Digital Mammography Update

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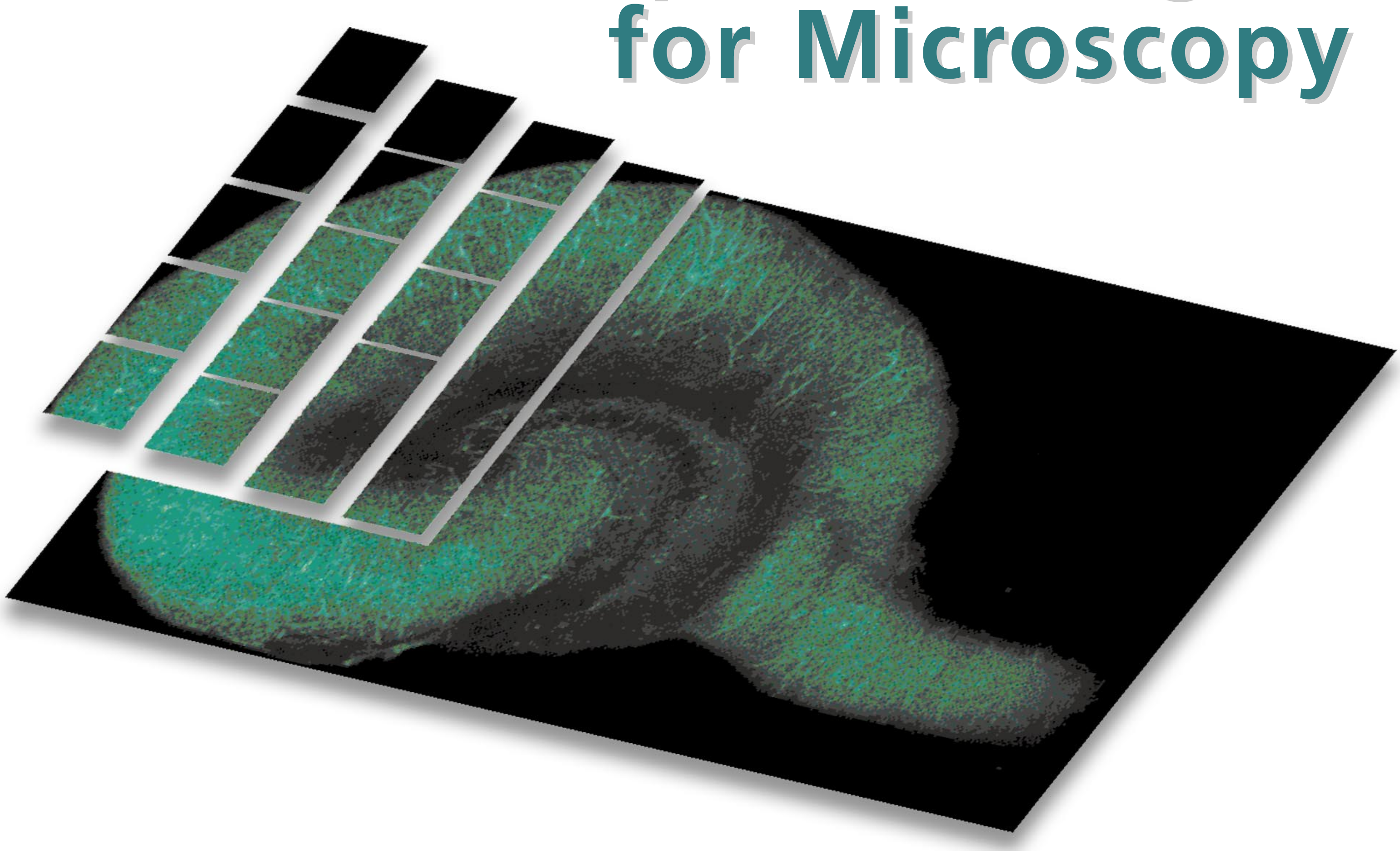
Micropositioning for Microscopy



Ultrafast Lasers Shrink

DISCOVERY Through Automation

by John Zemek, Dr. Colin Monks and Dr. Ben Freiberg

SUMMARY

Submicron positioning stages with resolutions in the 20- to 50-nm range and repeatability accuracies as low as 200 nm can be combined with powerful imaging software to make your microscope more effective.

Automated stage motion control is critical in high-performance microscopy techniques that require motion accuracy or speed that cannot be matched by human control. These techniques include six-dimensional imaging; montage imaging, or stitching; stereology; and automated multiwell plate screening.

To perform six-dimensional imaging, three-dimensional data (X-Y-Z) is collected across time at different fluorescent wavelengths or imaging modes and at different spots on an X-Y stage. Imaging in six dimensions is essentially multiplexing of 4-D multiwavelength imaging. It allows the collection of a vast amount of data from living cells during a single collection sequence. Researchers use this technique because biological conditions can be difficult to reproduce when working with living cells. Multiplexing allows them to perform robust statistical analysis because many examples of a condition are collected at the same time. Revisiting the same X and Y positions at multiple time points requires precise and rapid automated stage motion.

Montage imaging, or image stitching, is the seamless collection of data that is larger than the frame size of a single image (Figure 1). It is critical for constructing high-resolution images of tissue or exhaustive sampling of an individual biological sample. This involves revisiting the same site again and again to look for changes that the cell may be undergoing. Such changes may include growth in neurons, or changes in the physiology of the cell, such as calcium-ion concentrations used for intercellular communications. Accurate automated stage movement is required to move the correct distance between frames.

Stereology involves sampling, or counting, a specific type of cell so that an unbiased statistical analysis of an event can be made. For example, if a scientist is studying a neurological disorder and wants to statistically analyze the number

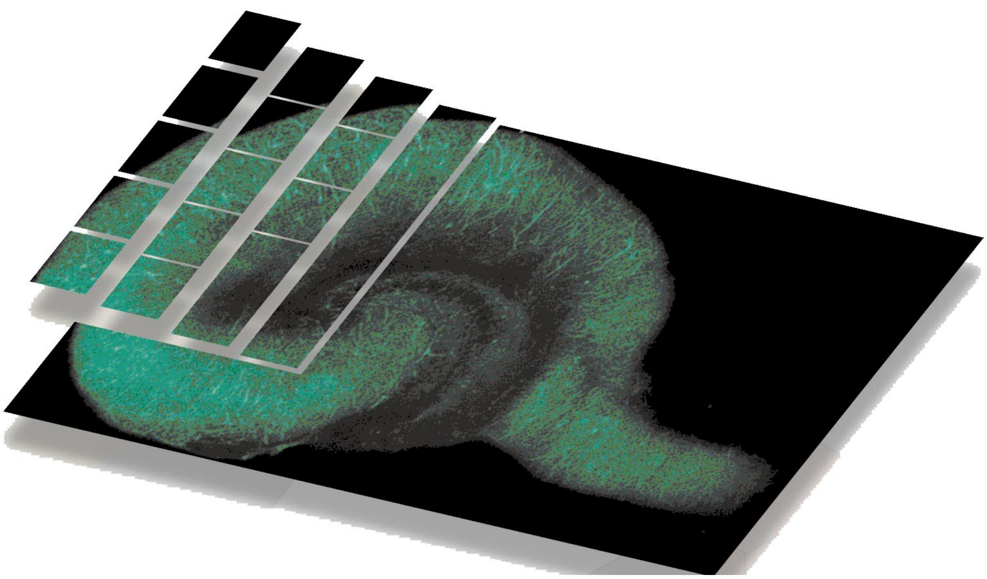
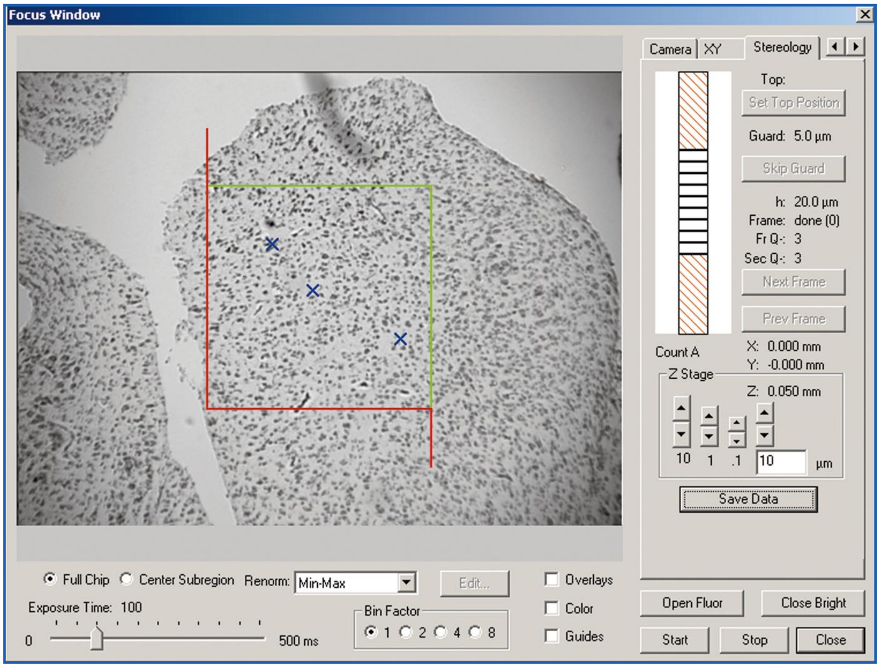


Figure 1. Montage imaging of this fluorescence-labeled rat hippocampus allowed data to be collected from an area larger than the frame size of a single image. Accurate automated stage movement is required to move the correct distance between frames in this technique. The final image consists of 80 individual frames stitched to form a seamless picture. Courtesy of Karl Kilborn, Intelligent Imaging Innovations Inc.

Figure 2. Stereology is a technique that allows counting of a specific type of cell for statistical analysis. Because software such as this from Intelligent Imaging Innovations determines the locations for scoring, precision X-Y-Z motorization ensures that counts are statistically unbiased.



of neurons in a brain, he can take multiple samples from the brain, prepare them and mount them on microscope slides. The stereology program will pick a random area of the sample to examine, and the researcher will count the number of neurons present. This is done to a number of samples, and because the exact volume/area of the random samples is known, the number of neurons within the complete volume of the brain can be statistically projected. Because a computer determines the exact locations within a sample that will be scored, it must control the position of the stage (Figure 2).

Automation of multiwell-plate imaging has become the basis of many biological screening procedures. Accurate and rapid automated stage positioning allows the same spot in each well to be imaged.

Many applications that can benefit from automated micropositioning systems in microscopy demand similar requirements from their motorized equipment. Some important specifications are repeatability, resolution and speed.

Repeatability is related to the accuracy of a micropositioning system. This specification should be clarified as either unidirectional or bidirectional. Unidirectional repeatability is the ability of the stage to return to a given point, always coming from the same previously defined direction. Bidirectional repeatability is the ability of the stage to return to a given point from any random previous point. The value for each should be the standard deviation of many moves to give a solid statistical specification.

In many applications, such as multipoint capture, multiwell screening and stereology, the stage must move precisely to a given X-Y coordinate. When imaging cells at high magnification, small shifts in positioning can lead to huge shifts in the location of a cell in the field. For example, if a user is trying to image many cells in multiple fields over time, it is vital that the motorized positioning system locate the same fields repeatedly. Analysis of images that have poor alignment due to poor stage repeatability is difficult be-

Glossary of Micropositioning Terms

Antibacklash nut — A nut that is spring-loaded, or held under tension, in some manner to prevent lost motion, or backlash, when the direction of travel is reversed.

DC stepper motor — A motor that moves a given amount when a DC pulse is applied to it. Typical stepper motors offer 200 to 400 steps per revolution, but the devices can also be microstepped to get finer resolutions. Stepper motors are usually used in an open-loop system that does not employ feedback encoders but, rather, relies on counting the pulses sent to the motor for positioning information.

DC servomotor — A motor that moves a given amount when a DC voltage is applied to it. Unlike a stepper motor, it has an infinite resolution. However, servomotors must be used with an encoder to provide positioning information.

Encoder — A device used to measure movement by counting pulses. The encoder can be optical and count pulses, via a grating etched in glass or plastic, or magnetic and count a magnetic field. There are two basic types, those that measure rotary movement and those that measure linear movement. Depending upon the resolution of the scale and the interpolation used, encoders can measure movement in the nanometer range.

Leadscrew — A precision ground screw that is machined with a constant known pitch. If the leadscrew is machined to exacting tolerances, a nut attached to it will travel an exact linear amount plus or minus the tolerance of accuracy when the leadscrew turns. An antibacklash nut is usually used with the leadscrew to prevent lost motion when the direction of travel is reversed. □

cause objects may be in vastly different locations at subsequent time points. To take this one step further, if the user wants to measure how much the cells are moving in different fields, repeatability becomes even more critical because errors in stage positioning will directly affect the distance the object apparently travels.

The resolution factor

The resolution — the smallest step size that the stage can move in a repeatable manner — is also extremely important to assess when purchasing micropositioning equipment. For example, if a scientist wanted to image a cell 100 µm in length and the image field captured only 50 µm of the cell at a time, a minimum of two fields would be needed to capture the entire cell. If the X-Y resolution of the stage were 100 µm, it would be impossible to image the entire cell.

However, if the resolution were 25 µm, the entire cell could easily be imaged. At an even finer resolution, the stage could be positioned with even less overlap between subsequent images. One thing to remember is that the resolution of micropositioning equipment is usually less than its repeatability. Therefore, it is essential to look at both numbers when evaluating devices.

The time it takes a stage to move between fields can determine whether a user observes or misses a phenomenon. Stages with a high degree of repeatability and resolution often sacrifice speed. Moving quickly and achieving high precision is difficult and is a major trade-off in micropositioning equipment because, in some systems, the pitch of the leadscrew determines the resolution of the stage. The finer the pitch, the finer the resolution and the slower the stage will move, because it takes many more turns for the leadscrew to cover the same area than with a coarser-pitch leadscrew.

Speed is also important in many screening applications. Faster movement of samples leads to higher-throughput systems. When imaging multiwell plates, speed becomes compounded between each well and each plate. At the end of the day, small differences in speed can mean the difference between capturing hundreds of images or just a few dozen.

Other major factors to consider include feedback, motor type, encoder resolution and backlash.

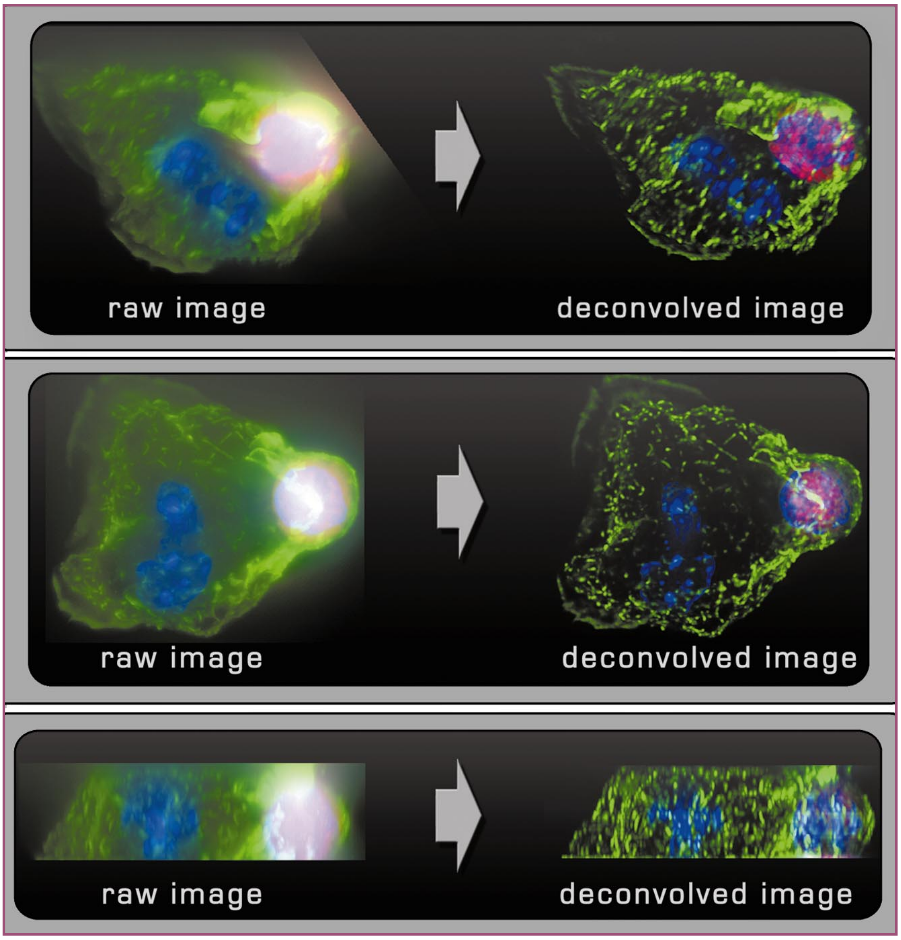


Figure 3. Precision Z-motorization allowed this 3-D triple fluorescent image of a macrophage engulfing an apoptotic cell. Courtesy of Jennifer Monks, National Jewish Hospital, Denver.

Closed loop, open loop

Closed-loop systems differ from open-loop systems in that the former use an encoder to feed back the position of the stage or fine focus shaft of the microscope. For example, in a microscope with a closed-loop automated Z-axis drive, the stage can be commanded to move 1.5 µm toward the objective. The command is converted into a DC voltage that powers a DC servomotor attached to the fine or coarse focus shaft. A high-resolution encoder allows the control electronics to know when the stage or the shaft has actually moved the 1.5 µm, and then the control electronics can tell the motor to hold its position. However, an encoder can add substantial cost.

An open-loop system uses no feedback. Instead, a DC stepper motor's controller keeps track of position by counting the pulses it sends to the motor because they usually result in the proper linear movement of the Z-axis drive or X-Y stage. Stepper motors are commonly used in low-cost positioners, but if something binds the stage's leadscrew or the stepper motor's shaft, motion is lost and the system can lose its true position.

Systems that utilize closed-loop feedback are usually more precise than open-loop systems because the device ensures that the commanded position is reached. DC stepper motor systems can be operated in either open- or closed-loop mode. To obtain fine resolutions, they are usually stepped in minute increments. DC servomotors, on the other hand, have a virtually infinite resolution and are almost always used with a feedback encoder to provide a precise closed-loop design.

The resolution of the encoder determines the accuracy of a closed-loop system. Encoders used in microscopy appli-

The Number Game

A number of manufacturers offer automated X-Y stages, and each publishes resolution, repeatability and accuracy values for them. Many factors affect positioning accuracy, so the actual specifications for each stage may vary slightly from the published specifications.

Most manufacturers use DC stepper motors in microstepping mode to control the movement of their stages. Because stepper motors are usually used in an open-loop configuration, it is important that the stage be well-designed and thoroughly tested to ensure accuracy and repeatability. In open-loop systems, mechanical wear or contaminants on the bearing surfaces can affect accuracy. Linear encoders in combination with DC servomotors can provide a very accurate system.

For precision stages, quality control testing should be performed using high-resolution equipment such as laser interferometers, or other test jigs such as optical imaging systems. In the latter, a CCD camera is controlled by software to take measurements from the images. One can measure positional accuracy of less than 20 nm.

The target can be any diffraction-limited sample, such as a speck of dust. The position of the center of mass of the speck image is simply measured after various move commands, and the statistical data stored in a file for the particular stage. This data can be used to compare the accuracy of the feedback device for a closed-loop stage or the accuracy of the stepper motor for an open-loop device. The raw data and statistical information for each stage should be available to the customer. □

cations measure either rotary or linear movement. Rotary encoders measure the position of the microscope's focusing shaft for Z-axis movement, the position of the motor shaft or the leadscrew for X-Y stage positioning. If properly placed on a particular microscope or stage, they can provide a resolution from 0.1 µm to 50 nm and a repeatability of around 1 µm rms. Rotary encoders hold the advantage of being easily installed, and they provide a very good price/performance ratio.

Encoders and backlash

However, they can introduce unaccounted backlash — systematic error created by lost motion in the drive mechanism that appears when changing direction. For the Z-axis, the backlash is the total of all of the gaps between the gears on the focusing mechanism that move the stage. It can vary between about 0.10 and 5.0 µm depending upon the design and adjustment of the microscope and the wear on these mechanical components. For X-Y stages, the backlash error is the total mechanical gap between the motor that drives the leadscrew to position the plates of the stage to the optical axis. Antibacklash gear heads in the motor and antibacklash nuts on the leadscrews should be used to reduce this error. The control electronics can use algorithms to reduce it as well.

Linear encoders also can account for backlash by measuring the actual linear movement of the stage. This provides a more accurate means of measuring stage position and allows the use of coarser-pitched leadscrews that, in turn, increase the speed with little or no loss in resolution. However, high-quality submicron linear encoders are relatively expensive and must be properly installed and aligned to provide optimal performance.

These devices are available with resolutions down to 5 nm, but because of the mechanical limits of cross-roller bearings and leadscrews used in high-quality X-Y stages, realistic resolutions at the optical axis are about 50 nm. Higher resolutions require air bearings or more sophisticated designs that offer less friction. Standard stage designs that use cross-roller bearings and leadscrews and well-designed controller electronics and linear encoders can achieve bidirectional repeatability accuracies on the order of less than 200 nm rms for moves of a few hundred microns or so, and about twice that on larger moves. When working at these levels, the thermal expansion of the metal from which the stage is built must also be considered.

There are a number of other factors to consider when selecting a stage, and if you went into all of the details, a complete textbook could be written on the topic. Any stage manufacturer or imaging system dealer should be able to help you with these points and aid you in selecting a stage that is right for your application. With the right stage and a well-designed imaging software package, you can make your microscope a more powerful research tool. □

Meet the authors

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Dr. Colin Monks is co-president and Dr. Ben Freiberg, vice president, at Intelligent Imaging Innovations in Denver.

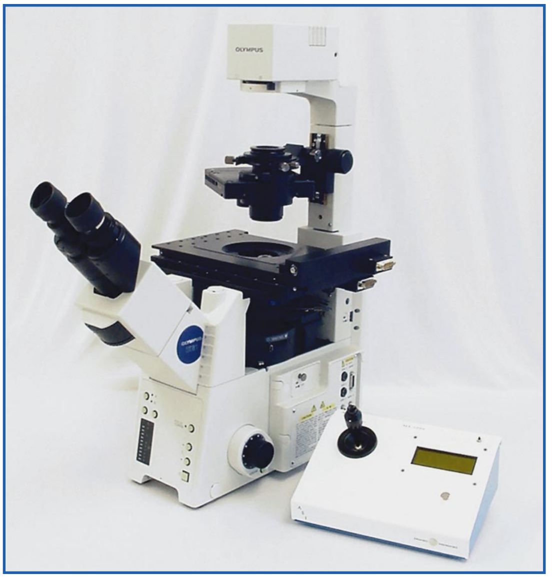

Figure 4. A closed-loop DC servomotor X-Y stage from Applied Scientific Instrumentation is mounted on an Olympus microscope. DC servomotors are almost always used with a feedback encoder to provide a precise closed-loop design.