Digital Image Tips: Adjusting Brightness And Contrast In Micrographs Using Adobe PhotoshopÆ

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This adjustment does gain enhanced contrast on the surface region, but the interior of the void became more washed out. Both approaches (using only a Levels Adjustment Layer or a Levels Adjustment Layer along with a Curves Adjustment Layer) require a trial and error approach to find the settings that most suitably portray the data of interest. This is where the tremendous power of Adjustments layers becomes so valuable, because they allow the user to freely apply and edit numerous different adjustments without permanently changing the raw data of the image.

In summary, when faced with a difficult charging problem, meaningful and useful information can still be obtained from a digital micrograph by using Adjustment Layers in Photoshop to edit the black point, white point, and transfer function of the histogram for the image. These adjustments are reversible, and do not alter the raw data of the image. Additionally, this approach can achieve dramatic improvements to the brightness and contrast of an image with only modest effort, and can be used to emphasize valuable information that might be otherwise obscured. ■

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Using the PMT in a Laser Scanning Confocal Microscope as a Digital Light Meter to Measure Detection Photon Efficiency

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If we accept that the optical layouts of single-beam laser scanning confocals are generally quite similar, the most important variable is "Photon Efficiency" (PE): the fraction of photons leaving the focal plane that actually contribute to the number stored in the image memory. Unfortunately, this parameter is a function of a very large number of operator settings, design decisions and optical performance specifications (BioTechniques The 39 Steps: A Cautionary Tale about "quantitative" 3D fluorescence microscopy, April 2000 28:884-7). As a result, although all manufactures claim excellent "Photon Efficiency" none actually provide any specifications. We have to determine the PE ourselves.

Let us first agree that, because the loss of excitation photons can always be made up by using more laser light, Detection Photon Efficiency is what we are really interested in. Now the problem becomes, how can we create a "specimen" that has a known and repeatable brightness.

I suggest that the best plan is to use the transmitted-light illumination source as "the specimen." All light microscopes have one, and it is usually adjustable in intensity. With the laser off, any confocal microscope becomes a flying spot detector that only collects photons from one pixel in the image plane at a time. Each number recorded in the confocal image memory should be proportional to the number of photons passing through a particular pixel in the focused plane during the few microseconds that

the scanning mirrors convey the image of this pixel to the pinhole.

The data recorded represent whatever image is transmitted from the focused plane. If no specimen is present, one will record a white field, as defined by the field diaphragm. Because only light passing through the pinhole reaches the detector, the effective light flux reaching the PMT (in photon/second) is about 250,000 times less than if one measures the whole widefield illumination beam at the specimen plane.

As the quantum efficiency of the PMT varies slightly with multiplier voltage, the PMT should be operated at approximately the same voltage that is normally used for viewing ones typical confocal specimens. This may require adjustment of the transmitted light source intensity. Line-frequency variations in the filament heating current may be visible as small intensity variations about 16 ms in duration. Likewise, any instability in the power delivered to the plasma of arc sources will produce "structure" in the recorded data. The best plan therefore may be to measure average signal level from the recorded image data.

As the light flux passing thorough the specimen is much larger than that measured by the scanned pinhole, it can easily be measured by using a simple light meter (use a carefully-placed black cloth to make sure that the meter isn't mostly measuring room light!). As long as the NA of the condenser is less than that of the objective (ensure that this condition is satisfied by closing the condenser aperture diaphragm down until just the edge of it is seen when viewing the objective back-focal plane using a Bertrand lens or a phase telescope), one can then use the meter reading to normalize the results between different confocals (if one is trying to make a purchase choice) or to detect changes in the performance of a specific microscope over time.

Set up the normal transmitted-light source system of the microscope for Köhler illumination (edge of field diaphragm, centered and in focus in the image plane) and adjust the field diaphragm for a known diameter in the image plane. This can be measured with a stage-micrometer specimen or determined later, as the diameter of the bright circle recorded in the confocal image memory with the lasers off. The arc or incandescent transmitted light source must be provided with a regulated power supply, and it is a good plan to add a bandpass filter below the condenser to normalize for the differences in spectral distribution that occur between different light sources or different power level settings of the same source. To test "normal operation" try an "interference green" filter, as used for DIC, and a Cy-5 excitation filter if red light performance is of interest.

Once the microscope has been set up, the only major variables affecting the number stored in the image memory (apart from the system "detection photon efficiency" that you are trying to measure!) are the illumination power setting, the amount of metal deposited on the inside of the glass tube surrounding the source, the bandpass effects of any other filters or beam splitters remaining in the light path (perhaps in the confocal scan head), the pinhole diameter and the PMT voltage. In many instruments, it is relatively easy to remove all of the dichroic and bandpass filters in the confocal head and let the light from the image plane pass directly to the PMT. With the Zeiss META, the signal from all channels will have to be summed; with the Leica SP series, the slit will have to be opened entirely and one has to rely on the interference green filter to determine the bandwidth.

Under these conditions, one should always get a standard reading with a given objective, a fixed PMT voltage, a standard pinhole size (say, one Airy disk diameter) and a fixed lamp power. Write these settings down in the Log Book!

By closing the pinhole, reducing the power to a tungsten source or introducing ND filters after an arc source, it should be possible to reduce the intensity of the transmitted light to the level at which photon-counting is appropriate (about 15 counts/pixel, maximum). On those instruments that have this ability (basically Bio-Rad instruments), one can easily measure a rough ratio between the actual number of photons being detected (using photon- counting) and the intensity-values stored in the image memory at the same PMT settings but using analog detection. This number will be found to be quite low.

It is worth knowing because Poisson Statistics limit the accuracy of all intensity measurements made with confocal fluorescent microscopes to an uncertainty of +/- the square root of the number of quantum mechanical events (photons?) detected. Although the number stored in the image memory should be proportional to this number, it is usually larger by a generally unknown factor (5 to 20 times?). Only if one can convert this stored number into photons can one gain any appreciation of the "accuracy" of data recorded in the image.

I realize that this procedure may intimidate some, but it really is the only fairly reliable way to conveniently measure this most important aspect of microscope performance. Start by turning off the laser and recording the image of the light coming through the specimen. This is a first estimate of the transmission photon efficiency. The remainder of the procedures described above is just an effort to standardize the other parameters that can effect the value recorded. Normalizing these parameters will make the rough measurement more reliable and representative.

This topic is discussed in more detail in the *Handbook of Biological Confocal Microscopy*, Chapter 2, pp 29 -30 (J.B. Pawley, ed 1995, 2nd ed. Plenum Pr NY xxiii + 632pp). ■