Keeping Life in Focus
New Systems Prevent Z-axis Drift in Time Lapse Studies

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Just two decades ago, life scientists studied biological structure, developmental anatomy and intracellular processes by describing individual snapshots of kinetic events. Today, with so much bioscience research focusing on dynamic processes that occur on the molecular, cellular and whole organ level, it is important to record events as they happen, over seconds, minutes or hours, in living cells. Photographs and camera lucida drawings of fixed, stained cells have given way to live cell imaging using fluorescent probes, warming trays to promote cell viability and cinemicrography as a method of recording events.

The increasing use of time-lapse imaging in optical microscopy is evidenced by the numerous reports in the literature of cellular activity monitored in tissue culture using fluorescence labeling techniques coupled to time-lapse sequence capture. Contrast-enhancing techniques such as differential interference contrast, phase contrast, and Hoffman modulated contrast can be used for recording a wide variety of specimens with time-lapse sequences. Time-lapse imaging can also be used to study crystal formation in biological, chemical, and geological systems, liquid crystalline phase transitions and structural analysis of new materials in metallography. In the life sciences, time-lapse photomicrography has been an important tool for studying protracted particle motion, cell migration, cell division and neurite outgrowth. The development of green and other fluorescent proteins has resulted in the discovery of countless cellular and intracellular events that can only be described by observation over time.

History

The first sophisticated time-lapse sequences were recorded when 16-millimeter movie cameras were attached to the microscope. Intervalometers were used to control the time interval between sequentially acquired images while densitometers were used to turn the illuminator on and off automatically to avoid overheating the specimen. These techniques were dependent on outside photo processing, which meant that the results of an individual experiment could not be immediately evaluated. In addition to being time consuming, outside processing was also costly and subject to inconsistencies caused by variability in film processing quality. In addition, the experiments were often compromised by slowly drifting stages that produced focus changes, inaccurate film exposure times, differential heating of the specimen, and vibrations originating from a variety of sources. Many of these problems still plague modern investigators who attempt to conduct time-lapse studies using digital equipment.

Another major advance in time-lapse cinemicroscopy occurred when video cameras and frame grabber boards were first integrated onto the microscope. Although the images produced by video cameras were lower in resolution than film, the video camera was immune to some of the exposure problems associated with film cameras. Moreover, the results of an experiment could be reviewed immediately by playing back videotape on a video recorder. Finally, the overall cost of an experiment could be reduced because film processing was eliminated and videotapes could be reused and easily duplicated.

As video time-lapse technology matured, real-time image processing electronics were added to the ensemble to enhance the overall appearance of image sequences. Software was later added to the mix, and the technique of background subtraction emerged to further enhance image quality. Quantitative imaging tools also came into wide use, making it possible to deal with the large amounts of temporal and spatial image data coming out of time-lapse image sequences. Video-enhanced differential interference contrast (DIC) microscopy enabled scientists to record structures not previously visible in the optical microscope. For example, the intracellular movement of microtubules was observed for the first time using such technology.

Simultaneously, still imaging has continued to be a widely used method of documenting changes in cells over time because of its advantages in resolution. The technological development of the personal computer has catapulted time-lapse cinemicrography to a new level of sophistication. RAM, cache and hard drive capacity are no longer limitations; today, complex image sequences can be captured for hours and stored locally on the host computer. In fact, gigabytes of data may be collected and displayed on the desktop. Hardware interfaces, once limited in number and in bandwidth, have evolved into high-throughput plug-and-play links capable of controlling stage motion (*x, y,* and *z*), illumination intensity, wavelength and support systems that promote cell viability. Even the simplest PC can control a scientific grade digital camera via IEEE interface.

Advanced fluorescence techniques, supported by developments in CCD technology, enable investigators to observe events utilizing a wide spectrum of fluorescent molecules and proteins targeted at specific cellular organelles and receptors. Multiple events can be simultaneously recorded in four dimensions (laterally, axially, temporally and spectrally) to provide a complete view of intracellular activity over a period of time. These techniques have helped to lead the current revolution in cell biology, which is providing scientists with dynamic information that was previously unavailable with older and less sophisticated methodology.

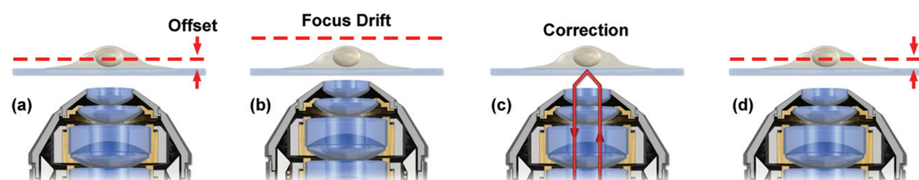

Figure 1. Images a-d show how focus drift compensation systems that take advantage of the reflection of light from the coverslip operate. A reference image is used as the basis of compensation (1a). If drift occurs (1b), the detected intensity of reflectance from the coverslip provides the information required for compensation (1c). The system compensates for the drift detected at the coverslip, and then offsets to the predefined value of Z, providing sharp focus for the specimen (1d). Figures 1 and 3 courtesy of Michael W. Davidson, The Florida State University, Tallahassee, FL.

Focus Drift Key Problem

In spite of the all the developments that have occurred in cinemicrography, axial fluctuation during the course of time-lapse imaging remains a complex problem. The

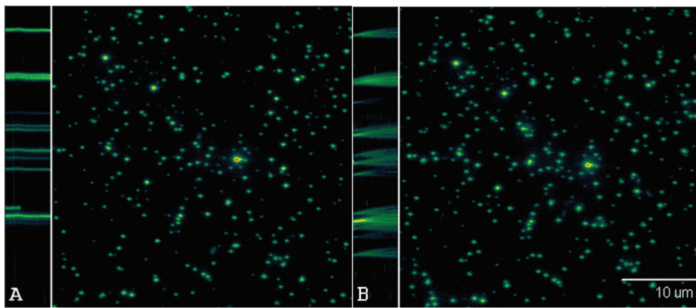

Figure 2. Kymography is a way of recording and measuring motion, most commonly used to show undulations of muscle activity or to show seismic activity during earthquakes. Here, kymography is used to show relative focus stability with and without Zero Drift focus compensation. Time lapse images of sub-resolution microspheres were captured with their corresponding kymographs (column to left of each image). The beads were imaged via total internal reflection with Zero Drift compensation enabled (A) and disabled (B). Individual images were acquired with the 60X 1.45 TIRFM objective mounted on the Olympus IX81-ZDC inverted microscope every 2 minutes for 8 hours using SlideBook (Intelligent Imaging Innovations) and a Hamamatsu 9100-02 (electron multiplication) CCD camera. When Zero Drift was enabled, the spatial position of the beads remained fixed, concentrated and undistorted as illustrated by the kymograph in Figure XX-A. With Zero Drift disabled, the spatial position over time decayed, resulting in distended kymographs that decayed in intensity over time, as shown in the kymograph in Figure XX-B.

inability of a microscope to maintain focus over time is called focus drift. Focus drift occurs independent of living specimens' movements. There are four main contributors to this axial flux:

- Thermal drift. As the temperature of the room or the instrument changes, focus drifts. Different rates of expansion and contraction in the specimen vessel and microscope components result in the distance between the objective and specimen changing, leading to a loss of focus. In fact, a change of just one degree Celsius may be enough to cause a drift of up to a full micron in focus. At 40× or 60× magnification, one micron of drift may be enough to bring the observer to a completely different part of the cell. This can be largely compensated for with environmental enclosures that insulate the microscope from its external environment and also promote cell viability. Unfortunately, these thermostatically controlled systems need to encompass not only the specimen but also the entire microscope in order to be effective. An objective heater and stage-top incubator might be used, but these devices are not always compatible with immersion-dependent objectives.
- System mechanics. Gravity, or the force that a fully loaded objective turret places on a focus mechanism, is one of the chief causes of focus loss. While gravity always wins, its affects may be addressed by placing fewer objectives on the turret during an experiment or ensuring that the microscope has a precision focusing system with a short mechanical length.
- Immersion media. Microscope objectives that are used in time-lapse experiments may require oil, water or glycerin. Over time, gravity and temperature may affect the properties of these immersion media. Oil viscosity may change or its refractive index may vary with temperature; water may evaporate or its surface tension may be reduced. Any one of these changes can lead to focus drift. One way of addressing this problem is to use a gasket that forms a seal between the objective and the coverslip.
- Vibration. This can be caused by electromechanical elements within the experimental setup, as well as by sounds or imperceptible motion in the environment, such as that by building elevators, air handling systems, foot traffic or heavy equipment. Isolation tables and similar vibration reduction systems can help resolve this problem.

Compensation systems

Traditionally, software autofocus routines have also been engineered into a variety of scientific imaging packages to help further correct for drift. These solutions rely heavily on edge-detecting algorithms used on images collected with the camera. While they can be effective tools for transmitted light imaging, they are considered even better suited for fluorescence imaging because of the stark contrast between a fluorescently labeled specimen and its background. Herein lies one of the chief shortcomings of the software-based compensation methodologies: in order to calculate focus, the system must first capture several images of the fluorescent specimen. However, photobleaching and phototoxicity increase each time the cell is exposed to light. In addition, software-based solutions are slow. A single routine can take up to as long as six seconds, depending upon how many wavelengths are being examined, how bright the emission wavelengths are, how much of the CCD is being read out, and how fast the camera's digitizer clock operates. Finally, software routines are not foolproof. While many routines might apply Boolean logic, they are contrast-based operations, and remain subject to reporting false-positives.

A number of manufacturers have recently introduced hardware solutions to further help prevent focus drift. Each solution is based on the premise that the relationship between the cell and the coverslip is fixed. That is, live cells are not freely floating. They are usually attached to the coverslip via laminin, poly-lysine or other glycoproteins. Each system finds the external coverslip face by measuring the reflectance of a weak infrared (IR) laser.

One system measures the reflectance of the IR beam via total internal reflection fluorescence (TIRF). This solution works particularly well for investigators studying dynamic events using high numerical aperture (NA) objectives. This solution can also be retrofitted to any microscope stand, including non-motorized

Figure 3. The Olympus Zero Drift IX81-ZDC can handle focus drift compensation using objectives with either high or low numerical apertures.

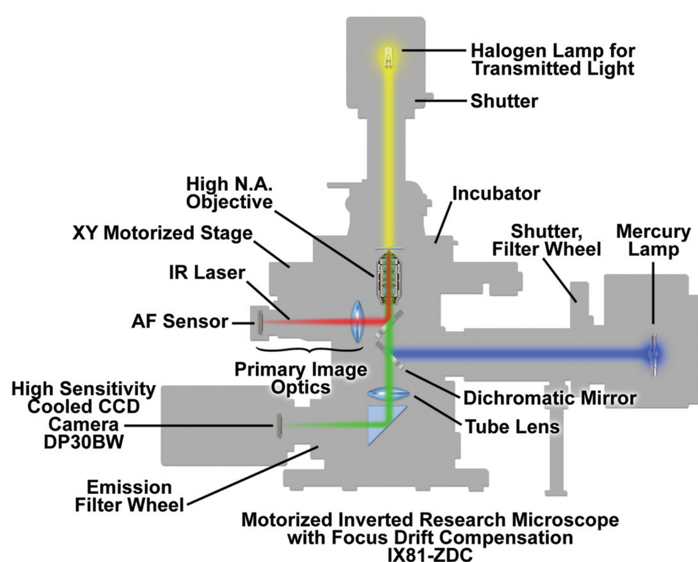

Figure 4. Typical configuration for an inverted microscope equipped for focus drift compensation.

microscopes. However, measuring reflectance via total internal reflection has its limitations. Low magnification or water immersion objectives that have lower NAs cannot be used because objective NAs must be higher than the refractive index of the specimen (typically 1.33-1.38 for live cells) in order for proper measurement of reflectance to occur. These systems are usually limited to oil immersion optics with NAs of 1.4 or higher.

Other companies have introduced focus drift compensation systems that take advantage of the natural reflection of light from the coverslip. These systems operate by comparing a "current image" with a "reference image." In most of these systems, the reference is reflectance from the coverslip whose intensity is measured by a CCD. Based on the intensity information acquired, the microscope compensates for thermal and mechanical drift to the coverslip, then offsets to some user-defined value of Z, providing sharp focus for the specimen that exists at a set distance from the surface of the coverslip. These systems can correct for drift in milliseconds – a distinct advantage over protracted software-based autofocus routines. Drift compensation timing can be defined by software, intimately synchronizing the compensatory operation with the acquisition of the image and peripherals such as wavelength switchers and motorized stages.

One such system, the Olympus Zero Drift IX81-ZDC, is integrated into an Olympus IX81 motorized microscope or FV1000 laser scanning confocal microscope. Both low and high magnification objectives can be used with the Zero Drift microscope for focus compensation. Working distances range from 150 microns to nearly 7 mm. The near-infrared laser used by the Zero-Drift system is introduced through its own special ray path so that all of the microscope's light path can be used for imaging.

While all of the drift compensating systems currently available are designed to measure reflectance from the coverslip, the various focus drift systems have a number of differences, providing a multitude of choices for researchers doing long-term time-lapse imaging. First, there are substantial price variations among the various companies' offerings, so scientists have considerable flexibility in configuring a system to their needs. Some companies provide a solution that may be retrofitted for use on an existing motorized microscope, while one solution can be retrofitted for use with non-motorized inverted microscopes. Still other companies provide unique, fully integrated fixed products of the LED/laser feedback type.

Another important difference is the capacity of various systems to handle complex imaging of multiple features at different *x, y,* and *z* locations within the confines of a single experiment. For instance, the Olympus Zero Drift system has the flexibility to handle complex sequences at each sequential time interval, including changes in *x, y,* and *z* locations for every point photographed, at every time interval, all within one experiment.

Still another difference involves how frequently the various systems' feedback loops "retune" focus. Some products offer a "continuous" compensatory mechanism, while others go through the autofocus process immediately before each image is captured. There are advantages to each type of approach. If the experiment involves real-time observation, then the devices using continuous feedback mechanisms may be more desirable as these systems will correct for drift independent of a software prompt. But for experiments where capturing images is the key consideration, image capture must be synchronized carefully with the measurement of the reference point, which is done each time focus is evaluated to determine if drift has occurred. In typical time-lapse scenarios where there is a significant time interval between image captures, the short interval required to measure reflectance and then offset to a *z* point above the coverslip is inconsequential. When collecting streams of images in rapid bursts of 30 frames per second or more, there is no compelling reason to believe that any drift compensating system would be advantageous. A continuous feedback system, for example, might not be able to complete the compensatory process between every exposure. In fact, there could be a risk of capturing an image while the system is still searching for the reference plane.

Another variable to consider in selecting between continuous and capture-driven focus compensation is total experiment time. Time-lapse experiments can last a few seconds to hours to days. For experiments that last several hours or days, continuous feedback systems themselves might be subject to their own mechanical drift, particularly due to hysterisis, and, in the case of systems that rely on piezo actuators, creep. Of course, for short-term experiments the effects of hysterisis or creep are negligible, but over protracted periods of use, their contributions could be untoward.

Defeating the problem of focus drift is a key consideration to be taken into account during configuration of an experimental system designed for time-lapse imaging. The problem is even more acute when using high-NA objectives where the narrow depth of focus requires very tight focal specifications. It is also a considerable challenge when imaging a complex sequence of *x, y,* and *z* points within the specimen at every time interval. Thermostatic controls, environmental and incubator chambers, lighter-weight objectives, a short nosepiece focus mechanism, isolation tables, and the new focus drift compensation systems each have an important place in making time-lapse imaging work for microscopists. ■

Acknowlegement

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