APPLICATION NOTE

Quantifying subtypes of the iPSC-derived cardiomyocytes and α-actinin fragmentation by drug treatments using automated cell imaging

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Introduction

Animal testing has been an indispensable necessity throughout the history of biomedical research, drug development, and preclinical testing. There have, however, been movements and efforts to steer away from the use of animal models or the conducting of animal testing in various fields, including drug evaluation, with the recent developments and advances in in vitro and in silico methods as well as other alternative methods.

The status quo of animal models and testing has changed as regulations surrounding the use of test animals are becoming ever more stringent year after year with the European Commission enforcing its 3R strategy to "reduce, refine, and replace," which ultimately subjects the usage of animals in selected toxicity tests under heavy surveillance.

In the midst of the ongoing regulation changes, many institutions/organizations are still conducting preclinical or toxicity tests in animal cells or models, with inconsistent results due to interspecies differences. Therefore, the development of a new toxicity study model to replace the use of animals in the non-clinical study is of the utmost urgency.

With the continued development of stem cell research, induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) can be used across multiple stages of drug development, which include but are not limited to efficacy tests, safety tests, metabolomic analysis, and toxicity assessment. In particular, the Cardiosight®-S, a highly pure iPSC-CM manufactured by NEXEL through their proprietary induced differentiation technology, is used in the comprehensive in vitro proarrhythmia assay (CiPA) for its ability to detect arrhythmia caused by test compounds using electrophysiological test equipment and has been proven to be a suitable cardiomyocyte for the evaluation of cardiac safety.

To investigate and analyze shifts in electrophysiological characteristics or muscular contraction of a drug in vitro, a cardiomyocyte that has been thoroughly investigated across multiple parameters is required, due to the nature of cardiomyocytes that differentiate into other various subtypes including atrial and ventricular myocytes as well as nodal cells. Therefore, strict quality control is also required for the cardiomyocyte types along with thorough investigation into physiological functions, expression conditions, and respective biological markers of the cell.

Benefits

- Providing a standard for quality control of cell line production through automatic image acquisition and simultaneous quantification of the iPSC-derived atrial and ventricular myocytes composition.
- Providing an automated method for evaluating drug-induced structural changes of hiPSC-derived cardiomyocytes and aiding in candidate selection by performing high throughput screening based on cardiac toxicity.

In the present study, the analysis of subtypes of iPSC-CMs and the investigation of drug effects on the CM structure were made possible through the ImageXpress PICO automated cell imaging system and CellReporterXpress software. The expression of each subtype-specific marker (ventricular myocytes: MLC-2V, atrial myocytes: MLC-2A) in the differentiated iPSC-CM and α-actinin fragmentation after drug treatment in the CMs was inspected qualitatively through fluorescence images. Subsequently, the quantitative analysis and the statistical significance of the marker expressions and changes were analyzed comparatively through the software analysis function.

In general, the conventional fluorescent microscopic equipment suffers from scope-limited and user-biased image acquisition. However, these drawbacks of the conventional method are overcome by the automated high-throughput system, which allows for the quantification of cellular characteristics and changes in multiple parameters, offering the system as a viable contender for a highly efficient 'high content screening' evaluation system. Therefore, we demonstrated the possibility of using the ImageXpress PICO automated cell imaging system to evaluate the cardiomyocyte subtypes differentiated from the human pluripotent stem cells (atrial vs. ventricular myocytes) and to assess drug toxicity by measuring the structural changes of the cardiomyocytes.

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Methods

Cell cultures

Cardiomyocytes used in the study were manufactured and provided by Nexel Co., Ltd. For the cardiomyocytes subtype analysis, Cardiosight®-S (#C-002) mature hiPSC-CM and an immature hiPSC-CM in the production line of identical cell were used. To confirm α-actinin fragmentation, Cardiosight®-S and Hypertrophied hiPSC-CM (R403Q) were used. All cell lines were thawed and cultured according to the user guide provided by Nexel. 30,000 cells were plated onto a 13 mm coverslip and cultured for 7 days. For the α-actinin fragmentation, cells were treated with compounds that induce sarcomere structure damage (3 µM Doxorubicin, 3 µM Sunitinib, 200 µM Cyclophosphamide in 0.1% DMSO) for 24 hours.

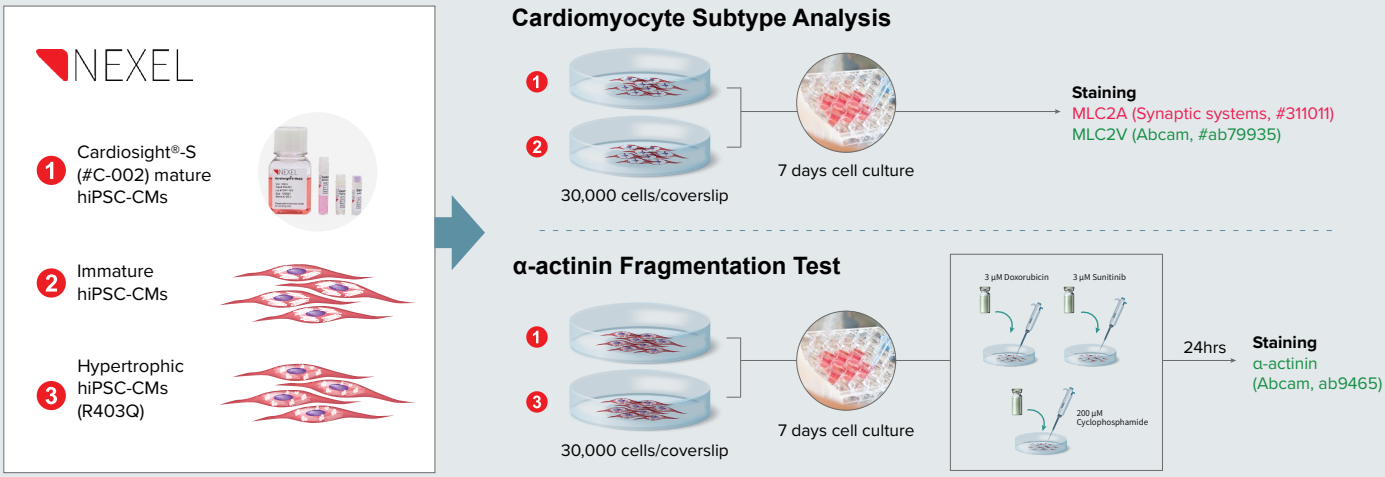
Immunostatining

Cardiomyocytes subtype analysis

To confirm the purity of the cardiomyocytes, cells were fixed in 4% paraformaldehyde on Day 7 and stained with atrial myocyte antibody MLC2A (Synaptic systems, 313011) and ventricular myocyte antibody MLC2V (Abcam, ab79935).

α-actinin fragmentation test

Cells were fixed in 4% paraformaldehyde after undergoing 24-hour drug treatment on Day 7, and stained with α-actinin (Abcam, ab9465).



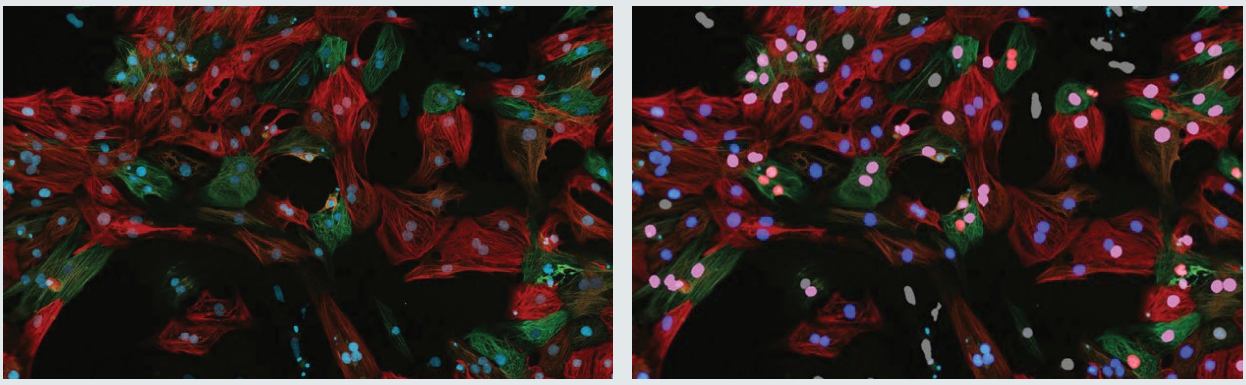


Image of the immunostaining of hiPSC-CM taken by the automated imaging system for subtype quantification. Left: blue-nuclei, green-MLC2A, red-MLC2V. Right: Image applied with white overlay mask processed by CellReporterXpress Cell Scoring Module.

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Automated cell imaging and analysis

Cardiomyocytes subtype analysis

In order to confirm whether hiPSC-CMs are atrial or ventricular myocytes, images were acquired by the ImageXpress PICO automated cell imaging system, which was then followed by an analysis of atrial and ventricular myocytes composition through CellReporterXpress software. Cells that gave off corresponding fluorescent signals above a set threshold for atrial and ventricular myocytes were selected and measured.

α-actinin fragmentation test

To analyze changes in the structure of both normal and hypertrophied cells treated with cardiomyocyte sarcomere structure damaging compounds, α-actinin structure images were taken and quantified. When cardiomyocyte sarcomere structures are damaged, linear structures break down into small fragments in the shape of granules. As such, the granularity analysis module was used to analyze α-actinin morphology and to quantify the degree of fragmentation.

Result

Cardiomyocytes subtype analysis

Mature cardiomyocytes generally contain a higher ratio of ventricular myocytes than atrial myocytes. Upon analyzing Cardiosight®-S, two different batches were revealed to have greater than or equal to 55% ventricular myocytes (MLC2V+++/MLC2A-) and less than or equal to 20% atrial myocytes (MLC2V-/MLC2A+++). Other than these two subtypes, cells that exhibited both markers were either classified as MLC2V++/MLC2A+ or MLC2V+/MLC2A++ depending on the intensity of the two markers. As a result, about 30% of all cells were revealed to show both types (MLC2V+/MLC2A+), and of those cells, those that displayed a ventricular characteristic (MLC2V++/MLC2A+) were 3 folds greater in number than those exhibiting an atrial characteristic (MLC2V+/MLC2A++). From the fluorescence expression comparison, it is expected that cells which exhibit both subtypes will transition into a ventricular myocyte through atrial-ventricular transition following further maturation achieved through a longer culture period (Figure 1, (a)).

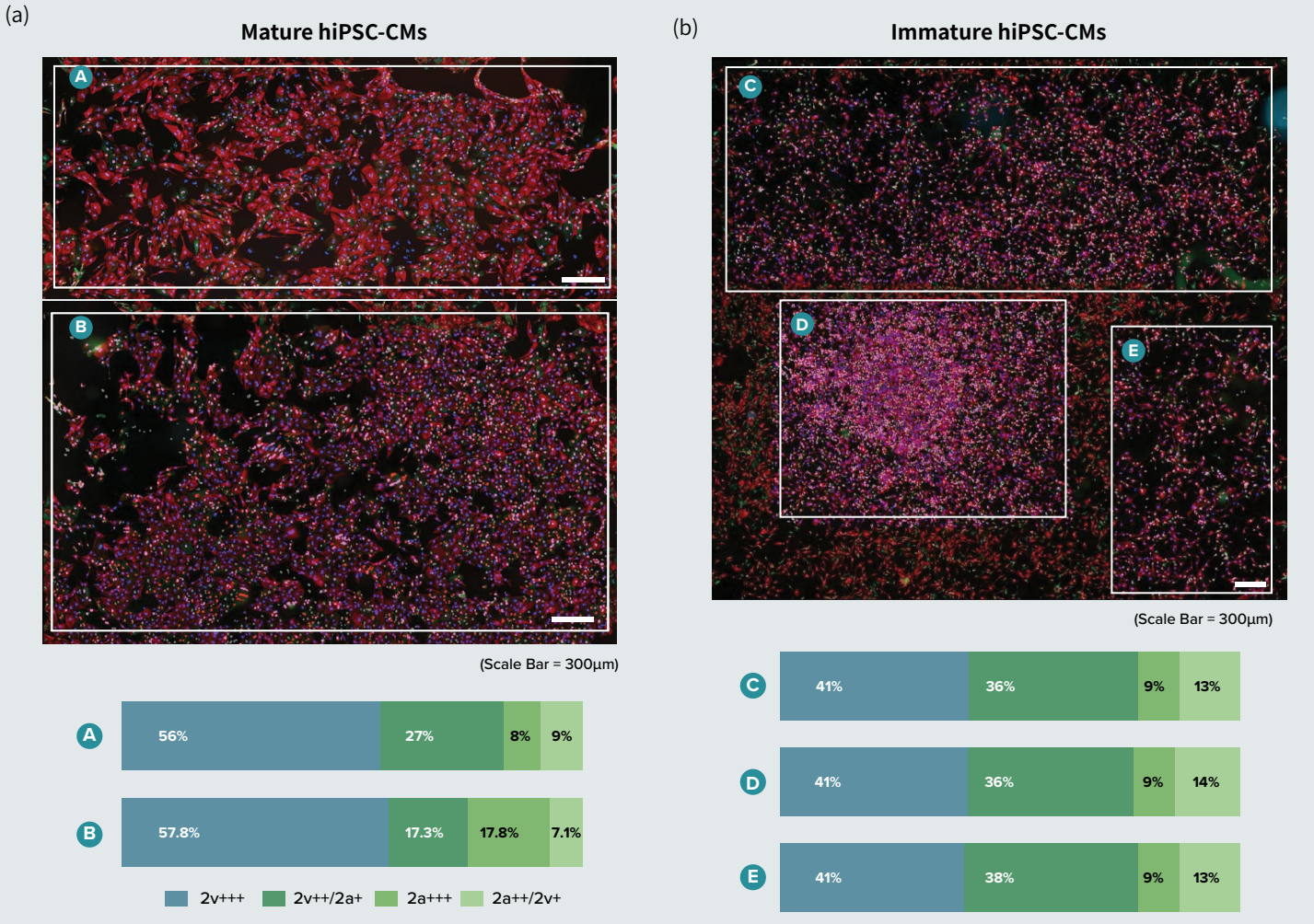

Figure 1. *Purity quantification of hiPSC-CM using the automated imaging system. Nucleus dyed in white, atrial antibody MLC2A in green, and ventricular antibody MLC2V in red. An analysis overlay from CellReporterXpress has been applied. Below are bar graphs representing the cell composition ratio by batch. Figure 1 (a) represents mature hiPSC-CM and figure 1 (b) represents immature hiPCS-CM.*

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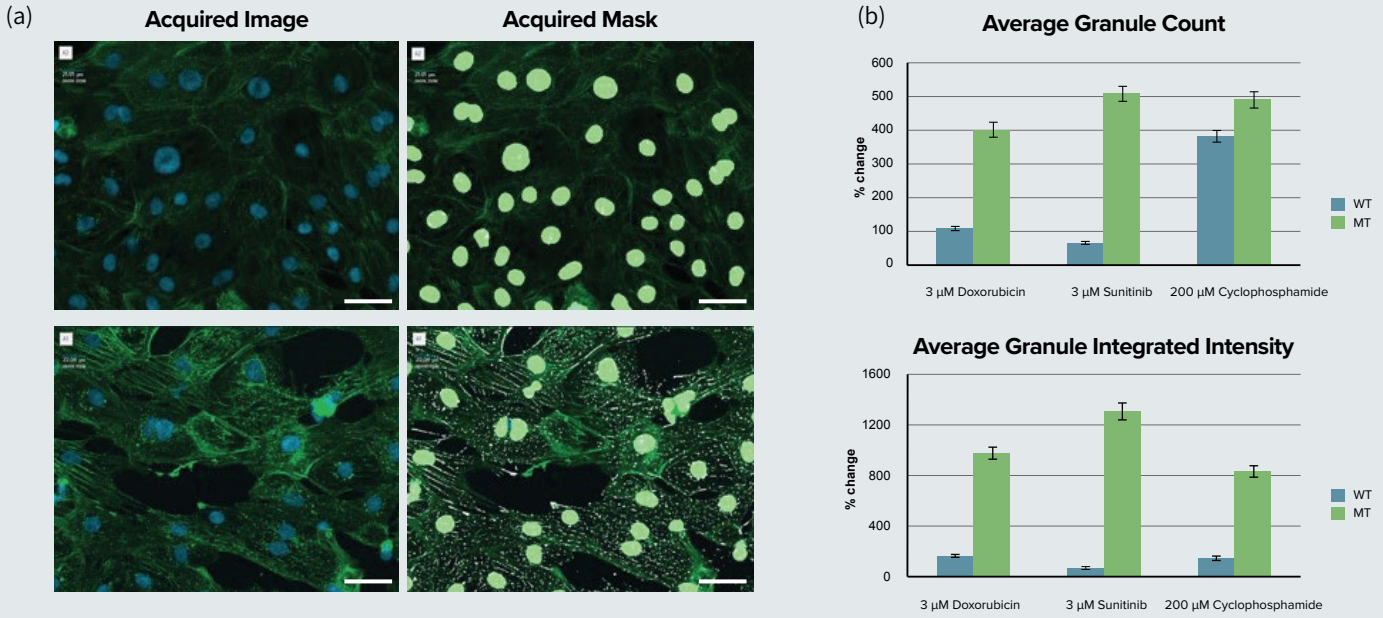

Figure 2. *Measurements of change in the cardiomyocyte structure and α-actinin caused by compounds in hiPSC-CM with the automated imaging system. (a) Left: nucleus in light blue, and α-actinin in green. Right: image with the white overlay mask. Nucleus in yellow-green, and the fragmentation of altered structure of α-actinin in white. (b) Graph exhibiting the number and the fluorescence intensity of fragmented α-actinin.*

The subtype of immature cardiomyocytes was analyzed by selecting three regions within a single sample. Upon confirming the analyzed data of the three regions, it was revealed that it contained 41% ventricular myocytes (MLC2V+++/MLC2A-) and 9% atrial myocytes (MLC2V-/MLC2A+++), 36.9% of ventricular-biased types (MLC2V++/MLC2A+), and 13.1% atrial-biased types (MLC2V+/MLC2A++) on average. The numbers maintain a constant ratio that is indifferent to the region analyzed. (Figure 1, (b)) There has, however, been a limitation on objective results analysis by interpreting the acquired image alone. The ImageXpress PICO automated cell imaging system allows for a quick and easy image acquisition, and also provides quantified analysis results through its software. Therefore, the cardiomyocyte subtype analysis demonstrates the feasibility of utilizing the ImageXpress PICO automated cell imaging system to measure whether the production maintains a constant cardiomyocyte quality.

α-actinin fragmentation test

Granule-like particles of α-actinin, which are unable to form a long chain due to the damage caused by the compounds administered, were observed in every group other than the negative control (0.1% DMSO). Specifically, normal cells that were treated with cyclophosphamide were revealed to have the most substantial damage to the α-actinin, which led to the highest granule count and fluorescence intensity. On the other hand, hypertrophied cardiomyocytes did not exhibit any significant differences in α-actinin damage between the compounds administered, but it a higher value of α-actinin damage compared to that of the normal cells was shown. In summary, it can be deduced that hypertrophied cardiomyocytes are more vulnerable to sarcomere structure damage by compounds than normal cardiomyocytes (Figure 2).

Conclusion

ImageXpress PICO automated cell imaging system was used successfully to analyze the subtypes of differentiating and differentiated cardiomyocytes from hiPSCs and calculate α-actinin fragmentation for a comparative analysis of structural cardiotoxicity caused by various compounds. The previous method of image analysis has cast uncertainty on data reliability due to the limited sample image scope representing the entirety of the data. The present study demonstrated the ability of the ImageXpress PICO automated cell imaging system, combined with CellReporterXpress software, to present analysis results and various comparative parameters based on the acquired fluorescent image. Furthermore, with the use of the system, objective analysis of cell composition provides a standard for maintaining quality control of the cardiomyocytes, which in turn extends the possibilities of the said cardiomyocytes to be used as a model for toxicity evaluation.

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