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Simultaneous Nomarski and fluorescence imaging during video microscopy of cells

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FOSKETT, J. KEVIN. *Simultaneous Nomarski and fluorescence imaging during video microscopy of cells.* Am. J. Physiol. 255 (Cell Physiol. 24): C566–C571, 1988.—A video microscope designed to allow low light level fluorescence imaging of cells during simultaneous high-resolution differential interference contrast (DIC) imaging, without the fluorescence light losses of 60–90% normally associated with this contrast-enhancement technique, is described. Transmitted light for DIC imaging, filtered at >620 nm, passes through standard DIC optical components, (¼ λ-plate, polarizer, and Wollaston prism) before illuminating the cells. Transmitted light and fluorescence emission pass through a second Wollaston prism but not through the analyzer, which is repositioned more distally in the optical path. Prisms designed to reflect light out a side port of the microscope to a video camera have been replaced with a dichroic mirror. This mirror reflects fluorescence emission out the side port to a low light-sensitive video camera. The spectrally distinct transmitted light continues through the dichroic mirror to an overhead camera through a polarizer (analyzer), which completes the DIC optical path. The fluorescence and DIC images can be viewed simultaneously on side-by-side video monitors, examined sequentially by an image-processing computer, or examined simultaneously using a video splitter/inserter. The ability to image cells with high resolution simultaneously with low light level fluorescence imaging should find wide applicability whenever it is necessary or desirable to correlate fluorescence intensity or distribution with specific cell structure or function.

differential interference contrast; low light level; fura-2

VIDEO IMAGING of fluorescence has recently become a very powerful tool in cell biology. The coupling of the video microscope to video image-processing systems has provided in the light microscope an instrument capable of quantitative fluorescence measurements within single cells. However, incorporation and illumination of fluorescent probes in living cells, especially at the single cell level, may not be without adverse physiological effects. For example, excited fluorescent probes (intrinsic and extrinsic) may generate toxic oxygen free radical species (6) and buffer intracellular ion levels and may have diverse metabolic or toxic effects (7). Two general strategies are employed to minimize these effects. First, attempts are made to minimize the amount of dye loaded into the cell. Second, the total excitation light flux is reduced, by exposing the cell to intermittent excitation and/or by attenuating the excitation intensity. Because fluorescence intensity is proportional to the amount of probe as well as the level of excitation illumination, these strategies require enhanced light-detection systems as well as the elimination of optical elements, including phase rings and polarizers, which attenuate fluorescence excitation and emission intensities. For example, an ideal polarizer transmits 50% of the incoming light, although in practice it generally transmits only 30–40%. In a microscope equipped for Nomarski differential interference contrast (DIC), the fluorescence emission will pass through a Wollaston prism and a polarizer. If the excitation light also passes through the same elements, then the resultant fluorescence intensity will be <10% compared with that of the same microscope without these elements. Thus low light level fluorescence imaging is generally associated with elimination of optical elements that provide high contrast in transmitted-light imaging. The loss of image resolution and contrast resulting from the elimination of these optical elements is further compounded by another necessity of video imaging. Because the video detector is operated in a low light level mode for the fluorescence imaging, the transmitted light intensity used for bright-field imaging must be greatly attenuated. This results in an image with poor signal characteristics that is degraded by noise in the camera. Even with digital image-processing systems where it is possible to integrate a number of sequential images, the resultant image is still degraded compared with that obtained under high light level conditions and with the expense of lost temporal resolution. As a result of the combined effects of eliminating contrast-enhancing optical elements and imaging under photon-poor conditions, low light level fluorescence is associated with poor transmitted light imaging, making correlation of fluorescence with simultaneous cell structure and function difficult.

I have developed a video microscope system to make quantitative fluorescence measurements in single living cells while they are simultaneously imaged with high contrast and resolution in Nomarski DIC optics, without the light losses normally associated with this contrast-

enhancement technique. This development allows good correlation between fluorescence and cell structure in real time.

METHODS

Microscope. The ability to image cells in DIC and fluorescence simultaneously without fluorescence light loss due to DIC optical components requires two video detectors, the means to separate spectrally the two images to the respective cameras, and a judicious placement of the polarizers in the optical path. The microscope (a Zeiss IM-35 inverted microscope equipped for epifluorescence) shown in Fig. 1 incorporates these elements. A standard 60-W halogen lamp is employed for transmitted light imaging, and a 75-W xenon arc is generally employed for fluorescence excitation. Most of my work employs a Zeiss Neofluor ×63, numerical aperture (NA) = 1.25 lens, since it combines high light-gathering capability with a fairly long (0.5 mm) working distance, which is helpful for examining thick specimens and in electrophysiology experiments. With an open-bath chamber a Zeiss water immersion ×40, NA = 0.75 lens is used as the condenser lens; for long-working distance applications, a Leitz ×32, NA = 0.40 lens is used instead. The transmitted light optical path incorporates the elements required for DIC imaging. The light from the halogen lamp is polarized by a calcite prism (Zeta International, Prospect, IL) and passes through a Wollaston prism before illuminating the preparation. Fluorescence excitation from the xenon arc is directed to the preparation by a standard Zeiss dichroic mirror encased in a Zeiss filter cube. Fluorescence emission as well as transmitted light that passes through the specimen pass through the objective lens and a second Wollaston prism associated with it. In the standard Zeiss configuration, the light would next pass through a second polarizer (analyzer). However, because this polarizer would be in the fluorescence emission light path, it was repositioned more distally in the optical path, as described later. In the standard microscope configuration, the light proceeds through the microscope to a set of prisms designed to either allow the light to continue to the oculars or to direct it at right angles through a side port to a video camera. With the use of a Zeiss Photochanger (or other trinocular), it is possible to mount, in addition to the one at the side port, a second camera above the oculars. The prisms can direct the light to either or both cameras. However, they cannot direct fluorescence emission to one camera and transmitted light to the other. To this end, I have replaced the prisms with a simple dichroic mirror mounted at 45° to the incident light path. The dichroic mirror can separate light based on spectral properties; thus it becomes possible to image the transmitted light and fluorescence separately if they are spectrally distinct. Because most biologically relevant probes have fluorescence emission <550 nm, the halogen light is initially filtered to allow only >620 nm light for transmitted light imaging. The dichroic mirror that reflects the fluorescence excitation to the preparation passes this red light without interference. The dichroic mirror that replaces the prisms to direct the light to the appropriate cameras is chosen to be able to pass >620 nm to the overhead camera for transmitted light imaging and reflect the fluorescence emission out the side port through an interference filter for fluorescence imaging. The analyzer (calcite prism) is positioned between this dichroic mirror and the transmitted light camera, completing the DIC light path. Thus the overhead camera views the preparation in high light, contrast, and resolution DIC optics, whereas the side-port camera views the low light level fluorescence. The only DIC optical element in the fluorescence light path is a Wollaston prism. My measurements indicate that each pass through this prism attenuates the light by 1–2% at wavelengths >~400 nm. At 340 nm, attenuation is ~5%. Thus the combination of DIC with fluorescence imaging results in a fluorescence diminution of ~2–4% for most wavelengths.

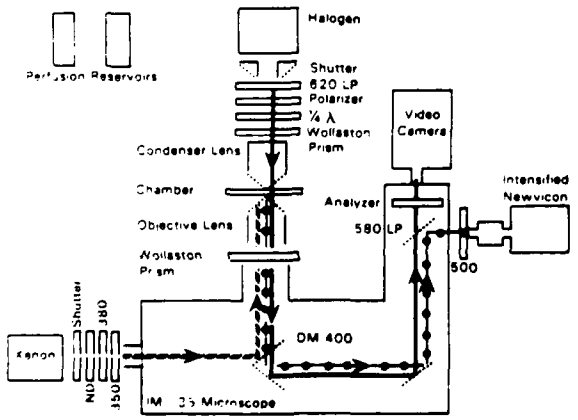

FIG. 1. Differential interference contrast fluorescence microscope. LP, long pass filter; DM, dichroic mirror; 350, 380, 500, band-pass interference filters; ND, neutral density filter. ■■■, Fluorescence excitation; ●—●—●, fluorescence emission; ———, transmitted light. See text for details.

Video image-processing system. The overhead camera that views the transmitted light image is coupled to the microscope by a Zeiss Photochanger (to direct light to the oculars or to the camera), a ×10 ocular, and a Hitachi zoom lens to adjust the magnification to that of the fluorescence camera. The ocular/zoom lens unit is adjusted to make the overhead, transmitted light camera parfocal with the side-port camera. The overhead camera currently used for transmitted light imaging is a silicon intensified target model (model 65 Dage-MTI, Michigan City, IN) simply because it was available. However, it is preferable to use a high-resolution, low-noise camera such as a Newvicon with gain and black-level controls.

The side-port camera that views the fluorescence emission is coupled to the microscope using the standard Zeiss coupling lenses incorporated into a tube that holds the 45° dichroic mirror in proper position. Although the local Zeiss representatives (Baltimore Instrument, Baltimore, MD) constructed this holder, a standard machine shop could easily make it. The camera should be sensitive to low light levels. I employ a two-stage, inverted-type image intensifier (model XX1380-FL, Amperex Electronic, Slatersville, RI) coupled by rely lenses (50-mm, f1.2, Nikon, Garden City, NY) to a Newvicon camera (model 65, Dage MTI) as described by Spring and Smith

(8). All automatic features (gain, black level) of this camera are disabled, and a provision for driving the sync signal from an external source is incorporated into it to allow images from the overhead (transmitted light) camera to be inserted into the video field of the intensified camera by the use of a video mixer (described below).

The video signal from the transmitted light camera is encoded for time and date (For-A, VTG-33, Los Angeles, CA), analog enhanced (For-A Contour Synthesizer, IV-530), and directed to a video tape recorder as well as a video switching device (Videotek, PVS-6, Pottstown, PA) (Fig. 2). The switching device feeds the signal to a video disk recorder (Precision Echo, EFS-1B, Santa Clara, CA) for recording and/or displaying on a video monitor. This image can be time-base corrected (Edutron, ccd 2h-1) and directed to the input of video image-processing boards (IP-512 series, Imaging Technology, Woburn, MA) housed in a Heurikon HK-68 computer (Madison, WI). The video signal from the fluorescence camera proceeds to a monitor and then to the image processor. Under software control, the image processor can select either camera as input and digitize images to 512 × 480 or 256 × 240 pixel resolution. The digitized images can be output to the video disk recorder. In a modified arrangement, the transmitted light image can be time/date coded and analog enhanced (as above) and inserted into the video signal of the fluorescence camera using a video splitter/inserter (RCA, TC1470A, Lancaster, PA). The advantage of this arrangement is that because the image processor can digitize signals from only one camera at a time, a signal containing both images results in no temporal dispersion between the time the image processor digitizes the images from the two cameras.

The fluorescence and DIC images can be viewed simultaneously in real time on side-by-side monitors or on one monitor if the splitter/inserter is used. However, the fluorescence image is inverted as a result of being reflected at right angles out of the microscope to the camera. Although this sometimes makes real time correlation of fluorescence with the DIC image somewhat difficult, it is advantageous for viewing both images on one monitor using the splitter/inserter, as shown in RESULTS. However, there are two ways to flip the image over in real time to facilitate viewing and comparing with the DIC image on side-by-side monitors. The first method is an optical approach. Placement of a dove prism in the optical path in front of the fluorescence camera will invert the image. I have not employed this prism in my microscope because of some difficulty in properly aligning it. The second method is an electronic approach. As an option on some video cameras, the raster scan can be initiated from the bottom of the field instead of the top.

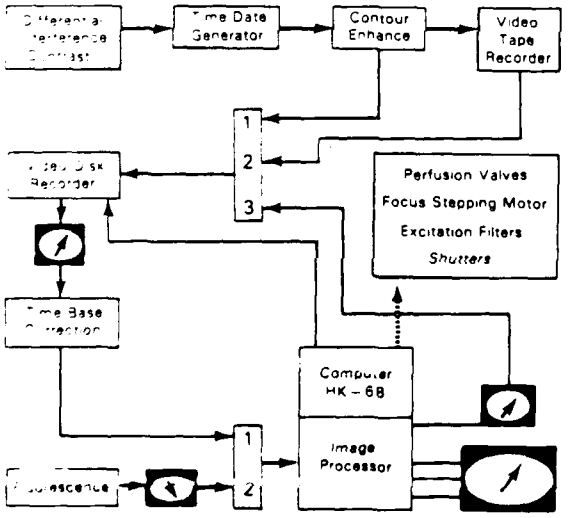

FIG. 2. Image-processing system. Video signals are from cameras viewing Nomarski image (*top*) and fluorescence image (*bottom*). Boxes with arrows, television monitors; larger box with arrow, a color monitor; arrows within boxes, indicate that fluorescence image is inverted from top to bottom compared with corresponding Nomarski image, until image processor creates an upright image to facilitate comparison between images. Boxes containing numbers, video switching devices. See text for further details.

Images digitized by this image-processing computer can be processed and/or stored in four video frame buffers (in real time), on video disk recorder (200 images in half real time), or laser disk recorder (24×10^3 images in real time), in 4-Mbyte random access memory (requiring a couple of seconds; it can be as fast as real time in some other image-processing systems), on 45-Mbyte Winchester disk, or on 65-MByte steaming tape (both requiring several seconds). The computer controls a stepping motor for microscope focus as well as shutters between the halogen and xenon lamps and the microscope and pinch valves to permit rapid changes of perfusion media. For examination of cells labeled with more than one dye, or for examination of dyes requiring more than one excitation wavelength, a custom-designed bank of solenoid-activated interference filter (1 in.; Ditric Optics, Hudson, MA) holders is positioned between the xenon arc and the microscope on a mechanically independent platform to prevent vibration transmission to the microscope. These filters can be called into and out of the light path under computer control. Switching between two wavelengths requires ~40 ms.

RESULTS

The advantages of DIC imaging have been reviewed (4). DIC images have a three-dimensional look, provide a shallow depth of field (out of focus information does not interfere), and provide high resolution and contrast. However, for low light level fluorescence imaging in a microscope with a single camera, DIC optical components are removed from the microscope, and the preparation is imaged in bright-field optics under low light level conditions. Figure 3 demonstrates the advantages of the two-camera system for imaging cells under low light level conditions. Figure 3*A* is a digitized fluorescence image of a field of living human monocytes recently plated onto a glass cover slip. The fluorescence is cell autofluorescence demonstrating the low light conditions in which the system is able to work and emphasizing the extreme sensitivity of the intensified Newvicon. Figure 3*B* shows the same field of cells imaged by the same camera at the same gain setting in low light level bright-field optics. In Figure 3*C*, the same field of cells is imaged in DIC optics with the second camera while simultane-

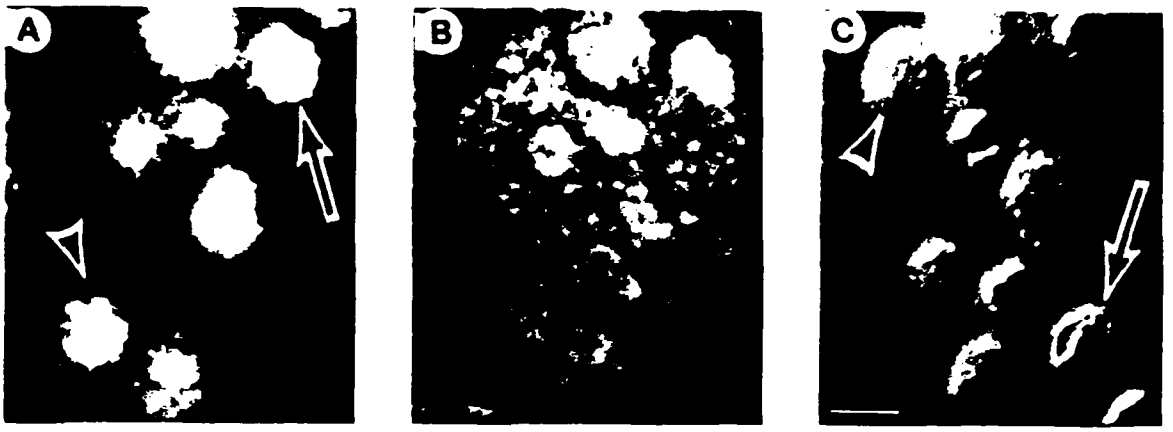

FIG. 3. Advantages of 2-camera system for imaging cells under low light level conditions. Human monocytes in fluorescence (*A*), low-light light level brightfield optics (*B*), and high light level differential interference contrast (DIC) optics (*C*). Fluorescence is cell autofluorescence (excitation 380 nm, emission 500 ± 20 nm). DIC image is inverted, from top to bottom, compared with other images, since it was obtained from a camera mounted at 90° to fluorescence bright-field camera. Fluorescence image was obtained on 1 camera while simultaneously imaging in DIC optics on another. Arrows and arrowheads identify same cells, for orientation. See text for details. Bar, 15 μm.

ously imaging the fluorescence with the first camera. Figure 3*C* demonstrates the remarkable gain in transmitted light resolution and contrast obtained in this system compared with the single camera system under low light level conditions.

In the simplest configuration, the video-microscope system consists of two cameras with outputs that are directed to two monitors. In Fig. 4*A*, side-by-side monitors display live images of human monocytes grown in culture on glass cover slips. The field on the right is imaged in DIC optics, whereas on the left the same field of cells is imaged simultaneously in fluorescence. The cells have been loaded with the Ca^{2+}-sensitive dye fura-2 and are being excited at 380 nm and observed at 500 ± 20 nm. The cells are imaged with high resolution and contrast in DIC while the fluorescence is likewise imaged in good detail. Figure 4*A* demonstrates the parfocality of the two images as well as the inverted (from *top* to *bottom*) nature of the fluorescence image compared with the DIC image as a result of being deflected at right angles out through the side port of the microscope.

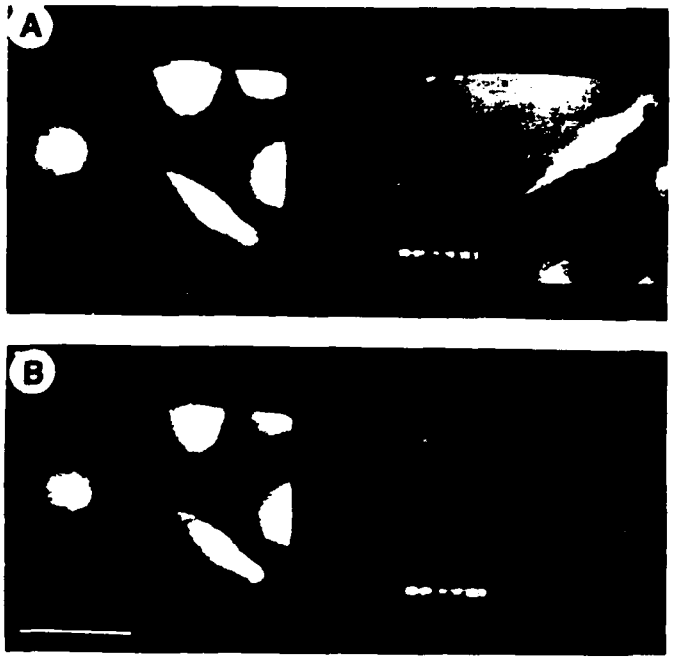

FIG. 4. Simultaneous differential interference contrast (DIC) and fluorescence imaging. Human monocytes, grown in culture, perfused on stage of microscope and loaded with Ca^{2+}-sensitive dye, fura-2. *Left monitor*: fluorescence emission. *Right monitor*: simultaneous DIC image. *Bottom*: transmitted light shuttered off. See text for details. Bar, 50 μm.

It is possible to make quantitative measurements of fluorescence despite the high light throughout the DIC optical path. This is evident in Fig. 4*B*, which demonstrates that the fluorescence intensity is unaffected by the presence of the transmitted light, indicating that there is no "spectral contamination" of the two light paths. However, this may not always be the case, depending on conditions including the degree of spectral separation of the two light paths, the quality of the dichroic filter than separates the light to the two cameras, the gain and sensitivity of the fluorescence camera, and the intensities of the fluorescence and transmitted lights.

Although the cameras permit simultaneous imaging of DIC and fluorescence, the image-processing computer that I use can digitize input from only one camera at a time. However, under software control the computer can rapidly switch between the two. Figure 5 represents digitized images of living human monocytes loaded with fura-2 and excited at 380 nm, as previously described. Figure 5*A* represents a digitized (512 × 480, 128-frame integrated average) DIC image of these cells. Figure 5*B* is a digitized 128-frame average fluorescence image of the same cells obtained beginning 33 ms after the DIC image was grabbed. The image processor has constructed the inverted image to make comparison with the DIC image easier. The acceptable amount of time separating the two images will depend on the physiology of interest, the signal-to-noise of the images, and the degree of cell

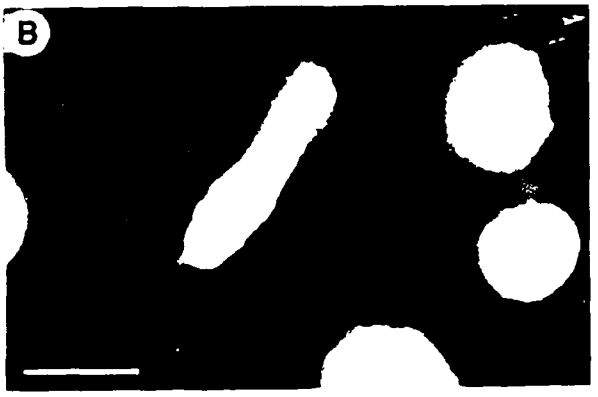

FIG. 5. Sequential differential interference contrast (DIC) (*A*) and fluorescence (*B*) imaging. Human monocytes perfused on stage of microscope and loaded with fura-2. Images are digitized 512 × 480, 32 frame integrated averages. See text for details. Bar, 25 μm.

movement. It may also depend on the amount of immediately available image-storage buffers. For example, to integrate two images, each to 16-bit resolution, requires three 8-bit frame buffers. If only two are available, the first image must be transferred out of one of the frame buffers into computer memory or an image-recording device such as a video disk or tape recorder. The amount of time required for such a transfer will also determine the minimum amount of time to separate acquisitions from each camera.

One solution to these problems is to mix the video signals from the two cameras with the use of a video splitter inserter. A video splitter inserter allows display of user-selected portions of video from two cameras on the video signal such that both can be simultaneously viewed, recorded, or digitized. In Fig. 6, fura-2-loaded monocytes are observed in DIC and fluorescence optics simultaneously digitized as a single video input from the video splitter inserter.

DISCUSSION

The application of video techniques to fluorescence microscopy has led to an increased use of fluorescent dyes to probe living cells. Photographic techniques for examining fluorescence distribution in living cells is severely limited by the sensitivity of emulsion to low light levels. The resultant long exposures result in poor temporal resolution, probe bleaching, possible photodynamic damage to the cell, and cell movement artifacts. The

FIG. 6. Video splitter inserted combined differential interference contrast (DIC) and fluorescence images. Cultured human monocytes loaded with fura-2. *Top*: DIC image. *Bottom*: simultaneous fluorescence image. See text for details. Bar, 25 μm.

speed and sensitivity of video overcomes these problems. Rapid changes in fluorescence intensity and distribution can be followed and recorded in real time. The use of *ultra low light level-sensitive* video detectors has made it possible to image fluorescence in living cells for relatively *long* periods of time with minimal photodynamic injury and dye bleaching. Furthermore, these cameras allow the cells to be loaded with less dye, thereby minimizing toxic or perturbing effects of the fluorescence probe. Thus video fluorescence microscopy affords the possibility to probe diverse intracellular environments simultaneously over time and under different physiological conditions. Because these capabilities largely accrue from the ability to work at very low light levels, the optical paths within the microscope should transmit excitation and emission wavelengths with as little light loss as possible. Therefore, a high numeral aperture objective is used, the number of reflecting surfaces within the microscope is minimized, the excitation lamp is focused and centered, and so forth. A generally employed technique has been to remove optical elements used to enhance contrast in transmitted light imaging. However, full advantage of the high spatial and temporal resolution provided by low light level video imaging of fluorescence is realized only when the cell structure can also be imaged with high contrast and resolution and with equivalent temporal resolution. In that way, a high correlation between cell structure and fluorescence can be achieved.

DIC imaging permits resolution of structural detail that surpasses other light microscopic methods. The use of video for DIC imaging has greatly extended the useful resolution and contrast provided by the light microscope (1, 4). Thus the combination of low light level fluorescence with DIC imaging provides the best means to

correlate fluorescence distribution with cell structure in living cells. However, at least one polarizer, and in some microscopes two polarizers, are present in the fluorescence light path, diminishing fluorescence intensity by 70–90%. The microscope described in the present paper allows simultaneous application of DIC imaging and low light level fluorescence without these light losses. This has been achieved by removing the DIC analyzer from the fluorescence optical path and by a modification of a standard Zeiss microscope to separate the transmitted light and fluorescence emission spectrally to two separate video cameras. This arrangement allows correlation of cell structure with fluorescence in real time. Because the two imaging modalities are not viewed with the same camera, different magnifications and geometrical distortions in the two cameras and optical paths do not permit a precise overlay of the fluorescence and DIC images (although sophisticated image-processing techniques could in fact precisely superimpose the two images). However, for most applications this will not be a serious limitation, since it will usually be possible to correlate fluorescence with the fine structure of the cell even when it is not possible to strictly superimpose the two images (see Figs. 5 and 6). For example, subtle retractions of the periphery of venous endothelial cells on stimulation with histamine have been observed during simultaneous spatial imaging of Ca^{2+} inside the same cells using the fluorescent dye fura-2 (3). In many instances, a correlation between fluorescence and cell fine structure is not important, although correlation with more macroscopic features of the cells may be important. For example, changes in cell volume and vacuole formation inside single cells and single acini isolated from rat parotid glands have been observed during simultaneous imaging of intracellular Ca^{2+} with fura-2 (2). At a somewhat more macroscopic level, this technique provides the ability to accurately identify different cell types in a morphologically heterogeneous population for correlation with single cell fluorescence. It should be pointed out that the fluorescence-imaging camera could be replaced with a photomultiplier tube. With an appropriate mask in an intermediate image plane, nonspatially resolved fluorescence can be correlated with cell structure and function. Finally, it seems worthwhile to consider the utility of this approach within the context of the recently described confocal microscope. In principle (see Fig. 1 of Ref. 9), the laser-scanning confocal microscope, appropriately modified, is an ideal method for imaging cells in Nomarski optics while simultaneously imaging them in fluorescence. The tremendously improved spatial resolution of fluorescence in the depth (z) and, to a lesser extent x–y-dimensions afforded by the confocal microscope allows fluorescence and Nomarksi optical sections to be perfectly resolved and registered. However, to this advancement must be weighed certain features of the present commercially available confocal microscope, of which the author is aware, that are less than ideal. First, the speed at which images are generated in the confocal microscope may be limiting in certain circumstances. Second, because the transmitted light wavelength is that used for epifluorescence illumination, continuous transmitted light imaging of the preparation will cause dye bleaching and possible direct and indirect phototoxic effects. Also, polarization of the fluorescence excitation beam may result in photoselection of fluorophores. Appropriate modifications to be used for the Nomarski imaging, such as including a second detector and allowing a second light beam filtered to longer wavelengths and polarized, will solve these problems. In either type of microscopy, the ability to image living cells in high resolution simultaneously with low light level fluorescence imaging should find wide applicability whenever it is necessary or desirable to correlate fluorescence intensity or distribution with specific cell structure and function.

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The views presented in this paper are those of the authors. No endorsement by the Defense Nuclear Agency has been given or should be inferred.

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