NEW TECHNOLOGIES FOR CSU-X1 CONFOCAL SCANNER UNIT

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This paper describes new technologies we have innovated for the CSU-X1 series Yokogawa confocal scanner unit. They are enhanced features such as high-speed image acquisition and low phototoxicity and photobleaching based on the microlens-enhanced Nipkow disk method popular for live cell imaging, thus achieving the world's highest scanning speed of 0.5 ms/frame, better light efficiency, and higher resolution. In addition, we have provided various peripheral devices such as high-speed filter wheel, by-pass optics, and second camera port, realizing various system configurations to meet the needs for multiwavelength measurement.

INTRODUCTION

A confocal microscope was invented in 1957 by Professor Marvin Minsky [(1)] of MIT, highly renowned for research on AI (artificial intelligence). The patent of Minsky suggested that clear microscopic images can be obtained by placing a point light source and a pinhole at positions conjugate with the focal point of an objective lens, because this design prevents the scattered light near the focal point from penetrating into a detector. This idea has been put into practice by the invention of laser. Confocal microscopes have high resolution and shallow focal depth, allowing for the generation of three-dimensional images based on multiple images taken at different focal planes. For this reason, currently confocal microscopes are widely used in the life science and semiconductor fields.

A confocal microscope, in principle, requires a scanning of the laser excitation light and its performance is largely dependent on the scanning method. In 1996, Yokogawa released the CSU10 confocal multi-beam scanner with microlens-enhanced Nipkow disk. Conventional single-beam galvano scanners usually require about one second for generating a 1000 × 1000 pixel image. Compared with this, the CSU10 is capable of generating such an image in a shorter time (normally in the order of milliseconds), enabling real-time observation of a dynamic live cell. In addition, since the power of each excitation light in a multi-beam is low, i.e. in the order of microwatts, the CSU10 provides less fluorescence photobleaching and is suited for continuous, long-time observations [(2)].

We have newly developed the CSU-X1 that has been enhanced in high-speed image generation and low photobleaching compared with the CSU10, and allows for various configurations including that for multiwavelength measurements. Figure 1 shows the entire view of the CSU-X1.

FEATURES OF CSU-X1

(1) World's Fastest Scanning Speed
The CSU-X1 has achieved the world's fastest scanning/imaging speed of 0.5 ms/frame, equaling to two times that of the previous product. This enables more accurate observation of bloodstream or more effective analysis in microPIV (Particle Image Velocimetry).

Figure 1 External View of the CSU-X

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Table 1 Main Specifications of the CSU-X1

Confocal scanning method	Microlens-enhanced Nipkow disk scanning
Scanning speed	360 frame/s (300-2000 frame/s, optional)
Excitation wavelength range	405 to 647 nm
Image size	10×7 mm
Laser beam input	Single mode polarization-maintaining fiber, connected using an FC connector
Connection with microscope	C-mount (an adaptor is needed for the bright field light path option)
Connection with camera	C-mount
Communication interface	RS232C

(2) Significant increase in the use efficiency of laser transmittance.
The CSU-X1 incorporates newly developed lenses, achieving a high use efficiency of the laser transmittance, about two times that of the previous product series. This improvement can reduce an exposure time even when using the laser of the same power, thus allowing a time resolution during measurement to be increased.

(3) Rich options
Some components or parts of the CSU-X1, such as the exchange unit of the camera port and a lens unit of the camera output component, are designed as modules in order to meet specific user configurations. For example, a bright field light path option is useful for obtaining brighter images when capturing differential interference images, or epifluorescence images using a mercury lamp. The CSU-X1 also offers a configuration that allows the user to use two cameras, which helps to perform a fast and accurate two-wavelength measurement where no time lag is permitted. Filters can be easily and quickly replaced according to the needs of various applications. Table 1 lists the main specifications of the CSU-X1.

PRINCIPLE AND METHOD OF SCANNING

Basic Principle of a Confocal Microscope

Generally, a conventional epifluorescence microscope uses Koehler lighting. This lighting illuminates an entire field of view of the specimen with parallel light, resulting in occurrence of fluorescence in the vicinity of the focal point, and secondary scattered light as well near there. When these lights are focused by the objective lens to form an image on the detector, resolution will decrease because the original image is overlapped by such fluorescence and scattered light.

On the other hand, a confocal microscope employs a point light source or laser beam to illuminate only a "spot" on the focal plane of the objective lens. This approach ensures the reduction of fluorescence from the area out of focus, which often is a principal cause of noise. Furthermore, the confocal microscope can achieve high resolution by blocking the fluorescence and scattered light occurred near the focal point in the specimen, which can be done by placing a pinhole at the objective lens focal point (conjugate points) in front of the light detector. This effect is especially significant in the direction of depth.

Figure 2 shows the fluorescence images of plant spores: one captured by an epifluorescence microscope with a mercury lamp, and the other captured by a confocal microscope using laser, which is discussed in this section. As seen in Figure 2, the image obtained using an epifluorescence microscope (left) contains spores located outside the focal plane, whereas those spores are eliminated in the image by a confocal microscope (right). It can also be observed that the confocal microscope's image depicts only a cross-section of the spherical spore.

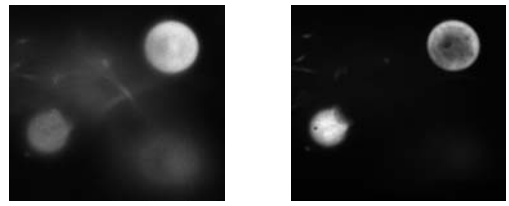

Figure 2 Fluorescence Images of Horsetail Spores: an epifluorescence microscope (left)/a confocal microscope (right)

Laser Beam Scanning

As described above, the confocal microscope is capable of eliminating the fluorescence and scattered light occurred outside the focal point. However, a single illuminated spot only provides a zero-dimensional signal. To obtain two-dimensional information, i.e. an image, it is required to move the focal point along specimen's focal plane and create an image based on the position and signal intensity obtained.

One typical technique for performing two-dimensional scanning is to use two galvano scanners (a scanner that can adjust the direction of the laser beam using a mirror installed on the shaft of a servo motor), and move the focus point two-dimensionally on the specimen by changing the incident angle of the laser beam. In this approach, the scanning time is determined by the mechanical response frequency of galvano scanners (several kHz); it takes about one second to achieve a resolution of 1000×1000.

Another technique is to use a rotating disk, on which a series of small pinholes are arranged in a spiral configuration, in order to scan the specimen using the light beam that passes through such pinholes and the objective lens. This disk was invented by Paul Nipkow in 1884 as an electrical image transmission device, called a Nipkow disk after his name [3].

As illustrated in Figure 3, as the Nipkow disk rotates, each pinhole which traverses in the area laser beam illuminated on the disk is works as a point light source. The point light sources shifts toward the radial direction and the rotating direction at the same time, because pinholes are arranged in a spiral pattern. When the focal

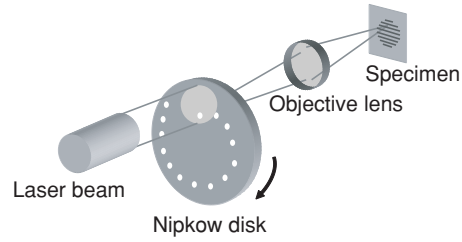

Figure 3 Nipkow Disk

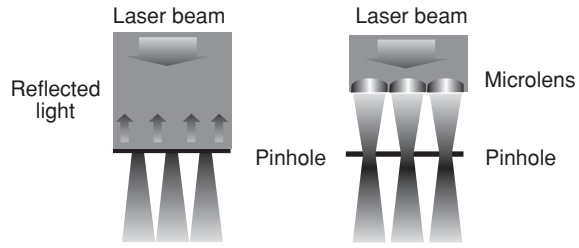

Figure 4 Improvement in Optical Characteristics Resulting from the Use of Microlenses; the backscattering light remains (left) and increase in the amount of the laser light passing through the pinholes (right)

plane of the objective lens is arranged at the Nipkow disk, each light emitted from each point light source, or pinhole, is converged by the objective lens to focus on the specimen's focal plane. Thus two-dimensional scanning is performed in such a way that a laser beam spot moves on the specimen's focal plane. In this approach, scanning time can be reduced by increasing rotation speed of the Nipkow disk and by using multiple spiral pinhole patterns [4].

A drawback associated with a confocal microscope using a Nipkow disk is low efficiency of laser transmittance. Only several percent of the laser beam passes through a pinhole on the disk because the area of pinhole opening is significantly smaller than the area irradiated by laser beam, resulting in insufficient excitation. In addition, part of the laser beam does not pass through pinholes and reflects back from the Nipkow disk (left, Figure 4). This generates stray light inside the optical system, causing a problem that the level of background against weak fluorescent signals will increase.

To address this problem, we have installed microlenses on a disk (microlens array) at positions corresponding to all pinholes on a Nipkow disk. This technique allows each laser beam that passes through a microlens to be focused at a corresponding pinhole and pass through it, which improves the use efficiency of laser beam over ten times greater than otherwise. Consequently, the amount of stray light generated due to the reflection of laser beam on the pinhole plane decreases. Using this technique we have succeeded in observing weak fluorescence emitted from cells [5].

CONFIGURATION OF OPTICAL SYSTEM

Figure 5 shows the configuration of the optical system used in the CSU-X1 confocal scanner. The laser beam introduced through an optical fiber is collimated before reaching a microlens array disk. Between the microlens array disk and the pinhole array disk, there is a fixed dichroic mirror that reflects fluorescence emitted from a specimen. Each laser beam that has passed through each microlens on the microlens array disk penetrates the dichroic mirror, and is focused at its corresponding pinhole which acts as a point light source in the optical system. As a set of the two disks rotates, each laser beam that has passed through each pinhole (i.e. point light source) moves in the direction of rotation. These moving laser beams are condensed by the microscope's optical system and projected on the specimen for scanning.

These individual laser beams excite the specimen which in turn

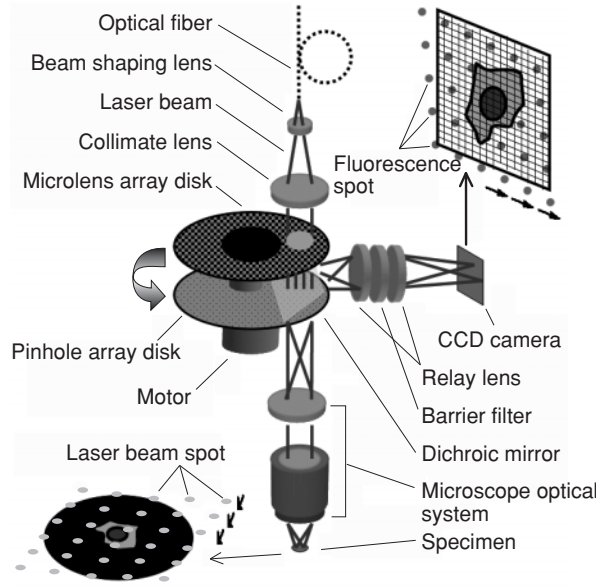

Figure 5 Configuration of the Optical System Used in the CSU-X1

emits fluorescence. This fluorescence is imaged on the pinhole plane, where scattered light is rejected by the confocal effect. This is the initial fluorescence image, which is reflected on the dichroic mirror and passed through a relay lens system before being imaged again on a CCD camera. Stray light that cannot be eliminated by means of microlenses mechanism on the disk (see above) will be removed using a barrier filter installed in the light path, because a CCD camera does not separate stray light by itself. A confocal scanner employing a Nipkow disk allows images to be captured directly by a CCD camera, and, unlike a galvano scanner, does not require image processing by a computer. These benefits help simplify the unit design. The following sections describe components of the CSU-X1 confocal scanner unit.

Excitation Optical System

The laser beam is introduced into the scanner through a single mode optical fiber. The core diameter of the fiber is approx. 3 µm, with the output laser beam having Gaussian intensity distribution. In a scanner using the Nipkow disk technique, the intensity

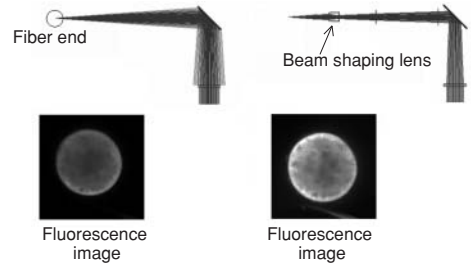

Figure 6 Intensity Distributions of the Laser Beam; an image captured using a conventional technique (left) and an image improved by using a beam shaping lens

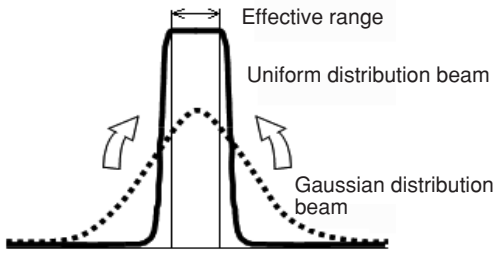

Figure 7 Adjustment of the Intensity Distribution in the Laser Beam

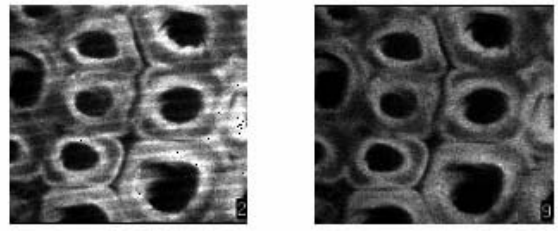

Figure 8 Suppression of Stripes by Adjusting Rotation Speed; stripes due to the fraction of time (left)/no stripes because an integral multiple is met (right)

distribution of excitation light affects the luminance distribution of a fluorescence image. Conventionally, the laser beam is broadened and only the central part where the intensity is relatively constant is used to obtain as uniform a distribution as possible (see the image on the left in Figure 6). However, this approach has presented a problem of low use efficiency of laser beam.

The CSU-X1 incorporates a beam shaping lens that is installed between the end of the optical fiber and the collimate lens to effectively use the laser beam (see the image on the right in Figure 6). As illustrated in Figure 7, the laser beam, which has been emitted from the fiber and has Gaussian intensity distribution, passes through the beam shaping lens to deflect its outer optical rays towards the center, and then is collimated by the collimate lens. This method has improved the use efficiency of laser beam by two times. As seen from Figure 6, it is understood that this approach provides a much brighter image than that obtained by conventional techniques on the same specimen. The beam shaping lens has been implemented using a spherical lens.

Nipkow Disk and Microlens

The microlens array disk is combined with the pinhole array disk, so that the focal point of each microlens comes at its corresponding pinhole. This set of two disks is rotated with a motor.

Each pinhole has a diameter of 50 μm, and is optimized for use with objective lenses having magnification ratio from 60× to 100×. Pinholes are arranged in equal-pitch spiral patterns to prevent luminance irregularity that may occur in inner and outer disk areas, and to minimize non-scanned portions. The pinhole spiral patterns are prepared so that one image is created with a disk rotation of 30 degrees. Usually, a scanner using a pinhole array disk has a problem that cross talk occurs between neighboring pinholes. The CSU-X1 has addressed this cross talk issue by means of placing pinholes at a spacing 5 times the pinhole diameter. This spacing value has been determined by taking into account the image generation speed and the resolution. The pinhole array disk contains about 20,000 pinholes, and about 1,000 are arranged in the image area [5].

In the Nipkow disk technology, it is important to adjust a disk rotation speed when exposure time is short, i.e. ranging from several milliseconds to several dozen milliseconds. The pinhole patterns on the CSU-X1's pinhole array disk are arranged so that one image is generated with a 30° rotation of the array disk. This means that a uniform scanning is performed for a image area if exposure time is an integral multiple of the time needed to create one image. However, when exposure time is not an exact integral multiple and contains any fraction of time, scanning continues for that length of time, resulting in stripes on the image as shown in Figure 8. Such stripes can be suppressed by adjusting the disk rotation speed to achieve an integral multiple of the unit image scanning time.

Motor Component

A DC brushless motor is used to revolve the set of microlens array and pinhole array disks, driven under the back electromotive voltage detection mode and the rotational speed sensorless control. The motor allows for fine adjustment of rotation speed so that exposure time would be an integral multiple of the time needed to generate one image. In addition, the CSU-X1 incorporates a dynamic balancing mechanism for rotating parts including the disks, in order to prevent vibrations, which may occur when the motor revolves at a high speed such as 10,000 rpm, from affecting the scanning process and the resulting image.

Dichroic Mirror and Barrier Filter

In the CSU-X1, the laser beam passes through microlenses to pinholes, and it also passes through the dichroic mirror located between the microlenses and the pinholes. On the other hand, the fluorescence must be reflected on the dichroic mirror. Therefore, the dichroic mirror have characteristics that are opposite to those of a dichroic mirror normally used for epifluorescence microscopes, that is, the dichroic mirror must transmit shorter wavelengths and reflect longer wavelengths.

Any remaining stray light, i.e. light that has not been eliminated at the microlens mechanism, may increase background in the camera. To address this problem, a barrier filter has been installed between the dichroic mirror and the camera.

To effectively collect fluorescent light, the dichroic mirror and the barrier filter must have short rise characteristics in the range longer than the excitation wavelength. This means that they need to be manufactured in the form of a multilayer film containing layers on the order of 100 levels, which often produces a warp due to the internal stress. In recent years, techniques developed for the filters used in optical wavelength multiplexing communications have also been applied in the field of biology/spectrum analysis. These techniques, in combination with spectral characteristics simulation, have provided a sputtering technology that enables precise online monitoring of the thickness of a film being formed. This sputtering technology has created a situation where mirrors and filters with desired sharp

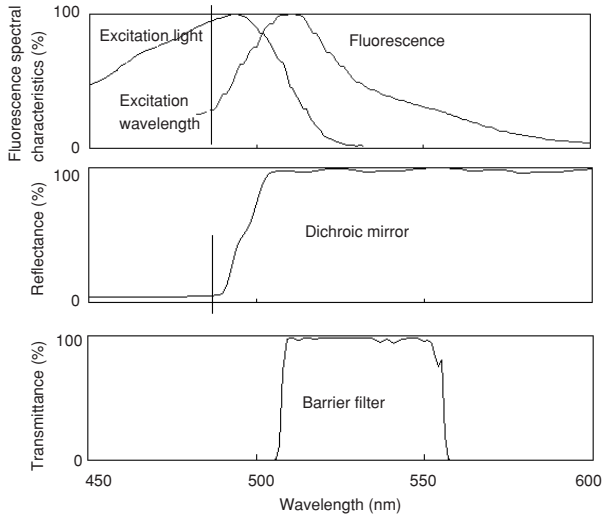

Figure 9 Example Spectral Characteristics of a Filter

spectral characteristics are available. Figure 9 shows the example spectral characteristics of a filter.

A high-end model of the CSU-X1 series is equipped with a computer-controlled dichroic mirror changer that allows the user to select one of up to three dichroic mirrors. This enables an automatic configuration for use with various experiments. There is a block that stores the dichroic mirrors, which the user can replace as needed.

Filter Wheel

The CSU-X1 incorporates a fast-moving filter wheel, enabling the user to continuously perform fluorescence observations at different wavelength ranges and capture images with a single camera, while changing the excitation wavelength and the fluorescence wavelength range. The drive system uses a stepping motor without loss of synchronism, lightweight pulleys and a timing belt, achieving a high-speed performance that it takes only 33 ms to move to a neighboring position. The filter wheel can include a maximum of six general-purpose filters of 25 mm in diameter.

Bright Field Light Path (Optional)

The CSU-X1 provides an optional bright field light path unit that gives the user a way to send the fluorescence images to the camera by bypassing the pinhole array disk, which enables the user to make the most of the product for purposes other than confocal microscope

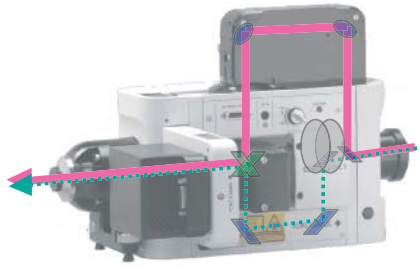

Figure 10 Bright Field Light Path

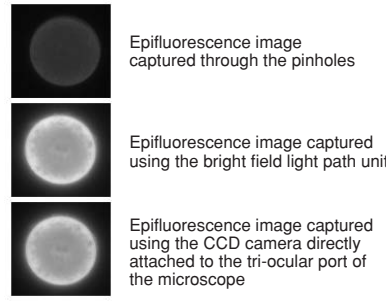

Figure 11 Image Captured Using the Bright Field Light Path Unit

observations. There are often cases in which differential interferometry or bright field illumination are used to capture the image of an entire cell(s) and compare it with the fluorescence image for analysis, since a single fluorescence image does not usually give enough information on the specimen. There also are cases where it is required to perform fluorescence observations using the light from a mercury lamp instead of the laser beam, because of the excitation wavelength used for that observation. When a halogen or mercury lamp equipped with the microscope is used to illuminate the specimen and the image is captured by the camera through the CSU-X1's pinhole array disk, only a dark image will be obtained. This is because each pinhole on the disk is extremely small, so merely several percent of the light from such a light source passes through the pinhole disk. The CSU-X1's optional bright field light path unit provides a light path that bypasses the pinhole disk and sends the image to the camera by means of relay lenses and mirrors that reflect light (Figure 10). This unit improves the light transmittance from several percent up to about 80%.

Figure 11 shows three example fluorescence images: the image captured through the pinhole disk, the image captured using the bright field light path unit, and the image captured using the CCD camera directly attached to the tri-ocular port of the microscope without using the CSU-X1. Since brightness decreases by the order of one digit when an image is shot through the pinholes, the broad dynamic range and the autoscale feature of the camera have been used to obtain brighter results. The bright field light path enables an image with less loss of the light to be produced.

Second Camera Port (Optional)

In observations at different wavelength ranges, usually the time-division method with a filter wheel is used. However, there have been increasing cases in which, depending on the specimen to be observed, simultaneous imaging is required and therefore the rotation time of the filter wheel cannot be ignored. One conventional approach includes installing a special optical system, which contains a dichroic mirror, in front of the camera to "divide" a fluorescence image based on wavelength ranges. This technique, however, has limitations in the available image size and the flexibility of exposure time setting.

The CSU-X1 provides an option where a dichroic mirror can be installed and a port to which a second camera can be attached. These features allow for fluorescence separation, for example the separation into GFP (Green Fluorescence Protein) and RFP (Red

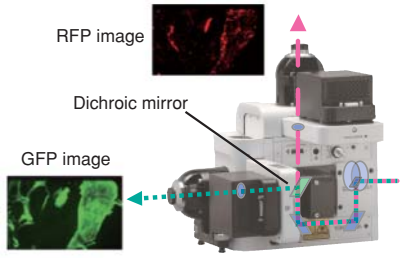

Figure 12 Second Camera Port

Figure 13 An Example System Configuration of a Confocal Microscope

Fluorescence Protein), as shown in Figure 12. This technique helps minimize optical loss during observation. If the intensity of fluorescence is different depending on the wavelength, it can be addressed by adjusting the settings of the camera used to capture that fluorescence. Also it is possible to achieve simultaneous imaging. Since the time needed for the filter wheel to rotate is eliminated, three-dimensional observations at two different wavelengths can be done easily and quickly. In addition, a high-sensitivity camera and a high-definition camera can be used to perform non-spectral observations.

SYSTEM CONFIGURATION

Figure 13 shows an example system configuration of a confocal microscope using the CSU-X1.

Laser

Commonly used wavelengths include 405, 445, 473, 488, 491, 514, 532, 560, 568, 635 and 647 nm. Recently it has become easier to obtain semiconductor lasers with 10 to 100 mW of power, with their operability being improved. For observations at different wavelength ranges, a beam combiner is used to select desired laser wavelengths offered by lasers (those described above). This beam combiner allows the user to control filters and shutters from a computer console to introduce the light of selected wavelength into the fiber.

Microscope

The CSU-X1 can be connected to an upright, inverted or stereo microscope using a C mount adaptor for cameras. To obtain brighter images, it is recommended to use a large NA's objective lens.

Objective Lens Actuator

In three-dimensional observations, a piezo actuator is widely used to precisely move the objective lens because it is excellent in resolution and speed. The piezo actuator is capable of moving the objective lens with a resolution of 10 to 20 nm over the range of 100 μm.

Camera

Because the intensity of the fluorescence emitted from cells is extremely low, it is common to use high-performance cameras such as a high sensitivity cooling CCD, an EMCCD and a CCD with the image intensifier[6]. Most of these cameras are monochromatic, because priority is given to sensitivity. In addition, these CCD cameras are often cooled to -20 to -100℃ by a Peltier element to reduce noise. When observing a fast moving specimen such as described above, it is advisable to use a following CCD camera with the image intensifier. This type of camera allows the user to temporarily store captured images in the camera's built-in memory and transmit those data to the computer after completion of image capturing.

CONCLUSION

This paper described the new technologies that have been incorporated into the new CSU-X1 confocal scanner unit. The scanner unit has succeeded in the implementation of high-speed and two-three-dimensional specimen observations through the improvement in the imaging speed and the use efficiency of laser beam. In addition, the CSU-X1 provides a broad application range by offering optional features such as a bright field light path unit and a second camera port. We will continue the development of this product as a more integrated system.

The CSU-X1 has been developed based on the research results of a health assurance program called "Program for Developing the Technology to Analyze the Dynamism of Inter-cell Network" in Japanese, conducted by the independent administrative agency, the New Energy and Industrial Technology Development Organization (NEDO). ◆

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