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# THREE-DIMENSIONAL LIGHT MICROSCOPE WORKSTATION FOR THE ANALYSIS OF THE CELL

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## ABSTRACT

We have developed a high-resolution, three-dimensional light microscope system for the non-destructive analysis of three-dimensional dynamics of living cells. The system is comprised of a fluorescence microscope, a cooled charge-coupled device (CCD) and a computer, and allows the three-dimensional image data collection, image processing and image analysis to be carried out on a single workstation. Substantial improvement in the microscopic resolution has been made by computational image processing and it has become possible to analyze small subcellular structures in three dimensions.

## 1. Introduction

Light microscopy provides a powerful tool to study structure-function relationship of the cell, allowing the entire cell to be examined in the living state. Especially molecular specificity of imaging in fluorescence microscopy, specific molecules can be selectively visualized within a cell[1]. Also fluorescent dyes that are dependent on intracellular environments, such as calcium concentration or pH, have been developed[2][3], and the information at the molecular level can be measured within an individual cell.

A microscopic image is basically two-dimensional whereas cellular structures are intrinsically three-dimensional. One way to obtain three-dimensional images on a light microscope is to take a set of two-dimensional images at different focal planes by stepping a microscope focus and stack them together to reconstruct the three-dimensional image (Fig. 1). However, a problem to be taken up here is that a microscopic image of a three-dimensional object contains not only the true information from the infocus plane, but also the out-of-focus information from neighboring regions of the object. This is due to the fact that the point light source does not focus onto a single point, but instead is three-dimensionally spread out by the passage through a microscope. Thus, the out-of focus image information contaminates the infocus image. This optical "blurring" of the microscopic image greatly reduces the resolution of the microscope. Thus, it is necessary to eliminate the out-of-focus information to obtain high resolution three-dimensional images[4].

To obtain high-resolution, three-dimensional images from a light microscope, we have developed a computer-controlled light microscope system in conjunction with computational image processing. This system can perform three-dimensional image data collection, image processing and image analysis on a single workstation and enables us to analyze small subcellular structures in three dimensions.

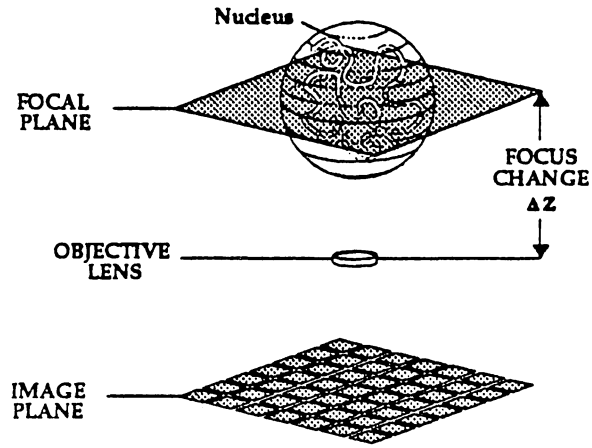


**Fig. 1 Three-dimensional optical section microscopy.**

## 2. Three-Dimensional Light Microscope Workstation

The three-dimensional light microscope system consists of three major components: a fluorescent microscope, a cooled CCD and a computer that controls the microscope and the CCD (Fig. 2). Three-dimensional images are taken on the CCD by stepping the microscope focus at constant intervals. Focus movement is made by a stepping motor with 50,000 steps per revolution attached to the microscope focus knob. The one step in the rotation corresponds to 4 nm in the microscope focus. A scientific-grade, cooled CCD is an ideal image detector for the three-dimensional image processing, having high geometrical and numerical accuracy[5][6]. CCD chip is kept at –40°C by the Pertier cooling to reduce thermal noise. The CCD is, with its high sensitivity and low noise, ideal for the live cell observation which requires low levels of illumination.

Fluorescence microscopy provides a powerful approach, allowing multiple specific molecules in a cell to be observed simultaneously. Our microscope system is designed so that the three-dimensional images can be recorded efficiently and accurately from the multiply-stained specimens[7]. As illustrated in Fig. 2, optical filters for several wavelengths are mounted on a revolving wheel and switch fluorescent wavelengths are switched by revolving the filter wheel under the computer control; a dychroic mirror is designed to allow 4 kinds of wavelength to be observed simultaneously without switching. Operations of optical filter switching, image collection, and focus movement is made under the computer control.

## 3. Three-Dimensional Image Formation in a Light Microscope

A microscopic image is not an exact representation of the real object, but instead is distorted by optical "blurring". As light from each of the individual points in the object is three-dimensionally spread out in a microscope, a microscope image is formed as a summation of such spread points of

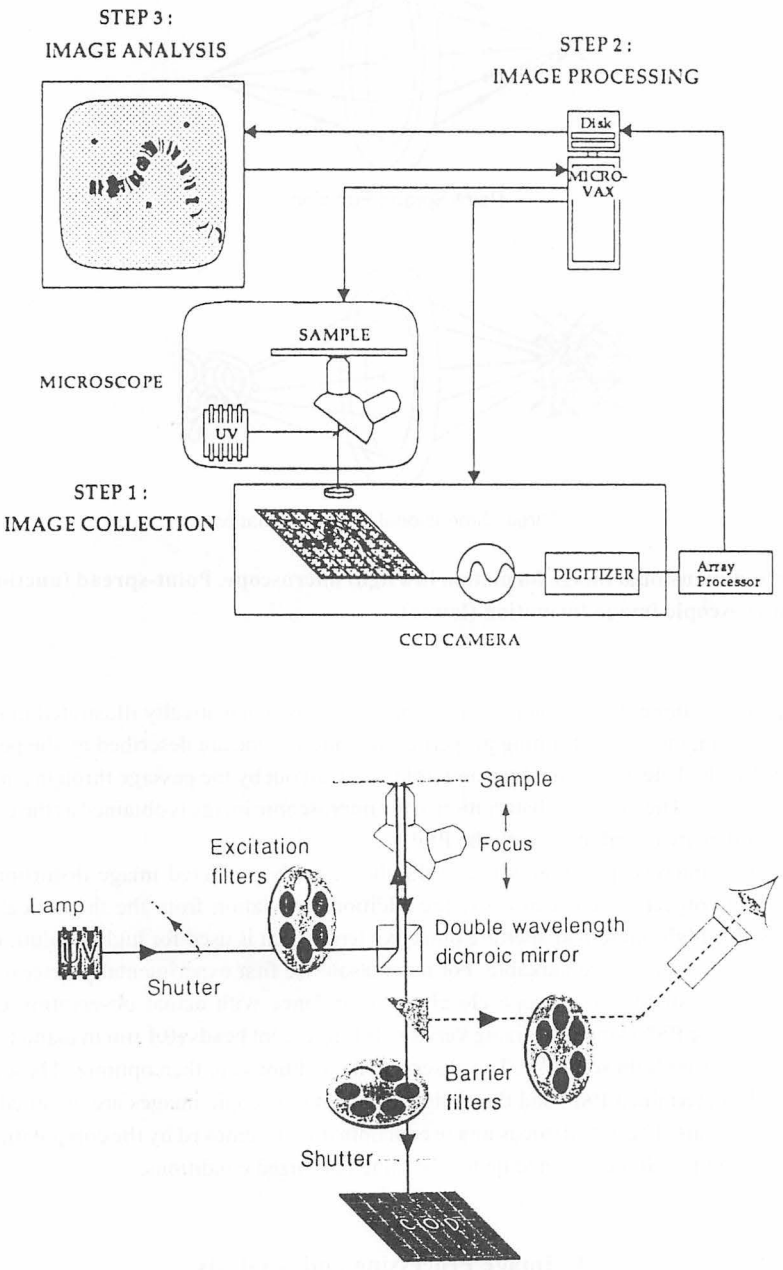


**Fig. 2 Microscope system description. System overview (upper panel) and microscope layout (lower panel).**

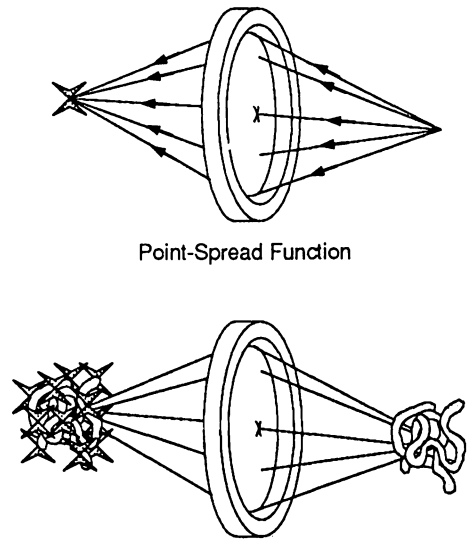


**Fig. 3 Three-dimensional image formation in a light microscope. Point-spread function (upper) and microscopic image formation (lower).**

light. Formation of three-dimensional microscopic images is schematically illustrated in Fig. 3. In a mathematical term, the image-forming properties of a microscope are described by the point-spread function (PSF) which decribes how the point light is spread out by the passage through a microscope in three dimensions. The intensity distribution of the microscopic image is obtained as the convolution of the object intensity distribution with the PSF.

In an actual microscope, in addition to the theoretically-predicted image distortion, residual aberration in the objective lens can cause the additional deviation from the theoretical behavior. Especially with a high-numerical aperture objective lens which is used for high-resolution analysis, deviation from the theory is remarkable. For this reason, we first experimentally determine image-forming properties of the microscope closely in accordance with actual observation conditions. Measurement of the PSF is made utilizing very small fluorescent beads (0.1 μm in diameter) that can be regarded as a point light source[8]. The observation conditions are then optimized based upon the experimentally-determined PSF, and three-dimensional microscopic images are obtained under the optimized conditions. The out-of-focus image contamination is removed by the computational image processing using the PSF determined under the same optimized conditions.

## 4. Image Processing and Analysis

A microscopic image contains the out-of-focus image information as described previously, and it is necessary to remove the out-of-focus information in order to analyze small subcellular structures in high resolution. After three-dimensional images are recorded on a computer, the out-of-focus information is removed by the computational image processing to improve the resolution. Several

kinds of software to remove the out-of-focus information have been developed. We routinely use either of the following two methods[4]. The first of these is the nearest-neighbor method where only the contribution from each plain above and below the infocus plane is taken into the computation. The advantage of this method is that the out-of-focus information can be removed almost in real time and thus processed images can be displayed simultaneously during the image data collection. This method is effective especially for the three-dimensional images of relatively large structures taken at large

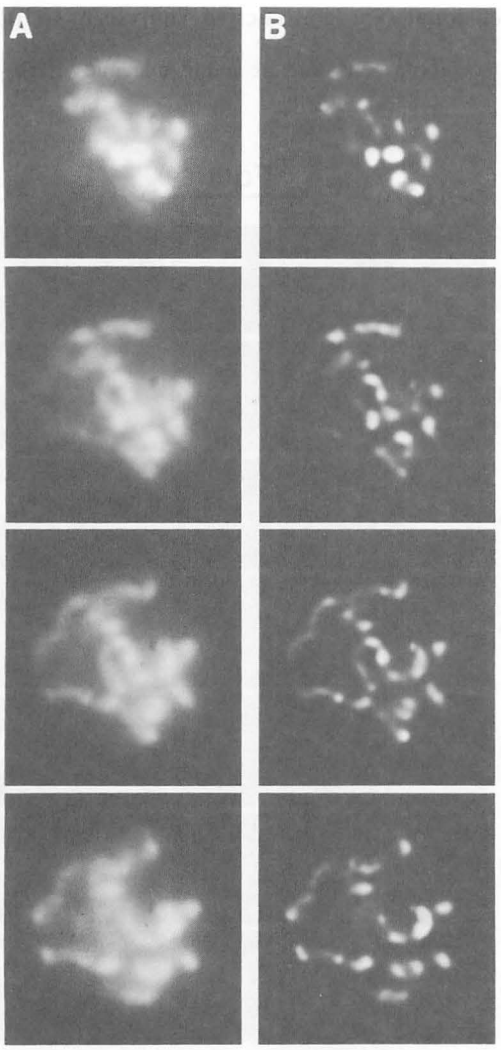


**Fig. 4 High-resolution optical sectioning in a fixed specimen. An example of optical sections from a three-dimensional data stack before (A) and after (B) the removal of out-of-focus image contamination. Three-dimensional image data were obtained at 0.25 μm focus intervals; each displayed section is separated by 0.5 μm in z. Reproduced from Journal of Cell Biology[10].**

focus intervals (larger than 0.5 μm). The second one is the iterative deconvolution method allowing the three-dimensional deconvolution to be done sequentially employing the entire three-dimensional stack of image data. This method is used, for high-resolution analysis of the small intracellular structures. In this case, three-dimensional images are collected at fine focus intervals (0.1–0.25 μm). Although high-resolution images can be obtained, a considerable extent of computaion is required. Thus it is desired to choose a method according to the resolution required.

## 5. Three-Dimensional, Non-Destructive Analysis of Cellular Structures

We have analyzed the chromosome structure and its dynamics during the cell cycle using this

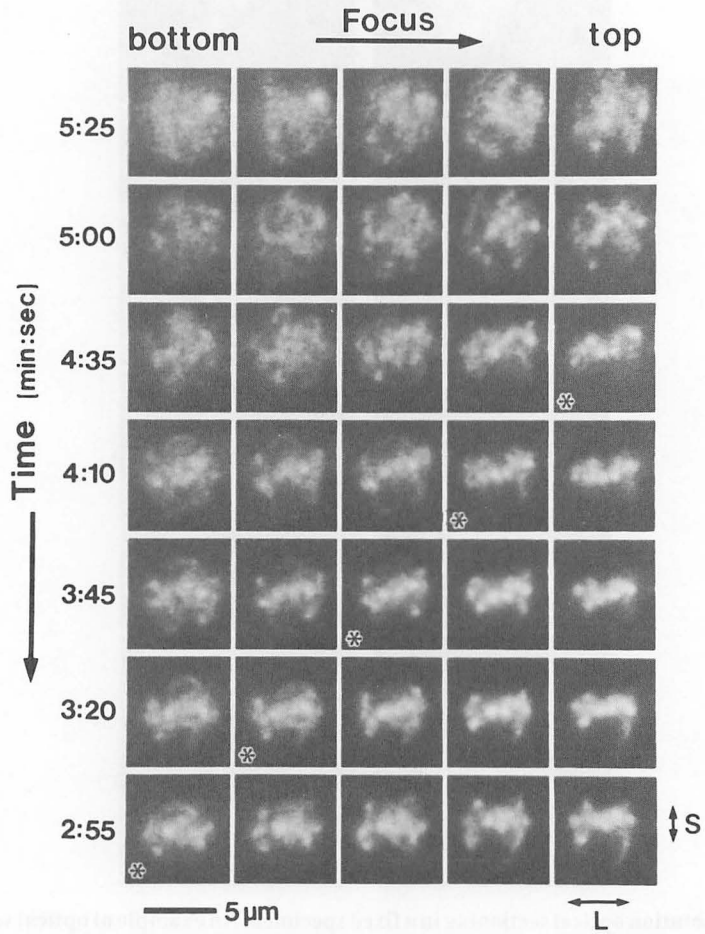


**Fig. 5 Time-lapse optical sectioning in a live specimen. Single nucleus displayed as a focus series (left to right) and as a time course (top to bottom). Optical section was taken at 1 μm focus intervals. Numbers on the left represent time to the next nuclear division in minute:second. Reproduced from Journal of Cell Biology[10].**

microscope system[9][10]. At first the high-resolution three-dimensional analysis was made with the chemically-fixed specimens stained with a DNA-specific fluorescent dye. Three-dimensional arrangement of the individual chromosomes was determined within a nucleus (Fig. 4). Furthermore fluorescent visualization of the chromosomes in live specimens has allowed dynamic behavior of the chromosomes to be observed continuously (Fig. 5).

In high-resolution analysis, specimens are chemically-fixed and three-dimensional images are collected at fine focus intervals of 0.1–0.25 μm. In the example shown in Fig. 4, the three-dimensional image data were collected at an interval of 0.25 μm and out-of-focus information was removed by the iterative deconvolution.

From the obtained high-resolution image data, a model of the chromosome arrangement was constructed. On the other hand, in case of living cell observation, the data collection rate is limited by, in many cases, the rate of the structural changes. This compels us to take a limited number of three-dimensional images (typically 5–10 planes) at relatively low resolution (focus intervals of 0.5–1 μm). Despite of the low resolution, observation of live cells not only eliminates the possibility of artifact accompanied with fixation, but also is useful for direct continuous observation of the structural changes in individual cases.

## 6. Perspectives

An attempt to visualize three-dimensional cellular structures directly in three dimensions using a light microscope is in international trends in the field of cell biology. Importance of such three-dimensional imaging technologies is becoming gradually perceived in this country as well. The three-dimensional microscope system described herein is not a goal of our research but instead a starting point. We have ahead of us several points to be improved in the technology. The greatest limitation in the three-dimensional microscopy at present is that the resolution in a direction of the optical axis is remarkably low compared with the one within the focal plane. The substantial solution for this problem can be found in a method to reconstruct the three-dimensional images by obtaining multiple data sets from different directions, ideally from directions separated by 90 degrees[11]. On the other hand, the technological difficulty in making real-time analysis of live cells resides with the fact that spatial resolution is liable to be sacrificed because it trades off with the rate of the structural changes. At present, the temporal resolution is limited by the CCD read-out rate. To raise the temporal resolution without sacrificing the spatial resolution, a higher rate of CCD read-out is required.

As a long term prospect, it is challenging to make possible the manipulation of the cells by means of light. In principle, it should be possible to manipulate cellular events without contact by inducing photochemical reactions in a small confined regions within a cell. To restrict the photochemical reaction in a small region, it is necessary for excitation to be restricted onto a focal point based on a two-photon absorption process[12]. The effects of the excited photo-active substances are analyzed with the three-dimensional microscope system in the cells. We believe that our three-dimensional microscope system described here has a wide range of applications in imaging and manipulating cellular events.

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