The Quantitative Quagmire of Confocal Microscopy

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Any reasonably modern confocal fluorescence microscope can produce beautiful images of cells and tissues without too much effort. It has generally been assumed that with thoughtful experimental planning and careful execution, confocal images can be used for **quantitative** measurements of fluorescence intensities (Figure 1). Microscope users are taught to prepare all of their specimens the same way, and to be diligent about using the same microscope settings (exposure time, laser power, etc.) for control and experimental conditions. Despite these precautions, and unbeknownst to the users, there are several relatively common issues lurking behind the colourful façades of their images that result in inaccurate or even incorrect quantification:

-Severe lamp/ laser intensity fluctuations.
-Dramatically non-uniform illumination across the field-of view.
-Uncorrected background, crosstalk, and bleedthrough.
-Varying degrees of photobleaching.

Particularly troubling are the relatively common laser fluctuations, which may change the illumination intensity by as much as 40% over a 3hr imaging session (Figure 2) even in a shiny new confocal microscope! Despite the fact that these (and other) issues are surprisingly widespread and also known to facility staff, users of these microscopes are often blissfully unaware of any problems and will likely go on to quantify the resulting images.

We present several case studies that illustrate the extent of these problems. Next, we make suggestions for users to help them recognize and potentially account for the quantification quagmire that these issues produce. Finally, we call upon manufacturers to use their built-in photodiodes (present in most laser-scanning confocals already) to calibrate and standardize the intensity dimension to truly make confocal microscopes tools for quantitative fluorescence microscopy.

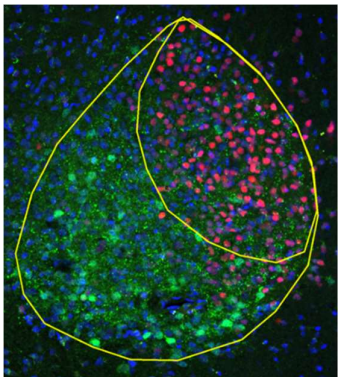

Figure 1. Quantifying the expression of c-FOS in the SCN of a rat brain.

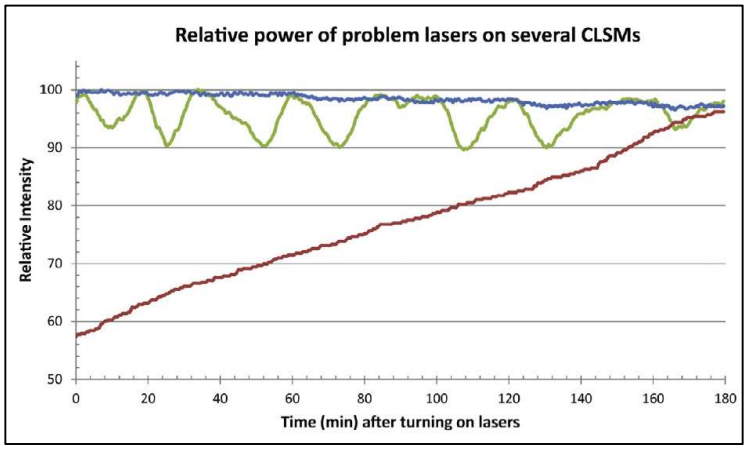

Figure 2. Laser fluctuations recorded using power meters on several relatively new confocal microscopes.