VIDEO MICROSCOPY

In video microscopy, the image produced by the light microscope is projected onto the detector of a video camera (Figure 1). Electronics in the video camera scan the faceplate of the detector in a raster pattern, converting variations in light intensity into variations in voltage which are encoded into a video signal. Conventional video cameras produce images at 30 frames/sec (the standard TV rate), while special cameras can acquire images at faster or slower rates. The video signal produced by the camera can be immediately displayed on a video (or TV) monitor and recorded on a video tape or laser disk recorder. Alternatively, prior to recording, the signal can be subjected to analog or digital processing to improve image contrast, obtain quantitative intensity measurements or display image information in useful ways.

Video technology has revolutionized the usefulness of bright field and fluorescence light microscopy in many ways (3,4,6,9,18,21-23,26,27,32,40,46,47) including: (a) Detection of low contrast objects such as single unstained macromolecular complexes, (b) detection of low intensity objects such as weakly fluorescent objects, (c) convenient image recording and retrieval, (d) automated analysis of motion and geometrical parameters, (e) image overlay and 3-D reconstruction, (f) 2-D photometry and quantitative fluorescence microscopy, and (g) ratio imaging to obtain 2-D distributions of the concentrations of molecules such as H⁺, Ca⁺⁺, Mg⁺⁺, and Na⁺.

This article will focus on the principles and practical aspects of achieving high contrast, high resolution images using differential interference contrast (DIC) light microscopy and video contrast enhancement methods. DIC was an immediate success in a variety of applications after its introduction in the late 1960’s. DIC can produce images of phase objects at the diffraction limit of the light microscope and it has a corresponding shallow depth of field when the objective aperture is fully illuminated (2). This shallow depth of field produces clear “optical sectioning” of relatively thick biological specimens without contamination due to out-of-focus object details or spurious light, a major problem with phase contrast systems. Analog and digital video techniques can produce enormous gains in image contrast. Objects or fine structural detail, invisible to the eye by direct viewing or conventional photographic techniques, can be displayed in high contrast and recorded at video frame rates (3,4,6,21,22,24,39). We have used unstained microtubule preparations in this paper to illustrate the principles, advantages and practical aspects of video-enhanced DIC microscopy. Microtubules are 25-nm diameter protein polymers of α,β-tubulin dimers, which can grow to be many μm long.
They are an order of magnitude smaller in width than the resolution limit of the light microscope at green wavelengths of light.

**BASICS OF DIC IMAGE FORMATION**

A DIC microscope is a bright field polarized light microscope containing two birefringent Wollaston prisms, in addition to the polarizer, analyzer and rotating stage used in polarization microscopy (Figure 1).

Contrast is generated by the interference of a pair of wavefronts passing through the specimen. Light passing through the polarizer is split into two diverging and orthogonally polarized light beams by one Wollaston prism at the condenser diaphragm plane. The diverging beams are converted by the condenser lens into two wavefronts, which pass through the specimen separated laterally from each other in one direction (termed the "shear" direction) by a distance less than the resolution limit of the microscope optics. These two wavefronts are recombined by a Wollaston prism mounted above the objective lens. When a bias retardation is added to the two waves (using a compensator or by sliding the upper Wollaston prism with respect to the lower Wollaston prism), images produced by DIC are perceived as though objects were shadow-cast (Figure 2). The bias retardation brightens the background light intensity while raising the intensity of one wavefront and lowering the intensity of the other with respect to the background intensity. Contrast in DIC images is a function of the gradient in phase-difference across objects and it depends on azimuth. Contrast is maximum in the shear direction and minimum in the orthogonal direction (Figure 2).

Resolution in DIC images is limited by the numerical apertures of the objective (NAₐₒᵢ) and condenser (NAᵢₐₒᵢ) lenses. The lens aperture produces diffraction images (Airy disks) of point sources in the specimen. Points in the specimen are seen as two overlapping diffraction patterns (Airy disks) in DIC images corresponding to the two wavefronts passing through the specimen (Figure 2). The radius of the Airy disk is given approximately by (16, 22, 24, 39):

$$ r = 1.22 \frac{\lambda}{(NA_{obj} + NA_{cond})} $$

where $\lambda$ is the wavelength of illumination. The maximum NA_{obj} available is 1.4. For NA_{obj} = NA_{cond} = 1.4 and $\lambda = 550$ nm, then $r = 200$ nm. For 25-nm diameter microtubules, the diffraction of light by the objective lens aperture broadens the width of the microtubule image more than 10 fold over the width predicted by the microscope magnification (Figure 2). Lateral and vertical resolution depend on distinguishing two overlapping Airy disk diffraction patterns as two different objects. The conventional lateral resolution limit is the radius of the Airy disk given in Eqn. 1. The lens point spread function which produces the Airy disk is three-dimensional (16), and vertical resolution is also inversely related to NA (16, 22).

The contrast of the Airy disk image depends on the mass of material separated by less than the resolution limit (24, 39). In Figure 3, a single microtubule is attached to the lower end of an axoneme fragment, which is a 150-nm diameter cluster of 9 doublet...
microtubules. Clearly, the contrast of the axoneme is an order of magnitude greater than that of the single microtubule.

BASICS OF VIDEO-ENHANCED CONTRAST

Video-enhanced contrast methods can make visible fine structural detail in specimens at the maximum resolution limit of the microscope optics and make visible molecular complexes, like microtubules, whose dimensions are more than an order of magnitude smaller than the diffraction limit of the microscope optics. This was initially demonstrated by Allen and coworkers (3,4,6) and Inoué (21) using the analog video enhancement capabilities of relatively inexpensive surveillance cameras. Although resolution increases with objective and condenser NA (Eqn. 1), optical noise in the lenses (e.g. scattered light, birefringence, rotation of the plane of polarization) also increases with NA so that when viewing by eye or with conventional photography, resolution was frequently sacrificed for image contrast. When using video contrast enhancement techniques, it is best to fully illuminate the objective aperture. This not only produces the maximum lateral resolution (Eqn. 1), but also the maximum vertical resolution. A shallow depth of field is important to obtain images free of out-of-focus mottle (6). Lateral resolution of about 100 nm (3) and vertical resolution of 150 nm (24,25) have been reported using video-enhanced contrast methods and fully illuminated objectives of NA = 1.4 and green light. These resolution values are about twice those seen by eye or photographic film. Inoué has been able to achieve 3-D reconstructions of objects from 150-nm thick optical sections (24,25).

Most video contrast enhancement is produced by analog electronics as illustrated for microtubule imaging in Figures 3a-b and 4. Contrast enhancement is produced by subtracting voltage (Black Level or Pedestal control) from the video signal and amplifying the difference (Gain control) for each horizontal line scan of the video raster image. These functions are usually available in the medium-to-high performance video cameras.

Increasing the contrast to the levels necessary to see protein complexes of the diameter of microtubules produces a new set of problems (6,22,27,44) (Figure 3b). Image contrast becomes limited by noise which is amplified as the gain is increased. There are two different types of noise. One is stochastic or random noise which is produced by the video detector and variations in photon flux. The second is fixed pattern noise. The image of weak contrast objects like microtubules is obscured by uneven background intensity in the image produced by a number of factors within the optical components and the video detector. These include out-of-focus dirt in the illumination or projection optics, uneven illumination intensity, optical noise like birefringence in the lenses, or uneven sensitivity across the surface of the detector in the video camera (shading).

Frequently, the amount of analog contrast enhancement that can be used is limited by the degree of uneven background intensity across the screen. As the gain is increased, the bright and dark regions of the image exceed the dynamic range of the electronics, and clipping occurs. Specimen detail is lost in clipped regions. Some high performance video cameras (e.g., Hamamatsu C2400) have analog electronic controls which can be used to flatten out the uneven background, thus allowing higher contrast enhancement. These devices are very useful for correcting the unidirectional gradient in background intensity produced by mismatched Wollaston prisms. However, the mottle produced by out-of-focus direct or uneven illumination reduces the effectiveness of the analog corrections.

The problems induced by the fixed pattern and the random noise sources can be rectified (Figure 3c) using digital image processors which operate at video frame rates and contain several frame memories and several arithmetic logic units (6,22,27,38,44). Frame grabbers in the digital image processor convert the analog video voltage into

![Figure 2. Microtubules are 25-nm diameter polymers composed of the proteins α- and β-tubulin. Although well below the limit of resolution of the light microscope, microtubules can be visualized (a) using the video-enhanced DIC microscope system described in Figure 1 (the arrow indicates the direction of shear). The lattice structure depicted in (b) was modified from the electron microscope studies of Mandelkow et al. (33).](image-url)
digital voltages (8 bits give 256 grey levels) and generate a picture element (pixel) array of the video image (typically 512H by 512V or 640H by 480V) which can be stored in frame memories, and redisplayed as video signal by digital-to-analog converters after arithmetical processing. Mottle can be subtracted from an image by storing an out-of-focus background image in a frame memory (Figure 5). We do this by defocusing enough so that the microtubule image disappears. Then we average 256 video frames to eliminate the random noise, and the out-of-focus background image is stored in a frame memory. We then refocus the microtubules. The fixed pattern noise is removed by continuously subtracting the out-of-focus background image from the live in-focus image (Figure 6). A grey value of 128 is added to this difference, to produce a uniform grey background. This procedure substantially reduces the fixed pattern noise, but has relatively little effect on the random noise.

Random noise can be reduced by various algorithms which average successive frames. Temporal averaging reduces time resolution, so care must be taken on how much averaging to use. We use an N frame exponential (or sliding) average for microtubule imaging, where N in our experiments is usually set to 2 or 4 (Figure 6). Further contrast enhancement is now possible, since the background signal is nearly constant throughout the image. We use the output look-up tables of the image processor to increase the contrast 6 fold before the image is converted from digital to analog (Figures 2, 3c, 7).

PRACTICAL ASPECTS OF IMPLEMENTING VIDEO-ENHANCED DIC MICROSCOPY

Achieving high contrast images of specimens, such as unstained single microtubules, requires the highest performance of both the optical and electronic components of the video microscope system. What follows is a brief presentation of the parameters we have found to have impact on image contrast and resolution.

Microscope Alignment

An intense source of light is required because of the nature of DIC contrast generation (reduction in light intensity due to crossed polarizers) and because of the high magnifications required to project microtubule images onto the video detector. We use either the HBO 200W or the HBO 100W mercury light sources with efficient collector lenses.

It is important to fully and evenly illuminate the objective aperture with constant light intensity in order to achieve high resolution images depleted of out-of-focus mottle, uneven background intensity and flicker (22,38). The arc of the HBO 200W Hg lamp is large (about 2 mm high), and it can be projected using the standard Koehler illumination so that the in-

Figure 3. An example of the enormous gain in contrast produced by video-enhanced contrast. All three images show the same field containing a sea urchin flagellar axoneme fragment and a single microtubule extending off the lower end of the axoneme fragment. (a): The typical image as viewed by eye through the microscope oculars. The axoneme is barely visible and the microtubule is invisible. (b): Analog contrast enhancement in the video camera (Figure 4) produces clear visualization of the axoneme fragment and allows detection of the single microtubule. However, note that the background intensity is very uneven and that out-of-focus mottle is now evident. (c): The image has been substantially improved with the use of background subtraction, exponential averaging, and digital contrast enhancement methods (Figure 6). This image, unlike Figures 1, 7, and 8, was obtained using a Zeiss Axiphot microscope coupled with an infinity-corrected 100X/1.3 NA Plan Neofluar objective, matching condenser and Wollaston prisms, and a 220W tin halide burner as the light source. Although images produced by the Axiphot are equivalent, if not superior, to our Photomicroscope I system when a 200W Hg burner is used, the tin halide burner exhibited more intensity variations and flicker. These variations make it extremely difficult to match background-image pixel intensities with incoming image pixel intensities. Hence, the image in (c) is not as "flat" as those shown in Figures 1, 7, and 8.
focus images of the tips of the electrodes are positioned just outside the condenser aperture, which is typically over 20 mm in diameter. This produces nearly full illumination of the condenser aperture and uniform illumination of the specimen. The only problem with the 200W Hg burner is that it tends to flicker, even with well regulated DC power supplies. The arc of the 100W Hg lamp is more stable and intrinsically brighter than the 200W Hg burner (22). However, it is much smaller (0.25 mm high) and conventional microscope illumination optics are unable to produce sufficient magnification of the arc to fill the condenser and, hence, objective apertures using Kohler illumination. There are two solutions. One is to use "critical illumination," where the arc is directly focused on the specimen by the condenser lens (the field diaphragm is left wide open) (31,38). The collector lens is adjusted so that the image of the arc is out-of-focus at the condenser diaphragm plane and it fully fills the objective aperture. This method works, but the intensity at the specimen can be very uneven. The other option is to use a light "scrambler" as designed by Ellis (13,22,38). In this device, the output from the collector lens of the 100W Hg burner is focused by another collector lens into a quartz optical fiber. The fiber is bent to scramble the light so the output surface of the fiber is evenly intense. This output surface then serves as the light source in a Kohler illumination (22) or critical illumination (38).

It is important for video-enhanced contrast that the transmission of light through the optical components of the microscope be as efficient as possible. This is not always the case for the DIC optics supplied by all manufacturers. In particular, care should be used in choosing the polarizing material for the polarizer and analyzer, the heat cut or reflecting filters, the band pass filters and the objectives for high transmission. Polarizing material with at least 30% transmission of incident unpolarized light should be used; calcite prisms can have 50% transmission, but high quality prisms are expensive ($1000 or more). We use a narrow bandwidth interference filter (540 nm, 85% peak transmission, Omega #540DF40) to isolate the green line of the mercury arc. DIC image contrast drops as the bandwidth of the illuminating light increases.

We use a Zeiss 100X NA=1.25 Plan Achromat objective, a Zeiss NA= 1.4 condenser with matched Wollaston prism for the objective and condenser. This optical combination gives excellent DIC contrast with very little shading. Higher NA Planapochromat Objectives will give better resolution, but care must be taken to make sure that the Wollaston prisms for the objective and condenser are matched. Otherwise, uneven background intensity in the image will limit contrast enhancement.

In our experiments, we have found that a bias retardation between the two DIC wavefronts of about 1/10 wave-length gives optimum contrast. A similar value was determined by Schnapp (38). This retardation is smaller than the 1/4 wavelength recommended by Allen et al. (3) from theoretical considerations.

A major source of noise or poor contrast is air bubbles in the immersion oil between the objective and the coverslip, and between the condenser and the slide. Care must be taken so there are no air bubbles in the oil!

The surfaces of the glass slides and coverslips must be cleaned free of dirt and other light-scattering material, particularly when viewing macromolecular complexes, such as microtubules in solution. In our in vitro microtubule studies, we pipette 5 µl of solution onto a cleaned slide and cover this drop with

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**Figure 4. Analog contrast enhancement.** Video cameras with black level and gain controls can improve image contrast by first subtracting voltage (termed black level or pedestal) from the video signal and then amplifying the difference (using the gain control) to stretch the remaining information in each horizontal scan line. This process also increases the magnitude of uneven background intensity and random noise.

**Figure 5. Background-image average and storage.** To obtain an image of the fixed pattern noise, such as dirt particles and uneven background in the video image, the microtubule image is slightly defocused, and 256 sequential video frames are acquired and averaged to produce a low noise image, which is saved in a framestore board (FSbc).
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a 22 mm x 22 mm #0 or #1 coverslip. When the solution spreads to the edge, the preparation is sealed with valap (22,44) making a chamber 10 μm thick.

Another major source of noise is dust on optical surfaces and the faceplate of the video camera. We use a negative projection lens (Zeiss 4x TV adapter) instead of an ocular to project the specimen image directly onto the faceplate of the camera. This reduces the number of glass surfaces scattering light between the objective and the camera.

We use a 3x - 4x projection magnification from the objective to the video camera. This magnification was chosen so that the diffraction-limited image of the microtubules was several times larger than the pixel size of the video detector.

Finally, a vibration-free platform for the microscope is required to ensure best results at the high magnification and resolution used in our experiments.

Video Camera

Video-enhanced contrast applications are usually distinctly different from low light level applications, which require electronic detectors to render visible images generated by few photons (9,22,32,40,46,47). In video-enhanced DIC microscopy, high light levels are used to provide diffraction-limited image detail at video frame rates, using high resolution video cameras which have much lower sensitivity than intensified video cameras. Ideally, cameras with 800 TV line resolution, low lag, low shading, high image stability, high linearity and superior signal-to-noise characteristics are desired. This last parameter is most important for temporal resolution. We have found that research-grade cameras using Newvicon tubes give satisfactory performance (DAGE/MTI models 67 and 70 with select Newvicon tubes and the Hamamatsu model C2400).

Digital Image Processor

Image processing speed and algorithms are application dependent as are methods of image recording and retrieval. We needed high temporal resolution in our microtubule studies, so we chose image processing equipment which could perform background subtraction and exponential averaging algorithms at video rates (30 frames/sec). This required image processing hardware with at least three framestore boards and the equivalent of three arithmetic logic units, in addition to the A/D and D/A framegrabbing and lookup table hardware which is available in most image processing systems. We use Max Video Image Processing boards (Datacube, Peabody, MA) housed in a VME-bus computer (Model CPU-6, Force, Los Gatos, CA). Many of the less expensive image processing boards take several frame times to do the multiplications and image manipulations which our system does in one frame time. Our system requires a host computer, which provides the facility for image analysis in other

Figure 6. Flow diagram of a real-time digital image processor. An arithmetic logic unit (ALU) subtracts a previously obtained background image (FSBG) from each incoming (every 33 msec) live video frame and sets the average background intensity at 128. The resulting frame is then averaged with a previously obtained frame (FSb) and stored in FSd. For digital contrast enhancement, the image in FSb is passed through an output look-up table (OLUT), which converts the grey scale values of each pixel according to the function stored in the OLUT. To accomplish these operations in real time, we use Max Video Image Processing boards (2 MAX-SP, 1 FRAMESTORE, 1 DIGIMAX; Datacube, Peabody, MA) housed in a VME-bus computer (model CPU-6, Force, Los Gatos, CA).
applications like fluorescence microscopy. For straight video processing, there are now available devices, such as the Hamamatsu DVS 3000, which have the hardware to do our real-time algorithms at a push of a few buttons.

**Recorder and Monitors**

We record most of our data on Sony VO 5800H 3/4-inch U-Matic videocassette recorders. These monochrome machines have about 450 TV lines of resolution, they are durable and they have excellent editing capabilities. The new Super-VHS and extended Beta 1/2-inch videocassette recorders provide similar horizontal resolution, are less expensive, but their durability is unproven. We use the Panasonic TQ-2028F optical memory disk recorder (OMDR) for time-lapse recording and single frame analysis. It is much easier to measure particle movement between frames with the OMDR than with video tape. The relatively inexpensive Panasonic model WV-5410 13” monitors do an excellent job, provided the horizontal and vertical deflections are adjusted properly (22).

**APPLICATIONS OF VIDEO-ENHANCED DIC MICROSCOPY**

The video-enhanced contrast methods described here for DIC microscopy have been able to reveal new information about a variety of dynamic processes in cells and in reconstructed preparations in vitro (22,32,40). More recent research applications in cell biology include: The cell surface membrane and cortical cytoskeleton assembly and translocation which occur during tissue cell motility (14, 15), endocytosis (11), cytoplasmic streaming in plants along the endoplasmic reticulum and actin filament cables (1,31), intracellular organelle transport along microtubules (5,19,41,43), the dynamic instability of microtubule assembly in living cells (8) and in reconstituted preparations in vitro (44,45), the formation of networks of endoplasmic reticulum driven by microtubule-dependent force producers (10), the identification and biochemical characterization of the microtubule dependent translocators, kinesin and cytoplasmic dynein (42), and the dynamic rearrangements in cell structure which occur during mitosis and cytokinesis (7,22).

A major advantage of video-enhanced DIC is the ability to record the dynamics of individual macromolecular assemblies at video frame rates. This has had a revolutionary effect on advances in membrane and cytoskeleton dynamics, particularly for microtubules. Video-enhanced DIC methods have been used to see the fast transport of unresolvable vesicles and other organelles along microtubules within living axons and individual...

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Figure 7. Microtubules serve as polarized roadways for vesicle transport driven by translocator molecules (e.g., kinesin, dynein (42)). Motility can be characterized by allowing the translocators to adhere to the glass surfaces of a slide-coverslip chamber. When microtubules are added they will attach to the translocators and be driven across the glass surface. Video-enhanced DIC microscopy provides the means to both visualize and record microtubule translocation which occurs at rates between 0.5 - 10 μm/sec depending on physiological buffer conditions and translocator species. The example shown here is microtubule gliding produced by sea urchin kinesin (36).

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Figure 8. Microtubule polymers assembled from purified tubulin exhibit alternating phases of elongation and shortening. This dynamic instability behavior is easily observed using video-enhanced DIC microscopy (44). Image processing at video frame rates is necessary to accurately establish rates of tubulin subunit association and dissociation, and the frequencies of transitions between the elongation and shortening phases.
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microtubules in extracts of axons (reviewed in 42). It turned out that the protein ATPases, which propel vesicles along microtubules, kinesin and cytoplasmic dynein, are active when stuck to a coverslip surface and propel isolated microtubules over the surface at the 0.5-10 μm/sec velocities typical of fast axonal transport and microtubule-dependent transport in non-neuronal cells (Figure 7). This gliding activity, seen by video-enhanced DIC microscopy, provided for the first time the functional assay needed to purify these ATPases from cells (42). Video-enhanced DIC microscopy has also provided the functional assay needed for biochemical studies of how these molecular translocators use ATP to drive vesicle transport along microtubules (42). Particle and microtubule translocation can be recorded at high magnification and the kinetics of movement determined using computer-assisted image analysis methods (36, 39,42). In addition, the high contrast enhancement produced by video-enhanced DIC has allowed the development of image analysis methods which can measure particle position with nanometer precision (17,24,39).

Our primary use of video-enhanced DIC microscopy has been for measurement of the biochemical parameters of microtubule assembly (35,44,45). Microtubules are novel polymers in that they exhibit dynamic instability (Figure 8). Following nucleation, they elongate by net subunit addition to their ends for a random period, then abruptly begin rapid shortening by subunit dissociation from their ends until they either abruptly revert to elongation or disappear altogether (Figure 8). Because the phase transitions are stochastic and asynchronous between different microtubules, measurements of the life-history of individual microtubules using a technique like video-enhanced DIC microscopy are necessary to accurately obtain the rate constants and transition frequencies of dynamic instability (44). This technique also provides an assay for exploring the biochemical basis of instability by manipulating solution conditions using perfusion chambers or by physically manipulating microtubules using UV microbeam irradiation (45).

The methods for video-enhanced contrast presented here for DIC microscopy also apply to other modes of bright field microscopy where optical contrast is generated by either absorption (11,22,27), phase contrast (22,25,37,48), birefringence (7,22,25), dichroism (22,27), oblique illumination (12,20,30) and interference reflection contrast (28,29).

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REFERENCES


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