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ANALYSIS OF STRUCTURAL DYNAMICS IN LIVING CELLS WITH FLUORESCENCE VIDEO MICROSCOPY

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INTRODUCTION

Recent advances in fluorescence microscopy and digital image processing have allowed direct observations of structural components in living cells (Wang and Taylor, 1989; Taylor and Wang, 1989). Specific targets in the cell are labeled with a tracer amount of fluorescently labeled ligands or structural components. Using low light level imaging systems, sufficient signals can be obtained while maintaining the viability of the cell. Images of a relatively high quality are generated with simple manipulations such as frame averaging and background subtraction. Quantitative studies can also be performed based on the distribution of fluorescence intensity.

Although the performance of such studies on living cells may appear similar to that of immunofluorescence, many new questions arise due to the fundamental difference of the two approaches: the involvement of time-dependent changes in living cells. For example, how should dynamic processes be recorded and analyzed? and how are living cells affected by the illumination? In simple qualitative analyses, one might attempt to record fluorescence images continuously with a video recorder, much like classical microcinematography. However, such an approach is usually limited by radiation damages to the living cell, and by the photobleaching of fluorophores. More typically, the samples are illuminated and recorded intermittently, and images are stored as computer files. Simple methods are thus required to identify dynamic events from a series of image files.

In addition to direct imaging, fluorescence photobleaching techniques can be used to study the movement of molecules, at a level beyond the resolution of the light microscope. The combination of photobleaching and imaging techniques has several important advantages: they allow direct observations of directional transport of molecules (Wang, 1985), and structural anisotrophy (Kapitza et al., 1985). However, there are also technical problems when imaging is combined with photobleaching. For example, the wavelength of the laser may not be optimal for image recording, and the temporal resolution may be limited by the time required for image processing and storage.

The purpose of this article is to describe methods, using relatively simple equipment, for studying structural dynamics. In the first part, a software approach is described that allows visualization of motions based on images stored as computer files. The second part discusses a simple method that combines image processing and photobleaching techniques.

INSTRUMENTATION FOR IMAGE PROCESSING

Our system is based on a Zeiss IM35 microscope coupled to a Dage-MTI (Michigan City, IN) ISIT-66 low light level video camera (Fig. 1). Hardware for video image processing includes 3 frame buffers, an ALU board, and an analog processor board (512 series Q-bus; Imaging Technology, Woburn, MA). Each frame buffer has a resolution of 480x512 and can hold 4 images of 240x256 pixels. The circuits are controlled by a host computer: a DEC PDP11/73 microcomputer with a 330 megabyte Winchester hard disk running the VENIX (VenturCom, Cambridge, MA) operating system. Devices, such as shutters and solenoids, are controlled by an Optomux controller (OPTO22, Huntington Beach, CA) connected to the PDP computer through the RS232 interface. In addition, a graphics tablet (Digi-Pad 5; GTCO, Rockville, MD) allows definitions of points and tracks, which are then used by the computer to perform site-specific operations, such as measurements of local or integrated intensities. The hardware is assembled by G.W. Hannaway and Associates (Boulder, CO). Most of the image processing software is developed in our laboratory using subroutines from Hannaway & Associates.

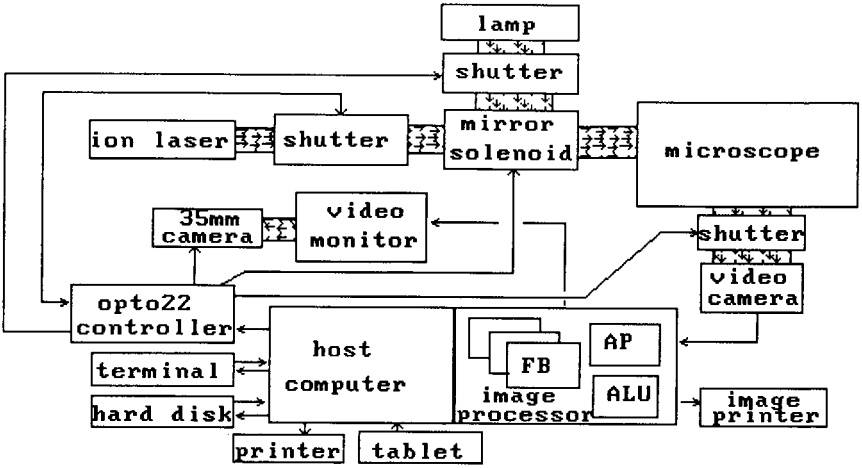

Figure 1. Simplified diagram of an integrated video microscopy-digital image processing-photobleaching system. Multiple arrows denote light paths. Single arrows denote electronic signal paths.

VISUALIZATION OF STRUCTURAL REORGANIZATION AND MOVEMENT

Although a large amount of information can often be obtained by simply comparing images taken at fixed intervals, as the complexity of the images increases, it becomes increasingly difficult to identify specific structures among different images. An example is shown in Fig. 2, which reveals hundreds of small, sometimes ill-defined, myosin filaments in a 3T3 fibroblast microinjected with fluorescently labeled myosin (McKenna et al., 1989). By comparing images taken at different time points, it is apparent that reorganization takes place. However, it is almost impossible to identify specific filaments with confidence and to determine whether the reorganization is caused by movement or by assembly-disassembly.

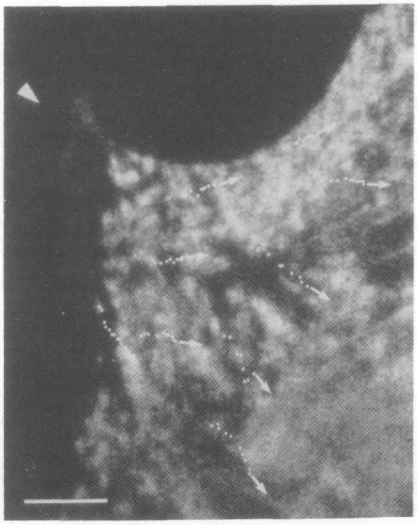

Figure 2. Fluorescence image of a 3T3 cell microinjected with rhodamine-labeled smooth muscle myosin. Each bead probably represents one myosin filament. Images of this cell were recorded at 2-3 min interval for ~20 min, then observed with the motion program to determine the behavior of specific beads. The positions of 9 beads are marked as small white dots. Arrows indicate the direction of translocation over the period of observation. Bar, 5 μm. Reproduced from J. Cell Biol. 1989, Vol. 109, pp. 1170, by copyright permission of the Rockefeller University Press.

The simplest way to analyze such complicated images is to rely on vision, provided that the sequence can be displayed in rapid succession as motion pictures. Unfortunately, the time required for loading the frame buffers is usually too long to allow for the sense of motion. One approach that we took previously is to load images sequentially into the viewing frame buffer, while recording with a video recorder in the time lapse mode. Motion can be sensed when the tape is played back at a high speed. However, this process is both time-consuming and inconvenient. Our current approach is to load images, in the 240x256 format, into the 480x512 frame buffers, so that up to 12 images are loaded into three frame buffers. The frame buffers are displayed in the zoom mode, allowing each image to fill the entire monitor screen. A simple program is then used to change the display pan and scroll registers, such that the four images in a frame buffer appear in succession. The display is then shifted to the second, and third, frame buffers, and the process repeated.

Several additional features greatly enhance the power of the program. The display of motion is repeated within an infinite loop. The speed of display is made variable. Between the change of images, the computer polls the keyboard for instructions, such as speeding up, slowing down, changing the direction of display, or freezing the

frame. The interactive change in speed is crucial since the sense of motion is affected by both the differences between different frames and the speed of display.

Using this approach, we have identified most myosin filaments shown in Fig. 2 through a sequence of images taken over 20 min, and determined their speed and direction of movement. The filaments appear to undergo a continuous assembly near the lamellipodia, then move away from the leading edge at a rate of ~0.18±0.09 um/min (McKenna et al., 1989). An attractive model involves a continuous cycling of myosin molecules between the assembled and unassembled forms, and between different regions of the cell (Fig. 3). The molecule may move centripetally along the cortex in the assembled form, disassemble in a more central area such as the perinuclear region, then diffuse in the unassembled form back toward the leading edge. This cycling process may play an important role in the control of the polarity of the cell and in the movement of surface receptors.

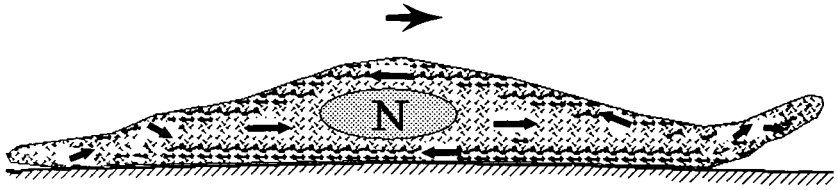

Figure 3. A hypothetical model showing cycling of myosin molecules between unassembled and assembled forms. The molecules assemble near the leading edge, move along the cortex toward the center of the cell, then disassemble in an unidentified region, possibly trailing edge or the perinuclear area. Disassembled molecules then move back to the leading edge.

IMAGE ANALYSIS OF FLUORESCENCE PHOTOBLEACHING

Conventional light sources are better than a laser in several regards for the purpose of imaging, such as the availability of a wide range of wavelengths and the relative ease to achieve uniform illumination. However, in order to use lamps for image recording before and after laser photobleaching, a mechanism allowing rapid

interchanges between the two light sources is required.

A simple approach used in our laboratory is to move the lamp away from the microscope, next to the laser light path (Fig. 1). A intercepting mirror is placed in the laser light path, blocking the passage of laser and reflecting light from the lamp into the microscope. The mirror is driven by a rotary solenoid controlled by the PDP computer, such that it rotates up by 45^{o} upon activation, allowing the laser beam to enter the microscope. Signals from the timer for the bleaching shutter are fed into the computer through the Optomux controller, allowing the computer to sense the completion of photobleaching, deactivate the mirror solenoid, and resume the image acquisition (see also Kapitza et al., 1985 and Wolf, 1989; for alternative designs).

Although images before and after photobleaching can be acquired and analyzed as for the time-lapse recording (Wang, 1985), the speed is limited by the time required for frame averaging and file storage, to one image per 5-25 sec. The time resolution can be improved by reducing the number of frame averaging and sacrificing the spatial resolution, and/or by storing only a portion of the image. As an alternative method for qualitative studies, we have used a 35mm camera equipped with an electric winder for image recording. The camera is mounted in front of the video monitor. The computer automatically triggers the camera after a processed image is acquired, allowing images to be recorded within one sec of photobleaching and thereafter at a rate of one frame per sec.

Quantitative analysis of the rate of recovery can be performed based on the distribution of fluorescence as a function of time (Kapitza et al.). However, a simpler approach can be taken if the purpose is to detect changes in mobility as a result of cell perturbation. The center and the radius of the bleached area are input into the computer, which then identifies pixels within the defined area. Dark images are acquired and dark counts integrated within the area of photobleaching. Averaged images are then obtained before and repeatedly, at a preset frequency, after photobleaching. Pixel intensities within bleached areas are integrated immediately after the acquisition of each image, and, after subtracting out the dark counts, the numbers are stored in a computer file. This process can be

carried out simultaneous with the recording of images. The recovery curve is displayed upon the termination of the measurements and the half recovery time estimated. The half time can be used as the criterion for comparison, as long as the bleached spot remains consistent throughout the experiments. With the averaging of 8 frames, 3 measurements can be performed per sec, fast enough for most recovery studies.

Compared to the measurements with photomultipliers, the accuracy of photometry with image detectors is limited by several factors. First, most video cameras have variable responses across the field and show linear responses only over a narrow range of intensities (Aikens et al., 1989). However, since the measurement is usually limited to a small, constant area, the variability across the field does not represent a problem. Second, since an aperture cannot be inserted on the secondary image plane, as has been done with photomultipliers (Koppel et al., 1976), contribution from out-of-focus signals is higher than that in spot measurements. This error should be limited if the measurement is performed in a thin area, such as the periphery of spread cultured cells. It should also be noted that, since the illumination profile during recovery measurements is uniform, rather than Gaussian, errors will be introduced if the integrated intensity is analyzed, for the calculation of diffusion coefficients, with equations derived for laser illuminations.

We have used video recording for studying photobleaching recovery in an experiment examining the effect of oncogenic transformation on the mobility of cytoskeletal components at cell-substrate adhesion sites (Stickel and Wang, 1987). The substrate adhesion in transformed cells is weaker than that in normal cells, although both contain similar protein components such as actin, alpha actinin, and vinculin. We have microinjected fluorescently labeled alpha actinin into transformed and normal cells, and examined the recovery of fluorescence after photobleaching molecules that are associated with cell-substrate adhesion structures. Fig. 4 shows a sequence of images, taken with a 35 mm camera as described above, of a transformed cell during the first 11 sec after photobleaching. Recovery is seen as a rapid process, even though the protein appears to be associated with a discrete structure. Consistent results are obtained with the

photometric approach (Fig. 5). The half recovery time is ~4 sec. When similar experiments are performed with non-transformed cells at focal contacts, <50% recovery is observed after 50 sec. It is difficult to apply the equations of diffusion here since the mechanism of recovery, most likely an on-off reaction at contact sites, is quite different from diffusion, and the steady-state distribution of molecules is non-homogeneous. Nevertheless, these results indicate that one of the effects of oncogenic transformation is to destabilize molecular interactions at the cell-substrate and possibly cell-cell adhesion sites. The reduced adhesion may then lead to an increase in cell motility and loss of contact inhibition.

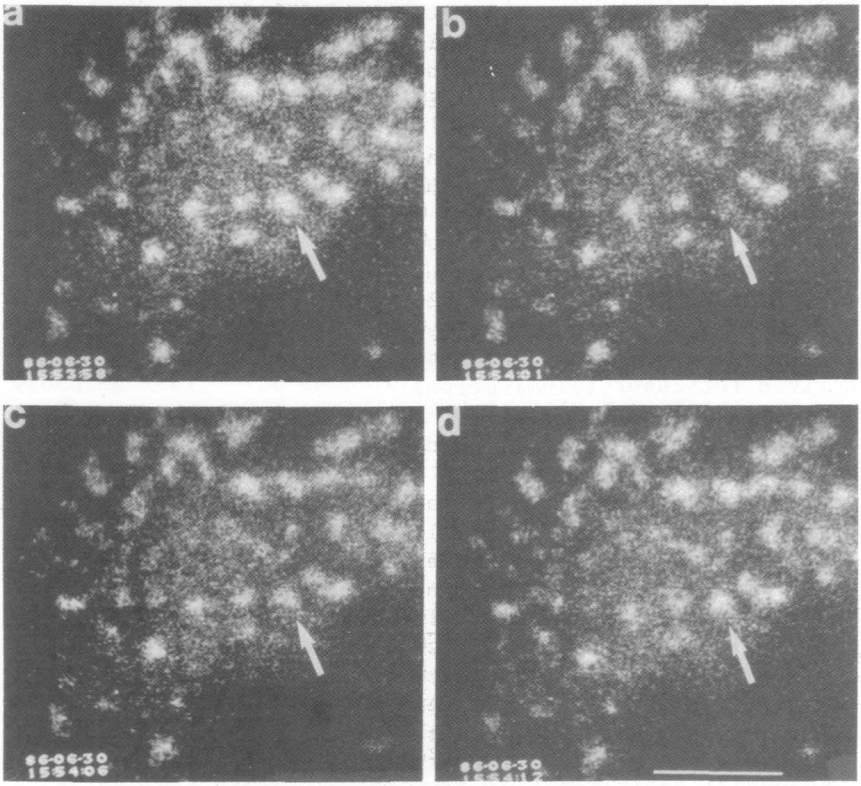

Figure 4. Photobleaching recovery of alpha actinin in substrate adhesion structures of transformed NRK cells. Images are recorded with a 35mm camera in front of a video monitor, before (a), immediately after (b), 5 sec after

(c), and 11 sec after (d) photobleaching. Extensive recovery is reached by the end of the sequence. Bar, 10 μm. Reproduced from J. Cell Biol. 1987, Vol. 104, pp. 1524 by copy copyright permission of the Rockefeller University Press.

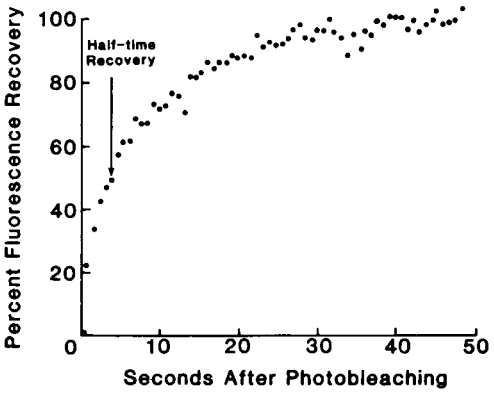

Figure 5. Time course of fluorescence recovery after photobleaching fluorescent alpha actinin associated with adhesion structures in transformed cells. Time 0 represents the first time point after bleaching. Half time of recovery is about 4 sec. Reproduced from J. Cell. Biol. Vol. 104, pp. 1525 by copyright permission of the Rockefeller University Press.

CONCLUSION

Over the past few years, fluorescence techniques have evolved from a morphological tool for fixed samples into physical chemical probes for living cells. The new dimension, time-dependence of molecular organization in living cells, has opened up numerous novel ways to study cellular processes. The methods discussed in this article illustrate that many tasks that appear to require expensive, sophisticated computers can nevertheless be achieved with a relatively simple setup. Although the development of electronic instrumentation clearly plays an essential role in the advancement of this field, equally important is how various kinds of instruments can be effectively used and combined to achieve an endless variety of new functions.

ACKNOWLEDGMENT

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