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The Video-enhanced Light Microscope: A Renaissance Tool for Quantitative Live-cell Microscopy

Charles E. Bracker
Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907-1057, USA

The in vivo dynamics of living cells, organelles, and other moving objects on a microscopic scale are especially amenable to study and quantitative analysis by video enhancement and real-time digital image processing of high magnification light microscopic images. Dynamic events in cell growth or other processes can be tracked and measured with unprecedented precision and efficiency. Videotaped images of cells growing in thin slide culture chambers are spectacular, and the results dramatically bring to life developmental processes at high resolution that are impossible to imagine, interpret, or accurately quantitate from still images. The processing of these images permits them to be measured precisely and analyzed. The magnification required as the resolution limit of the microscope is approached is in the range of 5,000 x to 20,000 x on video display monitors. While such magnifications necessarily involve empty magnification, the enlargement is essential for detecting positional changes in the 1-2 pixel range between video frames when using a microscaler or the digital measuring function of an image processor.

Images obtained through a video camera are processed before they are displayed on a monitor or recorded on magnetic storage media. Real-time digital image processing simulates the video-enhanced contrast championed by Robert Allen for DIC microscopy of living cells, microtubules, etc. and permits the ready detection and display of delicate objects that are well below the size range for Abbe resolution. This processing allows background equalization to remove optical irregularities, enables brightness control, provides gamma control and contrast stretching to optimize image contrast for parts of the object that are of greatest interest, and it permits pseudocoloring to discriminate specific gray levels. Real-time application of digital filters also allows special effects to be recorded in real time. Videotaped images can be analyzed frame by frame to precisely locate and plot the displacement rate, trajectories, and motion patterns of objects that are either moving or changing size and shape during a video sequence. Successive freeze-frame images obtained through a frame grabber can be subtracted from each other to reveal difference images which reveal patterns of dynamic change during a particular time interval.

Traditionally performed with differential interference contrast optics (DIC), video enhancement is especially effective with phase contrast images of living cells. Phase contrast often reveals more information about the specimens than does DIC because it reveals differences and dynamic changes in refractive index, and it displays information in shades of gray that are related to differential refractive index within the specimen. Thus, images that are otherwise revealed as undifferentiated "pseudo bumps and depressions" in DIC images, appear distinct from each other for more precise identification and characterization when phase contrast optics are used. Delicate objects in living cells, which may appear undetectable to the human eye in DIC images, can be seen, tracked, and measured with enhanced phase contrast. To take best advantage of phase contrast optics, cells should be mounted or grown in a medium with its refractive index adjusted to a value similar to that of the specimen, to minimize the distracting phase haloes at the boundaries of cells.

Measurements of motion or growth near the limits of optical detectability require appropriate compromise among competing variables to assure that true displacement is being measured and that artifacts of the measurement technique have been avoided. This requires attention to ac-

ceptable noise levels in digital images, and selecting appropriate time intervals between measurements in relation to pixel size and displacement rate. Without such precautions, non-linear displacement patterns can be corrupted artifactually to disclose artifactual changes in displacement rate or suppression of rate changes, both of which lead to erroneous conclusions about motion or growth.

The detection limits in low-noise images approach single pixels which represent image units as small as 30 nm. When gray-scale images are converted to pseudocolor, the positions of specific gray-scale values are obvious to the unaided eye and can be used as objective markers for locating and measuring positions of an object in successive images. This is especially evident for living fungal hyphae whose growth can now be measured at one-second intervals, roughly two orders of magnitude faster than was practical with prior methodology.

This advantage has permitted measurements that reveal cell elongation in altemating fast-slow pulses with periods ranging from 3 to 30 seconds depending on the organism and its overall cell elongation rate. Individual organelles can be tracked, their motion plotted, and dynamic processes interpreted from the displayed data in ways that were not previously possible.

One of the problems encountered in video microscopy is the potential image distortion caused by electronic devices in the chain of components leading from the original optical image created by the objective lens to the final image that is displayed or printed (e.g. frame grabbers, video monitors, printers, image processors, cameras, etc.). Further, because of the nature of video imaging, point-to-point resolution in a final image may differ from that obtainable by the optical system alone and will differ in the X and Y axes. Microfabricated test targets are being developed to: a) routinely record and measure resolution in X, Y, and Z axes, b) to judge detection capability in X and Y, c) to measure image distortion, and d) to establish accurate size calibration, by recording phase and/or amplitude test objects from the test targets onto videotapes under specific conditions of microscope alignment and operation. An angled ramp with microfabricated resolution patterns etched onto its surface yields a simple and direct measure of depth-of-field under any conditions of microscope alignment. This provides a simple means of accurately measuring the thickness of in-focus optical sections when it is necessary to acquire images of specimens at known focal intervals for three-dimensional reconstructions without overlap or missing information. These targets will function as routine calibration tools and as test targets for critically evaluating the capabilities of optical and electronic devices used in the total microscope technology package.

Another problem important to quantitative studies is artifactual drift, or creep, of the specimen image. This is caused by thermal drift of microscope parts as the instrument warms up after the illuminator and other electrical elements are turned on. Typically, the rate of drift is greatest in the Z axis (reaching more than 30 μm in 2 hrs.) and least in the X axis, and is influenced by the construction, design, and brand of the microscope as well as the time when the illuminator was turned on. Thermal drift declines to a minimum about two hours after the illuminator is turned on but still continues at a measurable rate that cannot be controlled with conventional microscope design. A robotic auto-tracking stage can be programmed to track several objects within the image independently, and when the drift of stable markers is automatically subtracted from the total motion of target objects, net displacement due to true motion or growth of the object can be plotted.

A recent development, which will be available in the near future, is a robotic motion and/or cell growth tracking stage for light microscopes that will automatically track moving objects in real time, in three dimensions, by analyzing the trajectories of objects identified in successive video images. The concept instrument will provide focus tracking as well as automatic X-Y tracking and data recording for displacement of the target objects. This technological advance turns the microscope stage from a relatively simple mechanical device for adjusting the position and focus of the object, into a sophisticated analytical tool for studying quantitative aspects of cellular dynamics at a level of accuracy, and with a response time, that lie beyond the capability of human control of a manual or motor driven stage. By tracking object(s) over a coordinate field that is many times larger than the window in view at any one time, tracking is seamless, and continuous data streams are not corrupted when the stage translates to track an object as it moves from one field of view to another.

The convenience and power of the modern video-enhanced microscope system has made it possible to quantitatively analyze patterns of cell morphogenesis as they are influenced by behavior of subcellular structures. These include coordination between the positions and migration rates

of secretory vesicles in fungal cells and sites of localized cell expansion and wall growth. But the technique is applicable to any object which changes its position, orientation, or size during observation. This approach has also provided an unprecedented opportunity to merge microscopy of real cell dynamics with computer simulations created by computer-driven cell growth models and to quantitatively compare predictions of the models with actual images of growing cells.