CellVoyager CV8000: A High-throughput Cytological Discovery System for Enhancing the Efficiency of Drug Discovery

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Yokogawa has developed the CellVoyager CV8000 high-throughput cytological discovery system, which can elucidate the functions of individual cells or tissues composed of multiple cells by acquiring and analyzing images of cells. This system can automatically observe processes within cells caused by the administration of drug-candidate compounds, at the world's highest resolution and measurement speed. This paper describes various functions of the CV8000, including a newly developed element technology for preserving culture environments, a water supply mechanism for water-immersion objective lenses, a digital phase contrast (DPC) technique for label-free imaging, and a function for correcting fluorescent crosstalk.

INTRODUCTION

The development of one drug typically takes at least ten years and tens of billions of yen. The process starts with basic research in bioscience and goes on to compound screening, preclinical and clinical tests, application for approval, and manufacturing. In the process of compound screening, promising compounds are selected from several hundred thousand to a million candidates. In this R&D process, it is crucial to obtain as much information as possible quickly from an experiment. Accordingly, Yokogawa developed the CellVoyager series, which delivers high throughput when testing a large number of compounds. The CellVoyager series has been used for a wide range of applications from bioresearch to drug discovery, with many successful results.

Recently, observation objects have expanded from fixed cells to living cells, and from fluorescence-labeled cells to label-free cells to avoid the toxicity of fluorescent stains caused by labeling.

Responding to such changes in market needs, and to offer greater potential for the field of life science, Yokogawa started to develop the CV8000 high-throughput cytological discovery system. Figure 1 shows an external view of the CV8000.

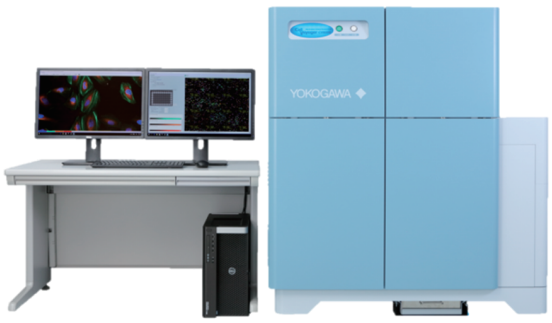

Figure 1 External view of the CV8000

The CV8000 comes with newly added, developed, or improved functions compared to the previous models. For example, a function to hold a container for compounds has

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been developed, a stage incubator function to maintain a constant incubation environment has been improved, new imaging functions for observing label-free cells have been added, and the number of selectable laser wavelengths and cameras has been increased to enable simultaneous illumination and imaging of cells at multiple wavelengths. Even with these added functions, the CV8000 is just 60% of the volume of the previous model. This paper introduces these new functions.

MEASUREMENT SOFTWARE

Figure 2 shows the block diagram of the measurement software that controls the CV8000 and Figure 3 shows its screen. The measurement software consists of a window for setting measurement (image acquisition) conditions, a window for creating various associated setting files, a window for controlling the system, a window for displaying measurement results, and the portal window that helps operators check the system condition and move to other windows.

The window for setting measurement conditions is designed to enable these conditions to be set freely. Operators can flexibly set desired conditions, including long-term time lapse imaging, 3D imaging by capturing slice images along the Z-axis, high-speed time-lapse imaging by capturing images at several hundred millisecond intervals, and a dispenser function.

The CV8000 supports simultaneous, multichannel imaging by using multiple cameras, and has an image display function with a programmable shader for fast display of pseudo-colored multichannel merged images.

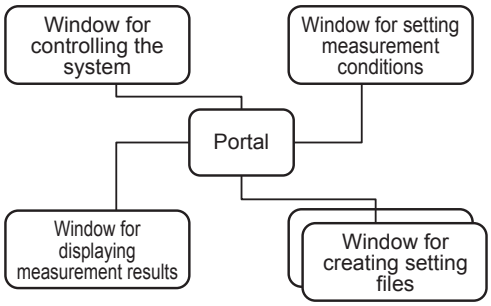


Figure 2 Block diagram of measurement software

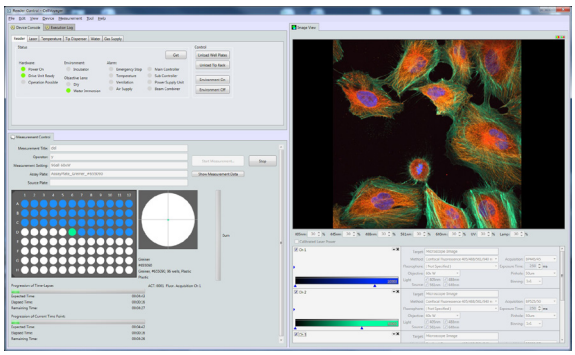


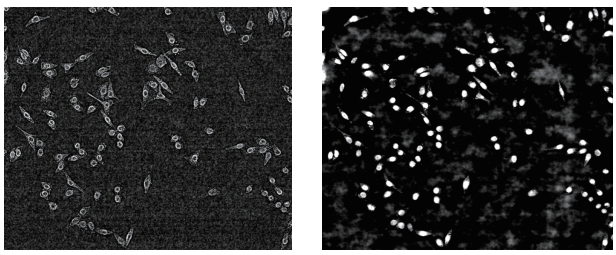
Figure 3 Screen of measurement software (during measurement)

Digital Phase Contrast (DPC)

Recently, assessment systems for observing label-free cells are attracting attention. However, since cells are clear and colorless, conventional images show low contrast between the cells and their background, making it extremely difficult to analyze cells separately from their background. For this reason, phase-contrast images are used for analyzing label-free cells. Such images generally have a high contrast, which makes it possible to distinguish cells from their background. However, with this method the dimension of the wells in a microplate imposes restrictions on the optics used for imaging and satisfactory images cannot be acquired with microplates of 96 or more wells.

The digital phase contrast (DPC) method was proposed to solve this problem in phase-contrast optics, and has achieved remarkable results. In this method, multiple images are taken under the optical system of a conventional microscope at various positions along the optical axis. Based on these images, an image with a phase of the sample emphasized is created. A slight difference between refractive indices of cells and the surrounding culture liquid causes misalignment of the phase of the transmitted light. DPC detects this phase misalignment and visualizes it in an image.

Yokogawa has developed its original DPC algorithm and implemented it in the CV8000. This function enables the acquisition of high-contrast images of label-free cells using 96-well or 384-well microplates, which are commonly used for screening applications. Furthermore, DPC offers two modes: phase type and fluor type. Phase type yields images resembling phase-contrast ones, and fluor type yields images resembling fluorescent ones. The phase type images have high contrast and retain detailed information on cells, while the fluor type images are easy to analyze. Figure 4 shows images of clear, colorless cells, taken in each mode.



(a) Phase type (b) Fluor type

Figure 4 DPC images

On-the-fly Image Correction

Since the CV8000 is a large system, it is not free from mechanical errors and electrical noises. Therefore, the images taken by the CV8000 contain incorrect information arising from such factors, degrading the reliability of analysis results. Major error factors are listed below.

- Dark component (dark current of cameras)
- Shading (non-uniformity in illuminating light)
- Pixel misalignment among multiple cameras

- Pixel misalignment due to the imperfect positional accuracy of the XY stage mechanism
- Crosstalk due to wide fluorescence bandwidths

Yokogawa offers a correction tool to eliminate errors arising from these factors from acquired images. However, in the previous CV7000 model, this correction tool started processing only after images were captured. Therefore, an image correction time was required in addition to the imaging time to obtain a corrected image.

To overcome this problem, the CV8000 uses a method of correcting an image (on-the-fly image correction) for dark components, shading, and pixel misalignment among cameras, on capturing the image (before saving the image). These corrections are not affected by images taken at other wavelengths. This method considerably reduces the time taken to obtain a corrected image and achieves high-speed image processing.

This image correction is performed in multithreading, because on-the-fly image correction imposes a heavy load on the processor, and many images from multiple cameras need to be corrected simultaneously. Figure 5 shows an example of the effect of the on-the-fly correction function. The time before starting analysis is shortened considerably. This method is especially effective for 3D images for which multiple Z-sliced images are captured in a short time.

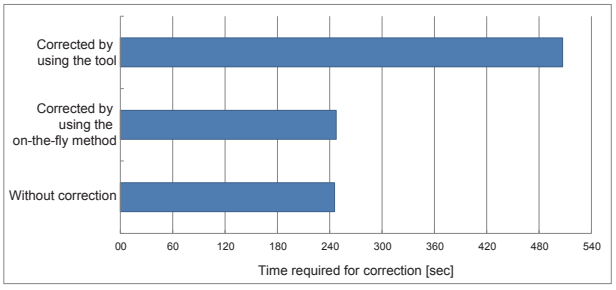


Figure 5 Effect of on-the-fly correction

Crosstalk Correction

For simultaneous multichannel (multi-wavelength) imaging using multiple cameras, an object cell is irradiated simultaneously by multiple laser beams of various wavelengths, and the multi-wavelength fluorescence excited by the lasers is spectroscopically selected by optical filters, and guided to each camera. As shown in Figure 6, however, since each fluorescence spectrum has a bandwidth of several ten to several hundred nanometers, part of the fluorescence spectrum is detected by unintended cameras, and images with desired fluorescence will contain undesired fluorescence. Such leaking is called crosstalk, which deteriorates the S/N ratio of images.

The image correction tool built into the CV8000 calculates the amount of crosstalk based on the spectral data of fluorescent substances, transmittance of the optical system, and the acquired image, and corrects its effect.

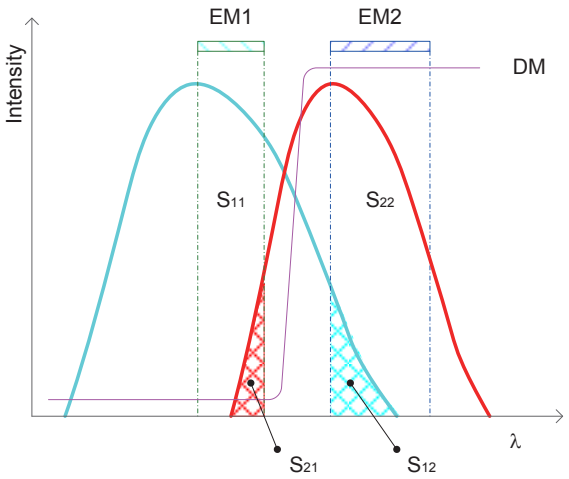


Figure 6 Conceptual diagram of crosstalk

The principle of crosstalk correction is described below. The total amount of fluorescence, *Chn*, detected by channel *n* can be expressed by Equation (1).

$$\begin{aligned} Ch_1 &= S_{11} + S_{21} \\ Ch_2 &= S_{22} + S_{12} \end{aligned} \quad \cdots (1)$$

See Figure 6 for S_x.

The ratio of leaked fluorescence to desired fluorescence is expressed by Equation (2).

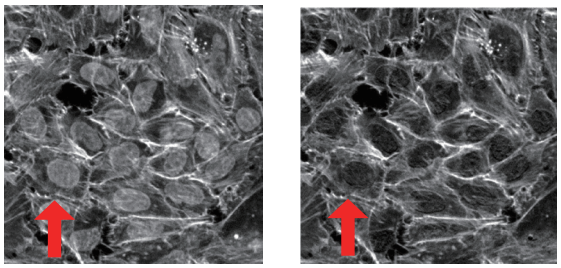
$$\begin{aligned} \frac{S_{12}}{S_{11}} \times \left(\frac{EM2 \times DM}{EM1 \times (1 - DM)} \right) \times \frac{ET2}{ET1} &= a \\ \frac{S_{21}}{S_{22}} \times \left(\frac{EM1 \times (1 - DM)}{EM2 \times DM} \right) \times \frac{ET1}{ET2} &= b \end{aligned} \quad \cdots (2)$$

where, ET*n* is the exposure time of channel *n*, and EM*n* × DM is the average value of the products of the transmittances of the optical filter and the dichroic mirror at the effective wavelength band of the optical filter of channel *n*.

Equation (3) is derived from Equations (1) and (2). By solving Equation (3) for S_{11} and S_{22}, the intensity without leak light is obtained.

$$\begin{aligned} Ch_1 &= S_{11} + b \times S_{22} \\ Ch_2 &= S_{22} + a \times S_{11} \end{aligned} \quad \cdots (3)$$

Figure 7 shows images before and after the crosstalk correction. Nuclei and cytoplasm were labeled by Hoechst 33342 and Azami-Green, respectively. Figure 7(a) shows that the fluorescence from nuclei leaks into the cytoplasm channel. Figure 7(b) shows that the crosstalk correction reduced the fluorescence leak and clearly distinguished between the nucleus and the cytoplasm.



(a) Before crosstalk correction (b) After crosstalk correction

Figure 7 Images before and after crosstalk correction

HARDWARE

The CV8000 consists of a main body and a controller workstation. Figure 8 shows the outline of the main body.

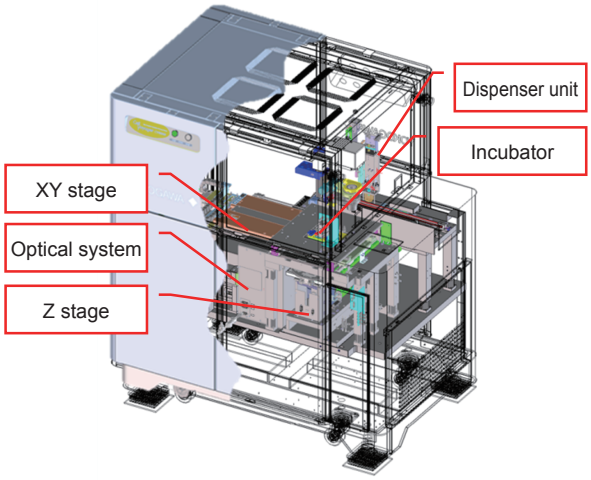


Figure 8 Inside of the CV8000 main body

The mechatronics of the main body include a confocal microscope optical system, an XY stage for moving samples, a Z stage for scanning the objective lens, a stage incubator for controlling sample temperature, humidity, and CO_2 concentration, and a dispenser unit for dripping reagents onto samples. In addition, the following components are mounted in the main body to drive the above units: a power supply, control boards, a pneumatic system, various controllers, a CO_2 gas mixer, a lamp for transmission illumination, a beam combiner with lasers for fluorescence observation, and a UV light source.

Highly sensitive digital CMOS cameras are used for observing samples. The number of cameras is increased from three in the previous model to four in order to increase the number of simultaneously captured images and shorten the image acquisition time. A larger number of cameras with improved performance is a crucial factor for multicolor imaging, in which multiple signals are visualized simultaneously by labeling cells with multiple fluorescent dyes having different wavelengths and introducing multiple fluorescent molecules into cells.

Conventional microscope systems require a significant amount of labor and high skills for the selection, setting up, installation, and adjustment of each unit; preparation of a darkroom; and mastering the manuals of each unit. In contrast, the CellVoyager series implements all these functions in a single box and thus does not require the adjustment or mastering of individual units, shortening the time from installation to full operation. Moreover, the CV8000 is only about 60% of the volume of the CV7000, allowing the system to be installed more freely.

Water Immersion Lens and Water Supply Mechanism

In the field of high content analysis (HCA) which automatically captures cell images, there are increasing needs for a wider field of view, 3D images with substantial penetration depth, and long-term observation of live cells. To respond to these needs, Yokogawa improved the performance of the microscope optical system, especially the objective lens.

Two types of objective lens are available for optical microscopes: the dry type and the water immersion type. In the water immersion type, the space between the sample and the objective lens is filled with pure water, oil, and other liquids of high refractive index. This filling makes the numerical aperture (NA) of the objective lens larger, leading to higher resolution, higher light-collecting capability, higher SN ratio, and brighter images than dry lenses.

Therefore, Yokogawa worked on developing new water immersion objective lenses of 20× and 40× magnifications. Aiming to develop a water immersion lens ideal for HCA, Yokogawa successfully achieved a high NA, minimal field curvature aberration, long working distance, and an automatic water supply mechanism.

Field curvature aberration distorts images; it blurs the peripheral area even if the center area is sharply focused. Therefore, images with large field curvature aberration cannot be used for analyses. When multiple images are connected to form a large image, joint areas are blurred and this makes the whole image unnatural. Therefore, even if an image is taken by a camera with a wide field of view, its peripheral area is not suited for analyses.

The water immersion lens developed by Yokogawa is designed to minimize the field curvature aberration, and can bring the whole image area into focus, enabling the analysis of wide-field images. This improves the system throughput. In addition, multiple images can be connected seamlessly, which enables multiple images to be connected for high-precision analyses of wide samples such as tissue slices.

HCA samples are often prepared on microplates. Although the width and length of microplates are standardized, there is no limitation on the thickness. To handle various microplates, the working distance of the objective lens should be as long as possible.

However, the working distance and the NA of an objective lens have a trade-off relation. To obtain a high-resolution image, the NA must be high, which inevitably makes the working distance short. In contrast, a longer working distance lowers the NA. To satisfy both requirements, we reviewed

the materials and other conditions of the objective lens and successfully developed an objective lens with a long working distance and high NA.

An objective lens with a long working distance can be used not only for various microplates but also for large samples such as spheroids (agglomerate of cells), which have become widespread in regenerative medicine.

The water immersion type is used with water in the space between the sample and the objective lens. Because the CV8000 is an automated system for continuous operation, the objective lens needs an automatic water supply mechanism.

This mechanism, which is mounted on the front end of the objective lens, could narrow the imaging area. The water supply mechanism of this system was designed to use capillaries for water supply and drainage so as not to affect the field of view of the objective lens.

Figure 9 shows the water immersion objective lens developed by Yokogawa, with water on the front end and a laser beam at the wavelength of 488 nm irradiating the sample. This water lens enables samples to be observed with a wide field of view, high resolution, and deep penetration depth.

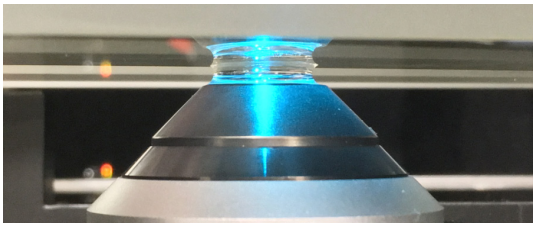


Figure 9 Newly developed water immersion objective lens

Improved Performance of Stage Incubator

One of the features of Yokogawa's HCA system is long-term observation of living cells. This was achieved by implementing the capability of the CSU confocal scanner, which is highly evaluated in the field of microscopes, as the core technology in the HCA field. As a result, an optical system that causes little damage to cells has been achieved. In addition, the stability of controlling and maintaining the environment of an incubator has been improved (normal conditions: temperature of 37°C, humidity of 80% or higher, CO_2 gas concentration of 5%). The cells to be observed are incubated on microplates, and the microplates are placed in this incubator.

The incubation environment of the CellVoyager series has been continuously improved. It is empirically known that the environmental stability depends on the seal performance of the incubator, so we aimed to achieve perfect sealing. A movable cover is arranged on a small hole of about 10 mm diameter, through which reagents are administered to samples. As a result, the amount of CO_2 supply to keep the concentration constant was reduced to 1/6 of the previous model without a cover.

The temperature distribution was made more uniform by dividing the incubator area into several segments and controlling individual heaters for each segment. Figure 10 shows the temperature control range of the CV8000. When the room temperature is 15-30°C, the incubator can be maintained at 34-40°C. This enables the CV8000 to be used for a wide range of drug discovery applications.

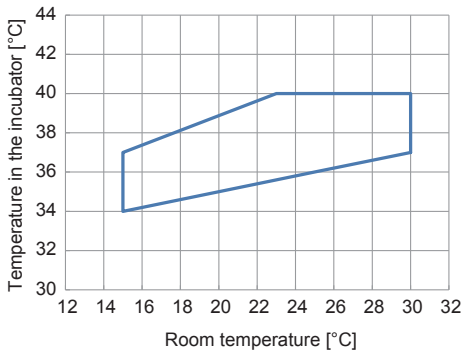


Figure 10 Incubator temperature control range

Low-oxygen Applications

For developing cancer drugs, cells are often tested in a low-oxygen gas, in which the concentration of oxygen is 1% or lower. It is extremely difficult to maintain low-oxygen conditions even with well-sealed incubators, because the concentration of atmospheric oxygen is as high as 21%, so oxygen can easily pass through any microscopic gaps, raising the concentration in the incubator.

The CV8000 achieves low-oxygen conditions by employing a dual incubator structure in which a small incubator is mounted in a stage incubator. The blue part in Figure 11 shows the small, inner incubator. In addition to maintaining low-oxygen conditions, this structure achieves a uniform distribution of oxygen across the microplate and its long-term stability.

The HCA system with low-oxygen performance offers an environment similar to that found in actual organs, helping elucidate pathological conditions and assess drug efficacy more precisely. This system is expected to assist drug discovery, especially the screening of cancer drugs.

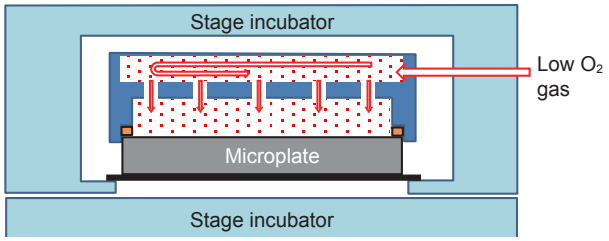


Figure 11 Structure for maintaining low-oxygen conditions

Dust Prevention Measures for Optical System

Dust causes various problems for optical systems. It affects the reliability of image analysis results, and considerably increases the cleaning time during maintenance.

The CV8000 maintains a positive inside pressure to prevent dust from entering and to keep the inside clean. Moreover, optical components and light paths are enclosed with walls. The interface of each wall was made a labyrinth structure to prevent the penetration of dust. In particular, the confocal scanner, which is the crucial part of the system, is tightly sealed with rubber packing, and the inside is purged with clean air to keep the pressure positive, minimizing the risk of dust. Figure 12 outlines the dust prevention structure.

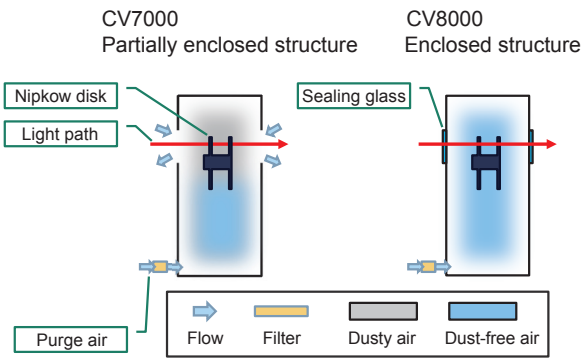


Figure 12 Dust prevention structure

CONCLUSION

This paper outlined the technologies, functions and performance of the CV8000 high-throughput cytological discovery system. The CV8000 is a user-friendly product with new functions, while maintaining the excellent features of Yokogawa's previous products: the CSU series, the CQ1, and the CV series[(1)(2)(3)].

Research and development in drug discovery and life science are expected to lead to new drugs and medical technologies, and help conquer diseases and extend healthy life expectancy. The microcosm of life is full of unknowns and will not be elucidated easily. The authors hope that the CV8000 will help tackle the unknowns and contribute to the elucidation of cell functions and the development of life science including drug discovery and regenerative medicine.

REFERENCES

(1) Hironori Takai, Kenji Hachiya, et al., "Confocal Scanner System for Long-term Live Cell Imaging," Yokogawa Technical Report English Edition, Vol. 55, No. 1, 2012, pp. 27-30
(2) Hideo Hirukawa, Hiroshi Nakayama, et al., "New Technologies for CSU-X1 Confocal Scanner Unit," Yokogawa Technical Report English Edition, No. 45, 2008, pp. 22-26
(3) Hirofumi Sakashita, Koji Ohashi, et al., "The CQ1 Confocal Quantitative Image Cytometer and its Application to Biological Measurement," Yokogawa Technical Report English Edition, Vol. 58, No. 1, 2015, pp. 29-33