5

Evaluation of Confocal Microscopy System Performance

Robert M. Zucker

Summary

The confocal laser scanning microscope (CLSM) has enormous potential in many biological fields. When tests are made to evaluate the performance of a CLSM, the usual subjective assessment is accomplished by using a histological test slide to create a "pretty picture." Without the use of functional tests, many of the machines could be working at suboptimal performance levels, delivering suboptimum performance and possibly misleading data. To replace the subjectivity in evaluating a confocal microscope, tests were derived or perfected that measure field illumination, lens clarity, laser power, laser stability, dichroic functionality, spectral registration, axial resolution, scanning stability, photomultiplier tube quality, overall machine stability, and system noise. These tests will help serve as a guide for other investigators to ensure that their machines are working correctly to provide data that are accurate with the necessary resolution, sensitivity, and precision. Utilization of this proposed testing approach will help eliminate the subjective nature of assessing the CLSM and allow different machines to be compared. These tests are essential if one is to make intensity measurements.

Key Words: Confocal microscope; lasers; coefficient of variation; photomultiplier tubes; field illumination; axial resolution; spectral registration; laser stability; beads; microscope lenses; quality assurance; quantification; spectroscopy

1. Introduction

The confocal laser scanning microscope (CLSM) has been evaluated by using the following biological test samples: beads, spores, pollens, diatoms, fluorescent plastic slides, fluorescence dye slides, silicone chips, and histological slides from plants or animals ***(1–10)***. In most cases, the test sample is of biological origin and is sometimes used in the course of research in the individual's research laboratory. This is a testing procedure recommended by the manufacturers of most CLSM equipment. In our opinion, this is too arbitrary a test when applications (i.e., intensity measurements or colocalization studies) other than "pretty pictures" are needed. Unfortunately, this technology differs from flow

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cytometry and does not have a universal standard with which to evaluate the CLSM or the image quality derived from a CLSM. It would be advantageous to have better methods to measure system performance and image quality.

The CLSM consists of a standard high-end microscope with very good objectives, different lasers to excite the sample, fiber optics to deliver the laser light to the stage, acoustical transmission optical filters (AOTFs) to regulate the laser light onto the stage, barrier filters, dichroics, and pinholes to control the light, electronic scanning devices (galvanometers), detection devices to measure photons (i.e., photomultiplier tubes [PMTs]) and various other electronic components. For this system to operate correctly, it is important for it to be properly aligned and to have all of the components function correctly. Instrument performance tests that have been devised include the following: laser power, laser stability, field illumination, spectral registration, lateral resolution, axial *Z* resolution, lens cleanliness, lens functionality, and *Z*-drive reproducibility ***(1–10)***. This list is not inclusive and additional parameters might be needed to assess if the CLSM is working properly.

Because a confocal microscope can provide spectacular three-dimensional (3D) data of biological structures, one can have a tendency to overlook many of the quality assurance (QA) parameters that might be necessary as controls. The CLSM could function at suboptimum conditions for long periods of time, delivering inferior data, with the problems being resolved only when the investigator cannot achieve the desired images or there is a hard failure of the system necessitating a service personnel visit. Inferior performance of a CLSM could be attributed to sample preparation. However, the image data might also be incorrectly interpreted if the CLSM is not working correctly. Because all CLSM images are digital and made with sophisticated optical equipment, it is now possible to derive tests that can evaluate some of the components installed in the machine. The CLSMs should not be evaluated by using only a subjective "pretty" image on a histological slide. QA on the CLSM is essential to ensure that it is performing properly and delivering accurate and reproducible data. This review attempts to incorporate QA procedures into the operation/maintenance of confocal microscopes. This review also emphasizes that scientists need to evaluate their CLSM system performance to ensure that it is working properly.

2. Materials

2.1. Field Illumination: Fluorescent Slides

The field illumination test slides consisted of three fluorescent plastic slides (Delta; Applied Precision Inc, Issaquah, Washington) that had excitation peak wavelengths of 408 nm (blue), 488 nm (orange), and 590 nm (red) and emission peak wavelengths of 440 nm, 519 nm, and 650 nm, respectively. The orange slides (488 nm) were used to test for visible field illumination and alignment. The blue

slides (408 nm) were used for ultraviolet (UV) field illumination and alignment. Field illumination can also be measured using four Fluor-ref slides (Microscopy Education, Springfield, MA) or four Chroma slides (Chroma, Brattleboro, VT). Although extensive tests were not made with these slides, in preliminary studies they appear to work well if the surface is clean and free of debris.

2.2. Power Meter

The power meter used to measure light on the microscope stage was either a Fieldmaster or a Lasermate Q (Coherent, Auburn, CA) with visible (LN36) and UV detectors (L818). A power meter (1830C) from Newport Corporation with an SL 818 visible wand detector can also be used for power measurements. A remote control box for the Coherent UV Enterprise laser was used to regulate UV laser power (model 0163-662-00; Coherent, Santa Clara, CA). On most confocal systems, there is a 10× lens: Zeiss uses a 10× Plan Neofluar (numerical aperture [NA] of 0.3) and a Leica has a 10× Plan Fluorotar (NA of 0.3) or 10× Plan Apo (NA = 0.4). The dry 10× lens was used to take power measurements. The machinist's plans for building the power detector holder are available by e-mailing the author.

2.3. Beads

Various bead types are useful as test particles to access machine functionality. The beads were obtained from Spherotech (Libertyville, IL), Molecular Probes (Eugene, OR) or Coulter electronics (Hialeah, FL).

Spherotech beads that were used included the 10-µm Rainbow (EX 365, 488, 568) fluorescent particles (FPS-10057). The following beads were used for preliminary field illumination tests: yellow beads (5.5 µm, FPS-5052) EX 488 for visible field illumination; UV beads (5.5 µm, FPS-5040) EX 365 for UV field illumination; blue beads (5.5 µm FPS-5070, EX 647) for 647 nm field illumination. The 6.2-µm Rainbow beads with three different intensities (FPS-6057-3) were used for early statistical PMT tests. The following PSF Rainbow beads were used: 0.16 µm, FP-02557-2s; 0.5 µm, FP 0857-2; and 1.0 µm, FP-0557-2. The polystyrene 10-µm beads (refractive index [RI] = 1.59) were mounted with optical cement (RI=1.56) on a slide using a no. 1.5 size cover glass. The Leica immersion oil has a refractive index of 1.518.

The following Molecular Probes beads were used: TetraSpeck™ beads (T7282 0.5 or 1 µm, EX 365, 488, 568, 647) were used for spectral registration tests and point spread functions (PSF); PS-Speck Microscope Point Source Kit, consisting of beads (175 nm, P-7220) of different wavelengths, were used for acute deconvolution PSF measurements; 15-µm FocalCheck microspheres (F24634 kit) consisting of an orange ring and blue throughout (F7236) for UV and visible colocalization or green, orange, and dark red ring stains (F7235) for visible colocalization of the 488, 543, and 633 laser lines. Fluorospheres (10 µm, Fullbright

Green II; Coulter, Hialeah, FL; EX 488) were used for preliminary statistical PMT tests. The 10-µm Spherotech Rainbow beads were substituted for these beads in later experiments.

Bead slides were made by dropping 3–5 µL of diluted beads onto a slide, allowing the liquid to dry and then covering the spot with Permount, glycerol, water, or oil and sealing it with a 1.5 cover glass. Antifade from Vector (Vectoshield H-1000) or from Molecular Probes (Slowfade light S-7461) is useful to decrease bleaching.

2.4. Biological Test Slides

FluoCells 1 (F-14780; Molecular Probes, Eugene, OR) were stained with three fluorochomes (Mitotracker Red CMXRos, BODIPY FL phalloidin, DAPI) and used as biological test slides. Additional slides were made in our laboratory with cells grown on cover slips, fixed with paraformaldehyde, and stained with DAPI for UV excitation or other suitable fluorochromes for visible excitation.

2.5. Axial (Z) Resolution Test

The axial resolution of the CLSM is tested using a single reflecting mirror obtained from Leica or Edmonds Scientific. A 21-mm square (#31008; Edmonds Scientific, Philadelphia, PA) was glued onto a microscope slide and a cover glass (#1.5; Fischer, Pittsburgh, PA) was placed on top of the slide with a drop of immersion oil (Leica Immersion oil, $n = 1.518$) The cover slip is placed firmly onto the mirror to remove all excessive oil. This type of standard test slide can also be obtained from a confocal manufacturer or Spherotech (Libertyville, IL).

2.6. Square Sampling Galvanometer Check

It is important to ascertain whether there was square sampling or rectangular sampling in an image. A computer chip was glued onto a glass slide and used as a test substrate. A commercial grid product can also be obtained from MicroBrightField (Williston, VT) or Geller MicroScientific (Topsfield, MA). A digital tagged image file format (TIFF) image was obtained using a dry 20× objective and the amount 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 they are not equivalent, then the sampling of pixels is rectangular, which is not desired. Problems with the galvanometer can also be detected with this grid.

2.7. Laser Beam Shape

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 diameter [id]; H1089,) and focusing the beam onto a white piece of paper to show its configuration mode.

2.8. Confocal Microscope

The majority of data presented in this chapter was derived on either a Leica TCS-SP1 or a Leica TCS4D (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 AOTF and the following three dichroics for visible light applications: single dichroic (RSP500); double dichroic (DD); and triple dichroic (TD). The Leica-derived tests were shown to be applicable to other point scanning systems that contain different types of laser, objective, or other hardware configuration. For comparison purposes, similar tests were made on two different Zeiss 510 units containing three lasers (argon 488 [25 mW], HENE 543 [1 mW], and HENE 633 [5 mW]) with a merge module and an AOTF. In addition, for comparison purposes, similar tests were made on a Leica SP2 unit that contained three lasers (argon 488 [50 mW], HENE 543 [1.2 mW], and HENE 633 [10 mW]) with a merge module and an AOTF.

2.9. Software Analysis

The analysis of the images was made on workstations that contained Leica, Zeiss, and Bitplane (Zurich Switzerland) software packages. If necessary, the TIFF images were imported into Image Pro Plus (Media Cybernetics, Silver Springs, MD) or Image J (NIH) for more intensive measurements and analysis.

2.10. PMT Spectral Check

The PMT spectral response was measured over a large spectral region using an inexpensive Pariss fluorescence calibration lamp consisting of a defined mixed-ion gas (model 816025; LightForm Inc, Hillsborough, NJ).

3. Methods

3.1. Field Illumination

The fluorescent slide was placed on the stage and the maximum intensity was found on the surface of the slide. It is important to measure the field illumination at a specific depth in the plastic slide, as the intensity distribution might change from the surface to the interior of the slide. The depth of focus was adjusted between 30 and 100 μm, depending on the objective that was used: (5× [100 μm], 10× [75 μm], 20× [50 μm], 40× [40 μm], 63× [30 μm], 100× [30 μm]). Investigators should also be careful not to observe an illumination field deep within the plastic slide samples, as it will usually yield a better field illumination than regions closer to the surface because of various optical distortion factors.

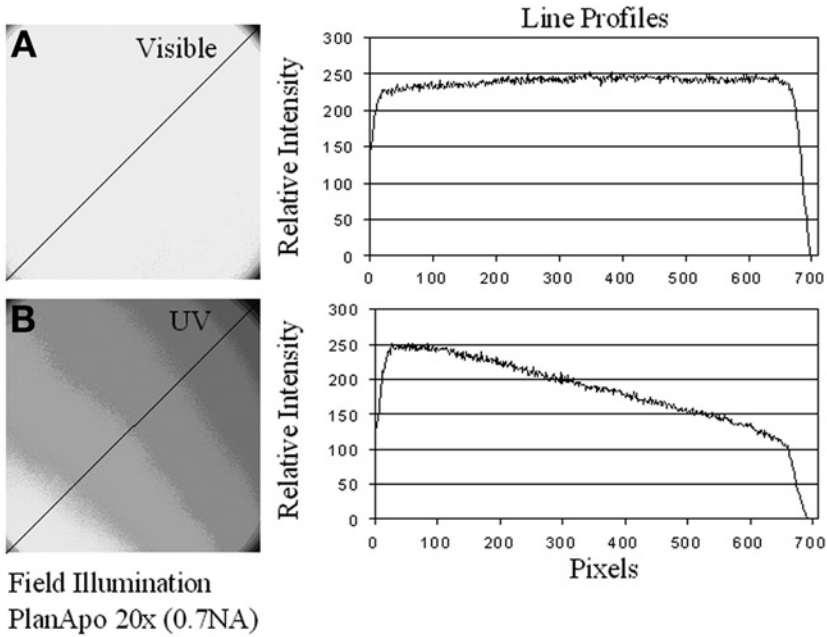


Fig. 1. Field illumination. Field illumination pattern of visible **(A)** and UV **(B)** excitation using a 20× (PlanApo; NA = 0.7) lens. The visible field illumination shows uniform illumination, with the brightest intensity being in the center of the objective. The line running diagonally in panels A and B measures the histogram intensity of the field illumination graphically represented in panels **C** and **D**. The variation in intensity from the left to right side of the field is less than 10% for visible excitation and more than 150% for UV excitation. Acceptable field illumination has the brightest intensity in the center of the objective, decreasing less than 25% across the field. The intensity regions were prepared by using Image Pro Plus to divide the grey scale value into 10 equal regions and a median filter was used for additional processing.

Data derived from a 20× PlanApo lens (NA = 0.7) zoomed to a factor of 1.2 is used to illustrate good visible field illumination (488 nm), and a misaligned UV (365 nm) system is used to yield a poor field illumination (*see* **Fig. 1**). The images were obtained with either a UV plastic slide (excitation [ex] 365 nm; emission [em] 440–480 nm) or a visible plastic slide (ex 488 nm; em 505–550 nm) located securely on the stage. The original images were contoured into 10 intensity ranges using Image Pro Plus software. The line running diagonally in **Fig. 1A,B** measures the histogram intensity of the field that is represented in the graphs in **Fig. 1C,D**.

The maximum intensity should be in the center of the objective and decreasing less than 25% across the field in all directions, as shown with visible 488-nm excitation in **Fig. 1A**. It should not be in the bottom corner, as illustrated with

UV illumination in **Fig. 1B.** As shown in **Fig. 1**, the visible light (**Fig. 1C**) had less than a 10% decease in intensity across the field, whereas the UV light (**Fig. 1D**) had a 150% decrease across the field. If the maximum light intensity is not located in the center of the field, there is an alignment problem that needs to be addressed. The nonuniform pattern shown in **Fig. 1** with UV illumination clearly illustrates a field illumination problem, which will affect intensity measurements in an image. Although **Fig. 1** was obtained with UV optics, it represents the type of field illumination that can also occur with visible excitation. This pattern is unacceptable with any CLSM optical system, as the maximum intensity should be in the center of the objective and not in a corner. Each laser line must be checked to ensure that they are aligned properly, as they use different dichroics to ensure that the beams are colocalized. In addition, the field illumination of one lens is not necessary identical to the field illumination of the other lenses, necessitating that each lens be checked with the suitable dichroic that will be used in the experiment.

In our Leica system, the three visible wavelengths of light are derived from one Omnichrome argon–krypton laser. This enables the field illumination to be tested at one wavelength (488 nm) and allows one to assume that 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 (**Fig. 1**) at the 365-nm excitation in addition to the 488-nm excitation. Newer designed confocal systems (Leica SP2, Leica AOBS, Zeiss 510, Zeiss 510 Meta, Nikon C1, Olympus FluoView FV1000, and Bio-Rad Radiance 2100) use three individual lasers with a merge module, which requires that all laser wavelengths have correctly aligned beams emitted from the merge modules. In these systems, all three lines have to be individually tested for field illumination. One laser line might be perfectly aligned, yielding acceptable field illumination, whereas the other laser lines might be misaligned, yielding intensity values for which the brightest region is not in the center of the field, as illustrated in **Fig. 1**.

3.2. Power Meter

The equipment used to acquire power readings include a detector, a machine-shop-built detector holder, and a portable power meter, which are illustrated in **Fig. 2.** To measure the power output of the different wavelengths, either a UV or visible probe (Coherent probe detectors [L818, LN36]) or Newport corporation wand visible probe detector (SL 818) is secured in a special holder that fits onto the microscope stage during the measurement of either UV or visible laser light (*see* **Fig. 2**). The test should be done with a dry objective (2.5× to 20×; preferably 10×) at a fixed position, usually at the top of its movable tract.

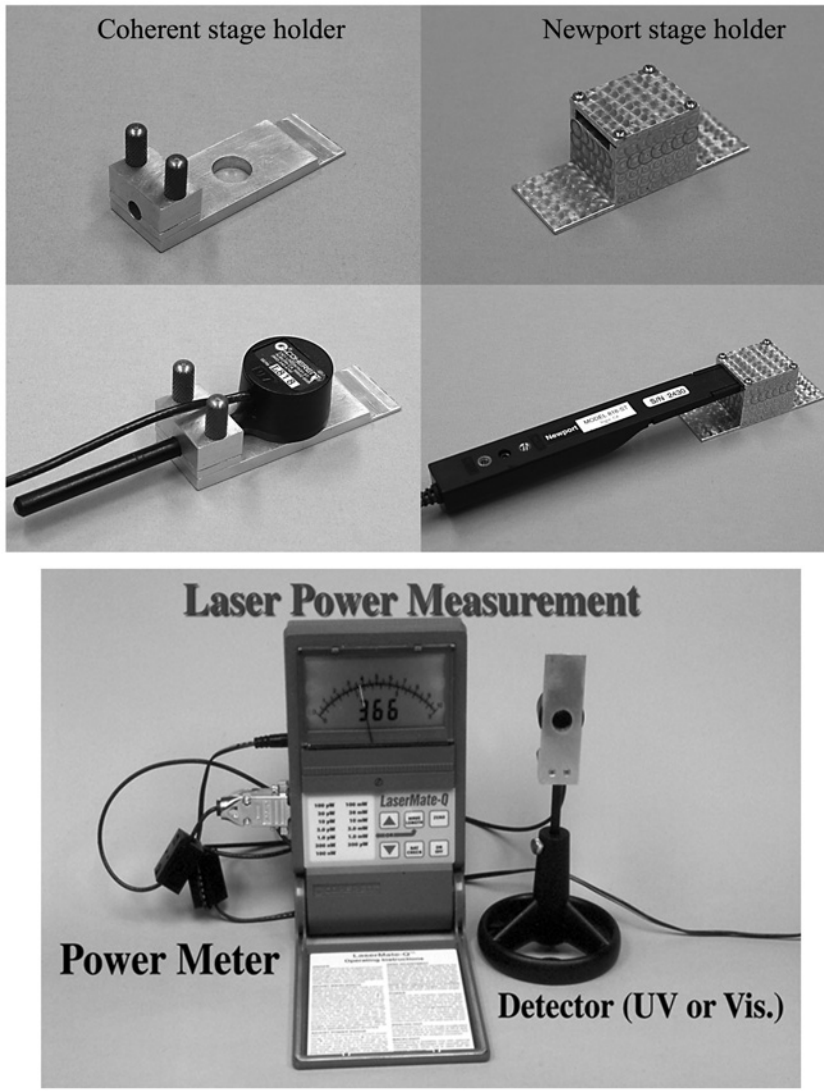


Fig. 2. Power meter and detector holders. A holder to secure detectors on the microscope stage was made in the machine shop in a size similar to a 3×1 microscope slide. The detector holders for the Coherent (LN 36) or Newport wand (SL818) are represented in the photographs. With small objective circumferences (i.e., Leica 10× PlanApo (NA = 0.4), the Coherent detectors can be placed on top of the objective. A Coherent power meter and detector are used to measure laser power on the microscope stage.

Currently, most systems will have a dry 10× lens (NA = 0.3 or 0.4) that can be used to access power using a power meter detector. The use of a lens that has a different lens design, magnification, or NA will affect the laser power transmission and measurement.

The test was made using a 10× (NA = 0.3) or a 10× (NA = 0.4) objective in the following manner. The lens is raised to its maximum specified height. The detector is secured on the stage and grossly centered using either laser light or mercury fluorescent light. The detector position is then more accurately adjusted to achieve maximum signal intensity by using the microscope's *x*/*y* joystick. The power meter is adjusted to the specific wavelength (365, 488, 568, or 647 nm) and the maximum power of laser light is read on the digital scale. The CLSM zoom factor is set from 8 to 32 to reduce the beam scan and to focus it into the "sweet spot" of the detector. The scanner is set at bidirectional slow speed to reduce the time period that the power meter is reading 0. The maximum digital reading from the power meter was recorded. The power derived from this measurement is dependent on the magnification and NA of the lens used. Each lens will have a unique set of values, which is dependent on the objective's NA and other transmission factors. The power meter diode was not reliable and could only be used as a crude estimate of the functioning of the laser. This might change in the future with better designed systems. The Coherent probe detectors LN818 and LN36 can also be placed directly on top of a 10× objective without a holder.

A comparison of the maximum power output derived from different lasers and different optical systems was made on a Zeiss 510, a Leica TCS-SP1, and a Leica TCS-SP2. The maximum power was measured with a Coherent power meter using two different 10× (NA = 0.3, SP1 and Z510) lenses and one 10× (NA = 0.4, SP2) lens on three different types of CLSM system: Zeiss 510, Leica TCS-SP2, and Leica TCS-SP1. The Leica TCS-SP1 system has one 75-mW argon–krypton laser (model 643) emitting three laser wavelength lines. The newer CLSM systems from all manufacturers are designed with three lasers that use different dichroic components to merge the different laser wavelengths. The Zeiss 510 contained three different lasers (30-mW argon, 1 mW HeNe [543 nm], and 5-mW HeNe [633 nm]) with the multiple wavelengths aligned with a merge module. The Leica SP2 contained three different lasers (50-mW argon, 1.2-mW HeNe [543 nm], and 10-mW HeNe [633 nm]) with the multiple wavelengths aligned with a merge module.

Table 1 shows a comparison of power with three different systems from Leica and Zeiss using a triple dichroic to reflect different wavelengths of laser light to the stage. The power meter values shown in **Table 1** can be used to determine both the maximum power output and dichroic reflectance and, thus, functionality in the system. The values reported in **Table 1** were acquired from functioning systems that were subjectively assessed to be problem-free, aligned correctly, and functioning properly. There are no assurances that these machines were producing ideal power readings or were perfectly aligned when these tests were made. However, we believe that the values demonstrate the type of data that can be achieved with this test. The acquired data can be used to compare

Table 1
Comparison of Relative Power on Microscope Stage

	Leica SP1	Leica SP2	Zeiss 510
Laser	Ar–krypton	(3-Laser)	(3-Laser)
488 nm	1.1	4.5	3.13
543–568 nm	1.45	0.22	0.26
633–647 nm	1.6	2.8	1.21

Note: The maximum power was measured in milliwatts with a LN 36 detector and a Coherent Lasermate power meter adjusted to the specific excitation wavelengths. The power was measured on the stage of a Zeiss 510, a Leica SP2, and a Leica SP1 system. This test can be used to determine if the system has acceptable laser power by measuring the power on the microscope stage and not opening up the scan head.

similar machines and internally control an individual machine for proper performance over a period of time.

3.3. UV Power Test

The test was carried out in a manner similar to that described for the visible power measurement. A power meter (Lasermate/Q with UV detector [L818]; Coherent, Santa Clara, CA) was used to measure the light emitted from a UV Enterprise laser. A Coherent UV, 60-mW, 3-yr-old Enterprise laser delivered normal power output at the laser head (over 40 mW of laser power), but only about 500 µW of maximum power through a Plan Fluor 10× (NA = 0.3). However, it should be noted that when our system had insufficient output under these conditions (approx 500 µW through a 10× Leica [NA = 0.3] lens), we also had insufficient light for many UV experiments using higher magnification objectives (40×, 63×, and 100×).

3.4. UV Beam Shape

In addition to power requirements of a UV system, it is important that the UV beam have the correct mode. The beam should be radially symmetric with a Gaussian intensity distribution. The beam should have a TEM00 configuration (transverse excitation mode or Gaussian mode). The UV laser beam can be checked using an inexpensive lens (12 mm od; B1099; Melles Griot) held in a lens holder (13 mm id; H1089) and focusing the beam onto a white piece of paper to show its configuration mode.

3.5. Bead or Histological Power Meter

The crude power of the system can also be assessed by recording the PMT voltage necessary to acquire an image at almost saturation values by using standard histological samples like the FluoCell 1 slide (F-14780; Molecular Probes,

Eugene, OR), beads like the 10-µm Spherotech beads (FPS-10057-100), or Applied Precision fluorescent plastic slides. If conditions are identical between machines, this PMT value can be used as a reference value to compare CLSM units and to establish their acceptable performance levels. Leica technicians routinely 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 PMT 1, the system is passed as having adequate power.

Because of the optical limitations of the stage, the power meter detector cannot be used with higher power optics (40×, 63×, 100×). Thus, it will be useful to use a test sample (fluorescence histological slide, colored plastic, or large beads) to assess UV and visible power with these higher-magnification lenses. Using maximum UV power, it was found that the 10-µm Spherotech bead saturates PMT 1 (low-noise PMT, R6857) at a voltage setting of 650 using a 100× PlanApo lens (NA = 1.4). When saturation occurred at the higher values, it indicated less power throughput in the CLSM system, and when saturation occurred at relatively lower PMT values, it indicated greater power throughput. The saturation values on other substrates or slides (biological or histological specimens) can also be used and have to be defined by the user's laboratory, as the degree of staining can be different.

3.6. Dichroic Functionality

The dichroic comparison test was accomplished in the following manner. An API fluorescent plastic slide (orange) was placed on the stage using either 488-nm or 568-nm excitation light. After the dichroic was switched into position, the PMT was kept constant and the mean gray scale value (GSV) of a region of interest (ROI) in the image was determined for each acquisition condition. The values for the three dichroics can be compared to determine which one has the highest reflectivity (*see* **Table 2**). The maximum values are normalized to 1 and the other values are reported as a percentage of the maximum GSV. It is important to use a bandpass or barrier filter to collect the desired light. Light at different regions of the spectra may have unique information and it is sometimes advisable to use a narrower bandpass with higher reflection to get the desired fluorescent information.

3.7. Axial (Z) Resolution (Mirror)

The axial resolution test is considered the "gold standard" of resolution in confocal microscopy (***1***,***3***,***7***,***8***). 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. The reflected surface of the mirror can be found in either *xy* or *xz* scanning by observing the brighter spot with an open aperture. Initially, axial resolution is tested in the reflection mode with the 100× objective

Table 2
Comparison of Relative Dichroic Reflectance

Wavelength	Dichroic	10× (0.3/NA)	63× (1.2/NA)
488	SD	0.95	0.97
	DD	1.00	1.00
	TD	0.84	0.80
568	SD	0.05	0.06
	DD	0.62	0.72
	TD	1.00	1.00

Note: The relative laser power was measured with the 488-nm and 568-nm wavelengths using six different magnification objectives and three dichroics. The 10× and 63× data are shown for clarity. The table demonstrates the relative reflectivity of the dichroics in the system. The test was accomplished by measuring the intensity in an ROI of an image using either 488-nm or 568-nm excitation light, a fluorescent plastic slide, and one of three specific dichroic and 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 are divided to yield a ratio that is expressed in the table as a fraction. The value of 1.00 is the maximum dichroic refection and is expressed as a bold number. The dichroic with the maximum reflection should be used when only one fluorochrome is required. Unexpectedly, the double dichroic (DD) yielded the best reflectivity with 488-nm wavelength light with most lenses and the triple dichroic (TD) yielded the best reflectively with the 568 nm wavelength light (30% more light reflected than the DD) with most objectives. This table can be used to choose the dichroic that should be used with each excitation wavelength for optimized reflection.

(NA = 1.4; PlanApo lens), zoom of 16× to 24×, a large pinhole diameter opening, and minimum laser power. After the reflected surface is found by scanning in the *xz* mode, the pinhole aperture is reduced to a minimum size. The reflected image is then obtained with a frame averaging of 2–4, and the intensity profile across the reflected surface is determined as shown in **Fig. 3.** The maximum of the peak is determined and then the half-maximum intensity value of the profile is obtained to determine the full width at half-maximum (FWHM) distance to determine the axial resolution. The data can be observed graphically or it can be transferred into Excel to measure the peak and the half-maximum values. The specification for axial registration in a Leica TCS-SP system is below 350 nm. The pattern of the axial resolution curve is also important. One looks for a symmetrical large peak and smaller peaks and valleys to the left of it, indicating diffraction patterns of an acceptable lens (**Fig. 3B**).

The axial *Z*-resolution of three different lenses on an aligned Leica TCS-SP1 system was the following: a 40× (Fluor, NA = 1.0) was 610 nm; a 63× water-immersion lens (PlanApo, NA = 1.2) was 390 nm; and a 63× oil-immersion lens (PlanApo, NA = 1.32) was 315 nm. These are good values for high-resolution work on any confocal microscope. These axial registration values will change depending on lens quality and system alignment.

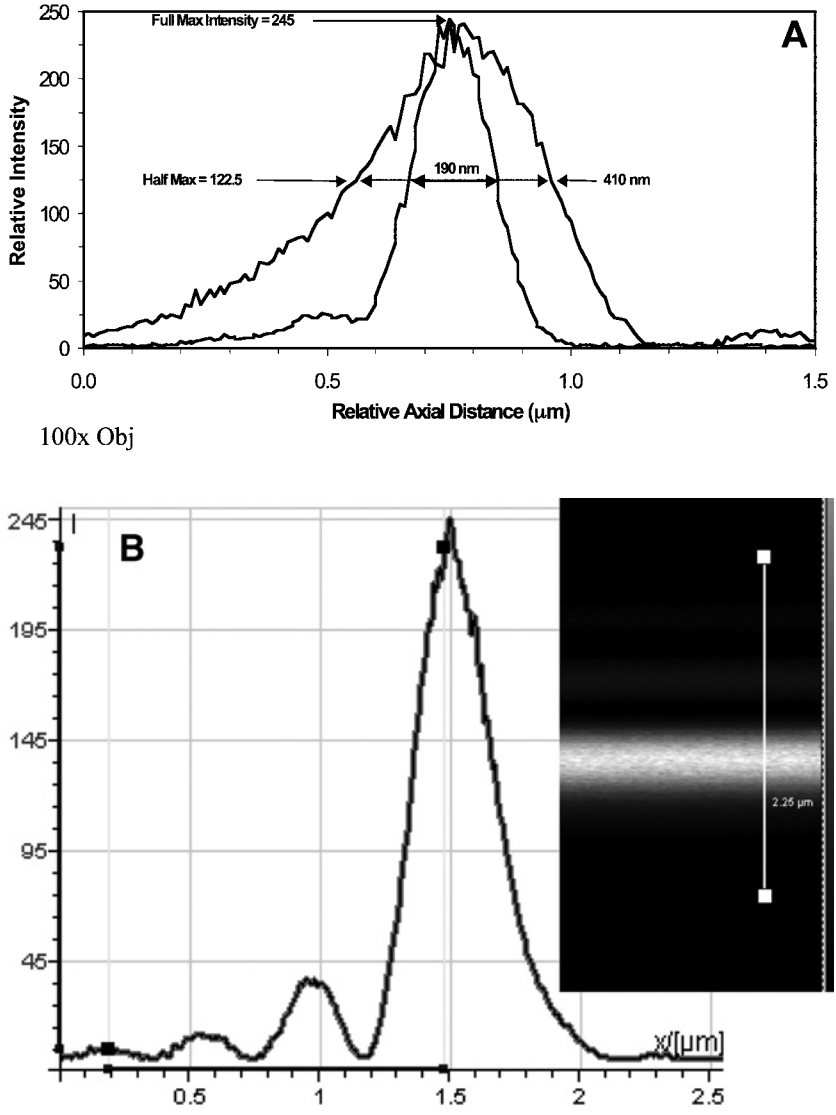


Fig. 3. **(A)** Axial resolution. The axial resolution was made with two 100× lenses (NA = 1.4) on the same Leica TCS-SP1 confocal system. The peak intensity of the histogram is 245 and the half-maximum intensity is measured at 122.5. One lens gave an excellent full width at half-maximum (FWHM) of 190 nm, whereas the other lens yielded a poor value of 410 nm. The system was aligned properly in both cases. **(B)** The axial resolution of a PlanApo 63× (NA = 1.32, 330 nm) showing a symmetrical major peak and a diffraction pattern consisting of smaller peaks and valleys. This pattern is suggestive of an excellent-quality lens.

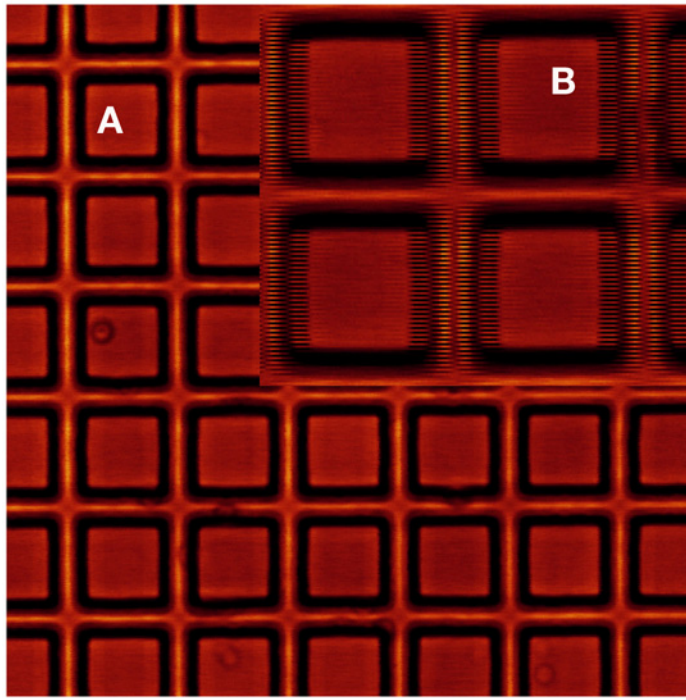


Fig. 4. Field alignment. A computer chip that is mounted on a slide shows 25 boxes per inch. The *x* and *y* difference should be equivalent, yielding square boxes and not rectangles. To align the system for bidirectional scanning, the scanning lines should show a smooth transition **(A)** and not the individual scan lines in the inset **(B)**.

3.8. Axial (Z) Registration (Beads)

One-micrometer beads from Molecular Probes (Tetraspec, T7284) or Spherotech (Rainbow, FP–0857-2) are first located in the *xy* direction and then scanned in the *xz* direction. The power is adjusted for saturation and then they are zoomed approx 8× and averaged: 4×. The size of the bead in the horizontal is compared to the vertical size. The difference between the two numbers will yield the axial resolution of the lens. This method is slightly more subjective than the gold standard axial mirror test, but it does yield similar values. Sometimes the values for unknown reasons are better and sometimes they are worse.

3.9. Square Pixels, Phase Alignment, and Galvanometer Evaluation

The pixel size and symmetry in *XY* directional field scanning can be checked by using a computer chip attached to a glass slide or a slide obtained from Microbright Field or Geller MicroScientific (*see* **Fig. 4**). The confocal should be set up in the reflective mode using a 10× or 20× dry lens. The small boxes in the

vertical and horizontal direction should be compared by counting or by a measured standard line. If there are the same number of boxes per inch in the vertical and horizontal directions, then it can be assumed that the sampling of pixels is a square. If they are not equivalent, then the sampling of pixels will be rectangular, which is not desired. This test ensures that the scanning in the *X* and *Y* directions yields a perfect square and the information will be registered correctly. This test can be used to assure that alignment exists in bidirectional scanning. This test can also be used to evaluate galvanometer function. The vertical lines should be straight without wiggles. One should be equivalent to multiple averaged scans.

3.10. Spectral Registration With Beads (1 μm) (UV and Visible)

The 1 μm multiple wavelength fluorescent beads (Tetraspec, T7284 Molecular Probes, or Rainbow beads, FP–0857-2 Spherotech) were used to monitor the visible spectral registration of lenses (100× PlanApo, NA = 1.4; 63× PlanApo, NA = 1.2; PlanFluor 40×, NA = 1.0) or the registration between multiple beams (UV and 568 nm in our case). The bead was located at low zoom in *xy* and the gain and offset were adjusted to their respective optimum image quality levels. Next, an *xz* scan was obtained at the proper zoom magnification. Care was made to make adjustments at the lower power levels to reduce possible bleaching effects. By balancing laser light intensity with the AOTF, the laser crossover between the detection channels was minimized. To check for laser crosstalk, one laser light line is closed with the AOTF and the signal is observed in the other channels with the PMT setting that is necessary to acquire the proper signal with the respective excitation lines. Subjectively, it can be ascertained how much crossover occurs. If too much crossover exists, the test will be invalidated. The bead was imaged (*xy* and *xz* scans) with an 8× to 24× zoom, a slow to medium scanning rate, and frame averaged four to eight times. The registration of bead fluorescence images between the 365-nm UV wavelengths and the 568-nm visible wavelengths in an aligned system was almost superimposable (*see* **Fig. 5A**). **Figure 5B** shows the *xz* registration between the 365-nm UV line and the 568-nm visible line in a misaligned system. The data for **Fig. 5B** were acquired at a different time using the same 100× lens, suggesting that the system is misaligned, as the difference between the peaks was 650 nm (acceptable difference is 210 nm). The 568-nm line was chosen instead of the 488-nm line to minimize the crossover fluorescence between the visible and UV wavelengths.

3.11. Focal Check Beads (UV and Visible Lines)

Molecular Probes produces a series of beads (Focal Check) that can be used to assess the different visible wavelengths from multiple lasers in a confocal system. These beads have different fluorescent excitation rings and core colors that can be used to assess colocalization of laser light from multiple lasers. We used these beads

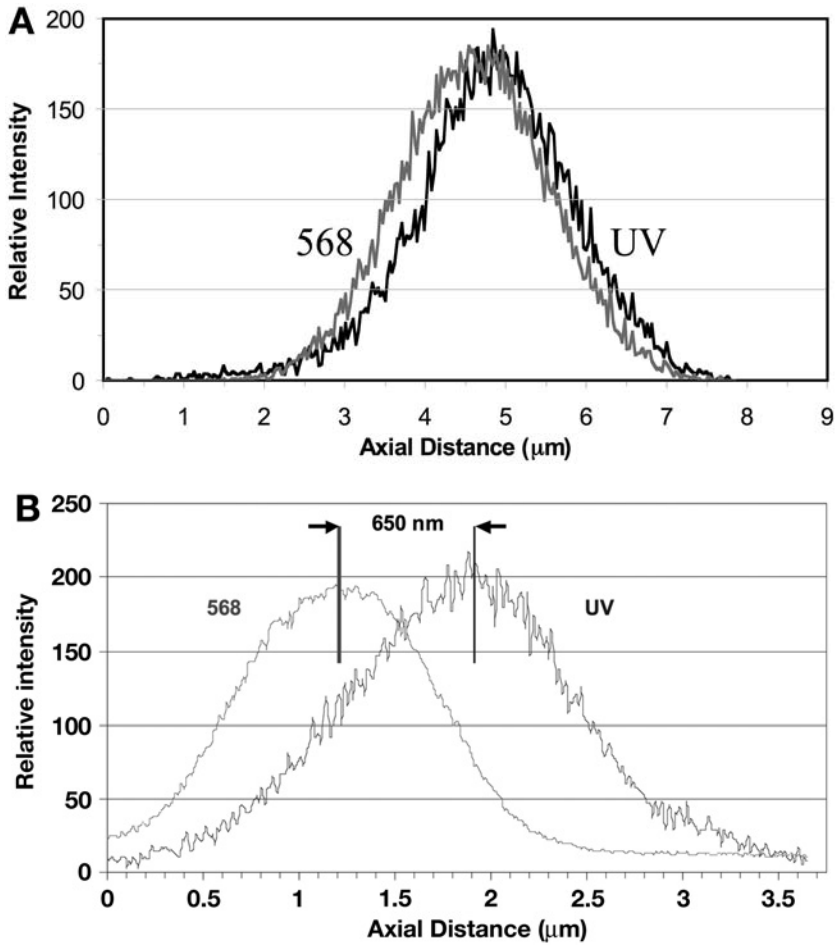


Fig. 5. Spectral registration (UV and visible). The *xz* spectral colocalization of this UV (365 nm) and visible wavelength (568nm) was evaluated with a 100× PlanApo NA = 1.4 lens using a 1-μm multiple wavelength fluorescent bead (TetraSpec T7284; Molecular Probes). An aligned system has a FWHM of less than 210 nm **(A)**, whereas a misaligned system has a FWHM difference of 650 nm **(B)**. The bead was imaged using *xz* scans with a 24× zoom, a slow scanning rate, and averaged eight times. The 568-nm line was chosen instead of the 488-nm line to minimize the crossover between the visible and UV wavelengths.

to examine the UV (365 nm) and visible lines (568 nm) in our TCS-SP1 confocal system that had an argon–krypton laser emitting three lines and a UV Enterprise laser. A 15-μm bead with UV interior and orange fluorescence ring exterior (F7236; Molecular Probes) was used to show that the UV and 568-nm lines were aligned (*see* **Fig. 6;** *see* Color Plate 4, following p. 274). In a similar manner, the F7237 bead consists of a UV blue core and a green ring and this can be used to show

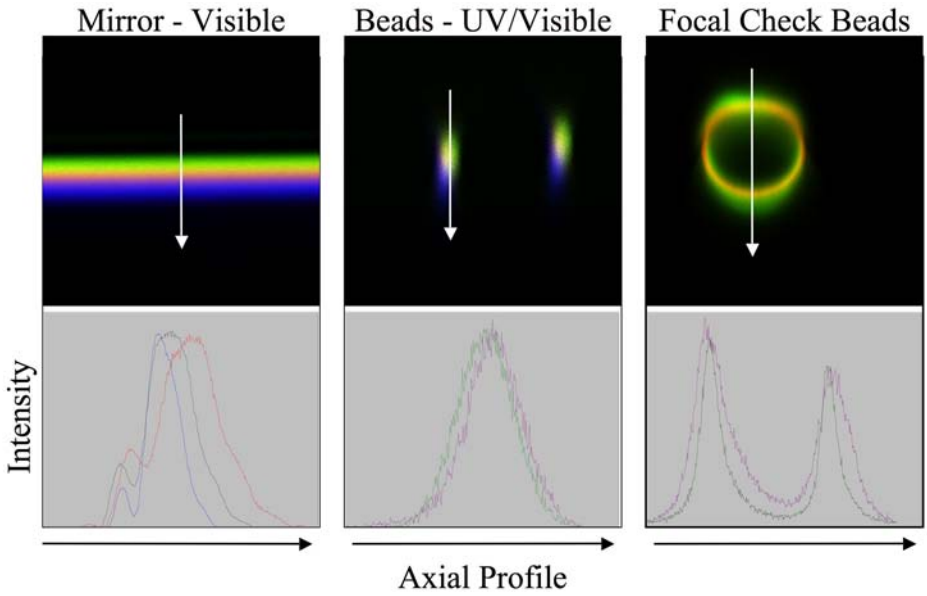


Fig. 6. Spectral registration methods. Three methods are used to check spectral registration of different laser lines. A mirror can be used for visible wavelengths on a Leica TCS-SP system (**left**). A bead scanned in the *xz* directions can be used on all confocal systems (**middle**) to show alignment of laser beams. Focal check beads (**right**) can be used to show alignment of multiple laser beams. In all cases, it is useful to make measurements on the peak height position to determine the spectral registration. (*See* Color Plate 4, following p. 274.)

colocalization of the 488-nm and 365-nm laser lines. This bead might have slightly more crosstalk than the blue core with a red ring (F7236). Normally, the bead should reveal concentric fluorescent rings that have maximum values in the same focal plane with either an *xy* or *xz* scan. The laser power and AOTF should be adjusted to reduce crosstalk between the emitted fluorescence. Any deviation between the concentric localization of the rings or the maximum diameter of the rings and core bead size suggests misalignment. In the newer confocal systems that have three lasers and a merge module, it is recommended to test for colocalization with a focal check bead (F7235) that has three rings representing green, orange, and red or a focal check bead (F7239) that has a red ring and a green core. Individual pinholes in a Zeiss system might have to be realigned monthly and this test can indicate if the pinhole needs adjustments. Smaller beads should yield more accuracy.

3.12. Lens or System Spectral Registration (Beads or Reflective Mirror)

This spectral registration test demonstrates the ability of the CLSM to colocalize different wavelengths of fluorescence in the same plane. To evaluate the spectral registration of the 365-, 488-, 568-, and 647-nm lines, either a 1-µm

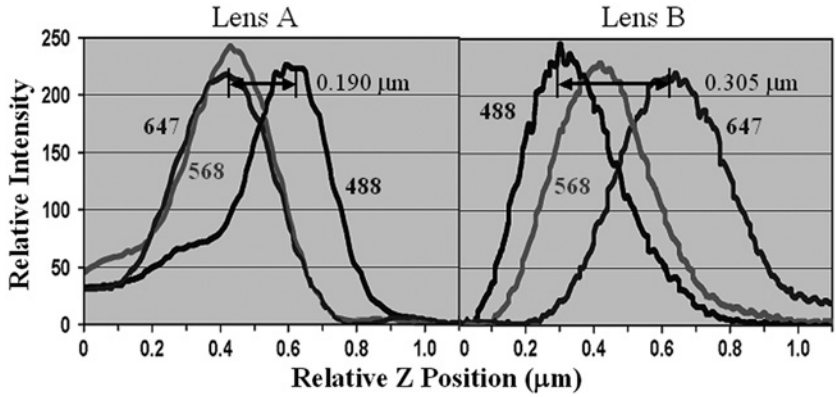


Fig. 7. Spectral registration (visible). The visible spectral registration of a 100× Plan Apo NA = 1.4 objective was evaluated using a front-surface, single-reflection mirror with the same lens at different times. A 10-nm slit was put over each wavelength and the reflection of each line was measured sequentially. The AOTF and PMT intensity was adjusted so that the maximum intensity of each line was 250 GSV. Lens B was sent back to the factory, as it did not meet the following: (1) spectral registration for UV (365 nm) and visible (568 nm); (2) spectral registration for the three visible lines; and (3) 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 will yield slightly better spectral registration than 1-µm bead data for the 647-nm excitation line, as 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-nm and 568-nm wavelength excitation.

multicolored bead (Tetraspec or Rainbow) or a front-surface, single-reflective mirror (*see* **Fig. 7**) was used. The front-surface, single-reflective mirror can be used to check visible spectral registration in a Leica TCS-SP system, in a similar manner to what was described for **Fig. 3** for axial *Z* registration. In the Leica SP system, a 10-nm refection bandwidth is put over each excitation wavelength and the reflection is measured sequentially. By tweaking the AOTF and PMT voltage adjustments, the intensity of each reflected line was adjusted so that the maximum intensity of the image was approx 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 are normally recorded at least 10–40 nm above the excitation wavelengths and not at the excitation wavelength. Many lenses have difficulty in colocalizing far-red fluorescence with the blue and green fluorescence, so measuring the emission at 647 ± 10 nm will yield better resolution than measuring the emission at 660–700 nm.

Figure 7 represents a 100× lens measured over a time period of approx 6 mo on the same CLSM system. On testing Lens B, problems in axial resolution and

spectral registration existed. The separation between the 488-nm and 647-nm line was 305 nm in lens B and the axial registration was 410 nm. This lens (B) was returned to the factory to correct this spectral registration problem in visible light and a spectral registration problem between UV and visible light. Upon repair, lens A showed perfect colocalization between the 488-nm and 647-nm lines and acceptable registration among the 488-, 568-, and 647-nm lines. The UV and 568 nm (*see* **Fig. 5**) also showed acceptable registration after repair.

Depending on the laser configuration, this test can reveal characteristics of the lens' spectral registration or system alignment. In an argon–krypton laser system, because all three lines are derived simultaneously, the test will reveal the lens characteristics. If a three-laser system with a merge module is used, it will reveal the combination of both lens characteristics and laser alignment. It is useful to make this test with more than one objective, as it will be rare that a system will contain multiple lenses with axial and spectral registration problems.

A 1-μm bead can be used to test visible wavelengths for colocalizing in a similar manner to that described for colocalizing UV and visible light. Using the bead test, it is necessary to reduce the laser light to reduce bead bleaching and then adjust the laser light with the AOTF and PMT voltage so fluorescence crosstalk between the emitted wavelengths from different laser lines is minimized. It is also useful to suspend the beads in an antifade medium to reduce bleaching for this test, as they will be zoomed at high magnification, which increases photobleaching.

3.13. Laser Stability (Visible, Long Term [Hours])

Laser stability measurements were made over hours to evaluate the possible fluctuations in power (*1,11*). The laser power fluctuations were initially measured both in PMT 1 (blue light sensitive, low noise, R6857) and in the transmission detector, using a fluorescent plastic slide with very low laser power that was reduced with either neutral-density filters or with the AOTF adjustments. The transmission optical system without a slide showed results similar to PMT 1 with fluorescent plastic slides, and this was the desired optical system to perform this test if it exists on the system, as it eliminated any possibility of bleaching or laser interaction with the substrate.

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 sequentially measuring the laser light with the 488-, 568-, and 647-nm wavelengths, with the power being 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 10–30 s over a period of 2 h. The intensity of a large ROI of the field is averaged and plotted

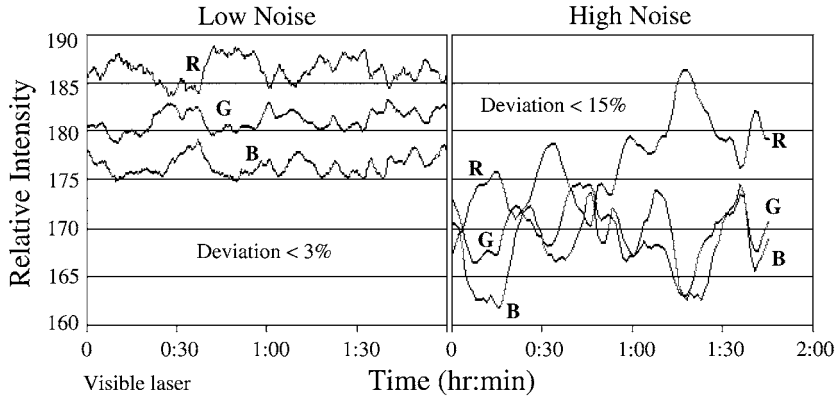


Fig. 8. Laser noise. The periodic change in laser power was measured using transmitted interference optics without a slide in the optical path. The two panels show a visible system delivering low-power fluctuations and one delivering high-laser-power fluctuations over time. The 488-nm and 568-nm lines are cycling periodically directly opposite the 647-nm line. Variations in this power intensity occur over hours and never seem to 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 sequentially measured 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 the following R (red, 647 nm), G (green, 568 nm), and B (blue, 488 nm). The laser power instability might be the result of either laser light entering a fiber with an incorrect laser polarization, thermal instability in the AOTF, or a poorly aligned system. The reason for the source of power instability is not known.

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. It is not necessary to save the scan, but it is useful to save the data measurements in an Excel file, text file, or equivalent.

The type of laser power stability data that can be achieved with this test is represented in **Fig. 8**. It shows a relatively stable visible system yielding low-power fluctuations (**Fig. 8A**; <3%) and an unstable visible system (**Fig. 8B**; >15%), yielding high-laser-power fluctuations (source of noise is not identified). The goal of this test is to achieve a flat stable line. There is periodic noise in the laser system that exceeds the manufacturer's (Ominichrome) laser stability fluctuations specifications of less that 0.5% over a 2-h time period. The 488-nm and 568-nm lines have a periodic cycle that is directly opposite the 647-nm lines, and stability is never achieved (**Fig. 8**). The AOTF may be reponsible for these power fluctuations.

The fiberoptics might influence artifacts resulting from deterioration with time or improper polarization alignment. Fiberoptical problems will attenuate the laser power and necessitate using higher laser power or higher PMT settings in the operation of the CLSM. One test procedure recommended by the manufacturer to

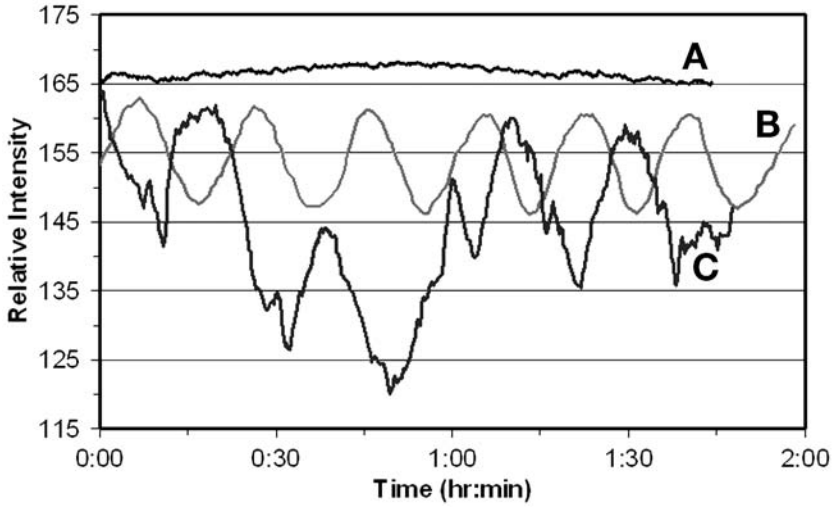


Fig. 9. Laser Stability of a Coherent Enterprise UV laser. The Coherent Enterprise UV laser delivers less than 1% peak-to-peak noise. The laser was connected to an LP 20 water–water cooler, which should be set at least 10°C above the cooling water of the building. It should also be set above the ambient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

ensure that polarization is correct after the alignment procedures is to wiggle the fiberoptic and see if the image returns to the same intensity, suggesting that the polarization is correct. This is a fairly crude test, but it will demonstrate whether the system fiberoptic needs further polarization alignment. Better procedures are being tested by the manufacturers to eliminate this polarization alignment problem.

3.14. Laser Power Stability (UV Long Term)

The Coherent Enterprise laser delivers less than 1% peak-to-peak noise and is considered a very quiet and stable laser. The Coherent laser was tested in a manner similar to that described for visible lasers using the transmitted optical system or the PMT system with blue-colored fluorescent plastic slides and very low laser power. A relatively stable line showing minimal fluctuations should be obtained (*see* **Fig. 9,** trace A). The temperature of the cooling system must be regulated properly or power fluctuations will occur (**Fig. 9,** traces B and C). One source of power instability appears to be way the laser is cooled and how the laser heat is dissipated. This was illustrated with our Coherent Enterprise

UV laser that 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 of the circulating cooling water in the laser, which, in turn, resulted in the improper regulation of the laser power (**Fig. 9,** trace B).

3.15. Laser Power Stability (Short Term [Seconds])

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 sequentially measuring the laser light with the 488-, 568-, and 647-nm wavelengths, with the power being adjusted by the AOTF transmission control so that the transmission detector voltage remains constant for all three wavelengths. In contrast to the long-term stability test, the short-term test uses only one scan for each wavelength. The intensity of a single-line scan is averaged and the mean, standard deviation, and coefficient of variation (CV) are determined (*see* **Fig. 10**). The stability of the laser and the system noise while scanning is determined by this test. This test will measure the noise in the laser and the system and how much averaging should be necessary to improve image quality at the PMT setting that is used. The goal of this test is to have a straight line with no variations in power. If power fluctuations exist, they can be averaged to increase image quality. Averaging will reduce the image CV. If transmission optics are unavailable on the system, this test can also be done with fluorescent plastic using very low laser power to reduce bleaching.

3.16. CV Principle

One of the major elements of a poor image in a confocal system is related to using the PMT at high voltage values. This can be the result of insufficient sample staining, a misaligned system, or a failing hardware component or attenuation of light through a fiberoptic. If the unit could be operated at lower PMT values, then the image quality would be improved and the PMT noise decreased. The noise present in the system can be evaluated using a large (10-µm) bead (Spherotech) or an Applied Precision fluorescent substrate slide. The 10-µm bead consisting of nearly uniform size and intensity (CV = 5% by flow cytometry) is zoomed 4× to increase the number of pixels contained in the ROI with either a 100× objective (PlanApo, NA = 1.4) or a 63× (water [NA = 1.2] or oil [NA = 1.32]) lens. The bead is located in the center of the field and the image of the bead is obtained at the center cross-section of the bead, which relates to its maximum diameter. This large bead suspended in an antifade solution permitted repeated measurements with minimal bleaching of the bead sample. A fixed ROI was

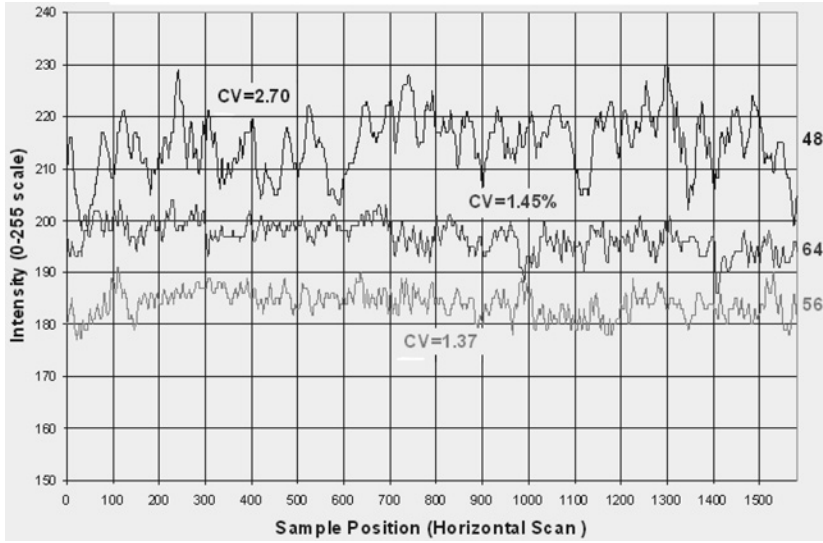


Fig. 10. Laser noise (short time). The transmission PMT was set to a constant value and the intensity of the laser lines was adjusted with the AOTF to bring the intensity to approximately the same point for graphic representation. The intensities of the three lines were made from one individual scan at each wavelength and it demonstrated that 488 nm (CV = 2.70), 568 nm (CV = 1.45), and 647 nm (CV = 1.37) had variations in intensity at very low PMT settings. The blue line (488 nm) was twice as noisy as the green (568 nm) or red (647 nm) lines. The more intensity variations, the more averaging that will be necessary to create a good image.

determined in the bead image that consisted of approximately half of the bead's area. The mean and standard deviation of the pixel intensities in the ROI of the bead were determined to yield the CV. This can also be done with a clean fluorescent substrate slide measured at a specific depth, so the intensity is reproducible.

The CV is defined as the standard deviation (σ)/mean (μ). It is important to maintain the machine variables (pinhole = size Airy disk, PMT voltage, averaging, etc.) at reproducible values for these studies. The laser power was set at a constant value that allowed the mean intensity level of the bead to be approx 150 (out of 255) for each PMT setting. The noise associated with the various settings can be evaluated by varying PMT settings, frame averaging, scan speed, image size, and laser power ***(1–3)***. The CV that we are measuring is actually the variation of pixel intensity within the bead, as opposed to the variation of intensity among a population of beads, as measured on a flow cytometer. An increase in CV might imply that there is either a decrease in laser power or a system alignment problem that results in higher PMT values. A noisy laser can also be detected by this test.

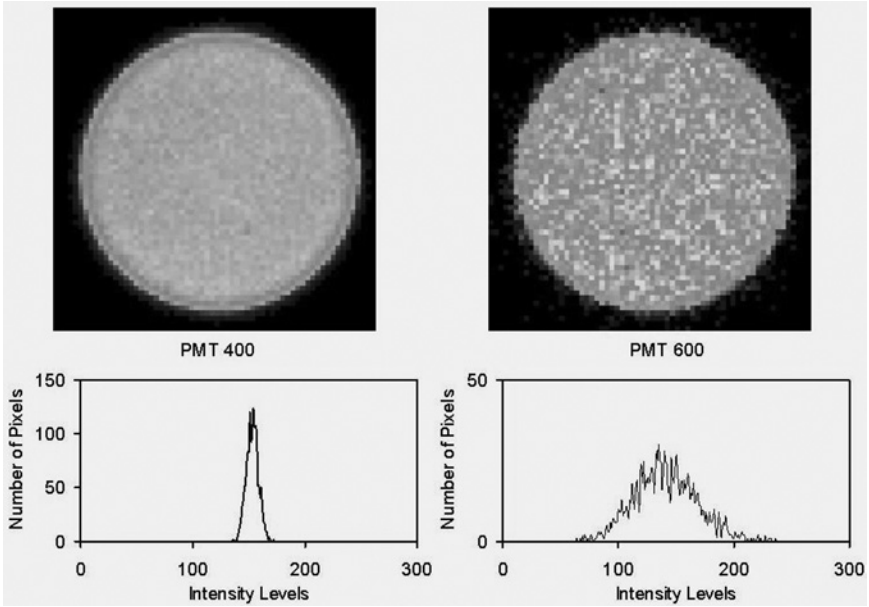


Fig. 11. Bead pixel variations. TIFF images of a 10-µm Spherotech bead were obtained with two different PMT settings (PMT = 400, PMT = 600) with a zoom of 4 and no frame averaging using a 100× PlanApo lens (NA = 1.4). A ROI was drawn in the interior of the bead and the histogram of the population of pixel intensities is displayed in the bottom panels. The mean pixel intensity in both images was approx 150 intensity levels and was obtained by keeping the PMT at 400 or 600 and adjusting the laser power with the AOTF.

Figure 11 illustrates the pixel distribution of a 10-µm bead that was measured with a PMT voltage setting of 400 and 600 and a zoom of 4× and an Airy disk of 1. These two bead images were obtained in the following manner. The mean intensity value in the ROI within the bead was set at channel no. 150 by adjusting the AOTF manipulation, instead of actually lowering/raising the laser power. The higher PMT voltage yielded a broader histogram, which translated into more pixel intensity variations. Because the CV (σ/µ) is defined as the standard deviation (σ) divided by the mean (µ), one can compare the quality of images using this technique. As the quality (less bead noise) of the images increases, the CV of the population of pixel intensities within the bead decreases. To compare image quality between different confocal microscopes, it is critical that as many variables as possible be kept constant ***(1,2)***.

The relationship between PMT voltages and frame averaging influenced the CV value and image quality. If the laser power was kept constant and the system power was adjusted with the AOTF, it was found that either increased frame averaging or lower PMT voltages could decrease the bead CV value and, thus, improve image quality. The relationship shown in **Fig. 12** demonstrates that at higher PMT set-

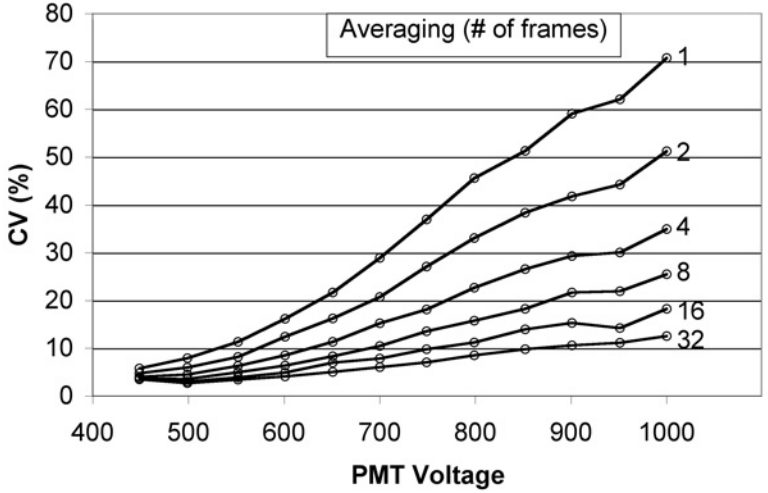


Fig. 12. Relationship among averaging, PMT, and CV. The noise present in the system was evaluated using a 10-µm bead with a 100× PlanApo objective (NA = 1.4). If the mean is assumed to be constant (channel no. 150), the histogram distributions generated by averaging can be produced and the CV and standard deviation (SD) calculated. This test was made by decreasing the PMT value and adjusting the laser power with the AOTF to ensure that the mean pixel intensity was at a value of 150. The higher PMT values were taken first to minimize possible bleaching. Images corresponding to 1, 2, 4, 8, 16, and 32 were obtained at each PMT setting. The CV is defined as the standard deviation (σ) of pixels in a bead divided by the mean (μ) intensity ($CV = \sigma/\mu$). The noise at a specific setting can be reduced if frame averaging is increased. The averaging decreases the pixel variation, which lowers the SD and decreases the CV. Theoretically, by averaging the image n times, the CV and SD are decreased by the square root of n.

tings, it is necessary to frame average more to reduce the CV and increase image quality. The same relationship exists in biological samples (*see* **Fig. 14**).

3.17. PMT Function

The PMT is the detecting system and there exists different quality PMTs of the same type in a confocal system. This CV bead test method can be used to access the operation and quality of the PMTs in the confocal system. The use of the Leica SP system easily allowed for switching of PMTs and pairing them with different excitation wavelengths. In effect, any PMT could be used in conjunction with any of the four excitation wavelengths. Although the PMT position will affect the CV, it is not considered to be a major contributor, and in this assessment, all of the PMTs were considered equivalent. PMT 1 and PMT 3 have an extra mirror present to reflect the light, whereas, PMT 2 collects the light directly after the prism. There are two types of PMT used in the Leica system: PMT 1 is considered low

Table 3
PMT Comparison and Noise

Excitation	Emission	PMT#	PMT Voltage	CV(%)	Relative CV
488 nm	505–555 nm	1	474	6.06	100
		2	428	6.58	108.65
		3	425	6.23	102.86
	555–600 nm	1	471	6.02	100
		2	432	7.00	116.25
		3	421	6.46	107.17
568 nm	580–630 nm	1	439	4.00	100
		2	411	4.88	122
		3	393	4.49	112.11
647 nm	665–765 nm	1	802	20.30	100
		2	732	22.70	111.68
		3	675	20.30	100.12

Note: The noise of the system was evaluated using a 10-µm bead (Spherotech) and a 100× PlanApo (NA = 1.4) objective. The intensity of a 10-µm bead was determined at a constant laser power, a zoom of 4, and no averaging using various PMT settings. The emitted light was measured in each of three PMTs. The pixels in each ROI were set to a mean of approx 150 and the standard deviation of pixel distribution was measured to determine the CV. The CV of the pixel intensity within the bead was measured at each PMT setting. PMT 1 is low noise and blue sensitive, whereas PMT 2 and PMT 3 are far-red sensitive. The quality and part of the performance of each PMT can be measured with this test.

noise (R6357) and PMT 2 and PMT 3 (R6358) have high efficiency and sensitivity in the far-red wavelength regions. Zeiss also has different types of PMT in their system. The system was set up with a triple dichroic (TD) using 488-nm, 568-nm, and 647-nm wavelength excitation. The three PMTs were adjusted to allow the mean pixel intensities to be at channel 150. The relative intensities were measured with the three PMTs for all conditions (*see* **Table 3**). Lower CVs will translate into better image quality and will require less frame averaging to produce the equivalent image quality. This is a test that is useful to determine system quality and identify a possible problem in PMT performance prior to a hard failure. The test can be done with dichroics and barrier filters to assess the efficiency of each filter in the light path. It is important to define the bandpass region that is being evaluated, as dichroics will eliminate specific regions in the transmission. A similar type of test can be made on filter systems to evaluate the PMTs.

3.18. Spectral Scanning: PMT Comparison

The PMT performance was measured using the Leica spectral scan feature. This feature allows for a sequential scan across the whole range of 400–800 nm in units as small as 5 nm. The test used an inexpensive, defined mixed-ion light source, (LightForm Inc., Hillsborough, NJ) to measure the spectral response of

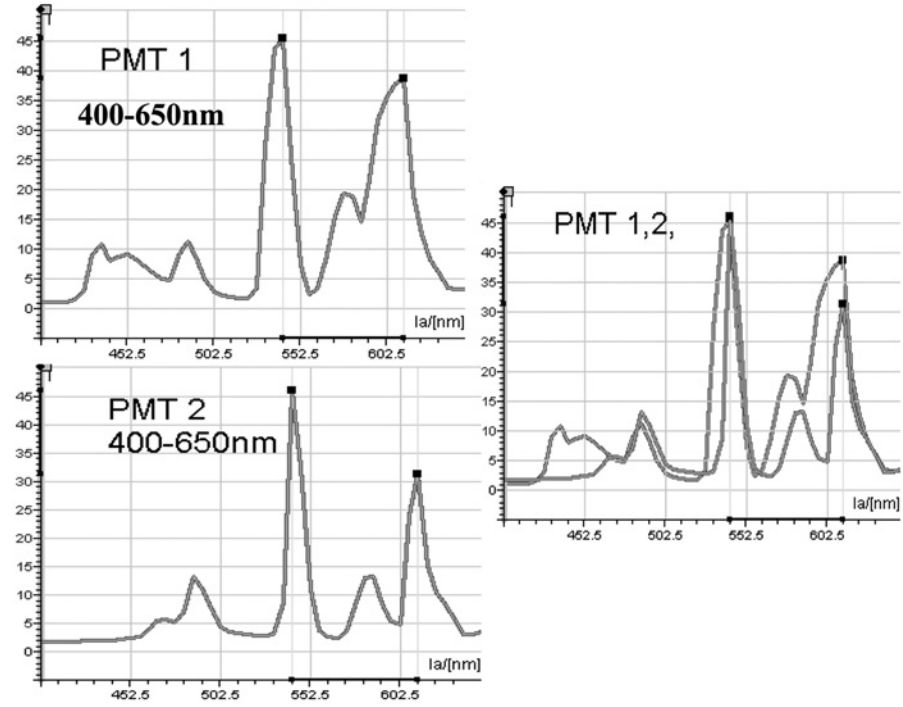
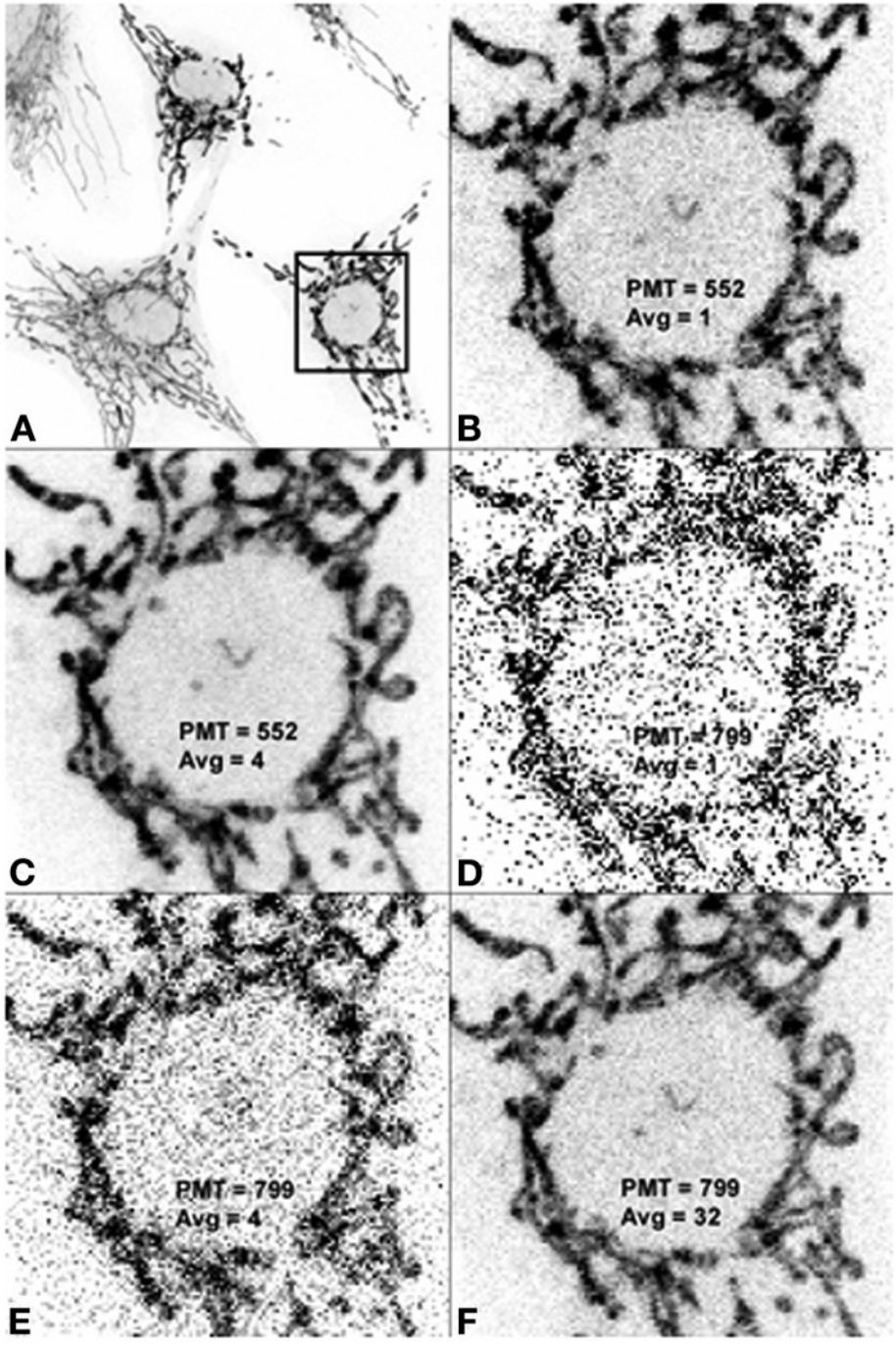


Fig. 13. Spectral scanning. The PMT performance was measured across the 400- to 650-nm spectral range using the Leica spectral scan feature. The slide holder was removed and the light source (LightForm Inc., Hillsborough, NJ) was positioned on the stage. A spectral scan consisting of 50 increments of 5 nm each was made between 400 and 650 nm using a 10× (NA = 0.4) PlanApo lens without averaging and an Airy disk of 1. A large ROI was used to analyze the intensity of each 5-nm scan and the data are displayed as an intensity curve between 400 and 650 nm. Note the broadness in the peaks acquired from PMT 1 relative to the tighter and smaller CVs in PMT 2. PMT 3 is similar to PMT 1 and is not shown for figure clarity. This scan shows a suppression of PMT 2 wavelengths below 450 nm. It also demonstrates a lack of resolution of the PMT 1 peaks relative to PMT 2 peaks ***(24)***.

a PMT across a large spectral region. The slide holder was removed and the light source positioned on the stage. A spectral scan consisting of 50 increments of 5 nm each was made between 400 and 650 nm using a 10× (NA = 0.4) PlanApo lens without averaging and an Airy disk of 1. The efficiency of the light-collection system was low, necessitating that a large PMT voltage setting be used. The intensity of each 5-nm scan was calculated as the mean from a large ROI and the data were displayed as an intensity graph between 400 and 650 nm (*see* **Fig. 13**). A similar test can be made on a Zeiss 510 Meta using a 10.7 nm scan size. If necessary, the pinhole can be opened to let in more light.


A
B
PMT = 552
Avg = 1
C
PMT = 552
Avg = 4
D
PMT = 799
Avg = 1
E
PMT = 799
Avg = 4
F
PMT = 799
Avg = 32

3.19. Biological Samples

The CV technique developed on beads was applied to biological specimens (MP FluoCells; chicken cells stained with Acridine Orange) to observe if the same relationship between PMT voltage and averaging described on beads are maintained on biological cells. FluoCells® (F-14780, Molecular Probes) were excited with a 568-nm laser line and detected with a 580 to 630-nm bandpass filter in PMT 2. The difference in image quality illustrated by averaging 1, 4, or 32 times at 2 different PMT settings (552 or 799) is shown in **Fig. 14**. Figure 13B–F shows images zoomed 4× using Image Pro Plus to clearly illustrate the individual pixels. The CVs of a selected ROI in the nucleus varied with the number of frames averaged and the PMT voltage. The best image quality (low CV) consisted of either low PMT voltages (**Fig. 14B,C**) with minimal amounts of frame averaging, or high PMT voltages with 32 frames averaged (**Fig. 14F**). High PMT settings (**Fig. 14D,E**) with minimal amounts of frame averaging (one or four) demonstrated high CVs and poor image quality. In all cases, the increase in averaging resulted in a decrease in the CV and a corresponding increase in image quality. In contrast, raising the PMT voltages increased the CV and decreased image quality. The higher PMT settings necessitated the use of greater frame averaging to increase image quality. **Figure 14** shows the relationship among PMT voltage, frame averaging, and CV on image quality on cells, which was similar to that described with beads in **Fig. 11.** The noise in this figure is also reduced as the square root of the frames averaged ***(12,13)***. The CVs of a ROI in the nucleus of the various panels of **Fig. 14** was the following: B, 49%; C, 40%; D, 212%; E, 109%; F, 49%.

3.20. Sensitivity

The sensitivity of a confocal microscope is an important parameter to determine, as its value influences the acquisition settings of the PMT voltage, laser power, and frame that are used to acquire images. The values derived will relate

Fig. 14. PMT and averaging of cells. FluoCells (F-14780; Molecular Probes) were excited with a 568-nm laser line and detected with a 580- to 630-nm bandpass filter in PMT 2. Averaging 1 measured the resolution, 4 or 32 times at two PMT settings (552 or 799). **(A)** shows the distribution of three cells at normal magnification, whereas **(B)–(F)** show one cell located in the box in **(A)** that was zoomed 4× with Image Pro Plus. The settings in the different panels were the following: (A) control (PMT = 552, AV = 1); (B) (PMT = 552, AV = 1), (C) (PMT = 552, AV = 4), (D) (PMT = 799 AV = 1), (E) (PMT = 799, AV = 4), and (F) (PMT = 799, AV = 32). Note the difference in pixel variations in the six panels of the same cell acquired at different PMT/averaging settings. The CVs of a ROI in the nucleus of the various panels were the following: (B) 49%; (C) 40%; (D) 212%; (E) 109%; (F) 49%.

Table 4
Confocal Laser scanning Microscope Sensitivity: Relationship Between Laser Power and CV

Laser type	Wavelength mW	Power mW	CV-Bead% SD/Mean
	Fixed Power Comparison		
Argon Krypton	488	1	4
(75mW, Leica)	568	0.2	4.6
Argon 25mW	488	1	1.3
HeNe 1mW	543	0.2	1.9
(Zeiss)			
	Maximum Power Comparison		
Argon Krypton	488	1.1	3.8
(75mW, Leica)	568	1.45	2.6
Argon 25mW	488	3.2	1
HeNe 1mW	543	0.23	1.9

Note: The sensitivity from a Leica TCS-SP1 containing an argon–krypton laser emitting three laser lines and a Zeiss 510 containing three individual lasers and a merge module are represented. The CVs were obtained from a 10-µm bead using a 100× PlanApo objective (NA = 1.4) The laser power was derived by using a 10× (NA = 0.3) objective and a power meter situated on the stage. By setting the power to a fixed value of either 1 mW of 488-nm laser light or 0.2 mW laser of 543-nm laser light on the stage, the sensitivity of two machines was measured. The CV of the bead was observed to be almost three times lower with the 488-nm laser lines using the Zeiss 510 system compared to the Leica TCS-SP1 system. By increasing the lasers to their maximum power, the CV values were decreased. Sometime maximum power measurements are useful to indicate alignment of the system and functionality of different components. There are many explanations for this difference.

to alignment, equipment functionality, and general performance of the CLSM. **Table 4** represents two confocal microscopes that have different configurations: a Leica TCS-SP1 containing one argon–krypton laser emitting three laser lines, and a Zeiss 510 system that contains three individual lasers with a merge module. The test particle was a 10-µm Spherotech bead and measurements were made using a 100× PlanApo objective (NA = 1.4) with a zoom factor of 4 with 488-nm excitation in both systems. The laser power in both systems was measured on the stage using a 10× (NA = 0.3) objective and a power meter detector secured on the stage. It is essential that the acquisition parameters be as equivalent as possible when trying to compare machines from the same or different manufacturer. Because there are considerable differences between the designs of different machines, extreme care must be made when interpreting the results of data obtained from different manufacturers' machines. The variables that

must be considered included specific wavelength, laser type, objective lens, optical components, pinhole size, scan speed, zoom, and pixel size. Other variables not mentioned could also affect the measurement. It should be emphasized again that it is extremely difficult to compare different manufacturers' machines because of the number of variables that must be kept constant, and conclusions drawn from this head-to-head test can only be made if all the variables are nearly equivalent.

To illustrate the test, we used a Leica machine that has an older Omnichrome argon–krypton laser emitting three laser lines and a new Zeiss 510 CLSM containing three relatively stable lasers. It would be expected that by using different lasers, the tests would reveal different sensitivity values (*see* **Table 4**). The sensitivity of two machines was made by maintaining the laser power at a constant value of 1 mW for 488-nm wavelengths and 0.2 mW for 568/543-nm wavelengths. The data from this test revealed that 1 mW of 488-nm power measured on the scan head yielded a CV value of 4% with a Leica TCS-SP1 and a CV value of 1.3% with a Zeiss 510. Comparable power readings on both systems showed the CV to be almost three times lower with the 488-nm and 568-nm lines with the Zeiss 510 system as with the Leica TCS-SP system containing an Omnichrome argon–krypton laser. The significance of a higher CV value means that the samples will have to be frame averaged to increase the image quality. These CV values will change if the laser power and PMT values are changed. The quality of lasers will also affect the CV values, as shown by the data in this test. Finally, increasing the laser power to a maximum value resulted in the CV being lowered with both the Zeiss 510 and Leica TCS-SP1 systems. However, it is not recommended to operate lasers at maximum power because of sample bleaching considerations and laser lifetime considerations.

This CV sensitivity data could be considered an initial reference point that can be used by other investigators to compare their CLSM performance with similar machines. It seems to be possible with this approach that the sensitivity of systems in different laboratories can be compared. Because of the number of variables that must be kept constant, extreme care must be made when interpreting this test in the comparison of different manufacturers' machines. However, there is no reason why similar machines from the same manufacturer cannot be compared if they are run under nearly identical conditions.

4. Notes

4.1. System Performance Tests

Image quality is an important parameter in the evaluation of a confocal microscope performance. Unfortunately, image quality is too often used as the "gold standard" to evaluate confocal microscope functionality and performance. Variables that effect image quality should be assessed to ensure that the

system is delivering its optimum performance. In the cases in which intensity measurements are required, it is essential that the machine be stable to deliver reproducible data. A series of tests were either adapted from the literature or devised in our laboratory to measure the system performance of the confocal microscope *(1–8)*. These tests include the following: 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 but represents what can be tested and interpreted to ensure that the machine is operating properly.

4.1.1. Field Illumination

Field illumination is one of the easiest and most important tests to make on a confocal microscope. Many CLSM units in laboratories that have been checked for field illumination using a plastic fluorescent slide (Applied Precision) have demonstrated unacceptable field illumination patterns. This test should be made with all objectives and all wavelengths of visible and UV light to ensure that the machine is delivering proper field illumination. 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 one manufacturer. The light should come in the center of the objective and it should decrease in all directions in a similar manner. The decrease is dependent on the characteristics of the objective and its magnification. Most alignment procedures are made using high-magnification objectives (40×). However, this does not always translate into good performance with different magnification objectives.

The illumination intensity across the observation field can be measured with different types of test specimen in order to ensure that a homogeneous field illumination exists. The following test substrates have been used: concentrated fluorescent dye suspended in a hanging-drop well slide, small concentrated fluorescent beads (1–3 µm) or large concentrated fluorescent beads (10 µm) (Spherotech), fluorescent specimens, uranyl glass slides, Altuglas, or plastic fluorescent slides (Applied Precision), 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) and mixed with immersion oil (Leica Immersion oil, $n = 1.518$) *(1)*. A histological sample derived from plant or animal, which is usually the choice of service field engineers *(1,3,8)*. The plastic slides (Applied Precision) were found to be the most consistent sample to test field illumination. We use the blue slide for UV excitation and the orange slide for 488-nm and 568-nm excitation. The red slide was found to bleach rapidly with 568-nm excitation; therefore, it is preferable to use the orange slide for this wavelength. The Chroma red slide was chosen for the 365-nm, 488-nm, 543-nm, and 568-nm excitations. The green slide can also be used for 488-nm excitation.

Initially, 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 the field illumination at a specific depth in the plastic slide, as the intensity distribution might change from the surface to the interior of the slide. Investigators should also be careful not to observe illumination fields deep within the plastic slide samples, as it will usually yield a better field illumination than regions closer to the surface because of various optical distortion factors ***(14)***. It is also important that the plastic slide be placed on a firm surface to eliminate any possibility of substrate flex. Although it has been proposed to use this fluorescent slide to take daily measurements for system stability, the factor of reproducible depth in addition to instability of laser power makes this application questionable.

There are many test substrates that have been used to measure field illumination. Some criticisms of these tests are as follows. Uranyl glass has previously been used to check field illumination, but it is currently difficult to obtain and we have observed that plastic slides have higher fluorescent efficiency than the uranyl glass at all wavelengths. A field of small or large beads on a slide (Spherotech) can be used, but it is essential that all of these beads be 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 the beads that represents field intensity. However, the downside of this method is that it is very time-consuming. Histological samples derived from plant or animal can also be used to measure field illumination, and these are usually the choice of service field engineers. Histological samples will show the illumination pattern indicating proper or uneven illumination. In our experience, histological samples are not sensitive enough to properly measure field illumination. It usually will yield a sense of false security for the investigator. Solutions of fluoresce fluid are unstable and may shift with time. If there is a discrepancy between the plastic substrate test slide and other test slides in measuring field illumination, it might be the result of a greater sensitivity on the plastic substrate.

In our system, the three visible wavelengths of light are derived from one Omnichrome argon–krypton laser, which allows us to test the field illumination at one wavelength (488 nm) and assume that 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 at the 365-nm excitation in addition to the visible 488-nm excitation. Newer designed confocal systems use three individual lasers with merge modules, requiring that all laser wavelengths have correctly aligned beams emitted from the merge modules. In these systems, each of the three lines has to be individually tested. One laser line might be perfectly aligned,

yielding acceptable field illumination, whereas the other laser lines might be misaligned, yielding intensity values in which the brightest region is not in the center of the field, as illustrated in **Fig. 1** with UV illumination.

4.1.1.1. Objectives

The lenses are the engines that drive this technology and their selection is very critical for optimum performance. Some of the factors to consider are the following: high numerical aperture (NA) relative to the specific magnification, flat field objectives, long working distance (WD) relative to NA, good fluorescence transmission, and good achromatic correction at desired wavelengths ***(12)***. Currently, microscope manufacturers make lenses that go though extratight QA procedures and have been classified as confocal objectives. These should be bought, as they are believed to be of a higher quality than ordinary fine microscope objectives. As a general rule, one should use the smallest magnification and the largest NA lens to acquire images ***(13)***. These lenses offer a larger field-of-view and better light transmission. Although it is critical to reject out-of-focus light for confocality, it is also necessary to have sufficient laser light entering the system, and lenses should be chosen that have good fluorescent transmission, characteristics. Opening the pinhole can increase transmission, but this should be done only if insufficient light is present, as this parameter decreases the confocality of the system. The choice of a good fluorescent transmission lens is sometimes chosen over PlanApo and spectral precision.

It is suggested that a confocal core lab should have a full range of high-quality microscope objectives consisting of air, water, multi-immersion, and oil lenses (typically planapochromat of the highest numerical apertures available) to satisfy the multiple applications in a core laboratory. It is necessary to match low-magnification oil and water objectives with higher-power water and oil objectives. Water-immersion objectives increase the working distance of the higher-power objectives. A complete set might be the following: 5× dry, 10× dry, 20× dry—used for low magnification imaging of macroscopic specimens (e.g., whole-mount mouse brains and fetuses), a 10× oil, 20×, 40× oil, 63× oil—used for conventional microscopy and 10× and 20× multi-immersion lenses combined with a 63× water lens and a 63× oil lens. A 100× lens has problems with alignment and low light yield and UV incompatibility. It is suggested that a 63× lens be used and zoomed slightly to address the 100× magnification range.

Objectives have unique characteristics and should be chosen for the specific applications accordingly. For instance, the Leica 100× (**Fig. 2;** NA = 1.4) is not recommended for UV applications, as it has a bull's-eye pattern with UV excitation. Because of the design of the 100× objective, it is recommended to use a zoom of 2× when using UV light in order to achieve a drop off of less than 25% across the field. Leica recommends that the PlanApo 63× (NA = 1.32) be used

for UV work, as it has more uniform UV field illumination and better UV light transmission.

Other objectives that are useful in conventional microscopy might have a fluorescence bull's-eye pattern, making these lenses unsuitable of confocal applications *(1,3)*. It is important to acquire lenses that are compatible for confocal microscopy applications and test the lenses for field illumination accuracy using UV and visible excitation wavelengths. This field illumination test allows for a system evaluation consisting of both the objective properties and the confocal microscope laser alignment. This bull's-eye intensity profile has been obtained with different magnification objectives using all manufacturers' systems (Biorad, Nikon, Leica, Zeiss). The incompatibility of different lenses with confocal microscope systems can increase this bull's-eye effect and this parameter should be considered in choosing lenses. The problem might be the result of the lasers under filling the objective, which results in operating a lens under suboptimum conditions, resulting in the field illumination problems. One recommended solution to poor field illumination or bull's-eye illumination is to increase the zoom factor. However, this enlarges the illumination center and pushes the lower intensities off the field-of-view. Increasing zoom also increases the magnification and bleaching rate of the sample and this might defeat the purpose of using a low-magnification objective to observe a large field-of-view or might rapidly bleach the sample. This field illumination effect has to be monitored with each laser wavelength and each objective, as the alignment, wavelength, and lens design can influence the field illumination pattern. In summary, 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 that 25% in all directions equally from the center with most lenses.

Not all problems with the field illumination test are the result of poor alignment, lens design/quality, or incompatibility of a lens with specific wavelengths of light. A dirty lens will yield both poor field illumination and poor resolution. If a lens is dirty or covered with dried oil, then it would yield a nonuniform pattern *(3)*. In one example, the intensity of the field from a 20× (NA = 0.6) dirty lens varied by as much as 70%, with the maximum intensity being off center on the right side of the image. After cleaning the lens to remove oil and other particles, an acceptable illumination pattern was obtained, with the maximum intensity being located in the center of the image and decreasing in intensity by less than 10% from the center maximum *(3)*.

4.2. Power Meter Readings

This power test appears to be one of the most useful tests because it quickly evaluates both the system alignment and performance. For the adequate operation of a CLSM, a sufficient laser power must excite the specimen. If a system

is misaligned or functioning suboptimally, it can be assessed by a test that measures laser power. The power test can indicate if the system is aligned properly up to the plane of excitation on the stage or if the machine has a defective component (i.e., a dying laser or a defective fiber). It should be emphasized that this test is performed at the microscope stage prior to the light reflecting the dichroics for a second time and penetrating through the emission pinhole and the emission barrier filters or prisms (if they exists in the system) and into the PMT. In our experience, without sufficient power throughput in the system, the voltages will have to be increased to high values to visualize fluorescence derived from specimens, which will introduce PMT noise. In addition, the cause of the decreased laser power might result in other problems (i.e., laser instability, loss of axial resolution, increased laser noise, increased PMT noise, fiber polarization, broken fibers, AOTF malfunction).

The laser power measurements are listed in **Table 1** and are useful to illustrate the maximum power that can be obtained from a CLSM having these different laser configurations. These measurements serve as a valuable reference for an individual lab to QA their system over time and as a comparison with other similar CLSM for adequate performance. In a three-laser system, it appears that there is sufficient power with the 633-nm and 488-nm lasers, but because the maximum power of the 543-nm laser is so low and the attenuation of the 543-nm laser is so great, at least 0.2 mW of power are necessary from the 1.2-mW HeNe 543-nm laser to provide sufficient light to excite the samples. If the system is not aligned properly, the laser output will be decreased. With insufficient power, the PMTs will need to be operated at high-voltage settings, which increases the system noise and produces unacceptable images. Less power throughput in the confocal system suggests a problematic laser, a fiber polarization problem, defective AOTF, or just a poorly aligned system. The maximum laser power is dependent on the laser, optical configuration, and the specific objective used. Using a 10× objective (NA = 0.3), it is desirable to have at least 1 mW of power on the microscope stage for each laser line derived from a 75-mW Omnichrome 643 argon–krypton laser. Other confocal systems with different laser configurations will naturally have different power values, as indicated in **Table 1**.

It is important to measure the power output to evaluate system performance for all three lines after installation to make sure that the system is aligned properly and the laser is functioning correctly. These power values will not only serve as a reference to ensure the system is performing properly but can be useful to notify the confocal manufacturer of deviations from acceptable values, which will 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; 647 nm, 1.60 mW. After time and proper laser

alignment, almost 3 mW for each line have been achieved in this system. However, on another day the maximum power with a Leica TCS-SP1 system using a Plan 10× Fluor (NA = 0.3) was the following: 488 nm (1.1 mW), 568 nm (1.45 mW), and 647 nm (1.65 mW). Similarly, the maximum power measured on installation with a Leica SP2 system using a Plan 10× Fluor (NA = 0.3) was the following: 4.6 mW (TD, 488 nm), 6.5 mW (DD, 514 nm), 0.22 mW (TD, 543 nm), and 2.8 mW (TD, 633 nm). The values on the same machine taken a few months later were the following: 2.87 mW (TD, 488 nm), 3.9 mW (DD, 514 nm), 0.093 mW (TD, 543 nm), and 1.45 mW (TD, 633 nm). The reason for the fluctuations is unknown, but it appears that they might be attributed to fiber polarization problems, and AOTF instability problems.

4.3. Bead/Histological Power Meter Sample

If a power meter is not available, the crude power of the system can be assessed using standard histological samples like the FluoCells slide (F-14780; Molecular Probes) or beads like the 10-µm Spherotech beads (FPS-10057-100) and recording the PMT value that is necessary to acquire an image at almost saturation values If conditions are identical between machines. This PMT value can be used as a crude 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 because of the large range of acceptable PMT voltage values. Another reason to doubt the data from using a histological sample is that individual PMTs and samples can vary greatly in quality. Two similar PMTs on our machine had almost 100 PMT unit differences in the 700 PMT range. What is even more troubling with this test is that the PMT voltage is expressed as a logarithmic relationship relative to an intensity increase, which means that the difference of only a few PMT units can be translated into a huge difference in intensity.

In our opinion, these tests are very crude and subjective because of the acceptability of such a large range of PMT amplification values, the variations in staining between different plant samples, variations in PMT characteristics, and the logarithmic relationship between PMT and intensity ***(1)***. It does, however, yield a rough estimate to determine if there is sufficient power in the system. It is useful for each laboratory to have a reference slide to determine PMT saturation values with different laser wavelengths to determine if there is adequate power in the system and if the CLSM is adequately performing.

The comparison of two lenses to transmit light at an Airy disk of 1 can be relatively compared using a saturation test with almost any uniformly stained sample. On a Leica system, the 100× (NA = 1.4) does not transmit as much light as a 63× (NA = 1.32) lens; thus, for low-light applications, this 100× lens should not be used when there is insufficient staining.

4.4. Laser Adjustments

Argon, UV, and argon–krypton lasers need to be aligned and adjusted on a regular basis, as they occasionally go out of alignment. The investigator can easily measure laser power over time using a power meter positioned on the stage (*see* **Table 1**). Either the loss of laser power or poor optical alignment will reduce the laser power in the system, necessitating an increase in PMT to compensate for lack of laser light intensity. If minor adjustments are made to the mirrors with the horizontal and vertical knobs located on the back of these lasers, the laser power sometimes can be increased. However, these visible lasers are usually enclosed in a box, with the rear knobs being inaccessible for adjustment by the investigator. In fact, most confocal manufactures do not allow the user to adjust these controls, as it is the responsibility of the manufacturer on a service contract to ensure that the lasers and system are 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, as it is enclosed in a box that the manufacturer required not be open or the service contract would be invalidated. The investigator usually will not notice a problem with laser power or alignment, but will continually have to increase 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 are indicated by reduced power. The reduced laser power might result in a system yielding poor resolution and system noise.

If there is insufficient light entering the system, a careful realignment of the laser beam might be 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**), then the laser might need to be replaced. Knowing specifications of laser power output on a stage is a critical parameter to assess system performance. In the future, it is suggested that the different manufacturers should specify these power values obtained on the microscope stage for different laser configurations, thus allowing investigators to determine their CLSM performance in their laboratories.

4.5. UV Power Test

One of the major problems that occur with UV confocal systems is that there is insufficient UV power output. The UV power transmission can be decreased from a number of factors, which include misalignment, aging fiberoptic, polarization mismatch between fiber and laser, unfocused collimator lens, and dying laser. This measurement of UV power helps assess the system performance and determines if adequate UV power is being transmitted through the system and if the fiber is in good condition. Although it is recommended to take the measurements

at the back objective aperture region to eliminate the characteristics of the lens from influencing the test, we were not able to mount our detector probes in a sufficiently stable manner to allow for repeatable measurements.

An objective designed with good UV transmission characteristics should be used to increase the power throughput. Attenuation of the laser light through the low-power optics of the system will still occur and the power values obtained are relative values that are highly dependent on the specific type of objective used. Because our power detector does not work with higher-power optics (40×, 63×, 100×) because of 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. For instance, by using maximum UV laser power, it was found that the 10-µm Spherotech bead saturates PMT 1 (low-noise PMT) at a setting of 650 using a 100× PlanApo lens (NA = 1.4). Leica technicians routinely 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 PMT 1, the system is passed as having adequate power. In our opinion, this test is very crude and subjective because of the acceptability of such a large range of PMT amplification values, the variations in staining between different plant samples, variations in PMT characteristics and the logarithmic relationship between PMT and intensity ***(1,3)***. It does, however, yield a rough estimate to determine if there is sufficient laser power in the CLSM. It would be useful to measure these values on a user histological test slide or bead when the machine is first installed and deemed working acceptably by the manufacturer's service and sales representative.

4.6. Dichroic Reflectance (Single-Wavelength Excitation)

Dichroic filters are made to reflect or reject specific wavelengths of light and pass the desired excitation/emission wavelengths of light. Dichroics do not always perform the way they were designed to perform in a CLSM (*see* **Table 2**). Dichroic tests should be made to determine the optimum system efficiency and ascertain the performance of individual dichroics with a variety of objectives and wavelengths. In certain images, it is important to have narrow bandpasses and not broad long passes in acquiring the fluorescent emission. It is important to use the dichroics that reflect the maximum amount of light at the desired wavelengths to increase the efficiency of the CLSM ***(1,3,6,7)***. By placing a fluorescent slide on the stage and measuring the relative intensity of an image, the efficiency to reflect light in a confocal system of the specific dichroic filter can be evaluated. The double dichroics (DD) and triple dichroics (TD) are more complicated dichroics than the single dichroics (SD) and, in theory, should reflect less light, as they were made to reject more light and pass fewer specific wavelengths of light. The placing of either

a single, double, or triple dichroics in the light path should reflect successively less light using the 488-nm excitation line. Using a Leica confocal filter system, normally the 488-nm line should use the SD (RSP500), the 568-nm line should use the DD (488/568), and the 647-nm line should use the TD (488/568/647). However, the DD reflected the 488-nm light best and the TD reflected the 568-nm light best with all objectives. In principle, the better the reflection, the more efficient the dichroic. Using 568-nm excitation, the TD reflected 30% more light than the recommended DD dichroic. Comparing the RSP500 and DD dichroics with 488-nm excitation shows the efficiency difference between the low-power and high-power objectives, necessitating the need to test all of the objectives. From these data, it can be surmised that when using single-wavelength excitation, the DD should be used in preference to the RSP for 488-nm excitation for all lenses, and the TD should be used instead of the DD for 568-nm excitation for all lenses. This is a QA test to determine the efficiency of the dichroics in CLSM and help in determining which dichroic should be used in a single-wavelength excitation experiment. This dichroic test is used with single-wavelength excitation and 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 be chosen to balance the power of the emitted fluorescence from each fluorochrome, but this test is helpful in making the decision. A lambda scan of the dichroics will indicate their actual spectra in the system and whether there are alignment problems.

4.7. Axial (Z) Resolution

The axial resolution test is considered the "gold standard" of resolution in confocal microscopy ***(1,3,7,8)***. The axial resolution test is made using a 100× PlanApo (NA = 1.4) objective and has yielded below 350 nm. It should be emphasized that this is the only performance specification in 2006 that a company has said it will guarantee on a confocal microscope. Normally in a functioning system, values between 280 nm and 350 nm with a 100× (NA = 1.4) lens were obtained. A 63× PlanApo (NA = 1.32) lens should meet the specification of 400 nm, although Leica does not currently guarantee this value on a TCS-SP system. If the laboratory does not have a 100× PlanApo (NA = 1.4) objective and it is not possible to borrow one for comparison purposes from another confocal facility, it is useful to have other lenses as a reference point. The axial *Z* resolution of three different lenses was the following: a 40× (Fluor, NA = 1.0) was 610 nm; a 63× water-immersion lens (PlanApo, NA = 1.2) was 390 nm; and a 63× oil-immersion (PlanApo, NA = 1.32) was 315 nm. 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. It is important that the lenses achieve good values or the resolution in the system will

be inadequate. It is also important that the pattern of the axial resolution be symmetrical with suitable diffraction regions (peaks and valleys) to the left of the major peak (*see* **Fig. 3B**). Normally, the axial registration does not change over time assuming the laser lines are stable. However if 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. The quality of the lens by this test will relate to the quality of the biological image and that is why it is called the "gold standard."

It is important to compare the user-determined test slide with that of the service technician's slide to ensure that both specimens are yielding the same value. It should be emphasized that not all lenses are created equal and some will yield better resolution than others, as clearly illustrated in **Fig. 3**. If possible, lenses should be chosen from the manufacturer for excellent quality. Currently, there is a grade of lenses defined as confocal grade by one manufacturer. These lenses should be acquired, as these lenses undergo higher QA procedures in the factory and they are guaranteed to show excellent axial resolution, spectral registration, and other excellent lens characteristics. Other manufacturers should let you evaluate the lenses that are purchased prior to acceptance of the CLSM system. It is very important to have the best quality objectives on a CLSM.

4.8. Axial Registration (Beads)

This method is slightly more subjective than the axial *z* mirror test, but it does yield similar values most of the time. For unknown reasons, the values might be better or worse than the mirror-derived values. The mirror test is more accurate and should be used if available.

4.9. Square Pixels

The changing of a galvanometer stage will require that the symmetry in *XY* directional field scanning be measured. Measurements of objects will be inaccurate if the *xy* scanning does not yield a perfect square. This value should stay constant, but it must be checked and adjusted periodically. The phase adjustment in bidirectional scanning can also be checked and adjusted by this test. A lack of adequate phase alignment will result in a decrease in resolution. In cases that require the highest-resolution image, unidirectional scanning should be done, knowing that the scanning time will double. The galvanometer stability can also be checked using the square pixel test

4.10. Spectral Registration (UV and Visible)

The 1-μm multiple wavelength fluorescent beads (Tetraspec, T7284, Molecular Probes; or Rainbow beads, Spherotech) were used to monitor the UV and visible colocalization. The registration of bead fluorescence images between

the 365-nm UV wavelengths and the 568-nm visible wavelengths in an aligned system was almost superimposable (*see* **Fig. 5A**), whereas in a misaligned system (*see* **Fig. 5B**), the difference between the peaks was 650 nm (acceptable difference is only 210 nm). The 568-nm line was chosen instead of the 488-nm line to minimize the crossover fluorescence between the visible and UV wavelengths. If the wavelengths are not aligned, then colocalization and fluorescence resonance energy transfer (FRET) studies cannot be effectively made on the machine. The image data must also be expressed as a maximum projection to eliminate the spectral mismatch. It appears for an unknown reason that if there is proper spectral registration between UV and visible wavelengths, then the UV field illumination might not be uniform, and vice versa. Both field illumination and spectral registration parameters must be checked. In addition, the confocal machines have separate collimator lenses that are used to align the UV light for different magnification lenses. It is very difficult to get all of the objectives to show proper spectral registration between UV and visible wavelengths.

The spectral registration of the 365-, 488-, 568-, and 647-nm lines can be made with either a small (0.5- or 1.0-µm multicolored bead) or a front-surface, single-reflective mirror (*see* **Fig. 7**). The spectral registration with the mirror on a Leica system is a superior test to the bead, as the laser light can be measured sequentially or simultaneously to eliminate any crosstalk between adjacent emission wavelengths. In addition, no bleaching occurs at high zoom magnifications with the mirror.

With new confocal systems that contain three visible lasers, the spectral registration test measures both the lens spectral registration and the laser spectral registration. It would be useful to measure a few different objectives to determine if the spectral registration of the lasers is correct or if a pattern of misalignment occurs. It is highly unlikely that different lenses will show the same spectral mismatch and, thus, the pattern observed should indicate if there are potential problems with either the lasers or with the objectives.

Molecular Probes produces a series of different-sized beads (Focal Check 1 µm, 6 µm, 15 µm) with different fluorescent rings and core bodies to assess colocalization from multiple lasers. These data should reveal if the laser lines are aligned correctly. The smaller bead should be more accurate but slightly harder to use.

4.11. Laser Power Stability

Power stability in a CLSM can be influenced by many factors, which include the lasers, PMTs, electronics, electronic component failure, fiberoptics transmission, fiberoptical polarizations incompatibility, AOTF thermal regulation, thermal heat dissipation, optical components, and galvanometers. The data obtained from this power stability test alert the investigator to possible errors that might exist in the acquisition of intensity measurements in biological and physiological

experiments ***(1,11)***. The reason for such high variations in CLSM systems is unknown. For an investigator, it is not initially important to know where the source of instability is being generated, but only that it exists. Once the problem is identified, trained microscope service personnel will be able to troubleshoot the system and hopefully remove the source of the power instability.

Laser stability measurements should be made on the CLSM for the investigator to have confidence that the CLSM is not introducing artifacts in experiments requiring intensity measurements or time-dependent physiological experiments. In our system, there was periodic noise in the laser power tested by the transmitted light detectors that exceeds the manufacturer's (Ominichrome) laser stability fluctuations specifications of less that 0.5% over a 2-h time period. The 488-nm and 568-nm lines have a periodic cycle that is directly opposite the 647-nm line (*see* **Fig. 9**). Argon lasers and helium–neon lasers (543 nm or 633 nm) are considerably quieter than the argon–krypton laser and are definitely preferable.

In a confocal microscope, there are different ways to measure power stability over time (hours). These include the following: (1) manufacturer-installed pin diodes; (2) laser meters on the microscope stage connected to a readout device; (3) fluorescent emission intensity from a plastic slide detected by a PMT; (4) transmission optical system detection. The pin diode test was not stable and should only be used as a subjective assessment of power. A laser power meter can be connected to a UV or visible (VIS) wavelength detector situated on the microscope stage and then be continuously used to monitor the power output with either a chart recorder or equivalent computer software (Coherent).

Simultaneous comparison of the measurements using a pin diode in the Leica SP CLSM and either a power meter on the stage or the transmission average intensity (not shown) demonstrated that the pin diode has unstable power readings, whereas the other two measurements (transmission optics detection and power meter detection) 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.

A UV or VIS detector situated on the microscope stage and connected to a suitable power meter can monitor the CLSM laser power. The power output intensity is continuously monitored with either a chart recorder or equivalent computer software. Manual measurements are deemed not accurate enough and are very time-consuming. If transmission optics is not available on the system, a 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 could bleach the sample, which will decrease the fluorescence intensity and increase the transmission intensity. Therefore, the 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 might result in laser instability. In addition, one must be aware of possible energy excitation of the fluorochrome in the slide.

In our experience, the most reliable method to measure the laser power stability consisted of using the transmission optics of the CLSM without a fluorescence slide in the optical path. If transmission optics is not present, a fluorescent-colored slide can be used, but extreme care must be made to reduce the laser power with an AOTF to reduce possible interaction of the laser beam on the sample. Sometimes the output fluorescence intensity might increase as a result of repetitive additive excitation or decrease resulting from bleaching.

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, which include the AOTF, laser polarization mismatch, heat dissipation, and power supplies. One of the most likely causes is fiber polarization, which might be mismatched with the polarization of the laser. 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 ***(15)***. Noise in a helium–neon laser might be found at frequencies of a few hundred kilohertz as a result of either radio-frequency energies used to pump the laser medium or of fluctuations in the medium itself ***(15)***. The DC power supply used 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) and not constant current mode ***(15)***. The 171B power supply had transformers and heating problems and has been replaced by the 176B model with rectifiers that regulate heat better. Typically, a Coherent Enterprise laser, Coherent 90-5 or Coherent 70-4, will have less than 1% peak-to-peak noise ***(15)*** and the power will not fluctuate over time. The air-cooled argon laser from Uniphase or Spectra Physics used in benchtop flow cytometers or confocal microscopes will also have less than 1% peak-to-peak noise according to the manufacturer's specifications. The argon–krypton (Melles Girot, Omnichrome 643) laser contains three simultaneous laser lines that yield power intensity fluctuations of less than 0.5% for 2 h (personal communication and website). If this is true, where do the fluctuations in power intensity in excess of 10% as shown in **Figs. 8** and **9** come from if the laser is not generating it? Is it the AOTF or fiber polarization? Lasers can be checked with power meters in front of the beam or by special electronic boards that connect to the power supply to determine laser stability. However, this testing might not be possible by the investigator, as the laser is in a sealed compartment and the investigator is not allowed into this compartment or the service contract will be canceled.

4.12. Laser Power Stability (UV)

The Coherent Enterprise laser delivers less than 1% peak-to-peak noise and is considered a very stable laser. The argon air-cooled lasers, HeNe lasers, and Spectrophysics argon–krypton laser are all rated at less than 1% peak-to-peak noise. However, even with the Coherent UV Enterprise laser or a HeNe laser (543 nm), periodic noise and large power fluctuations were observed. One source of power stability appears connected to the way the laser is cooled and how the laser heat that is generated is dissipated. This was illustrated with our Coherent Enterprise UV laser that 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 of the circulating cooling water in the laser, which, in turn, resulted in the improper regulation of the laser power (*see* **Fig. 9,** trace B). In addition, problems with proper fiber alignment also appeared to occur with the UV system, resulting in power fluctuations (*see* **Fig. 9,** trace C). The elimination of these temperature and polarization issues resulted in proper laser cooling and laser stability (**Fig. 9**, trace A; <3% noise). The water exchanger appears to have better thermal regulation than the noisier air–air (LP5) exchanges, but as illustrated in **Fig. 9**, the cooler must be set properly or thermal power instability might occur.

4.13. Laser Configurations (Fiberoptic Polarization)

This argon–krypton laser has been incorporated into the older-designed systems from which most of these measurements have been made. These argon–krypton lasers will deteriorate with time because of the gas escaping, resulting in a continuously reduced power output over time. The new systems from Leica, Zeiss, Nikon, Olympus, and Bio-Rad all have merge modules and individual lasers. 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. It is recommended to acquire a stable low-noise laser. 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 fiberoptics. This is in contrast to the older versions of confocal microscopes, which used direct coupling with only dichroics to deliver the light to the microscope's scan head. The use of the fiberoptics makes it critical to align the polarization in the fiberoptic with that of the laser's polarization value ***(1,15)***. Failure to do this could result in laser power instability in the CLSM system. The fiberoptics 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 fiberoptic and see if the image returns to the same intensity, suggesting that the polarization is correct. This is a fairly crude test, but it will demonstrate whether the system needs further alignment. Confocal microscope manufacturers are supposedly devising more reliable tests to check for polarization and ensure laser power stability within the CLSM.

4.14. Heat Dissipation

The dissipation of heat is a very important variable to consider in measuring laser stability. Improper heat dissipation with the air-cooled lasers will result in laser power fluctuations. In our case, we have observed fluctuations with an argon–krypton air-cooled laser that (1) had a restrictor in the exhaust line and (2) used a smaller exhaust duct (4 in. instead of 5 in.) to remove heat. In both cases, the heat was not dissipated correctly and the laser power in the CLSM fluctuated above 20%. Removal of these problems reduced the laser calculations to less than 5%. All lasers have to dissipate heat properly and their thermoregulators must be set correctly or the power to the lasers will fluctuate as illustrated using the Coherent Enterprise laser in **Fig. 9**.

4.15. AOTF

The AOTF could 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 and did not use the AOTF. However, the AOTF is enormously useful in operating a confocal microscope, as wavelength selection can be very easily and accurately controlled. It is also used to control the power that illuminates a sample, which effectively acts to reduce the crosstalk between detectors. The original AOTFs that were installed in older CLSM models were not temperature regulated and could have introduced some power instability into the confocal microscope systems. This problem is very disturbing to investigators expecting to make comparative intensity measurements on biological samples. The laser has 1% fluctuations, but there is over 10% observed on the stage. The AOTF is suspected of introducing these power fluctuations.

In summary, the laser power instability might be attributed to the power supply, AOTF thermal regulation, improper thermal heat dissipation, electronic component failure, or fiberoptical polarization incompatibility, but the definitive source of laser power fluctuations has not been identified at this time.

4.16. Standard Bead and Fluorescent Plastic Slide Tests

In flow cytometry, alignment beads, linearity beads, and chicken red blood cells (CRBCs) are used to ensure that the machine is functioning properly *(16,17)*. It would be useful if a suitable bead or fluorescence slide test sample were used for a similar assessment on the CLSM. Fluorescent-colored plastic slides can be used as a reference standard; however, the issue of the depth that the measurement is taken at and possible surface irregularities will both affect the measurements. If these two variables are controlled, the plastic slide can be effectively used. Most of the description will be on a bead, but the data are applicable to uniform plastic slides at a specific depth that are lying flat on a microscope stage.

4.17. Biological Test Slides

It is important to have a reliable sample that can be used to test the machine performance and image quality. The most useful histological test slide in our laboratory was a FluoCell slide (Molecular Probes; F-14780) stained with three fluorochromes. This slide allows for proper evaluation of resolution, crosstalk between detector channels, and observation of the emission from multiple excitation wavelengths (UV, 488 nm, and 568 nm). The resolution of biological structures such as mitochondria (Mitotracker red), nucleus (DAPI), and tubules (Alexa 488) can be assessed with the slide. The pollen or diatom slides (Carolina Biological) have also been 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 of the 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. Although a trained confocal person can evaluate many parameters on a confocal microscope, these observations on histological slides are subjective and cannot be confirmed, and the machine performance should not be totally based on this type of sample as a performance standard, as illustrated by the data described in this chapter.

A biological sample is a very subjective method of addressing total system performance. Similar to beads, biological test samples (FluoCells, F-14780, Molecular Probes, Acridine Orange-stained CRBCs) show a relationship between frame averaging and PMT voltage. The higher the voltage, the more averaging that is necessary to produce a desirable image. Because the relationship between PMT and CV (image quality) is logarithmic, very small changes in PMT values can indicate major changes in system performance *(1)*. In our opinion, the use of an acceptable range of hundreds of PMT units is far too large to effectively assess instrument performance.

4.18. General Confocal Principle

Generally, as the PMT is used at higher values, the sample needs to be averaged more to reduce the noise in the image. How much averaging is done is dependent on the amount of bleaching in the sample and the amount of time available to average. The more averaging, the better the image, but after a few averages, there is a limiting return, with factors of bleaching affecting image quality. We have found that averaging between three and six times dependent on PMT value yields good images. **Figure 12** shows the relationship between CV averaging and PMT value. In certain cases, the pinhole size might need to be increased to lower the PMT voltage and decrease the noise. This will sacrifice some confocality in the system.

4.19. CLSM Comparison

It is possible to compare similar and different systems using this CV test. This is done by first measuring a specific fixed power value on the stage, keeping all staining parameters constant and then measuring the noise of a single scan line. The amount of fluctuation that occurs in a line scan may indicate the instability of the system to produce a good image. It will be directly related to PMTool talk. The more line fluctuations that occur during scanning, the more averaging that will have to be used to create a desirable image.

4.20. Image Size

A sample should be adjusted using medium speed. A slower scan speed is equivalent to averaging more, as the laser spends a longer time on each pixel. Larger-sized images, (e.g., 1024×1024 or 2048×2048) give only slightly better detail than 512×512 but allow for extra zooming, which could be useful in visualization of structures. Compared to the 512 images the 1024 images are 4 times larger and the 2048 images are 16 times larger. They will take 4 and 16 times, respectively, longer to acquire, and it will need 4 and 16 times more disk space, respectively, to store the data. Most of our routine work is done at 512×512 resolution *(18)*.

4.21. PMT Factors

The PMT is the detection unit of a confocal microscope and its quality and function is extremely important to a confocal microscope. Not all PMTs are equal; therefore, the PMTs should be checked to determine if the unit in the confocal microscope has good sensitivity. The bead tests describe a procedure that can be used to measure PMT sensitivity. The PMT should have a large dynamic range, be linear, and show good sensitivity in the wavelengths measured *(7,19)*. Often, the confocal user will set the PMT at high voltages to observe an image and be unaware how this setting influences image quality.

Operating a system with high PMT values will generate poor image quality because of reduced signal and excessive noise. This noise will have to be eliminated by averaging to yield a good image. Frame averaging or high laser power will increase image quality, but bleaching usually occurs by repeated scans over the same sample or with high laser power. It would be useful to have the best PMT with the least noise and greatest dynamic range installed in the machine.

4.22. Spectral Scanning: PMT Comparisons

The PMT performance was also measured using the Leica spectral scan feature. This feature allows for a sequential scan supposedly across the whole range of 400–800 nm in units as small as 5 nm. Using the light form lamp, a spectral scan consisting of 50 increments of 5 nm each was made between 400 and 650 nm using a 10× (NA = 0.4) PlanApo lens or with an Airy disk of 1 and without averaging *(24)*. The efficiency of the system was low, necessitating a large PMT voltage setting. It is essential to use a reflecting mirror and not a dichroic to measure the spectral scans so that no attenuation of light will be introduced across the spectral range. In our system, PMT 2 has a direct path to the detector, whereas PMT 1 and PMT 3 need to be reflected off an additional mirror prior to entering the detector. PMT 2 showed sharper bands than PMT 1 and PMT 3. However, PMT had less response in both the lower blue regions and far-red regions. This might be the result part of the light path, quality of PMT, PMT alignment, or type of PMT. The PMT pattern with the tightest peaks and the largest valleys define the PMT with the best spectral response, which is located in the second position in our machine. PMT 2 should thus be used for spectral imaging experiments. This test has been adapted to the Zeiss 510 Meta system.

4.23. Interference Contrast and Confocal

Interference contrast is a very useful parameter in microscopy and it can be combined with fluorescence. However, because the microscope system was designed for light to traverse through two interference filters, when this optical system is applied to a confocal microscope there is distortion in the fluorescence signals. The fluorescent light traverses the interference contrast filter and excites the sample, and then the emitted fluorescence travels back down through the same interference contrast filter and back through the scan head. The resulting image shows a duplication of very small particles (0.17 µm, PSF beads) and a distortion of larger particles. PSF beads show two spots and 0.5 µm beads show an egg shaped image instead of a round image. The same distortion that is observed on beads will occur on biological structures in cells (*see* **Fig. 15**). For optimum resolution of data that will be deconvoluted later, it is recommended to remove the interference filters when acquiring an image.

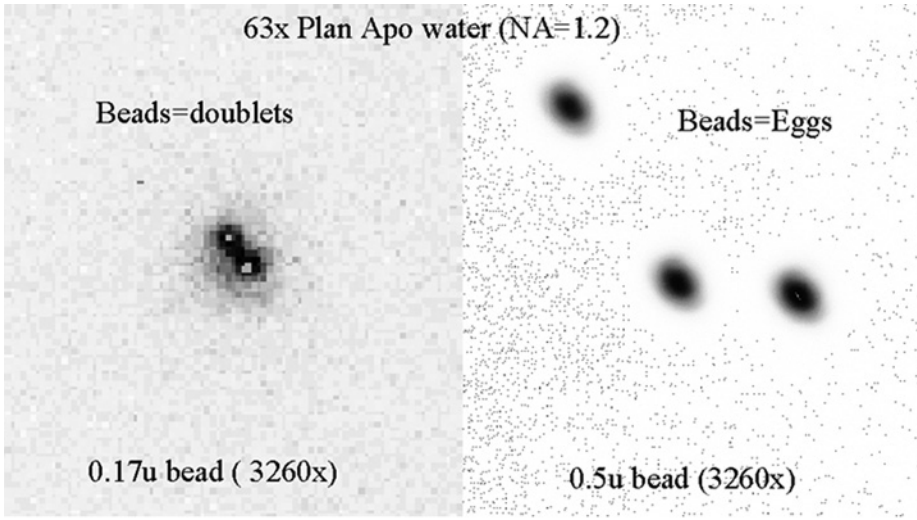


Fig. 15. Incompatibility of interference and CLSM. The Normarski interference contrast light path consists of an analyzer, a polarizer, and two interference filters. In acquiring an interference image simultaneously with a fluorescent image, the interference filters interfere with the fluorescent image, resulting in doublets using PSF beads (0.17 µm) and egg-shaped objects with 0.5-µm beads. All objectives show aberrations with the interference contrast transmission optics in place and it is assumed that the same aberrations will occur with biological structures.

4.24. Good Images: Take-Home Lessons

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 might be difficult to know how to balance these factors to produce an ideal image. Although all of these factors affect image quality, we have found that the zoom greatly bleaches samples and high PMT settings will introduce excessive noise that can only be eliminated through averaging, which, in turn, can also bleach the sample. The zoom factor increases the bleaching by the square of the zoom factor. If possible, magnification should be obtained with objectives, not with the zoom magnification *(13)*. 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. Although the pinhole diameter determines the confocality of the system and should ideally be set at the size of the first Airy disk, the pinhole diameter can be increased if insufficient fluorescent laser light is emitted from the sample, making visualization noisy and difficult. In a filter system, the detection bandpass barrier filter can also be replaced with a Schott long-pass filter to increase the amount of detected light,

or in a Leica spectrometer system (TCS-SP), the slit can be adjusted to wider values. However, the quality of the image can also be affected by the spectra of wavelengths that are recorded. A slow scan speed can be used, which increases the time the laser beam resides at each pixel. These acquisition factors should be regarded as starting points for using the CLSM and not as an absolute rule, as every sample might have unique qualities that must be considered prior to imaging.

The aim of acquiring a good image is to lower the CV of the image. The challenge for the investigator is to create this image using a standard pinhole (Airy disk = 1, eliminates light) 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). The use of lower PMT settings allows for less averaging because of the decrease in noise in the system (***1,3***) (**Figs. 11 and 12**). Antifade compounds are useful to reduce the bleaching, allowing for increased averaging with fixed samples. Another way to reduce bleaching is to reduce the size of the sample image field (e.g., from 512×512 to 128×128) acquired. 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 performance tests, the loss of minor details as a result of decreased resolution is not a significant issue, but for biological samples, structural details are very important and good images should be taken at pixel values of 512×512 or higher.

In summary, to visualize any fluorescent specimen, a sufficient amount of light has to be delivered onto the sample located on the stage. If the sample cannot be adequately visualized, then it will be necessary to increase the PMT voltage. As the PMT voltage is increased, the image noise also increases. At higher PMT settings, it is necessary to use more frame averaging in order to reduce the noise in the image. However this leads to longer exposure times and possible sample bleaching.

4.25. Laser Choices

The new configuration of CLSM uses the quiet helium–neon lasers (543 nm and 633 nm) that will yield lower power values at the microscope stage compared with the noisy 75-mW argon–krypton laser. These low-noise lasers should deliver better signal-to-noise performance, as they have less peak-to-peak noise than the argon–krypton laser (***15***). The advantages of these small air-cooled lasers are clear: They are more stable, less noisy, last longer, and create fewer problems for the field engineers. Lasers should be chosen dependent on power, dye excitation noise, and frequency of service. Currently, higher-power argon lasers (30, 50, or 100 mW) are being installed to allow one to use the following excitation lines: 458, 478, 488, 514, and 532 nm. The HeNe 543-nm laser is 1–1.2 mW and HeNe 633-nm laser is either 5 or 10 mW.

4.26. CV Principle

One aim of this research was to apply similar statistical procedures, used for many years in flow cytometry, as a standard to evaluate CLSM images and system performance *(16,17,20)*. One method to assess flow cytometry system performance is to use a population of uniform fluorescent beads and measure the fluorescence and light scatter of approx 10,000 beads. The measurement of 10,000 beads yields a distribution of fluorescent intensities and sizes, which correlates to the particle variation and system performance factors *(17,18)*. From this distribution, the CV can be measured. We have applied the principle of CV of a population of beads to evaluate confocal system noise, image quality, and system performance. Instead of using thousands of beads to produce a fluorescent histogram, this novel technique uses thousands of pixels from a single bead to generate a population distribution. From these pixel-intensity values, the population means and standard deviation, and thus the CV, can be determined. Given the impracticality of imaging tens of thousands of beads to get a distribution of fluorescence intensities or particle sizes with a CLSM, we have analyzed a large bead consisting of many pixels. The intensity deviation of these pixels represents the noise in the scanned image of the bead. If the beads are uniform in intensity and size, then they can represent a standard for the evaluation of image quality and the performance delivered by a specific manufacturer's system. Generally, it is assumed that the smaller CV represents a system that is properly aligned, stable, and yielding good resolution and system performance. The inherent sources of noise were analyzed by measuring the pixels contained within a large ROI in one large bead. By zooming the bead 4×, problems with the field illumination, focal excitation plane, and the presence of unequal illumination at the edges of the bead were eliminated.

Although the CV Spherotech bead noise test might be useful to evaluate the reproducibility of the system over weeks and months, its applicability in our laboratory was found to be limited as a daily/monthly test. The laser power and stability in the CLSM system were extremely variable for unknown reasons. Perhaps this can be attributed to the quality of the argon–krypton laser alignment instability, variable temperature dissipation, or the AOTF temperature instability.

4.27. CV Test

The CV that we are measuring in a confocal microscope is actually the variation of pixel intensity within the bead, as opposed to flow cytometry, where the variation of intensity is among a population of beads. The 10-µm bead appeared to be the correct size using an Airy disk of 1, as the image that was captured

contained relatively homogenous pixels throughout the bead area using two different microscope systems (Zeiss 510 and Leica TCS-SP1). The area inside the bead was of sufficient size to allow a uniform ROI to be defined within it. It was helpful to zoom the bead four times to increase the quantity of pixels contained within the ROI. Repeated sampling of the same bead resulted in minimal bleaching and the CV did not change significantly during subsequent scans. It is important to maintain the machine variables (pinhole size, PMT voltage, averaging, etc.) 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 approx 150 (out of 255) for each PMT setting.

The relationship between the PMT and calculated CV is the following: As the PMT voltage is raised, the distribution of the pixels in the bead increases, resulting in 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 evaluate the PMTs and, thus, determine the best PMT that should be used to acquire signal at the lowest voltage settings and possibly how much averaging will be necessary to achieve a desired image quality ***(1–3)***. By using the most efficient PMT, the image CV is decreased as lower PMT values are used. An increase in CV values might imply that there is a decrease in laser power after the stage measurement from misalignment, resulting in higher PMT values. This CV test on beads can detect a noisy laser. With the argon–krypton laser, fast scans actually yielded less noise than slower scans, which was interpreted as when the pixel dwell time was longer, the laser fluctuations were more detectable.

There are numerous factors that effect the CV measurement. These include the fluorescence of the bead at the emission and excitation wavelengths, 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 essentially 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 will deteriorate with time, it is important to measure the initial CV (bead) and then to periodically measure how the CV (image quality) changes over time as a reference point for possible replacement (*see* **Table 3**).

4.28. Sensitivity Test

The sensitivity of any fluorescence optical system depends on the intensity of the light source, the efficiency of the optical system, and the quality of the detection system ***(7,19,21)***. For confocal microscopes specifically, the sensitivity

comprises variables that include PMT noise, laser noise, alignment, and system efficiency. It would be extremely useful if there was a test that could assess sensitivity in this optical equipment. We believe that we have developed a fluorescent bead test that can be used to measure sensitivity over time, so that an assessment can be made on how the machine is performing over time. The test can also be used to compare the sensitivity of two machines from one manufacturer or compare the machines from different manufacturers with regard to sensitivity and performance. Extreme care must be made to ensure that all variables are equivalent when undertaking this exercise. We have shown that the CV bead test confirms principles of noise reduction by averaging sequential frames. The noise is reduced inversely as the square root of the number of frames averaged *(18,22)*.

The voltage setting of the PMT was the primary determinant of image quality and bead noise. An increase in PMT value was always accompanied by an increase in image noise and pixel CV distributions. This test can be used to assess the sensitivity of an individual machine, and if the acquisition variables are rigorously controlled, it could measure the sensitivity between different machines. For example, the best CV on a Leica TCS-SP1 with UV excitation that was obtainable using a 10-µm Spherotech bead was 19%, whereas the best CV obtainable for 488-nm excitation was 3.8%, and for 568-nm excitation, it was 2.5%. These percentages are invaluable to determine the sensitivity of our TCS-SP and compare its sensitivity to other CLSMs. The values will represent how well the system is aligned and how well each system is functioning regarding this sensitivity parameter. These values are dependent on the maximum power throughput in a system.

The CV bead test has been used to monitor sensitivity in a CLSM system. This test is important not only to detect defective lasers and system alignment problems but also to set some rational basis to determine how much averaging will be necessary to remove the noise from an image. 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 **(Subheading 3.)**. 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. Using this test, we found that 1 mW of power yielded a CV of 4% on a Leica TCS-SP and 1.3% on a Zeiss 510 system. With the 568-nm line, using 0.2 mW of power yielded 4% on a Leica TCS-SP system and 2% on a Zeiss 510 system. This test reveals, as expected, that the noisy argon–krypton laser yields a higher CV than the quieter three individual-laser systems.

The comparison of the CV from an argon–krypton laser system (Leica TCS-SP1) and air-cooled argon/HeNe system (Zeiss 510) at the same milliwatt

ranges were made and it was determined that the system with individual lasers was functioning better than the system with only a single Omnichrome argon–krypton laser emitting three wavelengths. The data can be attributed to the different style lasers. It should be emphasized that there are many variables that can affect these CV measurements and they should be used as approximate values, not absolute values. However, the approach appears to be very useful to compare like machines where there can be no argument that the test was done incorrectly. It should be emphasized that reducing the light to increase confocality will also decease the sensitivity of the system, as fewer photons hit the PMT and the PMT voltage is raised. It is important to do these tests at a uniform pinhole setting of one Airy disk.

The comparison of maximum power 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. Explanations for the difference in CV between the two excitation wavelengths include the following: (1) The bead might not be excited as well in an UV system as in a visible system; (2) the optical system might be more efficient and less attenuated with visible excitation compared to UV excitation; or (3) there is insufficient UV laser power necessitating the increase of the PMT value to observe the bead at channel 150 (pixel mean). This bead test appears to be a reproducible test with both wavelengths that can be used to compare the sensitivity in a CLSM machine and could also be used to compare different machines. However, in our experience, because of some unknown laser power fluctuations, daily tests on a confocal microscope do not appear to be as useful, simple, or efficient as they are on a flow cytometer.

4.29. AOTF and Spectrophotometer Sliders and Functional Wavelengths

The functioning of the AOTF and spectrophotometer sliders can be checked by the following two procedures. (1) Set the machine up in the reflection mode similar to that for an axial *Z*-resolution test. Positioning the 5-nm sliders over the individual laser lines indicates the approximate relative position of the laser line. By moving the 5-nm slider above or below the laser line, the laser light should eventually be attenuated. The laser light should be reflected from regions 2–3 nm below and above the desired laser line of choice. If light is reflected below or above the laser line being checked, it suggests that the sliders or AOTF might not be selecting the correct lines. The 488, 568, and 647 nm laser lines had 5 nm, 8 nm, and 13 nm of reflected bandpass, respectively. A lambda scan between 470 and 670 nm can be made to show the width of the reflected laser lines; (2) Fluorescent plastic can be placed on the stage and the sliders can be placed close to the excitation band to determine where the emission lines are and where the excitation lines are. This test will determine the acceptable detection wavelengths above and below

the excitation wavelength. It should be less than 10 nm below the excitation wavelength. It will be more difficult to test the functionality of the AOTF or proper functioning of excitation wavelengths in a filter containing the confocal system. However, by measuring the emission wavelengths of the laser line through different barrier filters from PMT 1, PMT 2, and PMT 3, a pattern of acceptable fluorescence can be determined.

4.30. Purchase Considerations

The purchase of a new confocal machine is a very difficult and complex decision *(23)*. 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. The pretty picture might be primarily determined by lens characteristics or the functionality of the demonstration unit. It is critical when comparing machines from different manufacturers that they be set up in a similar fashion. The laser power, objectives, scans speed, illumination and detection pinhole size, and other hardware components are factors that affect image quality. Other critical factors entering into the buying decision should include service and company support after the machine is delivered. QA, organizational, and service issues should not be overlooked in choosing which manufacturer's machine to buy, as the buyer will have a long-lasting relationship with the vendor and it should be based on trust and knowledge that the machine is operating at its top performance levels. The ability of a manufacturer to guarantee specifications on their machine and address the issues of QA would rate very high on the list of criteria for choosing which CLSM machine to buy. It does the investigator little good if he/she has the best designed machine that does not perform to those advertised high standards in his/her laboratory.

Microscopes connected to a confocal scanning apparatus can also be combined with digital cameras if a suitable port exists. The confocal microscope should contain excellent objectives and the microscope should be ordered with a 100-mW bulb light housing instead of a 50-mW housing so that the microscope can be used also for conventional fluorescence applications. Very high-quality lenses consisting of water, multi-immersion, and oil should be ordered. These should have the largest NA and greatest working distance possible. Units are sometimes ordered with lower-quality objectives because of budgetary constraints. This is one of the biggest mistakes in operating a confocal facility. The lenses are the engines that drive this technology and they should always be the best quality available.

4.31. Summary

We have described the following tests to measure system performance in a CLSM: 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 ***(1)***. The work of the field engineer also has to be checked carefully, as the field engineer might not be able to accomplish all of these tests during an installation or a preventative maintenance visit. Many sales and service representatives might have different levels of machine understanding, and without specifications provided by the manufacturers, the level of a correctly aligned and functional machine is open to question and debate. Unfortunately, even sales/service representatives from confocal companies can make mistakes in the judgment of what constitutes a correctly aligned machine; thus, it becomes necessary to use these tests to ensure that the machines are working correctly in the scientist's laboratories. Unfortunately, the CLSM might function at suboptimum conditions during its operation and problems are resolved only when the investigator cannot achieve the desired images or there is a hard failure of the system necessitating a service personnel visit. It is the responsibility of the core director in each lab to ensure that the machine is working at acceptable levels of performance.

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