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NEW TECHNIQUES IN MICROSCOPY AND IMAGE ANALYSIS

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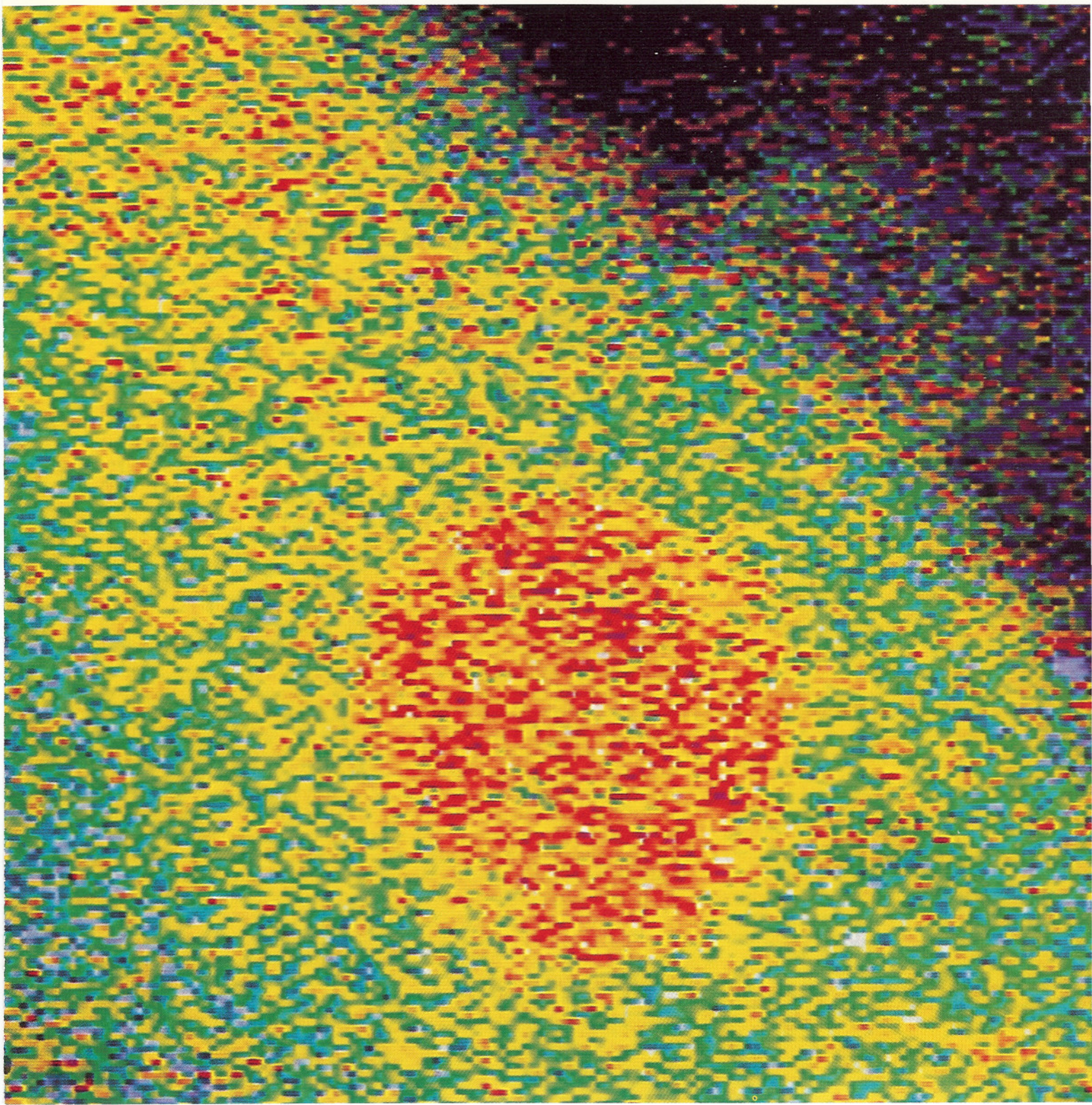

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By Stanley Schwartz

Real-time laser-scanning confocal ratio imaging

Molecular biology and biochemistry have long had as their goal an understanding of complex processes at the cellular level. The mechanisms of cell locomotion, cell division, pathogenesis, nutrition, energy cycles, intracellular transport, and other processes involve complex morphological and chemical changes;[1] the understanding of these changes could contribute immeasurably to the understanding of the cell and of life itself.

Among the most significant techniques for observing dynamic life processes is fluorescence ratio imaging. This technique has been researched and reported on in hundreds of published papers.[2] Technology in confocal microscopy now enables the use of fluorescence ratio imaging to observe and record life processes in real time.

A thorough discussion of fluorescence ratioing history and methodology is outside the scope of this paper; however, it is important to discuss the history of this technology and its advantages and limitations in order to understand the significance of the new developments.

Development of fluorescence ratio imaging methodology

In the middle of this century, microscopy, which had been used for pathology and morphology studies for more than a century, was first linked to molecular chemistry to study life processes in living cells. Since the 1960's, scientists have been studying the relationships of cytoplasmic free ions,[3] and today, scientists routinely use fluorescence techniques to observe concentrations and movement of hydrogen ions (pH); cell electrolytes such as calcium, magnesium, sodium, and potassium; intracellular messengers such as cAMP; hormone levels and neurotransmitters; as well as studying cellular events such as fertilization and mitosis.

The genesis of the methodology has been closely linked to the development of fluorescence probes that are indicators for specific aspects of cellular kinetics. These fluorochromes, especially FURA-2 and INDO-1, synthesized by Tsien, Poenie et al. in the 1980's,[4] have taken the place of the original dyes used to study in vitro processes. They are useful because of their sensitivity and specificity. They also offer high signal-to-noise ratios in comparison to absorption spectroscopy, since the signal is not buried in a high background and is generated only in regions containing the probe.[1]

The fluorophores can be used to collect either photometric or spatial information about the cells into which they are introduced, and by using different probes, many different parameters can be quantified—even, theoretically, in the same cell. Two wavelengths can be used for emission recording,[5] and a ratio can be created from the signal from any two wavelength pairs. For instance, in calcium (Ca^{2+}) studies, each ratio is uniquely determined by the relationship between the free and bound calcium ions in the cell. This ratio method normalizes and eliminates most of the variation caused by fluctuations and changes in the dye content of the cells due to bleaching, illumination intensity, leakage, cell thickness, dye concentration, and other anomalies.[6] Dynamic biochemical interactive processes within living cells can now be routinely visualized and measured.

Temporal and spatial techniques and limitations

In photometry, light is measured with a microspectrophotometer, providing data about changing fluorescence intensity levels over time as a cellular event occurs. Typically, photometry studies result in temporal data on a large dynamic range of relative intensities, but provide no spatial or visual detail on exactly where in the cell the change is taking place. Temporal data can, however, be acquired very quickly, for real-time study of events as they occur.

Information gathered via the microspectrophotometer could include average fluctuations over many cells within a population under a low-magnification objective field or intracellular changes of a single cell under high magnification, but not both simultaneously, since measurements are limited to one region at a time.

The alternative, acquiring spatial information, typically requires extensive image acquisition hardware and software, along with computerized image enhancement systems. Images of a cell as a kinetic process takes place are provided on video or film. Although there is no substitute for watching an event as it occurs, these traditional, nonconfocal systems have

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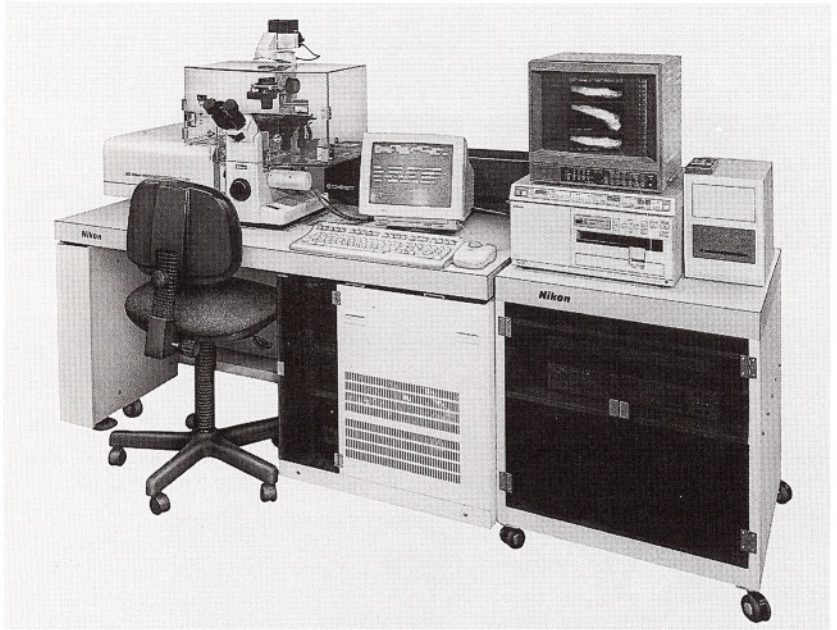

Figure 1 *The RCM-8000 version 2.5 real-time confocal microscope features laser scanning, UV optics, and dual emission ratio-imaging capabilities.*

had a number of limitations. They offer images that distinguish between events over the x and y dimensions of the cell, but reveal no discernment of depth, thus leaving confusion about the exact whereabouts of an event in the z-axis. Spatial imaging techniques permit measurements of single cells as well as cell populations and cell-to-cell interactions. Responses of multiple subcellular compartments as well as cell population distributions can be imaged simultaneously within a microscope field.

Also, since most ratiometric fluorochromes are excitable in the UV range, acquiring accurate and usable images in the emitted range of visible light can be problematic and can present an obvious design challenge. Finally, because of very low-light-level detection required and mechanical/hardware considerations, as well as the computational time required to assemble a visual image, the process can take up to 1–2 sec/frame. This is many times slower than photometric systems[7] and is not nearly fast enough to view certain rapid dynamic processes in action.

There are other constraints to fluorescence ratio imaging that affect both spatial and temporal data collection and interpretation. FURA-2, a commonly used fluorescence probe, requires complex light chopping (alternating excitation) techniques that actually prevent truly simultaneous, time artifact-free imaging to occur while ratioing excitation wavelengths. Furthermore, in order to acquire ratio pairs faster than 1 pair/sec with FURA-2, the researcher must give up some aspect of data collection.

INDO-1, another commonly used ratiometric fluorophore, can be used for true simultaneous emission wavelength studies, but even this probe, used with two low-light-level detecting video cameras, has its limitations. Unless the cell is very thin (approximately 5 μm in depth), the researcher cannot know for sure that light emitted from the specimen contains no reflections, fluorescence, or other confusing emissions from other parts of the cell. In short, all commonly used techniques for imaging have the inherent possibility of including aberrations or artifacts in the data, and preclude the ability to image specimens of any considerable depth.

Real-time ratio fluorescence laser-scanning confocal microscopy

The optimal system for fluorescence ratio imaging is one that provides both high signal-to-noise spatial and temporal information, eliminates all aberrations and artifacts, could be highly localized within thick specimens at any depth, and offers high repeatability.

The RCM-8000 laser scanning confocal system (Nikon Inc., Melville, NY) (*Figure 1*) offers researchers doing ratio fluorescence studies all the advantages of true pinhole confocal detection. It eliminates out-of-focus fluorescence from any area of the specimen that is not specifically focused on by the researcher and provides highly quantitative intensity and spatial location data. It also offers the possibility of acquiring images at two emission wavelengths that are from a single, precisely registered location and are truly simultaneous (acquired with no time artifacts), and instantaneously displays a spatial map of the ratios unlike the images usually available in conventional ratio imaging.

The confocal system scans full-field images (typically 175 μm with a 40× objective) at normal NTSC video rates of 30 frames/sec, with excitation at 351 nm from an argon ion laser for probes such as INDO-1. The two emission bands, at 405 nm and 485 nm from calcium-bound and calcium-free ions, respectively, are separated by a dichroic mirror, detected by parallel photomultipliers, ratioed in real time, displayed in pseudocolor, and recorded by appropriate software under interactive control.[8]

This methodology has significant quantitative advantages. Each discrete point (pixel) is only briefly illuminated, and its emission intensity is simultaneously detected via fast-reacting high-dynamic-range photomultipliers in approximately 70 nsec/pixel; thus, problems associated with UV excitation, cellular damage, photo bleaching, and slow detector readout times are greatly reduced or eliminated. In addition, tixel analysis (4-D analysis of a pixel over time) is easily obtainable and highly quantitative.

In contrast, intensified CCD video methodology would require a full microscope imaging field to be excited at nominal energy levels while integration and CCD pixel

readout occurs. Every pixel is thus subjected to at least 30 msec of UV excitation and is subject to probe photo bleaching dynamics. Using this nonconfocal methodology, thick specimens would be repeatedly exposed to prolonged excitation through many optical sections, and poor contrast due to very low signal-to-background fluorescence would occur, yielding limited quantitative data on a relatively gross time scale.

The system is suitable for use in the area of optophysiological imaging of thick specimens such as embryos or tissue slices, in which out-of-focus fluorescence has created blurring problems in the past; in motile cells, which in commonly used FURA-2 systems gave artifacts due to the sequential (not truly simultaneous) measurement of the two wavelengths; and in neural networks, in which calcium is an easily detected and intrinsically important signal of neuronal activation.[9]

In general, the design of the microscope is traditional, the four exceptions being increased scanning speed, chromatically correct UV optical path for confocal excitation and emission detection, specially designed diffraction limited objectives for UV confocal use, and real-time digital image ratio capabilities and software.

Fast, full-frame scanning

The system utilizes a resonant galvanometer operating at a resonating frequency of 7.875 kHz for the horizontal line scanning. A 60-Hz galvanometer is used for vertical positioning of the laser beam. The bidirectional scanning and descanning (patent applied for) through the resonant galvanometer is synchronized and linearized to provide high-resolution, full-frame imaging at the standard RS-170 video output rate of 30 frames/sec. Image resolution of 512 × 483 × 8 bits is attained in 33 msec. Line scans as fast as 64 μm per horizontal line are attainable. This is 30 times faster than comparable confocal systems acquiring images at the same resolution.

The traditional confocal laser scanning microscope (CLSM) will scan a single field in raster fashion in approximately 1 sec while exciting and reading one line at a time, starting at the left and progressing to the right. During the vertical blanking period, when the horizontal x mirror returns to its starting position, the photomultiplier is not read, but the laser continues to excite the specimen during the return period and before the vertical y mirror repositions to the next line, causing potential photo damage or bleaching.

In comparison, the RCM-8000's sinusoidal scan pattern excites and reads in both directions with minimal photomultiplier reading dead time. Conversion of the sinusoidal scan to the traditional raster needed for subsequent processing and display requires triggering of the signal digitization at nonuniform time intervals. This is accomplished by optical feedback from a reference timing beam and locked to synchronize with the 7.875-kHz resonant galvonometer at standard video rates. Therefore, every other scan line is laterally reversed by a last-in-first-out digital buffer. As a result, the system offers simple mirror scanning, compactness in design, optical simplicity, absolute achromaticity, no dispersive efficiency loss, high transmission efficiency, and a lack of pyramidal scan deflection errors.

Fast scanning speed allows real-time image capture of dynamic cellular events and almost completely reduces any time artifacts introduced by specimen movement. Dynamic confocal studies involving chemical, molecular, or other kinetic events can be imaged.

UV correction for confocal imaging

While other instruments have adapted objectives and the light detection systems in their confocal systems to adapt to UV studies, this instrument was designed for difficult work in the UV range and thus operates confocally and images properly in the UV.

The CF optical system provides for full correction of chromatic and spherical aberrations. Both lateral chromatic aberration, also known as chromatic difference of magnification, and longitudinal (axial) chromatic aberrations are virtually eliminated. Optical elements, scan angles, and detector positioning were designed for work in the UV range, and also allow good performance in the visible range with no compromises. Both UV and visible confocal images are achieved with variable-sized pinholes on the detector side.

In addition, a specially developed, high-power argon ion laser using a high-quality Gaussian beam providing 150 mW of power offers highly accurate optical alignment, providing laser lines at 351/364 nm for UV excitation and 488/514 nm for the visible blue excitation. An additional helium neon laser provides green (543.5 nm) excitation for multiple probe imaging.

High-NA, long-working distance objectives

Research using confocal techniques requires optics that have greater working distances and high numerical apertures (NAs), so that thick specimens can be imaged without having the objective touch the cover glass. A second consideration is that oil immersion microscope objectives suffer from increased spherical aberrations as they focus into thick tissue within an aqueous medium, resulting in significant image contrast loss.[10] As a result, objectives that utilize the immersion medium of water, which has a refractive index close to that of the aqueous specimen, and are outfitted with a correction collar to compensate for the thickness of the cover glass have long been needed by researchers. With them, full correction of spherical aberration can be maintained while sequential deep confocal optical sections are obtained.

High-NA water immersion objectives with effective free working distances of approximately 200 μm (Nikon) produce good contrast and diffraction-limited resolution. The CF Fluor 40× water immersion objective (Nikon) (NA of 1.2; working distance of 220 μm) provides for the highest possible light throughput

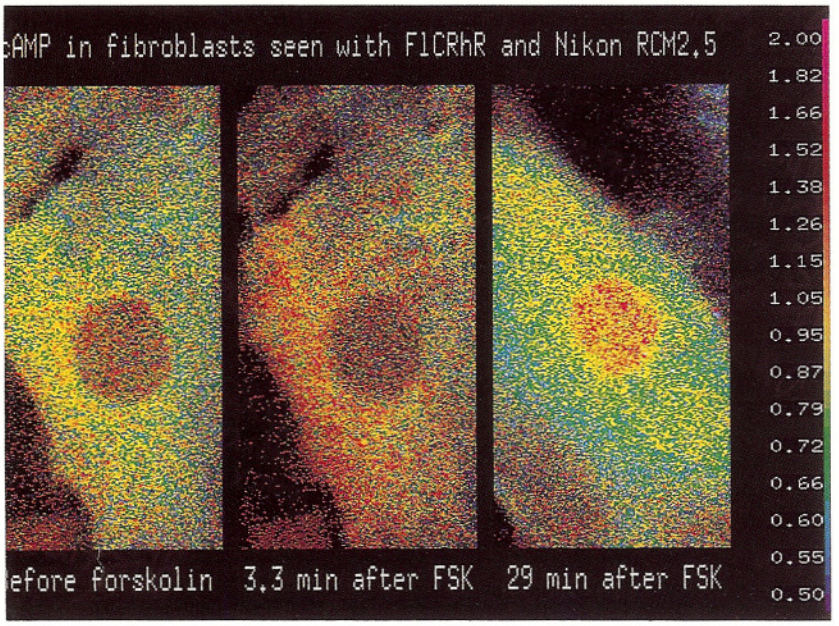

Figure 2 *Three images of real-time cAMP in fibroblasts, shown over time. Confocal image taken with the RCM-8000 laser scanning microscope system. Image taken by Roger Y. Tsien and Alec Harootunian of Howard Hughes Medical Institute, University of California, San Diego, CA, U.S.A.*

at down to 340-nm wavelengths.

A companion 20× Fluor water immersion objective (Nikon) will be available. In addition, a CF 60× water immersion Plan Apo objective (Nikon), with an NA of 1.2 and a similar long working distance, will be available in late 1993 for highly corrected images in the visible wavelengths.

The entire confocal arrangement is centered around the inverted microscope series, Diaphot 300/200 (Nikon). The system possesses all stability, ergonomic, and quantitative capabilities required of an inverted physiological confocal microscope workstation and includes a vibration isolation air table. The optical configuration allows for laser confocal imaging simultaneously with infrared transmission of brightfield, phase contrast, and differential interference contrast.

Accurate real-time ratios

The digital technology incorporated in the instrument is designed specifically to facilitate real-time ratio confocal imaging.

Two highly sensitive photomultipliers simultaneously detect both emission wavelengths produced through the use of ratioing probes such as INDO-1 for calcium, SNARF for pH (Molecular Probes, Eugene, OR), or FICRhR for cAMP (Dr. S. Adams, Univ. of California, San Diego, CA). The two analogue signals are then digitized, ratioed, and displayed in color as high-resolution spatial concentration images in real time.

Advanced high-speed digitizing and image-processing technology from Datacube (Danvers, MA) allows for the most powerful real-time analysis. Both channels of data are collected at video rate and stored separately for access at any time. Ratioed images can be viewed in real time or rerun with a few keystrokes; thus an entire sequence of full-frame ratio images can be reviewed, calibrated, analyzed, and manipulated in real time.

Images are stored via optically detected magnetic resonance (OMDR) and high-capacity magnetic hard disk, which are both included with the system for image archiving and documentation.

The ratioing software is optimized for confocal applications in obtaining optophysiological measurements in the real-time multitasking environment and documenting them. It provides for complete, easy control of specimen and user documentation, image processing, image storage and retrieval, ratioing, microscope Z-axis controls, shuttering, timing, control of peripheral devices, photomultiplier sensitivity, and all other parameters of the experiment in real time.

Applications

In combination with the principle of resonant energy transfer, probes are being developed to quantify the action of other important intracellular messengers. FICRhR is one such sensor that has an affinity for cAMP. This is accomplished by attaching a fluorescent label on the AMP-dependent protein kinase in such a way that cAMP not only triggers the normal activity of this enzyme, but produces an immediate optical signal that can be imaged microscopically. This labeled protein enables visualization of cAMP levels and shows that different regions of a single cell can have differing responses to neurotransmitters and drug stimulation. The image shows that a subunit of the enzyme can move in and out of the cell nucleus as the cAMP level rises and falls (see *Figure 2*).

Research in the areas of protein phosphorylation or gene transcription may be enhanced by these optical methods and allow imaging of the macromolecular biochemical signals.

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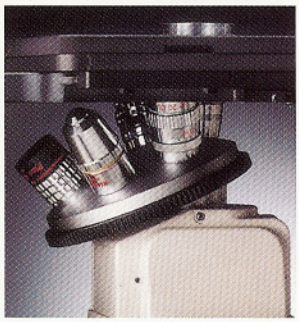

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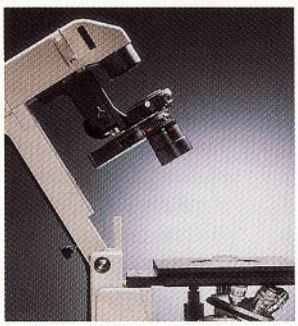

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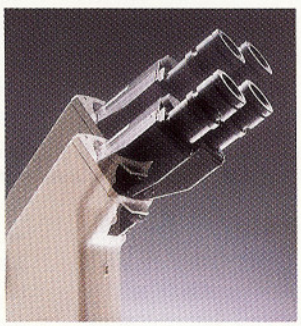

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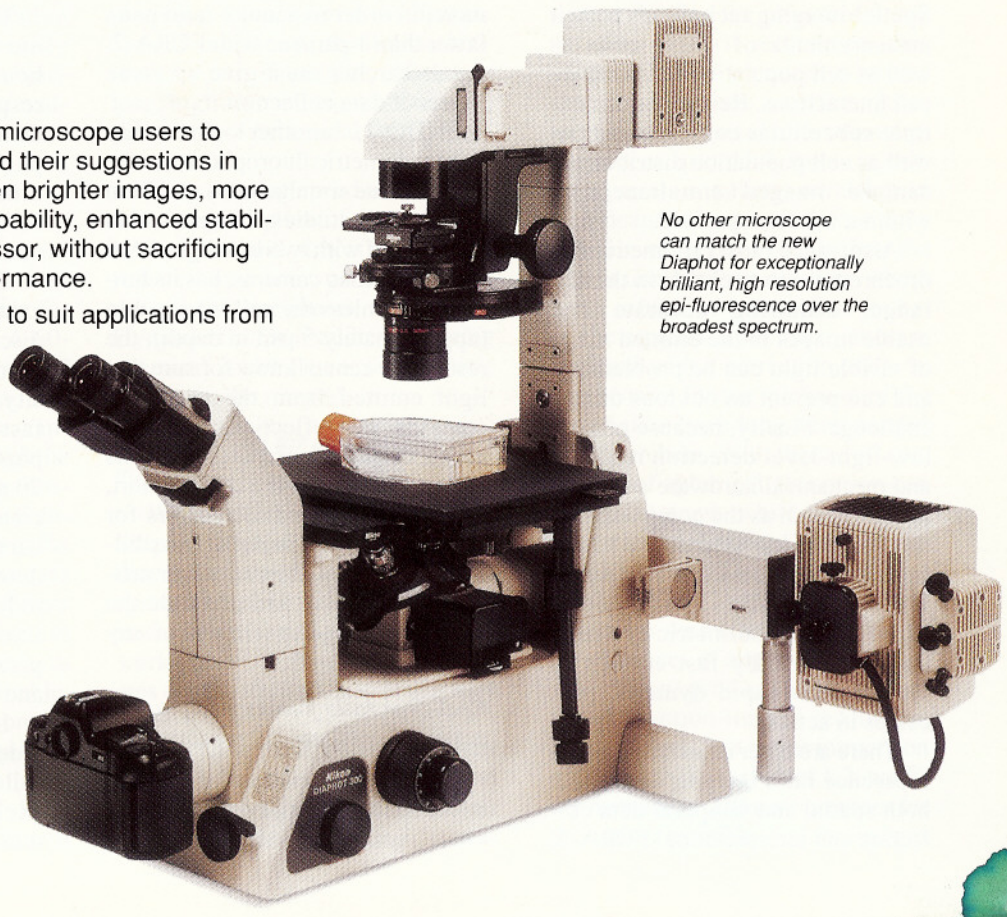

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