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Study on Robotic Cell Culture Systems for Autonomous Cultivation of Fibroblast Cells

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***Abstract*— Fibrosis is a key factor in diseases such as interstitial pneumonia and cirrhosis. Conventional approaches in cell therapy, which involves transplanting living cells into patients, are able to address these conditions. However, cell cultivation involves numerous intricate procedures. Given the demands for a substantial quantity of cells in the therapy, manual cultivation has its limitations. Furthermore, removing cells from a CO_2 incubator during culturing can cause damage to the cells, leading to reduced cell viability. To improve the efficacy of cell therapy, it is crucial to cultivate cells with minimal damage. The purpose of this study is to develop a robotic cell culture system that enables the automatic cultivation of fibroblast cells in large quantities for demands of cell therapy, in an environment similar to that of a CO_2 incubator and allows to monitor the culture environment. The system enables observation of cells under a microscope without altering the culture conditions and automates the medium exchange process. To confirm the feasibility of the system, we conducted the basic experiment set comparing the developed system to manual cultivation of fibroblast cells. The results showed the developed system improved the fibroblast viability and proliferation rate compared with manual cultivation. In conclusion, we confirmed the feasibility of the developed robotic cell culture system in its capability for cultivating fibroblasts with an efficiency equivalent to or greater than conventional manual cultivation.**

I. Introduction

Fibrosis is a pathology of chronic inflammation and organ injury, characterized by repeated cycles of biological repair and damage, ultimately leading to organ hardening due to excessive extracellular matrix production[1]. Major fibrotic diseases include pulmonary fibrosis and liver cirrhosis, both of which are chronic conditions that significantly reduce patients' quality of life (QOL). It has been established that fibrosis is driven by activated fibroblasts that accumulate at fibrotic sites and produce large amounts of type I collagen[2]. To address these diseases, research and development in cell therapy, which involves the transplantation of living cells into patients, is underway[3]. Cell therapy is applied to various diseases, including those in regenerative medicine and oncology, as the secreted factors from administered cells influence surrounding tissues and promote tissue regeneration. However, cell therapy faces several challenges, including efficacy, safety, and production methods[4]. Allogeneic cells, derived from donors, can be produced and stored in advance, but they carry risks of immune rejection and infection. Autologous cells, derived from the patient's own tissues, can bring a risk of oncogenic transformation although eliminate the risk of immune rejection and infection[5], and harvesting and expanding a sufficient number of autologous cells is a significant challenge. *In vitro* culture of mammalian cells, including human cells for autologous therapy, is typically conducted in a CO_2 incubator (37 °C, 5% CO_2, 95% humidity) to construct suitable environment for cell cultivation. To monitor the characteristics and changes in cultured cells, microscopy is essential, but this usually requires removing the culture vessel from the incubator. Additionally, during cell culture, nutrient supply and waste removal necessitate medium changes, which also require removing the culture vessel from the CO_2 incubator. Such operations lead to pH fluctuations due to changes in temperature and CO_2 concentration, placing stress on the cultured cells[6]. The stress can induce cell death, complicating the assurance of therapeutic efficacy in cell therapy. Moreover, manual culture operations involve varying medium exchange frequencies and times, and there is a risk of microbial contamination, all of which can affect cell function and therapy efficacy. Given the large number of cells required for cell therapy, scaling up culture operations manually is impractical and costly. To address these challenges, systems have been developed to automate culture operations using humanoid robots in sterile environments and to automate medium exchange within CO_2 incubators[7]. However, the system had the possibility of risk of cell death due to environmental fluctuations because the system required to remove cells from the incubator for observation , thereby hindering the reliability of cell therapy. To ensure the quality of cells used in therapy and to achieve reproducible and stable cell cultures, it is crucial to analyze environmental changes during culture and the associated operations. A comprehensive understanding of the cell culture process is, therefore, necessary. Consequently, to advance cell therapy, a robotic culture system is needed that can maintain a high cell survival rate while continuously monitoring and adjusting the culture environment in real-time. The purpose of this study is to develop a robotic cell culture system that enables cell observation in an environment similar to that of a CO_2 incubator while monitoring the culture conditions and exchanges the medium automatically, and to confirm its feasibility for culturing the fibroblast.

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II. Material and Method

A. System requirement

Contamination is one of the most common issue in cell culture and can significantly impact experimental results[8]. When contamination occurs, cells, culture media, and other materials, as well as culture vessels, must be discarded. A widely used preventive measure is to sterilize components that come into contact with the culture medium through heat treatment with high-pressure steam[9]. Therefore, it is essential to select heat-resistant materials for any parts that will come into contact with the medium. After cells have been seeded in a dish, it is crucial to maintain an environment similar to that of a CO_2 incubator, including the ability to automatically change the culture medium. This ensures continuous observation and culture of the cells without altering the culture environment. Additionally, to achieve stable cell culture, it is important to monitor the culture environment throughout the process. Therefore, the system must include functions for automatic medium exchange, observation of cells without disturbing the culture environment, and continuous monitoring of the culture conditions.

B. System configuration

The developed robotic cell culture system consists of three main components: a medium transport unit for adding and removing medium to and from a culture dish, a CO_2 incubation unit that enables cell observation using a microscope while maintaining an environment similar to a CO_2 incubator, and a cell culture environment monitoring unit for assessing the cell culture process. Figure 1 shows the overview of the system with microscope. A Canon EOS 7D camera was attached to the inverted microscope (Nikon Eclipse TS100) to capture images of the cells under observation. Fresh culture media are stored in a refrigerator (VS-430, VERSOS).

C. Medium transport unit

Perfusion pump units and control units have been developed for tissue construction and maturation via medium perfusion[10-12]. In this system, the medium transport unit was constructed by the developed perfusion pump unit and control unit. The operating algorithm of the tube pump was modified to determine the flow rate in ml/min by specifying the volume of liquid to be transported and the time, with control based on the flow rate.

Figure 2 shows overview of the medium transport unit. The unit consists of Tube pump (WPM2-P3EA-CP, Welco), Controller, Silicone tube (inner diameter φ2 mm, outer diameter φ4 mm), fresh medium container, waste medium container, deionized water container, humidifying tray, air filter, culture dish, winding component, tube connectors, and glucose/lactate sensor (B.LV5, Jobst Technologies). A silicone lid was custom-made to fit the dimensions of the culture dish by modeling the mold on a 3D printer (Fortus 250mc, Stratasys), filling it with silicone, and curing it in a dryer at 70 °C for 3 hours. Figure 3 shows system configuration of the medium transport unit. The components that come into direct contact with the culture medium, such as the tube pump, silicone tube, and silicone lid, are autoclave-sterilizable. While, the culture dish and glucose/lactate sensor are pre-sterilized.

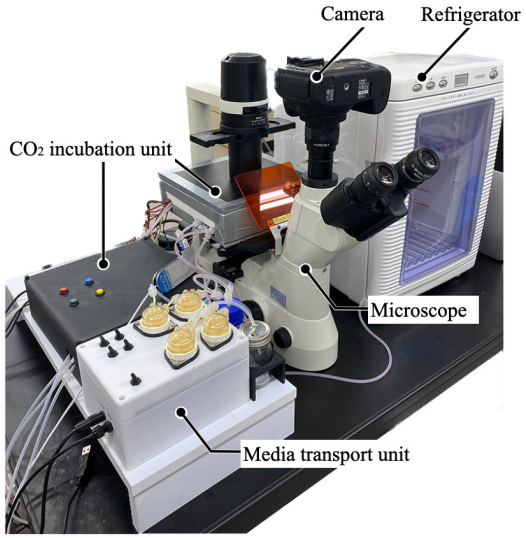

Fig.1 Developed robotized cell culture system

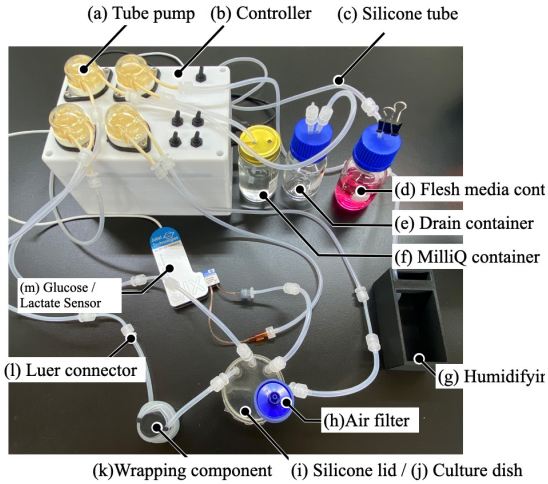

Fig.2 The overview of the media transport unit

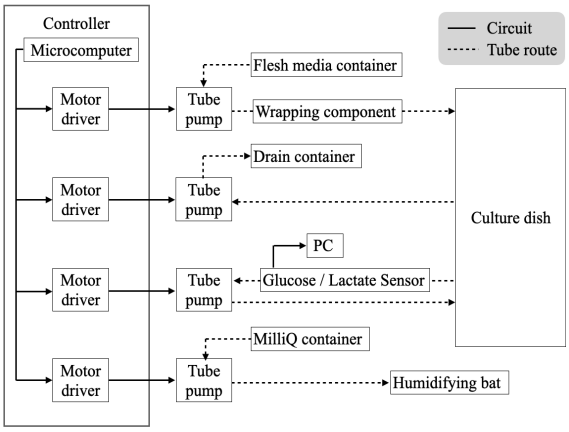

Fig.3 System configuration of the media transport unit

The four tube pumps in this unit serve the following functions:

1. Addition of culture medium to the culture dish
2. Removal of medium from the culture dish
3. Determination of the molar concentration of glucose and lactic acid in the culture medium by attached flow-through sensor at the tube
4. Addition of water to the humidifying tray in the CO_2 container

The process of adding culture medium to the dish (function 1) involves aspirating fresh medium from the container through the silicone lid, pausing for a predetermined period, and then transferring the medium from the lid into the culture dish. Fresh medium container was stored in a small refrigerator within the unit. However, since the culture dish is maintained at 37 °C, directly adding cooled medium could impose a significant thermal shock on the cells. Therefore, the medium must be pre-warmed to eliminate temperature differences. To achieve this, the fresh culture medium is warmed within the tube in the CO_2 container. Silicone tubes with an inner diameter of 2 mm were chosen to accommodate the size constraints of the CO_2 container. The addition of medium is executed by specifying the length of the silicone tube from the fresh medium container to the culture dish and the amount of medium to be added. For function 2, the process of removing medium from the culture dish follows a similar operation. The glucose and lactic acid levels in the medium are measured using a flow-through glucose/lactic acid sensor, which is integrated into the path of the silicone tubing. The medium is drawn into the sensor by a tube pump, held for a specific period, and then returned to the culture dish by reversing the pump. The addition of water to the humidifying tray in the CO_2 container (function 4) is performed as needed based on the water level in the tray.

D. CO_2, incubation unit

This unit enables the observation of cells while maintaining an environment similar to that inside a CO_2 incubator (37°C, 5% CO_2, and approximately 95% humidity). The developed unit consists of a controller, a CO2 container, and a CO_2 incubator (ASTEC). The CO_2 container includes the following components: (a) insulation, (b) DC fan, (c) air tube, (d) atomization component, (e) humidifying tray, (f) environmental sensor (BME280), (g) CO_2 sensor (SprintIR®-W 20%), and (h) heater. Figure 6 shows the structure of the CO_2 container. Transparent acrylic plates were attached to the bottom and top of the container to avoid obstructing the light emitted from the microscope during cell observation. The container was fabricated using a resin fused deposition modeling (FDM) 3D printer. Insulation was applied over the entire CO_2 container to maintain the temperature for cell incubation. Figure 7 shows a diagram of the unit. The air temperature, humidity, and atmospheric pressure inside the unit are measured using environmental sensors (BME280). The air temperature is kept at 37°C by switching the heater panel on and off based on the sensor readings. To increase the humidity inside the container to over 90%, a humidifying tray with a spray component was installed. In standard CO_2 incubators, humidity is typically maintained solely by the humidifying tray. However, when the container is small,

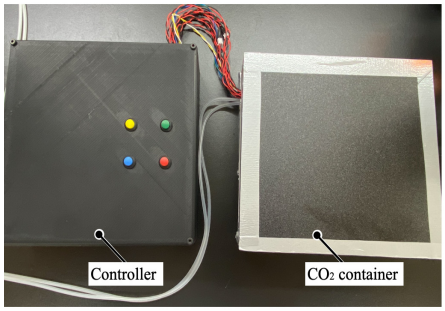

Fig.4 The overview of the CO_2 incubation unit

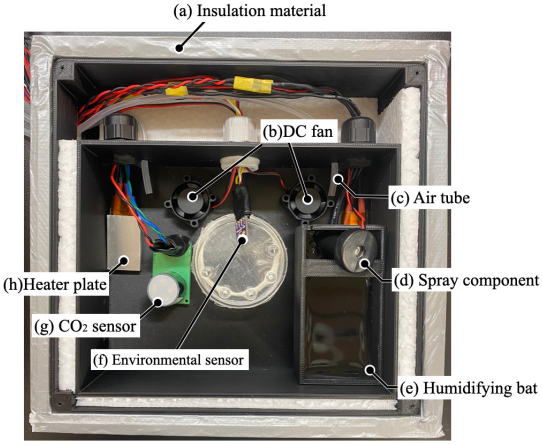

Fig.5 Component placement of the CO_2 container

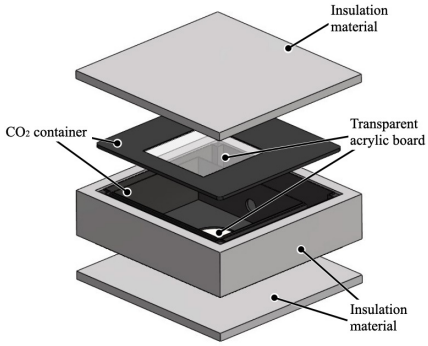

Fig.6 The structure of the CO_2 container

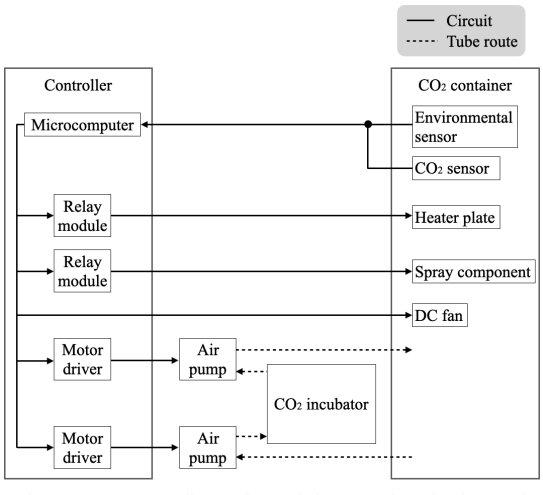

Fig.7 System configuration of the CO_2 incubation unit

external temperature fluctuations can affect the insulation properties, leading to condensation on the container walls due to saturated water vapor. This makes it difficult to maintain high humidity levels. Therefore, when the humidity drops below 90.5%, the system controls humidity by atomizing water onto the humidifying tray using the spray component. The CO_2 incubator supplies gas to maintain the CO_2 concentration in the container at 5%. The CO_2 incubator is connected to the CO_2 container via silicon tubes and an air pump, which is used for suction when the CO_2 concentration falls below 5% and for discharge when the concentration exceeds 5.15%. The operational criteria for the air pump were determined through trial and error.

F. Aseptic experiment

As a preliminary step to cell culture, aseptic experiments were conducted to ensure that cells could be safely applied to the developed system without contamination. All components of the system (Table 1) were sterilized by autoclaving prior to the experiment. A total of 5 ml of serum-free DMEM/Ham's F-12 was added to a culture dish, and the system was operated for 5 days. The color change (due to possible fluctuations in pH) and transparency of the medium in the dish, were visually inspected at the start of the experiment and after 5 days. The presence of contamination was also assessed using an inverted microscope.

G. Culturing experiment

fibroblast cells were cultured by seeding cell suspensions (10^4–10^5 cells/ml in DMEM medium supplemented with 10% fetal bovine serum and 1% GlutaMAX) onto 5.5 mm dishes. Two identical culture dishes were prepared to compare the results with and without the developed system (Fig. 10). One dish was placed in the CO_2 container of the developed system shown in Fig. 5, and the lid was replaced with a fabricated silicone lid. The other dish was placed in a standard CO_2 incubator (SANYO). The cultures were maintained for five days, during which cells were observed and photographed daily. The medium in manual culture dish was changed on the third day, following the typical schedule for medium exchange. Approximately half of the medium volume in each dish was replaced. For the dish in the developed system, an environment similar to that of a CO_2 incubator was maintained, and cell observation, photography, and medium exchange were performed automatically. In contrast, for the dish without the system, cells were manually removed from the CO_2 incubator, observed, photographed, and had their medium changed in a safety cabinet, according to standard procedures. The time required to complete the manipulations with the culture dish from the CO_2 incubator was recorded. Ten random microscopic images from the fields of view were taken, and the number of cells was counted from these images. The cell growth rate was calculated based on the cell count on the first day of culture to evaluate the effect of the developed system on cell growth. After five days of culture, fluorescence staining was performed using the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen). To prepare the staining solution, 20 µL of 2 mM Ethidium was added to 10 ml of sterile PBS and mixed by vortexing (TAITEC). Then, 5 µL of 4 mM Calcein AM was added, and the solution was mixed again by vortexing. Approximately 5 ml of the prepared solution was added to the

Fig.8 Temperature/Humidity/CO_2 monitoring

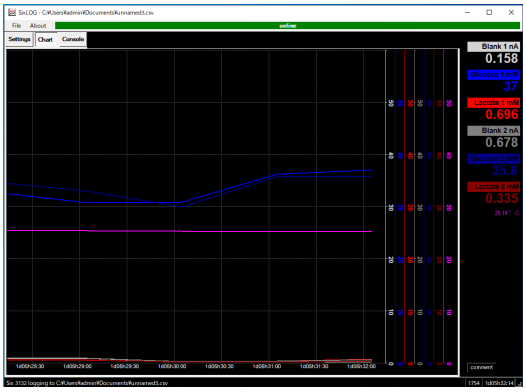

Fig.9 Glucose/Lactate monitoring

Table.1 Autoclaved components

Sterilized object	Material
Pump cassette	Polysulfone
Silicone tube	Silicone rubber
Silicone lid	Silicone resin
Flesh media container	Glass
Drain media container	Glass

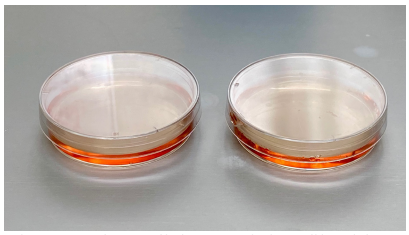
Fig.10 Culture dish containing fibroblasts

dishes cultured with and without the developed system. After that fluorescence staining was complete, 10 random images were taken. Cell viability was determined by counting the number of live and dead cells in the images. Since the data for comparison were obtained from the same cell suspension, a corresponding two-sample t-test was performed for statistical

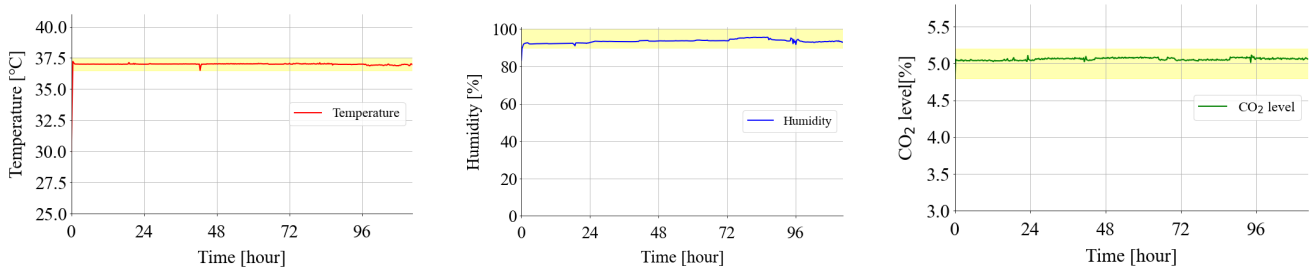

Fig.11 Measurement results of temperature/humidity/CO_2 level

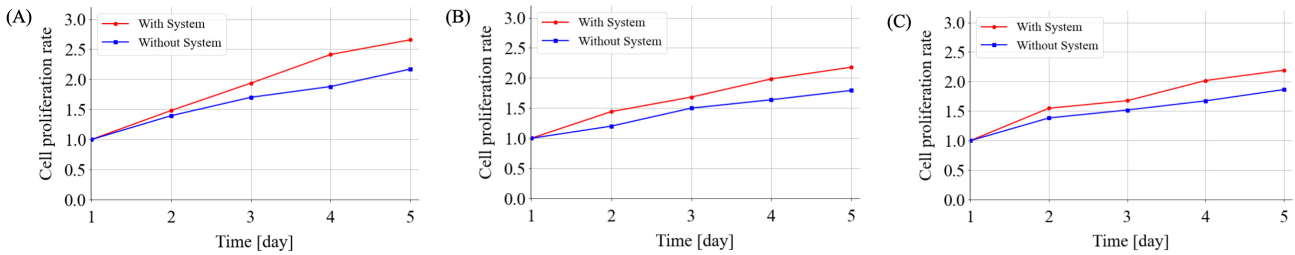

Fig.12 Trend of cell proliferation rate

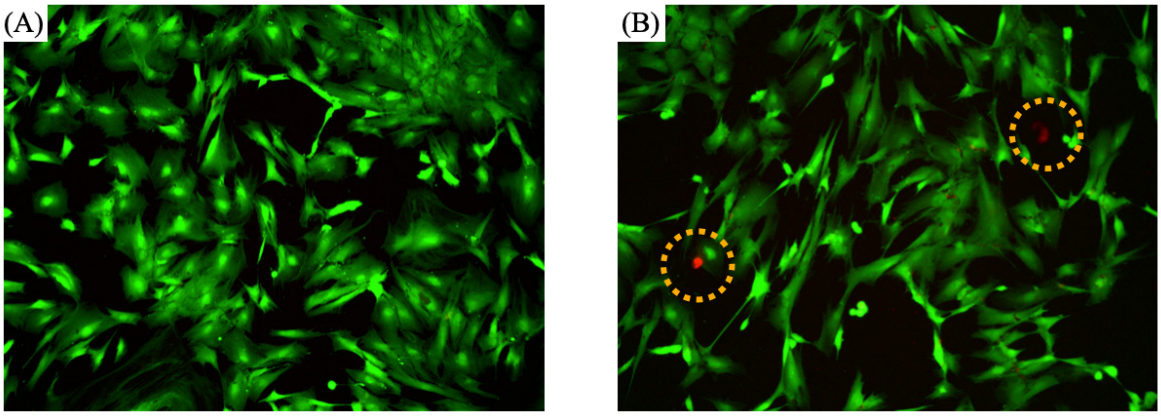

Fig.13 (A) Fluorescently stained fibroblasts (with system), (B) Fluorescently stained fibroblasts (without system)

analysis. To account for the issue of multiple comparisons in triplicate, the significance level was assessed using the Holm method with a significance level of 5%.

III. Results

In the aseptic experiments, the medium in the dish remained sterile with no signs of contamination. Figure 11 shows a graph of the changes in temperature, humidity, and CO_2 concentration during the five-day incubation period using the developed system. Throughout the incubation period, the temperature, humidity, and CO_2 concentration remained within the ranges typically maintained by standard CO_2 incubators: temperature (37 ± 0.25 °C), humidity (95 ± 5%), and CO_2 concentration (5 ± 0.15%). Every day, ten random images were taken from dishes cultured with and without the developed system, and cell counts were measured. Figure 12 shows the cell proliferation rate was calculated based on the cell count on day 1 to assess the rate of proliferation. The results confirmed that cell proliferation was enhanced when using the developed system. Figure 13 shows the results of fibroblasts stained with Calcein AM and Ethidium. Ten random field-of-view images were taken from dishes cultured with and without the developed system. Cell viability was calculated by counting the total number of cells and the number of dead cells, which were positive for Ethidium staining and appeared red (Table 2). In the first culture test, the mean cell viability was higher when using the system, but this difference was not statistically significant. However, in the second and third culture tests, cell viability improved significantly with the developed system, and this improvement was confirmed to be statistically significant.

Table.2 Survival rate

Trial	Survival rate (with system) [%]	Survival rate (without system) [%]	P-value	Significance level
1	98.8 ± 0.93	97.7 ± 2.0	0.22	0.05
2	98.9 ± 0.37	97.4 ± 0.75	0.0017	0.017
3	98.3 ± 0.56	97.2 ± 0.83	0.019	0.025

IV. Discussion

When contamination occurs caused by certain bacteria, the pH of the medium drops rapidly, the color is changed from red to yellow. Microorganisms can also be observed under a microscope. In the aseptic experiments, both visual and microscopic observations confirmed absence of contamination in the culture dishes when using the developed system. Therefore, it is considered that the developed system can safely culture cells without contamination. Through the experiment, we observed changes in cell growth rate and survival rate when the stress on the cells was reduced by observing the cells without altering the cell environment. The increase in cell viability is likely due to the suppression of fluid shear stress during medium exchange by maintaining a stable culture environment and gradually changing the medium, or improving delivery efficient of oxygen and nutrient in bioreactor. In standard cell culture, medium is typically changed using a micropipette, which can rapidly introduce or remove medium, subjecting the cells to significant stress. The medium transport unit has potential to reduce the physical stress on the cells compared to traditional micropipette-based methods because of precise control for the volume and timing of medium addition and removal. For the same reasons, the increased cell growth rate may also be attributed to this reduced stress, potentially coupled with the expression of growth factors such as fibroblast growth factor (FGF) [13]. During the monitoring of the culture environment, temperature, humidity, and CO_2 concentration were checked via application including Slack that is online communication tool. However, humidity was occasionally unstable when the external temperature dropped. This instability is thought to be due to the insufficient insulation of the CO2 container, causing condensation and fluctuations in humidity due to the temperature difference between the inside of the CO_2 container and the external environment. Regarding the glucose/lactate sensor, a decrease in glucose concentration and an increase in lactate concentration were observed during the five-day test, indicating that the system can monitor cellular metabolic activity.

As a future consideration, it is necessary to expand multiple dishes because the developed system currently supports a single culture dish although cell therapy requires the large number of cells. This would require configuring a new silicon tube route by increasing the number of tube pumps and solenoid valves used in the medium transport unit. Additionally, in the tests conducted during this study, the medium was changed on the third day of a five-day culture period, following general medium change practices. However, in typical culture operations, dead cells and debris visible under a microscope often guide the timing of medium changes. Incorporating an image processing system that can observe changes in cellular metabolic activity, in conjunction with measurements from glucose/lactate sensors, into the robotic cell culture system would enable the optimization of medium replacement timing. Although the study confirmed an improvement in cell proliferation rate, the analysis of medium-secreted growth factors, such as FGFs, has not yet been conducted. Further verification of the factors contributing to the improved proliferation rate is necessary.

V. Conclusion

We developed a robotic cell culture system that enables cell observation in an environment similar to that of a CO_2 incubator while monitoring the culture conditions and exchanges the medium automatically to facilitate the automated culture of fibroblasts cell with minimal damage, which are needed in large quantities for cell therapy. Through the experiment, both the survival rate and proliferation rate of fibroblasts were significantly improved. We confirmed the feasibility of the developed system about the capability of cultivating fibroblasts with an efficiency equivalent to or greater than conventional manual cell cultivation.

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