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Confocal Microscopy System Performance: Foundations for Quantifying Cytometric Applications with Spectroscopic Instruments.

Robert M. Zucker [1] and **Jeremy M. Lerner** [2],
[1] Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711; (919) 541-1585; zucker.robert@.epa.gov
[2] LightForm, Inc., 601 Route 206, Ste 26-479, Hillsborough New Jersey 08844; Tel: (908) 281-9098; jlerner@lightforminc.com

The confocal laser-scanning microscopy (CLSM) has enormous potential in many biological fields. The goal of a CLSM is to acquire and quantify fluorescence and in some instruments acquire spectral characterization of the emitted signal. The accuracy of these measurements demands that the system be in alignment with stable laser power and spectral registration. For many applications it is useful to confirm the system's spatial resolution, sensitivity and precision prior to acquiring image data. The characterization of FRET, "unmixing" co-localized mixed fluorescence spectra and the use of deconvolution necessitates that the system be correctly configured and be operating optimally.

The most common method to check the performance of a CLSM system is to characterize a histological slide to create a "pretty picture". We have developed tests to replace this subjective method with objective measurements of field illumination, lens function and clarity, spectral registration, total laser power, laser stability, dichroic reflectance, axial resolution, scanning stability, overall machine stability, system noise, and spectral tests (1-3). We have continued this system approach and have perfected some of our previous tests which hopefully will serve as guidelines for investigators to assess both the performance of their instruments as well as the quality of their data.

In flow cytometry it is generally assumed that small 2-3 micron beads can be used as alignment standard and multi intensity beads can be used to measure machine sensitivity and linearity. These tests use CV statistical endpoints to measure accuracy. The QA in Confocal microscopy is more complicated, as many different type of tests are needed to insure that the machine is working correctly at all levels of performance. It is essential that these tests be made prior to acquiring samples for cytometric measurements so the investigator can trust the accuracy of their experimental data. Similar to flow cytometry equipment measurements, we have tried to devise tests that could evaluate the CLSM equipment quantitatively instead of subjectively.

We developed a new spectral characterization test that is well suited to all wavelength dispersive CLSM systems including the Leica SP series (SP), the Zeiss LSM510 Meta (Meta), Olympus FV1000 and Nikon C1 confocal microscopes. We used an inexpensive, eye-safe, battery operated, multi-ion discharge lamp (MIDL) (LightForm, Inc., Hillsborough NJ) containing mercury ions and inorganic fluorophores as an absolute reference light source because it emits stable, reproducible, peaks between 400 and 650 nm.

The lamp is positioned on the microscope stage above (or below) the objective lens. The characteristics of an acquired spectrum enable us to measure wavelength accuracy, spectral sensitivity, contrast, wavelength ratios and spectral resolution. Significantly, it can also be used to contrast and compare the performance of one instrument against another in a different location.

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The spectral features of the MIDL lamp are shown using by an Olympus FV1000 "lambda scan" (figure 1). The MIDL characterization was accurate and showed significant detail in the 611 nm spectral feature. Figure 2 shows the same spectrum presented by a Zeiss LSM 510 Meta system. The wavelength accuracy is much broader due to the spectral sampling increment of ~10.7 nm.

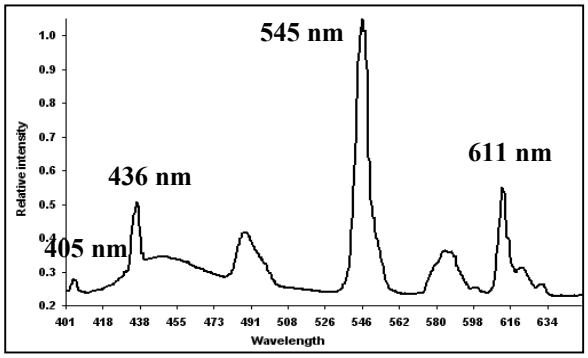

Figure 1: Olympus FV1000 characterization of a MIDL light source. The system is shown to accurately and optimally identify the emitted spectral features

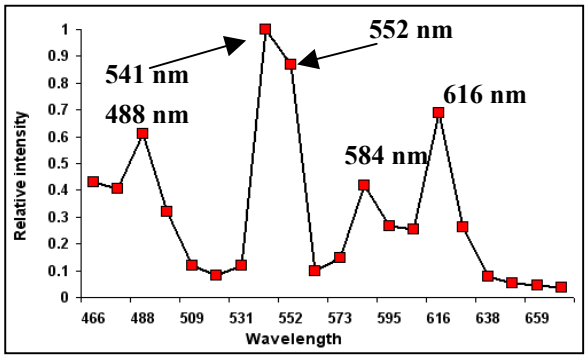

Figure 2: Zeiss LSM 510 Meta. The MIDL 545 nm line has been bisected with two data points. The wavelength accuracy is within the specified 10.7 nm per wavelength data point. The background is elevated below 520 nm.

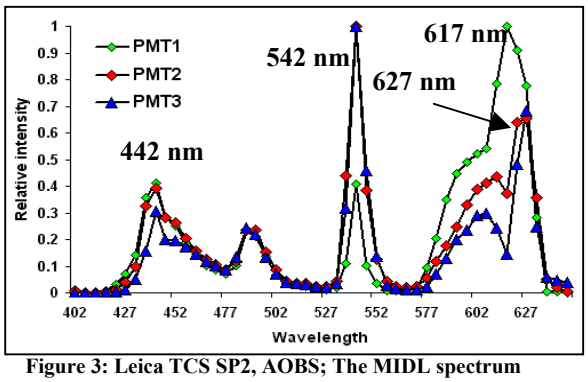

Figure 3: Leica TCS SP2, AOBS; The MIDL spectrum clearly reveals problems with the spectral profiles when compared to the spectrum in Figure 1. Note that the 545 nm line has a single wavelength data point at its peak.

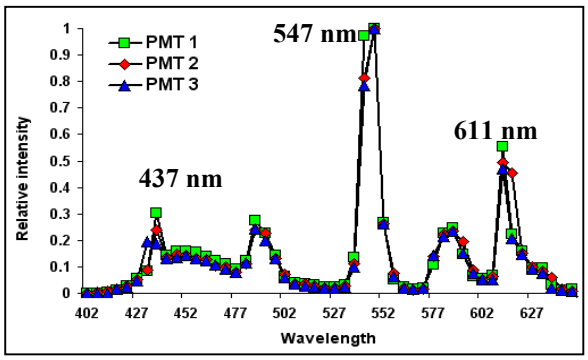

Figure 4: The same system shown in Figure 3 following adjustment by a Leica technician. Note that the 545 line is now bisected with two wavelength data points at its peak, but the wavelength accuracy is good

We also evaluated a Leica TCS SP2, AOBS system before (figure 3) and after (figure 4) service by a Leica technician. The only problem reported by the laboratory was that it suppressed light above 650 nm, but was thought to be in good working condition from 400-650 nm. Figure 3 shows that before adjustment each of the three PMT assemblies delivered inaccurate and mutually different MIDL spectra over the 400 to 650 nm range. Following adjustment the wavelength range increased to 750 nm and the output of all three PMTs was consistent (Figure 4). However, when compared with Figure 1, the spectral resolution and detail in the 611 nm feature was lacking in the Leica SP2 system. In summary, this MIDL lamp serves as an absolute reference light source, for any researcher to test and validate the performance of their instrument. It also serves as a stable universal reference spectrum to compare instrumental performance with colleagues in different laboratories.

References

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