a high-throughput drug toxicity screening system in future.

1. Introduction

The human heart plays an integral role in circulating blood throughout the body by repeated contraction and relaxation. In recent years, frequent occurrences of abnormal symptoms such as arrhythmia due to drug side effects (Norman et al., 2008) have resulted in various studies being conducted to identify the causes (Hersch et al., 2013; Agarwal et al., 2013; Haushalter et al., 2008; Braama et al., 2010). In these studies, one of the methods used for electrophysiological measurements is the patch-clamp assay (Park et al., 2013; Barry, 1994), in which pipettes are integrated with electrodes. The method assesses drug toxicity by measuring the changes in the potassium ions of cardiomyocytes (Stuart et al., 1993; Fenwick et al., 1982; Methfessel et al., 1986; Sakmann and Neher, 1984). However, because it is only applied to a single cardiomyocyte at a time, using this method for tissue unit assessment during drug experiment is time-consuming and expensive.

In an effort to overcome these drawbacks, a number of methods to directly observe the physiology of the heart's contraction force have been proposed (Tanaka et al., 2006; Feinberg et al., 2007; Lehto et al., 2003; Liu et al., 2012; Nishimura et al., 2004). In general, these methods utilize a simple polymeric structure (You et al., 2014; Rodriguez et al., 2014) such as a micropost or a cantilever. The micropost method measures the contraction force of cardiomyocytes through the bending of a cylindrical elastomer (Chang et al., 2012; Cheng et al., 2013; Zhao and Zhang, 2006). However, this method cannot be used for real-time measurements because it involves analysis of optical microscopy images (Kim et al., 2011; Rodriguez et al., 2011; Han et al., 2012) stored on a computer. Further, the structure of a micropost makes it impossible to use this method to measure heart tissues. In addition, because a micropost's displacement is too small, the experiment results obtained are often unreliable. The cantilever method also measures the bending displacement caused by the contraction of the heart tissue, which is formed by connection among numerous cardiac cells (Feinberg et al., 2012; Kim et al., 2008; Grosberg et al., 2011; Park et al., 2005). In contrast to the micropost method, this method produces large displacements. Consequently, analysis of

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the reactions to drugs can be easily accomplished with an optical microscope. However, as in the micropost method, because the displacements are analyzed from optical microscope images, real-time measurements and measurements involving micro-displacements of less than several micrometers are difficult. Further, miniaturization and mass production are difficult because the microposts and cantilevers are usually made from polydimethylsiloxane (PDMS). The use of impedance sensor arrays (Hu et al., 2015; Wang et al., 2013) is also one of good candidates for the high-throughput drug screening applications. However, the impedance-based detection method does not directly indicate the contraction force of cardiomyocytes and the method has the potential to induce undesired side effects by the current flow.

This paper proposes the utilization of micromachined SU-8 cantilever arrays to characterize the contractibility of cardiomyocytes *in vitro* and presents the experimental results of drug-induced cardiac toxicity test for the preliminary screening purpose. The proposed SU-8 cantilever is biocompatible and photosensitive, facilitates miniaturization and mass production. Further, microgrooves (μgrooves) on the surface of the SU-8 cantilever are used to maximize the contractions of cardiomyocytes (Kim et al., 2010; Entcheva and Bien, 2005; Yu et al., 2006). A SU-8 cantilever displacement measurement system consisting of a laser vibrometer and a micro-motorized stage was constructed to measure the changes in contractibility according to cell cultivation period *in vitro*. In addition, the physiology of cardiomyocytes was measured in real time for various drugs so that the influence of drugs on changes in their contraction force and beating frequency could be precisely analyzed. The proposed cantilever system can be utilized in various fields related to high-throughput drug screening.

2. Material and methods

2.1. Fabrication of SU-8 cantilever arrays

Figure S1(a) shows the proposed SU-8 cantilever, in which μgrooves and a reflective plate designed to measure the contraction force of cardiomyocytes are integrated. Figure S1(b) shows the SU-8 cantilever fabrication process flow. In the process, an n-type (100) silicon wafer with a diameter of four inches is used as a substrate (b-1). Next, a 300 nm-thick SiO_2 layer formed through wet oxidation is used as a sacrificial layer to separate the SU-8 cantilevers from the silicon wafer (b-2). Following patterning of the cantilever shape using a 2 μm-thick SU-8 layer (b-4), a 100 nm-thick Au reflective plate is fabricated through a lift-off process (AZ5214E) (b-5). Next, a process that precisely controls the thickness of the SU-8 cantilever is then conducted using SU-8 2007 (b-6). In order to form uniform μgrooves over the upper part of the fabricated SU-8 cantilever, μgrooves with a pitch of 3 μm are fabricated using SU-8 2002 following treatment of the surface with oxygen plasma. Next, an SU-8 cantilever body with approximately 120 μm-thick is fabricated using SU-8 2050 (b-8). Finally, SiO_2 is wet-etched over the body-formed substrate using a BHF (6:1) solution, and the SU-8 cantilever fabricated on the silicon wafer is separated as shown in Fig. S3. Subsequently, through an additional process such as post-baking and liquid-based treatment, SU-8 toxicity is completely removed. To improve the adhesion force of cardiomyocytes on the SU-8 cantilever surface, 50 μg/mL concentration fibronectin (Corning) solution was coated onto the SU-8 cantilever surface for one hour. Then, the SU-8 cantilever surface was washed three times with phosphate-buffered saline (PBS, Takara).

Fig. 1 shows optical images of fabricated SU-8 cantilevers. The single SU-8 cantilever was 4000 μm length, 2000 μm width, and 20 μm thickness. Each μgroove integrated on a cantilever was 0.8 μm depth and 3 μm width, and the gap between μgrooves was 3 μm. As shown in Fig. S4, the spring constant of the SU-8 cantilevers used in the cell experiment was measured using a nano-force calibrator and a micro-XYZ stage. The SU-8 cantilever was attached to the micro-XYZ stage, which was then driven 50 μm in intervals of 10 μm in the z direction, while its end was in contact with the nano-force calibrator. Because the nano-force calculator was used to measure the forces generated at the cantilever end, it was possible to measure the spring constant of the SU-8 cantilever, which was found to be 0.354 N/m within a 5% error range.

2.2. Preparation of cardiomyocytes and geometric effects on cell alignment

All animal experiments were approved by the Animal Ethics Committee of Chonnam National University. Ventricles were harvested from one-day-old neonatal Sprague-Dawley rats. In order to digest cardiomyocytes from the ventricular tissue, the ventricular tissue was digested with a mixture of 0.4 $mgmL^{-1}$ collagenase and 0.6 $mgmL^{-1}$ pancreatin solution. The digested solution was then divided into cardiomyocyte and fibroblast layers using percoll, and the separated layers pre-plated to prepare high purity cardiomyocytes. Cardiomyocytes were cultured on the SU-8 cantilever at a density of 1000 cardiomyocytes per mm^2 in a culture solution comprising 67% Dulbecco's modified Eagle medium (DMEM, LONZA), 17% Heparin sodium salt from porcine intestinal mucosa (M199, Sigma-Aldrich), 10% Horse serum (HS, Sigma-Aldrich), 5% fetal bovine serum (FBS, Sigma-Aldrich), and 1% penicillin streptomycin (P/S, Sigma-Aldrich). The cardiomyocytes were cultivated in an incubator that maintained the temperature at 37 °C and carbon dioxide (CO_2) at 5%, and the culture solution was replaced every three days. The cardiomyocytes began to beat and synchronize 24 hours and 48 hours after culture, respectively. PDMS substrates of various morphologies were used to conduct basic experiment related to the geometry importance in cardiomyocyte culture. Figure S5 shows the characteristics of the

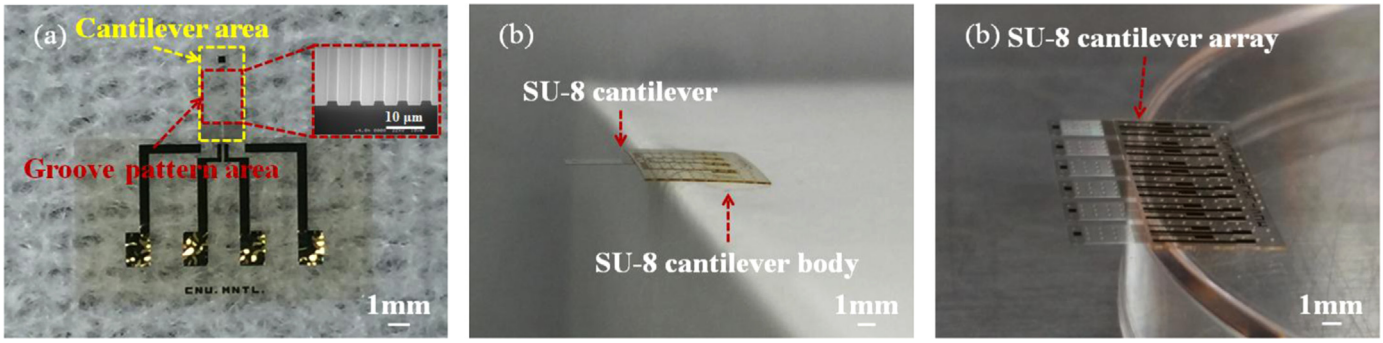

Fig. 1. Optical images of the fabricated SU-8 cantilevers: (a) Top view, (b) cross-sectional view, and (c) array of SU-8 cantilevers.

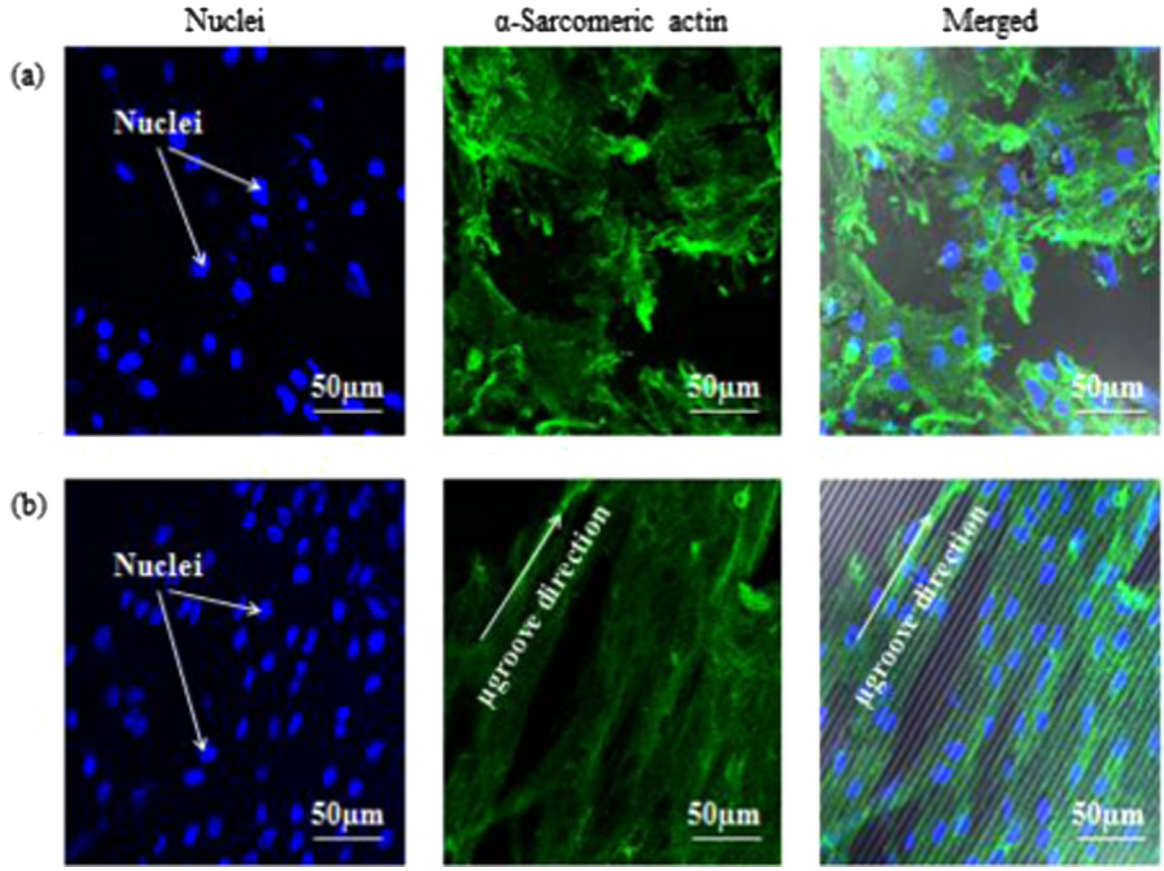

Fig. 2. Staining images of cardiomyocytes; rows: (a) cantilever without μgrooves, (b) cantilever with μgrooves showing cells aligning along the groove. The images in the leftmost column are nuclei (blue), center column images are α-sarcomeric actin (green), and rightmost column images are merged images. The grooved direction is indicated with white lines.

cardiomyocytes that grew on the PDMS substrates with and without μgrooves. As shown in the optical microscope image, the cardiomyocytes were aligned in the μgrooves' direction and generated a contraction force in the longitudinal direction of the μgrooves. In particular, after 48 hours, the contraction force increased further because of the synchronization of the cardiomyocytes. We also optimized the pitch distance by employing various μgrooves with pitches ranging from 1 μm to 10 μm and a depth of about 1 μm. We observed a slight difference in the contraction force, however, it was not able to find a significant difference between different μgrooves in the proposed range.

2.3. Laser-based measurement system

To measure the contraction force of the cardiomyocytes cultivated on the SU-8 cantilever, a laser vibrometer-based measurement system was constructed as shown in Fig. S6(a). The experimental setup for the measurement of cantilever displacement consisted of a laser vibrometer that measured displacements in nanoscale, a home-made table-top incubator that maintained the temperature of a well plate (Fig. S6(b)), and a motorized stage that moves various samples very quickly. The system precisely measured cantilever displacement and beating frequency resulting from the contractile forces of the cardiomyocytes using a position-sensitive photodiode (PSPD) sensor as a laser was irradiated on the reflective plate of the cantilever. More specifically, the laser vibrometer (Polytec GmbH) measures the projected component of the cantilever's vibration vector along the direction of the incident laser beam. For example, if aligned perpendicularly to surface of the SU-8 cantilever, the vibrometer measures the out-of-plane vibration to the surface. The vibrometer measures the amount of vibration at a single point on the surface and records the vibration characteristics as a function of time. The size of a laser spot was 1.5 μm with in-line illumination and this enables micro inspections. The minimum detectable displacement of the laser system is about 15 pm. Figure S6(c) shows the process flow employed with the laser-based cantilever displacement measurement system to characterize the contractile force of the cardiomyocytes. All devices used in the measurement system were controlled via LabVIEW and, because high-precision motor-driven stages were used, the SU-8 cantilever arrays could be measured very quickly. The contraction force of the cardiomyocytes was estimated by substituting the cantilever displacement into a theoretical equation.

3. Results and discussion

3.1. Surface-patterned SU-8 cantilever and preliminary experiment

Two different types of SU-8 cantilevers, with and without μgrooves, were used to measure the contraction force of the cardiomyocytes. First, 50 μg/mL of fibronectin (Corning) 1 mL was coated onto the prepared SU-8 cantilever for one hour at room temperature. Then, the SU-8 cantilevers were washed three times with PBS to remove the remaining fibronectin. Figure S2 shows an optical microscope images of the single SU-8 cantilever on which cardiomyocytes were grown. Figure S2(a) shows an image of the cultivation of cardiomyocytes while they were fixed on the SU-8 cantilever surface in the media. Figures S2(b) and (c) show enlarged photos of the cardiomyocytes fixed on the SU-8 cantilever

with and without μgrooves. For the SU-8 cantilever without μgrooves, the cardiomyocytes that were attached on the surface were grown isotropically, whereas for the μgroove-integrated SU-8 cantilever, cardiac cells were aligned in one direction along the μgrooves and grown anisotropically.

In order to identify the degree of alignment of the cardiomyocytes grown on the SU-8 cantilever, immunocytochemical staining was conducted. First, the cardiomyocytes were fixed using formalin solution (3.7%, 10 min, RT) and washed with PBS. Permeabilization was then accomplished with 0.2% Triton-X (Sigma-Aldrich) in PBS for 15 min at room temperature. To prevent non-specific binding of the antibody, cardiomyocytes were cultivated at room temperature for 40 min by adding 1% bovine serum albumin (1% BSA, Sigma-Aldrich). The primary antibody (monoclonal anti-actin antibody produced in mouse (α-sarcomeric, Sigma-Aldrich)) was mixed with 1% BSA in a ratio of 1:200, and the cardiomyocytes cultivated at room temperature for 90 min. Then, a secondary antibody (Alexa-Flour 488 goat anti-mouse IgG conjugate) was mixed with 1% BSA in a ratio of 1:500, and the cardiomyocytes cultivated at room temperature for 60 min. Finally, DAPI solution (4′, 6-Diamidino-2-phenylindole) was added and the cardiomyocytes cultured for 15 min at 37 °C to conduct nuclei staining. Fig. 2 shows the fluorescence staining image of the cardiomyocytes cultured on the SU-8 cantilever with and without the μgrooves. As shown in the image that merges stained nuclei and α-sarcomeric actin in Fig. 2, the cardiomyocytes and α-sarcomeric actin were aligned along the μgrooves, and the cardiomyocytes on the SU-8 cantilever with μgrooves remained in position more than those on the cantilever without μgrooves.

3.2. Cardiac toxicity screening using SU-8 cantilever arrays

The cardiomyocytes started beating 24 hours after cell culture, and synchronization between them was conducted 48 hours after culture. The cell contraction force was evaluated using the SU-8 cantilever from 96 hours to 10 days in intervals of 24 hours. In addition, the media were replaced every 72 h during the culture period of the cardiomyocytes. Fig. 3(a) shows the displacement of the SU-8 cantilever over cell culture time. The experiment results show that both SU-8 cantilevers, with and without μgrooves, had the largest contraction force on the eight day after cell cultivation. Further, the contraction force for the SU-8 cantilever with μgrooves was approximately 2.5 times that of the cantilever without μgrooves. This result occurred because the cardiomyocytes aligned in one direction along the μgrooves, thereby contracting anisotropically, as shown in the optical microscope images. This increased contraction force can increase the sensitivity of the SU-8 cantilever sensor in drug toxicity evaluations.

A stock solution comprising Isoproterenol (Calbiochem), Verapamil (Sigma-Aldrich), and Astemizole (Sigma-Aldrich) was prepared, by dissolving them in 100% ethanol and dimethyl sulfoxide (DMSO, Sigma-Aldrich) and stored at 4 °C refrigerator. Various concentrations of the drug were made by controlling the amount of ethanol. Further, the ethanol concentration of all drugs used in the experiment did not exceed 0.1%. Prior to the drug treatment test, changes in the physiology of the cardiomyocytes due to the ethanol concentration used for stock solution fabrication were verified. As shown in Fig. 3(b), the ethanol used to control the concentration of the drugs did not influence the contraction force and beating frequency of the cardiomyocytes.

To measure the reproducible contraction force of the cardiomyocytes, the size of the SU-8 cantilever, cardiomyocytes seeding density (~1,000/mm^2), and drug processing time (a few second) were made constant. In the drug treatment test, Isoproterenol, which increased the contraction force of the cardiomyocytes, Verapamil, which decreased the contraction force of the cardiomyocytes, and Astemizole known as hERG channel inhibitor, were used. It is important to choose the date of drug treatment because the contraction force of cardiomyocytes varies as a function of day as shown in Fig. 3(a). In order to verify the side effect of drugs, Verapamil was treated on day 6 (the contraction force is still increasing) after cell culture, whereas Isoproterenol was treated on day 9 (the contraction force is decreasing) after cell culture. The different date of drug treatment was due to the decided by the Changes in the contraction force and beating frequency of the cardiomyocytes due to drug concentration were measured immediately after drug treatment. Astemizole known as hERG channel inhibitor was treated on day 8 when the maximum contraction force was revealed after cell culture, and changes in the contraction force and beating frequency of the cardiomyocytes due to drug concentration were measured immediately after drug treatment. The μgroove-integrated SU-8 cantilever was used in the experiment. Changes in the physiology of the cardiomyocytes with respect to Isoproterenol (0.2, 0.5, 1, 2, 4 μM), a β-adrenalin agent; Verapamil (50, 100, 200, 500 nM), a Ca^{2+} channel inhibitor; and Astemizole (1, 2, 2.5, 5 nM), an hERG channel inhibitor, are shown in Figs. 4 and 5.

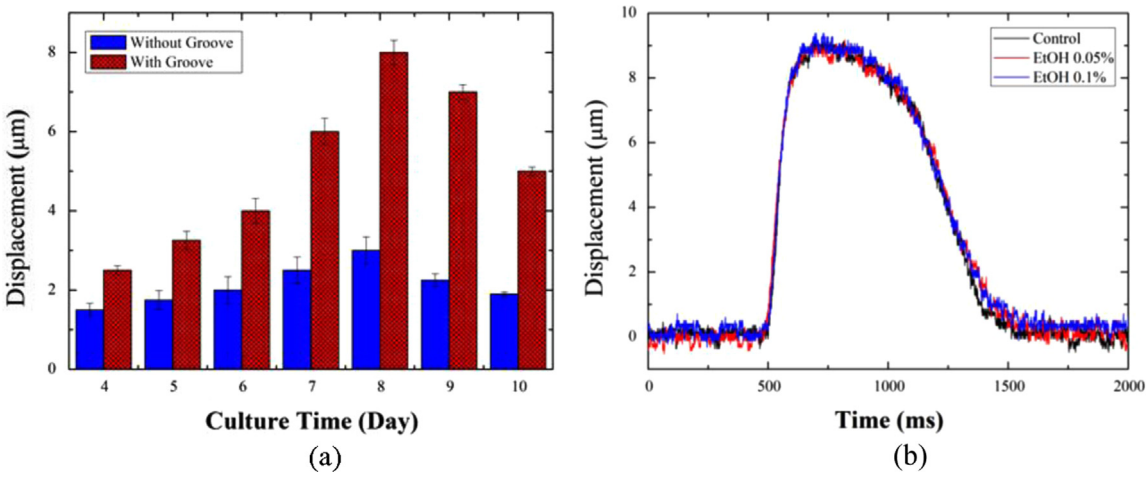

Fig. 3. (a) Changes in cantilever displacement from days 4 to 10. Contractile force was maximized on day 8 for both SU-8 cantilevers. The relative standard deviation for the cantilever with and without μgroove is 11.2, 13.5, 16.9, 13.3, 11.2, 7.1, 2.6% and 4.4, 7.1, 7.7, 5.6, 3.8, 2.6, 2.1% (from day 4 to day 10), respectively. (b) Changes in contraction force and one cycle of beating of cardiomyocytes with respect to ethanol concentration.

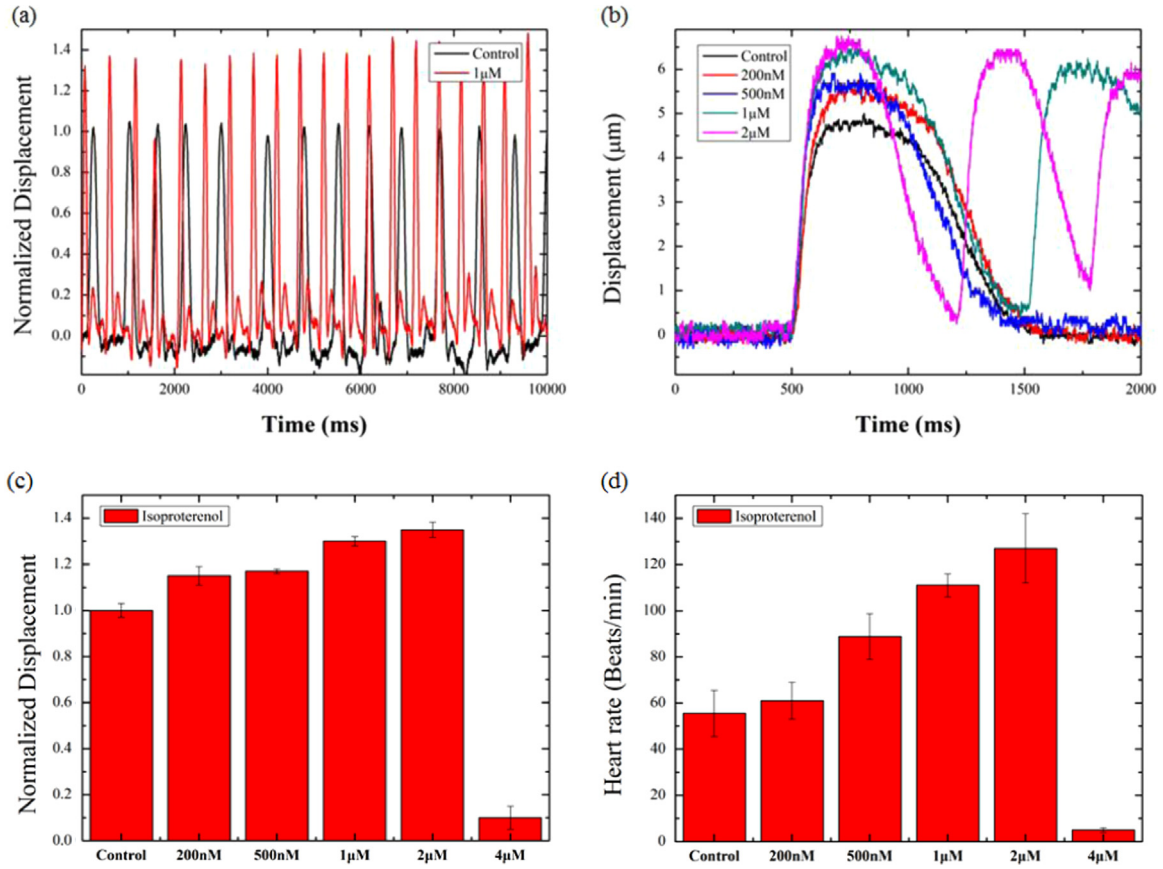

Fig. 4. Mechanical response of cardiomyocytes to Isoproterenol: (a) changes in cantilever bending displacement and beating frequency after treatment with 1 μM Isoproterenol, (b) one cycle at various drug concentrations, (c) cantilever displacement as a function of drug concentration (The relative standard deviation in each drug concentration is 3.0, 3.4, 0.8, 1.5, 2.4 and 50%, respectively), (d) heart rate in accordance with various drug concentrations. (The relative standard deviation in each drug concentration is 18.0, 13.1, 11.2, 4.5, 11.8 and 20.0%, respectively).

Fig. 4(a)–(d) shows the physiology of the cardiomyocytes according to the concentration of Isoproterenol. As shown in Fig. 4 (a), when 1 μM was treated, the displacement of the SU-8 cantilever increased by 30% and its beating frequency increased by 200%. Fig. 4 shows the results of contraction force and pulse duration of cardiomyocytes according to drug treatment concentration increase during one cycle. As drug concentration was increased, the displacement of SU-8 cantilever increased, and at 4 μM and higher concentrations, the displacement decreased. The normalized displacement test result is shown in Fig. 4(c). As shown in Fig. 4(d), the same result was also obtained for the heart rate. The contraction force of the cardiomyocytes increased for Isoproterenol, even with a small amount of the drug, but at a certain concentration and higher, it had a significant effect on the cardiomyocytes.

Fig. 5(a)–(d) depict the physiology of cardiomyocytes according to the concentration of Verapamil. The contraction force of the cardiomyocytes was reduced to 56% at approximately 500 nM concentration, and returned to the normal state following replacement of the old medium with a new media. Fig. 5(a) shows the changes in the displacement and beating frequency of the SU-8 cantilever when Verapamil was treared at 500 nM. Fig. 5 (b) shows the changes in the contraction force and beating frequency of the cardiomyocytes according to the concentration of Verapamil during one cycle. The contraction force decreased relatively linearly with drug concentration. Fig. 5(b) indicates that there was no significant change in the contractile time during one cycle, but as the drug concentration increased, the beating frequency varied significantly. Fig. 5(c) and (d) show the changes in the contraction force and beating frequency of the cardiomyocytes according to Verapamil concentration. These experimental results verify that the proposed cardiac toxicity screening method has the potential to support the patch clamp assay.

Astemizole, a known hERG channel inhibitor, can induce arrhythmia in the heart. To measure changes in the contraction force and beating frequency of the cardiomyocytes according to Astemizole concentration, the amounts of DMSO and ethanol were controlled to prepare a drug with 1 nM to 5 nM concentrations. Following treatment of various concentration of Astemizole on day 8 when the contraction force of the cardiomyocytes was greatest, changes in the displacement and beating frequency of the SU-8 cantilever were measured. Figure S7 shows the changes in the beating frequency and contraction force of the cardiomyocytes according to Astemizole concentration. The experiment results show that the pulse duration in one cycle of cardiomyocytes decreased for concentrations up to 2.5 nM of Astemizole. However, in contrast with the results for Isoproterenol and Verapamil, Astemizole concentration did not influence the contraction force of the cardiomyocytes. This result is different from the previous experiment result for an existing Ca^{2+} control drug; therefore, additional tests are required in the future to understand the relationship between the beating frequency and K^{+}. Fig. 6 shows the changes in the displacement and beating frequency of the SU-8 cantilever over time after 5 nM Astemizole was treated into the cardiomyocytes. The experiment result showed that the contraction and relaxation times of the cell decreased at Astemizole concentrations of 5 nM and higher, resulting in the beating frequency increasing non-uniformly. Consequently, there was no significant effect on the displacement due to the contraction force of the cardiomyocytes as was shown at a lower concentration. The abnormal

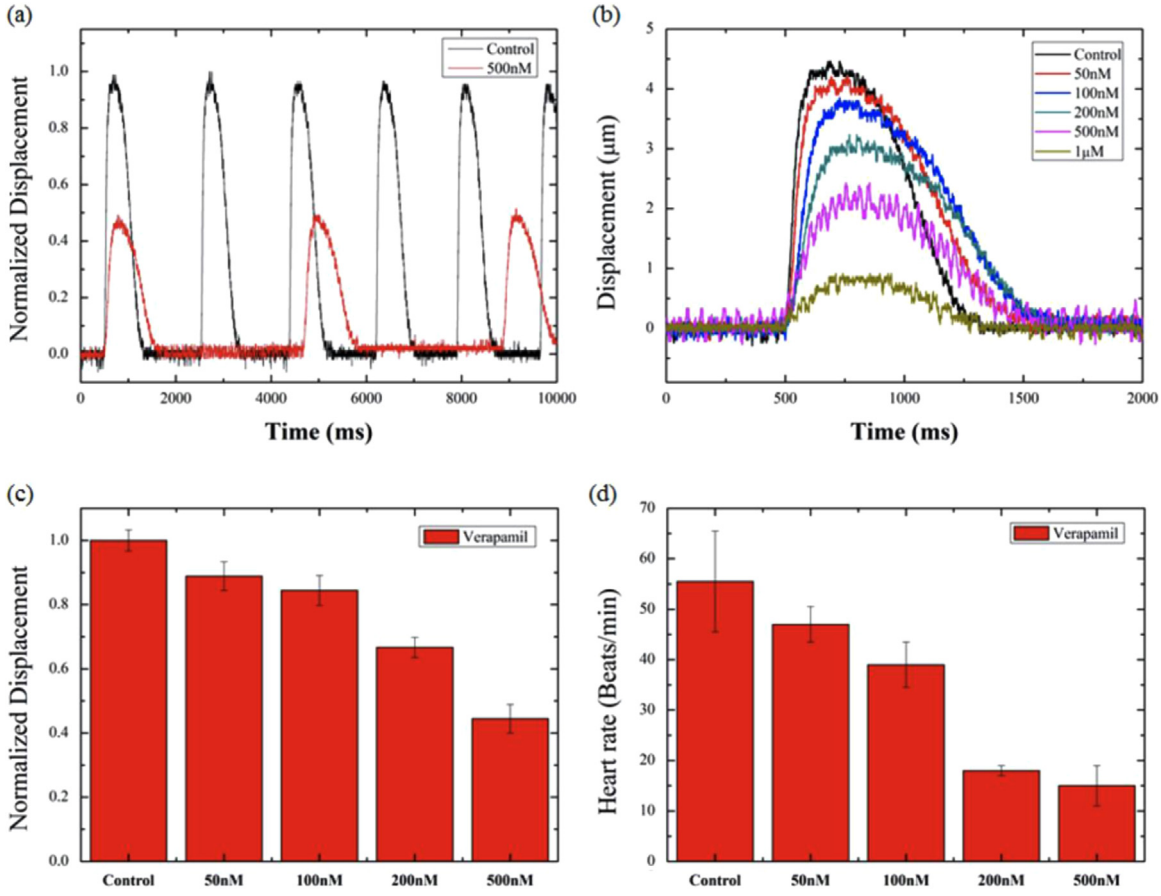

Fig. 5. Mechanical response of the cardiomyocytes to Verapamil: (a) changes in cantilever bending displacement and beating frequency following treatment with 500 nM Verapamil, (b) one cycle at various drug concentrations, (c) cantilever displacement as a function of drug concentration (The relative standard deviation in each drug concentration is 3.3, 5.0, 5.4, 4.6 and 9.9%, respectively), (d) heart rate in accordance with various drug concentrations (The relative standard deviation in each drug concentration is 18.0, 7.4, 10.7, 5.5, 26.6%, respectively).

features of the cardiomyocytes with respect to drugs are closely related to heart diseases such as arrhythmia. The effect of the arrhythmia due to the side effect of drugs was immediately observed after the Astemizole treatment (5 nM and higher). The phenomenon was maintained during the measurement of 5 min. In the future, a new high-speed drug toxicity screening system may be enabled by quantifying changes in the contraction force and beating frequency of cardiomyocytes due to drugs treatment into cardiomyocytes and by utilizing SU-8 cantilever arrays.

4. Conclusions

In this study, changes in the physiology of cardiomyocytes according to drug concentration were measured *in vitro* using a μgroove-patterned SU-8 cantilever and a high-sensitivity laser vibrometer. SU-8 cantilevers with and without μgrooves were used to verify the effect of three dimensional (3D) surface structure on cardiomyocytes contraction force, with the structure of the μgrooves optimized to maximize the contraction force of

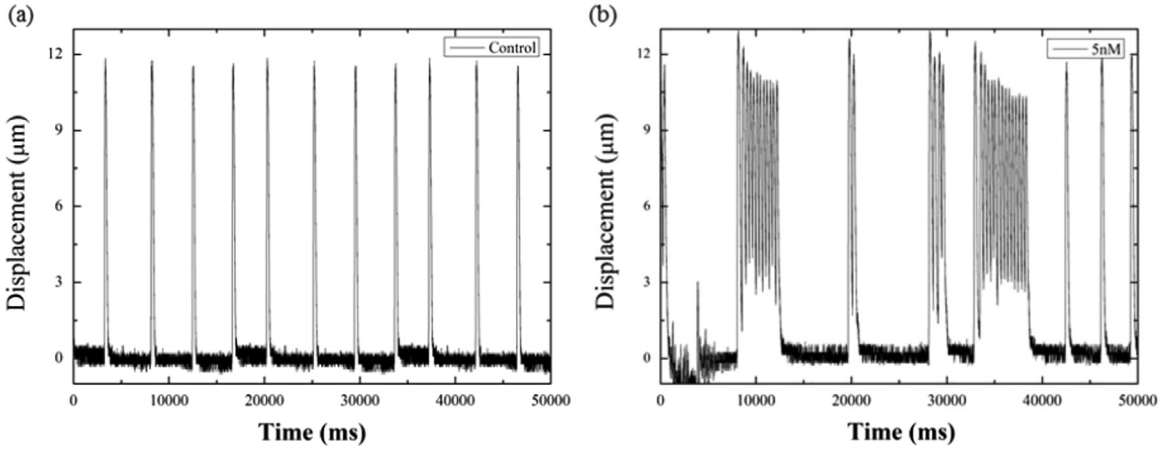

Fig. 6. Mechanical behavior changes due to 5 nM Astemizole: (a) before, (b) after.

cardiomyocytes. The SU-8 cantilevers fabricated using a conventional semiconductor process facilitates miniaturization and mass production. The maximum contraction force of the cardiomyocytes were on day 8 for both types of SU-8 cantilevers, with the contraction force of the cantilever with μgrooves measured as being 2.7 times that of the cantilever without μgrooves. This result is attributable to the alignment and growth of the cardiomyocytes in one direction along the 3D-shaped μgroove pattern. Following culture of the cardiomyocytes on the μgroove-integrated cantilever surface, changes in their characteristics due to drugs were analyzed using Isoproterenol, Verapamil, and Astemizole at various concentrations. At 1 μM of Isoproterenol, the contraction force and beating frequency increased by 30% and 200%, respectively, whereas at 500 nM of Verapamil, they were respectively reduced by 56% and 30%. Both drugs resulted in suspended heart rates above a certain concentration. Astemizole, a well-known hERG channel inhibitor, produced no changes in the contraction force but caused the beating frequency to become irregular and increase abnormally at 5 nM concentration. It is expected that this SU-8 cantilever-based system will in the future facilitate new preclinical high-throughput drug toxicity screening following further development.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.01.089.

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