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On-Chip Cellomics: Single-Cell-Based Constructive Cell-Network Assay for Quasi-*In Vivo* Screening of Cardiotoxicity*

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We have developed methods and systems of analyzing epigenetic information in cells, as well as that of genetic information, to expand our understanding of how living systems are determined. A system of analyzing epigenetic information was developed starting from the twin complementary viewpoints of cell regulation as an 'algebraic' system (emphasis on temporal aspects) and as a 'geometric' system (emphasis on spatial aspects). As an example of the 'geometric' system, we have developed an quasi-*in vivo* hiPS cardiomyocyte network assay and confirmed that it can predict the risk of lethal arrythmia correctly in 22 compounds. The knowlege acquired from this study may lead to the use of cells that fully control practical applications like cell-based drug screening and the regeneration of organs.

I. Introduction

Cells are minimum units determining their responses through genetic and epigenetic information like the history of interactions between them and fluctuations in environmental conditions affecting them. The cells in a group are also individual entities, and their differences arise even among cells with identical genetic information that have grown under the same conditions. These cells respond differently to perturbations.[1] Why and how do these differences arise? To understand the rules underlying possible differences occurring in cells, we need to develop methods of simultaneously evaluating both the genetic and epigenetic information not only for molecular level measurement but also for functional measurement. In other words, if we are to understand topics like variations in cells with the same genetic information, inheritance of non-genetic information between adjacent generations of cells, cellular adaptation processes caused by environmental change, the community effect of cells, we also need to analyze their epigenetic information. We thus started a series of studies to analyze epigenetic information among neighboring generation of cells and in the spatial structures of cell network to expand our understanding of how the fates of living systems are determined. As cells are minimum units reflecting epigenetic information, which is considered to map the history of a parallel-processing recurrent network of biochemical reactions, their behaviors cannot be explained by considering only conventional DNA information-processing events. The role of epigenetic information in the higher complexity of celluler groups, which complements their genetic information, is inferred by comparing predictions from genetic information with cell behaviour observed under conditions chosen to reveal adaptation processes and community effects. A system of analyzing epigenetic information should be developed starting from the twin complementary viewpoints of cell regulation as an 'algebraic' system (emphasis on temporal aspects; adaptation among generation) and as a 'geometric' system (emphasis on spatial aspects; spatial pattern-dependent community effect). The acquired knowlege should lead not only to understand the mechanism of the inheretable epigenetic memory but also to be able to control the epigenetic information by the designed sequence of the external stimulation.

As we can see in Fig. 1, the strategy behind our on-chip microfabrication method is constructive, involving three steps. First, we purify cells from tissue one by one in a nondestructive manner such like using ultrahighspeed camera-based real time cell sorting, or digestible DNA-aptamer labeling.[2] We then cultivate and observe them under fully controlled conditions (*e.g.*, cell population, network patterns, or nutrient conditions) using an on-chip single-cell cultivation chip [3-9] or an on-chip agarose microchamber system [10-13]. Finally, we do single-cell-based genome/proteome analysis through photothermal denaturation and single-molecule level analysis [14].

In this paper, we explain the aims of our single-cell-based study using the on-chip single-cell-based cultivation/analysis system and introduce a part of the results focusing on the

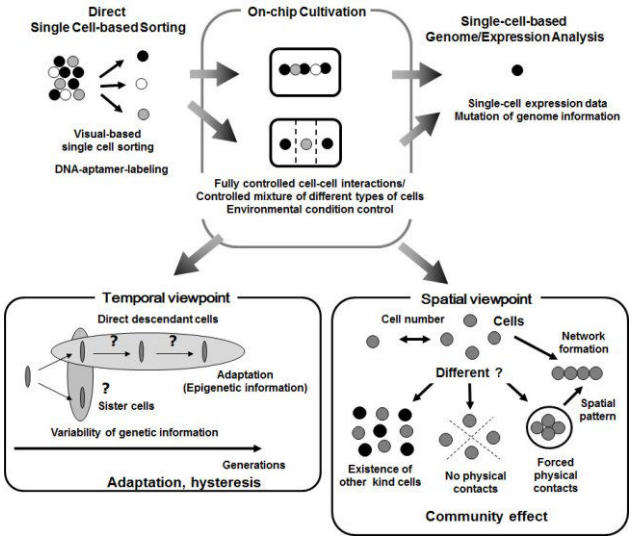

Figure 1. Our strategy: Three steps of on-chip single-cell-based constructive cellmoics analysis and the aim of this approach: temporal aspect and spatial aspect.

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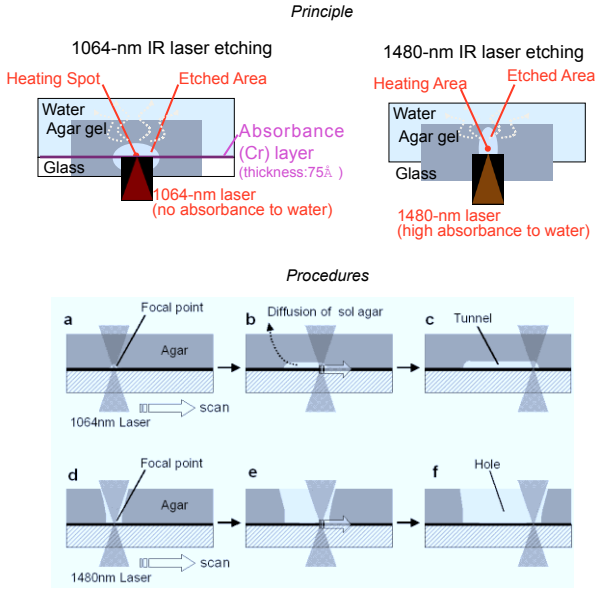
'geometric' understanding of cellular systems using cardiomyocyte network.

II. CULTIVATION SYSTEM FOR 'GEOMETRIC' VIEWPOINT: ON-CHIP AGAROSE MICROCHAMBER CULTIVATION

An approach to studying network patterns (or cell-cell interactions) and the community effect in cells was to create a fully controlled network by using cells on the chip (Fig. 1). For understanding the reaction of cells to the topography of the substratum, which occurs in the development and natural regeneration of tissue, a silicon wafer and a glass slide with holes and metal decorations have been created and tested [15-18]. Though these conventional microfabrication techniques provide structures with fine spatial resolution, it is still hard to change the shape of these structures during cell cultivation, which is usually unpredictable and is only defined during cultivation.

We therefore developed a system consisting of an agar-microchamber (AMC) array chip, a cultivation dish with a nutrient-buffer-changing apparatus, a permeable cultivation container, and a phase-contrast/fluorescent optical microscope with a 1064-nm/1480-nm focused laser irradiation apparatus



Figre 2. Schematic drawings of principles of 1064-nm infrared (IR) laser etching and 1480-nm IR laser etching, and their applications for microfabricatin formation. (a)-(c), Microtunnel formation using 1064 nm infrared focused laser beam, which does not have absorbance to water and agar; (d)-(f), microchamber formation using 1480 nm laser beam, which has absorbance to water and agar. The lasers melted the agar as follows: (a) When a 1064-nm infrared laser beam was focused on the chromium layer on the glass slide, the agar at the focal point near the chromium layer started to melt. (b) Then, when the focused beam was moved parallel to the chip surface, a portion of the agar at the heated spot melted and diffused into the water through the agar mesh. (c) After the heated spot had been moved, a tunnel was created at the bottom of the agar layer. (d) However, when a 1480-nm infrared laser beam was focused on the agar glass slide, the agar in the light path started to melt. (e) When the focused beam was moved parallel to the chip surface, a portion of the agar in the light path melted and diffused into the water. (f) Finally, after the heated spot had been moved, a hole was created on the glass slide.

to create photothermal spot heating (Fig. 2) [10, 11, 13, 19]. The most important advantage of this system was that we could change the microstructures in the agar layer even during cultivation, which is impossible when conventional Si/glass-based microfabrication techniques and microprinting methods are used.

Significant advances have been made in developing analytical methods to monitor cell activity on a single cell level (fluorescence imaging, voltammetry, ion-selective electrodes, microelectrode arrays, combination of separation techniques with mass spectrometry). To meet the spatial resolution of those single cell level-monitoring technologies, micropatterning techniques for controlling of adequate spatial arrangements of cardimoyocytes, neurons and neurites have been developed and applied. While most of micropatterning techniques such as microcontact printing and microetching-based fabrication techniques are suitable for controlling the populations of dissociated cells with randomly arranged network patterns, those conventional micropatterning techniques can just control the orientation of spatial arrangements of their connections in pre-fabricated (ready-made) micropatterns, and, in principle, cannot control the directions of their elongation and connections. To overcome those problems, agar-microetching technique has been developed to fully control of spatial arrangements of single cells and the direction of their connectivity by flexible stepwise-fabrication of additional microstructures [6, 7, 16]. This pioneering technique provides a constructive approach for spatial direction control and cell network formation during cultivation.

Agar microstructures can be photothermally etched by area-specific melting of agar microchambers by spot heating using a focused laser beam of 1480 nm (which is absorbed by water and agar gel), and of a thin layer made of a light-absorbing material such as chromium with a laser beam of 1064 nm (since water and agar itself have little absorbance at 1064 nm)[17]. For phase-contrast microscopy and μm-scale photo-thermal etching, three different wavelengths (visible light for observation, and 1480-nm/1064-nm infrared lasers for spot heating) were used simultaneously to observe the positions of the agar chip surface and to melt a portion of the agar in the area being heated. Using this non-contact etching, microstructures such as holes and tunnels can be created within a matter of minutes (Fig. 2).

III. QUASI-*IN VIVO* ASSAY FOR PREDICTIVE CARDIOTOXICITY

Lethal arrhythmia has been one of the major safety concerns for the pharmaceutical industry in selecting and developing drug candidates. Integrated assay systems using hERG-transfected HEK-293/CHO-cells (hERG assay), isolated animal tissues (APD or MAPD assay) and conscious and/or anesthetized whole animals (QT or MAPD assay), are currently used to identify QT prolongation, whereas those assay systems are not competent to fully predict the potential lethal arrhythmia such as Torsades de Pointes (TdP) or ventricular fibrillation (Vf) induced by drugs or candidates. In this context, there is a longstanding and urgent need for a surrogate marker that can distinguish the torsadogenic potential from the QT interval duration. We have proposed a

quasi-*in vivo* cardiotoxicity assay, which is a new *in-vitro* assay technology platform where human iPS/ES cell-derived cardiomyocytes and on-chip technology are combined and used as an assay tool to bridge the gap between pre-clinical studies and human clinical settings in terms of cardiotoxicity of new chemical entities for drug development (Fig. 3).

Potential advantages of the newly developed strategy of our quasi-*in vivo* assay include: 1) using a set of standard human cardiomyocytes prepared from human iPS/ES cells of different races, sexes and also from patients with various diseases to provide an ideal testing panel platform; 2) to predict lethal arrhythmia (TdP/VT/Vf) by evaluation of temporal fluctuation of single-cell-level ion channels kinetics, and by evaluation of spatial cell-to-cell conductance fluctuation using the on-chip cell network loop which can choose different conductance pathways of human cardiomyocytes among neighboring circulations; and 3) the capacity to quantitatively evaluate the correlation between calcium release and tension generation, and the inhibition on the trafficking pathway of ion-channel proteins by its long-term optical/electrical simultaneous measurement.

To study the re-entry cardiomyocyte cell network assay, we have developed the on-chip cell network cultivation system, and extra-cellular signals (field potentials: FP) of human embryonic cardiomyocytes in geometrically patterning chambers have been recorded with on-chip multi electrode array (MEA) system. Then, we have functionally reconstructed the normal and abnormal re-entry model of cardiomyocytes network loop from the viewpoint of propagation of contractile signals to be able to include the characteristics of heart into the chip like the functional spiral re-entry model (Figs. 3 and 4). And we found that the on-chip cardiomyocyte cell network assay is expected to be one of the candidates having the potential to measure the TdP and VF probability as pre-clinical testing for cardiac safety.

The data obtained in our laboratory indicates that the torsadogenic potential of 22 QT prolonging and non-QT prolonging drugs including false-negative/false-positive compounds in the current *in vitro* assays have been predicted correctly by quantitative evaluation of spatiotemporal fluctuation increase, and evaluation of hERG channel trafficking inhibition with longer exposure of compounds. Moreover, we have shown that the on-chip cell network loop model would offer the novel platform to assess the proarrhythmic (not only TdP but also VT/Vf) risks of compounds.

IV. CONCLUSION

We have demonstrated the on-chip cell network assay, We developed and used a series of new methods of understanding the meaning of genetic and epigenetic information in a life system exploiting microstructures fabricated on a chip. The most important contribution of this study was to be able to reconstruct the concept of a cell regulatory network from the 'local' (molecules expressed at certain times and places) to the 'global' (the cell as a viable, functioning system). Knowledge of epigenetic information, which we can control and change during cell lives, complements the genetic variety, and these two kinds are indispensable for living organisms. This new kind of knowlege has the potential to be the basis of cell-based biological and medical fields like those involving cell-based drug screening and the regeneration of organs from stem cells.

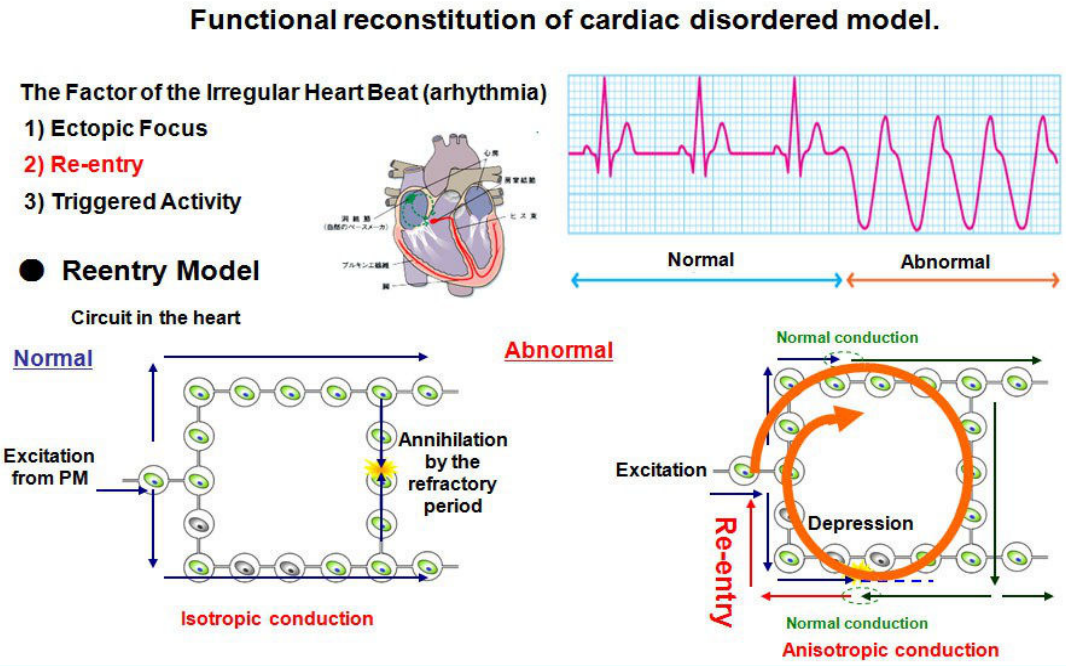


Figure 3. Concept of single-cell-based on-chip re-entry model as an example of quasi-*in vivo* assay for pre-clinical testing for TdP prediction. The network formation enables us to make a model of the signal propagation in the heart tissue.

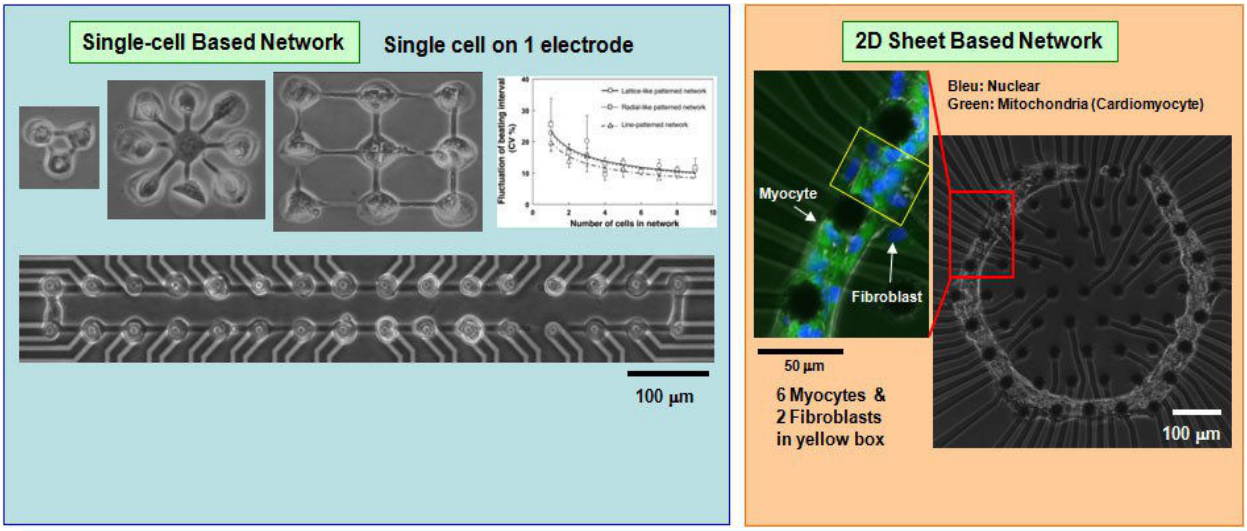


Figure 4. Formation of single-cell-based re-entry cell circuit in the agarose microstructures on a MEA chip.

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