Drug Discovery Applications of High-throughput Cytological Discovery Systems

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Yokogawa's range of high-throughput cytological discovery systems includes the CellVoyager CV8000 high-throughput cytological discovery system, which is a large high-end type, and the CQ1 confocal quantitative image cytometer, which is a small bench-top type, both of which use the CellPathfinder high content analysis (HCA) software. By using confocal scanning technologies based on microlens-enhanced Nipkow disks, both systems can automatically acquire a large amount of high-definition, 2D/3D confocal microscopic images at high speed and quantify the information contained in these images. These systems can also provide ideal culture conditions for cells through temperature control, CO_2 control, and a humidifier. These useful functions enable the systems to analyze various cellular responses and thus to be increasingly used in the drug discovery field. This paper introduces three examples of using these systems for drug discovery research: hepatotoxicity assay, cellular migration assay, and in vivo tumor metastasis assay using zebrafish.

INTRODUCTION

Quantifying the amount of protein expression in cells and elucidating molecular activities such as interaction between proteins are essential for understanding life phenomena. To observe the behaviors of cells and intercellular proteins, remarkable progress has been made in imaging technologies including fluorescence microscopy.

Yokogawa's high-throughput cytological discovery systems capture high-definition microscopic images of cells at high speed, by using Yokogawa's original confocal scanning technologies based on the microlens-enhanced Nipkow disk, and precisely measure the brightness and size of each cell from the captured images[(1)]. The systems are increasingly used by customers in various fields including drug discovery.

APPLICATION TO DRUG DISCOVERY

The high content screening (HCS) method is used widely in drug development, especially in the search process to screen a huge number of candidate compounds for a new drug, and also in the process of evaluating their pharmacological effects and safety[(2)]. HCS automatically acquires a large amount of image data at high speed using an imaging system based on a microscope, analyzes the data, calculates multiple parameters, and extracts compounds that meet the requirements using the method of data mining. Among Yokogawa's products, the CQ1, CellVoyager CV8000, and CellPathfinder analysis software fall into this category.

This paper introduces three examples of applications using Yokogawa's system: toxicity assay of two-dimensionally cultured cells; cellular migration assay; and *in vivo* tumor metastasis assay using zebrafish (zebrafish is a promising new test animal).

EXAMPLES OF APPLICATION TO DRUG DISCOVERY

Hepatotoxicity Assay

Suspending the development of a new drug, or receiving a warning on or discontinuing sales of a drug after its release, may cause severe damage to a pharmaceutical company and a substantial impact to society. Therefore, it is crucial to assess the safety of candidate compounds and exclude toxic compounds in the early stage of drug discovery.

An example of drug toxicity is hepatic disorder, for which safety is assessed by measuring various items using liver cells. We studied hepatotoxicity with HepG2 (human hepatoma cell line) and menadione (synthetic vitamin K). The detailed procedure of this assay is described below.

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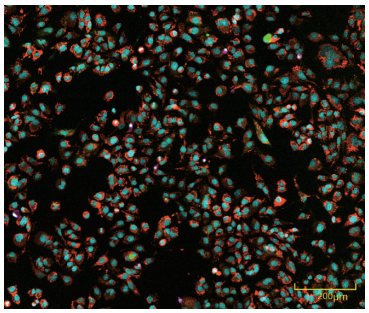
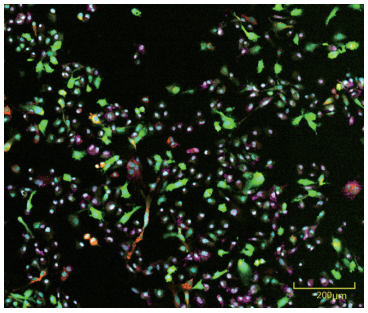
The HepG2 cells were cultured two-dimensionally and exposed to menadione for 24 hours. We measured the number of cell nuclei, production of reactive oxygen species (ROS), membrane potential of mitochondria, and the number of dead cells in images of the control and test samples. Cell nuclei were labeled with Hoechst 33342 (blue), ROS with CMH2DCFDA (green), mitochondria with MitoTracker Orange (red), and dead cells with TOTO-3 (purple), respectively. Figure 1 shows the images of labeled cells.

Figure 1(a) shows the image of the control. Dominant red fluorescence indicates that mitochondria maintain the normal membrane potential. In contrast, in the image of a sample with menadione treatment (Figure 1(b)), red fluorescence was weaker and green fluorescence was stronger than in Figure 1(a), indicating that ROS had been produced. Purple fluorescence was also seen in Figure 1(b), showing that the number of dead cells was increased by the menadione treatment.



(a) Menadione: 0 μM

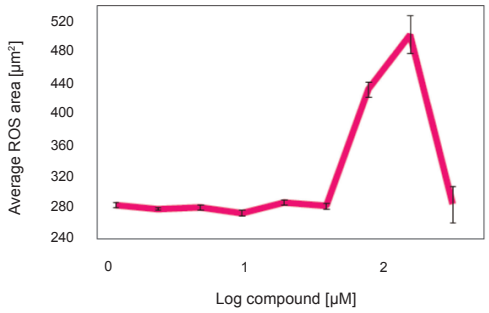


(b) Menadione: 400 μM

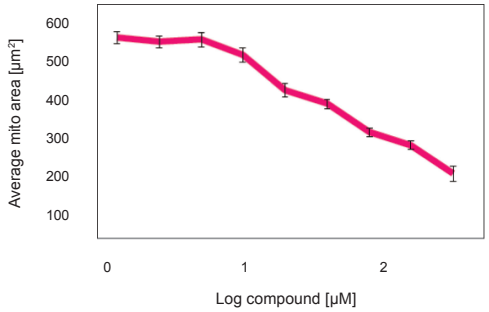
Blue : Cell nuclei
Green : ROS
Red : Mitochondria with normal membrane potential
Purple : Dead cells

Figure 1 Images of HepG2 with menadione treatment

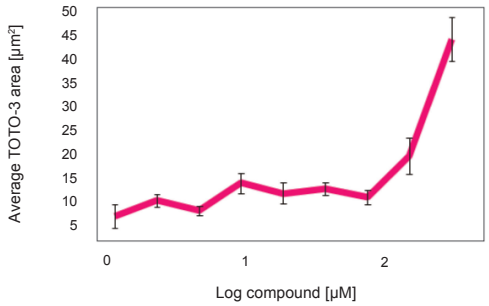
Figure 2 shows the analysis results of the image in Figure 1(b) in terms of the amount of ROS, level of mitochondrial membrane potential and number of dead cells in the samples with menadione treatment. The number of mitochondria with normal membrane potential decreased inversely with the menadione concentration while the amount of ROS and the number of dead cells increased sharply at high menadione concentration. These results indicate that menadione has toxicity to liver cells.



(a) Amount of ROS



(b) Mitochondria with normal membrane potential



(c) Number of dead cells

Figure 2 Results of hepatotoxicity assay

Thus, the high-throughput cytological discovery system can be conveniently used for imaging and analysis in hepatotoxicity assays.

Cellular Migration Assay

Cellular migration is a physiologically fundamental function, and is involved in various phenomena such as wound healing, differentiation, embryogenesis, and tumor metastasis. Cellular migration is also deeply involved in diseases such as osteoporosis, arthritis, and congenital abnormalities in the brain and heart. A proper understanding of cellular migration is important for the treatment of these diseases.

Scratch assay is a method of quantitatively evaluating cellular migration. In this assay, cells are cultured two-dimensionally to the confluent state (the whole surface of the culture dish is covered with cells). The cell layer is scratched by a tool to create a wound gap, and the healing of this gap by cellular migration is monitored.

We added mitomycin C (MMC) to HeLa cells labeled

with Fucci probes and observed and analyzed their images. The Fucci probe is a fluorescent probe that helps visualize the progress of the cell cycle. Cells labeled with Fucci probes emit red fluorescence in the G1 phase, and green fluorescence in other phases. MMC is an antitumor antibiotic that inhibits DNA replication and cell proliferation.

The wound gap is gradually filled with cells. There are two possibilities for this phenomenon: one is cell migration and the other is cells pushed out by the proliferation of other cells. The combination of the Fucci probe and MMC is a good way to distinguish these two possibilities. The procedure of the time lapse analysis of cellular migration and proliferation in the scratch assay is described below.

The HeLa cells were cultured up to the confluent state and scratched with a pipette tip to create a gap across the cell layer. MMC was added to the HeLa cells to reach a concentration of 3 µg/mL. Time lapse imaging of the area around the gap was carried out for three days. Figure 3 shows changes in the cell layer.

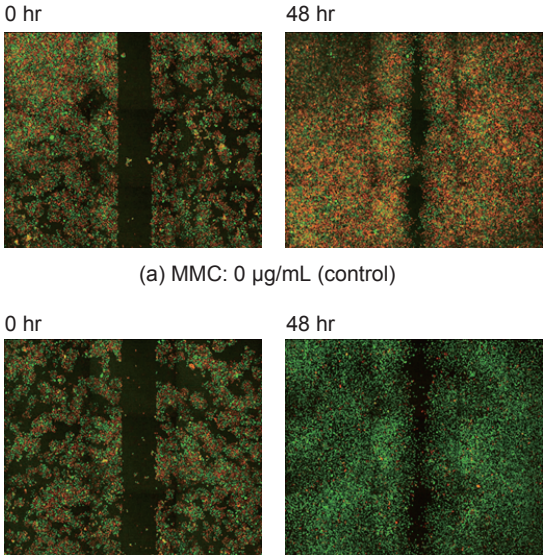


Figure 3 Time lapse images of HeLa cells with MMC treatment

As shown in Figure 3, the gaps were restored in both samples with and without MMC after 48 hours. Red fluorescence and green fluorescence were recognized in the control (without MMC) sample while only green fluorescence was seen in the sample with MMC. The number of cells in the gap area was counted by using the analysis software. Figure 4 shows the results.

The numbers of red and green cells in the control equally increased over time while in the MMC sample, the number of green cells increased with time but the number of red cells remained the same. These results suggest that the gap in the control was filled by both proliferation and migration while the gap in the MMC sample was restored mainly by cellular migration.

The time lapse observation showed how the gap was filled gradually by cells. The cells grew steadily in the control, which indicates that the system's environmental control mechanism for the growth of living cells worked stably. The fluorescence intensity remained the same 48 hours after the start of the assay. This suggests that phototoxicity was low in this system. Since the live cell imaging method is used in a broad range of research fields, the long-term observation capability of Yokogawa's system is expected to be extremely beneficial.

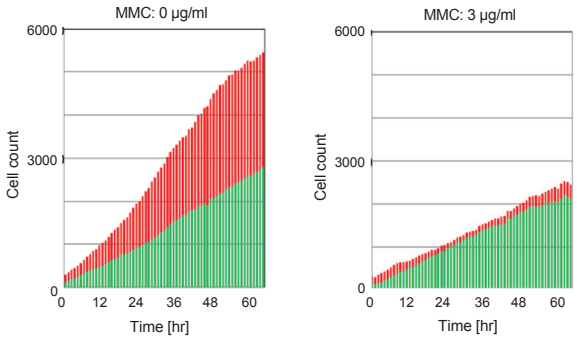


Figure 4 Analysis results of the number of cells in the gap

Assay Using Zebrafish

Zebrafish has been increasingly used for drug discovery research, replacing mammals. While its tissues are complex, zebrafish enables simple and rapid screening, and thus research results are easy to extrapolate to humans[3]. This section introduces imaging of a transgenic zebrafish with human tumor cells transplanted and the results of analyzing the images.

The vascular cells in the head of a zebrafish were labeled with GFP, and human tumor cells labeled with tdTomato were transplanted there. The sample zebrafish was observed three-dimensionally by sliced imaging along the Z axis. Figure 5 shows its maximum intensity projection (MIP) image.

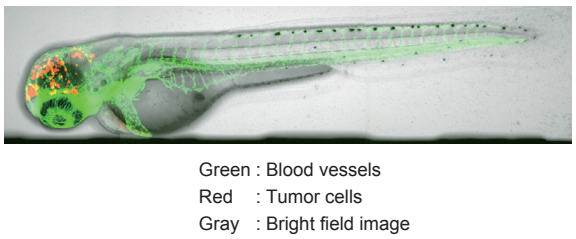


Figure 5 Fluorescent image of zebrafish

Through the image analysis, red fluorescent spots were recognized and the amount of tumor cells was determined. Figure 6 shows the result of the image analysis. We analyzed the red tumor cells in Figure 6(a) and calculated the

parameters such as the area and total brightness of tumor cells.

As shown in Figure 6, two-dimensional MIP images can be created from three-dimensionally captured images for simple planar analysis. Three-dimensional analyses are also possible using each slice image. Such three-dimensional analyses yield information on the spatial distribution and volume of tumor cells in the body.

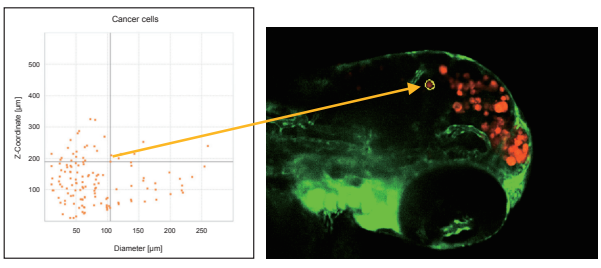
(a) Before analysis

(b) After analysis

Light blue line : Contours of zebrafish
Blue spots : Tumor cells

Figure 6 Recognition of zebrafish contours and tumor cells within (MIP image)

Furthermore, Yokogawa's system has a function for highlighting a spot in an image by clicking the corresponding point (measurement value) in a graph of analysis results on the software window. Figure 7 shows this linkage between the graph and the image. The conditions of tumor cells can be easily checked in images.



(a) Measurement results of tumor cells

(b) The cell selected in the left graph

Figure 7 Linkage between the graph and the image

The objective of this assay was to assess the efficacy of anti-cancer drugs. Zebrafishes with tumor cells transplanted were bred in wells and treated with various anti-cancer drugs. The number and metastasis of tumor cells in the zebrafish were quantified. This method is expected to be used in the future as a tool for personalized medicine in which the tumor cells of a patient are used to select the optimum anti-cancer drug for the patient. If similar experiments were carried out in mammals, a large amount of time and cost would be required. It is also difficult to acquire images of tumor cells that have metastasized throughout the body. Using zebrafish for experiments can solve these problems and is ideal for *in vivo* assays[i].

CONCLUSION

This paper described three applications regarding drug discovery. Recently, the need for HCS and high content analysis (HCA) using imaging technologies is increasing instead of high throughput screening (HTS) using the conventional plate reader and other tools. High content live cell analysis using living cells is also attracting attention. In drug development, many new drugs are not brought to market even after enormous resources are invested, due to safety problems found during the search of candidate compounds and clinical tests. Although drug efficacy and safety have been assessed based on little measurement information, many researchers now believe that the assessment must be based on more information using live cell imaging technologies and must be carried out under conditions closer to those of the living organism. This is why there is an increasing need for HCA and high content live cell analysis.

The features of Yokogawa's systems – high quality image, high throughput, three-dimensional analysis, and viable environment for living cells – meet the requirements described above. The CQ1, CV8000, and CellPathfinder can improve the efficiency of drug development while widening the scope of assays using imaging technologies. Furthermore, these systems will enable diverse evaluations of phenomena that have been overlooked, and will bring a great deal of knowledge to researchers.

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i Data regarding zebrafish was kindly provided by Dr. Toshio Tanaka, M.D., Ph.D., Professor of Department of Molecular and Cellular Pharmacology, Pharmacogenomics and Pharmacoinformatics, Mie University Graduate School of Medicine.