Confocal Microscopy System Performance: Field Illumination

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The confocal laser-scanning microscope (CLSM) has enormous potential in many biological fields. The reliability of the CLSM to obtain specific measurements and quantify fluorescence data is dependent on using a correctly aligned machine that contains a stable laser power. For many applications it is useful to know the CLSM system's performance prior to acquiring data images so the necessary resolution, sensitivity and precision can be obtained. Applications involving deconvolution, FRET and quantification necessitate that the confocal microscope is correctly configured and operating at the highest performance levels.

The most common method in many laboratories to measure system performance involves the use of a histological slide to create a "pretty picture". Although this test evaluates many parameters in a crude manner (laser power, field illumination and lateral resolution) that can influence a CLSM image, the interpretation of this histological image is subjective and the range of acceptability is variable. In fact, many confocal microscopes can indeed obtain "pretty pictures" even when they are functioning sub-optimally. Furthermore, it is impossible to compare similar machines for proper functionality when the reference standard is only an image. The methodology described in this study can be used to help provide quality assurance (QA) on a CLSM. Without the use of these various performance tests, it cannot be determined whether CLSM machines are working at appropriate performance levels.

The following test methods have been devised on the Leica TCS-SP confocal microscope system: field illumination, lens functionality and lens clarity, lens spectral registration, total laser power, laser stability, dichroic reflectance, spectral registration of the laser beams, axial resolution, spectral response, scanning stability, overall machine stability, and system noise (1-4). 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. These tests have been used in a similar manner to evaluate the performance of a Zeiss 510 confocal system. These tests will be described in Microscopy Today in a series of short articles over the next few issues. For clarity only one test will be described in each article. However, it is essential that more than one test be used to evaluate confocal performance.

Field illumination is one of the easiest and most important tests to make on a confocal microscope. Many CLSM units in laboratories settings have demonstrated unacceptable field illumination patterns using a plastic fluorescent slide (Applied Precision, MME) illumination. This test should be made with all objectives and all wavelengths of visible and UV light to insure the machine is delivering proper field illumination under all excitation conditions. Field illumination should be relatively uniform with the maximum intensity being in the center of the objective and decreasing less than 25% in all directions across the field, with most objectives according to one manufacturer. The range of intensity decrease is partially dependent on the characteristics of the objective and its magnification. Most alignment procedures by service technicians are made using one high magnification objective *i.e.* a 40x. However, this does not always translate into good performance with lower magnification objectives or even with other higher power objectives. All objectives at all wavelengths must be tested for proper field illumination.

Materials: Fluorescent Slides

The illumination intensity across the observation field can be measured with different types of test specimens in order to insure that a homogeneous field illumination exists. The following test substrates have been used: histological samples, concentrated fluorescent dye suspended in a hanging drop well slide, small concentrated fluorescent beads (1-3 micron), large concentrated fluorescent beads (10 micron, Spherotech Libertyville IL, FPS-10057-100), fluorescent specimens, uranyl glass slides, or plastic fluorescent slides (Applied Precision or MME), a piece of tissue paper stained with fluorescent dye or fluorescent dye solution consisting of {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 plant sample appears to be the choice of service field engineers (1,3,4). In our experience, beads, biological/histological samples, or fluorescent liquid in a well slide were not as reliable or sensitive as the fluorescent plastic slides to measure field illumination. It usually will yield a sense of false security of proper field illumination for the investigator.

The field illumination test slides used in this study consists of three fluorescent plastic slides (Delta, Applied Precision Inc, Issaquah, Washington), which have an excitation peak wavelengths of 408nm (blue), 488nm (orange), and 590nm (red), and emission peak wavelengths of 440nm, 519nm, and 650 nm respectively. The blue slides (408nm) were used for UV field illumination and alignment while the orange slides were used for 488nm and 568nm field illumination and alignment. The red slide was found to bleach rapidly with 568nm excitation and therefore, it is preferable to use the orange slide for this wavelength also. MME (Microscopy/Microscopy Education, MME, Microscopy/Microscopy Education, Springfield, MA) offers a similar kit with four slides.

Method:

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 may change from the surface to the interior of the slide. The depth of focus was adjusted between 40-100 microns, dependent on the objective that was used. {(5x (100 μ); 10x (75 μ); 20x (50 μ); 40x (40 μ); 63x (30 μ); 100x (30 μ). Investigators should also be careful not to observe an illumination fields deep within the plastic slide samples, as it will usually yield a better field illumination than regions closer to the surface due to various optical distortion factors.

Data derived from a 20x Plan Apo lens (0.7 NA) zoomed to a factor of 1.2 is used to illustrate good visible field illumination (488 nm) and a misaligned UV (365nm) system yielding bad field illumination (Figure 1). The images were obtained with either UV plastic slide (ex 365, em 440-480) or visible plastic slide (ex 488, em 505-550nm) located securely on the stage. The original images were contoured into 10 intensity ranges using Image Pro Plus software. The line running diagonally in Figures 1A, 1B measures

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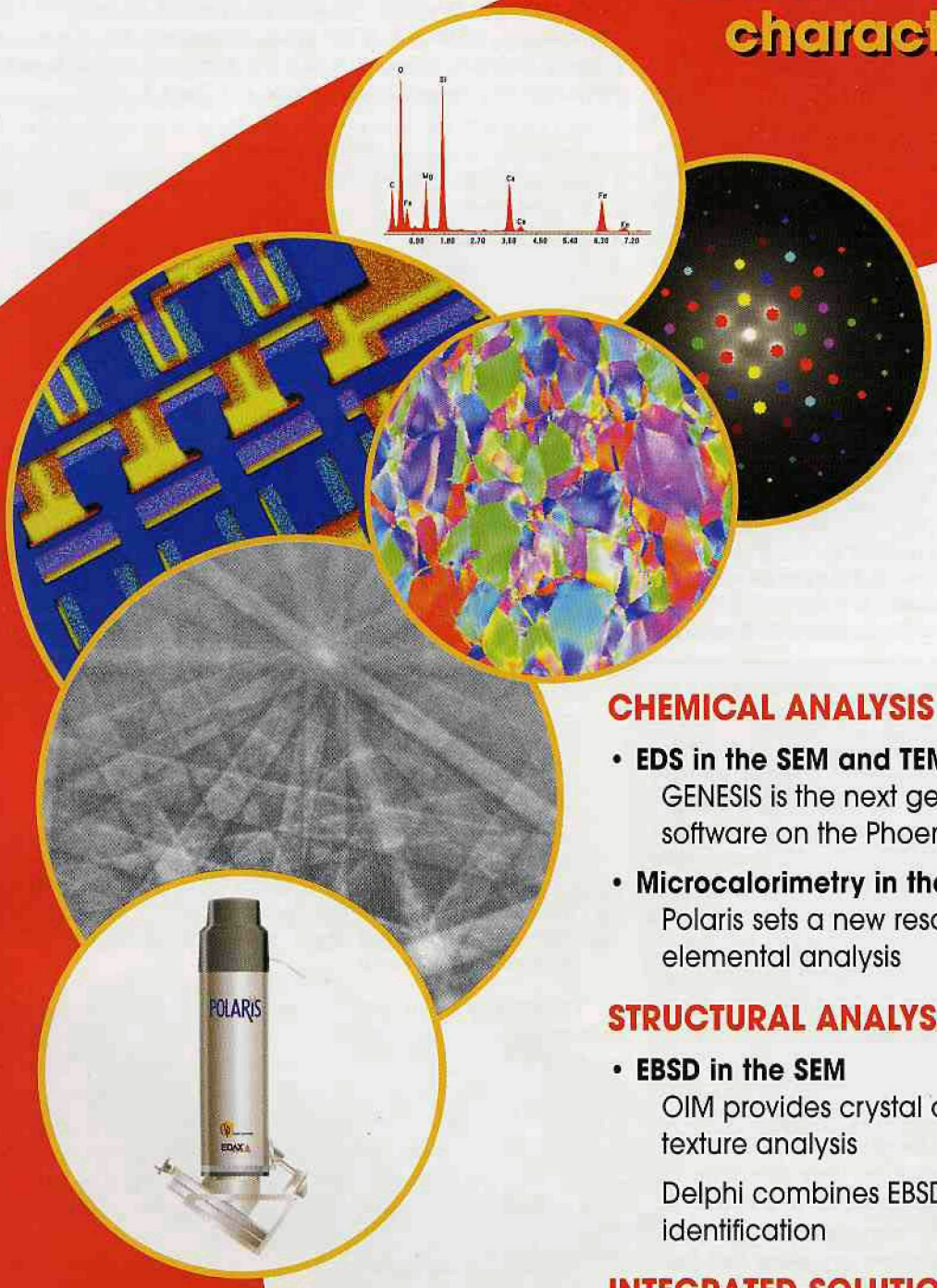

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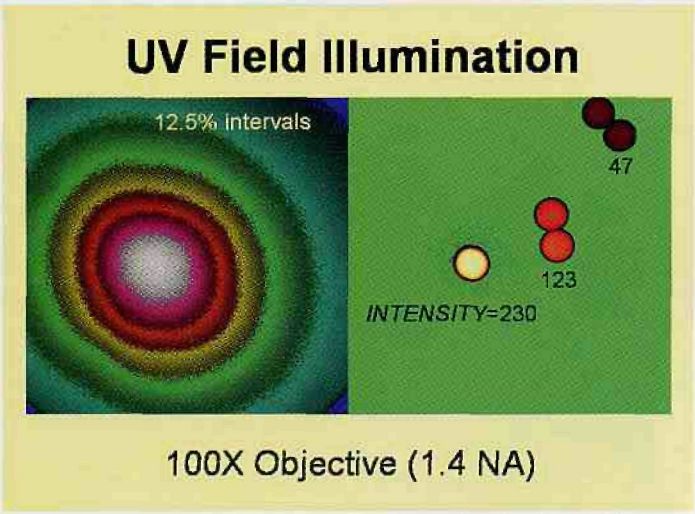

*Figure. 1. **Field illumination**. Field illumination pattern of visible (A) and UV (B) excitation using a 20x (Plan Apo, NA 0.7) lens. The visible field illumination shows uniform illumination with the brightest intensity being in the center of the objective. The line running diagonally in panels A and B measures the histogram intensity of the field illumination graphically represented in Figures C and D. The variation in intensity from the left to right side of the field is less than 10% for visible excitation and over 150% for UV excitation. Acceptable field illumination has 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 GSV into 10 equal regions and a median filter was used for additional processing.*

the histogram intensity of the field that is represented in the graphs in Figures 1C, 1D.

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 488nm excitation in Figure 1A. It should not be in the bottom corner as illustrated with UV illumination in Figure 1B. As shown in Figure 1, the visible light (Figure 1C) had less than a 10 % decease in intensity across the field while the UV light (Figure 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 non-uniform pattern shown in figure 1 with UV illumination clearly illustrates a field illumination problem, which will affect intensity measurements in an image. Although Figure 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 insure they are aligned properly as they use different dichroics to insure the beams are co localized. 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 us to assume it is equivalent to testing field illumination with the other wavelengths. Since the UV line is derived from a different laser (Enterprise, Coherent) it is essential to check all objectives for proper field illumination (Figures 1) at the 365 nm excitation in addition to the 488 nm excitation. Newer designed confocal systems (Leica SP2, Zeiss 510, Nikon C1, Olympus FluoView FV500 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 may be perfectly aligned yielding acceptable field illumination, while the other laser lines may be misaligned yielding intensity values in which the brightest region is not in the center of the field as illustrated in Figure 1.

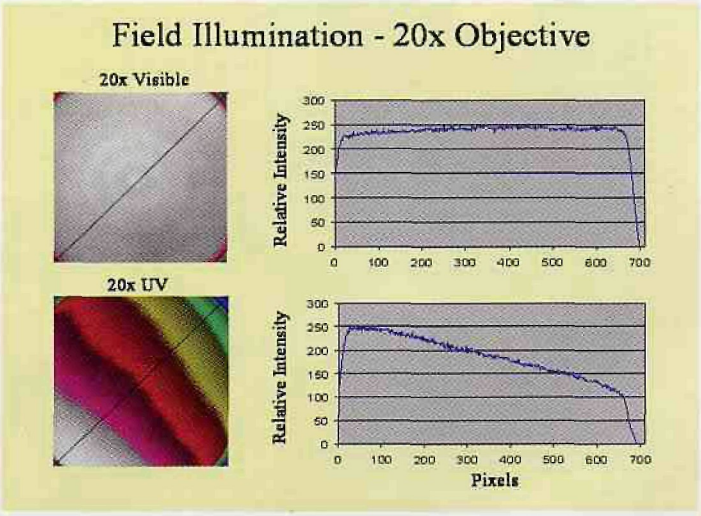

Figure 2. UV field illumination of a Plan Apo 100x lens (1.4NA) derived with a fluorescent plastic slide and the intensity measurement of 10-micron Spherotech beads. This illustrates the problem of using a lens with improper field illumination to make comparative measurements on a sample. The field illumination pattern shows a bull's eye intensity pattern slightly off center and the 5 beads located in different parts of the field to illustrate the variation in intensity occurring by using a lens that has improper field illumination. The intensity of beads was derived by a small ROI inside the bead. The 5 beads show a decrease in intensity of approximately a 100% (GSV=123) and 400% (GSV=47) relative to the bead in the center of the illumination (GSV=230)

Objectives have unique characteristics and should be chosen for the specific applications accordingly. For instance, the Leica 100x (figure 2, NA 1.4) is not recommended for UV applications as it has a bull's eye pattern. Due to the design of the 100x objective, it is recommended to use a zoom of 2x when using UV light in order to achieve a drop off of less than 25% across the field. Leica recommends that the Plan Apo 63x (1.32 NA) be used for UV work as it has more uniform UV field illumination and better light transmission.

Other objectives made for conventional microscopy may have a bulls eye pattern making these lens 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

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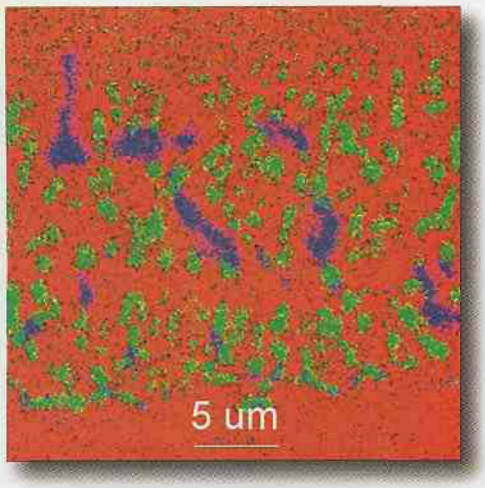
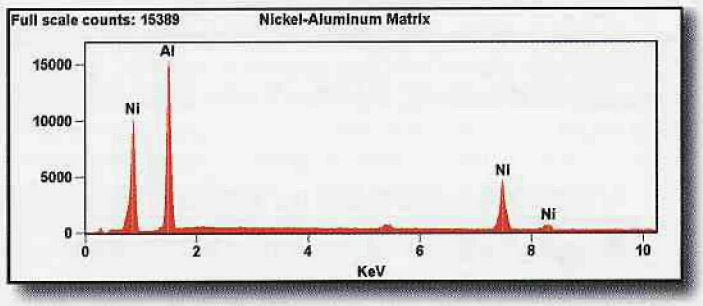
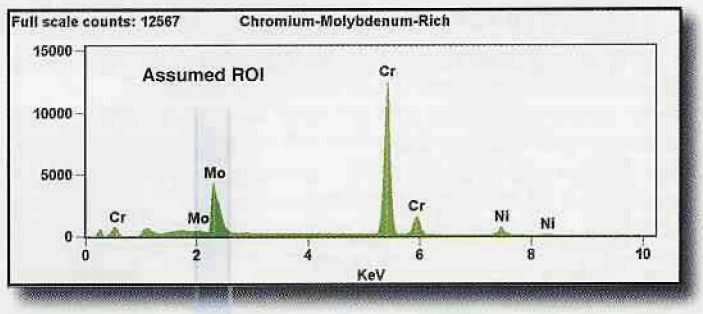


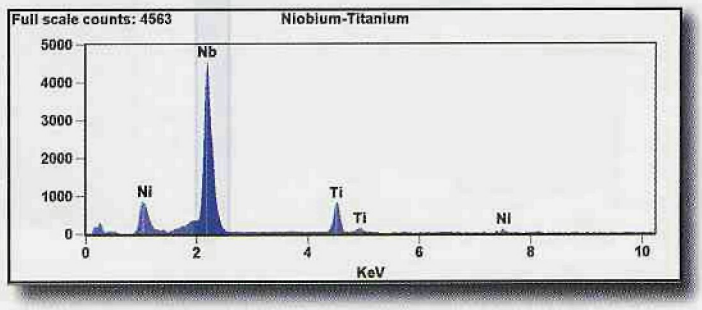
RGB overlay of COMPASS components (256 x 256 pixels) over a SEM image and corresponding spectra with coordinated RGB colors.

The red portions of the COMPASS overlay image show the sample's Nickel-Alumimum Matrix, while the green regions indicate a Chromium-Molybdenum Rich component, and the blue regions indicate a Niobium Rich component.

*With traditional x-ray mapping using ROIs, the Molybdenum and Niobium peaks cannot be separated, leading to an inaccurate analysis of the sample. COMPASS is able to deconvolute these true components and provide the **correct** answer.*







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(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 may be due to the lasers under filling the objective, which results in operating a lens under sub optimum conditions resulting in the field illumination problems. One recommended solution to bad 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 may defeat the purpose of using a low magnification objective to observe a large field of view or may 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.

Not all problems with the field illumination test are the result of bad alignment, lens design/quality or incompatibility of a lens with specific wavelengths of light A dirty lens will yield bad field illumination and bad resolution also If a lens is dirty or covered with dried oil, it would yield a non-uniform pattern (6). In one example, the intensity of the field from a 20x (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 (6). ■

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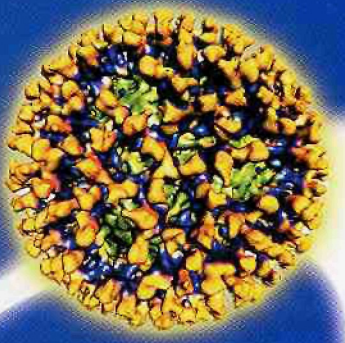

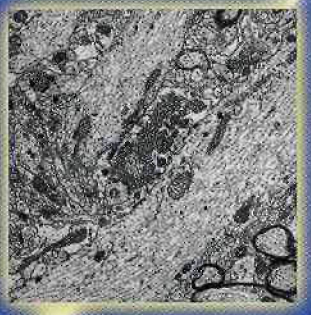

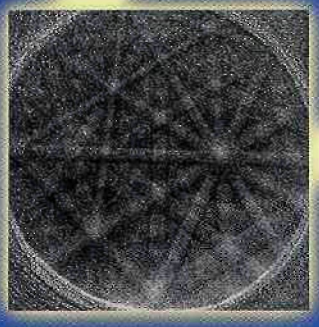

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