IMAGE PROCESSING IN VIDEO MICROSCOPY

KIYOSHI KAMIYA

Hamamatsu Photonics K. K., Jyokou-cho 812, Hamamatsu, Shizuoka 431-32

The technique called Video Microscopy has become very popular in the field of biomedical research, in such areas as the study of the behavior of calcium ion in single cells. The video microscopy utilizes advantages of both the light microscope and the video camera. There are two typical methods in video microscopy, one of them being Video Enhanced contrast microscopy (VEC) and the other, Video Intensified microscopy (VIM). The VEC technique is being used to visualize specimens far beneath the resolution limits of the optical microscope, such as the observation of moving particles along the single microtubule, the behavior of the microtubules in mitosis and the observation of colloidal gold (5 nm in size) in the living cell. VIM technique is a useful and powerful method used to observe or quantize features under limited light conditions such as fluorescence, bioluminescence and chemiluminescence. The typical application is to study the cytosolic free Ca concentrations in a living cell with a fluorescence microscope. Photon counting imaging (under the ultra-low light level, it had better count photons individually rather than measure the amplitude of the light) is finding applications in biological research to detect chemiluminescence and bioluminescence. This technique provides a means of directly visualizing gene expression in single cells, imaging metabolites in tumor tissue and visualizing the chemiluminescence associated with oxidative metabolism in phagocytic cells. It is clear that VEC and VIM techniques are very promising methods in biomedical research.

INTRODUCTION

Recently it has been getting more and more popular to use the technique called video microscopy in the biomedical field. This technique employs an optical microscope, video camera and image processing unit. It utilizes the advantages of the optical microscope and video camera. Optical microscopes allow researchers to study many features on a specimen which is being kept alive. This is a definite advantage they have over the electron microscope. There are two kinds of techniques in video microscopy. One of them is called video enhanced contrast microscopy (VEC). It is a powerful technique used to study features in a cell under conditions in which there is plenty of light but in which the specific objects are either barely visible or invisible because the modulation in light (contrast) is very slight.

The other technique is called VIDEO INTENSIFIED MICROSCOPY (VIM). It gives many opportunities for researchers to study living cells with limited light such as fluorescence, chemiluminescence and bioluminescence.

VEC

This technique is being used to visualize specimens far beneath the resolution limits of the optical microscope. Basic theory to improve the visuali-

ty or detectability is to increase the contrast of specimens as compared to the background. This is not so easy to do in optics, but it becomes an easier job in electronics. By employing gain and offset, contrast can be dramatically increased. This principle and effect, often called analog contrast enhancement, is illustrated in Figure 1. As a result of the additional gain and offset, signal to noise ratio is reduced a slightly, but the increase of contrast will have more impact for visualization of specimens. At the same time, the other optical components such as internal dust particles, lens defect, and shading from both uneven field illumination and detector nonuniformity are also amplified. These unwanted artifacts together appear as a fixed background (mottle) superimposed on the image which prevents clear visualization of specimens. By employing digital image processing, this mottle image may be easily removed.

This theory is illustrated in Fig 2. It is accomplished by defocusing the specimen and store it in the frame memory, then focusing the specimen and subtracting the mottle image.

Highly sophisticated digital image processors are capable of performing these functions in real time, thus allowing application to study static as well as dynamic events. The simultaneous use of signal averaging further improves image quality by reducing

random noise. The contrast of the image may be further enhanced by manipulation of the gray level. A variety of other enhancement operations for low contrast imaging may be conveniently available to the digitized image. They are image pseudo coloring, zooming, feature extraction, statistical analysis and morphometric measurement.

Typical applications of VEC

- Visualization of small particles along the single microtubule
- Visualization of colloidal gold down to 5 nm in diameter which are injected into the cell, has been reported (1).

VIM

This technique is a very useful and powerful method used to visualize specimens under very light limited conditions such as fluorescence, chemiluminescence and bioluminescence. It is usually required to improve signal to noise ratio (S/N) to visualize and quantitize the specimen, and is commonly done by employing integration of incoming signals. The longer integration improves S/N, but it limits images to dynamic events.

The detectors being used for VIM are the SIT camera, the Intensified CCD camera and the single photon counting camera.

The schematic diagram and the principle of the single photon counting camera is illustrated in Fig. 3.

A single photon event typically occurs when light intensity decreases to a point where the light flux per unit area becomes sufficiently low that the scene being imaged will no longer appear continuous. Instead it will be represented by individual photons which are temporally and spatially distinct from one another.

FIG. 1. Schematic diagram of contrast enhancement.

FIG. 2. Schematic diagram of mottle subtraction.

- (A) Video signal of mottle
- (B) Video signal of specimen
- (C) Video Signal of Specimen with mottle in focus
- (D) Video Signal of Specimen with mottle in defocus
- (E) (C)-(D)

At these light levels it should detect light as discrete particles (photons).

The single photon counting camera is a two-stage MCP image intensifier optically coupled to a low lag vidicon. The MCP itself is an array of glass fibers, each approximately $10\text{ }\mu\text{m}$ in diameter and internally coated with a voltage sensitive electron emissive substance.

When incoming photons strike the photocathode, each photon is converted into a photoelectron. These photoelectrons are amplified approximately 10^6 times in MCP and strike the output phosphor screen producing a bright spot which is then imaged by the low lag vidicon.

It is important that this image intensifier be capable of discriminating between real signals and noise components.

By establishing a threshold level based on this characteristic, it becomes easy to discriminate the noise element from the respective signal. There are several advantages which a single photon counting camera has and they are

- Wide dynamic range
- Quantitative measurement capability
- Low dark noise
- Little damage to specimen

Typical applications of VIM

The measurement of intracellular calcium levels in living cells has been of great interest since fluorescent dye (Fura 2) was introduced.

It is considered that calcium ions take an important role in biological phenomena in living cells such as muscle contraction and transmittance in neuron and hormone activities.

So it becomes essential to measure the concentration of calcium ions, both spatially and temporally. To measure calcium ion concentration, it is necessary to use a technique called ratio imaging. Usually, as a detector, a SIT camera and an image processor are required to perform the ratio imaging. There are advantages to ratio imaging, by taking a ratio between two images which are fluorescent images acquired with different wavelengths:

- No effect of the concentration in fluorescent dye

FIG. 3. Schematic diagram of single photon counting camera and signal processing.

FIG. 4. Chemiluminescent image of the respiratory burst in cultured neutrophils stimulated with opsonized zymosan in the presence of luminol. Upper left shows a transmitted light image of the cells. The remaining show the temporal sequence of chemiluminescent emission. Photo courtesy of Dr. M. Suematsu.

- No effect of the intensity in illumination
- No effect of the thickness in specimen

Ratio imaging is also a useful technique to study other ions in living cells, such as Mg^{++} , Na^+ , H^+ and so on.

Applications using the single photon counting camera are becoming widely spread because it may enable researchers to visualize things that have never been visualized before.

An attempt has been made to apply this camera to visualization of the calcium wave made by aequorin emission during fertilization in medaka egg (2).

Other applications are also reported, such as metabolic imaging (glucose, lactate and ATP in normal and tumor tissue (3)), visualization of gene expression by luciferase (4), and visualization of the respiratory burst (5) (see Fig. 4).

Conclusions

The technique of video microscopy is explained from the technical standpoint. We engineers believe that requests from researchers will improve instrument more and more. Researchers and engineers working together will produce better instruments for video microscopy.

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