Confocal Microscopy System Performance: Axial Resolution[1]

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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 may be working at sub-optimal performance levels, delivering sub optimum performance, and possibly misleading data. In order 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, PMT quality, overall machine stability, and system noise (1-3). It is anticipated by using this type of test data, performance standards for confocal microscopes will be obtained and the current subjectivity in evaluating CLSM performance will be eliminated. These tests will help serve as guidelines for other investigators to assess both the performance of their machines and the quality of data derived from their machines. Utilization of this proposed testing protocol will help eliminate the subjective nature of assessing the CLSM and may allow different machines to be compared. These tests are essential if one is to make intensity measurements. These tests have been used in a similar manner to evaluate the performance of a Zeiss 510, Zeiss 510 Meta, and a Leica AOBS confocal microscopy systems.

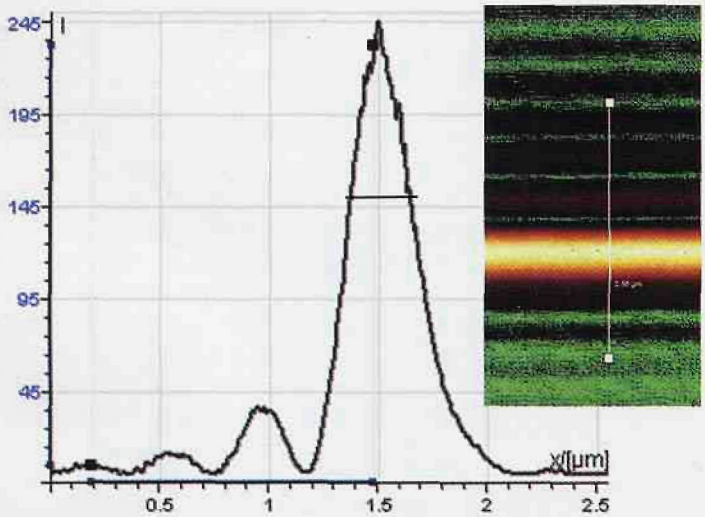


Figure 1: The axial resolution of a Plan Apo 63x (1.32NA, 330nm) showing a symmetrical major peak and a diffraction pattern consisting of smaller peaks to the left of the main peak. This pattern is suggestive of an excellent quality lens.

Confocal microscope

The majority of data presented in this manuscript was derived on either a Leica TCS-SP1 or a Leica TCS4D (Heidelberg, Germany) confocal microscope system. The Leica derived tests were shown to be applicable to other point scanning systems that contain different types of lasers, objectives or other hardware configurations.

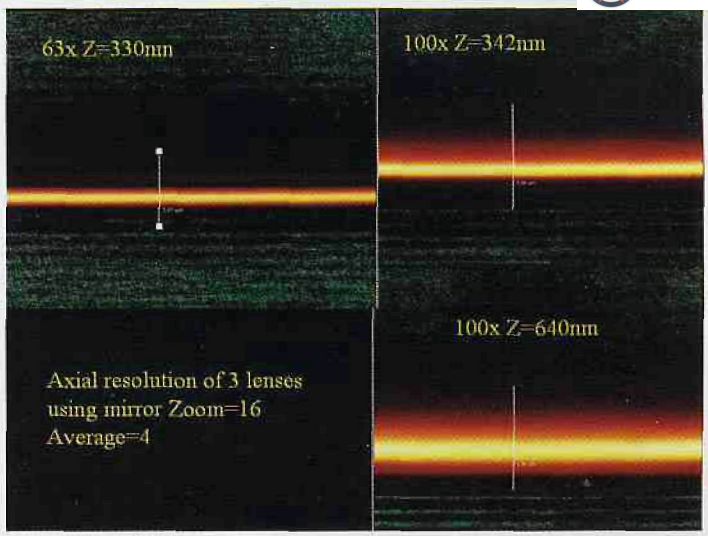


Figure 2: Axial resolution images of three lenses using the reflection mirror test.

For comparison purposes, similar tests were made on the Zeiss 510 system and the Leica AOBS system.

Axial (z) resolution test

The axial resolution of the CLSM is tested using a single reflecting mirror obtained from Leica or Edmonds Scientific. A 21mm 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 was 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, Illinois).

Axial (z) resolution (mirror).

The axial resolution test is considered the "gold standard" of resolution in confocal microscopy (1, 2, 4, and 5). Although it is not the only criteria 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 reflection mode with the 100x objective (1.4 NA Plan Apo lens), zoom of 16x-24x, a large pinhole diameter opening, and minimum laser power. After the reflected surface is found by scanning in xz mode, the pinhole aperture is reduced to a minimum size. The reflected image is then obtained with frame averaging of 2-4 times and the intensity profile across the reflected surface plotted, as shown in Figure 1. for a 63x Plan apo lens(NA=1.32). The maximum of the peak and the half-maximum intensity value of the profile is obtained to determine the full-width-half-maximum (FWHM) location, which is the measure of the axial resolution (330 nm in figure 1). The data can be observed graphically or it can be transferred into Microsoft EXCEL® to measure the peak and the FWHM values. The specification for axial registration in a Leica TCS-SP system using a 100x (1.4NA) is below 350 nm. The shape of the axial resolution curve is also important. One looks for a symmetrical large peak with smaller peaks to its left, which is indicative of the diffraction pattern of an acceptable lens.

Three different lenses are illustrated in figure 2. By drawing a line though the center of the reflected peak images (white line) the intensity distribution plots are obtained as shown in figure 3. It is

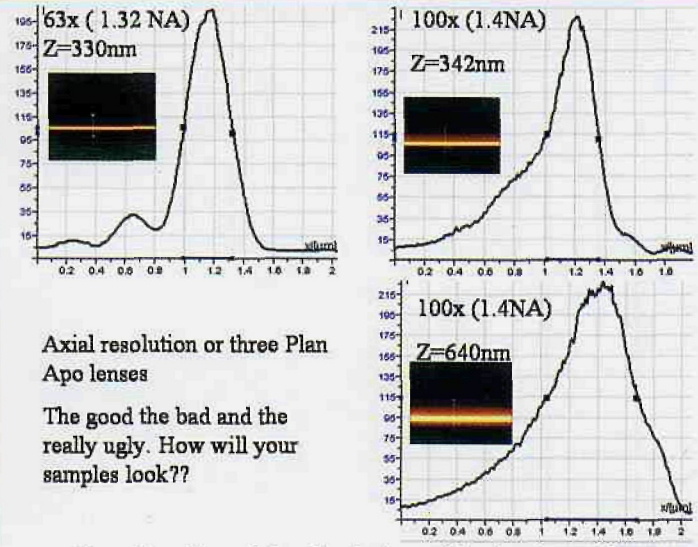


Figure 3: axial resolution distributions of the three lenses shown in figure 2.

clear that the 63x is the desired image and distribution. The axial resolution of this lens is excellent.

The axial *Z* resolution of 3 different lenses on an aligned Leica TCS-SP1 system was the following: a 40x (Fluor, NA 1.0) was 610 nm; a 63x water immersion lens (Plan Apo NA 1.2) was 390 nm; and a 63x oil immersion Plan Apo (1.32NA) was 315 nm. These are good values for high-resolution work on any confocal microscope. These axial resolution values will change as a function of lens quality and system alignment. They may also be used as a reference for other investigators to align their machines.

Axial (*z*) registration (beads)

One micron beads from Molecular probes (Tetraspec, T7284) or Spherotech (Rainbow, FP–0857-2) are located in the *xy* direction and then scanned in the *xz* direction. The power is adjusted for saturation and the image is zoomed approximately 8x and frame averaged 4 times. The size of the bead in the horizontal direction is compared to the size of the bead in the vertical direction. The difference between the two sizes 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. For unknown reasons the bead axial values can be better or worse than the mirror test.

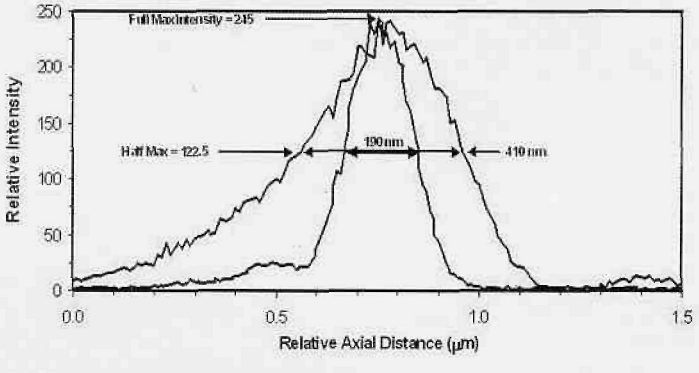
Bead slides were made by dropping 3-5 µl of diluted beads onto a slide, allowing the liquid to dry and then covering the spot with Per mount, glycerol, water, or oil and sealing it with a #1.5 cover glass. Antifade from Vector (Vectoshield H-1000) or from Molecular Probes (Slowfade light S-7461) is useful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

NOTES

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 insure the system is delivering its optimum performance. In the cases where intensity measurements are required, it is essential that the machine is 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). As stated previously, these tests include: laser power measured at the stage, field illumination, laser stability, dichroic performance, PMT performance, system linearity, axial resolution, spectral registration, sensitivity, and lens quality. This list is not inclusive but represents what can be tested and interpreted to insure the machine is operating properly.

Again, making the point that the axial resolution test is correctly considered to be the "gold standard" of resolution in confocal microscopy (1, 2, 4, 5), it should be emphasized that an axial resolution test made using a 100x Plan Apo (1.4NA) objective that yields 350nm is the only performance specification in 2003 that a company has said it will guarantee on their confocal microscope. Normally in a functioning system, values between 280 nm and 350 nm with a 100x (1.4NA lens) were obtained. A 63x Plan Apo (1.32NA) lens should meet the specification of 400 nm, although Leica does not currently guarantee this value on a TCS-SP system. If a laboratory does not have a 100x Plan Apo (1.4NA) objective, and it is not possible to borrow one for comparison purposes from another confocal facility, it is useful to have a reference point with other system lenses to eliminate the variable of the lens when measuring axial resolution. The axial *Z* resolution of 3 different lenses was the following: a 40x (Fluor, NA 1.0) was 610 nm; a 63-x water immersion lens (Plan Apo NA 1.2) was 390 nm; and a 63x oil immersion Plan Apo (1.32NA) was 315nm. The excellent resolution that was obtained with the 40x and 63x 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 (figure 1). 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 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 Figure 4. If possible, lenses should be chosen from the manufacturer that has 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 manufactures should let you evaluate the



100x Obj

Figure 4 : An axial resolution comparison was made using two 100x lenses (NA 1.4) on the same Leica TCS-SP1 confocal system. The peak intensity of the histogram is 245 yielding a half-maximum intensity at 122.5. One lens gave an excellent Full Width Half Maximum (FWHM) of 190 nm while the other lens yielded a bad value of 410 nm. The system was aligned properly in both cases. The value of offset while taking the image can affect the axial resolution, this may have resulted in a 10-30 nm error.

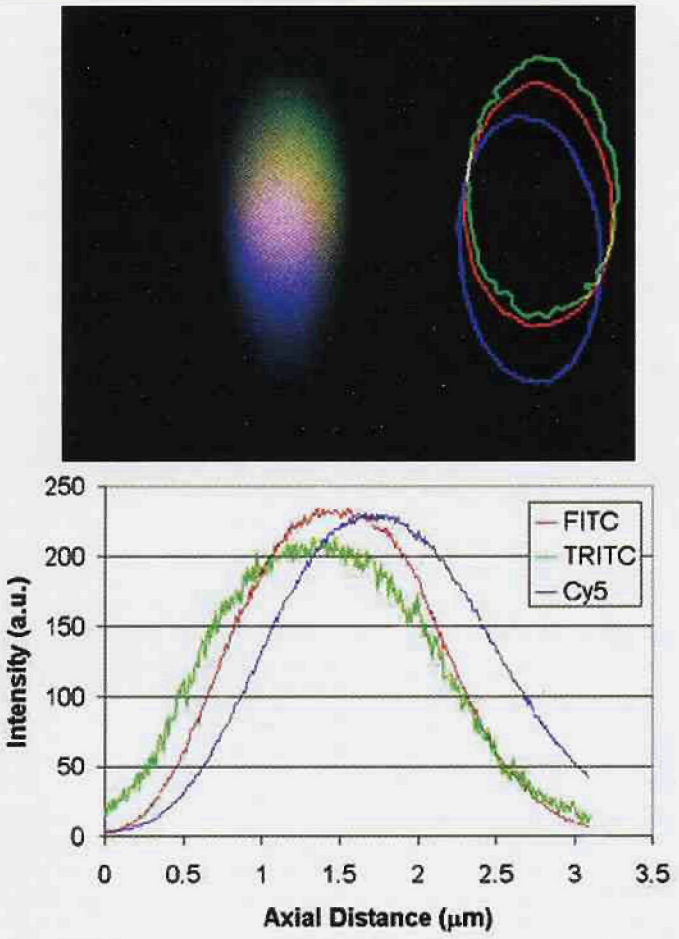


Figure 5. Axial resolution of a 1 µm bead obtained at 3 different wavelengths. A vertical line though the image will yield the axial resolution. The outline of the bead was made using Image Pro Plus software (Media Cybernetics). The excitation was 488, 568 and 647 which are refereed to as FITC, TRITC and Cy 5 in the figure caption.

lenses that are purchased prior to acceptance of the CLSM system. It is very important to have the best quality objectives on a CLSM or some experiments may not be able to be achieved.

Axial reolution (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 may be better or worse than the mirror derived values. Figure 5. shows an image of a bead taken using three wavelengths of light. The *xz* image was converted into an outline of the bead using Image Pro Plus. The distribution of intensities can be made in the *xz* (long axis) direction and this value can be compared to the *xy* (short axis) value to determine axial resolution. Naturally, the more circular the bead image is, the better the axial resolution. This test is also used to compare spectral registration of the system that will be described in a subsequent communication. In our opinion, the mirror test is more accurate and should be used where available.

Summary

We have described the critical axial resolution test using a mirror and a bead. We believe that it is the responsibility of the core director in each lab to insure that these instruments are working at acceptable levels of performance. Many sales and service representatives may have different levels of instrumental understanding and, without specifications provided by the manufacturers, the level of a correctly aligned and functional instrument is open to question and debate. Unfortunately, even sales/service representatives from confocal companies can make mistakes in judgment of what constitutes a correctly aligned machine, thus it becomes necessary to use these tests to insure the machines are working correctly in the scientist's laboratories. These tests are critical if one is to have optimum performance of their instruments. ■

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

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