TECHNICAL VIEWPOINT

Fluorescence imaging of mammalian living cells

Yasushi Hiraoka & Tokuko Haraguchi

Cellular events are accomplished by the coordinated interactions of molecular components within the three-dimensional context of a cell. Simultaneous observation of multiple components in three dimensions can be essential for understanding such interactions. The ability to observe living cells is one of the most powerful aspects of light microscopy for studying structure–function relationships. Fluorescence microscopy provides a unique opportunity to observe specific molecules in living cells with its molecular selectivity in imaging. A number of specific fluorescent probes are available (Waggoner *et al.* 1989). Especially since the advent of the green fluorescent protein (GFP; Chalfie *et al.* 1994), fluorescence microscopy techniques are of increasing importance for molecular cell biology. Several techniques of fluorescence microscopy have been developed to obtain improved resolution in three dimensions; among these are confocal microscopy (Brakenhoff *et al.* 1989), two-photon excitation (Denk *et al.* 1990), standing-wave excitation (Bailey *et al.* 1993) and computational image processing (Agard *et al.* 1989, Fay *et al.* 1989). In this short review, we focus on a computerized fluorescence microscope system in conjunction with computational image processing.

Microscope system description

Our aim has been to construct a fluorescence microscope system that is capable of recording multiple-wavelength, three-dimensional images as a function of time. High sensitivity of the image detector is required to observe fluorescently labelled living cells as their photoliability demands a low level of excitation light. For this reason, our microscope system uses a cooled charge-coupled device (CCD) as an image detector. In addition, a lamp shutter is essential to minimize the exposure of fluorescently stained living cells to excitation light. The excitation light level is reduced by neutral density filters when necessary. To observe specimens stained with multiple fluorescent probes, the microscope must be capable of changing its excitation and emission wavelengths during data collection. In an ordinary fluorescence microscope, switching wavelengths requires changing the combination of excitation filter, dichroic mirror and barrier filter. Manual switching of filter combinations will cause variations in the position of the dichroic mirrors, resulting in a translational displacement for the multiple-wavelength images. In our system, a single stationary dichroic mirror that has quadruple-wavelength band-pass properties (Chroma Technology, Brattleboro, VT, USA) is used for multiple wavelengths, avoiding the need to change the mirrors. High-selectivity excitation and barrier filters (Chroma Technology) are mounted on revolving filter wheels to permit switching under the computer control. For accurate control of the microscope focus, the focus is driven by a backlash-free stepping motor that is directly coupled to the microscope fine-focusing knob under the computer control.

In our microscope system, a Peltier-cooled CCD camera (Photometrics Ltd, Tucson, AZ, USA), with a 1317 × 1035 pixel CCD chip (KAF1400) is attached to an Olympus inverted microscope IX70; microscope lamp shutter, focus movement, CCD data collection and filter combinations are controlled by a Silicon Graphics Indigo2 XZ. Microscope control, data collection, image processing and analysis can be performed on the one workstation.

The microscope system was originally developed in Sedat's and Agard's laboratories in University of California, San Francisco, to examine three-dimensional arrangement and dynamics of chromosomes in embryos of *Drosophila melanogaster*. Basic configuration of the microscope system is documented in Hiraoka *et al.* (1991). The entire microscope system, together with a software package for image aquisition, processing and display, is now commercially available as a Delta Vision® system from Applied Precision (Seattle, WA, USA).

High-resolution, three-dimensional imaging in fixed cells

A three-dimensional image can be reconstructed by stacking a series of two-dimensional images obtained by stepping a microscope focus. However, a microscope

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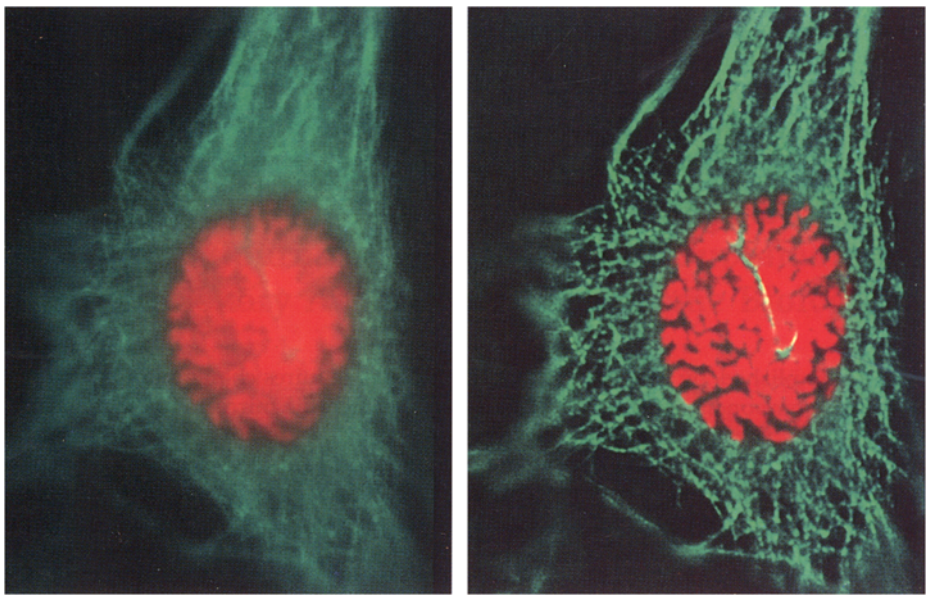

(A) (B)

Figure 1. Chromosomes and microtubules in a fixed HeLa cell. A single section in three-dimensional data is shown before (A) and after (B) the removal of out-of-focus information. Chromosomes stained with DAPI are displayed in red and microtubules stained with Texas red are displayed in green.

image of a thick specimen is not an exact representation of an object, but instead is the sum of in-focus information from a given focal plane and out-of-focus information from the remainder of the specimen. To obtain high-resolution three-dimensional images, it is necessary to remove the out-of-focus information. We remove the out-of-focus information by computational image processing using the point-spread function (PSF), which is determined experimentally for the optical system being used for data collection; PSF defines a three-dimensional function that describes how a point image is spread out by the passage through a microscope. Excellent geometric and photometric properties of the CCD ensure dimensional and numerical accuracy of the three-dimensional image data, which is important for image processing to remove the out-of-focus image information.

An example of the computational image processing for an image of chromosomes and microtubules is shown in Figure 1. In fixed preparations, microtubules were fluorescently labelled with Texas red using anti-tubulin antibody (coloured green in Figure 1), while chromosomes were stained with DAPI (coloured red in Figure 1). Optical section data were collected at 0.5-μm focus intervals by repeating the following sequence at each focal plane; two images were obtained sequentially for chromosomes and microtubules, and then microscope focus was stepped by 0.5 μm. Out-of-focus information was removed by computational image processing using an iterative deconvolution method (Agard *et al.* 1989).

Fluorescence imaging in living cells

An ideal complement to the high-resolution analysis in fixed preparation is a continuous observation in living cells. This technology allows us to examine the dynamic cellular events under the conditions free from artefact introduced during fixation and sample preparation. For fluorescence imaging of chromosomes and intracellular structures in living cells, we are currently using the combination of the following three methods: (1) the use of DNA-specific fluorescent dye, Hoechst33342; (2) microinjection of protein chemically tagged with a fluorescent dye; and (3) the use of the GFP fusion.

To examine living mammalian cells on a microscope stage, temperature control is necessary. Temperature control devices for a microscope stage are commercially available. In many such commercial devices, however, an objective lens is not temperature controlled and acts as a heat sink, making it difficult to control the temperature accurately at the specimen plane. Thus, for precise temperature control, we built the microscope system in a custom-made temperature-controlled room. The room temperature can be controlled in a range from 10°C to 50°C with a precision of 0.1°C. A computer and other control units are placed outside the room and remotely control the microscope.

Using this computerized fluorescence microscope system with a precise temperature control, we observed dynamics of chromosomes and microtubules during mitosis in living mammalian cells at 37°C (T. Haraguchi & Y. Hiraoka, unpublished). Briefly, HeLa cells were

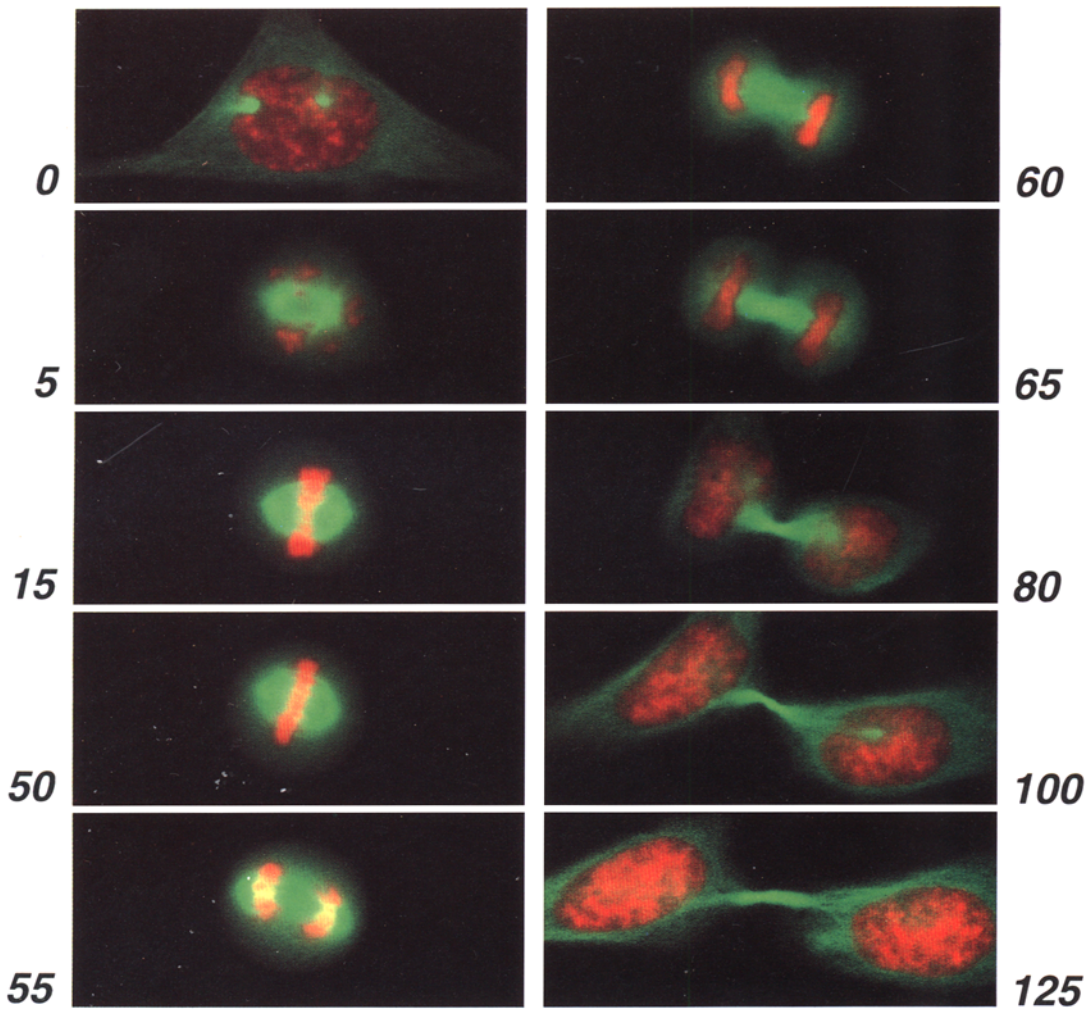

Figure 2. Chromosomes and microtubules in a living HeLa cell. Chromosomes stained with Hoechst33342 are displayed in red and microtubules stained with rhodamine are displayed in green. A series of images show continuous observation of chromosomes and microtubules in a single cell; number in each image indicates time in min. Chromosome condensation at prophase (0 min), chromosome movement toward metaphase plate at prometaphase (5 min), formation of metaphase plate at metaphase (15–50 min), chromosome segregation at anaphase (55–65 min) and chromosome decondensation at telophase (80 min). Images were obtained on an Olympus IX70 using an Olympus oil immersion objective lens UApo40 × /340 (NA = 1.35). Only one focal plane was recorded at each time point and no image processing was made in this example.

cultured in a 35-mm glass-bottom culture dish (MatTek, Ashland, MA, USA) and stained with Hoechst33342 in an appropriate medium; rhodamine-labelled tubulin was microinjected into the Hoechst33342-stained cells for double staining. An example of the dynamics of chromosomes and microtubules from prophase to telophase in a HeLa cell is shown in Figure 2. We are also studying chromosome dynamcs in fission yeast by a combination of Hoechst33342 staining (Chikashige *et al.* 1994) and GFP fusion technology (D.-Q. Ding & Y. Hiraoka, unpublished).

With its molecular selectivity in imaging, fluorescence microscopy can provide the potential capability of biochemistry under the microscope, or 'single-cell biochemistry', for analysing the behaviour of intracellular molecular components in the processes of dynamic biological phenomena. A key aspect of a microscopic approach is the ability to observe transient, ephemeral structures and interactions on a cell-by-cell basis that cannot be detected by a biochemical approach.

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