Generation of a growth curve for iPS cells in a feeder-free culture by noninvasive image analysis.

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Introduction

Maintaining the undifferentiated state of iPS cells is an essential, but difficult process to control. The process requires improvement in the following three areas:
1. Feeder-free culturing: To eliminate culture variability and to reduce culturing costs.
2. Automation: To reduce culturing costs, and to achieve consistency in the supply of iPS cells.
3. Establishment of a quantitative index: To quantify culturing status and provide a clear index for evaluation.

As a means for implementing these improvements, a system has been developed for time-lapse observation of feeder-free cultures within which iPS colonies can be automatically detected and measured.

The Nikon BioStation CT cell culture observation system was used to capture time-lapse images of the entire surface of a 6-well plate (100 images with a 2x objective (4x4mm)) over a 1 week period of feeder-free culturing (using ReproCELL ReproFF2), from seeding to passage. Once a culture dish is inserted and an observation schedule is set, the system automatically captures images while maintaining the observation position even with daily medium replacements, allowing the growth of each colony to be tracked with ease.

CL-Quant image analysis software was taught to detect iPS colonies, thus allowing iPS colonies to be identified within phase-contrast images. Changes within each colony were measured and plotted as a colony growth curve which could then be summarized per well, each representing a distinct culturing condition.

The system was used to generate growth curves for iPS cells cultured over a 1 week period (from seeding to passaging) while their undifferentiated state was maintained. Quantifying the rate of growth and comparing it to the undifferentiated state established a benchmark for this process.

Methods

iPS cell culture and growth

Image acquisition

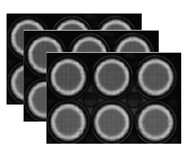
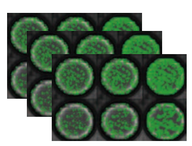


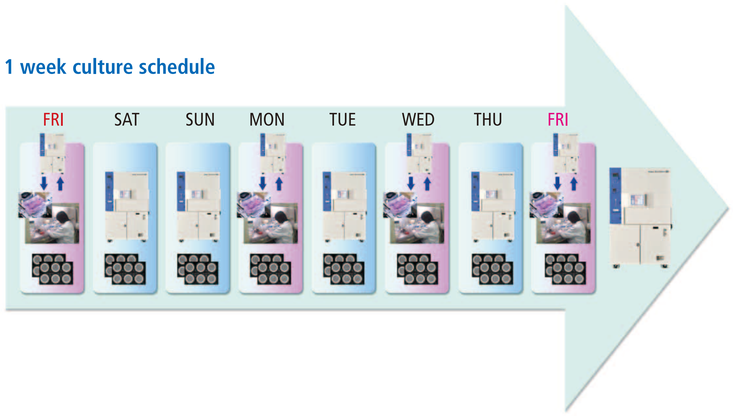
Image analysis



iPS cell culture and growth

- 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 feeder free cells using a 6-well plate. Repro FF2 medium ReproCELL was used.

1 week culture schedule



Basic condition (1 week)

- Culture conditions
  Cell: hiPSs:201B7, Coating: Matrigel, Culture vessel: 6-well plate, Culture medium: ReproFF2, Medium change: Mon, Wed, Fri/1 week
- Capture conditions
  Magnification: x2 (Full scan), Culture day: 1 week, Imaging interval: 3 or 12 hours

Image acquisition

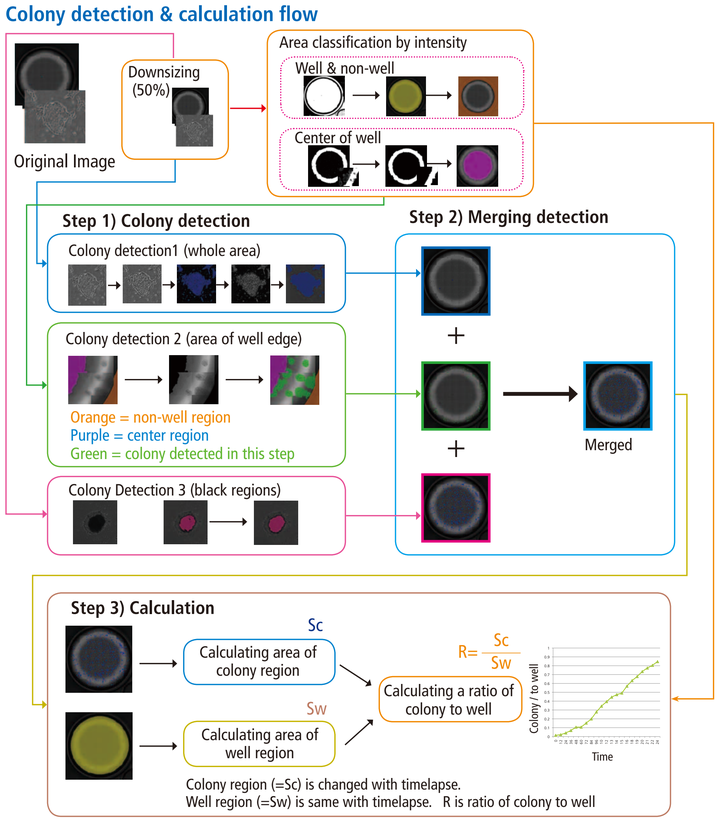
- Phase contrast images of a 6-well plate were acquired using BioStation CT (NIKON CORPORATION) at 37℃ , 5% CO_2.
- High-precision repeatability: Accurate tracing of same cells, as well as X-Y positions (±5μm) for each vessel.
- Full Scan mode (2x) was set for acquiring full-well tiling images.

Image analysis

- Full-well tiling images were loaded into CL-Quant software (NIKON CORPORATION) and stitched to create 10,000-by-10,000 pixel composite images for analysis.
- All image analyses (colony segmentation, measurement etc.) were performed using functions built into CL-Quant software [2].

Algorithm development

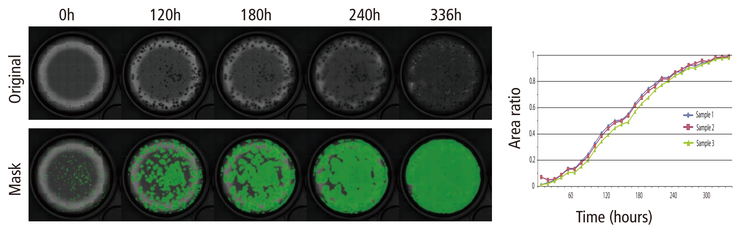
Colony detection & calculation flow



Confirmation of algorithm

Algorithm development

Nikon has developed an algorithm that enables temporal quantitative analysis of colony proliferation, from immediately after cell seeding to cell confluence.

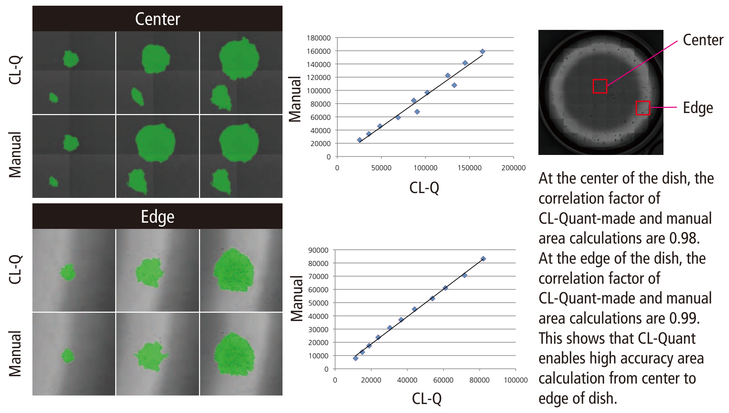


Experiment procedures

- Culture conditions
  Cell: hiPSs:201B7, Coating: Matrigel, Culture vessel: 6 well plate, Culture medium: ReproFF2
  Medium change: Mon, Wed, Fri/ 1 week
- Capture conditions
  Magnification: x2 (Full scan), Culture day: 2 weeks, Imaging interval: 12hours

Comparison of results from detection using algorithm and manual detection

Manual versus auto-detection with CL-Quant calculations were made. The results were similar from center to edge of dish.



At the center of the dish, the correlation factor of CL-Quant-made and manual area calculations are 0.98. At the edge of the dish, the correlation factor of CL-Quant-made and manual area calculations are 0.99. This shows that CL-Quant enables high accuracy area calculation from center to edge of dish.

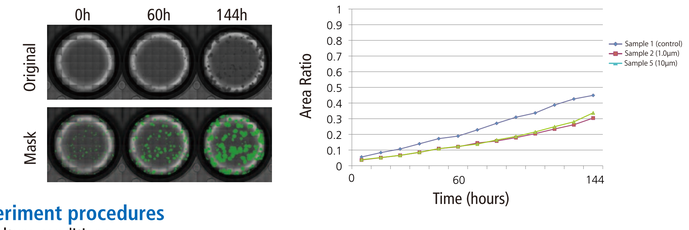
Experimental procedure

- Culture condition
  Cell: hiPSs:201B7, Coating: Matrigel, Culture vessel: 6 well plate, Culture medium: ReproFF2
  Medium change: Mon, Wed, Fri/ 1 week
- Capture condition
  Magnification: x2 (Full scan), Culture day: 1 week, Imaging interval: 3hours

Results

Experiment 1) Using the CL-Quant algorithm for standard cell culture

CL-Quant enables image analysis of standard 1 week iPS cell-culture sequence.

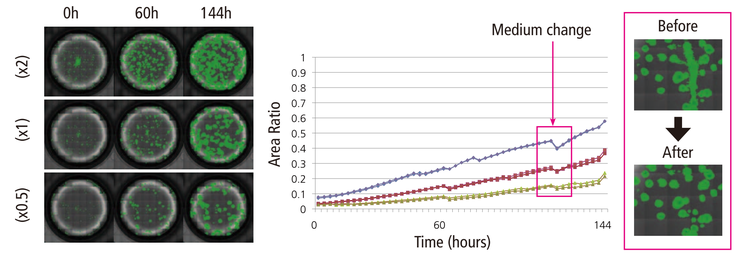


Experiment procedures

- Culture conditions
  Cell: hiPSs:201B7, Coating: Matrigel, Culture vessel: 6 well plate, Culture medium: ReproFF2
  Medium change: Mon, Wed, Fri/ 1 week
- Capture conditions
  Magnification: x2 (Full scan), Culture day: 1 week, Imaging interval: 12hours

Experiment 2) Comparison of cell seeding density

The results indicate that growth of colonies depends on seeding density (for 1 week cell cultivation).

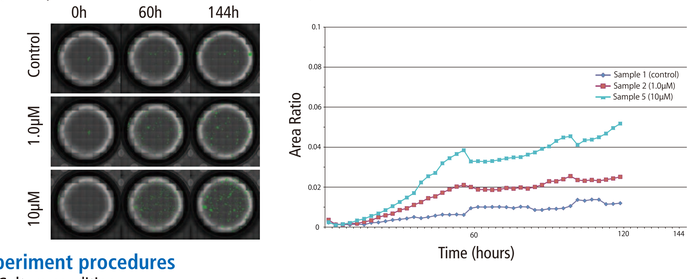


Experiment procedures

- Culture conditions
  Cell: hiPSs:201B7, Coating: Matrigel, Culture vessel: 6 well plate, Culture medium: ReproFF2
  Medium change: Mon, Wed, Fri/ 1 week
- Capture conditions
  Magnification: x2 (Full scan), Culture day: 2 weeks, Imaging interval: 3 hours

Experiment 3) Drug addition

CL-Quant quantifies the influence of Tzv-addition, which affects a cell's survival rate.



Experiment procedures

- Culture condition
  Cell: hiPSs:201B7, Coating: Matrigel, Culture vessel: 6 well plate, Culture medium: ReproFF2
  Medium change: Mon, Wed, Fri/ 1 week
- Capture conditions
  Magnification: x2 (Full scan), Culture day: 5 days, imaging interval: 3 hours

Thiazovivin Mechanism Overview

Description

Tzv is a novel ROCK inhibitor and Rho-ROCK axis regulates cell-ECM and cell-cell adhesion. Thiazovivin protects human embryonic stem cells (hESCs) in the absence of ECM by regulating E-cadherin mediated cell-cell interaction [3]. This observation suggests that Thiazovivin promotes cell survival. Thiazovivin, in combination with inhibitors of the TGF-ß receptor and MEK pathway, has shown to improve reprogramming efficiency by more than 200-fold [4].

Reference

1. Takahashi, K,. Yamanaka. S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. (2007) Nov 30;131(5):861-72.
2. Alworth, SV., Watanabe, H. & Lee, JSJ. Teachable, High-Content Analytics for Live-Cell, Phase Contrast Movies. J Biomol Screening. (2010) 8, 968-977.
3. Xu, Y., Ding, S. Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules. Proc Natl Acad Sci USA. (2010) 107: 8129-8134.
4. Lin, T., Ding, S. A chemical platform for improved induction of human iPSCs. Nature Methods (2009) 6: 805-808. (URL:https://www.stemgent.com/products/show/72)

Conclusion

1. Nikon has developed an algorithm that enables quantitative analysis of colony proliferation, from immediately after cell seeding to confluency. Comparing manmade and algorithm-detection, the correlation factors in full image areas have similar results: 0.98 at the center of the dish, and 0.99 at edge.
2. CL-Quant enables analysis of effects under various conditions.
- Colony area proliferation depends on cell seeding density.
- CL-Quant quantifies the effects of thiazovivin density.

CL-Quant has achieved non-invasive quantification of change of culture conditions of iPS cells in a feeder-free culture, based on the ratio of colony/bottom of dish area.