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Analysis of the Growth Characteristics of Cardiac Cells According to Mechanical Properties of Substrates Using the Simplified Measurement Technique of Tracker

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Abstract

To date, various techniques have been utilized to assess the contractility of cardiomyocytes and their response to drug-induced toxicity. However, these techniques are either invasive or involve complex fabrication methods and expertise. Here, we introduce the use of video-based analysis software to track the motion of cardiomyocytes and assess their contractility. The software, called "Tracker", is freely available and this is the first attempt at using it for cardiac contractility measurement. We used the software to measure the contractile properties of cells cultured on a rigid substrate and two flexible polydimethylsiloxane (PDMS) substrates having different elastic moduli day-wise up to eight days. Contractility was found to be highest in the most flexible substrate. Subsequently, the cardiotoxicity response of the cells on three different substrates was analyzed with verapamil. It was observed that the cells on rigid substrate were primarily affected by drug-induced toxicity, while the drug had a lesser impact on cells on the more flexible PDMS substrate. Evidently, the flexible substrate aided the maturation of cells and had lower drug toxicity, while the cells on PS could not fully mature. The assessment of cardiomyocytes using "Tracker" proved to be simple and reliable.

Keywords: Polydimethylsiloxane, Contractility, Young's modulus, Cardiomyocytes, Drug toxicity screening.

1. INTRODUCTION

The role of the heart is to pump the required blood and oxygen to the body, and during the process, the heart tissue undergoes sufficient contraction and relaxation. Serious health repercussions could occur if there is a problem in cardiac contractility [1]. The functioning of the heart is also assessed during the preclinical drug trials, mainly because drug-induced cardiotoxicity is a significant reason behind the withdrawal of numerous drugs from public consumption [2,3]. Therefore, cardiac contractility must be assessed during the preclinical phase.

To date, several techniques have been devised to measure the contractility of the cardiomyocytes. Atomic force microscopy (AFM) is one such technique that measures the contraction force of cardiomyocytes at the single-cell level [4]. However, since the cantilever tip can penetrate the cell membrane, it is classified as an invasive technique. The use of micro-posts for contractile measurements is a non-invasive technique, where the cells are cultured on top of micro-posts, and deflection of the posts is recorded and analyzed. However, recording the deflection of the micro-posts can be difficult and tedious. Traction force microscopy (TFM) is another technique for contractility assessment [5], where cardiomyocytes are cultured on the fluorescent beads attached to the substrate, and the contractility is measured using fluorescent microscopy. However, the fluorescent beads may interact with the cells, which may interfere with the cellular response. A non-interactive technique uses a cantilever, and the cantilever deflection resulting from the cardiac contraction is measured optically [6-9]. However, the fabrication of the cantilever is complex as it involves using photolithographic techniques.

ImageJ utilizes image analysis for contractile assessment is another optical technique [10,11], where contractile motion is measured frame-by-frame using complicated analyses and discrete 'macros'. The technique analyzes discreet images, thereby making it less reliable for contractility measurement. Video analysis is a better technique to analyze contractile activity owing to its higher number of frames per second, resulting in

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better resolution. Several techniques for video analyses using MATLAB and other software have been reported [12-15]. However, they either involve complex and time-consuming algorithms for data analysis, or cannot measure the contractility profile.

In the present work, we propose the use of a video-based analysis software called "Tracker" to assess cardiomyocytes contractility for the first time. "Tracker" is a freely available video analysis software that can track the motion of selected cardiomyocytes. Accordingly, contractile and beating parameters were assessed using three types of substrates with varying stiffness. A rigid substrate of polystyrene (PS) was used as the reference, and the other two substrates were composed of polydimethylsiloxane (PDMS) having varying stiffness. Neonatal rats' ventricular myocytes (NRVM) were cultured on the substrates and the contractile behavior was analyzed for up to 8 days. The contractile response recorded was highest in the PDMS with lower elastic modulus. The cells were then subjected to verapamil drug and the contractile response to measure the toxicity using the Tracker software. It was found that contractility changed the least in the cells cultured on the PDMS with a lower elastic modulus.

2. EXPERIMENTAL

2.1 Synthesis and characterization of PDMS substrates

A Sylgard 184 silicone elastomer base and a Sylgard 184 silicone elastomer curing agent manufactured by Dow Corning (Midland, MI) were used to create the PDMS substrate. In this study, we used a series of PDMS samples with different base/curing agent mass ratios to explore the relationship between the elastic modulus and a different amount of crosslinking of PDMS network, whose base/agent mass ratios were 10:1 and 20:1. Moreover, PS was maintained as a reference sample to compare the contractile properties of cardiomyocytes with a standard stiff substrate. The schematic is shown in Fig. 1 (a). PDMS substrate (2 mm thick) was created by thoroughly mixing the corresponding elastomer base and the curing agent mixtures before pouring the mixture into a flat bottom square polystyrene dish and degassing the PDMS under vacuum to remove the air bubbles. The PDMS was cured on a hot plate at 80°C for 2 h. Following the curing, the PDMS substrate samples were cut into square shapes of 20×20 mm using a medical knife.

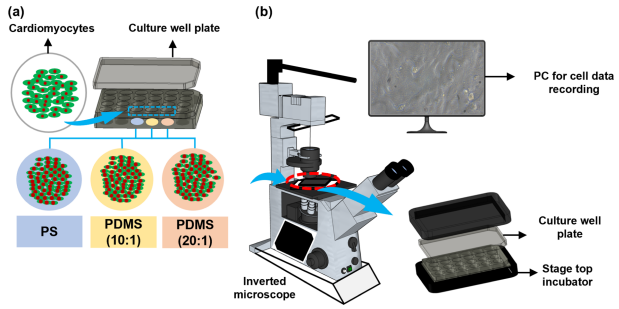

Fig. 1. (a) Schematic of the cardiomyocytes cultured on PS, PDMS (10:1), and PDMS (20:1) substrates used for analysis, (b) Schematic of the setup used for recording the motion of the cardiomyocytes.

The relationship between stress and strain was measured using a tabletop universal testing instrument (EZ-L 500N Shimadzu, Japan). The Young's modulus of elasticity was then calculated from the tensile testing data.

2.2 Isolation of NRVM

The ventricles were harvested from 1-3-day old neonatal Sprague-Dawley rats, which was approved by the Animal Ethics Committee of Chonnam National University. The ventricular tissue was digested in a mixture of collagenase (0.4 mg/mL) and pancreatin (0.6 mg/mL) to separate the cardiomyocytes from the tissues. Once the cardiomyocytes and the fibroblast layer were separated with centrifugal separation using Purcell, the cardiomyocytes with high purity were collected. The prepared cardiomyocytes were seeded at the PS and PDMS (10:1, 20:1) substrates with 1000 cells per mm^2 and cultured in an incubator at 37°C with 5% carbon dioxide. The culture medium was refreshed once every three days.

2.3 Measurement of contractility

An inverted microscope (Eclipse TS100) was used to optically measure the contractility of the cells cultured on the different PDMS substrate well plate, as shown in Fig. 1 (b). The focal length of the microscope was set to 40x to record the video (Fig. 2 (a)). The recorded video was utilized to evaluate the contractile properties of the cultured cardiomyocytes. The video was then processed using the Tracker free video analysis and modeling tool developed using the Open-Source Physics (OSP) Java framework to show the target and select the desired cell on the culture substrate. Subsequently, the processed video was read frame by frame to track the selected cells on the substrate set by the user.

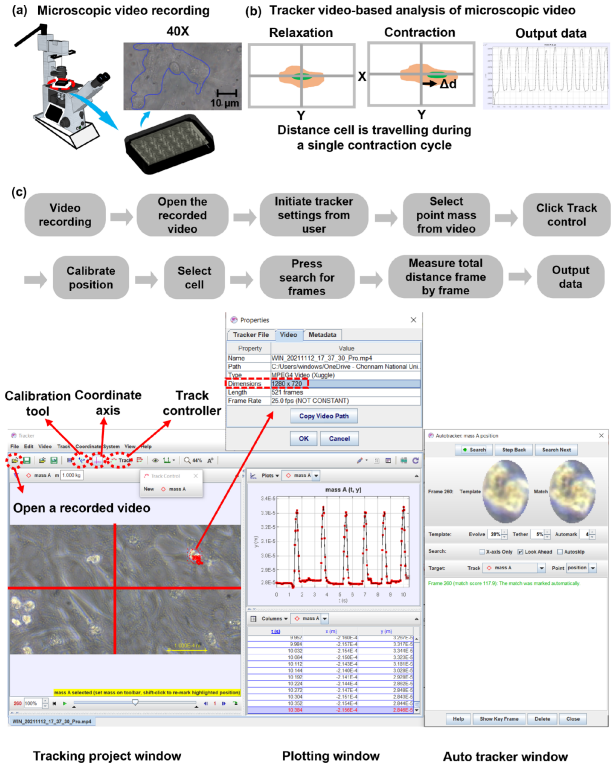

Fig. 2. (a) Schematic of the video recording and cell selection, (b) Concept of freely available Tracker software for analysis, (c) Flow chart for using the Tracker software with a screenshot of its dashboard.

The displacement data of the cell were derived from the tracking information, which contained the change in position of the cell for each frame (Fig. 2 (b)). The data was processed to convert the frame pixel into contractile distance. The step-by-step procedure for analysis using the Tracker software is presented in Fig. 2 (c).

2.4 Drug toxicity screening

Drug toxicity experiments were performed using Verapamil (Sigma-Aldrich), an L-type Ca2+ channel blocker. The drug was diluted in ethanol, and the total ethanol concentration was maintained below 0.1 % v/v. Drug concentrations were selected based on the IC50 values approved by the Food and Drug Administration. Contractile changes were assessed at various verapamil concentrations: 10 nM, 100 nM, 300 nM, 500 nM and 1000 nM.

2.5 Data analysis and statistical significance

In this study, results are presented as mean ± standard deviation (S.D.). All statistical analyses were performed using the GraphPad Prism 7 software. Statistical significance of the data was determined using one-way ANOVA followed by Tukey's honest significant difference test, with significant differences defined by *$p < 0.05$ and **$p < 0.001$.

3. RESULTS AND DISCUSSIONS

3.1 Characterization of PDMS substrates

First, the fabricated PDMS substrates were characterized to determine their mechanical properties. A 5 N force was applied up to PDMS (10:1) and a 3.5 N force was applied up to PDMS (20:1). The stress analysis with respect to displacement is shown in Fig. 3. PDMS (10:1) experienced stress up to 0.16 N/mm^2, while PDMS (20:1) experienced stress up to 0.07 N/mm^2. The corresponding Young's moduli for PDMS (10:1) and (20:1) were 1330 kPa and 706 kPa, respectively. The details are described in Table S1 in the supplementary information. The data for PDMS (10:1) indicate that the polymer has at least two distinct tangent moduli, and lower stiffness was observed at lower strain levels than at higher strains, and the particular transition was not observed in PDMS (20:1), although it is likely that this was because the samples failed before they reached the strain level at which the transition occurred.

3.2 Measuring Contraction Force

The cardiac tissue adhered and stabilized once the NRVM were cultured on the PS, PDMS (10:1) and PDMS (20:1), and started

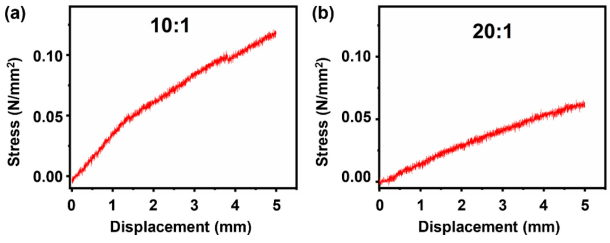

Fig. 3. Stress-strain profiles of (a) 10:1 PDMS, (b) 20:1 PDMS substrates.

Table 1. Stress-strain profiles of (a) 10:1 PDMS, (b) 20:1 PDMS substrates

Crosslinking	Force (N)	Stress (N/mm^2)	Strain	Young's modulus (kPa)
10:1	5	0.16	120	1330
20:1	3.5	0.11	120	706

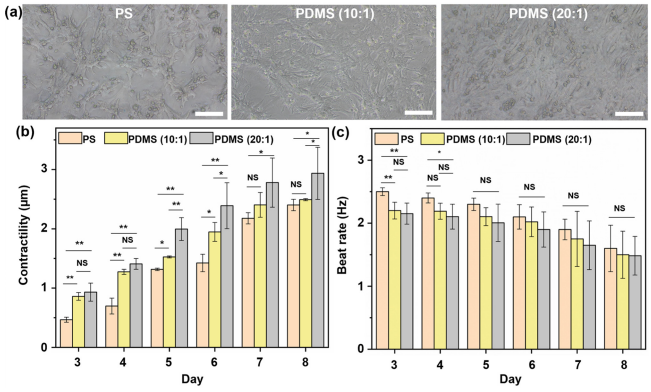

Fig. 4. (a) Optical images of cardiomyocytes on the different substrates on day 6 after cell culture (scale bar = 100 mm). Day-wise changes in (b) contractility and (c) beat rate as measured by Tracker. Results are displayed as mean ± S.D with n = 5. Statistical significance of data was determined using one-way ANOVA followed by Tukey's honest significant difference test, with significant differences defined by *p < 0.05 and **p < 0.001.

displaying beating activity on day 3 after cell culture. From day 3 to day 8, the contractile profile was recorded under the microscope and subsequently analyzed using the Tracker software. Fig. 4 (a) shows the cell condition on the three substrates on day 6 after cell culture. Fig. 4 (b, c) shows the day-wise contractility and beating rate trends. The contractile displacement for PS, PDMS (10:1), and PDMS (20:1) on day 3 were 0.466 ± 0.042 μm, 0.86 ± 0.065 μm, and 0.932 ± 0.152 μm, respectively, and by day 8, it had increased to 2.4 ± 0.089 μm, 2.49 ± 0.085 μm, and 2.936 ± 0.270 μm, respectively. When compared among the three substrates, contractility in PDMS (20:1) was highest followed by PDMS (10:1) followed by PS. The difference between PS and PDMS (10:1), and PS and PDMS (20:1) on days 3 and 4 was statistically significant. The contractile difference between PS and PDMS (10:1) was not significant on days 7 and 8, while the difference between PS and PDMS (20:1) remained significant throughout the culture period.

Meanwhile, the beating rate reduced from 2.5 ± 0.061 Hz, 2.2 ± 0.132 Hz, and 2.152 ± 0.167 Hz on day 3 to 1.6 ± 0.160 Hz, 1.5 ± 0.241 Hz, and 1.48 ± 0.272 Hz for PS, PDMS (10:1), and PDMS (20:1), respectively. Regarding the beating rate, the mean beating rate for PDMS (20:1) was lowest, which was followed by PDMS (10:1) followed by PS. On day 3, the difference between PS and PDMS (10:1), and PS and PDMS (20:1) was statistically significant, while on day 4, only the difference between PS and PDMS (20:1) was significant. The difference among the three substrates was not significant over the rest of the culture period. PDMS (20:1), having the lowest Young's modulus, exhibited a larger contractility on a daily basis as the culture grew. Substrates with lower Young's modulus are conducive for better cellular growth and adhesion, resulting in larger contractile displacements and lower beating rates [16-17].

3.3 Contraction forces change in response to drug treatment

Following cell culture, drug toxicity experiments were performed on day 9 on the three substrates using verapamil, which is an L-type Ca2+ channel blocker. The contractile patterns of the cardiomyocytes were recorded after adding sequentially increasing drug concentrations from 10 nM to 1000 nM from the three types of substrates with varying elastic moduli. The representative contractile motions as recorded by the Tracker software for control, 300 nM, and 1000 nM of verapamil for PS, PDMS (10:1), and PDMS (20:1) are depicted in Fig. 5. It is evident that both the contractility and beat rate are decreasing at higher concentrations of the drug.

Fig. 6 illustrates a detailed analysis of the contractility and beat rate changes after the drug was added. The contractility of all three substrates reduced after adding verapamil; however, there was no statistical significance between PS, PDMS (10:1) and PDMS (20:1) up to 100 nM. At 300 nM, PS, PDMS (10:1) and PDMS (20:1) reduced to 70.79%, 77.28%, and 79.64% of control,

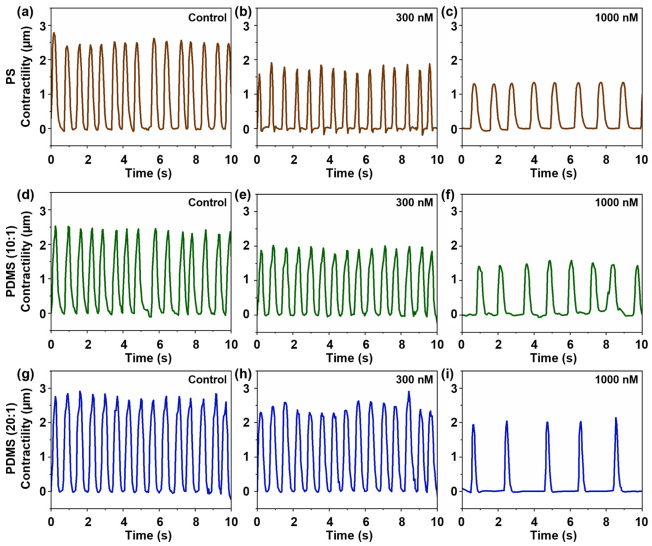

Fig. 5. Real-time changes of contractility as measured by Tracker in response to drug toxicity using verapamil. (a), (b), (c) display PS contractility, (d), (e), (f) illustrate PDMS (10:1) contractility, and (g), (h), (i) illustrate PDMS (20:1) contractility. The three concentrations showed are control, 300 nM, and 1000 nM of verapamil.

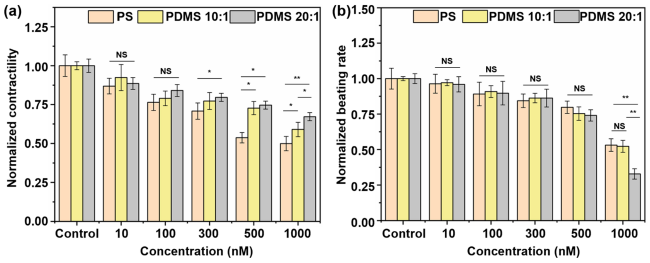

Fig. 6. Concentration-wise response to (a) normalized contractility and (b) normalized beating rate on addition of verapamil. Results are displayed as mean ± S.D with n = 5. Statistical significance of data was determined using one-way ANOVA followed by Tukey's honest significant difference test, with significant differences defined by *p < 0.05 and **p < 0.001.

and the change was statistically significant between PS and PDMS (20:1). The reduction after adding higher doses of verapamil was also statistically significant, with 49.88%, 58.98%, and 67.21% at 1000 nM for PS, PDMS (10:1), and PDMS (20:1), respectively. Moreover, the reduction in contractility was observed to be the highest for PS and lower for PDMS 20:1. In all substrates, the beating rate reduced in a concentration-dependent manner; however, there was no significant difference in the reduction among different substrates, except at a high concentration of 1000 nM. At 1000 nM, there was a reduction of 53.12% for PS, 52.22% for PDMS (10:1), and 32.84% for PDMS (20:1), which was statistically significant.

Generally, Verapamil is used to prevent supraventricular tachycardia by controlling the ventricular rate, particularly as the contraction and beat rate reduce [18]. As seen in Fig. 6, the cells predictably displayed negative chronotropy and negative inotropy in response to verapamil, while the rate of reduction changed with each substrate. Cells on the most flexible substrate PDMS (20:1) exhibited the lowest change in contractility, while the stiff substrate PS had the highest change. Accordingly, it could be inferred that the cellular maturation on PDMS (20:1) was the highest and PS was the lowest. Consequently, verapamil proved to be more toxic to the cells on PS, primarily owing to lesser maturity. Meanwhile, a more flexible substrate like PDMS (20:1) aided in higher maturation of cardiomyocytes. This was seconded by the day-wise trends (Fig. 4), where cells on PDMS (20:1) possessed the highest contractile motion and lowest beating rate.

The above data of the day-wise cellular trends and response to cardiotoxicity was successfully accomplished using the Tracker software. Tracker proved to be an easy-to-use video-based analysis tool to assess the cardiac contractility. Until now, this software was primarily used for motion tracking of macro-sized objects. The present study is the first successful demonstration of Tracker for cellular activity. Tracker can be further investigated for other cellular motion and behaviors.

4. CONCLUSIONS

In this study, we successfully demonstrated the use of Tracker software to accomplish video-based analysis of cardiac contractility. Specifically, contractility was measured using three different substrates – PS, PDMS (10:1), and PDMS (20:1) – with varying stiffness. From the measurement results of day-wise contractility, cells on PDMS (20:1) exhibited the highest contractility and lowest beating rate among the three, indicating that the flexible substrate aided in cellular maturation. Drug toxicity screening using verapamil showed that even though all the substrates exhibited reduced contractility and beat rate on adding verapamil, the least mature cells on PS were most prone to drug toxicity, while the cells on PDMS (20:1) had lesser effect to toxicity. In summary, cellular analysis was successfully realized using the Tracker software, and we believe this software can be further explored to better understand cellular activity.

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