High-speed 1-frame/ms scanning confocal microscope with a microlens and Nipkow disks

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We have developed a high-speed confocal laser microscope. A microlens-array disk set in front of a pinhole-array disk improved optical efficiency more than ten times compared with that of conventional Nipkow confocal microscopy. This new microscope achieves a high-speed measurement of 1 frame/ms. We expect that it will be used for measuring biological and industrial active samples.

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1. Introduction

Recently considerable progress has been made in the development of confocal microscopy, which is attracting increasing attention as a new-generation optical microscope. However, most of today's confocal microscopes are insufficient for high-speed measurements, such as needed for living cells and organs in biology or actuators for industry, which require real-time observation. We have developed an innovative compact high-resolution scanning confocal microscope, using a microlens and Nipkow disks. This microscope has attained a scanning speed as high as 1 frame/ms, which we believe to be the highest ever achieved for a confocal microscope, and 10–1000 times greater than that of conventional confocal microscopes. We report on its construction and features and provide some measurement data.

2. Principles and Features of Confocal Microscopy

A conventional optical microscope relies on both a light source, such as a halogen lamp, and an optical detector, such as a camera or human eyes, which are area sources and area detectors. As a result, the sensor receives scattered light from sources other than the point to be measured and also light from the optical axis. This leads to measurement that is out of focus and adversely affects resolution.

A confocal microscope with a pinhole in front of the detector functions as a spatial filter that reduces the effect of scattered light. Light from the focal point of the microscope passes through the pinhole, but light from other points on the focal plane does not. Light scattered from dust and similar on the optical axis is focused by the lens on a point somewhere in front of the pinhole, so the resultant image is spread around the pinhole and thus hardly reaches the detector. As a consequence, a confocal system offers a high spatial (three-dimensional; 3-D) resolution, of 1 μm or greater. This confocal microscopy focuses on a single point in 3-D space. However, since it focuses on only one point, it is necessary to scan the beam over the spatial plane to get a complete image. We developed a high-speed scanner for this purpose.

3. Construction and Principle of the Microscope

Figure 1 illustrates the construction of our microscope, which consists of two disks. The upper disk is a microlens disk consisting of ~20,000 microlenses. When collimated light from a laser illuminates the upper disk, the microlenses focus the light onto the lower disk, which has ~20,000 pinholes with the same pattern as that of the microlenses. The light passing through each pinhole is aimed by the objective lens at a spot on the specimen. Reflected light from the specimen passes back on the same path through the objective lens and pinhole, is reflected by a beam splitter, and is focused on the camera through a relay lens. The upper disk containing the microlenses and the lower disk containing the pinholes are

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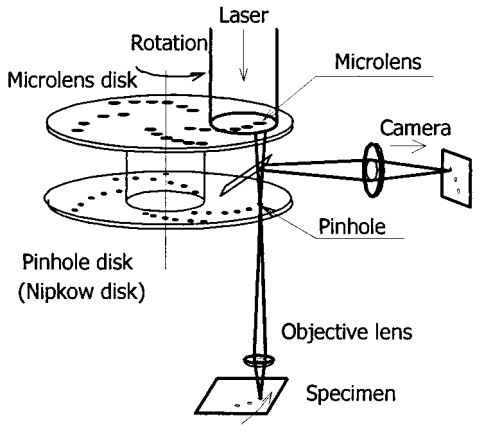


Fig. 1. Microlens- and Nipkow-disk scanning confocal microscope.

physically connected and rotated together by an electrical motor, thus raster scanning the specimen. This two-dimensional scanning of the specimen produces a two-dimensional confocal image on the camera.

4. Features

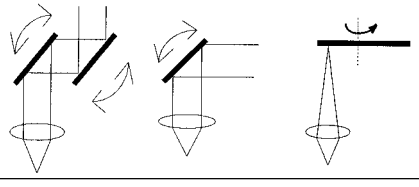
A method to produce images by rotating a disk with pinholes was invented by Paul Nipkow in 1884. In the early development stage of television, the disks were used in both the camera and the monitor.[1] Despite its long history, the Nipkow scanner has retained some special features, as described below, which later scanners do not have.

A. High Speed and High Resolution

Typical features of different scanning confocal microscopes are shown in Table 1. Many of these microscopes currently are category A, which uses scanning mirrors for both the X and the Y axes. Apart from resonance scanning, most two-axis scanning microscopes take 1–2 s to construct an image. Category B uses slit scanning rather than pinhole scanning, thus attaining relatively high speeds (video rate, 30 frames/s) but giving a confocal effect on only one axis. This means a low resolution.

Table 1. Typical Features of Confocal Scanners

Category	A:2 Axis mirror	B:1 Axis mirror	C: Nipkow
Speed	1 Frame/s	33 Frames/ms	33–1 Frames/ms
Confocal effect	Both X and Y axes	Single axis	Both X and Y axes



Both category A and B microscopes scan the specimen with a single light beam, but the Nipkow scanner, category C in Table 1, illuminates with 1000 beams at once. At the same linear velocity, scanning with 1000 beams is 1000 times brighter and faster than scanners with a single beam. Thus a Nipkow confocal microscope with its pinhole scanning can achieve a confocal effect on both the X and the Y the axes at a high speed of 1 frame/ms, as well as at the video rate. Studies on the response of neurons or heart cells in biology, and microlinear actuator systems in industry, indicate that a scanning speed of 1 frame/ms is desirable. Therefore the high speed of this Nipkow confocal microscope is a major advantage.

B. Simple Construction

Compared with category A and B microscopes, which have a complex construction for scanning mirrors and optical relay systems and require special actuators such as galvanometer mirrors, a Nipkow confocal microscope is simple and compact and runs on a simple electrical motor. Moreover, it has no optical relays between the pinhole and the objective lens, which is a big advantage in minimizing optical aberrations and distortions.

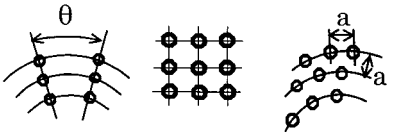
5. Problems and Solutions

A. Optical Signal-to-Noise Ratio

Apart from the above advantages, a major disadvantage of conventional Nipkow confocal microscope was a low signal-to-noise ratio. To attain a confocal effect, it is necessary to place the pinholes far enough apart. If the ratio of pinhole diameter to pinhole pitch is 1:10, the aperture ratio of the pinholes is only 1% of the disk area, which means that only 1% of the signal can pass through the pinhole. At the same time, 99% of the light impinging on the disk does not pass through the pinhole but is reflected from the disk surface, resulting in high background illumination or stray light, and reaches the camera as noise.

As shown in Fig. 1, we added microlenses in front of the pinholes, which greatly increased the signal and decreased the noise (scattered and reflected light from the disk surface), resulting in improving the signal-to-noise ratio significantly.[2–4] With our microlenses we attained an optical ratio—defined as the ratio of the light output from the pinholes to the total light impinging on the microlens disk—of more than 40%. Compared with a conventional Nipkow microscope with a pinhole disk alone, our microscope with microlenses achieves a light efficiency that is more than tenfold greater. In addition, there is an upper limit on the brightness with white light as a light source to focus the light onto these small pinholes. However, there is no limitation on light power when laser light is used as a light source for this construction.

Table 2. Comparison of Pinhole Patterns

	A:Fixed-angle spiral	B:Tetragonal	C:Equal spiral pitch
Category			
Pattern			
Uniformity	Non-uniform	Uniform	Uniform
Scanning pitch	Even	Uneven	Even

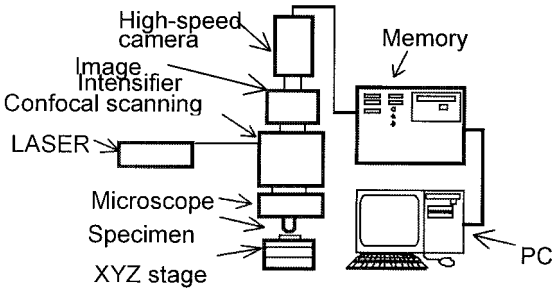


Fig. 3. System construction. PC, personal computer.

B. Nonuniformity of Illumination

On a conventional Nipkow disk multiple numbers of pinholes form in a spiral as seen in category A of Table 2, which is an equal angular spiral pattern. This particular placement of pinholes is used because, in fabricating a mask, it is necessary to copy the pattern of radial rows one by one at equal angles. Its disadvantage is that the light intensity at the outer periphery of the disk is lower than that at the inner periphery, since the pitch between pinholes gets wider toward the outer periphery of the disk. To lessen the effect of nonuniform illumination, it is necessary to make the disk large enough for illumination at the outer periphery of the disk to remain sufficient. With a tetragonal pattern as seen in category B of Table 2, there is no imbalance in the light intensity between the inner and the outer peripheries of the disk; however, when the disk is spun, the scanning pitch is not equal, resulting in stripes.

As shown in category C of Table 2, we designed a new pattern. The equal-pitch spiral pattern category C is designed with equal pitch along the spiral pattern of the pinholes (peripheral pitch) and equal track pitch of the spiral pattern (radial pitch) so as to give constant illumination and also equal scanning pitch regardless of the radius. As a result, there is almost no distortion with this pattern.

Category C of Table 2 illustrates the pinhole pattern where more than one thread of pinhole rows are disposed in a spiral shape and where the radial pitch of a track of an imaginary center line connecting the centers of a plurality of pinholes forming the pinhole rows, and the peripheral pitch along the spiral, are equal. This pattern is traced according to the following expressions.

As shown in Fig. 2, when the pinhole disk spins one full rotation, the radius becomes larger by $m \times a$ between the starting point and the ending point of the track of the spiral.

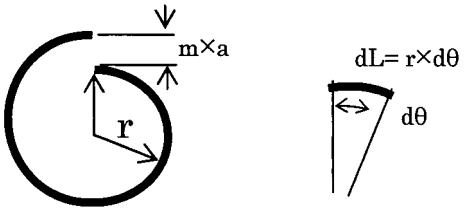


Fig. 2. Diagrams to explain the arrangement of pinholes.

Thus radius r is as follows:

$$r = r_o + \theta ma/(2\pi), \tag{1}$$

where

$i = 0, 1, 2, \ldots, n$ (i.e., in order starting from inside the spiral), ri is the radius of the ith pinhole, ro is the innermost radius, θi is the angle of the ith pinhole, m is the number of spirals, and a is the pinhole pitch.

The length of arc L is as follows:

$$L = \int_0^{\theta} r\,d\theta = r_o\theta + \frac{(ma\theta^2)}{(2 \times 2\pi)}. \tag{2}$$

When Eq. (2) is represented in terms of θ,

$$\theta = \left(\frac{2\pi}{ma}\right)\left[-r_o + \left(r_o^2 + \frac{maL}{\pi}\right)^{1/2}\right]. \tag{3}$$

From Eqs. (1) and (3), Eqs. (4)–(6) are obtained:

$$L = ia, \tag{4}$$

$$r_i = r_o + \theta_i ma/(2\pi), \tag{5}$$

$$\theta = \left(\frac{2\pi}{ma}\right)\left[-r_o + \left(r_o^2 + \frac{ima^2}{\pi}\right)^{1/2}\right]. \tag{6}$$

The pinhole pattern of category C of Table 2 is traced with Eqs. (5) and (6). This pinhole arrangement is formed by a pinhole pitch, a, which is the same in the radial and the peripheral directions as shown in category C of Table 2.

6. System

Figure 3 shows our high-speed confocal system. The confocal microscope illuminates the specimen with a laser beam, and the light signal reflected back to the microscope is detected and stored as digital data by the high-speed camera and memory unit.

7. Results

A. Resolution

The measurement result of the optical-axis resolution is shown in Fig. 4. Measurement was made in a

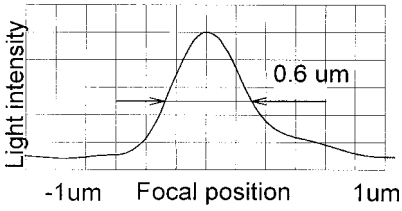


Fig. 4. Resolution along the optical axis.

reflection mode by use of a mirror as a specimen, which was vibrated continuously by a piezoelement in the direction of the optical axis, recording the light intensity. An objective lens with a numerical aperture of 0.9 and a laser light source with a wavelength of 488 nm were used. As shown in Fig. 4, the FWHM was 0.6 μm, which agrees well with the simulation value.

B. Signal-to-Noise Ratio of Images

There are two types of signal-to-noise ratio for images: camera noise and image intensifier noise.

1. Camera Noise

The main noise of a camera is shot noise caused by the intensity of photons and analog-to-digital noise caused by an analog-to-digital transform. We estimate these two types of noise. Figure 5 shows the results. We use a low-noise dc light source (noise, $<0.4\%$). The shot noise is

$$N_{\text{shot}} = (2q\ \text{Pin}\ B)^{1/2}, \tag{7}$$

where q is the electric charge [1.6×10^{-19} (Celsius)], Pin is the input light power (watts), and B is the bandwidth of noise (hertz).

The experimental result and calculation of shot noise fits well. It shows that the limitation of signal-to-noise ratio of this camera system is shot noise.

2. Camera and Image Intensifier Noise

Figure 6 shows the results of an entire system that includes a camera and an image intensifier. The parameters of the graph are the gain of image intensifier. This result means that this system has a 7-bit ($\sim 0.8\%$) signal-to-noise ratio of images if there is enough light to the camera and image intensifier.

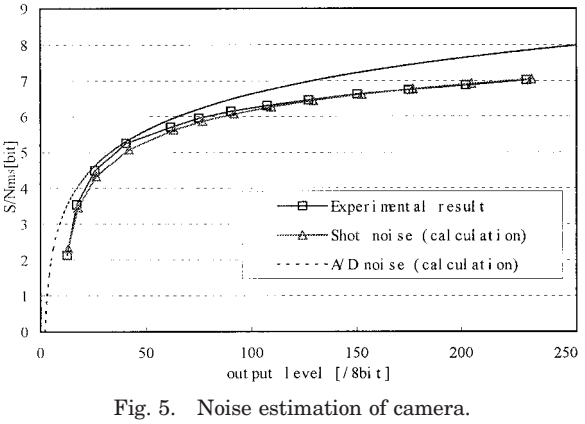


Fig. 5. Noise estimation of camera.

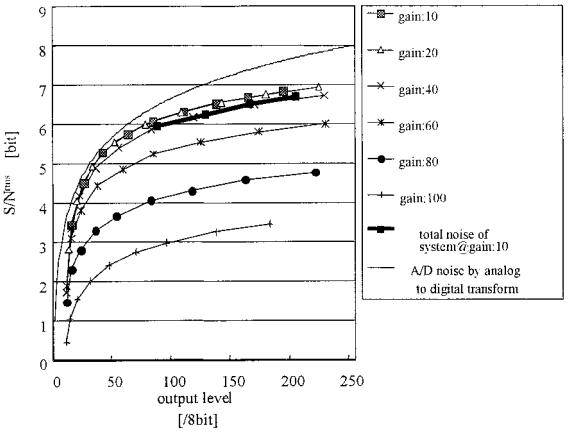


Fig. 6. Noise estimation of camera and image intensifier.

C. Image Data

An example of a high-speed (1-frame/ms) continuous measurement of a moving micromachine is shown in Fig. 7. Images of a moving linear encoder were captured a rate of 1 frame/ms. This encoder was part of a linear actuator, which moved 20 mm/s from the left-hand side to the right-hand side on the images. Our system is capable of continuously capturing 2700 images at the maximum.

The result of a biological application is shown in Fig. 8. We achieved observation of changes in intracellular positive calcium ions after electrical stimulation of adult rat ventricular cells, which were loaded by Ca^{2+} indicator, fluo-3.

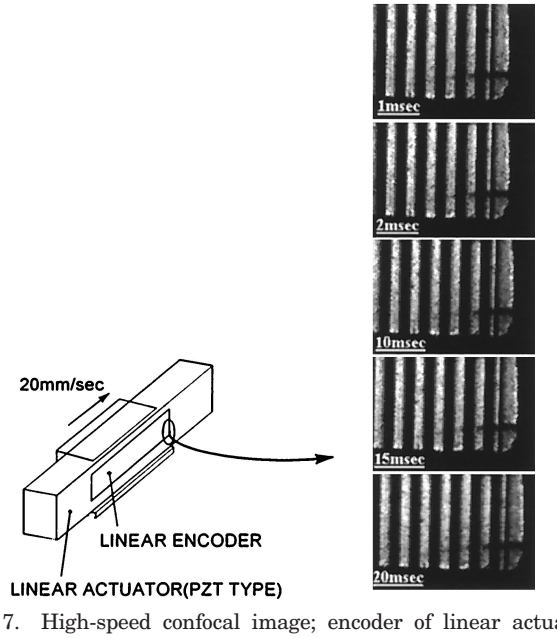


Fig. 7. High-speed confocal image; encoder of linear actuator (pitch, 0.17 mm).

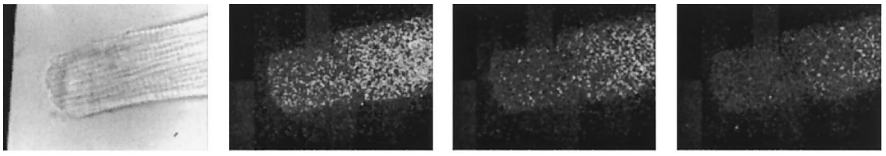


Fig. 8. Fluorescent images of ventricular cell from adult rat (1 ms/frame).

To our knowledge, these are the fastest confocal images ever observed.

8. Conclusion

By adding microlenses to a Nipkow pinhole-scanning unit, we attained an optical ratio—which is defined as ratio of light output from pinholes to the total light impinging on the microlens disk—of more than 40%. Using our system, we were able to capture images at 1 frame/ms or a video rate achieving a confocal effect on both the *X* and the *Y* planes. This system would be a useful tool for measuring not only industrial but also biological specimens.

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