Published 2001 Wiley-Liss, Inc.†

Original Articles

Evaluation of Confocal Microscopy System Performance

Robert M. Zucker* **and Owen Price**

Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina

Received 13 December 2000; Revision Received 24 April 2001; Accepted 1 May 2001

Background: The confocal laser scanning microscope (CLSM) has been used by scientists to visualize three-dimensional (3D) biological samples. Although this system involves lasers, electronics, optics, and microscopes, there are few published tests that can be used to assess the performance of this equipment. Usually the CLSM is assessed by subjectively evaluating a biological/histological test slide for image quality. Although there is a use for the test slide, there are many other components in the CLSM that need to be assessed. It would be useful if tests existed that produced reference values for machine performance. The aim of this research was to develop quality assurance tests to ensure that the CLSM was stable while delivering reproducible intensity measurements with excellent image quality.

Methods: Our ultimate research objective was to quantify fluorescence using a CLSM. To achieve this goal, it is essential that the CLSM be stable while delivering known parameters of performance. Using Leica TCS-SP1 and TCS-4D systems, a number of tests have been devised to evaluate equipment performance. Tests measuring dichroic reflectivity, field illumination, lens performance, laser power output, spectral registration, axial resolution, laser stability, photomultiplier tube (PMT) reliability, and system noise were either incorporated from the literature or derived in our laboratory to measure performance. These tests are also applicable to other manufacturer's systems with minor modifications.

Results: A preliminary report from our laboratory has addressed a number of the QA issues necessary to achieve CLSM performance. This report extends our initial work on the evaluation of CLSM system performance. Tests that were described previously have been modified and new tests involved in laser stability and sensitivity are described. The QA tests on the CLSM measured laser power, PMT function, dichroic reflection, spectral registration, axial registration, system noise and sensitivity, lens performance, and laser stability. Laser power stability varied between 3% and 30% due to various factors, which may include incompatibility of the fiber-optic polarization with laser polarization, thermal instability of the acoustical optical transmission filter (AOTF), and laser noise. The sensitivity of the system was measured using a 10-μm Spherotech bead and the PMTs were assessed with the CV concept (image noise). The maximum sensitivity obtainable on our TCS-SP1 system measured on the 10-μm Spherotech beads was approximately 4% for 488 nm, 2.5% for 568 nm, 20% for 647 nm, and 19% for 365 nm laser light. The values serve as a comparison to test machine sensitivity from the same or different manufacturers.

Conclusions: QA tests are described on the CLSM to assess performance and ensure that reproducing data are obtained. It is suggested strongly that these tests be used in place of a biological/histological sample to evaluate system performance. The tests are more specific and can recognize instrument functionality and problems better than a biological/histological sample. Utilization of this testing approach will eliminate the subjective assessment of the CLSM and may allow the data from different machines to be compared. These tests are essential if one is interested in making intensity measurements on experimental samples as well as obtaining the best signal detection and image resolution from a CLSM. Cytometry 44:273–294, 2001.

Key terms: confocal microscope; lasers; coefficient of variation; photo multiplier tubes; lens; field illumination; axial resolution; spectral registration; laser stability; beads; quality assurance

The research described in this article has been reviewed and approved for publication as an EPA document. Approval does not necessarily signify that the contents reflect the views and policies of the EPA, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

*Correspondence to: Robert M. Zucker, U.S. Environmental Protection Agency, Reproductive Toxicology Division (MD-72), National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC 27711.

E-mail: zucker.robert@epa.gov

Confocal laser scanning microscopy (CLSM) has emerged as a very useful technique to visualize biological structures. Small apertures (pinholes) are used in CLSM to eliminate the fluorescent light from above and below the plane of focus, which results in increased resolution compared with conventional fluorescence microscopes. By using lasers, the investigator can image structures within a tissue, which is not possible when using conventional fluorescence containing mercury arc lamps. However, as with other fluorescence microscopes, it is necessary and essential to control quality assurance (QA) variables to achieve ideal image resolution and reproducible performance. These sophisticated optical machines should be properly aligned and all components should function properly to achieve maximum efficiency (1–6). A preliminary report from our laboratory has addressed a number of the QA issues necessary to achieve confocal system performance (1). The report extends our initial work by providing additional details and by offering new tests that are essential to achieve reliable performance from confocal microscopes. The data will be useful when trying to apply this technology to make reproducible intensity measurements.

Quantitative fluorescence microscopy is concerned with acquiring measurements from fluorescence specimens by measuring the emission from a defined area of the specimen (7). It is generally assumed that the intensity of fluorescence will be proportional to the amount of fluorescence present. However, because the fluorescent image is weak compared with images from other microscopes, it is necessary to have good optical efficiency in the system to observe the specimen. The system must be aligned so the maximum number of photons hit the sample and the detection system must be efficient to capture the majority of photons emitted from the specimen. One goal in using the CLSM is to adjust the emission pinhole to exclude out-of-focus light, thus producing a confocal image. However, in the process, most photons are eliminated from reaching the detector, making the system less efficient (5,6). To compensate for the production of confocality, the photomultiplier tube (PMT) voltage is increased, which introduces more noise into the image. These factors seem to be opposed diametrically because the light emitted from the sample must be reduced for confocality while the light transmitted to the PMT must be maximized to reduce the system noise. Because the sensitivity of the optical system depends on the light source, the optical efficiency, and the ability to detect fluorescence, it would be extremely useful if the system's sensitivity could be maximized (2,3,6,8). Thus, the second goal of the CLSM instrument is to produce an image with the least amount of light hitting the specimen, so the bleaching is minimized. Prior to acquiring an image, the specimen may fade. The fading may result in errors in the intensity of the acquired image.

Ideal PMTs should show linearity over a large dynamic range and they should be very sensitive with low noise in the wavelengths measured. A CLSM should produce an image with the least amount of light hitting the specimen to minimize bleaching and efficient PMTs should be operated at low voltages to minimize noise. To visualize any fluorescent specimen, a sufficient amount of light has to be delivered onto the sample that is located on the stage. If the sample cannot be visualized adequately, it will be necessary to increase the PMT voltage. As the PMT voltage increases, the image noise increases. At higher PMT settings, more frame averaging is used to reduce the noise in the image. However, this leads to longer exposure times and possible sample bleaching.

Lasers used in a CLSM must have sufficient power to excite the sample. However, there is a trend to use low-powered air-cooled helium-neon (HeNe) lasers to obtain the 543 and 633 laser lines. However, the reduced power output may create problems because of the need to operate PMTs with high values to visualize samples. Low-power lasers are being used as confocal excitation sources because they are stable, reliable, and are low maintenance. Because these laser sources have a low power output, increased optical system efficiency becomes essential (1–6,8). An efficient optical system that is aligned correctly and functions properly will allow low-power lasers to be used effectively with low PMT voltage values (1). From both scientific and QA standpoints, optical system performance should be optimized (1–6).

To quantify fluorescence using a CLSM, a number of variables that affect instrument function and sample preparation have to be addressed. The specimen variables include the rate of bleaching, the environment of the sample, the concentration of the dye, mounting media, autofluorescence, energy transfer, and wavelength of excitation and emission. Although these sample variables are important, the focus of this paper is on instrument variability. These factors include the instability of a light source, homogeneity of illumination, background fluorescence, lens quality, optical components, light leak from stray room light, instability of photometer detection, and nonlinearity of photometer detection (7–13). These factors are not unique to CLSMs. They have been described for conventional fluorescence optical equipment connected to cameras and photometers (9–13). Due to the complexity, sensitivity, and increased capacity of confocal microscopes, other factors involving optical performance can now be evaluated better. Because all CLSM images are digital and made with sophisticated optical equipment, many tests can be performed to ensure adequate QA of the instrument. The tests determine if the machine is performing correctly while assessing components in the system for their proper functioning. Many studies compare intensity measurements of experimental samples. Therefore, it is important that the instruments used in laboratories deliver a similar performance. It is also important to implement performance standards so that machines are used correctly. We describe a series of new tests as well as modifications to exisiting tests that may be used as performance standards on a confocal microscope (1).

In this paper, we incorporate QA procedures into the operation/maintenance of confocal microscopes. We de-

scribe the test methods used successfully in our laboratory to evaluate the functioning of the Leica TCS-SP and TCS-4D confocal microscopes. These tests should not only be confined to the Leica equipment, but should be valid for other point scanning systems. Several other approaches to optimize CLSM system performances have been published (2–6). QA testing will help investigators ensure that their machines are operating in the manner that they were designed to operate.

MATERIALS AND METHODS

Field Illumination-Fluorescent Slides

The field illumination test slides consisted of three fluorescent plastic slides (Delta; Applied Precision, Issaquah, WA) that had excitation peak wavelengths of 408, 488, and 590 nm and emission peak wavelengths of 440, 519, and 650 nm, respectively. The 488 orange slides and the 408 blue slides were used to align visible and ultraviolet (UV) wavelengths, respectively. The slide was placed on the stage and the maximum intensity was found on the surface of the slide. The depth of focus was adjusted between 40 and 100 μm, depending on the objective used, i.e., 5× (100 μm), 10× (75 μm), 20× (50 μm), 40× (40 μm), 63× (30 μm), and 100× (30 μm). The depth of focus is below the slide surface to reduce the nonuniform fluorescent patterns on the slide's surface. Focusing too deep into the slide will result in more uniform illumination due to light scattering and other optical factors. Therefore, this depth parameter should be controlled rigorously. In our experience, beads, biological samples, or fluorescent liquid in a well slide were not as reliable as the fluorescent plastic slides to measure field illumination.

Power Meter

A Lasermate Q (Coherent, Auburn, CA) with visible (LN36) and UV detectors (L818) was used to measure light on the microscope stage. A power meter (1830C; Newport) with an SL 818 visible wand detector can also be used to obtain power measurements. A remote control box for the UV Enterprise laser was used to regulate UV laser power (0163-662-00; Coherent, Santa Clara, CA). The power test is performed with a low-magnification lens (2.5×–10×) in the following manner. The lens is raised to its maximum specified height. The detector is secured on the stage and centered grossly using either laser light or mercury fluorescent light. Using the microscope's x/y joystick, the detector position is then adjusted more accurately to achieve maximum signal intensity. The CLSM zoom factor is set from 8 to 32 to reduce the beam scan and to focus the beam into the "sweet spot" of the detector. The scanner is set at bidirectional slow speed to reduce the time period that the power meter reads "0." The power derived from this measurement depends on the lens type, lens magnification, and lens numerical aperture (NA). On most confocal systems, there is a 10× lens: Zeiss uses a 10× Plan Neofluar (NA 0.3) and Leica uses a 10× Plan Fluortar (NA 0.3). We have used both lenses for power measurements on their respective systems in addition to a 5× Zeiss Plan Fluortar on both systems. Normally, lower magnification lenses will deliver more laser power than higher magnification lenses in this test. However, each lens will have a unique set of values that depend on NA and other transmission factors. The Leica built-in power meter diode was not reliable or stable and was only used as a crude estimate of the laser power output.

Beads

Beads are useful as test particles to assess machine functionality. We obtained beads from three sources for the tests described in this study. Most of the beads were obtained from Spherotech (Libertyville, IL): Rainbow fluorescent particles (10-μm; FPS-10057-100 EX 365, 488, 568) were used for statistical PMT tests; Yellow beads (5.5-μm FPS-5052 EX 488) were used for visible field illumination; UV beads (5.5-μm FPS-5040 EX 365) were used UV field illumination; and Blue beads (5.5-μm FPS-5070 EX 647) were used for field illumination. The polystyrene beads (refractive index [RI] =1.59) were mounted with optical cement (RI = 1.56) on a slide using a 1.5-size coverglass. The Leica immersion oil has a refractive index of 1.51.

Fluorospheres (10-μm Fullbright Green II [Coulter, Hialeah, FL], EX 488) were used for preliminary statistical PMT tests. Tetraspeck beads (T7284 1-μm [Molecular Probes, Eugene, OR], EX 365, 488, 568, 647) were used for spectral registration tests. These beads are also used to observe point spread functions (PSF).

Biological Test Slides

FluoCells (F-14780; Molecular Probes) was stained with three fluorochomes (Mitotracker Red CMXRos, BODIPY FL phallacidin, DAPI) and used as biological test slides. Additional slides were made in our laboratory with cells grown on coverslips, fixed with paraformaldehyde, and stained with DAPI for UV excitation or other suitable fluorochromes for visible excitation.

Axial (Z) Resolution Test

The axial resolution of the CLSM is tested using a single 21-mm^{2}reflecting mirror (31008; Edmonds Scientific, Philadelphia, PA) that was glued onto a microscope slide. A 1.5-size coverglass (Fischer, Pittsburgh, PA) was placed on top of the slide with a drop of immersion oil (Leica immersion oil, n = 1.518). The coverslip is placed firmly on the mirror to remove all excessive oil. This standard test slide can also be obtained from a confocal manufacturer or from Spherotech. A test slide should be compared with that of the service engineer's slide to ensure that the reference slide delivers the proper values. Using this test system, Leica has designated that its TCS-SP confocal microscopes will achieve a minimum axial resolution value of 350 nm when a 100× Plan Apo lens (NA 1.4) is used (1). Failure to achieve this value suggests that there is a problem with system resolution or lens performance, which will require the attention of trained service personnel.

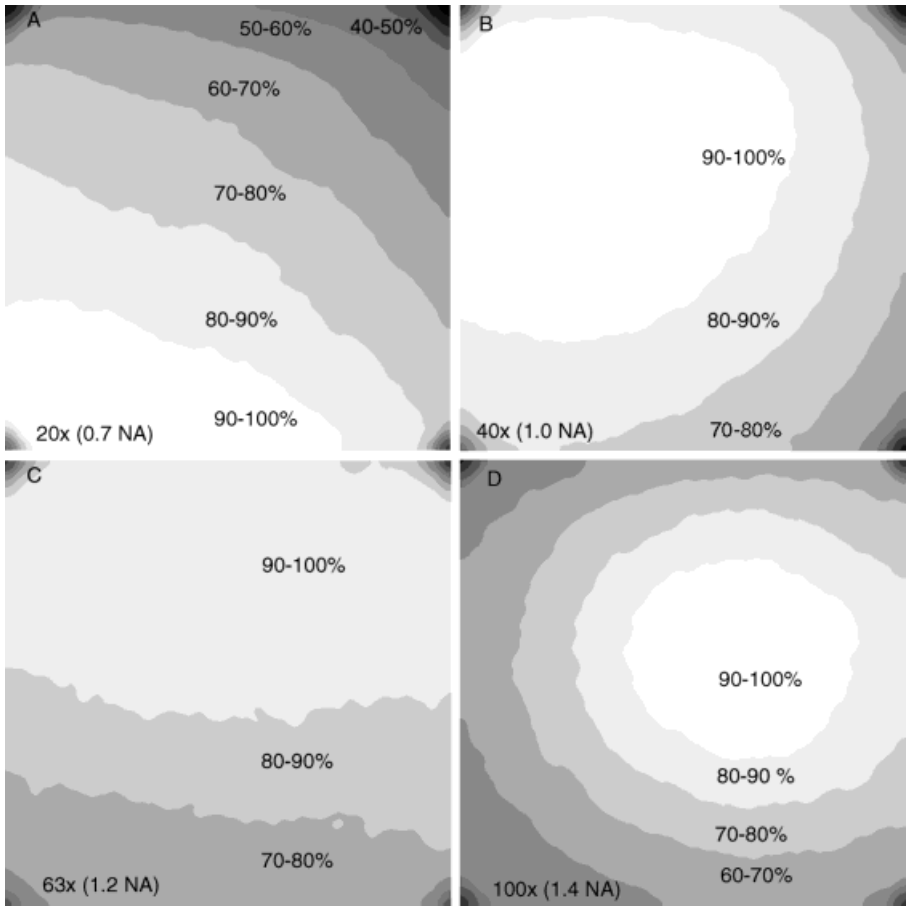

FIG. 1. **A–D:** Field illumination of UV (365 excitation) was made with the following lenses on the Leica DMIRBE inverted microscope: 20× (Plan Apo NA 0.7); 40× (Plan Fluotar NA 0.5–1.0); 63× (Plan Apo NA 1.2); 100× (Plan Apo NA 1.4). The illumination pattern was derived from a plastic fluorescent slide imaged between 20 and 50 μm below the surface. The UV field illumination shows representative data from a misaligned system with light intensity being off center and decreasing in excess of 25% across the field with all objectives. The lightest part has the most intense laser light and each intensity band represents a 10% decrease in laser light. The intensity regions were prepared by using Image Pro Plus to divide the GSV into 10 equal regions. The resulting image was processed using a median process filter that defined the intensity boundaries.

Square Sampling

It is important to ascertain whether there was square or rectangular sampling in an image. A computer chip was glued onto a glass slide and used as a test substrate. A digital TIFF image was obtained using a dry 20× objective and the number of small boxes observed was counted by eye in the vertical and horizontal directions. If there is the same number of boxes per inch in the vertical and horizontal directions, then it can be assumed that the sampling of pixels is square. If the number of boxes in both directions is not equivalent, then the sampling of pixels will be rectangular, which is not desired.

Confocal Microscope

Most of the data presented in this study were derived on either a Leica TCS-SP1 or TCS-4D (Heidelberg, Germany) confocal microscope system. These systems contained an argon-krypton laser (Melles Griot, Omnichrome) emitting 488, 568, and 647-nm lines and a Coherent Enterprise UV laser emitting 351 and 365-nm lines. The system contains an acoustical optical transmission filter (AOTF) and the following three dichroics for visible light applications: single dichroic (SD; RSP500); double dichroic (DD); and triple dichroic (TD). The Leica-derived tests were applicable to other point-scanning systems that contain other lasers, objectives, or hardware configurations. For comparison purposes, some of the tests were made on two Zeiss 510 units containing three lasers (argon 488, 25 mW; HENE 547, 1 mW; and HENE 633, 5 mW) with a merge module and an AOTF.

Software Analysis

The Leica statistical software package, which was developed as an MS Excel-based application, was used to evaluate most of the images. Some of the software features may not be present in the other confocal machines, which makes analysis of the data more difficult or necessitates the use of third-party software to analyze the data. If necessary, the TIFF images that were acquired with the TCS-SP1 hardware can be imported into Image Pro Plus (Media Cybernetics, Silver Springs, MD) for more intensive measurements and analysis. In this study (Figs. 1–3), the pixel intensity (gray scale value [GSV] = 255) was divided into 10 equal parts and the 10 regions were then processed using a median process filter to delineate the regions.

Laser Stability

Laser stability measurements were made over hours to evaluate fluctuations in power. The laser power fluctuations were measured initially both in PMT1 (blue light

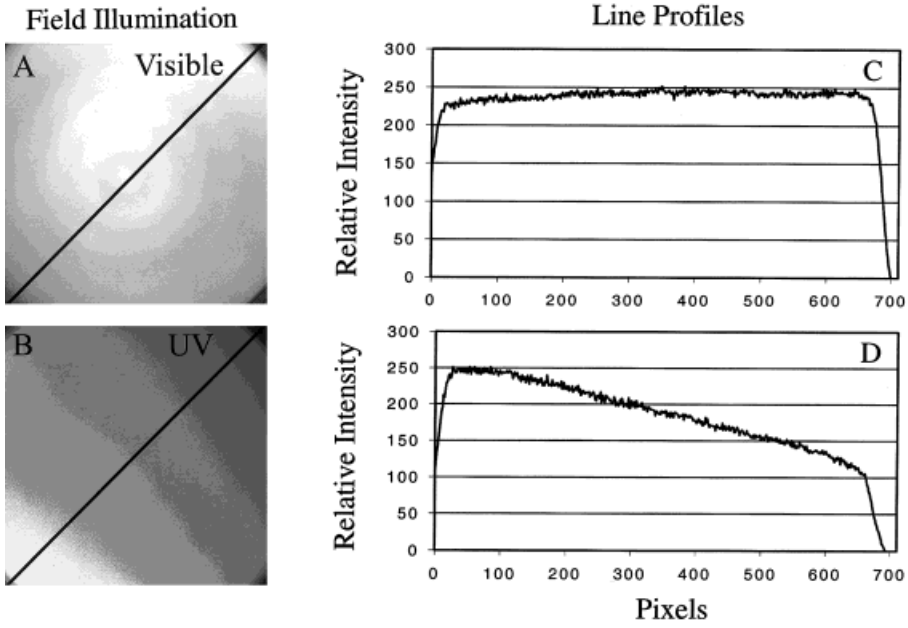

FIG. 2. Field illumination pattern of visible **(A)** and UV **(B)** excitation using a 20× (Plan Apo, NA 0.7) lens. The visible field illumination shows uniform illumination with the brightest intensity being in the center of the objective. **C,D:** The line running diagonally in A and B measures the histogram intensity of the field illumination graphically represented in C and D. The variation in intensity from the left to the right side of the field is less than 10% for visible excitation and over 150% for UV excitation. Acceptable field illumination has the brightest intensity in the center of the objective, decreasing less than 25% across the field. Image Pro Plus was used to define the 10 equal intensity regions and a median filter was used for additional processing.

sensitive, low noise) and in the transmission detector using a fluorescent plastic slide with low laser power that was reduced by using either neutral density filters or by adjusting the AOTF. The transmission optical system without a slide showed similar results to PMT1 with fluorescent plastic slides. This was the desired optical system to perform this test because it eliminated any possibility of bleaching or laser interaction with the substrate.

To measure laser stability using the transmission optical system, the microscope is aligned for Kohler illumination

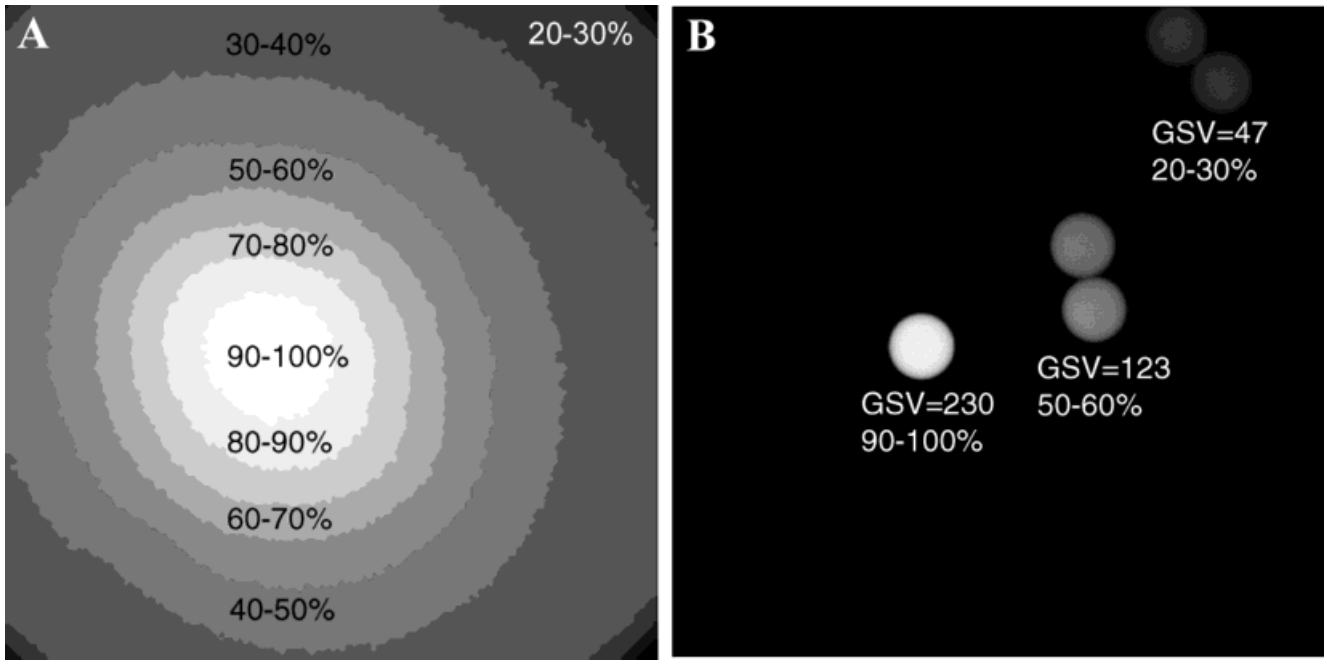

FIG. 3. UV field illumination of a Plan Apo 100× lens (NA 1.4) derived with a fluorescent plastic slide **(A)** and the intensity measurement of 10-μm Spherotech beads **(B)**. This illustrates the problem of using a lens with improper field illumination to make comparative measurements on a sample. The field illumination pattern shows a bull's eye intensity pattern slightly off center (A) and the five beads located in different parts of the field (B) to illustrate the variation in intensity that occurs by using a lens that has improper field illumination. The intensity of beads was derived by a small ROI inside the bead. The five beads show a decrease in intensity of approximately 100% (GSV = 123) and 400% (GSV = 47) relative to the bead in the center of the illumination (GSV = 230).

with a histological slide, which is then removed from the optical path. The image intensity is measured using the transmission detector for the three wavelengths of the argon-krypton laser by measuring sequentially the laser light with the 488, 568, and 647 wavelengths with the power being adjusted by the AOTF transmission control so the transmission detector voltage remains constant for all three wavelengths. The test usually takes a few hundred scans separated by 15–30 s over a period of 2 h. The intensity of a large region of interest (ROI) of the field is averaged and plotted over time for the three wavelengths. The goal of this test is to have a straight line with no variations in power to ensure accuracy in the intensity measurements.

RESULTS

Image quality is an important parameter when evaluating the performance of a confocal microscope. Unfortunately, it is used too often as a "gold standard" to assess microscope functionality and performance. Other variables that effect image quality and system operation should be assessed to ensure that the system is efficient and is delivering optimum performance. When intensity measurements are required, it is essential that the machine be stable to deliver reproducible data. An initial publication from our laboratory has described some tests to help evaluate machine performance (1). This publication extends our QA work by describing modifications of previously reported tests and introducing new tests. These various tests were either adapted from the literature or devised in our laboratory to measure the system performance of the confocal microscope (1–6). These tests include laser power measured at the stage, field illumination, laser stability, dichroic performance, PMT performance, system linearity, axial resolution, spectral registration, sensitivity, and lens quality. This list is not inclusive and represents what we believe can be tested and interpreted to ensure proper operation.

Power Meter Readings

This power test appears to be one of the most useful tests because it evaluates quickly both the system alignment and performance. For the adequate operation of a CLSM, sufficient laser power is needed to excite the specimen. A system that is misaligned or is functioning suboptimally can be assessed by a test that measures laser power. The power test indicates quickly whether the system is aligned properly up to the plane of excitation on the stage or whether the machine has a defective component (i.e., a dying laser or a defective fiber). In our experience, without sufficient power throughput in the system, major problems will occur with PMT noise because the voltages will have to be increased to high values to visualize fluorescence derived from specimens. It should be emphasized that these tests were done using a Zeiss 510 and a Leica TCS-SP1 in research laboratories without much technical support from either company. There are no assurances that these machines were producing ideal power readings or were perfectly aligned when these tests were made.

Table 1
*Dichroic Reflection Versus Wavelength Comparison (mW)**

λ (nm)	Dichroic	5× (NA .25)	10× (NA .3)	20× (NA .7)
488	RSP500	1.66	1.14	0.46
	DD	1.92	1.34	0.52
	TD	1.92	1.36	0.50
568	RSP500	0.24	0.15	0.05
	DD	2.15	1.42	0.62
	TD	2.22	1.48	0.64
647	RSP500	.83	.48	0.17
	DD	.03	.02	0.00
	TD	1.72	1.02	0.38

*The maximum power was measured in mW with an LN 818 detector and a Coherent Lasermate power meter adjusted to the specific excitation wavelengths. The three dichroics were tested. The data demonstrate the relative reflectivity of the dichroics in the system. This can be used to test QA in the dichroic and to assess the proper positioning of the dichroic in the system. This test can be used to determine if the system has acceptable laser power by measuring the power on the microscope stage. There should be at least 1 mW of power using a 5 or 10× objective in a Leica TCS-SP1 system.

Initially, the power was measured in our system using a 5× (Zeiss, NA 0.25) objective. The Zeiss lens has a higher NA and better fluorescence transmission than the equivalent 5× lens from Leica (NA 0.12). There is also different power output obtained from the same Zeiss lens that is used on either a Leica TCS-SP or a Zeiss 510 system. When the Zeiss 5× is used on a Leica system, there is a 20% increase in magnification due to the incompatibility of the two systems. There is also a 20% reduction in power readings. Not all investigators may want to purchase a Zeiss 5× lens for their systems. Therefore, we have also reported data using the 10× and 20×objectives (Table 1), which will help them compare their machines with the data produced on our machine. Currently, most systems use a 10× lens (NA 0.3) to access power. This should be used eventually as the standard in testing power on a CLSM. The use of an NA lens of the same magnification will affect the laser power transmission.

To measure the power output of the wavelengths, a UV or visible probe (Coherent probe detectors L818, LN36) or Newport wand visible probe detector (SL 818) is placed on the stage. A special holder secures the probe on the microscope stage during the measurement of either UV or visible laser light. The test should be done with a dry objective (2.5×–20×) at a fixed position, usually at the top of its moveable tract. Table 1 compares the three dichroics (RSP500, DD, TD) that reflect the three wavelengths of laser light (488, 568, and 647 nm) to the stage using a 5×, 10×, or 20× objective. The lens characteristics, lens magnification, and lens NA influence the power throughput. The power meter values (Table 1) are used to determine the maximum power output and dichroic functionality in the system. The comparison of dichroic reflectivity and power values derived from a Leica TCS-SP1 system may be used as a reference standard for other

investigators as what comprises a functional CLSM. It is important to use the proper dichroic when operating the machine because different dichroics reflect different amounts of laser light to the power meter, which demonstrates their reflective quality. This test measures the system power throughput for each wavelength and evaluates the manufactured dichroics in the system. A defective dichroic will not bounce enough light at the desired wavelength and should be replaced or substituted with one that has better reflective performance.

Using either a 5× Zeiss Fluor objective (NA 0.25) or other Leica objectives in the range of 2.5×–10× in a Leica TCS-SP1, it is desirable to have at least 1 mW of power on the microscope stage for each laser line derived from an Omnichrome 643 argon-krypton laser. At this power, the PMTs can be operated at low settings, which reduce the system noise and produce acceptable images. Less power may suggest a dying laser or a system that is badly aligned. When using less powerful lasers (e.g., the He-Ne lasers, 543 nm or 633 nm) in a CLSM, different performance characteristics will be achieved on the stage.

After installation, it is important to measure the power output to evaluate system performance for all three lines to make sure that the system is aligned properly and the laser is functioning correctly. The power values serve as a reference to ensure proper system performance and notify the confocal manufacturer of deviations from acceptable values that mean either laser failure or misalignment. A new Omnichrome 75-mW argon-krypton mixed-gas laser delivered the following power outputs: 488 nm, 1.10 mW; 568 nm, 2.68 mW; and 647 nm, 1.60 mW. After time and proper laser alignment, almost 3 mW for each line was achieved in our system. The maximum power derived from an argon-krypton laser fluctuates daily for unknown reasons.

A comparison of the maximum power output derived from different lasers and from different optical systems was made on a Zeiss 510 and on a Leica TCS-SP1. The maximum power was measured with a Coherent power meter using two 10× (NA 0.3) lenses on two CLSM systems. The Leica TCS-SP1 system has a 75-mW argon-krypton laser (model 643) that emits three laser wavelength lines. The newer CLSM systems are designed with three lasers that use different dichroic components to merge the laser wavelengths. The Zeiss 510 contained three lasers (25-mW argon, 1-mW HeNe [543 nm], 5 mW HeNe [633 nm]) with the multiple wavelengths aligned with a merge module. The maximum power was measured with a Coherent power meter using two 10× (NA 0.3) lenses on two CLSM systems, the Zeiss 510 and Leica TCS-SP1. The maximum power measured on a specific day with a Leica TCS-SP1 system using a Plan Fluor 10× (NA 0.3) included 488 (1.1 mW), 568 (1.45 mW), and 647 (1.65 mW). The maximum power measured on a specific day with the Zeiss 510 system using a Plan Neofluor 10× (NA 0.3) included 3.2 mW (488 nm), 0.234 mW (543 nm), and 0.650 mW (633 nm). These values illustrate the maximum power obtained from a CLSM with two laser configurations. These measurements serve as a valuable reference for this system over time and as a comparison with similar CLSMs. This power output test can help to determine if the system is misaligned, is functioning badly, or if the laser needs to be replaced. It should be emphasized that this test is performed at the microscope stage prior to the light reflecting the dichroics a second time and penetrating the emission pinhole and the emission barrier filters (if they exist in the system) and into the PMT.

If a power meter is not available, the crude power of the system can be assessed easily by recording the PMT voltage necessary to acquire an image at almost saturation values. This is achieved by using standard histological samples like the FluorCells slide (F-14780; Molecular Probes) or 10-μm Spherotech beads (FPS-10057-100). If conditions are identical between machines, the PMT value can be used as a reference value to compare CLSM units and to establish their acceptable performance levels. Scientists desiring a more accurate method to test performance will find major problems with this type of testing due to the wide range of acceptable PMT values. Another reason to doubt the data from a histological sample is that individual PMTs and samples vary greatly in quality. What is even more troubling with this test is that the PMT voltage is expressed as a logarithmic relationship relative to an intensity increase. This means that the difference of only a few PMT units translates into a huge difference in intensity and relative laser power measured at the stage. However, CLSM service representatives use histological samples to evaluate the crude power of the CLSM, as well as many other CLSM variables.

Argon, UV, and argon-krypton lasers need to be aligned and adjusted regularly. The investigator can measure laser power over time using a power meter positioning on the stage (Table 1). Either the loss of laser power or inaccurate optical alignment will reduce the laser power in the system, necessitating an increase in PMT to compensate for the lack of laser light intensity. Minor adjustments are made to the mirrors with the horizontal and vertical knobs located on the back of the lasers. However, the lasers are enclosed in a box, their rear knobs being inaccessible for adjustment by the investigator. In fact, most confocal manufacturers do not allow the user to adjust these controls. The manufacturer's service contract keeps the lasers and system functioning properly. For example, in our system, it is possible to tweak the Coherent UV Enterprise laser, but it is not possible to adjust the argon-krypton laser. It was enclosed in a box that the manufacturer required not to be opened or the service contract would be invalidated. The investigator usually will not notice a problem with laser power or alignment but will have to increase continually the laser power to compensate for the reduced system laser power. This use of increased laser power will not only shorten the life of the laser but will not correct the CLSM system problems that may be yielding poor resolution and system noise. If there is insufficient light entering the system, a careful realignment of the laser beam is required (a separate procedure done by qualified personnel) to increase the laser output. If this alignment does not solve the insufficient system

power values (similar to data shown in Table 1 with a Leica TCS-SP1 unit), it may be time to replace the laser. Knowing the specifications of laser power output on a stage is a critical parameter to assess system performance. Manufacturers should specify the power values obtained on the stage for different lasers configurations, which will allow investigators to determine CLSM performance in their laboratories.

UV Power Test

One of the major problems that occur with confocal systems is insufficient UV power output. A power meter (Lasermate/Q with UV detector (L818); Coherent) was used to measure the light emitted from a UV Enterprise laser. A Coherent UV, 60-mW, 3-year-old Enterprise laser delivered normal power output at the laser head (over 40 mW of laser power), but only about 500 μW maximum power through the 5× Zeiss lens (Fluar NA 0.25). This indicated that there was an attenuation of the laser light through the opticAL system. However, to perform this test properly, it is essential to have a UV-transmissible objective between 5× and 20× and to have the light reflected adequately by the optical system on to the stage where the power meter is located. The following reflected power values were measured on our Leica TCS-SP1 system: 5× Zeiss Fluar (NA 0.25), 500 μW; 10× Leica Fluotar (NA 0.3) 243 μW; 20× Leica Plan Apo (NA 0.7) 93 μW. We were not able to make a measurement at the back of the aperture due to problems in positioning the power detector and the spread of the unfocused laser light. In the Leica system, there are three UV focusing alignment lenses. The three measurements were taken with only one of these alignment lenses that were supposedly optimized for the 20× low-power objective. However, when our system did not have enough output under these conditions (approximately 500 μW with a 5× Zeiss lens), we also had insufficient light for many UV experiments with the higher magnification objectives (40×, 63×, and 100×).

Because our power detector does not work with higher power optics (40×, 63×, 100×) due to optical limitations of the stage, it will be useful to use a histological test slide sample, fluorescence slides, or bead sample to assess UV power with these higher power lenses. Experiments can also be done with histological test slides or fluorescent colored glass to approximate the laser output with higher power objectives. Using maximum UV power, 10-μm Spherotech beads saturated PMT1 (low-noise PMT) at a setting of 650 using a 100× Plan Apo lens (NA 1.4). Noise tests using beads will be described in a subsequent section that will be useful to assess UV power. Leica technicians use a 40× lens to measure the fluorescence saturation of a histological plant sample. If it saturates in the PMT range between 600 and 700 units in PMT1, the system is passed as having adequate power. In our opinion, this test is crude and subjective due to the acceptability of such a wide range of PMT amplification values, the variations in staining between plant samples, variations in PMT characteristics, and the logarithmic relationship between PMT versus intensity (1). It does, however, give a rough estimate to determine if there is sufficient power in the system.

Decreased UV power transmission may be caused by misalignment, aging fiber optics, a polarization mismatch between fiber and laser, an unfocused collimator lens, and a dying laser. This measurement of UV power helps to assess the system performance and determines if adequate UV power is being transmitted through the system and if the fiber is in a good condition. It is recommended that measurements be taken at the back objective aperture region to eliminate the characteristics of the lens from influencing the test. However, we were not able to mount our detector probes in a sufficiently stable manner to allow for repeatable measurements. When taking UV measurements, an objective designed with good transmission characteristics should be used to increase the power throughput and system performance.

In addition to the power requirements of a UV system, the UV beam should have the correct mode. The beam should be radial symmetric with a Gaussian intensity distribution and a TEM00 configuration (transverse excitation mode or Gaussian mode). The UV laser beam can be checked using an inexpensive lens (12 mm outer diameter [OD], B1099, Melles Griot) held in a lens holder (13 mm inner diamter [ID], H1089) and focusing the beam onto a white piece of paper to show its configuration mode.

Dichroic Reflectance (Reflectivity of Dichroic and Barrier Filters)

Dichroic filters are made to reflect or reject specific wavelengths of light and to pass the desired excitation/emission wavelengths of light. Placing a fluorescent slide on the stage and measuring the relative intensity of an image can evaluate the efficiency of the dichroic filter to reflect light in a confocal system. The relative fluorescent emission in our system from a fluorescent plastic slide was measured with the 488 and 568 wavelengths using six objectives and three dichroics. Table 2 compares the reflectivity of the three dichroic filters (SD, DD, TD) and six lenses with the three excitation wavelengths (488, 568, 647). The test was accomplished using a fluorescent plastic slide with a specific dichroic and by keeping the PMT constant and measuring the intensity in an ROI of an image using either 488 or 568 excitation light. The mean GSV of an ROI in the image was determined for each acquisition condition. The values for the three dichroics reported in Table 2 are relative to the dichroic with the highest reflectivity normalized to 1 and the other values are reported as a percentage of the maximum GSV.

These tests should be done to determine optimum system efficiency and ascertain the performance of individual dichroics with a variety of objectives and wavelengths. It is important that the dichroics reflect the maximum amount of light at the desired wavelengths to increase the efficiency of the optical system (1,6,7). The 488 line should use the SD (RSP500), the 568 line should use the DD (488/568), and the 647 line should use the TD (488/568/647). The placing of either a SD, DD, or TD in the light path should reflect successively less light using the

Table 2
*Comparison of Relative Dichroic Reflectance**

λ (nm)	Dichroic	5× (NA .25)	10× (NA .3)	20× (NA .7)	40× (NA 1.0)	63× (NA 1.2)	100× (NA 1.4)
488	SD	0.92	0.95	**1.00**	**1.00**	0.97	0.95
	DD	**1.00**	**1.00**	**1.00**	0.98	**1.00**	**1.00**
	TD	0.80	0.84	0.85	0.85	0.80	0.81
568	SD	0.05	0.05	0.06	0.07	0.06	0.07
	DD	0.70	0.62	0.69	0.69	0.72	0.69
	TD	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**

*The relative laser power was measured with the 488 and 568 wavelengths using six magnification objectives and three dichroics. The test was accomplished by measuring the intensity in a ROI of an image using either 488 or 568 excitation light, a fluorescent plastic slide, one of three specific dichroics and by maintaining the PMT at a constant voltage. An ROI of the image yielded the mean value for each acquisition condition (wavelength, objective, and dichroic). The GSV of the two images is divided to yield a ratio that is expressed as a fraction. The value of 1.00 is the maximum reflection and is expressed as a bold number. The dichroic with the maximum reflection should be used when only one fluorochrome is required. Unexpectedly, the DD yielded the best reflectivity with 488 nm wavelength light with all lenses and the TD yielded the best reflectivity with the 568 wavelength light (30% more light reflected than the DD) with most objectives. The data can be used to choose the dichroic that should be used with each excitation wavelength for optimized reflection.

488-nm excitation line. The DD and TD are more complicated dichroics than the SD and, in theory, should reflect less light as they were made to reject more light and pass fewer specific wavelengths of light. However, as shown in Table 2, the DD reflected the 488 light best and the TD reflected the 568 light best with all objectives. In principle, the better the reflection, the more efficient the dichroic. Using 568 excitation, the TD reflected 30% more light than the recommended DD. Comparing the RSP500 and DD dichroics with 488 excitation shows the efficiency difference between the low and high-power objectives, necessitating the need to test all objectives. Unfortunately, dichroics do not always perform as they were designed to perform in a CLSM (Table 2). From these data, it can be surmised that when using single wavelength excitation, the DD is preferred to the RSP500 for 488 excitation and the TD should be used instead of the DD for 568 excitation for all lenses. This is a QA test to determine the efficiency of the dichroics in CLSM and to help in determining which dichroic should be used in a single-wavelength excitation experiment. The dichroic test is used with single-wavelength excitation. Application of the data allows the system to be run at lower PMT values, which translates into less noise and better performance. With multiple excitation wavelengths, the dichroics have to chosen to balance the power of the emitted fluorescence from each fluorchrome, but this test is helpful in making the decision.

Field Illumination

The intensity of illumination across the observation field can be measured with test specimens in order to ensure that a homogeneous field illumination exists. Test substrates include a concentrated fluorescent dye suspended in a hanging drop well slide, small (1–3 μm) or large concentrated fluorescent beads (10 μm; Spherotech), fluorescent specimens, uranyl glass slides, Altuglas, or plastic fluorescent slides (Applied Precision; 1,4,14). Other tests include a piece of tissue paper stained with fluorescent dye or fluorescent dye solution (Fluorescein [F-7505] or Rhodamine B [R-6626]; Sigma, St. Louis, MO) mixed with immersion oil (Leica immersion oil, n = 1.518; ref. 1). Plant or animal-derived histological samples can also be used to measure field illumination and are usually the choice of service field engineers.

It is useful to have a samples to test field illumination because there can be problems with specific test slides or with specific protocols. Uranyl glass has been used previously to check field illumination, but it is difficult to obtain and we have observed that plastic slides have higher efficiency than the uranyl glass at all wavelengths. A field of small or large beads suspended in a slide (Spherotech) can be used, but it is essential that all beads are located at the same plane or the image will be inaccurate. To eliminate this potential error, a stack of images can be obtained from the beads followed by a maximum projection of the stack to obtain an image of bead that represents field intensity. However, the downside of this method is that it is very time consuming to perform. In our experience, histological samples are not sensitive enough to properly measure field illumination. They yield a sense of false security for the investigator. The plastic slides (Applied Precision) were the most consistent sample to test field illumination. We measure routinely the intensity between 30 and 100 μm beneath the surface, depending on the objective tested. The surface of the slide is determined as it is the region that emits the most intense fluorescence in the "z" axial direction. It is important to measure field illumination at a specific depth in the plastic slide because the intensity distribution may change from the surface to the interior of the slide due to various optical distortion factors. Investigators should also be careful not to observe illumination fields deep within the plastic slide samples as they yield a better field illumination than regions closer to the surface (15). The slide surface also contains irregularities, scratches, and possible warping. It is also important that the plastic slide be placed on a firm surface to eliminate any possibility of substrate flex.

Field illumination is one of the easiest and most important tests to make on a confocal microscope. From per-

sonal experience, many CLSM units that have been checked for field illumination using a plastic fluorescent slide (Applied Precision) have been found to have an unacceptable field illumination pattern. The CLSM system misalignment was caused by an inaccurate adjustment of field illumination using histological slides, the laser alignment drifted out of alignment, or the service engineer never made the proper adjustment. In any case, there is a discrepancy between the plastic substrate test and the histological test slide. This test should be carefully evaluated for proper representation of intensity in a microscopic field. It should be made with all objectives and all wavelengths of visible and UV light.

In our system, perfect illumination was observed with visible wavelengths using 20×, 40×, 63×, and 100× objectives. However, when using the identical objectives with UV excitation, the field illumination was poor (Fig. 1). The maximum light intensity is not located in the center of the field. The data in Figure 1 represent illumination in which the brightest spot was in a different quadrant of the image field with each objective used. The UV system consists of three collimator lenses. Therefore, the field illumination of one lens is not necessarily related to the field illumination of the other lenses. In our UV system, good field illumination was recorded with low-power (5× and 10×) objectives. However, poor field illumination was observed with all higher magnification (20×, 40×, 63×, 100×) objectives. This indicated that there were major problems with either the alignment, collimator lenses, or an incompatibility of the objective with UV laser light.

The nonuniform pattern (Fig. 1) with UV illumination illustrates a field illumination problem, which will affect the representation of accurate intensity in an image. Although Figure 1 was obtained with UV optics, it represents field illumination that can occur with visible excitation and should be considered unacceptable with any CLSM optical system. Maximum intensity should be in the center of the objective, not in a corner or at the top as was observed with the four objectives tested on the Leica TCS-SP1 confocal microscope. The Leica 20×, 40×, 63×, and 100× objectives pass this intensity criterion when using visible excitation light (1). Due to the design of the 100× objective, a zoom of 2× is recommended when using UV light in order to achieve a drop off of less than 25% across the field (Fig. 3). Leica recommends the use of the Plan Apo 63× (NA 1.2) for UV excitation because it has better UV field illumination. This field illumination test allows system evaluation that consists of both the objective properties and the confocal microscope alignment.

Field illumination should be relatively uniform, with the maximum intensity being in the center of the objective and decreasing less than 25% across the field according to the manufacturer. Data derived from a 20× Plan Apo lens (NA 0.7) zoomed to a factor of 1.2 illustrate good visible field illumination (488 nm) and a misaligned UV (365 nm) system yielding poor field illumination (Fig. 2). The images were obtained with either the UV (ex 408, em 447) or visible plastic slide (ex 488, em 505) located securely on the stage. The original images were contoured into 10 intensity ranges using Image Pro Plus software. The line running diagonally in Figures 2A,B measures the histogram intensity of the field that is represented in the graphs in Figures 2C,D. The maximum intensity should be in the center of the objective (visible, Fig. 2A) and not in the bottom corner (UV, Fig. 2B). The visible light (Fig. 2C) had less than a 10% decrease in intensity across the field whereas the UV light (Fig. 2D) had a 150% decrease across the field. The accepted intensity values derived from Leica engineers should decrease by less than 25% in intensity from the center maximum value. This value was high as we obtained variations in the 10% range with most of our Leica objectives.

When an identical field illumination test (Fig. 2) with a 20× Plan Apo (NA 0.7) was made on an identical CLSM system using a 20× Plan Apo lens (NA 0.6), the field illumination patterns were considerably better. Both 20× Plan Apo lenses yielded different field illumination patterns. The lower NA lens (NA 0.6) showed a better pattern than the higher NA (0.7 NA) lens. When using a higher NA lens, it becomes more difficult to align correctly the UV system (Fig. 1). These data suggest strongly that all the lenses in a system should be tested for field illumination accuracy using both UV and visible excitation wavelengths.

The three visible wavelengths of light in our system are derived from one Omnichrome argon-krypton laser, which allows us to test field illumination at one wavelength (488 nm) and assume it is equivalent to testing field illumination with the other wavelengths. Because the UV line is derived from a different laser (Enterprise, Coherent), it is essential to check all objectives for proper field illumination (Figs. 1, 2) at the 365 and 488-nm excitation wavelengths. Newer confocal systems use three lasers with merge modules, which require that all laser wavelengths have correctly aligned beams emitted from the merge modules. In these systems, the three lines have to be tested individually. One laser line may be aligned perfectly and yield acceptable field illumination. The other laser lines may be misaligned and yield intensity values in which the brightest region is not in the center of the field (Fig. 1).

Most alignment procedures use high magnification objectives, which does not always translate into good performance with lower magnification objectives. As shown previously (1), the system was aligned perfectly for visible fluorescence with all objectives except a 10× lens (NA 0.3). This optical problem was not observed during installation of the equipment because the system was tested with higher magnification lenses only. We replaced the lens with the newer 10× Plan Apo objective (NA 0.4), which has better specifications and yields perfect field illumination. This emphasizes that lenses of the same magnification can yield different field illumination patterns on the same CLSM, necessitating that each lens be tested for its proper field illumination.

Not all problems with the field illumination test are the result of poor alignment, lens design/quality, or incompat-

ibility of a lens with specific wavelengths of light. This test is also useful to identify and address the problem of a dirty lens. A dirty lens or a lens covered with dried oil yields a nonuniform pattern (1). In one example, the intensity of the field from a 20× (NA 0.6) dirty lens varied by as much as 70%. The maximum intensity was off center on the right side of the image. After cleaning the lens, an acceptable illumination pattern was obtained. The maximum intensity was in the center of the image and decreased less than 10% from the center (1).

Using visible light, the lower magnification objectives show highly concentrated illumination in the center of the field (bull's eye; 1). However, the bull's eye intensity pattern is present with different magnification objectives using all manufacturers' systems (Biorad, Leica, Zeiss). The incompatibility of different lenses with confocal microscope systems can increase this bull's eye effect. Therefore, this parameter should be considered when choosing lenses. The problem appears to be due to lasers underfilling the objective, which results in field illumination problems and suboptimum conditions for lens operation. Unfortunately, this field illumination effect has to be monitored with each laser wavelength and each objective because the alignment, wavelength, and lens design influence the field illumination pattern. One recommended solution to poor field illumination is to increase the zoom factor. However, this enlarges the illumination center and pushes the lower intensities off the field of view. Increasing the zoom also increases the magnification and bleaching rate of the sample, which may defeat the purpose of using a low-magnification objective to observe a large field of illumination. Leica recommends increasing the zoom by 20% (from 1.0 to 1.2) to eliminate known problems with illumination and yield a homogenous field illumination pattern. In summary, in order to eliminate field illumination problems, the system should be aligned correctly with the brightest light being focused into the center of the field and decreasing less than 25% in all directions equally from the center. This is a specification that should be required by the manufacturers of confocal microscopes and the CLSM units should be serviced to this specification.

Intensity Measurements

If the field illumination is not uniform, errors in comparative intensity measurements will be made. To illustrate the potential problems that can occur with improper field illumination, we imaged Spherotech 10-μm uniform Rainbow intensity beads with a 100× Plan Apo lens (Fig. 3). The 100× lens (Plan Apo NA 1.4) yields perfect field illumination with visible light but shows a bull's eye intensity pattern with UV illumination (Fig. 3). The beads in the center of the bull's eye have a GSV intensity of 230 on a scale of 0–255. The other beads located away from the center of the field show a GSV intensity value of 123 (approximately 100% reduction) and a GSV of 47 (approximately 400% reduction), depending on the distance away from the center and the decrease in light illuminating the bead. Any specimen or cells measured with such a field illumination would reveal the same nonuniform intensity and the accuracy of the intensity measurement would be compromised. The recommended solution is either to zoom the specimen to reduce the intensity differences in the field, use a small part of the field that shows uniform illumination, or buy a different lens with better UV field illumination properties. In place of the Plan Apo 100× lens, Leica recommends a Plan Apo 63× (NA 1.32) for UV applications as it produces good UV transmission and more uniform UV field illumination. The data from this test can be interpreted as the existence of some incompatibility between confocal illumination in a CLSM and a specific lens design. This necessitates the measurement of the field illumination for each objective with both visible and UV wavelengths to ensure the accuracy of field illumination.

Axial (Z) Resolution

The axial resolution test is considered the "gold standard" of resolution in confocal microscopy (1,5, 6,16,17). Although it is not the only criterion for a good image, the axial resolution of the system should be maximized to yield a minimal axial Z resolution value (1). In order to measure axial resolution, it is necessary to have a front surface, single reflective mirror (31008; Edmonds Scientific or Spherotech). The mirror is glued to a glass slide and a 1.5 coverslip (17 mm) is placed on top of it with a drop of the manufacturer's immersion oil (Leica, N = 1.5180). Initially, axial resolution is tested in reflection mode with a 100× objective (NA 1.4 Plan Apo lens), a zoom of 24×, a large pinhole diameter opening, and minimum laser power. After the reflected surface is found by scanning in xz mode, the pinhole aperture is reduced to a minimum. The reflected image is obtained with frame averaging and the intensity profile across the reflected surface is determined as shown in Figure 4. The half-maximum intensity value of the profile is obtained to determine the full width half-maximum (FWHM) distance to determine the axial resolution. The specification for axial resolution in a Leica TCS-SP system is 350 nm. It is important to compare the user-determined test slide with that of the service technician's slide to ensure that both specimens yield the same value. Once an acceptable value is obtained, it should remain constant and the scan head can be configured with either an upright or inverted microscope. When alterations are made in the scan head (i.e., galvanometer replaced) or when the lasers in the system are replaced, it will be necessary to realign the system and measure the axial resolution again. This test can be useful in comparing the axial resolution between different objectives (Fig. 4) and between CLSM units from the same or different manufacturers to test performance.

The axial resolution test is made using a 100× Plan Apo (NA 1.4) objective. If the laboratory does not have a 100×Plan Apo (NA 1.4) objective or if it is not possible to borrow one for comparison purposes from another confocal facility, it is useful to have as a reference point other system lenses to eliminate the variable of the lens when measuring axial resolution. The axial Z resolution of three

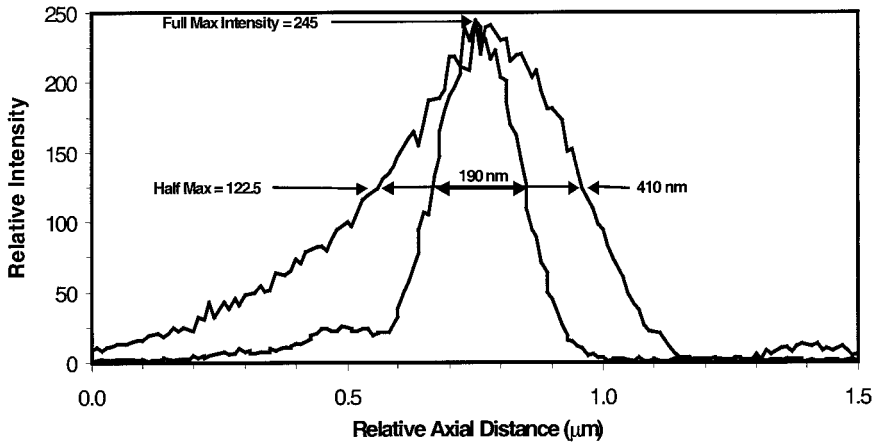

FIG. 4. The axial resolution was made with two 100× lenses (NA 1.4) on the same Leica TCS-SP1 confocal system. The peak intensity of the histogram is 245 and the half-maximum intensity is 122.5 One lens gave an excellent FWHM of 190 nm whereas the other lens yielded a value of only 410 nm. The system was aligned properly in both cases, but the lens failed to meet this strict performance test and was returned to the Leica factory for modification. The returned lens yielded a value below the specification of 350 nm, indicating a good lens delivering proper axial resolution in the confocal system.

lenses was 610 nm for a 40× (Fluor, NA 1.0), 390 nm for a 63× water immersion lens (Plan Apo NA 1.2), and 400 nm for a 63× oil immersion Plan Apo (NA 1.32) objective. A new 63× Plan Apo (NA 1.32) lens should meet the specification of 350–400 nm, although Leica does not guarantee this value on a TCS-SP system. The excellent resolution that was obtained with the 40× and 63× lenses on our aligned system can serve as a system standard for axial resolution in a correctly aligned machine for other investigators using Leica TCS-SP equipment.

When the machine did not achieve the manufacturer's specification of 350 nm, problems were found with the confocal scan head or the lens quality. In the first case, it was suspected that the performance problem might be in the lens and not the system alignment as the service engineer aligned the system according to protocol and it still failed to achieve the manufacturer's specification of 350 nm with our Plan Apo 100× lens (NA 1.4). Figure 4 shows a Z resolution test with two objectives tested on the same CLSM. One lens yielded an excellent value of 190 nm and the other yielded a suboptimal value of 410 Because both lenses were measured on the same system, the lens yielding the 410 nm value was considered defective and was returned to the factory for examination and repair. A Leica representative in the United States stated emphatically that Leica does not make defective lenses and it was thus reworked by the factory to ensure its proper performance levels. In contrast, the objective yielding a value of 190 nm illustrates superb resolution that exists with only very few lenses. Normally, we obtain values between 280 and 350 nm. The system using the repaired 100× objective now achieves an axial Z value under 350 nm with adequate power on the stage, thus meeting Leica's published specification.

In the second case, a value of only 370 nm was obtained with our confocal system. Opening up the scan head revealed a problem that needed the attention of service personnel. A nonsymmetrical diffraction ring pattern was observed by examining the light pattern derived from laser light traversing the excitation pinhole. This results in laser light attenuation and corruption. Further examination by the Leica service personnel revealed that the sheet of metal that contains the pinhole was slightly warped. By changing the excitation pinhole, the axial resolution decreased below the 350 nm specification. Although the difference between 370 and 350 is only 20 nm, values below 350 nm will increase system performance by yielding better resolution. Leica should be complimented for releasing these values and they should release additional axial resolution values for all other high-performance lenses used on their CLSM. Other manufacturers should provide similar values to ensure that their systems perform to these specifications.

Square Pixels

The pixel size and symmetry in XY directional field scanning can be checked with a computer chip attached to a glass slide. This ensures that the scanning in the X and Y directions yields a perfect square. If the image of the smaller squares on the chip is not represented as a square, then the pixels contained in a specimens image will not be square, but rectangular, and the information in the specimen's image will be distorted (data not shown).

Spectral Registration (UV and Visible)

Figure 5 shows the xz registration between the 365 UV line and the 568 visible line in a misaligned system. The 1-μm multiple wavelength fluorescent beads (Tetraspeck, T7284, or Rainbow beads) were used to monitor the visible spectral registration of lenses (100× Plan Apo, NA 1.4; 63× Plan Apo, NA 1.2; Plan Fluor 40×, NA 1.0) or the registration between multiple beams (UV and 568 nm in our case). By balancing laser light intensity with the AOTF, the laser crossover between the detection channels was minimized. The bead was imaged (xy and xz scans) with a 24× zoom and a slow scanning rate and frame averaged eight times. The registration of bead fluorescence images between the 365 UV wavelengths and the 568 visible wavelengths in an aligned system was almost superimposable (data not shown). In a misaligned system (Fig. 5), the difference between the peaks was 650 nm (acceptable difference is only 210 nm). The 568 line was chosen

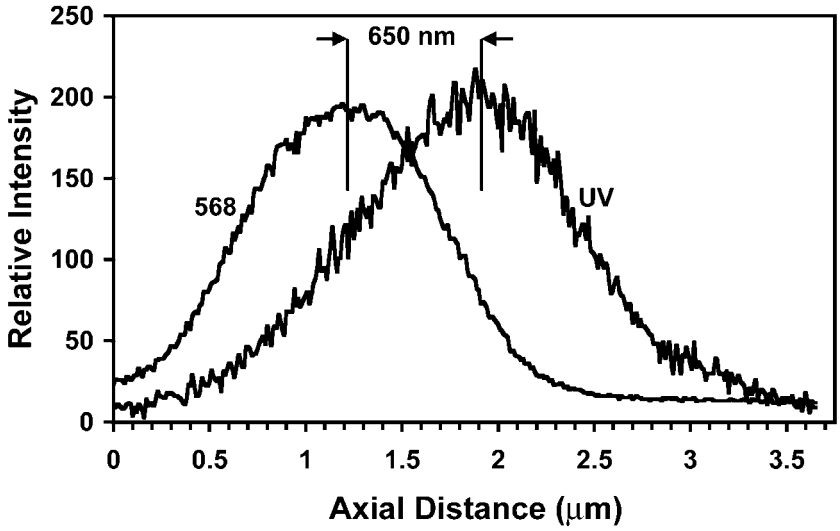

FIG. 5. Spectral registration (UV and visible). The xz spectral colocalization of the UV (365 nm) and visible wavelengths (568 nm) was evaluated with a 100× Plan Apo NA 1.4 lens using a 1μm multiple wavelength fluorescent bead (Tetra Spec T7284). An aligned system has a FWHM of less than 210 nm (not shown) whereas a misaligned system has a FWHM difference of 650 nm. The bead was imaged using xz scans with a 24× zoom, a slow scanning rate, and averaged eight times. The 568 line was chosen instead of the 488 line to minimize the crossover between the visible and UV wavelengths.

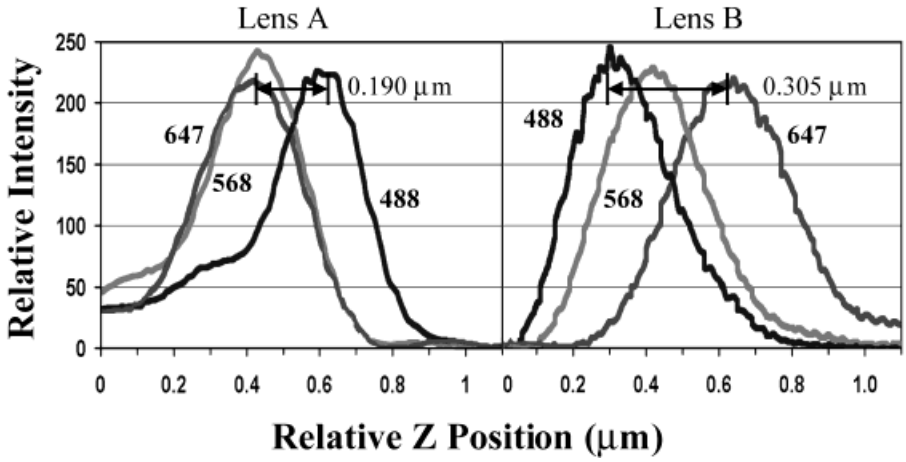

FIG. 6. Spectral registration (visible). The visible spectral registration of a 100× Plan Apo NA 1.4 objective was evaluated using a front surface, single reflection mirror with the same lens at different times. A 10-nm slit is put over each wavelength and the reflection of each line was measured sequentially. The AOTF and PMT intensity was adjusted so the maximum intensity of each line was 250 GSV. Lens B was sent back to the factory, as it did not meet spectral registration for UV (365) and visible (568) wavelengths, spectral registration for the three visible lines, and axial resolution specifications. The refurbished Lens A showed excellent registration among the three visible lines with the difference being less than 220 μm. Refurbished Lens A also had an axial registration below 350 nm. This single reflection mirror test yields slightly better spectral registration than 1-μm bead data for the 647 excitation line. This is because the fluorescence emission occurs in the far-red range (>660 nm) and many lenses have difficulty colocalizing this far red emitted light with the fluorescence emitted from the 488 and 568 wavelength excitation.

instead of the 488 line to minimize the crossover fluorescence between the visible and UV wavelengths.

Molecular Probes produces a series of beads (Focal Check) with fluorescent rings to assess colocalization from multiple lasers. With the proper dye excitation fluorochromes, the beads can be used to assess visible wavelengths from multiple lasers in a confocal system. We used these beads to examine the UV and visible lines in our TCS-SP1 confocal system. A 15-μm bead with a UV interior and a red fluorescence ring exterior (F7236; Molecular Probes) was used to show that the UV and 568 lines were aligned. The bead should reveal concentric fluorescent rings that are maximum in the same focal plane with either an xy or xz scan. We do not have information on how this bead identifies a misaligned system, but the test is easy to perform and should be tried using suitable fluorochome-stained beads in multilaser systems that have merge modules (data not shown).

This spectral registration test demonstrates the ability of the CLSM to colocalize wavelengths of varying fluorescence in the same focal plane. To evaluate the spectral registration of the 365, 488, 568, and 647 nm lines, either a 1-μm multicolored bead (1) or a front surface, single reflective mirror (Fig. 6) was used. The front surface, single reflective mirror can be used to check visible spectral registration in a Leica TCS-SP1 system, in a similar manner to that described in Figure 4 for axial registration. In the Leica system, a 10-nm reflection bandwidth is placed over each excitation wavelength and the reflection is measured sequentially. By tweaking the AOTF and PMT

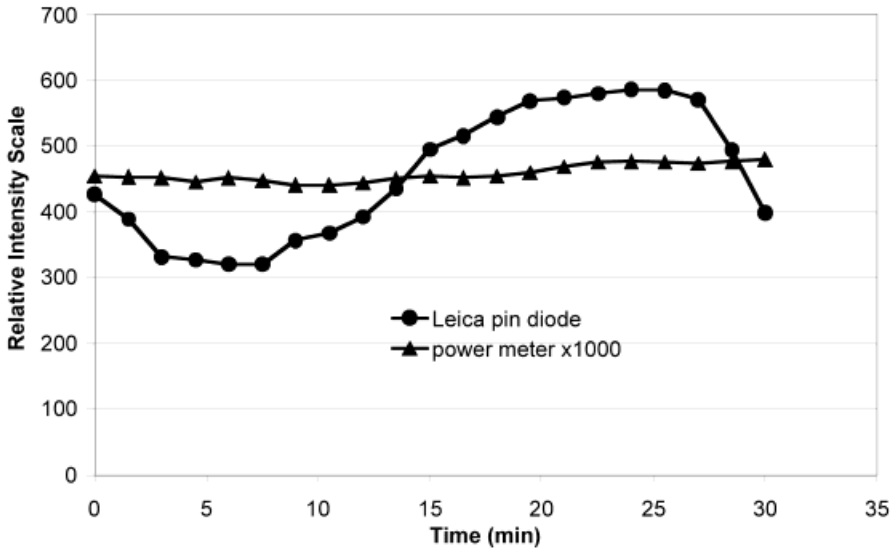

FIG. 7. A pin diode contained in the machine varied in intensity over time. Simultaneous comparison of the measurements using a pin diode and a Coherent power meter with an LN 818 visible detector on the microscope stage demonstrated that the pin diode had unstable power readings and was not reliable. The built-in pin diode should not be used as an absolute indicator for power or stability, as it varies greatly over time. It can, however, be used as a subjective assessment of the laser performance. This test indicated that the lasers in the system were relatively stable. However, the pin diode measurements fluctuated for unknown reasons.

voltage adjustments, the intensity of each reflected line was adjusted so that the maximum intensity of the image was approximately 250 GSV. This mirror test is more accurate than the bead tests, but the data obtained skew the results slightly toward better values. In normal operating conditions, the emission from either specimens or beads is recorded at least 10–40 nm above the excitation wavelengths, not at the excitation wavelength. Many lenses have difficulty in colocalizing far red fluorescence with the blue and green fluorescence. Therefore, measuring the emission at 647 ± 10 nm will yield better resolution than measuring the emission at 660–700 nm. Figure 6 represents the identical lens (A & B) using the same CLSM system measured after a period of months. Lens B revealed problems in axial resolution and spectral registration. The separation between the 488 nm and 647 nm line was 305 nm; it should be under 220 nm. Lens B was returned to the factory to correct the spectral registration problem in visible wavelengths, the problem in spectral registration between UV and visible wavelengths, and a problem in axial registration of 410 nm. Upon return, of Lens B (now named Lens A) it showed perfect colocalization between the 488 nm and 647 nm lines and acceptable registration between these lines and the 568 lines. In addition, the lens was corrected to yield an axial resolution of under 350 nm. This spectral registration test illustrates the spectral registration performances that can be obtained from two lenses from the same manufacturers on the same CLSM. Unfortunately, not all lenses meet manufacturer's specifications, which require that they all be individually checked to ensure proper performance. Systems that do not have the spectrophotometer head will most likely have to use a 1-μm bead to test the colocalizations of visible wavelengths.

Laser Power Stability (Visible)

Power stability in a CLSM can be influenced by the lasers, PMTs, electronics, AOTF, heat dissipation, fiber optics, optical components, and galvanometers. For an investigator, it is not important initially to know where the source of instability is being generated, but that it exists. Once the problem is identified, trained microscope service personnel will be able to troubleshoot the system to correct the problem and remove the power instability. In a confocal microscope, power stability over time (hours) can be measured by the manufacturer's installed pin diodes, laser meters on the microscope stage connected to a readout device, fluorescent emission intensity from a plastic slide to a PMT, and transmission optical system detection.

Simultaneous comparison of the measurements using a pin diode in the Leica SP CLSM and either a power meter (Fig. 7) on the stage or the transmission average intensity (not shown) demonstrated that the pin diode has unstable power readings. The other two measurements (transmission optics detection and power meter) were relatively stable over time. The pin diode should not be used as an absolute indicator for power or stability, as the power derived from it can vary in intensity over time. It can, however, be used as a subjective assessment of the laser performance and system alignment. This test indicated that the lasers in the system were relatively stable but the pin diode power measurements fluctuated and should not be trusted as an indicator of power stability.

One way to monitor CLSM laser power is to connect a UV or VIS detector to a suitable power meter situated on the microscope stage and then to monitor continuously the power output with either a chart recorder or equivalent computer software. Manual measurements are not accurate enough and are very time consuming. If transmission optics is not available, a similar power test can be made that uses a fluorescent slide sample placed in the light path. However, the investigator must be aware that repeated samplings of a fluorescence slide may bleach the sample, which will decrease the fluorescence intensity and increase the transmission intensity. Therefore, the

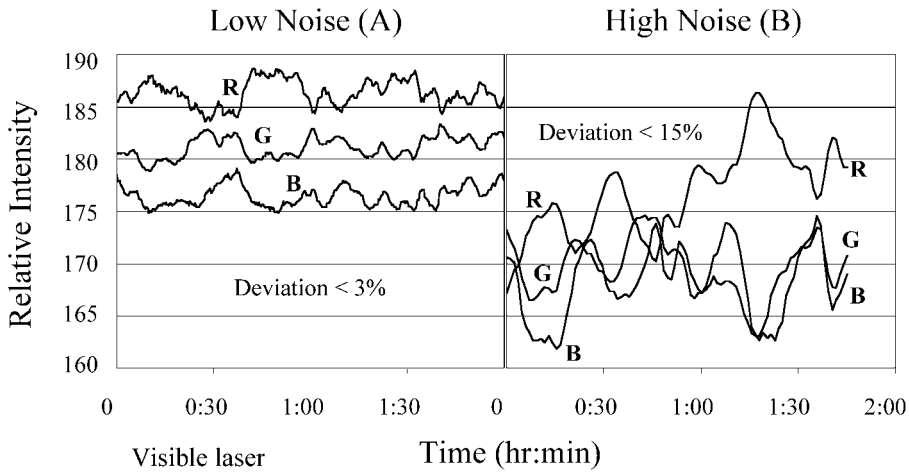

FIG. 8. Laser noise. The periodic change in laser power was measured using transmitted interference optics without a slide in the optical path. The two panels show a visible system delivering low power fluctuations and one delivering high laser power fluctuations over time. The 488 and 568 lines cycle periodically and directly opposite the 647 line. Variations in this power intensity occur over hours and never stabilize. To measure laser stability over time, the PMT was kept constant and the laser power of the three lines was adjusted with the AOTF. Next, over 200 samples were measured sequentially every 15 s. After the test was complete, the intensity of a large ROI was evaluated and plotted over time. The three lines are designated R (red, 647 nm), G (green, 568 nm), and B (blue, 488 nm). The laser power instability may be due to either laser light entering a fiber with an incorrect laser polarization, thermal instability in the AOTF, or to a badly aligned system. The reason for the source of power instability is not known.

laser power should be decreased with the AOTF to minimum values to help reduce slide bleaching as decreasing the laser power with the power supply may result in laser instability. In addition, one must be aware that energy excitation of the slide fluorchrome may occur.

The most reliable method to measure laser power stability consisted of using the transmission optics of the CLSM without a fluorescence slide in the optical path. To measure laser stability using the transmission optical system, the microscope is first aligned for Kohler illumination with a histological slide, which is then removed from the optical path. The image intensity is measured using the transmission detector for the three wavelengths of the argon-krypton laser by measuring sequentially the laser light with the 488, 568, and 647 wavelengths. The power is adjusted by the AOTF transmission control so that the transmission detector voltage remains constant for all three wavelengths. The test usually takes a few hundred scans separated by 15–30 s over a period of 2 h. The intensity of a large ROI of the field was averaged and plotted over time for the three wavelengths (Fig. 8). Power fluctuations in excess of 30% have been measured for both the UV and visible lines during a 2-h test of laser power stability.

Lasers used in flow cytometry or confocal microscopy equipment should be stable with low peak-to-peak noise and minimal power fluctuations over hours. Laser noise can originate from different sources, including the AOTF, laser polarization mismatch, heat dissipation, and power supplies. One of the most likely sources is a poor power supply regulation that results in light output fluctuations at the frequency of line current used to run the power supply (18). Noise in the He-Ne laser may be found at frequencies of a few hundred kilohertz due to either radio frequency energies used to pump the laser medium or to fluctuations in the medium itself (18). The DC power supply should be the correct type (Omnichrome power supply 171B or 176B with Omnichrome argon-krypton laser) to produce low noise and should be operated at "light mode" (constant power), not at a constant current mode (18). The 171B power supply had transformer and heating problems and was replaced by the 176B model with rectifiers that regulate heat better. Typically, a Coherent Enterprise laser (e.g., 90-5 or 70-4) will have less than 1% peak-to-peak noise (7) and the power will not fluctuate over time. The air-cooled argon laser from Uniphase or Spectra Physics used in bench top flow cytometers or confocal microscopes will also have less than 1% peak-to-peak noise according to the manufacturer's specifications. However, the argon-krypton (Melles Girot, Omnichrome 643) laser, which delivers three simultaneous lines, yields maximum peak-to-peak laser noise between 3% and 5% for the three lines under 100 kHz and 6%–8 % peak-to-peak noise for power under 1 MHz. Use of an argon-krypton laser is not recommended due to this peak-to-peak noise, unless it is needed to produce more than one excitation line. However, the power intensity fluctuations over 2 h will be less than 0.5% according to the manufacturer (personal communication and web site). Where do the fluctuations in power intensity (Figs. 7, 8) originate if the laser is not generating it? To determine their stability, lasers can be checked with power meters in front of the beam or by special electronic boards that connect to the power supply. However, this testing may not be possible because the laser is in a sealed compartment, which must not be violated to avoid nullifying the service contract.

The argon-krypton laser has been incorporated into the older systems from Leica, Zeiss, and Biorad, which used three excitation lines derived from one laser. It is sold by

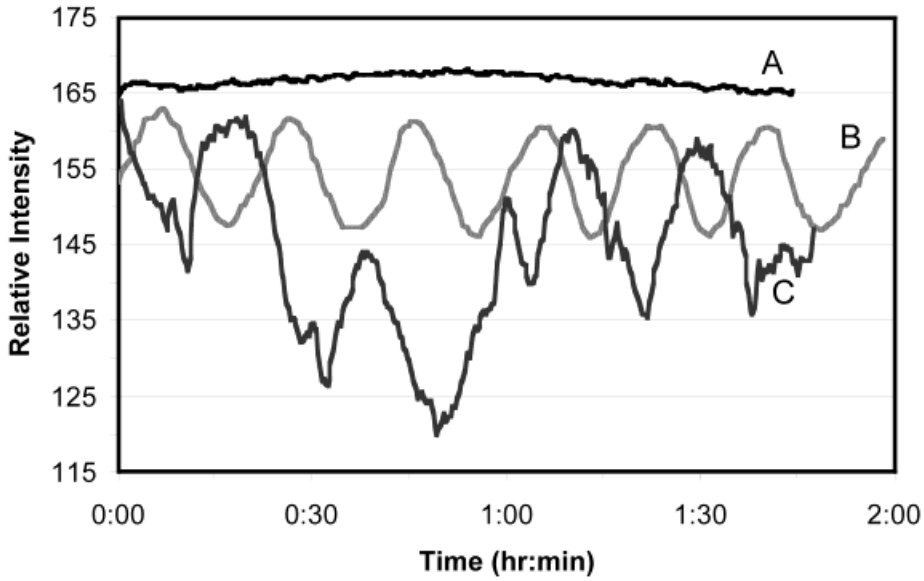

FIG. 9. Laser stability of a Coherent Enterprise UV laser. The Coherent Enterprise UV laser delivers less than 1% peak-to-peak noise. The laser was connected to an LP 20 water-water cooler, which should be set at least 10°C above the cooling water of the building. It should also be set above the ambient temperature of the room. Improper set points for laser cooling resulted in poor thermal regulation of the laser (B). Improper fiber alignment resulted in additional laser intensity variations (C). The elimination of the temperature and polarization issues resulted in proper laser stability (A, 3% power variation over time) The test was conducted by measuring the laser power stability in PMT1 using a fluorescent plastic slide. Neutral density filters were used to reduce the power and minimize slide bleaching. The transmission detector optics gave similar results to the UV fluorescent plastic slide and was also used to measure UV laser stability.

Biorad in the United States, but not by Leica and Zeiss due to Biorad's patent infringement. Spectra Physics has released an argon-krypton laser that has less than 1% peak-to-peak noise and its power is supposedly very stable over time. However, this laser only emits the 488 and 568 wavelengths. Therefore, another HeNe laser with a merge module will be necessary to produce the 633 wavelength. The argon-krypton lasers will also deteriorate with time due to escape of gas, which results in a continuously reduced power output over time. The merge module design allows for the incorporation of multiple lasers in a confocal microscope that are less noisy and more stable than the Omnichrome argon-krypton laser supplied by Melles Girot. One major problem with the design of the current version of confocal microscopes that use merge modules is that the laser lines are directed into the scan head with the fiber optics. This is in contrast to the older versions, which used direct coupling with dichroics to deliver the light to the microscope's scan head. The use of the fiber optics makes it critical to align the polarization in the fiber optics with that of the laser's polarization value (17). Failure to do this may result in laser power instability in the CLSM system. The fiber optics also deteriorate with time, which will attenuate the laser power and necessitate running the machine with higher laser power or higher PMT settings. One test procedure to ensure that polarization is correct after the alignment procedures is to wiggle the fiber optics. If the image returns to the same intensity, the polarization is correct. This is a crude test, but it demonstrates whether the system needs further alignment. Confocal microscope manufacturers are supposedly devising more reliable tests to check for polarization and to ensure laser power stability in the CLSM.

Laser stability measurements should be made to ensure that the CLSM does not introduce artifacts in intensity measurements in time-dependent physiological experiments. Power stability tests to measure the fluctuations in power are described in the Materials and Methods section. They are invaluable for research involving comparative intensity measurements or those that require sequential intensity measurements over time on the same sample. Laser power stability over time shows a stable visible system that yields low power fluctuations (Fig. 8A, $<3\%$) and an unstable visible system (Fig. 8B, $>15\%$) that yields increased laser power instability (source of noise is not identified). The goal of this test is to achieve a flat stable line, which was never achieved (Fig. 8). There is periodic noise in the laser system that exceeds the manufacturer's (Omnichrome) laser stability fluctuation specifications of less that 0.5% over a 2-h time period. The 488 and 568 lines have a periodic cycle that is directly opposite the 647 line (Fig. 8).

The dissipation of heat is an important variable to consider when measuring laser stability. Improper heat dissipation with the visible air-cooled lasers causes the laser power to fluctuate. This fluctuation occurred with our argon-krypton air-cooled laser, which had a restrictor in the exhaust line and used a smaller (4 in. instead of 5 in.) exhaust duct to remove heat. In both cases, the heat was not dissipated correctly and the laser power in the CLSM fluctuated above 20%. All lasers have to dissipate heat properly and their thermoregulators must be set correctly or the power to the lasers will fluctuate (Fig. 9).

The AOTF may also introduce power fluctuations in the CLSM system by improper thermal regulation. The AOTF is a birefringence crystal capable of rapid and precise wavelength selection. Earlier CLSM systems used dichroics, barrier filters, and neutral density filters to regulate the proper intensity of laser light that illuminates the samples. They did not use the AOTF. However, the AOTF is useful in operating a confocal microscope as wavelength selection can be controlled easily and accurately. It is also used to control the power that illuminates a sample, which effectively acts to reduce the crosstalk between detectors.

The original AOTF that was installed in older CLSM models was not temperature regulated and may have introduced power instability in confocal microscopes. The data presented in Figures 8 and 9 show that power instability occurs in some CLSMs, which may be due to faulty operation of the AOTF. If laser stability is due to the AOTF, extra care should be made to optimize the conditions of its use and to install the higher quality devise. This problem is disturbing to investigators making comparative intensity measurements on samples. The problem should be addressed by the manufacturers, who should correct and release specifications for proper laser stability.

We have presented a method to identify the problem if it exists in a system. The data obtained from this power stability test alert the investigator to possible errors that may exist in the acquisition of intensity measurements in biological and physiological experiments. It has been reported that some Zeiss 510 systems have fewer than 1% fluctuations over time (Kees Jalink, personal communication). The reason for such high variations in other CLSM systems is unknown. It may be attributed to the power supply, AOTF thermal regulation, improper thermal heat dissipation, electronic component failure, or fiber-optic polarization incompatibility. A major problem with this system is that large laser power intensity variations occur over hours and never stabilize.

Laser Power Stability (UV)

In contrast to the Melles Girot argon-krypton laser (3%–5% peak-to-peak noise), the Coherent Enterprise laser delivers less than 1% peak-to-peak noise and is quiet and stable. The argon air-cooled, HeNe, and Spectra Physics argon-krypton lasers are all rated at less than 1% peak-to-peak noise. However, even with the Coherent UV Enterprise or HeNe laser (543 nm), periodic noise and large power fluctuations were observed. One source of power stability is connected to the way the laser is cooled and heat is dissipated. This was illustrated with the Coherent Enterprise UV laser, which was connected to a Coherent LP 20 water-water exchange cooler. This cooler should be set at least 10°C above the circulating cooling water of the building and it should be set above the ambient temperature of the room. Improper set points for the LP 20 cooler resulted in temperature regulation problems, which resulted in the improper regulation of the laser power (Fig. 9B). In addition, problems with proper fiber alignment also occurred with the UV system, which resulted in power fluctuations (Fig. 9C). The elimination of these temperature and polarization issues resulted in proper laser cooling and laser stability (Fig. 9A, $<3\%$ noise). Although it is not the focus of this study, the water-water coolers for UV lasers in flow cytometry should also be adjusted with the proper set point values as they are sensitive to the same power variations (Fig. 9).

Bead Tests

In flow cytometry, alignment beads, linearity beads, and chicken red blood cells (CRBC) are used to ensure that the machine is functioning properly (19,20). It would be useful if a suitable bead or test sample were used for a similar assessment on the CLSM. This work has been described in depth in another publication (21). Because it is essential for the optimization of the CLSM and our QA assessment, the relevant issues are also discussed in this study. The major element of noise in a confocal system is related to the functionality of the PMT and the PMT voltage values used to obtain an image. The noise associated with various settings can be evaluated by varying PMT settings, frame average, scan speed, image size, and laser power (1,21).

The noise present in the system was evaluated with a 100× objective (Plan Apo NA 1.4) and the test sample was a large 10-μm bead (Spherotech) of nearly uniform size and intensity (coefficient of variation [CV] = 5% by flow cytometry) that was zoomed 4× to increase the number of pixels contained in the ROI. The bead was located in the center of the field and the image of the bead was obtained at the center cross-section of the bead, which relates to its maximum diameter. This large bead permitted repeated measurements without bleaching the sample. In the bead image, a fixed ROI was determined that consisted of approximately one half of the area of the bead. The Leica analysis software was used to determine the mean and standard deviation of the pixel intensities in the ROI of the bead. It is important to maintain the machine variables (pinhole size, PMT voltage, averaging) at reproducible values for all studies. The laser power was set at a constant value that allowed the mean intensity level of the bead to be approximately 150 (out of 255) for each PMT setting.

The CV of the population of beads or pixels is defined as $CV = \sigma/\mu$ (σ = standard deviation of intensity, μ = mean intensity; 20–22). This measurement is a a noise-to-signal ratio, where μ represents the true signal in the image and σ represents the noise in the image. In this test, μ is always at mean channel 150 whereas σ can vary, depending on the system variables. The CV being measured is the variation of pixel intensity within the bead, as opposed to the variation of intensity among a population of beads. An increase in CV may imply that there is either a decrease in laser power or a system alignment problem that results in higher PMT values. A noisy laser will also be detected by this test as fast scans yielded less noise than slower scans.

This CV test (derived from pixels within a bead) assesses CLSM sensitivity with multiple PMTs, as well as the quality of the PMTs in the CLSM. In the Leica system, there are three Hammatsu PMTs: PMT1 is a low-noise, blue sensitive type; PMT2,3 are higher noise but far red sensitive. The PMT voltages demonstrated a logarithmic increase in intensity values as the PMT voltages increased (1). The Leica TCS-SP system allowed easy switching of PMTs with the different excitation wavelengths. The system was set up with a TD with 488, 568, and 647 excitation. The three PMTs were adjusted so that the mean pixel of the ROI was at approximately channel 150. The relative intensities were measured with the three PMTs for all excitation and emission conditions (Table 3). Due to the physical location of the three PMTs and the way Leica

Table 3
Comparison of PMT Noise Versus Time

PMT	Excitation (nm)	Relative CV (T = 0)	Relative CV (T = 9 mo)
1	488	1	2%
2	488	9%	1
3	488	3%	9%
1	568	1	1
2	568	22%	6%
3	568	12%	8%
1	647	1	1
2	647	12%	4%
3	647	1	2%

*The quality and the performance of each PMT can be measured with this CV test. The sensitivity of the system was evaluated with a 10-μm bead (Spherotech) and a 100× Plan Apo (NA 1.4) objective using the CV concept. The pixel intensity of a 10-μm bead was determined with the following conditions: constant laser power, TD, zoom of 4, no averaging, and various PMT settings. The emitted light was measured in each of the three PMTs with each wavelength of light. The pixels in each ROI were set to a mean of approximately 150 and the SD of pixel distribution was measured to determine the CV (CV = SD/mean). The CV of the pixel distribution within the bead was measured at a PMT setting that yielded a mean value of 150. PMT1 is low noise, blue sensitive whereas PMT2 and 3 are far-red sensitive. PMT2 differed from PMT1 by 22% and differed from PMT3 by 12% with 568 excitation. After PMT2 was changed, better performance was obtained with all excitation wavelengths. The values are shown as decreased percentages relative to the individual PMT yielding the best CVs.

CLSM reflects light, the most efficient PMT should be PMT1. These tests on PMTs were made at a time interval of 9 months and after PMT2 was replaced. The first test showed that PMT1 had the least noise using the 488 and 568 laser lines. In setting up this machine, PMT1 would be used for 488 excitation, PMT2 for 568 excitation, and PMT3 for 647 excitation. This test showed that the least noise was derived from PMT1 under all conditions. PMT2 had 20% more noise than PMT1 for 568 excitation. After PMT2 was replaced, it produced better CVs with 488 and 568 excitation (Table 3).

As the PMT voltage is increased, the distribution of the pixels in the bead increases, which results an increased CV. The value gives the reference of each PMT relative to each other and suggests which one should be used for optimum performance. In a Leica system, this information is useful to determine which PMT should be used and possibly how much averaging will be necessary to achieve a desired image quality (1,21). By using the most efficient PMT, the image CV is decreased as lower PMT values are used.

Numerous factors effect the CV measurement, including bead fluorescence at the emission and excitation wavelength, optical components and efficiency of the system, maximum system laser power obtainable, and functionality of optical components in the system and electronic components (PMTs). In addition, many acquisition parameters, such as scan speed, pinhole setting, and objectives, will affect the CV value. When all of these factors are considered, the CV will be a measure of the system's relative sensitivity. Lower CVs will translate into better image quality with less image averaging and less bleaching. Because PMTs deteriorate with time, it is important to measure the initial CV (bead) and then to measure periodically how the CV (image quality) changes over time as a reference point for possible replacement (Table 3).

Laser Sensitivity: Noise Comparison

CV bead tests have been used to monitor sensitivity in a multilaser system. This test detects defective lasers and system alignment problems and establishes guidelines for determining how much averaging will be necessary to remove the noise from an image (1,21). It also provides criteria for selecting a laser for a confocal system.

To compare the sensitivity of CLSM system lasers, one can measure a defined milliwatt power on the stage and then conduct the bead noise test as described previously (1). Because the power on the stage is the same under different systems and configurations, the CV should be an indicator of system sensitivity that includes efficiency, laser noise, and PMT detector quality. We found that 1mW of power yielded a CV of 4% on the Leica TCS-SP and a CV of 1.3% on the Zeiss 510 system. With the 568 line, 0.2 mW of power yielded 4% on the Leica TC-SSP system and 2% on the Zeiss 510 system.

The CV value is affected by the excitation/emission characteristics of the fluorescence beads and the system performance. In a TCS-SP1 at approximate $\frac{3}{4}$ power value, the beads yielded CVs of approximately 6% (488 nm), 4% (568 nm), and 20% (647 nm). Increasing the laser power to maximum values changed the CV values to 4% (488 nm), 2.5% (568 nm), and 19% (365 nm). Using the same beads, we achieved the following CVs on a Zeiss 510 confocal system: 1% (488 nm) and 2% (568 nm). By comparing the CV from an argon-krypton laser system (Leica TCS-SP1) and the CV from an air-cooled argon/HeNe system (Zeiss 510) at the same milliwatt ranges, we determined that the system with individual lasers functioned better than the system with only a single Omnichrome argon-krypton laser emitting three wavelengths. It should be emphasized that there are many variables that can effect CV measurements. Therefore, they should be used as approximate values and not absolute values.

Comparison of UV (365 nm) and visible (568 nm) excitation on a 10-μm bead revealed the following PMT and CV values: UV (PMT 679 V, CV = 19%) and 568 nm (PMT = 382 V, CV = 5%) excitation. The difference in CVs between the two excitation wavelengths may be explained by the facts that the bead in a UV system may not be excited as the bead in a visible system, the optical system may be more efficient and less attenuated with visible excitation compared with UV excitation, and the UV laser power is insufficient, necessitating the increase of the PMT value to observe the bead at channel 150 (pixel mean). This bead test is a reproducible test with both wavelengths and can be used to compare the sensitivity in a CLSM or other machine. However, due to unknown laser power fluctuations, daily tests on a confocal

microscope are not as useful, simple, or efficient as those performed on a flow cytometer.

Biological Test Slides

It is important to have a reliable sample that can be used to test machine performance and image quality. Most CLSMs are assessed for proper performance with the user's slides. Although this is subjective, it does work in a crude way. We used hematoxylin/eosin (HE)-stained specimens (Goblet cells H215; Carolina Biological, Burlington, NC), mixed pollen grains (B690; Carolina Biological), diatoms (BA-29-5984, BA 29-6008; Carolina Biological), tissue culture cells stained with three fluorochromes (FL14780, Molecular Probes), CRBC (fixed with glutaraldehyde and stained with various probes), or other user-derived slides stained with DAPI and other miscellaneous fluorescent probes. The most useful histological test slide in our laboratory was a FluoCells slide (Molecular Probes, F-14780). This slide allows proper evaluation of resolution, crosstalk between detector channels, and observation of the emission from multiple excitation wavelengths (UV, 488 and 568). The resolution of biological structures of mitochondria (Mitotracker), nucleus (DAPI), and tubules (Alexa 488) can be assessed with the slide. The pollen or diatom slide was also used to demonstrate fine structure at various magnifications with different excitation wavelengths. Leica service engineers use a fluorescent plant tissue that can be excited using all wavelengths. The histological plant test sample has been used for a combination of power output, field illumination, resolution, and overall assessment of the machine. Most sales and service personnel use their favorite histological slide as their gold standard to determine if the system is functioning properly. This is a subjective assessment that cannot be challenged effectively or its accuracy confirmed. Observations on histological slides are subjective and machine performance should not be based totally on these samples as a performance standard.

Noise in Biological Specimens

The use of a biological sample is a subjective method of addressing total system performance. Similar to beads, biological test samples (FluoCells, AO-stained CRBC) have been used to demonstrate the relationship among frame averaging, laser power, and PMT voltage. Many factors influence increasing the PMT variables and image quality, which included a decrease in laser power, a smaller pinhole, scan speed, or a bad PMT. If the image cannot be visualized effectively with a specific PMT setting, it will necessitate averaging (1,21). Because the relationship between PMT and CV (image quality) is logarithmic, small changes in PMT values will result in major changes in system performance (1). Because of the wide range of PMT values between 500 and 600, PMT values cannot be used effectively to assess total system performance.

The aim of the CLSM is to lower the CV of the image and to produce a higher quality image. The challenge for the investigator is to create this image without bleaching the sample and without using excessive laser power or long exposure times. At a given PMT setting, increased frame averaging produces a greater reduction of CV (decreased noise). Lower PMT settings allow less averaging due to the decrease in noise in the system (1). Antifade compounds reduce the bleaching and maintain low PMT values or increased averaging with fixed samples. Another way to reduce bleaching is to reduce the size of the sample image field (e.g., from 512 $\times$ 512 to 128 $\times$ 128). This will result in shorter exposure times with less bleaching while keeping the PMT and laser power settings constant. However, this also results in a smaller sample size and less resolution. For system performance tests, losing minor details due to decreased resolution is not a significant issue. However, for biological samples, structural details are important and good images should be taken at pixel values of 512 $\times$ 512 or higher.

DISCUSSION

A confocal microscope can provide spectacular three-dimensional data of biological structures. QA on the CLSM is essential to ensure proper performance and delivery of accurate and reproducible data. The CLSM is a sophisticated microscope and a subjective "pretty" image on a histological slide is not the only relevant endpoint for system performance. This study emphasizes the need for scientists to perform other assessments of system performance because a malfunctioning machine cannot be determined by only comparing images from a biological test slide. We have described field illumination for individual lenses, laser power indicators, dichroic efficiency, chromatic lens aberration, axial resolution, spherical registration, bead noise, PMT performance, sensitivity, laser stability, and noise analysis. These tests have to be scrutinized to ensure that they are working correctly and providing the necessary and desired test data. The work of the field engineer also has to be checked carefully because he/she may not accomplish all of these tests during an installation or preventative maintenance visit. It is important to conduct these tests when the system arrives and routinely thereafter to ensure that it operates as it was designed to operate. In addition, many sales and service representatives may have different levels of machine understanding. Without manufacturer specifications, the level of alignment is open to question and debate. Even the sales/service representatives from confocal companies can make mistakes when aligning a machine. Therefore, these tests are necessary to ensure proper functioning of the machine. Unfortunately, the CLSM may function at suboptimum conditions during operation and problems (e.g., desired images cannot be obtained or hard failure of the system) are resolved by service personnel.

In order to obtain a good confocal image, it is necessary to balance laser power, PMT voltage, frame averaging, pinhole aperture size, scan speed, and zoom magnification (1–6). For a novice user, it is extremely difficult to balance these factors to produce an ideal image. Although all of these factors are essential, we have found that the zoom bleaches samples and high PMT settings introduce excessive noise that can only be eliminated through averaging,

which can also bleach the samples. Bleaching increases as the square of the zoom factor. If possible, magnification should be obtained with objectives and not with the zoom magnification (23). The PMT values should be kept as low as possible, even to the extent of increasing the pinhole diameter, decreasing the scan speed, and increasing the laser power. The pinhole diameter determines the confocality of the system and should be set ideally at the size of the 1st Airy disk. It can be increased if insufficient fluorescent laser light is emitted from the sample, making visualization difficult. If available, the detection barrier filter can also be replaced with a Schott long pass filter to increase the amount of detected light or the slit can be adjusted to wider values with the Leica spectrometer system. A slow scan speed should be used, which is equivalent to increasing the averaging as the laser beam resides for a longer time on each pixel. These acquisition factors should be regarded as starting points for using the CLSM and not as an absolute rule, as every sample may have unique qualities that must be considered prior to imaging.

In order to maximize performance in a CLSM, a series of tests should be made to ensure the machine is working correctly and delivering its proper test values. One of the major problems with confocal equipment is that the manufacturers do not provide specifications to the investigator. The QA on CLSMs for investigators and service personnel would be easier if the manufacturers would provide the necessary specifications. The specifications are either proprietary or they are not published because the companies would have to sell and service equipment to high standards. For example, the axial resolution value of 350 nm on a Leica TCS-SP1 system is one of the few published specifications for a confocal microscope that a manufacturer guarantees will meet or exceed their equipment. It was easy to determine whether the CLSM system meets or fails this performance test. By knowing this specification, we were able to detect and correct a serious problem in our CLSM when it failed the axial resolution test (1). Similar values for other parameters are needed for proper QA on a CLSM. Many of the tests could be simplified if manufacturers released specifications, provided standardized tests, and recommended standard accessory equipment required to accomplish these tests. Without these specification values, most of the test results can be questioned for accuracy and reliability. In our opinion, a pretty picture from a histological sample is too subjective a method to either align or buy a confocal microscope. Objective quantitative data and QA tests are necessary for the proper evaluation of a confocal microscope prior to purchase and in subsequent operation in a laboratory.

The purchase of a new confocal machine is a difficult and complex decision (24). Too often, it is decided on subjective criteria, such as whether a specific machine can observe a phenomenon on a slide or generate a pretty picture during the demonstration. It is critical when comparing machines from different manufacturers that they are set up the same. The laser power, objectives, scan speed, illumination, detection pinhole size, and other hardware components affect image quality. Other critical factors influencing the buying decision should be the service and support package provided by the manufacturer after the machine is delivered. QA, organizational, and service issues should not be overlooked when comparison shopping. The buyer is going to have a long-lasting relationship with the vendor and it should be based on trust and on the confidence of knowing that the machine will be serviced to ensure efficient operation. In our opinion, the ability of a manufacturer to guarantee specifications and address the issues of QA would rate very high on the list of criteria for purchasing a CLSM. It is worthless to have the best-designed machine if it does not perform to high standards.

We demonstrated the existence of a relationship among PMT voltages, laser power, and frame averaging and introduced the CV concept to evaluate image noise (1,21). In order to obtain good image quality, the PMT voltage should be minimized and,if necessary, averaging should be used to further reduce image noise and increase image quality. The aim in using CLSM is to produce a high quality image by lowering the CV (noise) of the image. By balancing the acquisition variables and sample staining, a lower CV can be obtained, which will result in better image quality. In contrast, if the system is operated at high PMT values, noise will be introduced into the image. This will be reflected by an increased CV, which will be detrimental to image quality. Averaging will reduce the CV, but bleaching must be controlled in the sample by using anti-fade agents on fixed samples or by optimizing the staining protocol. The challenge for the investigator is to create a good image without bleaching the sample that has minimal noise and a low CV.

Although the CV Spherotech bead noise test may be useful to evaluate the reproducibility of the system over weeks and months, its applicability in our laboratory was limited as a daily/monthly test. The laser power and stability in the CLSM system were extremely variable, the reasons for which are unkown. Perhaps this can be attributed to the instability of the argon-krypton laser alignment, variable temperature dissipation, or to the temperature instability of the AOTF. The voltage setting of the PMT was the primary determinant of image quality and bead noise. An increase in PMT values was always accompanied by an increase in image noise and pixel CV distributions. This test can be used to assess machine sensitivity and measure the sensitivity between different machines if the acquisition variables are controlled. For example, the best CV on a Leica TCS-SP1 with UV excitation that was obtainable using a 10-μm Spherotech bead was 19%. The best CVs obtainable for 488 nm and 568 nm excitation were 3.8% and 2.5%, respectively. These percentages are invaluable for determining the sensitivity of the TCS-SP and comparing its sensitivity to other CLSMs. The values will represent how well the system is aligned and functioning.

The manner in which a PMT functions is known to electronic engineers, but not to the end user, who may be a biologist who uses the CLSM to answer biological ques-

tions (7,19,25,26). The PMT should have a wide dynamic range, it should be linear, and it should show good sensitivity in the wavelengths measured (5,18,25). The confocal user may set the PMT at high voltages to observe an image, but may be unaware how this setting influences image quality. Operating a system with high PMT values will generate a poor image quality due to reduced signal and excessive noise. This noise will have to be eliminated by averaging to yield a good image. Frame averaging and high laser power will increase image quality. However, bleaching may occur with repeated scans over the same sample or with high laser power. It is best to optimize system performance so that the CLSM detection system can be operated at lower PMT values.

The newer CLSMs use HeNe lasers (543 nm and 633 nm) that yield lower power values at the microscope stage compared with the 75-mW argon-krypton laser. These low noise lasers should deliver better signal/noise performance as they have less peak-to-peak noise than the argon-krypton laser. The advantages of these small air-cooled lasers are many: they are more stable, less noisy, last longer, and create fewer problems for the field engineers. The disadvantage is that the systems contain more dichroics and more pinholes, which become harder for the field engineer to align properly. Because these HeNe lasers deliver less power to the stage, PMT performance and optical efficiency will become more critical in measuring CLSM system performance.

The lenses are the engines that drive this technology and their selection is critical for optimum performance. Some of the factors to consider are the high numerical aperture (NA) relative to the specific magnification, flat field objectives, long working distance relative to NA, good fluorescence transmission, and good achromatic correction at desired wavelengths. As a general rule, one should use the smallest magnification and the largest NA lens to acquire images (23). These lenses offer a larger field of view. The pinhole should be adjusted to the specific lens and specific NA of the lens to allow a sufficient amount of light to enter the system to maintain confocality. Although it is critical to reject out-of-focus light for confocality, it is also necessary to have sufficient laser light entering the system, even if it can only be achieved by opening the pinhole. In experiments that measure two and three-color fluorescence, it is important to use the apochromats for chromatic corrections even though they have less light collection efficiency. The far-red emission (647/633 excitation) is the most difficult to colocalize completely with the emission from the 488 and 568 wavelengths due to spectral characteristics of the objective (Fig. 6). If only one color is used, it is acceptable to use a Fluar lens with a higher NA that transmits more light.

This study has described a number of tests (field illumination, axial resolution, and spectral registration) to ensure proper functioning of the lenses in a CLSM system. Many lenses do not meet the current confocal specifications with regard to spectral registration and field illumination. We found a few defective lenses in our system before the manufacturers instituted higher standards for confocal lenses. The CLSM companies now sell a different class of confocal lenses that have higher specifications than normal lenses. In some cases, there is an incompatibility between lens design and confocal applications. This is illustrated in the Leica system with the 100× lens that shows a bull's eye pattern with UV illumination but demonstrates a uniform field illumination with visible excitation. To gain a more uniform illumination, Leica recommends zooming the image to 20%. These bull's eye patterns were also observed with objectives from other manufacturers, which limit severely the entire field from being used in creating an image. It makes sense that all objectives should be tested for field illumination and other specifications to ensure proper functioning in the laboratory. When making these measurements on a confocal system, it is important to allow the laser to warm up for 30 min and to stabilize.

In summary, to achieve maximum performance from a CLSM, it is necessary to use a series of tests that ensure optimal performance. Without using these tests, the CLSM may lack proper resolution, sensitivity, and precision and may yield inaccurate data. We described some new tests in this study and perfected some standard tests to assess the functionality of the CLSM. Tests measuring field illumination, lens clarity, laser power and stability, dichroic functionality, spectral registration, axial resolution, sensitivity, overall machine stability, and system noise were derived in order to determine the quality of the CLSM. These tests have provided us with the tools to eliminate the subjective nature of assessing the confocal microscope by only evaluating a user-defined histological sample. The ability to apply estimates of image noise (CV) provides a better way to assess the confocal system sensitivity (1,21). For unknown reasons, laser power stability over time (hours) has been shown to vary by as much as 30% with all lasers at different times in a TCS-SP1 system. If comparative intensity measurements on multiple samples or comparative intensity changes over time on a single specimen are required, it is essential to make a power stability test. It is anticipated that other investigators or manufacturers will improve these tests and derive additional QA tests to help assess CLSM functionality.

ACKNOWLEDGMENTS

We thank Jeff Wang of Spherotech for providing us with test particle slides to evaluate and produce a 10-μm bead that was uniform in size and fluorescence intensity and did not bleach readily with repeated samplings. We also thank Earl Puckett (USEPA instrument shop) for building a stage adapter for the Newport and Coherent power meter detectors and Keith Tarpley (CES Corporation) for providing excellent technical assistance in the preparation of the illustrations.

LITERATURE CITED

1. Zucker RM, Price OT. Practical confocal microscopy and the evaluation of system performance. Methods 1999;18:447–458.
2. Centroze V, Pawley J. Tutorial on practical confocal microscopy and

use of the confocal test specimen. In: Pawley J, editor. Handbook of biological confocal microscopy, 2nd edition. New York: Plenum Press; 1995. p 559–567.
3. Centroze V, Pawley J. Practical laser scanning confocal light microscopy: obtaining optimum performance from your instrument. In: Celis J, editor. Cell biology, 2nd edition. New York: Academic Press; 1998. p 149–169.
4. Carter D. Practical consideration for collecting confocal images. Methods Mol Biol 1999;122:35–57.
5. Sheppard CJR, Shotton DM. Confocal laser scanning microscopy. New York: Bios Scientific Publishing; 1997. p 15–44.
6. Pawley J. Fundamental limits in confocal microscopy . In: Pawley J, editor. Handbook of biological confocal microscopy, 2nd edition. New York: Plenum Press; 1995. p 19–36.
7. Rost FWD. Tutorial on practical confocal microscopy and the use of the confocal test specimen. In: Pawley J, editor. Quantitative fluorescence microscopy. Cambridge: Cambridge University Press; 1991. p 1–55.
8. Pawley J. The 39 steps: a cautionary tale of quantitative 3-D fluorescence microscopy. Biotechniques 2000;28:884–886.
9. Liljeborg A, Czader M, Porwit A. A method to compensate for light attenuation with depth in three-dimensional DNA image cytometry using a confocal scanning laser microscope. J Microsc 1995;177(Pt 2):108–114.
10. Jericevic Z, Wiese B, Bryan J, Smith LC. Validation of an imaging system: steps to evaluate and validate a microscope imaging system for quantitative studies. Methods Cell Biol 1989;30:47–83.
11. Van den Doel LR, Klein AD, Ellenberger SL, Netten H, Boddeke FR, van Vliet LJ, Young IT. Quantitative evaluation of light microscope based on image processing techniques. Bioimaging 1998;6:138–149.
12. Cardullo RA, Alms EJ. Introduction to image processing. Methods Cell Biol 1998;56:99–115.
13. Wolf DE. Introduction to image processing. Methods Cell Biol 1998; 56:117–134.
14. White NS, Errington RJ, Fricker MD, Wood JL. Aberration control in quantitative imaging of botanical specimens by multi dimensional fluorescence microscopy. J Microsc 1996;181:99–116.
15. Cogwheel CJ, Larkin KG. The specimen illumination path and its effect on image quality. In: Pawley J, editor. Handbook of biological confocal microscopy, 2nd edition. New York: Plenum Press; 1995. p 127–137.
16. Cox G. Measurement in the confocal microscope. Methods Mol Biol 1999;122:357–371.
17. Delaney PM, Harris MR. Fiber optics in confocal microscopy. In: Pawley J, editor. Handbook of biological confocal microscopy, 2nd edition. New York: Plenum Press; 1995. p 515–523.
18. Shapiro H. How flow cytometers work in practical flow cytometry. Practical flow cytometry, 3rd edition. New York: Wiley-Liss; 1995. p 106–135.
19. Hoffman R. Standardization, calibration, and control in flow cytometry. In: Robinson P, editor. Current protocols in cytometry. New York: John Wiley and Sons; 2000. p 1.3.1–1.3.18.
20. Zucker RM, Elstein KH, Gershey EL, Massaro EJ. Increased sensitivity of the Ortho analytical cytoflurograph by modifying the fluidic system. Cytometry 1990;11:848–851.
21. Zucker RM, Price OT. Statistical evaluation of confocal microscopy images. Cytometry 2001;44:295–308.
22. Watson JV. Flow cytometry data analysis: basic concepts and statistics. Cambridge: Cambridge University Press; 1992. p 94–100.
23. Piston DW. Choosing objective lenses: the importance of numerical aperture and magnification in digital optical microscopy. Biol Bull 1998;195:1–4.
24. Steyenger P. Assessing confocal microscopy systems for purchase. Methods 1999;18:435–446.
25. Pawley JB. Sources of noise in three dimensional microscope data sets. In: Stevens J, editor. Three dimensional confocal microscopy: volume investigations of biological specimens. New York: Academic Press; 1994. p 47–94.
26. Art J. Photon detectors for confocal microscopy. In: Pawley J, editor. Handbook of biological confocal microscopy, 2nd edition. New York: Plenum Press; 1995. p 183–195.