# Development of iPS Cell Automated Mass-Cultivation Instrument for Regenerative Medicine

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It is expected that the invention of iPS cells will lead to the realization of regenerative medicine. Because iPS cells are cultivated by hand, there are problems involving mistakes, contamination and the inability to culture the cells on a large scale. IHI is developing an iPS cell automated mass-cultivation instrument to solve this problem by applying large-scale culture and automation technologies. We have developed a novel bioreactor for large-scale suspension culture and a test instrument including the bioreactor. In this paper, we introduce these efforts.

## 1. Introduction

IHI has made advances into the medical field, with its group companies UNIGEN Inc. and IHI Plant Engineering Corporation (IPEC), operating businesses in animal cell-based vaccine drug substance manufacturing and pharmaceutical plant construction, respectively.

With the aging society, regenerative medicine is expected. In this field, we have been developing an automated cultivation instrument that draws on the automation techniques we have developed for industrial machinery and the cell culturing techniques developed for production of biopharmaceuticals. In order to contribute to expanding regenerative medicine, we are focusing on developing a technology that effectively cultures high-quality cells on a mass scale by reducing time spent on what is currently a manual procedure. In this paper, we will discuss the component technologies used in the automated mass-cultivation instrument currently under development.

## 2. Necessity of large-scale and automated cultures

In regenerative medicine, iPS cells or ES cells are cultured in order to generate tissues or organs from differentiated cells to restore or establish normal function. **Figure 1** shows the basic process of regenerative medicine. To generate large organs, a large number of cells are required as the source material of the organs. For example, a liver is made up of $2.50 \times 10^{11}$ cells, a heart $7.00 \times 10^{10}$ cells, and a kidney $2.60 \times 10^{10}$ cells.[1] To generate these organs and cells, approximately the same number of cells is required. **Table 1** shows the number of cells that compose different organs.

Above cells are cultured by the hand using petri dishes, which would require thousands to tens of thousands of petri dishes for culturing the numbers of required cells (see **Table 1**). Provided that one operator can process approximately 100 petri dishes in a day, performing maintenance culture in such a number of petri dishes is impossible by hand. Moreover, manual culturing may give rise to differences in the quality of cultured cells due to skill-level differences between operators. Therefore, an automated mass-cultivation instrument has been greatly required for advancement of regenerative medicine.

## 3. Current culturing method

iPS cells or ES cells, the source material for regenerative medicine, are adherent cells and are unable to survive or grow with cell-scaffolds (proteins such as collagen) attachment or cell-cell attachment. Currently, regenerative medicine research laboratories use a method of culturing such cells in which the bottom surface of a petri dish is coated with a cell adhesion matrix before the cells are cultured on it [2], [3] (dish culture). Another method suspends spheres of cells in a culture medium (suspension culture). Additionally, there has also already been a report of successfully culturing spheres of iPS cells by stirring using spinner flasks.[4], [5]

Culturing methods suitable for large-scale culturing were compared from the viewpoints of growth rate, ratio of undifferentiated cells, scalability, and technical difficulty.

The growth rate is considered superior in a suspension culture because the nutrients in the culture medium are delivered to the cells by convection, compared to diffusion in the case of a dish culture.

As for the ratio of undifferentiated cells, there appears to be a limit to the size of cell spheres that allow for stable culturing in a cell-sphere suspension culture, as factors and

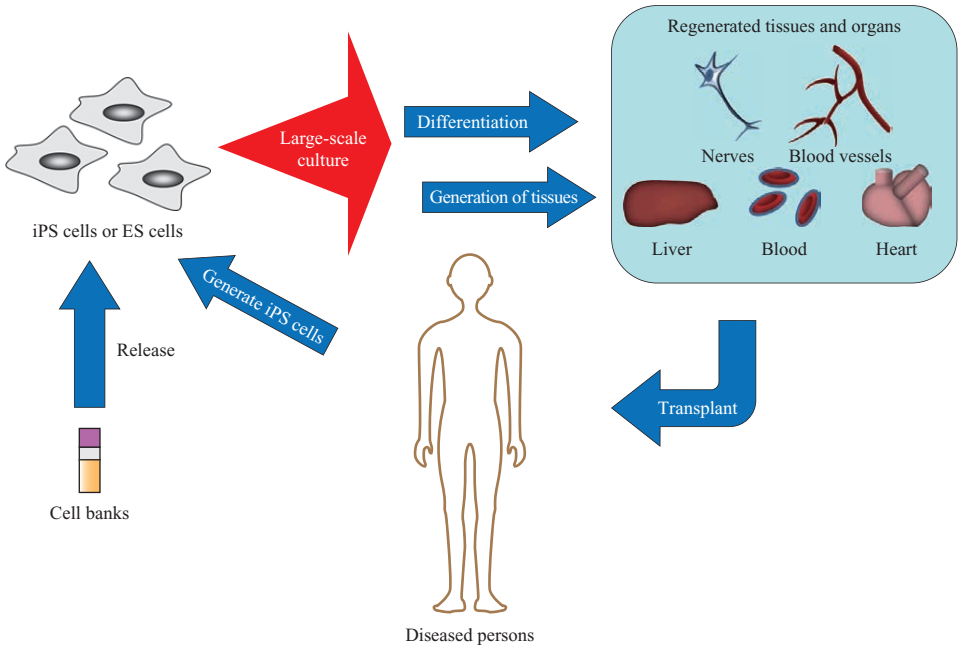



**Fig. 1 Basic process of regenerative medicine**

**Table 1 Number of cells in body organs**

| Organ | Weight (g) | Number of cells | Number of petri dishes equivalent*1 |
|---|---|---|---|
| Blood | 5 000-6 000 m*l* | $2.50 \times 10^{13}$ | 2 500 000 |
| Liver | 1 200-1 400 | $2.50 \times 10^{11}$ | 25 000 |
| Heart | 300-400 | $7.00 \times 10^{10}$ | 7 000 |
| Kidney | 150 | $2.60 \times 10^{10}$ | 2 600 |

(Note) *1: Assuming $1 \times 10^7$ cells are cultured in a $\phi$10-cm petri dish.

nutrients that prevent differentiation cannot penetrate to the interior of the sphere when the spheres become too large, causing inner cells to differentiate or die. Such a method of culturing therefore requires techniques that prevent spheres from becoming too big.

To culture cells on a large scale in a dish culture, a larger area is needed, requiring either larger or more petri dishes to be used. Neither of these options is suitable for large-scale cell culturing. On the other hand, a suspension method may be adapted for scale by using larger culture vessels. Approximately 1 to $2 \times 10^6$ cells/m*l* can be cultured in a normal suspension culture.[4), (6)]

Suspension culture requires a higher level of technical skill than dish culture. Because of the apparent need for controlling cell density and sphere size, and also because the shear stress caused by stirring may damage the cells, requiring accurate controls.

In addition there is also the method of suspending cells by attaching them to the surface of carriers[(7)] (carrier suspension culture). Although this culturing method combines the benefits of dish culture and suspension culture, a technique for consistently attaching cells to carriers has not been established. In addition, the size of the carriers to which cells are attached is approximately 200 µm in diameter, making them susceptible to shear stress. This method thus requires any high level technical skills.

**Table 2** shows the characteristics of each culture methods described above. As shown in **Table 2**, the carrier suspension culture is likely the most appropriate method, however, the level of technical difficulty is also too high. We have decided to begin our technology development by setting the suspension culture as our initial target for culturing cells automatically and on a large scale.

## 4. New bioreactor for suspension culture

In a suspension culture, it is important to reduce shear stress. For that purpose, we have been developing a new bioreactor. **Figure 2** shows a conceptual diagram of the new bioreactor. In this bioreactor, the medium flows from the bottom to the top, and the cells are cultured in this upward flow. Because the reactor is wider at the top, the upward flow is faster at the bottom and slower at the top. Fluid in a tube flows faster in the center and slower near the wall due to friction, but this reactor is designed to create a uniform extrusion flow (plug flow) in which the flow rate gradient in the tube is reduced (**Fig. 3**). As a result, cell spheres are held at the place where the sedimentation rate and the upward flow are balanced. This means that the larger spheres are held lower in the reactor, and the smaller spheres held higher. By forming a uniform plug flow, the old culture medium in the reactor can

**Table 2 Characteristics of culture methods**

| Culture method | Growth rate | Undifferentiated cell ratio | Scalability | Technical difficulty |
|---|---|---|---|---|
| dish | △ | ○ | △ | Low |
| Suspension (cell sphere) | ○ | △ | ◎ | Medium |
| Suspension (carrier adhesion) | ○ | ○ | ◎ | High |

(Note) *1 : ◎, ○, △ : Levels of suitability

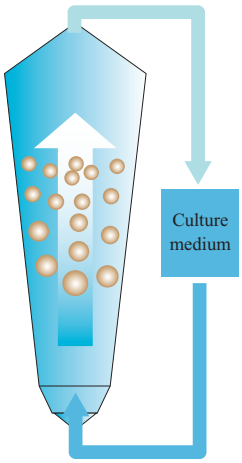



**Fig. 2** Conceptual diagram of new reactor

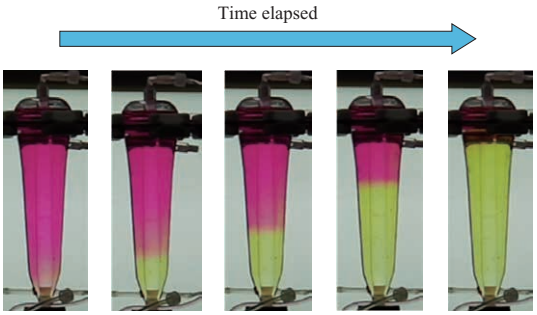



**Fig. 3** Plug flow formed in new reactor

be replaced to the new culture medium that flows in from the bottom of the reactor without mixing each other.

This culture system reduces the stress to cells, making it possible to culture cells without damaging them. Additionally, there is no need for a separation mechanism because the cells are held in place within the reactor, allowing for a simple culture system without a separation device such as a centrifuge separator or separation membranes.

## 5. Production of test instrument

We have created a test model of an automated cell culture instrument built-in the new bioreactor. **Figure 4** shows the automated cell culture component test instrument. This instrument consists of ① a $CO_2$ incubator with a built-in bioreactor, ② a refrigerator for storing medium, ③ a controller, ④ a touch screen for operation, and ⑤ a number of tube pumps for medium and chemical solution for delivery, and is designed to accommodate various culture processes by controlling these components. The culture vessels and pipes used here are expected to be sealed and sterile to reduce the possibility of microbial contamination without having a sterilization function. Our ultimate goal is to allow automated passage. The test instrument requires approximately 1.6 $m^2$ for installation, and we are currently working on downsizing it.

## 6. Culture test using mouse ES cells

First we tested culture performance using mouse ES cells. This was because human iPS cells cannot be used without establishment of an ethical committee (Japan rules). We also intended to utilize knowledge acquired from the mouse ES cells to the culture test using human iPS cells.

Spheres of mouse ES cells formed after one day of culturing in the new bioreactor. By continuing the culture, we could culture of mouse ES cells spheres can grow approximately 100 fold, from $1 \times 10^4$ cells/m*l* to $1 \times 10^6$ cells/m*l*.[8] **Figure 5** shows the culture test using mouse ES cells. As a result of studying the undifferentiation of the cultured mouse ES cells using an immunostaining method, Nanog, Oct4, and Sox2 markers, which are indicative of undifferentiation, were verified.[8]

## 7. Culture test using human iPS cells

After verifying that mouse ES cells can be cultured in the new bioreactor, we began of culture test using human iPS cells in Yokohama City University (with establishment of an ethical committee).

Before beginning the culture test, we created a culture

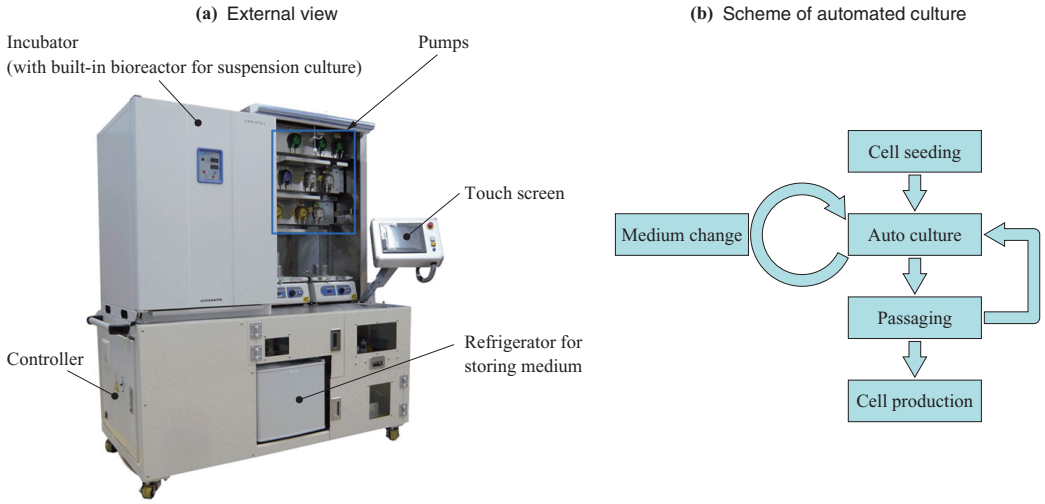
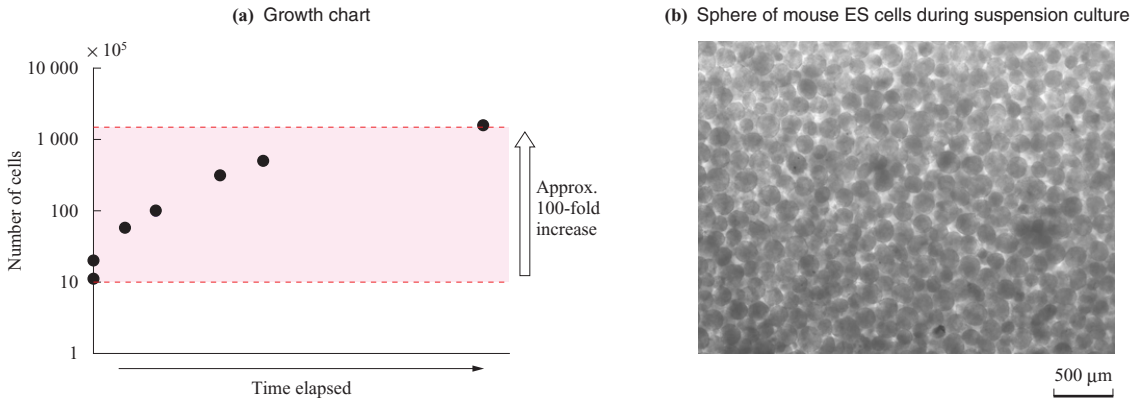



**Fig. 4** Test model of automated cell culture instrument



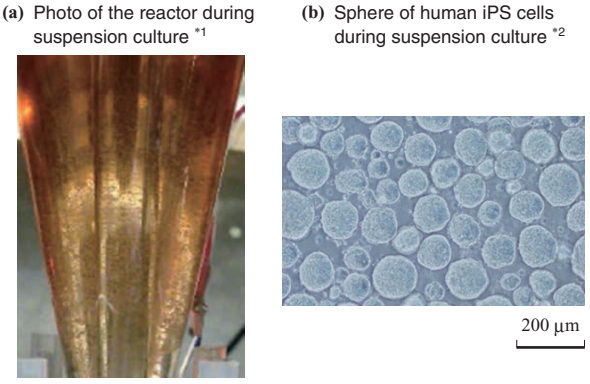

(Note) *1 : One sphere made from thousands to tens of thousands of Mouse ES cells.

**Fig. 5 Mouse ES cell culture test**

program for implementing the automated culture process by referring to the procedures for dish culture of human iPS cells.

First, we will describe the procedure for culturing human iPS cells in petri dishes recommended by Kyoto University.[9] In this procedure, human iPS cells are cultured by attaching them to petri dishes coated with a scaffolding protein called laminin. Because iPS cells die easily when the cell-to-cell adhesion or cell-to-matrix adhesion is dissociated, a reagent that suppresses cell death called ROCK inhibitor is added when dispersing spheres of cells into single cells. The added ROCK inhibitor is removed one day after the cells are seeded in petri dishes. After that, the medium is replaced daily, and the cells are detached from the petri dishes again in about 7 days, and are diluted and seeded in new petri dishes. Repeating this procedure is the protocol for culturing iPS cells in petri dishes.

We have created a culture program in which this protocol is adapted to the control of the pumps in the component test instrument we have developed. **Figure 6** shows the culture test using human iPS cells. Using this program allowed us to culture human iPS cells using a suspension culture method, as shown in **Fig. 6-(a)**. Sampling the culture fluid and observing it with a microscope, we found that the human iPS cells formed spheres of 50 to 150 µm in diameter as shown in **Fig. 6-(b)** (thousands to tens of thousands of human iPS cells, each of which is approximately 10 µm in diameter, formed one sphere).




(Notes) *1 : Each brown particle represents a sphere of iPS cells.
*2 : Human iPS cells are cultured in spheres of thousands to tens of thousands of cells.

**Fig. 6 Human iPS cell culture test**

As a result of studying the undifferentiation of the cultured human iPS cells using an immunostaining method, Nanog, Oct4, Sox2, SSEA4, TRA1-60, and TRA1-81 markers, which are indicative of undifferentiation, were verified (**Fig. 7**).

We are currently working to improve the culture program with the aim of enhancing the efficiency of culturing human iPS cells in suspension using the new bioreactor.

## 8. Conclusion

In this paper, we discussed the background, conceptual formulation, and development of the component technologies of a new bioreactor and automated test instrument for culturing human iPS cells on a mass scale, as well as the results of culture tests using this instrument. The results of the culture tests using mouse ES cells and human iPS cells suggest that the cultured cells maintained their undifferentiated state. In the future, we will overcome remaining challenges with the goal of developing an automated cultivation instrument product for use at medical facilities and research laboratories.

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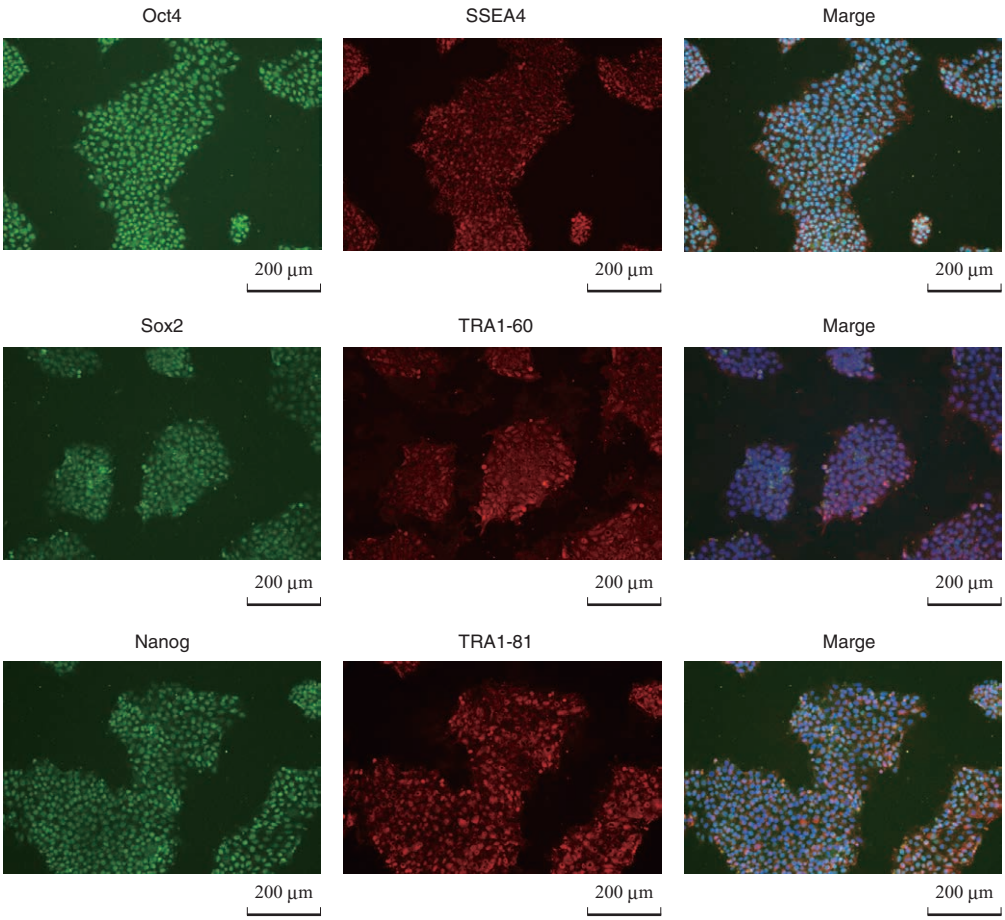


(Note) Verification method: Immunostaining method to color marker proteins which are indicative of undifferentiated human iPS cells.

**Fig. 7 Confirmation of iPS cell anaplasticity with immunostaining method**

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