Establishment of an algorithm for automated detection of iPS/non-iPS cells under a culture condition by noninvasive image analysis

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Introduction

There are great expectations for iPS cells as tools for drug development and resources for regenerative medicine. However, such medical applications demand a stable supply of iPS cells in large quantities and many issues for optimization of iPS cell generation remain to be resolved. In addition, evaluation of iPS cells is time-consuming and dependent on the skills of individual technicians.
We have developed a system that can observe a large quantity of iPS cells automatically and classify iPS colonies with a standardized algorithm.
In this experiment, a cell culture observation system (BioStation CT) was used to observe a culture, and image analysis software (CL-Quant) was used to scan phase-contrast images and automatically detect iPS/non-iPS colonies generated by reprogramming of normal somatic cells. In implementing the automatic detection an algorithm was designed to categorize cells of different origin based on parameters pertaining to morphological characteristics of the cells. For 20 samples consisting of various numbers of colonies the coefficient of correlation was extremely high between the number of iPS colonies counted by inspection and the number of iPS colonies counted by CL-Quant. Accuracy in iPS/non-iPS identification was next compared. For the cell type used for algorithm design, accuracy was high at an average of 79.9%. For three types of cells of different origin the average accuracy was 80.3%. This indicates the strength of the method in providing accurate iPS/non-iPS identification results. To confirm the consistency of analysis results, accuracy was compared with the results of analyzing cells stained with the TRA-1-60 antibody (marker specific to iPS cells). This experiment shows that iPS/non-iPS identification during the establishment stage can be automated eliminating the need for human intervention and allowing for non-invasive analysis without the use of fluorescent dyes. This provides several advantages. 1) Reduction of required time and effort. 2) Non-invasive analysis.
3) Homogenization. The method allows for consistent selection of good iPS cells without requiring training. 4) Historical management. By maintaining a record of the identification results, quality evaluations can be reanalyzed with ease.

Methods

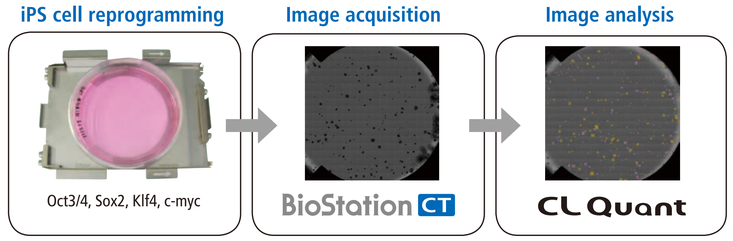

Fig. 1 Experimental step flow of iPS cell reprogramming, Image acquisition, and Image analysis

iPS cell reprogramming

- iPS cells were generated from adult human dermal fibroblast(HDF) by retroviral transduction of four factors: Oct3/4, Sox2, Klf4, and c-Myc.[1]
- Six days after transduction, the cells were harvested by trypsinization and plated onto mitomycin C-treated SNL feeder cells using 100 mm dish.

Image acquisition

- Phase contrast images and fluorescence images of 100 mm dish were acquired using BioStation CT (NIKON CORPORATION) at 37°C, 5% CO_2.
- Full Scan mode (2x) was set for acquiring full well tiling images.
- Tiling mode (10x) was set for acquiring partial well tiling images.

Fig. 2 BioStation CT
BioStation CT is a cell culture observation system that allows microscope observation of multiple vessels (max. 30) easily and automatically under culture conditions. It is possible to acquire full-well tiling 2x images by selecting Full Scan mode. Acquired images are automatically downloaded onto a PC for analysis.

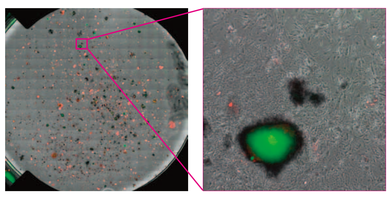

Image analysis

- Full well tiling images were loaded into CL-Quant software (NIKON CORPORATION) and stitched to create 18,000-by-18,000 pixel composite images for analysis.
- All image analyses (colony segmentation, measurement, colony classification etc.) were performed using functions built into CL-Quant software [2].

Algorithm development

Step 1) Total colony detection

To detect colonies accurately and quickly from stitched images, original stitched images are pre-processed and colony regions are recognized as colony masks using machine leaning technology (Soft Matching™). Created colony masks undergo post-processing as follows (Fig. 3).

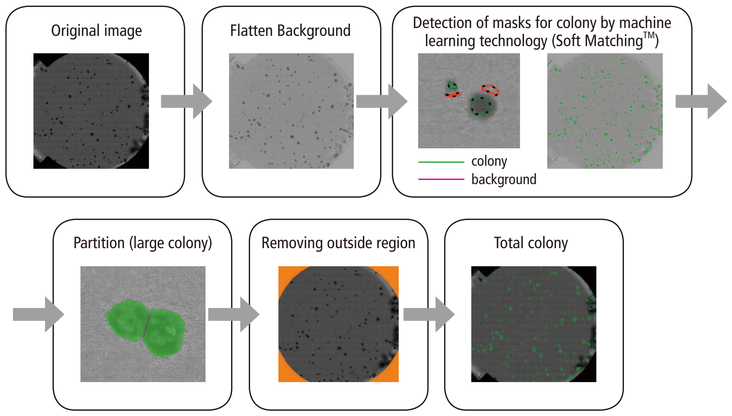

Fig. 3 Total colony detection step flow

Step 2) iPS colony identification

iPS colony identification steps are shown in Fig. 4. Colonies detected in Step 1) are separated by size. Additional colony boundary masks are given to each colony (Fig. 5). Each colony is then classified as iPS or non-iPS based on the morphological rules depicted by the decision tree. The decision tree was created by teaching iPS/non-iPS colonies to CL-Quant software (Fig.6). Classified colonies are referred to the total iPS/non-iPS colony number in the vessel.

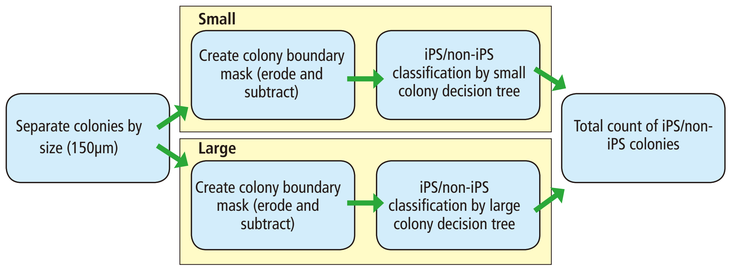

Fig. 4 iPS colony identification step flow

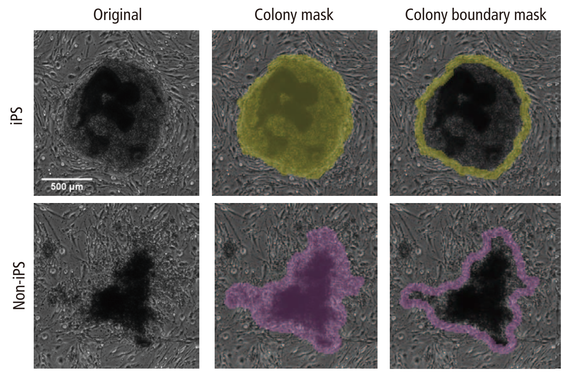

Fig. 5 Typical iPS/non-iPS colony images and created colony masks and colony boundary masks

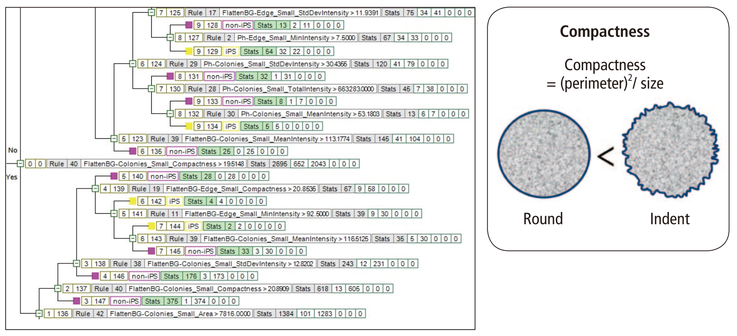

Fig. 6 Classification rule.
Left: A portion of a decision tree. 2695 colonies were taught (652 iPS, 2043 non-iPS) to create the small colony decision tree. 2013 colonies were taught (899 iPS, 1114 non-iPS) to create the large colony decision tree. Decision trees contain a variety of colony morphological and intensity information (size, intensity of boundary region, compactness, etc.) In both trees, the compactness of the colony mask is the primary rule (first decision node). This shows that iPS colonies have more rounded colony boundaries than non-iPS colonies.
Right: The compactness of the object.

Algorithm validation

1) iPS colony count evaluation

Fig. 8 shows plotted results of manual and CL-Quant-made iPS counts of iPS colonies (in 20 samples) used for creating the algorithm. The linear approximation of the graph shows a high correlation function of R=0.95. This indicates that CL-Quant-made iPS counts have similar results to manual counts of any number of colonies. The difference between target areas of manual and CL-Quant-made counts may be less than 1 linear approximation slope.

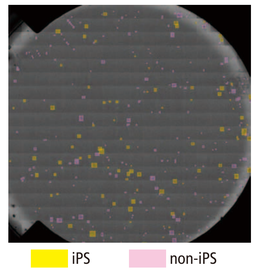

Fig. 7 Analysis result image

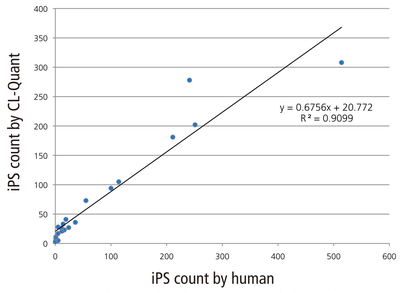

Fig. 8 Comparison of manual and CL-Quant-made iPS colony counts

2) Accuracy evaluation for different cell lines

Compare accuracies of manual and CL-Quant-made iPS/non-iPS identifications using iPS cell (Cell A), used for creating an algorithm, and other cell lines (Cell B, C, D). CL-Quant-made iPS/non-iPS identification using Cell A indicates an accuracy of 79.9%, and other cell lines 80.3%. This shows the strength of this analysis algorithm.

Accuracy

Type	Human	CL-Quant
A	iPS	iPS
B	non-iPS	non-iPS
C	iPS	non-iPS
D	non-iPS	iPS

$$\text{Accuracy (\%)} = \frac{A+B}{A+B+C+D} \times 100$$

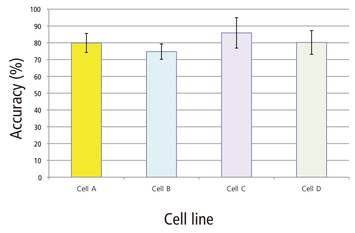

Fig. 9 Accuracy evaluation of iPS/non-iPS identification
Left: Definition of Accuracy.
Right: Accuracy comparison of iPS/non-iPS identification of several cell lines (use dozens of colonies for 1 data, N=3).

3) Comparison with iPS cell specific marker

Comparison results of TRA-1-60 positive/negative identification and CL-Quant-made iPS/non-iPS identification using TRA-1-60 immunofluorescent stain (an iPS cell specific marker).
Comparing results of TRA-1-60 stain and CL-Quant-made identifications of 237 colonies, the results coincide for 70.4% of colonies (167 colonies), indicating the high consistency of both identifications. (Fig. 10, Table 1)

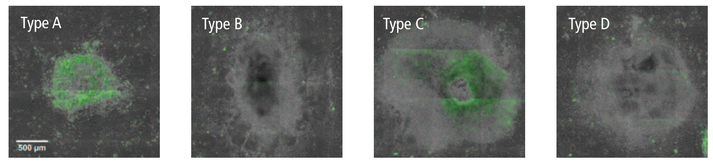

Fig. 10 TRA-1-60 immunofluorescent stained colony images (10x tiling images)

Table 1 Comparison of TRA-1-60 immunofluorescent stain result with CL-Quant iPS/non-iPS identification

Type	TRA-1-60	CL-Quant	Percentage	Accuracy
A	+	iPS	65.8% (156 colonies)	○ 70.4%
B	-	non-iPS	4.6% (11 colonies)	
C	+	non-iPS	28.7% (68 colonies)	× 29.5%
D	-	iPS	0.8% (2 colonies)	

Reference

1. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. & Yamanaka, S. 2007 Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131**, 861-872.
2. Alworth, SV., Watanabe, H. & Lee, JSJ. 2010 Teachable, High-Content Analytics for Live-Cell, Phase Contrast Movies. *J Biomol Screening* **8**, 968-977.

Conclusion

- Nikon has developed an image analysis algorithm that detects colonies in the entire area of a dish and identifies iPS/non-iPS colonies using certain standards, such as compactness.
- iPS/non-iPS identification using the analysis algorithm shows similar results to manual identification (R=0.95) and also shows similar results (70.4% of agreement rate) to identification using iPS cell specific markers. In addition, the algorithm proves it is highly accurate (80.3%) for different cell lines.
- These noninvasive analysis systems can automatically acquire a number of images of samples and analyze images based on certain standards. These functions reduce variations in evaluation during iPS cell generation and save time and work.