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Image-based cell quality evaluation to detect irregularities under same culture process of human induced pluripotent stem cells

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To meet the growing demand for human induced pluripotent stem cells (iPSCs) for various applications, technologies that enable the manufacturing of iPSCs on a large scale should be developed. There are several technological challenges in iPSC manufacturing technology. Image-based cell quality evaluation technology for monitoring iPSC quality in culture enables the manufacture of intact cells for further applications. Although several studies have reported the effectiveness of image-based evaluation of iPSCs, it remains challenging to detect irregularities that may arise using the same processing operations during quality evaluation of automated processing. In this study, we investigated the evaluation performance of image-based cell quality analysis in detecting small differences that can result from human measurement, even when the same protocol is followed. To imitate such culture conditions, by image-analysis guided colony pickup, we changed the proportions of morphologically different subpopulations: "good morphology, regular morphology correlated with undifferentiation marker expression" and "bad morphology, irregular morphology correlated with loss of undifferentiation marker expression". In addition, comprehensive gene-expression and metabolomics analyses were carried out for the same samples to investigate performance differences. Our data shows an example of investigating the usefulness and sensitivity of quality evaluation methods for iPSC quality monitoring.

[**Key words:** Quality control; Induced pluripotent stem cell; Morphology analysis; Cell quality evaluation; Gene expression profile; Metabolomic analysis]

Induced pluripotent stem cells (iPSCs) have been increasingly used in various applications, such as drug discovery and regenerative medicine studies (1,2). To expand their applications, industrial-scale cell manufacturing that enables production of iPSCs in large scale as cell-based products is becoming essential. Given these increasing demands, technologies for automating the cell culture process are urgently required. However, technological challenges remain in establishing controlled manufacture methods for iPSCs with stable quality.

First, during the culture of iPSCs, various factors easily alter the undifferentiated status of iPSCs (3,4). Because iPSC culture is currently mostly based on manual handling, it remains difficult to identify critical factors that disturb the final iPSC quality. Therefore, for effective automation of iPSC manufacture, methods for monitoring the daily status of iPSCs, such as undifferentiation rate, are important. Additionally, the heterogeneity of cells in the evaluation of cellular status remains unclear. It is technologically difficult to monitor the diversity of all sub-populations and the effect of their proportions during the culture process. However, previous studies have reported the importance of such heterogeneity derived from sub-populations in stem cells (5). Because one of the final goals of iPSC culture automation is to stably produce undifferentiated cells with low quality diversity, technologies for detecting heterogeneous transitions in iPSCs during culture should be developed.

Currently used iPSC culture processes greatly rely on the daily microscopic observation of colony morphology. The detection and monitoring of irregularity in colony morphologies is important for iPSC culture. This technology would enable both the daily monitoring of cells and detection of heterogenic irregularities. The automation of such technology would enable quality monitoring in cell manufacturing. Moreover, automated image analysis would enable high-throughput and low cost iPSC manufacture.

Given the importance of morphological evaluation in various cell cultures, we have been investigating image-based cellular morphology evaluation based on phase contrast microscopic images (6,7). We demonstrated that morphological analysis can be used for daily monitoring of cellular quality and can predict the future quality of mesenchymal stem cells (8). We also recently reported that the multi-parametric morphological classification of diverse colony populations could reveal morphologically irregular colonies that also reflect irregular gene expression profiles (9). An increasing number of reports have described the effectiveness of image-based evaluation of embryonic stem cell (ESC) and iPSC colonies for determining their qualities (10,11). Tokunaga et al. (12)

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found a correlation between the morphological features and characteristics in cellular components, and we reported that morphological features reflect the irregular pattern of gene expression profiles (9). Therefore, interest in evaluating colony morphology as a surrogate marker of quality of the final cell-based product is increasing.

However, studies in which image-based evaluation has been applied in iPSCs were focused on comparing different conditions, such as the difference of cell lines and culture conditions. To manufacture iPSCs, irregularities in similarly controlled culture processes must be detected. Image-based quality evaluation technology used with automated manufacturing should be able to detect irregularities that occur within the same operations. Such irregularity can be practically described as partial irregular cells increasing in a vessel. Therefore, in this study, we compared two morphological conditions that mimicked small differences, such as changes in the proportions of morphologically different cells even using the same culture protocol (same cell line, culture medium, culture period, and operator). Small differences in cellular status can easily occur because of differences in handler skills; cell culture experts can maintain iPSCs in good condition. To create such a culture condition, we controlled the proportion of two morphologies by objective removal of target type of cells assisted by image analysis-guided colony picking: cells with good morphology (GM), which is a regular morphology correlated with undifferentiation marker expression, and bad morphology (BM), which is an irregular morphology correlated with the loss of undifferentiation marker expression. We compared three different criteria for such evaluation: (i) colony morphological profile (phenotypic signatures), (ii) comprehensive gene expression profile (inter-cellular signatures), and (iii) metabolite profile (extra-cellular signatures), to determine the applicability of these measurements. Our results reveal the potential of morphological evaluation of iPSC colonies as a sensitive quality control technology in their manufacture and suggest the effective combination of quality evaluation methods for iPSC quality monitoring.

MATERIALS AND METHODS

Cells and cell culture Human iPSCs (201B7) was obtained from the RIKEN cell bank. The hiPSCs were cultured in TeSR-E8 (Stemcell Technologies, Vancouver, BC, Canada) on Vitronectin XF (Stemcell Technologies) at 37°C in a fully humidified atmosphere with 5% CO_2. Cells were passaged with a cell scraper and ReLeSR (Stemcell Technologies) each day. Human mesenchymal stem cells (MSCs) (P5, Lot. 01174, Lonza, Basel, Switzerland) were cultured in MSCGM (Lonza Japan Corp., Tokyo, Japan), incubated at 37°C with 5% CO_2, and used as control stem cells.

Evaluation of cells Cells were counted using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Absorbance (450 nm) of the supernatant was measured using an EnSpire (PerkinElmer, Waltham, MA, USA). For marker staining, cells were first fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), washed twice, and permeabilized with PBS containing 0.1% Triton X-100 (T-PBS) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 15 min at room temperature. After blocking with PBS containing 4% goat serum (16210-064, Life Technologies, Carlsbad, CA, USA) for 15 min, primary antibody (anti-OCT3/4 rabbit IgG H134, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was hybridized overnight. After washing with T-PBS three times, secondary antibody (Alexa Fluor 488 Goat Anti-Rabbit IgG, Thermo Fisher Scientific, Waltham, MA, USA) was hybridized for 1 h at room temperature. After immunohistochemical staining, nuclei were stained with SYTOX Blue fluorescent dye (500 μL per well; Thermo Fisher Scientific). To stain another marker in different well, rBC2LCN-635 (200-fold diluted; Wako Pure Chemical Industries, Ltd.) was applied with new medium for 2 h at 37°C and 5% CO_2. Before image acquisition, cell samples were washed once with new medium. The cell culture was conducted in 6-well plates (Corning, Corning, NY, USA).

Image acquisition and processing Phase contrast and fluorescent microscopic images were acquired using a Biostation CT (Nikon Corp., Tokyo, Japan) at a ×4 magnification. The image acquisition field was set to 1.6 cm^2 in the center position (consist of 8 × 8 tiling images. 1000 × 1000 pixel per image). Images were processed using CL-Quant software version 3.2 (Nikon Corp.). The cellular area in the images was recognized by a soft-matching algorithm following the manufacturer's protocol. The detailed image processing scheme with processed images are given in Supplementary Fig. S1. Briefly, three independent raw images including several colonies were selected as training images. By comparing the fluorescent images of undifferentiation marker staining (OCT3/4 and rBC2LCN-635), 5–8 positions were chosen from the raw images to train the soft-matching algorithm as "enhance (target)", "suppress (non-target)", or "background" area data. For the target area, a colony area corresponding to the stained area of OCT3/4 was selected. For the non-target area, cells were selected as cellular objects, but they corresponded to an area where the OCT3/4 was negative. For "background", areas not containing cellular objects were selected. Training was repeated while modifying the selected areas until all collected images showed good correlation with the targeted area, and also match to the area of rBC2LCN-635 positive. This process created a trained image recognition algorithm (recipe) in CL-Quant, which recognizes the cellular area and confirm both colony area and undifferentiation marker positive, but eliminates other areas. We defined the cellular area recognized by this recipe as good morphology (GM) and designated the recognition recipe as GM_recipe. Next, we enhanced the white area to flatten raw images to create a recipe that recognized the cell-occupying area. We subtracted the GM_recipe area from this cell existing area to develop a precise recognition recipe that only recognized the cellular area without the GM_recipe-recognized area. We defined such cells and colonies as bad morphology (BM), and designated the recipe as BM_recipe.

Enhancement of proportion of morphologically different subpopulations To mimic the slightly different culture conditions resulting from differences in human skills even when using the same protocol, we set the proportion of colonies with irregular morphologies to either increase or decrease using a combination of morphology analysis and colony pickup (schematic illustration in Fig. 1). First, when a certain number of colonies were obtained in 6-well plates during the preparation culture of iPSCs (before the step starts in Fig. 1), phase contrast images were acquired using a BioStation CT as described above. Acquired images were processed using CL-Quant with both the GM_recipe and BM_recipe to classify the existing colonies in each image as GM or BM. Two conditions showing altered morphological variation were set; BM area minimized condition (BM_min), and BM area enhanced condition (BM_enhance) (Supplementary Fig. S1). To prepare either condition in a well, cellular objects outside the field of view (1.6 cm^2 in the center position of a well) were scraped off using a scraper (with PBS washing). Next, the entire cellular area recognized by BM_recipe was scraped off with a pipette and scraper (with PBS washing). The remaining cells were then picked up for seeding into new wells (N = 6) using a scraper. This condition was designated as BM_min. In contrast, the cellular area recognized by GM_recipe was removed in the same manner and the remaining cells were picked up for seeding into new wells (N = 6) in the same manner, designated as BM_enhance. This manipulation day was counted as day 0. At day 4, when cell growth was in the logarithmic growth stage (Supplementary Fig. S2), images of cultured iPSC colonies were acquired and analyzed using GM_recipe and BM_recipe. On this day, the cellular objects outside the field of view were again scraped off to eliminate the cellular existence outside the analyzed images. Next, poly-dimethylpolysiloxane (PDMS) was inserted to fill the space except for the field of view (Supplementary Fig. S3). In the well containing the PDMS insert, the recognition and quantification of cellular area was analyzed using both recipes and the proportion of the GM or BM area was determined. In the well containing the PDMS insert, the existing cell number was counted as previously described. After counting the cell number, we washed the cells 3 times with fresh medium and added 800 μL fresh medium followed by 24 h incubation. Following incubation, 150 μL of the supernatant was collected for metabolomics analysis. From 6-well plates seeded for BM_min (1 plate) and BM_enhance (1 plate), 2 wells from each plate were first picked for marker staining (described in the Evaluation of cells section). In each plate, supernatant was collected from the 4 remaining wells; the cell number was determined, and lactate and glucose concentrations were measured by the F-kit (Roche Diagnostics K.K., Tokyo, Japan). By measuring the lactate production rate per cell (LacR) and the glucose consumption rate per cell (GLuR), the contrast value of LacR/GluR was calculated to select 2 wells for further evaluation by gene expression and metabolomics analyses. For example, in the BM_min plate, the 2 samples that showed higher LacR/GluR contrast were set as conditions A and B. In the BM_enhance plate, the 2 samples that showed lower LacR/GluR contrast were set as conditions C and D. The detailed sample setting is illustrated in Supplementary Fig. S4. To prepare new data for amino acid measurement, BM_min2 and BM_enahnce2 were newly set following the aforementioned protocol. Measurement of the BM_recipe recognition area described above demonstrated that this condition was consisted of 100% BM_recipe coverage ratio. We designated this condition as BM_only. To further emphasize the difference in the BM_enhance2 condition, cells of the BM_recognized area were disaggregated and ROCK inhibitor (Wako Pure Chemical Industries, Ltd.) was added to the culture medium during the preparation of BM_only.

Metabolomics data analysis The supernatants collected from the 6-well plate with the PDMS insert (which contained the same cell number for image analysis, gene expression analysis, and metabolomics analysis in each well) were analyzed by capillary electrophoresis-mass spectrometry with the Basic scan plan

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