Video Image Processing Greatly Enhances Contrast, Quality, and Speed in Polarization-based Microscopy

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Abstract Video cameras with contrast and black level controls can yield polarized light and differential interference contrast microscope images with unprecedented image quality, resolution, and recording speed. The theoretical basis and practical aspects of video polarization and differential interference contrast microscopy are discussed and several applications in cell biology are illustrated. These include: birefringence of cortical structures and beating cilia in Stentor, birefringence of rotating flagella on a single bacterium, growth and morphogenesis of echinoderm skeletal spicules in culture, ciliary and electrical activity in a balancing organ of a nudibranch snail, and acrosomal reaction in activated sperm.

In the last decade, closed circuit television has increasingly been applied to the light microscope for studies of cell structure and behavior. Exceedingly low light level microscope images have been detected and recorded by Reynolds and co-workers (21, 22), who pioneered in the use of electronic image intensifier tubes coupled to vidicon and other video cameras. For example, in a medaka (fish) egg previously injected with aequorin, a photoprotein that luminesces upon exposure to micromolar free Ca++, they were able to record the appearance of a faint luminescent patch at the point of entry of the sperm followed by the migration of a luminescent ring away from that point around the surface of the fertilized egg. Thus they succeeded in visualizing and demonstrating the presence of a wave of intracellular Ca++ release that traverses the surface of the egg after fertilization (7). Likewise, Rose and Lowenstein (24) used an image intensifier and video recording to demonstrate the limited diffusion of Ca++ in epithelial cells previously microinjected with aequorin (for additional references, see 22, 32, and 33).

Image intensifiers with video output have also been applied to dark-field microscopy, for example, for visualizing the sliding of individual microtubules in a Chlamydomonas flagellum or the shape change induced in isolated bacterial flagella (20). Also, in fluorescence microscopy, video cameras using very high sensitivity image tubes, such as the silicon intensified target tube, have been used to record images that are so dim as to be barely perceptible to the observer (18, 35).

For differential interference contrast microscopy, Dvorak et al. (5) devised a video system in 1975, using an image orthicon in a high-gain video camera whose output was processed with an image enhancer. With this system they were able to generate sufficient image contrast and resolution at the low light level needed to obtain time-lapse records of the invasion of human erythrocytes by live malaria parasites.

With the recent availability of moderately priced instrumentation, video cameras, and tape recorders, video systems are finding broader use in light microscopy. Schatten (29) and others have made effective use of moderately priced video equipment attached to phase-contrast and differential interference contrast microscopes. Recordings made in time-lapse and played back immediately allowed repeated observations, and discussion by several individuals, of experiments on extracted cell models and cells in division and early development (also see references 2 and 28). The general advantages of video microscopy are discussed in an informative survey by Butterfield (4).

I now report some striking improvements in the performance and utility of the polarized light and differential interference...
contrast microscopes that we were able to attain by the optimized application of video systems. The new combination provides clear images with outstanding contrast, resolution, diffraction image quality, and sensitivity. Objects with exceedingly weak birefringence and minute optical path differences can be visualized at the maximum resolution of the light microscope, and their rapid changes can be recorded and analyzed in real time, freeze-frame, slow motion, or time-lapse. Some gain is also made in visualizing weak birefringence and small optical path differences through opaque tissues and cells.

These improvements open up new possibilities for polarized light and differential interference contrast microscopy. Subtle, hitherto unobserved, dynamic changes in cellular organization and fine structure can now be analyzed with precision and speed in living cells. The improvements should also prove useful in the materials sciences and other related fields.

In the following I shall open with some general observations about the subject, describe the optical and electronic characteristics required for video high-extinction polarized light microscopy, illustrate the advances achieved, and discuss the limitations and advantages of the system.

The Polarizing Microscope in Cell Biology

In cell biology, the polarizing microscope has been used to detect crystalline inclusions as well as fibers and membranes that are regularly arrayed at a submicroscopic dimension. Examples include myofibrils, mitotic spindle fibers, chloroplasts, and retinal rods, etc. (27). Those structures display a birefringence (and dichroism) whose sign and magnitude reflect the nature of the constituent oriented molecules or fine structure and their concentrations (25, 26). The birefringence can be detected and measured without perturbing the cell, so that dynamic changes that occur at the molecular levels can be monitored directly in living cells, albeit with an image resolution limited to just below a half wavelength of light.

The polarizing microscope complements the electron microscope and X-ray diffractometer, which are used to resolve spacings far smaller than the wavelength of light. With the latter methods, however, the greater resolution is achieved at a cost of either the need to fix and dehydrate the cell or to utilize crystalline domains far greater than the wave length of light, often much greater than the size of a whole living cell.

To take full advantage of the polarizing microscope for studying the molecular and fine structural orientations in living cells, one needs to detect exceedingly weak birefringence (often <0.1 nm in retardation) at the very limit of resolution of the light microscope. Both theoretical and practical limitations tend to interfere with the achievement of these goals. Several biologists have investigated the factors commonly responsible for these limitations and have improved upon the performance of conventional polarizing microscopes (11, 31).

The ultimate limitations were found to originate in the rotation of polarized light at the interfaces of the lenses, slides, and cover slips, etc., lying between the crossed polarizers (1, 9, 36). As seen by examining the extinction pattern at the back aperture of the objective lens, the sense of rotation is reversed in the adjacent quadrants defined by the polarizer axes. The magnitude of rotation varies with azimuth angle and numerical aperture, and at the high numerical apertures essential for high resolution, the rotation can reach several degrees. The rotation thus drastically lowers the extinction factor, E.F. ([Intensity with parallel polarizers] + [Intensity with crossed polarizers]), of a polarizing microscope and increases the amount of stray light. This severely limits the image contrast of weakly birefringent objects under the very condition required to achieve high resolution.

The rotation of polarized light at the air-glass interface was substantially corrected by the introduction of the polarization rectifier (12). The image transfer function of the polarizing microscope was consequently improved, so that the diffraction anomaly and image error that used to accompany the images of weakly retarding objects viewed with an unrectified polarizing microscope were eliminated (14, 19).

Although the polarization rectifier substantially improved the combined resolution, sensitivity, and image quality of the polarizing microscope and expanded its utility in biological research (e.g., references 15, 16, and 23) it was still not possible to use objective lenses with the superior correction that gave the very best resolution and image quality. For example, plan apochromatic objectives that provide superior, low aberration images with objective and condenser numerical apertures (NA) of up to 1.4 utilize fluorite lens elements. These crystalline elements invariably contain heterogeneous crystalline domains that substantially reduce the extinction factor.

Furthermore, even with rectified optics, it has often been impossible to gain enough light to record the images of weakly birefringent motile cellular structures photographically even though they could be seen with the eye.

These practical but serious limitations of the polarizing microscope (and other instruments requiring high extinction conditions) have now been overcome by the use of certain video systems.

Comparison of Visual, Photographic, and Video Imaging

We shall now consider the differences between polarization optical images perceived visually, produced photographically, and aided by video.

For polarized light (and differential interference contrast) microscopes, the image brightness, I, produced by a retardation, R, located between crossed polarizers is given by: 

$$I = I_0 \times \sin^2(R/2) + I_1,$$

where I_0 is the image brightness with the analyzer and analyzer axes parallel and I_1, the brightness with their axes crossed (Fig. 1). Because the extinction factor E.F. is defined as $I_1/I_0$ (13, 31), 

$$I = I_0(\sin^2(R/2) + 1/E.F.)$$

(Eq. 1). The extinction factor varies with various parameters including the NA of the objective and condenser lenses, and the presence or absence of rectification.

The contrast and details detectable in the final image are affected by a number of factors, including the extinction factor. For direct visual observation, the detected contrast is proportional to the brightness difference, $\Delta I$, of the two image areas to be compared, divided by the average image brightness, I. Thus the eye responds linearly to $\Delta I/I$, or approximately to the log of the image brightness. This is true provided the
average brightness of the image is moderately high and the two areas compared (i.e., the image detail) subtend a sufficiently large area of view (3).

Therefore, in a polarizing optical system, the response of the eye to a small increment in retardation, $\Delta R$, is proportional to $\Delta \log I/\Delta R$, where $I$ is defined by Eq. 1. For a given $\Delta R$, the response is greater the higher the extinction factor (Fig. 2).

For a given extinction factor, the sensitivity ($\Delta \log I/\Delta R$, where $\Delta R$ approaches zero) varies with the amount of bias or offset retardation ($R_b$) by which $\Delta R$ is displaced from $R = 0$. At $R_b = 0$, the sensitivity becomes zero. As $R_b$ is increased, $\Delta \log I/\Delta R$ increases, reaches a maximum and gradually decreases again. The maximum is reached at a bias retardation that roughly doubles the brightness of the field, $I_s$, at maximum extinction (11, 13).

For the detection of weakly birefringent objects, it is therefore essential that a suitable compensator be used to introduce an appropriate amount of bias retardation. As evident from Fig. 2, the bias retardation that maximizes $\Delta \log I/\Delta R$ varies with the extinction factor.

In addition to altering the contrast of a birefringent specimen, the compensator also alters (increases) the average field brightness (Fig. 2). With high-extinction polarization microscopy, one inevitably works at a low field brightness even with a high intensity source. The gain in brightness provided by the compensator improves our ability to detect small retardations by raising the contrast discriminating ability of the eye. Thus we find that, even in a darkened room, the optimum bias retardation for visual observation is somewhat greater than that which provides maximum $\Delta \log I/\Delta R$.

Photographic emulsions respond more or less in the same manner as the eye. Over a moderate range of image brightness, characterized by the Hurter and Driffield (H and D) curve for the particular emulsion, the density is approximately proportional to $\log I$. However, compared to the eye, the photographic emulsion generally fails even faster to detect contrast as we exceed the optimal intensity ranges.

For photographing weakly birefringent cellular structures, one generally chooses emulsions and processing methods that provide moderately fine grain in order to record the needed image detail combined with an adequate grey scale range. The exposure is kept as short as possible to avoid image movement. Given these constraints, when weakly birefringent objects are photographed, the bias retardation is even further increased than the optimum bias retardation used for visual observation. Only then can the intensity range be made compatible with the emulsion (however, see Jones' remarks [17] on correcting the "underloading" of photographic emulsion). Even so, the ex-

\[ I/I_\text{II} = \sin^2 (R/2) + I_s/I_\text{II} \]

\[ \log (I/I_\text{II}) \]

Figure 1

Figure 2

**Figure 1** Brightness of retarders placed between crossed polarizers. For different extinction factors (E.F. = $I_p/I_\text{II}$; where $I_p$ is the field brightness with the polarizers oriented parallel to each other, and $I_\text{II}$ the brightness with zero retardation and the polarizers crossed), the curves are displaced vertically without change in shape. For a given bias retardation, $R_b$, the increment of brightness, $\Delta I$, introduced by an increment in retardation, $\Delta R$, is not affected by the extinction factor. For a very small $\Delta R$, $\Delta I$ converges to 0 at $R_b = 0$; $\Delta I$ increases with $R_b$ up to a maximum at $R_b = \lambda/4$, or as shown in the figure, $R_b/2 = \pi/4$. With an idealized photodetector that responds linearly without limit to the light intensity, $\Delta R$ would give rise to the same signal independent of E.F. at a given $R_b$ and the signal would be maximum at $\lambda/4$. The background brightness $I_\text{II}$ could be subtracted by setting appropriate pedestal levels. The response of actual vidicon cameras appears to follow curves lying part way between those in Figs. 1 and 2.

**Figure 2** Log plots of brightness of retarders placed between crossed polarizers. With the intensity plotted on a log scale, the curves for different E.F.s take on different shapes and slopes (cf. Fig. 1). Contrast detectors such as the eye and photographic emulsions respond more or less linearly to the incremental brightness, $\Delta I$, over the background brightness, $I_\text{II}$ (see Fig. 1). Thus they respond to the log of brightness, $\Delta \log I/\Delta R$ determines the sensitivity for such detectors. For contrast detectors, $\Delta \log I/\Delta R$ (where $\Delta R \to 0$) is also 0 at $R_b = 0$. With idealized detectors, the contrast reaches a maximum at $I_s = 2 \times I_\text{II}$. For systems with different E.F.s, the maximum $\Delta \log I/\Delta R$ as well as the optimum $R_b$ vary considerably.
posure commonly lies in the range of a few to many seconds, and significant image detail must often be recorded in the region of the H and D curve below the linear region, often in the very low negative density range. The contrast is regained up to a point during photographic processing, but this approach is limited by the increase of photographic noise, or graininess, that worsens as the contrast is raised.

With electronic detectors, including video imaging tubes, the voltage output or response is more nearly proportional to the light intensity. Therefore, in contrast to the approximately logarithmic response of the eye and photographic emulsion, we can treat the video camera as an approximately linear device.

With a device whose response is truly linear with the input light intensity, the response should follow Eq. 1, which relates light intensity to specimen or compensator retardation. The curves for different extinction factors would then not change in shape or slope as is the case for detectors with logarithmic responses (Fig. 2). Instead, as Fig. 1 shows, the curves should simply be displaced vertically along the intensity axis. Therefore, with an idealized linear detector, \( \Delta I \) could in principle remain unaffected by the extinction factor.

In a video system, the electrical signal arising from an increment of brightness, \( \Delta I \), is superimposed on an electrical signal that corresponds to the background brightness, \( I_0 \). Within a limited range, the background signal can be subtracted electrically by applying an offset—the “pedestal” or “black level” adjustment (Eq. 1). With an appropriate pedestal, the incremental brightness, \( \Delta I \), would then stand out in the video image against a dark grey or black background and we would have effectively obtained high extinction.

In addition to the pedestal level, the response of the video system to intensity can be regulated by adjustment of an amplifier gain (“gamma-control”) that affects contrast. In a video system equipped with gain and pedestal adjustments, one can regulate the contrast of the image by adjusting the gain and simultaneously cancelling out the signal originating in the stray light of the polarization optical system with the pedestal or black level control.

A suitable video system could thus be used to cure or minimize the extinction defects of a polarization optical system. A dark background and high-contrast video image should be attainable even under some conditions where the extinction factor cannot be made high enough for visual observation or photographic recording of weakly birefringent specimens. The same argument holds for differential interference contrast and other microscopes requiring high extinction. Conversely, with a microscope whose extinction factor is already high, the sensitivity for detecting and measuring small retardations and minute optical path differences could be made even greater by the use of video.

To a considerable extent, these theoretical considerations are materialized with some modern video equipment. With the video camera we used, the regulation of contrast and black level could be achieved automatically by the electronic circuit built into the video camera. Combined with our optical system, the auto-black effectively suppresses the stray background light, and together with the auto-gain control provides exceptionally crisp, sharp images of weakly retarding, minute specimen regions.

In fact the dynamic range for vidicon tubes such as the Newvicon and Chalnicon is quite limited. Their response (sensitivity to light) also varies considerably with face-plate illumination. See the Discussion for further clarification of these points.

**MATERIALS AND METHODS**

**The Microscope**

The microscope used in these studies is a specially built, inverted universal polarizing microscope designed jointly by Gordon W. Ellis, Edward Horn, and myself. The optical elements and mechanical components are arranged in a manner similar to that described earlier and generally used with Köhler illumination (10). In brief, a high-intensity 100-W concentrated arc mercury lamp is placed at the top of the optical bench microscope; the projected beam is collimated and then directed to remove heat rays and (for living cells) to provide monochromatic 546-nm green illumination; the beam is polarized with a Glan-Thompson high-extinction, high-transmission calcite prism (Karl Lambrecht Corp., Chicago, Ill.); passes a \( \times 10 \) Bruce-Köhler compensator (made to special order; Nikon Instrument Division, Garden City, N. Y.); and is collected to the specimen with a rectified strain-free condenser (e.g., Nikon 8-mm rectified oil-immersion condenser to which a second rectifier was added). The image of the specimen placed on a precision revolving stage is used with selected plano-apochromatic objectives, through a Glan-Thompson prism analyzer equipped with stigmatizing lenses, and projected by the ocular to the faceplate of the video camera.

For differential interference contrast, the rectified condenser is replaced with a low-strain-birefringence differential interference contrast condenser with built-in Senarmont compensator (Leitz-Smith T system). The objective lenses with built-in Wollaston prisms were carefully oriented to maximally darken the field.

**The Video System**

The video camera used in these studies (model 65 H; Dage-MTI Inc., Michigan City, Ind.) is equipped with a 1-inch Newvicon tube, circuitry with auto-gamma and auto-black controls, and an option for driving the sync signal from an external source (for split-screen comparison or insertion of a second image onto the same field by the use of a special-effects generator).

The output of the video camera feeds through a video processor, a sync stripper, and a video analyzer (nos. 604, 302-2, and 321; Colorado Video, Inc., Boulder, Colo.); a time-date generator (ET 202; Cramer Video, Boston, Mass.); and then to a time-lapse tape recorder and the main monitor (Fig. 3). The image analyzer permits the quantitation of image intensities and the determination of \( x-y \) coordinates of image points; the image processor provides a nonlinear gain function that allows manual control of image signal level, black level, and contrast; and the sync stripper allows the concurrent use of these components.

The 4-inch cassette, time-lapse video tape recorder (TV0 9000; Sony Corp., Long Island City, N. Y.) permits real-time and time-lapse recording, and playback or freeze-frame display of the recording. Although the recorder output is of good quality, the horizontal image resolution is limited by the recorder circuitry to \( \sim 320 \) TV lines (or \( 160 \) line pairs). Therefore, in addition to the main monitor (4290; Sanyo Electric, Inc., Compton, Calif.) displaying the signal coming from the recorder, another monitor is placed in the circuit before the video recorder. The second monitor provides an image with a horizontal resolution of \( \sim 700 \) lines (350 line pairs) nearly matching the capability of the video camera itself. The monitor chosen (VV 5510; Panasonic Co., Secaucus, N. J.) has a "undercase" capability, so that we can photograph the full field scanned by the video camera rather than that trimmed down by 20% or even more in many monitors. The second monitor also permits comparison of events occurring under the microscope at one time with a scene that had taken place some time earlier, which is displayed from the recorder onto the main monitor. From either monitor, photographs of good image quality could be recorded with a 35-mm camera (Nikon FM equipped with an \( f/3.5 \) 55-mm Macro lens) with a \( \times 4 \) exposure on Kodak Plus-X film, developed according to Kodak prescription with Microdol-X diluted 1:3. A third monitor with a built-in disk recorder (Sony SVM-1010 motion analyzer) allows us to slow down rapid motion or to analyze the recorded events frame-by-frame.

**RESULTS**

**Polarized Light Microscopy**

Fig. 4 shows a diatom, embedded in a medium with nearly matched refractive index, viewed in polarized light through a 40-power 0.95 NA plan apochromatic objective. The picture to the left depicts the appearance of the low-contrast image that is seen through the ocular. The picture to the right was taken without readjusting the microscope, except that the picture was taken from the monitor connected to the video camera attached to the microscope. The video camera was equipped with auto-black and auto-gain circuits. The improvements introduced by the video system are clear.
In taking these pictures, the rectifier in the 1.15 NA oil-immersion condenser was doubled so that the rotation of polarized light introduced by the microscope slide and the nonrectified objective lens were also eliminated. The iris of the condenser was adjusted to match the NA of the objective lens, and a $\lambda/10$ Brace-Köhler compensator, located between the crossed polarizers, was oriented to provide a bias retardation of $\approx 15$ nm.

The extinction factor of the microscope used with the 40/0.95 plan apochromatic objective was $1.25 \times 10^3$ with the system rectified as described above. A higher extinction factor could not be attained at full objective and condenser NAs because of the nonuniform crystalline fluorite elements present in (even this selected) plan apochromatic objective. Nevertheless, the image on the video monitor provides a very high contrast for the birefringent regions of the specimen and in general a high resolution and good correction.

Figs. 5 and 6 show recorded video images taken with the same polarization optical system as for Fig. 4. The cilia and cirri on the Stentor were captured by photographing single frozen frames of the recorded video tape. While I used a $1/4$-s photographic exposure to record the image frozen on the video monitor, the scene on the monitor is formed by a single frame of video scan. Two $1/50$-s fields are interlaced to make up a single video frame so that these photographs represent video exposures of $1/50$-s each. I do not believe such detailed birefringence of organellar structures in swimming protozoa has even been captured photographically or perhaps even been observed before.

Fig. 7 illustrates the high resolution achieved in video polarized light microscopy with a 100/1.35 oil-immersion plan apochromatic objective lens. The surface ridges of the human oral epithelial cell (about one quarter of the cell is visible) is depicted with unprecedented image detail. The contrast in this image reflects the detailed distribution of birefringence of the surface ridges, whose retardation measured $<0.2$ nm. Although I have observed many oral epithelial cells as test objects for microscopy, I have never before observed such detailed surface architecture with any mode of microscopy.

Not only do we obtain a fine image, but the contrast of the ridges is precisely reversed without shift in focus when the compensator is turned. In an unrectified system, the rotation of polarized light by the lenses introduces a diffraction image anomaly. The rotation modifies the aperture transfer function of the objective lens; thus fine specimen details may be improperly represented in the image (14, 19). Also, the focus of a weakly birefringent object shifts and necessitates the refocusing of the objective lens when contrast of a weakly birefringent specimen is reversed by turning the compensator. In the present video optical system, the rotation of polarized light and the
diffraction image anomaly have been corrected by introducing a stronger rectifier in the condenser without placing a rectifier in the objective lens. The extinction factor of the 100/1.35 plan apochromatic objective lens combined with the doubly rectified condenser of 1.15 NA was $8.5 \times 10^2$.

Fig. 8 illustrates another example of high-resolution polarized-light video images obtained with the 100/1.35 plan apochromatic objective. The photographs show a live bacterium attached to the surface of an anaerobic protozoan ("rubberneckia").

The very weak birefringence of the rotating bacterial flagella is clearly seen in alternating contrast trailing below the main body of the bacterium. As the spiral bundle of flagella rotates, the birefringent black and white stripes travel "away" from the bacterial body as in a rotating barber pole. Each black and white region corresponds to a portion of the flagellar spiral tilted to the right or the left. The contrast is produced because the local flagellar axes alternately lie in the opposite and same quadrant as the slow axis of the compensator.

The width of this bacterium as well as the wavelength of the flagellar spiral is only 0.5 μm, while the average amplitude of the wave is ~0.1 μm. Even though the amplitude of the flagellar wave is well below the Abbe limit of resolution, the alternately tilted regions of the wave clearly define the contrast of those flagellar regions in the compensated polarized-light video image. In the past I have occasionally observed birefringent waves of live bacterial flagella with high-resolution rectified optics, but never before these video recordings has it been possible to photographically document or analyze the birefringent waves.

For images of the video monitor magnified to the extent shown here, the scan lines can detract considerably, as in the upper row of pictures in Fig. 8. In the lower row, the distracting scan lines have been nearly eliminated, by rephotographing the upper pictures through a Ronchi grating.

4 A Ronchi glass grating with 50-100 black lines ruled per inch (Rolyn Optics Co., Arcadia, Calif.) is oriented with the rulings parallel to the video scan lines and placed 4–10 inches above the print or video monitor to be copied. The Ronchi grating is initially positioned so that diffraction by the grating roughly doubles the frequency of the video scan lines. The final position of the grating is set to where the scan lines disappear from the image in the viewfinder of the copy camera. The Ronchi grating can also be placed in the projection beam of the enlarger. The use of the Ronchi grating was kindly suggested to me by Raymond E. Stephens of the Marine Biological Laboratory, who reminded me of a related use that I had made of the grating earlier (6, 8).

Besides using Ronchi gratings, which invariably introduce some image degradation, the scan lines can be made less conspicuous by photographing the video screen just when the scan lines (whose positions periodically fluctuate) are not prominent, and by photographing several sequential or frozen video frames with a $\frac{1}{8}$- to $\frac{1}{64}$-s photographic exposure.

The most dramatic removal of the scan lines, without loss of even very fine video image details, was achieved by a method of optical filtration devised by Gordon W. Ellis of the University of Pennsylvania. This method will be reported elsewhere.

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FIGURE 5 Birefringence of ciliary rootlets and beating cilia in Stentor. The video-polarized light microscope image was taken with a 40 x, 0.95 NA nonrectified Nikon plan apochromatic objective lens with correction collar. The objective lens was coupled with a doubly rectified 1.15 NA oil-immersion condenser, illuminated with a narrow-band 546-nm mercury green line. The image was photographed off a 9-inch monitor displaying, in freeze-frame mode, a selected scene from the ¾-inch video cassette tape record. Two ½o-s interlaced fields make up this single video frame (Inoué and Chen, unpublished photograph). Bar, 10 μm x 1,300.

FIGURE 6 Swimming Stentor showing birefringence of km fibers, ciliary rootlets, and beating aboral membranelle. Single frame from ¾-inch video cassette record as in Fig. 5 (Inoué and Chen, unpublished photograph). Bar, 10 μm x 1,300.

Differential Interference Contrast Microscopy

As noted earlier, image contrast in differential interference contrast microscopy is also affected by the extinction factor of the system and the setting of the compensator. We would therefore expect the differential interference contrast images also to be improved by the use of video, which could at the same time provide several other practical advantages characteristic of electronic recording.

Fig. 9 shows the growth of a calcareous skeletal spicule isolated from a prism-stage sea urchin embryo. The three mesenchyme cells that support the in vitro growth and mor-

FIGURE 7 High resolution, high extinction video-polarized light microscope image of an oral epithelial cell. The 1-inch Newvicon video camera output was coupled directly to a 9-inch video monitor to minimize the loss of image resolution. The birefringent ridge structure on the cell surface is resolved in great detail. A Nikon 100/1.35 oil-immersion plan apo (nonrectified) objective was combined with a doubly rectified 1.15 NA oil-immersion condenser and illuminated with the mercury green line. Bar, 10 μm x 2,000.
phogenesis of the spicule, the growing spicule itself, and the mesenchymal pseudopod that envelopes the spicule, are all clearly recorded in the differential interference contrast image. Without video, the very high birefringence of the spicule, which is composed of a single crystal of calcite, can overwhelm the considerably lower contrast of the mesenchyme cells, especially their fine pseudopods.

The original time-lapse video image, recorded on a ¾-inch tape, which showed the behavior of the mesenchymal pseudopods, could easily be transferred again in time-lapse mode to another video tape. In this way, the very slow process of spicule growth and the more rapid changes in the mesenchymal cell behavior could both be analyzed at appropriate framing rates. These video images record the first observations ever made on skeletal spicules that were isolated from the embryo and grown in culture.

Fig. 10 shows a differential interference contrast image of a balancing organ within the excised brain tissue of a nudibranch snail *Hermissenda crassicornis* (30). The (out of focus) micropipette to the upper left (arrow), penetrating a hair cell of the statocyst, is used for iontophoretic microinjection of reagents that affect ciliary motion and for monitoring the electrical activity of the ciliated epithelial cell. A video image of the oscilloscope that traces the electrical activity is electronically superimposed with the microscope image aided by a special effects generator. Despite the thickness of the preparation and the presence of considerable overlying connective tissues, the individual cilia in the statocysts can be seen reasonably well, especially as the scene is played back onto the monitor.

With the video tape record, the activity of the hair cell cilia, their change with microinjected reagents, and the varying...
mechanical impact of the statoconia on the cilia, can all be correlated directly with the electrical response of the ciliated sensory cell.

Fig. 11 shows high-contrast differential interference contrast images of a Thyone briareus (sea cucumber) sperm that is extending its acrosomal process. This very fine process, measuring only ~50 nm in diameter at its thinner parts, is extended when the sperm meets the egg jelly or, as shown here, is artificially activated by a calcium ionophore (34). The acrosomal process grows up to 90 μm long in <10 s as actin molecules polymerize inside its growing tip and lengthen a slender axial filament.

The photographs in Fig. 11 were taken from a sequence used to analyze the kinetics of acrosomal process growth and the changing morphology of the growing acrosomal process. Such photographs would have been all but impossible to take without the aid of video. Video can provide both the exceptionally high contrast required to clearly visualize the thin acrosomal process and the short exposure time (60 fields/s) required to obtain the sharp images.

Fig. 12 shows a high magnification view of the Thyone sperm head taken with a 100/1.30 Smith T system differential interference contrast objective. The contents of the acrosome are expressed as the acrosomal process extends.

In the far left scene, the acrosome that has just become swollen appears as a 1.2-μm-diameter hollow region located near the tip of the sperm head. In the next scene, taken 1.8 s later, the acrosomal process, indicated by an inverted v, has grown out approximately one sperm head length (but is blurred because of the rotation of the process). Within the acrosome, two compartments start to show. The spherical forward compartment and the acrosomal vesicle have both lost their solids content.

The changes in the acrosome that are so clearly seen here reflect the swelling of the rear acrosomal compartment by a mere 1 μm in just 3 s. These events that take place inside the acrosome of an activated sperm have never been observed before. Nor has it been possible, so far, to capture these dynamic events and structures with the electron microscope.

DISCUSSION

Earlier, in the general observations about video imaging, we treated the video camera as though it had certain idealized qualities: we assumed that the image tube responded truly linearly to the image brightness over an arbitrarily wide brightness range. Although such assumptions help qualitatively to grasp the virtues of video imaging, they do not fit the properties of real video cameras nor adequately explain the performance of actual video systems.

In actuality, the microscope and the video system had to be adjusted with some care in order to match their performances with the actual characteristics of the video components and to achieve the type of image improvements that we report in this paper.

First, the sensitivity of our video camera, equipped with the sensitive Newvicon tube, was just barely adequate to operate very near the maximum extinction on our microscope. With the selected 40/0.95 plan apochromatic objective lens combined with a matched oil-immersion rectified condenser, the image near extinction was so close to the threshold sensitivity of the Newvicon camera that the light source and the condenser had to be aligned with considerable care. Even then, the video image of a 1-nm retardation specimen right at extinction was buried in noise (snow) when the microscope image was magnified sufficiently to match the video resolution. With the λ/10 Brace-Köhler compensator turned to introduce a bias retardation of a few to >10 nm, a clear, sharp image of the weakly birefringent specimen appeared on the screen. At these compensator settings, the visual image formed by the plan apochromatic objectives and viewed through the ocular was vir-
ultimately washed out, as illustrated in the left-hand part of Fig. 4. 

Second, increasing the bias compensation beyond ~15 nm reduced, rather than increased, the video image contrast. An increase of contrast would have been expected from Fig. 1 if the video camera were truly responding linearly to the image brightness and did not saturate at high light levels.\(^4\) Because the reduction of image brightness with neutral density filters only slightly improved the video image contrast, the lowered contrast at higher compensation is apparently not caused by image tube saturation. Rather, it must be that the response of the Newvicon camera is more closely represented by a curve shaped part way between those shown in Figs. 1 and 2.

Third, in contrast to polarized light microscopy of weakly retarding specimens, the image brightness in differential interference contrast microscopy was often too high for the Newvicon camera. For differential interference contrast, it was often necessary to reduce the illumination; only then could the video image contrast be raised to the desired high level.

Fourth, while image contrast in video could be raised electronically by greater amplifier gain, nonuniform sensitivity of the image tube and uneven illumination of the microscope field became increasingly more limiting. Likewise, dirt, flare, and reflections in the optics became increasingly more conspicuous. Reduction of optical noise as well as electrical noise thus became increasingly more demanding.

Fifth, the resolution of the video system, especially of the recorder, tended to be limited as described earlier. Therefore, to avoid losing image detail, we were obliged to provide the video camera with a high magnification image. Increased magnification results in lower image brightness and reduced field size.\(^7\) We often found it necessary, lacking appropriate zoom lenses, to adjust the ocular magnification and image projection distance in order to find an optimum choice (or compromise) between image brightness, detail, and field coverage.

The considerations listed here impose practical constraints in applying modern vidicon cameras to polarized light and differential interference contrast microscopes. Nevertheless, as illustrated in the Results, by applying video we have been able to make very significant improvements in the image quality attained and information gained by light microscopy.

With polarized light microscopy, high-NA well-corrected objectives can finally be used and the very weak birefringence of cellular details provide sharp, high-resolution images with excellent contrast. The birefringence of moving organelles such as beating cilia and flagella, even the rotating flagellar spiral on a single bacterium, can now be sequentially recorded in %o-s fields and their behavior analyzed in detail.

With differential interference contrast microscopy, we gain clear, high-resolution images of dynamically changing, minute cellular structures. Slow changes in embryonic development, as well as the rapid changes within an activated spermatozoon, are vividly displayed with remarkable image detail.

In both modes of microscopy, contrast provided by objects considerably below the Abbe limit of resolution are clearly detected (on objects separated from their neighbors by a distance larger than the resolution limit). Rapid or slow events are now readily and economically recorded, and analyzed repeatedly, in real time, freeze-frame, slow motion, or time-lapse.

The major improvements that have been realized stem from: (a) the approximately linear response of the video image tubes; (b) the relatively high sensitivity and low noise level of modern vidicon cameras; (c) video camera circuitry that allows control and enhancement of image contrast and black level; (d) convenience, economy, and reliability of modern video equipment; and (e) optimized combination of video and polarization optical microscope parameters.

Thus, moderately priced modern video equipment now allows us to use plan apochromatic objectives on polarized light microscopes and obtain well-corrected, bright, high-resolution images of weakly birefringent objects. Appropriate rectifiers built into the condenser lens alone, and used with adequate compensation, provide high-resolution images free from spurious diffraction.

In general, polarization and differential interference contrast microscopes can be used with less demanding extinction factors, compensated by the electronic contrast and offset controls provided by video. The greatest sensitivity for detecting very small retardations is, however, still achieved with a polarizing microscope that gives the highest extinction factor. For high extinction microscopes, the image sensitivity is further improved by the use of highly sensitive video cameras.\(^3\)

In obtaining the results reported in this paper, we used a specially built inverted polarizing microscope as described earlier. This should not be taken to mean that the improvements demonstrated can only be made on such a special microscope. On the contrary, the basic improvements with video should be attainable with most research-grade polarizing microscopes equipped with a bright source and optical components more or less similar to ours. For differential interference contrast microscopes, the use of other instruments should pose little problem so long as the microscope image mag
cation and brightness are adjusted to match the performance of the video camera.

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REFERENCES

1. Berek, M. 1953. Titine-Berek: Anleitung zu Optischen Untersuchungen mit dem Polarisationsmikroskop. Schweizerbart'sche Verlagshandlung, Stuttgart, W. Germany.
2. Berns, M. W. 1972. Partial cell irradiation with a tunable dye laser. Nature (Land.). 240:483-485.
3. Blackwell, R. H. 1946. Contrast threshold of the human eye. J. Opt. Soc. Am. 36:624-643.
4. Butterfield, J. F. 1978. Video microscopy. Microscope. 26:171-182.
5. Dvorak, J. A., L. H. Miller, W. C. Whitehouse, and T. Shiroishi. 1975. Invasion of erythrocytes by malaria merozoites. Science (Wash. D. C.). 187:748-750.
6. Erickson, R. O. 1973. Tubular packing of spheres in biological fine structure. Science (Wash. D. C.). 181:705-716.
7. Gilkey, J. C., L. F. Jaffe, E. B. Ridgway, and G. T. Reynolds. 1978. A free calcium wave traverses the activating egg of the medaka, Oryzias latipes. J. Cell Biol. 76:448-466.
8. Gilhui, V. P. 1979. A simple method of optical filtration. Ultramicroscopy. 4:323-336.
9. Inoue, S. 1952. Studies on depolarization of light at microscope lens surfaces. I. The origin of stray light by reflection at lens surfaces. Exp. Cell Res. 3:199-208.
10. Inoue, S. 1961. Polarizing microscope design for maximum sensitivity. In Encyclopedia of Microscopy. G. L. Clarke, editor. Reinhold Publishing Corp., N. Y. 480-485.
11. Inoue, S., and K. Dan. 1951. Birefringence of the dividing cell. J. Morphol. 89:423-456.
12. Inoue, S., and W. L. Hyde. 1957. Studies on depolarization of light at microscope lens surface. II. The instantaneous realization of high resolution and high sensitivity with the polarizing microscope. J. Biophys. Biochem. Cytol. 3:831-838.
13. Inoue, S., and C. J. Koester. 1959. Optimum halfshade angle in polarizing instruments. J. Opt. Soc. Am. 49:556-559.
14. Inoue, S., and H. Kubota. 1958. Diffraction anomaly in polarizing microscopes. Nature (Land.). 182:1723-1726.
15. Inoue, S., and H. Ritter. Jr. 1978. Mitosis in Barbulanympha. II. Dynamics of a two-stage anaphase, nuclear morphogenesis, and cytokinesis. J. Cell Biol. 77:655-684.
16. Inoue, S., and H. Sato. 1966. Deoxyribonucleic acid arrangements in living sperm. In Molecular Architecture in Cell Physiology. T. T. Hayashi and A. G. Szern-Gyorgy, editors. Prentice-Hall, Englewood Cliffs, N. J. 209-248.
17. Jones, R. C. 1959. Quantum efficiency of detectors for visible and infrared radiation. Advan. Electron Electromm Phys. 11:87-183.
18. Kokaia, G. B. 1978. Image intensification comes to biology. Science (Wash. D. C.). 201:396.
19. Kubota, H., and S. Inoue. 1959. Diffraction images in the polarizing microscope. J. Opt. Soc. Am. 49:191-198.
20. Nakamura, S., and R. Kamiya. 1978. Bending motion in split flagella of Chlamydomonas. Cell Struct. Func. 3:141-154.
21. Reynolds, G. T. 1972. Image intensification applied to biological problems. Q. Rev. Biophys. 5:299-347.
22. Reynolds, G. T. 1978. Application of photoconductive devices to bioluminescence studies. Photochem. Photobiol. 27:405-421.
23. Ritter, H., Jr., S. Inoue, and D. Kubai. 1978. Mitosis in Barbulanympha. I. Spindle structure, formation, and kinetochore engagement. J. Cell Biol. 77:638-654.
24. Rose, B., and W. Lowenstein. 1975. Calcium ion distribution in cytoplasm visualized by aequorin: diffusion in cytosol restricted by energized sequestering. Science (Wash. D. C.). 190:1204-1206.
25. Sato, H., G. W. Ellis, and S. Inoue. 1975. Microtubular origin of mitotic spindle form birefringence. Demonstration of the applicability of Wiener's equation. J. Cell Biol. 67:501-517.
26. Schmidt, W. J. 1934. Polarizationsoptische Analyse des submikroskopischen Baues von Zellen und Geweben. In Handbuch der biologischen Arbeitsmethoden. E. A bearer, editor. Urban and Schwarzenberg, Berlin, W. Germany. 6(10):465-466.
27. Schmidt, W. J. 1937. Die Doppelbrechung von Karyoplasma, Zytoplasma und Meta-plasma. Protoplasma Monographien, Vol. 11. Bern, Switzerland.
28. Selgall, R. R., and E. D. Salmon. 1979. ,Ca** induces microtubule depolymerization and spindle fiber shortening in isolated mitotic cytoskeletons. J. Cell Biol. 83(2 Pt 2):171a (Abstr.).
29. Schatten, G. The movements and fusion of the pronuclei at fertilization of the sea urchin Lytechinus variegatus: time-lapse video microscopy. J. Morphol. In press.
30. Steinmetz, E. W. R., E. Stephens, and D. L. Alkon. 1980. Monile stalk cilium transmits rather than directly transduce mechanical stimuli. J. Cell Biol. 87:652-662.
31. Swanz, M. M., and J. M. Mitchison. 1950. Refinedness in polarized light microscopy. J. Exp. Biol. 28:434-444.
32. Taylor, D. L., J. R. Blinks, and G. T. Reynolds. 1980. Contractile basis of ameboid movement. VIII. Aequorin luminescence during ameboid movement, endoeytokinosis, and capping. J. Cell Biol. 86:590-598.
33. Tilney, L. G., D. P. Kiehart, C. Sardet, and M. Tilney. 1978. Polymerization of actin. IV. Role of Ca** and H* in the assembly of actin and in membrane fusion in the acrosomal reaction of echinoderm sperm. J. Cell Biol. 77:536-550.
34. Tilney, L. G., D. P. Kiehart, C. Sardet, and M. Tilney. 1978. Polymerization of actin. V. Role of Ca** and H* in the assembly of actin and in membrane fusion in the acrosomal reaction of echinoderm sperm. J. Cell Biol. 77:536-550.
35. Willingham, M. C., and I. Pastan. 1978. The visualization of fluorescent proteins in living cells by video intensification microscopy. Cell. 13:501-507.
36. Wright, F. E. 1911. The Methods of Petrographic-Microscopic Research. Publ. no. 158. Carnegie Institute, Washington, D. C.