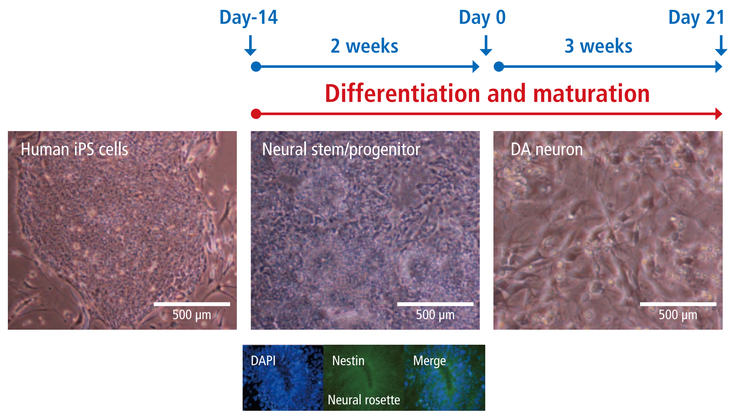
Automated, non-invasive culture, and evaluation system for iPS cells under neural differentiation process

Chieko Nakada[1], Momotaro Ishikawa[1], Hiroaki Kii[1], Takayuki Uozumi[1], Hirotada Watanabe[1], Yoichi Yamazaki[2], Takayuki Kitogo[3], Yasuyuki Asai[3], Yasujiro Kiyota[1]
[1] Instruments company, NIKON CORPORATION, Yokohama, Japan, [2] NIKON SYSTEMS, Inc., Tokyo, Japan, [3] ReproCELL, Inc., Kanagawa, Japan

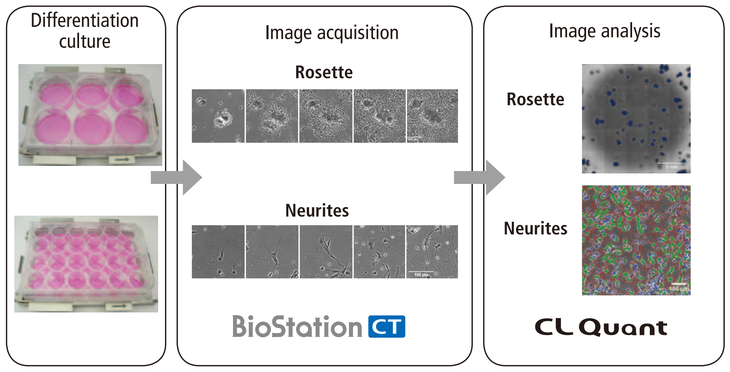
Introduction

Differentiation process of human iPSCs toward DA neuron



- It takes more than ~4 weeks in general to induce neurons from iPS/ES cells.
- "Neural rosette" and "neurites" are the two morphological checkpoints in the neural differentiation process.
- We aimed to develop an integrated culture, observation and evaluation system which enables quantitative and label-free analysis of cells being differentiated from iPS cells into neurons under incubation condition.
- New image processing methods were developed to detect neural rosette structures and neurons (cell bodies and neurites);
- The developed methods were then tested and applied to estimate the differentiation status.

Methods



Schematic drawing showing experimental workflow.

Cell culture

- iPS cells were differentiated from different lines of iPSCs into Dopaminergic neurons.
- Cells at rosette stage were plated on 6 well-plates. To culture neural precursors for further differentiation and maturation, rosette-stage cells were collected, dissociated, and plated on 24-well plate at densities of 15 and 7.5 x 104 cells/well. Cells were kept in BioStation CT (NIKON CORPORATION) at 37°C, 5% CO2.

Time-lapse image acquisition

- All imaging was performed using BioStation CT (NIKON CORPORATION) at 37°C, 5% CO_2.
- For rosette-stage cells, phase contrast images were captured at a magnification of 2x. To obtain high contrast at any height of the cell structure, z-stack of 5µm x 40 images was acquired at each x-y position. These z-stack images were used in the image analysis to create EDF (extended depth of focus) image. 25 x-y positions were observed at each well, so that the squared area of 19.2 x 19.2 mm was covered. Images were captured every 6 hours for 5 to 7 days.

BioStation CT

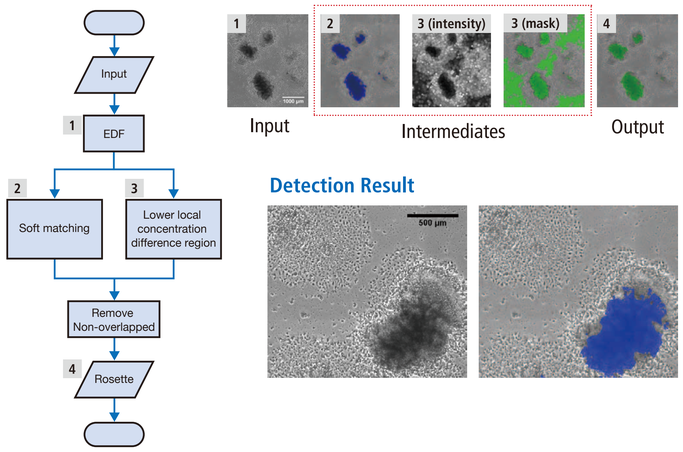
- For observation of neurons and precursors, phase contrast images were obtained at 10x and 20x. Neurite outgrowth was observed by capturing images at every 30 min or 60 min for 5 days for 10x and 20x observation, respectively. After time lapse observations, neurons were fixed, stained with a monoclonal antibody against MAP2 (a neurite marker protein) followed by Alexa 488-labeled secondary antibodies, and the fluorescence signals were observed with the GFP channel.

Image analysis

- All image analysis was performed using functions built-in software (CL-Quant, NIKON CORPORATION) [1].

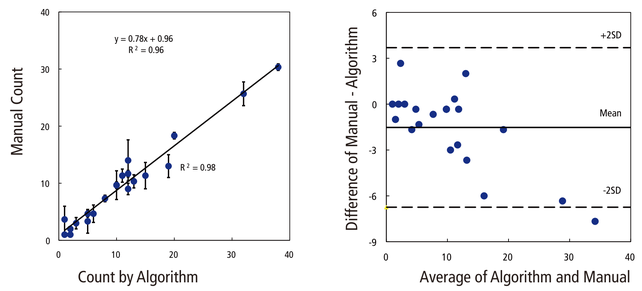
Results 1.
Neural rosette structure detection and analysis

Algorithm Development



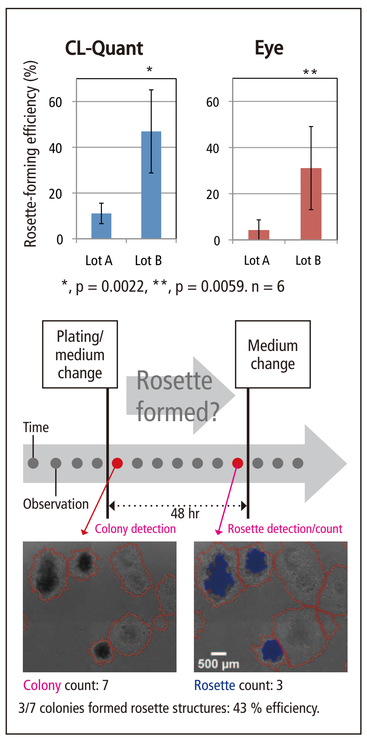
Detection Result

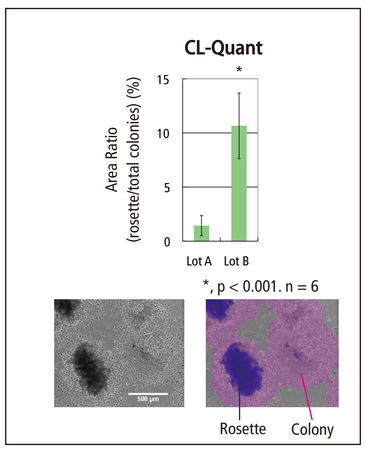
Algorithm Validation



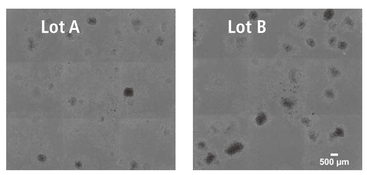
Validation of the developed algorithm. Left, correlation in the number of rosette-like structures between human eye (manual count, n=3) and the algorithm. It showed a high correlation efficient of 0.98. Right, Bland-Altman plot.

Application: Rosette-forming efficiency: lot-to-lot variation





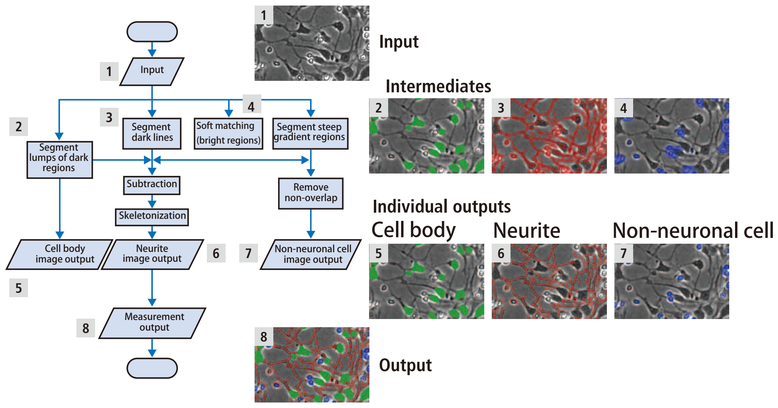
The rosette-detection method was applied to estimate the efficiency of culture protocols in terms of rosette formation, and to compare the results between two different lots (A and B).
In the top left box, number of colonies were counted right after plating and evaluated if each of the colony possessed rosettes afterward (measured ~48 hr later, right before medium change). Colonies and rosettes were counted by algorithm (blue) or eye (red), and then % colonies that formed rosettes later were calculated (rosette-forming efficiency). In the top box, total area of rosette and colonies were calculated by software to obtain the ratio as an index of rosette abundance at one time point (green). Both parameters indicated that more rosettes appeared in lot B than lot A, being consistent with the result by human eye-based detection (three experienced investigators).



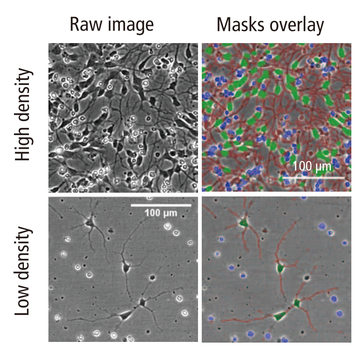
Representative image of lot A and B in a rosette-forming stage. Lot B has more rosette structures than Lot A.

Results 2.
Neurite detection and outgrowth analysis

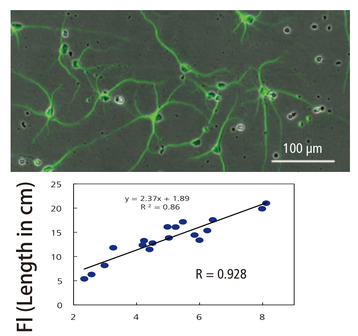
Algorithm Development



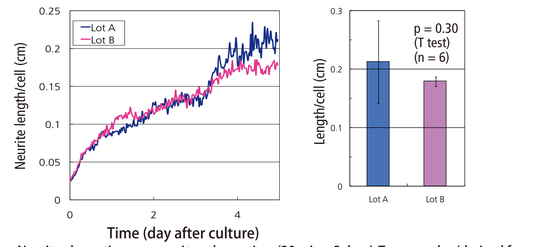
Detection Results



Algorithm Validation



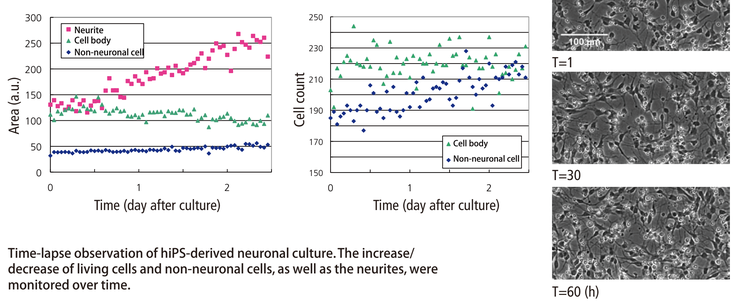
Detection Results



Algorithm Validation. After neurons were cultured for 14 days, cells were fixed and immuno-stained with MAP2. Neurites detection was performed based both on phase and fluorescence images to see their correlation. The correlation coefficient was high at 0.93.

Neurite elongation was monitored over time (30 min x 5 days). Two samples (derived from different rosette samples (lot A and B) were compared and there was no significant difference throughout the observation period. The top bar graphs show the values at the final time point. Based on neurite and cell body measurements, average neurite length per cell was calculated as the total neurite length divided by the total number of cell bodies in a field of view.

Application: Monitoring cell survival over time



Time-lapse observation of hiPS-derived neuronal culture. The increase/ decrease of living cells and non-neuronal cells, as well as the neurites, were monitored over time.

Reference

1. Alworth, SV., Watanabe, H. & Lee, JSJ. Teachable, High-Content Analytics for Live-Cell, Phase Contrast Movies. J Biomol Screening 8, 968-977, 2010.

Conclusion

- Phase contrast, live cell, Image-based analysis was performed with new algorithms developed for neural rosette-structure and neurite detection.
- These algorithms detected rosettes in high correlation with human eye.
- The neurites detected based on the phase-contrast images highly matched with those based on fluorescent signals from neurite marker.
- These analysis would become useful tools for variety of applications, such as estimation of differentiation efficiency in different protocols, toxicity testing, and benchmarks for quality controls.