Figure 1. Fluorescent test beads sample: do you see them—or don't you?

Video Microscopy Benchmarks:

Ten Quick Tests for Evaluating Scientific Imaging Systems

By Barbara Foster

Although the age of video has landed four-square in the realm of microscopy, routine use of benchmarks for evaluating video microscopy components is still foreign except to a handful of gurus.

To most microscopists, after all, video and computer-based image analysis systems are simply their *tools*—and often fairly new tools, at that. In some instances, the nature of microscopy is also a culprit. Microscopes are used in so many venues from high level, basic research facilities to routine industrial production and clinical labs that it is relatively difficult for information to cross from one type of lab to another. Also, microscopy uses a broad universe of samples: from stained tissue sections to polyurethane foams used in seat cushions, from cells undergoing drug testing to semiconductor devices. Being a pragmatic lot, most microscopists tend to rely on a representative sample from their everyday work; they'll choose a stable, challenging specimen which carries with it the specific problems of gray level segmentation, intensity, or spatial resolution inherent in their own application. All of that said, there *are* some quick tests which can provide benchmarks for system comparison.

Benchmarking *what*?

The move from simple direct observation in microscopy to video imaging brings together a sophisticated mix of components. In every instance (light, SEM, TEM, Confocal, SPM, etc.), the system actually starts with good sample preparation and correct microscope alignment. For example, light microscopes should be set up for Koehler illumination and should then insert a diffuser or light scrambler to make the background as even as possible. To establish a baseline, use an image analysis system to run a "Null" test on a blank field. The histogram of intensities should have one, narrow, well-defined peak. Alternatively, apply pseudocolor to image variations in intensity and look for changes in color.

Electronic components of a video system include the camera or detector, digitizer (on board in the camera or in the computer), imaging/image analysis software, output device and medium, and storage device and medium. The major concerns of microscopists relating to this part of the system center on resolution (both grayscale and spatial) and contrast. For low light systems, noise and intensity response (quantum efficiency) are also critical.

Clearly, each application has its own secondary parameters: microscopists using color as a marker are interested in good color rendition; for those involved with moving objects or transitory processes like fading fluorescence, response speed is paramount. Common to all these systems is the interrelationship of the hardware and software. Since the final image is only a representation of the object, the microscopist's job is to represent the object as accurately and faithfully as possible, and that means testing both the individual system components as well as the total, integrated package. Here are ten quick tests which can be used as benchmarks when comparing either individual pieces or the full video microscopy system.

1. Spatial res and edge fidelity

Like many microscopists, Ted Inoue (of Universal Imaging, West Chester PA) likes to use diatoms for testing spatial resolution in the optical train, since they're very regular and reproducible. Since different species exhibit different hole spacings in their surfaces, a small collection of diatoms provides a variety of resolutions .

Other common test targets are the Air Force resolution target, USAF1951, which provides patterns from 1mm to 5 microns in both vertical and horizontal stripes and the NBS1010A target which tests resolution to 631 line pairs/mm (0.8 µm bars and spaces).

For horizontal resolution on cameras, monitors, and output devices, most microscopists rely on the specifications provided by the manufactures. For those practitioners interested in really putting these components through their paces, detailed procedures for measuring both horizontal and vertical resolution are provided in the newest edition of *Video microscopy: The Fundamentals* by Inoue and Spring.

2. Contrast

Contrast is defined several ways in video microscopy. For brightfield microscopy, it is simply the visibility of the object against the background as calculated by differences in intensity of specimen to background, divided by the intensity of the background.

In the video system, it is defined by the modulation transfer function (MTF) which correlates the grayscale range or modulation presented by the actual specimen compared to the modulation presented by the image. That's really an output/input relationship.

Typically, the video image presents flatter contrast than the actual object; it exhibits less contrast or modulation between bright and dark portions than truly exists in the specimen. To test contrast, Dr. Guy Cox (University of Sydney, New South Wales) uses a fast/slow muscle slide which has been stained with immunoperoxidase and cryosectioned. The ice crystal damage makes the sample "stippled" and there are light, dark and "in-between" cells which provide a good range of contrasts.

Since MTF is a function of both intensity and spatial frequency, the ideal test target for benchmarking includes both sinusoidal variation in intensity and differences in frequencies. These test plates are now available through companies such as Sine Patterns (Penfield NY). As with the horizontal resolution test, the target is aligned East-west on the microscope stage and scanned horizontally. To calculate the MTF, measure the peak-to-valley intensities. For statistical valid-

Resulting T	100%	80%	50%	40%	30%	24%	15%	12%
Filters Used:	no filter	0.1	0.3	0.1+0.3	0.5	0.1+0.5	0.3+0.5	0.1+0.3+0.5

Table 1. Creating a Photometric Test from Neutral Density Filters.

ity, the measurement is usually conducted for several sets of oscillations which appear in the center of the field.

Ideally, MTFs should be tested for the detector or camera and image processing electronics as well as for image storage and display components. The system, from camera to processor, recorder and monitor, will have an MTF which is a product of the individual component values. Since MTF affects rise time, a poorly reacting system will respond more sluggishly to rapid changes in intensity.

3. Sensitivity and S/N

While sensitivity and signal-to-noise (S/N) ratios are more important in low light situations, they can have an impact on nearly any image and can be tested quickly and easily. The major symptom of poor S/N ratio is high background level compared to specimen information so that as noise increases, the specimen features seem to merge with or disappear into the background.

For fluorescence systems, the typical benchmark is a simple plastic bead which has been treated with a fluorochrome or dye which mimics experimental conditions. These beads, shown in Figure 1 on page 37, are readily available from a number of sources such as Molecular Probes (Eugene OR), which provided the image shown, or Bangs Laboratories (Carmel IN).

The test is simple: *can you see the bead or not*? Since pre-prepared slides can be very costly, Dr. James Pawley (University of Wisconsin, Madison WI) suggests purchasing beads with a single fluorescent species attached and making your own slides. To minimize bead fading during alignment and focus process, he recommends covering the preparation with Lucite film. The film limits exposure to oxygen and reduces photobleaching.

For typical brightfield applications, the system can be tested with the target containing the horizontal pattern of equally spaced black and white bars. Place the pattern so that it runs east-west (vertical bars) and scan horizontally across the pattern. Ideally, where the scan passes over a black bar, the background should go cleanly to zero and where it passes over a white bar, it should rise sharply to a maximum and exhibit a well defined, flat plateau. A background which does not go to zero, and rough upper surfaces on both the flat and the plateau signal, poor S/N performance.

4. Geometric accuracy

Simple square grids are the best targets to test geometric accuracy for optics, electronics and software. Homemade targets can be made from a piece of ultra fine screening or even a TEM grid mounted on a microscope slide. Many years ago, the microscopy lab at Baylor School of Medicine had a grid made with 10 micron spacings to test the flatness and accuracy of confocal scans but the expansion of microprinting techniques has made it easier to obtain commercially available grids and special patterns such as the Ball Chart.

5. Photometric accuracy

For those microscopists interested in photometry, the linearity of a video microscopy system is critical. This test is especially important for practitioners who need to perform accurate photometric measurements at either end of the grayscale where the response curves are non-linear.

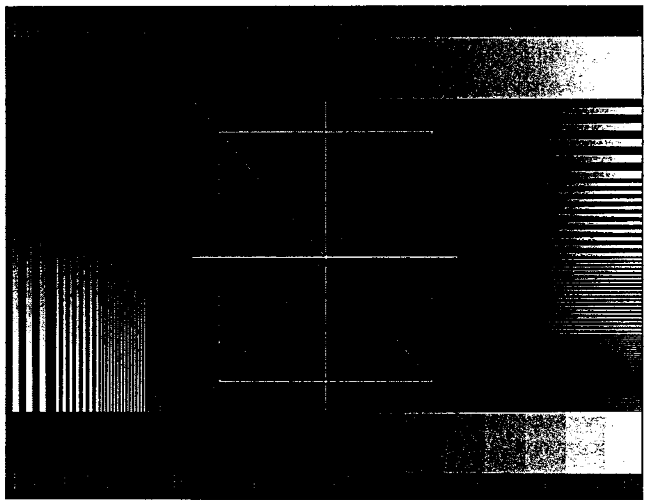

Figure 2. Grayscale test pattern used for testing printers and displays for video microscopy.

The quickest way to test for photometric accuracy is to use a set of three neutral density filters typically found in any lab: 0.1 (80% Transmittance), 0.3 (50% T), and 0.5 (30% T). They can be used individually and in combination to generate the test points shown in Table 1, above. Measure the photometric response using light meter, photometer, or, for CCD- based systems, optical density software such as the OD measurement in Media Cybernetics' Image-Pro Plus.

Neutral density filters are engineered to generate these transmissions for a specific wavelength (usually around 550nm). For more critical testing, use a narrow band 546 interference filter in combination with the neutral density filters.

An alternative approach involves purchasing a commercially available step wedge. In either case, this approach has two benefits: it provides a test for photometric accuracy as well as generating a multi-point calibration curve for photometric measurement.

6. Spectral response

Whether a camera, recorder, printer, or software can really isolate *color* may also be an important benchmark. This simple test uses narrow band interference filters to segment a specific spectral band.

First, set the white balance on the camera using a blank, evenly illuminated field. Next, insert the filter over the light path. Green interference filters (546 nm) are common around most microscopy labs: they come routinely with Phase or Modulation Contrast kits or may be part of the standard filter set in the microscope. Run a histogram on the field and examine the histogram of the signal on all three color channels: red, green and blue. For a well-behaved system, using a 546 nm narrow band (+/- 10 nm) green filter should generate signal just in the green channel; there should be no cross talk in the red or blue channels. To really understand the spectral response of the camera and other electronics, it is very important to repeat the process using red and blue filters. Cameras, especially, often present surprises in terms of their spectral response.

7. General camera performance

Since the camera is really the link between the microscope and the rest of the imaging system, its performance is paramount to the microscopist's success. (Don Lake has presented a broad array of ways to look at electronic imaging cameras in years of the "Getting the Picture" column in these pages.)

I haven't located specific targets purely for these tests, but several mentioned above will give good, consistent results. The pattern of bright and dark lines can be used to test for lag and blooming. Lag appears as a persistent image, smear, or white comet tail when a bright object moves in a video scene, so simply using the stage movement controls to move the target is a good test. This target will also reveal the camera's tendency to bloom or oversaturate at higher light levels.

Try just an empty, evenly illuminated field to test for blemishes. Look for hot spots, missing pixels, any irregular patterns such as smudges and streaks or a mottled or grainy background. For wavelength dependence and gamma (the slope of the grayscale response for input versus output), follow the tests for spectral and photometric accuracy described above.

Before you enter into any testing, remember that today's camera technologies have developed systems with specific functionality. Color cameras should give good color rendition, but don't expect them to provide premium spatial or grayscale resolution. Conversely, if you can live without color, a good quality black-and-white camera will give high sensitivity, high resolution in both grayscale and spatial arenas, and high or variable contrast.

8. Accuracy of software

There's a subject that will raise a few eyebrows! But Dr. Cox has contributed the following test for the accuracy of software: Draw a filled circle then skeletonize it. The result should be a single dot. He indicates that a cross is another arguably "correct" answer with some structural elements but that some systems produce very strange results indeed.

A word about automatic or on-board signal processing. This software option may provide edge enhancement which may be important for visualization, but may totally confound automated image analysis. As with most other microscopy tests, try a representative sample before going too far.

9. Test the output device!

Printers, films and displays devices all have their own idiosyncrasies. Mary Mager of the University of British Columbia (Vancouver BC) uses the gray-scale image shown in Figure 2 on page 38 to evaluate printers and displays for video microscopy.

This test pattern is also helpful if you're going to print in black and white. Different black-and-white films have radically different sensitivities which have profound impacts on the contrast of the image. As a matter of fact, image contrast can be enhanced or suppressed with clever and knowing use of the exposure curves for film.

Obviously, the key for color film is color fidelity. Some films (ex., Kodak's Kodachrome 64) tend to be more sensitive in the red region of the spectrum, while Polaroid instant films are well known to have a slight greenish-gray cast. A simple color test pattern can be used to test anything from camera and monitor to processor, recording and archiving components. The test kit comes with a microscope slide with a representative band of colors and a matching test print. To test any component of the system, simply image the object on the slide and compare the output to the test print. One caveat: when testing color it is imperative that all individual components be set to their respective baselines. For example, the microscope should be set for proper photographic illumination, camera should be white balance, and printers set for correct color rendition.

10. Consider the environment.

If you are using benchmarks, either to calibrate existing equipment or to compare potential purchases, do not rule out impact from the local environment. Both floor and air can transmit disturbing and distorting vibrations from equipment cooling fans and air conditioners. Electrical interference, often seen as a regular, corrugated overlay on an image, can come from nearby process equipment or even from large generators on the other side of the wall. Well defined and well-behaved test patterns help distinguish problems within the system from these extraneous environmental problems.

One last thought: Using low-light level cameras in these applications presents another set of issues. We'll take *those* up at a later date. ■

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